This report presents the recommendations of a WHO Expert Committee commissioned to coordinate activities leading to the adoption of international recommendations for the production and control of vaccines and other biological substances, and the establishment of international biological reference materials.

Following a brief introduction, the report summarizes a number of general issues brought to the attention of the Committee. The next part of the report, of particular relevance to manufacturers and national regulatory authorities, outlines the discussions held on the development and revision of WHO Guidelines for a number of vaccines, blood products and related substances. Specific discussion areas included WHO guidance on the production and evaluation of the quality, safety and efficacy of monoclonal antibodies as similar biotherapeutic products (SBPs); blood and blood components as essential medicines; estimation of residual risk of HIV, HBV or HCV infections via cellular blood components and plasma; snake antivenom immunoglobulins; human pandemic influenza vaccines in non-vaccine-producing countries; and clinical evaluation of vaccines: regulatory expectations. In addition, the following WHO guidance documents were also adopted: WHO manual for the preparation of secondary reference materials for in vitro diagnostic assays designed for infectious disease nucleic acid or antigen detection: calibration to WHO International Standards; and Human challenge trials for vaccine development: regulatory considerations. One WHO addendum document – Labelling information of inactivated influenza vaccines for use in pregnant women – was also adopted.

Subsequent sections of the report provide information on the current status, proposed development and establishment of international reference materials in the areas of: biotherapeutics other than blood products; blood products and related substances: cellular and gene therapies; in vitro diagnostics; and vaccines and related substances.

A series of annexes are then presented which include an updated list of all WHO Recommendations, Guidelines and other documents on biological substances used in medicine (Annex 1). The above nine WHO documents adopted on the advice of the Committee are then published as part of this report (Annexes 2–10). Finally, all additions and discontinuations made during the 2016 meeting to the list of International Standards, Reference Reagents and Reference Panels for biological substances maintained by WHO are summarized in Annex 11. The updated full catalogue of WHO International Reference Preparations is available at: http://www.who.int/bloodproducts/catalogue/en/.
The World Health Organization was established in 1948 as a specialized agency of the United Nations serving as the directing and coordinating authority for international health matters and public health. One of WHO's constitutional functions is to provide objective and reliable information and advice in the field of human health, a responsibility that it fulfills in part through its extensive programme of publications.

The Organization seeks through its publications to support national health strategies and address the most pressing public health concerns of populations around the world. To respond to the needs of Member States at all levels of development, WHO publishes practical manuals, handbooks and training material for specific categories of health workers; internationally applicable guidelines and standards; reviews and analyses of health policies, programmes and research; and state-of-the-art consensus reports that offer technical advice and recommendations for decision-makers. These books are closely tied to the Organization’s priority activities, encompassing disease prevention and control, the development of equitable health systems based on primary health care, and health promotion for individuals and communities. Progress towards better health for all also demands the global dissemination and exchange of information that draws on the knowledge and experience of all WHO’s Member countries and the collaboration of world leaders in public health and the biomedical sciences.

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WHO Expert Committee on Biological Standardization

Sixty-seventh report

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization.
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Annex 1  
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Annex 3  
Guidelines on management of blood and blood components as essential medicines

Annex 4  
Guidelines on estimation of residual risk of HIV, HBV or HCV infections via cellular blood components and plasma

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Biological substances: WHO International Standards, Reference Reagents and Reference Panels
WHO Expert Committee on Biological Standardization
17 to 21 October 2016

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17 Participated via teleconference.
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20 Participated via teleconference.
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²⁸ Participated via teleconference.
²⁹ Participated via teleconference.
³⁰ Participated via teleconference.
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31 A maximum of two representatives of the Developing Countries Vaccine Manufacturers Network and two representatives of the International Federation of Pharmaceutical Manufacturers & Associations were present in the meeting room during discussion of any one agenda item.

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# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>anti-HBc</td>
<td>Antibodies to hepatitis B core protein</td>
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<tr>
<td>anti-HBs</td>
<td>Antibodies to hepatitis B surface antigen</td>
</tr>
<tr>
<td>ASfBT</td>
<td>Africa Society for Blood Transfusion</td>
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<tr>
<td>BGTD</td>
<td>Biologics and Genetic Therapies Directorate</td>
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<tr>
<td>BRN</td>
<td>WHO Blood Regulators Network</td>
</tr>
<tr>
<td>CBER</td>
<td>Center for Biologics Evaluation and Research</td>
</tr>
<tr>
<td>CEG</td>
<td>Core Expert Group</td>
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<tr>
<td>CHIKV</td>
<td>Chikungunya virus</td>
</tr>
<tr>
<td>CTP</td>
<td>Cell therapy product</td>
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<tr>
<td>DENV</td>
<td>Dengue virus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTP</td>
<td>Diphtheria, tetanus and pertussis</td>
</tr>
<tr>
<td>EBOV</td>
<td>Ebola virus</td>
</tr>
<tr>
<td>ECSPP</td>
<td>WHO Expert Committee on Specifications for Pharmaceutical Preparations</td>
</tr>
<tr>
<td>EDQM</td>
<td>European Directorate for the Quality of Medicines &amp; HealthCare</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>EMP</td>
<td>WHO Department of Essential Medicines and Health Products</td>
</tr>
<tr>
<td>EVAL</td>
<td>WHO emergency use assessment and listing (procedure)</td>
</tr>
<tr>
<td>EV</td>
<td>Enterovirus</td>
</tr>
<tr>
<td>EVD</td>
<td>Ebola virus disease</td>
</tr>
<tr>
<td>EQA</td>
<td>External quality assurance</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drugs Authority (Ghana)</td>
</tr>
<tr>
<td>FV</td>
<td>Blood coagulation factor V</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FXI</td>
<td>blood coagulation factor XI</td>
</tr>
<tr>
<td>FXIa</td>
<td>activated blood coagulation factor XI</td>
</tr>
<tr>
<td>FXI:Ag</td>
<td>blood coagulation factor XI (antigen value)</td>
</tr>
<tr>
<td>FXI:C</td>
<td>blood coagulation factor XI (functional activity)</td>
</tr>
<tr>
<td>FXII</td>
<td>blood coagulation factor XII</td>
</tr>
<tr>
<td>FXIII</td>
<td>blood coagulation factor XIII</td>
</tr>
<tr>
<td>GACVS</td>
<td>WHO Global Advisory Committee on Vaccine Safety</td>
</tr>
<tr>
<td>GAPIII</td>
<td>WHO Global Action Plan to minimize poliovirus facility-associated risk after type-specific eradication of wild polioviruses and sequential cessation of oral polio vaccine use</td>
</tr>
<tr>
<td>GBS</td>
<td>Group B streptococcus</td>
</tr>
<tr>
<td>GCV</td>
<td>geometric coefficient of variation</td>
</tr>
<tr>
<td>GMP</td>
<td>good manufacturing practice(s)</td>
</tr>
<tr>
<td>GPP</td>
<td>good preparation practice(s)</td>
</tr>
<tr>
<td>HAV</td>
<td>hepatitis A virus</td>
</tr>
<tr>
<td>HBsAg</td>
<td>hepatitis B surface antigen</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HER</td>
<td>human epidermal growth factor receptor</td>
</tr>
<tr>
<td>HI</td>
<td>haemagglutination inhibition</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>HPV</td>
<td>human papillomavirus</td>
</tr>
<tr>
<td>ICDRA</td>
<td>International Conference of Drug Regulatory Authorities</td>
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<tr>
<td>INN</td>
<td>International Nonproprietary Name(s)</td>
</tr>
<tr>
<td>INR</td>
<td>international normalized ratio</td>
</tr>
<tr>
<td>IPV</td>
<td>inactivated poliomyelitis vaccine</td>
</tr>
<tr>
<td>ISI</td>
<td>International Sensitivity Index</td>
</tr>
<tr>
<td>ISTH</td>
<td>International Society on Thrombosis and Haemostasis</td>
</tr>
<tr>
<td>IU</td>
<td>International Unit(s)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>---------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>IVD</td>
<td>in vitro diagnostic</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MERS-CoV</td>
<td>Middle East respiratory syndrome coronavirus</td>
</tr>
<tr>
<td>MFDS</td>
<td>Ministry of Food and Drug Safety</td>
</tr>
<tr>
<td>MN</td>
<td>microneutralization</td>
</tr>
<tr>
<td>MPN</td>
<td>myeloproliferative neoplasm</td>
</tr>
<tr>
<td>MSF</td>
<td>Médecins Sans Frontières</td>
</tr>
<tr>
<td>NAPTT</td>
<td>non-activated partial thromboplastin time</td>
</tr>
<tr>
<td>NAT</td>
<td>nucleic acid amplification technique</td>
</tr>
<tr>
<td>NBS</td>
<td>National Blood Service (Ghana)</td>
</tr>
<tr>
<td>NCL</td>
<td>national control laboratory</td>
</tr>
<tr>
<td>NDU</td>
<td>NAT-detectable unit</td>
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<tr>
<td>NIBSC</td>
<td>National Institute for Biological Standards and Control</td>
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<tr>
<td>NIFDC</td>
<td>National Institutes for Food and Drug Control</td>
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<tr>
<td>NIFDS</td>
<td>National Institute of Food and Drug Safety Evaluation</td>
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<tr>
<td>NIID</td>
<td>National Institute of Infectious Diseases</td>
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<tr>
<td>NIST</td>
<td>National Institute for Standards and Technologies</td>
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<tr>
<td>NRA</td>
<td>national regulatory authority</td>
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<tr>
<td>OMCL</td>
<td>Official Medicines Control Laboratory (network)</td>
</tr>
<tr>
<td>OPV</td>
<td>oral poliomyelitis vaccine</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDMP</td>
<td>plasma-derived medicinal product</td>
</tr>
<tr>
<td>PEESP</td>
<td>WHO Polio Eradication and Endgame Strategic Plan 2013–2018</td>
</tr>
<tr>
<td>PEI</td>
<td>Paul-Ehrlich-Institut</td>
</tr>
<tr>
<td>PT</td>
<td>prothrombin time</td>
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<tr>
<td>rDNA</td>
<td>recombinant DNA</td>
</tr>
<tr>
<td>RDT</td>
<td>rapid diagnostic test</td>
</tr>
<tr>
<td>rhPTH1-34</td>
<td>parathyroid hormone 1-34 (recombinant, human)</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
<tr>
<td>SAGE</td>
<td>WHO Strategic Advisory Group of Experts</td>
</tr>
<tr>
<td>SBP</td>
<td>similar biotherapeutic product</td>
</tr>
<tr>
<td>sIPV</td>
<td>Sabin inactivated poliomyelitis vaccine</td>
</tr>
<tr>
<td>SoGAT</td>
<td>Standardisation of Genome Amplification Techniques (group)</td>
</tr>
<tr>
<td>SSC</td>
<td>Scientific and Standardization Committee (of ISTH)</td>
</tr>
<tr>
<td>TAFI</td>
<td>Thrombin-activatable fibrinolysis inhibitor</td>
</tr>
<tr>
<td>TGA</td>
<td>Therapeutic Goods Administration</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VIFFP</td>
<td>virus-inactivated fresh frozen plasma</td>
</tr>
<tr>
<td>WHO CC</td>
<td>WHO collaborating centre</td>
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<tr>
<td>WNV</td>
<td>West Nile virus</td>
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<tr>
<td>YF</td>
<td>yellow fever</td>
</tr>
<tr>
<td>YFV</td>
<td>yellow fever virus</td>
</tr>
<tr>
<td>ZIKV</td>
<td>Zika virus</td>
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1. Introduction

The WHO Expert Committee on Biological Standardization met in Geneva from 17 to 21 October 2016. The meeting was opened on behalf of the Director-General of WHO by Dr Suzanne Hill, the recently appointed Director of Essential Medicines and Health Products (EMP). Dr Hill welcomed the Committee, meeting participants and observers. She informed the meeting of several staff changes in the Department over the previous year, with a number of further senior WHO staff changes expected in the near future. This would include the Secretary to the WHO Expert Committee on Biological Standardization, Dr David Wood, who would be retiring in early 2017.

Dr Hill referred to the United Nations overarching strategic direction entitled “Transforming our world: The 2030 Agenda for Sustainable Development”1 and indicated that this was being translated into a new vision for EMP. One new initiative would be a greater focus on access to biotherapeutics. It is envisaged that by 2030, biological substances will be used more widely than at present and ensuring sustainable access to biotherapeutics of assured quality for public health-care systems will be a key challenge for all countries, rich and poor alike. Dr Hill emphasized that the development and adoption of norms and standards to regulate the quality, safety and efficacy of medical products and guide their cost-effective use would be a critical foundation on which future aspirations would be built. A global approach to this core normative work is facilitated by the coordinated efforts of WHO Expert Committees and the vital support of WHO collaborating centres (WHO CCs) and partner organizations.

Dr Hill reminded meeting participants that the Committee had a mandate, enunciated in the WHO Constitution, to develop, establish and promote international standards for biological products. In addition to supporting the development and use of biological medicines, other goals now needed to be considered – specifically, promoting access to essential medicines and regulatory strengthening. Furthermore, the world of regulatory science was changing, particularly as new products came to the market, and there was an expectation concerning the role of WHO in providing regulatory guidance and promoting regulatory strengthening. Norms and standards underpin and reflect these expectations but need to be regularly reviewed to ensure they reflect the best regulatory science. The convergence of norms and standards internationally is recognized as a key driver in addressing these needs.

After norms and standards have been established there was then a need for proactive technical support from WHO to its Member States in order

to obtain maximum understanding and impact, and to facilitate consistent application. There were many emerging issues to be dealt with, including learning the lessons of the recent Ebola public health emergency, particularly in relation to the rolling out of candidate vaccines and other health products during an epidemic. Regulatory preparedness for future emergency situations was crucial. There was also a need to consider the issue of access to new products in a timely way.

After touching on the extremely heavy workload of the Committee and acknowledging the difficulty of running a two-track meeting – with the blood products and in vitro diagnostics track and the vaccines and biotherapeutics track running in parallel – Dr Hill moved on to the election of meeting officials. In the absence of dissent, Professor Klaus Cichutek was elected as Chair and Dr Elwyn Griffiths as Rapporteur for the plenary sessions, and for the track considering vaccines and biotherapeutics. Dr Harvey Klein was elected as Chair and Dr Clare Morris and Dr Jens Reinhardt as Rapporteurs for the track considering blood products and in vitro diagnostics. Dr Klein was also elected as Vice-Chair for the plenary sessions of the Committee.

Finally, Dr Hill expressed her thanks on behalf of WHO to the Committee, to WHO CCs, and to all the experts, institutions and professional societies working in this area whose efforts provided vital support to WHO programmes in global public health. She concluded by reminding participants that Committee members acted in their personal capacities as experts and not on behalf of their organizations or countries.

Dr David Wood then gave a brief overview of WHO Expert Committees and of their important and greatly valued role in providing assistance to WHO Member States. He noted that two Expert Committees were meeting during the week – the Expert Committee on Specifications for Pharmaceutical Preparations and this Expert Committee on Biological Standardization – and that the 63rd Consultation on International Nonproprietary Names for Pharmaceutical Substances was also taking place. Dr Wood then introduced the members of the 2016 Expert Committee on Biological Standardization, and highlighted the new requirement initiated in 2015 that biographical summaries of all the members must be posted for public review and comment prior to the meeting. All biographical summaries had been posted and no comments had been received. Dr Wood then outlined the organization of the meeting and the major issues to be discussed. Declarations of Interests made by members of the Committee, Temporary Advisers and participants were then presented. Following prior

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2 Dr E. Griffiths (consulting); Dr P. Minor (public statements; research support); Professor S. Efstathiou (research support); Dr J. Ferguson (investments); Dr R. Sheets (consulting); Dr J. Southern (consulting); and Dr D. Williams (public statements).
evaluation, WHO had concluded that none of the declarations made constituted a significant conflict of interest, and that the individuals concerned would be allowed to participate fully in the meeting.

Following participant introductions, the Committee adopted the proposed agenda (WHO/BS/2016.2303 Add.1).
2. General

2.1 Current directions

2.1.1 Strategic directions in biological standardization: WHO priorities

Dr Wood reminded the Committee of the core activities of WHO and that its Constitution required it...to develop, establish and promote international standards with respect to biological and pharmaceutical products. WHO had been doing this for over 60 years through a programme which included the development of global written standards, global measurement standards and International Nonproprietary Names (INN). In the field of biological substances there were now over 70 WHO written standards and 300 reference preparations, all of which make a significant contribution to global public health. Indeed, the first international biological reference preparation, for insulin, had been established in 1925 under the auspices of the League of Nations. Current global public health priorities include responding to public health emergencies of international concern, access to biotherapeutics and the strengthening of regulatory systems – the latter two being supported by two World Health Assembly resolutions: one on biotherapeutic products (WHA67.21, 2014) and the other on regulatory systems strengthening (WHA67.20, 2014). Resolution WHA67.21 requests WHO to support Member States in the regulation of biotherapeutic products, including similar biotherapeutic products (SBPs). In particular, the Resolution requested WHO to convene the Expert Committee on Biological Standardization in order to update its 2009 Guidelines in this area, taking into account technological advances in the characterization of biotherapeutic products and considering national regulatory needs and capacities. WHO had recently reported on progress in this area to its Executive Board and to the sixty-ninth World Health Assembly in May 2016.

As part of this programme, the Committee had adopted key written guidelines on the regulatory assessment of approved recombinant DNA (rDNA) biotherapeutics, and a series of WHO implementation workshops had been held over a period of 5 years on current WHO Guidelines for biotherapeutic products and SBPs. These workshops had involved participants from over 50 countries. The workshops had included case studies, with the outcomes published in the scientific literature. Dr Wood highlighted the continuing and growing need for international measurement standards for the calibration of bioassays, especially for biotherapeutics. He noted that the number of INN applications for biological and biotherapeutic substances had increased enormously in recent years and now accounted for over 50% of all such applications. The nature of the standards required was also evolving and WHO would need to adapt its standardization programme for biotherapeutics as these gained market authorization through the biosimilars route. There was thus a
need to consider very carefully the potential use and extent of applicability of such reference preparations.

In considering the workload in the biologicals field, Dr Wood raised the issues of the increasingly packed agendas of meetings of the Committee, the difficulties in minimizing scheduling conflicts in the two-track system and the insufficient time allocated for discussing strategic issues. Various proposals were being considered to optimize the use of Committee time during face-to-face meetings, such as holding meetings biannually, and the introduction of more pre-meeting technical discussion of proposals for reference preparations. Discussion at the face-to-face meetings could then be reserved for the more complex proposals or those that were precedent-setting. Increasing the inputs from WHO CC networks (see sections 2.2.2 and 2.2.3 below) was also under consideration. Currently, WHO CC networks exist for vaccines and for blood products and in vitro diagnostics (IVDs) but not for biotherapeutics. Dr Wood also added that the WHO Secretariat is currently understaffed but that additional staffing was being sought through secondments. Linkages with other Expert Committees and Expert Groups were also considered important, for example in developing both biological and chemical reference preparations for biotherapeutic medicines, along with efforts to raise the visibility of the work carried out in the area of biological standardization.

The Committee thanked Dr Wood for his overview and expressed support for the focus and priorities outlined. The Committee drew attention to standardization needs in potential new areas of work, such as vaccine platforms, companion diagnostics and cell therapies, and highlighted the need to consider where such activities might fit into the WHO programme of work. Such consideration should include a careful review of the scope of the Committee, which currently includes vaccines, biotherapeutics, blood products and IVDs. The Committee also agreed that there was a need to improve the visibility of the WHO biological standardization programme and its key role in supporting global public health. This should involve not only reporting on the work of the Committee in the scientific media but also keeping medical prescribers and practitioners aware of the WHO biological standardization programme and its significance in assuring the quality, safety and efficacy of biological medicines. Indeed, the Committee considered that improving visibility and explaining the crucial need for biological standardization should start during the education of medical and pharmaceutical science students.

2.1.2 Vaccines and biotherapeutics: recent and planned activities in biological standardization

Dr Ivana Knezevic reported on activities relating to the standardization and regulatory evaluation of vaccines and biotherapeutics and discussed several
strategic issues. During the period 2013–2016 seven measurement standards had been established for biotherapeutics and 11 for vaccines.

Vaccine development work was being carefully monitored to ensure that standardization needs were being met in a timely way. This was achieved by ensuring links between the Committee, the WHO Strategic Advisory Group of Experts (SAGE) on Immunization and the relatively new WHO Product Development for Vaccines Advisory Committee. There had been significant recent developments in the infectious diseases area, including: (a) regulatory approval of the first malaria and dengue vaccines; (b) licensure in China of an enterovirus 71 (EV71) vaccine; (c) the first Phase III trials of a respiratory syncytial virus (RSV) vaccine in pregnant women and the elderly; and (d) the continuing problems of Middle East respiratory syndrome coronavirus (MERS-CoV) and Zika viruses (ZIKVs), and associated vaccine development work. Seven written standards related to vaccine regulation and evaluation were under development, with five being submitted to the Committee for consideration in this meeting (see sections 3.5.2–3.5.6 below), one planned for 2017 (see section 3.5.1 below) and another on RSV vaccines scheduled for consideration by the Committee in 2018. The development of three new written standards (for meningitis B vaccines, EV71 vaccines and hepatitis E vaccines) was being considered.

Two written standards for biotherapeutic products and SBPs were also under development – with guidelines on the evaluation of monoclonal antibodies as SBPs being submitted to the Committee in this meeting (see section 3.1.1 below), and guidelines on the regulatory expectations for post-approval changes for biotherapeutic products expected to be submitted for adoption in 2017. A revision of the 2009 WHO Guidelines on evaluation of similar biotherapeutic products (SBPs) was also under consideration, and the possibility of developing guidelines for cellular and gene therapies would be discussed separately (see section 3.3 below).

Dr Knezevic reported that between 2010 and 2015 a number of WHO implementation workshops on standards for biotherapeutics, including SBPs, had been held (in Canada, China, Colombia, Ghana and the Republic of Korea) and these had been considered to be extremely useful to both national regulatory authorities (NRAs) and manufacturers. Thanks to the support of the Ministry of Food and Drugs, Republic of Korea, three workshops had been held in Seoul. In 2016, implementation workshops on standards for human papillomavirus (HPV) had been held in China and Thailand, and on typhoid conjugate vaccines in Indonesia – with plans in place for a further four workshops during 2017–2018.

The work of WHO CCs was becoming increasingly important in supporting the WHO biological standardization programme, and the third
meeting of the WHO network of collaborating centres on standardization and regulatory evaluation of vaccines (see section 2.2.2 below) was held in the Republic of Korea in July 2016.

Improvements in testing methods are considered to be crucial for the effective regulation of biologicals. Efforts had previously been made to improve neurovirulence safety tests used in the quality control of oral poliomyelitis vaccine (OPV) and yellow fever (YF) vaccines, and the potency tests used for the lot release of diphtheria, tetanus and pertussis (DTP), rabies and YF vaccines. However, more remains to be done in the case of rabies vaccines. One particularly active area of investigation concerns the possibility of replacing the NIH potency test for rabies vaccines – which is a test in animals established in the 1950s – with alternative or complementary tests based on enzyme-linked immunosorbent assays (ELISAs) or serological assays. These replacement efforts are based both on a sound scientific basis and on increasing recognition of the 3Rs concept (Replacement, Reduction, Refinement), which stresses the importance of minimizing the use of animal testing in research.

A number of international and regional initiatives for promoting regulatory convergence were also highlighted by Dr Knezevic. This is an area in which many stakeholders recognized the unique role of WHO and had called for action. Although there was a need to prioritize WHO activities, it was considered important to assist Member States in making their national regulatory requirements more consistent with each other by using WHO standards as common ground. The International Conference of Drug Regulatory Authorities (ICDRA) provides a forum to meet and discuss ways to strengthen collaboration and Dr Knezevic reported that several relevant topics had been selected for discussion at the 17th ICDRA to be held in Cape Town, South Africa. These included SBPs, good regulatory practices, regulatory convergence initiatives, maternal immunization and regulatory responses to shortages of medicines and vaccines.

The Committee thanked Dr Knezevic for her overview and supported the proposed initiatives. It considered that EV71 was of major regional significance and the joint effort of the National Institutes for Food and Drug Control (NIFDC) and the National Institute for Biological Standards and Control (NIBSC) in developing the First International Standard for anti-EV71 serum (human)\(^3\) had been timely and very successful. Consideration should now be given to the development of a written standard for EV71 vaccine.

Developments in methodology were recognized as an area where WHO had a comparative advantage. In that context, the Committee urged WHO to continue to support activities aimed at replacing the NIH in vivo assay for rabies vaccine potency with an alternative in vitro assay. In line with this development, WHO was also strongly encouraged to consider deleting the abnormal toxicity test (sometimes called General Safety or Innocuity test) from all WHO Recommendations, Guidelines and other guidance documents (see also section 2.3.1; EDQM). It was noted that this is particularly important for global manufacturers who would greatly welcome WHO initiatives in this direction.

The future development of cancer treatments based on combination products consisting of active biological and chemical components (as well as nanomedicines) was also highlighted as an area to be monitored. The Committee agreed and noted that any future regulatory guidance for such products would need both biological and pharmaceutical input and supported the idea of increased involvement of academia in this area of work.

2.1.3 Blood products and in vitro diagnostics: recent and planned activities in biological standardization

Dr Micha Nuebling reviewed recent WHO activities in blood products and in vitro diagnostics, highlighting the following four activity areas: (a) antivenoms; (b) ZIKV; (c) the Achilles project; and (d) standardization issues.

Dr Nuebling reported that each year there are more than 100,000 fatalities from snake-bites caused by difficulties in accessing antivenoms. WHO had established a database showing the distribution of venomous snakes, their respective antivenoms and the relevant manufacturers. However, the manufacturing and distribution of antivenoms remains widely unregulated and many products are of unknown quality. Moreover, in 2014 there had been a production stop for “Fav-Afrique” by Sanofi Pasteur. In order to address the need for antivenoms of assured quality, safety and efficacy, WHO had initiated an assessment procedure for products intended for use in sub-Saharan Africa. Applications for eight products, including polyvalent and monovalent preparations, were received. For the assessment exercise, a panel of experts (which included two regulators from Africa) was asked to evaluate the dossiers at WHO headquarters in Geneva. Differences in the quality of the products were obvious and plans are in place to supplement the evaluation of at least some products by laboratory testing and/or inspection.

During the evaluation process, it became apparent that there was a need to revise the 2008 WHO Guidelines for the production, control and regulation of snake antivenom immunoglobulins. Expert revision was promptly undertaken and, following public consultation, the revised guidelines were
Dr Nuebling indicated that if antivenom assessment is to become a sustainable process in the future then funding issues will need to be addressed. Planned next steps include the completion of first-round assessment based on laboratory testing of promising products, funded by Médecins Sans Frontières (MSF), and inspections of manufacturing sites. A side event on antivenoms took place at the 2016 World Health Assembly, initiated by Costa Rica and supported by 17 Member States, with the intention of raising awareness and potential donor interest in this important public health issue. Moreover, snake-bites will be proposed for re-entry into the WHO List of Neglected Tropical Diseases and the topic has been proposed as an agenda item of the WHO Executive Board. There are also plans to promote snake-bite prevention and treatment initiatives, including antivenom technology transfer, early case management and research.

Due to the 2015–2016 ZIKV epidemic in the Americas, and the suspected association in Brazil between ZIKV infection in pregnant women and microcephaly in newborns, WHO guidance on blood donations was urgently required. Following close cooperation between EMP, the WHO Department of Service Delivery Systems and the WHO Regional Office for the Americas, with support from the WHO Blood Regulators Network (BRN), a WHO guidance document on blood collection was published in February 2016. This document advises on situations with and without active ZIKV transmission and evaluates potential measures such as donor deferral, quarantining of blood components, pathogen inactivation and testing. There was also an urgent need for reference materials in this area, and projects to develop reference preparations for ZIKV IVDs had therefore been initiated. This resulted in the rapid development of a candidate WHO standard for ZIKV RNA to be considered for establishment by the Committee this year (see section 7.1.1 below). Problems had been encountered however in obtaining materials for serology standards.

Dr Nuebling also reported on the progress of the Achilles project for improving access to safe blood products through local production and technology transfer in blood establishments. Indonesia had been chosen as a pilot country for the Achilles project. Project activities included the evaluation of IVDs related to blood safety since many tests were available but their performance data were not usually assessed. A workshop was held in Jakarta in August 2016 to review current practices. Workshop participants included representatives of Ministry of Health departments, the hepatitis surveillance programme, the Indonesian Red Cross and the IVD laboratory of the Indonesian Centers for Disease Control. The main aim was to evaluate > 40 different rapid diagnostic tests (RDTs) for the detection of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. RDTs are still used for blood screening for 15% of the national blood supply and are the main surveillance tool in the country. Protocols for evaluating all HBV and HCV RDTs were reviewed with an initial focus on sensitivity.
Aspects of blood regulation were also covered in WHO workshops conducted in the WHO African Region and WHO Eastern Mediterranean Region, resulting in respective regional strategies. Following a request from Ghana, an in-country expert assessment of its new blood regulations was undertaken involving WHO headquarters, the WHO Regional Office for Africa and the BRN, using the new BRN Assessment Criteria for Blood Regulatory Systems. This initial field test of the suitability of the assessment tool resulted in the identification of gaps in the national blood regulatory system and the generating of a number of recommendations (see section 3.2.1 below).

In the field of IVD standardization, concerns had been raised regarding the suitability of the current First WHO International Standard for Anti-Rubella Immunoglobulin (established in 1996) for use with more recent IVDs. There appeared to be some uncertainty over whether the associated recommendation of using 10 IU/ml as a general threshold for the decision to vaccinate young women was still valid. Recent anti-rubella IVDs can show quite inconsistent quantitative results in the low-titre range of anti-rubella antibodies, leading to potentially discrepant decisions on the vaccination or re-vaccination of individuals. A meeting was planned for June 2017 to review the current situation and develop appropriate recommendations. Dr Nuebling concluded by reporting that several new reference preparations in the area of blood products and IVDs were to be considered for establishment by the Committee in 2016 (see sections 5.1.1–5.1.5 and sections 7.1.1–7.1.6 below) and a number of new projects considered for endorsement.

The Committee thanked Dr Nuebling for his report and looked forward to hearing of further progress at its next meeting.

2.2 Reports

2.2.1 Report from the WHO Blood Regulators Network

Dr Christian Schaerer reported on the activities of the BRN during the past year. Following its face-to-face meeting during the previous Committee meeting in October 2015, five teleconferences had been held. There had also been several changes in BRN member representatives as well as a minor revision of the BRN Terms of Reference. This now allowed “alternate” representatives to be eligible candidates for the BRN Chair. Dr Schaerer himself had been elected BRN Chair for a second term, until October 2017.

BRN activities during 2015–2016 had included discussion of the ongoing revision of the WHO NRA assessment tools, and of the draft WHO Guidelines on estimation of residual risk of HIV, HBV or HCV infections via cellular blood components and plasma, proposed for adoption at this meeting (see section 3.2.4 below). Discussions were also held on the first results of clinical trials
performed in Guinea using convalescent plasma from Ebola virus (EBOV) survivors, particularly in light of the infrastructure conditions under which the trial was performed. During the above teleconferences, regular updates on the Ebola disease situation and the activities under way to strengthen the blood systems in Ebola-affected countries were provided. Revision of the BRN position paper on *Collection and use of convalescent plasma or serum as an element in filovirus outbreak response* was still pending. It was expected that data and results on the antibody titres and other characteristics of the convalescent plasmas used would soon become available and would be reflected in the revised position paper.

Discussions also continued to be held on: (a) national decision-making in relation to donor deferral for men who have sex with men; (b) the Alliance of Blood Operators project on risk-based decision-making for blood safety; and (c) the WHO expert assessment of antivenoms. One additional item on the BRN agenda was the development of the draft WHO Guidelines on management of blood and blood components as essential medicines, in response to recommendations made at the 16th ICDRA. Following an extensive development process, the final draft was now being proposed for adoption at this meeting (see section 3.2.3 below). Other work products in 2015–2016 included: (a) BRN consultation with WHO on the development of emergency guidance on yellow fever virus (YFV) vaccination and blood donation following outbreaks in Angola, the Democratic Republic of the Congo and Uganda and; (b) the sharing of information on the ZIKV situation, including discussion of potential measures to protect the blood supply; and (c) discussing and sharing information on the hepatitis E virus situation based on the results of a standardized survey of BRN members.

BRN activities also included participation in the WHO in-country assessment of the blood regulatory system in Ghana – representing the first real-life application of the BRN assessment criteria to an external NRA assessment process. The BRN was also involved in the WHO Regional training workshop on regulatory systems for blood and blood products held in Benin in July 2016, and would be assisting in a workshop on blood products at the upcoming 17th ICDRA in South Africa.

The Committee thanked Dr Schaerer for his report and raised the issue of BRN membership. While the Committee recognized the valuable support provided by the BRN to WHO there was an impression that the composition of the BRN did not take into account the experience of developing countries. Regarding the criteria for BRN membership, Dr Schaeerer pointed out that in principle any country could join provided it met the conditions laid out in the BRN Terms of Reference, and provided the example of Japan as a non-founding member of the BRN. However, it was pointed out by the Committee
that the Terms of Reference make it clear that candidates should be experienced blood regulating authorities. It was therefore suggested that the BRN develop mechanisms for observing and taking into consideration the perspectives of other countries. Dr Schaerer referred to already existing processes for such interactions, such as the consultation phases for guidance documents.

It was also suggested that the BRN, in addition to responding to new demands or emergencies, should also strengthen its support for the educating and training of regulators in NRAs less experienced in blood regulation. It was again emphasized that BRN members, despite limited capacities, were already active in this endeavour through workshops in developing countries, for example in Africa.

2.2.2 Report from the WHO network of collaborating centres on standardization and regulatory evaluation of vaccines

Dr Yeowon Sohn and Dr Paul Stickings reported on the third meeting of the network. One of the main objectives of this meeting was to discuss how the network might best support the streamlining of the work of the Committee and contribute to the priority-setting process for both written and measurement standards. The possibility of setting up a working group to provide advice to WHO on norms and standards priorities had been discussed. It was acknowledged however that long-term planning was not easy, and had to reflect a range of drivers both internal and external to WHO. The possibility of the network playing a role in pre-reviewing selected proposals for measurement standards intended for submission to the Committee was also considered. At present, the review of large numbers of proposals for new or replacement measurement standards consumed a significant amount of Committee time. It may be that relevant expertise within the network could be utilized to support the Committee by focusing on selected proposals and reducing some of the workload. It had therefore been proposed that a “Core Expert Group” (CEG) from the network be formed. As new measurement standards have strategic considerations (in addition to scientific issues) it was felt that these should remain the responsibility

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4 The network currently consists of the following eight WHOCCs: (a) National Institute for Biological Standards and Control (NIBSC), Medicines and Healthcare products Regulatory Agency, England; (b) Center for Biologics Evaluation and Research (CBER), Food and Drug Administration, the USA; (c) Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases (NIID), Japan; (d) Immunobiology and Biochemistry Group, Office of Laboratories & Scientific Services, Therapeutic Goods Administration (TGA), Australia; (e) National Institute of Food and Drug Safety Evaluation (NIFDS), Ministry of Food and Drug Safety (MFDS), Republic of Korea; (f) Biologics and Genetic Therapies Directorate (BGTD), Health Canada, Canada; (g) Institute for Biological Product Control of the National Institutes for Food and Drug Control (NIFDC), China; and (h) Division of Virology, Paul-Ehrlich-Institut (PEI), Germany.
of the Committee itself. However, proposals for replacement measurement standards were likely to be more straightforward, with few strategic or scientific issues, and could be the initial focus of a proposed CEG. It was further suggested that this process might initially cover measurement standards for vaccines, with potential expansion to other standards categories in the future. As any CEG pre-review would be likely to affect the current timelines for submission of proposals this would need to be taken into account. A potential process overview plan had been suggested which would involve the submission of a one-page summary for CEG review of a replacement standard. Where no issues were raised, the standard would be recommended to the Committee for endorsement without further review. Submissions for new standards would then be reviewed directly by the Committee in the usual way.

The Committee thanked Dr Sohn and Dr Stickings for their report, and welcomed the idea of creating a CEG to support its work. As a pilot study, the Committee agreed that the CEG could pre-review selected measurement standards in the vaccines area in order to streamline the current review process. However, it was proposed and agreed that the CEG would include two or three Committee members. The CEG would then prepare a one-page summary for each of the recommended and rejected proposals for which there were no issues to be resolved, for consideration by the Committee. A more detailed summary would be needed where issues were identified that required further discussion by the Committee. For measurement standards for which no issues were noted, and for which the advice was simply to endorse or reject the proposal, it was proposed that the one-page summary be considered by the Committee in its closed meeting, without discussion in the open sessions. Proposals for which issues had been identified would be considered in the usual way. The public posting of all proposals (in the form of WHO/BS documents) would be maintained. If successful, the pilot study could be expanded to cover all tracks, and the Committee was informed that similar proposals were being explored by the WHO network of collaborating centres for blood products and in vitro diagnostics (see section 2.2.3 below).

2.2.3 Report from the WHO network of collaborating centres for blood products and in vitro diagnostics

Since 2007 the network has held biennial 2-day meetings, with core delegates attending in person and other stakeholders joining via teleconference facilities. At the last meeting, held at the NIBSC in July 2015, it had been decided that more frequent meetings were required and it was agreed that in the years between the biennial meetings, two half-day WebEx meetings might be helpful. Two such meetings were therefore held in 2016 to review progress on current projects, discuss potential issues in advance of making presentations to the
Committee and to discuss new project areas. Dr Morris summarized the agenda topics discussed, which in the case of two items – namely the dengue virus (DENV) RNA reference reagent and the anti-CMV collaborative studies – had led to a re-evaluation of data and alteration of the final proposals made to the Committee.

Comments from users of current standards, which are not normally considered at routine meetings of the Committee, had also been discussed. Dr Morris gave the example of the B19 DNA standard, explaining that some users of one specific test (Roche DPX) had reported under-quantification of the International Standard. On further investigation a single base-pair mismatch was identified in the current standard compared to that of the manufacturer’s primers/probes. Despite calls for the revision of the assigned IU of the standard, it was agreed by the network, and by the delegates of the annual Standardisation of Genome Amplification Techniques (group) (SoGAT) meeting, that the unit should remain. Difficulties were also reported in reconstituting the current HCV standard. This standard had been produced from a donation that on freeze-thawing showed signs of insoluble lipid particles. These also appeared following reconstitution. Users have now been advised to ensure thorough mixing immediately prior to use to ensure that particulate matter is also incorporated into the extraction.

Dr Morris reported that the WebEx meetings were considered overall to have been a valuable addition to the network meeting calendar, allowing pertinent issues to drive the agenda in a timely way while assisting in the streamlining of the main process of standards approval by the Committee.

During discussion, it was suggested that where a project is considered straightforward, a one-page summary could be submitted to the Committee for its review and endorsement without requirement for a presentation of the project itself. Where a project was considered more complex and issues remained to be addressed, a two-page proposal could be submitted and the Committee could also seek further clarification in the form of a presentation. However, it was also made clear that the work of the WHO CCs could not supplant the role of the Committee in establishing standards and endorsing new work. The Committee thanked Dr Morris for her presentation and agreed that network meetings of the kind outlined should be pursued and further explored as a means of facilitating the work of the Committee.

2.3 Feedback from custodian laboratories

2.3.1 Developments and scientific issues highlighted by custodians of WHO biological reference preparations

The Committee was informed of recent developments and issues identified by the following custodians of WHO biological reference preparations.
National Institute for Biological Standards and Control (NIBSC), Potters Bar, England

Dr Christian Schneider, the new Director of the NIBSC, presented a brief overview of the scientific activities of the NIBSC and then drew attention to a number of current issues in the field of biological standards.

He first drew attention to an issue raised at the last meeting of the Committee, namely the increasing difficulty experienced in publishing data from collaborative studies of candidate biological reference materials in peer-reviewed journals in cases where those data had already been presented to the Committee, incorporated into its report and published as part of the WHO Technical Report Series. Dr Schneider considered that publishing data in the public domain was crucial in both promoting the visibility of individual studies and enhancing appreciation of the highly technical nature of biological standardization in general. He urged WHO and the Committee to explore ways of ensuring that data are published in both domains in order to convey standardization messages and their importance to as broad an audience as possible. Dr Schneider gave the example of the European Medicines Agency (EMA) which had published a reflection paper on the management of clinical risks deriving from insertional mutagenesis both in a peer-reviewed scientific journal and on the EMA website. This had been achieved by the two parties working together within specified timelines.

Dr Schneider then raised the issue of biotherapeutics, which he considered had revolutionized modern medicine in numerous ways. The field was now a major component of pharmaceutical business and was still expanding on a global scale, especially with regard to SBPs. There was an essential need to maintain the high standards of quality, safety and efficacy to which these complex medicines are produced and licensed. Such standards support innovation and facilitate global access to new medicines by supporting the introduction of high-quality SBPs. As biotherapeutics are currently not well covered by existing WHO CC networks there was a case to be made for establishing a new dedicated group.

Dr Schneider also addressed the issue of alternative fill formats for biological standards; in particular, alternatives to the existing formats for freeze-dried preparations which had several recognized limitations. Flame-sealed glass ampoules have been the preferred format for many years but these are ill-suited for some materials – such as low fill volumes of DNA and viral marker standards for nucleic acid amplification technique (NAT)-based assays. Alternative approaches that do not use freeze-drying (as well as smaller freeze-drying formats using 96-well plates with lyocluster caps) are being explored at the NIBSC to deal with smaller volume formats and could be suitable for wider use if successfully established. Work on seal integrity and moisture content was ongoing but not straightforward.
The Committee noted Dr Schneider’s comments and thanked him for an interesting, practical and forward-looking overview.

**European Directorate for the Quality of Medicines &
HealthCare (EDQM), Strasbourg, France**

Dr Karl-Heinz Buchheit outlined a number of recent EDQM activity areas in biological standardization, including the European Pharmacopoeia, international standards for antibiotics and the biological standardization programme, in which WHO has Observer status.

The Committee was reminded that EDQM is the custodian centre for international standards for antibiotics – a responsibility it assumed from the NIBSC in 2006. Twenty-three international standards for old antibiotics are available and there had been eight replacement batches established between 2006 and 2015. There was no requirement for any replacement batches this year and no issues had been identified since the last meeting of the Committee. Eight of the above antibiotics are on the WHO Model List of Essential Medicines and the international standards are indispensable in the calibration of regional and in-house standards. Although there is no cost recovery for these standards (and an average of 10–20 vials being distributed annually) the work is in line with WHA67.25 and EDQM is happy to continue acting in this capacity.

Dr Buchheit then discussed a number of recent activities of the EDQM biological standardization programme – the goal of which is to establish European Pharmacopoeia biological reference preparations and to standardize methods. The programme of work is established by a Steering Committee and, whenever possible, collaboration and common projects were undertaken with WHO and non-European partners. Current EDQM projects on human and veterinary vaccines, blood-derived products and contaminants, and biotechnology products were briefly described. Dr Buchheit reiterated that across its programme of work, the development of alternatives to animal experiments remained a major EDQM commitment in line with European Union directives. WHO was once again strongly urged to consider the incorporation of the 3Rs principles (Replacement, Reduction, Refinement) into its written standards and other guidance where appropriate.

Specific projects of potential interest to the Committee included work on rabies vaccines in which the intention was to replace the current in vivo NIH potency test with an ELISA assay (see also section 2.1.2 above). A G-protein-based ELISA for non-adjuvanted rabies vaccines for human use was reported to be suitable for all European and some non-European vaccines. This project, initiated by the European Partnership for Alternative Approaches to Animal Testing, had now been handed over to the EDQM biological standardization programme for further validation and inclusion into the European Pharmacopoeia. As the
assay is based on two commercial monoclonal antibodies a number of issues concerning licensing agreements will need to be resolved with manufacturers.

Dr Buchheit reminded the Committee that one of the main outcomes of a conference held in 2015 by the International Alliance for Biological Standardization on the 3Rs concept was a formal request to WHO to initiate steps to delete the abnormal toxicity test (sometimes called General Safety or Innocuity test) from all WHO Recommendations, Guidelines and other guidance documents. Recently, the European Pharmacopoeia Expert Group on Vaccines agreed to the deletion of the abnormal toxicity test from all European Pharmacopoeia monographs.

The Committee noted that deletion of the abnormal toxicity test from the European Pharmacopoeia will provide additional impetus for the global elimination of this test, although this may take some time to achieve. It was pointed out that the US Code of Federal Register had already taken this step and no longer required the General Safety test. Furthermore, the recently revised WHO Recommendations for HPV vaccines adopted by the Committee in 2015 note in small print that some countries no longer require this test. Further consideration of this issue will be required by the Committee at its future meetings.

The Committee also encouraged WHO to reflect on how to maintain the IU for international standards currently calibrated using in vivo assays once these assays are replaced by quite different in vitro assays. Examples would include the assigned IU for the potency of diphtheria and tetanus vaccines. As the number of laboratories performing in vivo assays is expected to be reduced, technical competency in performing the in vivo test may be lost. Any associated need for new reference standards should be considered in good time in light of the time required to calibrate and establish international reference preparations.

Paul-Ehrlich-Institut (PEI), Langen, Germany

Dr Heidi Meyer reported on the activities of the two PEI WHO CCs and discussed scientific issues of interest. There had also been two organizational changes with Dr Dorothea Stahl succeeding Professor Rainer Seitz as Head of the WHO CC for Quality Assurance of Blood Products and In Vitro Diagnostic Devices, and Dr Meyer succeeding Dr Michal Pfleiderer as Head of the WHO Collaborating Centre for the Standardization and Evaluation of Vaccines.

PEI WHO CC activities of interest included supporting the establishment of WHO reference materials through participation in collaborative studies, developing new standards – such as a new chikungunya virus (CHIKV) international standard – and contributing to the development of WHO technical documents. The PEI also provided support for the WHO prequalification programme for IVDs and vaccines through dossier review, quality control
testing and participation in on-site inspections. The PEI was also involved in activities related to: (a) the ongoing ZIKV public health emergency, including the development of a WHO international standard for ZIKV RNA for NAT-based assays (see section 7.1.1 below); (b) the WHO emergency use assessment and listing (EUAL) procedure for ZIKV diagnostics; and (c) the safe use of plasma-derived medicinal products (PDMPs). With regard to the latter, the bloodborne transmission of ZIKV had raised the issue of PDMP safety and PEI researchers therefore investigated the effectiveness of the most commonly used virus-reduction/inactivation methodologies. Pasteurization and solvent/detergent treatment both led to the rapid inactivation of ZIKV. Retentive virus filtration was also shown to be effective, with ZIKV infectivity removed by filters with nominal pore sizes ≤ 40 nm. Dr Meyer also reported that immunoglobulins sourced in Europe or the USA had failed to neutralize ZIKV.

Dr Meyer acknowledged the excellent cooperation of its partners in the two WHO CC networks in which it was involved, and highlighted PEI support for the implementation of documents issued by the BRN. Topics highlighted for future consideration by the Committee included: (a) considerations in advancing the scientific evaluation of convalescent plasma collection and use beyond the Ebola outbreak; (b) implementation of the assessment criteria for national blood regulatory systems (which had already been applied in Ghana); (c) assessment of the significance of hepatitis E virus for blood safety; (d) the quality and safety standards for haematopoietic stem cells; and (e) the role of the Committee in ensuring sustainable vaccine supply (see section 2.4.4 below). The PEI also supported a proposal to facilitate the work of the Committee by streamlining the process for evaluating new projects in the area of vaccines and biotherapeutics, including the establishment of a process for project prioritization.

It was also reported that the PEI was providing support for two health-system strengthening and capacity-building efforts. The first involved the establishment of bilateral interactions with Ghana and Liberia to strengthen their blood regulatory systems, which had included the development of a partnership with the Ghanaian Food and Drugs Authority and the initiation of a twinning project with Liberia. The second activity had been developed in light of the recent Ebola epidemic and as a consequence of the G7 summit in June 2015. The German Ministry of Health had agreed to fund two PEI projects to facilitate access to medical countermeasures in low- and middle-income countries. These focused on the availability, safety and quality of blood and blood products, and on regulatory training in the evaluation and approval of clinical trials of vaccines and biomedicines. Dedicated PEI personnel would be assigned to these projects, which are expected to deliver short-, medium- and long-term outcomes.

The Committee thanked Dr Meyer for her presentation and raised the question of the suitability of current ZIKV polymerase chain reaction (PCR) assays for Asian strains. Dr Meyer referred this point to the later presentation on
the proposed establishment of a WHO international standard for ZIKV RNA for NAT-based assays.

**Center for Biologics Evaluation and Research (CBER), Silver Spring, MD, the USA**

Dr Jay Epstein informed the Committee of recent and current developments at CBER, including the appointment of Dr Peter Marks as Director following the retirement of Dr Karen Midthun. Organizational restructuring had resulted in three product line offices: the Office of Blood Research and Review, the Office of Vaccines Research and Review and the Office of Tissues and Advanced Therapies. Products regulated by the latter will include all purified and recombinant versions of therapeutic proteins for use in haematology, as well as antivenins. CBER had also been redesignated as a WHO CC for the period 2016–2020 and an umbrella agreement had been put in place with WHO on vaccines, blood and blood products, relevant IVDs and cell and tissue therapies. This agreement will support the development of norms and standards, regulatory systems strengthening, the WHO prequalification programme, product safety and vigilance, and regulatory science in order to increase access to safe and effective biological products.

Dr Epstein outlined new United States Food and Drug Administration NAT-testing policies with regards to blood donations (which are intended to reduce the transfusion risk from ZIKV) as well as revised donor-deferral criteria for HIV risk which permit men who have sex with men to donate blood under certain circumstances. A Transfusion Transmissible Infections Monitoring System had also been established to assess infectious disease risks based on marker rates, incidence and risk factors in blood donors. Dr Epstein also described CBER’s global involvement with other WHO CCs in the standardization of plasma-derived coagulation factors, and in the development of reference reagents and panels for the standardization of assays for transfusion-transmitted infectious agents such as DENV, CHIKV, West Nile virus, HIV and ZIKV, as well as for Babesia microti antibodies.

Recent CBER activities related to vaccines included participation in an international study to evaluate new methods for measuring the potency of inactivated influenza virus vaccines compared with the traditional single radial immunodiffusion assay. Following completion, study results indicated that despite the general feasibility of all alternative assays, additional studies would be needed to identify the most promising ones for further development and possible implementation. A second collaborative study was now under development and scheduled for early 2017. An international study to evaluate the inter-laboratory variability of influenza virus serological assays had also been conducted. Coordinated by the Consortium for the Standardization of Influenza Seroepidemiology, this study compared the inter-laboratory variability of the standardized haemagglutination inhibition (HI) and microneutralization
(MN) assay protocols, with CBER performing the HI, MN and pseudotype neutralization assays. Twenty laboratories provided data, and statistical analysis of the results was ongoing at the NIBSC. Preparations for dealing with new inactivated poliomyelitis vaccines (IPVs) based on Sabin vaccine strains instead of conventional IPVs were also under way. Conventional IPV and Sabin IPV differ antigenically and the current D-antigen potency ELISAs for conventional IPV may not be suitable for Sabin IPV vaccines. A PATH-sponsored collaboration with the Lankenau Institute of Medical Research to evaluate human monoclonal antibodies in new D-antigen potency ELISAs has therefore been initiated. In addition, CBER is supporting the development of new vaccine technologies by participating in studies led by the Advanced Virus Detection Technologies Interest Group (which is supported by the Parenteral Drug Association) to develop next-generation sequencing controls for detecting adventitious viruses in biological substances. CBER was responsible for preparing the large well-characterized virus stocks to be used as controls in spiking studies for the evaluation and standardization of next-generation sequencing platforms.

The Committee thanked Dr Epstein for his presentation and asked whether the standardization materials being developed for next-generation sequencing would be applicable across all available platforms. Dr Epstein confirmed that this was the intention.

2.4 Cross-cutting activities of other WHO committees and groups

2.4.1 WHO Global Model Regulatory Framework for Medical Devices including IVD medical devices

The Committee was informed of progress in the development of this framework and invited to comment on the current draft (QAS/16.664/rev2), particularly on those aspects concerning biological substances. This framework was regarded as part of the response to resolution WHA67.20 on regulatory systems strengthening for medical products in general, including medical devices and diagnostics. A survey of the regulation of medical devices globally had shown that only 58% of WHO Member States had any such regulations in place, with low-income countries often having no regulations at all. The target audience of the proposed document was countries with no or limited regulatory frameworks in place but that wished to improve upon the situation. Since the implementation of regulations would require political commitment, the document was also specifically aimed at the legislative, executive and regulatory branches of government. Two rounds of public consultations had resulted in over 900 comments being received, indicating a high level of engagement with the aims of the document and the need for such guidance.

The Committee raised a number of points concerning the draft document, including the need for a more appropriate classification of medical devices,
and highlighted the importance of emphasizing the responsibilities of device manufacturers and users. In addition, medical devices that affected the safe and effective use of a medicine (such as a companion diagnostic) or those used in the manufacture of a biological substance (such as an apheresis machine or pathogen-inactivation technology) require special considerations. These include careful assessment of the level of risk of the device and of the potential need for clinical studies of the related drug or biological substance as part of device validation. The difference between IVDs and other medical devices was debated extensively, with the view of some Committee members being that examples of each type of device should be provided in the document. However, it was brought to the Committee's attention that country-specific requirements may contradict each other and that too many examples would lead to confusion. Variations in terminology were also discussed and it was pointed out that, in the field of blood regulation, the terms validation and verification have precise meanings and dictate specific courses of action. Instances where these terms had been used interchangeably in the draft document should be addressed.

The Committee was informed that document QAS/16.664/rev2 was currently under consideration for endorsement by the WHO Expert Committee on Specifications for Pharmaceutical Preparations (ECSPP). The Committee asked that the above points and others raised during its discussions be transmitted to the ECSPP and taken into consideration during its review. The Committee was subsequently informed that the ECSPP had taken note of the points raised and amendments to the text made. Following review of the proposed amendments and further minor alterations, the Committee indicated its agreement with the revised text. The Committee also took note of plans to organize regional training workshops to promote the implementation of this guidance.

The ECSPP adopted the amended WHO Global Model Regulatory Framework for Medical Devices including IVD medical devices, and agreed that this much needed guidance be annexed to its report.5

2.4.2 Report of a WHO ad hoc consultation on International Nonproprietary Names for biological substances

The Committee was informed that in 2002 only 18% of all INN applications were for biological substances. By 2016, this figure had risen to 50% of all applications – with 45% of such applications being for monoclonal antibodies.

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This significantly increased level of activity in the biological/biotechnological sector of the pharmaceutical industry was likely to be a pointer to future trends in the work of the Committee.

The Committee was further informed that meetings have been held at intervals since 2002 to address the general and specific aspects of the nomenclature used for biological substances, which now included gene therapies, cell therapies, monoclonal antibodies and SBPs. A review of this area: *International nonproprietary names (INN) for biological and biotechnological substances* was now available as a WHO working document (http://www.who.int/medicines/services/inn/BioReview2016.pdf).

The objectives of the 2016 ad hoc consultation were to review the current INN approach to naming specific classes of biological substances and to discuss whether existing policies and established nomenclature were applicable to emerging biological medicines. Recommendations arising from this ad hoc consultation would be considered at the 63rd INN Consultation. Areas of discussion included cell and advanced biotherapies, vaccine-like substances, monoclonal antibodies and therapeutic proteins – the latter including fused and conjugated substances as well as glycosylated biotherapeutics and monoclonal antibodies. There are currently three sub-schemes for the nomenclature of advanced therapies: (a) two-word names for existing gene therapies; (b) one-word names for cell therapies; and (c) two-word names for genetically modified cells.

It was further noted that vaccines had generally been excluded from INN assignment and that the Committee had a system in place for assigning international names to prophylactic vaccines for infectious diseases. Immunization communities were familiar with the international names so assigned and this was an important consideration since the major use of prophylactic vaccines was by public health bodies. The INN programme had also not assigned INN to defined recombinant proteins used as active substances in vaccines but could do so on request. Substances for anticancer immunotherapy (so-called cancer vaccines) could be handled within existing INN policies, and INN could also be assigned to engineered live viruses and bacteria.

### Monographs on capreomycin sulfate and capreomycin for injection in *The International Pharmacopoeia*

Antibiotics produced by fermentation often consist of complex mixtures of structurally related components with different activities. Microbiological methods have historically been used to quantify the total activity of these mixtures. As knowledge of their structure and composition increased, a transition from microbiological to physicochemical assays became possible. The latter assays are considered to be more discriminative and easier to perform in quality control.
laboratories. Microbiological assays, on the other hand, measure the total activity of antibiotics, integrating all moieties that contribute to the antibiotic effect.

At the 2009 meeting of the ECSPP the decision was taken to replace microbiological assays, where possible and appropriate, by physicochemical methods (in particular, chromatographic methods) in *The International Pharmacopoeia*. The Committee was informed that this transition from microbiological to physicochemical assays had been largely completed for single-component antibiotics. However, for multicomponent compounds the use of physicochemical methods often poses a challenge as the total antimicrobiological response of these substances is not only a function of their concentration but also of their composition.

Capreomycin is a mixture of four structurally related compounds (capreomycin IA, IB, IIA and IIB) with different individual activities. In *The International Pharmacopoeia* monograph on capreomycin sulfate the active substance is defined on a mass basis, whereas capreomycin is assayed using high-performance liquid chromatography which discriminates between IA, IB, IIA and IIB. In 1969, the activities of these four main components were determined by a manufacturer of capreomycin. The results indicated that there was a significant difference between the activities of components IA and IB and between I and II. The United States, Indian and Chinese pharmacopoeias all have similar requirements regarding the composition of capreomycin sulfate but in these pharmacopoeias the capreomycin content is determined using microbiological methods. Although *The International Pharmacopoeia* limits capreomycin II content to a maximum of 10%, the ratio between capreomycin IA and IB is not defined. There is therefore at least a theoretical possibility that capreomycin samples with significant differences in their IA and IB concentrations comply with the requirements of *The International Pharmacopoeia*, but may be determined as sub- or super-potent when analysed according the United States, Indian or Chinese pharmacopoeias.

The issue had been referred to the Committee and to the ECSPP for advice as to whether a revision of the current International Pharmacopoeia monograph is recommended. Prior to such an amendment, it was proposed that the secretariat of *The International Pharmacopoeia* should contact manufacturers of capreomycin sulfate and powders for injection to request information regarding the composition of their products – as determined for example by the chromatographic methods described in *The International Pharmacopoeia*. This information would then be evaluated by a group of experts to see whether amendments to the monograph were appropriate. Such amendments could include the provision of a specification for the ratio between capreomycin IA and IB and/or the establishment of a correlation between the results of the
chromatographic and microbiological assays described in the above-mentioned pharmacopoeias.

Following discussion and clarification of various points, the Committee agreed that more information was needed before any decision could be made on revising *The International Pharmacopoeia*.

2.4.4 **Yellow fever vaccine shortages and outbreak response**

The Committee was informed that a recent YF outbreak in Africa had greatly increased the demand for YF vaccine, exhausting the global stockpile and putting routine immunization in endemic regions at risk. There was now a shortage of such vaccine which could worsen if additional immunization campaigns were required on a large scale. The risk of YF is also increasing, especially in metropolitan areas with growing human population densities and urban environments that provide the mosquito vector, *Aedes aegypti*, with an ideal habitat. Increased urbanization, in particular among poorer groups of the population without access to a clean water supply, and increased international travel, could also potentially lead to the increased spread of the disease.

Potential strategies to improve YF vaccine supply include extension of vaccine shelf-life, increasing the number of available vaccine doses by immunizing with a fractional volume (0.1 ml instead of 0.5 ml) as an emergency response and, in the longer term, the development of more flexible production technologies based on cell cultures.

In response to the serious YF outbreak and acute vaccine shortages, an emergency immunization campaign was conducted in Kinshasa, Democratic Republic of the Congo, and along the border with Angola in August 2016 using the fractional volume given by the usual route. This campaign was conducted following a recommendation from WHO, based on WHO SAGE advice on the use of the fractional volume as a dose-sparing measure. It was recognized that the recommendation constituted an off-label use of the vaccine and that a fractional volume should be used only as an exceptional response where there was a large disease outbreak and vaccine shortage – and not in routine immunization.

The WHO SAGE advice was based on limited clinical studies of fractional dose administration. While the data support the recommendation, important data gaps remained – such as on vaccination responses among children and immunocompromised populations to fractional doses and on the duration of immunity. The Committee was reminded that there were currently only four WHO-prequalified YF vaccines (all live-attenuated) which differ in their properties. As batch records for these vaccines have shown excess potency (albeit with some variability) there was potential scope for reducing the dose required to achieve an acceptable seroconversion threshold. However, the available data are based mainly on product from one manufacturer and the long-term duration of immunity beyond one year following a fractional dose
of vaccine is unknown. There was therefore a need to follow up on the recent emergency campaign in Africa.

The Committee supported the use of fractional volumes of YF vaccine in emergency situations. However, discussion highlighted the need for caution in extrapolating data on one vaccine to all YF vaccines given the known differences in the manufacturing and release titres of the different vaccines, and the need for good supporting clinical data. The importance of assay standardization when comparing products from different manufacturers was emphasized. The Committee also noted the need to address relevant regulatory aspects, which could include updating the vaccine potency recommendations given in current WHO guidance. As a medium-term strategy to increase vaccine supply, exploration of the introduction of an upper potency limit might be considered by manufacturers and NRAs. It was recalled that previous Committee discussions had taken place on an upper potency specification from a safety perspective but such a specification had not been introduced. The Committee was informed that the issues of YF vaccine supply and the use of fractional volumes and doses were also due to be considered at a concurrent WHO SAGE meeting.

2.4.5 Vaccines for public health emergencies

The Committee was informed of a new WHO initiative – the Blueprint for Research and Development: Responding to Public Health Emergencies of International Concern (R&D Blueprint) – developed in light of lessons learnt from the Ebola epidemic of 2014–2016 and similar previous public health emergencies. Following a request from its Member States, WHO had convened a broad coalition to develop the R&D Blueprint as a sustainable platform for accelerating research and development “in epidemics or health emergency situations where there are no, or insufficient, preventive, and curative solutions, taking into account other relevant work streams within WHO”. The R&D Blueprint was subsequently endorsed by the sixty-ninth World Health Assembly in May 2016.

The overarching vision of the R&D Blueprint is that the research and development response to public health emergencies of international concern caused by emerging pathogens will be faster and more effective than ever before, and that continuous efforts will be made to accelerate the results of research

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adapted to the scientific, logistic and social challenges specific to epidemics. Four principles underpin the project: (a) an inclusive process with a clear mandate and milestones; (b) building on the efforts of others; (c) a collaborative effort with affected Member States at the core of public health emergency responses; and (d) a process driven by scientific knowledge. The R&D Blueprint aims to reduce the time between the declaration of an international public health emergency and the availability of effective tests, vaccines and medicines that can be used to save lives and avert a crisis.

The R&D Blueprint has three work streams: (a) improving coordination and fostering an enabling environment – for example, by building effective governance and coordination frameworks; (b) accelerating the research and development processes – for example, by assessing epidemic threats and defining priority pathogens – and then in a second step by developing R&D roadmaps to accelerate evaluation of diagnostics, therapeutics and vaccines; and (c) developing new norms and standards adapted to the epidemic context – for example, supporting expansion of capacity to implement adequate study designs, developing guidance and tools to frame collaborations and exchanges, and anticipating the evidence needed to inform regulatory review and policy development. With regard to research strategy aspects, the Committee was informed that a recent call for platform technologies for vaccine production, medicines and diagnostics had been made by WHO. Forty-five proposals were subsequently submitted, of which six were selected for presentation to potential funders.

Priority pathogens and diseases listed in 2016 were: Crimean Congo haemorrhagic fever, filovirus disease (EBOV and Marburg virus diseases), Lassa fever, highly pathogenic emerging coronaviruses relevant to humans (MERS-CoV and severe acute respiratory syndrome), Nipah, Rift Valley fever and ZIKV. Two other diseases listed as serious are chikungunya and severe fever with thrombocytopenia syndrome. The list will be reviewed annually or when a new disease emerges. It was intended that R&D roadmaps for all priority pathogens would be developed to guide the R&D response to large-scale public health challenges, with such a roadmap having been developed for MERS-CoV as a test case. The R&D Blueprint was also intended to link together other international efforts, such as The Coalition for Epidemic Product Innovation established following the Annual Meeting of the World Economic Forum in Davos in January 2016.

The Committee welcomed this WHO initiative and noted that considerable efforts were already under way in the area of norms and standards. Guidelines on the quality, safety and efficacy of Ebola vaccines were being developed (see section 3.5.6 below) – the principles of which could inform the evaluation of similar vaccines against other priority pathogens. In addition, the WHO CCs at NIBSC and PEI had developed international reference reagents
for pathogens with epidemic potential, including standards for ZIKV RNA and for EBOV antigen and antibodies. However, the Committee also agreed that more remained to be done and there were still many challenges to be resolved. One problem was that of obtaining clinical material from priority pathogens with which to produce international reference materials. Although efforts were now under way to facilitate the sharing of clinical material, sending biological materials across national and state borders, especially convalescent sera and infectious materials, was often problematic. The Committee recommended that WHO should play a role in facilitating procedures and ensuring that the pathways for providing material to the relevant WHO CC were made clear.

The Committee also raised the possibility of using blood donations as a source material for research. Despite challenges in ensuring informed consent, this had been shown to be a viable option – for example, for following virus evolution using next-generation sequencing technologies or for better understanding epidemics with respect to pre-symptomatic patients via screening programmes. The possibility of using material from deferred blood donations to prepare reagents might also be considered.

The question was raised as to whether the use of convalescent plasma as a treatment option could realistically benefit from a platform approach given that in the case of the Ebola epidemic several issues could not be addressed due to a lack of time and infrastructure. Such issues included determining the ideal time point after infection or during convalescence to procure plasma with high neutralizing titres.
3. International Recommendations, Guidelines and other matters related to the manufacture, quality control and evaluation of biological substances

All WHO Recommendations, Guidelines and guidance documents adopted at the meeting are included in Annex 1, which provides an updated listing of all current WHO Recommendations, Guidelines and other documents related to the manufacture, quality control and evaluation of biological substances used in medicine.

3.1 Biotherapeutics other than blood products

3.1.1 Guidelines on evaluation of monoclonal antibodies as similar biotherapeutic products (SBPs)

Monoclonal antibodies (mAbs) are a major class of rDNA technology-derived biotherapeutic products that have achieved outstanding success in treating many life-threatening and chronic diseases. Some of these targeted therapy products are ranked in the top-10 lists of annual global pharmaceutical revenue successes. As patents and data-protection measures on mAb products have expired, or are nearing expiry, considerable attention has turned towards producing SBPs (also termed “biosimilars”) based upon the approved mAb innovator products with a view to making these products more affordable and globally accessible.

WHO Guidelines on evaluation of similar biotherapeutic products (SBPs) were adopted by the Committee in 2009 and have served well as a basis for setting national requirements for SBPs. These Guidelines provide the scientific principles, including the stepwise approach, for evaluating similarity between an SBP and its reference biotherapeutic product. High similarity at the quality level is regarded as a prerequisite for the use of a tailored nonclinical and clinical data set for licensure. However, because of the structural complexity of mAbs, comparability studies between a candidate biosimilar mAb and a reference product mAb are challenging for both developers and regulators.

In 2014, the World Health Assembly adopted a resolution (WHA67.12) on Access to biotherapeutic products, including similar biotherapeutic products, and ensuring their quality, safety and efficacy. This resolution requested WHO through its Expert Committee on Biological Standardization to update the 2009 Guidelines, taking into account technological advances in the characterization of biotherapeutic products, and considering national regulatory needs and capacities. A corresponding request was then made at the 16th ICDRA. In response, WHO organized an informal consultation in 2015 to review the 2009 Guidelines and to consider ways of improving its guidance in this important pharmaceutical sector. Participants included NRAs and national control
laboratories (NCLs) from 26 countries in the six WHO regions, together with
developed and developing country manufacturers’ associations and individual
manufacturers. It was concluded there was no need to revise the overarching
2009 WHO Guidelines since the evaluation principles described still applied
and were valuable in facilitating the convergence of SBP requirements globally.
However, it was also agreed that, because of their complexity, there was a need for
additional WHO guidance on the evaluation of biosimilar mAbs.

Consequently, class-specific guidance on special considerations for the
evaluation of mAbs developed as SBPs was prepared and subjected to global
public consultation. The resulting draft Guidelines (WHO/BS/2016.2290)
covered rDNA-derived biosimilar mAbs, as well as mAb-derived fragments and
Fc fusion proteins, used in the treatment of human diseases. These Guidelines
are intended to be read in conjunction with the existing WHO Guidelines on
the quality, safety and efficacy of biotherapeutic protein products prepared
by recombinant DNA technology and the WHO Guidelines on evaluation of
similar biotherapeutic products (SBPs), and are intended to complement existing
relevant regulatory documents from other bodies.

The Committee reviewed the small number of comments that had
been received during a final round of public consultation. After agreeing
upon a number of further amendments to improve the clarity of the text, the
Committee recommended that the WHO Guidelines be adopted and annexed
to its report (Annex 2).

3.2 Blood products and related substances
3.2.1 Blood regulation activities

The Committee was informed of the outcome of a WHO BRN in-country
assessment of the Ghana Food and Drugs Authority (FDA) performed on
21–22 July 2016 using the BRN NRA assessment criteria compared against a
WHO Regional Office for Africa assessment tool. Ghana has a population of
25 million people and a national blood policy has been in place since 2006, with
the National Blood Service (NBS) Ghana being the responsible entity for blood
donation and transfusion. In 2015, 155,250 blood units were collected; with
54% of the units collected by the NBS coming from voluntary non-remunerated
blood donations, compared with 23% of private blood facility collections. More
than 150 blood facilities exist in Ghana (with a possible total number in excess
of 400) and all but one are hospital based.

The Ghana FDA regulates food, medical devices, medicines and
biologics (including blood and blood components), with blood regulation
having been incorporated into this portfolio only recently. Blood products and
PDMPs have been covered to date by the biologicals regulations, whereas the
regulation of blood itself remains at an early interim stage. Due to the small
workforce currently dedicated to blood at the Ghana FDA (two full-time staff) synergies achieved through interaction with other relevant Ghana FDA units are imperative. In order to understand the status quo, the Ghana FDA had previously visited more than 50 blood facilities across the country.

The in-country assessment identified areas for improvement and led to several recommendations. These included making good use of already existing structures for pharmaceutical regulation and generating synergies with additional departments, such as those responsible for enforcement. However, blood-specific training is also necessary, especially in relation to facility inspection and haemovigilance. For blood-screening IVDs, a reliance on approvals made by other NRAs or via WHO prequalification should be considered. If IVD testing is to be performed at the Ghana FDA then the use of regional specimens for test panels is recommended, rather than simply repeating the tests performed by manufacturers.

The assessment report, and associated recommendations, were well received by the Ghana FDA and were used during negotiations with the Ghana Ministry of Health for further support. Both training (potentially in the form of twinning projects) and funding will be essential in supporting the currently understaffed but highly motivated team at Ghana FDA. WHO plans to follow up with a reassessment after a certain time, but this was dependent upon funding. Other countries that had expressed an interest in undergoing an external WHO BRN assessment were Kenya and Zambia.

The Committee noted the assessment report and requested to be kept informed of future assessments and their outcomes.

3.2.2 Africa Society for Blood Transfusion

The Committee was informed that at present only 20 of 46 WHO African Region countries include PDMPs in their Essential Medicines List, with almost all products currently being imported from abroad. Whole blood or fresh frozen plasma is mostly used in place of PDMPs, which both increases the risk of transfusion-transmitted infections and prolongs treatment. Only South Africa manufactures products through the fractionation of plasma collected in the country, and also exports surplus products to neighbouring countries.

During a presentation made to the Committee on behalf of the Africa Society for Blood Transfusion (ASfBT), an action plan was outlined for possible adoption by WHO as a set of concrete next steps to further advance the WHO Achilles project on improving access to safe blood products through local production and technology transfer in blood establishments. As proposed by the ASfBT, this action plan for Southern Africa aimed to increase the plasma supply and to improve access to PDMPs in Angola, Botswana, Lesotho, Malawi, Mozambique, Namibia, South Africa, Swaziland, Zambia and Zimbabwe.
Although not distributed to the Committee, a specific proposal to WHO for support in this activity had been submitted jointly by the ASfBT, National Bioproducts Institute of South Africa, South African National Blood Service, Western Province Blood Transfusion Service, Medicines Control Council of South Africa and the International Plasma Fractionation Association. The essential elements of the proposed action plan were:

- that WHO should encourage countries in Africa to participate in the ASfBT Step-Wise Accreditation Programme, as a measurable means of helping countries to achieve standards consistent with WHO good manufacturing practice (GMP) standards for blood establishments;
- that the WHO Guidelines now being prepared on the management of blood and blood components as essential medicines should be used by the ASfBT to help educate and train blood establishment personnel and blood regulators in Africa;
- that WHO should provide the ASfBT with full access to its training materials on the quality production of blood;
- that WHO should routinely consider the ASfBT in global and regional meetings in which the agenda impacts on blood safety and availability in Africa.

The Committee was asked to provide a statement endorsing all of these initiatives as actions appropriately designed to help meet the long-term goals of self-sufficiency and regional collaboration towards ensuring the availability of plasma derivatives as essential medicines.

The Committee acknowledged that this ASfBT initiative was clearly constructive and accorded very well with the objectives of the Achilles project. However, it was not clear what it was that was specifically to be endorsed, as normally there would be a specific plan proposed, which included cost calculations. In addition, the element of accreditation had previously been discussed by the Committee and was considered to be controversial, particularly because of the different levels to be evaluated. Nevertheless, a consensus was reached on the following statement of support for this initiative:

The WHO Expert Committee on Biological Standardization agrees that there is a need for WHO to establish an Action Plan to assure continued progress of the Achilles Project. The Committee finds that the elements of the ASfBT proposed Action Plan to define next steps for the Achilles Project are well aligned with the goals of that project and encourages WHO to adopt the plan subject to its normal review of external cooperative engagements. The Committee encourages WHO to support development of, and engagement with, similar initiatives in other WHO regions.
3.2.3 Guidelines on management of blood and blood components as essential medicines

The development history of these WHO Guidelines was briefly summarized for the Committee. Following its initial drafting, early consultations with BRN members and subsequently with several members of the Committee were held. After consolidation of the comments received, a revised draft (WHO/BS/2016.2285) was released for public consultation between July and September 2016. Final consolidation of the document was then conducted via teleconferences and also at the BRN face-to-face meeting in October 2016.

The topics covered in the proposed Guidelines were presented and the list of organizations and individuals who had submitted comments was provided. Of the 236 comments received, almost 50% had been submitted by the European Blood Alliance, with other comments being received from a range of different parties. Some of these comments reflected perceived differences between the contents of the Guidelines and blood regulation practices in Europe. Clarification was given that the Guidelines were primarily intended to support countries lacking a highly developed blood regulatory system.

A number of comments received during the public consultation had also led to changes in the text. For example, the inclusion of explicit reference to the principles of biomedical ethics, and an exclusive focus on the requirements for blood and blood components only, and not for the plasma collected in excess of transfusion needs and used as a source material for fractionation. As the latter is not considered an essential medicine, such considerations were perceived to lie outside the scope of the Guidelines. Other comments were not accepted, for example in relation to making a recommendation that NRAs should be tasked with monitoring the supply of blood and dealing with shortages.

During discussion, the distinction between current good manufacturing practice (cGMP) as used for small-molecule drugs and good preparation practice (GPP) as used for blood components was raised. As use of the latter term in this context was in line with other relevant resources and with European Union terminology, its addition to the Guidelines was accepted. Other discussion topics raised included the requirement for obtaining specific consent for research, and the possibility of using accreditation and monitoring systems instead of NRAs for blood systems. With respect to the latter, it was noted that governmental NRAs are considered essential for the oversight of blood establishments, whereas accreditation, despite its potential in enhancing the quality of blood establishments, was generally voluntary and should only be viewed as a supplemental system. Although there was also some discussion of the concept of national self-sufficiency margins defined in terms of annual blood units per population size, it was decided that all issues pertaining to blood supply lie outside the scope of the Guidelines.
Following incorporation of the comments made the Committee recommended that the WHO Guidelines be adopted and annexed to its report (Annex 3).

### 3.2.4 Guidelines on estimation of residual risk of HIV, HBV or HCV infections via cellular blood components and plasma

During the initial phase of the Achilles Project the development of WHO guidance on residual risk estimation had been requested by blood transfusion services in a number of low- and middle-income countries, and a proposal to develop such guidance had subsequently been endorsed by the Committee in 2012. The goal of these WHO Guidelines is to define the impact of screening algorithms on blood safety in order to be able to analyse the cost–benefit characteristics of different testing algorithms, primarily in low- and middle-income countries. The Guidelines should also allow for risk estimations to be made on less-detailed databases than are usually available in more highly developed blood regulatory systems that employ computerized data-management systems.

Following initial development of a first draft by an international working group and subsequent additional inputs by experts in the field, the document was presented and discussed at the 2015 meetings of the Committee and the BRN. Further inputs were also obtained during workshops held in the WHO African Region and the WHO Eastern Mediterranean Region, and during several scientific conferences. A revised document (WHO/BS/2016.2283) was posted on the WHO Biologics website for public consultation between August and September 2016.

The Committee was presented with the current version of the Guidelines and informed of the comments received from a variety of stakeholders in the blood field, including CBER, EDQM, the European Blood Alliance, the International Plasma Fractionation Association, the International Society of Blood Transfusion, PEI, the Plasma Protein Therapeutics Association and the WHO IVD prequalification programme. In addition, the Recipient Epidemiology and Donor Evaluation Study (REDS) III group had comprehensively commented upon the proposed estimation model and provided comparisons with other estimation methods. The comparison results confirmed that the model proposed in the Guidelines can provide similar results to the other systems, especially in settings in which less detailed data were available. However, where more detailed data were available, some of the other methods would be more accurate. The comments received led to a number of significant changes to the guideline text, which were presented to the Committee. Such changes included the addition of text on the risk of HBV transmission via cellular blood components arising from occult HBV infection, a statement on the limitations of the approach taken and provision of simplified formulae for calculation.
The Committee discussed and accepted the proposed revisions to the document, and recommended that the WHO Guidelines be adopted and annexed to its report (Annex 4).

3.2.5 **WHO assessment of antivenoms**

Snake-bites have a significant global impact on health with an estimated 5 million people bitten by snakes every year, resulting in around 100,000 deaths and leaving 400,000 people permanently disabled or disfigured. Currently, antivenoms are the only effective therapy. These immunoglobulin preparations are manufactured from equine (or ovine) plasma and can be either monospecific to individual snake species or polyspecific. Against a backdrop of regulatory deficiencies in affected regions, falling numbers of antivenom producers, increasing fragility of the remaining production systems and the 2014 announcement by Sanofi Pasteur of a production stop for “Fav-Afrique” due to economic reasons, the inappropriate marketing of products is taking place.

In a 2015 editorial *The Lancet* highlighted snake-bite envenoming as a neglected tropical disease and urged the world to increase antivenom production. WHO took the lead by calling on manufacturers to propose antivenoms for WHO assessment – the first round of which took place in 2016. This initiative built on previous WHO efforts, such as the inclusion of antivenoms in its 2007 Model List of Essential Medicines, and adoption of the WHO Guidelines for the production, control and regulation of snake antivenom immunoglobulins by the Committee in 2008. In addition, WHO developed a database, summarizing the distribution of snake species and listing corresponding antivenoms and their manufacturers. Intended to allow, in the case of a snake-bite, for the rapid identification of snakes endemic to that region and of the respective antivenoms and their manufacturers, this database is outdated and requires revision.

The minimal data requirements for the 2016 WHO antivenom assessment were based on the 2008 WHO Guidelines for the production, control and regulation of snake antivenom immunoglobulins. These requirements ranged from aspects of manufacturing and quality control, to nonclinical and clinical data requirements, and post-marketing activities. The assessment was performed by external experts and regulators from Australia, Brazil, Cuba, France, Kenya, Nigeria and South Africa, as well as WHO staff members, based on a risk–benefit evaluation, and was therefore different to the prequalification scheme for procurement decisions. During the assessment it became clear that several chapters of the 2008 WHO Guidelines on which the assessment requirements had been based required urgent revision (see section 3.2.6 below).

In total, nine product applications were received, with one that targeted Northern Africa not being further considered. The remaining eight products came from manufacturers from Costa Rica, Egypt, India, Mexico, South Africa, Spain and the United Kingdom. It was pointed out that although some
manufacturers appeared to be based in countries with sufficient regulatory experience to handle market authorization without WHO assistance, the target regions for which the products were proposed lacked the necessary regulatory structures, and products may therefore be unregulated. The eight product dossiers were highly variable in terms of depth of information provided and on occasion included less than reliable data. Following assessment, questions were sent to the manufacturers, with the responses received to date now under evaluation. Initial desk review indicates that three to five products may be suitable for listing by WHO. Next steps will include laboratory testing to confirm product features, and possible inspection visits to manufacturers. Inspection decisions will be based upon the respective regulatory environment, for example, on whether prior inspection by a stringent regulatory authority has already been conducted or not.

The Committee discussed the issues raised and acknowledged that WHO capacity to deal with antivenoms and their assessment is constrained as this activity area is significantly under-resourced.

3.2.6 Guidelines for the production, control and regulation of snake antivenom immunoglobulins

The first version of these WHO Guidelines was established in 2008. However, in 2016 it was proposed that, in light of changes in technology, the identification of new snake species, taxonomic name changes and the need to ensure relevance and accuracy, the document be reviewed and revised. A proposed revised document (WHO/BS/2016.2300) was therefore presented to the Committee which included the following major changes:

- updates to the lists of medically important snakes to reflect new species discoveries and nomenclature changes;
- revision of methodologies for the production of venoms to ensure traceability and quality control;
- specific mention of the need for national reference venom collections that are independent of manufacturers;
- recommending of research into new adjuvants;
- updating of text relating to equine viruses;
- greater emphasis on controlling the health of donor animals prior to and during bleeding sessions;
- a strong emphasis placed on the importance of addressing animal welfare and associated ethical issues, including adoption of the 3Rs concept and principles in relation to all animals used in antivenom production and quality control processes;
- redrafted sections on quality control and preclinical testing, and updating of recommendations on stability studies;
- inclusion of antivenomics as an additional preclinical testing methodology that can supplement conventional approaches;
- updated section on clinical assessment, with expanded information on the role of regulatory authorities in antivenom production.

The use of poorly produced low-quality medicines and the distribution of products with inappropriate specificities for a given region are among the major reasons why many snake-bite victims do not seek medical treatment with antivenom. It is intended that the improvements that could be achieved through application of the guidance set out in these WHO Guidelines would address this issue and lead to both the increased use of antivenoms and improved outcomes.

The Committee discussed the issue of the under-utilization of automated equine plasmapheresis and the basis for this. Although cost is an issue, some manufacturers also believe that animals may not tolerate the longer duration of this procedure. On the other hand, improved horse husbandry and increased training of animal handlers in appropriate methods of control could potentially solve this issue, and lead to improvements in husbandry, welfare and plasma production. It was suggested that this topic could be addressed in the section referring to the 3Rs concept and principles. Although the increased use of automated plasmapheresis could prompt a rise in the cost of antivenoms, this was considered to be unlikely given the expected productivity increases and herd size reductions that might follow its implementation.

Improving the quality and safety of antivenoms by addressing current production and quality control weaknesses in relation to the key starting materials would be a crucial step in restoring confidence in antivenom immunotherapy, particularly in resource-poor settings. It was hoped that these WHO Guidelines would contribute to the dissemination and adoption of best practices in the manufacture of snake antivenoms and hyperimmune plasma.

After making a number of minor changes to the text, the Committee recommended that the revised WHO Guidelines be adopted and annexed to its report (Annex 5).

### 3.3 Cellular and gene therapies
#### 3.3.1 Regulation of cell therapy products

The Committee was provided with an outline of current activities in the development and regulation of cell therapy products (CTPs). Despite being at an early stage of development, the field was now very active worldwide, with many products at different stages of clinical evaluation in a number of countries. Technological breakthroughs and research advances had led to increasing
expectations that novel cell-based investigational products will become useful new therapies. In addition to the regulatory oversight of clinical studies and licensing, there was also a demand from medical researchers and manufacturers in this area for proactive scientific and regulatory advice.

The need for WHO international guidelines on CTPs had already been raised in various forums, such as the 16th ICDRA. ICDRA participants had recommended the development of regulatory expertise for CTPs appropriate to the specific nature of these products. As part of this, ICDRA urged WHO to consider developing guidance on the manufacture, and nonclinical and clinical development of CTPs, taking into account existing guidelines and in collaboration with established regulatory authorities. In 2014, the World Health Assembly resolution WHA67.20 on regulatory system strengthening also recognized the need for increased support and guidance in strengthening the capacity to regulate increasingly complex biological products, including somatic-cell therapies. Furthermore, a proposal that WHO define the scientific and regulatory considerations for CTPs as part of promoting their standardization and regulatory convergence had emerged following several international conferences organized by the International Alliance for Biological Standardization. Such issues had also been considered at various international forums, including a Cell Therapy Working Group initiated in 2011 by the International Pharmaceutical Regulatory Forum to address issues related to this emerging product class. As the principal technical agency in the area of biological standardization, WHO is well placed to provide the leadership now required to address a range of key needs in this area.

The Committee recognized that new cell-based medicinal products had great potential in the treatment of various diseases, and that CTPs would become important future public health interventions. Action was therefore needed to promote global-level standardization of both technical and regulatory approaches to these novel biotherapies. However, it was noted that the application of cell therapy is generally undertaken in hospital settings where over-regulation at this stage of development could potentially overburden those developing novel products. The issue of stem cell tourism was also brought up as an issue requiring raised awareness since there was little or no evidence of efficacy but numerous safety concerns associated with such ventures.

It was pointed out that WHO involvement in the early stages of other emerging technologies – for example, rDNA-derived biotherapeutics, including mAbs – had in the past been helpful in their development and regulation. Early guidelines and points to consider produced by the EMA, the United States Food and Drug Administration and WHO on rDNA-derived products had been instrumental in establishing expectations for their quality, safety and efficacy. Although several of the organizations mentioned above were active in developing guidelines on various aspects of cell therapies there was a need for
further harmonization at the global level. It was considered that WHO is in a unique position to provide such guidance, especially to developing countries.

There was a clear consensus within the Committee that global harmonization in the cell therapy field is needed and that WHO should become engaged in this area. The Committee recommended that WHO collaborate with the range of international groups active in cell therapy with the goal of providing a common guideline. A focus should be placed on somatic and not stem cell therapy and should include quality considerations. An agreed definition of cell therapy would also be helpful, along with clarification of whether genetically modified cells should be included or considered under gene therapy. Harmonized definitions and terminology would be particularly helpful for countries that are now setting their own national requirements. It was however considered premature to consider measurement standards for CTPs at this time, and the issue of WHO resources for engaging in the cell therapy field should not be overlooked.

3.3.2 Reference preparations for gene therapy products

The Committee was provided with an outline of the current situation concerning gene therapy, and their advice sought on proposed new work in this area, including the need, if any, for international biological reference preparations. The scope of the discussion was confined to reference preparations for gene therapy products as the possibility of WHO developing written standards to guide regulatory activities in this area would be the subject of a future Committee discussion paper.

The technical aim of initiating work on such reference preparations – which would be developed as WHO biological standards – would be to provide global quality assurance tools to help regulatory authorities and public health authorities make decisions on approving and monitoring gene therapy clinical trials and products both now in high-income countries and, in the future, in low- and middle-income countries. The long-term objective would then be to help improve access to gene therapies of assured quality, safety and efficacy by 2030.

The clinical development of gene therapies – which are at a more advanced stage than cellular therapies – began over 20 years ago. Some of the early clinical trials involved gene therapy vectors which caused leukaemia in some immunodeficient children through unwanted insertional mutagenesis. More recent trials, which used different vectors considered to carry less risk of such adverse events, have led to more encouraging results and to a resurgence of optimism about these products. The first retroviral gene therapy to treat the rare inherited disease adenosine deaminase-deaminase-severe combined immunodeficiency (ADA-SCID) is now licensed in Europe. Tools to minimize future risk and
maximize potential benefits would be beneficial and would help realize the public health benefits of this technology.

Reference preparations would potentially be used by product developers and manufacturers of gene therapies to calibrate the gene dosage in clinical trials and thus assist NRAs in making decisions on clinical trial (and eventually product) approvals. It was also foreseen that hospital diagnostic laboratories would require tools to help monitor gene therapy patients and the outcomes of the therapies. The impact of standardization could be the assurance that the same virus vector is applied under standardized conditions and/or that potential integration into chromosomes is determined in a standardized way. The use of reference preparations would also be expected to improve comparability between clinical studies.

In 2002, WHO established a Clinical Gene Transfer Monitoring Group which met in 2002 and 2003 and then reported to the Committee. At that time there was no consensus on the most appropriate approach to standardizing gene therapy vectors and as a result no WHO reference preparations have yet been established. However, WHO reference preparations have been established for genetic tests and the methodology proposed to develop standards for gene therapy vectors would build upon experience with the genomic reference materials. In 2005, the WHO INN Expert Group established a naming policy for gene therapy products and has, to date, provided names for 24 candidate products. Analysis of the applications being made to the WHO INN programme provides a means of identifying trends in the field and the potential need for biological standards. NIBSC has identified the standardization of gene copy number for candidate products being tested in human clinical trials as a sufficiently mature area of work to potentially benefit from the establishment and maintenance of WHO biological standards. Based on a scientific workshop convened by NIBSC in June 2016 a proposal has been made to develop a lentiviral vector copy number standard (see section 6.1.1 below). Lentiviral vectors are emerging as a platform technology for gene therapies. If other such platform technologies are developed, and if WHO initiates work on reference preparations, then WHO would need to consider establishing appropriate additional biological standards for these other platform technologies. The National Institute for Standards and Technologies (NIST) has also organized workshops on standardization in the field of advanced therapy medicinal products but there is no overlap with potential WHO projects on gene therapy.

The Committee agreed that the development of reference preparations for gene therapy products should be explored further and considered the proposed development of a lentivirus reference to be a good model exercise. It also noted that the need for a corresponding written standard would be considered at a future meeting.
3.4 In vitro diagnostics

3.4.1 Preparation of secondary reference materials for in vitro diagnostic assays designed for infectious disease nucleic acid or antigen detection: calibration to WHO International Standards

It is a principle of standardization that biological samples often cannot be fully characterized by a physicochemical reference method. Moreover, biological assays are heterogeneous and the lack of a reference method does not permit their results to be expressed in absolute values according to the SI system. Instead, WHO International Standards are defined that are expressed in arbitrary International Units (IU). These are the highest order reference materials as defined in ISO 17511: international convention calibrators, and WHO guidance documents limit the use of WHO International Standards to the calibration of other biological materials to minimize the need for their regular replacement. The calibration of a secondary reference material is a complex process and the effort involved in setting up such standards should not be underestimated. In light of the current lack of published guidance on the production and evaluation of secondary standards for use in IVD assays, WHO guidance has been developed on the preparation and calibration of secondary reference materials against WHO International Standards in this area, with a specific focus on in vitro measurement procedures used for the diagnosis, detection and management of infectious diseases.

It was expected that due to the complexity of secondary standard preparation in this field such practical guidance would facilitate appropriate standard design, manufacture and use, and would contribute to the global harmonization and quality assurance of IVDs. The proposal to develop the WHO guidance had been endorsed by the Committee in 2012. Following a series of presentations at a number of SoGAT and WHO CC meetings, a draft text was developed and sent out for comments. Based on the feedback received a second draft was prepared and more widely circulated among relevant parties. Following consideration of this document by the Committee in 2015, incorporation of the points raised, and additional stakeholder discussions the proposed document (WHO/BS/2016.2284) was submitted to the Committee for adoption.

The Committee was informed that the document was intended to provide practical guidance on the preparation and calibration of secondary standards, and would be of use to bodies that prepare and establish secondary standards, IVD manufacturers, and providers of external quality assurance or proficiency testing programmes, as well as to other laboratories using reference materials for NAT-based and serological infectious disease assays. Due to their inherent complexity – for example, inter-individual differences in antibody response and inter-assay design differences – the scope of the document did not cover materials
used for antibody detection methods. The Committee was then provided with an overview of the structure and content of the proposed document.

The Committee considered that the document as a whole provided a better understanding of calibration than part B of the current WHO Recommendations in this area.\(^8\) It was also agreed that the document should be established as a stand-alone WHO manual instead of being integrated into the current WHO Recommendations to allow for more rapid and flexible adaptations to real-world changes than would otherwise be possible in the context of a full revision of the latter.

The Committee recommended that the WHO manual be adopted and annexed to its report (Annex 6). Additionally, the Committee also recommended that part B of the current WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards be revised so that it referred to the new guidance.

3.4.2 **WHO prequalification of in vitro diagnostic devices**

The WHO prequalification programme for IVD devices aims to promote and facilitate access to safe, appropriate and affordable IVDs of good quality in an equitable manner. IVD prequalification is coordinated through the WHO Prequalification (PQ) Team which undertakes the comprehensive assessment of individual IVDs using a standardized procedure to determine if products meet prequalification requirements. Currently the focus is placed on IVDs for priority diseases (including HIV/AIDS, malaria, hepatitis B, hepatitis C, syphilis and HPV). In future, and dependent upon funding, additional diseases such as cholera, dengue, and possibly tuberculosis will be added to the scope of the programme. The provision of an essential diagnostics list is also under discussion which would serve in prioritizing fields of work.

The aim of IVD prequalification is to provide independent technical information on the quality, safety and performance of IVDs, principally to other United Nations agencies but also to WHO Member States and other interested organizations such as MSF. In conjunction with other procurement criteria WHO IVD prequalification status is used to guide IVD procurement. IVD prequalification consists of three components, namely: (a) the review of a product dossier; (b) a performance evaluation, including operational characteristics; and (c) inspection of manufacturing site(s).

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This prequalification process is complex and time intensive, and in a best-case scenario may take 5 months from reception of the dossier to issuing of eligibility – more typically, the procedure takes around one year. Prequalification is then followed by post-qualification activities, including post-marketing surveillance, change reporting and annual reporting. In addition to the prequalification scheme, the WHO EUAL procedure had been established to allow for a rapid response during an emergency situation – where demands cannot be dealt with in the time frame of the usual prequalification procedure. This slimmed-down approach was introduced during the Ebola crisis and involves risk-based decision-making. The EUAL procedure lacks the inspection component and has a shortened laboratory evaluation programme.

The WHO PQ Team also formulates technical specification documents, and has prepared EUAL requirements and additional guidance documents. As discussed at the previous meeting of the Committee, biological reference materials will also be needed for IVD prequalification. These will include both international standards and international reference panels for the purpose of IVD verification and validation studies, performance evaluations and post-marketing surveillance in countries. The materials most needed are for quantitative and qualitative PCR – for example, for detecting HIV, HCV and HPV. As appropriate, international reference preparations would need to cover all major subtypes and mutants, and their commutability for use in whole blood would be important. Moreover, reference standards are needed for rapid diagnostic tests – for example, for detecting HIV Ab, HIV Ab/Ag, HIV/syphilis, HCV Ab, HCV Ab/Ag, HBsAg, malaria Pf Ag, malaria Pf Pv Ag, malaria Pf Pan Ag, EBOV (Zaire), ZIKV Ab, ZIKV Ag, and G6PD. Materials are also needed for use in enzyme immunoassays to detect HIV and HCV. Previous experience in this field has demonstrated the need for clear requirements and appropriate standards.

The Committee asked how often IVD manufacturers are inspected and were informed that inspections take place following every new application. However, if a production line of interest had been inspected recently, an inspection lasting 1 day (instead of 3 days) may be sufficient, depending on the risk-based decision made. Further discussion included the observation that for the EUAL procedure, post-marketing follow-up is currently not funded making it difficult to perform, and the clarification that quality control laboratories can apply to perform appropriate evaluations should they wish.

The Committee agreed that the relevant Technical Specification Series documents being produced by the WHO PQ Team in the area of IVD prequalification should be sent to the Committee for review and endorsement.
3.5 Vaccines and related substances

3.5.1 Revision of WHO Guidelines for the safe production and quality control of inactivated poliomyelitis vaccines manufactured from wild polioviruses

The Committee was reminded that the *WHO Polio Eradication and Endgame Strategic Plan 2013–2018* (PEESP) published by the Global Polio Eradication Initiative sets the goal of achieving a polio-free world by 2018. One important component of this goal is to minimize the risk of facility-associated reintroduction of wild or attenuated oral poliomyelitis vaccine (OPV, Sabin) polioviruses following eradication. In the third edition of the *WHO Global Action Plan to minimize poliovirus facility-associated risk after type-specific eradication of wild polioviruses and sequential cessation of oral polio vaccine use* (GAPIII) published in May 2015, the safe handling and containment of poliovirus infectious and potentially infectious materials has been aligned with the PEESP.

Noting these developments, the Committee at its previous meeting had expressed its support for the revision of the above WHO Guidelines, which had been published as a 2003 Addendum to the earlier WHO Recommendations. The Committee was informed that this revision process was now under way and that a WHO working group meeting had been held in September 2016. Meeting participants had agreed that the current Guidelines should be revised to provide concise information on the safe production and quality control of poliomyelitis vaccines, and should be aligned with other relevant WHO documents, particularly GAPIII. The importance of the revised document was emphasized since many WHO Member States will largely depend on it to ensure the safe production of poliomyelitis vaccines. However, the full and immediate implementation of GAPIII is difficult since it requires major changes in facilities and operating procedures. Certification through the GAPIII Containment Certification Scheme has three levels indicating increasing degrees of compliance, namely: Certificate of participation; Interim certification; and Full certification. Working group meeting participants had agreed that the issues identified should be resolved on the basis of scientific evidence and risk-management principles.

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There was also agreement that the proposed scope of the revised document should encompass: (a) containment of the manufacturing and quality control facilities for inactivated poliomyelitis vaccine (IPV) derived from both Sabin and wild-type poliovirus; and (b) a risk assessment of new safer strains of poliovirus to be used in manufacturing or quality control testing. The routine production of OPV using Sabin vaccine strains would be outside the scope of the document because as long as it is being used for routine immunization it does not have to be contained. When it is not in routine use it will not be made, leaving only the problem of manufacturing a post-eradication OPV for emergency response – assuming that the ability to make OPV is retained. It should also be possible to store contained poliovirus, such as monovalent OPV, outside GAPIII-compliant facilities which would greatly simplify future work for manufacturers and other stakeholders. Several other issues had yet to be resolved, such as the need for routine showering upon exit from the containment area as required by GAPIII.

The Committee noted with interest the information provided and, following discussion and clarification of a number of points, expressed its support for the developments to date and looked forward to hearing of further progress at its next meeting.

3.5.2 Guidelines on regulatory preparedness for provision of marketing authorization of human pandemic influenza vaccines in non-vaccine-producing countries

Strategies to shorten the time between the emergence of a human pandemic influenza virus and the availability of safe and effective pandemic influenza vaccines are among the highest priorities in global health security due to the urgent public health need for vaccine in such situations. The WHO Guidelines on regulatory preparedness for human pandemic influenza vaccines were adopted by the Committee in 2007 and provide NRAs and vaccine manufacturers with: (a) guidance on regulatory pathways for approving pandemic influenza vaccines; (b) regulatory considerations to be taken into account in evaluating the quality, safety and efficacy of candidate pandemic influenza vaccines; and (c) guidance on effective post-marketing surveillance of pandemic influenza vaccines. However, these Guidelines apply mainly to countries in which influenza vaccine production takes place and where pandemic influenza vaccines are likely to be given market authorization first in the event of a pandemic. Consultation with stakeholders following the 2009 H1N1 influenza pandemic identified regulatory delays due to a lack of regulatory preparedness as a significant factor in delaying or preventing the deployment of pandemic vaccine in non-vaccine-producing countries. This was especially the case for
vaccine destined for donation or deployment by United Nations agencies in response to the pandemic emergency.

The Committee was informed that proposed WHO Guidelines had now been drafted in response to requests from non-vaccine-producing countries for guidance on the identification of appropriate regulatory approaches to the marketing authorization of pandemic influenza vaccines, and on arrangements for lot release of such vaccines during a public health emergency. The Guidelines were developed in the context of the Pandemic Influenza Preparedness (PIP) Framework’s Partnership Contribution Implementation Plan 2013–2016 for supporting regulatory capacity-building and strengthening pandemic preparedness and response activities. The document had then undergone considerable public consultation.

The resulting draft Guidelines (WHO/BS/2016.2289) provide guidance to the NRAs of non-vaccine-producing countries on the regulatory oversight of pandemic influenza vaccines for use in public health emergencies. The document is aimed to assist such countries in preparing and putting in place, in advance of a pandemic influenza emergency, a regulatory process for pandemic influenza vaccines. Such a process should enable countries to expedite the provision of marketing authorization and lot release of influenza vaccines in response to a pandemic emergency. It is acknowledged that each country will have national legislation and policies on the regulation of medicines, vaccines and other health products. Some countries may also have regulations in place on accepting donations of vaccines and ancillary products. The proposed document is intended to provide additional and specific guidance to the NRAs of non-vaccine-producing countries and emphasizes the need to put in place appropriate decision-making processes that minimize duplication and make much-needed vaccines available for use without unnecessary delay during pandemic emergencies. In particular, it recommends that these processes be established during the interpandemic phase.

The Committee reviewed the document WHO/BS/2016.2289 and made a number of important changes. One table that had caused some difficulties with respect to clarity was replaced by a simpler diagram and parts of the text, including the title, were amended to improve the guidance given. Following these changes the Committee recommended that the WHO Guidelines be adopted and annexed to its report (Annex 7). The Committee additionally highlighted the need for NRAs to interact closely with other stakeholders when addressing issues related to pandemic influenza vaccines. It would also be important that WHO follows up the adoption of these Guidelines with implementation meetings in countries which most require assistance in this area, including countries without a functional NRA.
3.5.3 Labelling information of inactivated influenza vaccines for use in pregnant women

Enhancing the uptake of vaccines during pregnancy is an important element in ongoing WHO efforts to improve maternal and child health. Levels of morbidity and mortality due to seasonal influenza are considered to be substantial worldwide, and pregnant women are especially vulnerable. Such women also have an increased risk of severe disease and death from influenza and the infection may also increase fetal complications. In 2012, the WHO position paper on vaccines against influenza, endorsed by the WHO SAGE, recommended the immunization of pregnant women with trivalent inactivated influenza vaccine (IIV) at any stage of pregnancy. WHO SAGE also recommended that pregnant women should have the highest priority in countries considering the initiation or expansion of seasonal influenza vaccination programmes. In addition, the WHO Global Advisory Committee on Vaccine Safety (GACVS), following careful analysis of data worldwide, concluded that there was no evidence of adverse pregnancy outcomes associated with the vaccination of pregnant women with several inactivated viral or bacterial vaccines, including IIVs.

Nevertheless, the implementation of influenza immunization during pregnancy remains suboptimal. One reason for this is the ongoing perceived risk of administering influenza vaccine – or any vaccine – to this population group, especially in view of the precautionary language used on some product labels, which is open to misinterpretation. Such product labels also carry no explicit indication for vaccine use during pregnancy. Furthermore, as pregnant women are usually excluded from clinical studies during vaccine development, licensure dossiers generally do not include information on their safety and efficacy in this group. A recent survey had indicated that health-care providers perceived package insert information as contradicting national immunization recommendations, and that this affected their decision on whether or not to use IIVs in pregnant women.

A proposal had therefore been made to develop an explanatory addendum to the current WHO Recommendations for the production and control of influenza vaccine (inactivated)\(^\text{10}\) to clarify and interpret the labelling information provided in the product insert of IIVs. This proposal had arisen from the 2012 WHO SAGE recommendations and from the discussions at several WHO consultations and meetings, including the previous meeting of the Committee. The addendum was intended to be a clear statement

indicating that, on the basis of current evidence, the use of IIV in pregnant women is not contraindicated. It was expected that this would in turn facilitate maternal immunization programmes by raising awareness of the convergence of regulatory positions on this matter. The Committee was informed that the proposed document (WHO/BS/2016.2280) had been the subject of extensive international public consultation with regulators, manufacturers, academia and vaccine users, which had resulted in editorial changes to improve clarity.

Following review of the document, the Committee considered it to be a suitable explanatory document without further revision, and therefore recommended that the proposed addendum be adopted and annexed to its report (Annex 8).

3.5.4 Revision of the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations

The current WHO Guidelines on clinical evaluation of vaccines: regulatory expectations were adopted by the Committee in 2001 and since then have provided guidance to NRAs and manufacturers, and informed WHO vaccine prequalification activities. More than 20 vaccine-specific documents that include a section on clinical evaluation have subsequently been adopted by the Committee – all of which were intended to be read in conjunction with the 2001 WHO Guidelines. The Committee was informed, however, that the current text was now outdated and did not address issues of vaccine development that have emerged since that time, and there was also no dedicated safety section in the current document. Furthermore, the nonclinical evaluation section had become redundant with the subsequent adoption in 2003 of new WHO Guidelines specifically addressing this aspect. As a result of these and other considerations, the Guidelines had now been revised and updated to reflect the scientific and regulatory experience gained from vaccine clinical development programmes since 2001, and to take into account the content of clinical development programmes, clinical trial designs, the interpretation of trial results and post-licensing activities.

The main changes in this revision included more information on the general principles of comparative immunogenicity studies, more details on trial designs and analysis, a clarification of terminology (such as the distinction between vaccine effectiveness and efficacy) and a new section on vaccine safety evaluation. The structure of the document had also changed, with a number of

methodological considerations having been incorporated into relevant sections and subsections rather than being described in a separate section. In particular, the revised document was not organized along the lines of the traditional pre-licensure phases of drug clinical development (Phases I, II and III) given that vaccine clinical development programmes are very variable and depend upon: (a) what is already known about the antigen and adjuvant content; (b) the epidemiological situation regarding the disease to be prevented; (c) any prior vaccine efficacy studies; and (d) the existence of immune correlates of protection. There was however a section on the different phases of pre-licensure development, and one on post-licensure clinical evaluations.

The Committee was informed that the revised document had undergone three rounds of public consultation, including discussion at consultation meetings, and had been well received. The Committee reviewed the proposed document (WHO/BS/2016.2287) and despite some initial concerns regarding a document structure not based on the traditional phases of clinical development, recognized the variable nature of modern clinical development programmes for vaccines and the advantages of the new document structure. After making a number of amendments to the text, including clarification of what was absolutely necessary for licensing as opposed to what might be desirable to know, the Committee recommended that the revised WHO Guidelines be adopted and annexed to its report (Annex 9). The Committee once again emphasized the importance of WHO implementation workshops in ensuring that NRAs, researchers, public health officials and others involved in vaccine development were made aware of and understood the new Guidelines and modern approaches to vaccine clinical development programmes. Strong support was also expressed by representatives of manufacturer associations for the running of such workshops.

3.5.5 Human challenge trials for vaccine development: regulatory considerations

During the course of the revision process for the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (see section 3.5.4 above) it became clear that one subject area that was not covered by the original 2001 document, and which might be considered for addition during revision, was human challenge trials. Human challenge trials are studies in which immunized and non-immunized volunteers are intentionally challenged with an infectious disease-causing organism to see whether immunization affords any protection against the challenge strain. The challenge organism may be close to wild-type and pathogenic (but adapted and/or attenuated from wild-type, with possibly less or no pathogenicity) or genetically modified in some way. During subsequent public consultations, it was agreed that since human challenge trials are not required for licensing, any guidance on this matter should be developed as a
separate WHO guidance document to be read in conjunction with the updated WHO Guidelines.

A document on human challenge trials (WHO/BS/2016.2288) was therefore developed to highlight the standardization, regulatory and ethical aspects of such trials, and to make clear that such studies, although not essential, could nevertheless be helpful for many reasons during vaccine development. All principles relating to the conduct of clinical evaluations of vaccines in humans should apply, including appropriate study design, approval by an NRA and ethical committees, and compliance with good clinical practice.

The Committee reviewed the document WHO/BS/2016.2288 and, following discussion, requested that a number of clarifications be made to the text and title. The Committee then recommended that the WHO guidance document be adopted and annexed to its report (Annex 10). The Committee also recommended that the topic of human challenge trials in clinical development programmes for vaccines should be included in the WHO implementation workshops on the revised WHO Guidelines on clinical evaluation of vaccines: regulatory expectations.

3.5.6 Guidelines on the quality, safety and efficacy of Ebola vaccines

The unprecedented scale and severity of the Ebola virus disease (EVD) epidemic in West Africa in 2014–2016 had led to calls for the urgent development and licensing of an Ebola vaccine, and considerable efforts had been made towards achieving this goal over a very short time. The Committee was reminded that as part of ongoing WHO measures to support the development of Ebola vaccines, draft WHO Guidelines had been prepared on the scientific and regulatory considerations relating to their quality, safety and efficacy. In March 2015, WHO convened an informal consultation, attended by scientific experts, regulatory professionals and other stakeholders involved in Ebola vaccine development, production, evaluation and licensure, to review the draft Guidelines and seek consensus on key technical and regulatory issues. The draft document was then revised in the light of comments made and underwent a round of public consultation, resulting in a large number of further comments and suggestions.

At its 2015 meeting, the Committee reviewed the comments received and agreed upon the proposed content, scope and style of the proposed document. Following another round of consultation and discussion a revised draft document (WHO/BS/2016.2279) was again subjected to public consultation, resulting in only a few new comments and suggestions.

One major challenge in the development of the Ebola vaccine Guidelines was that they had initially been prepared during the rapidly evolving epidemic situation when the need for a vaccine was most urgent. However, the epidemic was later brought under control by infection-control measures not involving
vaccines. The first drafts of the Guidelines were thus concerned with an epidemic situation with a focus on accelerating product development and availability during a public health emergency. The end of the large-scale EVD outbreak in Africa and the post-emergency epidemiological situation made the assessment of Ebola vaccine efficacy challenging due to the now sporadic nature of the disease.

The Committee was informed that document WHO/BS/2016.2279 provides scientific and regulatory guidance for NRAs and vaccine manufacturers on the quality, and nonclinical and clinical aspects of Ebola vaccines relevant to marketing authorization applications. The Guidelines particularly apply to Ebola vaccines based on viral vectors as these are the vaccines currently at the most advanced stages of development and for which no specific WHO guidance is available. For the first time in any WHO Guidelines of this type, opportunities to accelerate product development and availability during a public health emergency are discussed.

Although the Committee recognized that ongoing clinical studies may generate more data on vaccine performance, it agreed that any new data were unlikely to significantly alter the technical guidance given. Furthermore, the early availability of such WHO Guidelines in the public domain would aid the evaluation of applications for clinical trials and for the licensure of Ebola vaccines in the near future. The Guidelines may also be of benefit in the evaluation of other viral vectored vaccines and could help guide future activities in outbreak situations, especially those which constituted a Public Health Emergency of International Concern. At the same time, specific outbreaks have unique features and there would always be a need for flexibility of response.

The Committee reviewed the draft document WHO/BS/2016.2279 and after extensive discussion agreed that the guidance given on multivalent Ebola vaccines and on the clinical evaluation of candidate vaccines using newer clinical trial designs should be expanded. A revised document will therefore be submitted to the Committee in 2017.
4. International reference materials – biotherapeutics other than blood products

All reference materials established at the meeting are listed in Annex 11.

4.1 Proposed new projects and updates – biotherapeutics other than blood products

4.1.1 Proposed Second WHO International Standard for parathyroid hormone 1-34 (recombinant, human)

The Committee was informed that stocks of the First WHO International Standard for parathyroid hormone 1-34 (recombinant, human) (rhPTH 1-34) were likely to be exhausted within 2 years. The International Standard defines the mass unit for rhPTH 1-34 and is essential for the correct potency labelling of therapeutic rhPTH 1-34 products used to treat osteoporosis. The establishment of a replacement International Standard should therefore commence as soon as possible to ensure the continuous availability of this WHO International Standard.

The standard is used by manufacturers, and regulatory, quality assurance and academic laboratories for the calibration of therapeutic rhPTH 1-34 potency assays, with an anticipated demand of 250–300 ampoules per year. Currently there is one licensed rhPTH 1 34 product in the United States and Europe but several SBPs have already been licensed in India and it is anticipated that the expiry of the United States/European patent in 2018 will lead to the global emergence of more.

It is expected that rhPTH 1-34 (expressed in *Escherichia coli*) will be donated by the United States/European manufacturer and assigned a mass value via a collaborative study using a primary calibrant approach. NIBSC intends to dispatch ampouled material and the primary calibrant (previously mass value assigned) to more than 10 collaborating laboratories for mass value assignment using the reversed-phase high-performance liquid chromatography (RP-HPLC) assay. Bioassays will also be performed where available to confirm bioactivity according to a given study protocol. The accelerated thermal degradation of samples will be analysed in order to predict long-term stability.

The Committee recognized the importance of replacing the current International Standard and endorsed the proposal (WHO/BS/2016.2296 Rev.1) to establish a Second International Standard for parathyroid hormone 1-34 (recombinant, human).
4.1.2 Proposed First WHO international standards for vascular endothelial growth factor antagonists

Vascular endothelial growth factor-A (VEGF-A) is the most important angiogenic growth factor of the VEGF family and has several isoforms (VEGF-A121, VEGF-A145, VEGF-A165, VEGF-A183, VEGF-A189 and VEGF-A201). VEGF-A165 is the major isoform required for angiogenesis through binding to its signalling receptor, VEGF receptor-2 (VEGFR2), to promote the growth and differentiation of the endothelial cells which compose the inner lining of the vasculature. Over-expression of VEGF is associated with pathogenesis in the form of neovascular disorders (including solid neoplasms and intraocular diseases). Consequently, several VEGF antagonists are approved as therapeutics in various cancers and/or eye disorders, including neovascular age-related macular degeneration. Such antagonists include mAbs, for example, bevacizumab (a humanized mAb) and ranibizumab (a Fab fragment derived from the same parent antibody as bevacizumab). Both these mAbs bind with high affinity to all VEGF-A isoforms and prevent the binding of VEGF specifically to VEGFR1 and VEGFR2. Bevacizumab is approved for various cancers while ranibizumab is indicated for ocular diseases. Also approved are the Fc-fusion proteins aflibercept and ziv-aflibercept which consist of the extracellular domain 2 of VEGFR1 and the extracellular domain 3 of VEGFR2 fused to the Fc portion of human immunoglobulin G1, which act as soluble decoy receptors to VEGF-A, VEGF-B and placenta growth factor. Aflibercept and ziv-aflibercept are identical in structure but differ in their purification process and formulation. The former is indicated for treating ocular diseases whereas the latter is approved as an anticancer drug.

As the patent expiry of originator products is imminent, biosimilar VEGF antagonists (particularly bevacizumab and ranibizumab) are currently under development worldwide. There is therefore an urgent need for standards to control the performance of assays for bioactivity evaluations of these products to ensure their clinical safety and efficacy. The intended users of such standards would be manufacturers and regulatory authorities. Ideally, candidate materials from both originator and SBP manufacturers would be included in a collaborative study. The proposed NIBSC plan is to initiate activities with bevacizumab or ranibizumab (depending on the availability of materials) and to follow on with other molecules as available. Pilot lyophilizations would be conducted to determine a suitable formulation for the molecule of interest. Following definitive fills, the different candidate preparations would then be included in an international collaborative study. The range of assays likely to be used would include binding assays and bioassays involving both primary and engineered cell lines.

The Committee regarded this as a longer term project with somewhat unclear timelines, and it was envisaged that securing candidate materials for the
collaborative study might be an issue. Nevertheless, the Committee considered this to be a worthwhile project and endorsed the proposal (WHO/BS/2016.2296 Rev.1) to develop standards in this area.

4.1.3 Proposed First WHO international standards (or reference panels) for antibodies for use in immunogenicity assessments of biotherapeutic products

Testing the potential immunogenicity of a biotherapeutic product is a regulatory expectation for product approval. The bioanalytical testing strategy adopted for antibody testing requires a multi-tiered approach which includes a screening assay for the detection of binding antibodies, a confirmatory step and subsequent analysis of positive samples for the presence of neutralizing antibodies. This approach has been universally adopted and used in the immunogenicity assessment of most biotherapeutic products. Testing for antibodies is however a specialized field. Despite recent advances in new and evolving technologies, antibody assays remain very challenging and antibody detection difficult, with low-affinity antibodies having the potential to be missed. With the sole exception of the First WHO Reference Panel for antibodies to erythropoietin (human), there are currently no reference materials for antibody assays for biotherapeutic products, and it is difficult for laboratories involved in this work to determine the performance of their assays in the clinical setting.

There is thus a need for reference antibody standards/panels for use as positive controls to standardize antibody testing across different assay platforms and different laboratories, as this would provide a consistent basis for antibody detection and measurement. This requirement was also emphasized at a recent EMA workshop on immunogenicity assessment. The provision of such antibody-positive controls would also facilitate the immunogenicity assessment of emerging SBPs and thus promote wider access to safe and effective medicines, while potentially also improving clinical decision-making to the benefit of the patient.

This NIBSC-proposed collaborative project would aim to make available either reference antibodies or antibody reference panels as positive control(s) to standardize antibody testing across assay platforms and laboratories, where possible for a range of biotherapeutic products. Purified human mAbs, originating from isolated B-cells from patients treated with an innovator product (such as adalimumab, infliximab, natalizumab, rituximab or interferon-β) would be sourced and lyophilized. In some cases there was likely to be only one antibody, whereas in others panels of antibodies with different characteristics (for example, low or high affinity, or non-neutralizing/neutralizing) would need to be procured and made available. Following pilot fills and selection of an appropriate formulation, definitive fills would be conducted and multicentre collaborative studies undertaken. Participants would be required to test the
antibody/panel in different assay platforms for evaluation of both binding activity and neutralizing activity. It was anticipated that some patient samples (subject to availability) would be included in the studies. The expected users of the reference antibodies or antibody panels would be regulatory agencies, industry, clinical laboratories, independent investigators and researchers in academic and scientific organizations worldwide.

Despite appreciating that the timelines of the proposed project were somewhat uncertain, the Committee recognized the importance of this work and endorsed the proposal (WHO/BS/2016.2296 Rev.1) to develop international standards and/or reference panels for antibodies for use in this area.

4.1.4 Proposed First WHO international standards (or reference reagents) for monoclonal antibodies to the ErbB/HER receptor family

The ErbB/HER family of receptor tyrosine kinases (ErbB-1/HER-1, ErbB-2/HER-2, ErbB-3/HER-3 and ErbB-4/HER-4) are structurally related to epidermal growth factor receptors and are important therapeutic targets. Increased expression of members of the ErbB/HER family occurs in a number of solid tumours, and mAbs such as trastuzumab (directed to ErbB2/HER-2) and cetuximab (targeting ErbB1/HER-1) are currently used as mono- or combined therapies in a number of oncology indications. Their mechanism of action and clinical efficacy rely upon the inhibition of ErbB signalling by preventing ligand binding and promoting receptor internalization. In addition, Fc-effector functions such as antibody-dependent cellular cytotoxicity often contribute to their therapeutic activity.

Consisting of structurally complex molecules comprising several functional domains, mAbs are sensitive to small quality differences that may arise through changes in the manufacturing process. Bioassays that measure the direct or indirect mAb-induced cytotoxic effects are generally used for the potency testing and lot release of these products. To date, developers, manufacturers and regulators have relied solely on the use of in-house qualified reference standards and the reference clinical product during development and manufacturing, and no higher-order of mAb standard for bioassays is currently available.

Given that mAbs are the fastest-growing class of biotherapeutic product, and with increasing numbers of SBPs now in development, there is a widely recognized need for the global standardization of biotechnology products in this field. Preliminary data from ongoing collaborative studies for the development of WHO international standards and reference reagents for mAbs such as rituximab are encouraging, and highlight the value of such reagents in assessing, and ensuring the consistency of, bioassay performance by different stakeholders.

The proposal to develop WHO international standards or reference reagents for
use in the calibration, evaluation and validation of bioassays for measuring the biological activity of mAbs to the ErbB/HER receptor family is aligned with these recognized needs.

NIBSC therefore proposed an international multicentre collaborative study to evaluate the suitability of candidate preparations to act as biological standards/reference reagents in one or more relevant direct or indirect functional assays. Depending on the specific mAb under study, these could include bioassays for measuring cell binding, inhibition of receptor signalling, inhibition of proliferation of target cells and/or antibody-dependent cytotoxicity.

Candidate reference materials would now be sought from an innovator or SBP manufacturer. As this may be challenging, the first study to be carried out will be determined by the ability to source suitable candidate material. Other preparations may also be tested in parallel for comparison. It was expected that an international collaborative study would be likely to take place in 2017 and 2018.

The Committee agreed that there was a need for the standardization of biotechnology products globally and endorsed the proposal (WHO/BS/2016.2296 Rev.1) to develop international standards and/or reference reagents for use in this area. It noted that, ideally, the relevant reference materials should become available as SBPs were being developed and licensed, and that there was now a need for a catch-up programme of work.
5. International reference materials – blood products and related substances

All reference materials established at the meeting are listed in Annex 11.

5.1 WHO International Standards and Reference Reagents – blood products and related substances

5.1.1 Second WHO International Standard for ancrod

Ancrod and batroxobin are snake venom thrombin-like serine proteases produced by Calloselasma rhodostoma and Bothrops atrox moojeni, respectively. Both ancrod and batroxobin clot fibrinogen but lack the other functions of thrombin so do not activate or cleave other proteins or present a target for inhibitors. Because of the nature of the fibrin produced by ancrod and batroxobin they physiologically promote fibrinogen depletion and have been investigated as potential treatments to reduce clot formation under various circumstances. Ancrod has been demonstrated to be effective in the treatment of sudden sensorineural hearing loss and is also believed to have anticoagulant properties, whereas batroxobin is primarily used clinically as a diagnostic reagent for reptilase time. Despite being used in different clinical applications the two materials are derived in similar ways and assayed in the same tests.

The Committee was informed that there was now a need to replace both standards, and in the interests of efficiency it was decided that this would best be achieved by conducting a combined international collaborative study which included both the current First WHO International Standard for ancrod and the current Second British Standard for batroxobin. This study had now assessed the suitability of: (a) the proposed candidate material (NIBSC code 15/106) for use as the Second WHO International Standard for ancrod; and (b) a proposed reference reagent (NIBSC code 15/140) for use as the First WHO Reference Reagent for batroxobin.

A total of 17 laboratories returned data, with each asked to perform four independent assays. A range of assay methods were encouraged and suitable protocols provided. Potency estimates for ancrod were made relative to the current standard using parallel-line analysis and an unweighted geometric mean potency of 53.9 IU/ampoule obtained, consistent with the expected potency of 55 IU/ampoule. Greater variability was observed in the batroxobin values, with additional data from assays independent of the study confirming an apparent 10% higher potency when using plasma as the substrate compared to fibrinogen, thus highlighting a small but significant discrepancy between the proposed and current materials – potentially due to degradation of the current standard.
Overall, study data indicated a geometric mean value of 49.8 U/ampoule – which it was proposed be rounded up to 50 U/ampoule.

The Committee considered the report of the study (WHO/BS/2016.2282) and as no issues were raised recommended that the candidate material 15/106 be established as the Second WHO International Standard for ancrod with a potency of 54 IU/ampoule.

5.1.2  **First WHO Reference Reagent for batroxobin**

The Committee considered the report of the study (WHO/BS/2016.2282) and as no issues were raised recommended that the candidate material 15/140 be established as the First WHO Reference Reagent for batroxobin with a potency of 50 U/ampoule (see section 5.1.1 above).

5.1.3  **Second WHO International Standard for blood coagulation factor XI (plasma, human)**

Blood coagulation factor XI (FXI) deficiency is generally mild and bleeding is most often associated with surgery or trauma. Although deficiency is most common among Ashkenazi Jews (with approximately 1 in 190 homozygous for an FXI gene mutation and around 1 in 8 heterozygous) the condition has now been identified in a wide variety of populations.

The First WHO International Standard for blood coagulation factor XI (plasma, human) was established in 2005. This standard is used to aid the diagnosis of FXI deficiency and to assign potency to licensed FXI concentrates and to virus-inactivated plasma products, both of which are used as treatments. As stock levels of this standard were now nearing depletion a replacement standard was required. For continuity of the IU, the replacement standard for FXI functional activity (FXI:C) was value assigned relative to the current international standard. In addition, this study had also aimed to establish an antigen value for FXI (FXI:Ag) for the same candidate material relative to the local normal pooled plasma of participating laboratories.

The candidate material (NIBSC code 15/180) was frozen normal plasma purchased from the the UK NHS Blood and Transplant. Following thawing at 37 °C and filling at room temperature (avoiding contact with glass) the material was lyophilized into siliconized glass ampoules (with around 6000 ampoules being available). For the value assignment of FXI:C, potency was assigned relative to the current international standard, and the relationship with local normal pooled plasma assessed. The assignment of FXI:Ag – to help assess patient antigen value in addition to functional activity – was performed relative to local normal plasma pools as no former value existed. For FXI:C value assignment, 29 laboratories from 11 countries participated, with 11 laboratories from eight
countries taking part in the assignment of FXI:Ag values. In all cases, the use of four independent assays was requested, each with four or more dilutions in duplicate. All data were analysed by parallel-line assay, for which only assays valid for linearity were used \((p > 0.01)\). Parallelism was assessed as the ratio of standard and test slopes. Potency estimates were calculated using the unweighted geometric mean.

Excellent inter-laboratory agreement was observed for the assignment of FXI:C value \((\text{geometric coefficient of variation (GCV)} = 1.8\%)\) and no discrepancy was observed between the IU and the plasma unit \((0.71 \text{ versus } 0.72 \text{ IU/ampoule})\). For FXI:Ag value assignment, there was also good agreement between laboratories, albeit with wider variation due to differences between plasma pools. A small method bias was also seen, which is unlikely to be significant.

During discussion, the Committee noted that although enzyme activity had remained stable during stability studies, the antigenicity had dropped. Although this was the opposite of what would be expected, this may have resulted from the different reference products used or to the higher variability of the antigenicity test results. It was also commented that in future such validation studies of international reference preparations should involve laboratories from a wider selection of WHO regions.

The Committee considered the report of the study \((\text{WHO/BS/2016.2281})\) and recommended that the candidate material 15/180 \((6000 \text{ ampoules})\) be established as the Second WHO International Standard for blood coagulation factor XI \((\text{plasma, human})\) with an FXI:C value of 0.71 IU/ampoule, and an FXI:Ag value of 0.78 IU/ampoule.

5.1.4 Fifth WHO International Standard for thromboplastin (recombinant, human, plain)

International standards for thromboplastins are used to determine the International Sensitivity Index \((\text{ISI})\) for commercial or local prothrombin time \((\text{PT})\) test reagents and instruments, which is needed to determine the international normalized ratio \((\text{INR})\) in patients receiving vitamin K antagonist. Stocks of the current Fourth WHO International Standard for thromboplastin \((\text{recombinant, human, plain})\) and the Fourth WHO International Standard for thromboplastin \((\text{rabbit, plain})\) were now running low. An international collaborative study involving 20 laboratories was therefore conducted to assign ISI values to two candidate materials \((\text{NIBSC codes 14/001 and 15/001, respectively})\) and to assess their overall suitability as replacement standards.

PT determination was performed by manual tilt tube technique on 10 different days using serum from two healthy subjects and six patients per day – with the patients selected to have an INR between 1.5 and 4.5. In addition,
four freeze-dried control plasmas were used. To avoid bias in the testing system, the order of testing was different from day to day. The mean ISIs obtained were 1.11 for candidate 14/001 (inter-laboratory coefficient of variation = 5.7%) and 1.21 for candidate 15/001 (inter-laboratory coefficient of variation = 4.6%). Accelerated degradation studies were also performed to assess the stability of the freeze-dried candidate materials. There was no significant change in PT following storage of the candidate materials at 5 °C for 56 days. After storage at elevated temperatures (31–42 °C) there was a slight but significant change in PT. Stability after reconstitution was assessed at room temperature, over a time interval of 0.5–4 hours. Between 1 and 4 hours after reconstitution there was no change in PT for candidate material 14/001 and a slight change (−1.5%) for candidate material 15/001. Should these materials be established for use as international standards, it was proposed that the provisional codings used above be replaced by rTF/16 and RBT/16 respectively.

The results revealed significant variation in ISI results between laboratories, indicating that further standardization of the manual tilt tube technique is needed. It was intended that a document would be prepared that provided full details of the technique for approval by the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (SSC/ISTH). The document was then to be submitted as an approved reference method to the Joint Committee for Traceability in Laboratory Medicine of the Bureau International des Poids et Mesures.

The Committee noted that an automated method would be a good way of standardizing results as the manual tilt tube technique was apparently prone to variability. Despite a number of potential methodological challenges, a strong recommendation was made by the Committee to include automated methods in future validation studies of reference preparations for thromboplastins. Furthermore, given the observed instability of the candidate materials at temperatures > 30 °C, it was proposed that written advice to ship them with cooling packs to maintain temperatures at or below controlled room temperature be added to the product Instructions for Use.

The Committee considered the report of the study (WHO/BS/2016.2294) and recommended that the candidate material 14/001 (to be re-coded as rTF/16) be established as the Fifth WHO International Standard for thromboplastin (recombinant, human, plain) with an assigned value of 1.11 IU/ml.

5.1.5 **Fifth WHO International Standard for thromboplastin (rabbit, plain)**

The Committee considered the report of the study (WHO/BS/2016.2294) and recommended that the candidate material 15/001 (to be re-coded as RBT/16) be established as the Fifth WHO International Standard for thromboplastin (rabbit, plain) with an assigned value of 1.21 IU/ml (see section 5.1.4 above).
5.2 Proposed new projects and updates – blood products and related substances

5.2.1 Proposed Second WHO International Standard for blood coagulation factor V (plasma, human)

The measurement of blood coagulation factor V (FV) in human plasma is important for the diagnosis of FV and combined FV/FVIII deficiency, as well as for the quality control of virus-inactivated fresh frozen plasma (VIFFP). Users include manufacturers of VIFFP and commercial plasma calibrators, clinical laboratories and regulatory laboratories. Stocks of the First WHO International Standard for blood coagulation factor V (plasma, human) were now running low and a replacement was needed.

The proposed source material would be normal human plasma donations purchased from the UK NHS Blood and Transplant. The material will be pooled and buffered with HEPES (final concentration 40 mmol/l). Aliquots of 1 ml pooled plasma filled into 5 ml DIN ampoules will yield 10,000 ampoules. For value assignment a multicentre international collaborative study will be conducted, involving approximately 20 laboratories using one-stage clotting assays relative to the current international standard and local normal plasma pools. Raw data analysis will then be performed at NIBSC and a consensus mean value calculated relative to the current international standard. It was also planned to incorporate a “commutability” sample (for example, virus-inactivated plasma) into the study and to include the calibration of the SSC/ISTH Secondary Coagulation Standard Lot #5.

The Committee endorsed the proposal (WHO/BS/2016.2297) to develop a Second WHO International Standard for blood coagulation factor V (plasma, human).

5.2.2 Proposed First WHO International Standard for blood coagulation factor XII (plasma, human)

In vitro, blood coagulation factor XII (FXII) is involved in the contact phase of the coagulation cascade and has also been shown to influence the complement system. For many years, FXII was not considered to be important for haemostasis because its deficiency is not associated with excessive bleeding – with some patients paradoxically predisposing to thrombosis. Recent advances indicate that in vivo FXII does have a role to play in thrombosis, especially in events that are driven through interactions with endogenous FXII activators such as mast cell heparin, RNA and platelet polyphosphate. In addition, a missense mutation of the gene for FXII has been associated with hereditary angioedema, a life-threatening disorder.

The lack of certified reference materials currently hinders both the diagnosis of FXII deficiency and the comparison of research results in this
emerging area. In addition, the development of WHO international standards encompassing both FXII:C and FXII:Ag was now essential to aid patient diagnosis and the development of assay methods for FXII. It is intended that normal plasma will be sourced from the UK NHS Blood and Transplant. Since the candidate material has the same processing requirement as the recently established Second WHO International Standard for blood coagulation factor XI (plasma, human) (see section 5.1.3 above) it is further proposed to assign FXII:C and FXII:Ag values as additional analyte values to the proposed factor XI international standard. Stocks of the replacement international standard for FXI are estimated to be sufficient for approximately 15 years (6000 ampoules) – and are thus sufficient to allow for the assignment of FXII values as additional analytes.

The Committee discussed whether potential variability in antigenicity, which had been an issue in the FXI collaborative study, may also occur during the evaluation of FXII antigenicity. However, the proposed use of local plasma pools that overall contain > 20 000 donations should mitigate this risk. The Committee then endorsed the proposal (WHO/BS/2016.2297) to develop a First WHO International Standard for blood coagulation factor XII (plasma, human) and to assign FXII:C and FXII:Ag values as additional analyte values.

5.2.3 Proposed assignment of factor XIII-B subunit (total and free) values to the First WHO International Standard for factor XIII plasma

The First WHO International Standard for factor XIII plasma is currently used to measure the potency (functional activity and antigen value) of blood coagulating factor XIII (FXIII) in patient plasma during the diagnosis of FXIII deficiencies, and also to evaluate FXIII therapeutic concentrates. FXIII circulates in plasma as a heterotetramer of two A and two B subunits (FXIII-A2B2) in a 1:1 complex. The active A subunit functions by cross-linking fibrin and stabilizing the fibrin structure. The B subunit is a carrier protein without activity. However, FXIII-B is in excess over FXIII-A with around 50% of total FXIII-B existing in complex with FXIII-A and around 50% in free form. The half-life of FXIII-A depends upon the amount of available FXIII-B. Congenital and acquired FXIII deficiencies are severe bleeding disorders, and FXIII-B measurements are crucial in correctly diagnosing and characterizing the type of FXIII deficiency. Furthermore, therapy for FXIII-A deficiency with recombinant FXIII-A relies on available FXIII-B. Free FXIII-B may also have other so far unknown functions.

It was proposed that an international multicentre study involving manufacturers, clinical and research laboratories, and regulatory authorities be conducted to calibrate both the total and free levels of the FXIII-B subunit in the First WHO International Standard for factor XIII plasma relative to levels in locally collected and pooled normal plasma. Calibration will be performed by specific ELISAs with value assignment for the B subunit made in IU.s. In order to
determine both total and free FXIII-B levels, mAb to the free FXIII-B subunit is required and has recently been developed – although availability was restricted to a single supplier. These antibodies have been made available free of charge for the proposed value-assignment study but have also been commercialized and will thereafter be available for purchase. This proposal could also be viewed as part of the standardization of a companion diagnostic for informing the use of a recombinant FXIII-A product (Novo XIII) which should not be administered without assessing the patient’s FXIII-B subunit levels.

The Committee noted that approximately 150–200 ampoules are currently dispatched each year and that this level of demand was likely to increase with the new proposed value assignment. The Committee also emphasized that FXIII deficiency can also be treated with plasma. The Committee then endorsed the proposal (WHO/BS/2016.2297) to assign additional analyte potencies to the current First WHO International Standard for factor XIII plasma.

5.2.4 Proposed First WHO International Standard for thrombin-activatable fibrinolysis inhibitor (plasma, human)

Thrombin-activatable fibrinolysis inhibitor (TAFI – also proCPU or CPB2) is a human plasma protein circulating as a zymogen. TAFI is activated by thrombin to TAFIa, a basic carboxypeptidase that cleaves C-terminal lysines from partially degraded fibrin, thereby inhibiting fibrinolysis. High and low levels of TAFI and high and low levels of TAFIa have been shown to correlate with thrombotic diseases and inflammation. However, the results from a large number of studies investigating TAFI as a disease marker are confusing and often contradictory. In addition, estimates of plasma TAFI concentration vary widely (4–15 µg/ml in normal plasma) due in part to the use of different calibrators across the range of commercial and in-house methods for measuring TAFIa in plasma currently in use. ELISA-based assays show differences in detection between some antibodies and do not allow for the measurement of TAFIa. Assays based on functional activity measurement are available – either direct-activity-based assays (complicated in plasma by carboxypeptidase N activity) or indirect measurements that require the quantitative activation of TAFI. Both these assay types present challenges, including lower specificity when using direct assays.

To obtain reliable results from these different assays, standardized methods are required, and a common reference material for TAFI is now needed to harmonize global measurements. Such a material would be intended for use by manufacturers of commercial kits and by academic and clinical laboratories investigating TAFI as a disease marker. It was therefore proposed that a study be conducted involving all available antigen and activity assay methods. It was recognized that such an approach may yield highly variable results, particularly as ELISA-based assays measure concentration and not activity, and that the
value-assignment strategy would be challenging across the different assay platforms. Initially, a single candidate material (pooled human plasma) will be calibrated relative to local plasma pools. Eventually, the calibration would be performed in IUs (1 IU = amount of TAFI in 1 ml plasma) where a specific concentration would correlate with a given level of activity.

The Committee enquired whether the labelling of antigen content of the candidate material was envisaged to be in ng, consensus ng or IUs. The Committee was informed that the intention was to label the product in IUs both for functional activity and antigen content. The Committee then endorsed the proposal (WHO/BS/2016.2296 Rev.1) to develop a First WHO International Standard for thrombin-activatable fibrinolysis inhibitor (plasma, human) with assignment of both functional and antigenic units.

5.2.5 Proposed Third WHO International Standard for anti-D immunoglobulin

The Committee was informed that stocks of the Second WHO International Standard for anti-D immunoglobulin were nearing exhaustion. This international standard is used in potency assays of anti-D immunoglobulin products by manufacturers of blood products, the Official Medicines Control Laboratory (OMCL) network and blood transfusion laboratories.

In light of the frequent global demand for the current international standard, and the need to ensure both continuity of supply and ongoing comparability of test results across different laboratories, an international collaborative study had been proposed to develop a replacement international standard. The source material to be used would be purified anti-D immunoglobulin donated by manufacturers. The candidate replacement preparation would then be calibrated against the current international standard by end users.

The Committee endorsed the proposal (WHO/BS/2016.2297) to develop a Third WHO International Standard for anti-D immunoglobulin.

5.2.6 Update on the use of WHO reference materials in assays to detect activated blood coagulation factor XI in immunoglobulins

In 2010, a cluster of thrombotic events associated with intravenous immunoglobulin administration was reported. Root-cause analysis indicated that the thrombotic component was activated FXI (FXIa) which was a process-related impurity. Regulators and manufacturers therefore began to develop and validate procedures for removing FXIa and other procoagulant components from intravenous immunoglobulins.

Two panels of immunoglobulins – each with three different levels of procoagulant activity – were produced for use in the development of assay methods and for the investigation of assay discrepancies. These panels were
made available to stakeholders such as immunoglobulin manufacturers, kit manufacturers and regulators. In 2012, the First WHO Reference Reagent for activated blood coagulation factor XI (human) was established, followed in 2014 by the First WHO International Standard for activated blood coagulation factor XI.

There are a number of challenges associated with the three assay methods currently used, with each exhibiting different degrees of sensitivity: (a) the FXIa functional activity assay; (b) the non-activated partial thromboplastin time (NAPTT); and (c) the thrombin generation assay. In addition, inter-laboratory agreement continues to be poor, especially for NAPTT and the thrombin generation assay. This is mainly due to the non-usage or misuse of the current International Standard for FXIa. Moreover, there are local variations in assay procedures and a lack of system-suitability samples for the validation of methods, as well as product-specific matrix and/or excipient interference. Moreover, the inconsistent handling of pre- and post-analytical variables (for example, pre-dilution steps) and a lack of agreement on acceptable readouts and on the statistical analysis of data add to the observed inconsistencies.

To address these challenges, a global working group consisting of CBER, EDQM, NIBSC and the US Pharmacopoeial Convention agreed to develop and provide guidance on the harmonization of test methods, and to generate appropriate reference materials. The first step will be to conduct a survey of manufacturers and the OMCL network on current practices for measuring the procoagulant activity of immunoglobulin products. It is then intended that 2–3 laboratories will further develop and refine assay procedures within the time frame Q4–2016 to Q2–2017. This will then be followed by large collaborative studies (from Q2–2017 to Q4–2018) with the aim of testing the agreed assay procedures and identifying appropriate reference materials and/or system-suitability samples for subsequent production.

The Committee enquired whether the only options available to measure FXIa were functional assays or if, for example, antibodies with the ability to distinguish FXI from FXIa were available. The Committee was informed that although such antibodies do exist, reliable results in comparison to functional assays have not yet been obtained. Nor are FXI antigen assays sufficiently sensitive to be used as routine control assays in the immunoglobulin manufacturing process. The Committee looked forward to receiving an update in due course on the reference material selected for development and on whether this material would be a suitable replacement for the current reference materials.

All reference materials established at the meeting are listed in Annex 11.

6.1 Proposed new projects and updates – cellular and gene therapy

6.1.1 Proposed First WHO International Standard for lentiviral vector copy number quantitation

The first retroviral gene therapy to treat the rare inherited disease ADA-SCID is now licensed in Europe. The clinical trials pipeline for other rare inherited diseases such as X-linked severe combined immunodeficiency (X-SCID), Wiskott-Aldrich Syndrome (WAS), X-linked chronic granulomatous disease (X-CGD), beta-thalassemia, adreno-leukodystrophy (ALD) and metachromatic leukodystrophy (MLD) includes the use of lentiviral vectors. As the diseases treated are rare, patients need to be followed up worldwide. The same platform technology has also been used in clinical trials using genetically modified T-cells such as chimeric antigen receptor T-cells (CAR-T-cells) to treat cancer. Here there is potential for much larger numbers of patients. Regulatory authorities generally require that a minimum gene copy number of approximately one copy per cell should be used in gene transfer to ensure efficacy, with an upper limit of four copies per cell to minimize the risk of toxicity.

The proposed development of a First WHO International Standard for lentiviral vector copy number quantitation would build upon the experience of NIBSC in developing genomic reference materials. Genomic DNA from lentiviral vector transduced cells would be produced from a cell clone with a single lentivirus integrant which would contain sequences essential for vector function and which could be detected by various methods. It was expected that candidate integration cell lines would be characterized by the end of 2017, with the filling of candidate material and a collaborative study involving 10 or more hospitals and manufacturers being initiated in early 2018. It was expected that results would be presented to the Committee in October 2019.

The Committee heard that the anticipated use of this reference material would be as a primary standard for the quantitation of lentiviral vector integration copy number – an issue related to both product safety and efficacy. It would also be used as a calibrant in assays and for the calibration of secondary genomic DNA, plasmid or synthetic DNA standards. It was expected that the reference material would be future-proof with regard to novel lentivirus designs, and disease-independent. Potential users were likely to include vector manufacturers (potency), manufacturers of genetically modified cells (potency
and safety), hospital diagnostic laboratories following patients (clinical efficacy) and NCLs worldwide.

Following discussion, the Committee agreed that the availability of a First WHO International Standard for lentiviral vector copy number quantitation would be useful for the standardization of vector copy number – an issue with both safety and efficacy implications for gene therapy – and endorsed the proposal made (see also section 3.3.2 above).

All reference materials established at the meeting are listed in Annex 11.

7.1 WHO International Standards and Reference Reagents – in vitro diagnostics

7.1.1 First WHO International Standard for Zika virus RNA for NAT-based assays

ZIKV is a mosquito-borne flavivirus that was first identified in Rhesus monkeys in the Ugandan Zika forest in 1947. The first human cases were reported in Nigeria in 1954 and up until 2007 only 14 cases were known. In 2007, an outbreak occurred in Yap Island, Micronesia, followed by a new outbreak in French Polynesia in 2013 which was associated with an approximately 20-fold increase in reported Guillain-Barré Syndrome cases. Between 2014 and 2016 a widening outbreak occurred, first in the Pacific and then in central and South America. Although associated with mainly mild symptoms, increased numbers of Guillain-Barré Syndrome cases were again observed, along with cases of microcephaly and other congenital abnormalities, and fetal loss. On 1 February 2016, WHO declared a Public Health Emergency of International Concern following the recognition of clusters of microcephaly cases and other neurological disorders associated with ZIKV infection.

In 2016, an international collaborative study had been conducted to determine the suitability of a candidate material (PEI code 11468/16) for use as an international standard. The candidate material consisted of a heat-inactivated, lyophilized ZIKV preparation (4092 vials) formulated in a stabilizing neutral solution, and was intended for dilution using a range of different types of sample matrix. The virus strain used originated from a ZIKV-infected patient in 2013 from French Polynesia, closely related to the ZIKV strains currently circulating in the Asia-Pacific region and in central and South America.

Test materials for the collaborative study were made available from different outbreak locations, representing different subclades of all ZIKV sequences available at the time of the study, and obtained from different body fluids. In addition, two virus samples from previous outbreaks underwent extensive laboratory cultivation and were also included in the panel.

Results were obtained by 24 laboratories in 11 countries using a range of assays (in-house and commercial). Three sets of study materials were sent out, with laboratories performing three independent assay runs. Harmonization of results was observed for all biological reference materials and clinical samples when potencies were expressed relative to 11468/16 with the candidate material being detected by all assays evaluated in the study. However, such harmonization
for in vitro transcripts only occurred when expressed relative to other in vitro transcripts, with no harmonization observed when the candidate material was expressed relative to the universal in vitro transcript.

Discussion then took place of possible explanations for an observed variability between two laboratories in the degree of harmonization of results against the candidate material when material of African lineage was assayed. The Committee considered that the discrepant results might have been due to the different methods of virus inactivation used or to the matrix used for lyophilization, rather than the African origin of the isolates per se. In relation to the results observed for in vitro transcripts, it was noted that armored RNA was not available at the time of the study, but may provide a solution for improving harmonization in this regard. It was agreed that the continued fitness for purpose of the candidate material should be monitored against new isolates as they occur and that further data should be generated using NAT-based assays of African isolates.

The Committee considered the report of the study (WHO/BS/2016.2286) and recommended that the candidate material 11468/16 be established as the First WHO International Standard for Zika virus RNA for NAT-based assays with an assigned content of 50 000 000 IU/ml.

7.1.2 \textbf{First WHO Reference Panel for Ebola virus VP40 antigen}

Although the EBOV outbreak in West Africa has now ended, WHO preparedness activities are continuing to ensure that all countries are operationally ready to effectively and safely detect, investigate and report potential EVD cases, and to mount effective responses. There is thus a need for the development of point-of-care rapid tests for the specific and accurate EVD diagnosis, which do not require complex laboratory equipment or highly trained staff.

An international collaborative study was therefore conducted to evaluate the suitability of candidate recombinant protein preparations for use as WHO reference materials. Three potential candidate materials were available: (a) a full-length VP40 based upon EBOV Mayinga sequences and expressed in \textit{E. coli} (the preferred candidate); (b) a full-length native viral protein (VP40) based on 2014 Kissidougou-C15 and expressed in \textit{E. coli}; and (c) EBOV virus-like particles produced by co-transfection of three plasmids (pCMV3-codon optimized VP40, pCMV3-codon optimized nucleoprotein and pCAGGS-codon-optimized glycoprotein into HEK293T cells using polyethylenimine. A novel lyophilization method was used for the preferred candidate. Due to the low fill volume it was not possible to use conventional flame-sealed 5 ml ampoules and the material was therefore freeze-dried in 1 ml plastic screw-cap vials containing volumes of 120 µl. A negative sample was also included in the collaborative study along with other antigen-positive materials of differing strengths. A range of assays
were used, which included point-of-care tests, research use only tests and tests approved for use in the WHO EUAL procedure.

Eight laboratories from four countries each received nine blinded study samples and were asked to reconstitute these and test them without dilution. Each sample was assessed in three different runs and the results reported as either positive or negative. In general, results were consistent across the different VP assays with few exceptions. Assays of nucleoprotein and glycoprotein contained in the preferred candidate material almost invariably produced negative results for samples demonstrated as positive for VP40. As only limited stability data were available, it was acknowledged that additional studies would be required before any long-term stability predictions could be made. It was also noted that the moisture content in the novel vials was higher than is usually observed in ampoules. It was proposed that the preferred candidate medium- and low-titre VP40 samples plus a negative sample should be used to form a reference panel for use by laboratories in the qualitative assessment of assay performance in detecting VP40, with the potential limitations of the materials to be made clear to end users. The panel would not be suitable for assessing assays used to detect EBOV antigens other than VP40, and further work would be needed to produce reference materials for glycoprotein and nucleoprotein assays.

The Committee discussed the suitability of VP40 expressed in bacterial cells as a reference material for assays for point-of-care use. It was recommended that this aspect should be highlighted in the Instructions for Use of the proposed reference panel, and that the future inclusion of reference reagents for VP40 expressed in mammalian cells should be considered.

The Committee considered the report of the study (WHO/BS/2016.2302) and recommended that the candidate materials be established as the First WHO Reference Panel for Ebola virus VP40 antigen with the provision that it is clearly indicated that the material was only suitable for VP40 assays and that some assays might fail to detect a non-envelope-associated antigen.

7.1.3 First WHO reference reagents for dengue virus serotypes 1–4 RNA for NAT-based assays

Dengue is a mosquito-borne disease that affects more than 100 tropical and subtropical countries. An estimated 390 million infections occur each year caused by any one of four closely related flaviviruses (DENV serotypes 1–4). Infection with any of the four serotypes can be asymptomatic in approximately 80% of infected individuals, or can result in dengue fever, an influenza-like illness that may progress to severe dengue, a potentially life-threatening condition. DENVs are also transmissible through the transfusion of blood and blood components, and by solid organ transplant, thus posing a risk to recipients. NAT-based assays are considered to be the most appropriate approach for blood donor screening
for recent DENV infection, and a proposal by the United States Food and Drug Administration to prepare standards for use in this area had been endorsed by the Committee in 2009.

Four candidate materials (one for each of the serotypes 1–4) had been prepared, heat-inactivated and diluted into a base matrix at a concentration of approximately 6 log10 PCR-detectable units/ml. Both liquid frozen and lyophilized materials were prepared for each serotype (2000 and 8000 vials respectively). The materials were then evaluated in an international collaborative study during which 21 laboratories in 15 countries returned data. A wide range of extraction and amplification methods were used, with most assays using a different target region for amplification. Observed variations in intra-laboratory estimates indicated a lack of reproducibility even within the same test, and considerable variability was also observed in data sets produced by two of the four laboratories that had used quantitative methods. However, despite these issues, all assays were able to detect the viral RNA of each serotype, with negative samples also correctly identified. Proposed potency estimates were assigned to the proposed reference materials for each serotype with values reported as NAT-detectable units (NDUs) and a 95% confidence interval established for each data value.

The Committee agreed that there was a need for these materials. During discussion, the subject of value assignment was raised, and in particular the potential for confusion which may arise from the use of the term “NDU”. There was also much discussion as to whether the materials should be established as an international reference panel, as four separate reference reagents or as international standards for each serotype, with a case for each option being made. Following further deliberation, the Committee concluded that the materials should be established as four separate WHO reference reagents, each with an assigned value (“unit”) without a cited confidence interval. It was noted that these reference materials could be further developed as DENV serotype-specific international standards based on future commutability studies.

The Committee considered the report of the study (WHO/BS/2016.2299) and recommended that the candidate materials be established as four separate WHO reference reagents for DENV RNA for NAT-based assays with the following assigned values: (a) DENV-1 RNA – 13 500 units/ml; (b) DENV-2 RNA – 69 200 units/ml; (c) DENV-3 RNA – 23 400 units/ml; and (d) DENV-4 RNA – 33 900 units/ml.

7.1.4 **Fourth WHO International Standard for hepatitis B virus DNA for NAT-based assays**

HBV infection remains a major public health problem worldwide despite the availability of an effective vaccine and antiviral therapies. More than 240 million
people worldwide are estimated to be chronically infected, with 0.5–1 million people dying annually as a result of serious liver disease. The virus is transmitted in blood and body fluids, perinatally and through close person-to-person contact in early childhood (in regions with high HBV prevalence), as well as through infected needles and sexual contact (in regions with low HBV prevalence). NAT-based assays for HBV were first introduced for blood screening in 1997, and are now implemented in at least 30 countries worldwide. The current WHO international standard is used by in vitro diagnostic device manufacturers, blood transfusion centres, clinical laboratories and regulatory authorities to calibrate secondary reference materials and to validate HBV NAT-based assays.

During production of the current Third WHO International Standard for hepatitis B virus for NAT-based assays, established in 2011, a second batch of identical bulk material was produced but was lyophilized at a different time point. This material (NIBSC code 10/266) was assessed as part of the 2011 study and was deemed at the time to be a suitable candidate material for developing a replacement standard when required.

The candidate material was therefore included – along with the current international standard, three commutability samples and three materials for secondary reference material calibration – in an international collaborative study. Thirteen laboratories participated in the study with 14 data sets returned (13 from quantitative assays and one from a qualitative assay). Potency was assessed against the current international standard, with the overall mean estimates derived for both it and the candidate material being very similar to those seen in the 2011 study. Ongoing stability studies suggested that the proposed material remains stable. Given the close alignment of data between the two studies it was proposed that the unit assigned to the replacement material should be derived from the study data of 2011 – namely, $5.98 \log_{10} \text{IU/ml}$ – to reduce any potential drift in the IU value.

The Committee noted that this was a good example of a project that could be assessed in principle during meetings of the WHO network of collaborating centres for blood products and in vitro diagnostics (see section 2.2.3 above) with the aim of submitting a short one-page proposal to the Committee for final approval. The WHO Secretariat commented that the study had indeed been presented at the network meeting held earlier in the year. As no issues had been raised at that time, a reduced presentation time had been allocated for the topic during the current Committee meeting, thus demonstrating the potential efficiency gains of such an approach.

The Committee considered the report of the study (WHO/BS/2016.2291) and recommended that the candidate material 10/266 be established as the Fourth WHO International Standard for hepatitis B virus DNA for NAT-based assays with an assigned value of $5.98 \log_{10} \text{IU/ml}$.
7.1.5 Fourth WHO International Standard for prolactin (pituitary, human)

Prolactin immunoassays are used to evaluate pituitary gland function and to monitor prolactinomas. The majority of immunoassays are calibrated with respect to the current Third WHO International Standard for prolactin, human, stocks of which were now running low. A candidate material (NIBSC code 83/573) was therefore prepared under similar conditions to the current international standard but using a different donated source of pituitary prolactin. Following its initial formulation in the mid 1980s this candidate material had previously been assessed twice in different collaborative studies – once in 1986 during evaluation of both the second and third international standards by radioimmunoassay, and once in 2001 during the assessment of a recombinant reference reagent immunoassay.

In the current international collaborative study, 10 laboratories from seven countries provided data using 11 different methods. In addition to the candidate study samples received by all laboratories, which included the First WHO Reference Reagent for prolactin (recombinant, human), some laboratories also received human serum samples with normal and high prolactin concentrations. Study results indicated that the evaluated candidate material gave a value of 67.2 mIU/ampoule with a GCV of 8.1%. The observed stability of the material was also good with a predicted loss of immunoreactivity of 0.007% per year at −20 °C.

The issue of commutability was addressed by assessing the inter-laboratory variability of results obtained for serum standards reported in terms of the kit standards and by evaluating sources of potential bias. This analysis was performed for both the candidate material and the recombinant reference material. The outcomes of both assessments showed that the candidate material was commutable – as was the recombinant material, which could act as a replacement preparation in the future.

The Committee noted that one laboratory had reported only limited commutability and suggested that further work should be undertaken with the assay manufacturer to understand the reason for this. The Committee also questioned whether manufacturers use recombinant materials for their kit controls and were informed that end users are not provided with this information. The Committee commended the commutability work undertaken for this study but noted that problems in sourcing suitable materials for commutability assessments in this area should not be underestimated.

The Committee considered the report of the study (WHO/BS/2016.2292) and recommended that the candidate material 83/573 be established as the Fourth WHO International Standard for prolactin (pituitary, human) with an assigned value of 67 mIU/ampoule.
7.1.6 First WHO Reference Panel for the Janus kinase 2 V617F gene mutation

Janus kinase 2 is a non-receptor tyrosine kinase encoded by the JAK2 gene and is involved in cytokine receptor signalling in the JAK/STAT pathway – particularly in relation to the production of blood cells from haematopoietic stem cells. Chronic myeloproliferative neoplasms (MPNs) are associated with malfunctioning blood cell production and include polycythaemia vera (PV), essential thrombocythaemia (ET), idiopathic myelofibrosis (IM) and chronic myeloid leukaemia (CML). The JAK2 V617F mutation was discovered in 2005 and is present in > 95% of PV patients and in 50–60% of patients with either ET or IM. The mutation results in constitutive activation of JAK2 and increased blood cell production. WHO classification criteria for MPNs include JAK2 analysis as a requirement. Furthermore, ruxolitinib – a small-molecule JAK2 V617F inhibitor – is approved by several NRAs, and other drugs and therapies are in development. The clinically actionable nature of such genotyping highlights the need to identify JAK2 V617F-positive patients, and to monitor treatment response and remission via JAK2 V617F quantitation. To achieve this goal, accurate and sensitive testing is essential.

During an international collaborative study involving 29 laboratories, a panel of materials (NIBSC panel code 16/120) comprising seven members of differing wild-type/mutant ratio was formulated from a lymphoblastoid cell line (wild-type) and a UKE-1 cell line established from the peripheral blood of an acute myeloid leukaemia patient. The final formulation of each panel member was approximately 5 µg of genomic DNA freeze-dried into glass ampoules, with each panel member assigned a separate code number and the study conducted blind. The panel was assessed using a range of assays, where possible using quantitative methods. All data sets returned accurately quantified both extremities of the panel (that is, 100% and 0%) with small deviations from the expected values observed for other ratios. The final panel, of which 1300 were produced, was proposed for establishment with genomic JAK2 V617F DNA concentrations of 0, 0.03, 1.0, 10.8, 29.6, 89.5 and 100%. The stability of all panel members was assessed for up to 8 months and shown to be acceptable.

The Committee queried the apparent large gap in the proposed concentration range of the panel – with the range between 30% and 90% lacking representation. It was clarified that this range only accounted for 0.5 log₁₀, which equated to only a very small factorial difference. It was also highlighted that the panel was intended for use in the calibration of secondary materials, and therefore such a range should allow for a suitable calibration curve to be generated.
The Committee considered the report of the study (WHO/BS/2016.2293) and recommended that the proposed materials be established as the First WHO Reference Panel for the Janus kinase 2 V617F gene mutation with the genomic DNA concentrations proposed above.

7.2 Proposed new projects and updates – in vitro diagnostics

7.2.1 Update on the proposed First WHO International Standard for Ebola virus antibodies (plasma, human)

The Committee was provided with an update on recent efforts to develop a First WHO International Standard for Ebola virus antibody (plasma, human) and associated reference panel (WHO/BS/2016.2301). The Committee was reminded that a convalescent plasma from the American Red Cross had been established as an interim First WHO Reference Reagent for Ebola virus antibodies in 2015. However, the proposed candidate material for establishment as a first international standard, subject to satisfactory outcomes from a recent international collaborative study, was pooled patient plasma from recovered Ebola patients in Sierra Leone, where treatment history is unknown but where a greater volume of material was available. Other study materials had included a series of convalescent plasmas from four patients who had received different drug therapies, and two purified monoclonal antibodies.

The study involved 17 laboratories from four countries with a total of 26 data sets being returned using a range of different assay methods. Each laboratory had received nine blinded study samples and was asked to perform serial dilutions of these in three independent assays. As not all studies had yet been completed, additional data would soon become available for analysis. In the meantime, results collated to date indicate that most assays targeted the glycoprotein region of the virus genome, with significant variability in the results obtained. This was unexpected based on the initial in-house validation of the materials used. Additional data also need to be generated on the stability of the candidate reference preparation. Once all analysis is completed, a report will be prepared.

The Committee suggested that in the final report potency should be assigned relative to the interim standard. It was then proposed that, as the finalization of the report would occur well in advance of the 2017 meeting of the Committee, the material could be made available before its formal establishment as an international standard. Following some discussion around this point, it was agreed that the material should remain labelled as a “candidate standard” with a value assigned in units and not IUs – which could be changed if the Committee formally established the material in 2017. Accordingly, the Committee recommended that, following the completed analysis, the candidate
material be made available to the community in advance of its meeting in 2017. The material should be labelled as a candidate standard with a value assigned in units until its formal establishment.

7.2.2 Proposed First WHO International Standard for Zika virus antibodies (immunoglobulin G and immunoglobulin M) (human)

The accurate diagnosis of ZIKV infection, particularly in pregnant women, is a crucial step in making appropriate health-care decisions. However, the virus has a short period of detection in plasma, and PCR-based tests of plasma are thus only useful during this period (approximately 9 days following the onset of symptoms). After a negative PCR result, diagnosis is made on the basis of serological testing that measures antibody responses to the virus. Current serological tests are prone to high cross-reactivity against other flaviviruses (particularly DENVs) spread by the same mosquito vector. To ensure accurate diagnosis, the standardization of tests is required to improve both their sensitivity and specificity. Both immunoglobulin G and immunoglobulin M are currently measured to determine prior exposure, where immunoglobulin M would indicate a recent infection. To support the WHO response to the ZVD outbreak NIBSC had initiated development of an international standard for use in the calibration and control of ZIKV antibody assays.

The intended use of such a material would be the calibration of ELISAs and neutralization assays used to measure ZIKV antibody levels (immunoglobulin G and immunoglobulin M) in human serum. The recommendation to use the candidate material for this purpose will be subject to the satisfactory demonstration of commutability. Primary users will be public health and other clinical laboratories, kit manufacturers and ZIKV vaccine manufacturers (for clinical trials).

The Committee expressed concern over reported delays and problems encountered in receiving positive plasma donations from countries with infected individuals. It was noted that difficulties were also encountered in sourcing ZIKV antibody-positive plasma that was negative for antibodies to other flaviviruses. The Committee endorsed the proposal (WHO/BS/2016.2298 Rev.1) to develop a First WHO International Standard for Zika virus antibodies (immunoglobulin G and immunoglobulin M) (human).

7.2.3 Proposed First WHO International Standard for chikungunya virus antibodies (immunoglobulin G and immunoglobulin M) (human)

The mosquito-borne CHIKV is a member of the Alphavirus genus in the Togaviridae family. Chikungunya was first identified in the United Republic of Tanzania in the early 1950s. The virus is present not only in Africa but also in
Asia and the Indian subcontinent and, since 2013, has spread to the Americas, particularly central and southern areas. Small outbreaks have also occurred recently in Europe. The diagnosis of CHIKV infection requires a variety of tests, including the detection of immunoglobulin M and immunoglobulin G antibodies. The co-circulation of CHIKV with DENV and ZIKV frequently occurs and infections caused by these viruses share common signs and symptoms in infected patients.

The accurate diagnosis and discrimination of CHIKV infection from other virus infections is thus vital for patient care. Analysis of immunoglobulin M antibodies is particularly useful for the confirmation of acute infection. Anti-CHIKV immunoglobulin G may also be detectable during acute infection, but is also a marker of past CHIKV infection and of seroprevalence. Anti-CHIKV assays vary in their performance, and Alphavirus serological cross-reactivity is known to exist with members of the Semliki Forest serocomplex.

A collaborative study was therefore proposed to evaluate sera from CHIKV-infected patients (and potentially from blood donors) for their suitability for use as an international standard. The samples would include both immunoglobulin G and immunoglobulin M reactive sera to distinguish between recent and past infections.

The Committee was informed that sera would be sourced through national and international collaborations, with the proposed project complementing a previously endorsed and ongoing CHIKV RNA project. The Committee endorsed the proposal (WHO/BS/2016.2298 Rev.1) to develop a First WHO International Standard for chikungunya virus antibodies (immunoglobulin G and immunoglobulin M) (human).

7.2.4 Proposed Second WHO International Standard for syphilitic plasma (immunoglobulin G and immunoglobulin M) (human)

Syphilis is a sexually transmitted disease caused by spirochetes of the species *Treponema pallidum* subsp. *pallidum*. The Committee was informed that recent reports indicate that the incidence of syphilis had risen in both developed and developing countries in recent years. This fact, plus the projected imminent depletion of both the current First International Standard for human syphilitic plasma (immunoglobulin G and immunoglobulin M) and the First International Standard for human syphilitic plasma (immunoglobulin G), necessitated the replacement of reference materials in this area.

A number of candidate materials were therefore currently being evaluated at NIBSC for their suitability as replacement standards. A proposed international collaborative study was anticipated to take place in 2017, with submission of its report to the Committee in 2018. Anticipated users of these
The Committee recognized the need for the proposed replacement standards and enquired whether the proposed study would include specific and nonspecific assays, and if so whether any difference between the two was expected. The Committee was informed that as was usual practice a wide range of assays would be included in the collaborative study and any differences between them highlighted in the outcome report. The Committee then endorsed the proposal (WHO/BS/2016.2298 Rev.1) to develop a Second WHO International Standard for syphilitic plasma (immunoglobulin G and immunoglobulin M) (human).

7.2.5 Proposed Second WHO International Standard for syphilitic plasma (immunoglobulin G) (human)

The Committee endorsed the proposal (WHO/BS/2016.2298 Rev.1) to develop a Second WHO International Standard for syphilitic plasma (immunoglobulin G) (human) (see section 7.2.4 above).

7.2.6 Proposed Second WHO International Standard for human immunodeficiency virus type 2 RNA for NAT-based assays

The incidence of HIV type 2 (HIV-2) is lower than that of HIV-1 and typically follows a different course of infection, with lower viral loads and a higher percentage of longer term non-progressors. As not all treatments established for HIV-1 are effective against HIV-2, the accurate and timely diagnosis of HIV-2 infection is crucial in ensuring use of the correct therapy and in informing individuals of their infection status to prevent the risk of unknown transmissions. In addition, although screening for HIV-2 RNA is not mandated in as many countries as for HIV-1 due to the lower incidence of infection and lower titres observed in infected individuals, accurate and sensitive assays are vital in ensuring the safety of the blood supply in countries where HIV-2 is prevalent.

Although supplies of the First International Standard for HIV-2 RNA established in 2009 remain plentiful, reports from users have suggested that the assigned unitage of 103 IU/ml is too low to create a calibration curve. This standard is required to calibrate HIV-2 NAT-based assays and secondary HIV-2 NAT standards and is used by test kit manufacturers, blood fractionators, reference and diagnostic laboratories, external quality assurance (EQA) providers and the OMCL network.

An international collaborative study was therefore proposed in order to formulate a replacement standard at a high titre (in the order of $5 \times 10^6 \log_{10}$). Where possible the collaborative study would also include a clinical sample for commutability assessment, although sourcing such material may prove problematic. The stock material to be used would be derived from the same
batch of tissue-culture-grown virus (HIV-2 CAM-2) as was used for the current international standard. Approximately 500 vials of lyophilized material would be produced.

The Committee endorsed the proposal (WHO/BS/2016.2298 Rev.1) to develop a Second WHO International Standard for human immunodeficiency virus type 2 RNA for NAT-based assays.

7.2.7 Proposed First WHO International Standard for respiratory syncytial virus RNA for NAT-based assays

Respiratory syncytial virus (RSV) and influenza virus types A and B cause respiratory tract infections which are often more severe in vulnerable populations such as the elderly or the very young. In both these cases, the diagnostic field is moving towards molecular methods of detection due to their greater sensitivity compared to other methods. However, EQA data have indicated large variations in reported titres, highlighting the need for improved standardization in this area.

The Committee was informed that preliminary work was required to determine the most suitable influenza A variants for inclusion as candidate materials in the proposed parallel collaborative study. As the conserved regions of the genome appear to be used for molecular detection, a panel of variants could be used to assess this in a small pilot study. There was also a need to determine whether a single RSV type (A or B) could be used for the harmonization of both types. In addition, the sourcing of suitable clinical samples in relevant matrix types in sufficient volumes to assess commutability may be problematic given the limited supply of such materials. The candidate reference materials themselves would be formulated from tissue-culture-grown virus, spiked into a universal buffer.

The Committee discussed the impact of the genetic diversity of these viruses on detection efficiency, and a panel-based approach was proposed as one way of addressing this issue. However, the longevity of such a panel for influenza was questioned should conserved regions of virus variants exhibit differing amplification abilities. Variants chosen for a standard may not in this instance be future-proof. This point was accepted and it was suggested that producing batches to last up to 5 years (instead of > 10 years) may be prudent to allow for reformulation. In addition, a different form of continued analysis may be required to determine whether the selected strain remained suitable for standardization purposes. It was suggested that this may be achieved by annual inclusion in an EQA scheme. It was further proposed that egg-grown influenza viruses be used as candidate materials instead of the originally proposed laboratory-adapted tissue-culture-grown viruses.

The Committee endorsed the proposal (WHO/BS/2016.2298 Rev.1) to develop a First WHO International Standard for respiratory syncytial virus RNA for NAT-based assays.
7.2.8 **Proposed First WHO International Standard for influenza virus type A RNA for NAT-based assays**

The Committee endorsed the proposal (WHO/BS/2016.2298 Rev.1) to develop a First WHO International Standard for influenza virus type A RNA for NAT-based assays (see section 7.2.7 above).

7.2.9 **Proposed First WHO International Standard for influenza virus type B RNA for NAT-based assays**

The Committee endorsed the proposal (WHO/BS/2016.2298 Rev.1) to develop a First WHO International Standard for influenza virus type B RNA for NAT-based assays (see section 7.2.7 above).

7.2.10 **Proposed First WHO Reference Panel for the BRAF V600E gene mutation**

BRAF V600E is a mutation of the BRAF gene known to be responsible for a high percentage of malignant melanomas as well as other solid tumours. As several drugs are approved for the treatment of metastatic melanoma patients with known BRAF V600 mutations, accurate and sensitive assays for screening melanoma cases are required. As the incidence and mortality rates of melanoma have risen sharply throughout the world over the past few decades – with > 130 000 new cases of melanoma diagnosed globally each year – there is a strong public health need to improve the quality of diagnosis and treatment.

It is proposed that two genomic DNA materials are produced – the BRAF wild-type and the BRAF V600E – with a view to evaluating the suitability of a candidate panel based on ratio dilutions of the two materials as a reference material. By providing purified genomic DNA prepared from cell lines, a reproducible replacement strategy would appear to be possible. Envisaged users include manufacturers (for the calibration of diagnostic kits) and clinical and reference laboratories (for the calibration of secondary standards used in multiple routine diagnostic assays for BRAF V600E detection). The project is predicted to progress such that the results for the panel to be established would be submitted to the Committee in 2019.

The Committee queried the suitability of the proposed format of the study as a mimic of the way in which material would normally be assessed in the laboratory – with users typically extracting diagnostic material from formalin-fixed tissue blocks. In response, it was pointed out that this method of tissue preparation is known to be highly variable and therefore introducing such a step would be likely to add undesirable variability to the proposed evaluation. It was therefore agreed that the candidate standardized DNA material would be provided as initially proposed and then used in assays with material obtained post-extraction from formalin-fixed blocks. The inclusion of an additional panel
member at a 10–20% dilution ratio was suggested. There was also some discussion concerning the decision not to include lyophilized cells, with the exclusive use of extracted DNA viewed as a positive step in assay standardization. It was clarified that the cell line used in the development of the BRAF material had been derived from an Epstein-Barr virus-positive cell line and it would not be appropriate to supply laboratories with a class-2 pathogen in addition to the genetic material, with variability in DNA extraction methods additionally compromising the reference value of the DNA standard.

The Committee endorsed the proposal (WHO/BS/2016.2298 Rev.1) to develop a First WHO Reference Panel for the BRAF V600E gene mutation.

7.2.11 Proposed First WHO Reference Panel for ErbB2 copy number and mRNA expression

Human epidermal growth factor receptor 2 (HER2) is a member of the epidermal growth factor receptor family of receptor tyrosine kinases and is coded for by the ErbB2 gene. This gene is amplified (and thus the HER2 protein over-expressed) in 18–20% of breast cancers, and is associated with increased tumour aggressiveness. HER2 over-expression is also associated with more aggressive forms of ovarian, stomach and uterine cancer. The patent for the biotherapeutic product Herceptin (the approved treatment for HER2 over-expression) has now expired in Europe and will expire in the United States in 2019. It is therefore likely that SBPs will now become available. Nucleic-acid-based screening methods are increasingly used for diagnosis and have proved to be more sensitive than immunohistochemistry and in situ hybridization.

The proposed study will investigate several candidate HER2-positive cells lines as well as a wild-type cell line. The intention is that one HER2-positive high mRNA expression cell line will be selected and then serially diluted in the wild-type cell line prior to lyophilization. This approach will allow for analysis of both genomic DNA copy number and mRNA expression. Although another agency (NIST) has prepared a genomic standard for HER2 this is for genomic DNA measurement only. Envisaged users include manufacturers (for the calibration of diagnostic kits) and clinical and reference laboratories (for the calibration of secondary standards used in multiple routine diagnostic assays for ErbB2 characterization). The project is predicted to progress such that the results for the panel to be established would be submitted to the Committee in 2020.

The Committee endorsed the proposal (WHO/BS/2016.2298 Rev.1) to develop a First WHO Reference Panel for ErbB2 copy number and mRNA expression.
8. International reference materials – vaccines and related substances

All reference materials established at the meeting are listed in Annex 11.

8.1 Proposed new projects and updates – vaccines and related substances

8.1.1 Proposed First WHO International Standard for Sabin inactivated poliomyelitis vaccine

The production of IPV using Sabin live-attenuated strains (sIPV) instead of wild-type poliovirus strains is considered to be safer in the context of containment requirements for the endgame of polio eradication. However, in contrast to traditional IPV, the human dose for sIPV products is significantly different between manufacturers. There is no current international standard for sIPV – complicating the standardization of both in vitro and in vivo potency assays and making the comparison of different sIPV products difficult.

A preliminary collaborative study was undertaken to assess whether the current Third WHO International Standard for inactivated poliomyelitis vaccine would be suitable for use with sIPV. Results showed that this current international standard was not universally suitable for determining the D-antigen potency of sIPV products as consistency between laboratories was only achieved when using common reagents in the ELISA method. It was concluded that an international standard specific for sIPV would be more appropriate.

The sIPV vaccine plays a key role in the global eradication of polio and there are numerous sIPV manufacturers worldwide. The provision of an international standard will support the standardization of potency assays of both licensed and newly developed sIPVs and assist manufacturers, NCLs and the OMCL network in evaluating this crucial vaccine. Ensuring the availability of safe and effective vaccines during the endgame of polio eradication, and in the post-eradication era, is a recognized key public health goal.

Following discussion and clarifications, the Committee endorsed the proposal (WHO/BS/2016.2295) to develop a First WHO International Standard for Sabin inactivated poliomyelitis vaccine, and agreed that NIBSC should seek donations of sIPV concentrate from manufacturers worldwide as soon as possible.

8.1.2 Proposed First WHO international standards for Group B streptococcus (polysaccharide and antiserum)

Two Group B streptococcus (GBS) polysaccharide-conjugate vaccines based on the five most common serotypes (Ia, Ib, II, III and V) were now being developed by two different manufacturers. Given the very low incidence of this disease
(around 3 per 1000 live births) these vaccines were likely to be licensed on the basis of surrogates of protection (immunoglobulin G level and assessment of functional antibody by opsonophagocytosis assay) rather than through very large clinical protection studies. There is, therefore, an urgent need to establish standardized assays for the quality control of such vaccines and to measure the concentration and functional activity of antibodies against GBS in the sera of immunized individuals.

The development of several standards and reference reagents (polysaccharide standards and human reference serum) is required. The polysaccharide standards would be used to calibrate internal controls in various physicochemical and immune assays used in the quality control of GBS vaccines (identity, total and free polysaccharide) and as antigens to measure antibody responses. Human reference sera will be used as standards in assays for the quantification of antibody response and for measurement of functional activity in clinical trials materials. It was expected that vaccine manufacturers would donate the polysaccharides and that human serum reference materials would be obtained from volunteers immunized with the conjugate vaccine. It was proposed that monovalent sera already available at NIBSC should be used to calibrate the human reference serum. These monovalent sera have been calibrated by radio-immunoassays for antibody concentration and have been looked upon as gold standards in various studies to establish correlates of protection, and by manufacturers in evaluating immune responses during clinical trials.

It was expected that the availability of relevant international standards would expedite the development and licensing of GBS vaccines for immunizing pregnant women in order to prevent GBS in newborns, as well as facilitate ongoing quality control testing should such vaccines be licensed. Envisaged users include vaccine and diagnostic kit manufacturers, public and national health authorities, academic researchers and NCLs.

Following discussion and clarifications, the Committee endorsed the proposal (WHO/BS/2016.2295) to develop First WHO international standards for Group B streptococcus (polysaccharide and antiserum).

8.1.3 Proposed Second WHO International Standard for diphtheria antitoxin (equine)

Diphtheria antitoxin is an essential medicine for diphtheria therapy and is used in countries where disease is endemic. In countries with good vaccination coverage, it is stockpiled for emergency use. In some parts of the world equine diphtheria antitoxin is used for diphtheria therapy and prophylaxis against suspected cases. It is licensed nationally in some countries and imported by others for stockpiling for emergency use. Stockpiled antitoxin is periodically assessed for potency by a number of national regulatory authorities and an antitoxin standard is essential for this purpose.
The current First International Standard for diphtheria antitoxin (equine) is a dried hyperimmune horse serum standard that was prepared in 1934 at the Statens Serum Institut. The IU is defined as the activity contained in 0.0628 mg of the dried serum. Approximately every 2 years this dried serum is used to prepare a standard preparation in 66% v/v glycerol/saline. The standard is then distributed in vials containing 10 ml at 10 IU/ml. The stock of the original dried serum is now running low. Despite being an old standard, a replacement is needed as it is still used in some parts of the world. Although a First WHO International Standard for human diphtheria antitoxin was established in 2012 there is a need for an equine standard for the calibration of equine products. As minimum requirements for antitoxin potency (expressed in IU) exist in many national and regional pharmacopoeias it is important to ensure continuity of the IU for equine diphtheria antitoxin through the establishment of a second international standard.

NIBSC proposes to prepare a freeze-dried standard to provide a single homogeneous batch that can be used for a number of years. The standard is intended to be used to calibrate potency assays for diphtheria antitoxin. These assays include lethal and non-lethal toxin neutralization tests performed in guinea-pigs and are used to determine the antitoxin potency of therapeutic antitoxin products produced from equine serum. A global shortage of equine diphtheria antitoxin has meant that source material has been difficult to identify and cannot be freely donated. It was now expected to be sourced from an Indian manufacturer. The proposed batch size was ≥ 2500 vials, which at the current rate of use (approximately 50–100 vials per year) would be sufficient for more than 20 years.

The Committee endorsed the proposal (WHO/BS/2016.2295) to develop a Second WHO International Standard for diphtheria antitoxin (equine).

8.1.4 Proposed First WHO international standards for antibodies against human papillomavirus types 6, 11, 31, 33, 45, 52 and 58

A second-generation vaccine against human papillomavirus (HPV), the cause of cervical cancer, containing nine different HPV types had now been licensed and other second-generation vaccines were under clinical development. Assessing their immunogenicity will be crucial in defining a correlate of protection and in monitoring their quality and performance in different populations. There were also increasing demands to standardize HPV serological methods for measuring past or present HPV infection in epidemiological studies as this is a key element in the planning and follow-up of optimal HPV-control programmes.

WHO international standards for antibodies against the high-risk types HPV16 and HPV18 were established in 2009 and 2012, respectively, and their use has been shown to improve inter-laboratory comparisons. The development and establishment of international standards for antibodies against low-risk types
HPV6 and HPV11 and against high-risk types HPV31, HPV33, HPV45, HPV52 and HPV58 would provide the full complement of international standards for use in standardizing HPV serology assays. The users of such reference materials would include vaccine developers and manufacturers, epidemiologists, research laboratories, public health laboratories and developers of assay kits.

For each anti-HPV type, donations will be obtained from at least two individuals naturally infected with the HPV type of interest. Sera would preferably be reactive with only one genital HPV type, with donation suitability assessed by testing for antibodies against multiple genital HPV types.

The International HPV Reference Center, Karolinska Institute will be involved in enrolling and selecting suitable donors, with candidate sera having also been obtained from collaborators at the National Institutes for Food and Drug Control, China. NIBSC will undertake filling and freeze-drying operations for each candidate standard according to standard operating procedures. The freeze-dried candidates will then be assessed in an international collaborative study involving 10–15 laboratories using a range of assays. Assay data will be analysed at NIBSC using standard statistical techniques. Stability studies on the candidate standards will also be carried out in the usual way. It was expected that sourcing suitable samples from naturally infected individuals to produce the seven international standards may be the rate-limiting step for this project.

Following discussion and clarifications, the Committee endorsed the proposal (WHO/BS/2016.2295) to develop WHO international standards for antibodies against human papillomavirus types 6, 11, 31, 33, 45, 52 and 58.

8.1.5 Proposed Second WHO International Standard for hepatitis A vaccine

Vaccines against the hepatitis A virus (HAV) play a crucial role in the prevention of HAV infection and are used globally, predominantly as travel vaccines. The potencies of these vaccines are expressed in units unique to each manufacturer and standardization requires the availability of an appropriate reference material. The First WHO International Standard for inactivated hepatitis A vaccine established in 1999 is used by manufacturers and control laboratories for the calibration and validation of in-house standards for use in the determination of the HAV antigen content of commercial vaccines. Potency determination is usually carried out by ELISA or the mouse potency immunogenicity test. As stocks of the current international standard were now running low there was a need to consider a replacement strategy.

The collaborative study proposed by NIBSC would assign potency in IU to the replacement standard using in vitro ELISAs and the mouse immunogenicity assay. One issue to be addressed is the sourcing of the candidate material. Originally, the only suppliers were European, but since there were now several global vaccine manufacturers it will be important to see
whether a candidate material can be used to evaluate vaccine produced using different HAV strains, different cell lines and different manufacturing processes. The stability profile of candidate bulk material obtained from new producers during storage at −70 °C will also need to be studied since concentrated bulk is normally stored at 4 °C.

The Committee agreed that there was a need to start the replacement process for this important international standard before stocks of the current international standard are exhausted, and endorsed the proposal (WHO/BS/2016.2295) to develop a Second WHO International Standard for hepatitis A vaccine.

8.1.6 Proposed WHO international standards for oral poliomyelitis vaccine

Following the significant progress made towards eliminating wild-type poliovirus transmission, and the global eradication of serotype 2 polio, OPV2 has now been withdrawn and the trivalent OPV (a blend of poliovirus serotypes 1, 2 and 3) replaced by monovalent and bivalent vaccines without the serotype 2 component. In addition, containment requirements for serotype 2 poliovirus mean that the current trivalent WHO international standard for the assay of OPV can no longer be used. In order to provide suitable OPV reference materials for current vaccines, vaccine stockpiles and vaccines in development a number of new OPV international standards are now required.

NIBSC intends to produce international standards for bivalent OPV (containing serotypes 1 and 3) and for monovalent OPV1, 2 and 3 vaccines. It was expected that these new international standards would be used: (a) in potency assays of current OPV; (b) in the assessment of monovalent bulk titres; (c) for the titration of dose preparations for neurovirulence testing; (d) for the validation of in-house reference preparations; and (e) as an important tool in the preparation of new sIPV bulks. Expected users of the new OPV international standards included manufacturers, NCLs and consortia developing new and safer vaccine strains for use following polio eradication. The proposed collaborative study would assign potency in $\log_{10}/CCID_{50}$ for each of the serotypes based on infectivity assays using the Hep2C cell system.

Provision of these international standards will therefore support the standardization of potency assays of new OPVs globally, aid NCLs in the control of OPV and facilitate the maintaining of vaccine stockpiles, all of which will help to ensure that safe and effective vaccines are available for disease prevention and control during the endgame of polio eradication and in the post-eradication era. The Committee was informed that monovalent poliovirus serotypes 1, 2 and 3 are already available at NIBSC and it was proposed that this project should take a fast-track approach, while recognizing that containment issues linked to GAPIII would be a potential issue, particularly for the polio serotype 2 standards.
The Committee fully supported the adoption of a fast-track approach bearing in mind the urgent need for such standards and their importance to the global polio eradication programme, and endorsed the proposal (WHO/BS/2016.2295) to develop WHO international standards for oral poliomyelitis vaccine.
Annex 1

WHO Recommendations, Guidelines and other documents related to the manufacture, quality control and evaluation of biological substances used in medicine

WHO Recommendations, Guidelines and other documents are intended to provide guidance to those responsible for the production of biological substances as well as to others who may have to decide upon appropriate methods of assay and control to ensure that products are safe, reliable and potent. WHO Recommendations (previously called Requirements) and Guidelines are scientific and advisory in nature but may be adopted by a national regulatory authority (NRA) as national requirements or used as the basis of such requirements.

Recommendations concerned with biological substances used in medicine are formulated by international groups of experts and are published in the WHO Technical Report Series¹ as listed below. A historical list of Requirements and other sets of Recommendations is available on request from the World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland.

Reports of the WHO Expert Committee on Biological Standardization published in the WHO Technical Report Series can be purchased from:

WHO Press
World Health Organization
20 avenue Appia
1211 Geneva 27
Switzerland
Telephone: + 41 22 791 3246
Fax: +41 22 791 4857
Email: bookorders@who.int
Website: www.who.int/bookorders

Individual Recommendations and Guidelines may be obtained free of charge as offprints by writing to:

Technologies Standards and Norms
Department of Essential Medicines and Health Products
World Health Organization
20 avenue Appia
1211 Geneva 27
Switzerland

¹ Abbreviated in the following pages to TRS.
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Annex 2

Guidelines on evaluation of monoclonal antibodies as similar biotherapeutic products (SBPs)

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Abbreviations

ACR20  American College of Rheumatology 20% improvement criteria
ADA    anti-drug antibody
ADCC   antibody-dependent cellular cytotoxicity
ADCP   antibody-dependent cellular phagocytosis
AUC    area under the curve
CDC    complement-dependent cytotoxicity
CHO    Chinese hamster ovary
CLB    competitive ligand-binding (assay)
CR     complete response
CRP    C-reactive protein
DAS28  disease activity score in 28 joints
DCVMN  Developing Countries Vaccine Manufacturers Network
EGFR   epidermal growth factor receptor
ESR    erythrocyte sedimentation rate
IFPMA  International Federation of Pharmaceutical Manufacturers & Associations
IgE    immunoglobulin E
IgG    immunoglobulin G
IGPA   International Generic Pharmaceutical Alliance
mAb    monoclonal antibody
MOA    mechanism of action
NRA    national regulatory authority
ORR    overall response rate
pCR    pathological complete response
PD     pharmacodynamics
PK     pharmacokinetics
RBP    reference biotherapeutic product
rDNA   recombinant deoxyribonucleic acid
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<td>similar biotherapeutic product</td>
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<td>TK</td>
<td>toxicokinetics</td>
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<td>TMD</td>
<td>target-mediated disposition</td>
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<td>tumour necrosis factor</td>
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<td>TOST</td>
<td>two one-sided test</td>
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<td>VEGF</td>
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1. Introduction

Monoclonal antibodies (mAbs) are a major class of recombinant deoxyribonucleic acid (rDNA) technology-derived biotherapeutic products that have achieved outstanding success in treating many life-threatening and chronic diseases. Some of these targeted therapy products are ranked in the top-10 lists of annual global pharmaceutical revenue sources. As patents and data-protection measures on mAb products have expired, or are nearing expiry, considerable attention has turned towards producing similar biotherapeutic products (SBPs, also termed “biosimilars”) based upon the approved mAb innovator products, with a view to making more affordable products that could improve global access to these so-called blockbusters.

Therapeutic mAbs are preparations of an immunoglobulin or a fragment of an immunoglobulin with specificity for a target ligand and are derived from a single clone of cells. Each full-length molecule of a mAb consists of two heavy and two light polypeptide chains which are linked by disulfide bonds. MAbs have several possible functional domains within a single molecule. The defined specificity of a mAb is based on the binding region for an antigen that is located in the antigen-binding fragment (Fab) region. For full-length mAbs, their crystallizable fragment (Fc) region has the ability to bind to specific receptors, potentially leading to immune effector functions such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and antibody-dependent cellular phagocytosis (ADCP). Full-length mAbs are glycoproteins with glycosylation sites in the Fc region of the heavy chains, with further possible glycosylation sites depending on the type of molecule. Therefore, mAbs are highly complex biological macromolecules with size and charge variants, various post-translational modifications including different glycosylation patterns and N- and C-terminal heterogeneity, long half-lives and the potential to induce immunogenicity. Each individual mAb may therefore present a unique profile, which should be taken into consideration during the evaluation of such products as SBPs.

The WHO Guidelines on evaluation of similar biotherapeutic products (SBPs) were adopted by the WHO Expert Committee on Biological Standardization in 2009 (1). This document set out the scientific principles, including the stepwise approach, which should be applied for the demonstration of similarity between an SBP and the reference biotherapeutic product (RBP). High similarity at the quality level is regarded as a prerequisite for enabling the use of a tailored nonclinical and clinical programme for licensure. The goal of the clinical comparability exercise is to confirm the similarity established at previous stages of development and to demonstrate that there are no clinically meaningful differences between the SBP and the RBP – and not to re-establish safety and efficacy, as this has been done already for the RBP. The decision on
licensure of the SBP should be based upon evaluation of the totality of evidence from quality, nonclinical and clinical parameters. It should be noted that clinical studies cannot be used to resolve substantial differences in physicochemical characteristics and biological activity between the RBP and the SBP. If substantial differences in quality attributes are present, a stand-alone licensing approach may be considered.

The set of globally acceptable key principles outlined above for the regulatory evaluation and licensing of SBPs has served well as a basis for setting national requirements for SBPs. However, because of the structural complexity and heterogeneity of mAbs, their quality attributes can vary from product to product. Furthermore, one mAb product may have multiple indications. Therefore, biosimilar comparability studies between a candidate biosimilar mAb and a reference product mAb are challenging for both developers and regulators. Consequently, in 2014, WHO was requested to update its 2009 SBP Guidelines to take into account technological advances in the characterization of rDNA-derived products, and particularly mAbs. In response, WHO organized an informal consultation in 2015 on the possible amendment of the Guidelines, with an additional focus placed on SBPs containing mAbs. All participants, including national regulatory authorities (NRAs) and industry, recognized and agreed that the evaluation principles described in the WHO Guidelines were still valid, valuable and applicable in facilitating the harmonization of SBP requirements globally. It was therefore concluded that there was no need to revise the main body of the existing WHO Guidelines on SBPs. However, it was also agreed that, rather than an amendment, there was a need for additional guidance on the evaluation of biosimilar mAbs.

2. Purpose and scope

The intention of this class-specific document is to set out the specific considerations involved in the evaluation of mAbs developed as SBPs. These WHO Guidelines cover rDNA-derived biosimilar mAbs used in the treatment of human diseases. The principles discussed in this document also apply to mAb-derived proteins – for example, mAb fragments and Fc fusion proteins.

From a regulatory perspective, mAb assessment is based on the same principles as those used for the evaluation of other rDNA-derived biotherapeutic proteins. On the other hand, biosimilar mAbs should also comply with the criteria established for demonstration of similarity. Therefore this document should be read in conjunction with both the WHO Guidelines on evaluation of similar biotherapeutic products (SBPs) and the WHO Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (1, 2).
Guidance on various aspects of rDNA-derived medicines, SBPs and mAbs is also available from several other bodies. These WHO Guidelines are not intended to conflict with, but rather to complement, existing relevant regulatory documents.

3. Terminology

The definitions given below apply to the terms as used in these WHO Guidelines. These terms may have different meanings in other contexts.

**American College of Rheumatology 20% improvement criteria** (ACR20): a combined index that measures disease activity in patients with rheumatoid arthritis, and which corresponds to at least a 20% improvement in both the tender joint count and the swollen joint count, and at least a 20% improvement in 3 of 5 other score-set measures.

**Antibody-dependent cellular cytotoxicity** (ADCC): an immune mechanism through which Fc receptor-bearing effector cells can recognize and kill antibody-coated target cells expressing tumour- or pathogen-derived antigens on their surface.

**Antibody-dependent cellular phagocytosis** (ADCP): an immune mechanism which relies on Fc receptors, especially FcγRIIa, on macrophages or other phagocytic cells which bind to antibodies that are attached to target cells, followed by the phagocytosis and destruction of target cells, including tumour cells.

**Anti-drug antibodies** (ADAs): host antibodies that are capable of binding to a therapeutic antigen (recombinant protein or mAb). This may or may not inactivate the therapeutic effects of the treatment and, in rare cases, induce serious adverse effects.

**Area under the curve** (AUC): the area under the curve in a plot of concentration of drug in serum or plasma against time.

AUC\(_i\): the area under the concentration-time curve of drug in serum or plasma from zero up to a definite time \(t\).

AUC\(_{tau}\): the area under the concentration-time curve of drug in serum or plasma during a dosage interval.

**Biological activity**: the specific ability or capacity of a product to achieve a defined biological effect.

**Biosimilar mAb**: a mAb product that is similar in terms of quality, safety and efficacy to an already licensed reference product.

C\(_{max}\): the maximum (peak) serum or plasma concentration observed that a drug achieves in a tested area after the drug has been administered and prior to the administration of a second dose.
$C_{\text{min}}$: the minimum serum or plasma concentration observed that a drug achieves in a tested area after the drug has been administered and prior to the administration of a second dose.

$C_{\text{trough}}$: the measured serum or plasma concentration of a drug in a tested area at the end of a dosing interval prior to the administration of the next dose.

**Complement-dependent cytotoxicity (CDC):** the immune process by which an antibody–antigen complex activates complement that ultimately results in the formation of a terminal lytic complex that is inserted into a cell membrane, resulting in lysis and cell death.

**Complete response (CR):** the disappearance of all signs of cancer in response to treatment. This does not always mean the cancer has been cured.

**Disease activity score in 28 joints (DAS28):** a combined index that measures disease activity in patients with rheumatoid arthritis, which assesses the number of swollen and tender joints, and the erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) levels indicating how active the rheumatoid arthritis is, along with a patient’s global assessment of their health.

**Equivalence margin:** a pre-specified value in the equivalence trials, which is the largest difference that can be judged as being clinically acceptable and which should be smaller than differences observed in superiority trials of the active comparator.

**Equivalence trial:** a trial with the primary objective of showing that the response to two or more treatments differs by an amount which is clinically unimportant. This is usually demonstrated by showing that the true treatment difference is likely to lie between a lower and an upper equivalence margin of clinically acceptable differences.

**Mechanism of action (MOA):** the specific biochemical interaction through which a product produces its pharmacological effect.

**Monoclonal antibody (mAb):** antibody derived from a single clone of cells.

**Non-inferiority trial:** a trial with the primary objective of showing that the response to the investigational product is not clinically inferior to that of a comparative agent.

**Overall response rate (ORR):** the overall percentage of patients whose cancer shrinks or disappears after treatment; this includes the rate of complete response (CR) and partial response (PR).

**Potency:** the quantitative measure of biological activity based on the attribute of the product which is linked to the relevant biological properties and is expressed in units.

**Similarity:** absence of a relevant difference in the parameter of interest.
4. Special considerations for characterization and quality assessment

The WHO Guidelines on evaluation of similar biotherapeutic products (SBPs) set out the principle that demonstrating the similarity of a candidate SBP with respect to the RBP in terms of quality is a prerequisite for moving forward to comparative nonclinical and clinical studies (1). In particular, studies should be comparative in nature and should be performed with an appropriate number of batches of the reference product and of the SBP that is representative of the material intended for clinical use. The RBP should be extensively tested by analysing multiple batches, preferably over an extended period, in order to detect possible changes in the quality profile of the RBP over time. The minimum number of batches that should be tested will depend on the extent of the variability of the reference product and on assay variability. The number tested should be sufficient for drawing meaningful conclusions on the variability of a given parameter for both the SBP and the RBP, and on the similarity of both products. To obtain unambiguous results, the methods used should be sufficiently sensitive, scientifically valid and suitable for their purpose.

In comparison to many other proteins, mAbs are complex glycoproteins with distinct structural features which contribute to their diverse and variable biological functions. Specific carbohydrates can also have an impact on the biological activity of mAbs. For example, fucose bound by an α1–6 linkage to the core portion of N-linked carbohydrate chains interferes with the ability of the antibody to bind well to certain Fc receptors, resulting in diminished Fc-mediated activities, including ADCC, whereas an increase in non-reduced terminal galactose can enhance FcγRIIIa binding and ADCC activity. Consequently, the assessment of biological activity of biosimilar mAbs is particularly important and has some unique characteristics. The expression system used for the production of mAbs can, in some cases, considerably affect the structure and function of the mAb product. The general principles for quality assessment of biosimilar mAbs, including physicochemical characterization, are already described in the WHO SBP and rDNA guidelines (1, 2). Thus the quality aspects covered in this document will focus only on specific considerations for the assessment of mAb biological activity and on the impact of the expression system selected for production.

4.1 Strategy for assessment of mAb biological activity

Biological activity of mAb products is an important parameter and should be appropriately assessed. Since changes of higher-order structure could alter the biological activity of the mAb and may not be detected by physicochemical
methods, the analysis of bioactivity is additionally useful for confirming the comparability of higher-order structure.

An understanding of the mechanism of action (MOA) and receptor interactions of the mAb is important when considering the strategy for biological activity assessment in both the characterization study and the comparability study. MAbs exert their action by various mechanisms ranging from simple binding to antigen (which alone mediates the clinical effect) to binding antigen and mediating one or more immunobiological mechanisms that combine to produce the overall clinical response. These properties may play a role in the MOA and/or have an impact on product safety and efficacy. Therefore, a detailed analysis of the biological activity of the mAb – demonstrating the MOA (including Fab- and/or Fc-mediated functions) and ability to bind to Fcy and neonatal Fc receptors (as well as to complement C1q) – should be provided (see section 5.1.2 below).

Although simple antigen binding may seem to be the only mechanism operating to achieve clinical efficacy, other effects may also play a role. In some cases multiple functions of the mAb may be involved in an additive or synergistic manner to produce an overall combined clinical effect. This combined effect may be hard to dissect experimentally when seeking a clear understanding of how the mAb mediates its clinical efficacy. Therefore, if intact mAbs are used, care should be taken not to assume that the Fc-mediated immunobiological effects of the product are not involved in clinical efficacy, even in situations where simple antigen binding is considered to be the primary MOA. For example, rituximab (a chimeric mAb specific for CD20) requires Fc function, including ADCC, for its clinical efficacy. Assessment of Fc functions is therefore paramount for this mAb. For infliximab (a tumour necrosis factor alpha (TNFα) antagonist) the neutralization of soluble TNF is the primary MOA while Fc function seems less important. However, ADCC along with other Fc- and Fab-related functions (for example, reverse signalling) also need to be considered as potential secondary MOAs.

Assays for measuring Fc functions can be technically demanding. Differences in both assay formats and cell combinations have significant impact on assay sensitivities. Assays for investigating ADCC activity require appropriately responsive target cells and efficient effector cells. Although the use of primary cells may provide a more physiologically relevant model, the criteria of low assay variability and robustness may not be satisfied. Continuously growing cell lines may overcome these limitations in some cases provided they are more sensitive and more capable of detecting minor differences between the RBP and the SBP. However, identifying or producing a suitable cell line can be difficult and arduous. Furthermore, the clinical relevance of data generated by engineered/artificial cell lines may also be challenged because of the use of a homogeneous cell population over-expressing the targets/receptors.
Therefore, selection of an appropriate assay for the intended purpose should always be considered as a priority in developing the strategy for assessing mAb biological activity. Additional data may be generated by the use of different assay formats and cell combinations to obtain results that are more relevant to the physiological/pathophysiological conditions in patients. Although biological assays used in characterization or for demonstrating similarity may not be as robust as release assays, the assays should be qualified for the intended use and should be sufficiently sensitive to detect minor differences between the RBP and the SBP.

4.2 Considerations for selection of the expression system

The WHO SBP Guidelines (1) allow for the use of different expression systems for production of the SBP compared to the reference product, as long as the manufacturer can convincingly demonstrate that the structure of the molecule is not affected or that the clinical profile of the product will not change. However, this may pose a challenge in the context of biosimilar mAb development. Therefore, the expression system should be carefully selected, taking into account expression system differences that may result in undesired consequences such as an atypical glycosylation pattern or a different impurity profile when compared to the RBP.

Differences in glycoforms present on products may or may not have clinical consequences. For example, production cells based on mouse cell lines (such as SP2/0 and NS0) secrete mAbs with the carbohydrate structure alpha-gal-1,3-gal present on the carbohydrate moiety. Humans cannot produce the alpha-gal-1,3-gal structure as they lack the necessary enzyme for its synthesis; however, many humans produce antibodies against this. In a proportion of these individuals the antibodies are of the immunoglobulin E (IgE) class and this sensitization can result in anaphylactic reactions (often serious) if they are treated with mouse-cell-line-derived mAbs containing alpha-gal-1,3-gal. Such pre-existing antibodies are particularly evident for cetuximab – an inhibitor of epidermal growth factor receptor (EGFR), which contains an additional glycosylation site on the Fab region that is accessible for IgE binding. Anaphylactic responses may potentially be avoided by using cell substrates of human origin or selected clones of Chinese hamster ovary (CHO) cells for mAb production since these cells normally cannot synthesize alpha-gal-1,3 gal. This type of phenomenon can have important implications for biosimilar mAb development. For example, producing an SBP of cetuximab in mouse cells would probably show the same alpha-gal-1,3-gal-related anaphylaxis problems as the reference product. Although production of the mAb in CHO cells may avoid the anaphylaxis problem (since the alpha-gal-1,3-gal structure would not be likely to be present on the mAb) the differences in glycosylation, and possibly
other modifications, could have an impact on the extent of studies needed for demonstration of biosimilarity. Therefore, the selection of an expression system for a biosimilar mAb requires careful consideration, with various potential issues needing to be thoroughly assessed to ensure that an expression system difference does not result in changes to critical quality attributes.

### 4.3 International standards for biological assays used in the characterization

The development of assays for the determination of biological activity of mAbs will be facilitated by WHO International Standards or WHO Reference Reagents when available. Importantly, a clear distinction exists between reference products and WHO International Standards or Reference Reagents since they serve different purposes and cannot be used interchangeably. The key difference between their uses reflects the fact that the RBP is used for all the comparability studies, whereas WHO International Standards and Reference Reagents are used for calibrating procedures, particularly bioassays, and cannot be used as RBPs. The distinct roles of reference products and international standards are described elsewhere (1, 3).

### 5. Special considerations for nonclinical evaluation

As with all SBPs undergoing nonclinical evaluation, a stepwise approach should be applied to evaluate the similarity of biosimilar and reference mAbs. In vitro studies should be conducted first and a decision then made regarding the extent to which, if necessary, in vivo studies will be required. When deemed necessary, in vivo nonclinical studies should be performed before initiating clinical trials.

The following approach may be considered and should be tailored on a case-by-case basis to the SBP concerned. The approach used should be scientifically justified in the nonclinical overview.

#### 5.1 In vitro studies

##### 5.1.1 SBP – general aspects

In order to assess any difference in biological activity between the SBP and the RBP, data from a number of in vitro studies, some of which may already be available from quality-related assays, should be provided.

As for all SBPs, the following general principles apply to biosimilar mAbs:

- The studies should be sensitive, specific and sufficiently discriminatory to provide evidence that observed differences in quality attributes, as well as possible differences that may not have
been detected during the comparative analytical assessment, are not clinically relevant. Functional studies should be comparative and should be designed to be sufficiently sensitive to detect differences in the concentration–activity relationship between the SBP and the RBP.

- Together, these assays should cover the whole spectrum of pharmacological/toxicological aspects with potential clinical relevance for the reference product and for the product class.
- The manufacturer should discuss to what degree the in vitro assays used are representative/predictive of the clinical situation according to current scientific knowledge.

Since in vitro assays may often be more specific and sensitive for detecting differences between the SBP and the reference product than studies in animals, these assays can be considered as paramount for the nonclinical biosimilar comparability exercise.

5.1.2 **Biosimilar mAbs – specific aspects**

For biosimilar mAbs, the nonclinical in vitro programme should usually include relevant assays for the following specific evaluations:

- Binding studies:
  (a) binding to soluble and/or membrane-bound target antigen(s); and
  (b) binding to representative isoforms of the relevant Fc receptors (that is, for immunoglobulin G (IgG)-based mAbs to FcγRI, FcγRII and FcγRIII), FcRn and complement (C1q).

- Functional studies/biological activities:
  (a) Fab-associated functions (for example, neutralization of a soluble ligand, receptor activation or blockade, reverse signalling via activation of membrane-bound antigen); and
  (b) Fc-associated functions (for example, ADCC, ADCP, CDC), as applicable.

These assays are often technically demanding and the models chosen should be appropriately justified by the applicant (see section 4.1 above). Together, these assays should broadly cover the functional aspects of the mAb even though some may not be considered essential for the therapeutic MOA. However, an evaluation of ADCC, ADCP and CDC may be waived for mAbs directed against non-membrane-bound targets if appropriately justified.
Additional note: as indicated in the ICH Guideline S6(R1) (4), tissue cross-reactivity studies with mAbs are not suitable for detecting subtle changes in critical quality attributes and are thus not recommended for assessing biosimilar comparability.

5.2 In vivo studies

5.2.1 Determination of the need for in vivo studies

- As for SBPs in general, on the basis of the totality of quality and nonclinical in vitro data available and the extent to which there is residual uncertainty about the similarity of the test mAb and the reference mAb, it is at the discretion of NRAs to waive or not to waive a requirement for nonclinical in vivo studies. If the biosimilar quality-comparability exercise and nonclinical in vitro studies are considered satisfactory, and no issues are identified that would block direct entrance into humans, an in vivo animal study may not be considered necessary.

5.2.2 General aspects to be considered for all SBPs, including biosimilar mAbs

- If there is a need for additional in vivo information, the availability of a relevant animal species or other relevant models (for example, transgenic animals or transplant models) should be considered.
- If a relevant in vivo animal model is not available the manufacturer may choose to proceed to human studies, taking into account the principles for mitigating any potential risk.
- When the need for additional in vivo nonclinical studies is evaluated, the factors to be considered include but are not restricted to:
  (a) (the presence of potentially relevant quality attributes that have not been detected in the reference product (for example, new post-translational modification structures);
  (b) the presence of potentially relevant quantitative differences in quality attributes between the SBP and the RBP; and
  (c) relevant differences in formulation (for example, use of excipients not widely used for mAbs).

  Although not all of the factors mentioned here necessarily warrant in vivo testing, these factors should be considered together to assess the level of concern and to determine whether or not there is a need for in vivo testing.

- If product-inherent factors that have an impact on pharmacokinetics (PK) and/or biodistribution (such as glycosylation) cannot
sufficiently be characterized on a quality and in vitro level, the manufacturer should carefully consider if in vivo animal PK and/or pharmacodynamics (PD) studies should be performed in advance of clinical PK/PD testing.

5.2.3 Performance of in vivo studies

The following guidance applies to all SBPs, including biosimilar mAbs.

5.2.3.1 General aspects

If an in vivo evaluation is deemed necessary, the focus of the study/studies (PK and/or PD, and/or safety) depends on the need for additional information to address residual uncertainty from the quality and in vitro nonclinical evaluation.

Animal studies should be designed to maximize the information obtained. The duration of the study (including observation period) should be justified, taking into consideration the PK behaviour of the reference mAb, the time to onset of formation of anti-drug antibodies (ADAs) in the test species and the clinical use of the reference mAb.

The effects of SBPs are often species-specific. In accordance with the WHO Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (2) and ICH Guideline S6(R1) (4), in vivo studies should be performed only in relevant species – that is, species which are pharmacologically and/or toxicologically responsive to the SBP.

5.2.3.2 PK and/or PD studies

When the model allows, the PK and/or PD of the SBP and the RBP should be compared quantitatively, including, if feasible, through a dose–response assessment that includes the intended exposure in humans.

In vivo assays may include the use of animal models of disease to evaluate functional effects on PD markers or efficacy measures.

5.2.3.3 Safety studies

If in vivo safety studies are deemed necessary on the basis of a need for additional information, a flexible approach should be considered. The conduct of repeated dose-toxicity studies in non-human primates is usually not recommended. If appropriately justified, a repeated dose-toxicity study with refined design (for example, using just one dose level of SBP and RBP, and/or just one biological sex and/or no recovery animals) and/or an in-life evaluation of safety parameters (such as clinical signs, body weight and vital functions) may be considered. Depending on the end-points needed it may not be necessary to kill the animals at the end of the study.
For repeated dose-toxicity studies, where only one dose is evaluated and the focus of the study is an evaluation of potential qualitative differences in the toxicity profile between RBP and SBP, the dose would usually be selected at the high end of the known dosing range of the RBP. Where the focus of the study is an evaluation of potential quantitative differences with regard to the known toxicity profile of the RBP, the dose level most likely to reveal differences between the RBP and SBP should be chosen as justified on the basis of the known toxicity and/or pharmacodynamic response of the RBP.

The conduct of toxicity studies in non-relevant species (that is, to assess nonspecific toxicity only, based on impurities) is not recommended. Because of the different production processes used by the SBP and reference product manufacturers, qualitative differences in process-related impurities will occur (for example, host cell proteins). Such impurities should be kept to a minimum in order to minimize any associated risk.

5.2.3.4 Immunogenicity studies
Qualitative or quantitative difference(s) in product-related variants (for example, glycosylation patterns, charge variants, aggregates and impurities such as host-cell proteins) may have an effect on immunogenic potential and on the potential to cause hypersensitivity. These effects are usually difficult to predict from animal studies and should be further assessed in clinical studies.

However, while immunogenicity assessment in animals is generally not predictive of immunogenicity in humans, it may be needed for the PK/toxicokinetics (TK) interpretation of in vivo animal studies. Therefore, adequate blood samples should be identified and stored for future evaluations if needed.

5.2.3.5 Local tolerance studies
Studies on local tolerance are usually not required. If excipients are introduced for which there is little or no experience with the intended clinical route, local tolerance may need to be evaluated. If other in vivo studies are performed the evaluation of local tolerance may be part of the design of those studies to avoid the need for separate local tolerance studies.

5.2.3.6 Other studies
In general, safety pharmacology and reproductive and development toxicity studies are not warranted in the nonclinical testing of biosimilar mAbs.

In accordance with the WHO Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (2) and ICH Guideline S6(R1) (4), genotoxicity and (rodent) carcinogenicity studies are not required for (similar) biotherapeutic products. This guidance also applies to biosimilar mAbs.
6. Special considerations for clinical evaluation

In general, the goal of the clinical evaluation programme is to confirm that any residual uncertainty about quality attributes or related to the preclinical assessment would not result in clinically meaningful differences – and not to establish the product's efficacy and safety in a particular indication. The clinical comparability exercise is a stepwise procedure that should begin with PK/PD studies and usually continues with one controlled clinical trial addressing comparative safety and efficacy. In exceptional circumstances, data obtained in clinical PK/PD studies may suffice in confirming biosimilarity established in preceding steps (see section 6.2.1 below). If relevant differences between the SBP and the RBP are detected at any stage, the reasons for the differences should be explored and justified. In reaching a conclusion as to whether a product qualifies as an SBP, the totality of evidence should be considered.

If the original development programme demonstrated that the reference product performed the same in different ethnic groups then there is no scientific rationale for conducting a comparative clinical study of the SBP in each ethnic group.

6.1 Pharmacokinetics studies

6.1.1 Aim of comparative PK studies

Comparative clinical PK studies are always required and should be used to further confirm the similarity of a biosimilar mAb to the RBP already established through comparative structural, functional and nonclinical studies. In general, factors to consider include whether the mAb is targeting a soluble antigen or a membrane-bound antigen, and whether it is dependent on FcRn binding and/or dependent on target-mediated clearance or non-target-mediated clearance. For example, a biosimilar mAb may differ in its affinity for FcRn receptors from its RBP which may lead to either a shorter or longer half-life. As a consequence of a shorter half-life, drug exposure would be reduced, which may lead to lower efficacy (5). Comparative PK studies may be useful in monitoring the impact of the formation of ADAs on efficacy and safety – while exploring the impact of ADAs on PK is also necessary. Both approaches contribute to establishing evidence in support of extrapolation. It is not necessary to study the PK of the biosimilar mAb in every indication that is being sought. In general, one comparative PK study under sensitive conditions that allow any potential differences between the SBP candidate and the RBP to be detected should be sufficient to bridge across the indications for which the reference mAb has been authorized. The design of comparative PK studies depends on various factors, including clinical context, safety profiles and PK characteristics of the reference product (for example, target-mediated disposition (TMD), linear or nonlinear PK, time-dependency and half-life).
6.1.2 Study design and population

A single-dose PK study in healthy volunteers is generally recommended as they can be considered a sensitive and homogeneous study population (6). A parallel-group design, which generally requires a higher number of subjects, is usually required for mAbs since a single-dose cross-over design may not be appropriate due to the long half-lives of mAbs and the potential influence of immunogenicity on the PK profile. However, for mAb fragments or mAbs that are not administered systemically, alternative approaches may also be applied.

A number of key issues should be taken into account regarding the use of healthy volunteers to study the PK of mAbs. First, healthy subjects are generally preferred, if possible, because of their higher sensitivity and homogeneity as compared to patient populations. Second, administering a clinically relevant dose of some mAbs (for example, bevacizumab) may not be considered ethical in healthy volunteers because of safety concerns, and in these cases a sub-therapeutic dose on the linear part of the dose–response curve may be required. Third, it may be necessary to perform the PK study for some biosimilar mAbs (for example, rituximab) in a sensitive patient population rather than in healthy volunteers for safety reasons. Unnecessary exposure to risk (because of safety or medical reasons) would be viewed as unethical. Fourth, it may sometimes be necessary to perform the PK study in a different population to that selected for the comparative clinical efficacy study in order to establish similar clinical efficacy. In such scenarios, population PK measurements should be collected during the clinical efficacy trial since such data may add relevant information on similarity. Measurement of PK parameters (especially trough levels, along with sampling for immunogenicity) is also recommended for the evaluation of clinical correlates of possible ADAs. Furthermore, the choice of a particular population for PK analysis also depends on the range of therapeutic indications of the mAb under development. For example, if a reference mAb is authorized both as an anti-inflammatory agent and as an anticancer antibody (as for example with rituximab) then PK data in one therapeutic area may complement clinical data obtained in another therapeutic area and thus can also strengthen the evidence for indication extrapolation.

6.1.3 Regimen

MAbs are often indicated both for monotherapy and as a part of combination regimens that incorporate immunosuppressants or chemotherapeutics. It may be sensible to study the comparative PK in the monotherapy setting in order to minimize sources of variability. When concomitant therapy alters PK, it may be appropriate to study comparative PK both in the monotherapy setting and in combination, particularly where differences cannot be excluded with regard to
quality attributes that might specifically have an impact on how the drug was cleared when used in combination.

6.1.4 **PK characteristics of the reference mAb**
The PK of the mAb may be affected by factors such as the antigen/receptor level (for example, related to tumour burden in oncology), the existence of target-mediated clearance, and/or receptor shedding which has an impact on the variability of PK measurements. These factors should be considered when selecting the population in which to compare the PK of the SBP to the reference product.

6.1.5 **Doses**
A dose should be selected that will enable detection of potential PK differences between the biosimilar mAb and the reference mAb. MAbs generally possess a high degree of target selectivity, with many exhibiting nonlinear distribution and elimination, influenced by binding to their target. In general, it is recommended that the PK profiles should be compared using the lowest recommended therapeutic dose. A higher (or the highest) therapeutic dose may be required where the nonspecific clearance mechanism dominates. For mAbs that are eliminated by TMD, a low dose (that is, one at which TMD is not saturated) may be particularly useful for detecting differences in PK (7).

6.1.6 **Routes of administration**
Administration via a route that requires an absorption step is preferred unless intravenous administration only is intended. Where the route of administration requires an absorption step, such as the subcutaneous route, standard comparisons of $C_{\text{max}}$, $AUC_t$ and $AUC_{0-\text{inf}}$ may be used to assess PK comparability.

6.1.7 **Sampling times and parameters**
Primary PK comparability studies should include early time points to accurately measure $C_{\text{max}}$ and should also include sufficient sampling time points in the later phases to adequately characterize the late elimination phase. This will allow for reliable estimation of the terminal disposition rate constant and sufficient characterization of any ADA response. In single-dose studies, optimal sampling should continue past the expected last quantifiable concentration ($AUC_t$), and the concentration–time curve should cover at least 80% of $AUC_{0-\text{inf}}$.

If a multiple-dose study is performed in patients, sampling should be carried out at first dose and at steady state. Steady state is typically reached after five half-lives of the mAb. PK parameters that should be evaluated include
AUC_{0-t}, AUC_{tau}, C_{max} and C_{trough}, clearance and half-life. For mAbs that are administered only intravenously, the aforementioned parameters should be compared, as should parameters that reflect the clearance of the product.

6.1.8 Specific assays for serum drug concentration

It is preferable to have a single, validated bioanalytical assay to detect both the biosimilar mAb and the reference mAb. The bioanalytical assay should be appropriate for the detection and quantification of mAbs, and should be demonstrated to be bioanalytically comparable with respect to its ability to quantify precisely and accurately both the biosimilar mAb and the reference mAb \(^{(8)}\). The production of ADAs may interfere with assays for test products. Therefore, ADAs should be measured in parallel with PK assessment, using the most appropriate sampling time points and a subgroup analysis by ADA status should be performed. PK analysis on the ADA-negative samples is of particular interest as it provides the clearest picture of PK similarity.

6.1.9 Equivalence margin

In general, a comparability margin of 80–125% for the primary parameters may be acceptable but should be justified. In some circumstances, narrowing or widening of this margin may be required and this too should be justified.

6.2 Pharmacodynamics studies

In general, it is advisable to include PD markers as part of the clinical comparability exercise.

6.2.1 PD markers and PD assay

For some mAbs it may even be possible to perform confirmatory PD studies instead of controlled clinical safety and efficacy studies with conventional clinical outcome measures. When clinical studies using PD markers are planned to provide the main clinical evidence to establish similarity, it is recommended that such an approach is discussed with the regulatory authorities.

The characteristics of PD markers that would support clinical efficacy, and that manufacturers should pay attention to, are \(^{(6)}\):

- The PD marker should be sufficiently sensitive to detect relevant differences, and should be measurable with sufficient precision.
- The use of multiple PD markers, if they exist, is recommended.
- The study dose–concentration–response relationships or time–response relationships of the selected doses should be within the linear part of the established dose–response curve of the RBP.
• A clear dose–response relationship is shown.
• The PD marker is an acceptable surrogate marker and is related to patient outcome.
• An equivalence margin should be predefined and justified.
• The PD assay should at least be relevant to a pharmacological effect of the biological product (PD assay is highly dependent on the pharmacological activity of the product; the approach for assay validation and the characteristics of the assay performance may differ depending on the specific PD assay).

In general, the principles regarding study design, conduct, analysis and interpretation that are relevant to equivalence trials with a clinical outcome as the primary end-point are applicable to equivalence trials with a PD marker as the primary outcome.

6.3 **Comparative clinical efficacy study**

The confirmatory efficacy trial is the last step of the comparability exercise, thus confirming that the clinical performance of the SBP and the RBP are comparable. Typically, one randomized, adequately powered and preferably double-blinded clinical efficacy study should be performed.

The manufacturer of a biosimilar mAb should perform a thorough analysis of the publicly available clinical data for the reference product to determine the most appropriate study population and primary end-point combination likely to provide a relevant and sensitive model for detecting clinically meaningful differences in efficacy and safety – and for extrapolating efficacy and safety to therapeutic indications that are not investigated. The type of comparative clinical trial required for the proposed biosimilar mAb could be influenced by several factors, including:

• the nature and complexity of the mAb and derived products;
• the behaviour of the reference product in the clinic;
• the degree of understanding of the MOA of the mAb and disease pathology, and the extent to which these vary in different indications – including MOA, site of action, antigen load, drug administration (dose, route, regimen and duration), concomitant medications, and target population sensitivity to drug effects.

The clinical data obtained in a sensitive model can also be used to support extrapolation to other indications of the RBP for which the proposed biosimilar mAb has not been tested.
6.3.1 Clinical trial design

Clinical trial design and statistical analysis of equivalence and non-inferiority trials that are already addressed in the WHO Guidelines on evaluation of similar biotherapeutic products (SBPs) also apply to biosimilar mAbs (1). The Guidelines stress the importance of clearly stating the specific design selected for a given study and include details on the determination of the equivalence/non-inferiority margin, sample size determination and statistical analyses. For biosimilar mAbs, extrapolation to other indications is especially important, and additional considerations are required in order to design a meaningful trial to support additional indications.

Although equivalence or non-inferiority studies would be acceptable for the comparative clinical studies of a biosimilar mAb against the RBP, equivalence trials are generally preferred. Detailed explanations of the advantages and disadvantages of equivalence and non-inferiority trial designs for SBPs are provided in the WHO Guidelines and in guidance developed by other agencies (1, 9–11). Special considerations for clinical trial design in relation to biosimilar mAbs are explained below.

A demonstration of equivalence, as opposed to non-inferiority, is especially important given that extrapolation to other indications may be one of the goals of the development programme for the biosimilar mAb. Non-inferiority trials are one-sided and hence do not exclude the possibility that the biosimilar mAb could be found to be superior to the RBP. Such a finding would create challenges in justifying extrapolation to other indications of the RBP. From a statistical perspective, assay sensitivity is important to provide some confidence that the trial, as planned and designed, will be able to detect differences between the biosimilar mAb and the RBP, if such differences exist (12). A trial that lacks sensitivity could lead to the erroneous conclusion of equivalence of the biosimilar mAb to the RBP. The selected study population should not only be representative of the approved therapeutic indications of the RBP, but should also be sufficiently sensitive to detect potential differences between the biosimilar mAb and the RBP. Hence, historical scientific evidence should be provided which shows that appropriately designed and conducted trials with the RBP against placebo for the approved indication have reliably demonstrated the superiority of the RBP over placebo.

Study population or study end-points may deviate from those which led to approval of the RBP for the specific indication as long as the primary end-points are sensitive to the detection of clinically meaningful differences between the biosimilar mAb and the RBP. Whatever approach is taken, applicants should always justify their selection of end-points, time points for analysis and the predefined margin, irrespective of whether this follows the RBP approach or not. If in doubt, applicants may wish to consult relevant regulatory authorities during the planning and design stage of the trial.
The efficacy of the RBP compared to placebo will have been demonstrated previously. Therefore, it is considered clinically important to ensure that the biosimilar mAb retains a substantial fraction of the effect of the RBP. As a consequence, an equivalence margin that preserves a fraction of the smallest effect size that the RBP can be expected to have relative to a placebo control is the most suitable. The fraction of the effect size of the RBP that should be retained by the biosimilar mAb should be clearly justified in each case, and should take into account the smallest clinically important difference in a given setting. Once the margin has been selected, determination of the required sample size should be based on methods specifically designed for equivalence and non-inferiority trials.

Statistical analysis of data from equivalence trials is typically based on the indirect confidence interval comparison which requires specification of the equivalence limits (13). Equivalence is demonstrated when the confidence interval for the selected metric of the treatment effect falls entirely within the lower and upper equivalence limits. If a $P$-value approach is used then the $P$-values should be computed on the basis of the two one-sided test (TOST) procedure, testing simultaneously the null hypotheses of inferiority and superiority. When using the TOST procedure, equivalence is demonstrated when the $P$-values obtained are less than the significance level used.

### 6.3.2 Study population

In order to detect differences between the biosimilar mAb and the reference mAb, clinical trials of the biosimilar mAb should be carried out in an appropriately sensitive patient population using end-points that are sensitive to the detection of clinically meaningful differences between the SBP and the reference product for the indication (see section 6.3.3 below). The rationale for the study population selected should be provided. In general, using a homogeneous population of patients (for example, same line(s) of therapy, severity or stage of disease progression) will minimize inter-patient variability and thus increase the likelihood of detecting differences between the biosimilar mAb and the reference mAb, if such differences exist. Patients who have not received previous treatment (for example, first-line therapy) are considered to be more homogeneous than patients who have previously received several or different lines of therapy. Ideally, the observed clinical effects should be triggered by the direct action of the biosimilar mAb/reference mAb without interference by other medications, as concomitant medications may affect or mask differences in the PK/PD, efficacy, safety and/or immunogenicity of the tested products. To validate the effect of the reference mAb and the sensitivity of the chosen study population, historical data should be used to justify the selection of the study population and equivalence margin. This could generally be done through a systematic review and/or meta-analysis of the relevant studies.
MAbs can function through various MOAs, including agonist activity or receptor blockade (for example, of vascular endothelial growth factor (VEGF) and EGFR), induction of apoptosis, delivery of a drug or cytotoxic agent, and immune-mediated mechanisms (for example, CDC, ADCC and regulation of T-cell function). Because the mechanisms involved in one disease may differ from those involved in another, extensive consideration should be given to the setting in which clinical comparability is to be tested, particularly if functional differences are identified in sensitive assays, and especially where it is known that extrapolation to other indications and uses will be sought.

Clinical studies in an unauthorized population (for example, with respect to line of therapy, combination therapy, disease severity or indication authorized in some but not all jurisdictions) may be acceptable for demonstrating “no clinically meaningful differences” for biosimilar mAbs. However, manufacturers of biosimilar mAbs should consult the relevant regulatory authorities prior to conducting such studies.

6.3.3 Primary study end-point
Clinitically relevant and sensitive study end-points within a sensitive population should be selected to improve the likelihood of detection of potential differences between the biosimilar mAb and the reference product. In general, clinical outcomes, surrogate outcomes or a combination of both can be used as primary end-points in biosimilar mAb trials. The same study end-points used for the innovator products may be used because a large body of historical data is generally available in the public domain for setting the equivalence margin and calculating the sample size. Alternatively, the study end-points used may be different from those traditionally used or from the end-points recommended by study guidelines for innovator products, as more sensitive end-points and/or time points may exist for detecting clinically meaningful differences in an equivalent trial setting where the objective is assessing similarity of efficacy, safety and immunogenicity, and not re-establishing the clinical benefit already demonstrated by the originator. A surrogate end-point can be used as the primary end-point when surrogacy to the clinical outcome is well established or generally accepted, as is the case, for example, with pathological complete response (pCR) in neoadjuvant treatment of breast cancer. The choice of study end-point should always be scientifically justified. More sensitive clinical end-points could be used as secondary end-points for the innovator product, primary or secondary end-points for the innovator products at different time points of analysis, and/or new surrogates. For example, overall response rate (ORR) or complete response (CR) rate can be considered as end-points for clinical efficacy studies of biosimilar mAbs in oncology trials because these end-points may be more sensitive and are not time related. However, if progression-free survival (which is one of the end-points frequently used for clinical efficacy testing for innovator products)
is considered more sensitive than ORR, then this may be the preferred option. Likewise, both continuous outcomes (for example, changes in DAS28 from baseline) and dichotomous outcomes (for example, ACR20) are considered in rheumatoid arthritis trials for determining clinical comparability (14).

When the primary efficacy end-points that were used for the RBP cannot be used for the SBP it is advisable to include some common end-points as secondary end-points to facilitate comparisons between the SBP and the RBP. The role of these secondary end-points in the overall interpretation of the study results should be clearly defined, particularly in terms of whether the secondary end-points are used to support or to confirm equivalency or similarity.

NRAs may not always agree on the choice of study end-points. For an SBP manufacturer with a global development programme that is guided or required by various NRAs to fulfil local regulatory or clinical practice requirements it may be possible to pre-specify different primary study end-points with the statistical power in the same trial to comply with various regulatory requirements.

6.3.4 **Safety**

6.3.4.1 **General considerations**

Comparative safety data should normally be collected pre-authorization. The extent of data collection depends on the type and severity of known safety issues for the reference product. The SBP study population should be followed to provide information on safety events of interest according to experiences with the reference mAb. Care should be taken to compare the nature, severity and frequency of adverse events between the biosimilar mAb and the reference product in clinical trials that enrolled a sufficient number of patients treated for an acceptable period. Clinical safety issues should be captured throughout clinical development during initial PK and/or PD evaluations and also in the primary clinical study establishing comparability.

6.3.4.2 **Immunogenicity of a biosimilar mAb**

Therapeutic mAbs, like other rDNA-derived biotherapeutics, may be recognized by the human immune system leading to an unwanted immune response. As mAbs may often be immunogenic in patients, the goal during development of a biosimilar mAb is to demonstrate similar immunogenicity to the reference product. There are some special considerations regarding the immunogenicity of mAbs as compared to other biotherapeutics. For example, mAbs do not evoke cross-reacting antibodies against the body’s endogenous proteins, as some growth factors and proteins for replacement therapy do. However, developing assays to test for anti-mAb-antibodies can be challenging.

From the regulatory point of view, animal data are not sufficiently predictive of the human immune response against a therapeutic protein. Thus,
immunogenicity generally needs to be investigated as part of the clinical trial programme of a biosimilar mAb. The analysis of the immunogenicity of DNA-derived biotherapeutics is outlined in the WHO Guidelines on evaluation of similar biotherapeutic products (SBPs) and the WHO Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (1, 2). These general guidance resources should be taken into account when assessing biosimilar mAbs. In addition, further details regarding the advantages and disadvantages of particular assays, as well as some considerations on the interpretation of the results and on the decision-making process, are provided in several review articles (15–18).

The basic data package contains the incidence, titre, neutralization ability and persistence of antibodies against the product/mAb, determined by appropriate assays, as well as their pharmacokinetic and clinical correlations. The immunogenicity programme needs to be tailored to each product. Thus, the evaluation of immunogenicity requires a multidisciplinary approach, including considerations of product-, process-, patient- and disease-related factors that will form the basis of a risk-based immunogenicity programme. It is recommended that the application for marketing authorization of a mAb includes a summary of the immunogenicity programme in support of the selected approach to immunogenicity. This summary should address the following topics as appropriate:

- risk assessment
- risk-based immunogenicity programme
- comparative immunogenicity
- assays and mAb characterization
- clinical immunogenicity assessment.

6.3.4.2.1 Risk assessment

- previous knowledge of the immunogenicity of the reference product, such as the presence of immunogenic structures in the active substance as well as the incidence, type, persistence and clinical correlations of the antibodies;
- findings of the physicochemical and structural comparisons between the biosimilar mAb and its reference product, including process-related impurities and aggregates;
- differences in formulation and packaging (for example, potential impurities and leachables);
- route and/or mode of administration of the product;
- patient- and disease-related factors such as the state of the immune system, concomitant immunomodulatory therapy and potential pre-existing immunity, antigenicity and sensitivity.

6.3.4.2.2 Risk-based immunogenicity programme

The manufacturer should present a risk-based immunogenicity assessment programme.

- The basis of the immunogenicity assessment is the testing of patient samples pretreatment, during treatment and, if needed, post-treatment in an appropriate set of assays that are suitable for the product in question. The measurement of antibodies to mAbs is methodologically challenging since standard assay formats involving anti-immunoglobulin reagents are inappropriate for this product class; therefore alternative methods should be used. As with other biotherapeutic products a multi-tiered assessment approach is needed. The developer has to validate assays for screening, confirmation and neutralization ability. Special attention should be paid to the choice of the control matrix, determination of cut-off points and the estimation of interference by matrix components, including the drug target and the residual drug in the sample. To mitigate this potential interference, corrective measures should be implemented. For example, drug interference may be overcome by allowing time for clearance of the drug from the circulation prior to sampling, or by dissociating immune complexes, and/or removal of the drug. Inclusion of any of these measures should not compromise the detection of antibodies or patient treatment.

- With regard to the integration of the product antibody testing into the comparative clinical trials, it is particularly important to synchronize the sampling schedule and duration of the follow-up for product antibody determination and PK measurements, as well as for assessments of safety and efficacy.

- Special emphasis should be placed on the potential association of product antibodies with loss of efficacy, with infusion reactions, and with acute and delayed hypersensitivity. The manufacturer should systematically use terminology and definitions to characterize potentially immune-mediated symptoms, in accordance with relevant publications (19, 20).

- The manufacturer should take into account the dose and dosing schedule, including re-administration, after discontinuation of treatment.
- The vulnerability of the patient population(s) and the expected risks of immunogenicity should be taken into account in planning for the intensity of monitoring.

- The manufacturer should provide a description and analysis of the use of pre-medication or de-immunization measures to mitigate acute infusion/injection-related reactions and other possibly immune-mediated reactions.

After discontinuation of the therapy, it is important to investigate the persistence of product antibodies formed during drug administration, as well as the emergence of product antibodies that may have escaped detection because of the immunosuppressive action of the product or because of technical problems (notably drug interference). The timing of the post-treatment samples should be justified.

6.3.4.2.3 Comparative immunogenicity

The lack of standardization and rapid evolution of the assay methodology makes it difficult to compare immunogenicity studies. Therefore, pre-licensing comparative immunogenicity data are generally needed in the development of SPBs (1, 11). Immunogenicity testing of the SBP and the reference product should be conducted within the biosimilar comparability exercise by using the same assay format and sampling schedule. A parallel-group design is recommended because of the long half-life of antibodies and because it may be difficult to interpret immunogenicity after a switch.

6.3.4.2.4 Assays and mAb characterization

ADA assays should ideally be capable of detecting all antibodies against both the reference and biosimilar molecule. Thus assays can be performed with both the reference and biosimilar molecule as the antigen/capture agent in parallel in order to measure the immune response against the product received by each patient. The challenge is to develop two assays with similar sensitivity. Cross-testing all serum samples by both tests is useful for exploring assay performance and antigen epitopes. The use of a single assay with the active substance of the SBP as the antigen/capturing agent for evaluation of all samples (including those from reference-product-treated patients) will be able to detect all antibodies developed against the biosimilar molecule (that is, the conservative approach). In general, the manufacturer should justify the chosen assay approach and should demonstrate the suitability of the method(s) used to measure similarly the immune response against the product received by each patient, irrespective of whether the patient was treated with the RBP or the SBP.
Following identification of confirmed antibody-positive samples, characterization of the antibodies is required. Determination of their neutralizing potential is essential and deviation from this requires justification. Although a functional (usually cell-based) bioassay or a binding assay (for example, a competitive ligand-binding (CLB) assay) can be used alone, the latter should be used only if relevant to the MOA of the product. For example, a CLB assay is appropriate in a scenario where a therapeutic mAb acts by binding to a soluble ligand, thereby blocking it from interacting with its receptor and thus inhibiting the biological action of the ligand. Since the assay procedure measures binding to the target and inhibition of the binding activity if neutralizing antibodies are present, it is reflective of the MOA of the therapeutic mAb. For intact mAbs where effector functions are likely to contribute to the clinical effect, functional cell-based bioassays are recommended because the MOA cannot be reflected adequately in a CLB assay. Nevertheless, such cell-based assays may not be sufficiently sensitive and a CLB assay may give a more accurate assessment of the incidence of neutralizing antibody induction.

Additional studies beyond the standard data package, such as immunoglobulin class, epitope mapping and IgG subclass, may be useful in specific situations (for example, occurrence of anaphylaxis or use of certain assay formats). It may also be necessary to locate the antigenic sites (for example, antigen-binding region versus constant region of the antibody molecule). The banking of patient samples is necessary in order to retain the possibility for retesting in case of technical problems in the original assay.

6.3.4.2.5 Clinical immunogenicity assessment

The selected patient population should be sensitive for the detection of differences in immunogenicity. It is also important that the controlled safety and efficacy study will include both immunogenicity and PK measurements (especially C_{trough} levels) in order to establish the clinical impact of immunogenicity. If the study includes patients previously treated with the reference mAb, a subgroup analysis of previously treated patients should be performed. The sampling schedule should be optimized for the demonstration of similar onset and persistency of antibodies to the test and reference products.

The duration of follow-up of immunogenicity depends on the duration of exposure and should be sufficient to demonstrate similar persistence and clinical impact of the antibodies. In chronic administration, the minimum follow-up is 6 months.

Immunogenicity should be followed after licensing by monitoring possible immune-mediated adverse effects. Special immunogenicity studies may be necessary in high-risk situations (for example, when the reference product is known to have serious but rare immune-mediated effects, such as anaphylaxis).
Evaluation of immunogenicity includes antibody incidence, titre, neutralization capacity and persistency, as well as correlations to exposure, safety and efficacy. Currently, there is no generally accepted statistical methodology that could be used to define the limits of comparable immunogenicity. In general, an increase in immunogenicity of an SBP when compared with the RBP is incompatible with the biosimilarity principle unless the sponsor can show that the product antibodies have no clinical relevance and that the underlying difference between the SBP and the reference product does not signal an otherwise important problem.

### 6.4 Indication extrapolation

Indication extrapolation is the regulatory and scientific process of extending information and conclusions available from one patient population in order to make inferences for other populations. In the context of SBPs, it refers to granting a clinical indication to an SBP for which the reference product is authorized, without conducting clinical efficacy and safety studies to support that indication. Extrapolation cannot be claimed automatically for all indications of the reference product and requires sound scientific justification based on the totality of evidence. The starting point for extrapolation is that the physicochemical and structural analyses, nonclinical tests and clinical studies have demonstrated comparability. Thus, extrapolation should be considered in the light of the totality of evidence of biosimilarity. Current WHO guidance on SBP evaluation \(^1\) sets out a number of recommended principles regarding the extrapolation of clinical data across indications which also apply to biosimilar mAbs. Extrapolation is possible when the following requirements are fulfilled:

- a sensitive clinical test model has been used that is able to detect potential differences between the SBP and the RBP;
- the clinically relevant MOA and/or involved receptor(s) are the same;
- safety and immunogenicity of the SBP have been sufficiently characterized and there are no unique or additional safety issues expected for the extrapolated indication(s).

MAbs have both Fab and Fc-effector functions and may exert their clinical effect through a variety of mechanisms – for example, ligand blockade, receptor blockade, receptor down-regulation, cell depletion (via ADCC, CDC or apoptosis) and signalling induction. A particular mAb may act through one or a combination of these or other mechanisms. Where a therapeutic mAb is indicated for a variety of diseases, various MOAs may be important, depending on the indication in question. In order to support extrapolation, the mechanisms that contribute to the efficacy of the mAb in each indication should ideally be well...
understood and clearly defined. In practice, this is often not the case. Therefore, extrapolation may pose additional challenges when a mAb is indicated for a variety of diseases in which the MOAs are not the same or are not well understood for each indication. In this situation, it is important to explore the comparability of in vitro functions of the mAb. In cases where significant functional differences exist, further nonclinical or clinical data are needed to support extrapolation. Therefore it is essential that the basic functions of the antibody are considered when relevant. The tests should be selected according to their relevance for a particular product and therapeutic indication and, if possible, tailored accordingly (for example, ADCC assays under different conditions). If minor quality differences are found, and the affected mechanism is not considered active in the studied indication, additional steps may be necessary to reach a conclusion on biosimilarity. Additional data, with appropriate supporting scientific rationale, could include quality, preclinical and/or PK/PD data and might impact on the selection of the final clinical, safety and efficacy study. Special post-marketing measures may be used to monitor aspects of safety and/or immunogenicity in the extrapolated therapeutic indications.

6.5 Pharmacovigilance and post-approval consideration

A risk-management plan should be put in place once a biosimilar mAb is approved, in order to ensure its long-term safety and efficacy. The general requirements for pharmacovigilance are the same as for any approved new drug. As described in WHO guidelines (1, 2) it is essential to record the product brand name, batch number and manufacturers’ name, and, where it exists, the International Nonproprietary Name (INN). In many cases, clinically important adverse events occur at a relatively low frequency and the probability of them occurring during the time frame of the clinical trial is also low. Additionally, because of their relatively small sample size, biosimilar mAb clinical trials may have the statistical power to detect only common adverse events. Thus, as for any biological medicine, pharmacovigilance is essential in order to detect potential overt new or rare biosimilar mAb-specific safety issues and to allow for the identification and assessment of potential post-marketing risks.

Authors and acknowledgements

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References


Annex 3

Guidelines on management of blood and blood components as essential medicines

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Appendix Examples of existing legislation, regulations and guidance 160
Guidelines published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for blood establishments/banks that prepare blood and blood components intended for transfusion. If an NRA so desires, these WHO Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA.
## Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>EM</td>
<td>essential medicine</td>
</tr>
<tr>
<td>GMP</td>
<td>good manufacturing practice(s)</td>
</tr>
<tr>
<td>GPP</td>
<td>good preparation practice(s)</td>
</tr>
<tr>
<td>GvHD</td>
<td>graft versus host disease(s)</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>NRA</td>
<td>national regulatory authority</td>
</tr>
<tr>
<td>PDMP</td>
<td>plasma-derived medicinal product</td>
</tr>
<tr>
<td>PRP</td>
<td>platelet-rich plasma</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RTTI</td>
<td>relevant transfusion-transmitted infection(s)</td>
</tr>
<tr>
<td>SOP</td>
<td>standard operating procedure</td>
</tr>
</tbody>
</table>
1. Introduction

Essential medicines (EMs) are defined by WHO as those medicinal products that satisfy the health-care needs of the majority of the population. They should therefore be available at all times, in adequate amounts and in appropriate dosage forms, with assured quality and affordability. The WHO Model List of Essential Medicines\(^1\) was first generated in 1977 and has been updated every 2 years since then.

This list of EMs includes a core list of minimum medicine needs for a basic health-care system (that is, the most efficacious, safe and cost-effective medicines for priority conditions that are selected based on current and estimated future public health relevance), as well as a complementary list of medicines for priority diseases for which specialized diagnostic or monitoring facilities, specialist medical care and/or specialist training are needed. A number of human plasma-derived medicinal products (PDMPs) – namely, factor VIII concentrate and factor IX complex concentrate (coagulation factors II, VII, IX and X) – were added to the 2nd edition of the complementary list of EMs in 1979, followed by the addition of human normal immunoglobulin to the 15th edition in 2007. In the 18th edition of the complementary list published in 2013, factor VIII concentrate and factor IX complex concentrate were replaced with coagulation factor VIII and coagulation factor IX respectively. Furthermore, anti-D, anti-rabies and anti-tetanus immunoglobulins were added to the 19th edition of the core list of EMs in 2015.

In the 2010 World Health Assembly resolution WHA63.12 concern was expressed about the unequal levels of access globally to blood products,\(^2\) particularly PDMPs (also called plasma derivatives). Such inequality of access left many patients without needed treatment, and many of those with severe congenital and acquired disorders without adequate plasma-derivative treatments. In this resolution, the World Health Assembly urged WHO Member States:

\[
\text{...to take all the necessary steps to update their national regulations on donor assessment and deferral, the collection, testing, processing, storage, transportation and use of blood products, and operation of regulatory authorities in order to ensure that regulatory control in the area of quality and safety of blood products across the entire transfusion chain meets internationally recognized standards.}
\]

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\(^1\) See: http://www.who.int/medicines/publications/essentialmedicines/en/

\(^2\) The term “blood products” used in resolution WHA63.12 means blood, blood components and plasma derivatives/PDMPs.
Requirements for implementing effective national blood regulation are described in the WHO Assessment criteria for national blood regulatory systems (1).

In accordance with resolution WHA63.12, and in recognition of the fact that achieving self-sufficiency in the supply of safe blood is an important national goal in preventing blood shortages and meeting the transfusion needs of the patient population, blood and blood components (whole blood, red blood cells (RBCs), platelets and fresh frozen plasma) were added to the 18th edition of the core list of the WHO Model List of Essential Medicines in 2013. Self-sufficiency in this context means that the national needs of patients for safe blood and blood components, as assessed within the framework of the national health system, are met in a timely manner, and that patients have equitable access to safe blood for transfusion, and that this can be accomplished by promoting voluntary non-remunerated blood donation. Defining blood and blood components as EMs (that is, as biological therapeutic products or simply “therapeutics”) could also contribute to self-sufficiency by: (a) drawing attention to the role of national governments in providing the necessary organizational and other support required for assuring a safe and adequate blood supply; and (b) encouraging countries to develop and ensure compliance with safety and quality standards, as well as good practices, in product preparation for transfusion.

Assuring the quality, safety and availability of blood and blood components is additionally linked to promoting self-sufficiency in essential PDMPs. If more plasma is collected by apheresis or recovered from whole blood than is needed for transfusion, it may be used as a starting material for fractionation and thereby support self-sufficiency in PDMPs – provided that the plasma meets required quality standards. As noted above, PDMPs such as coagulation factors and human immunoglobulins have been recognized as EMs since 1979 and 2007 respectively, and have been regulated in several countries as biological therapeutic products for decades to ensure they meet internationally recognized standards for safety, quality and efficacy. However, given the unequal access globally to PDMPs, some countries still rely primarily on the use of whole blood and plasma for various diseases and conditions that could be treated with PDMPs (for example, fresh frozen plasma used instead of factor VIII and factor IX for the treatment of patients with haemophilia A and B respectively), contributing to an essential need for plasma components. Furthermore, plasma components are used for the treatment of several plasma protein deficiency diseases that are not treated with PDMPs.

Effective blood regulation is crucial for the establishment of blood components as EMs. However, blood and blood components may not meet the legal definition of medicines in all countries and this could have an impact on the approach that must be taken to assure their quality, safety and availability (compared to the approach employed for conventional medicines). Consequently, in 2014, the International Conference of Drug Regulatory Authorities (ICDRA)
recommended that WHO undertake a project to provide guidance on the management of blood and blood components as EMs. This project involved the WHO Blood Regulators Network (BRN) in cooperation with the WHO Expert Committee on Biological Standardization.

Blood and blood components are either prepared by blood establishments and distributed to hospitals and other facilities, or prepared by hospital blood banks for use in the treatment of various diseases – with the latter in some cases being perceived as part of medical practice rather than the preparation of a biological therapeutic product. There is therefore concern that blood and blood components could be prepared in facilities (including hospitals) that are not subject to appropriate regulatory oversight. Consequently, the regulatory system should apply to all facilities.

In the context of blood and blood components for transfusion, quality requirements for the preparation of blood components may in some jurisdictions not be called “good manufacturing practices” (GMP) – for example in Europe they are called “good practices”. However, in general, WHO recognizes, and has developed, specific GMP for the preparation of blood components (2). In this GMP document WHO defines the relevant aspects of quality system requirements for blood establishments, including the relevant aspects of GMP that are applicable and necessary for the preparation of blood components for transfusion. In order to support the implementation of comparable regulatory systems for blood components for transfusion, the alternative term “good preparation practices” (GPP) will be used in the current document. The implementation of GPP that are equivalent to the WHO GMP for blood establishments (2) will ensure that blood components have similar safety and quality profiles regardless of where they are prepared.

2. Purpose and scope

These WHO Guidelines are intended to provide a framework for establishing regulatory oversight of blood and blood components for use in transfusion as EMs. The underlying concept is that blood and blood components are biological therapeutic products of human origin whose preparation should be subject to regulatory standardization and oversight to assure their quality, safety and efficacy. The framework provided in these Guidelines is similar to that which is widely applied to the regulation of drugs produced under current GMP (cGMP) but is adapted to address the specific attributes of blood and blood components for transfusion that distinguish them from PDMPs and from pharmaceutical medicines (drugs) in general. In jurisdictions where the legal frameworks in place for medicines manufactured under cGMP for pharmaceuticals would not apply to blood and blood components, parallel regulation based on the model
provided in these Guidelines would involve application of the analogous “GPP” for such products.

The scope of these Guidelines includes elements that:

- make reference to resolution WHA63.12 (2010) regarding the approach that must be taken to assure the quality, safety and availability of blood and blood components for transfusion (see section 1 above);
- clarify the specific nature of blood and blood components as biological therapeutic products of human origin (see section 3 below);
- focus on the ethical aspects of blood donation, such as the need to protect donors against exploitation, and to establish voluntary non-remunerated donations of blood and blood components for transfusion (see section 4.1 below);
- recognize the necessity to implement standards and controls, a quality assurance system and good practices for blood and blood component preparation (see sections 4.2 and 4.3 below);
- highlight the similarities and differences between blood and blood components and conventional biological medicines and biopharmaceuticals (see section 5 below);
- focus on the need to sustain nationally regulated blood systems (see sections 6 and 7 below).

3. Blood and blood components as biological therapeutic products

3.1 Historical background of blood transfusion

The first successful transfusion of human blood, as a treatment for postpartum haemorrhage, was performed in 1818 by a British obstetrician, Dr James Blundell, who drew blood from the patient’s husband and, to prevent the blood from coagulating ex vivo, infused it directly into the patient. This was followed by several technological advancements in transfusion medicine (3, 4), which included:

- A number of discoveries in the early 1900s that led to the introduction of blood typing, cross-matching and antibody identification in order to prevent the immunological risks associated with blood transfusion.
- The development of blood banks in the early to mid 20th century following the discovery that blood collected in anticoagulant solution can be stored for several days when refrigerated.
Developments in blood component manufacturing between the mid and late 20th century, which included the use of interconnected, sterile, disposable plastic containers for collection and preparation of blood and blood components, collection by apheresis and the storage of platelets at 22 ± 2 °C.

The implementation of specific serological and nucleic-acid-based tests for various infectious disease pathogens, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) and syphilis in the mid to late 20th century, to reduce transfusion-transmitted diseases.

Over time, blood collection and component preparation have become increasingly complex, and currently include: (a) donor selection using questionnaires to elicit risk factors for relevant transfusion-transmitted infections (RTTI) (5); (b) aseptic collection (6); (c) laboratory testing, and quarantine measures (2); (d) bacterial detection in platelets and pathogen reduction (7, 8); and (e) the use of data-management software. Further information on these aspects is provided below in section 4.

### 3.2 Indications for essential blood and blood component therapy

Human blood is a complex fluid which circulates in the vascular system and is composed of plasma (the liquid portion which contains proteins and a variety of small molecules) and cellular elements that include RBCs, white blood cells and platelets.

Blood and blood components perform numerous vital functions in the body (9, 10). Consequently, severe blood loss could result in life-threatening conditions such as hypovolaemic/haemorrhagic shock, which requires immediate blood transfusion in order to prevent organ failure and death. Blood transfusion is also used as a supportive therapy for surgery, chemotherapy, and stem cell and organ transplantation, as well as the treatment of serious acute and chronic diseases caused by deficiencies or defects in plasma proteins or cellular blood components, in order to avoid complications such as life-threatening haemorrhage or to improve quality of life by reducing anaemia-related symptoms. As blood systems developed, transfusion evolved from whole blood transfusion to targeted therapy with specific blood components. This is because several of these diseases are due to deficiencies or defects in a single blood component or plasma protein (for example, abnormal or low RBC counts for anaemia (including abnormal haemoglobin for thalassaemia); low platelet counts for thrombocytopenia; and clotting factor deficiency for haemophilia). Plasma derived from whole blood or apheresis can also serve as the starting material for the manufacturing of PDMPs. In this regard, the transfusion of cellular blood components instead of whole
blood could serve to generate additional plasma for further manufacturing of PDMPs, thereby providing one of the possible pathways towards self-sufficiency in PDMPs. Examples of diseases and conditions that are treated with blood or blood component transfusion are listed in Table A3.1 (11, 12).

The increasing global demand for access to safe blood and blood components for transfusion has led to the manufacturing or development of various types of equipment and tests used for their preparation. These technological advancements, along with the large number of components prepared annually, have resulted in a significant increase in the complexity of blood and blood component preparation, which further underlines the need for the development of standards for blood banking, and for the inspection of blood establishments/banks to verify compliance with these standards. However, the implementation of internationally accepted standards such as the WHO GMP Guidelines (2) is currently not mandatory in all countries. Regulatory controls should be established worldwide in order to enhance the safety and quality of blood and blood components intended for transfusion.

Table A3.1
Examples of indications for use of essential blood and blood components

<table>
<thead>
<tr>
<th>Blood/blood component</th>
<th>Examples of indications*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td>RBC replacement in acute active blood loss with hypovolaemia.</td>
</tr>
<tr>
<td></td>
<td>Therapy in the indications below when no specific blood components are available.</td>
</tr>
<tr>
<td>RBCs</td>
<td>Supplement oxygen-carrying capacity (for example, RBC replacement to treat symptomatic anaemia; blood loss during surgical intervention, trauma and haemolysis; and bone marrow failure; and to support patients with haemoglobinopathies).</td>
</tr>
<tr>
<td>Platelets</td>
<td>Prevention or treatment of bleeding due to platelet deficiency or dysfunction, or massive blood loss (for example, in patients with decreased platelet production due to congenital or acquired bone marrow failure; platelet-destructive conditions; dilutional thrombocytopenia; and functionally abnormal platelets).</td>
</tr>
</tbody>
</table>
Table A3.1 continued

<table>
<thead>
<tr>
<th>Blood/blood component</th>
<th>Examples of indications*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>Management of patients who require multiple coagulation factors (for example, bleeding patients; and patients undergoing invasive procedures). Treatment of patients with clinically significant coagulation abnormalities. Treatment of patients with selected coagulation factor or rare specific plasma protein deficiencies for which a more appropriate alternative therapy such as specific coagulation concentrate or recombinant products is not available. Plasma exchange in patients with thrombotic thrombocytopenic purpura. In order to preserve factor VIII, plasma frozen within 8 hours of collection is preferable for indications requiring labile coagulations, or for the preparation of cryoprecipitate for use in the correction of factor VIII deficiency. Plasma frozen within 24 hours could also be used.</td>
</tr>
</tbody>
</table>

* Additional specific medical indications apply to further processed blood components such as washed and irradiated components and cryoprecipitate.

3.3 Risks of blood and blood components

Blood transfusion carries the risk of transmitting infections if the donated blood contains pathogens.

- As the collection of blood requires a venepuncture to be performed, pathogenic bacteria could be transferred into the donation from a contaminated skin area and subsequently proliferate (particularly in platelets) to clinically significant numbers capable of causing an RTTI. This risk must be minimized by the use of standardized and validated techniques and disinfectants for aseptic venepuncture. Moreover, thorough adherence to aseptic technique with closed systems and appropriate microbiological sterility testing should be implemented.

- In the case of several pathogens causing severe disease (including HIV, HBV and HCV) an exposed donor harbouring an RTTI may feel well and wish to donate despite being at risk of transmitting
infections to patients. Therefore, it is crucial to: (a) collect blood from voluntary non-remunerated donors, as they are known to have lower rates of RTTI; (b) exclude from donating, through enquiry, any person who has been at increased risk of acquiring such an infection; and (c) test all donors for RTTI using validated assays that have been approved by relevant regulatory authorities.

- Donors who have tested positive on a first or previous donation must be systematically deferred – that is, the donation must not enter the processing and testing cycle. To achieve this it is recommended that: (a) a national blood donor registry (for example, as part of the blood management system) is maintained at all points of donation; and (b) donors are registered using a unique-identifier system. Conditions for the potential re-entry of donors (for example, after proven clearance of the infection or demonstration of a false-positive test) may be defined.

There are also a number of adverse reactions due to immunological mechanisms; the most relevant of these is blood group incompatibility. Therefore, careful blood group typing and documentation is essential to avoid errors (for example, giving the wrong blood to the patient).

The risks associated with blood transfusion necessitate traceability from donor to patient and vice versa, and a system of haemovigilance – that is, documenting and reporting adverse events and reactions, and initiating corrective actions where necessary. The management of risks associated with blood transfusion needs to be part of the quality management system developed by the blood establishment.

4. Preparation of blood and blood components

4.1 Ethical aspects of blood donation

To ensure the safety of both donors and patients, transactions of human blood and blood components should comply with the well-acknowledged principles of biomedical ethics – namely, autonomy, beneficence, non-maleficence and justice. Dignity also applies to donors in the sense of prohibiting the use of the human body as a source of financial gain (13). Respecting the rights and ensuring the safety and well-being of both donors and patients is fundamental. Consequently, blood donation should begin with a consideration of a number of ethical issues, which include:

- Encouraging voluntary non-remunerated blood donation which serves as an important foundation for a safe and sustainable blood supply.
- Providing information to the donor regarding the potential risks associated with the donation, the risk of donating infected blood, and the donor’s responsibility with respect to patient safety and well-being.

- Obtaining the donor’s consent to the donation and to the use of the donation either for transfusion or for further manufacturing of PDMPs. The donor must be mentally competent and the consent given voluntarily. Collection of plasma for PDMPs should be undertaken only after ensuring sufficient plasma for transfusion. The use of blood and blood components for other purposes should only be allowed when self-sufficiency in blood and blood components for transfusion is already ensured. Their use in research requires ethics approval and a separate and specific informed consent. Under national laws, exceptions may apply in situations where the donation is anonymized.

- Encouraging “non-directed donations” (that is, donations made independently of the needs of a particular patient) in order to prevent coercion by known donors/family members, as such coercion could result in a reluctance to disclose behaviours associated with infectious risks. An exception could be made for designated donations based on medical reasons (for example, for patients with rare blood types where no compatible non-directed donations are available).

- Minimizing the impact of deferrals on donors (for example, health concerns, or feelings of rejection or discrimination) by educating staff on donor-deferral criteria and communication to ensure they are able to explain the reasons for deferral to donors and to follow up with deferred donors as appropriate.

- Protecting donor health and safety during the collection of blood and blood components and, if needed, dealing with donor adverse reactions and obtaining medical care for the donor for an appropriate period of time after the collection.

- Informing donors of abnormal test results and ensuring that reactive infectious disease test results are confirmed and the donors counselled with respect to further investigation and management by an appropriately specialized physician.

- Protecting donors against exploitation.

- Avoiding incentives that could influence an individual’s decision to donate.

- Protecting personal data and making them accessible only to authorized personnel such as physicians or the responsible person.
4.2 Description of key product preparation steps

4.2.1 Donor suitability assessment

Blood and blood component preparation begins with the health screening of carefully recruited donors. Risk-based health criteria and acceptable limits should be taken into consideration during the donor-selection phase of the donation procedure. The measurement of haemoglobin is essential and a limited physical examination, including vital signs (for example, pulse, blood pressure and temperature), may be performed either routinely or where the donor’s condition raises suspicions of any possible anomaly, in accordance with national standards. Each time donors donate, standardized donor-screening questionnaires should be used to elicit information on their medical and social history in order to determine that: (a) they are in good health and will not be harmed by donating blood; and (b) they are not at increased risk of infection with communicable bloodborne diseases. A confidential interview should be conducted by trained personnel to clarify the answers obtained in the questionnaires.

The standard operating procedures (SOPs) of the blood establishment should specify the donor-exclusion criteria as well as the donor-deferral time frames, taking into account both the specific local epidemiology and internationally accepted standards and guidance, such as the Pan American Health Organization guidelines on prospective donor education and selection (14). Donors should also be informed about the necessity to provide post-donation information to the collection facility on any illness or any other information relevant to the safety of donated blood that was unknown prior to donating.

Donors should be tested for selected transfusion-transmissible infectious agents such as HIV, HBV, HCV and syphilis to prevent the use of blood and blood components from infected donors. The testing requirements for additional infectious disease agents should be based on epidemiological data for the geographical region in which the donations are made.

It should be noted that while donor suitability assessment significantly reduces the risk of disease transmission to recipients, there are still concerns about the residual risks that can result from: (a) limitations associated with the donor-screening process (for example, inaccurate responses to screening questions); (b) recent ("window-period") infections (15); (c) assay failures; (d) known pathogens for which testing is not performed; and (e) unknown pathogens. Essential measures for maintaining and/or enhancing the safety of the blood supply should be implemented, and should include quality management as well as the continuous monitoring of new infectious disease threats and timely implementation of appropriate risk-mitigation strategies involving donor screening and/or infectious-disease testing.
4.2.2 Collection and component preparation

After local skin disinfection using a defined and validated disinfection procedure, blood should be collected aseptically into single-use blood bags that meet a suitable regulatory standard. The blood bags, which contain anticoagulant solutions (and preservative/additive solutions where applicable), constitute a closed system. The use of blood bags with diversion pouches can further reduce the risk of contamination with skin microbiota by preventing the initial blood flow from entering the blood bags.

Blood components may be prepared using either a manual or automated procedure. The manual method involves the centrifugation of a unit of whole blood at low speed to obtain RBCs and platelet-rich plasma (PRP), the transfer of the PRP into a satellite blood bag and centrifugation of the PRP at high speed to obtain the platelets and plasma. Alternatively, whole blood can be centrifuged at high speed to obtain three layers consisting of RBCs, plasma and a buffy coat containing platelets and leukocytes. The buffy coats derived from approximately 4–6 units are then pooled and centrifuged at low speed to separate the platelets from the leukocytes. The RBCs, whole blood and platelet components should be leukocyte reduced by the use of pre-storage filters. Leukocyte reduction is needed to reduce the risk of:

- platelet refractoriness due to alloimmunization against human leukocyte antigen (HLA) and platelet-specific antigens in multiply transfused patients;
- febrile non-haemolytic transfusion reactions (FNTRs);
- transmission of leukocyte intracellular pathogens such as human cytomegalovirus (HCMV);
- transmission of variant Creutzfeldt-Jakob disease.

The second method of component preparation is an automated procedure that involves the use of apheresis machines that separate whole blood into its components, transfer the desired components into containers and return the remaining components to the donor. Some apheresis machines have built-in leukocyte-reduction mechanisms.

Blood and blood components may also be subject to additional processing steps such as: (a) pooling; (b) irradiation for the prevention of graft versus host diseases (GvHD); (c) the use of filter systems for the reduction of micro-aggregates; (d) washing to remove plasma; and (e) applying pathogen reduction (currently using photochemical methods) to enhance safety from infections. Donations from family members should be leukocyte-reduced and irradiated to prevent transfusion-associated GvHD.
The SOPs used by blood establishments/banks should specify limits for the volumes collected at each donation, as well as the frequency of donation, in order to protect donor health and safety.

4.2.3 Additional testing

In addition to testing donors for RTTI, blood and blood components should also be subject to the following testing:

- Each donation intended for transfusion should be tested for ABO and RhD blood groups. Testing for red cell antibodies of potential clinical significance is also recommended, particularly for first-time donors and donors with a history of pregnancy or transfusion since their last donation. Additional testing is required for specialized products such as HLA-matched and phenotyped components.

- Quality control testing should be performed on a statistically based proportion of components to ensure ongoing assessment of the quality of the procedures used for product preparation. The frequency of quality control testing, the test parameters (for example, haemoglobin, haematocrit, platelet count, factor VIII concentration and sterility) and acceptance criteria should all be established for each type of component. Test results should be analysed on an ongoing basis and appropriate corrective action taken when values deviate from acceptable limits. Bacterial detection in platelets may also be performed. Note: in some countries each platelet component is subject to bacteriological testing for the detection of bacterial contamination.

4.2.4 Labelling

The “labelling” of blood and blood components refers to both information appearing on the direct product label and/or contained in accompanying documentation. More specifically, the product label should include the product type, blood groups, a unique donation code that is traceable to the donor, the site of product preparation, the list of pathogens for which discretionary testing is performed (for example, cytomegalovirus or hepatitis E virus), the storage conditions and expiry date (and time of day if applicable). Standardized labels that can be universally read (such as those printed using machine-readable ISBT 128 standard terminology) should be used. The list of pathogens for which testing was performed and found to be negative should appear either on the product label or in accompanying documentation.
4.2.5 **Storage**

Blood and blood components should be stored under specified conditions in order to maintain their safety and quality. Units determined to be safe and released for transfusion should be segregated from untested units, and access to storage areas should be restricted to designated personnel. Plasma components should be frozen within a specified period after collection (preferably within 8 hours for fresh frozen plasma, or within 24 hours). Whole blood and RBCs should be refrigerated (at 1–6 °C) and platelets should be stored at 20–24 °C under agitation.

4.2.6 **Distribution and shipping**

To ensure the safety and quality of blood and blood components they should be formally released to hospitals for further storage in hospital blood banks, or for transfusion, after verifying that they meet all safety and quality standards, and are appropriately packaged prior to transportation. The shipping containers should be validated to maintain acceptable storage conditions for the blood and blood components.

4.2.7 **Haemovigilance**

4.2.7.1 **Documentation**

There should be a documentation system that assures bidirectional traceability of blood components between donors and patients as a foundation of haemovigilance.

4.2.7.2 **Adverse reaction reporting and investigating**

There should be a system in place for reporting and investigating serious donor reactions, and serious or unexpected adverse recipient reactions reported by hospitals. In the case of recipient reactions, measures should be taken to notify those in possession of co-components when applicable, and to quarantine and/or recall the co-components. NRAs should also be notified as required. System-wide corrective actions should be implemented where feasible and appropriate.

4.2.7.3 **Look-back and trace-back**

Blood establishments should have a look-back procedure in place in order to identify previous donations (and related blood components) from a donor who, on subsequent testing, is confirmed positive for a transfusion-transmissible infectious agent – and to identify recipients who received blood or blood components from a donor who is later confirmed positive for such an infectious agent.

Trace-back procedures should also be established to investigate any report of a suspected RTTI in order to: (a) identify a potential implicated donor;
(b) determine whether any donor who contributed to the transfusion is infected with (or positive for serological markers of) the implicated infectious agent; (c) trigger a recall of in-date blood or blood components contributed by that donor; and (d) notify consignees and recipients of components collected from that donor. The NRA should also be notified as required.

4.2.8  **Good preparation practices/quality systems**

4.2.8.1  **Key requirements**

It is recommended that blood establishments/banks comply with relevant elements of GPP to assure the quality and safety of blood and blood components (2). These elements include:

- organization and personnel (including training);
- maintenance of facilities/premises;
- equipment qualification, calibration and maintenance;
- quality control programme for products, supplies and services;
- donor selection, blood collection, testing, processing, storage and distribution, and record-keeping;
- SOPs containing step-by-step instructions for all activities undertaken during product preparation, as well as specifications for the resulting blood components;
- process validation;
- change control;
- corrective and preventive measures;
- quality monitoring;
- management of risks, documentation, nonconformities, audits and contracts.

4.2.8.2  **Nonconformity and deviation reporting and investigating**

There should be a system in place to ensure that any nonconformities and deviations that occur during blood and blood component preparation are documented, investigated for their causative factors and followed up by corrective actions. This should include a system for notifying those in possession of the implicated products (and the NRA, if applicable), and for quarantining and/or recalling products whose safety may have been compromised.

4.3  **Associated substances and equipment**

Associated substances and equipment used during blood and blood component preparation include:
- anticoagulant solutions and additive solutions for RBCs and platelets;
- blood pressure and pulse monitors, thermometers, haemoglobin analysers, etc. that are used to assess donor health;
- apheresis equipment, automated blood processors, blood bag collection systems, centrifuges, automated red cell washers, gamma and X-ray irradiators, sterile connection devices, automated blood extractors, plasma freezers, etc. that are used for blood and blood component collection and/or processing;
- in vitro screening test kits used for donor testing, and systems for microbial detection, compatibility testing and quality control testing (including automated systems);
- pathogen-reduction technology systems;
- computerized blood management systems, specifically systems that analyse data regarding the suitability of blood and blood components for transfusion (note: the classification of blood management systems as medical devices depends on the specifications of the product and the national medical devices legislation).

These substances and equipment are generally regulated as medical devices, except for the anticoagulant and additive solutions, which may be regulated as either drugs or devices. Blood establishments/banks need to ensure that the materials and devices being used for the preparation of blood and blood components are approved by their regulatory authorities. Furthermore, even though device manufacturers are responsible for the validation of the software in automated devices, in some cases additional validation is required prior to implementation – particularly when the equipment needs to be programmed according to the specific needs of the blood establishment/bank. This further complicates the preparation process for blood and blood components and underscores the need to comply with internationally recognized standards.

5. Comparison of blood components with PDMPs

5.1 General
These WHO Guidelines propose the regulation of blood and blood components under GMP, which consist of cGMP that have been adapted to address the attributes of blood and blood components that distinguish them from PDMPs. PDMPs may already be regulated as medicines under an existing framework. The following sections highlight both the similarities and the differences between blood and blood components and PDMPs to assist in determining quality requirements that could be applied to blood and blood components.
5.2 **Product safety and quality**

Conventional biological medicines are typically manufactured on an industrial scale using complex proprietary processes that vary between manufacturers. One example is the manufacturing of PDMPs, which may involve, among other steps: (a) the pooling of thousands of plasma units; (b) the concentration and/or purification of one or more plasma proteins using methods such as cryoprecipitation and various fractionation procedures that utilize chromatographic, precipitation and filtration techniques; (c) viral inactivation/removal techniques to enhance product safety; and (d) formulation, filling and lyophilization. In-process testing is performed at various steps to monitor the manufacturing process, and final product testing of each lot is performed to ensure that product specifications are met.

The manufacturing of PDMPs is similar to that of other biopharmaceuticals with respect to the complexity of the manufacturing process and its potential impact on the biological characteristics of the final products. Thus, like other biopharmaceuticals, PDMPs are subject to GMP regulations to ensure the products are consistently safe, efficacious and of high quality.

The preparation of blood and blood components differs from PDMP manufacturing in that: (a) closed single-use systems are used for product preparation to reduce the risk of contamination/cross-contamination; (b) each component is derived from one donation or from a limited number of donations; and (c) in some cases, the preparation techniques employed are limited to mechanical or physical methods such as centrifugation, separation and cryoprecipitation (for cryoprecipitates). Additional methods such as leukocyte reduction, pooling, washing, irradiation and photochemical methods for pathogen inactivation are also employed. Consequently, blood and blood components can be produced at various facilities, ranging from large blood establishments to small hospital blood banks. There are concerns that not all blood establishments/banks are regulated by an NRA. Even in settings where unregulated blood establishments/banks have adopted manufacturing standards developed by professional organizations, there is no mechanism for verifying compliance with these standards.

Notwithstanding the differences in the complexity of the processes used for the manufacturing of PDMPs and those used for the preparation of blood and blood components, there are also similarities with respect to the following:

- a reliance upon the availability of healthy donors and the need to protect donors;
- the risks associated with RTTI and the donor screening and testing measures required to mitigate these risks;
the importance of linking the donor with each lot of product manufactured or prepared through appropriate labelling and record-keeping to facilitate recalls and, where applicable, look-backs and trace-backs;

- the need to validate new or modified procedures employed for product manufacturing or preparation;

- the use of appropriately validated automated systems, particularly when there is a need to track a large number of donors/donations and the results of their screening and infectious-disease tests;

- the need for segregation and holding (that is, quarantine) of donations/products until they are released for distribution to prevent the release of potentially unsafe products;

- the need for product storage and transportation at appropriate temperatures and conditions.

These similarities lead to the underlying concept that blood and blood components should be prepared within a quality management system based on the principles of GMP (adapted to blood and blood components) when relevant and appropriate – and which includes elements such as the testing of starting materials, in-process quality testing and controls (for example, bacterial detection and other quality control tests), labelling that reflects product identity and assures traceability, and adverse event reporting (see section 4.2.8 above). Consequently, the regulation of blood and blood components as biological therapeutic products would ensure the consistent implementation of appropriate standards for product quality, safety and efficacy. Such regulation would apply to all blood establishments/banks involved in the preparation of these products.

5.3 Product efficacy

As with other biopharmaceuticals, PDMPs are subjected to clinical trials in the target population to establish their safety and efficacy before approval for clinical use. Such trials are typically not required for conventional blood and blood components because: (a) their efficacy has been established through historical use; and (b) they are prepared and stored using established procedures that are published in standards developed by professional organizations. However, clinical trials are currently required for blood and blood components when they are prepared using new technologies or processing steps (for example, pathogen-reduction technologies) as these could potentially alter their biological characteristics.
6. The blood regulatory system

6.1 Guiding principles
The management of blood and blood components as EMs should take into consideration the need to:

- sustain nationally regulated self-sufficient blood systems;
- protect donors against exploitation and prohibit financial gain;
- base blood and blood component standards and controls on a quality management system derived from GPP in order to assure the quality, safety and availability of these products;
- ensure that the regulations for blood and blood components and for PDMPs are complementary, and incorporate the essential elements and core functions specified in the WHO Assessment criteria for national blood regulatory systems (1).

6.2 Regulatory framework
6.2.1 General
Blood and blood components should be controlled under an appropriate regulatory system in order to promote and enhance their quality, safety and availability. The elements and functions of an effective national blood regulatory system have been described by WHO and are applicable both in developed and developing countries (1).

The regulatory system should consist of a regulatory framework administered by an NRA that is responsible for regulating the activities associated with the preparation of these products. Regulatory frameworks consist primarily of legislative instruments such as legislation (or act) and regulations that can be supplemented by non-legislative instruments such as policies, guidelines and guidance documents. Collectively, these instruments allow for the categorization of risk to an appropriate level of control and the capacity to respond quickly to rapid technological advances, while providing the required authority and capacity to take immediate action during crises and emergencies.

6.2.2 Legislation
The legislation or law serves as the first level of a comprehensive regulatory framework and provides a legal basis for the establishment of a regulatory system. A law is needed that governs the preparation of blood and blood components, as well as the use of associated substances and relevant medical devices. The law should define the scope of regulations and provide the legal authority for
their development. The following are examples of the kinds of provisions that could be included in legislation:

- Definition of the therapeutic products and devices to be regulated.
- Prohibitions that prevent the preparation or sale of potentially unsafe products (for example, products that are adulterated or prepared under unsanitary conditions).
- Assignment of an NRA with legal powers to administer, enforce and verify compliance with the legislation and regulations (for example, powers for inspection, seizure and forfeiture and for the establishment of a list that sets out the classes of products to be regulated).
- The offences and punishment of persons who deliberately contravene the legislation or regulations.
- Definition of the areas for which regulations should be developed and granting of the authority to develop the regulations necessary for carrying the purposes and provisions of the legislation into effect. These areas should include those covered below in section 6.2.3.

Detailed guidance regarding provisions that could be included in national acts or legislation may be found in the list of documents provided in the Appendix to these Guidelines.

6.2.3 Regulations

Regulations form the second level of the regulatory framework. They are developed under the authority of the legislation and serve to interpret the legislation and to provide policies and standards/technical requirements that are legally binding.

Regulations can be developed using different approaches. In the traditional risk-management approach, good practices and standards are written directly into regulations. The process for developing and amending regulations can be lengthy and can take up to several years in some jurisdictions, thus making it difficult to ensure that they remain current with regard to technological advances and emerging threats.

An alternative and more flexible approach is the development and use of standards that are not directly incorporated into regulations, but can be referenced in the regulations. For example, instead of specifying the requirements for donor screening and infectious-disease testing in regulations, the sections of voluntary or mandatory national or internationally recognized standards containing these requirements could be referenced in regulations to give them the force of law. Since the standards are a stand-alone document, they can be
amended rapidly when required without amending the regulations themselves. This approach is particularly useful for standards/technical requirements that are likely to require frequent amendments in response to rapid technological advances and emerging threats.

The incorporation of standards into regulations by reference may be achieved using one of the following approaches:

- Static or fixed – this approach references requirements in a specific version of a standard at a defined date to ensure that amendments to the standard do not automatically become part of the regulations. In this approach, a regulatory amendment will be required to reference subsequent versions of the standard.

- Ambulatory or flexible – this approach references requirements in the standard as amended from time to time to automatically make any amendments part of the regulations. In this approach, the regulations do not need to be amended to reference subsequent versions of the standard.

This standards-based approach to regulation could be adopted, at least in part, for blood and blood components for which the procedures used for their preparation and storage are well established.

Regulations for blood and blood components should focus on managing risk in four key areas:

- protection of donor health and safety;
- prevention of infectious-disease transmission from donors to recipients;
- prevention of adverse reactions due to immunological mechanisms in transfusion recipients;
- prevention of improper handling or processing that could affect product safety, efficacy and quality.

This can be accomplished by including requirements for the following elements in blood regulations:

- Standards for the collection and processing of blood and blood components, which include the methods used for their preparation (see section 4 above).
- GPP (consistent with GMP in some jurisdictions) to assure the quality, safety and availability of these products.
- The use of test kits, blood-collection sets, anticoagulant/additive solutions and other collection equipment that have been approved by the NRA.
Importation and exportation of blood and blood components – although self-sufficiency in blood and blood components is a basic principle.

The definition of clinical trials and the requirement for clinical trials of blood and blood components prepared using new technologies (for example, pathogen-reduction technologies) that could potentially alter their biological characteristics.

Pre-approval of applications/submissions to determine if the data submitted support the claims made for product safety and quality and, if applicable, for efficacy (this may include on-site evaluations of the facility and processes used for product preparation).

The issuance of authorization by the NRA to carry out product preparation activities.

The review of applications/submissions for post-approval changes.

The submission of applications for (or amendments to) registration, accreditation or blood establishment licensing.

The registration of blood establishments/banks and importers or the issuance of licences to such facilities based on evidence of compliance with GPP.

The authority of the NRA to issue, refuse, suspend, reinstate or cancel an authorization, registration or accreditation, or facility licence.

The provision of information to the NRA regarding serious reactions in donors and recipients by the holders of blood and blood component registration or authorization and licences.

The performance of risk–benefit evaluation and investigation of the root cause of nonconformity, deviation and adverse events reports.

Powers of inspectors, which allows for the performance of compliance and enforcement activities such as inspection of blood establishments/banks to assess compliance with regulatory requirements, investigation of nonconformities and follow-up of corrective actions.

Consideration should be given to the adoption of internationally recognized standards, such as the examples cited in these Guidelines (2, 5, 6, 14), that set out detailed requirements for the activities described in section 4 above. Regardless of the approach taken, all stakeholders should be given an opportunity to comment on the regulations before they are finalized.

There should also be regulations that define “investigational test” and that require the investigational testing and pre-approval of applications for associated substances (such as anticoagulant, additive and preservative solutions)
and relevant medical devices (such as in vitro screening and diagnostic test kits, blood-collection equipment and blood bag systems) that are used during the preparation of blood and blood components. Systems should be put in place to ensure compliance with these regulations.

6.2.4 Non-binding instruments
The third level of the regulatory framework consists of policies, guidance documents/guidelines and voluntary standards that can be used to supplement regulations. Typically, these documents may be simpler and faster to introduce than regulations, and can be used to interpret regulations and/or provide details to blood establishments/banks on how to meet regulatory requirements. Since they are not legally binding, they allow flexibility with respect to their interpretation and are adaptable to change. However, if a failure to implement these non-binding instruments were to result in a serious adverse event, the blood facility in question would need to explain why the guidance was not followed.

6.3 The regulatory authority
6.3.1 Organization of the regulatory authority
The regulatory authorities in different countries may currently be organized at a local, regional or national level. The establishment of regulatory authorities at the local or regional level could lead to differences in the standards and regulatory requirements applied to blood and blood components, as well as in the level of regulatory oversight. While recognizing that huge difficulties exist in some regions, it is recommended that countries move towards the establishment of an NRA in order to ensure consistency across the country in both regulatory requirements and oversight.

6.3.2 Functions of the NRA
The key functions of the NRA with respect to blood and blood components are described in the WHO Assessment criteria for national blood regulatory systems (1). Such functions include the development of regulations and standards for the preparation of blood and blood components, and the provision of regulatory oversight to verify compliance with regulatory requirements (for the individual elements of such requirements see section 6.2.3 above).

The WHO assessment criteria document also contains additional information on: (a) the essential elements necessary to establish the legal basis, authority and general characteristics of an NRA; (b) the core functions of an NRA required for the comprehensive oversight of blood and blood components; and (c) major criteria, indicators and associated ratings to assist NRAs in assessing their performance and identifying areas for improvement.
7. The blood supply system

7.1 Organization of the blood supply system

The blood supply systems in different countries may currently be organized at a local, regional or national level with respect to blood collection, testing and processing. The establishment of blood systems at the local or regional level could lead to differences in the implementation of standards and regulatory requirements, and consequently to blood and blood components with different safety and quality profiles. Where possible, it is recommended that countries move towards a nationally regulated and coordinated blood supply system in order to: (a) harmonize procedures and best practices at the national level; and (b) provide assurance that blood and blood components from different areas are of equivalent safety and quality and thereby facilitate the exchange of these products across the country.

7.2 Functions of blood establishments/banks

The blood supply system consists of blood establishments/banks that collect, test, process (including washing, pooling and irradiation) and distribute whole blood and blood components intended for transfusions, as well as plasma intended for further manufacturing into PDMPs. Such facilities are responsible for: (a) performing the activities described in sections 4.1, 4.2 and 4.3 above; and (b) implementing the regulations and standards developed by NRAs for these activities. All facilities that perform these activities (including hospital blood banks that prepare blood and blood components for use within their hospitals) should implement the regulations and standards developed by the NRA.

8. The blood transfusion system

The blood transfusion system consists of care centres (hospitals, surgical centres and outpatient facilities; and sometimes ambulances) that utilize blood and blood components for the treatment of patients. Such centres are responsible for carrying out the following activities:

- storing blood and blood components at appropriate temperatures and conditions;
- developing appropriate procedures for further processing of the blood and blood components prior to transfusion – for example, pooling, washing and irradiation, where applicable;
- appropriate pre-transfusion testing of patients and cross-matching to ensure compatibility of the blood component to be transfused;
- maintaining appropriate records to ensure that blood components can be traced to their recipients and from recipients back to their donors;
- documenting and investigating nonconformities and deviations related to the handling of blood and blood components;
- quarantining of blood and blood components that are under investigation by the blood establishments/banks and hospitals;
- reporting adverse events and reactions that are related to the quality of blood components to the blood establishments/banks;
- investigating, evaluating and documenting all adverse transfusion reactions;
- ensuring the appropriate use of blood and blood components by clinicians.

9. Stepwise implementation of a nationally regulated blood system

The implementation of a nationally regulated blood system is fundamental to assuring the quality, safety and availability of blood and blood components in accordance with their listing as EMs. A risk-based strategy is recommended when considering the development of a regulatory model for the blood system and a national roadmap for its implementation.

It is recognized that, when implementing a nationally regulated blood system, the starting situation may vary considerably from one country to another. In some countries, the blood system may be fragmented and central coordination completely lacking, whereas, in other countries, national or regional bodies may perform a coordinating function within the blood services. In any case, the political commitment of the ministry of health is necessary to establish a roadmap for implementing a nationally regulated blood system. The main elements of this roadmap should be developed and agreed upon in cooperation with the key stakeholders. This implementation plan may also incorporate an initial review and improvement of the existing structure of the blood system in a country. The assignment of the main tasks for implementing this roadmap and the role and mandate of key personnel should be defined and agreed upon as part of the political process. In any case, a cooperative and step-by-step process to restructure the blood system in a country (if needed) and to implement a nationally regulated blood system is encouraged in order to foster more success over time.

The key stakeholders in the blood system (that is, the blood regulatory system, the blood supply system and the blood transfusion system) should
be involved from the beginning in order to understand and define their individual responsibilities and expected contributions. Regular interaction among stakeholders is essential.

The legislative body should define a legal framework (regulation) applicable to blood and blood components. This would include assigning the NRA to oversee all institutions and health-care professionals supplying blood and blood components.

An NRA is an essential element of a regulatory system. The decision to adopt a particular regulatory model should take into account existing regulatory structures, capacities and expertise. Establishing the regulation of blood and blood components under the NRA for medicines may be the most effective and rapid way to accomplish this in settings where blood regulation is otherwise lacking. Regulatory frameworks for blood and blood components and for PDMPs should be complementary.

Blood establishments, other related health institutions, and health-care professionals supplying blood and blood components for transfusion should be engaged and their experience used to inform the establishment of standards and procedures. This may result in improving the existing structure of the blood system. The initial use of existing standards as a starting point for establishing a common language between all key players may provide an acceptable approach for all parties.

Where applicable, representatives of the plasma fractionation industry should be invited to participate as additional key players to support this process. This should ensure that appropriate standards are implemented and that the quality of surplus plasma as a starting material for further manufacturing will meet the necessary requirements.

The development of national blood standards covering donor-selection criteria, infectious disease marker testing strategies, quality system requirements and standards for the final products (specifications and/or monographs) will be an essential step. Both during and after the legislative process, these initial standards may continuously be improved and implemented on a national basis. The establishment of such standards should take into account existing national and international guidelines such as the WHO guidelines on good manufacturing practices for blood establishments (2). As soon as possible, blood establishments should apply these standards in a consistent manner by implementing appropriate procedures (including SOPs and training) within their quality system.

A parallel national implementation process and regular interaction among the key players will be essential in accelerating the implementation of standards and regulatory functions. Since the process of implementing a regulatory system and reaching an acceptable compliance status may take several years, a political decision will be required to define the time frames
for reaching full compliance with standards and for effective enforcement of regulations by the NRA. A possible stepwise implementation plan for a nationally regulated blood system is outlined in Fig. A3.1.

Fig. A3.1
Stepwise implementation plan for a nationally regulated blood system

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<td>• Development and/or adoption of blood standards</td>
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<td>• Review/improve organization, infrastructure and sustainable funding mechanisms</td>
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<td>• Increasing availability and ensuring supply</td>
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Authors and acknowledgements

The development of these WHO Guidelines was initiated in 2014 following a recommendation made by the International Conference of Drug Regulatory Authorities (ICDRA) to the WHO Blood Regulators Network (BRN). The BRN (Chair: Dr C. Schaerer) then undertook the project in cooperation with the WHO Expert Committee on Biological Standardization.

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The BRN reviewed all comments received for incorporation into the revised final draft document WHO/BS/2016.2285.

Further changes were subsequently made to document WHO/BS/2016.2285 by the WHO Expert Committee on Biological Standardization.

References


## Appendix

### Examples of existing legislation, regulations and guidance

The documents listed here are provided as examples of existing legislation, regulations and guidance that may be helpful in establishing a national regulatory framework.

<table>
<thead>
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<th>Country or region</th>
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|                   |   • This Act applies to food, drugs (including blood and blood components), cosmetics and devices.  
|                   |   • These are stand-alone regulations for blood and blood components intended for transfusion and further manufacturing, and were developed under the authority of the Food and Drugs Act.  
|                   | 4. Food and Drug Regulations:  
|                   |   • These regulations apply to food and drugs. The requirements for drugs (which may apply to associated substances such as anticoagulants/additive solutions) can be found in Part C, Divisions 1, 1A, 2, 5 and 8 of these regulations.  
|                   | 5. Medical Devices Regulations:  
|                   |   • These regulations apply to medical devices, for example, infectious disease test kits, blood-collection sets and apheresis equipment. |
### Table continued

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<th>Country or region</th>
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Guidelines published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA.
**Abbreviations**

anti-HBc  antibodies to hepatitis B core protein  
anti-HBs  antibodies to hepatitis B surface antigen  
CE  *Conformité Européenne* (conforms to European requirements)  
CLIA  chemiluminescence immunoassay  
EIA  enzyme immunoassay  
FDA  Food and Drug Administration  
HBsAg  hepatitis B surface antigen  
HBV  hepatitis B virus  
HCV  hepatitis C virus  
HIV  human immunodeficiency virus  
ID-NAT  individual donation nucleic acid amplification technique  
IDI  interdonation interval  
IU  International Unit(s)  
IVD  in vitro diagnostic  
MP-NAT  minipool nucleic acid amplification technique  
NAT  nucleic acid amplification technique  
OBI  occult hepatitis B infection  
P  probability  
PCR  polymerase chain reaction  
PDMP  plasma-derived medicinal product  
RDT  rapid diagnostic test  
RR  residual risk (used in mathematical formulae)  
vDWP  viraemic phase of the diagnostic window period
1. Introduction

The course that a viral infection may take in an individual and the different phases of viral infections are described in the following sections – together with the advantages and limitations of using different blood-screening assays for the different infection phases. Blood-screening assays are differentiated by distinct categories. The residual risk of missing viral infections using any screening assay is mainly due to the viraemic phase of the diagnostic window period (vDWP) for each assay – the mean size of which varies between different assay categories. Another component of the residual risk is the virus epidemiology of the donor population (consisting of repeat and first-time donors) with the rate of new infections (incidence) in donors determining the probability of window-period donations. The residual risk per donation from the repeat-donor subpopulation may be used to extrapolate the respective risk for the first-time donor subpopulation, for which incidence data are often unavailable. The residual risk affects recipients of non-pathogen-inactivated blood components to whom viruses may be transmitted. It also determines the potential viral load of plasma pools used for the manufacturing of plasma-derived medicinal products (PDMPs); this potential contamination level needs to be assessed against the viral inactivation or reduction strategies in the manufacturing process.

2. Purpose and scope

These WHO Guidelines provide advice on estimating the residual risk of human immunodeficiency virus (HIV), hepatitis B virus (HBV) or hepatitis C virus (HCV) being present in cellular blood components and plasma. This estimation has implications for the safety of non- (or incompletely) pathogen-inactivated blood components or plasma products. There are large differences in the prevalence and incidence of viral infections in blood donors around the world. The impact of such epidemiological differences on blood safety needs to be assessed together with the sensitivity of the testing strategy applied. Such assessments may be used to guide strategic decisions on the choice of assays to detect virus-positive blood donations and as a basis for cost–benefit analysis of the different testing scenarios most suitable in the region. The factors influencing the risk of virus transmission by blood components are described, as well as simple mathematical formulae to calculate its probability. These estimates may also be used to counsel recipients on the risks of transfusion. Similarly, the probability and potential level of viral contamination of plasma pools used for the manufacture of PDMPs can be calculated. The infectivity risk of plasma products can then be estimated in relation to the inactivation and reduction capacity of the manufacturing process.
Currently, recovered plasma from whole blood donations is often not used for plasma fractionation because of perceived potential virus risks and quality concerns. This is true for (but not limited to) many blood establishments in low- and middle-income countries, where specific data (for example, on interdonation periods of individual donors) are often not available due to a lack of computerized systems. These WHO Guidelines therefore aim to enable the approximate estimation of residual risks based on limited data, while recognizing that more precise models have been published in the scientific literature. Nevertheless, it is hoped that this document can help in rationalizing decision-making on the use of plasma units for fractionation.

Since the performance of screening assays is one of the key elements in minimizing the residual risk of blood components and guaranteeing the safety of plasma products, these WHO Guidelines also contain advice on the assessment of in vitro diagnostics (IVDs) in studies using specimen panels from the region (Appendix 1). Such targeted performance evaluations for new assays may be performed prior to the acceptance of a new blood-screening assay in a country.

3. Terminology

The definitions given below apply to the terms as used in these WHO Guidelines. These terms may have different meanings in other contexts.

Analytical sensitivity: the smallest amount of the target marker that can be precisely detected by an IVD assay; it may be expressed as the limit of detection and is often determined by testing limiting dilutions of a biological reference preparation.

Apheresis: the process by which one or more blood components are selectively obtained from a donor by withdrawing whole blood, separating it by centrifugation and/or filtration into its components, and returning those not required to the donor. The term “plasmapheresis” is used for a procedure dedicated specifically to the collection of plasma.

Blood collection: a procedure whereby a single donation of blood is collected in a sterile receptacle containing anticoagulant and/or stabilizing solution, under conditions designed to minimize microbiological contamination, cellular damage and/or coagulation activation.

Blood component: a constituent of blood that can be used directly or after further processing for therapeutic applications. The main therapeutic blood components are red blood cell concentrates, platelet concentrates, plasma for transfusion and cryoprecipitate.

Blood establishment: any structure, facility or body that is responsible for any aspect of the collection, testing, processing, storage, release and/or distribution of human blood or blood components when intended for transfusion.
or further industrial manufacturing. It encompasses the terms “blood bank”, “blood centre”, “blood transfusion unit”, “blood service” and “blood transfusion service”. The definition of this term may differ between legislations.

**Blood product**: any therapeutic substance derived from human blood, including whole blood, blood components and PDMPs.

**Diagnostic sensitivity**: the probability that an assay gives a positive result in human specimens containing the target marker (that is, being true positive).

**Diagnostic window period**: the time interval from infection to the time point when a blood specimen from that infected person first yields a positive result in a diagnostic or screening assay for that agent (for example, for specific antibodies); in the context of residual risk this is often simply called the “diagnostic window” or “window period”. The diagnostic window period consists of two phases – the first period of viral replication in the target tissue without presence in peripheral blood is called the eclipse period; the eclipse period is then followed by the ramp-up phase during which the virus concentration increases exponentially in the blood (viraemic phase). Blood components prepared from a blood donation made during the viraemic phase of the diagnostic window period (vDWP) (the potentially infectious window period) can transmit infection to the transfusion recipient, or the respective plasma may contaminate the plasma pool used for manufacturing PDMPs.

**Donor**: a person in defined good health conditions who voluntarily donates blood or blood components.

**First-time (tested) donor**: a donor whose blood or plasma is tested for the first time for infectious disease markers in a blood establishment.

**Fractionation**: the (large-scale) process by which plasma is separated into individual protein fractions that are further purified for medicinal use. The term “fractionation” is usually used to describe a sequence of processes, including plasma protein separation steps (typically precipitation and/or chromatography) and purification steps (typically ion-exchange or affinity chromatography). These steps may also contribute to the inactivation or removal of bloodborne infectious agents (most specifically viruses and, possibly, prions).

**Hepatitis B virus (HBV)**: an enveloped double-stranded DNA virus; causative agent of hepatitis B.

**Hepatitis C virus (HCV)**: an enveloped single-stranded RNA virus; causative agent of hepatitis C.

**Human immunodeficiency virus (HIV)**: an enveloped diploid single-stranded RNA virus; causative agent of acquired immunodeficiency syndrome (AIDS).

**Incidence**: the number of newly acquired infections per unit of time in a defined population.
NAT conversion: the time period during which specific nucleic acids (for example, viral nucleic acids after a recent virus infection) become detectable by a nucleic acid amplification technique.

Nucleic acid amplification technique (NAT): a testing method to detect the presence of a targeted area of a defined nucleic acid sequence (for example, viral genome) using amplification techniques such as polymerase chain reaction (PCR) or transcription mediated amplification (TMA).

Plasma: the liquid portion remaining after separation of the cellular elements from blood – collected in a receptacle containing an anticoagulant, or separated by the continuous filtration or centrifugation of anticoagulated blood.

Plasma for fractionation: plasma (from whole blood or apheresis) used for the production of PDMPs.

Plasma for transfusion: plasma (from whole blood or apheresis) used for direct infusion into patients without a prior fractionation step. It can be subjected to treatment for inactivating a broad range of pathogens.

Plasma-derived medicinal products (PDMPs): a range of medicinal products obtained by the fractionation of human plasma. Also called plasma derivatives, plasma products or fractionated plasma products.

Plasmapheresis: see “Apheresis” above.

Prevalence: the proportion of past infections identified over a specified period in a defined population.

Recovered plasma: plasma recovered from a whole blood donation and used for transfusion or for fractionation into PDMPs.

Repeat donor: a person who has donated blood/plasma previously in the blood establishment. The definition of this term may differ between legislations.

Sensitivity: see “Analytical sensitivity” and “Diagnostic sensitivity” above.

Seroconversion: the time period during which specific antibodies develop (for example, after a recent virus infection) and become detectable in the blood; this term is sometimes also used for the time period during which viral antigens, such as hepatitis B surface antigen (HBsAg), or viral nucleic acids become detectable in the blood after recent infection. See also “NAT conversion” above.

Source plasma: plasma obtained by apheresis for further fractionation into PDMPs.

Viraemic phase of the diagnostic window period (vDWP): the part of the diagnostic window period during which viruses are present in the blood; the beginning of the viraemic phase is defined by the putative presence of one virus particle in a blood component (20 mL plasma for packed red blood cells) and can be extrapolated using viral replication kinetics (viral doubling time).

Window period: see “Diagnostic window period” above.
4. Course of HIV, HBV and HCV infections

The course of infection in humans differs for HIV, HBV and HCV depending on the biological features of the virus and on the individual immunological response to the infection. In principle, chronically persistent virus infections can be distinguished from infection courses leading to clearance of the virus. Both courses have in common an acute phase which is associated with viral replication, detectable viraemia and sometimes with clinical symptoms. A chronically persisting infection without viral clearance almost always occurs with HIV, frequently with HCV and sometimes with HBV.

4.1 Acute infection

The acute viraemic phase of infection is followed by the humoral and cellular immune responses, resulting in seroconversion and potential clearance of the virus. For some infections the immunity also protects against reinfection. The acute viraemic phase of virus infection in blood donors may be detected by antigen assays or, more sensitively, by assays based upon the nucleic acid amplification technique (NAT). Antibody assays are not useful for the detection of acute infections, but have long been used for the detection of persistent infection (HIV, HCV). Usually there is an overlap of immunoglobulin detection (for example, of immunoglobulin M) and the declining phase of viraemia.

For HBV, both acute resolving and chronic persistent infection courses occur. The frequencies of either are dependent upon different factors (such as the age of the individual becoming infected). It has been estimated that in 70% of HBV-infected donors hepatitis B surface antigen (HBsAg) may be detected transiently in blood, 5% develop chronic HBV infection with continuous antigenaemia and 25% do not show detectable antigenaemia. In principle the marker HBV DNA follows the same transient pattern as HBsAg but the median length of viraemia detection is longer. The transient nature of these HBV blood-screening markers requires the use of an adjustment factor when calculating rates of new infections (1).

4.2 Chronic persistent infection

HIV causes persistent infection in almost all infected individuals, while HCV infection becomes chronic in approximately 70% of cases (2). A minority of HBV-infected adults (around 5%) become chronic carriers, depending on the age and immune status of the infected subjects. These chronic infections of HIV, HBV and HCV are usually lifelong active infections associated with viral replication characterized by continuous or reappearing (undulating) phases of viraemia, despite the presence of specific antibodies.
Persistent viraemic infections are usually detectable by both serology and NAT-based assays. An exception is HBV where low-level HBV-DNA-positive carriers (HBsAg negative; antibodies to hepatitis B core protein (anti-HBc) positive) have been associated with so-called occult hepatitis B infection (OBI) (3, 4). In some low-prevalence countries the potential OBI transmission risk has been greatly reduced by the introduction of anti-HBc testing. However, in large parts of the world where HBV is endemic, screening for this marker would lead to the loss of an unacceptable proportion of donors. Blood components from donors with OBI have transmitted HBV at a low frequency (approximately 3%), while the presence of detectable levels of antibody against HBsAg (anti-HBs) has been found to protect against infection, with few exceptions (5–9). The OBI-associated input of HBV into plasma pools used for the manufacture of PDMPs appears negligible when compared to the potential viral loads in diagnostic window period donations.

### 5. Residual risk origins

Predominantly, the residual risk of HIV, HBV or HCV infections in blood or plasma donations is defined as the probability of collecting a donation from an asymptomatic viraemic donor infected with one of these bloodborne viruses, and this not being detected by the routine screening assays.

Such an undetected blood donation may transmit the infection to a recipient if the blood components are not pathogen inactivated. If the pathogen inactivation and removal capacity of the production process is not sufficient an infectious unit of plasma may also contaminate a manufacturing plasma pool and pose a risk to the recipients of the plasma-derived products.

The non-detection of virus infection in blood or plasma donors may be caused by assay failures or by donors being in the diagnostic window period.

#### 5.1 Assay failures

Assay failures in blood screening can occur due to viral variants escaping detection (for example by oligonucleotide mismatches in NAT-based methods, monoclonal antibodies not detecting the antigen of a mutant virus, or recombinant antigens/peptides not detecting antiviral antibodies) (10–12). The contribution of assay failures to the residual risk is considered negligible for “state-of-the-art” assays and will not be factored into the residual risk calculation suggested by these Guidelines. Nevertheless, it is important to continuously survey the quality features of screening assays and to identify potential causes of false test results. Post-marketing surveillance of assay safety,
quality and performance is a mechanism for detecting, investigating and acting on any issues and failures identified, and for addressing the need for continuous assay improvement (13).

Another potential root cause of assay failure is an inadequate quality management system in place within the testing laboratory. Quality assurance aspects include: (a) participation in external quality assessment (proficiency testing and on-site supervision); (b) the conduct of process (quality) control; (c) maintaining adequate documentation (through standard operating procedures) and record-keeping (testing logbooks, registers); (d) maintaining proper inventory and purchasing systems; (e) equipment maintenance; (f) safe facilities; (g) appropriate organization; and (h) measures to ensure adequately trained and competent testing personnel.

5.2 Diagnostic window periods

Historically, the phase elapsing between the time point of infection and the point of first detectability of the viral marker by the screening assay has been called the diagnostic window period. All types of screening assays are associated with a diagnostic window, the length of which is dependent upon the screening marker, the screening assay category, the sensitivity of the assay used and the replication kinetics of the virus during early infection.

The diagnostic window of HIV, HBV and HCV infections begins with the eclipse phase during which the virus is not yet detectable in blood (even by highly sensitive NAT-based assays). This non-viraemic phase is followed by the viraemic ramp-up phase during which virus concentration in the plasma increases in an exponential fashion. For each of the three bloodborne viruses covered in these Guidelines (HIV, HBV and HCV) a specific constant replication rate is apparent until a peak or plateau phase of maximal viral concentration is reached.

In the context of blood safety, the viraemic phase within the diagnostic window period is relevant. The start of the potentially infectious window period during the early ramp-up phase of viraemia can be defined as the point at which one virus particle is present in a blood component. A generally accepted worst-case assumption for cellular components is to define the start of the infectious window period as the point at which the concentration reaches one virus particle in 20 mL of plasma (the volume co-transfused with a red blood cell unit suspended in additive solution) (14). Viral replication characteristics in the early phase of infection are rather consistent among recently infected individuals. This phenomenon results in characteristic doubling times of plasma viral concentration for HIV, HBV and HCV. By knowing the viral replication kinetics of HIV, HBV or HCV in the early infection phase, along with the diagnostic sensitivity of the screening assay, the length of the viraemic phase can be extrapolated for each screening assay.
5.2.1 HIV
HIV replicates with an average doubling time of 20 hours (0.83 days) to reach a peak level of viraemia of up to \(10^7\) IU HIV RNA/mL (15). This virus concentration decreases in parallel with the development of specific antibodies detectable by anti-HIV assays. The currently most sensitive antigen assays can detect HIV p24 antigen at a level corresponding to \(10^4\) IU HIV RNA/mL. Most HIV antigen-antibody combination ("combo") assays are less sensitive in their detection of p24 antigen when compared to antigen assays – with the corresponding HIV RNA concentration for detection by state-of-the-art combo assays being around \(10^5\) IU/mL (15, 16). Attention should be paid to donors having taken early antiretroviral treatment or pre-exposure antiretroviral treatment which could reverse seroconversion and lower viral load (17).

5.2.2 HBV
The replication rate of HBV in the early infection phase as determined by the increase in viraemia is significantly lower when compared to HIV or HCV, with an HBV average doubling time of 2.6 days (18, 19). HBV viraemia in the early infection phase is detected earlier by NAT-based assays than by HBsAg assays. In the absence of NAT-based assays the use of HBsAg assays with a high analytical sensitivity is key for the detection of early infection.

5.2.3 HCV
For HCV an average doubling time of 10.8 hours (0.45 days) during the ramp-up phase has been determined, followed by an anti-HCV-negative plateau phase of several weeks characterized by high-level viraemia of up to \(10^8\) IU HCV RNA/mL (20, 21). HCV core antigen appears to be detectable by core antigen assays during the major part of this anti-HCV-negative phase, namely the entire plateau phase and the last part of the ramp-up phase. Similar to HIV, the antigen detection efficiency of current HCV combo assays is less than that of the antigen assays. Combo assays have an overall detection rate of approximately 40% of anti-HCV-negative window period specimens, and preferentially detect those with virus concentrations above \(10^6\) IU HCV RNA/mL (22).

6. Screening assay categories and diagnostic window periods

6.1 Screening assay categories
In these Guidelines screening assays are discussed according to the following categories:

- NAT-based
- antigen
- combo
- antibody
- rapid diagnostic test (RDT).

While antibody assays are designed to detect both recent and chronic persistent infections, the additional benefit of antigen or viral genome detection lies mainly in further reducing the diagnostic window period. The length of the diagnostic window period varies greatly between the different assay categories.

### 6.1.1 NAT-based assays

NAT-based assays detect viral nucleic acids after in vitro amplification of a target region of the viral genome. Such assays are performed on individual donations (ID-NAT) or in small minipools of donations (MP-NAT). A true infection may not be detectable by NAT-based assays if the concentration of viral genomes is below the detection limit of the assay. Without virus-enrichment steps (for example, ultracentrifugation) in pooled specimens the length of the window period increases with the minipool size and is shortest with ID-NAT. At low virus concentrations in the early ramp-up phase of the window period the amount of virus in a defined volume follows a Poisson distribution, with higher virus concentrations associated with increasing detection probabilities by NAT-based assay. The concentration range between a 5% and a 95% probability of detection may be 100-fold, and this complicates the estimation of window-period reduction that can be achieved by the use of NAT-based assays. In these WHO Guidelines the three-fold concentration of the 95% detection probability has been taken as worst-case assumption for reliable NAT detection for estimating virus concentration in a potentially contaminated plasma pool (Table A4.1; normal font). However, NAT-based assay window periods may be significantly shorter at the lower bound of uncertainty range. The vDWP corresponding to the 50% NAT-detection probability is considered a more accurate estimate for virus transmission risk by blood components without pathogen inactivation (Table A4.1; bold italic font) (23, 24).

### 6.1.2 Antigen assays

Antigen assays have been optimized for the detection of viral proteins (antigens), which are part of the virus particle, such as viral capsids (for example, HIV p24 or HCV core) or virus envelopes; or are subviral particles (for example, HBsAg). For recently infected individuals, non-reactive test results using antigen assays are caused by an absence of viral proteins, the presence of mutated antigen or the presence of antigens with concentrations below the detection limit of the assay.
6.1.3 **Combo assays**

Combo assays are designed to simultaneously detect specific antibodies and viral proteins; non-reactive combo assay test results for a true infection may be caused by the absence (or too low a concentration) of antibodies and/or viral antigens in the test sample, or by hidden epitopes in the immune complexes. The antigen-detection potency of combo assays is often lower than that of assays optimized for exclusive antigen detection.

6.1.4 **Antibody assays**

Antibody assays report infection through the detection of specific antibodies against the pathogen; for recently infected individuals, non-reactive test results using antibody assays can be caused by the absence of specific antibodies, an antibody concentration that is insufficient for obtaining a signal in the immunoassay or low binding strength (avidity) of antibodies. The design of the antibody assay determines its sensitivity and capacity to detect low-avidity antibodies.

6.1.5 **RDTs**

RDTs are diagnostic devices of simple design, often based on immunochromatographic (lateral flow) or immunofiltration (flow-through) technologies. RDTs do not require complex equipment and provide the test result within a short time (15–30 minutes). Although often not claimed by the manufacturer as suitable for use in blood screening, these devices are sometimes used for blood-safety testing in resource-limited settings or in emergency situations. RDT technology is associated with a lower sensitivity than that of more sophisticated immunoassays developed specifically for blood screening (25, 26).

6.2 **Diagnostic window periods**

NAT-based assays are generally able to detect a recent infection sooner than antigen assays, followed by combo assays and antibody assays. These differential capacities for detecting recent infections result in different lengths of the diagnostic window period for different assay categories. Within each of the assay categories, individual assays from different manufacturers may have different sensitivities. These differences sometimes result in overlapping diagnostic sensitivities in detecting early infection when less sensitive assays of one category are compared with the more sensitive assays in another category. For example, currently the most sensitive HIV1/2 antibody assay provides a shorter diagnostic window period than the least sensitive HIV1/2 combo assay. This is true both for assays prequalified by WHO and for CE-marked assays. Furthermore, assays may
have differing sensitivities for different viral genotypes and/or for viral subtypes. The vast majority of commercial seroconversion panels used for diagnostic sensitivity studies originate from regular plasma donors, and mainly represent viral genotypes and subtypes prevalent in Europe and the United States (namely HIV subtype B, HCV genotypes 1–3 and HBV genotype A). However, the sensitivity of assays observed with these seroconversion panels may not always be representative for early infection with viral genotypes prevalent elsewhere in the world (27). Further details on this and other considerations in the evaluation of new blood-screening assays are provided in Appendix 1.

Mean estimates of the length of the vDWP for so-called state-of-the-art assays are presented by assay category in Table A4.1. These estimates should be used for risk calculation unless more detailed information is available on the sensitivity and corresponding window period of the assay used for blood screening. Hence, if comparative data obtained with multiple seroconversion panels indicate that the sensitivity of a specific assay is clearly different from the mean value shown in Table A4.1, the more accurate data for this assay should be used for the estimation of residual risk.
### Table A4.1
Length of the vDWP for different assay categories (days)\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>ID-NAT(^b)</th>
<th>MP16-NAT(^b, c)</th>
<th>Antigen EIA/CLIA(^d)</th>
<th>Combo EIA/CLIA(^d)</th>
<th>Antibody EIA/CLIA(^d)</th>
<th>Antigen RDT(^e)</th>
<th>Combo RDT(^e)</th>
<th>Antibody RDT(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV</td>
<td>8</td>
<td>11</td>
<td>14</td>
<td>16</td>
<td>21</td>
<td>–</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV</td>
<td>27</td>
<td>37</td>
<td>42</td>
<td>–</td>
<td>–</td>
<td>55</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCV</td>
<td>5</td>
<td>7</td>
<td>9</td>
<td>38</td>
<td>60</td>
<td>–</td>
<td>–</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) vDWP: defined here as the period with a virus concentration of \(\geq 1\) virus particle in 20 mL plasma. 1 virus particle has been assumed to correspond to 1 (HCV, HBV) or 2 (HIV) viral genome copies. 1 IU HCV RNA has been assumed to correspond to 4 genome copies HCV RNA; 1 IU HBV DNA to 5 genome copies HBV DNA; and 1 IU HIV-1 RNA to 0.5 genome copies HIV-1 RNA.

\(^b\) NAT-based assays: to date, only a limited number of NAT-based assays claiming blood screening as an intended use have been CE-marked or approved by the United States Food and Drug Administration (FDA). For a worst-case scenario, diagnostic window periods of less sensitive NAT-based assay versions have been taken as examples in Table A4.1.

**Plasma pool contamination:** for estimating the maximal virus concentration in a contaminated plasma pool the three-fold concentration of the 95% detection probability has been taken as a worst-case assumption for reliable and consistent (“100%”) NAT detection. This approach is analogous to the determination of the whole system failure rate in the European Commission’s common technical specifications for in vitro diagnostic medical devices (28). The respective sizes of the vDWP in days are indicated in normal font.

**Transmission risk by non-pathogen-inactivated blood components:** the Poisson distribution property of analyte detection by NAT-based assay is considered suitable for more accurate estimation of virus transmission risk by blood components without pathogen inactivation. NAT-based assay window periods may be significantly shorter at lower bound of uncertainty range. The probability of 50% detection in the early ramp-up phase of viraemia may be taken as the basis for the respective vDWP (indicated in bold italic font) (23, 24).

\(^c\) MP16-NAT = MP-NAT of 16 donations.

\(^d\) EIA/CLIA: for these assay types United States FDA-approved, CE-marked and/or WHO-prequalified assays of medium sensitivity have been selected as examples (20, 22, 25, 26, 29, 30).

\(^e\) RDT: there is a wide range of sensitivity among different RDT assays; values for medium-sensitivity RDTs have been used for Table A4.1 (25, 26).
7. Virus concentrations during diagnostic window period

For risk modelling of plasma pool contamination the maximal virus concentrations that can be found during the respective window period are relevant. Viral loads in viraemic plasma units undetected by screening assays define the extent of initial contamination of the plasma pool. Other parameters for calculation of potential contamination of plasma pools are the number of viraemic donations expected per pool and the individual plasma unit volume relative to the pool size. The maximal viral loads of window-period donations are listed in Table A4.2 as worst-case scenarios for each of the different assay categories correspondingly shown in Table A4.1.

Table A4.2
Maximal concentration of viral genomes in the vDWP (IU/mL)\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>ID-NAT</th>
<th>MP16-NAT</th>
<th>Antigen EIA/CLIA</th>
<th>Combo EIA/CLIA</th>
<th>Antibody EIA/CLIA</th>
<th>Antigen RDT</th>
<th>Combo RDT</th>
<th>Antibody RDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV</td>
<td>150</td>
<td>2400</td>
<td>2 \times 10^4</td>
<td>10^5</td>
<td>10^7</td>
<td>10^7</td>
<td>10^7</td>
<td></td>
</tr>
<tr>
<td>HBV</td>
<td>24</td>
<td>384</td>
<td>10^3</td>
<td>3 \times 10^4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCV</td>
<td>30</td>
<td>480</td>
<td>10^4</td>
<td>5 \times 10^6</td>
<td>10^8</td>
<td>10^8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) IU/mL = International Units per millilitre

8. Confirmation of reactive screening results

The residual risk estimations rely on reactive screening assay results representing true infection events. Initially reactive screening results obtained by antibody or antigen tests should be checked by repeat testing in duplicate in the same assay. Even when reactivity is repeatedly obtained in the routine screening assay, the test result should still be checked by a confirmation strategy (31).

Confirmation strategies may include the use of more specific assays (for example, HIV Western blot or immunoblot, HCV immunoblot and HBsAg neutralization assay) or another screening or diagnostic assay for the same marker, but of different design.

NAT results should be checked by testing an independent aliquot of the donation to exclude contamination and/or by testing replicates to overcome potential Poisson distribution of the analyte present at low concentration. Follow-up investigations of the donor may further assist in differentiating false-positive from true-positive test results.
Only reactive screening test results subsequently confirmed as true positive should be taken for the estimation of residual risk. If no confirmation is performed, residual risk estimations based on reactive test results represent a worst-case scenario and may considerably overestimate the risks.

9. Virus epidemiology of donor populations

Donor populations consist of first-time donors (individuals donating for the first time) and repeat donors (donors with previous donation(s) having tested negative). Blood systems aim towards having an established population of repeat donors undergoing constant selection for absence of infectious markers.

9.1 First-time donors

Positive screening test results in first-time donors may be an indication of infections that occurred either a longer time ago (prevalent infections) or more recently (incident infections). Prevalent infections in first-time donors are expected to be easily detected by high-quality screening assay(s) without assay failures; in contrast, incident infections represent the major contribution to the residual risk of window-period infections. Making the distinction between prevalent and incident infections will require more detailed investigation – recently infected donors may be identified by NAT-only or antigen-only positive results. Furthermore, for antibody-positive donors, modified antibody assays (“detuned” or “recency” assays) can be used to determine the antibody binding strength (avidity). As antibody avidity increases with maturation of the humoral immune response it is possible to differentiate first-time donors with more recent (incident) infections (low-avidity antibodies) from donors with past (prevalent) infections (high-avidity antibodies) and thus determine the specific incidence of infection in this subpopulation (14, 32). If results from these investigations are not available for a specific first-time donor population, the incidence of infection in these donors can be derived from the rate among repeat donors by applying an adjustment factor. A number of scientific studies on HIV, HBV and HCV infections in different donor populations have investigated their incidence among both first-time and repeat donors. Although some of these studies found a two- to three-fold higher rate of recent infections among first-time donors (compared to the corresponding repeat donors) other studies have not found such a difference between the two donor subpopulations (33–38). In the absence of incidence data specific to the first-time donor population, one option is to assume a three-fold higher incidence of virus infections as the worst-case scenario for this subpopulation when compared to the corresponding repeat-donor subpopulation of the same blood establishment. This factor will be referred to as the “first-time donor incidence adjustment factor”.
First-time donor incidence (and corresponding adjustment factor) does not have to be calculated for blood establishments in which newly registered donors are routinely tested for bloodborne infections prior to their first donation of blood or blood components.

9.2 Repeat donors
For repeat donors any confirmed positive screening test result indicates a new infection, which is likely to have occurred during the interdonation interval (IDI) – defined as the time period between the most recent donation (which in this case will have tested positive) and the previous donation (which will have tested negative). However, it is also possible that the previous donation (tested negative) was drawn during the diagnostic window period of the screening assay. The relative frequency of this possibility depends on the length of the IDI, with shorter IDIs increasing the probability of a vDWP donation that tested negative in the screening assay.

10. Estimation of incidence and window period modelling of risks

10.1 Incidence
The rate of new infections of repeat donors (incidence) is defined as the number of NAT conversions or seroconversions (number of infected donors) divided by the total number of person years of observation of all donors during the study period (14, 39, 40). Determining the person years of observation requires a computer system that records the follow-up periods for each individual donation. This kind of information management system is often not available in resource-limited blood establishments.

For the purpose of these Guidelines, both the estimation of incidence and the estimation of the residual risk per blood donation are derived from data from the repeat-donor population for the period of one calendar year (365 days). Incidence is calculated by dividing the number of newly infected repeat donors by the total number of repeat donors, usually expressed as the number of new infection cases per 100 000 repeat donors. If one calendar year is taken as the observation period then the incidence is expressed as per 100 000 person years. This simplification assumes that each repeat donor has been followed for one year during the calendar year and that differences in follow-up periods for individual donors will average out at one person year of observation per donor.

In low-incidence regions the number of positive donors may show strong year-to-year variation. For these situations longer periods may be chosen for the calculation of residual risks.
Screening-positive donations that were excluded for other reasons (for example, donor self-exclusion) may be excluded from the calculation (adjusted incidence).

**Formula 1: Incidence (per 100,000 person years)**

\[
\text{Incidence} = \frac{\text{number of repeat donors tested positive during one year}}{\text{total number of repeat donors in the year}} \times 100,000
\]

10.2 **Residual risk per blood donation in repeat donors**

For calculating the probability that a blood donation has been collected during the vDWP different factors are involved:

- the rate of new infections (incidence) in the repeat-donor population
- the length of the vDWP for the assay used (Table A4.1).

The residual risk of a blood donation from a repeat donor having been collected during the vDWP of the screening assay used can be calculated as follows:

**Formula 2: Residual risk (RR) per donation**

\[
\text{RR per donation} = \text{vDWP} \times \text{incidence}
\]

RR is usually expressed as per million donations (for which one has to multiply the RR figure calculated above by 1 million.

Formula 2 can be directly used to calculate RR for HIV and HCV infections in repeat donors; for HBV infections RR calculated by this formula has to be multiplied by an HBV incidence adjustment factor.

10.2.1 **HBV incidence adjustment factor**

An adjustment factor of \( \geq 1 \) is necessary because HBV (sero)conversions in repeat donors may be missed due to the transient nature of viraemia and antigenaemia in HBV infections that resolve after the acute phase. Such a transient infection course is seen in adults for the majority of HBV infections (95%) while 5% become chronic carriers. The probability of missing transiently detectable HBsAg or HBV DNA in repeat donors by respective screening assays depends on the length of the IDIs and on assay sensitivity. The donation frequency of repeat donors (average number of donations per repeat donor) determines the average length of the IDI. The average IDI (in days) can be calculated by dividing the observation period of one calendar year (365 days) by the average number of donations per repeat donor. For each assay category
a mean detection period for the transient HBV marker (HBsAg or HBV DNA) can be factored into the adjustment. Further contributions to the adjustment factor originate from HBV infections without detectable antigenaemia (assumed to be 25%; transiently picked up by sensitive HBV NAT-based assays) (1). The scientific literature provides several different estimates for the length of transient antigenaemia (1, 19, 41). The differences observed between the underlying studies may be explained by different infection routes, different inoculum, different HBV genotypes, and HBsAg or HBV-DNA assays of different sensitivity.

The lengths of the HBV marker detection periods have been estimated from the available data for the different assay categories and are listed in Table A4.3.

<table>
<thead>
<tr>
<th>NAT ID</th>
<th>MP16-NAT</th>
<th>HBsAg EIA/CLIA</th>
<th>HBsAg RDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>70</td>
<td>60</td>
<td>44</td>
</tr>
</tbody>
</table>

The probability $P$ (in %) of detection by HBsAg assays (Table A4.3) may be calculated as:

$$P = 70\% \times \frac{\text{HBsAg detection period}}{\text{IDI}} + 5\%$$

The probability $P$ (in %) of detection by NAT-based testing (Table A4.3) may be calculated as:

$$P = 95\% \times \frac{\text{HBV DNA detection period}}{\text{IDI}} + 5\%$$

The HBV incidence adjustment factor is calculated as $100/P$. For results where $P \geq 100\%$ no adjustment is necessary.

To determine the RR per donation for HBV infection, the figure obtained for HBV using Formula 2 in section 10.2 above is then multiplied by the adjustment factor for the specific assay category used.

**10.2.2 Adjustment for IDIs**

The incidence/window period modelling of residual risk, as described above, assumes that donation behaviour with regard to donation timing and frequency is the same for both infected and non-infected donors. However, evidence can be found in the scientific literature indicating that seroconverting or
NAT-converting donors sometimes delay their return to blood donation, and therefore have larger average IDIs than non-infected donors, resulting in a lower residual risk (42). Mathematical models are available to reflect this difference in donor behaviour (43). For high-incidence settings (that is, settings in which a higher number of repeat donors have tested positive (seroconverters or NAT converters) for HIV, HBV or HCV infection) the harmonic mean of individual IDIs (in days) of the converting repeat donors (that is, the period between the last negative donation and the first positive donation after infection with the respective virus) may be compared with the mean IDI of non-infected repeat donors (36). Respective functions for calculating mean or harmonic mean values are part of commonly used statistical software (for example, Excel). The residual risk calculation may then include the IDI adjustment factor $S$.

$$S = \frac{\text{mean IDI of all donors}}{\text{harmonic mean IDI of converters for virus X}}$$

If, however, only a few acute infections are found it is advised to take the average IDI of all repeat donors.

10.2.3 First-time donor incidence adjustment factor

In the absence of specific incidence data for first-time donors, a three-fold higher residual risk may be assumed for blood donations from such donors when compared to repeat donors of the same donor population.

Accordingly, the residual risk of a blood donation from a first-time donor having been collected during the vDWP of the screening assay may be assumed to be three-fold higher than the risk calculated for a blood donation obtained from the corresponding repeat donors of the same blood establishment.

11. Residual risks

The approach to residual risk estimation proposed by these Guidelines requires less detailed data on individual donors when compared to other models published in the scientific literature. A recent comparison of seven different models for estimating HIV incidence was performed by simulating donor populations with different donation frequencies combined with different incidence rates (44). The approach proposed by these Guidelines was retrospectively included in the same simulation scenarios. In summary, this exercise revealed a slight overestimation of incidence (by up to 20%) in the scenarios with low donation frequency. This finding confirms the validity of the approach proposed in these Guidelines and is in line with the worst-case scenarios chosen for the different parameters, for example: (a) the proposed lengths of the vDWP (Table A4.1);
(b) the assumption of one virus particle in 20 mL plasma being infectious; or (c) the use of the maximal viral concentration for all vDWP donations for the calculation of potential plasma pool contamination (Table A4.2).

11.1 **Infection of recipients of non-pathogen-inactivated blood components**

The actual infection risk in recipients of non-pathogen-inactivated blood components is dependent on factors such as the amount of intact viruses transmitted, the presence of potentially neutralizing antibodies in the donation or recipient, virus properties and recipient immunological factors (30). Using worst-case scenarios, the probability of viraemic donations escaping screening can be estimated using Formula 2 in section 10.2 above. For whole blood donations, different blood components (red cells, platelets and plasma) may be obtained from the same donation and transfused to recipients, each contributing to the residual risk. The amount of plasma in the blood component, the probability of non-detection by the screening assay(s) and the infectivity of the virus after storage of the blood component are all important factors influencing the infection risk but are beyond the scope of these Guidelines (24, 30).

11.2 **Contamination of plasma pools**

Plasma prepared from whole blood donations (recovered plasma) or obtained by plasmapheresis may be used as source material for plasma-derived products manufactured from plasma pools (such as immunoglobulins, albumin and clotting factors). These pools may be contaminated with HIV, HBV or HCV as a result of the inclusion of plasma units originating from window-period donations not detected by the screening assays. The extent of potential plasma-pool contamination depends upon a number of factors:

- the expected probability of obtaining donations during the vDWP of the screening assay used;
- the (maximal) amount of virus contamination in vDWP plasma units;
- the volume of contaminated plasma unit(s) relative to pool size.

The proportion of viraemic plasma units is estimated by the residual risk calculation. The (maximal) level of virus contamination in respective plasma units can be calculated from the individual plasma volume and its virus concentration. For these calculations, the maximal viral load of window-period donations (shown above in Table A4.2 for the different assay categories) should be taken as the worst-case scenario, even though only a minority of window-period plasma units will reach the maximal viral load.
Authors and acknowledgements

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Between September 2015 and May 2016 the draft document was presented and extensively discussed at a range of international workshops and other forums. The resulting revised draft document was then published on the WHO Biologicals website during a round of public consultation (11 July–26 September 2016). During this phase further comments were received from the following organizations, institutions and individuals: European Blood Alliance (EBA); International Plasma Fractionation Association (IPFA); International Society of Blood Transfusion (ISBT); Permanent United Nations Representation of France; Plasma Protein Therapeutics Association (PPTA); Dr P. Akolkar and Dr I. Hewlett, United States Food and Drug Administration, the USA; Dr D. Brambilla, Dr S. Kleinman, Dr M. Busch, Dr R. Dodd, and S. Glynn for the Recipient Epidemiology and Donor Evaluation Study (REDS)-III; Dr N. Lelie, Consultant, Amsterdam, the Netherlands; Dr F. Moftah, Egyptian Society for Blood Services, and Arab Transfusion Medicine Forum, Egypt; Dr M-L. Hecquet, European Directorate for the Quality of Medicines & HealthCare, France; Dr E. Lindberg, Medical Products Agency, Sweden; Dr M. Jannsssen, University Medical Center Utrecht, the Netherlands; Dr G. Praefcke, Paul-Ehrlich-Institut, Germany; A. Sands, World Health Organization, Switzerland; M. Vermeulen, South African National Blood Service, South Africa;

In October 2016, following review and incorporation of all comments received, the final draft document WHO/BS/2016.2283 was prepared.

Further changes were subsequently made to document WHO/BS/2016.2283 by the WHO Expert Committee on Biological Standardization.
References


Appendix 1

Evaluation of new blood-screening assays

Depending on the legal structure in a country, a regulatory body or the national blood system itself may be responsible for decisions on the acceptability of new blood-screening assays. It is recommended that previous assessments of quality features of the assay performed by experienced regulatory authorities (for example, United States FDA approval, European CE certification, and Australian Therapeutic Goods Administration (TGA) or Health Canada marketing authorizations) or by the WHO Prequalification Programme for IVDs should be taken into account. Previous assessments by such stringent regulatory bodies will have included the review of analytical and clinical performance data submitted by the manufacturer, and of the manufacturer’s quality management system and batch-to-batch consistency – and in the case of WHO prequalification, an independent performance evaluation.

As a result, a country’s assessment of manufacturer documentation, with a focus on the specific regional situation and needs, may be sufficient for assays already approved elsewhere under stringent regulation.

If local regulation requires a performance evaluation of new assays (for example, by a national reference laboratory) prior to their implementation, it is recommended that the evaluation focuses on essential assay features through a targeted performance evaluation.

Assessment of documents

Documents provided by the IVD manufacturer may be assessed, with a special focus placed on the specific regional situation and needs. Such a focus may include assessing whether or not the stability studies performed by the manufacturer cover the regional environmental conditions (for example, with regard to temperature and humidity) or whether the Instructions for Use are appropriate for the target users.

In addition, performance evaluation studies documented by the IVD manufacturer may be reviewed to evaluate the extent of representation of specimens reflecting the regional situation (for example, with regard to viral genotypes or variants) or to assess potential interference with the test result by other regionally more prevalent infections.
Targeted performance evaluation of new assays used for blood screening

If laboratory testing of a new IVD is a component of the national or regional evaluation and approval scheme, it is advisable not to repeat evaluation elements already performed by other bodies, but to focus instead on regionally important quality aspects. This would involve, for example, a focused assessment of performance data with respect to viral variants or genotypes prevalent in the region.

Well-characterized specimen panels representing the regional epidemiological situation with regard to viral variants/genotypes of HIV, HBV or HCV may be helpful for comparative independent evaluation of new assays. A comparative database obtained using a number of assays may then be the scientific basis for the definition of acceptance criteria for new assays and for the identification of less suitable assays.

The preconditions for the suitability of such panels are the inclusion of specimens differentiating between different assays (for example, low-positive specimens or positive specimens previously tested discrepantly by different assays) and the availability of sufficient volumes to allow a number of evaluations to obtain comparative data. The recommended size of such a panel strongly depends on its composition, with more critical panel members (for example, low-positive or early infection specimens) able to differentiate between assays being more important than a high number of strong positive specimens. Panels used for this type of exercise typically comprise 20–50 members collected from different phases of the infection. A strategy for the replacement of panel members should be in place.

Furthermore, WHO offers through its IVD standardization programme a range of biological reference preparations that may be useful in the confirmation of basic assay features. WHO International Standards (expressed in IU) are available for the confirmation of analytical sensitivity, while WHO Reference Panels representing the major viral genotypes could be used to check genotype-detection efficiency.

These WHO reference preparations are usually lyophilized to facilitate worldwide shipping and are listed in the WHO online catalogue (http://www.who.int/bloodproducts/catalogue/en/). They can be obtained from the WHO Collaborating Centres which act as WHO custodians in this field – namely, the National Institute for Biological Standards and Control (NIBSC), England, or the Paul-Ehrlich-Institut (PEI), Germany.
Table A4A1.1 summarizes the most important WHO reference preparations currently available in the field of blood screening.

Table A4A1.1

<table>
<thead>
<tr>
<th>Marker</th>
<th>Preparation</th>
<th>Details</th>
<th>Custodian</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-HIV-1/2</td>
<td>1st International Reference Panel</td>
<td>HIV-1 subtypes A, B, C, CRF01_AE; group O; HIV-2</td>
<td>NIBSC</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>No unitage</td>
<td></td>
</tr>
<tr>
<td>HIV-1 p24</td>
<td>1st International Reference Reagent</td>
<td>–</td>
<td>NIBSC</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>1000 IU/ampoule</td>
<td></td>
</tr>
<tr>
<td>HIV-1 RNA</td>
<td>3rd International Standard</td>
<td>–</td>
<td>NIBSC</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>185 000 IU/mL</td>
<td></td>
</tr>
<tr>
<td>HIV-1 CRFs</td>
<td>1st International Reference Panel HIV-1 circulating recombinant forms (CRFs)</td>
<td>HIV-1 CRFs 11GJ, 02AG, 01AE, 01AGJU,BG24; subtypes J, G, C; group O</td>
<td>NIBSC</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>No unitage</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>No unitage</td>
<td></td>
</tr>
<tr>
<td>HIV-2 RNA</td>
<td>1st International Standard</td>
<td>–</td>
<td>NIBSC</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>1000 IU/vial</td>
<td></td>
</tr>
<tr>
<td>HBsAg</td>
<td>3rd International Standard</td>
<td>–</td>
<td>NIBSC</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>50 IU/mL</td>
<td></td>
</tr>
<tr>
<td>Dilutional panel</td>
<td>–</td>
<td>8.25; 2.06; 0.52; 0.13 IU/vial</td>
<td>NIBSC</td>
</tr>
<tr>
<td>HBV genotypes</td>
<td>1st International Reference Panel HBV genotypes</td>
<td>HBV genotypes A–F, H</td>
<td>PEI</td>
</tr>
<tr>
<td></td>
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<td>No unitage</td>
<td></td>
</tr>
</tbody>
</table>
### Table A4A1.1 continued

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<th>Details</th>
<th>Custodian</th>
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<td>HBV DNA</td>
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<td>–</td>
<td>NIBSC</td>
</tr>
<tr>
<td></td>
<td>1st International Reference Panel HBV genotypes Lyophilized No unitage</td>
<td>HBV genotypes A–G</td>
<td>PEI</td>
</tr>
<tr>
<td>anti-HBc</td>
<td>1st International Standard Lyophilized 50 IU/vial</td>
<td>–</td>
<td>NIBSC</td>
</tr>
<tr>
<td>HCV core</td>
<td>1st International Standard Lyophilized 3200 IU/mL</td>
<td>–</td>
<td>PEI</td>
</tr>
<tr>
<td>HCV RNA</td>
<td>5th International Standard Lyophilized 100 000 IU/mL</td>
<td>–</td>
<td>NIBSC</td>
</tr>
</tbody>
</table>
Appendix 2

Examples for estimation of residual risks

Example 1: HCV screening by anti-HCV EIA

Centre A; observation period 01.06.2011–31.05.2012
49 660 repeat donors; 100 313 donations; 45 anti-HCV pos (EIA)
11 452 first-time donors; 11 452 donations; 89 anti-HCV pos (EIA)

Table A4.1 (see section 6.2 main text) – anti-HCV EIA: vDWP = 60 days = 0.164 years
Table A4.2 (see section 7 main text) – anti-HCV EIA: maximal virus concentration: $10^8$ IU HCV RNA/mL plasma of vDWP donation

A. Residual risk (RR) per blood donation from repeat donors

Incidence = \[
\frac{\text{number of repeat donors tested positive during one year}}{\text{total number of repeat donors in the year}} \times 100 000
\]

\[
= \frac{45}{49 660} \times 100 000
\]

= 90.61 HCV infections per 100 000 donor years

RR per blood donation = vDWP × incidence

\[
= 0.164 \times 0.000 \, 906 \, 1 = 0.000 \, 148 \, 600
\]

= 148.60 per million donations

Number (N) of vDWP blood donations from repeat donors

\[
N = 100 \, 313 \times \frac{148.60}{1 \, 000 \, 000} = 14.90
\]

B. Residual risk (RR) per blood donation from first-time donors

Positive screening test results for first-time donors represent mainly old (prevalent) infections. The rate of recent infections can be determined by specific investigations (for example, recency assays or NAT-only positive results).

In the absence of incidence data, the worst-case assumption is a threefold incidence in first-time donors compared to the corresponding repeat donors.
RR = 0.000 148 61 × 3 = 0.000 445 = 445 per million donations

Number (N) of vDWP blood donations from first-time donors:

\[ N = 11\,452 \times \frac{445}{1\,000\,000} = 5.10 \]

C. Expected number (N) and risk of window-phase donations for repeat and first-time donors combined (Centre A; observation period of 1 year)

\[ N = 14.90 + 5.10 = 20.00 \]

\[ RR = \frac{20}{100\,313 + 11\,452} = 0.000 \, 179 = 179 \text{ per million donations} \]

Example 2: HBV screening by HBsAg RDT; HBV adjustment factor

Centre A; observation period 01.06.2011–31.05.2012

49 660 repeat donors; 100 313 donations; 184 HBsAg RDT pos

11 452 first-time donors; 11 452 donations; 291 HBsAg RDT pos

Table A4.1 (section 6.2 main text) – HBsAg RDT: vDWP = 55 days = 0.15 years

Table A4.3 (section 10.2.1 main text) – HBsAg RDTs: HBV marker detection period = 44 days

Average number of donations per repeat donor: 100 313/49 660 = 2.02

Interdonation interval (IDI)

\[ \text{IDI} = \frac{365 \text{ days}}{\text{average number of donations per repeat donor}} = 180.69 \text{ days} \]

A. Residual risk (RR) per blood donation from repeat donors (without adjustment for transient HBsAg)

Incidence = \[ \frac{\text{number of repeat donors tested positive during one year}}{\text{total number of repeat donors in the year}} \times 100\,000 \]

\[ = \frac{184}{49\,660} \times 100\,000 \]

\[ = 370.52 \text{ HBV infections per 100 000 donor years} \]
vDWP = 55 days = 0.15 years

RR = vDWP × incidence
  = 0.15 × 0.003 705 2 = 0.000 555 78
  = 555.78 per million donations

B. HBV incidence adjustment factor

Probability (P) for HBsAg detection

\[ P = 70\% \times \frac{\text{HBV marker detection period}}{\text{IDI}} + 5\% \]

\[ = 70\% \times \frac{44 \text{ days}}{180.69 \text{ days}} + 5\% = 70\% \times 0.24 + 5\% = 21.8\% \]

HBV incidence adjustment factor =

\[ \frac{100\%}{P} = \frac{100\%}{21.8\%} = 4.58 \]

C. Residual risk (RR) per blood donation from repeat donors (with adjustment for transient HBsAg)

Adjusted RR = 4.58 × 0.000 555 78 = 0.002 545 = 2545 per million donations.
Annex 5

Guidelines for the production, control and regulation of snake antivenom immunoglobulins


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Guidelines published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA.
Abbreviations

ASV anti-snake venom
BVDV bovine viral diarrhoea virus
CK creatine kinase
CPD citrate phosphate dextrose solution
CTD Common Technical Document
ds-DNA double-stranded deoxyribonucleic acid
ds-RNA double-stranded ribonucleic acid
ED$_{50}$ effective dose 50%
EIA enzyme immunoassay
ELISA enzyme-linked immunosorbent assay
EMCV encephalomyocarditis virus
FCA Freund’s complete adjuvant
FIA Freund’s incomplete adjuvant
GCP good clinical practice
GMP good manufacturing practice(s)
Hb haemoglobin
HPLC high-performance liquid chromatography
ICH International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IgG immunoglobulin G
IgM immunoglobulin M
LD$_{50}$ lethal dose 50%
MCD minimum coagulant dose
MDD minimum defibrinogenating dose
MHD minimum haemorrhagic dose
MHD$_{50}$ MHD-median effective dose
MMD minimum myotoxic dose
MMD$\text{}_{50}$  MMD-median effective dose
MND   minimum necrotizing dose
MND$\text{}_{50}$  MND-median effective dose
Mr    relative molecular mass
NRA   national regulatory authority
PCV   packed cell volume
RCT   randomized controlled trial
SDS-PAGE sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SOP   standard operating procedure
ss-DNA single-stranded deoxyribonucleic acid
ss-RNA single-stranded ribonucleic acid
TPP   total plasma protein
TSE   transmissible spongiform encephalopathy
VSV   vesicular stomatitis virus
WNV   West Nile virus
1. Introduction

Snake antivenom immunoglobulins (antivenoms) are the only therapeutic products for the treatment of snake-bite envenoming. The lack of availability of effective snake antivenom immunoglobulins to treat envenoming by medically important venomous snakes encountered in various regions of the world has become a critical health issue at global level. The crisis has reached its greatest intensity in sub-Saharan Africa, but other regions, such as South and South-East Asia, are also suffering from a lack of effective and affordable products.

The complexity of the production of efficient antivenoms, in particular the importance of preparing appropriate snake venom mixtures for the production of hyperimmune plasma (the source of antivenom immunoglobulins), the decreasing number of producers, and the fragility of the production systems in developing countries further jeopardize the availability of effective antivenoms in Africa, Asia, the Middle East and South America. Most of the remaining current producers are located in countries where the application of quality and safety standards needs to be improved.

In October 2005, the WHO Expert Committee on Biological Standardization recognized the extent of the problem and asked the WHO Secretariat to support and strengthen world capacity to ensure the long-term and sufficient supply of safe and efficient antivenoms. In March 2007, snake antivenom immunoglobulins were included in the WHO Model List of Essential Medicines (1), acknowledging their role in a primary health-care system.

WHO recognizes that urgent measures are needed to support the design of immunizing snake venom mixtures that can be used to make appropriate antivenoms for various geographical areas of the world. Sustainable availability of effective and safe antivenom immunoglobulins must be ensured and production systems for these effective treatments must be strengthened at global level. Meaningful preclinical assessment of the neutralizing capacity of snake antivenom immunoglobulins needs to be done before these products are used in humans and medicines regulatory authorities should enforce the licensing of these products in all countries, before they are used in the population.

The first edition of the WHO Guidelines for the production, control and regulation of snake antivenom immunoglobulins was developed in response to the above-mentioned needs and approved by the WHO Expert Committee on Biological Standardization in October 2008. These Guidelines covered all the steps involved in the production, control and regulation of venoms and antivenoms. The Guidelines are supported by a WHO antivenoms database website\(^1\) that

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features information on all the venomous snakes listed in Appendix 1, including distributions and photographs, as well as information on available antivenoms.

It is intended that these updated Guidelines, by comprehensively describing the current existing experience in the manufacture, preclinical and clinical assessment of these products, will serve as a guide to national regulatory authorities (NRAs) and manufacturers in support of worldwide production of these essential medicines. The production of snake antivenoms following good manufacturing practices (GMP) should be the aim of all countries involved in the manufacture of these life-saving biological products.

In addition to the need to produce appropriate antivenoms, there is a need to ensure that antivenoms are appropriately used and that outcomes for envenomed patients are improved. This entails improving availability and access to antivenoms, appropriate distribution policies, antivenom affordability, and training of health workers to allow safe, selective and effective use of antivenoms and effective management of snake-bite envenoming. These important issues are beyond the scope of this document and will not be further addressed specifically here, but should be considered as vital components in the care pathway for envenoming.

This second edition of the Guidelines was prepared in 2016 in order to ensure that the information contained in these sections remains relevant to the current production of snake antivenom immunoglobulins and their subsequent control and regulation.

Major updates in this second edition include:

- inclusion of stronger animal welfare and ethical compliance messages (section 4) to reinforce the importance of humane use of animals in the production of antivenoms;
- updates to lists of medically important snakes to reflect new species discoveries and recent nomenclatural changes (section 7; and Appendix 1);
- revision of methodologies for serpentariums that produce venoms to emphasize traceability and quality control, including the recommendation to discontinue use of wild-capture/release strategies for ethical and quality control reasons (section 9);
- increased emphasis on the specific health control of plasma donor animals, particularly prior to, and during plasma collection session (sections 12 and 14);
- updated lists of known potential equine virus contaminants (section 16);
redrafting and reorganization of sections on the quality control (section 17), stability studies (section 18) and preclinical assessment (section 19) of antivenoms, to incorporate new approaches and technologies, and eliminate repetition;

- revised information on the clinical assessment of antivenoms (section 20), as well as an expanded and strengthened discussion on the role of NRAs and the need for national reference venom collections independent from antivenom manufacturers (section 21; see also section 10.3).

2. Purpose and scope

These WHO Guidelines provide guidance to NRAs and manufacturers on the production, control and regulation of snake antivenom immunoglobulins. It should however be recognized that some sections, such as: those dealing with immunogen quality control, reference materials, and the production, purification and testing of antibodies (sections 10–19); as well as most of the guidance which deals with regulatory oversight (section 21); and the ethical use of laboratory animals and plasma donor animals (section 4); may also apply to other types of antivenoms, such as those produced for the treatment of envenoming caused by spiders, scorpions and other organisms. There are also other immunoglobulin products of animal origin for which some of the production methodologies described here may be similar or identical — for example, the selection and veterinary health care of animals; immunization regimens and use of adjuvants; collection and control of animal plasma for fractionation; purification of immunoglobulins; and control of infectious risks. These WHO Guidelines may therefore have application beyond providing information for the production of snake antivenom immunoglobulins, and may be applicable also to other antivenoms or animal-derived immunoglobulin products (for example, equine-derived botulism antitoxins).

3. Terminology

The definitions given below apply to the terms as used in these WHO Guidelines. These terms may have different meanings in other contexts.

**Antivenom** – also called antivenin or anti-snake venom (ASV): a purified fraction of immunoglobulins or immunoglobulin fragments fractionated from the plasma of animals that have been immunized against one or more snake venoms.
**Apheresis**: procedure whereby blood is removed from the donor, separated by physical means into components and one or more of them returned to the donor.

**Batch**: a defined quantity of starting material or product manufactured in a single process or series of processes so that it is expected to be homogeneous.

**Batch records**: all documents associated with the manufacture of a batch of bulk product or finished product. They provide a history of each batch of product and of all circumstances pertinent to the quality of the final product.

**Blood collection**: a procedure whereby a single donation of blood is collected in an anticoagulant and/or stabilizing solution, under conditions designed to minimize microbiological contamination of the resulting donation.

**Bulk product**: any product that has completed all processing stages up to, but not including, aseptic filling and final packaging.

**Clean area**: an area with defined environmental control of particulate and microbial contamination constructed and used in such a way as to reduce the introduction, generation, and retention of contaminants within the area.

**Contamination**: the undesired introduction of impurities of a microbiological or chemical nature, or of foreign matter, into or on to a starting material or intermediate during production, sampling, packaging, or repackaging, storage or transport.

**Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES)**: an international agreement between governments that aims to ensure that international trade in specimens of wild animals and plants does not threaten their survival.

**Cross-contamination**: contamination of a starting material, intermediate product or finished product with another starting material or product during production.

**Cross-neutralization**: the ability of an antivenom raised against a venom, or a group of venoms, to react and neutralize the toxic effects of the venom of a related species not included in the immunizing venom mixture.

**Common Technical Document (CTD) format**: a specific format for product dossier preparation recommended by WHO and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

**Desiccation**: a storage process where venoms are dehydrated under vacuum in the presence of calcium salts or phosphoric acid.

**Effectiveness**: the effectiveness of an antivenom is a measure of its ability to produce a clinically effective outcome when used to treat snake-bite envenoming.

**Efficacy**: the efficacy of an antivenom is a measure of the in vivo or in vitro neutralizing potency against a specific activity of a venom or venoms.
**Envenoming**: injection of venom by an organism (for example, venomous snake) into another organism, leading to pathological manifestations (also called envenomation).

**Fab**: an antigen-binding fragment (Fab) of an immunoglobulin comprising a heavy chain and a light chain that each have a single constant domain and a single variable domain. Fab fragments result from the proteolytic digestion of immunoglobulins by papain (or pepsin after F(ab’)_2 digestion).

**F(ab’)_2**: an immunoglobulin fragment comprising a pair of Fab fragments connected by a protein hinge, and produced by proteolytic digestion of whole immunoglobulins with pepsin.

**Fractionation**: large-scale process by which animal plasma is separated to isolate the immunoglobulin fraction that is further processed for therapeutic use or may be subjected to digestion with pepsin or papain to generate immunoglobulin fragments. The term fractionation is generally used to describe a sequence of processes, usually including plasma protein precipitation and/or chromatography, ultrafiltration and filtration steps.

**Freund’s complete adjuvant** (FCA): an adjuvant that may be used in the immunization process of animals to enhance the immune response to venoms. It is composed of mineral oil, an emulsifier and inactivated *Mycobacterium tuberculosis*.

**Freund’s incomplete adjuvant** (FIA): an adjuvant that may be used in the immunization process of animals to enhance the immune response to venoms. It is composed of mineral oil and an emulsifier.

**Good clinical practice** (GCP): an international standard for rigorous, ethical and high quality conduct in clinical research, particularly in relation to all aspects of the design, conduct, analysis, record-keeping, auditing and reporting of clinical trials involving human subjects. GCP standards are established by the ICH under Topic E 6 (R1).

**Good manufacturing practice** (GMP): that part of quality assurance which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the marketing authorization or product specification. It is concerned with both production and quality control.

**Immunization process**: a process by which an animal is injected with venom(s) to produce a high-titre antibody response against the lethal and other deleterious components in the immunogen.

**Immunoglobulin**: immune system forming protein produced by B-cells in plasma that can recognize specific antigens. These can be generated by immunizing an animal (most often a horse) against a snake venom or a snake venom mixture. Immunoglobulin G (IgG) is the most abundant immunoglobulin fraction.
Immunoglobulin G (IgG): one of the five classes of antibodies produced by the B-cells. It is synthesized in response to invasions by bacteria, fungi and viruses. IgG crosses the placenta and protects the fetus. It is a complex protein composed of four peptide chains – two identical heavy chains and two identical light chains arranged in a typical Y shape of antibody monomers. Representing approximately 75% of serum antibodies in humans, IgG has a molecular mass of approximately 150 kDa.

Immunoglobulin M (IgM): another type of antibody. It is an immunoglobulin of high molecular weight that is released into the blood early in the immune response to be replaced later by IgG and is highly efficient in binding complement. IgM antibodies make up about 5 to 10% of all the antibodies in the body; they have a polymeric form, mostly as pentamers. IgM has a molecular mass of approximately 970 kDa.

In-process control: checks performed during production to monitor and, if necessary, to adjust the process to ensure that the antivenom conforms to specifications. The control of the environment or equipment may also be regarded as part of in-process control.

Manufacture: all operations of purchase of materials and products, production, quality control, release, storage and distribution of snake antivenom immunoglobulins, and the related controls.

Median effective dose – or effective dose 50% (ED50): the quantity of antivenom that protects 50% of test animals injected with a median lethal dose of venom.

Median lethal dose – or lethal dose 50% (LD50): the quantity of snake venoms, injected intravenously or intraperitoneally, that leads to the death of 50% of the animals in a group after an established period of time (usually 24–48 hours).

Minimum coagulant dose (MCD): the minimum amount of venom (in mg/L or µg/mL) that clots either a solution of bovine fibrinogen (2.0 g/L) in 60 seconds at 37 °C (MCD-F) and/or a standard citrated solution of human plasma (2.8 g/L fibrinogen) under the same conditions (MCD-P).

Minimum coagulant dose-F-effective dose (MCD-F100) and MCD-P-effective dose (MCD-P100): the minimum volume of antivenom or venom/antivenom ratio, which completely prevents clotting induced by either one MCD-F or MCD-P dose of venom.

Minimum defibrinogenating dose (MDD): the minimum amount of venom that produces incoagulable blood in all mice tested within one hour of intravenous injection.

Minimum defibrinogenating dose-effective dose (MDD100): the minimum volume of antivenom or venom/antivenom ratio, at which the blood
samples of all injected mice show clot formation after administration of one or more MDD doses of venom.

Minimum haemorrhagic dose (MHD): The minimum amount of venom (in µg) that when injected intradermally in mice causes a 10 mm haemorrhagic lesion within a predefined time interval (for example, 2–3 hours).

Minimum haemorrhagic dose-median effective dose (MHD₅₀): the minimum volume of antivenom (in µL) that reduces the diameter of haemorrhagic lesions by 50% compared to those induced in animals who receive a control solution of venom/saline.

Minimum myotoxic dose (MMD): the minimum amount of venom that produces a four-fold increase in serum or plasma creatine kinase (CK) activity above that of control animals.

Minimum myotoxic dose-median effective dose (MMD₅₀): the minimum amount of antivenom (in µL or the venom/antivenom ratio) that reduces the serum or plasma CK activity by 50% compared to those induced in animals who receive a control solution of venom/saline.

Minimum necrotizing dose (MND): the minimum amount of venom (in µg) that when injected intradermally in groups of lightly anaesthetized mice results in a necrotic lesion 5 mm in diameter within 72 hours.

Minimum necrotizing dose-median effective dose (MND₅₀): the minimum amount of antivenom (in µL or the venom/antivenom ratio) that reduces the diameter of necrotic lesions by 50% compared to those induced in animals who receive a control solution of venom/saline.

Monospecific antivenom: antivenoms that are raised from venom of a single species, and are limited in use to that species or to a few closely related species (typically from the same genus) whose venoms show clinically effective cross-neutralization with the antivenom. The term “monovalent” is often used and has the same meaning.

Nanofilter: filters, most typically with effective pore sizes of 50 nm or below, designed to remove viruses from protein solutions.

National regulatory authority (NRA): WHO terminology to refer to national medicines regulatory authorities. Such authorities promulgate medicine regulations and enforce them.

Plasma: the liquid portion remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant, or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure.

Plasmapheresis: procedure in which whole blood is removed from the donor, the plasma is separated from the cellular elements by sedimentation, filtration, or centrifugation, and at least the red blood cells are returned to the donor.
**Polyspecific antivenom**: antivenoms that are obtained by fractionating the plasma from animals immunized with a mixture of venoms from more than one species of venomous snake. The term “polyvalent” is often used and has the same meaning.

**Prion**: a particle of protein that is thought to be able to self-replicate and to be the agent of infection in a variety of diseases of the nervous system, such as scrapie, mad cow disease and other transmissible spongiform encephalopathies (TSEs). It is generally believed not to contain nucleic acid.

**Production**: all operations involved in the preparation of snake antivenom immunoglobulins, from preparation of venoms, immunization of animals, collection of blood or plasma, processing, packaging and labelling, to its completion as a finished product.

**Quality manual**: an authorized, written controlled document that defines and describes the quality system, the scope and operations of the quality system throughout all levels of production, management responsibilities, key quality systems processes and safeguards.

**Quarantine**: a period of enforced isolation and observation typically to contain the spread of an infectious disease among animals. The same terminology applies to the period of isolation used to perform quality control of plasma prior to fractionation, or of antivenom immunoglobulins prior to release and distribution.

**Randomized controlled trial (RCT)**: randomized controlled trial of a pharmaceutical substance or medical device.

**Serpentarium**: a place where snakes are kept, for example, for exhibition and/or for collection of venoms.

**Serum**: a liquid portion remaining after clotting of the blood. Serum has a composition similar to plasma (including the immunoglobulins) apart from fibrinogen and other coagulation factors which constitute the fibrin clot.

**Site Master File**: an authorized, written controlled document containing specific factual details of the GMP production and quality control manufacturing activities that are undertaken at every site of operations linked to products that a company produces.

**Standard operating procedure (SOP)**: an authorized written procedure giving instructions for performing operations not necessarily specific to a given product or material (for example, equipment operation, maintenance and cleaning; validation; cleaning of premises and environmental control; sampling and inspection). Certain SOPs may be used to supplement product-specific master and batch production documentation.

**Toxin**: a toxic substance, especially a peptide or protein, which is produced by living cells or organisms and is capable of causing disease when introduced into the body tissues. It is often also capable of inducing neutralizing antibodies or antitoxins.
**Traceability**: ability to trace each individual snake, venom, immunized animal, or unit of blood or plasma used in the production of an antivenom immunoglobulin with each batch of the final product. The term is used to describe forward and reverse tracing.

**Validation**: action of proving, in accordance with the principles of GMP, that any procedure, process, equipment, material, activity, or system actually leads to the expected results.

**Venom**: the toxic secretion of a specialized venom gland which, in the case of snakes, is delivered through the fangs and provokes deleterious effects. Venoms usually comprise many different protein components of variable structure and toxicity.

**Venom extraction** – or **venom collection** or **“milking”**: The process of collecting venom from live snakes.

**Viral inactivation**: a process of enhancing viral safety in which viruses are intentionally “killed”.

**Viral reduction**: a process of enhancing viral safety in which viruses are inactivated and/or removed.

**Viral removal**: a process of enhancing viral safety by partitioning viruses from the components of interest.

4. **The ethical use of animals**

Current methods of antivenom production rely on the use of animals to manufacture these life-saving products. For all animals, whether they are venomous snakes from which venom is obtained for use as an immunogen; the horses, sheep or other large animals that are injected with the venom, and serve as living antibody factories, producing hyperimmune plasma from which antivenom is derived; or the small laboratory animals sacrificed in order to test the preclinical efficacy and safety of antivenoms, there is an absolute necessity for all manufacturers to use animals humanely and ethically.

It is imperative that venom producers, antivenom manufacturers and quality control laboratories that make use of animals in venom or antivenom research, production, or in the preclinical evaluation of antivenoms adhere to the highest ethical standards. The *International guiding principles for biomedical research involving animals* (2012) developed by the International Council for Laboratory Animal Science and the Council for International Organization of Medical Sciences provide an international benchmark for the use of animals in research. Compliance with national guidelines, laws and regulations is also essential. All animal experimentation should be subject to regulatory oversight at an institutional and national level. In many jurisdictions, the 3R principles of Replacement, Reduction and Refinement have been adopted as cornerstones
of ethical use of animals, and WHO strongly recommends that every effort be made to reduce pain, distress and discomfort to experimental animals – for example, by the routine use of analgesia in mice used in these assays.

4.1 Ethical considerations for the use of venomous snakes in the production of snake venoms

Venomous snakes kept in serpentariums for use in venom production should be maintained according to nationally and internationally accepted ethical standards. All relevant local regulations should be strictly adhered to, and where required the use of venomous snakes in venom production should be conducted in accordance with ethics approvals obtained from responsible authorities in the jurisdiction. This particularly applies to the collection of wild specimens and their transportation to serpentariums. It is important that specimens be sourced from legal suppliers, and venom producers should ensure that the collection localities of all specimens are known, and that evidence of legal collection is supplied. As discussed in section 9.1.4.2 the practice of capturing wild venomous snakes, extracting venom and releasing the snakes after translocation into new habitat must be discontinued. This is not just because of issues relating to traceability and quality control, which are fundamental to production of antivenoms in accordance with GMP, but also because mounting evidence demonstrates unacceptably high mortality among translocated venomous snakes. Compliance with local ethical requirements for the keeping of venomous snakes in captivity, the humane handling of specimens, veterinary care and supervision, and euthanasia (when necessary for humane reasons) should be maintained. Another important consideration for serpentariums is the necessity to use other animals as food sources for venomous snakes. The types of animals used as food, their production, humane euthanasia, or in some cases, presentation to snakes as live prey, require appropriate ethical considerations, and specific licences and ethics approvals may be required to keep, breed and use some animals as sources of food for venomous snakes. Venom producers must ensure that their operations comply with all necessary regulations and requirements in this regard.

4.2 Ethical considerations for the use of large animals in the production of hyperimmune plasma

The use of large animals (for example, horses, ponies, mules and sheep) in the production of hyperimmune plasma requires constant veterinary supervision and strict adherence to approved national and international ethical standards for these animals. Equines are the most commonly used for production of
hyperimmune plasma in antivenom production and have specific physiological and psychological requirements for good health and the minimization of pain and distress. Manufacturers must recognize these needs and structure their use of animals to ensure that their social, physical and environmental needs are appropriately met. Relevant guidelines and regulations established by competent authorities should be implemented. Veterinary care of animals should meet the highest standards, and the health and welfare of individual animals used for plasma production should be closely monitored at all times. The process of immunizing donor animals with snake venoms raises important ethical considerations, particularly because of the potential harm that can be caused by some venoms (for example, neurotoxins, necrotic or cytotoxic venoms) and by the adjuvants that are used in most immunization protocols, particularly Freund’s complete adjuvant (FCA) or Freund’s incomplete adjuvant (FIA). Animals used in plasma production may suffer considerable distress, pain or discomfort as a result of the immunization process and all manufacturers have an obligation to strictly comply with animal welfare and ethical use requirements and actively work to minimize these deleterious effects. Similarly, the bleeding of animals to collect hyperimmune plasma can be traumatic for donor animals if appropriate techniques are not used to minimize negative effects, including fear, pain, distress and physical harm. Manufacturers are encouraged very strongly to proactively improve the welfare of large animals used in plasma production, and to develop protocols that reduce suffering and improve the health of animals.

4.3 Ethical considerations for the use of animals in preclinical testing of antivenoms

The preclinical testing of new or existing antivenoms necessitates the use of experimental animals, typically rodents, particularly for essential median lethal venom dose (LD$_{50}$) and median effective antivenom dose (ED$_{50}$) determination. Despite reservations about the physiological relevance of these animal models to human envenoming and the harm that these in vivo assays cause to the animals (sections 19.2 and 19.3), they are used by both manufacturers and regulatory authorities worldwide for determining venom lethality (LD$_{50}$) and antivenom neutralizing capacity (ED$_{50}$) as these are currently the only validated means of assessing venom toxicity and antivenom neutralizing potency. Non-sentient or in vitro assays as alternatives to the standard venom LD$_{50}$ and antivenom ED$_{50}$ in vivo tests have been promoted (2). Unfortunately, such systems have not been developed to the point where they can fully replace the above-mentioned preclinical assays. In the absence of effective alternatives, the continued use of experimental animals is still justified by the considerable benefits to human health of these preclinical assays.
4.4 Development of alternative assays to replace murine lethality testing

In vivo murine assays cause considerable suffering and a 3R approach involving innovation and validation should be applied in the development of standardized LD$_{50}$ and ED$_{50}$ test protocols. Designing protocols that use the minimal number of animals necessary and introducing procedures to minimize pain and suffering is essential. The development of alternative methods to replace animal testing in the preclinical evaluation of antivenoms should be encouraged. When tests on live animals are absolutely necessary, anaesthesia or analgesia should be considered and evaluated to ensure that the humane benefits of these interventions to the experimental animals do not invalidate the objectives of the assay by altering relevant physiological processes (3). In particular, the use of analgesia is recommended when working with venoms that induce tissue damage, and experimental evidence demonstrates convincingly that opioid drugs relieve suffering without altering critical end-points such as LD$_{50}$ and ED$_{50}$ (4). The establishment of humane end-points to reduce suffering and limiting the duration of the assays to reduce the period of animal suffering is encouraged; this requires appropriate standardization and validation within a quality assurance framework.

4.5 Refinement of the preclinical assay protocols to reduce pain, harm and distress to experimental animals

The substantial suffering caused to small animals by the preclinical assays is outweighed by the considerable benefits to human health. Nevertheless, WHO strongly encourages that opportunities to implement alternatives to the essential and supplementary tests, according to the 3R, to reduce pain, harm and distress be tested. Thus, designing protocols that use the minimum number of animals necessary and introducing procedures to minimize pain and suffering is essential. Analgesia should be considered, and evaluated to ensure that the humane benefits of analgesia to the experimental animals do not invalidate the objectives of the assay by altering relevant physiological processes (3). In particular, the use of analgesia is recommended when working with venoms that induce tissue damage (4). The establishment of humane end-points, instead of using survival/death as the assay metric, is encouraged to reduce suffering and limit the duration of the assays. The use of humane end-points also offers the opportunity to introduce ‘dose-staging’ into the experimental design (in which multiple doses are prepared for the assays, one dose given and the next dose(s) selected based on the results of giving the previous dose) to reduce the number of mice required for these assays. All such efforts towards 3R require appropriate standardization and validation within a quality assurance framework.
4.6 Main recommendations

- It is imperative that venom producers, antivenom manufacturers and quality control laboratories that use animals in venom or antivenom production, in research or in the preclinical evaluation of antivenoms adhere to the highest ethical standards.
- Relevant national and international animal welfare and ethical use guidelines and regulations should be adhered to.
- Wherever possible, alternative protocols and procedures that minimize pain, suffering and physical or psychological distress to animals should be developed and validated.
- The 3R approach should be applied in the development of standardized and validated LD$_{50}$ and ED$_{50}$ test protocols.

5. General considerations

Snake antivenom immunoglobulins – antivenoms, antivenins, anti-snake-bite serum and anti-snake venom (ASV) – are the only specific treatment for envenoming by snake-bites. They are produced by the fractionation of plasma that is usually obtained from large domestic animals hyperimmunized against relevant venoms. Important but seldom used antivenoms may be prepared in smaller animals. In general, when injected into an envenomed human patient, an effective antivenom will neutralize toxins in any of the venoms used in its production, and in some instances, will also neutralize venoms from closely related species.

5.1 Historical background

Shortly after the identification of diphtheria and tetanus toxins, von Behring and Kitasato reported the antitoxic properties of the serum of animals immunized against diphtheria or tetanus toxins and suggested the use of antisera for the treatment of these diseases (5). In 1894, von Behring diphtheria antitoxin was first successfully administered by Roux to save children suffering from severe diphtheria. Thus, serum therapy was born and the antitoxin was manufactured by Burroughs Wellcome in the United Kingdom. The same year, Phisalix and Bertrand (6) and Calmette (7) simultaneously, but independently, presented during the same session of the same meeting their observations on the antitoxic properties of the serum of rabbits and guinea-pigs immunized against cobra and viper venoms, respectively. Immediately after his discovery of “antivenin serum therapy”, Calmette became actively involved in proving its effectiveness in the
treatment of human envenoming. The first horse-derived antivenom sera that he prepared were in clinical use in 1895 by Haffkine in India and by Lépinay in Viet Nam. The latter reported the first successful use of antivenin serum therapy in patients in 1896 (8).

5.2 The use of serum versus plasma as source material

Historically, the pioneers, Calmette, Vital Brazil and others, used serum separated from the blood of hyperimmunized horses for the preparation of antivenom (“antivenin serum therapy”). Later, antibodies (immunoglobulins) were demonstrated to be the active molecules responsible for the therapeutic action of “antivenom serum”. Subsequently, immunoglobulins, or immunoglobulin fragments (F(\text{ab}')_2, Fab), purified from serum were used instead of crude serum (9, 10). Nowadays, plasmapheresis, whereby red blood cells are re-injected into the donor animal within 24 hours of blood collection, is commonly employed to reduce anaemia in the hyperimmunized animal that donates the plasma. Accordingly, it is almost exclusively plasma rather than serum, which is used as the starting material for the extraction of the immunoglobulin or its fragments (11–13). Thus “snake antivenom immunoglobulin” is the preferred term, rather than “anti-snake-bite serum” or “antiserum” which are no longer accurate.

5.3 Antivenom purification methods and product safety

The recognition of their role, and the purification of immunoglobulins from other components of the serum or plasma of donor animals, was pioneered in the earliest years of the last century using simple chemical reactions (14–18). The subsequent discovery, more than half a century later, of the structure of antibodies opened new doors to the fractionation of immunoglobulins. It became possible to produce antibody fractions (F(\text{ab}')_2 or Fab) that were believed to potentially reduce the frequency of early and late antivenom reactions by removing the Fc fragment from IgG (19). This was subsequently believed to prevent complement activation and perhaps reduce the intensity of immune-complex formation responsible for late antivenom reactions (serum sickness). For 60–70 years, immunoglobulin F(\text{ab}')_2 fragments have been widely used. However, antivenom protein aggregation, and not Fc-mediated complement activation, was increasingly identified as a major cause of antivenom reactions. Thus, a critical issue in antivenom safety probably lies in the physicochemical characteristics of antivenoms and not exclusively in the type of neutralizing molecules constituting the active substance. It is also important to ensure that the current methods of producing antivenoms provide a sufficient margin of safety with regard to the potential risk of transmission of zoonoses.
5.4 Pharmacokinetics and pharmacodynamics of antivenoms

Rapid elimination of some therapeutic antivenoms (for example, when Fab fragments are used) has led to recurrence of envenoming in patients. However, the choice of preparing specific IgG or fragments appears to depend on the size and toxicokinetics of the principal toxin(s) of the venoms. Large relative molecular mass (Mr) bivalent antibodies (IgG and F(\(ab'\))\(_2\) fragments) may be effective for the complete and prolonged neutralization of intravascular toxins (for example, procoagulant enzymes), which have a long half-life in envenomed patients. Low Mr and monovalent IgG fragments, such as Fab, may be more appropriate against low-molecular-mass neurotoxins which are rapidly distributed to their tissue targets and are rapidly eliminated from the patient’s body, for example, scorpion and spider toxins (20).

5.5 Need for national and regional reference venom preparations

Antivenom production is technically demanding. The need to design appropriate monospecific or polyspecific antivenoms (depending on the composition of the snake fauna) is supported by the difference in venom composition among venomous animals, in particular bearing in mind that:

- many countries can be inhabited by several medically important species;
- there may be wide variation in venom composition (and hence antigenicity) through the geographical range of a single species;
- in some circumstances there is no distinctive clinical syndrome to direct the use of monospecific antivenoms.

However, similarities in the venom toxins of closely related venomous species may result in cross-neutralization (paraspecific neutralization), thus reducing the number of venoms required for the preparation of polyspecific antivenoms. Cross-neutralization should be tested in animal models and ideally by clinical studies in envenomed patients. Preclinical testing of antivenoms against medically important venoms present in each geographical region or country is a prerequisite for product licences and batch approval, and should always precede clinical use in envenomed patients. This requires efforts by manufacturers and/or regulators to establish regional or national reference venom preparations that can be used to test the neutralization capacity of antivenoms. The national control laboratory of the country where the antivenom will be used, or the manufacturer seeking a licence for the antivenom, should perform such preclinical testing using reference venom preparations relevant to the country or the geographical area.
6. Epidemiological background

The incidence of medically important snake-bites in different parts of the world and the recognition of the species of greatest medical importance is fundamental to the appropriate design of monospecific and polyspecific antivenoms in countries and regions. Up-to-date epidemiological and herpetological information is therefore highly relevant to antivenom manufacturers and regulators, especially for the selection of the most appropriate venoms or venom mixtures to be used in the production and quality control of antivenoms.

6.1 Global burden of snake-bites

Envenoming and deaths resulting from snake-bites are a particularly important public health problem in rural tropical areas of Africa, Asia, Latin America and Papua New Guinea (21). Agricultural workers and children are the most affected groups. Epidemiological assessment of the true incidence of global mortality and morbidity from snake-bite envenoming has been hindered by several well recognized problems (22, 23). Snake-bite envenoming and associated mortality are underreported because many victims (20–70% in some studies) do not seek treatment in government dispensaries or hospitals and hence are not recorded. This is compounded by the fact that medical posts in regions of high incidence are unable to keep accurate records of the patients who do present for treatment, and because death certification of snake-bite is often imprecise (24, 25).

Correctly designed population surveys, in which questionnaires are distributed to randomly selected households in demographically well-defined areas, are the only reliable method for estimating the true burden of snake-bites in rural areas. The results of the few such surveys that have been performed have shown surprisingly high rates of bites, deaths and permanent sequelae of envenoming (25–29). However, because of the heterogeneity of snake-bite incidence within countries, the results of surveys of local areas cannot be extrapolated to give total national values. Most of the available data suffer from these deficiencies and, in general, should be regarded as underestimates and approximations.

However, the true burden of national snake-bite morbidity and mortality in three South Asian countries has recently been revealed by the results of three well-designed community-based studies. In India, a direct estimate of 46 000 snake-bite deaths in 2005 was derived from the Million Death Study (30), in Bangladesh there were an estimated 589 919 snake-bites resulting in 6,041 deaths in 2009 (31), and in Sri Lanka in 2012–2013, 80 000 bites, 30 000 envenomings and 400 deaths in one year (32). Published estimates of global burden, employing highly controversial methodologies, suggest a range from a minimum of 421 000
envenomings and 20 000 deaths up to as many as 2.5 million cases and more than 100 000 deaths each year (23, 33). In view of the recent data from South Asia, these figures would seem to be underestimates. In addition, the number of people left with permanent sequelae as a result of envenoming is likely to be higher than the number of fatalities (21). As already identified, most of the estimated burden of snake-bite is in sub-Saharan Africa, Central and South America and South and South-East Asia.

The current literature on snake-bite epidemiology highlights the inadequacy of the available data on this neglected tropical disease. There is clearly a need to improve reporting and record-keeping of venomous bites in health facilities, to support high-quality epidemiological studies of snake-bite in different regions, and to improve the training of medical personnel. Wherever possible, recording the species that caused the bite as well as death or injury would greatly assist in documenting which species are of clinical significance in individual countries. Making venomous bites notifiable and fully implementing the use of the International Statistical Classification of Diseases and Related Health Problems 10th Revision (34) in official death certification (for example, T 63.0 snake venom) would further help to determine the burden of snake-bite more accurately.

6.2 Main recommendations

- In most parts of the world, snake-bites are underreported and in some parts are completely unreported. This deficiency in surveillance and the paucity of properly designed epidemiological studies explain why the impact of this important public health problem has remained for so long unrecognized and neglected.

- National health authorities should be encouraged to improve the scope and precision of their epidemiological surveillance of this disease by:
  - improving the training of all medical personnel so that they are more aware of the local causes, manifestations and treatment of venomous bites;
  - making venomous bites notifiable;
  - setting up standardized and consistent epidemiological surveys;
  - improving the reporting and record-keeping of venomous bites by hospitals, clinics, dispensaries and primary health-care posts, and relating the bites to the species of venomous snake that caused the bite wherever possible; and
fully implementing the use of the International Statistical Classification of Diseases and Related Health Problems 10th Revision (2007) (22) in official death certification (for example, T 63.0 snake venom).2

7. Worldwide distribution of venomous snakes

7.1 Taxonomy of venomous snakes
Recognizing the species causing the greatest public health burden, designing and manufacturing antivenoms and optimizing patient treatment are all critically dependent on a correct understanding of the taxonomy of venomous snakes. Like other sciences, the field of taxonomy is constantly developing. New species are still being discovered, and many species formerly recognized as being widespread have been found to comprise multiple separate species as scientists obtain better information, often with new technologies. As the understanding of the relationships between species is still developing, the classification of species into genera is also subject to change. The names of venomous species used in these guidelines conform to the taxonomic nomenclature that was current at the time of publication. Some groups of venomous snakes remain under-studied and poorly known. In these cases, the classification best supported by what evidence exists is presented with the limitation that new studies may result in changes to the nomenclature.

Clinicians, toxinologists, venom producers and antivenom manufacturers should endeavour to remain abreast of these nomenclatural changes. These changes often reflect improved knowledge of the heterogeneity of snake populations, and may have implications for venom producers, researchers and antivenom manufacturers. Although taxonomic changes do not necessarily indicate the presence of “new” venoms, they strongly suggest that toxinological and epidemiological research into these “new” taxa may be required to establish their medical relevance, if any.

Since some of the names of medically important species have changed in recent years, the following points are intended to enable readers to relate the current nomenclature to information in the older literature:

- The large group of Asian arboreal pit vipers, which in recent years had been split from a single genus (Trimeresurus), into a number of new genera (for example, Cryptelytrops, Parias, Peltopelor, Himalayophis, Popeia, Viridovipera, Ovophis and Protobothrops, with a few species retained in Trimeresurus) based on prevailing

views of the interrelationships between these groups, have now largely been returned to *Trimeresurus*. There are divergent views on this approach to the taxonomy of these snakes, and interested parties should consult the literature. Some changes made in the early 1980s have gained acceptance and been retained (that is, *Protobothrops*). Medically important species formerly classified in *Cryptelytrops* include *Trimeresurus albolabris*, *T. erythrurus* and *T. insularis*. *Viridovipera stejnegeri* has been returned to *Trimeresurus*.

- It is likely that new species of cobra (*Naja* spp.) will be identified within existing taxa in both Africa and Asia. Three new species (*N. ashei*, *N. mandalayensis* and *N. nubiae*) have been described and several subspecies elevated to specific status since 2000 (for example, *N. annulifera* and *N. anchietae*, from being subspecies of *N. haje*), in addition to the synonymization of the genera *Boulengerina* and *Paranaja* within the *Naja* genus. Such changes may hold significance for antivenom manufacturers and should stimulate further research to test whether existing antivenoms cover all target snake populations.

- Several medically important vipers have been reclassified: *Daboia siamensis* has been recognized as a separate species from *Daboia russelii*; *Macrovipera mauritanica* and *M. deserti* have been transferred to *Daboia*; the Central American rattlesnakes, formerly classified with *Crotalus durissus*, are now *Crotalus simus*; and *Bothrops neuwiedi* has been found to consist of a number of different species, three of which (*B. neuwiedi*, *B. diporus* and *B. mattogrossensis*) may be of public health importance.

It is recognized that there have been many accepted revisions of taxonomy over the past few decades. These WHO Guidelines are aimed at a very wide range of readers, and to assist in matching some old and familiar names with the current nomenclature, Tables A5.1 and A5.2 summarize the major changes made between 1999 and 2016. A list of relevant herpetological references is provided at the end of Appendix 1 of these Guidelines.

### Table A5.1
Genus-level name changes (1999–2016)

<table>
<thead>
<tr>
<th>Currently accepted name</th>
<th>Previous name(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bothrocophias hyoprora</em></td>
<td><em>Bothrops hyoprora</em></td>
</tr>
<tr>
<td><em>Bothrocophias microphthalmus</em></td>
<td><em>Bothrops microphthalmus</em></td>
</tr>
<tr>
<td><em>Trimeresurus albolabris</em></td>
<td><em>Cryptelytrops albolabris</em></td>
</tr>
</tbody>
</table>
### Table A5.1 continued

<table>
<thead>
<tr>
<th>Currently accepted name</th>
<th>Previous name(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trimeresurus erythrurus</em></td>
<td>Cryptelytrops erythrurus</td>
</tr>
<tr>
<td><em>Trimeresurus insularis</em></td>
<td>Cryptelytrops insularis, <em>Trimeresurus albolabris insularis</em></td>
</tr>
<tr>
<td><em>Trimeresurus macrops</em></td>
<td>Cryptelytrops macrops</td>
</tr>
<tr>
<td><em>Trimeresurus purpureomaculatus</em></td>
<td>Cryptelytrops purpureomaculatus</td>
</tr>
<tr>
<td><em>Trimeresurus septentrionalis</em></td>
<td>Cryptelytrops septentrionalis, <em>Trimeresurus albolabris septentrionalis</em></td>
</tr>
<tr>
<td><em>Daboia deserti</em></td>
<td><em>Macrovipera deserti, Vipera mauritanica deserti, Vipera lebetina deserti</em></td>
</tr>
<tr>
<td><em>Daboia mauritanica</em></td>
<td><em>Macrovipera mauritanica, Vipera lebetina mauritanica</em></td>
</tr>
<tr>
<td><em>Daboia palaestinae</em></td>
<td><em>Vipera palaestinae</em></td>
</tr>
<tr>
<td><em>Daboia russelii</em></td>
<td><em>Vipera russelii</em></td>
</tr>
<tr>
<td><em>Himalayophis tibetanus</em></td>
<td><em>Trimeresurus tibetanus</em></td>
</tr>
<tr>
<td><em>Montivipera raddei</em></td>
<td><em>Vipera raddei</em></td>
</tr>
<tr>
<td><em>Montivipera xanthina</em></td>
<td><em>Vipera xanthina</em></td>
</tr>
<tr>
<td><em>Naja annulata</em></td>
<td><em>Boulengerina annulata</em></td>
</tr>
<tr>
<td><em>Naja christyi</em></td>
<td><em>Boulengerina christyi</em></td>
</tr>
<tr>
<td><em>Trimeresurus flavomaculatus</em></td>
<td><em>Parias flavomaculatus</em></td>
</tr>
<tr>
<td><em>Trimeresurus sumatranus</em></td>
<td><em>Parias sumatranus</em></td>
</tr>
<tr>
<td><em>Protobothrops mangshanensis</em></td>
<td><em>Zhaermia mangshanensis, Ermia mangshanensis, Trimeresurus mangshanensis</em></td>
</tr>
<tr>
<td><em>Trimeresurus stejnegeri</em></td>
<td><em>Viridovipera stejnegeri</em></td>
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</tbody>
</table>

### Table A5.2

**Changes resulting from new species descriptions or redefinitions (1999–2016)**

<table>
<thead>
<tr>
<th>Currently accepted name</th>
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</tr>
</thead>
<tbody>
<tr>
<td><em>Acanthophis crytamydros</em></td>
<td>Previously part of <em>Acanthophis rugosus</em></td>
</tr>
<tr>
<td><em>Acanthophis laevis</em></td>
<td><em>Acanthophis antarcticus laevis</em>, confused with <em>A. antarcticus</em> or <em>A. praelongus</em></td>
</tr>
</tbody>
</table>
### Table A5.2 continued

<table>
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<tr>
<th>Currently accepted name</th>
<th>Previous name(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acanthophis rugosus</em> (New Guinea)</td>
<td><em>Acanthophis antarcticus rugosus</em>, confused with <em>A. antarcticus</em> or <em>A. praelongus</em></td>
</tr>
<tr>
<td><em>Agkistrodon howardgloydi</em></td>
<td><em>Agkistrodon bilineatus howardgloydi</em></td>
</tr>
<tr>
<td><em>Agkistrodon russeolus</em></td>
<td><em>Agkistrodon bilineatus russeolus</em></td>
</tr>
<tr>
<td><em>Agkistrodon taylori</em></td>
<td><em>Agkistrodon bilineatus taylori</em></td>
</tr>
<tr>
<td><em>Bitis gabonica</em></td>
<td><em>Bitis gabonica gabonica</em></td>
</tr>
<tr>
<td><em>Bitis harenna</em></td>
<td>New species</td>
</tr>
<tr>
<td><em>Bitis rhinoceros</em></td>
<td><em>Bitis gabonica rhinoceros</em></td>
</tr>
<tr>
<td><em>Bothrops diporus</em></td>
<td><em>Bothrops neuwiedi diporus</em></td>
</tr>
<tr>
<td><em>Bothrops mattogrossensis</em></td>
<td><em>Bothrops neuwiedi mattogrossensis</em>, <em>B.n. bolivianus</em></td>
</tr>
<tr>
<td><em>Bothrops pubescens</em></td>
<td><em>Bothrops neuwiedi pubescens</em></td>
</tr>
<tr>
<td><em>Bungarus persicus</em></td>
<td>New species</td>
</tr>
<tr>
<td><em>Cerrophidion sasai</em></td>
<td>Previously part of <em>Cerrophidion godmani</em></td>
</tr>
<tr>
<td><em>Cerrophidion wilsoni</em></td>
<td>Previously part of <em>Cerrophidion godmani</em></td>
</tr>
<tr>
<td><em>Crotalus oreganus</em></td>
<td>Previously considered part of <em>Crotalus viridis</em></td>
</tr>
<tr>
<td><em>Crotalus ornatus</em></td>
<td>Previously considered part of <em>Crotalus molossus</em></td>
</tr>
<tr>
<td><em>Crotalus simus</em></td>
<td><em>Crotalus durissus durissus</em> (Central American populations of <em>C. durissus</em> complex)</td>
</tr>
<tr>
<td><em>Crotalus totonacus</em></td>
<td><em>Crotalus durissus totonacus</em></td>
</tr>
<tr>
<td><em>Crotalus tzabcan</em></td>
<td><em>Crotalus simus tzabcan</em>, <em>Crotalus durissus tzabcan</em></td>
</tr>
<tr>
<td><em>Daboia russelii</em></td>
<td><em>Daboia russelii russelii</em>, <em>Daboia r. pulchella</em></td>
</tr>
<tr>
<td><em>Daboia siamensis</em></td>
<td><em>Daboia russelii siamensis</em>, <em>D.r. limitis</em>, <em>D.r. sublimitis</em>, <em>D.r. formosensis</em></td>
</tr>
<tr>
<td><em>Echis borkini</em></td>
<td>Previously part of <em>Echis pyramidum</em></td>
</tr>
<tr>
<td><em>Echis omanensis</em></td>
<td>Previously known as NE population of <em>Echis coloratus</em></td>
</tr>
<tr>
<td><em>Gloydius intermedius</em></td>
<td>Previously named <em>Gloydius saxatilis</em></td>
</tr>
<tr>
<td><em>Hypnale zara</em></td>
<td>New species</td>
</tr>
</tbody>
</table>
Table A5.2 continued

<table>
<thead>
<tr>
<th>Currently accepted name</th>
<th>Previous name(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lachesis acrochorda</em></td>
<td>Previously part of <em>Lachesis stenophrys</em></td>
</tr>
<tr>
<td><em>Naja arabica</em></td>
<td>Previously part of <em>Naja haje</em></td>
</tr>
<tr>
<td><em>Naja anchietae</em></td>
<td><em>Naja annulifera anchietae</em>, <em>Naja haje anchietae</em></td>
</tr>
<tr>
<td><em>Naja ashei</em></td>
<td>Previously part of <em>Naja nigricollis</em></td>
</tr>
<tr>
<td><em>Naja nigricincta</em></td>
<td><em>Naja nigricollis nigricincta</em>, <em>Naja nigricollis woodi</em></td>
</tr>
<tr>
<td><em>Naja nubiae</em></td>
<td>Previously part of <em>Naja pallida</em></td>
</tr>
<tr>
<td><em>Naja senegalensis</em></td>
<td>Previously part of <em>Naja haje</em></td>
</tr>
<tr>
<td><em>Pseudechis rossignolii</em></td>
<td><em>Pailsus rossignolii</em>, previously part of <em>Pseudechis australis</em></td>
</tr>
<tr>
<td><em>Pseudonaja aspidorhyncha</em></td>
<td>Previously part of <em>Pseudonaja nuchalis</em></td>
</tr>
<tr>
<td><em>Pseudonaja mengdeni</em></td>
<td>Previously part of <em>Pseudonaja nuchalis</em></td>
</tr>
<tr>
<td><em>Thelotornis mossambicanus</em></td>
<td><em>Thelotornis capensis mossambicanus</em></td>
</tr>
<tr>
<td><em>Thelotornis usambaricus</em></td>
<td><em>Thelotornis capensis mossambicanus</em></td>
</tr>
<tr>
<td><em>Trimeresurus cardamomensis</em></td>
<td>Previously part of <em>Trimeresurus macrops</em></td>
</tr>
<tr>
<td><em>Trimeresurus rubeus</em></td>
<td>Previously part of <em>Trimeresurus macrops</em></td>
</tr>
<tr>
<td><em>Tropidolaemus philippensis</em></td>
<td>Previously part of <em>Tropidolaemus wagleri</em></td>
</tr>
<tr>
<td><em>Tropidolaemus subannulatus</em></td>
<td>Previously part of <em>Tropidolaemus wagleri</em></td>
</tr>
<tr>
<td><em>Vipera renardi</em></td>
<td>Previously part of <em>V. ursinii</em></td>
</tr>
<tr>
<td><em>Walterinnesia morgani</em></td>
<td>Previously part of <em>Walterinnesia aegyptia</em></td>
</tr>
</tbody>
</table>

7.2 Medically important venomous snakes

Based on current herpetological and medical literature, it is possible to partially prioritize the species of snakes that are of greatest medical importance in different regions. Detailed statistics on the species of snakes responsible for morbidity and mortality throughout the world are lacking, except for a few epidemiological studies which include rigorous identification of the biting snake in a few scattered localities. Thus, establishing a list of medically important species for different countries, territories and other areas relies, at least in part, on extrapolation from the few known studies, as well as on the biology of the snake species concerned: for example, where species of a group of snakes are known to be of public health
importance, based on epidemiological studies, it seems reasonable to deduce that closely related species with similar natural history occurring in hitherto unstudied regions are also likely to be medically important. Examples include Asian cobras in several under-studied regions of Asia, lowland *Bungarus* species in Asia, and spitting cobras in Africa.

Tables A5.3–A5.6 list the species of venomous snakes of greatest medical importance in each of four broad geographical regions. Species listed in these tables are either:

- those that are common or widespread in areas with large human populations and which cause numerous snake-bites, resulting in high levels of morbidity, disability or mortality among victims; or
- poorly known species that are strongly suspected of falling into this category; or
- species that cause major and life-threatening envenoming responsive to antivenom, but are not common causes of bites.

The venoms of these species should be considered a starting point for establishing the most important targets for antivenom production. The need for additional epidemiological and toxinological research to better define which venoms to include and exclude for antivenom production in various regions, territories and countries around the world is emphasized. Detailed data from countries, territories and other areas on species believed to contribute most to the global burden of injury, and/or which pose the most significant risk of morbidity or mortality are provided in Appendix 1 of these Guidelines. Illustrations of some important venomous snakes of Africa and the Middle East are shown in Figs A5.1 and A5.2.

**Table A5.3**

**Medically important venomous snakes: Africa and the Middle East**

<table>
<thead>
<tr>
<th>North Africa/Middle East</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atractaspidae: <em>Atractaspis andersonii</em>; Elapidae: <em>Naja arubica, Naja haje, Naja oxiana</em>; Viperidae: <em>Bitis arietans, Cerastes cerastes, Cerastes gasperetti, Daboia mauritanica, Daboia palaestinai, Echis borkini, Echis carinatus, Echis coloratus, Echis omanensis, Echis pyramidum; Macro vipera lebetina, Montivipera xanthina, Pseudocerastes persicus</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Central sub-Saharan Africa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elapidae: <em>Dendroaspis jame soni, Dendroaspis polylepis; Naja anchietae, Naja haje, Naja melanoleuca, Naja nigrilolli; Viperidae: Bitis arietans, Bitis gabonica, Bitis nasicornis, Echis leucogaster, Echis ocellatus, Echis pyramidum</em></td>
</tr>
</tbody>
</table>
### Table A5.3 continued

#### Eastern sub-Saharan Africa

**Elapidae:** *Dendroaspis angusticeps, Dendroaspis jamesoni, Dendroaspis polylepis; Naja anchietae, Naja annulifera, Naja ashei, Naja haje, Naja melanoleuca, Naja mossambica, Naja nigrigollis; Viperidae:** *Bitis arietans, Bitis gabonica, Bitis nasicornis; Echis pyramidum*

#### Southern sub-Saharan Africa

**Elapidae:** *Dendroaspis angusticeps, Dendroaspis polylepis; Naja anchietae, Naja annulifera, Naja mossambica, Naja nigricincta, Naja nivea; Viperidae:** *Bitis arietans*

#### Western sub-Saharan Africa

**Elapidae:** *Dendroaspis jamesoni, Dendroaspis polylepis, Dendroaspis viridis; Naja haje, Naja katiensis, Naja melanoleuca, Naja nigrigollis, Naja senegalensis; Viperidae:** *Bitis arietans, Bitis gabonica, Bitis nasicornis, Bitis rhinoceros; Cerastes cerastes; Echis jogeri, Echis leucogaster, Echis ocellatus*

### Table A5.4

**Medically important venomous snakes: Asia and Australasia**

#### Central Asia

**Elapidae:** *Naja oxiana; Viperidae:** *Echis carinatus; Gloydius halys; Macrovet vera lebetina*

#### East Asia

**Elapidae:** *Bungarus multicinctus; Naja atra; Viperidae:** *Trimeresurus albolabris; Daboia russellii; Deinagkistrodon acutus; Gloydius blomhoffii; Gloydius brevicaudus; Protobothrops flavoviridis, Protobothrops mucrosquamatus; Trimeresurus stejnegeri*

#### South Asia

**Elapidae:** *Bungarus caeruleus, Bungarus ceylonicus, Bungarus niger, Bungarus sindanus, Bungarus walli; Naja kaouthia, Naja naja, Naja oxiana; Viperidae:** *Trimeresurus erythrus, Daboia russellii; Echis carinatus; Hypnale hypnale; Macrovet vera lebetina*

#### South-East Asia (excluding Indonesian West Papua)

**Elapidae:** *Bungarus candidus, Bungarus magnimaculatus, Bungarus multicinctus, Bungarus slowinski; Naja atra, Naja kaouthia, Naja mandalayensis, Naja philippinensis, Naja samarensis, Naja siamensis, Naja sputatrix, Naja sumatrana; Viperidae:** *Calloselasma rhodostoma; Trimeresurus albolabris, Trimeresurus erythrus, Trimeresurus insularis; Daboia siamensis; Deinagkistrodon acutus*
Table A5.4 continued

**Australo-Papua** (includes Indonesian West Papua)

Elapidae: *Acanthophis laevis*; *Notechis scutatus*; *Oxyuranus scutellatus*; *Pseudechis australis*; *Pseudonaja affinis*, *Pseudonaja mengdeni*, *Pseudonaja nuchalis*, *Pseudonaja textilis*

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Table A5.5

**Medically important venomous snakes: Europe**

<table>
<thead>
<tr>
<th>Region</th>
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<tbody>
<tr>
<td><strong>Central Europe</strong></td>
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<tr>
<td>Viperidae:</td>
<td><em>Vipera ammodytes</em></td>
</tr>
<tr>
<td><strong>Eastern Europe</strong></td>
<td></td>
</tr>
<tr>
<td>Viperidae:</td>
<td><em>Vipera berus</em></td>
</tr>
<tr>
<td><strong>Western Europe</strong></td>
<td></td>
</tr>
<tr>
<td>Viperidae:</td>
<td><em>Vipera aspis</em>, <em>Vipera berus</em></td>
</tr>
</tbody>
</table>

Table A5.6

**Medically important venomous snakes: the Americas**

<table>
<thead>
<tr>
<th>Region</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>North America</strong></td>
<td></td>
</tr>
<tr>
<td>Viperidae:</td>
<td><em>Agkistrodon bilineatus</em>, <em>Agkistrodon contortrix</em>, <em>Agkistrodon piscivorus</em>, <em>Agkistrodon taylori</em>; <em>Bothrops asper</em>, <em>Crotalus adamanteus</em>, <em>Crotalus atrox</em>, <em>Crotalus horridus</em>, <em>Crotalus oreganus</em>, <em>Crotalus simus</em>, <em>Crotalus scutulatus</em>, <em>Crotalus molossus</em>, <em>Crotalus viridis</em></td>
</tr>
<tr>
<td><strong>Caribbean</strong></td>
<td></td>
</tr>
<tr>
<td>Viperidae:</td>
<td><em>Bothrops cf. atrox</em> (Trinidad), <em>Bothrops caribbaeus</em> (St Lucia), <em>Bothrops lanceolatus</em> (Martinique); <em>Crotalus durissus</em> (Aruba)</td>
</tr>
<tr>
<td><strong>Central America</strong></td>
<td></td>
</tr>
<tr>
<td>Viperidae:</td>
<td><em>Bothrops asper</em>, <em>Crotalus simus</em></td>
</tr>
</tbody>
</table>

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3 *Pseudechis australis* is common and widespread and causes numerous snake-bites; bites may be severe, although this species has not caused a death in Australia since 1968.
Table A5.6 continued

<table>
<thead>
<tr>
<th>South America</th>
</tr>
</thead>
</table>

**Viperidae:** Bothrops alternatus, Bothrops asper, Bothrops atrox, Bothrops brazili, Bothrops bilineatus, Bothrops diporus, Bothrops jararaca, Bothrops jararacussu, Bothrops leucurus, Bothrops matogrossensis, Bothrops moojeni, Bothrops pictus, Bothrops venezuelensis; Crotalus durissus; Lachesis muta

### 7.3 Minor venomous snake species

In many countries, territories and other areas there are species of snakes that rarely bite humans but are capable of causing severe or fatal envenoming. Their medical importance may not justify inclusion of their venoms in the immunizing mixture for production of polyspecific antivenoms but the need to make antivenoms against these species needs to be carefully analysed.

In some cases, such as with some Central American pit vipers (genera Agkistrodon, Porthidium, Bothriechis, Atropoides among others), there is clinically effective cross-neutralization of venoms by standard national polyspecific antivenoms (35).

In other cases, there is no effective cross-neutralization and manufacturers may therefore consider that the production of a monospecific antivenom is justified for use in potentially fatal cases of envenoming, provided that such cases can be identified. Such antivenoms are currently available for envenoming by the boomslang (Dispholidus typus), desert black snake (Walterinnesia aegyptia), Arabian burrowing asp (Atractaspis andersonii) (36), king cobra (Ophiophagus hannah), Malayan krait (Bungarus candidus) (36) “yamakagashi” (Rhabdophis tigrinus) and red-necked keelback (R. subminiatus), Martinique’s “Fer-de-lance” (Bothrops lanceolatus), St Lucia’s B. caribbaeus, and some species of American coral snake (Micrurus).

No antivenoms are currently available for envenoming by species such as African bush vipers (for example, Atheris, Proatheris), berg adder (Bitis atropos) and several other small southern African Bitis spp. (for example, B. peringueyi), Sri Lankan and south-west Indian hump-nosed vipers (Hypnale spp.) (37, 38), many Asian pit vipers (“Trimeresurus” sensu lato), some species of kraits (for example, B. niger) and all but one species of burrowing asp (genus Atractaspis).

An alternative to antivenom production against species that cause few, but potentially severe envenomings, is to manufacture polyspecific antivenoms for broadly distributed groups that have similar venom compositions (for example, African Dendroaspis and Atractaspis; Asian “green pit vipers”; American Micrurus). This may result in antivenoms that offer broad protection against
venoms from minor species within genera, or species whose bites are less frequent than those of others in the same taxonomic groups (that is, genus, subfamily or family).

7.4 **Sea snake venoms**

Although venomous marine sea snakes have not been included in the tables of medically important venomous snakes, it should be recognized that there are a number of species of marine snakes with potent venoms that can cause illness or death. Available evidence, particularly clinical experience, indicates that the major sea snake antivenom that is currently commercially available, which uses venom of a single sea snake, *Hydrophis schistosus* (previously known as *Enhydrina schistosa*), in the immunizing venoms mixture, is effective against envenoming by other sea snake species for which there are clinical data. Further research would be needed to better define the full extent of cross-neutralization offered by this antivenom against other sea snake species.

7.5 **Main recommendations**

- Clinicians, toxinologists, poison centres, regulators, venom producers and antivenom manufacturers should be well informed about current nomenclature and new changes to taxonomy, so as to ensure the currency of information, correct identification of species in their countries, and correct selection and sourcing of venoms used in the manufacture of antivenoms.

- Identification of the medically important venomous snakes (those that cause the greatest burden of injury, disability and/or mortality) is a critical prerequisite to meeting the need for efficacious antivenom. Improving the quality of the available data and correcting and amplifying the level of geographical detail and precision of attribution should be important priorities.

- Support for establishment of local capacity for venom production as a means of ensuring that venom immunogens from geographically representative populations of medically important snake species are used in antivenom production would improve antivenom specificity.
Fig. A5.1
Medically important North African and Middle Eastern venomous snakes:
(A) Egyptian cobra (*Naja haje*), (B) East Africa carpet viper (*Echis pyramidium*), (C) puff adder (*Bitis arietans*), (D) Saharan horned viper (*Cerastes cerastes*) and (E) Levant viper (*Macrovipera lebetina*)
Fig. A5.2
Medically important sub-Saharan African venomous snakes: (A) West African carpet viper (*Echis ocellatus*), (B) Gaboon viper (*Bitis gabonica*), (C) Black mamba (*Dendroaspis polylepis*), (D) Black-necked spitting cobra (*Naja nigricollis*), (E) Mozambique spitting cobra (*Naja mossambica*)
8. Antivenoms design: selection of snake venoms

Venomous snakes exhibit significant species- and genus-specific variation in venom protein composition (39). The clinical effectiveness of antivenom is therefore largely restricted to the venom(s) used in its manufacture. It is therefore imperative that antivenom manufacturers carefully consider the venoms used in antivenom manufacture by first defining the geographical area where the antivenom will be deployed, and sequentially:

- identifying the most medically important snakes in that region;
- examining the venom protein composition of the snakes, including information from relevant literature;
- conducting antivenom preclinical efficacy tests on venoms of all the most medically important snakes in that region.

8.1 Selection and preparation of representative venom mixtures

Appendix 1 presents an up-to-date list of the most medically important venomous snake species by country, region and continent. The venoms from Category 1 snakes must be included for antivenom production and venoms from Category 2 snakes only excluded after careful risk–benefit assessment.

It is important to appreciate that there are variations in venom composition and antigenicity: (a) within the geographical range of a single species; and (b) between snakes of different ages (40, 41). Therefore, venom should be collected from specimens of different geographical origins and ages, and mixed before being used for immunization (see section 9 on venom preparation). The greater the intra-specific variation, the more snake specimens of distinct origin and age are required to create an adequate venom immunization mixture.

Cross-neutralization of venoms with similar protein composition profiles to the venoms used for immunization may extend the effectiveness of some antivenoms, but requires, minimally, preclinical efficacy testing to identify the potential cross-neutralization capacity of an antivenom. In vitro preclinical immunological cross-reactivity testing alone is NOT an adequate measure of antivenom efficacy.

8.2 Manufacture of monospecific or polyspecific antivenoms

Antivenom manufacturers face an early, critical decision as to whether the antivenom should possess monospecific or polyspecific effectiveness.
8.2.1 **Monospecific antivenoms**

Monospecific antivenoms are manufactured with venoms from a single venomous snake species, and their effectiveness is largely restricted to that snake species. These conditions apply in areas where:

- there is only one medically important species (for example, *Vipera berus* in Scandinavia and the United Kingdom) or where one species is responsible for the majority of cases (for example, *Oxyuranus scutellatus* in southern Papua New Guinea);
- a simple blood test, suitable for use even in under-resourced health-care centres, can define the biting species (for example, detection of incoagulable blood by the 20-minute whole blood clotting test in the northern third of Africa, where only *Echis* spp. cause coagulopathy);
- a simple algorithmic approach allows the species to be inferred from the pattern of clinical and biological features;
- there is a reliable and affordable rapid immunodiagnostic test readily available allowing the toxins to be identified unambiguously (currently only available in Australia).

Monospecific antivenoms can be effective in treating envenoming by a few closely related species whose venoms show clinically effective cross-neutralization – but this requires preclinical and clinical confirmation.

8.2.2 **Polyspecific antivenoms**

Most tropical countries are inhabited by several medically important snake species, and it is commercially unrealistic to develop multiple monospecific antivenoms. In these cases, the manufacture of polyspecific antivenoms is highly recommended. Polyspecific antivenoms are designed to contain IgG effective against venoms from multiple species or genera of venomous snakes in a defined region. Manufacturing protocols of polyspecific antivenom include:

1. Mixing venoms from multiple snake species or genera (sometimes in amounts quantitatively associated with medical importance, immunogenicity etc.) and immunizing donor animals with this mixture. Immunizing an animal with venoms from several taxonomically related snakes (for example, different vipers) can have the advantage over monospecific antivenom of increasing the titre of neutralizing IgG to any one snake venom (42).
2. Immunizing groups of donor animals with distinct venom mixtures and then mixing the hyperimmune plasma from each group of animals.

3. Immunizing groups of donor animals with distinct venom mixtures and then mixing the monospecific antivenom IgGs to formulate the final polyspecific antivenom.

When using options 2. and 3. it is important to monitor the efficacy for each monospecific antivenom to ensure that the efficacy of the mixed final product is consistent, reproducible and in line with the product specification for each individual venom. This “combined monospecific antivenoms” approach anticipates that the amount of neutralizing IgG targeting each individual venom will be proportionally diluted – necessitating administration of more vials to reverse venom pathology, which in turn increases the risks of adverse reactions.

In some regions, it is possible to differentiate envenoming by detecting distinct clinical syndromes: neurotoxicity, haematological disturbances (haemorrhage or coagulopathy) and/or local tissue damage. Such situations justify the preparation of syndrome-specific polyspecific antivenoms by immunizing donor animals with mixtures of either neurotoxic venoms or venoms causing haemorrhage and/or coagulopathy and local tissue damage.

In most tropical regions where snake-bite is a significant medical burden, polyspecific antivenoms offer significant clinical advantages and their production should be encouraged. They can also offer greater commercial manufacturing incentives (economies of scale) than monospecific antivenoms because of their significantly greater geographical and snake-species cover – increasing the likelihood of their delivery to victims residing in regions where antivenom manufacture is not government subsidized.

8.3 Main recommendations

- Prior to importing antivenoms, national health authorities should carefully consider their regional threat from venomous snakes to guide their antivenom requirements.
- The design of the venom mixture used in immunization, and the decision to prepare monospecific or polyspecific antivenoms, must be guided by the epidemiological and clinical information on snake-bites in the defined country, region or continent.
- In most tropical countries polyspecific antivenoms are likely to have significant clinical and logistical advantages over monospecific antivenoms, particularly in the absence of rapid, affordable snake venom diagnosis.
- Polyspecific antivenom may be prepared from IgG of donor animals immunized with a mixture of venoms, or by mixing monospecific antivenoms.
- Manufacturers seeking marketing authorization for antivenoms in a given country should provide experimental evidence from preclinical testing that the product exhibits a neutralization capacity against different local venoms (see section 19).
- National health authorities should organize independent preclinical efficacy testing prior to importation of any antivenom to avoid national distribution of dangerously ineffective products.

9. Preparation and storage of snake venom

Venom preparations are used both to hyperimmunize animals as part of antivenom production, and to provide reference venom samples for routine and/or preclinical potency assessment of antivenoms. According to GMP for pharmaceutical products, snake venoms are starting materials, and therefore ensuring their quality is critical, and their preparation should follow the principles and recommendations stated below. The essential principles of quality systems should be applied to venom production including traceability, reproducibility, taxonomic accuracy and hygiene control. Manufacturers of snake venoms used in antivenom production should strive to comply with WHO’s Guidelines on GMP for biological products and Guidelines for good manufacturing practices for pharmaceutical products.4

Venoms used for antivenom manufacture should be representative of the snake population living in the area where the antivenom is to be used. To take account of the variability in venom composition within a species (43–47), it is imperative that the venom of an adequate number of individual snakes (generally no fewer than 20 specimens, including males and females) collected from various regions covering the entire geographical distribution of the particular venomous snake species should be collected together. Consideration should also be given to including venom from juvenile or sub-adult snakes in these venom pools as there is strong evidence of age-related venom variation within individual specimens and populations (48). A similar approach should be used in the preparation of Standard Reference Venoms (national or regional) for use in the validation of antivenom products by reference laboratories and

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regulatory agencies (see section 10) or in preclinical testing of antivenoms by manufacturers (see section 19).

Venom producers should ensure that they fully document, and can provide evidence of:

- geographical origin and the length or age (juvenile or adult) of each individual snake used for venom production;
- taxonomic details of each snake species used;
- correct implementation of compliance with local wildlife legislation, and the Convention on International Trade in Endangered Species (CITES) documents in the case of endangered species;
- application of appropriate withholding rules (for example, not collecting venom from animals under quarantine, or which are gravid, injured, sick or in poor condition);
- individual identification of snake specimens contributing to each venom batch;
- traceability of each venom batch;
- appropriate handling and stabilization of venoms (for example, rapid freezing of the venom after collection and lyophilization for long-term stable storage);\(^5\)
- quality control confirmation of batch-to-batch consistency of venoms of each species and country of origin (for example, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or high-performance liquid chromatography (HPLC) profiling of venoms, measurement of residual moisture in lyophilized venom);
- confirmation of batch-to-batch similarity of venom of the same origin.

9.1 Production of snake venoms for immunization

The maintenance of a serpentarium and the handling of snakes used for antivenom production should comply with quality systems principles.

9.1.1 Quarantine of snakes

All new accessions should be quarantined for at least 2 months in a special “quarantine room” which should be located as far as possible from the “production rooms” where snakes qualified for venom production are kept.

\(^5\) Desiccation or vacuum-drying may be acceptable if proven to ensure stability of the preparation.
On arrival, snakes should be examined by a specialized veterinary surgeon (or experienced person) for ectoparasites, wounds and fractures. Endoparasites (nematodes, cestodes, trematodes and pentastomids) should be eliminated using broad-spectrum antiparasitic drugs and any injury must be adequately treated by a veterinarian (49–51). Some viruses can be transmitted between different species, and between different families of snakes. Therefore, different families should be kept in different rooms.

Sick snakes should be treated and their quarantine extended for 1–2 months after complete clinical recovery. Sick animals found in “production rooms” may be treated in situ (although quarantine is preferable) but they cannot be used for venom production. If an antibiotic treatment is given, the snake should not be used to obtain venom for 4 weeks following the end of the treatment. When housed in good conditions, adult snakes collected from the wild can live in captivity for 10 years or more. When handling snakes, the risk of infection with human mosquito-borne viruses such as Japanese encephalitis should be prevented, since arbovirus infections have been reported in some snakes (52).

9.1.2 Maintenance of captive snakes for venom production

Individual snakes should preferably be housed in separate cages large enough to allow them to move about, according to local and international standards. There are several acceptable options for the design of the cages. Transparent or black (for burrowing snakes) plastic boxes are recommended. Cage materials should be impermeable, free from fissures, and inert to disinfectants, cleaning chemicals and common solvents. Cleaning and disinfecting agents should be carefully selected to ensure they do not have adverse effects on the snakes. Cages should be adequately ventilated but perforations or mesh must be small enough to prevent escape. Ventilation holes should be clearly marked as hazard areas since there is a risk of accidental envenoming (for example, spitting cobras have been known to spray venom through such openings, and large vipers have fangs which can extend through a small hole if the snake strikes). In the case of gravid viviparous snakes, the ventilation holes or mesh should be sufficiently fine to prevent escape of their tiny, liveborn offspring. The cage interior should be visible from the outside to allow safe maintenance and handling. Access to cages through doors, lids or sliding panels should facilitate management without compromising safety or allowing snakes to escape. Be wary of cages with internal ledges or lips above doors, as some snakes can conceal themselves above them out of sight of the keepers. A disposable floor covering (for example, newspaper) is recommended. Cryptic and nocturnal species should be provided with a small shelter where they can hide.

The use of “hide boxes” is increasingly common as these provide both a more reassuring environment for the snake, and increased safety for keepers.
Hide boxes should be designed to be slightly larger than the curled snake, with an entrance/exit hole large enough to allow a recently fed snake easy access, plus some simple closure device to lock the snake in the hide box. This will allow removal of the snake from the cage without hazard to the keeper, making routine cage maintenance simpler and safer. Hide boxes can be made from plastic, wood or even cardboard (which is inexpensive and can be discarded and replaced regularly). Permanent hide boxes should be readily cleanable or autoclavable. The roof or side of the hide box should be removable, to allow easy and safe extraction of the snake when required.

Cages should be thoroughly cleaned and disinfected when soiled (daily if necessary). Faeces and uneaten or regurgitated food items should be removed as soon as possible. To avoid misidentification of the snake, a microchip should be implanted in the hypodermal layer of the snake’s posterior region and a label bearing its individual data should be attached to the cage and transferred with the snake when it is moved to another cage. Water should be freely available, and for species from humid climates more frequent watering or misting may be required, particularly when sloughing. Water should be changed regularly and as soon as it becomes contaminated. Water treatment by ultraviolet (UV) sterilization or acidification may be considered.

Tens of cages may be accommodated in the same “production room”, provided that there is enough space for maintenance and venom extraction. This room should be kept as clean as possible at all times, and thoroughly cleaned at least weekly. Measures should be taken to minimize or eliminate contamination or spread of diseases. The use of antiseptic hand washes, disposable over-clothing, antiseptic foot wash trays at entry and exit points, and other measures should be routine. The temperature and humidity of the snake room should be controlled according to the climatic requirements of the particular snake species. Ventilation should be ensured using fans, air-conditioning, or air renewing systems.

Access to snake rooms should be restricted to personnel responsible for their maintenance. The rooms should be kept locked, with any windows permanently closed or protected by bars and mosquito proofing. Access should be via a safety porch not allowing simultaneous door opening and with a transparent panel allowing a view of the entire snake room for pre-entry safety inspections. The spaces below the doors should be less than 3 mm and all openings to the exterior (for example, water pipes, drainage conduits, ventilation entrances and exits) should be protected by grilles having holes smaller than 3 mm. Natural light is often used; however, when not available, artificial light should be turned on for 12 hours during the day and turned off during the night for tropical species, but species from temperate zones may have different requirements. Snakes of the same species, collected at the same time in the same area should be placed in the same racks. The same “production room”
can contain snakes of different species, provided that they require similar living conditions (that is, temperature and humidity).

When kept under favourable housing and climatic conditions, and if left undisturbed, snakes will reproduce in captivity (53). Animals should be mated only with specimens from the same species, subspecies and local origin (54, 55). Sexing can be difficult, but is helped by the use of intra-cloacal probes. The male and the female should be individually identified and separated soon after copulation. The female should be kept under careful surveillance. Eggs from oviparous snakes and neonates from viviparous snakes should be removed from the female’s cage as soon as possible. Differences in the venom composition of adult and juvenile snakes have been reported in some species (43, 48, 56–58), and where this is known to occur or is suspected, the venom of a certain proportion of juvenile snakes might be mixed with that of adults during the production of venom batches.

The ideal frequency of feeding captive snakes depends on the species and age of the snake, varying from twice per week to once per month. Snakes are usually fed after venom extraction, ideally with dead mice or other appropriate prey according to the snake species. Animals such as rats and mice that are raised to feed snakes should be produced under appropriate quarantine standards in facilities designed for this task. Humane euthanasia should be employed in the killing of food animals, and ideally these food animals should be frozen for at least 7 days before being thawed for use. Some snakes will only accept living prey, but attempts should be made to wean them onto dead prey, and all local ethical standards should be followed in the production and use of food animals. Snake-eating species, such as kraits, coral snakes and king cobras, can be enticed to take dead mice if the prey is first flavoured with snake tissue fluids, although any such material should be frozen first for at least 7 days to kill parasites, before it is thawed for use. Some coral species can be fed with fish strips (59). Living, dead or regurgitated prey should not be left in the cage for more than a few hours. Force feeding may be necessary for neonates and snakes that persistently refuse to feed. Feeding time affords an opportunity to carefully check the snake for abnormal behaviour, wounds, and possible infections and to give dietary supplements when necessary. Individual feeding records are crucial. They should include details of what, when and how prey was offered, when it was consumed and whether it was regurgitated. The health of captive snakes can be estimated and recorded by observing regular feeding and by measuring their weight and length. These data are best stored on a computer system, using a “barcode” for each snake, or, alternatively, using a reliable manual recording system, and constitute useful records related to the venom batches produced. Venom extraction rooms should be equipped with emergency eyewash stations and safety showers as is the case in laboratories where there is a risk of chemical contact hazards.
9.1.3 General maintenance of a serpentarium

Serpentariums should be designed to comply with appropriate GMP principles. Quarantine facilities should be isolated in all respects from the main animal housing area, and should have separate air-handling systems, or be in a separate building. Maintenance areas such as storeroms, rooms for cleaning and sanitizing cages and racks, animal houses for production of food animals (for example, rodents or invertebrates), and rooms used for administration or for venom processing, venom quality control and secure storage of venoms, should also be separated by appropriate barrier systems from the main snake housing and venom extraction rooms. The main housing rooms for snakes used in venom production should be designed with security, hygiene and disease control needs in mind. Separate rooms for accommodation of snake egg incubators and both neonates and juvenile snakes should be included in the design of the serpentarium.

The cage cleaning rooms should be large enough to hold all the cages that are being cleaned and sanitized. Dirty cages and other items should be kept separate from clean cages and equipment being stored ready for use. Furthermore it is desirable to have two sets of washing and sanitizing rooms, a larger one for equipment from the venom production room and a smaller one for equipment from the quarantine area. These rooms should be secure in case a snake is inadvertently left in its cage when the container is placed in the cleaning room. The cleaning procedures for production rooms and for cages in which snakes are kept, and the cleaning schedule, should be established and documented.

Food animals, usually rodents, should be purpose bred in clean conventional animal houses, and kept, handled and killed in accordance with ethical principles. The rooms, exclusively used for rodent production, should be large enough to provide sufficient numbers of rats or mice to feed the snakes. Ideally, rodent production should be performed in facilities meeting the corresponding international guidelines. Alternatively, rodents can be purchased from qualified commercial sources. Breeding of rats and mice cannot take place in the same room, because of the stress induced by the rats in the mice. The diets required by young snakes may differ from those of adults (for instance, frogs and tadpoles are preferred to rodents by some species), and facilities for producing these food animals may also be required.

When possible, it is useful to have a small laboratory for performing quality control on the venoms. All serpentariums need to be designed with separate laboratories where venom can be processed after extraction and quality control performed (see section 10). An area for repairing broken equipment and for other miscellaneous purposes is also required. The administrative area should be sufficiently large and adequately equipped with computer facilities so that the traceability requirements needed for venom production can be met. The whole venom production facility should be made secure against unauthorized intrusion.
9.1.4  **Snake venom production**

The collection of venom is an inherently dangerous task; therefore specific safety protocols for operators must be applied and rigidly enforced (see section 9.2). All operations should be fully described in written procedures and SOPs, which should be checked and revised periodically according to a written master document. Pools of venom require unique batch numbers, and should be traceable to the individual specimens from which venom was collected for that batch.

9.1.4.1  **Venom collection in serpentariums**

Venom can be extracted from snakes according to a regular schedule, depending on the species. The interval between extractions varies among producers and ranges from every 2 or 3 weeks to every 3 months. Specimens that are quarantined, are gravid, are undergoing treatment for sickness or injury, or in the process of sloughing their skins should not be used for venom production.

Handling equipment must be appropriate for the particular species of snake to minimize risk of stress, discomfort and injury to both the snake and the operator. Staff must be familiar with the equipment and properly trained in its use. Common methods of restraint include gently removing the snake from its cage with a hook and either placing it on a foam rubber pad before being pinned behind the head, or encouraging the snake to crawl into a transparent plastic tube in which it can be restrained. Developing innovative methods that enable safe restraint of venomous snakes and minimize the risk of injury both to operators and snakes is strongly recommended. For very dangerous species, the use of short-acting general anaesthesia or moderate cooling (15 °C) during venom extraction can be considered (for example, inhaled isoflurane or sevoflurane or even carbon dioxide) as it reduces the risk of accidents both to the snake and to the snake handler. Excessive cooling of the snake in a refrigerator is potentially harmful and is not recommended. For the collection of venom, the snake's head is grasped in one hand just behind the angle of the jaw, while the snake's body is held with the other hand, or by an assistant snake handler. Individual techniques for holding the head of the snake vary and each operator should use the method that works best for them. An assistant should gently occlude the snake's cloaca to prevent messy contamination of the locality by spraying of faeces.

Different techniques are used to collect venom. Many rely on encouraging the snake to open its mouth and either bite through a plastic- or parafilm-covered membrane, which provides a barrier to contaminants such as saliva and blood (from minor oral trauma), or to release venom into a container over which the fangs have been hooked by the operator. In the case of large vipers, the dental sheath may be retracted when necessary with sterile forceps. Although it is common practice to squeeze the sides of the snake's head to try to force venom
from the glands, this may cause traumatic bruising to the animal and should be avoided. The use of brief electrical impulses of moderate intensity to stimulate venom secretion is not recommended. Any venom sample contaminated with blood should be centrifuged. After venom extraction, the fangs are carefully withdrawn from the collection vessel, while preventing damage to the mouth and dentition and avoiding the snake impaling itself with its own fangs. Then, the oral cavity should be sprayed with an antiseptic solution to avoid stomatitis. After each venom extraction, all materials used in the process should be sterilized.

Peptides and proteins in venom are amphiphatic and will adhere to most common surfaces including glass and plastic (60) resulting in the potential loss of toxins from the venom used to produce hyperimmune plasma. The use of polypropylene vessels and the addition of 1% bovine serum albumin can help reduce such losses, but different peptides may have variable affinity for being retained on vessel surfaces regardless of the approach taken to minimize loss.

Special procedures that avoid direct handling should be employed in the case of burrowing asps (genus Atractaspis) because they cannot be held safely in the way described above (61). For some species with small fangs and small venom yields, the use of sterile pipette tips or capillary tubes which are slipped over each fang one at a time, and pressure applied to the base of the fang to stimulate venom release into the tube, is recommended. In the case of colubrid snakes, special techniques are required, such as application of foam rubber pads (from which venom is recovered in the laboratory) or pipette tips/capillary tubes to the posteriorly placed fangs and the use of secretagogue drugs. Similarly, some elapid snakes have small fangs and the pipette tip or capillary tube technique is required to collect venom. At the time of venom extraction, there is an opportunity to remove broken or diseased fangs and to examine the snake for ectoparasites (for example, ticks and mites), wounds, dermatitis, areas of adherent dead skin and retained spectacles over the eyes. The snake can be treated with drugs and/or vitamins at the same time and, if necessary, can be force fed. When force fed with rodents, the rodent’s incisors must be cut out so as not to cause any injury to the snake’s oesophagus. The process of venom extraction is often combined with cage cleaning and disinfection and the feeding of the snake. Avoiding trauma to the snake’s mouth and dentition is critical to prevent infection and “mouth rot” and the venom extraction process should be performed in accordance with clean practices.

Several snakes from the same group (same species and subspecies collected at the same time in the same area) can have their venom collected into the same venom collection vessel. The vessel should be kept in an ice bath between individual extractions, and the venom aliquoted into labelled storage tubes or vials and snap-frozen at −20 °C or colder within 1 hour. For venoms with high proteolytic activity, the collected venom pool should be transferred into a vial maintained at ultra-low temperature (−70 to −80 °C) or at least
−20 °C, every 10–30 minutes, before continuing extractions from that group of specimens. Another method is to transfer the collected venom into a vial maintained in an ice bath. Refrigerated centrifugation of freshly collected venom is recommended, for instance at 1000 g for 5 minutes (4 °C), to remove cellular debris.

It is important to identify the vial into which the venom has been collected or transferred for storage, with an appropriate reference number. Primary indelible identification must be on the vial. This allows the identification of all the snakes used during venom extraction, the name of the operator and any other relevant information. To obtain large venom batches for the preparation of antivenom, especially from species with low yields, one approach is to use the same vial over several months for extractions performed with the same specimens, providing the cold chain is never broken. Pools of venom require unique batch numbers, and the individual venom extractions contributing to the pool must be traceable. When a pool is sufficient in volume, the venom should be either freeze- or vacuum-dried and kept in the dark at a low temperature (either −20 °C or 4 °C) in a well-sealed flask, precisely identified with a number, up to the time of delivery. Some producers use an alternative system, keeping dried venom at 20–25 °C in a desiccator. Regardless of the method used, the procedures for drying venom should be well established, documented, validated and incorporate appropriate quality control steps (for example, periodic determination of residual moisture against established standards). The potency of venom stored for considerable periods of time must be tested at least annually to ensure that no degradation or loss of activity has occurred (see section 10), and if a loss of potency is observed the batch must be replaced.

The equipment used for storage of frozen venom (freezers) and for venom drying, should be cleaned using established procedures, and the cleaning documented, in order to minimize cross-contamination. Likewise, equipment requiring calibration, such as freezers, balances and freeze-driers, should be calibrated according to a defined schedule.

9.1.4.2 Venom collection from wild snakes

The practice of collecting venoms from wild-caught snakes that are subsequently released in either the same or a different location should be discontinued, and is not recommended due to the lack of traceability and difficulties in ensuring effective quality control of venoms. There is also evidence of high levels of mortality among relocated snake species particularly if they are released at a distance from the capture site (62–65). In jurisdictions where it is current practice for collectors to go to designated localities in the wild, catch snakes and collect venom before releasing them elsewhere, strong efforts must be made to replace this approach with regulated production using captive snakes maintained in well-designed serpentariums.
9.2 **Staff responsible for handling snakes**

9.2.1 **Safety and health considerations**

Handling and extracting venom from snakes is a dangerous operation. One envenoming occurred every two years in each of the 15 extraction facilities reviewed by Powell et al. (66). At a commercial venom production plant in Uberlândia, Brazil between 1981 and 1999, 25 technicians performed 370,768 venom extractions from *Bothrops moojeni*. Twelve bites were recorded, 10 with envenoming, and one case of venom squirted into the eye of a worker (67).

Venom extractions should be performed according to well-designed and documented SOPs by well-trained snake handlers. All personnel involved in snake handling and venom collection should be fully informed about the potential dangers of being bitten and envenomed. They should be thoroughly trained, and the training procedures must be documented and specific protocols practised as a team. A minimum of two people should be present during snake handling for venom collection. For safety reasons, it is recommended that venom extraction sessions should be interrupted at least every 2 hours for a rest period, before restarting the process.

Personnel involved in snake handling and venom extraction should observe established hygiene standards (see below) to minimize the impact on snakes and the potential transfer of pathogens between snakes.

9.2.2 **Personal protective equipment (PPE) for snake or venom handling**

Protective clothing should include appropriate eye protection (safety glasses or face shields), face masks, nitrile gloves and a laboratory coat or gown. Eye protection is especially important when handling spitting elapids capable of squirting their venom. The wearing of puncture-resistant gloves designed to prevent an effective bite is unpopular among many keepers who fear that it impairs manual dexterity and sense of touch, but the use of nitrile gloves is advisable to prevent cross-contamination. Puncture-resistant gloves should be mandatory as protective equipment for assistants helping to restrain or handle snakes during procedures such as venom extraction.

When lyophilized or desiccated venom is being handled, the safety of operators is paramount, since dried venom can easily be aerosolized and affect people through skin breaks, eyes or mucous membranes, or by sensitizing them to the venom (68). Appropriate gowning is necessary when handling dried or liquid venom, to prevent contact of the venom with skin or mucous membranes. It is highly recommended that a biological safety cabinet (for example, Class II, B2), be used while handling lyophilized or desiccated venom.
9.2.3  Procedures to be followed if a bite occurs

There are several important measures to be put in place for dealing with a bite (69), as described below.

9.2.3.1  Procedures and alarms

Clearly defined, prominently displayed, well-understood and regularly rehearsed procedures should be in place in case of a bite. An alarm should be sounded to summon help, the snake returned safely to its cage or box and the victim should withdraw to an area designated for first aid.

9.2.3.2  First aid protocols

Clearly understandable first aid protocols should be established for each species. These should be available in printed form adjacent to each cage. Immediate application of pressure-immobilization may be appropriate for treating the bites of rapidly neurotoxic elapids. However, the technique is not easy and, if they are to use the method properly, staff will need extensive training and regular practice, and must be provided with the necessary materials (a number of crepe bandages, 10 cm wide × 4.5 m long, and splints). Analgesia should only be provided for pain during the pre-hospital period upon the advice of an attending physician. Provision of appropriate analgesia for first aid should be considered. If venom enters the eyes, immediate irrigation with generous volumes of clean water is an urgent necessity.

9.2.3.3  Hospital admission

As a precaution, all victims of bites, scratches by snakes’ fangs or teeth, and those in whom venom has entered the eye, or anyone else suspected of a snake-bite or venom exposure injury (for example, through aerosolized dried venom), should be transferred as quickly as possible to the designated local hospital, by prearranged transport, for medical assessment. It may be helpful to remove from the cage, and take to the hospital with the victim, the label identifying the snake responsible for the bite, so that accurate identification of the snake species and of the antivenom to be administered is ensured. Staff members should wear, or carry a card detailing their personal medical information (including drug allergies) at all times and the card should be taken with them to hospital in the event of an injury. The contact details of a recognized clinical toxinology expert should be included on this card.

It is highly recommended that all serpentariums stock in-date supplies of antivenom appropriate to the species of snakes being held, so that an adequate
supply of the correct antivenom can accompany the victim to hospital. Hospital staff should be warned in advance by telephone of the arrival of the casualty and informed about the species responsible and any background medical problems and relevant medical history, such as past reactions to antivenom or other equine sera (for example, anti-tetanus serum), and known allergies.

9.2.3.4 Snake venom hypersensitivity

Snake venom hypersensitivity is an occupational hazard of snake handlers that occurs due to sensitization to venom proteins. Two out of 12 snake-bites in a commercial venom production plant in Brazil resulted in venom anaphylaxis (67). Hypersensitivity is usually acquired by mucosal contact with aerosolized dried venom. Important early evidence of evolving sensitization is sneezing, coughing, wheezing, itching of the eyes or weeping when working around snakes and snake enclosures, or even upon entering the snake room. No one with established venom allergy should be permitted to continue working with snakes. Venom-induced anaphylaxis should be treated with self-injectable adrenaline (epinephrine) 0.5 ml of 0.1% solution by intramuscular injection (adult dose), which should be stocked in adequate doses in each room holding snakes, or where snakes are used for procedures such as venom extraction.

9.2.3.5 Medico-legal and health insurance aspects

The occupational exposure to venomous snake-bites in commercial venom production units is the responsibility of the employers and requires their formal attention.

9.3 Main recommendations

- Well-managed serpentariums are a key element in the production of venom preparations meeting the quality requirements for the production of effective antivenoms.
- The quality of snake venoms used for animal immunization, as material for both preclinical and batch release assessment of neutralization efficacy, or for the development of national or regional reference preparations is of critical importance.
- The procedures used in snake maintenance, handling and venom extraction, as well as in all aspects of venom collection should be properly documented and scheduled.
- Venoms used for antivenom preparations should be representative of the entire snake population living in the area for which the polyspecific and/or monospecific antivenoms are intended to be used. Because of regional and individual variations in
venom composition within snake species, the venoms used for immunization should be collected from a large number of individuals (generally at least 20, including males and females of different ages) collected from various regions covering the entire geographical distribution of the particular venomous snake species.

Venom producers should adhere rigorously to the following recommendations and should be able to demonstrate their application:

- Taxonomic identity and geographical origin of each individual animal used for venom production should be known and recorded.
- Housing, feeding and handling of snakes should meet the highest veterinary and ethical standards, and follow documented protocols.
- Adequate training should be provided to personnel involved in venom production in all procedures, and implementation of health and safety measures.
- Formal guidelines and procedures for emergency response in the event of any suspected snake-bite or venom exposure should be established and well documented.
- Venom should not be collected from animals under quarantine, or which are gravid, injured, sick or in poor condition.
- Full traceability of each venom batch should be ensured.
- Venoms should be frozen as soon as possible after collection, and at least within 1 hour.
- Freeze-drying or desiccation of the venoms should be done under conditions that ensure stability for long-term storage.
- Batch-to-batch consistency of venoms of the same origin should be confirmed.

10. Quality control of venoms

10.1 Records and traceability

It is critical to accurately identify the species (and subspecies, if any) of each individual snake used for venom production and the taxonomic status should be validated by a competent herpetologist. Increasingly, DNA taxonomy is replacing conventional morphological methods, but this technique is impracticable in most venom production units which will continue to rely on well-established physical features such as colour pattern and scale count to distinguish the principal medically important species.
Internationally recognized scientific names should be used and the biogeographical origin of each snake should be specified, since differences in venom composition may occur between different populations of the same species or subspecies (43–48, 70). Venom producers can consult academic zoologists who have appropriate skill and experience.

Data pertaining to each numbered venom batch should include the information considered to be key for traceability, quality and specific characteristics of the venom (for example, identification of all the snakes used, the species, subspecies and biogeographical origin, feeding, health care, date of each extraction, number of specimens used to prepare the batch, and quantity of venom produced). This information should be made available upon request to any auditor or control authority.

For long-term storage, venom should be appropriately aliquoted to minimize wastage and must then be stored in sealed vials until use. Liquid venoms should be stored frozen at −80 °C, while lyophilized or dried venoms may be stored at −20 °C. After opening the vial, the venom required should be used and any surplus product discarded. Unused venom should not be re-lyophilized, re-dried or refrozen (in the case of liquid venom).

10.2 National reference materials

The quality of snake venoms used as a reference standard by quality control laboratories and NRAs is crucial. WHO recommends that national reference venom collections be established and that these cover each medically important snake species used in antivenom production. Such reference venoms should be prepared as described elsewhere in these Guidelines (see sections 9 and 21), and must be tested for potency at least annually to ensure they comply with the original specifications.

Owing to the large variations in venom composition even within a single species it is recommended that national reference venom collections should be established, which cover the entire interspecies variability. Regional reference materials could be used when countries within a region share a similar distribution of venomous snakes.

Establishing a collection of reference venoms ensures that the antivenoms produced will be tested against the same relevant venoms in the specific countries or regions. The characterization and maintenance of reference venom collections should be performed under oversight from NRAs and other competent agencies with technical expertise to ensure that reference venoms are produced to international reference material standards.

Venom batches may be prepared following the procedure outlined in section 9. Whatever their origin, the snakes used for these reference standards should be accurately authenticated (species, subspecies) by a qualified person and
the place of capture recorded. Genetic samples (for example, tissue and blood) should be routinely collected from all specimens for DNA analysis if questions arise regarding the validity of the identification of specimens. Photographs of individual specimens may also have value.

10.3 Characterization of venom batches

It is the responsibility of the venom producer to provide clear information pertaining to the species, the subspecies and the geographical origin of the snakes used for the production of the venoms supplied for antivenom production, quality control and preclinical studies. This information should be included in the technical dossier supporting the marketing authorization of any antivenom. In addition to the certificate which details the scientific name of the snake species (and subspecies if any), the geographical origin and the number of animals used for preparing the batch, and the date of collection of the venom as well as additional biochemical and biological information may be provided for each venom batch as evidence of consistency.

This information may include analysis of:

- biochemical characteristics of the venom;
  - protein concentration per gram,
  - scans or pictures of SDS-PAGE (in reducing and non-reducing conditions), and
  - size-exclusion or reverse-phase chromatographic profiles (for example, reverse-phase HPLC);
- enzymatic and toxicological activities of the venoms;
  - for example, LD$_{50}$ and, depending on the particular venom, in vitro procoagulant activity, proteinase activity or phospholipase A2 activity;
- for lyophilized, vacuum-dried or desiccated venoms, analysis of residual moisture.

If the venom producer is not able to perform these determinations they can be subcontracted. Alternatively (depending on the agreement), the antivenom manufacturer can perform relevant assays to confirm compliance of venoms with specifications as part of the quality control of the raw material.

10.4 Main recommendations

- Quality control of snake venoms is essential to provide assurance that the venoms are representative of venomous snakes inhabiting the region for which the antivenoms are manufactured.
National reference venom collections should be established covering each of the medically important snake species for which antivenoms are produced.

Traceability of each venom batch is important for rapid detection of any errors that might occur during the preparation process.

For each venom batch, a certificate stating the scientific names of the snake species (and subspecies if any), their geographical origin and the number of animals used in collecting the batch, the date of collection of the venom, and any other relevant information, must be provided by the venom supplier to the antivenom manufacturer and also to the regulatory authority if required.

Consistency (within established limits of composition and quality) of venom batches produced over time for the same venomous species of the same origin should be guaranteed. Specific tests should be performed on each venom sample, and data recorded for traceability, including: the protein concentration per g (or mg), an assessment of biochemical and biological activity, scans or pictures from SDS-PAGE (in reducing and non-reducing conditions), and/or size-exclusion or reverse-phase HPLC chromatographic profiles of the venom sample. This information has proved useful to confirm the origin and the integrity of the venom.

11. Overview of the production process of antivenoms

Antivenoms are obtained following a complex production process (Fig. A5.3), which involves several steps critical to their effectiveness, quality and safety (71). These steps are summarized below:

- Collection of representative venom pools from correctly identified individual venomous snakes which have been confirmed to be in good health. They should be representative of the snake populations (for example, males/females, adults/juveniles) and region(s) where the resulting antivenom immunoglobulins are intended to be used.
- Preparation of the venom(s) mixture(s) used for the programme of immunization of animals.
- Immunization regimens of animals (most often horses). Animals should be selected and controlled carefully, and subjected to continuous health surveillance.
- Collection of blood or plasma from the immunized animals, once the immune response to the immunizing venom mixture has yielded satisfactory antibody levels.
- Preparation of the pool of plasma for fractionation.
- Fractionation of the plasma to extract and to purify the antivenom immunoglobulins if applicable.
- Formulation of the bulk antivenom immunoglobulins and aseptic filling.
- Quality control tests, including potency assessment by in vivo assay.
- Labelling, packaging, boxing and release.
- Distribution within the region(s) where the snakes used to prepare the venoms to immunize the animals are prevalent.
Fig. A5.3
General manufacturing process of antivenoms

- Collection of venoms (venom extraction)
- Preparation of venom mixtures
- Quality control of venom mixtures
- Preparation of immunizing doses of venoms
- Selection of animals (horses, sheep)
- Quarantine, vaccination and veterinary
- Inclusion in the herd
- Immunization programme for each animal
- Control of animal immune responses
- Collection of blood or plasma
- Storage and pooling of plasma for fractionation to isolate immunoglobulins
- Fractionation of plasma
- Formulation and filling
- Labelling, packaging, boxing and release
- Quality control of plasma for fractionation
- Quality control of antivenom
12. Selection and veterinary health care of animals used for production of antivenoms

12.1 Selection and quarantine period

Animals selected for antivenom production should comply with specific selection criteria relating to breed, size, age, health status and history, and should preferably be purchased from known accredited suppliers. The use of animals in the production of hyperimmune plasma should follow strict ethical standards in accordance with national and international conventions on the use and welfare of animals. Animals must be transported according to local transport standards. Before an animal is introduced into the herd used for a production programme, it should be subjected to a period of quarantine (which, in most countries, lasts from 6 to 12 weeks), depending upon the source of the animal. During the quarantine period an appropriate veterinary assessment should be performed to ensure the animal’s suitability for the programme. The quarantine facility should be separate from the main animal housing facility or farm and a biosecurity plan for all animal premises is recommended. Each animal should have an individual monitoring record system created on its entry into the quarantine facility, which will remain with the animal throughout its life at the facility or farm. All activities and information on all aspects of its life, including husbandry, health, antivenom immunization, bleeding and emergency care must be recorded in this file, which should be accessible for external review.

When an animal is imported from a country or region with different ecological characteristics, a period of acclimatization to the local environment of about 3 months is needed. Each individual animal should be unambiguously identified using, for example, a microchip, branding or ear clipping.

In the case of horses and other equines, animals aged between 3 and 10 years are usually included in an immunization programme, but in some cases older animals may also be suitable as long as they exhibit a satisfactory immune response to the immunization programme. In the case of sheep, animals retired from wool production have proved capable of useful antibody production for a number of years (beyond the age of 10 years). No particular breed is preferred, but in general large horses or sheep are chosen because they yield larger individual volumes of blood.

12.2 Veterinary care, monitoring and vaccinations

The veterinary examination will include a complete physical examination and blood tests including serological testing for the most prevalent infectious diseases for that type of animal in that particular geographical location (for example, equine infectious anaemia).
Depending upon the local epidemiological situation, animals should be vaccinated against tetanus and, possibly other endemic diseases, such as rabies, equine influenza, anthrax, brucellosis, glanders, African horse sickness and equine encephalitides. Animals should go through a treatment programme to eliminate gut helminths and other locally prevalent parasites. All vaccinations and health information should be recorded on the animal’s individual record.

Staff in contact with the animals should be vaccinated against tetanus and rabies.

### 12.3 Animal health and welfare after inclusion in the herd

After the quarantine period, if the animal is in good health according to a veterinary examination and blood parameters and body condition score, and the results of relevant serological tests are negative, the animal may be incorporated into the herd of animals used for immunization.

An individual record should be kept for each animal being used in an immunization programme for antivenom production. In addition to surveillance by a veterinary professional, the staff in charge of the animals should be well trained, and the operations related to animal care, emergency care and use should be clearly specified in the standard operating procedure (SOP).

Throughout the time an animal is used for immunization aimed at antivenom production, careful veterinary monitoring should be maintained, including continued vaccination regimes, and the performance of regular clinical examinations, together with clinical laboratory tests such as packed cell volume, haemogram, clotting tests and other tests associated with the possible clinical effects of venoms (72) and of successive large-volume blood collection (73). Possible anaemia, resulting from excessive volume or frequency of bleeding (when red blood cells are not re-infused into the animals after the whole blood bleeding session) or from the deleterious action of venoms should also be tested for.

The immune response against venom components should, when feasible, be followed throughout the immunization schedule, in order to detect when animals reach an acceptable antivenom titre. However, the monitoring of the immune response can be done on a pool of sera from various animals. This response may be followed by in vivo potency assays of neutralization of lethality or by in vitro tests, such as enzyme immunoassays (EIAs) (provided that a correlation has been demonstrated between these tests and the in vivo potency tests).

Whenever an animal develops any manifestation of sickness, it must be temporarily withdrawn from the immunization programme to allow it to receive appropriate veterinary examination and treatment. If the disease is controlled, the animal may return to the immunization programme after a suitable length
of time, usually 4 weeks. If an animal is receiving any type of antibiotic or drug, it should be withdrawn from the immunization programme for a period that would depend on the elimination kinetics of the particular drug(s) concerned. In the case of vaccination, this withdrawal period should not be shorter than 1 month. Any blood, plasma or serum obtained from the animal during the incubation period of any contracted disease should be excluded from use for the production of antivenoms. Animals should have appropriate physical exercise and routine husbandry (hoof care, teeth rasping etc.). Their feed should originate from a controlled source and should be free of ruminant-derived material. Ideally, the diet should include both hay and grass, or alternative plant material, and concentrated food preparations containing vitamins including folic acid, iron and other mineral supplements. The routine quality control of the food and water is recommended, in order to assure a consistent composition and an adequate level of nutrients.

As a consequence of immunization with venoms (see section 13) a common problem in antivenom-producing animals is the development of local ulcers or abscesses (sterile and infected) at sites of venom injection. This is a particular problem when necrotic venoms and FCA are used. All injections should be given under aseptic conditions and administered subcutaneously. There should be a limit to the total volume and dose of venom injected at a single site. Infected or ulcerated areas should be treated appropriately. Abscesses should be lanced and drained and the affected skin site should not be used again. In the event of the death of an animal being used for antivenom production, a careful analysis of the causes of death should be performed, including, when necessary, the performance of a necropsy and histopathology. All deaths should be recorded together with the necropsy report and made available for external review.

Some animals show declining titres of specific venom antibodies over time, despite rest or increasing doses of immunizing venoms. Such animals should be retired from the immunization programme. In agreement with the principles of GMP and to avoid impact on the composition and consistency of the antivenom produced, it is, in principle, not considered good practice to move animals from a given venom immunization programme to another one. If, however, the animal has been used in the preparation of a monospecific antivenom that is included in a polyclonal preparation, or if it was used for the production of other animal-derived antisera (for example, anti-rabies, anti-tetanus or anti-botulism), moving it to another programme may be acceptable.

When an animal is withdrawn from the herd, it may be kept on the horse farm. If it is sold, continued good care should be ensured.

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6 In some areas, legislation stipulates that animals used for production of plasma cannot be treated with penicillin or streptomycin.
12.4 Main recommendations

- A thorough biosecurity plan should be developed and implemented for each farm and facility.
- All staff working with the animals should be trained and qualified to care for them. Staff training records and history should be available for review.
- An emergency care protocol is essential especially during procedures – for example, during sensitization to venoms, blood collection and post-plasmapheresis. Adverse events must be reported and tracked appropriately.
- Animals intended for antivenom production programmes should be identified to ensure full traceability and health monitoring.
- Animals should go through a quarantine period of 6–12 weeks during which they are submitted to veterinary scrutiny and are vaccinated against specific diseases and treated for internal and external parasites.
- Following the quarantine period, animals may be introduced into the immunization programme. Animals should be appropriately housed, fed and managed according to best practice in veterinary, animal welfare and ethical standards.
- During immunization, the clinical status of each animal must be followed by a veterinarian through clinical and laboratory assessments which are recorded on the animals’ records. If an animal develops clinical signs of disease, it should be temporarily separated from the immunization programme to receive appropriate care and treatment. Particular care must be paid to local lesions that develop at the site of venom injections and to development of anaemia.
- The immune response of each animal to venoms should, when possible, be monitored during the immunization schedule (alternatively, the antivenom titres can be monitored indirectly by testing the plasma pool).
- An animal receiving an antibiotic or drug should be withdrawn from the immunization programme for a period depending on the elimination kinetics of the drug concerned. In the case of vaccination, this withdrawal period should not be shorter than 1 month.
13. Immunization regimens and use of adjuvant

One of the most crucial steps in antivenom production involves the immunization of animals with venom(s) to produce a long-lasting and high-titre antibody response against the lethal and other deleterious effects of the immunogenic toxins. To achieve this goal, the following considerations are important:

- Venom(s) used should be prepared as described in section 9, and should be in an optimal condition for inducing specific and neutralizing antibodies.
- Immunogen and the immunization regimens used should not seriously affect the health of the animal.
- Preparation of immunogens and the immunization protocol should be technically simple and economical and use a minimal amount of venom. The procedures followed must be included in a protocol and their performance must be documented.

The antivenom manufacturer is responsible for defining the appropriate immunization programme (choice of doses, selection of adjuvants, sites of immunization, and bleeding schedule) able to generate the best immune response and plasma production, while also ensuring optimal animal care. GMP principles should be applied in the preparation of the immunizing doses as well as in the immunization process.

13.1 Animals used in antivenom production

Numerous animal species have been used on various scales in antivenom production (horse, sheep, donkey, goat and rabbit) or for experimental purposes (camel, llama, dog and hen) (74, 75). However, the production of large volumes of antivenom from large animals such as equines is an advantage compared to the smaller species. The selection of the animal species should be based on several considerations, such as locally prevalent diseases, availability in the region, adaptation to the local environment, and cost of maintenance. The information in these Guidelines refers mostly to horse-derived immunoglobulins.

The horse is the animal of choice for commercial antivenom production. Horses are docile, thrive in most climates and yield a large volume of plasma. Antivenoms made from horse plasma have proven over time to have a satisfactory safety and efficacy profile (3). Sheep have also been used as an alternative source for antivenom production because they are cheaper, easier to raise, can better tolerate oil-based adjuvant than horses, and their antibodies may be useful in patients who are hypersensitive to equine proteins (75, 76). However, increasing concern about prion diseases may limit the use of sheep...
for commercial antivenom production. Larger animals are preferable to smaller ones because of their greater blood volume, but breed and age are less important. Any animals used should be under veterinary supervision (see section 12). When sheep or goats are to be used, manufacturers should comply with regulations to minimize the risk of transmissible spongiform encephalopathies (TSEs) to humans, such as the WHO Guidelines on tissue infectivity distribution in transmissible spongiform encephalopathies (77).

13.2 Venoms used for immunization
Venoms used as immunogens in antivenom production are chosen based on criteria discussed in section 9. Priority should be given to venoms from snakes responsible for frequent and severe envenomings. The quality, quantity and biological variation of venoms are important considerations (see sections 9 and 10).

13.3 Preparation of venom doses
Venom doses used for the immunization of animals should be prepared carefully in a clean environment, maintained according to an established, scheduled and documented cleaning regime. All venom manipulations should be performed using aseptic techniques under a hood; for highly toxic venoms, a cytotoxic cabinet may be used. Batch process records should be completed for each dose preparation session. The venom batches used and the animals to be immunized should be recorded and the containers in which the venom is dissolved should be appropriately identified. Ideally, the calculations and operations related to the dose of venom to be used, as well as dilutions, require verification by a second person to ensure accuracy and to prevent errors that may lead to animals receiving overdoses.

Venoms, when freeze-dried, are highly hygroscopic and allergenic, thus care should be taken when manipulating them. When taken out of the refrigerator or freezer, the venom should be allowed to warm up to room temperature before the bottle is opened. Otherwise, condensation may occur causing inaccuracy in weighing and, more seriously, proteolytic degradation of the venom proteins by venom enzymes. Venom should be dissolved in distilled water or buffer, but care should be taken not to shake the solution too vigorously since excessive foaming may cause protein denaturation.

The solvents used to dissolve venoms should be sterile and should be used before the established expiry dates. A stock solution of each venom should be prepared separately, rather than being mixed with other venoms. This is to allow flexibility of dosage and to avoid proteolytic degradation by one venom component of other venom proteins. Venom solutions can be sterile filtered (where this is known not to affect the potency of the preparation), aliquoted
and labelled. It is recommended that venoms used for immunization be freshly prepared at the time of use. The storage conditions of venom solutions before immunization must be validated by properly conducted stability tests.

All the equipment used for venom storage (freezers and refrigerators) and preparation (for example, balances) should be calibrated and validated for their intended purpose. Balances should be calibrated at least annually and calibration should be checked daily. Where possible, laboratory items used in venom preparation, that is, pipettes, syringes and other such items should be pre-sterilized, single-use, disposable items. The siliconization of venom solution containers may be considered to avoid the adherence of venom components to the surfaces of containers. Venom solutions/suspensions must be safely transported to the facilities where animals are going to be injected and the venom solutions/suspensions should be kept cold at about 2–8 °C.

Care should be taken to avoid accidents that may result in envenoming of the personnel preparing the venom solutions. Protective equipment (for example, eyewear, gloves and gowns) should be worn. Procedures for cleaning up broken glass or plastic containers that have held venom should be established and the personnel should be trained to follow them.

13.4 Detoxification of venom

Some snake venoms can cause local and/or systemic toxicity when injected into naive horses at the beginning of an immunization course. Various physical or chemical means have been adopted to decrease venom toxicity, for example, treatment with aldehydes (formaldehyde or glutaraldehyde), hypochlorite, ultraviolet or gamma radiation, and heat, among others. However, in most cases, not only the toxic sites, but also the antigenic sites of the toxins are destroyed after these treatments (78). For example, when glutaraldehyde is used, the protein polymerization is often extensive and is difficult to control and reproduce. Thus, although the detoxified toxin (toxoid or venoid) induces vigorous antibody response, the antibodies usually fail to neutralize the native toxin. In fact, no detoxification is usually necessary if inoculation is made with a small dose of venom that is well emulsified in an adjuvant such as FCA or FIA. Furthermore, traces of chemicals, especially aldehyde, can have deleterious effects on animals’ vital organs, for example, the liver.

13.5 Immunological adjuvants

Various types of immunological adjuvants have been tested, for example, FCA and FIA, aluminium salts (hydroxide and phosphate), bentonite and liposomes (79). The choice of adjuvant is determined by its effectiveness, side-effects, ease of preparation (especially on a large scale) and cost. It may vary depending upon the type of venoms and following manufacturers’ experience. FIA contains
mineral oil and an emulsifier. FCA, which contains mineral oil, an emulsifier and inactivated *Mycobacterium tuberculosis*, has been shown in experimental animals to be one of the most potent adjuvants known. However, horses are quite sensitive to FCA which tends to cause granuloma formation. For this reason, some producers prefer to use other adjuvants. It is recommended that when using FCA and FIA, they be utilized only at the beginning of the immunization schedule, and not during the rest of the immunization, nor during booster injections of venom; this significantly reduces the formation of granulomas in horses.

It has been noted that the granuloma caused by FCA is caused by the injection of a large volume (5–10 mL) of the emulsified immunogen at one or two sites. The large granuloma formed usually ruptures, resulting in a large infected wound. If the emulsified immunogen is injected subcutaneously in small volumes (50–200 µL/site) at multiple sites, granuloma formation may be avoided. Manufacturers are also encouraged to adopt an innovative approach with regard to adjuvants used for antivenom production, and should strive to replace FCA and FIA with new compounds of low toxicity and high adjuvant effect. The advances in the vaccine field concerning new adjuvants should be transferred to the antivenom field; for example, the use of microbial-derived products of low toxicity or of Toll-like receptor 4 (TLR4) ligand-based adjuvants (80).

**13.6 Preparation of immunogen in adjuvants**

To minimize infection at the immunization sites, all procedures should be carried out under aseptic conditions. Venom solutions are prepared in distilled water or phosphate-buffered saline solution and filtered through a 0.22 µm membrane. The venom solution is then mixed and/or emulsified with adjuvant, according to the instructions of the supplier. An example for the preparation of venom immunogen in FCA, FIA and aluminium salts is described in Box A5.1. To facilitate the injections, the immunogen suspension is filled in tuberculin syringes (Fig. A5.4).

**13.7 Immunization of animals**

The areas to be immunized should be thoroughly scrubbed with a disinfectant, shaved and rubbed with 70% ethanol before venom immunogen injection.

In general, the sites of immunization (Fig. A5.5) should be in areas close to major lymph nodes, preferably on the animal’s neck and back. The route of injection should be subcutaneous so as to recruit a large number of antigen-presenting cells resulting in a high antibody response. Some procedures call for a small volume of injection at each site (50–200 µL) so that the total surface area of the immunogen droplets is maximized, enhancing the interaction with the antigen-presenting cells and the immune response (81, 82). An example
of immunization of a horse using venom emulsified in FCA is described in Box A5.2.

Other immunization protocols, using larger amounts of venoms devoid of local-tissue-damaging activity (such as those of some elapids) and/or adjuvants other than FCA may be used with satisfactory results, as long as the schedule does not compromise the health of the animals. In situations where the main toxins of a given venom have a low molecular mass and would not induce a sufficient immune response if injected together with the other venom components, isolating such toxins using mild chromatographic procedures or ultrafiltration can be useful. Such isolated fractions can then be used for immunization.

Fig. A5.4
Tuberculin syringes are filled with immunogen suspension and used for the subcutaneous injection of the horse

Box A5.1
Example of preparation of venom immunogen in FCA, FIA and aluminium salts

Since FCA can cause severe irritation, precautions should be taken to avoid contact with the eyes, and protective eyewear and gloves are recommended. The vial containing FCA is shaken to disperse the insoluble *Mycobacterium tuberculosis*. The venom solution is mixed in a stainless steel container with an equal volume of FCA at 4 °C.
Box A5.1 continued

The emulsification is achieved by vigorous blending in a high-speed blender at a speed of approximately 3000 rpm for 15 minutes. The container is put in ice-water to dissipate the heat generated. The resultant emulsion should be quite thick and remain stable when dropped on the surface of cold water. The highly viscous emulsion is then transferred into a sterile 50 ml syringe with the plunger removed. The plunger is then put into the syringe to expel any air pocket inside. By means of a three-way stopcock, the emulsion from the 50 ml syringe is then transferred into tuberculin syringes to give a volume of 0.1–0.2 ml/syringe. After the tuberculin syringe is fitted with a 38 mm no. 21 gauge disposable needle, the needle cover with its end cut off is attached so that only 2–3 mm of the needle tip is exposed and penetrates the horse’s skin (Fig. A5.5). With each filled tuberculin syringe, immunization at a particular site can be performed by injection and expulsion of the immunogen almost simultaneously in one single step. This immunization procedure makes multiple subcutaneous injections with small immunogen volume easier, faster and requires minimal restraint of the horse.

Immunogen in FIA is prepared by a process similar to that described above except that FIA is used in place of FCA. Both the FCA and FIA emulsified immunogens may, if necessary, be stored at 4 °C, preferably for a maximum of 2 weeks, but re-emulsification is necessary before their injection. When the immunogen is prepared in aluminium hydroxide (Al(OH)₃) or aluminium phosphate (Al(PO)₄), a sterile venom solution and a suspension of aluminium salts are mixed in a ratio of 1:3 (v/v) and homogenized. When using other adjuvants, the preparation of the solution or emulsion should follow the manufacturer’s instructions for that type of adjuvant.

Fig. A5.5
Recommended areas of immunization in horses
Box A5.2
Example of immunization of horses using FCA, FIA and aluminium salts

The primary immunization could be done with venom(s) mixed with FCA as described in Box A5.1. The initial dose of each venom could be as low as 1–4 mg/horse with a total combined volume of injection of about 2 ml. The immunogen is filled in several 1 ml tuberculin syringes with 21G needles as described in Box A5.1 and Fig. A4.5. Subcutaneous injections of 100–200 µL of immunogen are made at each site, up to as many as 8–12 sites, although some producers may use only 3–4 injection sites. The neck of the horse, supplied with extensive lymphatic vessels and large lymph nodes, is a preferred area for immunization. If inoculation is made on the lateral sides of the neck, the animal tends to rub itself causing skin blisters. Thus, injections should be made to the upper (dorsal) part of the neck, close to the mane. About 4–6 injections can be made at each side of the neck. If injection in the rump is possible, 1–2 injections can be made in the area between the outer hip bone and the top of the thigh bone. The scratching of injected sites by animals can be partially alleviated by massaging the injection site after venom injection to disperse the dose material.

Immunization using FCA is usually done only once; in most cases, repeated use of this adjuvant can cause serious reactions which affect the horse’s health. After 2 weeks, the horses should receive a booster injection with the same venom(s) well emulsified in FIA. Similar volumes and areas of injection to those described above can be used. Subsequent booster immunizations at 2-week intervals can be administered, with higher doses (5–10 mg) of venom(s) in saline or mixed with aluminium salts or any other suitable adjuvant. In this case, subcutaneous injections of 1 ml of immunogen at each site in a total of 4 sites are recommended. Blood (10–20 ml) should be drawn before each immunization. Serum or plasma is prepared and EIA titres and/or lethality potency are determined. When the EIA titres reach a plateau, usually about 8–10 weeks after the primary immunization, an in vivo potency assay may be performed to confirm that the horse could be bled. After bleeding for antivenom production, the horses are allowed 4–8 weeks rest, depending on their physical condition. After the rest period, a new round of immunization can be performed as described above, but without the use of FCA.

13.8 Traceability of the immunization process

The traceability of the immunization process is critical for the quality control of the antivenoms produced and the steps to ensure traceability should be performed very accurately. Each immunized animal should be identified by its code number (see section 12) and the details of each immunization step should be recorded precisely. The details to be recorded include:

- date of immunization;
- batch(es) of venom(s) used with its (their) reference number(s) (see section 10);
- venom dose(s);
- adjuvant and/or salt used;
- names of the veterinary and supporting staff in charge of the immunization;
- occurrence of reaction and/or sickness.

The antivenom titre of the immunized animals should be monitored throughout the immunization procedure either in vitro, using EIA, during the immunization phase, or in vivo, by neutralization potency assays of lethality when the immunization plateau is reached or before each blood collection.

Each plasma batch should be assigned a unique reference number (for example, a barcode), which should allow complete traceability to the donor animal. Information (such as the date of collection, the unique identification number of the immunized donor animal, and the reference number of the venom(s) used for immunization) should be recorded to allow traceability to all venoms. Computer-based databases are very useful for properly recording these data, which are crucial for the traceability of the antivenoms produced. Standard procedures should be used to protect the integrity of data stored on a computer, including regular, frequent backup, protection against unauthorized access and storage of backup copies securely off-site.

13.9 Main recommendations

- Venom solutions should be prepared in such a way as to minimize proteolytic digestion and denaturation of the venom proteins.
Venom solution should be prepared under aseptic conditions to avoid infection at the injection sites.
- The type of adjuvant used is selected on the basis of its effectiveness, side-effects, ease of preparation and cost.
- Primary immunization should be done by subcutaneous injections of small volumes at multiple sites close to the animal’s lymphatic system to favour the recruitment of antigen-presenting cells and involvement of anatomically different groups of lymph nodes for antibody production.
- Subsequent booster injections can be made using venom immunogen doses, at volumes and intervals depending on the type of adjuvant used, until the antivenom titre reaches a plateau or a pre-established minimum accepted titre.
- After collection of blood for antivenom production, animals should have a resting period of 4–8 weeks. After this, a new round of immunization can be performed as above without the use of FCA.
All steps in the immunization of the donor animal, as well as the collection of blood or plasma, should be traceable.

14. Collection and control of animal plasma for fractionation

Historically, serum separated from the blood of hyperimmunized horses was the basis of “antivenin serum therapy”, but today plasma is used, almost exclusively, as the starting material and undergoes a fractionation process for the separation of purified antivenoms. Thus “antivenom immunoglobulins” is the preferred term, rather than “anti-snake-bite serum” or “antiserum”, which are imprecise and confusing terms that refer to a crude therapeutic preparation.

Plasma as a starting material is preferred to serum largely because red blood cells can be returned to the animal, thus preventing anaemia and hypovolaemia in the donor animal and allowing more frequent bleeding. Some laboratories have found that using plasma enables higher recovery of antibodies per donation and it is less contaminated with haemoglobin (Hb) than serum. Separation of plasma from anticoagulated blood is much faster than separation of serum from clotted blood. Plasma for fractionation can be obtained either from the collection of whole blood or by the apheresis procedure.

14.1 Health control of the animal prior to and during bleeding sessions

When an immunized animal has developed an antivenom antibody titre that meets the necessary specifications it can be bled. The animal should be in a satisfactory clinical condition and blood parameters and biochemistry need to be within the normal range for the animal type and breed. Before bleeding is performed, the animals should be evaluated by a veterinarian or other qualified person and declared healthy. Individual blood chemistry parameters – packed cell volume (PCV); Hb and total plasma protein (TPP) – must be within specified parameters. Animals showing evidence of clinical deterioration, such as weight loss, altered horse body condition score, a drop in Hb or serum protein concentration below a critical predefined value for the animal type and breed, or evidence of infection, should not be bled. It is recommended that animals to be bled have no contact with potentially infectious animals. Human beings can be a potential source of fomite infection in horses and therefore a biosecurity plan is essential.
14.2 Premises for blood or plasma collection

The bleeding of animals should be performed in designated rooms or areas dedicated to this activity and equipped with appropriate restraining devices. Some producers may design the bleeding rooms so that they can be closed, if needed, during the bleeding sessions, but this is not general practice. The rooms or areas should be thoroughly washed and cleaned before and after each bleeding session and their design should facilitate such cleaning procedures, which should be clearly established. The room or area should be inspected before the confinement of the animal. Animals need to be made as safe and comfortable as possible, in a quiet environment, during bleeding to minimize the chance of injury to the animal or its handlers. Individual animals should be confined in circumstances that reduce the potential for stress as much as possible. It is recommended that these rooms allow the simultaneous bleeding of several animals to reduce the time required for this operation as well as the stress.

To avoid bacterial and fungal contamination, animals should be cleaned and injection sites and jugular catheter sites clipped in a separate room before bleeding. Humidity control of the surrounding bleeding area should be ensured.

14.3 Blood or plasma collection session

Animals are bled by venepuncture from the external jugular vein. The area surrounding the venepuncture site should be clipped before bleeding and thoroughly cleaned and disinfected, using a disinfectant that has not reached the end of its recommended shelf-life, and, depending on the type of disinfectant, it should be allowed to dry. The disinfected area should not be touched or palpated before the needle has been inserted.

Before venepuncture, all containers and tubing should be inspected for defects (for example, abnormal moisture or discoloration as these may suggest a defect). There should be means to determine the volume of blood or plasma collected (such as a weighing machine).

When using disposable plastic bags to collect blood (which may take about one hour) it is recommended that the blood should be gently and continuously mixed to ensure a homogeneous distribution of the anticoagulant and avoid formation of clots.

Horses should be weighed before bleeding, if possible, and their weight recorded on their record. The clinical condition of the animals being bled should be closely monitored at the time of bleeding and during the days that follow, and bleeding should be suspended in the event of any adverse effect on the animal. If an animal shows signs of distress during the operation, the collection procedure should be terminated. In addition, animals should be kept under observation for at least 1 hour after the bleeding to allow any evidence of
physical alterations to be detected. Horses can be fed during blood collection depending on the horse crush set-up.

After bleeding for antivenom production and depending on whether red blood cells are re-transfused, the horses should not be re-bled for 4–8 weeks. Horses should have moderate (5 out of 9) body condition score and normal range haemogram (PCV, TPP and Hb) and be normal on clinical examination by the antivenom production veterinarian.

14.4 Labelling and identification

The identity of the animal should be recorded immediately before venepuncture. Labels on all bottles or bags of blood or plasma should be marked with the animal’s unique identification number. The label should be waterproof and heat resistant, and contain the following information: specificity of antivenom, plasma unit number and date of collection.

A document to register all steps of the production of the plasma lot should be maintained to guarantee traceability of the process.

14.4.1 Collection and storage of whole blood

14.4.1.1 Collection

The volume of blood to be obtained depends on the species and size of the immunized animal. It is recommended that approximately 13–15 mL of blood per kilogram body weight are collected in one bleeding session, or 1.5 to 2% of the weight of the animal. For sheep, 0.5 L is a typical yield, whereas for horses, the volume of blood may range between 3 and 6 L, depending on the size of the animal. The use of automated plasmapheresis may enable larger volumes of plasma to be collected, and has benefits for animal health and plasma quality. Manufacturers are encouraged to evaluate and implement automated plasmapheresis subject to approval from local regulators and in accordance with local regulations and standards.

Blood is collected, ideally, in disposable plastic bags containing sterile citrate anticoagulant or other preparations containing citrate phosphate dextrose solution (CPD), to prolong the durability of red blood cells. Usually, the volume ratio of anticoagulant to blood is 1:9 to 1:15, depending on the anticoagulant. Use of double plastic bags containing anticoagulant is recommended to avoid bacterial contamination and for ease of use. When plastic bags are not available, disposable polypropylene plastic bottles, or sterilized glass bottles containing anticoagulant may be considered.

While the bleeding is taking place, a constant flow of blood should be ensured. Blood should be gently and continuously mixed with the anticoagulant solution to ensure a homogeneous distribution of the anticoagulant, to avoid the risks of activation of the coagulation cascade and, therefore, avoid the formation
of clots. The duration of a bleeding session per animal is usually between 30 and 45 minutes depending upon the weight of the animal and the total volume collected. Care should be taken to avoid contamination of the blood by exposing the needle to contaminated surfaces. It is recommended to seal or occlude the device before removing the needle from the animal.

14.4.1.2 Storage
The bags or bottles in which the whole blood has been collected should be appropriately cleaned and sanitized on their external surfaces. They should be put into a refrigerated room (2–8 °C) for the plasma and blood cell separation procedure. They should be stored for no more than 24 hours before the reinfusion of the red cells, unless CPD is used. In this case, blood cells may be stored for up to 72 hours. Alternatively, aseptically collected blood can be stored for a maximum of 7 hours at 20–25 °C to allow for sedimentation. Under such circumstances, great care should be taken to avoid bacterial contamination.

14.4.1.3 Separation of plasma from whole blood
Hyperimmune plasma should be separated from blood cells under aseptic conditions and should be transferred into sterile containers (plastic bags, bottles or stainless steel containers). A designated room, designed to allow proper cleaning and sanitization, should be used for separation. When bottles are used, separation of plasma from blood cells should be performed in a laminar flow cabinet located in a room separated from the plasma fractionation area.

14.4.1.4 Reinfusion of the red blood cells
Reinfusion of the red blood cells after whole blood collection is recommended. Blood cells, most specifically red blood cells, should be separated from plasma by validated centrifugation or sedimentation procedures. Red blood cell reinfusion should take place within 24 hours after blood collection (or 72 hours if CPD anticoagulant is used), and after being suspended in sterile saline solution at room temperature for 1 hour (or 32–37 °C for less than 1 hour) prior to infusion. This procedure in which whole blood is collected and red blood cells are re-infused into the animal is commonly referred to as “manual apheresis”.

14.4.2 Plasma collection by automatic apheresis and storage
14.4.2.1 Plasma collection
In some laboratories, plasmapheresis machines are used to perform automatic plasma collection. This has proved a useful investment in some facilities; it ensures that the donor animal does not become hypovolaemic, increases plasma yield and purity, and reduces the risks of handling errors, in particular
during reinfusion of the red blood cells to the donor. Plasma from automatic apheresis tends to be less contaminated by blood cells (red blood cells, leukocytes and platelets). In the experience of some laboratories the plasma is easier to fractionate, as the filtration steps, in particular, are more readily performed, resulting in higher yields.

In such procedures, whole blood is collected from the animal, mixed with anticoagulant, and passed through an automated cell separator. The plasma is separated from the cellular components of the blood, which are returned to the animal in a series of collection/separation and return cycles. The plasma is separated from the red blood cells by centrifugation or filtration, or a combination of the two. The operational parameters of the plasmapheresis equipment are provided by the manufacturers of the equipment. In general, the anticoagulant is delivered at a rate yielding a specified ratio of anticoagulant to blood. The anticoagulant solutions used include AB16 (35.6 g sodium citrate, 12.6 g citric acid monohydrate, 51.0 g glucose monohydrate per litre using water for injection) and anticoagulant citrate dextrose formula A (ACDA) (22.0 g sodium citrate, 8.0 g citric acid, 24.5 g dextrose monohydrate, per litre using water for injection). The number of collection/separation and return cycles for each donor animal depends on the total volume of plasma that is to be harvested. For horses, the average volume of plasma collected may be about 6 litres per session. The number of cycles ranges from 10 to 20 depending upon the haematocrit of the horses. The collection process lasts for 1–4 hours. The apheresis equipment and apheresis procedures should be validated, maintained and serviced. Machine plasmapheresis can take several hours and animals can be fed during the operation.

14.4.2.2 Plasma storage

Bags or bottles containing apheresis plasma should be stored in a refrigerated room (2–8 °C) in the dark until the fractionation process starts. Individual or pooled plasma should be stored at 2–8 °C in a cold room dedicated for this purpose. This refrigerated storage room should be designed to allow proper cleaning and sanitization. To prevent microbial contamination of plasma, preservatives (phenol or cresols) can be added at a dose of less than 3 g/L at this stage and kept during storage of plasma. Care should be taken to dilute the phenol or cresols with water or saline solution before they are added to plasma with gentle stirring, to avoid denaturation of plasma proteins. The transportation of containers or bottles containing pooled plasma within the production facility or between facilities should be performed in such a way that contamination is

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7 In these Guidelines cresol isomers are referred to as “cresols”.
avoided and the cold chain is maintained. To avoid the risk of contamination, it is recommended that individual or pooled plasma is not stored for too long before fractionation, that is, the plasma should be fractionated as soon as possible after pooling.

If plasma is stored for prolonged periods, the storage time and conditions should be validated to ensure that there is no detrimental impact on the quality of the plasma material, on the fractionation process, or on the quality, efficacy and stability of the antivenoms.

Manufacturers of human plasma have found that plasma can be stored frozen at −20 °C or colder for 2 years without addition of a preservative, and with no observed detrimental effects on the fractionated plasma products.

### 14.5 Pooling

Plasma from individual animals should be pooled into sterile and sanitized containers before fractionation. For traceability purposes each plasma pool should be identified with a unique number. The number of plasma units collected from individual animals and used in the pool should be recorded. Before the large pool of plasma is prepared, it is recommended to prepare a small-volume pool and to test it for microbial contamination. If there is no contamination, the large pool can be prepared. If microbial contamination is detected, the plasma from the individual animals should be checked, and the contaminated plasma should be discarded to ensure that the pool is prepared with plasma free of microbial contamination. It may also be advisable to test small pools using a cytotoxicity assay, which can reveal the presence of unanticipated viruses or toxins (for example, following the US Code of Federal Regulations, 9 CFR 113.53 “Requirements for ingredients of animal origin used for production of biologics”).

Such pooling should be performed in an environment suitable to prevent microbial contamination, for example, classified areas (class D (83)) and pools should be adequately identified. The room should be designed to allow for appropriate cleaning and sanitization of all surfaces. Individual or pooled plasma should be stored at 2–8 °C in a room dedicated for this purpose. To ensure the prevention of microbial contamination of plasma, follow the recommendation given in section 14.4.2.2.

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14.6 Control of plasma prior to fractionation

Before fractionation, pools of plasma should be checked for macroscopically evident precipitates, gross haemolysis and bacterial contamination (bioburden assay). The neutralizing potency of the starting plasma should be ensured so that the resulting antivenoms will be within potency specifications. Additional checks may include, when relevant, a test for pyrogenic substances and total protein content.

Plasma pools should be discarded if the bioburden exceeds a defined limit stipulated in the marketing dossier or if the neutralizing potency is below a minimum limit established by the producer. Cloudy plasma, below this defined bioburden limit, may still be used for fractionation provided the fractionation process and product quality has been proven not to be impaired. Grossly haemolysed plasma should not be used for fractionation. Cloudy plasma may also reflect the increased level of chylomicrons in the animal plasma; therefore it is recommended to fast the animal before bleeding for a few hours (for example, 8 hours).

14.7 Main recommendations

- When animals have developed an adequate immune response against venoms, and if they are in good health, they can be bled for antivenom production. Bleeding should be performed in enclosed rooms which should be kept scrupulously clean. Traceability of the donations should be ensured.
- Plasma is preferred to serum as a source material. Animals should be bled from the external jugular vein. Plasma can be obtained either from whole blood or by automated plasmapheresis and using approved anticoagulants. Blood or plasma should ideally be collected into closed plastic bags. When this is not possible, glass or plastic bottles can be used, if they can be readily cleaned and sterilized.
- Plasmapheresis is recommended using either automatic or manual procedures. When manual apheresis is used, blood cells should be sedimented, separated from the plasma, re-suspended in saline solution and returned to the animals within 24 to 72 hours. Plasma separation should be performed in a designated room with a controlled environment.
- Plasma containers should be thoroughly cleaned on their external surfaces, adequately identified and stored in refrigerated rooms until further fractionation.
Plasma should be checked prior to fractionation to establish compliance with relevant acceptance criteria for fractionation, in particular the neutralizing potency and lack of bacterial contamination.

Special attention should be paid to ensuring traceability between individual animal donors and the plasma pool.

A certificate from a veterinarian or other qualified person should be issued stating that the donor animals were checked periodically to ensure that they were in good health at the time of plasma collection and during the follow-up observation period.

15. Purification of immunoglobulins and immunoglobulin fragments in the production of antivenoms

15.1 Good manufacturing practices

The purification of immunoglobulins and immunoglobulin fragments for the production of antivenoms should aim at obtaining products of consistent quality, safety and clinical effectiveness. The fractionation processes used should adhere to the GMP principles developed for medicinal products. All operations should therefore be carried out in accordance with an appropriate system of quality assurance and GMP. This covers all stages leading to the finished antivenoms, including the production of water, the production of plasma (animal selection and health control, production of venoms and immunization protocols, containers used for blood and plasma collection, anticoagulant solutions and quality control methods) and the purification, storage, transport, processing, quality control and delivery of the finished product. Of particular relevance is the control of microbiological risks, contamination with particulates and pyrogens, and the existence of a documentation system that ensures the traceability of all production steps. To establish satisfactory traceability of the antivenom produced, all the steps of the purification procedure used for the preparation of the antivenom batch should be recorded carefully in pre-established and approved batch record documents, and sampling should be done at established critical steps for in-process quality control tests.

WHO Guidelines on good manufacturing practices for medicinal products are available (83) and the main principles of GMP for the manufacture of blood plasma products of human origin have also been published (84, 85). These Guidelines can serve as a general guide for manufacturing practices in the production of antivenoms. A useful reference in the field of antivenoms is the Note for guidance on production and quality control of animal immunoglobulins and immunosera for human use (CPMP/BWP/3354/99) (86).
15.2 **Purification of the active substance**

Antivenoms are prepared from the starting plasma pool using diverse methods to obtain one of the following active substances:

- intact IgG molecules
- F(ab’)₂ fragments
- Fab fragments.

In general, fractionation procedures should not impair the neutralizing activity of antibodies; they should yield a product of acceptable physicochemical characteristics and purity with a low content of protein aggregates, which is non-pyrogenic and which should provide good recovery of antibody activity. If possible, the process should be simple (with few steps) and economical.

The characteristics of a batch of plasma to be fractionated should be clearly established. The methods used to purify the active substance and the in-process controls should be described in detail in the relevant SOPs. In the following sections, examples of basic protocols used for the production of IgG, F(ab’)₂ and Fab antivenoms are described. Some additional methodologies introduced to further purify the active substance of antivenoms are also discussed. Variations in these manufacturing procedures have often been developed by individual fractionators and should be considered as acceptable when shown to yield consistently safe and effective preparations of antivenoms.

15.2.1 **Purification of intact IgG antivenoms**

15.2.1.1 **Ammonium sulfate precipitation**

In the past, most laboratories that produced whole IgG antivenoms have used fractionation protocols based on salting-out procedures employing ammonium sulfate or sodium sulfate (87). Two precipitation steps are included using two different salt concentrations in addition to the elimination of “euglobulins” by precipitation in a diluted acidic solution.

Such fractionation protocols generally lead to a recovery of antibodies of between 40 and 50% and to the formation of protein aggregates. The final product of this procedure used to contain a relatively high proportion of contaminating proteins, such as albumin (88). This compromised the safety of the product, since a high incidence of early adverse reactions has been described in response to protein aggregates (89).

15.2.1.2 **Caprylic acid precipitation**

The use of caprylic acid (octanoic acid) as an agent for precipitating proteins from animal plasma has been described in the literature (90). Several procedures for the purification of whole IgG antivenoms with a good physicochemical
profile and purity using caprylic acid precipitation of non-immunoglobulin proteins have been developed (88, 91, 92) and are now used for the production of licensed antivenoms.

Fig. A4.6 illustrates a particular process in which caprylic acid is added slowly to undiluted plasma, with constant stirring, to reach a concentration of 5% (v/v) and pH 5.5. The mixture is stirred at 22–25 °C for a minimum of 1 hour. The precipitated proteins are removed by filtration or centrifugation and discarded. The filtrate or the supernatant containing the immunoglobulins is then submitted to tangential flow filtration to remove residual caprylic acid and low-molecular-mass proteins, depending on the molecular cut-off of the ultrafiltration membranes, and to concentrate the proteins. The immunoglobulin solution is then formulated by adding sodium chloride solution (NaCl), an antimicrobial agent and any other excipient(s) needed, such as stabilizers. The pH is then adjusted to a neutral value and finally subjected to sterile filtration through a filter of pore size 0.22 µm, and dispensed into final containers (vials or ampoules). Variations of this procedure have been introduced by various manufacturers, and include dilution of plasma, changes in caprylic acid concentration, pH, and temperature among others.

Caprylic acid fractionation allows the production of antivenoms of relatively high purity and with a low protein aggregate content, because the immunoglobulins are not precipitated during the process. The yield may reach up to 60–75% of the activity in the starting plasma, depending upon the particular procedure and/or the equipment used. The effectiveness and safety profiles of caprylic acid-fractionated antivenom immunoglobulins have been demonstrated in clinical trials (89, 93, 94).

15.2.2 Purification of F(\(ab\)'\(\_\)\(2\)) antivenoms

Many manufacturers follow the classical protocol for F(\(ab\)'\(\_\)\(2\)) antivenom production developed by Pope (9, 10), with a number of recent modifications (12, 13, 95).

The method of pepsin digestion (see Fig. A4.7) involves the digestion of horse plasma proteins by pepsin, leading to the degradation of many non-IgG proteins, and to the cleavage of IgG into bivalent F(\(ab\)'\(\_\)\(2\)) fragments by removal and digestion of the Fc fragment into small peptides. A heating step and the purification of F(\(ab\)'\(\_\)\(2\)) fragments by salting out using ammonium sulfate are also key elements of this methodology. Some procedures involve performing the pepsin digestion step on a pre-purified IgG fraction that is obtained by treatment of plasma with ammonium sulfate to obtain an IgG-enriched precipitate, whereas albumin is not precipitated.

Pepsin digestion is accomplished at a pH of 3.0–3.5. A typical protocol is based on incubation at pH 3.3 for 1 hour, at 30–37 °C in a jacketed tank, with a pepsin concentration of 1.0 g/L. Other procedures can be used which give similar
results. Each manufacturer should adjust the pepsin concentration to achieve the required enzymatic activity.

15.2.2.1 Downstream processing using ammonium sulfate

After pepsin digestion, the pH is adjusted to 4.5–5.0 by adding sodium hydroxide (or a weak alkaline buffer; then ammonium sulfate is added with stirring to a final concentration usually close to 12% (w/v). The precipitate is eliminated by filtration or centrifugation, and the filtrate, or supernatant, is heat treated (usually at 56 °C for 1 hour; this is known as “thermocoagulation”). After thermocoagulation, the preparation is cooled down to less than 30 °C, for example, by passing cold water through a jacketed vessel. The resulting fraction is filtered or centrifuged to remove the precipitate. The pH is then adjusted to 7.0–7.2 with sodium hydroxide, and a solution of ammonium sulfate is added with stirring to a final concentration high enough to precipitate the F(ab')\(_2\) fragments (usually 23% (w/v) or higher). After an additional filtration step, or following centrifugation, the F(ab')\(_2\) precipitate is dissolved, and then desalted (to remove the ammonium sulfate) and concentrated preferentially by tangential flow diafiltration. Care should be taken to avoid aggregate formation by ensuring gentle mixing and rapid dissolving of the precipitate. Alternatively, the 23% (w/v) step is bypassed by some manufacturers and, directly after the heating step, the filtrate obtained is subjected to ultrafiltration. Additional precipitation may also be applied on the starting material at low ionic strength and acid pH to remove “euglobulins” (10).

The F(ab')\(_2\) solution is then formulated by adding sodium chloride (NaCl), an antimicrobial agent, and any other excipient needed for formulation, such as protein stabilizers, and the pH is adjusted, generally to a neutral value. Finally, the preparation is sterilized by filtration through 0.22 µm filters, and dispensed into final containers (vials or ampoules). Such a process, or similar ones developed by other manufacturers, using pepsin digestion, ammonium sulfate precipitation and tangential diafiltration, is the most often used for the manufacture of F(ab')\(_2\) fragments. The yield of this fractionation protocol usually ranges between 30% and 40%.

15.2.2.2 Downstream processing using caprylic acid

Purification of F(ab')\(_2\) has also been shown, on an experimental scale, to be achievable by caprylic acid precipitation of non-F(ab')\(_2\) proteins after pepsin digestion, with an improved yield (~60%) (96). However, the yield obtained on a large scale has not been reported. Fig. A4.8 shows a fractionation scheme for F(ab')\(_2\) using caprylic acid. F(ab')\(_2\) is not precipitated, therefore reducing the formation of aggregates. Some manufacturers have introduced additional or alternative processing steps such as ion-exchange chromatography or ultrafiltration to eliminate low-molecular-mass contaminants.
15.2.3 Purification of Fab antivenoms

Production of monovalent Fab fragments is performed by some manufacturers (97), currently using hyperimmunized sheep plasma. Papain is used to carry out the enzymatic digestion in the presence of L-cysteine as a promoter, and the process of preparation of the fragment may use ammonium sulfate, sodium sulfate or caprylic acid. Fig. A5.9 shows a process in which immunoglobulins are precipitated from plasma by adding ammonium sulfate or sodium sulfate to a concentration of 23%. After filtration the filtrate is discarded and the immunoglobulin precipitate is dissolved in a sodium chloride solution at pH 7.4. Papain is added and digestion performed at 37 °C for 18–20 hours in a jacketed tank. Reaction is stopped by adding iodoacetamide. The product is then applied to a diafiltration system to remove iodoacetamide, salts and low-molecular-mass peptides and equilibrated with a buffered isotonic NaCl solution. The preparation is then chromatographed on an anion exchanger (usually in quaternary aminooethyl (QAE)-based or diethylaminoethyl (DEAE)-based media). Fc fragments and other impurities are bound on the column, whereas Fab fragments pass through. After an additional diafiltration/dialysis step, the product is formulated by adding NaCl, antimicrobial agents (when used) and any other excipients needed, and the pH is adjusted. Finally, the preparation is sterile filtered and dispensed into the final containers.
Fig. A5.6
Example of a fractionation process in which intact IgG is prepared by caprylic acid precipitation of non-immunoglobulin proteins

Fractionation of plasma for purification of IgG

Hyperimmune plasma

Acidification at pH 5.5, addition of caprylic acid to a concentration of 5% with stirring for 1 hr

Filtration or centrifugation

Discard the precipitate

Filtrate or supernatant

Adjust the pH to 7.0

Tangential diafiltration and concentration

Concentrated IgG solution

Formulation and sterile filtration

Bulk preparation

Dispensing in final container

Final product
Fig. A5.7
Example of a fractionation process in which F(ab’)₂ fragments are prepared by pepsin digestion and ammonium sulfate precipitation

Fractionation of plasma for purification of F(ab’)₂ fragments

1. **Hyperimmune plasma**
   - Acidification at pH 3.0–3.5, digestion with pepsin at 30 °C for 1 hr
   - Adjust pH to 4.5–5.0
   - Addition of 12% ammonium sulfate, with stirring for 1 hr
   - Filtration or centrifugation
   - Discard the precipitate

2. **Filtrate or supernatant**
   - Heating to 56 °C for 1 hr with stirring
   - Filtration or centrifugation
   - Discard the precipitate

3. **Filtrate or supernatant**
   - Addition of ammonium sulfate to reach 23% concentration; stirring for 1 hr
   - Filtration or centrifugation
   - Discard the supernatant

4. **F(ab’)₂ paste**
   - Solubilization of precipitate
   - Tangential flow diafiltration and concentration

5. **Concentrated F(ab’)₂ solution**
   - Formulation and sterile filtration

6. **Bulk preparation**
   - Dispensing in final container

7. **Final product**
Fig. A5.8  
Example of a fractionation process in which $F(ab')_2$ fragments are prepared by pepsin digestion and caprylic acid precipitation

Fractionation of plasma for purification of $F(ab')_2$ fragments

Hyperimmune plasma

Acidification at pH 3.0–3.5, digestion with pepsin at 30°C for 1 hr

Adjust pH to 5.5, Addition of caprylic acid to a concentration of 2 to 5% with stirring for 1 hr

Filtration or centrifugation

Discard the precipitate

Filtrate or supernatant

Adjust pH to 7.0

Tangential flow diafiltration and concentration

Concentrated $F(ab')_2$ solution

Formulation and sterile filtration

Bulk preparation

Dispensing in final container

Final product
Fig. A5.9
Example of a fractionation process in which Fab fragments are prepared by papain digestion and ammonium sulfate precipitation

Fractionation of plasma for purification of Fab fragments

Hyperimmune plasma
- Addition of ammonium or sodium sulfate to 23% concentration, with stirring for 1 hr
- Filtration or centrifugation
- Discard the supernatant

IgG-rich precipitate
- Solubilization of precipitate in buffered saline solution
- Digestion with papain at 37 °C
- Addition of iodoacetamide to stop the reaction

Fab solution
- Tangential flow diafiltration
- Anion-exchange chromatography
- Tangential flow diafiltration and concentration
- Formulation and sterile filtration

Bulk preparation
- Dispensing in final container

Final product
15.2.4 **Optional additional or alternative steps used by some manufacturers**

When performed following GMP and using validated fractionation protocols, the basic methodologies described above for the manufacture of IgG, F(ab’)2 and Fab antivenoms allow the production of antivenoms of adequate purity, safety and preclinical efficacy. Nevertheless, some manufacturers include additional steps to enhance product purity. The methodologies include those described below.

15.2.4.1 **Ion-exchange chromatography**

Ion-exchange chromatography can be successfully used for antivenom purification based on charge differential of the contaminants. Anion-exchange columns of DEAE or QAE gels or membranes, such as quaternary ammonium cellulose microporous membranes, can be used at neutral pH to adsorb protein contaminants and endotoxins (13, 95, 98). Alternatively, cation-exchange columns, for example, carboxymethyl or sulfopropyl gels, have been used for purification of IgG or F(ab’)2 fragments (96). The column is equilibrated at acid pH, for example, pH 4.5, to bind the antivenom IgG or its fragments, whereas protein contaminants are eluted in the break-through.

Chromatographic procedures should follow GMP. Columns should be adequately regenerated, sanitized and stored to prolong their useful lifetime. The reproducibility of columns over cycles should be validated. Measures to avoid batch-to-batch contamination should be in place. Specific SOPs should be developed and followed.

15.2.4.2 **Affinity chromatography**

Affinity chromatography using either immobilized venom or other ligands can be designed to bind immunoglobulins or their fragments (99). However, columns usually deteriorate rather rapidly, and meticulous care should be taken to wash, sanitize and store them under appropriate conditions. Procedures should be followed to ensure that any substances leaching from the columns do not affect the quality and safety of the product or else are completely removed during downstream processing; this is especially critical in affinity chromatography using immobilized venom. Affinity processes may affect recovery and high-affinity antibodies may be lost and/or denatured as a result of the harsh elution conditions needed to elute them from the chromatographic material.

15.2.4.3 **Process improvement**

Some manufacturers have introduced process improvements to enhance the quality or the yield of antivenoms. These include the use of a depth-filtration system combined with filter aids to facilitate filtration steps and improve antivenom recovery. Other additional manufacturing steps may be introduced to ensure inactivation or removal of infectious agents (see section 16).
15.2.5 **Formulation**

During formulation of antivenoms after diafiltration steps one should consider the addition of salts to adjust the osmolality, addition of preservatives, other excipients, if needed for protein stability, and the adjustment of pH.

In general, antivenoms are formulated at neutral pH (pH 7.0 ± 0.5) although some manufacturers are exploring the feasibility of formulation at more acidic pHs to improve stability and/or to reduce aggregate formation.

Formulation at a pH higher than 7.5 may not be recommended, since the stability of immunoglobulins and their fragments at alkaline pH may be poor, and the formation of aggregates may be favoured.

15.2.6 **Analysis of bulk product before dispensing**

The biological, physical and chemical characteristics of the final bulk product should meet pre-established specifications before dispensing. Analysis may include tests required to demonstrate:

- the purity and potency of the product
- product sterility
- compliance with the specifications for the aggregate content
- the pyrogen limit and/or the bacterial endotoxin content
- the formulation, that is, the concentration of excipients and the pH.

When the product is a stored liquid, some of these tests (such as the potency assay) may not need to be duplicated on the final container if the processing after the bulk preparation has been validated and shown not to alter this activity.

The sterilization equipment and the integrity of the membrane should be guaranteed before and verified after sterilization; the aseptic filling should also be validated.

15.2.7 **Dispensing and labelling of final product**

Once compliance of the final bulk product with the quality control specifications is established, the final product is bottled. For this, final glass containers (vials or ampoules) should be used. General principles pertaining to the dispensing of parenteral medicinal products should be applied. The dispensing should be performed in class A (83) clean room conditions, usually under a laminar flow hood. The equipment used for dispensing should be calibrated beforehand to ensure that the correct volume is delivered. European GMPs now recommend that sterile filtration be carried out at the closest point immediately before filling.

In the case of ampoules, the dispensing system should ensure an aseptic closure and the sealing of the ampoule should prevent risk of protein denaturation.
due to heat. For vials, insertion of rubber stoppers should be done inside this clean dispensing area. The quality of the rubber stoppers should be such as to guarantee inertness and to prevent leaching. Thereafter, aluminium seals should be placed on each vial in a clean area outside the class A area. Ampoules or vials containing the final product should then be properly identified and stored in a quarantine area maintained under proper storage conditions. Samples of the antivenoms should be sent to the quality control laboratory for analysis.

When an antivenom complies with all the quality control tests established for the final product, it should be properly labelled and identified.

- The vial or ampoule should be labelled with, at least, the following information:
  (a) name of the product and of the producer;
  (b) animal species used to produce the antivenom;
  (c) batch number;
  (d) pharmaceutical presentation (liquid or freeze-dried);
  (e) volume content;
  (f) administration route;
  (g) specificity – venoms neutralized by the antivenom, including both the common and the scientific name of the snake(s);\(^9\)
  (h) neutralizing potency;
  (i) storage conditions; and
  (j) expiry date.

Additional information may be requested by NRAs.

- The package, which is usually a cardboard box, in which the vials or ampoules are packed, should include the same information as is given on the primary container.
- The package insert should include all the information relating to the product, as established by NRAs, including:
  (a) the neutralizing potency;
  (b) the recommended dosage;
  (c) reconstitution procedure, if lyophilized;
  (d) the mode of administration (for example, the dilution of antivenom in a carrier fluid such as saline);

\(^9\) Special care should be taken to consider potential changes in snake species taxonomy.
(e) the rate of administration;
(f) details on the symptoms and treatment of early and delayed adverse reactions;
(g) snake species against which the antivenom is effective;
(h) recommended storage conditions; and
(i) an indication that the product is for single use.

15.2.8 **Use of preservatives**

The addition of preservatives to prevent bacterial and fungal contamination should be kept to a minimum during plasma storage and during fractionation. Their inclusion during the manufacturing process should be clearly justified, and should never substitute for any aspect of GMP. Preservatives can be considered in the final product, especially if it is manufactured in liquid form. Antimicrobial agents currently used in antivenom formulation include phenol and cresols. In general, phenol concentration is adjusted to 2.5 g/L, and concentration of cresols should be less than 3.5 g/L. The concentration of preservatives should be validated by each production laboratory on the basis of assays to test their efficacy and keeping in mind that they may degrade with time and cease to be effective. It is necessary to ascertain that any agent used has no potential detrimental interaction with the active substance and excipients of antivenoms. Any change in the formulation involving preservatives, or the elimination of preservatives from the final product, requires a very careful risk–benefit assessment on various microbial safety aspects, as well as a detailed validation procedure. Mercury-containing preservatives are not recommended in antivenom manufacture. The volume of antivenom required for the treatment of envenoming (in excess of 50 mL) might lead to an exposure to mercury far higher than the amounts currently used for other biological preparations and the levels at which they are toxic, especially in young children, are not known (100, 101).

15.2.9 **Freeze-drying**

Antivenoms are available either as liquid or as freeze-dried preparations. Freeze-dried antivenoms, which may usually be stored at a temperature not exceeding 25 °C, are generally distributed to markets where the cold chain cannot be guaranteed, such as in many tropical regions of the world. The absence of guarantee of a cold chain during distribution highlights the need for manufacturers to demonstrate the stability of the antivenoms under the high temperatures found in tropical climates.

Freeze-drying is a critical operation. Careful attention should be paid to the rate of freezing as well as to the protocol used for the primary and secondary drying cycles (102). The details of the freeze-drying protocols are product-
specific and should be adjusted according to the particular formulation of each antivenom. Inadequate freeze-drying protocols may affect the physicochemical quality of the product, inducing protein precipitation and denaturation, as well as aggregate formation, and altering stability and reconstitution. Specific stabilizers, such as sugars or polyols, aimed at protecting proteins from denaturation and aggregation, may be added to the final formulation of the antivenom (103). Bulking agents, frequently used for some biological products, are generally not required in antivenoms owing to their relatively high protein concentration; however, they are sometimes used for high-titre monospecific antivenoms.

15.2.10 Inspection of final container
All of the vials or ampoules in each batch of liquid antivenoms should be inspected, either visually or using a mechanical device. Any vial or ampoule presenting turbidity, abnormal coloration, presence of particulate matter, or defects of the vial, stopper or capsule should be discarded. In the case of freeze-dried products, a representative sample of the whole batch should be first tested dry for meltback and contamination with foreign matter, then dissolved in the solvent and inspected further as described. Turbidity can be assessed quantitatively using a turbidimeter.

15.2.11 Archive samples of antivenoms
In compliance with GMP, manufacturing laboratories should archive a number of vials of each antivenom batch, under the recommended storage conditions, in an amount that would enable the repetition of all quality control tests, when required.

15.3 Pharmacokinetic and pharmacodynamic properties of IgG, F(ab’)2 and Fab
Owing to their different molecular mass, the pharmacokinetics of heterologous IgG molecules (approximately 150 kDa) and F(ab’)2 (approximately 100 kDa) and Fab (approximately 50 kDa) fragments differ significantly. In envenomed patients, Fab fragments have the largest volume of distribution and readily reach extravascular compartments. Fab fragments are, however, rapidly eliminated, mainly by renal excretion, thus having a short elimination half-life (from 4–24 hours) (104, 105). In contrast, F(ab’)2 fragments and intact IgG molecules are not eliminated by the renal route (they are eliminated by phagocytosis and opsonized by the reticuloendothelial system) and therefore have a longer elimination half-life (between 2 and 4 days) (20, 106, 107). Such different pharmacokinetic profiles have important pharmacodynamic implications, and the selection of the ideal type of active substance in an antivenom should rely on a careful analysis of the venom toxicokinetics and antivenom pharmacokinetics.
Another difference between low-molecular-mass fragments, such as Fab and those with a higher molecular mass, such as F(ab’)
2 and IgG, is the number of paratopes of each molecule: Fab has one antigen-binding site whereas IgG and F(ab’)
2 each have two binding sites. Thus they will be able to form large and stable complexes or precipitates with antigens carrying several epitopes, while Fab will form small, reversible non-precipitable complexes.

Ideally, the volume of distribution of an antivenom should be as similar as possible to the volume of distribution of the main toxins in a particular venom; however, this is rarely the case. In venoms composed of low-molecular-mass toxins, such as some elapid snake venoms, low-molecular-mass neurotoxins are rapidly absorbed into the bloodstream and are rapidly distributed to the extravascular spaces where toxin targets are located. Furthermore, low-molecular-mass toxins are eliminated from the body in a few hours. In these cases, an antivenom of high distribution volume that readily reaches extravascular spaces, such as Fab, might be convenient, although its action is then eliminated within a few hours. It should be noted, however, that a number of elapid venoms contain some high-molecular-mass toxins of great clinical significance, such as procoagulants and pre-synaptic phospholipase A2 neurotoxins.

In contrast, in the case of viperid snake venoms and other venoms made up of toxins of larger molecular mass, including a number of elapid venoms, many of which act intravascularly to provoke bleeding and coagulopathy, the situation is different. The time required for toxins to distribute to extravascular spaces is longer than in the case of low-molecular-mass neurotoxins, and the targets of some of these toxins are present in the vascular compartment. In addition, the toxins of viperid venoms have a long half-life in vivo and can remain in the body for several days (108, 109). In this case, an antivenom made by Fab fragments neutralizes the toxins that reach the circulation but, after a certain time has elapsed, the Fab fragments are eliminated and non-neutralized toxins reach the circulation. This gives rise to the well-known phenomenon of recurrent envenoming, that is, the reappearance of signs and symptoms of envenoming at later time intervals after the initial control of envenoming. This situation demands repeated administration of antivenom to maintain therapeutic levels of Fab in the circulation (110). Therefore, in such envenomings, antivenoms made of IgG or F(ab’)
2 may be more appropriate because of their longer elimination half-lives. Moreover, it has been proposed that formation of venom–antivenom complexes in the circulation results in the redistribution of venom components from the extravascular space to the blood compartment, where they are bound and neutralized by circulating antivenom, provided that the dose of antivenom is sufficient (111, 112). Consequently, the maintenance of a high concentration of specific antivenom antibodies in the circulation for many hours is required for complete neutralization of toxins.
reaching the bloodstream during both early and late phases of envenoming (redistribution of toxins) present in the extravascular space. In conclusion, IgG and F(ab’)2 antivenoms have a pharmacokinetic profile that makes them more effective in many types of snake-bite envenoming.

15.4 Main recommendations

- Antivenoms should be manufactured using fractionation procedures that are well established, validated, and shown to yield products with proven safety and effectiveness. Fractionation processes used for the manufacture of antivenoms should adhere to the principles of GMP for parenteral medicinal products.

- Antivenoms can be composed of intact IgG molecules, F(ab’)2 fragments or Fab fragments. Intact IgG antivenoms are mainly produced by caprylic acid precipitation of non-IgG plasma proteins, leaving a highly purified IgG preparation in the supernatant or filtrate.

- F(ab’)2 fragment antivenoms are produced by pepsin digestion of plasma proteins, at acidic pH, usually followed by F(ab’)2 purification by salting out with ammonium sulfate solutions or by caprylic acid precipitation. Fab monovalent fragments are obtained by papain digestion of IgG at neutral pH.

- Further to ultrafiltration to remove low-molecular-mass contaminants, preparations are formulated, sterilized by filtration and dispensed in the final containers. Formulations of antivenoms may include preservative agents. Additional steps, such as chromatography, can be added to the fractionation protocols to enhance purity.

- Antivenoms can be presented as liquid or freeze-dried preparations. Freeze-drying of antivenoms should be performed in conditions that ensure no denaturation of proteins and no formation of protein aggregates.

- IgG, F(ab’)2 and Fab antivenoms exhibit different pharmacokinetic profiles: Fab fragments have a larger distribution volume and a much shorter elimination half-life. Thus, for viperid envenomings, IgG or F(ab’)2 antivenoms have a more suitable pharmacokinetic profile, whereas Fab fragments may be useful for the neutralization of venoms rich in low-molecular-mass neurotoxins which are rapidly distributed to the tissues. However, in general terms, IgG and F(ab’)2 antivenoms have shown a better pharmacokinetic profile than Fab antivenoms.
16. Control of infectious risks

16.1 Background

The viral safety of any biological product results from a combination of measures to ensure a minimal risk of viral contamination in the starting raw material (plasma), together with steps to inactivate or remove potential contaminating viruses during processing.

There are currently several recognized complementary approaches used for virus risk reduction for biological products. These are:

- minimizing the potential initial virus content by implementing a quality system for the production of the raw material;
- contribution of the manufacturing processes to inactivating and/or removing residual viruses during manufacture of the biological product; such a contribution can be inherent to the existing production technology or may result from the introduction of dedicated viral reduction steps;
- adherence to GMP at all steps of the manufacturing process;
- appropriate and timely response to any infectious events recognized during the clinical use of the product.

Production steps to inactivate and/or remove viruses have long been shown to play a powerful role in ensuring the safety of biologicals (84). Similarly, keeping to a minimum the potential viral load at the stage of the plasma pool, through appropriate epidemiological surveillance and health control of the donor animals, is also an important safety measure (see section 12).

Based on experience with human plasma products, a production process for antivenoms that includes two robust steps for viral reduction (preferably comprising at least one viral inactivation step) should provide a satisfactory level of viral safety. However, it should be kept in mind that non-enveloped viruses are more difficult to inactivate or remove than lipid-enveloped viruses.

16.2 Risk of viral contamination of the starting plasma

The main structural characteristics of viruses reported to possibly infect horses, sheep and goats are presented in Tables A5.7 and A5.8. They include viruses with a DNA or RNA genome, with and without a lipid envelope, and vary widely in size (22 to 300 nm).

A few of these viruses have been identified as possibly present, at least at some stages of the infection cycle, in the blood, or are considered pathogenic to humans. Special attention should be paid to these viruses.
16.3 **Viral validation of manufacturing processes**

An understanding of how much a manufacturing process may contribute to the viral safety of antivenoms is fundamental to both manufacturers and regulators. Such an understanding can only be achieved by viral validation studies. These studies are complex and require well-established virology laboratory infrastructure and cell culture methodologies. They are usually carried out by specialized laboratories, outside the manufacturing facilities. The principles guiding such studies have been described in WHO Guidelines (84) and are summarized below.

Table A5.7

**Viruses identified in horses (86, 113)**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Size (nm)</th>
<th>Genomea</th>
<th>Presence in blood reportedb</th>
<th>Classified as pathogenic to humans (86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid-enveloped viruses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borna virus</td>
<td>Bornaviridae</td>
<td>70–130</td>
<td>ss-RNA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Equine arteritis virus</td>
<td>Arteriviridae</td>
<td>50–60</td>
<td>ss-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equine encephalitis virus, Eastern and Western</td>
<td>Togaviridae</td>
<td>40–70</td>
<td>ss-RNA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Equine coronavirus</td>
<td>Coronaviridae</td>
<td>75–160</td>
<td>ss-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equine foamy virus</td>
<td>Retroviridae</td>
<td>80–100</td>
<td>ss-RNA</td>
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<tr>
<td>Equine herpesvirus 1–5</td>
<td>Herpesviridae</td>
<td>125–150</td>
<td>ds-DNA</td>
<td>Yes</td>
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<tr>
<td>Equine infectious anaemia virus</td>
<td>Lentiviridae</td>
<td>80–100</td>
<td>ss-RNA</td>
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<tr>
<td>Equine influenza virus</td>
<td>Orthomyxoviridae</td>
<td>80–120</td>
<td>ss-RNA</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Virus</td>
<td>Family</td>
<td>Size (nm)</td>
<td>Genome</td>
<td>Presence in blood reported(^b)</td>
<td>Classified as pathogenic to humans (86)</td>
</tr>
<tr>
<td>--------------------------------------</td>
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<td>---------------------------------</td>
<td>----------------------------------------</td>
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<tr>
<td>Equine morbillivirus (Hendra virus)</td>
<td>Paramyxoviridae</td>
<td>150</td>
<td>ss-RNA</td>
<td></td>
<td>Yes</td>
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<tr>
<td>Japanese encephalitis virus</td>
<td>Flaviridae</td>
<td>40–70</td>
<td>ss-RNA</td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Equine hepacivirus</td>
<td>Flaviridae</td>
<td>40–70</td>
<td>ss-RNA</td>
<td>Yes (114)</td>
<td></td>
</tr>
<tr>
<td>Equine pegivirus</td>
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<td>40–70</td>
<td>ss-RNA</td>
<td>Yes (115)</td>
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<td>Nipah virus</td>
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<td>Rabies virus</td>
<td>Rhabdoviridae</td>
<td>75–180</td>
<td>ss-RNA</td>
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<td>Salem virus</td>
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<td>St Louis encephalitis virus</td>
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<td>ss-RNA</td>
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<td>Theiler's disease-associated virus</td>
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<td>40–70</td>
<td>ss-RNA</td>
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<td>Tick-borne encephalitis virus (116, 117)</td>
<td>Flaviridae</td>
<td>40–70</td>
<td>ss-RNA</td>
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<td>Venezuelan equine encephalitis virus</td>
<td>Togaviridae</td>
<td>40–70</td>
<td>ss-RNA</td>
<td></td>
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<tr>
<td>Vesicular stomatitis virus</td>
<td>Rhabdoviridae</td>
<td>50–80</td>
<td>ss-RNA</td>
<td></td>
<td>Yes, Yes</td>
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<tr>
<td>West Nile virus</td>
<td>Flaviridae</td>
<td>40–70</td>
<td>ss-RNA</td>
<td></td>
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Table A5.7 continued

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<th>Virus</th>
<th>Family</th>
<th>Size (nm)</th>
<th>Genome&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Presence in blood reported&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Classified as pathogenic to humans&lt;sup&gt;86&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Non-lipid-enveloped viruses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equine encephalosis viruses</td>
<td>Reoviridae</td>
<td>80</td>
<td>ds-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equine rhinitis A and B viruses</td>
<td>Picornaviridae</td>
<td>22–30</td>
<td>ss-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equine rotavirus</td>
<td>Reoviridae</td>
<td>60–80</td>
<td>ds-RNA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> ss-RNA, single-stranded ribonucleic acid; ds-DNA, double-stranded deoxyribonucleic acid; ds-RNA, double-stranded ribonucleic acid.

<sup>b</sup> Absence of a report does not mean that the virus may not be found in the blood at certain stages of the cycle of infection.

<sup>c</sup> Recent studies have suggested that Borna virus is non-pathogenic to humans (118).

Table A5.8

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Size (nm)</th>
<th>Genome&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reported presence in blood&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Classified as pathogenic to humans&lt;sup&gt;86&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid-enveloped viruses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Adenoviridae</td>
<td>80–110</td>
<td>ds-DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Akabane virus</td>
<td>Bunyaviridae</td>
<td>80–120</td>
<td>ss-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bluetongue virus</td>
<td>Reoviridae</td>
<td>80</td>
<td>ds-RNA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Border disease virus</td>
<td>Flaviviridae</td>
<td>40–70</td>
<td>ss-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borna virus&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Bornaviridae</td>
<td>70–130</td>
<td>ss-RNA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Bovine herpesvirus types 1, 2, 4</td>
<td>Herpesviridae</td>
<td>120–200</td>
<td>ds-DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus</td>
<td>Family</td>
<td>Size (nm)</td>
<td>Genome</td>
<td>Reported presence in blood</td>
<td>Classified as pathogenic to humans</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-----------------</td>
<td>-----------</td>
<td>--------</td>
<td>----------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Bovine viral diarrhoea virus</td>
<td>Togaviridae</td>
<td>40–60</td>
<td>ss-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Looping ill virus</td>
<td>Flaviviridae</td>
<td>40–50</td>
<td>ss-RNA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Nairobi sheep disease</td>
<td>Bunyaviridae</td>
<td>80–120</td>
<td>ss-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovine/bovine papillomavirus</td>
<td>Papillomaviridae</td>
<td>40–55</td>
<td>ds-DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovine herpesvirus 2</td>
<td>Herpesviridae</td>
<td>120–200</td>
<td>ds-DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parainfluenza virus type 3</td>
<td>Paramyxoviridae</td>
<td>150–300</td>
<td>ss-RNA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Peste des petits ruminants (Morbillivirus)</td>
<td>Paramyxoviridae</td>
<td>150–300</td>
<td>ss-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poxviruses (Parapox, Capripox, Cowpox)</td>
<td>Poxviridae</td>
<td>140–260</td>
<td>ds-DNA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>Paramyxoviridae</td>
<td>150–300</td>
<td>ss-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retroviruses (Caprine arthritis encephalitis virus, Maedi-Visna virus, Jaagsiekte virus, Bovine leukaemia virus)</td>
<td>Retroviridae</td>
<td>80–100</td>
<td>ss-RNA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table A5.8 continued

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Size (nm)</th>
<th>Genome&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reported presence in blood&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Classified as pathogenic to humans&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rift Valley fever complex</td>
<td>Bunyaviridae</td>
<td>80–120</td>
<td>ss-RNA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Ross river virus</td>
<td>Togaviridae</td>
<td>70</td>
<td>ss-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotavirus</td>
<td>Reoviridae</td>
<td>80</td>
<td>ds-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tick-borne encephalitis virus</td>
<td>Flaviviridae</td>
<td>40–50</td>
<td>ss-RNA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Vesicular stomatitis virus</td>
<td>Rhabdoviridae</td>
<td>50–380</td>
<td>ss-RNA</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Wesselbron virus</td>
<td>Flaviviridae</td>
<td>40–50</td>
<td>ss-RNA</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

**Non-lipid-enveloped viruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Size (nm)</th>
<th>Genome&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reported presence in blood&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Classified as pathogenic to humans&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epizootic haemorrhagic disease virus</td>
<td>Reoviridae</td>
<td>80</td>
<td>ds-RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foot and mouth disease virus</td>
<td>Picornaviridae</td>
<td>27–30</td>
<td>ss-RNA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Reovirus 1-3</td>
<td>Reoviridae</td>
<td>60–80</td>
<td>ds-RNA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> ds-DNA, double-stranded deoxyribonucleic acid; ss-RNA, single-stranded ribonucleic acid; ds-RNA, double-stranded ribonucleic acid.

<sup>b</sup> Absence of a report does not mean that the virus may not be found in the blood at certain stages of the cycle of infection.

<sup>c</sup> Recent studies have suggested that Borna virus is non-pathogenic to humans (118).
16.3.1 Down-scale experiments

The contribution of manufacturing processes to inactivation and/or removal of potential viral contamination should be demonstrated. For this purpose, viral validation studies should be performed using at least three viruses exhibiting different structural characteristics. The antivenom manufacturer should first identify the steps that, based on the existing literature, are likely to remove or inactivate viruses and, then, provide evidence and quantitative assessment of the extent of virus reduction achieved for the specific process evaluated.

Validation should be done by down-scale experiments. The accuracy of the down-scale process should be assessed by comparing the characteristics of the starting intermediate and the fraction resulting from that step, for both the laboratory and the production scales. It may be more appropriate to use manufacturing intermediates for spiking in viral validation studies. Selected physical factors (for example, temperature, stirring or filtration conditions) and chemical factors (for example, pH or concentration of precipitating agents such as caprylic acid) should be as close as possible to those used at manufacturing scale.

Once the step is accurately modelled, the antivenom fraction derived from the fractionation process just prior to the step being evaluated (for example, the starting plasma to be subjected to a low pH treatment, or to caprylic acid precipitation, or a F(ab’)_2 fragment fraction to be subjected to ammonium sulfate heat treatment) should be spiked with one of the model viruses selected. Viral infectivity, most often determined using cell culture assays (less frequently animal models), should be quantified before (for example, prior to pH adjustment and addition of pepsin) and immediately after (for example, following low pH adjustment and incubation at that pH for a known period of time in the presence of pepsin) the steps evaluated to determine the viral clearance achieved. The results are conventionally expressed as the logarithm (log) of the reduction in infectivity that is observed. Total infectivity or viral load is calculated as the infectious titre (infectious units per mL) multiplied by the volume. For a viral inactivation step, it is highly recommended that the kinetics of the virus kill be evaluated. Such inactivation kinetics of the infectivity provide an important indication of the virucidal potential of the step and enables comparison of the data obtained to those from published studies.

Typically, a viral reduction of 4 logs or more is considered to represent an effective and reliable viral safety step.

Establishing the relative insensitivity of a manufacturing step to changes or deviations in process conditions is also important in evaluating its robustness, in addition to adding to the level of understanding of its contribution to the overall viral safety of the preparation. This can be achieved by validating the same step using a range of conditions deviating from those used in production (such as an upper pH limit applied to a pepsin digestion or to a caprylic acid precipitation step).
Virus validation studies are subject to a number of limitations (84), which should be considered when interpreting the results.

16.3.2 Selection of viruses for the validation of antivenom production processes

Viruses selected for viral validation studies should resemble as closely as possible those which may be present in the starting animal plasma material (Tables A5.7 and A5.8). It is usual to select a wide variety of viruses, some enveloped and some non-enveloped. At least 1–2 non-enveloped, relatively small viruses should be selected for validation. When possible, viruses known to potentially contaminate animal plasma (called “relevant viruses”) should be used.

Table A5.9 gives examples of a few viruses that have been used for the validation of animal-derived immunoglobulins. Vesicular stomatitis virus (VSV) and West Nile virus (WNV) are relevant lipid-enveloped horse plasma-borne viruses. Bovine viral diarrhoea virus (BVDV), a lipid-enveloped flavivirus, can be used as a model for WNV, tick-borne encephalitis virus, and for the Eastern, Western, and Venezuelan equine encephalitis togaviruses. Pseudorabies virus is a lipid-enveloped virus that can serve as a model for pathogenic equine herpesvirus. Encephalomyocarditis virus (EMCV), a picornavirus, can serve as a model for non-lipid-enveloped viruses. Porcine parvovirus can also be selected as a model for small resistant non-lipid-enveloped viruses or as a relevant virus when pepsin of porcine origin is used in the manufacture of F(ab’)2 fragments.

This list is not exhaustive and other model viruses can be used for validation studies of animal-derived antivenoms, in particular taking into account the characteristics of the viruses that may be present in the animal species used to generate antivenoms.
Table A5.9
Examples of laboratory model viruses that can be used for validation studies of horse-derived antivenoms

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Lipid-enveloped</th>
<th>Size (nm)</th>
<th>Genome(^a)</th>
<th>Resistance</th>
<th>Model for</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal parvovirus (for example, porcine)</td>
<td>Parvoviridae</td>
<td>No</td>
<td>18–26</td>
<td>ss-DNA</td>
<td>High</td>
<td>Relevant virus (when pepsin of porcine origin is used)</td>
</tr>
<tr>
<td>Bovine viral diarrhoea virus</td>
<td>Togaviridae</td>
<td>Yes</td>
<td>40–60</td>
<td>ss-RNA</td>
<td>Low</td>
<td>Eastern, Western and Venezuelan equine encephalitis virus; tick-borne encephalitis virus</td>
</tr>
<tr>
<td>Parainfluenza virus</td>
<td>Paramyxoviridae</td>
<td>Yes</td>
<td>100–200</td>
<td>ss-RNA</td>
<td>Low</td>
<td>Hendra virus; Nipah virus; Salem virus</td>
</tr>
<tr>
<td>Poliovirus; encephalomyocarditis virus; hepatitis A virus</td>
<td>Picornaviridae</td>
<td>No</td>
<td>25–30</td>
<td>ss-RNA</td>
<td>Medium-high</td>
<td>Equine rotavirus</td>
</tr>
<tr>
<td>Pseudorabies virus</td>
<td>Herpes</td>
<td>Yes</td>
<td>100–200</td>
<td>ds-DNA</td>
<td>Medium</td>
<td>Equine herpesvirus</td>
</tr>
<tr>
<td>Reovirus type 3</td>
<td>Reoviridae</td>
<td>No</td>
<td>60–80</td>
<td>ds-RNA</td>
<td>Medium</td>
<td>Equine encephalosis virus</td>
</tr>
<tr>
<td>Sindbis virus</td>
<td>Togaviridae</td>
<td>Yes</td>
<td>60–70</td>
<td>ss-RNA</td>
<td>Low</td>
<td>Eastern, Western and Venezuelan equine encephalitis virus</td>
</tr>
<tr>
<td>Vesicular stomatitis virus</td>
<td>Rhabdoviridae</td>
<td>Yes</td>
<td>50–200</td>
<td>ss-RNA</td>
<td>Low</td>
<td>Relevant virus</td>
</tr>
<tr>
<td>West Nile virus</td>
<td>Flaviridae</td>
<td>Yes</td>
<td>40–70</td>
<td>ss-RNA</td>
<td>Low</td>
<td>Relevant virus and model for Eastern equine encephalitis virus</td>
</tr>
</tbody>
</table>

\(^a\) ss-DNA, single-stranded deoxyribonucleic acid; ss-RNA, single-stranded ribonucleic acid; ds-DNA, double-stranded deoxyribonucleic acid; ds-RNA, double-stranded ribonucleic acid.
16.4  **Viral validation studies of antivenom immunoglobulins**

There is no documented case of transmission of zoonotic infections, including viral diseases, by antivenom immunoglobulins, or any other animal-derived immunoglobulins. Absence of reports of viral transmission may result from a lack of long-term surveillance of the patients receiving antivenoms. Alternatively, this may reveal that current processes for the manufacturing of antivenoms include processing steps that contribute to viral safety.

Among the various processing steps used in the production of antivenoms, caprylic acid and low pH treatments are known to contribute to safety against lipid-enveloped viruses. This information is based on well-established experience in the fractionation of human plasma with a production step comprising caprylic acid (119–121) or low pH treatment (84, 122–124).

Although information is still limited, there is growing evidence that similar steps used in the production of animal-derived immunoglobulins may also inactivate or remove viruses. In addition, some manufacturers have implemented dedicated viral reduction procedures. After the introduction of a new step in the process, the stability of the product must be verified.

16.4.1  **Caprylic acid treatment**

The conditions used for caprylic acid treatment of antivenoms (88, 113) and of human immunoglobulins (119–121) are similar, in particular the pH range, duration of treatment, temperature, and the caprylic acid/protein ratio, as summarized in Table A5.10.

<table>
<thead>
<tr>
<th>Product</th>
<th>Protein concentration (g/L)</th>
<th>Caprylic acid (g/kg solution)</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Duration (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human IgG</td>
<td>35</td>
<td>7.45</td>
<td>5.5</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>Human IgM enriched</td>
<td>43</td>
<td>15</td>
<td>4.8</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>Human IgM</td>
<td>25</td>
<td>20</td>
<td>5.0</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>Antivenoms</td>
<td>60–90</td>
<td>50</td>
<td>5.5–5.8</td>
<td>18–22</td>
<td>1</td>
</tr>
</tbody>
</table>
16.4.1.1 Validation studies with human immunoglobulins

Unsaturated fatty acids, most specifically caprylic acid, have long been known to have the capacity to inactivate lipid-enveloped viruses in human plasma protein fractions (125, 126). The non-ionized form of caprylic acid is thought to disrupt the lipid bilayer and membrane-associated proteins of enveloped viruses. Utilizing the dissociation reaction and varying the concentration of the ionized form of caprylate, a specific amount of the non-ionized form of caprylate can be maintained over a wide pH range.

The robustness of caprylic acid treatment applied to human IgG, human IgM and IgM-enriched preparations has been investigated using various enveloped viruses (human immunodeficiency virus (HIV), BVDV, Sindbis virus and Pseudorabies virus (120). Under the conditions applied during manufacture, caprylic acid leads to robust inactivation of lipid-enveloped viruses; pH is a particularly critical parameter and should be less than 6.

Another investigation studied the viral reduction achieved during treatment by caprylate of a human IgG product (119). At pH 5.1, 23 °C, in the presence of 9 mM caprylate, ≥ 4.7 and ≥ 4.2 log of HIV and pseudorabies virus, respectively, were inactivated during the 1 hour treatment, but only 1.5 log for BVDV was inactivated. At 12 mM caprylate, ≥ 4.4 log of BVDV were inactivated within this period. At pH 5.1, 24 °C, and 19 mM caprylate, and pH 5.1, 24 °C, and 12 mM caprylate, complete inactivation of BVDV and of HIV and pseudorabies virus was achieved in less than 3 minutes.

Treatment of cryoprecipitate-poor plasma with 5% caprylic acid/pH 5.5 at 31 ± 0.5 °C (a condition close to that used to prepare antivenoms) was shown to inactivate ≥ 5 log of HIV, BVDV and pseudorabies virus in less than 5 min (127).

16.4.1.2 Validation studies with antivenom immunoglobulins

Virus inactivation studies have been carried out on an F(ab’)₂ fraction obtained from pepsin-digested plasma subjected to ammonium sulfate precipitation. The F(ab’)₂ fraction was subjected to precipitation by drop-wise addition of caprylic acid to 0.5% (final concentration) and the mixture was maintained under vigorous stirring for 1 hour at 18°C. Rapid and complete reduction of BVDV, pseudorabies virus and VSV (> 6.6 log₁₀, > 6.6 log₁₀, and > 7.0 log₁₀, respectively) was observed. No significant reduction (0.7 log₁₀) of the non-enveloped EMCV (126) was observed.

In another process used to prepare equine immunoglobulins, serum is thawed at 4 °C, subjected to heating at 56 °C for 90 minutes, brought to 20 ± 5 °C, adjusted to pH 5.5 and subjected to 5% caprylic acid treatment for 1 hour. This process led to fast reduction of infectivity of > 4.32 and
4.65 \log_{10} for pseudorabies virus and BVDV, respectively. The caprylic acid step was confirmed to have only limited impact on the infectivity of EMCV and minute virus of mice (MVM) non-lipid-enveloped viruses (128). Data suggest that significant reduction in the infectivity of lipid-enveloped viruses can be obtained during caprylic acid treatment of antivenoms. The reduction of viral infectivity may result from both viral inactivation and partitioning during the precipitation step. No significant inactivation of non-enveloped viruses is expected.

16.4.1.3 Recommended actions
Further studies of the viral reduction achieved during caprylic acid treatment of antivenoms are recommended; in particular, robustness studies to define the impact on process variations should also be performed.

16.4.2 Acid pH treatment
The conditions used for low pH treatment of equine-derived antivenom immunoglobulins and of human immunoglobulins are summarized in Table A5.11.

Table A5.11
Typical conditions for acid pH treatment of human IgG preparations and equine antivenoms (113)

<table>
<thead>
<tr>
<th>Product</th>
<th>Protein concentration (g/L)</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Duration (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human IgG</td>
<td>40–60</td>
<td>4.0</td>
<td>30–37</td>
<td>20–30</td>
</tr>
<tr>
<td>Antivenoms</td>
<td>60–90</td>
<td>3.1–3.3</td>
<td>30–37</td>
<td>0.6–24</td>
</tr>
</tbody>
</table>

16.4.2.1 Validation studies with human immunoglobulins
Many studies have demonstrated that the low pH 4 treatment used in the manufacture of human intravenous IgG has the capacity to inactivate lipid-enveloped viruses (122–124). The rate and extent of inactivation may differ depending upon the virus. Inactivation is temperature dependent, and is influenced by the formulation of the IgG solution. Pepsin is sometimes added in trace amounts (to reduce anticomplementary activity and content of aggregates) but, at this low concentration, contributes little to virus kill (86). Most non-lipid-enveloped viruses are resistant to acid pH treatment.
16.4.2.2  Virus inactivation studies performed with antivenom immunoglobulins

As described in section 15, peptic cleavage of horse plasma IgG at pH 3.0–3.3 for 60 minutes is a common procedure for the preparation of F(\(\text{ab}'\))\(_2\). More than 4 logs of inactivation of WNV and of Sindbis virus has been observed in horse plasma subjected to peptic digestion at pH 3.2 for 60 minutes (129). WNV was very sensitive whether pepsin was added or not, whereas the rate and extent of inactivation of Sindbis virus was higher in the presence of pepsin. This suggests that pH 3.2 alone inactivates WNV, while other phenomena involving the action of pepsin contribute to Sindbis virus inactivation at low pH.

Confirmation of the significant inactivation of lipid-enveloped viruses during peptic cleavage of plasma at pH 3.2 was obtained by another study (126). In this study process, plasma was diluted with two volumes of saline, pH was adjusted to 3.3, and pepsin was added to a final concentration of 1 g/L. The mixture was incubated at pH 3.3 for 1 hour. Inactivation of pseudorabies virus > 5.1 log\(_{10}\) occurred in less than 6 minutes and > 7.0 log\(_{10}\) in 60 minutes. There was > 3.1 log\(_{10}\) and > 4.5 log\(_{10}\) inactivation of VSV after 6 and 20 minutes, respectively. The reduction of infectivity of BVDV was less: 1.7 log\(_{10}\) after 60 minutes. Inactivation of EMC, a non-enveloped virus, was relatively slow but reached between 2.5 and 5.7 log\(_{10}\) after 60 minutes of pepsin incubation. This showed that reduction of infectivity of at least some non-lipid-enveloped viruses may take place during peptic digestion of diluted horse plasma. This does not mean, however, that other non-lipid-enveloped viruses would be inactivated to the same extent under such conditions.

16.4.2.3  Recommended actions

Manufacturers of F(\(\text{ab}'\))\(_2\) antivenoms must validate the pepsin digestion process since virus inactivation is likely to be influenced by pH, time, temperature, pepsin content and protein content. Robustness studies to define the impact on process variations are also recommended.

16.4.3  Filtration steps

Other steps used in antivenom production may contribute to viral safety through nonspecific virus removal. The virus removal capacity of two depth-filtration steps performed in the presence of filter aids and used in the production of equine-derived immunoglobulins prepared by ammonium sulfate precipitation of pepsin-digested IgG has been evaluated (130). Clearance factors of 5.7 and 4.0 log\(_{10}\) have been found for two lipid-enveloped viruses (infectious bovine rhinotracheitis virus and canine distemper virus, respectively) and of 5.3 and 4.2 log\(_{10}\) for two non-lipid-enveloped viruses (canine adenovirus virus and poliovirus type I, respectively). However, it should be kept in mind that viral reductions obtained by non-dedicated removal steps are usually regarded
as less robust than those resulting from dedicated viral inactivation or removal steps (84).

16.4.4 Validation of dedicated viral reduction treatments

16.4.4.1 Pasteurization

Pasteurization is defined as the treatment of a liquid protein fraction for 10 hours, usually at 60 °C. It is a well-established viral inactivation treatment of human plasma products, such as IgG (84). It is being used in the production process of a few equine-derived immunoglobulins (13).

Validation studies have shown that heating a purified equine immunoglobulin at 58 °C ± 0.1 °C without stabilizers inactivates ≥ 4.8 log₁₀ of pseudorabies virus and ≥ 4.3 log₁₀ of BVDV in less than 30 minutes, and > 4.7 log of EMCV in less than 1 hour. In contrast, infectivity of MVM, a non-enveloped virus, was still detected after 9 hours and 30 minutes of treatment; only 1.59 log₁₀ were inactivated (128).

16.4.4.2 Nanofiltration

Nanofiltration is a technique of filtration specifically designed to remove viruses, based on size, while permitting flow-through of the desired protein (131). Effective virus removal requires, in principle, that the pore size of the filter be smaller than the effective diameter of the virus particles.

16.4.5 Other viral inactivation treatments currently not used in antivenom manufacture

Other methods of viral inactivation have been developed to ensure the viral safety of biological products. These include, in particular, a treatment with a combination of an organic solvent (tri-n-butyl phosphate or TnBP) at concentrations between 0.3 and 1%, and detergents such as Triton™ X-100 or Tween® 80, also at concentrations generally between 0.3% and 1%. Such solvent–detergent (S/D) procedures have proven very efficient and robust in the inactivation of lipid-enveloped viruses in human plasma products (84). However, use of this method for antivenoms has not been reported.

Implementation of dedicated viral inactivation treatments, such as S/D or other methods, should be encouraged for processes which, based on risk assessment, would offer an insufficient margin of viral safety. Process changes associated with the introduction of new viral reduction steps, and the subsequent removal of any toxic compounds needed for viral inactivation, should be demonstrated not to affect the quality and stability of antivenoms, and most particularly the neutralization efficacy of venoms. Preclinical assessment of the possible impact of newly introduced viral inactivation treatments should be mandatory.
16.4.6 Possible contribution of phenol and cresols

The antibacterial agents, phenol and cresols, and more rarely formaldehyde, are added by most manufacturers to the starting plasma donations as well as to the final liquid antivenom preparations, at a maximum final concentration of 0.25–0.35%. Compounds like phenol are known to be very lipid soluble and lipophilic.

Addition of 0.25% (final concentration) phenol to concentrated antivenom bulk at pH 6.5–6.7, prior to its dilution and formulation, was found to inactivate eight enveloped and non-enveloped viruses very efficiently within 30 minutes (132).

Performing additional validations of the virucidal effect of antimicrobial agents as added to the starting hyperimmune plasma and to the final antivenom preparations is encouraged. More information is needed on the potential impact of these antimicrobial agents on the viral safety of antivenoms.

16.5 Production-scale implementation of process steps contributing to viral safety

As there is increasing, although preliminary, evidence that at least some of the existing steps in the production of antivenoms contribute to viral reduction, it is already recommended that specific care should be taken to ensure their appropriate industrial implementation so as not to compromise any possible benefits they provide in terms of viral safety.

Measures should therefore be taken to ensure that such steps are correctly carried out in a manufacturing environment and that cross-contamination and downstream contamination are avoided. Such important aspects of product safety have been highlighted in WHO Guidelines (84) and should also be taken into consideration for large-scale manufacture of antivenoms. Specific attention should be paid to:

- **Process design and layout:** in particular the production floor area needed to carry out such treatment safely, minimizing the chance of cross-contamination between pre-viral-treatment steps product and post-treatment product; the justification for creating a safety zone to avoid risk of downstream contamination, and the procedures used for cleaning and sanitization of the equipment to avoid batch-to-batch cross-contamination.

- **Equipment specifications:** having in mind the potential contribution to viral reduction. For instance, vessels used for low pH incubation or caprylic acid treatment should be fully enclosed and temperature-controlled. There should be no “dead points” where the temperature
defined in the specification or the homogeneity of mixing cannot be ensured. A poor equipment design could compromise the viral safety potentially afforded by a given production step.

- **Qualification and validation**: should verify that the equipment conforms to predefined technical specifications and relevant GMP.

- **Process implementation**: production steps contributing to viral safety such as low pH treatment and caprylic acid treatments could be implemented in two stages performed in two distinct enclosed tanks. Care should be taken to ensure complete process segregation before and after the completion of these treatments to avoid risks of downstream contamination.

- **Process control**: is a critical part of the manufacturing process since completion of viral inactivation and removal cannot be guaranteed by testing the final product. Samples should be taken to confirm that the process conditions of claimed inactivation steps meet the specified limits (for example, for pH, concentration of stabilizers and concentration of virus inactivating agent, such as caprylate). When this is technically feasible and intermediates are stable, samples can be kept frozen for possible additional analysis prior to the release of the batch. It is the responsibility of the manufacturer to ensure that the execution of steps contributing to virus inactivation and removal in a production setting conforms to the conditions that contribute to such virus reduction.

- **SOPs**: steps contributing to viral reduction should be described in approved SOPs. These should contain critical process limits for the viral inactivation and removal methods.

- **Role of the quality assurance department**: because of the critical nature of the viral inactivation and removal steps, quality assurance personnel should review and approve the recorded conditions for viral inactivation and removal while the batch is being processed; that is, not just as part of the final overall review of the batch file.

### 16.6 Transmissible spongiform encephalopathy

Transmissible spongiform encephalopathy (TSE) has not been identified in any equine species. There has been no case of transmission of TSE linked to antivenoms or other equine-derived blood products.

Of particular concern, however, is that TSEs include scrapie in sheep, a ruminant species that is used, although much less frequently than horses, in the manufacture of antivenoms. Scrapie is a disease similar to bovine spongiform encephalopathy (BSE or “mad cow disease”), but is not known to infect humans.
However, the blood of sheep with experimental BSE or natural scrapie can be infectious and, because scrapie and BSE prion agents behave similarly in sheep and goats, the use of the blood of small ruminants in preparing biologicals should either be avoided or the animals should be selected very carefully from sources known to be free of TSEs. The findings of disease-associated proteins in muscle tissue of sheep with scrapie and the recognition of BSE itself in a goat, reinforce the need for manufacturers of biologicals, including antivenoms, to maintain the precautionary safety measures recommended in the WHO guidelines on TSE tissue infectivity (77). According to these recommendations, the use of tissues or body fluids of ruminant origin should be avoided in the preparation of biological and pharmaceutical products. When sheep-derived materials must be used, they should therefore be obtained from sources assessed to have negligible risk from the infectious agent of scrapie. Documented surveillance records should be available. The feed of animals used for production of hyperimmune plasma should be free of ruminant-derived material.

The infectious agent is thought to be a misfolded, abnormal, prion protein (PrPTSE). It is not yet known whether manufacturing processes used to produce antivenoms from sheep plasma include steps that can contribute to the removal of PrPTSE. Experimental prion clearance studies, based on spiking experiments, can be performed to assess the capacity of the process to remove prions. However, there is still uncertainty about the validity of such experimental studies since the biochemical features of PrPTSE in blood and plasma are not known.

16.7 Main recommendations

- The viral safety of antivenoms results from a combination of measures:
  - to ensure satisfactory health status of the animals;
  - to reduce the risk of contamination in the starting raw material;
  - to ensure the contribution of the manufacturing process towards inactivation and/or removal of viruses; and
  - to ensure compliance with GMP along the entire chain of production.

- Manufacturing processes should include at least two steps contributing to robust viral reduction. A virus inactivation step that can be easily monitored is usually preferred to other means of viral reduction, such as nonspecific removal.
Manufacturers must evaluate the capacity of their current manufacturing processes (in particular low pH pepsin digestion, caprylic acid treatment, ammonium sulfate or heat precipitation, and possibly other steps) to inactivate or remove viruses and validate them, if necessary. These studies should be done following existing international guidelines and using relevant and/or model viruses that are representative of the viruses that could affect the animals used for the production of the antivenom immunoglobulins.

The removal of antimicrobial agents from the final formulation of antivenoms should be carefully weighed against the likely benefits these agents may have on the viral safety.

Should the viral reduction processes used be found to be insufficient to ensure a margin of safety, the introduction of dedicated viral reduction methods should be considered. The impact of such process changes on product efficacy and safety should be carefully analysed in vitro as well as in preclinical studies before performing clinical evaluations in humans.

Great attention should be paid to the production-scale implementation of all steps contributing to viral safety to ensure a consistent and reproducible batch-to-batch viral reduction and an absence of risks of cross-contamination and downstream recontamination that would jeopardize the viral safety of the product.

When materials originating from sheep must be used for the production of plasma, they should be obtained from sources assessed to have negligible risk from the infectious agent of scrapie.

17. Quality control of antivenoms

Quality control of the final product is a key element in the quality assurance of antivenoms. Quality control tests should be performed by the manufacturer or under its responsibility before the product is released. In addition, relevant analyses should be performed on any intermediate steps of the manufacturing protocol as part of the in-process quality control system.

The results obtained should meet the specifications approved for each antivenom product or its intermediates, and constitute part of the batch record. For a liquid preparation, some quality control tests, such as the venom-neutralizing efficacy test or the detection of residual reagents used during fractionation, can be performed on the final bulk and may not need to be repeated.
on the final bottled product if the processing after the bulk preparation has been validated and shown not to have any impact. Quality control assessment of the final antivenom product includes the tests described below.

17.1 **Standard quality assays**

17.1.1 **Appearance**

The appearance of the product (for example, colour and clarity of the liquid, appearance of the powder) should comply with the description in the marketing dossier.

17.1.2 **Solubility (freeze-dried preparations)**

The time from the addition of solvent to the complete dissolution of freeze-dried antivenom, under gentle mixing, should be determined. Antivenoms should be completely dissolved within 10 minutes at room temperature. The solution should not be cloudy. Shaking of the container should be avoided to prevent the formation of foam.

17.1.3 **Extractable volume**

The volume of product extractable from the container should be in compliance with that indicated on the label.

17.1.4 **Venom-neutralizing efficacy tests**

These tests determine the capability of an antivenom to neutralize the lethal effect of the snake venom(s) against which the antivenom is designed. It is first necessary to determine the lethal potency of the venom, using the LD\(_{50}\) assay. The exact volume of antivenom required to neutralize venom lethality can then be determined using the antivenom effective dose (ED\(_{50}\)) assay.

The outputs of these tests provide globally applicable standard metrics of: venom lethality and antivenom efficacy, which enable internal monitoring and external, independent auditing of antivenom efficacy – thereby preventing the distribution of ineffective antivenom.

Consistent use of outbred strains of mice, of a defined weight range (for example, 18–20 g) that receive a defined challenge dose, is recommended for all the assays. Some producers use other test animals, such as guinea-pigs. While weights will clearly vary between animal species, a series of principles, specified for mice, will still apply to these alternative test animals. It should be borne in mind that there are variations in the susceptibility of different strains of mice to the lethal effect of venoms.

The venom-neutralizing potency tests are used for quality control and preclinical assessment, so protocol details are described in section 19, while ethical issues are discussed in section 4.
17.1.5 **Osmolality**

Osmolality is used to measure the tonicity of the antivenom solution, and should be at least 240 mOsmol/kg. Determination of osmolality is also an indirect means to determine the quantity of salts or excipients added for formulating the batch.

17.1.6 **Identity test**

When several types of antivenoms are produced by a single production facility, a system to identify each batch of antivenom should be established for monitoring and auditing purposes. Identity tests may include biological assays as well as physicochemical and immunological tests. Double immunodiffusion assays, confronting the antivenom with the venoms against which the antivenom is designed to act, are often used. In the case of laboratories that use various animal species to raise antivenoms, that is, horses and sheep, an immunological identity test should be used to identify the mammalian species in which the antivenoms are produced.

17.1.7 **Protein concentration**

The total protein concentration of antivenoms can be determined using a variety of approaches, including:

- the Kjeldahl method to determine nitrogen content;
- several colorimetric procedures; and
- spectrophotometric (280 nm) assays.

The presence of preservatives should be taken into account since they may interfere with some protein determination methods (133).

The total protein concentration of antivenoms should preferably not exceed 10 g/dL, since administration of higher amounts of protein may be associated with higher adverse reaction rates, although some jurisdictions have authorized higher concentrations.

17.1.8 **Purity and integrity of the immunoglobulin**

The purity and integrity of the active substance, that is, intact immunoglobulin or immunoglobulin fragments, should be assessed to identify contaminants and immunoglobulin degradation. Immunoglobulins or their fragments should constitute the great majority of the preparation, ideally greater than 90%. Evidence suggests, however, that although antivenoms may have physicochemical purity > 90% (for example, immunoglobulins or their fragments), immunochemical purity (for example, specificity for the snake venoms they are produced from) can be lower than 40% (134). These findings have emphasized the need to incorporate
both physicochemical and immunochemical analyses in the assessment of antivenom purity.

Electrophoretic methods in polyacrylamide gels (SDS-PAGE run under reducing or non-reducing conditions) are suitable for this purpose, since these techniques allow the detection and monitoring of IgG, F(ab’)2, Fab, non-immunoglobulin plasma protein contaminants (in particular albumin) and degradation products. The electrophoretic pattern should be compared to that of a reference preparation. A semi-quantification can be performed by calibration of the procedure. Of particular relevance is the assessment of the albumin content, which ideally should not exceed 1% of total protein content. The following approach can serve as a guide in assessing the purity of antivenoms:

- SDS-PAGE under non-reducing conditions – this analysis provides qualitative (or, at best, semi-quantitative) information on the amounts of intact immunoglobulins, digestion products and, importantly, on the presence of high-molecular-mass oligomers (soluble aggregates) and low-molecular-mass contaminants (which are expected in the case of enzymatically digested antivenoms).
- SDS-PAGE under reducing conditions – analysis under these conditions can provide information on the amount of immunoglobulins and their fragments by direct visualization of intact and/or digested immunoglobulin heavy chains.

17.1.9 Molecular-size distribution

The presence of aggregates and other components in antivenoms can be assessed by size-exclusion liquid chromatography (gel filtration) in fast protein liquid chromatography (FPLC) or HPLC systems.

Densitometric analyses of chromatographic profiles allow the quantification of protein aggregates and of the relative abundances of: intact immunoglobulins, divalent immunoglobulin fragments (F(ab’)2, monovalent immunoglobulin fragments (Fab) and dimers, as well as low-molecular-mass enzymatic digestion products.

In intact immunoglobulin-based antivenoms this method allows quantitation of albumin, as its molecular mass (~66 kDa) can be resolved from the ~160 kDa peak of intact immunoglobulins.

17.1.10 Test for pyrogen substances

Antivenoms should comply with the rabbit pyrogen test where required by the local regulations. This test is based on intravenous injection of antivenoms in the ear vein of rabbits. The dose of antivenom must be calculated by dividing the threshold pyrogenic dose in rabbits by the endotoxin although other doses might
Annex 5

be used depending on the pharmacopoeia), followed by the measurement of rectal temperature at various time intervals after injection. The detailed procedures are described in various pharmacopoeias. Bacterial lipopolysaccharides can also be detected by the Limulus amoebocyte lysate (LAL) test. The test should be validated for each type of antivenom, since there have been reports of false-positive and false-negative reactions when testing antivenoms and other plasma-derived products. The sensitivity of this LAL test should be correlated with the rabbit pyrogen test, and the endotoxin limits established (135). When regulation allows, a validated LAL test is used in place of the rabbit pyrogen test.

17.1.11 Abnormal toxicity test

The abnormal toxicity test (7 day observation of the effects of intraperitoneal injection of 0.2 mL and 0.5 mL antivenom into mice and guinea-pigs, respectively), is still required by some pharmacopoeias and is performed at the stage of product development.

However, because of the very limited quality control value of this assay, it is increasingly being abandoned by most regulatory authorities. Correct implementation of GMP should provide evidence that the product would comply with the test for abnormal toxicity.

17.1.12 Sterility test

Antivenoms should be free of bacteria and fungi, that is, they should be sterile. The sterility test is performed following methodologies specified in various pharmacopoeias such as the European Pharmacopoeia.

Since antivenoms may contain preservatives in their formulation, it is necessary to "neutralize" the preservatives before the samples are added to culture media. This is usually done by filtering a volume of antivenom through a 0.45 µm pore-size membrane, and then filtering through the same membrane a solution that neutralizes the bacteriostatic and fungistatic effects of the preservatives used in antivenom. The membrane is then aseptically removed and cut into two halves. One half is added to trypticase soy broth and the other is added to thioglycolate medium. Control culture flasks are included for each medium. Flasks are incubated at 20–25 °C (trypticase soy broth) or at 30–35 °C (thioglycolate) for 14 days. Culture flasks are examined daily for bacterial or fungal growth. The number of vials tested per batch should be in compliance with local regulations.

17.1.13 Concentration of sodium chloride and other excipients

The concentration of the various excipients or stabilizers added during formulation should be determined using appropriate chemical methods.
17.1.14 **Determination of pH**
The pH of antivenom should be determined using a potentiometer.

17.1.15 **Concentration of preservatives**
Phenol concentration should not exceed 2.5 g/L and cresols 3.5 g/L.

Phenol concentration can be determined spectrophotometrically on the basis of the reactivity of phenol with 4-aminoantipyrine, under alkaline conditions (pH 9.0–9.2) in the presence of potassium ferrocyanide as oxidant. Other methods are also available. Phenol and cresols can be determined by HPLC methods.

17.1.16 **Chemical agents used in plasma fractionation**
The chemical reagents used in the precipitation and purification of antivenoms, such as ammonium sulfate, caprylic acid and others, should be removed from the final product during diafiltration or dialysis. Limits should be established and their residual amount quantified in the final product. Likewise, the elimination of pepsin or papain from the final preparations should be guaranteed, especially for preparations that are maintained in liquid form, to avoid proteolytic activity that may damage the antivenoms.

The determination of the residual amount of agents used in plasma fractionation could be excluded from routine release testing if the process of manufacturing has been validated to eliminate these reagents. The detection of residual reagents can also be performed on the final bulk rather than in the final product.

17.1.17 **Residual moisture (freeze-dried preparations)**
Residual moisture content can be determined by several methodologies, such as:

- a gravimetric method assessing the loss of weight on heating;
- the Karl-Fischer titration, based on the principle that iodine, together with pyridine, sulfur dioxide and methanol from the reagent react quantitatively with water;
- thermogravimetric methods.

The methodology most commonly recommended is the Karl-Fischer titration. Every manufacturing and quality control laboratory must establish the accepted maximum residual moisture for their antivenom ensuring the stability of the product over its claimed shelf-life. A residual moisture content of less than 3% is usually recommended for most freeze-dried therapeutic biological products.
17.2 **Antivenom reference preparations**

The use by manufacturers of in-house reference preparations of antivenoms, instead of national or regional standards, is recommended, since venom-neutralizing efficacy, specificity, and purity can only be compared with antivenoms of similar specificity and neutralizing profile. In-house reference antivenom preparations should be obtained from a suitable batch of a product that has been fully characterized and evaluated by the quality control laboratory. For assays not related to venom-neutralizing efficacy or specificity, such as the quantification of proteins, preservatives and excipients, national or regional standards can be used. The preparation of national or regional reference antivenom preparations should be undertaken by relevant national or regional drug control laboratories and regulatory agencies. These also require comprehensive characterization and evaluation by drug control laboratories and appropriate validation prior to acceptance and establishment.

17.3 **Main recommendations**

- Quality control of antivenom preparations, both for intermediate and final products, as part of the batch release, must be performed by the manufacturers and results disclosed in the documentation.
- Results from the following quality control tests need to be provided by the manufacturer as part of the batch release documentation:
  - (a) venom-neutralizing efficacy test against the most relevant venoms to be neutralized;
  - (b) identity test;
  - (c) protein concentration;
  - (d) purity of the active substance;
  - (e) content of protein aggregates and non-IgG contaminants;
  - (f) pyrogen test;
  - (g) sterility test;
  - (h) concentration of excipients;
  - (i) osmolality;
  - (j) pH;
  - (k) concentration of preservatives;
  - (l) determination of traces of agents used in plasma fractionation;
  - (m) appearance, and, for freeze-dried preparations, residual moisture and solubility; and
  - (n) labelling validation and confirmation.
Antivenom reference preparations reflecting specific characteristics of antivenoms produced should be prepared by each manufacturer to be used as standards in their laboratory settings, in particular to measure neutralization capacity of their specific antivenom products against targeted venoms. Relevant standards are also used to establish conformity of purity and integrity. When possible, a national reference antivenom should be established.

It is the ethical responsibility of the manufacturer to use only the minimum number of experimental animals to measure the efficacy of an antivenom.

The development of in vitro methods validated for replacing animal experiments is strongly encouraged.

18. Stability, storage and distribution of antivenoms

18.1 Stability

Stability studies should be performed to determine the stability of antivenoms. These studies should be done when a new product, a process change, or a new formulation is developed. They are essential to define the shelf-life of the product and are intended to prove that the antivenom remains stable and efficacious until the expiry date. During the developmental stages, stability studies should be included. Their design should take into consideration that they are a complex set of procedures involving considerable cost, time and expertise necessary to build in quality, efficacy and safety of a product.

Most liquid antivenom preparations have a shelf-life of up to 3 years when stored refrigerated at 2–8 °C, whereas freeze-dried antivenoms have shelf-lives of up to 5 years when kept in the dark at room temperature. It is essential, however, that manufacturers determine the actual stability of each antivenom formulation under appropriate conditions using validated methodologies. It is also highly recommended that manufacturers perform stability studies to evaluate the possibility that their preparations could be stored for a long period without refrigeration (for instance at 30 °C).

Real-time stability tests should be performed under the expected storage conditions of the antivenom. In addition, these tests could be performed under worst-case storage conditions. Quality control parameters are determined at regular pre-established time intervals, normally extending the test period in order to allow significant product degradation under recommended storage conditions. Essential parameters include venom neutralization potency, turbidity and content of aggregates, among others, since these are especially prone to alter upon storage.
Accelerated stability studies may be performed to provide early useful information on the product stability profile, but are not a substitute for real-time data. In accelerated studies, the antivenom is exposed to harsher conditions than usual, such as a higher temperature, and the stability is assessed over a shorter time span. This is done to assess the conditions that accelerate degradation of the product and this information is then used to predict shelf-life.

Retained sample stability testing is usual practice for every product for which stability data are required. In such a study, retained samples from at least one batch a year are selected and tested at predetermined intervals – that is, if a product has shelf-life of 5 years, it is conventional to test samples at 3, 6, 9, 12, 18, 24, 36, 48, and 60 months.

Cyclic temperature stress testing is not routinely performed, but it may be useful since it is designed to mimic likely conditions in field place storage. It is recommended that the minimum and maximum temperatures are selected on a product-by-product basis and taking into account factors like recommended storage temperatures as well as specific chemical and physical degradation properties.

18.2 Storage
Antivenoms should be stored at a temperature within the range that assures stability, as found by stability tests. This is particularly critical for liquid formulations, which usually require storage at between 2 and 8 °C. Therefore, deviations from this temperature range, due to interruptions in the cold chain during transportation or storage, are likely to result in product deterioration. The design of adequate cold chain programmes, as part of the public health systems in every country, is critical, and national protocols should be developed. The distribution policies for national vaccination programmes can be adopted for the transportation and storage of antivenoms. The stability of liquid preparations at temperatures higher than 2–8 °C should be evaluated and, if needed, new formulations allowing such storage conditions should be developed.

18.3 Distribution
Adequate distribution of antivenoms is a matter of great concern in many regions of the world. Since most of the antivenoms available are liquid preparations, the maintenance of an adequate cold chain must be guaranteed, despite the difficulties to be encountered in rural areas of some developing countries. National and regional health authorities should develop distribution strategies to ensure that antivenoms are allocated to the areas where they are needed or use the distribution channels in place for other national primary health-care programmes. Both the specificity of the antivenom and the number
of vials or ampoules to be distributed should be taken into consideration. This is particularly relevant in countries that use monospecific antivenoms, since distribution of these products should be guided by the known distribution of the species and the epidemiological data. To ensure an appropriate supply for clinical use, inventories should be in excess of the estimated number of cases, to allow for unpredictable surges in local demand, accepting that some antivenoms will not have been used before their expiry date.

18.4 **Main recommendations**

- The quality control of each antivenom batch prepared by a manufacturer should include the potency test for neutralization of lethality (ED$_{50}$).
- In general, liquid preparations require a cold chain, whereas freeze-dried preparations do not. However, storage conditions are product- or formulation-specific and may vary. Manufacturers should therefore determine the stability of each antivenom pharmaceutical preparation by conducting real-time stability studies.
- Manufacturers should study the stability of antivenoms at the ambient temperatures in the areas where the product will be used.
- The distribution of antivenoms by health authorities should rely on a proper assessment of the epidemiology of snake-bite envenomings, and on the proper knowledge of the geographical distribution of the most relevant venomous species. This is particularly important for monospecific antivenoms.
- NRAs should ask manufacturers to provide information obtained from the preclinical assessment of all antivenom used in their territories against the venoms found in the region or country where the product is intended to be used.

19. **Preclinical assessment of antivenom efficacy**

Efficacy testing of antivenoms is one of a suite of assessments required for the quality control of antivenoms (see section 17 where further quality control tests are described) performed for each new batch. Efficacy testing of antivenoms is also part of the preclinical programme to be performed for new antivenoms, where the respective data are used for licensing or registration of antivenoms by regulatory agencies. The details of efficacy testing in the preclinical phase of antivenoms and for quality control purposes are described below. The testing of antivenoms on animals raises important ethical considerations (section 4) and it is essential that manufacturers and others apply the highest standards of ethical
conduct, including appropriate 3R steps, and use of analgesia or anaesthesia for the minimization of pain and discomfort.

It is a fundamental regulatory and ethical requirement that all new therapeutic agents for human use are tested for their safety and efficacy – initially by in vitro LD$_{50}$ laboratory tests and then in vivo ED$_{50}$ preclinical tests and, if the results of these prove satisfactory, by clinical trials in human patients. The preclinical efficacy tests must therefore be performed on new antivenoms, and newly manufactured batches of existing antivenoms.

19.1 Preliminary steps that may limit the need for animal experimentation

To prevent unnecessary animal use, careful perusal of existing literature for data on venom lethality may help to refine the experimental design and thereby reduce the number of experimental animals required.

Manufacturers may also investigate the immunological venom-binding capability of an antivenom by performing immunological assays (for example, ELISA, to identify, and exclude from experimentation, antivenoms that do not possess the requisite titre of venom-binding immunoglobulins. It is crucial to note, however, that: (a) a high venom-binding titre in an ELISA result for an antivenom cannot be used to infer venom-neutralizing efficacy; and (b) the failure of an antivenom to bind venom in an ELISA result suggests very strongly that the antivenom should be considered ineffective at neutralizing the effects of that venom – and withdrawn from ED$_{50}$ testing. This step can further limit non-productive animal experimentation. There is no single ELISA metric that enables stop/go decisions to be made for all the possible snake venom and antivenom combinations. These will therefore be in-house decisions.

An additional immunological cross-reactivity technology that can inform the preclinical assessment process before animal experiments are undertaken is the use of a proteomics-centred platform, termed antivenomics, which has been developed to assess the immunological reactivity of antivenoms against homologous and heterologous venoms (136–139). Antivenomics complements the in vitro and in vivo venom activity neutralization assays and substitute for the traditional, essentially qualitative, immunological methods, such as ELISA and Western blotting. Antivenomics uses an affinity chromatography approach to investigate the immuno-capturing ability of immobilized IgG, F(ab’)$_2$, or Fab antibody molecules followed by the proteomic identification of the venom components recovered in both the retained and the non-bound fractions. The fraction of non-immuno-captured protein “i” (%NRi) is estimated as the relative ratio of the chromatographic areas of the same protein recovered in the non-retained (NRi) and retained (Ri) affinity chromatography fractions using the equation:
\[
\% \text{ NRi} = 100 - \left( \frac{\text{Ri}}{\text{Ri} + \text{NRi}} \right) \times 100
\]

The antivenomic analysis provides both qualitative and quantitative information on the types of venom proteins presenting antivenom-recognized epitopes and those exhibiting impaired immunoreactivity. Although the level of immune recognition gathered from antivenomics should not be absolutely relied upon to predict the in vivo neutralization capacity of an antivenom (since both experiments involve radically different protocols), an immuno-capture capability of \( \geq 25\% \) generally correlates with a good outcome in homologous in vivo neutralization tests. If immuno-capture by this method is \(< 25\% \) the further testing of an antivenom using in vivo methods should be reconsidered.

As the degree of immuno-recognition of a given toxin by the immunoglobulins present in antivenom represents a measure of the capability of that particular antivenom to neutralize the toxic activity of that toxin, the antivenomics analysis may assist in assessing the range of clinical applications of current commercial or experimental antivenoms, and in the development of improved antivenoms on an immunologically sound basis. Growing evidence shows the potential of the combination of antivenomics and neutralization assays for analysing at the molecular level the preclinical efficacy of antivenoms against homologous and heterologous venoms. This is particularly so where antivenoms from more than one manufacturer, or from more than one batch of antivenom produced by a manufacturer require preclinical evaluation against the same venoms (136, 140).

Manufacturers should take steps to incorporate these approaches into their preliminary screening of antivenoms before in vivo animal testing is conducted.

19.2 **Essential preclinical assays to measure antivenom neutralization of venom-induced lethality**

These tests determine the capability of an antivenom to neutralize the lethal effect of the snake venom(s). It is first necessary to determine the lethal potency of the venom using the LD_{50} assay. The exact volume of antivenom, or the venom/antivenom ratio, required to neutralize venom lethality can then be determined using the antivenom effective dose (ED_{50}) assay.

Preclinical testing of antivenom is required:

- for routine quality control of efficacy of each newly manufactured batch of an existing antivenom;
- to test the ability of a new antivenom to neutralize the lethal effects of venoms from snakes from the country or region where it is going to be introduced;
to test the ability of an existing antivenom to neutralize the lethal effects of venoms for which no prior ED$_{50}$ data exist (for example, prior to introducing an antivenom to treat envenoming in a new geographical region or country).

The outputs of these tests provide globally applicable standard metrics of venom lethality and antivenom efficacy. This enables internal/manufacturer monitoring and external/independent auditing of antivenom efficacy – thereby providing manufacturers and NRAs worldwide with a standardized mechanism for preventing the distribution of dangerously ineffective antivenom.

Consistent use of venom standards and outbred strains of mice of a defined weight range (for example, 18–20 g), are recommended for all the assays. Some producers use other test animals, such as guinea-pigs. While weights will clearly vary between animal species, the following principles, specified for mice, will still apply to these alternative test animals. It should be borne in mind that there are variations in the susceptibility of various strains of mice to the lethal effect of venoms.

19.2.1 LD$_{50}$ range-finding test
For venoms whose LD$_{50}$ is unknown, it is recommended that a range dose-finding study, using one mouse per venom dose, is performed to set a narrow range of dose parameters for the full LD$_{50}$ test – reducing the total number of animals required. Range-finding tests are not required for venoms from validated suppliers where the lethal potency across successive batches of venom is established. If venom is sourced from a new supplier, re-establishment of LD$_{50}$ should be performed.

Various venom doses are prepared using saline as diluent, and aliquots of a precise volume (maximum 0.2 mL) of each dose are injected, using one mouse per dose, by the intravenous route, in the tail vein (or, alternatively, by the intraperitoneal route (using injection volumes of maximum 0.5 mL)). Deaths are recorded at 24 hours (intravenous test) or at 48 hours (intraperitoneal test). On the basis of this preliminary dose-finding experiment, a range of venom doses causing 0% to 100% lethality is established and thus narrows the range of venom doses required for the full venom LD$_{50}$ assay.

19.2.2 The LD$_{50}$ assay
Venom doses are prepared in saline and intravenously injected (maximum 0.2 mL) into the tail vein of groups of 5–6 mice (of a defined weight range). A group size of five mice is the smallest number recommended for obtaining a statistically significant result. In some laboratories the LD$_{50}$ is estimated by the intraperitoneal route using an injection volume of a maximum of 0.5 mL.
Deaths are recorded at 24 hours (for assays involving intravenous injections) or at 48 hours (when intraperitoneal injections are used), and \( \text{LD}_{50} \) is estimated by Probit analysis (141), Spearman-Karber (11) or alternative procedures (such as non-parametric methods). One venom \( \text{LD}_{50} \) dose is defined as the amount of venom causing death in 50% of injected mice.

19.2.3  **Antivenom efficacy assessment**

The venom \( \text{LD}_{50} \) results provide the information necessary to test the venom-neutralizing efficacy of an antivenom – using the median effective dose (ED\(_{50}\)) assay. It is important that the venoms used in the ED\(_{50}\) assays are from the same batch (lot) as that used to determine the venom \( \text{LD}_{50} \) result. It is equally important that all the \( \text{LD}_{50} \) and ED\(_{50}\) assays utilize mice of identical strain and weight.

19.2.3.1  **ED\(_{50}\) range-finding test**

For an antivenom whose ED\(_{50}\) against a specific venom is unknown, it is recommended that a range dose-finding study, using one mouse per venom dose, is performed to set a narrow range of dose parameters for the full ED\(_{50}\) test – reducing the total number of animals required. Range-finding tests are not required for an antivenom whose venom-neutralizing efficacy is established.

The selected multiple of the venom \( \text{LD}_{50} \) (3–6 \( \text{LD}_{50} \)) is mixed with different doses of antivenom, incubated at 37 °C for 30 minutes, and each mixture then intravenously injected into a single mouse. This preliminary test establishes a range of antivenom volumes that result in 100% survival and 100% death of the injected mice and thus narrows the range of doses required for the formal antivenom ED\(_{50}\) test.

19.2.3.2  **The median effective dose (ED\(_{50}\)) assay**

A fixed amount of venom (“challenge dose”, usually corresponding to 3–6 \( \text{LD}_{50} \)) is mixed with various volumes of the antivenom and adjusted to a constant final volume with saline (3, 142, 143). The mixtures are incubated for 30 minutes at 37 °C, and then aliquots of a precise volume (maximum 0.2 mL intravenously; maximum 0.5 mL intraperitoneally) of each mixture are injected into groups of 5–6 mice\(^{10}\) (of the same strain and weight range used for the LD\(_{50}\) assay). A control group injected with a mixture of the venom “challenge dose” with saline solution alone (no antivenom) should be included to confirm that the venom “challenge dose” induces 100% lethality. Centrifugation of the antivenom–venom

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\(^{10}\) Ten mice may be needed for some venoms.
mixtures before use is not recommended because residual venom toxicity may remain in the immuno-precipitate.

After injection, deaths are recorded at 24 hours (intravenous test) or at 48 hours (intraperitoneal test) and the results analysed using Probit analysis, Spearman-Karber or alternative procedures (such as non-parametric methods). One antivenom ED\textsubscript{50} dose is defined as the amount of antivenom, or the venom/antivenom ratio, resulting in the survival of 50% of mice injected with a mixture of antivenom and a lethal quantity of venom.

The ED\textsubscript{50} result can be expressed in various ways:

- mg of venom neutralized by mL of antivenom;
- µL antivenom required to neutralize the “challenge dose” of venom used;
- µL of antivenom required to neutralize 1 mg of venom.

The practice of defining the ED\textsubscript{50} by the number of murine LD\textsubscript{50}s of venom neutralized per mL of antivenom is inaccurate and has little clinical usefulness. Since LD\textsubscript{50} values for the same venom may vary from one manufacturer to another, this representation of ED\textsubscript{50} should be avoided in favour of one of the approaches listed above.

19.2.4 General recommendations

Before any antivenom is used therapeutically in humans, its efficacy against the relevant snake venoms should be confirmed in the essential preclinical LD\textsubscript{50} and ED\textsubscript{50} assays. Where minimum standards for venom-neutralizing efficacy exist in geographically relevant pharmacopoeias, or have been established by NRAs, these requirements must be met.

In some regions, no minimum acceptable levels of therapeutic efficacy that are clinically relevant to human envenoming have been established that take into account the need to deliver a therapeutic dose of antivenom in a realistic volume for administration. In such cases, NRAs in consultation with other organizations should establish such standards as a matter of priority for the various antivenoms produced or distributed in these jurisdictions.

The essential tests of preclinical efficacy, the venom LD\textsubscript{50} and antivenom ED\textsubscript{50}, should be standardized by NRAs and national quality control laboratories, and common protocols adopted to avoid variation in methodology between production facilities. Therefore, manufacturers should disclose details of their ED\textsubscript{50} protocol to the corresponding NRA as part of the licensing or registration application to demonstrate compliance.

Quality control laboratories need to establish national reference venom collections (venoms representing the taxonomic and geographical range of
snake species in a country), and these must periodically be independently evaluated to ensure that they have not deteriorated (see section 10 on quality control of venoms).

19.3 Supplementary preclinical assays to measure antivenom neutralization of specific venom-induced pathologies

Snake venoms generate a wide range of systemic pathologies, including a variety of haemostasis-disruptive (haemorrhage, pro- and anti-coagulopathic effects), neurotoxic, myotoxic, nephrotoxic and cardiac effects. Supplementary tests are therefore recommended for new antivenoms and for new applications of existing antivenoms to determine whether they are effective in eliminating the most clinically relevant pathophysiological effects induced by the specific venom(s) of interest.

For example, a new antivenom developed against Echis ocellatus envenoming should, in addition to preclinical LD$_{50}$ and ED$_{50}$ testing, be tested for its ability to eliminate venom-induced coagulopathy and haemorrhage – the most medically important effects of envenoming by *E. ocellatus*.

In this context it may be useful to consider that postmortem observations of mice from LD$_{50}$ and associated ED$_{50}$ experiments can provide a wealth of pathophysiological information as to antivenom neutralization of venom-induced pathology. Postmortem observations from LD$_{50}$ and associated ED$_{50}$ experiments may prove useful in reducing the need for and frequency of some of the supplementary assays recommended here; however, their use requires the same degree of scientific and procedural validation as other supplementary preclinical assays.

These supplementary preclinical tests are outlined below.

19.3.1 Neutralization of venom haemorrhagic activity

Many venoms, especially those of vipers, exert powerful local and systemic haemorrhagic activity effected primarily by snake venom zinc-dependent metalloproteinases. These enzymes damage the basement membrane that surrounds the endothelial cells of capillaries resulting in bleeding into the tissues. Bleeding into the brain and other major organs is considered to be the major lethal effect of envenoming by many viperid species (144). The minimum haemorrhagic dose of a venom (MHD) quantifies this venom-induced pathology, and is defined as the amount of venom (in µg dry weight) which, when injected intradermally, induces in mice a 10 mm haemorrhagic lesion after a predefined time interval, usually 2–3 hours, after injection (145, 146).

The venom MHD test is carried out by preparing aliquots of 50 µL of physiological saline solution containing a range of venom doses. Mice (18–20 g
body weight; five mice per group) are held securely and the hair surrounding the injection site is shaved. Venom solutions (50 µL) are injected intradermally into the shaved skin of lightly anaesthetized mice. After a defined time interval (usually 2–3 hours), mice are killed using an approved humane procedure, the area of the injected skin is removed, and the size of the haemorrhagic lesion in the inner side of the skin is measured using calipers in two directions with background illumination. Care should be taken not to stretch the skin. The mean diameter of the haemorrhagic lesion is calculated for each venom dose and the MHD estimated by plotting mean lesion diameter against venom dose and reading off the dose corresponding to a 10 mm diameter (145, 146).

The assay measuring the efficacy of antivenom to neutralize venom-induced haemorrhage is termed the MHD-median effective dose (MHD$_{50}$), and is defined as the volume of antivenom, in microlitres, or the venom/antivenom ratio, which reduces the diameter of haemorrhagic lesions by 50% when compared with the diameter of the lesion in animals injected with the control venom/saline mixture (146). A “challenge dose” of venom is selected – between one and five venom MHDs have been used as the challenge dose by different laboratories. The test is carried out as above, using five mice per group. Mixtures of a fixed amount of venom and various dilutions of antivenom are prepared so that the challenge dose of venom is contained in 50 µL. Controls must include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 minutes, and aliquots of 50 µL are injected intradermally in lightly anaesthetized mice. The diameter of haemorrhagic lesions is quantified as described above, and the neutralizing ability of antivenom is expressed as the MHD$_{50}$.

19.3.2 Neutralization of venom necrotizing activity

Venom-induced local dermonecrosis is a major problem in humans bitten by snakes and it has long been considered important to have an assay system to evaluate the effect of an antivenom on this pathology. However, the value of antivenoms in overcoming the cytolytic effects of venoms has not yet been established; indeed, there is considerable doubt as to whether antivenom is useful in obviating such effects in human victims of snake-bite. This is because venom-induced dermonecrosis occurs quickly after a bite and there is usually a considerable delay between the envenoming of a victim and his or her arrival in hospital for treatment. Consequently, antivenom therapy can have little or no effect in reversing the damage (147, 148). Animal experiments in which the antivenom was administered to the test animal at different times after the venom support this opinion (148–150). The minimum necrotizing dose (MND) of a venom is defined as the smallest amount of venom (in µg dry weight) which, when injected intradermally into groups of five lightly anaesthetized
mice (18–20 g body weight), results in a necrotic lesion of 5 mm diameter 3 days later. The method used is the same as that for the MHD, except that the skin is examined 3 days after the intradermal injection of the venom (145).

The assay measuring the ability of an antivenom to neutralize venom-induced dermonecrosis is termed the MND-median effective dose (MND$_{50}$), and is defined as the volume of antivenom, in microlitres or the venom/antivenom ratio, which reduces the diameter of necrotic lesions by 50% when compared with the diameter of the lesion in mice injected with the control venom/saline mixture. A challenge dose of venom is selected, usually between one and two MNDs. The test is carried out as above, using five mice per group. Mixtures of a fixed concentration of venom and various dilutions of antivenom are prepared so that the venom challenge dose is contained in 50 µL. Controls include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 minutes, and aliquots of 50 µL are injected intradermally in lightly anaesthetized mice (151, 152). The diameter of dermonecrotic lesions is quantified 3 days post-injection, as described above, and the neutralization by antivenom, expressed as the MND$_{50}$.

19.3.3 Neutralization of venom procoagulant effect

Many venoms, especially from some vipers, cause consumption of coagulation factors, which results in incoagulable blood. This, combined with the haemorrhagic nature of some of these venoms, can result in a very poor prognosis for severely envenomed patients. Simple in vitro methods exist to measure this venom-induced pathophysiological effect and the ability of an antivenom to eliminate it. The minimum coagulant dose (MCD) of a venom is defined as the smallest amount of venom (in mg dry weight per litre of test solution or µg/mL) that clots either a solution of bovine fibrinogen (2.0 g/L) in 60 seconds at 37 °C (MCD-F) and/or a standard citrated solution of human plasma (fibrinogen content 2.8 g/L) under the same conditions (MCD-P).

For measurement of the MCD-F, 50 µL of physiological saline with final venom concentrations ranging from 240 to 0.5 mg/L is added to 0.2 mL of bovine fibrinogen solution (2.0 g/L) at 37 °C in new glass clotting tubes. The solutions are mixed thoroughly and the clotting time recorded. The MCD-P is estimated by adding the same venom concentrations to 0.2 mL of the standard citrated human plasma solution under identical conditions and recording the clotting time. In each case, the MCD is calculated by plotting clotting time against venom concentration and reading off the level at the 60 second clotting time (145).

To estimate the ability of an antivenom to neutralize venom procoagulant activity, a challenge dose of venom is selected, which corresponds to one MCD-P or one MCD-F. Mixtures of a fixed concentration of venom and various dilutions
of antivenom are prepared so that the challenge dose of venom is contained in 50 µL. Controls include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 minutes, and aliquots of 50 µL are added to 0.2 mL of plasma or fibrinogen solution, as described. The formation or absence of clots is observed during a maximum period of 30 minutes. The minimum volume of antivenom, or venom/antivenom ratio that completely prevents clotting is estimated and is known either as the MCD-F-effective dose (MCD-F\textsubscript{100}) or MCD-P-effective dose (MCD-P\textsubscript{100}).

19.3.4 **Neutralization of in vivo venom defibrinogenating activity**

This test is a direct measure of the in vivo defibrinogenating effect of certain venoms. To measure the minimum venom defibrinogenating dose (MDD), a wide range of venom doses is selected and each dose, in a volume of 0.2 mL, is injected intravenously into five mice (18–20 g body weight). One hour after injection, the mice are placed under terminal general anaesthesia and bled by cardiac puncture. The blood from each animal is placed in a new glass clotting tube, left at room temperature for 1 hour and the presence or absence of a clot recorded by gently tilting the tube. The MDD is defined as the minimum dose of venom that produces incoagulable blood in all mice tested within 1 hour of intravenous injection.

Antivenom neutralization of the venom component(s) responsible for in vivo defibrinogenation is estimated by incubating a challenge dose of venom, corresponding to one or more MDD, with different dilutions of the antivenom. Controls should include venom solutions incubated with saline solution instead of antivenom. Mixtures are incubated at 37 °C for 30 minutes before injection of 0.2 mL by the intravenous route in groups of five mice (18–20 g body weight). After 1 hour, mice are bled as described above, and the blood is placed in new glass clotting tubes and left undisturbed for 1 hour at room temperature, after which the presence or absence of a clot is recorded. Neutralizing ability of antivenoms is expressed as MDD-effective dose (MDD\textsubscript{100}), corresponding to the minimum volume of antivenom, or venom/antivenom ratio at which the blood samples of all injected mice showed clot formation (152, 153).

19.3.5 **Neutralization of venom myotoxic activity**

The presence of myotoxic components in a venom results in the degeneration of skeletal muscle by myonecrosis of muscle fibres. Damage is characterized by the disruption of the muscle cell plasma membranes, myofilament hypercontraction, local infiltration of inflammatory cells and oedema. Myotoxicity is characterized by the appearance of myoglobin in urine and by increments in the serum levels of muscle-derived enzymes, such as creatine kinase (CK). Myotoxic phospholipase A2 (PLA2) enzymes are found in a wide range of snake venoms. Some of these
PLA2s may be primarily myotoxic, or neurotoxic or both. Cytotoxic proteins of the three-finger toxin family present in some elapid venoms also cause myonecrosis. In addition, myotoxicity may occur as a consequence of ischaemia induced in muscle fibres by the effect of haemorrhagic venom components in the microvasculature (154).

Venom myotoxic activity is determined by injecting mice with various doses of venom in a constant volume of 50 µL (using saline solution as diluent) into the right gastrocnemius muscle. Groups of five animals of 18–20 g body weight are used per dose. Control animals are injected with the same volume of saline solution. Tail-snip blood samples are collected after a specified time interval (3 hours in mice), and the CK activity of serum or plasma is determined using commercially available diagnostic kits (155, 156). Myotoxic activity is expressed as the minimum myotoxic dose (MMD), defined as the amount of venom that induces an increment in serum or plasma CK activity corresponding to four times the activity in serum or plasma of animals injected with saline solution alone. Myotoxicity can also be assessed by histological evaluation of muscle damage after venom injection, although this is a more expensive and more time consuming method than the CK determination.

To estimate the ability of an antivenom to neutralize venom myotoxicity, a challenge dose of venom is selected, which corresponds to 3 MMDs. The test is carried out as above, using five mice per group. Mixtures of a fixed concentration of venom and various dilutions of antivenom are prepared so that the challenge dose of venom is contained in 50 µL. Controls include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 minutes, and aliquots of 50 µL are injected into the gastrocnemius muscle, as described above. Blood samples are collected 3 hours after injection (in the case of mice) and serum or plasma CK activity is quantified. The neutralizing ability of antivenom, expressed as MMD-median effective dose (MMD<sub>50</sub>) is estimated as the volume of antivenom in microlitres, or the venom/antivenom ratio, which reduces the serum or plasma CK activity by 50% when compared to the activity in animals injected with venom incubated with saline solution only (143).

19.3.6 Neutralization of venom neurotoxic activity

Several laboratory methods for assessing venom-induced neurotoxicity have been developed (for example, chick biventer cervicis nerve-muscle preparation (157, 158) and the mouse hemidiaphragm phrenic nerve preparation (2, 159–162). However, they are difficult to perform, require costly equipment and technological expertise and are unlikely to be practicable for most antivenom producers. Mouse lethality tests are usually reliable in predicting the neutralization of neurotoxic effects of venoms.
19.4 Limitations of preclinical assays

It is acknowledged that the in vivo and in vitro essential and supplementary preclinical tests have physiological limitations. Venom and venom/antivenom injection protocols do not represent the natural situation, and the physiological responses of rodents to envenoming and treatment may differ from those of humans. Even comparing the levels of immune recognition gathered from antivenomic or ELISA data with the in vivo neutralization capacity of an antivenom, is not straightforward. Such limitations make the rodent model of human envenoming and treatment less than ideal. Care should therefore be taken to avoid simplistic extrapolations from this assay to the clinical situation. Nevertheless, the LD\(_{50}\) and ED\(_{50}\) tests represent the methods most widely used for assessment of antivenom potency, and a number of clinical trials have demonstrated that the ED\(_{50}\) test is useful (2, 163), but not infallible (164, 165), at predicting the efficacy of antivenoms in the clinical setting. In some cases, it is recommended to test the ability of antivenoms to neutralize pathophysiologically relevant effects other than the lethal effect. Examples include the neutralization of haemorrhagic and in vitro coagulant effects in the case of *Echis* sp. venoms, and of dermonecrotizing effect in the case of cytotoxic *Naja* sp. venoms. An additional value of these tests is the assurance that antivenoms are manufactured with an accepted, quantifiable and uniform neutralizing potency.

19.5 Main recommendations

- The estimation of the ability of an antivenom to neutralize the lethal activity of venom(s) (LD\(_{50}\) and ED\(_{50}\)) is a critical preclinical assessment and should be performed by manufacturers for all antivenoms, and enforced by the NRAs as part of the antivenom licensing procedure.
- All practitioners of these preclinical tests must prioritize the implementation of 3R to reduce the substantial number of mice used, and their collective pain, harm and distress.
- In vitro methods such as ELISA, antivenomics or other emerging technologies that enable antivenoms to be screened for immune recognition of venom components prior to in vivo evaluation should be adopted by manufacturers.
- The development of improved in vivo assay protocols to reduce pain and suffering of animals, such as routine use of opioids or other analgesics, and of in vitro alternatives to the in vivo assays to reduce the number of animals used in preclinical testing, is encouraged. The results of any modified in vivo, or new in vitro
protocols, should be rigorously compared with results from existing protocols and validated to ensure statistical reliability of the newly developed methods.

- All new antivenoms, as well as existing antivenoms to be used in new geographical areas, should furthermore be assessed for their ability to eliminate specific pathologies caused by the venoms of the snakes for which the antivenom has been designed.
- The selection of which preclinical supplementary test(s) to perform will depend on the predominant pathophysiological effects induced by the specific snake venom and be appropriately adapted for each antivenom. These supplementary tests are not required for quality control assessment of subsequent batches of antivenom.

20. Clinical assessment of antivenoms

20.1 Introduction

Antivenoms are unusual among pharmaceutical agents in that they have been used in human patients since 1896 with little attention being paid to clinical trials of their effectiveness and safety. However, since the 1970s it has been clearly demonstrated that it is possible to carry out dose-finding and randomized controlled trials (RCTs) in human victims of snake-bite envenoming. These studies have yielded invaluable information, as in the case of clinical trials of other therapeutic agents for which clinical trials are generally regarded as the essential basis for regulatory approval.

The conduct of clinical studies is guided by the principles set down in the international regulations governing good clinical practice,\(^\text{11}\) comprising European Union, United Kingdom and USA regulations, summarized in ICH Topic E 6 (R1) Guideline for good clinical practice (GCP), an international ethical and scientific quality standard for designing, conducting, recording and reporting trials that involve the participation of human subjects.\(^\text{12}\) These principles emphasize the responsibilities of the researcher and of the organization sponsoring the research to protect participants in the research and to ensure that the conduct of the trial is likely to lead to reliable results.


Clinical trials should be registered with an appropriate registration body, before they commence.\(^{13}\)

The conventional pathway for clinical evaluation of new therapeutic products is:

- Phase I: healthy volunteer studies – detection of unanticipated adverse events;
- Phase II: limited effectiveness and safety studies, often dose-finding;
- Phase III: full-scale clinical evaluation, often using blinded RCTs to avoid potential introduction of bias;
- Phase IV: post-marketing surveillance.

The appropriateness of this pathway for antivenoms depends upon a number of factors, including whether an antivenom is new or has been previously used in human patients, the ethical basis for the study, the trial’s practicability as well as ethical and national regulatory considerations. So far, most antivenoms have been registered without prior clinical studies. This situation should not persist: it is desirable, first, to collect the existing clinical data on antivenoms already marketed, and second to promote Phase II or III clinical trials before registering new antivenoms. In the absence of clinical data for antivenoms already in use, appropriate clinical trials should be quickly implemented.

20.1.1 **Identification of biting species in clinical studies of antivenoms**

It is absolutely essential that all clinical studies of antivenom effectiveness or safety, including clinical trials incorporate robust methodologies for ensuring the identification of the biting species. This can be achieved through:

- expert identification of the dead snake responsible for the bite, or of a photographic image of that snake; or
- the identification of specific venom components unique to particular species (from bite site swabs, wound exudate, serum or urine samples) through the use of EIAs (ELISA) or other immunological methods.

Failure to properly identify the species of snakes that are responsible for cases of envenoming included in clinical trials and other studies of snake antivenoms significantly diminishes the value of the research, and renders the

results unreliable. NRAs should be cautious about accepting the results of clinical evaluations of antivenom where robust, reliable identification of the biting species is not available.

20.1.2 **Phase I studies**

Conventional clinical studies using healthy volunteers are not appropriate in the case of antivenoms\(^\text{14}\) because of the risk of anaphylactic and other reactions (for example, pyrogenic or serum sickness and, rarely, hypersensitivity reactions to equine or ovine plasma proteins) and the risk of sensitization to equine or ovine plasma proteins in the volunteers. Phase I studies are primarily designed to detect unanticipated adverse reactions. This can be done only in human subjects as it is not possible in an animal model. Such studies are an essential protection against severe and even fatal effects of a new medication, before it is tested in the much larger numbers of subjects demanded for Phase II and III studies. Recent disasters or near disasters during Phase I studies of new therapeutic monoclonal antibodies have emphasized not only the need for such studies but also their potential dangers. A similar situation exists in the early testing of cytotoxic drugs and antibodies used in oncology. A preliminary open-label dose-finding study can establish both the effectiveness and safety of an initial dose of a new antivenom in small groups of adult, non-pregnant patients with systemic envenoming, but excluding those with features of severe envenoming. The aim is to assess clinical safety and effectiveness, as a prelude to full-scale Phase II or III RCTs. This modified Phase I approach can be combined with a preliminary dose-finding study. The “3 + 3” dose-escalation design (166) can be used to determine the minimum dose capable of achieving a defined end-point in two-thirds or more of a small group of patients. An additional stopping rule is added to ensure that patients are not exposed to doses likely to cause reactions in more than one-third of them – for details see Abubakar et al., 2010 (94). Currently, there is no alternative for ethical Phase I studies.

20.1.3 **Phase II and III studies**

Phase II studies are usually conducted to optimize doses, establish or confirm the relative safety of a product and give an indication of effectiveness. Phase III studies are normally used to establish effectiveness of a product, often in comparison with an existing product, or occasionally a placebo. Since antivenoms are so well established in the treatment of snake-bite envenoming, the use of placebo controls is ethically acceptable only where there is genuine uncertainty

\(^{14}\) Immunoglobulins derived from animal plasma.
about whether the benefit (degree of clinical improvement) from the antivenom outweighs the risk (potential incidence and severity of adverse events). Depending on the speed of evolution of envenoming, immediate treatment might be compared to delayed antivenom treatment. A new antivenom with demonstrable preclinical potency (see above) can be compared with an established product, or two markedly different initial doses or regimens of the same antivenom can be compared. In RCTs, non-inferiority, rather than superiority of a new antivenom or regimen, compared to an existing treatment, requires smaller numbers of trial participants to achieve acceptable power (94). Basic requirements for any clinical antivenom RCT are that the participants should be reasonably homogeneous as far as the species of snake responsible and their pretreatment characteristics (for example, interval between bite and treatment) are concerned, and that objective clinical end-points should be selected to judge effectiveness, and measure rates of adverse events.

20.1.4 Phase IV studies

Phase IV studies are clinical surveillance studies that occur after market authorization of the product. In view of the difficulty in performing standard clinical trials of antivenom in some situations, this may be the only way to study safety and effectiveness of an antivenom in a large number of patients. In practice, such studies have rarely if ever been attempted for antivenoms, but they are strongly recommended for the future.

20.2 Clinical studies of antivenom

Although preclinical testing may be valuable in ensuring that antivenoms neutralize the venoms of interest, the complex effects of venoms in humans and the need to consider venom pharmacokinetics mean that, ultimately, the effectiveness and safety of antivenoms for the treatment of human envenoming can only be determined by well-designed clinical studies. Clinical studies of antivenoms primarily address three main issues:

- assessment of the optimal initial dose of antivenom;
- assessment of effectiveness of the antivenom;
- assessment of the safety of an antivenom, particularly the incidence and severity of early and late reactions.

Antivenom safety and tolerance depend on manufacturing factors (immunoglobulins composition, purification of immunoglobulin fragments, protein concentration, and presence of preservatives) (167). Consequently, incidence and severity of adverse reactions to similar doses of a given batch of antivenom are unlikely to vary in different geographical locations. Conversely,
the effectiveness depends on both manufacturing factors (choice of venoms, immunological titre) and circumstantial factors (quality and quantity of inoculated venom, patient’s physical condition, delay of treatment, etc.). However, following initial preclinical testing, both effectiveness and dose-finding studies may need to be repeated for a new geographical location, depending upon the similarity of the snake species in the new location with those where the antivenom was initially tested. If the species are similar, if preclinical testing indicates good neutralization, and if evidence of clinical effectiveness has been reported in other places, post-marketing surveillance studies may be adequate.

20.2.1 Dose-finding studies
Dose-finding studies seek to establish the optimum initial dose of an antivenom required to control envenoming in patients with different severities of envenoming. The therapeutic dose of an antivenom administered by the intravenous route depends on:

- the quantity of venom injected (assessed by clinical and laboratory outcomes);
- the neutralizing potency of the antivenom (given by preclinical tests); and
- the dose regimen.

The dose is calculated to neutralize a certain amount of venom and does not vary between adults and children. Preclinical testing may be used to estimate starting doses and these dosage regimens may be evaluated in a number of ways using standard effectiveness and safety end-points. Dose regimens can be assessed approximately by using prospective observational studies (105). High-quality observational studies may extend evidence over a wider population and are particularly useful in defining safety or when RCTs are unethical or impracticable. In these, the proportion of patients with good clinical outcomes (for example, restoration of blood coagulability or failure to develop local wound necrosis) can be observed with different, escalating or de-escalating doses of antivenom. However, the many weaknesses of observational studies must always be borne in mind.

As part of the design of the study, it is important to determine the minimum number of patients required to establish meaningful results by using sample size calculations (168). Results may sometimes be compared to those of previous studies (historical controls) to determine how the effectiveness or safety of a newly introduced antivenom compares with previously used antivenoms (169). However, such comparisons are susceptible to many kinds of confounding variables and are potentially unreliable. Subsequently, the minimum dose that
appears to be effective can be evaluated in larger Phase II trials or compared to another antivenom or a different dose in Phase III RCTs.

20.2.2 Randomized controlled trials
Definitive Phase III RCTs may require large numbers of patients because of considerable individual variation in the clinical manifestation of envenoming (or the great variability in the quantity and quality of venom injected in different patients). The new antivenom is compared with the existing standard antivenom treatment or, if none exists, two different doses of the test antivenom may be compared. Placebo controls are rarely justified unless there is genuine uncertainty about the risk and benefits of antivenom treatment. In this situation, as a safeguard against unnecessary morbidity in either treatment group, a restricted sequential plan might be incorporated (170) which allows evaluation of results as the trial progresses, as in the early trials of therapeutic tetanus antitoxin (171).

To avoid bias, patients should be randomly allocated to the groups and the study should be blinded, at a minimum to those research personnel who are assessing the clinical response and ideally to both investigators and participants. The number of patients required in each trial arm should be calculated to give the study sufficient statistical power. These power calculations are based on the expected difference in outcome between the treatment groups if the study is designed to demonstrate superiority of one treatment over another. Alternatively, predefined limits of the acceptable performance compared to an existing product are set if the trial is designed to demonstrate that the new antivenom is not worse than existing products (non-inferiority). All patients enrolled in an RCT and randomly allocated to treatment should be included in the analysis of results according to the principle of “intention to treat”, so that any deleterious effects of an antivenom are not concealed by the recipients dropping out of the trial.

20.2.3 Effectiveness end-points for antivenom trials
The assessment criteria (end-points) used for antivenom studies should be predefined a priori and objective. They may be clinical or assessed by laboratory investigations. Common end-points include mortality, development of local tissue effects of envenoming such as necrosis, time taken to restore blood coagulability (assessed by the 20 minute whole blood clotting test) (172), other laboratory parameters such as the prothrombin time, halting of bleeding or objective clinical improvement in neurotoxicity. Surrogate markers such as platelet count are less suitable as they may be affected by complement activation resulting from the antivenom treatment itself. Patients should be observed carefully for long enough to reveal evidence of recurrent envenoming (seen particularly with short half-life Fab antivenoms) (173).
However, owing to the high variability of the mode of action of venoms and of the individual patient’s responses, as well as the diagnostic capacity of health centres, particularly in developing countries, it is necessary to promote clinical research to identify appropriate clinical and laboratory criteria.

20.2.4 Safety end-points for antivenom trials

Because antivenoms consist of foreign proteins/fragments that are liable to aggregation, adverse effects are an inevitable risk in therapy. Appropriate manufacturing steps can reduce the rate of adverse reactions. Rates of reaction are correlated with the purity of the antivenom product and the amount of protein infused. Continuous clinical observation at the bedside is necessary for several hours after treatment to detect acute reactions; late adverse reactions may occur several weeks later. Accurate reaction rates can only be assessed prospectively. Reaction rates may differ considerably between different antivenoms, but in most cases only a small proportion of reactions are life-threatening. Although there is no consensus on classifying or grading early adverse reactions, studies should aim to detect both early adverse events (anaphylaxis and pyrogenicity) occurring at the time of, or within 24 hours of, antivenom administration (such as urticaria itching, fever, hypotension or bronchospasm) and late reactions such as serum sickness occurring between 5 and 24 days after antivenom administration (for example, fever, urticaria, arthralgia, lymphadenopathy, proteinuria or neuropathy).

20.2.5 Challenges in clinical testing of antivenoms

Several particular features of snake-bite make clinical testing of antivenoms challenging. These features include the large variation in the consequences of envenoming between individuals that make it necessary to study large numbers of patients, difficulties in identification of the species responsible for envenoming and the inaccessibility and logistical challenges of areas where snake-bite is sufficiently common to provide sufficient numbers of patients to study. Clinical studies may also be expensive, particularly multicentre studies, with the attendant additional complexity and logistics of between-centre variations. However, despite these difficulties, a number of RCTs have been undertaken and published since 1974 (89, 93, 104, 172, 174–178).

20.3 Post-marketing surveillance

Phase IV studies may be of much greater importance for antivenoms than for other products. A period of active post-licensing surveillance should follow:

- the introduction of a new antivenom (often a regulatory requirement);
- the introduction of changes in manufacturing processes or in the use of raw materials (for example, switching from the use of venoms produced in wild-caught snakes to venoms from captive specimens in a serpentarium), which may result in changes in the quality or effectiveness of an established antivenom;\textsuperscript{15}
- the introduction of an established antivenom into a new geographical area.

Post-marketing studies of antivenoms examine effectiveness as well as the frequency of immediate or delayed side-effects. The combination of preclinical testing and post-marketing surveillance studies is a minimum acceptable clinical evaluation when an existing antivenom is used in a new region.

20.3.1 Possible approaches

Passive surveillance is currently practised by some antivenom manufacturers. However, approaches that rely upon voluntary return of questionnaires about safety and effectiveness are unlikely to provide the high quality data that are necessary. There are three potential approaches to obtaining such data as outlined below.

20.3.1.1 National or regional system for post-marketing surveillance

Countries using antivenoms should establish a national or regional system for the post-marketing surveillance of antivenoms. Clinicians and health workers (such as those working in poison centres) should be encouraged to report actively to national control authorities and manufacturers any unexpected lack of clinical effectiveness and any adverse reactions. These should include both early adverse events, occurring at the time of, or within 24 hours of, antivenom administration, and late reactions occurring between 5 and 24 days later. The mechanism for reporting (such as the use of standardized forms), the receiving body (for example, the national control authority), the deadline for reporting and the type of adverse events that are reportable need to be clearly defined by the authority and will depend on its structure and resources. The manufacturer of the antivenom and the authorities should assess these reports and, in consultation with one another and with specialists in the field, attempt to evaluate their significance. This assessment may require the testing of products already released and the

\textsuperscript{15} Major changes in the design, manufacturing process, or source of venoms used for production of antivenom may necessitate new preclinical and clinical trials of a product. Such changes may also have licensing implications depending on the legislated regulations in the country of manufacture, or the countries where the product will be marketed and used.
inspection of production and control facilities and local distribution channels. If an imported product is associated with adverse reactions, the manufacturer and the national control authorities both in the country of distribution and the country of origin should be notified.

20.3.1.2 Observational studies

In certain situations, for example, the first use of an established antivenom in a new geographical area, or when routine surveillance has identified safety or effectiveness concerns, there is a rationale for setting up observational studies to ensure adequate effectiveness and safety. In the case of first use of an established antivenom in a new geographical area, such studies should follow preclinical testing that ensures neutralization of locally important venoms. Observational studies should carefully document the clinical responses to antivenom, the clinical outcomes and the frequency of reactions in a substantial cohort of patients (179). However, owing to inherent weaknesses of these non-randomized trials, results of observational studies may be misleading.

20.3.1.3 Sentinel sites

In some settings, where post-marketing surveillance of the whole of a country may be problematic, the use of sentinel sites may allow focusing of limited resources to maximize surveillance effectiveness.

20.3.2 Responses to results of post-marketing studies

High-quality post-marketing studies will allow clinicians, public health officials and manufacturers to identify antivenoms with poor effectiveness (batch variations in potency and safety), instances of incorrect use and dosage of antivenoms, and serious safety issues arising from the use of antivenoms. In some situations, these issues may be addressed by improving training of staff in the management of snake-bite, but these studies may also allow identification of the use of an inappropriate antivenom (180).

20.4 Main recommendations

- Preclinical and clinical testing of antivenoms has been largely neglected in the past. Despite challenges, clinical trials of antivenoms in human patients have proved feasible and useful. As far as possible, trials should adhere to the principles of WHO and ICH GCP and should measure robust, objective end-points.
- NRAs should expect producers either to provide data confirming the clinical effectiveness and safety of their antivenoms against
envenoming by local species of venomous snakes or, to support in-country clinical testing of these products.

- Incorporating robust methodologies for reliable identification of the biting snake species is absolutely essential to the design of all clinical trials and other clinical studies of antivenoms.
- Prospective observational studies are of some use in monitoring the effectiveness and safety of an antivenom when first used in a new geographical region.
- Post-marketing surveillance studies should play a major role in the evaluation of effectiveness and safety of antivenoms.

21. Role of national regulatory authorities

NRAs or medicines regulatory authorities play a crucial role in ensuring that pharmaceuticals, vaccines, biological and other medicinal products that are available for use in a country have been carefully and thoroughly evaluated against internationally recognized standards of safety and quality. These agencies of government are vital to the process of strengthening health systems by providing regulatory controls based on legislative frameworks and technical expertise. NRAs therefore have a pivotal role in ensuring the quality, safety and efficacy of antivenoms.

WHO Guidelines for national authorities on quality assurance for biological products and on regulation and licensing of biological products in countries with newly developing regulatory authorities (181, 182) state that NRAs should ensure that available biological products, whether imported or manufactured locally, are of good quality, safe and efficacious, and should thus ensure that manufacturers adhere to approved standards regarding quality assurance and GMP. The responsibilities should also include the enforcement and implementation of effective national regulations, and the setting of appropriate standards and control measures. The evaluation and control of the quality, safety and consistency of production of animal-derived blood products involve the evaluation of the starting material, production processes and test methods to characterize batches of the product.

This requires the regulatory authorities to have appropriate expertise. WHO provides Member States with support in the establishment of NRAs and with the development of regulatory functions, technical abilities and adoption of standards and best practice guidelines, such as this document. A model protocol for the production and testing of snake antivenom immunoglobulins to assist NRAs in reviewing the quality of antivenom batches is provided in Appendix 2.
21.1 Regulatory evaluation of antivenoms

The regulatory evaluation and control of the quality, safety and consistency of production of antivenoms is summarized in Fig. A5.10 and involves the evaluation and approval of:

- the preparation of the starting plasma material from immunized animals (including the preparation of snake venom batches representative of the venomous animals of the geographical region the antivenom is made for), and the animal husbandry, control and traceability of the immunized animals and of the immunization process;
- the fractionation process used to produce the antivenoms;
- the test methods used to control batches of the product including realistic and validated potency tests based on neutralization of likely maximal envenomation;
- shelf-life and stability testing of intermediates and final product;
- the preclinical data supporting the expected effectiveness of the products for treatment of local envenomings;
- the clinical effectiveness of locally manufactured or imported antivenoms against the species of snakes found in the country, through active marketing surveillance.

21.2 Establishment licensing and site inspections

Many NRAs implement control systems based on licensing manufacturing establishments, inspecting them regularly, and enforcing the implementation of the legal requirements and applicable standards. This applies to the preparation of snake venoms, production of animal hyperimmune plasma for fractionation, and the manufacturing process of the antivenoms. Establishments involved in any or all stages of the manufacture of antivenoms need to have an establishment licence and to be inspected by the competent NRA before operations commence. To obtain the licence, the establishments have to fulfil a defined set of requirements to guarantee that their operation ensures the safety, quality and clinical effectiveness of the antivenoms.
Fig. A5.10
Schematic diagram for the regulatory evaluation and control of the quality, safety and standards of production of antivenoms

21.3 **Impact of good manufacturing practices**

Implementing the principles of GMP in the production of therapeutic products is acknowledged as essential for assuring the quality and safety of biological medicinal products. For antivenoms, GMP becomes even more important and more complex due to the biological nature of the production process and the complexity and local specificities of snake envenoming.

Therefore, taking into account the principles of GMP, and the existence of an appropriate quality assurance system to address and implement these requirements at all stages of manufacture, should be pivotal in ensuring the quality and safety of antivenoms. The following benefits are expected:

- ensures the application of quality assurance principles at all steps involved in the preparation of snake venoms, the production of animal plasma and the fractionation process of antivenoms;
- reduces errors and technical problems at all stages of manufacture of plasma for fractionation and antivenoms;
- contributes to the release of products that comply with quality and safety requirements;
- ensures adequate documentation and full traceability of plasma for fractionation and antivenom production stages;
- enables continuous improvement in production of plasma for fractionation and antivenoms;
- provides suitable tools for NRAs to assess the compliance status of a manufacturer of antivenoms, either local or abroad;
- supports regional cooperation networks that may result in the formation of competence centres by centralizing activities to enable compliance to be achieved at the required level.

An establishment licensing system for antivenom manufacturers operated by the responsible and competent NRA should therefore exist. The main requirements to be met to obtain an establishment licence may include in particular:

- quality assurance system and GMP applied to all steps of venom and antivenom production;
- personnel directly involved in collection, testing, processing, storage and distribution of antivenoms are appropriately qualified and provided with timely and relevant training;
- adequate premises and equipment are available;
- an adequate control system to ensure traceability of antivenoms manufacture is to be enforced through accurate identification procedures, record maintenance, and an appropriate labelling system;
- the existence of a post-marketing information system.

21.4 Inspections and audit systems in the production of antivenoms

The ongoing operations of antivenom manufacturers need to be subject to regulatory authority control and supervision in accordance with legislation. Regulatory authorities can also make use of WHO technical support services, monographs and guidelines to assist them in developing the capacity to undertake ongoing inspection activities, audits and reviews of manufacturing, quality control and other production process systems.

All manufacturers must have in place a quality assurance system for manufacture of animal-derived plasma products that comprehensively covers
all stages leading to the finished product, from production of plasma (including venom sourcing and preparation, production animal sourcing, selection, immunization and animal health control) to the collection and fractionation of the plasma into the finished products and their control. Manufacturers of antivenoms must maintain complete Site Master Files containing specific, factual details of the application of GMP to production and quality control activities that are undertaken at every site of operations linked to the products they produce. Manufacturers should also maintain quality manuals that define and describe the quality system, the scope and operations of the quality system at all levels of production, management responsibilities, key quality systems processes and safeguards. For individual products a product dossier in CTD format as recommended by WHO and ICH may also be required. NRAs should make full use of these three forms of production documentation in preparing for and conducting site inspections and audits.

For local producers, the NRA should enforce the implementation of GMP with the aim of ensuring the compliance of the manufacturer with the existing provisions. It is the responsibility of the NRA inspector to ensure that manufacturers adhere to the approved standards of GMP and quality assurance. The inspection and enforcement of control measures for venom producers, immune plasma producers, fractionation facilities and final product producers and distributors should be carried out by officials representing the competent NRA. They should be familiar with biological product technologies and trained in GMP inspections.

Inspections should follow common inspection procedures. These include:

- an opening meeting with management and key personnel;
- a tour of the facility, including inspection of the main areas and activities, such as:
  (a) serpentariums;
  (b) animal husbandry practices;
  (c) animal identification and suitability for blood or plasma collection;
  (d) process of collection and storage of blood or plasma;
  (e) plasma fractionation process;
  (f) testing and availability of test results for venoms, antivenoms and raw materials;
  (g) storage, transportation and shipment; and
  (h) quality assurance (including internal audits and change control);
- documentation (SOPs, records, donor record files, log books); personnel and organization;
- qualification and process validations;
- error and corrective action systems, recalls and complaints and product quality controls;
- a final meeting summarizing the inspection outcome.

A thorough inspection includes the observation of staff during performance of operations and comparison with established SOPs. The inspection should not only be considered as checking compliance with GMP, but also as an indirect product quality assessment by checking product-specific validation and quality control data.

A written report should summarize the main findings of the inspection including its scope, a description of the company, the deficiencies listed, specified and classified (for example, as critical, major or minor), and a conclusion. The written report is sent to the manufacturer. The manufacturers are requested to notify the NRA of the specific steps being taken, or which are planned, to correct the failures and to prevent their recurrence. If necessary, follow-up inspections should be performed – for example, to check the successful implementation of specific corrective actions. The NRA should have the mandate to withdraw an establishment licence in a case where inspection results show critical noncompliance with the requirements or product specifications. In the procedure for granting marketing authorization for an antivenom, information on the collection and control of the venoms and of the starting animal blood or plasma needs to be documented as part of the dossier. In summary, the enforcement and implementation of licensing and inspection regulatory systems for antivenoms constitute fundamental tools to ensure the quality of antivenoms produced or distributed to treat envenomings in a country.

21.5 Antivenom licensing

All antivenoms that are in use in a country must be approved and licensed by the appropriate NRA or another competent authority with legal jurisdiction. The process for applying for, considering and making a decision on the merits of an application should follow established processes and be subject to transparency and review. The process of product dossier assessment and review should be defined by legislation and appropriate regulations. Marketing authorization (licensing) of antivenoms should be subject to a thorough review of the product dossier, Site Master File and quality management system. For a product that is to be imported, NRAs should communicate directly with the licensing authority in the country of manufacturer to ensure that claims in the documents are factual and the product meets licensing requirements in its country of origin.
21.6 National reference venoms

As discussed in section 10.2 countries or regions should establish collections of reference venoms (“standard venoms”) against which antivenom products and the venoms used by manufacturers can be assessed. The establishment of reference venoms for release control of final product should be reviewed and monitored by the regulatory authority or by other competent authorities with technical expertise in the production of international reference material standards. Antivenom manufacturers should not be involved in the production of reference standards in order to ensure transparency. The task should be assigned to a central quality control laboratory, or to a third-party organization with specific expertise. The potency of each batch of final product should be confirmed by specific neutralization of a standard venom of each species of snake for which the antivenom is indicated.

A system of control for the reference venoms and for the design of the venom pools (for example, the geographical selection of animals) should be in place as part of the procedures for the management of reference venom collections.

21.7 Main recommendations

- NRAs should regulate and supervise local antivenom manufacturers.
- NRAs are responsible for market authorization of antivenoms distributed in the country.
- Inspection and audit processes are fundamental to the effective regulation and control of antivenoms and NRAs should seek appropriate assistance to develop both the legislative and technical expertise necessary to undertake these functions.
- Only antivenoms that pass stringent applications processes should be granted market authorization.
- National reference collections of “standard venoms” should be established according to accepted international reference material standards, and used to independently assess antivenoms, or to validate venoms used by manufacturers.

Authors and acknowledgements

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Prior to their presentation to the sixty-seventh meeting of the WHO Expert Committee on Biological Standardization, held in Geneva, Switzerland, from 17 to 21 October 2016, the draft Guidelines were published on the WHO Biologicals website as part of a process of public consultation. The draft document was also emailed to: all second edition section reviewers; antivenom manufacturers; regulatory authorities in countries producing antivenoms; WHO regional offices (for further distribution in each region); and all members of the Committee, with a request to provide feedback and comments.

In response, submissions were received from: Emeritus Professor D.A. Warrell, Oxford University, England; Dr L. Bowen, Sanofi US, the USA; Dr G. Leon, Instituto Clodomiro Picado, Costa Rica; Professor J.M. Gutiérrez, Instituto Clodomiro Picado, Costa Rica; Dr G. Habib, VACSERA, Egypt; Dr R.H. Harrison, Liverpool School of Tropical Medicine, England; Dr M. del Pilar Álvarez, Centro para el Control Estatal de Medicamentos, Equipos y Dispositivos Médicos (CECMED), Cuba; Dr E. Griffiths, WHO Expert Committee on Biological Standardization; Dr J. Southern, South Africa; Professor K. Ratanabanangkoon, Mahidol University, Thailand; Dr D. Garcia, Agence Nationale de Sécurité du Médicament et des Produits de Santé, France; Dr A. Britton, Ultimate Efficacy Consulting Pty Ltd, Australia; Dr A. Fernandes, Bharat Serums and Vaccines Ltd, India and Dr D. Scott and Dr O. Simakova, United States Food and Drug Administration, Center for Biologics Evaluation and Research, the USA. The resulting document WHO/BS/2016.2300 was then prepared in light of all comments received.

Further changes were subsequently made to document WHO/BS/2016.2300 by the WHO Expert Committee on Biological Standardization.
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Appendix 1

Worldwide distribution of medically important venomous snakes

Venomous snakes are widely distributed, especially in tropical countries, from sea level to altitudes of up to 4900 metres (Gloydius himalayanus). The European adder (Vipera berus) enters the Arctic Circle, and the Argentine Yaranata (Bothrops ammodytoides) occurs to 47 °S and is the most southerly occurring venomous snake. No other venomous species occur in cold regions such as the Arctic, Antarctic and north of around latitude 51 °N in North America (Newfoundland, Nova Scotia).

This Appendix lists venomous snake species considered to represent the greatest threat to public health in various countries, territories and other areas or regions around the world. Only species which fall into one of the two categories listed below are shown, and category listings are in alphabetical order according to taxonomic family, genus and species. The intention in categorizing these medically important snakes into two groups is to provide users of these WHO Guidelines with a prioritized listing. Snakes in both Category 1 and Category 2 are species for which antivenom production is important; however species listed in Category 1 within a country, territory or area should be considered as being of highest priority for antivenom production on the basis that available knowledge implicates them as being responsible for the greater burden in that particular setting.

Definitions of the categories used in this listing are:

- CATEGORY 1 (CAT 1): Highest medical importance
  Definition: highly venomous snakes which are common or widespread and cause numerous snake-bites, resulting in high levels of morbidity, disability or mortality.

- CATEGORY 2 (CAT 2): Secondary medical importance
  Definition: highly venomous snakes capable of causing morbidity, disability or death, but: (a) for which exact epidemiological or clinical data may be lacking; and/or (b) are less frequently implicated (owing to their activity cycles, behaviour, habitat preferences or occurrence in areas remote from large human populations).

There are numerous other venomous species that rank as lesser threats in countries territories and other areas listed here, and interested readers should
refer to the herpetological references provided in these Guidelines. It should be noted that over time, as more information becomes available, new species will doubtlessly be added to these lists, and some species, currently defined within Category 1 or Category 2 will be re-ranked.

It should also be noted that the organization of countries, territories and other areas in this Appendix does not follow the WHO regional organization, but instead is arranged biogeographically in alphabetical order of country, territory or geographical area. This approach was necessary to reflect the geographical distribution of major groups of venomous snakes throughout the world. For example, the venomous snakes of the eastern Indonesian Province of Papua have biogeographical origins in Australo-Papua, and are evolutionarily distinct from the venomous snakes of Asian origin that occur west of Wallace’s Line, which runs south of the Philippines, between Borneo and Sulawesi, and between Bali and Lombok, and which separates the zoogeographical regions of Asia and Australia. For this reason, the medically important snakes of Indonesian Papua are listed in the Australo-Papuan region, rather than the South-East Asian region.

Users of this Appendix should also recognize that the relative risk of injury from a particular species may vary from one country, territory or area to another. For this reason, some species that have been listed under Category 1 in one country, territory or area may have been listed under Category 2 in another country, territory or area to reflect the different risk posed by that species in different locations. Assignment to Category 1 or Category 2 was based in some cases on the relative importance of a species as a cause of snake-bite. In Europe, for example, the overall incidence of snake-bite is trivial compared to that in West Africa or India, but where a European species (such as *Vipera berus*) is a major (or sole) cause of envenoming where it occurs, this warrants ranking it as a medically important species in that setting.

**AFRICA AND THE MIDDLE EAST**

**Island populations**

Off the coast of Africa, there are no medically important snakes in Mauritius, Réunion, Rodrigues, the Comoros, the Canary Islands, the Cabo Verde Islands or the Seychelles. The islands that do have venomous snakes include the Lamu group, Zanzibar, Pemba and Mafia Islands, the Bazaruto Archipelago and Inhaca Island, São Tomé, Príncipe, Bioko (Fernando Po) and Dahlak Islands. The venomous snakes on these islands tend to be similar to those on the adjacent mainland. A colubrid, *Madagascarophis meridionalis*, and perhaps other species of the same genus, are the only terrestrial snakes of possible, if minimal, medical importance found in Madagascar.
North Africa/Middle East

Algeria:

| Cat 1: | Elapidae: *Naja haje*; Viperidae: *Cerastes cerastes; Daboia mauritanica* |
| Cat 2: | Viperidae: *Daboia deserti; Echis leucogaster; Macrovipera lebetina; Vipera latastei* |

Cyprus:

| Cat 1: | None |
| Cat 2: | Viperidae: *Macro vipera lebetina* |

Egypt:

| Cat 1: | Elapidae: *Naja haje*; Viperidae: *Cerastes cerastes; Echis coloratus* (east), *Echis pyramidum* |
| Cat 2: | Atractaspidae: *Atractaspis engaddensis* (Sinai); Elapidae: *Naja nubiae; Walterinnesia aegyptia* (Sinai); Viperidae: *Pseudocerastes fieldi* |

Iraq:

| Cat 1: | Viperidae: *Echis carinatus; Macrovipera lebetina* |
| Cat 2: | Elapidae: *Walterinnesia morgani*; Viperidae: *Cerastes gasperettii; Pseudocerastes fieldi, Pseudocerastes persicus* |

Iran (Islamic Republic of):

| Cat 1: | Elapidae: *Naja oxiana*; Viperidae: *Echis carinatus; Macrovipera lebetina; Pseudocerastes persicus* |
| Cat 2: | Elapidae: *Bungarus persicus* (south-east); Walterinnesia morgani (west); Viperidae: *Eristicophis macmahonii* (east); *Gloydius halys caucasicus; Montivipera raddei; Vipera spp.* |

Israel:

| Cat 1: | Viperidae: *Daboia palaestinae; Echis coloratus* |
| Cat 2: | Atractaspidae: *Atractaspis engaddensis*; Elapidae: *Walterinnesia aegyptia*; Viperidae: *Cerastes cerastes, Cerastes gasperettii; Pseudocerastes fieldi* |

Jordan:

| Cat 1: | Viperidae: *Daboia palaestinae; Echis coloratus* |
| Cat 2: | Atractaspidae: *Atractaspis engaddensis*; Elapidae: *Walterinnesia aegyptia*; Viperidae: *Cerastes gasperettii; Macrovipera lebetina; Pseudocerastes fieldi* |
Kuwait and Qatar:

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<tr>
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<td>Elapidae: Walterinnesia morgani (Kuwait only)</td>
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Libya:

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<td>Viperidae: Daboia deserti</td>
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Morocco:

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<tbody>
<tr>
<td>Cat 2:</td>
<td>Viperidae: Pseudocerastes fieldi</td>
</tr>
</tbody>
</table>
### Tunisia:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Viperidae: <strong>Daboia mauritanica</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Elapidae: Naja haje; Viperidae: Cerastes cerastes; Daboia deserti; Echis leucogaster; Macroipera lebetina; Vipera latastei</td>
</tr>
</tbody>
</table>

### Turkey:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Viperidae: <strong>Macrovipera lebetina; Montivipera xanthina</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Elapidae: Walterinnesia morgani (south); Viperidae: Montivipera raddei; Vipera ammodytes, Vipera eriwanensis, Vipera spp.</td>
</tr>
</tbody>
</table>

### United Arab Emirates:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Viperidae: <strong>Echis carinatus</strong> (east); <strong>Echis omanensis</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Viperidae: Cerastes gasperetti; Pseudocerastes persicus</td>
</tr>
</tbody>
</table>

### West Bank and Gaza Strip:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Viperidae: <strong>Daboia palaestinae; Echis coloratus</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Atractaspididae: Atractaspis engaddensis; Elapidae: Walterinnesia aegyptia; Viperidae: Cerastes cerastes; Pseudocerastes fieldi</td>
</tr>
</tbody>
</table>

### Western Sahara:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Viperidae: <strong>Cerastes cerastes</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Elapidae: Naja haje; Viperidae: Bitis arietans</td>
</tr>
</tbody>
</table>

### Yemen:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Atractaspididae: <strong>Atractaspis andersonii</strong>; Elapidae: Naja arabica; Viperidae: Bitis arietans; Echis borkini, Echis coloratus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Viperidae: Cerastes cerastes, Cerastes gasperetti; Echis khosatzkii</td>
</tr>
</tbody>
</table>

### Central sub-Saharan Africa

#### Angola:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <strong>Dendroaspis jameoni, Dendroaspis polylepis; Naja anchietae, Naja melanoleuca, Naja nigrigollis</strong>; Viperidae: Bitis arietans, Bitis gabonica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Atractaspididae: Atractaspis bibronii, Atractaspis irregularis; Colubridae: Dispholidus typhus; Thelotornis capensis, Thelotornis kirtlandii (north); Elapidae: Naja christyi (Cabinda), Naja mossambica (south), Naja nigriconota (south-west); Pseudohage goldii; Viperidae: Atheris squamigera; Bitis nasicornis (Cabinda)</td>
</tr>
</tbody>
</table>
Burundi:

**Cat 1:** Elapidae: *Naja nigricollis, Naja melanoleuca*; Viperidae: *Bitis arietans*

**Cat 2:** Atractaspididae: *Atractaspis bibronii, Atractaspis irregularis*; Colubridae: *Dispholidus typus, Thelotornis mossambicanus*; Elapidae: *Dendroaspis jamesoni*; Viperidae: *Bitis gabonica, Bitis nasicornis*

Central African Republic:

**Cat 1:** Elapidae: *Dendroaspis jamesoni, Dendroaspis polylepis, Naja haje, Naja nigricollis*; Viperidae: *Bitis arietans, Bitis gabonica, Echis ocellatus, Echis pyramidum*

**Cat 2:** Atractaspididae: *Atractaspis irregularris*; Colubridae: *Dispholidus typus, Thelotornis kirtlandii*; Elapidae: *Naja annulata, Naja melanoleuca*; *Pseudohaje goldii*; Viperidae: *Atheris broadleyi, Atheris squamigera, Bitis nasicornis*

Chad:

**Cat 1:** Elapidae: *Naja haje, Naja nigricollis*; Viperidae: *Bitis arietans* (south); *Echis ocellatus* (south)

**Cat 2:** Colubridae: *Dispholidus typus*; Elapidae: *Naja katiensis, Naja nubiae*; Viperidae: *Cerastes cerastes*

Republic of the Congo:

**Cat 1:** Elapidae: *Dendroaspis jamesoni, Naja melanoleuca*; Viperidae: *Bitis gabonica, Bitis nasicornis*

**Cat 2:** Atractaspididae: *Atractaspis irregularris*; Colubridae: *Dispholidus typus, Thelotornis kirtlandii*; Elapidae: *Naja annulata, Naja christyi, Naja nigricollis, Pseudohaje goldii*; Viperidae: *Atheris squamigera, Bitis arietans*

Democratic Republic of the Congo:

**Cat 1:** Elapidae: *Dendroaspis jamesoni, Naja melanoleuca, Naja nigricollis*; Viperidae: *Bitis arietans, Bitis gabonica, Bitis nasicornis*

**Cat 2:** Atractaspididae: *Atractaspis bibronii, Atractaspis irregularris*; Colubridae: *Dispholidus typus, Thelotornis capensis, Thelotornis kirtlandii*; Elapidae: *Dendroaspis polylepis, Naja anchiietae* (Katanga pedicle), *Naja annulata, Naja christyi, Naja haje* (north); *Pseudohaje goldii*; Viperidae: *Atheris squamigera*

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1 The medical importance of this species may be higher in the primary forest zone of the south-western Central African Republic, and in some secondary forest mosaic zones elsewhere in the Central African Republic.
### Equatorial Guinea:

**Cat 1:** Elapidae: *Dendroaspis jamesoni; Naja melanoleuca*; Viperidae: *Bitis gabonica, Bitis nasicornis*

**Cat 2:** Atractaspididae: *Atractaspis irregularis*; Colubridae: *Thelotornis kirtlandii*; Elapidae: *Naja annulata; Pseudohaje goldii*; Viperidae: *Atheris squamigera*

### Gabon:

**Cat 1:** Elapidae: *Dendroaspis jamesoni; Naja melanoleuca, Naja nigricollis*; Viperidae: *Bitis gabonica, Bitis nasicornis*

**Cat 2:** Atractaspididae: *Atractaspis irregularis*; Colubridae: *Thelotornis kirtlandii*; Elapidae: *Naja annulata; Pseudohaje goldii*; Viperidae: *Atheris squamigera*; *Bitis arietans*

### Rwanda:

**Cat 1:** Elapidae: *Dendroaspis jamesoni; Naja nigricollis*; Viperidae: *Bitis arietans*

**Cat 2:** Atractaspididae: *Atractaspis bibronii, Atractaspis irregularis*; Colubridae: *Dispholidus typus; Thelotornis kirtlandii*; Elapidae: *Dendroaspis polylepis; Naja annulata, Naja melanoleuca; Pseudohaje goldii*; Viperidae: *Bitis gabonica, Bitis nasicornis*

### East Sub-Saharan Africa

#### Djibouti:

**Cat 1:** Viperidae: *Echis pyramidum*

**Cat 2:** Atractaspididae: *Atractaspis fallax*; Colubridae: *Dispholidus typus*; Elapidae: *Naja pallida*; Viperidae: *Bitis arietans*

#### Eritrea:

**Cat 1:** Elapidae: *Dendroaspis polylepis; Naja haje*; Viperidae: *Bitis arietans*; *Echis pyramidum*

**Cat 2:** Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; Elapidae: *Naja nubiae*; Viperidae: *Echis megaloccephalus*

#### Ethiopia:

**Cat 1:** Elapidae: *Dendroaspis polylepis; Naja ashei* (south-east), *Naja haje, Naja nigricollis*; Viperidae: *Bitis arietans; Echis pyramidum*

**Cat 2:** Atractaspididae: *Atractaspis fallax, Atractaspis irregularis* (Mt Bizen); Colubridae: *Dispholidus typus; Elapidae: Naja melanoleuca, Naja pallida*; Viperidae: *Bitis parviocula, Bitis harenna*
**Kenya:**

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Dendroaspis angusticeps, Dendroaspis polylepis; Naja ashei</em> (north &amp; east), <em>Naja haje, Naja nigricollis</em>; Viperidae: <em>Bitis arietans; Echis pyramidum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Atractaspididae: <em>Atractaspis bibronii, Atractaspis fallax, Atractaspis irregularis</em>; Colubridae: <em>Dispholidus typus; Thelotornis mossambicanus, Thelotornis usambaricus</em> (east coast); Elapidae: <em>Dendroaspis jamesoni; Naja melanoleuca</em> (west and coastal forest), <em>Naja pallida</em> (north and east); <em>Pseudohaje goldii</em>; Viperidae: <em>Atheris squamigera; Bitis nasicornis, Bitis gabonica</em> (west)</td>
</tr>
</tbody>
</table>

**Malawi:**

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Dendroaspis angusticeps, Dendroaspis polylepis; Naja annulifera, Naja mossambica, Naja nigricollis</em>; Viperidae: <em>Bitis arietans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Atractaspididae: <em>Atractaspis bibronii</em>; Colubridae: <em>Dispholidus typus; Thelotornis capensis, Thelotornis mossambicanus</em>; Elapidae: <em>Naja melanoleuca</em>; Viperidae: <em>Proatheris superciliaris</em></td>
</tr>
</tbody>
</table>

**Mozambique:**

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Dendroaspis angusticeps, Dendroaspis polylepis; Naja annulifera, Naja mossambica</em>; Viperidae: <em>Bitis arietans, Bitis gabonica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Atractaspididae: <em>Atractaspis bibronii</em>; Colubridae: <em>Dispholidus typus; Thelotornis capensis, Thelotornis mossambicanus</em>; Elapidae: <em>Hemachatus haemachatus, Naja melanoleuca</em>; Viperidae: <em>Proatheris superciliaris</em></td>
</tr>
</tbody>
</table>

**Somalia:**

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Dendroaspis polylepis; Naja ashei</em> (south), <em>Naja haje</em>; Viperidae: <em>Bitis arietans; Echis pyramidum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Atractaspididae: <em>Atractaspis fallax</em>; Colubridae: <em>Dispholidus typus; Thelotornis mossambicanus</em>; Elapidae: <em>Naja pallida, Naja melanoleuca</em>; Viperidae: <em>Echis hughesi</em> (north)</td>
</tr>
</tbody>
</table>

**South Sudan:**

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Naja haje, Naja nigricollis</em>; Viperidae: <em>Bitis arietans; Echis pyramidum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Atractaspididae: <em>Atractaspis fallax, Atractaspis irregularis</em>; Colubridae: <em>Dispholidus typus; Elapidae: Dendroaspis jamesoni, Dendroaspis polylepis; Naja melanoleuca, Naja nubiae, Naja pallida</em>; Viperidae: <em>Bitis gabonica, Bitis nasicornis</em></td>
</tr>
</tbody>
</table>
### Sudan:

**Cat 1:** Elapidae: *Naja haje*; Viperidae: *Bitis arietans*; *Echis pyramidum*

**Cat 2:** Colubridae: *Dispholidus typus*; Elapidae: *Dendroaspis polylepis* (east); *Naja nubiaea*; Viperidae: *Cerastes cerastes*; *Echis coloratus* (east)

### United Republic of Tanzania:

**Cat 1:** Elapidae: *Dendroaspis angusticeps*, *Dendroaspis polylepis*; *Naja mossambica* (including Pemba Island), *Naja nigricollis*; Viperidae: *Bitis arietans*

**Cat 2:** Atractaspididae: *Atractaspis bibronii*, *Atractaspis fallax* (north), *Atractaspis irregularis* (north-east); Colubridae: *Dispholidus typus*; *Thelotornis capensis*, *Thelotornis kirtlandii* (Mahali and Udzungwa Mountains), *Thelotornis mossambicanus*, *Thelotornis usambaricus* (East Usambara Mountains); Elapidae: *Naja ashei* (poss. north-east), *Naja annulata*, *Naja haje* (north), *Naja melanoleuca* (west and coast, including Mafia Island), *Naja pallida*; Viperidae: *Atheris squamigera*; *Bitis gabonica* (west and south-east), *Bitis nasicornis* (north); *Proatheris superciliaris*

### Uganda:

**Cat 1:** Elapidae: *Naja ashei* (north-east), *Naja haje* (north), *Naja nigricollis*, *Dendroaspis jamesoni*, *Dendroaspis polylepis*; Viperidae: *Bitis arietans*, *Bitis gabonica*

**Cat 2:** Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis kirtlandii*; Elapidae: *Naja melanoleuca*; *Pseudohaje goldii*; Viperidae: *Atheris squamigera*; *Bitis nasicornis*

### Zambia:

**Cat 1:** Elapidae: *Dendroaspis polylepis*, *Naja anchietae*, *Naja annulifera*, *Naja mossambica*, *Naja nigricollis*; Viperidae: *Bitis arietans*, *Bitis gabonica*

**Cat 2:** Atractaspididae: *Atractaspis bibronii*; Colubridae: *Dispholidus typus*; *Thelotornis capensis*, *Thelotornis kirtlandii*, *Thelotornis mossambicanus*; Elapidae: *Naja annulata*, *Naja melanoleuca*

### South sub-Saharan Africa

#### Botswana:

**Cat 1:** Elapidae: *Dendroaspis polylepis*, *Naja anchietae* (west), *Naja annulifera* (east), *Naja mossambica*, *Naja nivea* (south-west); Viperidae: *Bitis arietans*

**Cat 2:** Atractaspididae: *Atractaspis bibronii*; Colubridae: *Dispholidus typus*; *Thelotornis capensis*
<table>
<thead>
<tr>
<th>Country</th>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesotho:</td>
<td>Elapidae: <em>Naja nivea</em>; Viperidae: <em>Bitis arietans</em></td>
<td>Elapidae: <em>Hemachatus haemachatus</em></td>
</tr>
<tr>
<td>Namibia:</td>
<td>Elapidae: <em>Dendroaspis polylepis</em>, <em>Naja anchietae</em>, <em>Naja nivea</em> (central and southern), <em>Naja mossambica</em> (north-east), <em>Naja nigricincta</em>; Viperidae: <em>Bitis arietans</em></td>
<td></td>
</tr>
<tr>
<td>South Africa:</td>
<td>Elapidae: <em>Dendroaspis angusticeps</em> (Natal), <em>Dendroaspis polylepis</em>, <em>Naja annulifera</em> (north-east), <em>Naja nivea</em>, <em>Naja mossambica</em> (north-east); Viperidae: <em>Bitis arietans</em></td>
<td></td>
</tr>
<tr>
<td>Swaziland:</td>
<td>Elapidae: <em>Dendroaspis polylepis</em>, <em>Naja annulifera</em>, <em>Naja mossambica</em>; Viperidae: <em>Bitis arietans</em></td>
<td>Atractaspididae: Atractaspis bibronii; Colubridae: Dispholidus typus; Thelotornis capensis; Elapidae: <em>Naja nigricollis</em> (Caprivi)</td>
</tr>
<tr>
<td>Zimbabwe:</td>
<td>Elapidae: <em>Dendroaspis polylepis</em>, <em>Naja anchietae</em> (west), <em>Naja annulifera</em>, <em>Naja mossambica</em>; Viperidae: <em>Bitis arietans</em></td>
<td>Atractaspididae: Atractaspis bibronii; Colubridae: Dispholidus typus; Thelotornis capensis; Elapidae: <em>Hemachatus haemachatus</em>; Elapidae: <em>Dendroaspis angusticeps</em> (east); Thelotornis mossambicanus; Viperidae: <em>Bitis gabonica</em> (east)</td>
</tr>
</tbody>
</table>
## West sub-Saharan Africa

### Benin:

| Cat 1: | Elapidae: *Naja nigricollis*; Viperidae: *Bitis arietans*; *Echis ocellatus* |
| Cat 2: | Atractaspidae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; Elapidae: *Dendroaspis jamesoni*; *Naja katiensis*, *Naja melanoleuca*, *Naja senegalensis*; Pseudohaje nigra; Viperidae: *Bitis rhinoceros*; *Echis leucogaster* (far north) |

### Burkina Faso:

| Cat 1: | Elapidae: *Naja nigricollis*, *Naja katiensis*; Viperidae: *Bitis arietans*; *Echis ocellatus* |
| Cat 2: | Colubridae: *Dispholidus typus*; Elapidae: *Dendroaspis polylepis*; *Naja melanoleuca*, *Naja senegalensis*; Viperidae: *Echis leucogaster* |

### Cameroon:

| Cat 1: | Elapidae: *Dendroaspis jamesoni*; *Naja haje*, *Naja nigricollis*, *Naja melanoleuca*; Viperidae: *Bitis arietans*, *Bitis gabonica*, *Bitis nasicornis*; *Echis ocellatus* |
| Cat 2: | Atractaspidae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; Thelotornis kirtlandii; Elapidae: *Dendroaspis polylepis*; *Naja annulata*, *Naja katiensis*; Pseudohaje goldii; Viperidae: *Atheris broadleyi* (East Province), *Atheris squamigera* |

### Côte d'Ivoire:

| Cat 1: | Elapidae: *Dendroaspis viridis*; *Naja nigricollis*, *Naja melanoleuca*, *Naja senegalensis*; Viperidae: *Bitis arietans*, *Bitis nasicornis*, *Bitis rhinoceros*; *Echis ocellatus* |
| Cat 2: | Atractaspidae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; Thelotornis kirtlandii; Elapidae: *Dendroaspis polylepis*; *Naja katiensis*; Pseudohaje goldii, Pseudohaje nigra; Viperidae: *Atheris chlorechis* |

### Gambia:

| Cat 1: | Elapidae: *Dendroaspis viridis*; *Naja nigricollis*; Viperidae: *Bitis arietans*; *Echis jogeri* |
| Cat 2: | Colubridae: *Dispholidus typus*; Elapidae: *Naja katiensis*, *Naja melanoleuca*, *Naja senegalensis* |

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2 This large, highly venomous snake is common in forested areas of south-west Cameroon and a high burden of injury may be expected, although clinical data with direct attribution are not yet available.
### Ghana:

**Cat 1:** Elapidae: *Dendroaspis viridis*, *Naja nigricollis*, *Naja senegalensis*; Viperidae: *Bitis arietans*, *Echis ocellatus*

**Cat 2:** Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*, *Thelotornis kirtlandii*; Elapidae: *Naja katiensis*, *Naja melanoleuca*; *Pseudohaje goldii*, *Pseudohaje nigra*; Viperidae: *Atheris chlorechis*, *Bitis nasicornis*, *Bitis rhinoceros*

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### Guinea:

**Cat 1:** Elapidae: *Dendroaspis polylepis*, *Dendroaspis viridis*, *Naja katiensis*, *Naja nigricollis*, *Naja melanoleuca*, *Naja senegalensis*; Viperidae: *Bitis arietans*, *Echis jogeri*

**Cat 2:** Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*, *Thelotornis kirtlandii*; Elapidae: *Pseudohaje nigra*; Viperidae: *Atheris chlorechis*, *Bitis nasicornis*, *Bitis rhinoceros*

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### Guinea-Bissau:

**Cat 1:** Elapidae: *Dendroaspis viridis*, *Naja nigricollis*, *Naja melanoleuca*, *Naja senegalensis*; Viperidae: *Bitis arietans*, *Echis jogeri*

**Cat 2:** Colubridae: *Dispholidus typus*, *Thelotornis kirtlandii*; Viperidae: *Bitis rhinoceros*

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### Liberia:

**Cat 1:** Elapidae: *Dendroaspis viridis*, *Naja melanoleuca*, *Naja nigricollis*

**Cat 2:** Atractaspididae: *Atractaspis irregularis*; Colubridae: *Thelotornis kirtlandii*; Elapidae: *Pseudohaje nigra*; Viperidae: *Atheris chlorechis*, *Bitis nasicornis*, *Bitis rhinoceros*

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### Mali:

**Cat 1:** Elapidae: *Naja katiensis*, *Naja nigricollis*, *Naja senegalensis*; Viperidae: *Bitis arietans*, *Echis jogeri* (west), *Echis leucogaster*, *Echis ocellatus*

**Cat 2:** Colubridae: *Dispholidus typus*, Elapidae: *Naja melanoleuca*; Viperidae: *Cerastes cerastes*

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### Mauritania:

**Cat 1:** Elapidae: *Naja senegalensis* (south-east); Viperidae: *Cerastes cerastes*, *Echis leucogaster*

**Cat 2:** Viperidae: *Bitis arietans*

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3 The medical importance of this species may be higher in the forested zone of southern Ghana.
Niger:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Naja nigricollis</em>; Viperidae: <em>Bitis arietans; Echis leucogaster, Echis ocellatus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Colubridae: <em>Dispholidus typus</em>; Elapidae: <em>Naja haje</em> (south-central), <em>Naja katiensis, Naja nubiae, Naja senegalensis</em> (south-west); Viperidae: <em>Cerastes cerastes</em></td>
</tr>
</tbody>
</table>

Nigeria:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Dendroaspis jamesoni; Naja haje</em> (north-east), <em>Naja nigricollis</em>; Viperidae: <em>Bitis arietans, Bitis gabonica; Echis ocellatus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Atractaspididae: <em>Atractaspis irregularis</em>; Colubridae: <em>Dispholidus typus; Thelotornis kirtlandii</em>; Elapidae: <em>Naja katiensis, Naja melanoleuca</em>&lt;sup&gt;4&lt;/sup&gt; <em>Naja senegalensis</em> (north-west); Pseudohaje goldii, Pseudohaje nigra; Viperidae: <em>Atheris squamigera; Bitis nasicornis; Echis leucogaster</em> (north)</td>
</tr>
</tbody>
</table>

Sao Tome and Principe:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Dendroaspis jamesoni; Naja melanoleuca</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
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</tr>
</tbody>
</table>

Senegal:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Naja katiensis, Naja nigricollis</em>; Viperidae: <em>Bitis arietans; Echis leucogaster, Echis jogeri</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Colubridae: <em>Dispholidus typus</em>; Elapidae: <em>Dendroaspis polylepis, Dendroaspis viridis; Naja melanoleuca, Naja senegalensis</em></td>
</tr>
</tbody>
</table>

Sierra Leone:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Dendroaspis viridis; Naja nigricollis</em>; Viperidae: <em>Bitis arietans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Atractaspididae: <em>Atractaspis irregularis</em>; Colubridae: <em>Dispholidus typus; Thelotornis kirtlandii</em>; Elapidae: <em>Naja melanoleuca</em>&lt;sup&gt;5&lt;/sup&gt; Pseudohaje nigra; Viperidae: <em>Atheris chlorechis; Bitis nasicornis, Bitis rhinoceros</em></td>
</tr>
</tbody>
</table>

<sup>4</sup> The medical importance of this species may be higher in the southern rainforest belt of Nigeria, from Ibadan in the west to Oban and Eket in the east, and in the forested southern quarter of Sierra Leone.

<sup>5</sup> The medical importance of this species may be higher in the forested southern quarter of Sierra Leone.
**Togo:**

| Cat 1: | Elapidae: *Naja nigricollis, Naja senegalensis*; Viperidae: *Bitis arietans* (south); *Echis ocellatus* |
| Cat 2: | *Atractaspididae: Atractaspis irregularis*; *Colubridae: Dispholidus typus, Thelotornis kirtlandii*; *Elapidae: Dendroaspis jamesoni, Dendroaspis viridis, Naja katiensis, Naja melanoleuca*; *Pseudohaje goldii, Pseudohaje nigra*; *Viperidae: Atheris chlorechis, Bitis nasicornis, Bitis rhinoceros* |

**ASIA AND AUSTRALASIA**

**Central Asia**

**Armenia:**

| Cat 1: | Viperidae: *Macrovipera lebetina* |
| Cat 2: | Viperidae: *Montivipera raddei, Vipera eriwanensis, Vipera spp.* |

**Azerbaijan:**

| Cat 1: | Viperidae: *Macrovipera lebetina* |
| Cat 2: | Viperidae: *Gloydius halys, Vipera eriwanensis, Vipera spp.* |

**Georgia:**

| Cat 1: | Viperidae: *Macrovipera lebetina, Vipera ammodytes* |
| Cat 2: | Viperidae: *Vipera kaznakovi, Vipera renardi, Vipera spp.* |

**Kazakhstan, Kyrgyzstan, Tajikistan, Uzbekistan and Turkmenistan:**

| Cat 1: | Elapidae: *Naja oxiana* (except Kazakhstan and Kyrgyzstan); *Viperidae: Echis carinatus* (except Kyrgyzstan); *Macrovipera lebetina* (except Kazakhstan and Kyrgyzstan); *Gloydius halys* (throughout) |
| Cat 2: | Viperidae: *Vipera renardi* (except Turkmenistan) |

**Mongolia:**

| Cat 1: | Viperidae: *Gloydius halys* |
| Cat 2: | Viperidae: *Vipera berus, Vipera renardi* |
### East Asia

#### China:

**China mainland**

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Elapidae: <em>Bungarus multicinctus; Naja atra</em>; Viperidae: <em>Trimeresurus albolabris; Daboia siamensis; Deinagkistrodon acutus; Gloydius brevicaudus; Protobothrops mucrosquamatus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2</td>
<td>Colubridae: <em>Rhabdophis tigrinus</em>; Elapidae: <em>Bungarus bungaroides</em> (south-east Tibet), <em>Bungarus fasciatus; Naja kaouthia; Ophiophagus hannah</em>; Viperidae: <em>Trimeresurus septentrionalis</em> (south Tibet); <em>Gloydius halys</em>, <em>Gloydius intermedius, Gloydius ussuriensis; Himalayophis tibetanus</em> (south Tibet); <em>Protobothrops jerdonii, Protobothrops kaibacki, Protobothrops mangshanensis; Vipera berus</em> (Jilin, western Xinjiang), <em>Vipera renardi</em> (western Xinjiang); <em>Trimeresurus stejnegeri</em></td>
</tr>
</tbody>
</table>

**Hong Kong, Special Administrative Region**

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Elapidae: <em>Bungarus multicinctus; Naja atra</em>; Viperidae: <em>Trimeresurus albolabris</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2</td>
<td>None</td>
</tr>
</tbody>
</table>

**Taiwan Province**

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Elapidae: <em>Bungarus multicinctus; Naja atra</em>; Viperidae: <em>Protobothrops mucrosquamatus; Trimeresurus stejnegeri</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2</td>
<td>Viperidae: <em>Deinagkistrodon acutus; Daboia siamensis</em></td>
</tr>
</tbody>
</table>

**Democratic People’s Republic of Korea:**

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Viperidae: <em>Gloydius brevicaudus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2</td>
<td>Viperidae: <em>Gloydius intermedius, Gloydius ussuriensis; Vipera berus</em></td>
</tr>
</tbody>
</table>

**Japan (including Ryukyu Islands):**

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Viperidae: <em>Gloydius blomhoffii</em> (main islands); <em>Protobothrops flavoviridis</em> (Ryukyu Islands)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2</td>
<td>Colubridae: <em>Rhabdophis tigrinus</em>; Viperidae: <em>Gloydius tsushimaensis</em> (Tsushima); <em>Protobothrops elegans</em></td>
</tr>
</tbody>
</table>

**Republic of Korea:**

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Viperidae: <em>Gloydius brevicaudus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2</td>
<td>Colubridae: <em>Rhabdophis tigrinus</em>; Viperidae: <em>Gloydius intermedius, Gloydius ussuriensis</em></td>
</tr>
</tbody>
</table>
## South Asia

### Afghanistan:

**Cat 1:** Elapidae: *Naja oxiana*; Viperidae: *Echis carinatus; Macrovipera lebetina*

**Cat 2:** Elapidae: *Bungarus caeruleus* (east), *Bungarus sindanus* (east); *Naja naja* (poss. south-east); Viperidae: *Eristicophis macmahonii* (south-west); *Gloydius halys* (north)

### Bangladesh:

**Cat 1:** Elapidae: *Bungarus caeruleus, Bungarus niger, Bungarus walli; Naja kaouthia; Viperidae: Trimeresurus erythrurus*

**Cat 2:** Elapidae: *Bungarus bungaroides, Bungarus fasciatus, Bungarus lividus; Naja naja; Ophiophagus hannah; Viperidae: Trimeresurus albolabris* (far north-west); *Daboia russelii* (west)

### Bhutan:

**Cat 1:** Elapidae: *Bungarus niger, Naja naja*

**Cat 2:** Elapidae: *Bungarus caeruleus, Bungarus fasciatus, Bungarus lividus; Naja kaouthia; Ophiophagus hannah; Viperidae: Trimeresurus erythrurus; Daboia russelii; Protobothrops jerdonii*

### India:

**Cat 1:** Elapidae: *Bungarus caeruleus; Naja kaouthia* (east), *Naja naja* (throughout); Viperidae: *Daboia russelii; Echis carinatus; Hypnale hypnale* (south-west)

**Cat 2:** *Bungarus bungaroides, Bungarus fasciatus, Bungarus lividus, Bungarus niger, Bungarus sindanus, Bungarus walli; Naja oxiana* (west), *Naja sagittifera* (Andaman Islands); *Ophiophagus hannah* (south, north-east, Andaman Islands); Viperidae: *Trimeresurus albolabris, Trimeresurus erythrurus, Trimeresurus septentrionalis; Gloydius himalayanus; Protobothrops jerdonii, Protobothrops kaulbacki, Protobothrops m ucrosquamatus; *Trimeresurus gramineus* (south India), *Trimeresurus malabaricus* (south-west),

### Nepal:

**Cat 1:** Elapidae: *Bungarus caeruleus, Bungarus niger; Naja naja, Naja kaouthia; Viperidae: Daboia russelii*

**Cat 2:** Elapidae: *Bungarus bungaroides, Bungarus fasciatus, Bungarus lividus, Bungarus walli; Ophiophagus hannah; Viperidae: Trimeresurus septentrionalis; Gloydius himalayanus; Himalayophis tibetanus; Protobothrops jerdonii*
Pakistan:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Bungarus caeruleus</em>, <em>Bungarus sindanus</em>; <em>Naja naja</em>, <em>Naja oxiana</em>; Viperidae: <em>Daboia russelii</em>, <em>Echis carinatus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viperidae: <em>Eristicophis macmahonii</em> (west); <em>Gloydius himalayanus</em> (north); <em>Macrovipera lebetina</em> (west)</td>
</tr>
</tbody>
</table>

Sri Lanka:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Bungarus caeruleus</em>; <em>Naja naja</em>; Viperidae: <em>Daboia russelii</em>, <em>Hypnale hypnale</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Elapidae: <em>Bungarus ceylonicus</em>; Viperidae: <em>Echis carinatus</em>, <em>Hypnale nepa</em>, <em>Hypnale zara</em>, <em>Trimeresurus trigonocephalus</em></td>
</tr>
</tbody>
</table>

South-East Asia

Brunei Darussalam:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Naja sumatrana</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Elapidae: <em>Bungarus fasciatus</em>, <em>Bungarus flaviceps</em>, <em>Calliophis bivirgatus</em>, <em>Calliophis intestinalis</em>; Ophiophagus hannah; Viperidae: <em>Trimeresurus sumatranus</em>, <em>Tropidolaemus subannulatus</em></td>
</tr>
</tbody>
</table>

Cambodia:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Bungarus candidus</em>, <em>Naja kaouthia</em>, <em>Naja siamensis</em>; Viperidae: <em>Calloselasma rhodostoma</em>, <em>Trimeresurus albolabris</em>; <em>Daboia siamensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Elapidae: <em>Bungarus fasciatus</em>, <em>Bungarus flaviceps</em>; Ophiophagus hannah; Viperidae: <em>Trimeresurus cardamomensis</em></td>
</tr>
</tbody>
</table>

Indonesia (Sumatra, Java, Borneo, Sulawesi and Lesser Sunda Islands):

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Elapidae: <em>Bungarus candidus</em> (Sumatra and Java); <em>Naja sputatrix</em> (Java and Lesser Sunda Islands), <em>Naja sumatrana</em> (Sumatra and Borneo); Viperidae: <em>Calloselasma rhodostoma</em> (Java); <em>Trimeresurus albolabris</em>; <em>Daboia siamensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Elapidae: <em>Bungarus fasciatus</em>, <em>Bungarus flaviceps</em> (Sumatra and Borneo); <em>Calliophis bivirgatus</em>, <em>Calliophis intestinalis</em>; Ophiophagus hannah (Sumatra, Borneo &amp; Java); Viperidae: <em>Trimeresurus insularis</em>, <em>Trimeresurus purpureomaculatus</em> (Sumatra), <em>Trimeresurus sumatranus</em>; <em>Tropidolaemus subannulatus</em></td>
</tr>
</tbody>
</table>
### Lao People's Democratic Republic:

| Cat 1: | Elapidae: *Bungarus candidus, Bungarus multicinctus; Naja atra* (north), *Naja siamensis* (south and east); Viperidae: *Calloselasma rhodostoma; Trimeresurus albolabris* |
| Cat 2: | Elapidae: *Bungarus fasciatus; Naja kaouthia* (south and east); *Ophiophagus hannah*; Viperidae: *Trimeresurus macrops; Protobothrops jerdonii, Protobothrops mucrosquamatus* |

### Malaysia:

| Cat 1: | Elapidae: *Bungarus candidus* (Peninsular Malaysia); *Naja kaouthia* (northern Peninsular Malaysia), *Naja sumatrana* (Peninsular Malaysia, Sabah and Sarawak); Viperidae: *Calloselasma rhodostoma* |
| Cat 2: | Elapidae: *Bungarus fasciatus, Bungarus flaviceps; Calliophis bivirgatus, Calliophis intestinalis; Ophiophagus hannah*; Viperidae: *Trimeresurus purpureomaculatus, Trimeresurus hageni; Tropidolaemus subannulatus* |

### Myanmar:

| Cat 1: | Elapidae: *Bungarus magnimaculatus, Bungarus multicinctus; Naja kaouthia, Naja mandalayensis*; Viperidae: *Trimeresurus albolabris, Trimeresurus erythrus; Daboia siamensis* |
| Cat 2: | Elapidae: *Bungarus bungaroides* (Chin State), *Bungarus candidus* (Thaninthayi Div.), *Bungarus flaviceps* (east Shan State), *Bungarus niger*; *Ophiophagus hannah*; Viperidae: *Calloselasma rhodostoma* (Thaninthayi Div.); *Trimeresurus purpureomaculatus; Protobothrops jerdonii, Protobothrops kaulbacki, Protobothrops mucrosquamatus* (Kachin) |

### Philippines:

| Cat 1: | Elapidae: *Naja philippinensis* (Luzon), *Naja samarensis* (Mindanao), *Naja sumatrana* (Palawan) |
| Cat 2: | Elapidae: *Calliophis intestinalis; Ophiophagus hannah*; Viperidae: *Trimeresurus flavomaculatus; Tropidolaemus philippensis, Tropidolaemus subannulatus* |

### Singapore:

| Cat 1: | Elapidae: *Bungarus candidus; Naja sumatrana* |
| Cat 2: | Elapidae: *Bungarus fasciatus; Calliophis bivirgatus, Calliophis intestinalis; Ophiophagus hannah*; Viperidae: *Trimeresurus purpureomaculatus* |
Thailand:

Cat 1: Elapidae: *Bungarus candidus; Naja kaouthia, Naja siamensis*
Viperidae: *Calloselasma rhodostoma; Trimeresurus albolabris; Daboia siamensis*

Cat 2: Elapidae: *Bungarus fasciatus, Bungarus flaviceps; Calliophis bivirgatus, Calliophis intestinalis; Naja sumatrana; Ophiophagus hannah*
Viperidae: *Trimeresurus macrops, Trimeresurus hageni*

Timor-Leste:

Cat 1: Viperidae: *Trimeresurus insularis*
Cat 2: None

Viet Nam:

Cat 1: Elapidae: *Bungarus candidus, Bungarus multicinctus, Bungarus slowinskii* (north); *Naja atra* (north), *Naja kaouthia* (south)
Viperidae: *Calloselasma rhodostoma; Trimeresurus albolabris* (throughout)

Cat 2: Elapidae: *Bungarus fasciatus, Bungarus flaviceps* (south); *Naja siamensis* (south); *Ophiophagus hannah*
Viperidae: *Trimeresurus rubeus; Protobothrops jerdonii, Protobothrops mucrosquamatus* (north); *Trimeresurus stejnegeri; Deinagkistrodon acutus*

Australo-Papua (including Pacific Islands):

There are no medically important land snakes in American Samoa, Cook Islands, Fiji, French Polynesia, Guam, Kiribati, Marshall Islands, Nauru, New Caledonia, New Zealand, Northern Mariana Islands, Pitcairn Island, Samoa, Tokelau, Tonga, Tuvalu, or Wallis and Futuna Islands. Fiji possesses a single terrestrial venomous snake species (*Ogmodon vitianus*) while the Solomon Islands possess three terrestrial venomous species (*Salomonelaps par; Loveridgelaps elapoides* and *Parapistocalamus hedigeri*) associated with no and few snake-bites, respectively.

Australia:

Cat 1: Elapidae: *Notechis scutatus; Pseudechis australis;* 6 *Pseudonaja affinis, Pseudonaja mengdeni, Pseudonaja nuchalis, Pseudonaja textilis*

Cat 2: Elapidae: *Acanthophis antarcticus, Acanthophis cryptamydros, Acanthophis spp.; Austrelaps spp.; Hoplocephalus spp.; Oxyuranus microlepidotus, Oxyuranus scutellatus, Oxyuranus temporalis; Pseudechis spp.; Pseudonaja aspidorhyncha, Pseudonaja spp.; Tropidechis carinatus*

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6 *Pseudechis australis* is common and widespread and causes numerous snake-bites; bites may be severe, although this species has not caused a fatality in Australia since 1968.
Indonesia (West Papua and Maluku):

<table>
<thead>
<tr>
<th>Cat</th>
<th>Family</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Elapidae</td>
<td>Acanthophis laevis</td>
</tr>
<tr>
<td>2</td>
<td>Elapidae</td>
<td>Acanthophis rugosus; Micropechis ikaheka; Oxyuranus scutellatus; Pseudechis papuanus, Pseudechis rossignolii; Pseudonaja textilis</td>
</tr>
</tbody>
</table>

Papua New Guinea:

<table>
<thead>
<tr>
<th>Cat</th>
<th>Family</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Elapidae</td>
<td>Acanthophis laevis; Oxyuranus scutellatus</td>
</tr>
<tr>
<td>2</td>
<td>Elapidae</td>
<td>Acanthophis rugosus; Micropechis ikaheka; Pseudonaja textilis; Pseudechis papuanus, Pseudechis rossignolii</td>
</tr>
</tbody>
</table>

EUROPE

There are no venomous snakes in Iceland, Ireland, Isle of Man, Outer Hebrides, Orkney or the Shetland Islands. Crete and most of the islands of the western Mediterranean are also free of venomous snakes.

Central Europe

Albania, Bulgaria, Romania, Serbia, Montenegro, Slovenia, The former Yugoslav Republic of Macedonia:

<table>
<thead>
<tr>
<th>Cat</th>
<th>Family</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Viperidae</td>
<td>Vipera ammodytes</td>
</tr>
<tr>
<td>2</td>
<td>Viperidae</td>
<td>Vipera berus</td>
</tr>
</tbody>
</table>

Bosnia and Herzegovina:

<table>
<thead>
<tr>
<th>Cat</th>
<th>Family</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Viperidae</td>
<td>Vipera ammodytes</td>
</tr>
<tr>
<td>2</td>
<td>Viperidae</td>
<td>Vipera berus</td>
</tr>
</tbody>
</table>

Croatia:

<table>
<thead>
<tr>
<th>Cat</th>
<th>Family</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Viperidae</td>
<td>Vipera ammodytes</td>
</tr>
<tr>
<td>2</td>
<td>Viperidae</td>
<td>Vipera berus</td>
</tr>
</tbody>
</table>

Czechia:

<table>
<thead>
<tr>
<th>Cat</th>
<th>Family</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Viperidae</td>
<td>Vipera berus</td>
</tr>
</tbody>
</table>

Greece:

<table>
<thead>
<tr>
<th>Cat</th>
<th>Family</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Viperidae</td>
<td>Vipera ammodytes</td>
</tr>
<tr>
<td>2</td>
<td>Viperidae</td>
<td>Macrovipera schweizeri; Montivipera xanthina; Vipera berus</td>
</tr>
</tbody>
</table>
Hungary:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Viperidae: <em>Vipera berus</em></td>
</tr>
</tbody>
</table>

Poland:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Viperidae: <em>Vipera berus</em></td>
</tr>
</tbody>
</table>

Slovakia:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Viperidae: <em>Vipera berus</em></td>
</tr>
</tbody>
</table>

Eastern Europe

Belarus, Estonia, Latvia, Lithuania, Republic of Moldova:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Viperidae: <em>Vipera berus, Vipera nikolskii</em> (Moldova)</td>
</tr>
</tbody>
</table>

Russian Federation:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viperidae: <em>Vipera berus</em></td>
<td>Viperidae: <em>Gloydius halys, Gloydius intermedius, Gloydius ussuriensis</em> (far-east Russia); <em>Macrovipera lebetina</em> (Dagestan); <em>Vipera nikolskii, Vipera renardi, Vipera spp.</em></td>
</tr>
</tbody>
</table>

Ukraine:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Viperidae: <em>Vipera berus, Vipera nikolskii, Vipera renardi</em></td>
</tr>
</tbody>
</table>

Western Europe

Austria:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Viperidae: <em>Vipera ammodytes, Vipera berus</em></td>
</tr>
</tbody>
</table>

Belgium:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Viperidae: <em>Vipera berus</em></td>
</tr>
</tbody>
</table>
### Denmark:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td><strong>Viperidae: Vipera berus</strong></td>
</tr>
</tbody>
</table>

### Finland:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td><strong>Viperidae: Vipera berus</strong></td>
</tr>
</tbody>
</table>

### France:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viperidae: Vipera aspis</strong></td>
<td><strong>Viperidae: Vipera berus</strong></td>
</tr>
</tbody>
</table>

### Germany:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td><strong>Viperidae: Vipera berus</strong></td>
</tr>
</tbody>
</table>

### Italy:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viperidae: Vipera aspis</strong></td>
<td><strong>Viperidae: Vipera ammodytes, Vipera berus</strong></td>
</tr>
</tbody>
</table>

### the Netherlands:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td><strong>Viperidae: Vipera berus</strong></td>
</tr>
</tbody>
</table>

### Norway:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td><strong>Viperidae: Vipera berus</strong></td>
</tr>
</tbody>
</table>

### Portugal:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td><strong>Viperidae: Vipera latastei, Vipera seoanei</strong></td>
</tr>
</tbody>
</table>

### Spain:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Cat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td><strong>Viperidae: Vipera aspis, Vipera latastei, Vipera seoanei</strong></td>
</tr>
</tbody>
</table>
Sweden:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Viperidae: <em>Vipera berus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2</td>
<td>None</td>
</tr>
</tbody>
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Switzerland:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2</td>
<td>Viperidae: <em>Vipera aspis</em>, <em>Vipera berus</em></td>
</tr>
</tbody>
</table>

United Kingdom of Great Britain and Northern Ireland:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Viperidae: <em>Vipera berus</em> (not Northern Ireland)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2</td>
<td>None</td>
</tr>
</tbody>
</table>

THE AMERICAS

North America

Canada:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2</td>
<td>Viperidae: <em>Crotalus oreganus</em>, <em>Crotalus viridis</em>; <em>Sistrurus catenatus</em></td>
</tr>
</tbody>
</table>

Mexico:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Viperidae: <em>Agkistrodon bilineatus</em>, <em>Agkistrodon taylori</em>; <em>Crotalus atrox</em>, <em>Crotalus scutulatus</em>, <em>Crotalus simus</em>, <em>Crotalus molossus</em>; <em>Bothrops asper</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2</td>
<td>Elapidae: <em>Micruroides euryxanthus</em>, <em>Micrurus nigrocinctus</em>, <em>Micrurus tener</em>, <em>Micrurus spp.</em>; Viperidae: <em>Agkistrodon contortrix</em>, <em>Agkistrodon ruseolus</em>; Atropoides mexicanus, Atropoides occiduus, Atropoides spp.; <em>Bothriechis schlegelii</em>, <em>Bothriechis spp.</em>; <em>Cerrophidion godmani</em>, <em>Cerrophidion spp.</em>; <em>Crotalus basiliscus</em>, <em>Crotalus totonacus</em>, <em>Crotalus oreganus</em>, <em>Crotalus ruber</em>, <em>Crotalus tzabcan</em>, <em>Crotalus viridis</em>, <em>Crotalus spp.</em>; <em>Ophryacus spp.</em>; <em>Porthidium nasutum</em>, <em>Porthidium spp.</em>; <em>Sistrurus catenatus</em></td>
</tr>
</tbody>
</table>

United States of America:

<table>
<thead>
<tr>
<th>Cat 1</th>
<th>Viperidae: <em>Agkistrodon contortrix</em>, <em>Agkistrodon piscivorus</em>; <em>Crotalus adamanteus</em>, <em>Crotalus atrox</em>, <em>Crotalus horridus</em>, <em>Crotalus oreganus</em>, <em>Crotalus scutulatus</em>, <em>Crotalus viridis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2</td>
<td>Elapidae: <em>Micrurus fulvius</em>, <em>Micrurus tener</em>; Viperidae: <em>Crotalus molossus</em>, <em>Crotalus ornatus</em>, <em>Crotalus ruber</em>, <em>Crotalus spp.</em>; <em>Sistrurus catenatus</em>, <em>Sistrurus milianii</em></td>
</tr>
</tbody>
</table>
Central America
The most medically important species are Crotalus simus and Bothrops asper.

Belize:

| Cat 1: Viperidae: **Bothrops asper** |
| Cat 2: Elapidae: Micrurus spp.; Viperidae: Agkistrodon russeolus; Atropoides mexicanus; Bothriechis schlegelii; Crotalus tzabcan; Porthidium nasutum |

Costa Rica:

| Cat 1: Viperidae: **Bothrops asper, Crotalus simus** |
| Cat 2: Elapidae: Micrurus nigrocinctus, Micrurus spp.; Viperidae: Agkistrodon howardgloydi; Atropoides mexicanus, Atropoides picadoi; Bothriechis schlegelii, Bothriechis lateralis, Bothriechis spp.; Cerrophidion sasai; Lachesis melanocephala, Lachesis stenophrys; Porthidium nasutum, Porthidium ophrymegas, Porthidium spp. |

El Salvador:

| Cat 1: Viperidae: **Crotalus simus** |
| Cat 2: Elapidae: Micrurus nigrocinctus, Micrurus spp.; Viperidae: Agkistrodon bilineatus; Atropoides occiduus; Bothriechis spp.; Cerrophidion wilsoni; Porthidium ophrymegas |

Guatemala:

| Cat 1: Viperidae: **Bothrops asper, Crotalus simus** |
| Cat 2: Elapidae: Micrurus nigrocinctus, Micrurus spp.; Viperidae: Agkistrodon bilineatus, Agkistrodon russeolus; Atropoides mexicanus, Atropoides occiduus; Bothriechis schlegelii, Bothriechis spp.; Cerrophidion godmani, Crotalus tzabcan, Porthidium nasutum, Porthidium ophrymegas |

Honduras:

| Cat 1: Viperidae: **Bothrops asper** |
| Cat 2: Elapidae: Micrurus nigrocinctus, Micrurus spp.; Viperidae: Agkistrodon howardgloydi; Atropoides mexicanus, Atropoides spp.; Bothriechis marchi, Bothriechis schlegelii, Bothriechis spp.; Cerrophidion wilsoni; Crotalus simus; Porthidium nasutum, Porthidium ophrymegas |
Nicaragua:

| Cat 1: | Viperidae: Bothrops asper, Crotalus simus |
| Cat 2: | Elapidae: Micrurus nigrocinctus, Micrurus spp.; Viperidae: Agkistrodon howardgloydi; Atropoides mexicanus; Bothriechis schlegelii; Cerrophidion godmani; Lachesis stenophrys; Porthidium nasutum, Porthidium ophryomegas |

Panama:

| Cat 1: | Viperidae: Bothrops asper |
| Cat 2: | Elapidae: Micrurus m impartitus, Micrurus nigrocinctus, Micrurus spp.; Viperidae: Atropoides mexicanus, Atropoides spp.; Bothriechis lateralis, Bothriechis schlegelii, Bothriechis spp.; Cerrophidion sasai; Lachesis acrochorda, Lachesis stenophrys; Porthidium nasutum, Porthidium lansbergii, Porthidium spp. |

Caribbean

No medically important snakes occur naturally in Anguilla, Antigua and Barbuda, the Bahamas, Barbados, Bermuda, The British Virgin Islands, Cayman Islands, Cuba, Dominica, the Dominican Republic, Grenada, Guadeloupe, Haiti, Jamaica, Montserrat, the Netherlands Antilles, Saint Kitts and Nevis, Saint Vincent and the Grenadines, and Turks and Caicos Islands.

Aruba, Martinique, Saint Lucia, Trinidad and Tobago, and offshore islands:

| Cat 1: | Viperidae: Bothrops cf. atrox (Trinidad), Bothrops caribbaeus (St Lucia), Bothrops lanceolatus (Martinique); Crotalus durissus (Aruba) |
| Cat 2: | Elapidae: Micrurus cirinalis (Trinidad), Micrurus lemniscatus (Trinidad); Viperidae: Lachesis muta (Trinidad) |

South America

No venomous snakes are occur naturally in the Falkland Islands and no dangerously venomous snakes occur naturally in Chile.

Argentina:

| Cat 1: | Viperidae: Bothrops alternatus, Bothrops diporus; Crotalus durissus |
| Cat 2: | Elapidae: Micrurus corallinus, Micrurus lemniscatus, Micrurus spp.; Viperidae: Bothrops ammodytoides, Bothrops jararaca, Bothrops jararacussu, Bothrops mattogrossensis, Bothrops neuwiedi, Bothrops pubescens |
Bolivia (Plurinational State of):

<table>
<thead>
<tr>
<th>Cat 1: Viperidae: Bothrops atrox, Bothrops mattrasshi; Crotalus durissus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2: Elapidae: Micrurus lemniscatus, Micrurus spixii, Micrurus surinamensis, Micrurus spp.; Viperidae: Bothrocophias hyoprora, Bothrocophias microphthalus; Bothrops bilineatus, Bothrops brasili, Bothrops jararacussu, Bothrops jonathani, Bothrops moojeni, Bothrops sanctaecrucis, Bothrops spp., Bothrops taeniatus; Lachesis muta</td>
</tr>
</tbody>
</table>

Brazil:

<table>
<thead>
<tr>
<th>Cat 1: Viperidae: Bothrops atrox, Bothrops jararaca, Bothrops jararacussu, Bothrops leucuru, Bothrops moojeni; Crotalus durissus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2: Elapidae: Micrurus corallinus, Micrurus lemniscatus, Micrurus spixii, Micrurus surinamensis, Micrurus spp.; Viperidae: Bothrocophias hyoprora, Bothrocophias microphthalus; Bothrops alternatus, Bothrops bilineatus, Bothrops brasili, Bothrops diporus, Bothrops mattrasshi; Bothrops neuwiedi, Bothrops pubescens, Bothrops taeniatus, Bothrops spp.; Lachesis muta</td>
</tr>
</tbody>
</table>

Colombia:

<table>
<thead>
<tr>
<th>Cat 1: Viperidae: Bothrops asper, Bothrops atrox, Bothrops bilineatus; Crotalus durissus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2: Elapidae: Micrurus lemniscatus, Micrurus m impartitus, Micrurus nigrocinctus, Micrurus spixii, Micrurus surinamensis, Micrurus spp.; Viperidae: Bothriechis schlegelii; Bothrocophias hyoprora, Bothrocophias microphthalus, Bothrocophias spp.; Bothrops brasili, Bothrops taeniatus, Bothrops spp.; Lachesis acrochorda, Lachesis muta; Porthidium nasutum, Porthidium lansbergii</td>
</tr>
</tbody>
</table>

Ecuador:

<table>
<thead>
<tr>
<th>Cat 1: Viperidae: Bothrops asper, Bothrops atrox, Bothrops bilineatus; Lachesis muta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2: Elapidae: Micrurus lemniscatus, Micrurus m impartitus, Micrurus spixii, Micrurus surinamensis, Micrurus spp.; Viperidae: Bothriechis schlegelii; Bothrocophias hyoprora, Bothrocophias microphthalus, Bothrocophias spp.; Bothrops brasili, Bothrops taeniatus, Bothrops spp.; Lachesis acrochorda; Porthidium nasutum, Porthidium spp.</td>
</tr>
</tbody>
</table>

French Guiana (France):

<table>
<thead>
<tr>
<th>Cat 1: Viperidae: Bothrops atrox, Bothrops bilineatus; Crotalus durissus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2: Elapidae: Micrurus lemniscatus, Micrurus surinamensis, Micrurus spp.; Viperidae: Bothrops brasili, Bothrops taeniatus; Lachesis muta</td>
</tr>
</tbody>
</table>
### Guyana:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Viperidae: Bothrops atrox, Bothrops bilineatus; Crotalus durissus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Elapidae: Micrurus lemniscatus, Micrurus surinamensis, Micrurus spp.; Viperidae: Bothrops brazili, Bothrops taeniatus; Lachesis muta</td>
</tr>
</tbody>
</table>

### Paraguay:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Viperidae: Bothrops alternatus; Crotalus durissus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Elapidae: Micrurus corallinus, Micrurus lemniscatus, Micrurus spixii, Micrurus surinamensis, Micrurus spp.; Viperidae: Bothrops diporus, Bothrops jararaca, Bothrops jararacussu, Bothrops mattogrossensis, Bothrops moojeni, Bothrops neuwiedi, Bothrops spp.</td>
</tr>
</tbody>
</table>

### Peru:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Viperidae: Bothrops atrox, Bothrops bilineatus, Bothrops pictus; Crotalus durissus; Lachesis muta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Elapidae: Micrurus lemniscatus, Micrurus mipartitus, Micrurus spixii, Micrurus surinamensis, Micrurus spp.; Viperidae: Bothriechis schlegelii; Bothrocophias hyopora, Bothrocophias microphthalmus; Bothrops asper; Bothrops brazili, Bothrops mattogrossensis, Bothrops taeniatus, Bothrops spp.</td>
</tr>
</tbody>
</table>

### Suriname:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Viperidae: Bothrops atrox, Bothrops bilineatus; Crotalus durissus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Elapidae: Micrurus lemniscatus, Micrurus surinamensis, Micrurus spp.; Viperidae: Bothrops brazili, Bothrops taeniatus; Lachesis muta</td>
</tr>
</tbody>
</table>

### Uruguay:

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Viperidae: Bothrops alternatus; Bothrops pubescens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Elapidae: Micrurus corallinus, Micrurus spp.; Viperidae: Crotalus durissus</td>
</tr>
</tbody>
</table>

### Venezuela (Bolivarian Republic of):

<table>
<thead>
<tr>
<th>Cat 1:</th>
<th>Viperidae: Bothrops atrox, Bothrops cf. atrox, Bothrops venezuelensis; Crotalus durissus (including Isla de Margarita)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 2:</td>
<td>Elapidae: Micrurus circinalis, Micrurus lemniscatus, Micrurus mipartitus, Micrurus spixii, Micrurus surinamensis, Micrurus spp.; Viperidae: Bothriechis schlegelii; Bothrops asper, Bothrops brazili, Bothrops bilineatus; Lachesis muta; Porthidium lansbergii</td>
</tr>
</tbody>
</table>
Herpetological references


Buys PJ, Buys PJC. Snakes of Namibia. Windhoek; Gamsberg Macmillan; 1983.


---

7 Major regional guides have author names italicized.


Murphy JC. Amphibians and Reptiles of Trinidad and Tobago. Malabar, FL: Krieger; 1997.


Appendix 2

Model protocol for the production and testing of snake antivenom immunoglobulins

Identification of the lot
Name and address of manufacturer ________________________________
Lot number of antivenom ________________________________
Date of filling ________________________________
Liquid or freeze-dried ________________________________
Expiry date ________________________________
Number of vials or ampoules ________________________________
Temperature of storage ________________________________

Control of the venom batch(es) used for animal immunization
Producer of venom and location ________________________________
Information on the snake contributing to the
venom batch ________________________________
Scientific names of the snake species ________________________________
Number of snakes ________________________________
Geographical origins of the snakes ________________________________
Dates of collection of the venoms ________________________________
Expiry date of the venoms preparation ________________________________
Biochemical and biological characterization
of the venoms ________________________________
  – Test performed ________________________________
  – Results ________________________________

Control of plasma donor animals
Location of the animal herd ________________________________
Animal species used for immunization ________________________________
Vaccinations performed on animals ________________________________
Dates of immunization ________________________________
Control of antivenom antibody titre of animal ________________________________
Veterinary certificate of health of animal donor ________________________________
### Collection and storage of plasma

**Method of collection**

**Date of collection**

**Date of storage**

**Type of containers**

**Temperature of storage**

**Type and content of preservatives added (if any)**

### Transport of plasma to fractionation facility

**Date of transport**

**Temperature of transport**

**Date of arrival**

### Plasma pooling and fractionation

**Temperature of plasma storage at fractionation facility**

**Volume of plasmas of different specificity pooled for the production of polyspecific antivenoms (if applicable)**

**Date of plasma pooling**

**Volume of the manufacturing plasma pool**

**Number of animal donors contributing to the manufacturing plasma pool**

**Quality control of manufacturing plasma pool**

- **Test performed**
- **Results**

**Type of active substance (intact IgG, fragments)**

### Preparation and control of final bulk

**Volume of bulk antivenoms of different specificity pooled for the production of polyspecific antivenoms (if applicable)**

**Concentration of preservatives (if used)**

- **Type**
- **Method**
- **Result**

**Quality control of manufacturing plasma pool**

- **Test performed**
- **Results**
### Filling and containers

<table>
<thead>
<tr>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of filling</td>
<td></td>
</tr>
<tr>
<td>Quantity of containers</td>
<td></td>
</tr>
<tr>
<td>Volume of antivenoms per container</td>
<td></td>
</tr>
<tr>
<td>Date of freeze-drying (if applicable)</td>
<td></td>
</tr>
</tbody>
</table>

### Control tests on final product

<table>
<thead>
<tr>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td></td>
</tr>
<tr>
<td>Solubility (freeze-dried product)</td>
<td></td>
</tr>
<tr>
<td>Extractable volume</td>
<td></td>
</tr>
<tr>
<td>Venom-neutralizing potency assay</td>
<td></td>
</tr>
<tr>
<td>- Method</td>
<td></td>
</tr>
<tr>
<td>- Venom used</td>
<td></td>
</tr>
<tr>
<td>- Results</td>
<td></td>
</tr>
<tr>
<td>Osmolality</td>
<td></td>
</tr>
<tr>
<td>Identity test</td>
<td></td>
</tr>
<tr>
<td>- Method</td>
<td></td>
</tr>
<tr>
<td>- Result</td>
<td></td>
</tr>
<tr>
<td>Protein concentration</td>
<td></td>
</tr>
<tr>
<td>- Method</td>
<td></td>
</tr>
<tr>
<td>- Result</td>
<td></td>
</tr>
<tr>
<td>Purity</td>
<td></td>
</tr>
<tr>
<td>- Method</td>
<td></td>
</tr>
<tr>
<td>- Result</td>
<td></td>
</tr>
<tr>
<td>Molecular size distribution</td>
<td></td>
</tr>
<tr>
<td>- Method</td>
<td></td>
</tr>
<tr>
<td>- Result</td>
<td></td>
</tr>
<tr>
<td>Test for pyrogenic substances</td>
<td></td>
</tr>
<tr>
<td>- Method</td>
<td></td>
</tr>
<tr>
<td>- Result</td>
<td></td>
</tr>
<tr>
<td>Sterility test</td>
<td></td>
</tr>
<tr>
<td>No. of containers examined</td>
<td></td>
</tr>
<tr>
<td>- Method</td>
<td></td>
</tr>
<tr>
<td>- Date at start of test</td>
<td></td>
</tr>
<tr>
<td>- Date at end of test</td>
<td></td>
</tr>
<tr>
<td>Concentration of sodium chloride and other excipients</td>
<td></td>
</tr>
<tr>
<td>- Method</td>
<td></td>
</tr>
<tr>
<td>- Result</td>
<td></td>
</tr>
</tbody>
</table>
Determination of pH
  - Result ________________________________

Concentration of preservatives (if used)
  - Type ________________________________
  - Method ______________________________
  - Result ______________________________

Chemical agents used in plasma fractionation
  - Type ________________________________
  - Method ______________________________
  - Result ______________________________

Inspection of final containers
  - Results ______________________________

Residual moisture in freeze-dried antivenoms
  - Method ______________________________
  - Result ______________________________

**Internal certification**

*Certification by person taking overall responsibility for production of the antivenom*

I certify that batch no. __________________ of __________________ snake antivenom immunoglobulin meets all national requirements and/or satisfies the 2016 WHO Guidelines for the production, control and regulation of snake antivenom immunoglobulins.¹

Signature ________________________________

Name (typed) ________________________________

Date ________________________________

Annex 6

WHO manual for the preparation of secondary reference materials for in vitro diagnostic assays designed for infectious disease nucleic acid or antigen detection: calibration to WHO International Standards

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   5.3 Tertiary standards 400
   5.4 Other control material 400
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Guidance documents published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>% CV</td>
<td>percentage coefficient of variation</td>
</tr>
<tr>
<td>Ct</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EQA</td>
<td>external quality assurance</td>
</tr>
<tr>
<td>IS</td>
<td>International Standard(s)</td>
</tr>
<tr>
<td>IU</td>
<td>International Unit(s)</td>
</tr>
<tr>
<td>IVD</td>
<td>in vitro diagnostic</td>
</tr>
<tr>
<td>MU</td>
<td>measurement uncertainty</td>
</tr>
<tr>
<td>NAT</td>
<td>nucleic acid amplification technique</td>
</tr>
<tr>
<td>NRA</td>
<td>national regulatory authority</td>
</tr>
<tr>
<td>PT</td>
<td>proficiency testing</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>S/CO</td>
<td>sample-to-cut-off (ratio); also signal-to-cut-off (ratio)</td>
</tr>
<tr>
<td>SI</td>
<td><em>Système international d’unités</em> (measurement system using metric units)</td>
</tr>
<tr>
<td>SoGAT</td>
<td>Standardisation of Genome Amplification Techniques (group)</td>
</tr>
</tbody>
</table>
1. Background

Through its Expert Committee on Biological Standardization, WHO developed its Guidelines for the preparation and establishment of reference materials and reference reagents for biological substances in 1978 (1). This document was last revised in 2004 (2). In the revised WHO Recommendations document, secondary standards are defined as reference preparations established by regional or national authorities, or by other laboratories, that are calibrated against and traceable to WHO reference materials. Part B of the 2004 Recommendations deals with general considerations for the preparation, characterization and calibration of regional or national biological reference standards.

Feedback from manufacturers and providers of secondary (for example, regional) standards used for in vitro diagnostic (IVD) devices, and from regulatory authorities, international trade organizations, IVD manufacturers, providers of external quality assurance (EQA) or proficiency testing (PT) programmes and laboratories using diagnostic assays, indicated a need for more specific guidance on the preparation of secondary standards; it was therefore concluded that a practical manual focusing on IVD needs would be helpful. This topic was discussed at the 2012 meeting of the WHO Expert Committee on Biological Standardization, at which the proposal to generate a WHO document on secondary standards for use in the IVD field was endorsed.

2. Purpose and scope

This WHO document provides practical guidance on the preparation of secondary biological reference materials and on their calibration to WHO International Standards (IS) where available. The document focuses on the in vitro measurement procedures used for diagnosis, detection and management of infectious diseases where the typical analytes (measurands) are nucleic acid or antigen (Ag). These IVD tests cover nucleic acid amplification technique (NAT)-based assays for detecting the DNA or RNA of infectious agents and immunological tests for the detection of Ag(s) of infectious agents. Currently, there are only a small number of IS with an assigned unitage available where the analyte is an antibody directed to an infectious agent. Due to their complexity (that is, the epitope spectrum represented by polyclonal antibodies in the serum of a patient) this document does not cover antibody-based secondary standards. However, several principles outlined in this manual may also apply to antibody assays. Where applicable, the document integrates existing guidance, referenced accordingly.

The document is intended for use by manufacturers of secondary reference materials, IVD manufacturers, providers of EQA or PT programmes
and other laboratories using reference materials for NAT-based and serological infectious disease assays. Analogous guidance has already been issued by WHO on secondary standards for vaccines (3) and chemical reference substances (4).

3. Terminology

The definitions given below apply to the terms as used in this WHO guidance document. These terms may have different meanings in other contexts.

**Accuracy**: (measurement) closeness of agreement between a measured quantity value and the true quantity value of a measurand (5).

**Biological matrix**: a discrete material of biological origin that can be sampled and processed in a reproducible manner. Examples include blood, serum, plasma, urine, faeces, saliva, sputum and various discrete tissues (6).

**Calibration**: a process that, under specified conditions, establishes as a first step a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication (5).

**Calibration hierarchy**: a sequence of calibrations from a reference to the final measuring system, where the outcome of each calibration depends on the outcome of the previous calibration (5).

**Commutability (of a reference material)**: a property of a reference material, demonstrated by the equivalence of the mathematical relationships among the results of different measurement procedures for a reference material and for representative samples of the type intended to be measured (7).

**Control material**: a substance, material or article used to verify the performance characteristics of an in vitro diagnostic (IVD) medical device (8).

**Diagnostic specificity**: the probability that the device gives a negative result in the absence of the target marker (9).

**End-point titre**: the reciprocal of the highest analyte dilution that gives a reading above the assay cut-off (10).

**International measurement standard**: a measurement standard recognized by signatories to an international agreement and intended to serve worldwide, for example a WHO International Standard (IS) (5).

**International conventional calibrator**: a calibrator whose value of a quantity is not metrologically traceable to SI units but is assigned by international agreement (11).

**International Unit(s) (IU)**: the unitage assigned by WHO to an International Biological Standard (2).

**Linearity (of a measuring system)**: the ability to provide measured quantity values that are directly proportional to the value of the measurand in the sample (12).
Potency: the specific ability or capacity of a product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result (13).

Precision: (measurement) closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions (5).

Relative potency: a measure obtained from the comparison of a test to a standard on the basis of capacity to produce the expected potency (13).

Reference material: a material, sufficiently homogeneous and stable with regard to specified properties, which has been established to be fit for its intended use in measurement or in the examination of nominal properties (5).

Reference standard: a measurement standard designated for the calibration of other measurement standards for quantities of a given kind in a given organization or at a given location (5).

Sample-to-cut-off (S/CO) ratio (also signal-to-cut-off ratio): S/CO ratios are calculated by dividing the signal value (for example, optical density or relative light unit) of the sample being tested by the signal value of the enzyme immunoassay or chemiluminescence assay cut-off for that run. If the signal produced by a given test sample is equal to or greater than the calculated cut-off value then the specimen is considered to be reactive in the test. In competitive assays the relationship between the signal value of the sample and the signal value of the cut-off is reversed (CO/S ratio) (14).

Secondary (reference) standards: reference standards established by regional or national authorities, or by other laboratories, that are calibrated against and traceable to WHO reference materials (2).


Traceability: (metrological) property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty (5).

Threshold cycle: the polymerase chain reaction (PCR) cycle at which the gain in fluorescence generated by the accumulating amplicon exceeds a threshold over baseline – for example, defined as 10 standard deviations of the mean baseline fluorescence using data taken from cycles 3 to 15 (15).

Working standard: a measurement standard used routinely to calibrate or verify measuring instruments or measuring systems (5).
Uncertainty: a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand (16).

4. Principles of biological standardization

The aim of metrological traceability is to enable the results obtained by the calibrated routine measurement procedure to be expressed in terms of the values obtained at the highest available level of the calibration hierarchy (11). This is usually achieved in the clinical chemistry field by physicochemical reference methods, obtaining values in SI units.

The majority of biological samples, containing for example nucleic acids, antigens or antibodies, are substances that cannot be fully characterized by a physicochemical reference method. Instead, biological assays are used for measurement of the potency or content of the analyte of interest. These methods are heterogeneous and the lack of a reference method does not permit the results to be expressed in absolute values according to the SI system.

The approach taken by the WHO Expert Committee on Biological Standardization to quantify biological materials is to first establish a highest order reference reagent – the IS. The procedure for the preparation, characterization and establishment of WHO IS preparations is described in detail elsewhere (2). Such material plays a crucial role in the standardization, harmonization and quality control of IVD assays, as was demonstrated in the 1990s when WHO IS were introduced for human immunodeficiency virus, hepatitis C virus and hepatitis B virus. These reference materials were fundamental in the regulation of IVD assays used for blood safety and for improving patient management in the clinical setting (17).

5. Calibration hierarchy of biological standards

“Reference material” is a generic term which refers to a material or substance whose property values are sufficiently homogeneous and stable, and whose fitness for purpose is well established, for its intended use in a measurement process (for example, the assessment of a measurement method or the assigning of values to materials) (18, 19). Biological reference materials for a given analyte can be related through a sequence of comparisons to create a calibration hierarchy traceable to the highest order material – the WHO IS (Fig. A6.1).
Furthermore, all biological standards have a range of key properties as summarized in Table A6.1.

**Table A6.1**  
**Key properties of WHO IS, secondary standards and tertiary standards**

<table>
<thead>
<tr>
<th>Property</th>
<th>WHO IS</th>
<th>Secondary standard</th>
<th>Tertiary standard</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alternative names</strong></td>
<td>Highest order, international conventional calibrator</td>
<td>Regional or national reference materials, laboratory or manufacturer’s working calibrator</td>
<td>Working reagents or standards, manufacturer’s product calibrator, control material</td>
</tr>
<tr>
<td><strong>Calibration</strong></td>
<td>Evaluated in an international collaborative study, involving laboratories worldwide, different assays and different types of test laboratories (usually 15–30 participants)</td>
<td>Calibrated against the WHO IS</td>
<td>Calibrated against the secondary standard</td>
</tr>
<tr>
<td>Property</td>
<td>WHO IS</td>
<td>Secondary standard</td>
<td>Tertiary standard</td>
</tr>
<tr>
<td>------------------</td>
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</tr>
<tr>
<td>Unitage</td>
<td>IU/mL</td>
<td>IU/mL</td>
<td>IU/mL</td>
</tr>
<tr>
<td>Traceability</td>
<td>N/A</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Uncertainty of measurement</td>
<td>No</td>
<td>Yes (assay-specific)</td>
<td>Yes (assay-specific)</td>
</tr>
<tr>
<td>Commutability</td>
<td>Must be determined experimentally relative to clinical specimens</td>
<td>Should be determined experimentally relative to clinical specimen</td>
<td>Consideration should be given to experimentally determining relative to clinical specimen</td>
</tr>
<tr>
<td>Material</td>
<td>Should resemble, as closely and as feasibly as possible, the analyte being measured – for example, for assays for viral nucleic acids the standard will be the wild-type patient-derived virus in plasma (the normal sample type analysed)</td>
<td>Should resemble, as closely as possible, the analyte to be measured. However, for assay-specific secondary standards, synthetic materials such as armored RNA, plasmids and recombinant proteins, may be used and laboratories are encouraged to address commutability</td>
<td>Should resemble, as closely as possible, the analyte to be measured. Biological material similar to the tested sample, or non-biological materials, such as armored RNA, plasmids and recombinant proteins may be used, and laboratories are encouraged to address commutability</td>
</tr>
<tr>
<td>Typical final format of standard</td>
<td>Lyophilized</td>
<td>Lyophilized or liquid</td>
<td>Liquid</td>
</tr>
<tr>
<td>Usage</td>
<td>Calibration of secondary standards; initial validation of new assay/platform</td>
<td>Calibration of tertiary standards; working standards; run control; calibrator</td>
<td>Working standards; run control; calibrator</td>
</tr>
</tbody>
</table>
### Table A6.1 continued

<table>
<thead>
<tr>
<th>Property</th>
<th>WHO IS</th>
<th>Secondary standard</th>
<th>Tertiary standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Establishment of standard</td>
<td>International agreement through a WHO international collaborative study, proposal for adoption and subsequent establishment by the WHO Expert Committee on Biological Standardization</td>
<td>May be calibrated in several ways:</td>
<td>1. Assay-specific study, normally by a single laboratory for use with a specific test/platform.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1. In parallel with a study to establish the IS</td>
<td>2. Small study by a limited number of laboratories with a single assay or a limited number of different assays/platforms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Example: <strong>Appendix 1</strong></td>
<td></td>
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<td></td>
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<td>2. Regional or national collaborative study similar to the WHO collaborative study but with fewer participants</td>
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<td>Example: <strong>Appendix 2</strong></td>
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<td></td>
<td>3. Small study by one or a limited number of laboratories with a single assay or a limited number of different assays/platforms</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Example: <strong>Appendix 3</strong></td>
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</tbody>
</table>

### 5.1 WHO International Standards

WHO IS are defined by ISO17511 as International Conventional Calibrators and are the highest order of standard for biological references. They are solely established by the WHO Expert Committee on Biological Standardization following specific guidance (2).

Establishment of a WHO IS follows a collaborative study involving various users of the material (including national control laboratories, IVD manufacturers and other certified laboratories) and as many different, well-established assays as feasible. The laboratories should be chosen to reflect the global use of the standard and consideration should be given to the expertise of laboratories with a proven track record (perhaps through EQA schemes).
As with all biological references, the material used should resemble as closely as possible the natural analyte of the clinical sample to be measured. An assessment of commutability should be performed as part of the collaborative study where appropriate and feasible (2).

By definition, an IS has a specified value expressed in International Units (IU). This value is arbitrarily assigned based on the results of the collaborative study. The assigned IU value of each IS (new and replacement) does not carry an uncertainty associated with calibration (see section 8.1 below). The uncertainty is considered to be the variance of the vial weight determined during the filling process (20, 21). The collaborative study design attempts to ensure continuity of the IU as far as possible. As explained further in section 8.1 there is no uncertainty value associated with replacements.

As the highest-order standard for biological material, the use of a WHO IS should be limited to the calibration of secondary biological reference materials in order to minimize the need to replace the IS on a regular basis. Unfortunately, the limited availability of secondary calibrator standards and the lack of specific guidance on the establishment and calibration of more readily available standards have resulted in the overuse of WHO IS for more routine procedures such as validation of assays and as run controls.

5.2 Secondary standards

A secondary standard is a material that has been directly calibrated against the IS. These preparations usually include regional or national reference preparations. The titre, composition and method of production of secondary standards will vary but should be suitable for obtaining sufficient measurements, when dilution is needed, to achieve an accurate calibration. Regardless of the method of production, each calibration will have a stated measurement uncertainty (see section 8.1 below).

Secondary standards should be used for the calibration of tertiary standards. They should also be used for the calibration and validation of assay systems.

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1 Biological stock materials assigned a potency based on calibration against the IS by using exclusively one specific test, and used, for example, for the preparation of calibrators or run controls for this test, are also considered as in-house secondary standards. Non-biological preparations such as synthetic preparations (for example, plasmid preparations, transcripts, armored RNA and antigens produced by recombinant DNA technology) are often used for test calibration. If this calibration is done against an IS, these materials will also be considered as secondary standards under the scope of this document. Nevertheless, these materials have a number of limitations compared to the biological preparations, for example, in terms of commutability.
5.3 Tertiary standards
Tertiary standards are calibrated against secondary standards using the same calibration procedure. These standards usually include working standards or calibrators established for one specific assay used by a laboratory or other institution.

The standard may be formulated from either biological (for example, patient-derived) or non-biological material. However, regardless of the material used, all references in the traceability chain should also demonstrate commutability to the clinical sample of the tested analyte. Many of the principles discussed in this document also apply to the development of tertiary standards.

Tertiary standards are typically formulated as a liquid preparation and may comprise a concentration of the analyte that is detected without dilution in the linear range of the assay it is intended for. They will often be used as an external control material in addition to that normally supplied by the assay manufacturer. Regular monitoring of such material may allow for the early detection of problems with assay performance.

5.4 Other control material
Control material that does not follow the path of traceability back to the highest-order reference material (that is, to the IS) may be produced by commercial and in-house laboratories where no higher-order standard is available for the analyte.

The material may be used as a run control, whereby the unit of measurement (for example, signal-to-cut-off ratio (S/CO) values, threshold cycle values for real-time nucleic acid amplification technique (NAT)-based assays, copies/mL or genome equivalents/mL) can be used for intra-laboratory monitoring and may provide valuable trending data in a similar way to tertiary standards. However, such material has not been designed to allow for the comparison of results between different assays.

6. Commutability of biological standards
Commutability is a property of a reference material such that values measured for that reference material and for representative clinical samples have the same relationship between two or more measurement procedures for the same measurand (10) – that is, reference materials should behave in the same way as the native analyte itself. Producing biological reference materials that are commutable can be challenging because the matrix of the analyte may vary in different clinical conditions, or the analyte may be modified during preparation or processing of the reference material. Commutability can therefore only be demonstrated for particular combinations of assays, with particular clinical samples. It is not a generic property of the reference materials. Thus,
demonstrating commutability for two assay methods does not guarantee that there will be commutability with other methods. Similarly, if a set of samples demonstrate commutability with each other for particular assay methods then this does not guarantee that this will apply to all samples (22, 23).

However, ISO17511:2003 states that calibrators are to be commutable at each step in the traceability chain (11). There are established recommendations for the assessment of commutability of reference materials used in laboratory medicine (7).

7. Selection and characterization of materials for the preparation of secondary standards – and application to tertiary standards

The basic parameters to be considered when selecting and characterizing a material for the preparation of a secondary standard are described in this section. In general, these considerations also apply to the process of developing tertiary reference materials to be used daily as control materials. In both scenarios, the selection of materials is dependent upon the type of technology to which they are to be applied, for example, NAT-based or immunological test systems. If necessary, expert scientific advice should be sought to support the development of any secondary standard.

The following should be considered:

- analyte – type, source and specificity
- immunological and genetic diversity
- type of matrix
- target concentration
- volume of final aliquots and storage temperature
- diagnostic specificity
- infectivity/inactivation
- physical appearance
- homogeneity
- commutability of the material
- stability – real-time and accelerated degradation.

7.1 Analyte – biological versus synthetic material

Consideration must be given to the most suitable form of the analyte to be established as a secondary standard – for example, whole organism, purified nucleic acid, recombinant protein, or laboratory-derived or clinical isolate.
Ideally, the material selected should resemble the analyte in the IS and in usual clinical specimens as closely as possible. The decision may be based on the availability of a sufficient volume of the material to enable preparation of a single batch of secondary standard that, when frozen, will last several years. In most cases, laboratory strains of microorganisms are better characterized and available in larger volumes than clinical strains. However, the latter may better represent the samples that are routinely tested. Where a whole organism is unavailable in sufficient quantities, a laboratory-derived material (such as a purified nucleic acid preparation) may be the only option. Demonstrating the commutability of such a material to different clinical samples may be challenging. This will need to be addressed on a case-by-case basis and should be done in cases where, through experimental assessment, it is proven that the use of laboratory-derived material improves agreement between assays. Analytes derived directly from human origin (such as a clinical sample) or the matrix of a biological sample (such as plasma or whole blood) should be tested and confirmed to be negative for the presence of pathogens other than the analyte of interest. This should be done to exclude potential cross-reactivity with the specific target analyte. If it is necessary to prepare a bulk material by pooling from more than one source, each component of the bulk material must be characterized and where possible all components should be identical – for example, in molecular detection the sequence of the target regions should be the same. All samples pooled must be mixed thoroughly and the pool should be homogeneous. It should be noted that pooling may not be appropriate in all circumstances. A biological bulk material with a high analyte concentration could if needed be diluted in a suitable matrix.

7.2 Immunological and genetic diversity

The detectability of an analyte by a particular assay may vary due to the immunological and genetic variability (serotypes, strains, variants, genotypes or subtypes and so on) of the organism being tested, resulting in suboptimal detection of particular variants. Therefore, the candidate material that best reflects the samples being tested should be chosen for the preparation of the secondary standard. Consideration should be given to the local geographical patterns of genetic diversity.

Where a standard is being prepared for nucleic acid detection, a well-characterized strain should be used for which the full genome sequence (or at least the sequence covering the most frequently amplified regions) is available. In principle, the same holds true for antigen standards where well-characterized antigen variants should be chosen. In both cases consideration should be given to the diagnostic implications of variant detection.
7.3 **Matrix**

The matrix in which the standard is formulated is crucial to creating a material that is fit for purpose. For NAT-based assays, the matrix needs to be appropriate for the assay or range of assays for which it is intended. Multiple sample types may be considered. It follows that for commutability purposes, a biological matrix would be preferred over a synthetic matrix. It should be taken into consideration that some sample matrices include inhibitory factors that interfere with the performance of specific types of assays (7). Furthermore, the chosen matrix of the reference preparation should be compatible with further matrices into which it may be spiked. For example, where a pathogen may be screened for in plasma, whole blood or urine, it may not be appropriate to formulate a material in a matrix that cannot be further diluted in clinical samples. In the case of plasma, consideration should also be given to any anticoagulant treatment and to any additional treatments such as cryoprecipitation or recalcification.

7.4 **Concentration**

Secondary standards will often be used in a quantitative capacity. Therefore, the concentration used should be high enough to permit the preparation of dilutions across the dynamic range of the assay and to allow for dilution into further matrices. It must be noted that dilutions performed on a high-titre secondary standard will contribute to the overall uncertainty arising from the dilution process. This will be the case for most secondary standards.

The target concentration of a standard will be dependent upon its final intended use and whether any clinical decision points exist when testing for the analyte. The detectable/quantitative range of all well-established assays for that pathogen must be taken into consideration. Reference materials that perform within the dynamic range of an assay (where changes in signal correspond to changes in analyte concentration) will typically be the norm.

In the case of tertiary standards where the material may be used as an external run control in qualitative tests, the concentration should ideally be at the lower end of the range of detection, at a concentration which will appropriately challenge the assays (for example, three times the 95% limit of detection of a NAT-based assay, or within the dynamic range of serological assays).

7.5 **Volume**

The aliquot volume may vary depending on the typical assay input volume for that analyte and the final storage temperature. The suitability of the container used for the filling of the aliquots should be validated in terms of the integrity and stability of the analyte. Where the standard is intended for single use, as defined in the Instructions for Use provided with the material, sufficient
volume should be provided for use in the assays for which it is intended and any remaining material must be discarded by the laboratory as no in-use stability testing will have been performed. If the standard is for multiple uses, the volume will depend on the required number of tests, and on stability at the recommended storage temperature. Where the manufacturer intends a material to be used on multiple occasions, suitable stability testing should be carried out to indicate the number of times the material can be removed from its storage temperature, allowed to warm to ambient temperature and then be re-cooled before the stability of the material is compromised.

7.6 Diagnostic specificity
Samples derived directly from human origin (for example, clinical samples or where the matrix is a biological component such as plasma or whole blood) should be tested and confirmed to be negative for the presence of pathogens other than the analyte of interest in order to minimize risk and the potential for cross-reactivity with the target analyte.

Where human material is used as the diluent, the diluent should be tested and found to be negative for both the analyte and common high-risk pathogens.

7.7 Infectivity
Use of infectious materials will impact on processing, handling and shipping of the standard. Where samples are prepared from infectious material, it is important to provide clear information to the end users about the exact nature of the material and origin of the pathogens. Import regulations differ from country to country and can vary depending on the origin of the pathogen. For example, tissue-culture-derived viral specimens may be subjected to different shipping regulations by some countries than a patient sample infected with this virus.

Where standards are prepared from inactivated materials it is important to confirm the success of the inactivation procedure and to determine potential effects of the inactivation on the performance of the final standard. The use of established or proven inactivation methods (such as published methods) is preferred. In addition, in cases where standards are prepared from inactivated material but are diluted in a biological matrix (for example, human plasma) the matrix should be screened by NAT-based and serological assays for the most common bloodborne viruses.

7.8 Physical appearance
A number of factors will determine the most appropriate physical appearance for the standard. Typically, IS are lyophilized preparations which have a better stability and longer shelf-life than, for example, liquid preparations. They are
also required to be shipped around the world, preferably at ambient temperature. This may also be the case for some secondary standards, especially national or regional secondary standards, such as the European Pharmacopoeia Biological Reference Preparations for NAT-based assays. A feasibility study should be conducted to demonstrate that the freeze-drying procedure does not have an adverse effect on the integrity of the target analyte. However, lyophilization is costly and specialized, and, for some pathogens, maintaining a stable product is problematic. In these cases, a liquid preparation stored and shipped at a suitable temperature may be more appropriate. Lyophilization also requires additional validation work to determine the potential impact of such a technique on the biological activity of the standard and on the commutability of the standard with clinical samples. In the case of nucleic acid extraction, lyophilization can lead to the formation of aggregates which reduces extraction efficiency. Lyophilized preparations should be evaluated against the liquid bulk preparation in different assays as part of the commutability assessment. Where standards are used frequently, such as tertiary control materials, a liquid or single-use frozen preparation is probably more suitable so that the standard is ready for use without the need for reconstitution.

### 7.9 Homogeneity

It is important to confirm the consistency of the filling procedure and to confirm that the bulk was dispersed (for example, stirred) sufficiently throughout. Homogeneity is assessed in two ways – by determining both the biological and the physical content (weight or volume) of multiple vials across the batch. The latter is particularly important prior to lyophilization, and can be addressed by weighing a proportion of vials before and after filling and then calculating the filled weight and associated coefficient of variation as a percentage (% CV). The % CV will be higher for a more viscous matrix. It is also important to determine the biological homogeneity by assessing the concentration of the analyte in multiple vials across the batch. It is known that homogeneity may be impaired by genetic quasi-species heterogeneity or antibody complexation. The number of vials used for testing will depend on the batch size. As a minimum, typically 1–2% of the vials should be tested (17).

### 7.10 Stability

A stability testing programme should be implemented to monitor the potency of the secondary standard over time. Stability monitoring can be based on real-time data. However, additional data from accelerated thermal degradation studies are helpful in characterizing the robustness of lyophilized reference materials. Such data are also important for assessing the suitability of the material after extreme shipping conditions.
Factors affecting stability will be dependent upon the physical appearance and final storage temperature of the material. For example, for final long-term storage at 2–8 °C, preservatives might need to be included in the final formulation to prevent fungal or bacterial growth. Likewise, lyophilized preparations may require different additives, such as inert sugars, which might aid preservation and protect pathogen viability upon reconstitution. It should be noted that the effect of any additives on downstream performance or commutability should be evaluated. Factors affecting the stability of lyophilized preparations also include residual moisture and oxygen, both of which can compromise the integrity of the lyophilized product. It is advisable to assess the levels of residual moisture and oxygen in such preparations following lyophilization and if possible during storage of the material. This is particularly important for stoppered vials which may permit the ingress of air during the lifetime of the product, causing displacement of the vacuum or inert gas which the material is held under. Loss of potency may occur as a result.

7.11 Stability assessment during product lifetime

7.11.1 Real-time stability
Materials should be periodically removed from their designated storage temperatures for testing in the routine laboratory assay of choice. The required frequency of testing will be dependent upon the physical appearance and final storage temperature of the material. For example, a frozen liquid product stored at −20 °C may require more frequent testing than a lyophilized product stored at −20 °C. Real-time monitoring may be more frequent following production of a new material, but by monitoring over time the frequency of testing could be reduced. For example, a liquid-filled preparation stored at −20 °C could be tested every 3 months for the first year following production. If the data suggest good stability then the testing interval could be increased to 6 months. Likewise, a lyophilized product may be tested every 6 months for the first year following production, but further testing could be reduced to annually. Any assessment of stability and associated outcome should be referenced on the Instructions for Use distributed with the materials, for example where an acceptable number of freeze–thaw cycles has been determined, this should be referenced.

7.11.2 Accelerated thermal degradation studies
Real-time stability studies may not demonstrate loss in analyte concentration over the testing period. For lyophilized material, accelerated thermal degradation studies can be used to predict the long-term stability of a product from its performance at elevated temperatures. For example, data demonstrating a loss in titre after 3 months at 37 °C can be used to predict the time it would take
for the same amount of degradation to occur at −20 °C or at another chosen baseline storage temperature of the product. In addition, accelerated thermal degradation studies cover the validation of the use of the reference material after extreme shipment conditions.

A chosen number of samples should be stored in temperature-controlled environments at for example, −20 °C, 4 °C, 20 °C and 37 °C. Studies at 45 °C and 56 °C may also be suitable for some pathogens and matrices but not all. For example, a lyophilized plasma matrix will generally not reconstitute following long-term storage at the higher temperatures indicated; however, this may not fully reflect the degradation of the analyte. Vials should be periodically removed and tested alongside vials that have been continuously stored at the recommended storage temperature (reference point).

The Arrhenius equation can be applied to the resulting data in order to predict the rate of degradation of the material at the recommended storage temperature (24, 25).

While accelerated thermal degradation studies have considerable usefulness in the production of secondary reference materials, they may be less useful for tertiary standards, for which real-time monitoring will provide the most valuable data set. Note that for tertiary standards used as external quality control IVDs, accelerated stability testing is usually performed prior to release. In all cases, stability testing protocols should be designed in conformity with ISO 23640:2013.

7.12 In-use stability

In-use stability testing of the standard measures the stability of the standard once it has been thawed, opened or reconstituted (depending on storage conditions and physical appearance). It is important to establish in-use stability if the material is intended to be used on multiple occasions and if it is stored under different conditions during this period. Where multiple freeze–thaw events are likely, the effect of these should be evaluated.

7.12.1 Lyophilized preparations

Materials should be reconstituted as defined in the Instructions for Use, aliquoted and stored at an appropriate temperature (for example, −80 °C for materials containing free RNA as the analyte).

Reconstituted materials should be subjected to freeze–thawing cycles and tested at predetermined intervals – for example, weekly, monthly, annually or biannually, and monitored by suitable assays (preferably quantitative assays if available). Tests should be performed on at least three replicates per time point to determine any loss in concentration.
7.12.2 Frozen preparations

If the total volume in each vial allows for multiple uses, at least three vials should be freeze–thawed on multiple occasions. At each time point the samples should be tested at least in duplicate in three replicate tests. If no degradation is observed then further time points should be added up to the limit of volume remaining in the vial.

7.12.3 Liquid preparations

Where materials are routinely held at 2–8 °C, exposure to short periods at ambient temperature may occur when the product is in use. Where this is the case multiple exposures to ambient temperatures should be assessed. Three vials should be removed from storage, left at room temperature for up to 1 hour and tested at least in duplicate in triplicate tests. These vials should then be returned to 2–8 °C and the process repeated with the same vials at frequent intervals. Interval frequency can be determined by the accumulation of data points and may be reduced following an observation of good stability, as discussed in section 7.11.1 above.

8. Calibration: testing and statistical analysis

8.1 Principles of calibration

Calibration is the process by which a concentration is assigned to a reference by the direct comparison of measurements with a higher-order reference, and represents one of the crucial stages of the establishment of a secondary standard. Each calibration of a candidate secondary standard has to be performed in parallel with the higher-order reference, in this case the WHO IS. The following sections describe the minimum requirements for the calibration of secondary standards intended for either one specific method in one laboratory (single assay calibration) or for multiple methods (collaborative study calibration). In both cases, several independent runs with the candidate standard and the IS in parallel have to be performed (same assay using the same test conditions). For each run, a new vial of each standard should be used and diluted in the matrix validated for the respective assay (for example, negative human plasma).

This WHO guidance document reflects the common statistical methods used for the calibration of reference materials. Any other statistical method that has been demonstrated to be a reliable approach to the calibration of such materials may also be applied. Appropriate software for the statistical analysis should be available for evaluation of the data, and the statistical analysis should be performed by staff with expertise in this field.

The calibration study data should be analysed using the relevant statistical models for bioassays, for example, using the methods recommended...
by the European Pharmacopoeia (26). The design of the study should take into consideration that:

- each analyte, IS and the candidate secondary standard should be tested with the same number of dilutions and replicates per dilution;
- the adjacent dilution steps should be equally spaced.

The statistical validity of the fitted model should be assessed for each individual assay. For the parallel-line and Probit models – as the most appropriate and proven statistical methods for this analysis – the linearity and parallelism of the logarithmic dose–response relationships between the IS and secondary standard should be evaluated. If the assay response is linear and the response lines are parallel then the relative potency of the candidate secondary standard against the IS can be calculated. Using the parallel-line model, validity criteria for linearity could include the coefficient of determination ($r^2$) or a test for nonlinearity (26). Parallelism could be demonstrated, for example, by means of a test for non-parallelism or an equivalence approach for the difference or ratio of slopes (that is, the 95% confidence interval for the ratio of slopes must lie entirely between predefined equivalence margins). In addition, the precision with which the potency has been estimated should be provided.

Each calibration will have a stated measurement uncertainty. This estimate can be determined by identifying all sources of variation, calculating the extent of variation and using established methods to combine the uncertainty. The measurement uncertainty associated with assigning a value to the standard is test-system specific (7, 8). It should be noted that an IS, by definition, has a specified value which has been assigned and expressed in IU per millilitre (IU/mL). As a consequence of defining the IU as a fraction of the contents of the container of the current IS, and because the units defined by any previous IS formally cease to exist, an uncertainty value is not given to the assigned IU (2). The variability of the vial weight during filling for each IS is quoted in the study report and in the Instructions for Use accompanying the standard.

8.2 Single assay calibration using qualitative tests

8.2.1 NAT-based assays

For qualitative NAT-based assays, four independent runs should be performed. The first run involves testing serial ten-fold dilutions (until negative) of the IS and the candidate secondary standard in duplicate, and is intended to determine the end-point dilution of both standards. In the subsequent three assays, two half-log10 dilutions either side of the predetermined end-point (5 dilutions in total) of the standards should be tested. Each dilution (runs 2–4) should be tested at least in duplicate, giving in total 6 replicates per dilution across all runs. For each assay, data from all runs at each dilution step will be pooled to
give a number of positive results out of the total number of tests performed. The Probit analysis will estimate the concentration at which 63% of the samples tested were positive (that is, the dilution at which on average one single copy per sample tested could be expected under the assumption of an underlying Poisson distribution). The calculated end-point is used to give estimates expressed in NAT-detectable units/mL after correcting for an equivalent volume of the test sample. It should be noted that these estimates are not necessarily directly equivalent to a genuine genome equivalent number or copy number per mL. The software for the Poisson distribution will calculate the proportion of potency of the test sample (candidate secondary standard) relative to the potency of the standard sample (that is, the IS) so long as the dose–response curves fit within the statistical model.

Using a real-time NAT-based assay, the calibration can be determined from the cycle threshold (Ct) values by applying a parallel-line analysis, conditional on the assumption that the slope fulfils the requirement of \(-1/\log_2\). The number of dilutions and the number of replicates per dilution should follow the instructions given below in section 8.3.1.

### 8.2.2 Antigen assays

The IS and the candidate secondary standard should be tested in three independent assays. Both standards should be diluted, using serial half-log\(_{10}\) or two-fold dilutions, in the diluent appropriate for the assay. The dilution ranges should be within the detection range of the assays used for the study and should span the end-point titre (intercept with the cut-off of the assays). The analytical sensitivity of each assay can be calculated by linear interpolation using the two dilutions of the dilution series having values below and above the assay cut-off. The (log-transformed) data should be evaluated against the results obtained for the secondary standard using a parallel-line assay analysis to estimate the potency (IU/mL) of the secondary standard relative to the IS. A logarithmic transformation of the assay response may be necessary if the dilution range was chosen around the sample cut-off rather than the dynamic range of the assay.

### 8.3 Single assay calibration using quantitative tests

#### 8.3.1 NAT-based assays

For the calibration of secondary standards tested in a quantitative NAT-based assay, the candidate material should be tested neat (where possible) and at two or three further (for example, ten-fold) dilutions within the linear range of the assay to obtain at least three concentrations giving quantitative values. The same methodology applies to the IS, with the exception that this material should be diluted starting from a concentration as close as possible to the estimated potency of the secondary standard (as indicated by preliminary tests). All standards
should be tested in duplicate and at least three independent runs should be performed. Where possible, multiple assay lots and reagents should be included in this testing. The calculation of the potency of the secondary standard may be performed in one of several ways:

- The assay output (for example, copies, genome equivalents\(^2\) or IU/mL) should be analysed by the parallel-line method using, if necessary, log-transformed data to obtain a “relative potency” in IU/mL of the secondary standard against the IS (where the slope fulfils the requirement of \(-1/\log_2\) corresponding to a value of \(S = -3.322\)). The parallel-line method should be the preferred option for the data analysis.

- The difference in estimated potency (using the test software) between the candidate secondary standard and the IS (log-transformed data) can be used to determine the potency of the secondary standard. The difference is then subtracted from the log-transformed nominal IU/mL of the IS to obtain the potency of the secondary standard.

- A standard curve generated by the instrument software using the IS as the standard may be used to determine the potency of the secondary standard.

### 8.3.2 Antigen assays

As with the quantitative NAT-based assays, the candidate material and the IS should be tested neat and at two or three further (for example, ten-fold) dilutions within the linear range of the assay, using the dilution matrix appropriate for the assay. All samples should be tested in duplicate in at least three independent runs. The calculation of the potency of the secondary standard can be performed in one of several ways:

- The results obtained with the parallel-line analysis (if necessary on log-transformed data) should be used to give a “relative potency” of the secondary standard against the IS in IU/mL. The parallel-line analysis should be the preferred option for the data analysis.

- A standard curve generated by the instrument software using the IS as the standard may be used to determine the unitage of the secondary standard.

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\(^2\) In the case of assays not yet calibrated against the IS.
8.4 **Collaborative study calibration using multiple assays**

Secondary standards that are intended to be used in different assays by multiple end users should be calibrated using a collaborative study approach. The amount of work and resources required to perform such a study should not be underestimated. The collaborative study should be organized by/or with advice from a body with experience in this field, such as a WHO collaborating centre. If necessary, a scientific adviser from the field should be identified to support the collaborative study, including the selection of study participants. Owing to the complexity of the reported data, which typically include data from many different types of assays, the statistical analysis should be performed by a statistician. The general principles of planning and executing these types of collaborative study are described in section 6 of the WHO *Recommendations for the preparation, characterization and establishment of international and other biological reference standards* (2).

Results from all participants should be analysed by statistical methods described and considered appropriate by the responsible statistician. This analysis typically requires access to suitable computing facilities and statistical software. The testing requirements and protocol of each laboratory/test should follow the protocol described for the single assay calibration depending on the assay type (qualitative or quantitative). The results of each assay method should be analysed separately and should provide an estimate of the relative potency and precision of the candidate secondary standard against the IS.

The variation in results between test methods, and between laboratories, should be described and assessed as part of the statistical analysis (precision and consistency of the results). An assessment should be made of any factors causing significant heterogeneity of the estimated potency, nonlinearity and differences in slopes. Although there is no generic outlier detection rule from the statistical point of view, the exclusion of data should be taken into account in subsequent analysis where striking differences in results within assays, between assays, or between participants or test methods are observed. All valid potency estimates for the candidate secondary standard should be combined to produce a final geometric mean potency/content with 95% confidence limits. The results of the tests should be displayed graphically, for example, as histograms or scatter plots. Tables of the quantitative and qualitative raw data should be included in the report annexes.

8.5 **Calculation of uncertainty of measurement**

The assignment of an uncertainty value must be applied to secondary reference materials. The uncertainty of an observed value is a property of the test system and is not the effect of mistakes introduced through human error. The
measurement of uncertainty is complex and, where possible, advice should be sought from a statistician. This section aims to give an overview of the area and to highlight that consideration must be given to the assignment of an uncertainty value to secondary references.

The uncertainty – often referred to as “measurement uncertainty” (MU) – expresses the 95% confidence limits either side of the observed value assigned to a product. By estimating the MU of a product the confidence in the final value assigned is shown. Uncertainty should be calculated using log-transformed data. For example, a value of \(3 \log_{10} \text{IU/mL}\) may be assigned to a standard. Following estimation of uncertainty this value may be displayed as \(3 \log_{10} \text{IU/mL} \pm 0.2\) – that is, the value of the material could range from 2.8–3.2 \(\log_{10} \text{IU/mL}\).

There are many aspects to uncertainty and well-documented examples of how to estimate uncertainty (20, 27–29). However, one simple approach to estimating MU for a secondary standard is to test the material multiple times on different occasions (but always using the same test system) in parallel with the WHO IS – that is, test the two standards under exactly the same conditions.

As a guide, a minimum of 30 test results for both the secondary standard and the IS should be generated from a combination of at least three independent tests. The more times the sample is tested the better. The test system used should be of the highest order possible, that is, a commercial assay, or, in the absence of such, a well-validated laboratory-developed test.

After determining the mean and standard deviation of the data points, dividing the standard deviation by the square root of the number of samples tested gives the standard uncertainty (or standard error) of measurement. This approach demonstrates the imprecision involved and does not account for MU derived from inherent bias.


In the absence of a WHO IS, an established regional or national reference standard may become the standard of comparison for the candidate assay. Current examples include: West Nile virus NAT reference reagent (Health Canada), and Chikungunya virus standard (Center for Biologics Evaluation and Research, United States Food and Drug Administration). These standards should be characterized through extensive analysis. The methods for their characterization, preparation and storage should ideally be published in peer-reviewed journals.

The calibration of reference standards where no IS and secondary standards are available should follow the WHO-recommended principles for
the preparation of international biological standards (2). Where no IS exists but a regional standard is available the calibration of a reference standard of lower order should follow the guidance provided in this document.

In the absence of an IS or a national reference standard, a candidate reference material may be assigned a value using either a commercial assay or a laboratory-developed test. Alternatively, the material may be calibrated against other commercially available preparations or samples from EQA programmes with assigned values, or a manufacturer’s working calibrator. For such reference materials, reserving a separate proportion of vials (baseline samples) to be used in the calibration of subsequent batches is strongly advised. This batch should be stored at the lowest possible temperature validated for the vials, preferably at a lower temperature than that at which the bulk of the reference material is stored (for example, if a reference material is routinely stored at −70 °C, the baseline samples should be stored under nitrogen vapour or liquid). Reference materials may also be assigned a value based on a range of assays (collaborative study) as described above rather than a single assay. In such cases, the assigned value will be the mean of the results reported by all the assays. Whichever approach is used, the method and assigned value need to be documented (20).

10. Post-production considerations

10.1 Storage of the material

Following the development and production of a batch of secondary reference materials, the material should be stored at an appropriate temperature which ensures stability throughout the lifetime of the product. The temperature of the cold-storage unit should be monitored and recorded. A protocol should be developed for the real-time monitoring of each product and should include details of testing frequency, number of replicates, methodology used and statistical analysis (see section 7.11 above).

10.2 Distribution of the material

Consideration should also be given to the method by which the material is to be distributed. Where material is potentially infectious, specialist couriers may be required. Some countries have import-permit requirements for infectious materials. These requirements are country-specific and should be discussed with the recipient in advance of the shipment.

Stability of the product during transportation can be addressed by distributing the material at appropriate temperatures, employing where necessary the use of dry ice or cold packs. The use of dry ice may be considered a hazard by some couriers and may require the use of specialized companies.
10.3 **Instructions for Use**

Detailed Instructions for Use should be supplied with every shipment. These should include the following information:

- characterization details of the analyte
- storage conditions
- procedures prior to use (for example, reconstitution)
- appropriate use
- stability information
- safety information
- references to any publications relating to the material (for example, study report).

10.4 **Replacement batches**

The need for replacement batches should be addressed in the development stages of the initial product. Consideration should be given to the acquisition of material for future replacement of the standard. For example, if the analyte comprises tissue-culture-derived material, large batches of stock material could be cultured in the first instance. Detailed records of the production of the first batch should also be documented to allow replication of the production method at some point after the initial material was produced.

11. **End user advice**

Manufacturers are encouraged to include details of the production and calibration process in the Instructions for Use provided with each material, or to provide a reference to where this information can be found. Any additional information not supplied could be requested from the manufacturer, and could include:

- number of replicates and methods used to assess repeatability and reproducibility;
- the metrological traceability of an assigned unit;
- whether a collaborative study was performed, and if so the number of participants and range of assays evaluated;
- assessment of performance in a different matrix;
- stability assessments including of shelf-life and in-use stability;
- assessment of specificity;
- validation of limit of detection or cut-off.
Authors and acknowledgements

This WHO guidance document was jointly drafted by Dr M. Chudy, Paul-Ehrlich-Institut, Germany; Dr C. Morris, National Institute for Biological Standards and Control, England; Dr J. Fryer National Institute for Biological Standards and Control, England; Dr W. Dimech, National Reference Laboratory, Australia; and Dr J. Saldanha, Immucor, Inc., the USA.

The first draft was then discussed at a Standardisation of Genome Amplification Techniques (SoGAT) workshop in 2015 and presented to the WHO Expert Committee on Biological Standardization in the same year. Further comments on the draft were received from IVD manufacturer associations, individual IVD manufacturers, regulatory bodies and experts in the field. A revised draft was then discussed at a 2016 SoGAT workshop. Acknowledgement is made to all delegates of the SoGAT 2015 and 2016 workshops for their critical reviewing of the draft versions and other inputs.

The second draft version was published on the WHO Biologicals website for a round of public consultation between 16 June and 16 September 2016, and the comments received were incorporated to produce the document WHO/BS/2016.2284.

Further changes were subsequently made to document WHO/BS/2016.2284 by the WHO Expert Committee on Biological Standardization.

References


Appendix 1

Example of the parallel calibration of a secondary standard in a study to establish the International Standard

WHO/BS/2016.2291

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EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 17 to 21 October 2016

Collaborative Study to Evaluate the Proposed 4th WHO International Standard for Hepatitis B Virus (HBV) DNA for Nucleic Acid Amplification Techniques (NAT)

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by 16 September 2016 and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: Dr C M Nübling at email: nueblingc@who.int.

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Summary
This report describes the collaborative study evaluation of the replacement 4th WHO International Standard for hepatitis B virus (HBV) DNA for the calibration of secondary reference preparations for HBV nucleic acid amplification techniques (NAT). The candidate 4th HBV International Standard was prepared and evaluated as part of the collaborative study to establish the 3rd HBV WHO International Standard in 2011. The lyophilized preparation comprises a dilution of the same HBV DNA-positive plasma sample as used for the previous HBV International Standards, in pooled human plasma. In this collaborative study, thirteen laboratories from seven countries evaluated the suitability of the candidate using their routine HBV NAT-based assay. The candidate NIBSC code 10/266 (sample 2) was evaluated alongside the 3rd HBV WHO International Standard, NIBSC code 10/264 (sample 1), three HBV reference preparations (samples 3-5) and three HBV-positive plasma samples comprising different HBV genotypes (samples 6-8). A range of HBV NAT assays were used in the evaluation, the majority of which were commercial quantitative assays based on real-time PCR technology.

The overall mean potency estimates for samples 1 and 2, were 5.94 and 5.97 log_{10} IU/mL respectively. These values are very similar to the values obtained for these samples relative to the pre-existing 2nd HBV WHO International Standard in the 2011 collaborative study (5.93 and 5.98 log_{10} IU/mL respectively). The standard deviation in individual laboratory mean estimates for samples 1 and 2 was 0.13 log_{10} IU/mL. The overall mean potency estimate for sample 2, relative to sample 1 was 5.96 log_{10} IU/mL. The results obtained from ongoing accelerated thermal degradation studies indicate that the candidate sample 2 has remained stable over the 5 years post-manufacture.

The results of the study indicate the suitability of sample 2 as the replacement 4th HBV WHO International Standard. Since the overall mean potency obtained for the candidate in this collaborative study is very similar to the overall mean potency obtained in the 2011 collaborative study, relative to the pre-existing 2nd HBV WHO International Standard, it is proposed that the value assigned to the candidate sample 2 is that obtained in the 2011 collaborative study. This approach would minimize any potential drift in the value of the IU during the replacement. It is therefore proposed that candidate sample 2 (NIBSC code 10/266) is established as the 4th WHO International Standard for HBV DNA for NAT with an assigned potency of 955,000 IU/mL (~5.98 log_{10} IU/mL) when reconstituted in 0.5 mL of nuclease-free water.

Introduction
Hepatitis B virus (HBV) remains a major public health problem worldwide, despite the availability of an effective vaccine and antiviral therapies. More than 240 million people worldwide are chronically infected, with 0.5-1 million dying annually as a result of serious liver disease. The virus is transmitted in blood and body fluids, perinatally and through close person-to-person contact in early childhood (in regions with high HBV prevalence), and through infected needles and sexual contact (in regions with low HBV prevalence). Nucleic acid amplification techniques (NAT) for HBV were first introduced for blood screening in 1997, and are now implemented in at least 30 countries worldwide. However, there remains a residual risk of transfusion-transmitted infection, through occult HBV infection and vaccine breakthrough infections. NAT is routinely used in the diagnosis and management of HBV infections, particularly, to guide the initiation of and monitor the response to antiviral therapy in chronically-infected patients. A range of both commercial and laboratory-developed NAT-based assays are currently in use. The WHO International Standard for HBV DNA was established in 1999, and is used by manufacturers of in vitro diagnostic devices (IVDs), blood transfusion centres, control authorities, and clinical laboratories, to calibrate secondary reference materials for NAT in terms of the International Unit (IU).
The 1st and 2nd WHO International Standards for HBV were prepared by dilution of a Eurohep R1 sample (Genotype A2, HBsAg subtype adw2, derived from a single donor) in HBV-negative pooled human plasma. Both materials were prepared from the same bulk (filled and freeze-dried on two separate occasions) and evaluated in parallel in a worldwide collaborative study using a range of NAT-based assays for HBV. The first candidate (NIBSC code 97/746) was established as the 1st WHO International Standard for HBV DNA in 1999, with an assigned potency of 1,000,000 IU/mL when reconstituted in 0.5 mL nuclease-free water. In 2006, the WHO Expert Committee on Biological Standardization (ECBS) established the second candidate (NIBSC code 97/750) as the replacement 2nd WHO International Standard for HBV DNA following a smaller collaborative study. In 2011, two replacement batches (NIBSC codes 10/264 and 10/266) were prepared from the same original HBV Eurohep R1 stock as the 1st and 2nd WHO International Standards, diluted in pooled human plasma, and were evaluated in a worldwide collaborative study in parallel with the 2nd WHO International Standard for HBV (NIBSC code 97/750). The first candidate (10/264) was established as the 3rd WHO International Standard for HBV DNA in October 2011 with a unitage of 850,000 IU/mL. It was noted that the second candidate (10/266) would be a suitable replacement HBV International Standard in due course, depending on ongoing stability assessment.

The established use of the HBV IU as the unit of measurement for HBV DNA highlights the importance of maintaining the availability of this International Standard. This report describes the collaborative study evaluation of the second candidate 10/266 as the replacement 4th WHO International Standard for HBV DNA. The proposal to replace the 3rd WHO International Standard for HBV DNA was endorsed by the WHO ECBS in October 2015. The collaborative study results were presented to the Scientific Working Group on the Standardization of Genome Amplification Techniques (SoGAT) in London in June 2016. The proposed standard is intended to be used in the in vitro diagnostics field and relates to ISO 17511:2003 Section 5.5.

**Aims of study**

The aim of this collaborative study was to evaluate the suitability of the candidate lyophilized preparation in parallel with the 3rd HBV WHO International Standard (NIBSC code 10/264) using a range of NAT-based assays.

**Materials**

**Candidate standard**

The candidate preparation (NIBSC code 10/266) comprises lyophilized human plasma and HBV. The HBV was sourced from a stock of the Eurohep R1 reference material stored at NIBSC and is a genotype A2, HBsAg subtype adw2 virus from a single donor. The pooled human plasma diluent was sourced from UK blood donations and had been tested and found negative for HIV antibody, HCV antibody, HBsAg and syphilis. It was also tested at NIBSC and found negative for HCV RNA by NAT. The preparation was lyophilized to ensure long-term stability.

The filling and lyophilization of the bulk material was performed under contract at eQAD, UK NEQAS (Colindale, UK), in March 2011 and has been described previously. The bulk was dispensed in 0.5 mL volumes into 3 mL screw-cap glass vials (Adelphi Tubes, Haywards Heath, UK). The homogeneity of the fill was determined by performing check-weighing of approximately every fiftieth vial, with vials outside the defined specification being discarded. Filled vials were partially stoppered, lyophilized and then fully stoppered in the freeze dryer. A total of 2700 vials were prepared for 10/266. The percentage coefficient of variation (%CV) of...
the fill weight was 0.36%. The sealed vials were returned to NIBSC for storage at -20 °C under continuous temperature monitoring for the lifetime of the product. Evaluation of multiple aliquots of 10/266 (n=30) at NIBSC indicated that the HBV content was homogeneous (2SD of 0.12 log_{10} IU/mL). Comparison of the liquid bulk versus the lyophilized product indicated that there was a minimal loss in potency of approximately 0.04 log_{10} IU/mL upon freeze-drying. Assessments of residual moisture and oxygen content, as an indicator of vial integrity after sealing, were determined for 20 vials of 10/266 as previously described 11, and were 0.29% (Karl Fischer, 0.45% NIR units, CV=14.66%) and 0.17% (CV=63.81%) respectively.

**Stability of the lyophilized candidate**

Ongoing accelerated thermal degradation studies have been underway at NIBSC since 2011 in order to predict the stability of 10/266 when stored at the recommended temperature of -20 °C. Vials of lyophilized product have been held at -70 °C, -20 °C, +4 °C, +20 °C, +37 °C and +45 °C. At 11 weeks, 12, 34 and 60 months post-manufacture, three vials have been removed from storage at each temperature and HBV DNA quantified by NAT using the COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, version 2.0 (Roche Diagnostics GmbH, Mannheim, Germany).

**Study samples**

The lyophilized candidate 10/266 was evaluated alongside the 3rd HBV WHO International Standard (NIBSC code 10/264), a HBV Secondary Reference Reagent (SRR), a HBV National Standard (NS) and Working Reagent (WR), and three individual HBV-positive plasma donations comprising different genotypes. These plasma donations were sourced from HBV-positive plasma packs rejected by the UK NHS Blood and Transplant authority (Colindale, UK). The HBV genotype was determined using a multiplex PCR assay with genotype-specific primers 13. Lyophilized and liquid frozen study samples were stored at -20 °C and -70 °C, respectively, prior to shipping to participants by courier on dry ice.

Study samples were coded as samples 1-8 and were as follows:

- Sample 1- Lyophilized 10/264 3rd HBV WHO International Standard in a 3 mL crimp-cap glass vial.
- Sample 2 - Lyophilized candidate 10/266 in a 3 mL screw-cap glass vial.
- Sample 3 - Liquid frozen HBV Secondary Reference Reagent (genotype A) in a 2 mL Sarstedt tube.
- Sample 4 - Liquid frozen HBV National Standard (genotype C) in a 2 mL Sarstedt tube.
- Sample 5 - Liquid frozen HBV Working Reagent (genotype C) in a 2 mL Sarstedt tube.
- Sample 6 - Liquid frozen HBV plasma (genotype D) in a 2 mL Sarstedt tube.
- Sample 7 - Liquid frozen HBV plasma (genotype E) in a 2 mL Sarstedt tube.
- Sample 8 - Liquid frozen HBV plasma (genotype A) in a 2 mL Sarstedt tube.

**Study design**

The aim of this collaborative study was to evaluate the suitability of the candidate 4th HBV WHO International Standard in parallel with the 3rd WHO International Standard for HBV using a range of NAT-based assays. Three HBV reference reagents were included in the study with the intention of calibrating these reagents in IU. Three HBV plasma samples were included in the study in order to provide a limited assessment of commutability 12,14. Three vials of each study sample were sent to participating laboratories, with specific instructions for storage, reconstitution and testing. Samples 6-8 were only sent to laboratories performing quantitative HBV NAT.
Study protocol
Participants were requested to test dilutions of each sample using their routine HBV NAT assay on three separate occasions, using a fresh vial of each sample in each independent assay. In accordance with the study protocol (Appendix 2), the lyophilized samples were to be reconstituted with 0.5 mL of deionized, nuclease-free molecular-grade water and left for a minimum of 20 minutes with occasional agitation before use. Samples 6-8 were to be tested neat and were therefore only evaluated by laboratories performing quantitative assays.

For quantitative assays, participants were requested to test samples 1-8 neat and to test samples 1-5 at a minimum of two serial ten-fold dilutions (10^-1 and 10^-2). For qualitative assays, participants were requested to test ten-fold serial dilutions of samples 1-5, around the assay end-point (in order to determine the actual assay end-point). For subsequent assays, participants were asked to test the dilution at the predetermined end-point, and a minimum of two half-log10 serial dilutions either side of the end-point (i.e., at least five dilutions in total). Participants were requested to perform dilutions using the sample matrix specific to their individual assay (e.g. HBV DNA-negative human plasma), and to extract samples prior to HBV DNA measurement.

Participants were requested to report the concentration of each sample in IU/mL (positive/negative for qualitative assays) for each dilution of each sample and to return results, including details of the methodology used, to NIBSC for analysis.

Participants
Study samples were sent to 13 participants representing 7 countries (Appendix 1). Participants were selected for their experience in HBV NAT, geographic distribution and participation in previous evaluation studies. They represented IVD manufacturers, Official Medicines Control Laboratories (OMCLs) and WHO collaborating centres. All participating laboratories are referred to by a code number, allocated at random, and not representing the order of listing in Appendix 1. Where a laboratory returned data using different assay methods, the results were analyzed separately, as if from different laboratories, and are referred to as, for example, laboratory 01A, 01B etc.

Statistical methods
Qualitative and quantitative assay results were evaluated separately. In the case of qualitative assays (from laboratory 12), data from all assays were pooled to give a number positive out of number tested at each dilution step. A single ‘end-point’ for each dilution series was calculated, to give an estimate of ‘NAT detectable units/mL’, as described previously 15. It should be noted that these estimates are not necessarily directly equivalent to a genuine genome copy number/mL. In the case of quantitative assays, analysis was based on the results supplied by the participants. Results were reported as IU/mL. For each assay run, a single estimate of log10 IU/mL was obtained for each sample, by taking the mean of the log10 estimates of IU/mL across replicates, after correcting for any dilution factor. A single estimate for the laboratory and assay method was then calculated as the mean of the log10 estimates of IU/mL across assay runs.

All analysis was based on the log10 estimates of IU/mL or ‘NAT detectable units/mL’. Overall mean estimates were calculated as the means of all individual laboratories. Variation between laboratories (inter-laboratory) was expressed as standard deviation (SD) of the log10 estimates and % geometric coefficient of variation (%GCV) 16 of the actual estimates. Potencies relative to sample 1, the current HBV WHO International Standard (10/264), were calculated as the difference in estimated log10 ‘units per mL’ (test sample – standard) plus the value in log10 IU/mL for the International Standard. Therefore for example, if in an individual assay, the test
sample is 0.5 log10 higher than the International Standard, assigned 5.93 log10 IU/mL, the relative potency of the test sample is 6.43 log10 IU/mL.

For the quantitative assays, variation within laboratories, and between assays, (intra-laboratory) was expressed as SDs and %GCVs of the individual assay mean log10 estimates. These estimates were pooled across all samples. The significance of the inter-laboratory variation relative to the intra-laboratory variation was assessed by an analysis of variance.

Results and data analysis
Validation of study samples and stability assessment
Production data for the candidate 10/266 from 2011 showed that the CV of the fill weight and mean residual moisture were within acceptable limits for a WHO International Standard 17. The residual oxygen content was within the NIBSC working limit of 1.1%.

Samples of 10/266 were stored at elevated temperatures, and assayed at NIBSC in parallel with samples stored at -20 °C and -70 °C by HBV NAT (as described for the stability of the lyophilized candidate). Three vials of each sample were evaluated after storage at each temperature for 11 weeks, 12, 30 and 60 months. It was not possible to reconstitute the vials stored at +37 °C and +45 °C for 12, 30 and 60 months. The mean estimated log10 IU/mL and differences (log10 IU/mL) from the -20 °C baseline samples are shown in Table 1. A negative value indicates a drop in potency relative to the -20 °C baseline. The 95% confidence intervals for the differences are ±0.08 log10 based on a pooled estimate of the standard deviation between individual vial test results. As there is no observed drop in potency it is not possible to fit the usual Arrhenius model for accelerated degradation studies, or obtain any predictions for the expected loss per year with long-term storage at -20 °C. All available data indicates adequate stability. Stability testing of 10/266 will be ongoing.

The stability of 10/266 when reconstituted has not been specifically determined. Therefore, it is recommended that the reconstituted material is for single use only.

Data received
Data were received from all 13 participating laboratories. Participants performed a variety of different assay methods, with some laboratories performing more than one assay method. In total, 15 datasets were received from 14 quantitative assays and 1 qualitative assay. Apart from the cases noted below, there were no exclusions of data.

Quantitative Assays:
Data were returned with dilutions ranging from neat to 10^-8 from different laboratories, although only dilutions between neat and 10^-2 were used in the analysis. For Sample 5, one or two dilutions were removed from the following laboratories’ data, 01, 02B, 03, 05, 06, 07, 08, 10 and 13 because the results were below the limit of detection. Samples not demonstrating dilutional linearity (i.e. a linear relationship between reported HBV content against log10 dilution with fitted slope between 0.80 and 1.25) were excluded. Non-linearity was assessed visually and determined in the following cases; laboratory 11A, Sample 5 on day 2, Samples 3 and 5 on day 3; laboratory 11B, Sample 5 on day 3; laboratory 13, Sample 3 on day 2. Laboratories 04, 05, 07, 09, 11B and 13 had one to three samples with slopes outside the range 0.80-1.25, with the majority of these cases (7 out of 10) for Sample 5.

Qualitative Assays:
Laboratory 12 tested multiple dilutions, from $10^{-1}$ down to $10^{-8}$ for different samples. Only dilutions that were tested in at least two of the three assays were used in the analysis.

**Summary of assay methodologies**

The majority of participants prepared dilutions of study samples 1-5 using negative human plasma, however, Basematrix 53 (SeraCare Life Sciences, Inc., Milford MA, USA) was also used. Assay methodologies for qualitative and quantitative assays are summarized in Table 2. Ten different commercial HBV NAT assays were represented. Five new assays were included that were not represented in the 2011 collaborative study (assay codes; c68, APT, VER, SKB and SaB). Only one of the commercial assays was qualitative (assay code cTSM). No participant used laboratory-developed tests.

**Estimated IU/mL or ‘NAT detectable units/mL’**

The laboratory mean estimates of IU/mL ($\log_{10}$) from the quantitative assays and ‘NAT detectable units/mL’ ($\log_{10}$) from the qualitative assay (shaded in grey) are shown in Table 3. The individual laboratory mean estimates are also shown in histogram form in Figure 1. Each box represents the mean estimate from one laboratory, and the boxes are labeled with the laboratory and assay code. They are also colour coded by assay. For samples 1, 4 and 5, there is good agreement between qualitative and quantitative assays, however, for samples 2 and 3 there is not. This may reflect variability in the dilution of study samples required by the study protocol for qualitative assays rather than actual variability in the detection of HBV in the different study samples. There was good agreement between the estimates from the quantitative assays, particularly for samples 1-3 which all comprise the same genotype A virus. Laboratory 6 appears to underquantify samples 4-8 compared to other quantitative assays. There also appears to be variability in the quantification of samples 4-8 by the different assay methods, with some assays showing either under or over-quantification compared to the overall mean estimate for each of the samples. This may represent variability in the quantification of different HBV genotypes as has been reported previously. However, for sample 8, the pattern of individual laboratory mean estimates is different to that for samples 1-3 despite all comprising genotype A viruses.

Table 4 shows the overall mean estimates of $\log_{10}$ IU/mL from the quantitative assays, along with the SD (of $\log_{10}$ estimates) and the %GCV (of actual estimates). The overall mean estimates for samples 1 and 2 were 5.94 and 5.97 $\log_{10}$ IU/mL respectively. These values are very similar to the values obtained for these samples in the 2011 collaborative study (5.93 and 5.98 $\log_{10}$ IU/mL respectively), despite some differences in the participants and assays involved in each study. For samples 1-3, the SDs and %GCVs are 0.11-0.13 $\log_{10}$ and 29-35% respectively. The overall SDs for samples 1 and 2 are slightly higher than those reported in the 2011 collaborative study. However, this still represents good agreement between laboratories and assay methods. For samples 4 and 5, the SDs and %GCVs are 0.22-0.27 $\log_{10}$ and 65-86% respectively. For samples 6-8, the SDs and %GCVs are 0.29-0.42 $\log_{10}$ and 95-160% respectively. The increased SDs and %GCVs for samples 4-8 are principally due to the outlying results of laboratory 6 (see Figure 1). The overall mean estimates, SDs and %GCVs for samples 1-8 excluding the dataset for laboratory 6 are shown in Table 5. The SDs and %GCVs for samples 4-8 are all reduced (by approximately 2-fold) when the results for laboratory 6 are excluded. Five laboratories reported results using the cobas® AmpliPrep/cobas® TaqMan® HBV Test, v2.0 (assay code cTSM). This assay is over-represented in comparison to the other assays. However, removing datasets from 3 laboratories using this assay did not greatly alter the overall laboratory results (Table 6).
Potencies relative to the 3rd WHO International Standard for HBV (Sample 1)
The estimated concentrations of samples 2-8 were expressed in IU, by direct comparison (relative potencies) to the current International Standard (10/264, sample 1), which has an assigned unitage of 850,00 IU/mL ($5.93 \log_{10}$), as described in the statistical methods section. The laboratory mean estimates are shown in Table 7 for the quantitative and qualitative assays. Units are $\log_{10}$ IU/mL in both cases. The results are also shown in histogram form in Figure 2. The overall mean relative potencies, along with SDs and %GCVs, are shown in Table 8. The overall mean relative potency for the candidate sample 2 is $5.96 \log_{10}$ IU/mL, based on the quantitative assays. This value compares well to the direct estimate of $5.97 \log_{10}$ IU/mL from the quantitative assays which are all calibrated in IU/mL.

Figure 2 and Table 8, show an improvement in the agreement between laboratories for samples 2-5 for the quantitative assays. The SD and %GCV between laboratories has reduced for these samples when compared to the values in Table 4. The reduction in these values for samples 4 and 5 (both genotype C) is less marked than for samples 1-3 (all genotype A), possibly because of increased variability in the quantification of genotype C viruses between the assays. For samples 6-8 there is no improvement in the agreement between laboratories when compared to the values in Table 4. This may be due to variability in the quantification of different HBV genotypes, although for sample 8 there is no improvement in the agreement between laboratories, despite samples 2 and 8 comprising genotype A viruses. Again, the over-representation of the cTM assay in the study did not greatly alter the overall laboratory results (Table 9).

Potencies relative to the candidate 4th WHO International Standard for HBV (Sample 2)
The estimated concentrations of samples 3-8 were expressed in IU, by direct comparison (relative potencies) to the candidate International Standard (10/266, sample 2), based on a candidate unitage of 955,000 IU/mL ($5.98 \log_{10}$), as described in the statistical methods section. This candidate unitage was based on the overall mean potency obtained for 10/266, relative to the 2nd HBV WHO International Standard (97/750), in the 2011 collaborative study.

Overall mean relative potencies, along with SDs and %GCVs, are shown in Table 10. Table 10 shows an improvement in the agreement between laboratories for samples 3-5 for the quantitative assays. The SD and %GCV between laboratories has reduced for these samples when compared to the values in Table 4. For samples 6-8 there is no improvement in the agreement between laboratories when compared to the values in Table 4. Again, this may be due to variability in the quantification of different HBV genotypes, although for sample 8 there is no improvement in the agreement between laboratories, despite samples 2 and 8 comprising genotype A viruses. The over-representation of the cTM assay in the study did not greatly alter the overall laboratory results (Table 11).

Inter and intra-laboratory variation
For all samples, the inter-laboratory variation was greater than the intra-laboratory variation ($p<0.01$). Table 12 shows the intra-laboratory SDs and %GCVs for each laboratory, calculated by pooling the estimates for samples 1-8. There are differences between the repeatability of laboratory estimates across assays. In general, the repeatability is good for assays of this type and the average SD is $0.07 \log_{10}$ or a %GCV of 18%. These values are slightly improved compared to the equivalent values obtained in the 2011 collaborative study (average SD of $0.08 \log_{10}$, %GCV of 20%) [1]. These figures represent the variability between individual assay mean estimates of IU/mL. Since each assay tested multiple replicates of samples at different dilutions,
the resulting between-assay variability is lower than would be expected if only a single replicate was tested in each assay. The ‘NAT detectable units’ from the qualitative assays are obtained by pooling all assay data to give a single series of number positive out of number tested at each dilution. As a result, there is no comparable analysis of intra-assay variation for the qualitative assay.

Conclusions
In this study, a range of NAT-based assays for HBV have been used to evaluate the suitability of the candidate standard (NIBSC code 10/266) as the replacement 4th WHO International Standard for HBV DNA for NAT-based assays. The candidate was prepared from the same virus stock used for previous HBV WHO International Standards and was diluted in a similar pooled human plasma material.\textsuperscript{6,7,9,10} Production data suggests that the batch is homogeneous and contains residual moisture and oxygen levels that are within WHO and NIBSC limits for lyophilized standards.\textsuperscript{11,17} Comparison of the liquid bulk versus the lyophilized product indicates that there was minimal loss in potency upon freeze-drying (0.04 log\textsubscript{10} IU/mL). The results of ongoing accelerated thermal degradation studies at 60 months indicate that the candidate is stable and suitable for long-term use.

In the collaborative study, the lyophilized candidate (sample 2) was evaluated alongside the 3rd HBV WHO International Standard (sample 1). The overall mean estimates for samples 1 and 2 were 5.94 and 5.97 Log\textsubscript{10} IU/mL, respectively, based on the calibration of quantitative assay kits in IU/mL. These values are very similar to the values obtained for these samples relative to the 2nd HBV WHO International Standard in the 2011 collaborative study,\textsuperscript{11} despite laboratories reporting results using different HBV NAT-based assays. In the present study, the agreement between laboratories for sample 2 was improved when the potency was expressed relative to the 3rd HBV WHO International Standard (sample 1). There was some evidence for variability in the quantification of different HBV genotypes present in the different study samples. This has been reported previously.\textsuperscript{18} Inter-laboratory variability was higher than intra-laboratory variability for the quantitative assays. This highlights the continued need for standardization of HBV NAT-based assays, and the importance of accurate calibration to the WHO International Standard.

A full assessment of commutability of the candidate standard for HBV-positive samples has not been possible in this study, due to the limited number of clinical samples that could be included. Three HBV-positive plasma samples comprising HBV genotypes A, D and E, from rejected blood donations were included in the study. There was no overall improvement in the agreement between laboratories when the estimated concentrations of the three HBV plasma samples were expressed relative to the candidate 4th HBV WHO International Standard (same formulation as previous HBV WHO International Standards), compared to the uncorrected values. This is principally due to variability in the quantification of different HBV genotypes between different assays, and outlying results from one or two laboratories for samples 6-8. There is no evidence of non-commutability with the three plasma samples that were included in the study.

In summary, the results of the study indicate the suitability of the candidate sample 2 as the replacement 4th WHO International Standard for HBV DNA for NAT. Since the overall mean potency obtained for the candidate in this collaborative study is very similar to the overall mean potency obtained in the 2011 collaborative study, relative to the pre-existing 2nd HBV WHO International Standard, it is proposed that the value assigned to the candidate sample 2 is that obtained in the 2011 collaborative study. This approach would minimize any potential drift in the value of the IU during the replacement.
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Proposal
It is proposed that the candidate standard, NIBSC code 10/266, is established as the 4th WHO International Standard for HBV DNA for use in NAT-based assays, with an assigned potency of 955,000 IU/mL (~5.98 log_{10} IU/mL) when reconstituted in 0.5 mL of nuclease-free water. This potency is based on the value that was assigned in the collaborative study in 2011 where the candidate was assessed alongside the 2nd HBV WHO International Standard. The uncertainty can be derived from the variance of the fill weight and is 0.36%. The proposed standard is intended to be used by IVD manufacturers, blood transfusion centres, control authorities, and clinical laboratories, to calibrate secondary reference materials used in HBV NAT assays. Proposed Instructions for Use (IFU) for the product are included in Appendix 3.

Comments from participants
8 of 13 participants responded to the report. There were no disagreements with the suitability of the candidate standard (NIBSC code 10/266) to serve as the 4th WHO International Standard for HBV DNA for NAT-based assays. Some comments suggested minor editorial changes and these have been implemented.

Acknowledgements
We gratefully acknowledge the important contributions of the collaborative study participants.

References


Table 1. Thermal stability of 10/266 at different storage temperatures. * Mean results from single extractions from 3 vials at each time point and temperature.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>11 weeks</th>
<th>12 months</th>
<th>34 months</th>
<th>60 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>-70</td>
<td>6.04</td>
<td>6.05</td>
<td>5.96</td>
<td>5.77</td>
</tr>
<tr>
<td>-20</td>
<td>6.03</td>
<td>6.02</td>
<td>5.92</td>
<td>5.77</td>
</tr>
<tr>
<td>4</td>
<td>6.07 (0.04)</td>
<td>6.01 (-0.01)</td>
<td>5.91 (-0.01)</td>
<td>5.80 (0.03)</td>
</tr>
<tr>
<td>20</td>
<td>6.06 (0.03)</td>
<td>6.01 (-0.01)</td>
<td>5.91 (-0.01)</td>
<td>5.89 (0.12)</td>
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<tr>
<td>37</td>
<td>6.09 (0.06)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>45</td>
<td>6.13 (0.10)</td>
<td>-</td>
<td>-</td>
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Table 2. Collaborative study assay methods and codes.

<table>
<thead>
<tr>
<th>Assay Code</th>
<th>Assay</th>
<th>No. of datasets</th>
</tr>
</thead>
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<tr>
<td>kPCR</td>
<td>VERSANT HBV DNA 1.0 Assay (kPCR) (Siemens Healthcare Diagnostics)</td>
<td>1</td>
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<tr>
<td>cTM</td>
<td>cobas® AmpliPrep/cobas® TaqMan® HBV Test, v2.0 (Roche Molecular Systems, Inc.)</td>
<td>5</td>
</tr>
<tr>
<td>c68</td>
<td>cobas® HBV test for use on the cobas® 6800/8800 Systems (Roche Molecular Systems, Inc.)</td>
<td>2</td>
</tr>
<tr>
<td>AbRT</td>
<td>Abbott RealTime HBV (Abbott Molecular, Inc.)</td>
<td>1</td>
</tr>
<tr>
<td>ArQS</td>
<td>artus® HBV QS-RGQ Kit, Version 1 (QIAGEN)</td>
<td>1</td>
</tr>
<tr>
<td>APT</td>
<td>Aptima HBV Quant assay on the Panther system (Hologic, Inc.)</td>
<td>1</td>
</tr>
<tr>
<td>VER</td>
<td>VERIS HBV Assay (Beckman Coulter, Inc.)</td>
<td>1</td>
</tr>
<tr>
<td>SKB</td>
<td>HBV DNA real-time PCR detection kit (Shanghai Kehua Bio-Engineering Co., Ltd.)</td>
<td>1</td>
</tr>
<tr>
<td>SaB</td>
<td>Hepatitis B Viral DNA Quantitative Fluorescence Diagnostic Kit (PCR Fluorescence Probing) Mag (Sansure Biotech, Inc.)</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Assay Code</th>
<th>Assay</th>
<th>No. of datasets</th>
</tr>
</thead>
<tbody>
<tr>
<td>cTSM</td>
<td>cobas® TaqScreen MPX Test, v2.0 (Roche Molecular Systems, Inc.)</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 3. Laboratory mean estimates from quantitative assays (log$_{10}$ IU/mL) and qualitative assays (log$_{10}$ "NAT detectable units/mL"). Qualitative results are shaded in grey. nd, not determined (either not tested or data excluded).

<table>
<thead>
<tr>
<th>Lab Assay</th>
<th>Sample</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>S1</td>
</tr>
<tr>
<td>1 kPCR</td>
<td>6.01</td>
</tr>
<tr>
<td>2A c68</td>
<td>6.00</td>
</tr>
<tr>
<td>2B cTM</td>
<td>5.95</td>
</tr>
<tr>
<td>3 AbRT</td>
<td>5.86</td>
</tr>
<tr>
<td>4 ArQS</td>
<td>6.15</td>
</tr>
<tr>
<td>5 cTM</td>
<td>5.91</td>
</tr>
<tr>
<td>6 VER</td>
<td>5.79</td>
</tr>
<tr>
<td>7 APT</td>
<td>5.86</td>
</tr>
<tr>
<td>8 c68</td>
<td>5.92</td>
</tr>
<tr>
<td>9 cTM</td>
<td>5.95</td>
</tr>
<tr>
<td>10 cTM</td>
<td>5.94</td>
</tr>
<tr>
<td>11A SKB</td>
<td>6.23</td>
</tr>
<tr>
<td>11B SaB</td>
<td>5.75</td>
</tr>
<tr>
<td>12 cTSM</td>
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</tr>
<tr>
<td>13 cTM</td>
<td>5.88</td>
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</tbody>
</table>

Table 4. Overall mean estimates and inter-laboratory variation for quantitative assays (log$_{10}$ IU/mL).

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of datasets</th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>SD</th>
<th>%GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1: 10/264 (gt.A)</td>
<td>14</td>
<td>5.94</td>
<td>5.75</td>
<td>6.23</td>
<td>0.13</td>
<td>35</td>
</tr>
<tr>
<td>S2: 10/266 (gt.A)</td>
<td>14</td>
<td>5.97</td>
<td>5.78</td>
<td>6.26</td>
<td>0.13</td>
<td>35</td>
</tr>
<tr>
<td>S3: SRR (gt.A)</td>
<td>14</td>
<td>5.68</td>
<td>5.48</td>
<td>5.91</td>
<td>0.11</td>
<td>29</td>
</tr>
<tr>
<td>S4: NS (gt.C)</td>
<td>14</td>
<td>6.03</td>
<td>5.45</td>
<td>6.30</td>
<td>0.22</td>
<td>65</td>
</tr>
<tr>
<td>S5: WR (gt.C)</td>
<td>12</td>
<td>3.05</td>
<td>2.33</td>
<td>3.25</td>
<td>0.24</td>
<td>75</td>
</tr>
<tr>
<td>S6: HBVpl (gt.D)</td>
<td>14</td>
<td>3.32</td>
<td>1.98</td>
<td>3.70</td>
<td>0.42</td>
<td>160</td>
</tr>
<tr>
<td>S7: HBVpl (gt.E)</td>
<td>14</td>
<td>3.28</td>
<td>2.50</td>
<td>3.54</td>
<td>0.29</td>
<td>95</td>
</tr>
<tr>
<td>S8: HBVpl (gt.A)</td>
<td>14</td>
<td>3.53</td>
<td>2.39</td>
<td>3.94</td>
<td>0.39</td>
<td>145</td>
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Table 5. Overall mean estimates and inter-laboratory variation for quantitative assays (log10 IU/mL), excluding the dataset from laboratory 6.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of datasets</th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>SD</th>
<th>%GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1: 10/264 (gt.A)</td>
<td>13</td>
<td>5.96</td>
<td>5.75</td>
<td>6.23</td>
<td>0.13</td>
<td>34</td>
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<tr>
<td>S2: 10/266 (gt.A)</td>
<td>13</td>
<td>5.99</td>
<td>5.78</td>
<td>6.26</td>
<td>0.13</td>
<td>34</td>
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<tr>
<td>S3: SRR (gt.A)</td>
<td>13</td>
<td>5.69</td>
<td>5.48</td>
<td>5.91</td>
<td>0.11</td>
<td>30</td>
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<tr>
<td>S4: NS (gt.C)</td>
<td>13</td>
<td>6.07</td>
<td>5.78</td>
<td>6.30</td>
<td>0.14</td>
<td>39</td>
</tr>
<tr>
<td>S5: WR (gt.C)</td>
<td>11</td>
<td>3.06</td>
<td>2.55</td>
<td>3.26</td>
<td>0.19</td>
<td>54</td>
</tr>
<tr>
<td>S6: HBVpl (gt.D)</td>
<td>13</td>
<td>3.42</td>
<td>3.09</td>
<td>3.70</td>
<td>0.17</td>
<td>47</td>
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<tr>
<td>S7: HBVpl (gt.E)</td>
<td>13</td>
<td>3.34</td>
<td>2.86</td>
<td>3.54</td>
<td>0.19</td>
<td>55</td>
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<tr>
<td>S8: HBVpl (gt.A)</td>
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<td>3.61</td>
<td>3.11</td>
<td>3.94</td>
<td>0.22</td>
<td>66</td>
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</table>

Table 6. Overall mean estimates and inter-laboratory variation for quantitative assays (log10 IU/mL), excluding datasets from laboratories 9, 10 and 13 using the cTM assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of datasets</th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>SD</th>
<th>%GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1: 10/264 (gt.A)</td>
<td>11</td>
<td>5.95</td>
<td>5.75</td>
<td>6.23</td>
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<td>S2: 10/266 (gt.A)</td>
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<td>5.98</td>
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<td>6.26</td>
<td>0.15</td>
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<td>S3: SRR (gt.A)</td>
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<td>5.68</td>
<td>5.48</td>
<td>5.91</td>
<td>0.13</td>
<td>34</td>
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<tr>
<td>S4: NS (gt.C)</td>
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<td>5.99</td>
<td>5.45</td>
<td>6.30</td>
<td>0.23</td>
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<td>S5: WR (gt.C)</td>
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<td>2.95</td>
<td>2.33</td>
<td>3.19</td>
<td>0.28</td>
<td>91</td>
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<tr>
<td>S6: HBVpl (gt.D)</td>
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<td>3.28</td>
<td>1.98</td>
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</tr>
<tr>
<td>S7: HBVpl (gt.E)</td>
<td>11</td>
<td>3.22</td>
<td>2.50</td>
<td>3.51</td>
<td>0.31</td>
<td>102</td>
</tr>
<tr>
<td>S8: HBVpl (gt.A)</td>
<td>11</td>
<td>3.47</td>
<td>2.39</td>
<td>3.94</td>
<td>0.42</td>
<td>165</td>
</tr>
</tbody>
</table>
Table 7. Laboratory estimates of potency relative to the 3rd WHO International Standard for HBV (sample 1) from quantitative assays and qualitative assays (log_{10} IU/mL) - based on an assigned unitage of the International Standard of 850,000 (5.93 log_{10}) IU/mL. nd, not determined (either not tested or data excluded).

<table>
<thead>
<tr>
<th>Lab</th>
<th>Assay</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S2</td>
</tr>
<tr>
<td>1</td>
<td>kPCR</td>
<td>6.00</td>
</tr>
<tr>
<td>2A</td>
<td>c68</td>
<td>5.93</td>
</tr>
<tr>
<td>2B</td>
<td>cTM</td>
<td>5.96</td>
</tr>
<tr>
<td>3</td>
<td>AbRT</td>
<td>5.93</td>
</tr>
<tr>
<td>4</td>
<td>ArQS</td>
<td>5.97</td>
</tr>
<tr>
<td>5</td>
<td>cTM</td>
<td>6.01</td>
</tr>
<tr>
<td>6</td>
<td>VER</td>
<td>5.95</td>
</tr>
<tr>
<td>7</td>
<td>APT</td>
<td>5.95</td>
</tr>
<tr>
<td>8</td>
<td>c68</td>
<td>5.97</td>
</tr>
<tr>
<td>9</td>
<td>cTM</td>
<td>5.94</td>
</tr>
<tr>
<td>10</td>
<td>cTM</td>
<td>5.94</td>
</tr>
<tr>
<td>11A</td>
<td>SKB</td>
<td>5.96</td>
</tr>
<tr>
<td>11B</td>
<td>SaB</td>
<td>5.97</td>
</tr>
<tr>
<td>12</td>
<td>cTSM</td>
<td>6.44</td>
</tr>
<tr>
<td>13</td>
<td>cTM</td>
<td>5.98</td>
</tr>
</tbody>
</table>

Table 8. Overall mean estimates and inter-laboratory variation for potency relative to the 3rd HBV WHO International Standard (sample 1) log_{10} IU/mL for quantitative assays - based on an assigned unitage of the International Standard of 850,000 (5.93 log_{10}) IU/mL.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of datasets</th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>SD</th>
<th>%GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2: 10/266 (gt.A)</td>
<td>14</td>
<td>5.96</td>
<td>5.94</td>
<td>6.01</td>
<td>0.02</td>
<td>5</td>
</tr>
<tr>
<td>S3: SRR (gt.A)</td>
<td>14</td>
<td>5.67</td>
<td>5.61</td>
<td>5.76</td>
<td>0.04</td>
<td>10</td>
</tr>
<tr>
<td>S4: NS (gt.C)</td>
<td>14</td>
<td>6.02</td>
<td>5.59</td>
<td>6.21</td>
<td>0.16</td>
<td>46</td>
</tr>
<tr>
<td>S5: WR (gt.C)</td>
<td>12</td>
<td>3.03</td>
<td>2.48</td>
<td>3.23</td>
<td>0.22</td>
<td>67</td>
</tr>
<tr>
<td>S6: HBVpl (gt.D)</td>
<td>14</td>
<td>3.30</td>
<td>2.13</td>
<td>3.89</td>
<td>0.41</td>
<td>159</td>
</tr>
<tr>
<td>S7: HBVpl (gt.E)</td>
<td>14</td>
<td>3.27</td>
<td>2.63</td>
<td>3.52</td>
<td>0.30</td>
<td>100</td>
</tr>
<tr>
<td>S8: HBVpl (gt.A)</td>
<td>14</td>
<td>3.51</td>
<td>2.54</td>
<td>3.94</td>
<td>0.38</td>
<td>138</td>
</tr>
</tbody>
</table>
**Table 9.** Overall mean estimates and inter-laboratory variation for potency relative to the 3rd HBV WHO International Standard (sample 1) $\log_{10}$ IU/mL for quantitative assays - based on an assigned unitage of the International Standard of 850,000 (5.93 $\log_{10}$) IU/mL, excluding datasets from laboratories 9, 10 and 13 using the cTM assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of datasets</th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>SD</th>
<th>%GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2: 10/266 (gt.A)</td>
<td>11</td>
<td>5.96</td>
<td>5.93</td>
<td>6.01</td>
<td>0.02</td>
<td>5</td>
</tr>
<tr>
<td>S3: SRR (gt.A)</td>
<td>11</td>
<td>5.66</td>
<td>5.61</td>
<td>5.76</td>
<td>0.04</td>
<td>10</td>
</tr>
<tr>
<td>S4: NS (gt.C)</td>
<td>11</td>
<td>5.98</td>
<td>5.59</td>
<td>6.19</td>
<td>0.16</td>
<td>43</td>
</tr>
<tr>
<td>S5: WR (gt.C)</td>
<td>9</td>
<td>2.97</td>
<td>2.48</td>
<td>3.19</td>
<td>0.23</td>
<td>69</td>
</tr>
<tr>
<td>S6: HBVpl (gt.D)</td>
<td>11</td>
<td>3.26</td>
<td>2.13</td>
<td>3.89</td>
<td>0.46</td>
<td>189</td>
</tr>
<tr>
<td>S7: HBVpl (gt.E)</td>
<td>11</td>
<td>3.21</td>
<td>2.63</td>
<td>3.52</td>
<td>0.31</td>
<td>106</td>
</tr>
<tr>
<td>S8: HBVpl (gt.A)</td>
<td>11</td>
<td>3.46</td>
<td>2.54</td>
<td>3.94</td>
<td>0.41</td>
<td>155</td>
</tr>
</tbody>
</table>

**Table 10.** Overall mean estimates and inter-laboratory variation for potency relative to the candidate 4th HBV WHO International Standard (sample 2) $\log_{10}$ IU/mL for quantitative assays - based on a candidate unitage of 955,000 (5.98 $\log_{10}$) IU/mL.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of datasets</th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>SD</th>
<th>%GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3: SRR (gt.A)</td>
<td>14</td>
<td>5.69</td>
<td>5.64</td>
<td>5.79</td>
<td>0.04</td>
<td>10</td>
</tr>
<tr>
<td>S4: NS (gt.C)</td>
<td>14</td>
<td>6.04</td>
<td>5.62</td>
<td>6.24</td>
<td>0.16</td>
<td>45</td>
</tr>
<tr>
<td>S5: WR (gt.C)</td>
<td>12</td>
<td>3.05</td>
<td>2.51</td>
<td>3.27</td>
<td>0.22</td>
<td>66</td>
</tr>
<tr>
<td>S6: HBVpl (gt.D)</td>
<td>14</td>
<td>3.32</td>
<td>2.16</td>
<td>3.90</td>
<td>0.41</td>
<td>157</td>
</tr>
<tr>
<td>S7: HBVpl (gt.E)</td>
<td>14</td>
<td>3.28</td>
<td>2.64</td>
<td>3.56</td>
<td>0.30</td>
<td>98</td>
</tr>
<tr>
<td>S8: HBVpl (gt.A)</td>
<td>14</td>
<td>3.53</td>
<td>2.57</td>
<td>3.95</td>
<td>0.38</td>
<td>138</td>
</tr>
</tbody>
</table>
Table 11. Overall mean estimates and inter-laboratory variation for potency relative to the candidate 4th HBV WHO International Standard (sample 2) log_{10} IU/mL for quantitative assays - based on a candidate unitage of 955,000 (5.98 log_{10}) IU/mL, excluding datasets from laboratories 9, 10 and 13 using the cTM assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of datasets</th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>SD</th>
<th>%GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3: SRR (gt.A)</td>
<td>11</td>
<td>5.68</td>
<td>5.64</td>
<td>5.79</td>
<td>0.04</td>
<td>10</td>
</tr>
<tr>
<td>S4: NS (gt.C)</td>
<td>11</td>
<td>5.99</td>
<td>5.62</td>
<td>6.16</td>
<td>0.15</td>
<td>41</td>
</tr>
<tr>
<td>S5: WR (gt.C)</td>
<td>9</td>
<td>2.99</td>
<td>2.51</td>
<td>3.18</td>
<td>0.22</td>
<td>66</td>
</tr>
<tr>
<td>S6: HBVpl (gt.D)</td>
<td>11</td>
<td>3.28</td>
<td>2.16</td>
<td>3.90</td>
<td>0.46</td>
<td>186</td>
</tr>
<tr>
<td>S7: HBVpl (gt.E)</td>
<td>11</td>
<td>3.22</td>
<td>2.64</td>
<td>3.52</td>
<td>0.31</td>
<td>103</td>
</tr>
<tr>
<td>S8: HBVpl (gt.A)</td>
<td>11</td>
<td>3.47</td>
<td>2.57</td>
<td>3.95</td>
<td>0.40</td>
<td>153</td>
</tr>
</tbody>
</table>

Table 12. Intra-laboratory SD of log_{10} IU/mL and %GCV for quantitative assays.

<table>
<thead>
<tr>
<th>Lab</th>
<th>Assay</th>
<th>SD</th>
<th>%GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>kPCR</td>
<td>0.05</td>
<td>13</td>
</tr>
<tr>
<td>2A</td>
<td>c68</td>
<td>0.03</td>
<td>7</td>
</tr>
<tr>
<td>2B</td>
<td>cTM</td>
<td>0.05</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>AbRT</td>
<td>0.03</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>ArQS</td>
<td>0.17</td>
<td>47</td>
</tr>
<tr>
<td>5</td>
<td>cTM</td>
<td>0.04</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>VER</td>
<td>0.06</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>APT</td>
<td>0.08</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>c68</td>
<td>0.03</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>cTM</td>
<td>0.05</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>cTM</td>
<td>0.07</td>
<td>16</td>
</tr>
<tr>
<td>11A</td>
<td>SKB</td>
<td>0.09</td>
<td>24</td>
</tr>
<tr>
<td>11B</td>
<td>SaB</td>
<td>0.05</td>
<td>13</td>
</tr>
<tr>
<td>13</td>
<td>cTM</td>
<td>0.05</td>
<td>13</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>0.07</td>
<td>18</td>
</tr>
</tbody>
</table>

Figure legends

Figure 1. Individual laboratory mean estimates for study samples 1-8 (a-h respectively) obtained using qualitative or quantitative NAT assays. Each box represents the mean estimate from each laboratory assay and is labeled with the laboratory and assay code. Boxes are also colour coded by assay.

Figure 2. Relative potencies of samples 2-8 against sample 1 (a-g respectively), for each qualitative or quantitative assay. Units are expressed as candidate log_{10} IU/mL. Each box represents the relative potency for each laboratory assay and is labeled with the laboratory and assay code. Boxes are also colour coded by assay.
Figure 1

a  Sample 1

b  Sample 2
c  Sample 3

\[ \text{Sample 3} \]

\[ \begin{align*}
&\text{\hspace{1cm}}
\end{align*} \]

\[ \begin{align*}
\text{Sample 4} \hspace{1cm}
\end{align*} \]

\[ \text{Sample 4} \]

\[ \begin{align*}
&\text{\hspace{1cm}}
\end{align*} \]
e  Sample 5

![Sample 5 Diagram]

f  Sample 6

![Sample 6 Diagram]
g  Sample 7

h  Sample 8
Figure 2

a  Sample 2 relative to Sample 1

b  Sample 3 relative to Sample 1
c  Sample 4 relative to Sample 1

d  Sample 5 relative to Sample 1
e  Sample 6 relative to Sample 1

f  Sample 7 relative to Sample 1
g  Sample 8 relative to Sample 1
Appendix 2

Example of the calibration of a national standard
(collaborative study calibration using multiple assays)


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A collaborative study to establish the first National Standard for HIV-1 RNA nucleic acid amplification techniques (NAT) in Taiwan

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1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome, commonly known as AIDS (Weiss, 1993). According to the UNAIDS report on the global AIDS epidemic in 2012, an estimated 34 million people are infected HIV (UNAIDS report, 2012) after 30 years of a very complex epidemic. Almost 30 million people have died from HIV-related diseases so far. In Taiwan, the number of reported cases of HIV/AIDS was approximately twenty four thousand from 1984 to 2012 December (CDC of Taiwan, 2012). Increasing sexual activity and needle sharing activity among drug abusers has resulted in HIV/AIDS becoming a severe public health problem.

HIV-1 is a member of the Retrovirus family and belongs to the Lentivirus genus. The RNA genome of HIV-1 is approximately 9.7 kb, containing three structural genes (gag, pol, and env) and six regulatory genes (tat, rev, nef, vif, vpu, and vpr) (Pluta and Kacprzak, 2009; Bolinger and Boris-Lawrie, 2009; Karlsson Hedestam et al., 2008). HIV-1 strains are categorized as major group (M group), outlier group (O group), new group (N group), or P group on the basis of differences in the envelope region. The M group is further divided into 9 genetic subtypes (A–D, F–H, J, and K) and circulating recombinant forms (CRFs). In Taiwan, subtype B was found to be the predominant genotype in homosexual males and in the intravenous drug abuser population. In recent years, subtype CRF07_BC has been the major group in intravenous drug abuser population (Spira et al., 2003; Robertson et al., 2000; Simon et al., 1998; Lin et al., 2007; Plantier et al., 2009).

Screening of blood and plasma products for blood–borne viruses has usually been performed using sensitive antibody-detection assays. In recent years, the nucleic-acid amplification techniques (NAT) have been widely applied in blood safety screens to enhance the sensitivity of detection of HIV-1 when present in low concentrations and at earlier stages of infection (Piatak et al., 1993; Murthy et al., 1999; Busch and Dodd, 2000).

To improve the safety of plasma products, a requirement that the plasma pools used to manufacture plasma products should be screened for HIV RNA by NAT was announced by the Department of Health in Taiwan on December 19, 2002. The development of a calibrated national reference standard that could be used routinely in assays would give assurance as to the validity of the test results and therefore fulfillment with such regulations. In addition to this national requirement for plasma screening, the HIV viral load assay is very critical in the management of antiretroviral therapy. Recently, numerous in vitro diagnostic devices (IVDs)
based on NAT technology have been developed to detect HIV qualitatively or quantitatively for blood screening or viral load measurement. Such NAT-based IVDs are highly related to blood safety, the quality and performance of the IVDs are of great importance. In order to ensure the continued fitness for purpose of the IVD, both the pre-market approval testing and the performance evaluation are crucial in post-marketing surveillances. The first and second World Health Organization (WHO) International Standard (IS) for HIV-1 RNA was established by the WHO Expert Committee on Biological Standards (ECBS) in 1999 and 2008, and the National Institute for Biological Standards and Control (NIBSC) code numbers are 97/656 and 97/650, respectively (Holmes et al., 2001; Davis et al., 2008). One of the main purposes of an International Standard is to facilitate the calibration of secondary working reagents developed at a local level, i.e. by individual laboratories. The calibration of secondary working reagents from a higher order standard would help reduce test result variability from different laboratories, aid in comparing the different commercial and ‘in-house’ assays, and make it easier to compare the proficiency of different laboratories (Revets et al., 1996; Schuurman et al., 1996). In order to ensure the correct use of the International Standard by the end user, for example for secondary working reagent calibration and not for use as a routine run control, the WHO IS for HIV-1 RNA is available only in limited amounts, several control laboratories (such as National Institute for Biological Standards and Control (NIBSC), Food and Drug Administration (FDA) and Istituto Superiore di Sanità (ISS)) have already prepared an in house or national secondary HIV-1 NAT working reagent themselves (Davis et al., 2003; Lee et al., 2006; Pisani et al., 2007). It is known that the distribution of HIV subtypes may differ by geographic region. It is therefore critical to use the major genotype of the HIV-1 as a source material for a National Standard. Since subtype B was found to be the predominant genotype in Taiwan, the genotype of the National Standard would select to be subtype B, the same as WHO IS. Therefore, the objective of this study was to establish the first National Standard for HIV-1 RNA NAT assays and to calibrate the HIV-1 RNA content of the candidate standard against the WHO IS for HIV-1 RNA NAT assays (97/650). The procedure for the development of a National Standard was based on the previous experience of the development of the National Standard for human parvovirus B19 DNA nucleic acid amplification techniques (NAT) in 2008 (Yang et al., 2008).

3. Materials and methods

3.1. Preparation of the candidate standard

The candidate standard for HIV-1 RNA NAT assays was liquid preparation and stored at or below −70 °C. It was prepared by diluting HIV-1 RNA positive plasma in pooled human cryopreparant. The proposed titer was approximately 10^4 IU/mL. The original HIV-1 RNA positive plasma had a titer of HIV-1 RNA of approximately 4.7 × 10^5 IU/mL and was negative for HBsAg, HBV DNA, anti-HCV, HCV RNA, HAV RNA as well as B19V DNA. The genotype of the HIV-1 RNA positive plasma was confirmed as subtype B by sequencing. The cryopreparant was negative for HBsAg, HBV DNA, B19V DNA, anti-HCV, HCV RNA, anti-HIV 1/2, HIV-1 RNA, and HAV RNA.

3.2. Design of the international collaborative study

The aim of the international collaborative study was to calibrate the titers of the HIV-1 RNA National Standard that was prepared by the Taiwan Food and Drug Administration (TFDA). Including our laboratory, a total of eleven laboratories from five different countries have participated in this study. Participants received the proposed national candidate standard and WHO IS for HIV-1 RNA (97/650) and were requested to perform three independent assays for HIV-1 RNA using the candidate standard and the WHO IS. For each assay, serial dilutions of the WHO IS were prepared using the appropriate diluent. The recommended diluent for the study was HIV-1 RNA negative human plasma. The serially diluted IS were used to calibrate the candidate standard (Sample A) by creating a standard curve. If a commercial kit was used, the IS could be treated as a second unknown sample (sample B) and quantitated in parallel with sample A. A single estimate was obtained for each sample in each laboratory and for each assay method by calculating the geometric mean of repeat data within a single assay. The overall mean and SD were then calculated from the results of all participants.

3.3. Stability study on the candidate standard

Vials of the proposed national candidate standard were incubated at +4 °C, +24 °C, −20 °C, and −80 °C, three vials were removed at regular intervals for three independent tests. Two different commercial assays were used for quantitative analysis: the Abbott RealTime HIV-1 (Abbott Molecular Inc., Des Plaines, IL, USA) and the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v1.0 (Roche Molecular Systems, Inc., Branchburg, NJ, USA). Here, 500 ul of the HIV-1 RNA candidate standard was used in Abbott RealTime HIV-1 Kit and 1 ml 5× pre-diluted candidate standard was used in COBAS AmpliPrep/COBAS TaqMan HIV-1 Test Kit. Both real-time PCR systems, the Abbott m2000 RealTime system and the COBAS AmpliPrep/TaqMan 48 system, were used according to the manufacturer’s instructions.

3. Results

3.1. Assay methods

Ten of the participants performed quantitative assays: four laboratories used the Abbott RT HIV-1 (Abbott Molecular Inc., Des Plaines, IL, USA); two laboratories used the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v1.0 (Roche Molecular Systems, Inc., Branchburg, NJ, USA); two laboratories used the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0 (Roche Molecular Systems, Inc., Branchburg, NJ, USA); two laboratories used the COBAS TaqMan HIV-1 Test, for use with High Purity System Viral Nucleic Acid Kit, v1.0 (Roche Molecular Systems, Inc., Branchburg, NJ, USA); two laboratories used the VERSANT HIV-1 RNA 3.0 (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA); and one laboratory used the COBAS Amplicor HIV-1 Monitor Test, v1.5 (Roche Molecular Systems, Inc., Branchburg, NJ, USA). Between them, lab code 1 used two different methods to detect HIV-1 RNA and analyzed the data separately (1A, 1B), and lab code 2 used three different methods to detect HIV-1 RNA and reported the results separately (2A, 2B, 2C). The overall results were therefore based on a maximum of 13 data sets. All these data sets were obtained by commercial assays. The quantitative methods used are summarized in Table 1. The other one participant used the COBAS HIV-1 AmpliScreen Test, v1.5 (Roche Molecular Systems, Inc., Branchburg, NJ, USA), which is a qualitative assay that was only give “positive” results (Detection limit: 78.4 IU/mL) and could not be calculated.

3.2. Estimated value of the HIV-1 RNA for the candidate standard

The estimated values of HIV-1 RNA, relative to the International Standard, for the candidate standards from each laboratory are listed in Table 2 and shown in Fig. 1. All the values have shown a good agreement with each other, except one laboratory has submitted an outlying result. The value of HIV-1 RNA estimate from each laboratory is shown in Fig. 2. Each box represents the estimate from
Table 1
An overview of the quantitative assays used in the collaborative study.

<table>
<thead>
<tr>
<th>Lab code</th>
<th>Assay method</th>
<th>Region for primer design</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B</td>
<td>Abbott RT HIV-1</td>
<td>A highly conserved region in HIV-1</td>
</tr>
<tr>
<td>2A</td>
<td>COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v1.0</td>
<td>A highly conserved region in HIV-1 gag gene</td>
</tr>
<tr>
<td>3</td>
<td>COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0</td>
<td>Two highly conserved regions of the HIV-1 genome–gag and long terminal repeat (LTR)</td>
</tr>
<tr>
<td>4</td>
<td>COBAS TaqMan HIV-1, for use with High Pure System Viral Nucleic Acid Kit, v1.0</td>
<td>A highly conserved region in HIV-1 gag gene</td>
</tr>
<tr>
<td>5</td>
<td>COBAS Amplipcr HIV-1 Monitor Test, v1.5</td>
<td>A highly conserved region in HIV-1 gag gene</td>
</tr>
<tr>
<td>6</td>
<td>VERSANT HIV-1 RNA 3.0 (b DNA)</td>
<td>A highly conserved region in HIV-1 pol gene</td>
</tr>
</tbody>
</table>

a Two laboratories (lab code 1 and 2) returned data from two and three different assay methods, respectively. The results are reported separately.

b pol-int gene is the integrase region of the polymerase gene.

Table 2
The estimated values of HIV-1 RNA (Log IU/mL) for candidate standard from 10 laboratories.

<table>
<thead>
<tr>
<th>Lab code</th>
<th>Mean</th>
<th>Minimum</th>
<th>Maximum</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>4.04</td>
<td>4.03</td>
<td>4.06</td>
<td>0.37</td>
</tr>
<tr>
<td>1B</td>
<td>3.98</td>
<td>3.96</td>
<td>4.01</td>
<td>0.58</td>
</tr>
<tr>
<td>2A</td>
<td>4.13</td>
<td>4.06</td>
<td>4.23</td>
<td>1.37</td>
</tr>
<tr>
<td>2B</td>
<td>4.11</td>
<td>4.01</td>
<td>4.18</td>
<td>1.63</td>
</tr>
<tr>
<td>2C</td>
<td>4.13</td>
<td>4.06</td>
<td>4.24</td>
<td>1.79</td>
</tr>
<tr>
<td>3</td>
<td>4.15</td>
<td>4.12</td>
<td>4.17</td>
<td>0.63</td>
</tr>
<tr>
<td>4</td>
<td>3.73</td>
<td>3.67</td>
<td>3.79</td>
<td>1.04</td>
</tr>
<tr>
<td>5</td>
<td>4.03</td>
<td>3.99</td>
<td>4.08</td>
<td>1.09</td>
</tr>
<tr>
<td>6</td>
<td>3.98</td>
<td>3.84</td>
<td>4.08</td>
<td>0.42</td>
</tr>
<tr>
<td>7</td>
<td>4.18</td>
<td>4.04</td>
<td>4.26</td>
<td>2.83</td>
</tr>
<tr>
<td>8</td>
<td>3.95</td>
<td>3.90</td>
<td>4.03</td>
<td>1.84</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>4.42</td>
<td>–</td>
<td>0.59</td>
</tr>
<tr>
<td>10</td>
<td>3.68</td>
<td>3.65</td>
<td>3.70</td>
<td>0.59</td>
</tr>
</tbody>
</table>

The measurements were performed using WHO International Standard for human HIV-1 RNA (WHO IS, 97/650) as the standard.

b Only one assay result was available from this laboratory, not three independent assay results.

One laboratory and/or assay method. All data were within a range of 1.0 Log for each sample, indicating that all the laboratories were in good agreement with the estimates. A comparison of the different commercial kit results is shown in Table 3; the results showed that the COBAS Amplipcr HIV-1 Monitor Test was significantly different from other commercial kit assays. The data generated from the COBAS Amplipcr HIV-1 Monitor Test was therefore excluded from the overall means for the candidate standard. Therefore, the
4. Discussion

There was a clear variation between the results from earlier HIV-1 viral load assays such as nucleic acid signal branch amplification (NASBA), PCR end point detection and branched DNA (bDNA) signal amplification compared to more recent tests such as the Abbott real-time assay. The limitation of these assays has previously been reported (Church et al., 2011) and it is known that are optimized to target subtype B group M viruses. New-generation real-time PCR assays for HIV-1 RNA quantification include the Abbott RT HIV-1 assay and the Cobas AmpliPrep/Cobas TaqMan HIV-1 assay (CAP-CTM). These real-time PCR assays have been improved and are now able to detect HIV-1 group M, non-B subtype viruses, group N viruses and O viruses. In addition, the Abbott RealTime HIV-1 assay has been reported to successfully detect HIV-1 group P infection (Plantier et al., 2009). However, it has also been reported that multiple mismatches in gag primers and probe binding regions for the first version of the CTM assay (CTM1) exist, which can result in an underestimation of the CTM1 values for some patients infected with HIV-1 group M, non-B subtypes. To overcome this problem, Roche Diagnostics have already upgraded their test to CTM version 2.0 (CTM2), which uses a dual-target strategy (Damond et al., 2010; Church et al., 2011; Wirden et al., 2011).

In this international collaborative study, most of the participants performed quantitative assays: four laboratories used the Abbott RT HIV-1; six laboratories used the COBAS TaqMan HIV-1 Test, COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v1.0 & 2.0, and COBAS TaqMan HIV-1 Test, for use with High Pure System Viral (Nucleic Acid Kit, v1.0); two laboratories used the VERSANT HIV-1 RNA 3.0; and one laboratory used the COBAS Amplipcr HIV-1 Monitor Test, v1.5. The results showed that all the laboratories were in good agreement with the estimates, except one laboratory submitted an outlying result, which is generated from COBAS Amplipcr HIV-1 Monitor Test, v1.5. By comparing the results from different commercial kits, indicated that the COBAS Amplipcr HIV-1 Monitor Test, v1.5 was significantly different from other commercial assay kits. The data generated from the COBAS Amplipcr HIV-1 Monitor Test was therefore excluded from the overall means for the candidate sample. Since there is only one assay result available in this collaborative study, it does not represent the performance of the kit. Interestingly, a similar result was also shown in an earlier collaborative study to establish a replacement International Standard for HIV-1 RNA nucleic acid assays (Davis et al., 2008).

All data points received from laboratories were within a range of 1.0 Log from this collaborative study, furthermore, most of the data were within in a range from 3.9 Log IU/mL to 4.1 Log IU/mL. In conclusion, a high level of agreement among the results obtained from the participating laboratories was observed. The first National Taiwan Standard for HIV-1 RNA NAT assays, with an assigned value of 1.0 × 10^4 IU/mL, was recognized. In order to reflect the predominant HIV-1 subtype found in Taiwan, this National Standard was formulated from a subtype B plasma. The results of the stability study indicated that the HIV-1 RNA National Standard is stable long-term when stored at −20°C and −80°C. Therefore, the first National Standard for HIV-1 RNA NAT assays in Taiwan was established. This standard could be used for quality control of HIV-1 RNA assays and as a quantitative reference material for HIV-1 NAT assays. Moreover, the standard could be used nationally for pre-market approval testing and the performance evaluation in post-marketing surveillances of NAT-based IVDs and facilitating to ensure the continued fitness for purpose of the IVD, either imported or domestic.

In recent years, subtype CRF07_BC has been the major group of HIV-1 found in the intravenous drug abuser population in Taiwan. As HIV-1 strain diversity and viral recombination events increase, the need for surveillance using commercial assays to...
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Table 3
Comparison of different commercial kits.

<table>
<thead>
<tr>
<th>Assay method</th>
<th>Lab code</th>
<th>Result</th>
<th>Mean</th>
<th>SD</th>
<th>CV(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbott RT HIV-1</td>
<td>1</td>
<td>3.98</td>
<td>4.05</td>
<td>0.10</td>
<td>2.52</td>
</tr>
<tr>
<td></td>
<td>2A</td>
<td>4.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3.95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COBAS Amp/Prep/COBAS TaqMan HIV-1 test, v2.0</td>
<td>1A</td>
<td>4.04</td>
<td>4.01</td>
<td>0.04</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.98</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COBAS Amp/Prep/COBAS TaqMan HIV-1 test, v1.0</td>
<td>2C</td>
<td>4.13</td>
<td>4.08</td>
<td>0.07</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COBAS TaqMan HIV-1 test, for use with High Pure System Viral Nucleic Acid Kit, v1.0</td>
<td>2B</td>
<td>4.11</td>
<td>3.90</td>
<td>0.30</td>
<td>7.81</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COBAS Amplicor HIV-1 Monitor Test, v1.5</td>
<td>9</td>
<td>4.42a</td>
<td>4.42a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VERSANT HIV-1 RNA 3.0 (HIV-1 DNA)</td>
<td>4</td>
<td>3.73</td>
<td>3.96</td>
<td>0.32</td>
<td>8.05</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4.18</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05. Please note that a single assay result does not represent the performance of the kit.

Table 4
Overall mean estimates of HIV-1 RNA (Log IU/mL) for candidate standard.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>95% confidence interval (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log IU/mL</td>
<td>U/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candidate standard</td>
<td>4.01</td>
<td>1.01E+04</td>
</tr>
</tbody>
</table>

Disadvantages

The findings and conclusions in this article have not been formally disseminated by Taiwan FDA and should not be construed to represent any agency determination or policy.

Acknowledgements

We would like to express our sincere thanks to all the participants of the collaborative study group (Appendix A) for their valuable contribution to this collaborative study and to Ai-Zhen Yang and Yu-Chen Pao for their technical assistance for formulating the candidate standard. We would like to thank Clare Morris for providing language help. In addition, we also acknowledge the funding support from Taiwan FDA.

Appendix A

List of participants:
- Brian Erickson, Abbott Molecular Inc., USA.
- Chen-Feng Sun/Shuan Yang, Chang-Gung Memorial Hospital, Taiwan.
- Clare Morris, National Institute for Biological Standards and Controls (NIBSC), UK.
- Chih-Yuan Yang/Cheng-Feng Kao, Centers for Disease Control (TCDC), Taiwan.
- Der-Yuan Wang/Yi-Chen Yang, Food and Drug Administration (TFDA), Taiwan.
- Eric T. Natoli, Siemens Healthcare Diagnostics, USA.
- Indira Hewlett/Sherwin Lee, Center for Biologics Evaluation and Research/Food and Drug Administration (CBER/FDA), USA.
- John Saldamala/Matthew Lin, Roche Molecular Systems, USA.
- Lena Panagiotopoulos/Stirling Dick, National Serology Reference Laboratory (NRL), Australia.
- Micha Nühling/Michael Chudy, Paul-Ehrlich-Institute, Germany.
- Yi-Li Shih, E-Da Hospital, Taiwan.

References


Appendix 3

Example of the calibration of a reference preparation by a single NAT assay

The reference preparation (RP) is calibrated against the WHO IS by testing dilutions of both samples using a real-time PCR, collecting raw data (threshold cycle values Ct) and performing a valid statistical analysis (e.g. parallel line). Study performed under the supervision of G. Pisani, ISS, Rome, Italy.

Study samples:

- RP for HIV RNA with a presumptive titre of 15 000 IU/mL of HIV RNA
- 3rd WHO IS HIV RNA batch 10/152 with a concentration of 185 000 IU/mL (5.26 log IU/mL).

Test the following dilutions of WHO IS and RP (in triplicate) on three separate days. Collect the raw data.

Day 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Final dilution (log)</th>
<th>Concentration</th>
<th>Raw data (Ct value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Replica 1</td>
</tr>
<tr>
<td>WHO IS</td>
<td>−1.09</td>
<td>15 000 IU/mL</td>
<td>28.5</td>
</tr>
<tr>
<td></td>
<td>−1.59</td>
<td>4 700 IU/mL</td>
<td>29.8</td>
</tr>
<tr>
<td></td>
<td>−2.09</td>
<td>1 500 IU/mL</td>
<td>31.6</td>
</tr>
<tr>
<td></td>
<td>−2.59</td>
<td>470 IU/mL</td>
<td>32.9</td>
</tr>
<tr>
<td>RP</td>
<td>Not diluted</td>
<td>–</td>
<td>28.4</td>
</tr>
<tr>
<td></td>
<td>−0.50</td>
<td>–</td>
<td>30.9</td>
</tr>
<tr>
<td></td>
<td>−1.00</td>
<td>–</td>
<td>32.1</td>
</tr>
<tr>
<td></td>
<td>−1.50</td>
<td>–</td>
<td>33.6</td>
</tr>
</tbody>
</table>
### Day 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Final dilution (log)</th>
<th>Concentration</th>
<th>Raw data (Ct value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Replica 1</td>
</tr>
<tr>
<td>WHO IS</td>
<td>−1.09</td>
<td>15 000 IU/mL</td>
<td>28.7</td>
</tr>
<tr>
<td></td>
<td>−1.59</td>
<td>4 700 IU/mL</td>
<td>29.7</td>
</tr>
<tr>
<td></td>
<td>−2.09</td>
<td>1 500 IU/mL</td>
<td>31.5</td>
</tr>
<tr>
<td></td>
<td>−2.59</td>
<td>470 IU/mL</td>
<td>33.3</td>
</tr>
<tr>
<td>RP</td>
<td>Not diluted</td>
<td>−</td>
<td>29.1</td>
</tr>
<tr>
<td></td>
<td>−0.50</td>
<td>−</td>
<td>30.1</td>
</tr>
<tr>
<td></td>
<td>−1.00</td>
<td>−</td>
<td>32.9</td>
</tr>
<tr>
<td></td>
<td>−1.50</td>
<td>−</td>
<td>34.5</td>
</tr>
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</table>

### Day 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Final dilution (log)</th>
<th>Concentration</th>
<th>Raw data (Ct value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Replica 1</td>
</tr>
<tr>
<td>WHO IS</td>
<td>−1.09</td>
<td>15 000 IU/mL</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td>−1.59</td>
<td>4 700 IU/mL</td>
<td>29.1</td>
</tr>
<tr>
<td></td>
<td>−2.09</td>
<td>1 500 IU/mL</td>
<td>31.1</td>
</tr>
<tr>
<td></td>
<td>−2.59</td>
<td>470 IU/mL</td>
<td>33.4</td>
</tr>
<tr>
<td>RP</td>
<td>Not diluted</td>
<td>−</td>
<td>28.8</td>
</tr>
<tr>
<td></td>
<td>−0.50</td>
<td>−</td>
<td>30.1</td>
</tr>
<tr>
<td></td>
<td>−1.00</td>
<td>−</td>
<td>32.1</td>
</tr>
<tr>
<td></td>
<td>−1.50</td>
<td>−</td>
<td>33.5</td>
</tr>
</tbody>
</table>

Perform statistical analysis: parallel-line assay.

Acceptance criteria: linearity and parallelism should be fulfilled.

It is possible that on each day (each experiment) one replicate or one dose may be deleted in order to fulfil the acceptance criteria.
Example calibration: Combistat (EDQM).

### Substance
- HIV RNA

### Method
- TaqScreen MPX

### Assay number
- 1

### Technician
- 

### Date of assay
- DAY 1

---

<table>
<thead>
<tr>
<th>Standard</th>
<th>Sample 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ass. pot.</td>
<td>HIV RNA Ref Prep</td>
</tr>
<tr>
<td>185,000IU/mL</td>
<td>lU/mL</td>
</tr>
<tr>
<td>Pre-dil. 1</td>
<td>Pre-dil. 1</td>
</tr>
<tr>
<td>1 mL/12.33mL</td>
<td>Doses</td>
</tr>
<tr>
<td>Doses</td>
<td>1/1</td>
</tr>
<tr>
<td>(1) 28.5</td>
<td>(2) 28.4</td>
</tr>
<tr>
<td>(3) 28.3</td>
<td>(3) 30.3</td>
</tr>
</tbody>
</table>

#### Model:
- Parallel lines

#### Design:
- Completely randomized

#### Transformation:
- $y' = y$

#### Variance:
- Observed residuals

#### Source of variation

<table>
<thead>
<tr>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F-ratio</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparations</td>
<td>1</td>
<td>2.10042</td>
<td>2.10042</td>
<td>22.504</td>
</tr>
<tr>
<td>Regression</td>
<td>1</td>
<td>8.8933</td>
<td>8.8933</td>
<td>941.714</td>
</tr>
<tr>
<td>Non-parallelism</td>
<td>4</td>
<td>0.290027</td>
<td>0.290027</td>
<td>3.107</td>
</tr>
<tr>
<td>Non-linearity</td>
<td>2</td>
<td>0.0305883</td>
<td>0.0152942</td>
<td>0.164</td>
</tr>
<tr>
<td>Standard</td>
<td>2</td>
<td>0.0819547</td>
<td>0.0409774</td>
<td>0.439</td>
</tr>
<tr>
<td>Sample 1</td>
<td>2</td>
<td>90.3963</td>
<td>12.9138</td>
<td>138.362</td>
</tr>
<tr>
<td>Residual error</td>
<td>16</td>
<td>1.49333</td>
<td>0.0933333</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>91.8496</td>
<td>3.99520</td>
<td></td>
</tr>
</tbody>
</table>

#### Common slope (factor) = -1.48691 (-1.57151 to -1.40232)

#### Correlation $r = 0.989630$

---

### Sample 1

<table>
<thead>
<tr>
<th>HIV RNA Ref Prep</th>
</tr>
</thead>
<tbody>
<tr>
<td>(IU/mL)</td>
</tr>
<tr>
<td>Lower limit</td>
</tr>
<tr>
<td>Estimate</td>
</tr>
<tr>
<td>Upper limit</td>
</tr>
</tbody>
</table>

#### Potency
- 83595.2

#### Rel. to Ass.
- 1

#### Rel. to Est.
- 83.3%

### All samples

#### Standard
- WHO IS 10/102

### Sample 1
- HIV RNA Ref Prep

---

### Executed by:

### Calculated by:

### Approved by:

---

### ID:
- IST1549/ITA

---

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### Table: Calibration of Reference Preparation for HIV RNA against the WHO IS

<table>
<thead>
<tr>
<th>Substance</th>
<th>HIV RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>TaqScreen MPX</td>
</tr>
<tr>
<td>Assay number</td>
<td>2</td>
</tr>
<tr>
<td>Technician</td>
<td>DAY 2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Standard</th>
<th>Sample 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV WHO IS 10/102</td>
<td>HIV RNA Ref Prep</td>
</tr>
<tr>
<td>Ass. pot.</td>
<td>1850000IU/mL</td>
</tr>
<tr>
<td>Pre-dil. 1</td>
<td>1 mL/12.33mL</td>
</tr>
<tr>
<td>Doses</td>
<td>(1)</td>
</tr>
<tr>
<td>1/1</td>
<td>28.7</td>
</tr>
<tr>
<td>1/3.16</td>
<td>29.7</td>
</tr>
<tr>
<td>1/10</td>
<td>31.5</td>
</tr>
<tr>
<td>1/31.6</td>
<td>33.3</td>
</tr>
</tbody>
</table>

**Model:** Parallel lines  
**Common slope (factor):** $-1.38413$ ($-1.50796$ to $-1.26029$)  
**Correlation ($r$):** 0.978440  
**Transformation:** $y = y$  
**Variance:** Observed residuals

### ANOVA Table:

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F-ratio</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparations</td>
<td>1</td>
<td>2,281,67</td>
<td>2.28167</td>
<td>114.08</td>
<td>0.004 (***)</td>
</tr>
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<td>Regression</td>
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<td>380.808</td>
<td>0.000 (***)</td>
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<td>0.00533467</td>
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<td>0.872</td>
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<td>0.125761</td>
<td>0.0628605</td>
<td>0.314</td>
<td>0.735</td>
</tr>
<tr>
<td>Sample 2</td>
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<td>0.163923</td>
<td>0.0819616</td>
<td>0.410</td>
<td>0.671</td>
</tr>
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<td>Treatments</td>
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<td>78.7383</td>
<td>11.2483</td>
<td>54.242</td>
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### Limits Table for Sample 1:

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<tr>
<td>(IU/mL)</td>
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<tr>
<td>----------</td>
</tr>
<tr>
<td>Potency</td>
</tr>
<tr>
<td>Rel. to Ass.</td>
</tr>
<tr>
<td>Rel. to Est.</td>
</tr>
</tbody>
</table>

**ID:** ISTISAN/ITA
WHO Expert Committee on Biological Standardization

Sixty-seventh report

Substance: HIV RNA

Method: TaqScreen MPX

Assay number: 3

Technician:  

Date of assay: DAY 3

Remarks: Calibration of Reference Preparation for HIV RNA against the WHO IS

EXAMPLE DAY 3

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<td>28.8</td>
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<td>1/10</td>
<td>31.1</td>
<td>31.2</td>
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</tr>
<tr>
<td>1/10</td>
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Model: Parallel lines

Design: Completely randomized

Transformation: y = y

Variance: Observed residuals

Common slope (factor) = -1.40151 (-1.49990 to -1.30312)

Correlation | r | 0.984946

Source of variation | Degrees of freedom | Sum of squares | Mean square | F-ratio | Probability |
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Sample 1

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<tr>
<th>HIV RNA Ref Prep</th>
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<tbody>
<tr>
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<tr>
<td>Potency</td>
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<tr>
<td>Rel. to Ass.</td>
</tr>
<tr>
<td>Rel. to Est.</td>
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</table>

ID: ISTASAN/ITA


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### Annex 6

**Substance**: HIV RNA  
**Method**: TaqScreen MPX  
**Technician**:  
**Ass. pot.**: 1IU/mL

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**Geometric combination**

**Homogeneity**: p = 0.879

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<td>112.7%</td>
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<table>
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<th><strong>Unweighted combination</strong></th>
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<td>Lower limit</td>
<td>Estimate</td>
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</tr>
<tr>
<td>Rel. to Est.</td>
<td>91.9%</td>
<td>100.0%</td>
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</table>

**Executed by**:  
**Calculated by**:  
**Approved by**:  

ID: ISTISAN/ITA
Annex 7

Guidelines on regulatory preparedness for provision of marketing authorization of human pandemic influenza vaccines in non-vaccine-producing countries

1. Introduction
2. Purpose and scope
3. Terminology
4. General considerations for regulatory preparedness for pandemic influenza vaccines
   4.1 Acknowledgement of the role of the NRA in the national pandemic influenza preparedness plan
   4.2 Considerations for national regulatory preparedness
   4.3 Reliance on the decisions and expertise of other regulatory authorities
   4.4 Seasonal influenza vaccines and pandemic preparedness influenza vaccines
5. Regulatory evaluation processes
   5.1 Expected basic documentation according to the source of pandemic influenza vaccine
   5.2 Possible regulatory review processes in a pandemic emergency
   5.3 WHO collaborative procedure for prequalified vaccines
   5.4 Final evaluation
   5.5 Emergency approval
   5.6 Post-marketing risk management and surveillance
6. Quality control preparedness

Authors and acknowledgements

References

Appendix 1 Checklist of regulatory actions for pandemic influenza preparedness and response

Appendix 2 Examples of information and documentation that may be required for the evaluation of a seasonal influenza vaccine annual virus strain change
Guidelines published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA.
Abbreviations

CTD Common Technical Document
GMP good manufacturing practice(s)
NCL national control laboratory
NRA national regulatory authority
PIP Pandemic Influenza Preparedness (Framework)
1. Introduction

An influenza pandemic occurs when a novel influenza A virus emerges against which most people do not have immunity, and spreads rapidly around the world. A pandemic influenza A virus is significantly different from normally circulating human influenza A viruses, with a widespread absence of immunity against the virus observed in the population. As with seasonal influenza viruses, pandemic influenza viruses have the ability to spread easily from human to human and cause disease. This may result in several simultaneous epidemics worldwide with high numbers of cases of clinical disease and deaths, leading to considerable social disruption. Pandemic influenza viruses may evolve from subtypes that previously only circulated in animals or from subtypes currently circulating in humans but sufficiently different antigenically for pre-existing immunity in the population to be low or minimal (an example of the latter case is the 2009 H1N1 influenza pandemic). Influenza viruses that have caused past pandemics have typically originated from animals. Owing to the urgent public health need, strategies to shorten the time between the emergence of a human pandemic influenza virus and the availability of safe and effective pandemic influenza vaccines are one of the highest priorities in global health security.

The WHO Guidelines on regulatory preparedness for human pandemic influenza vaccines (1) were adopted by the WHO Expert Committee on Biological Standardization in 2007. These Guidelines provide national regulatory authorities (NRAs) and vaccine manufacturers with:

- guidance regarding regulatory pathways for approving pandemic influenza vaccines;
- the regulatory considerations to take into account when evaluating the quality, safety and efficacy of candidate vaccines;
- guidance on effective post-marketing surveillance of pandemic influenza vaccines.

The Guidelines apply mainly to countries where influenza vaccine production takes place, but also contain much information that can be useful for countries in which vaccines are not produced (hereafter referred to as non-vaccine-producing countries). However, consultations with stakeholders following the 2009 H1N1 influenza pandemic identified lack of regulatory preparedness as one of the factors that delayed or prevented the deployment of pandemic influenza vaccine in non-vaccine-producing countries. This was especially the case for vaccine destined for donation or deployed by United Nations agencies in response to the pandemic emergency (2–4).

The present Guidelines were developed in response to requests from non-vaccine-producing countries for guidance on the identification of
appropriate regulatory approaches to the marketing authorization of pandemic influenza vaccines, and on arrangements for the lot release of these vaccines in public health emergency conditions. The Guidelines were developed in the context of the Pandemic Influenza Preparedness (PIP) Framework’s Partnership Contribution Implementation Plan 2013–2016 for regulatory capacity-building and strengthening of pandemic preparedness and response (5).

2. Purpose and scope

These WHO Guidelines provide guidance to NRAs of non-vaccine-producing countries on the regulatory oversight of pandemic influenza vaccines for use in public health emergencies. The document focuses in particular on the needs of countries that are not producing influenza vaccines, including countries supplied with vaccines through United Nations agencies and countries which self-procure vaccines.

This guidance is aimed to aid such countries in preparing and putting in place a regulatory process for pandemic influenza vaccines in advance of a pandemic influenza emergency. Such a process should enable countries to expedite the provision of marketing authorization and lot release of influenza vaccines in response to a pandemic emergency. It is acknowledged that each country will have national legislation and policies on the regulation of medicines, vaccines and other health products. Some countries may also have regulations in place on accepting donations of vaccines and ancillary products. This document is intended to provide additional and specific guidance to the NRAs of non-vaccine-producing countries when dealing with pandemic influenza emergencies.

The document specifically provides NRAs of non-vaccine-producing countries with the general principles for evaluating influenza vaccines and establishing basic emergency procedures for regulating pandemic influenza vaccines. A strong emphasis is placed on the need to prepare decision-making processes which minimize duplication and make much-needed vaccines available for use without unnecessary delay during pandemic emergencies. The need to establish such appropriate regulatory processes during the interpandemic phase is also emphasized.

These WHO Guidelines apply to all pandemic influenza vaccines. They are intended for use by NRAs, but will also be of interest to national immunization technical advisory groups (NITAGs), as well as manufacturers and authorities in the private and public sectors responsible for planning and managing vaccine deployment and vaccination operations at all levels.

Other relevant WHO guidelines should also be consulted as appropriate.
3. Terminology

The definitions given below apply to the terms as used in these WHO Guidelines. These terms may have different meanings in other contexts.

Alert phase: the phase during which influenza caused by a new strain is identified in humans. Increased vigilance and careful risk assessment at local, national and global levels are characteristic of this phase (6).

Influenza pandemic: an influenza pandemic (or global epidemic) occurs when a novel influenza virus strain appears which is significantly different from circulating strains and against which almost no one is immune. The Director-General of WHO may, as appropriate, declare a public health emergency of international concern under the International Health Regulations (2005) following the identification and determination of global spread of human influenza caused by a new virus strain (6, 7).

Interpandemic phase: the period between influenza pandemics (6).

Marketing authorization: a formal authorization for a medicine to be marketed. Once an NRA approves a marketing authorization application for a new medicine, the medicine may be marketed and made available for physicians to prescribe. Also referred to as “licensing” or “registration” in this and other documents (8).

National pandemic influenza preparedness plan: a national plan that aims to set out country-specific priorities and actions, and to identify the major components that must be put in place (for example, coordination, resource identification and allocation, and capacity-building) along with response actions that should be strengthened to respond to a pandemic (9).

Non-vaccine-producing country: a country in which vaccines are not produced.

Pandemic influenza vaccine: a monovalent vaccine containing the human influenza A virus strain recommended by WHO for use either when a pandemic is considered by WHO to be imminent or during a pandemic (1).

Pandemic phase: the period of global spread of human influenza caused by a new virus strain. Progression from the interpandemic to the alert and pandemic phases may occur quickly or gradually, as indicated by the global risk assessment, principally based on virological, epidemiological and clinical data (6).

Pandemic preparedness influenza vaccine: an influenza vaccine developed and tested in anticipation of an influenza pandemic, and manufactured using an influenza virus strain that is believed to have similar characteristics to a potential pandemic virus strain (also referred to as “mock-up pandemic influenza vaccine” or “vaccine against novel human influenza virus” in other documents) (1, 10, 11).
**Risk-management plan:** a document submitted as part of the marketing authorization dossier that is evaluated by regulatory authorities before a medicine can be authorized and which is regularly updated as new information becomes available. Risk-management plans include information on a medicine’s safety profile and explain the measures that are taken in order to prevent or minimize any risks associated with the use of the medicine in patients.

**Seasonal influenza vaccine:** a trivalent (or tetravalent) vaccine containing the two influenza A virus strains and one (or two) influenza B virus strains recommended by WHO at its biannual influenza vaccine composition meetings (once for the northern hemisphere and once for the southern hemisphere) (1).

**Supporting NRA:** an NRA selected by the NRA of a receiving country as suitable to support licensing decisions for pandemic influenza vaccines. The eligibility of supporting NRAs could be decided upon after consultation with WHO for guidance.

**Transition phase:** the phase during which the de-escalation of global actions occurs as the assessed global risk of influenza reduces; a corresponding reduction of response activities or movement towards recovery actions by countries may be appropriate, according to their own risk assessments (6).

### 4. General considerations for regulatory preparedness for pandemic influenza vaccines

Countries should have laws requiring that all medicinal products, including influenza vaccines procured or donated in normal or emergency circumstances, be licensed before being placed on the market.

All countries should prepare for public health emergency situations, including influenza pandemics that may cause high morbidity and mortality leading to considerable social disruption. In 2013, WHO revised and updated its pandemic preparedness guidance to reflect experience gained from the 2009 H1N1 influenza pandemic and to support further efforts at national and subnational levels. The updated guidance (6) provides for a risk-based approach that: (a) enables a more flexible response to different scenarios; (b) emphasizes reliance on multisectoral participation; and (c) uses a simplified pandemic phase structure that includes the interpandemic and pandemic (alert and transition) phases.

Regulatory preparations for an influenza pandemic should also be undertaken in the interpandemic phase (6) in order to strengthen the legal and regulatory requirements for importing and approving a vaccine in emergency situations. This would include improving NRA capacity and clearly defining the regulatory pathways for licensing the use of a new vaccine under emergency conditions (12).
NRAs should review the options available to them during a public health emergency and choose the appropriate procedures to fit the situation. The emergency procedures should include processes for ensuring information management, and effective communication and cooperation between different branches of the NRA and relevant stakeholders such as public health authorities (9, 13).

Plans should be developed to address the need for official communication from the NRA relevant to specific audiences – such as the public, health-care workers, national and subnational authorities and international collaborators when needed. Principles set out in relevant WHO communication guidelines (14, 15) should be followed. Communication and information-sharing systems should be established and need to be implemented for all stakeholders (12).

NRAs together with the national immunization programme and other stakeholders should develop post-marketing surveillance plans (including consideration of a risk-management plan which is part of marketing authorization) to monitor the safety and efficacy of pandemic influenza vaccines used during a pandemic. For guidance on safety monitoring and post-marketing surveillance plans, NRAs should refer to the WHO Guidelines on regulatory preparedness for human pandemic influenza vaccines (1) and the WHO Global manual on surveillance of adverse events following immunization (16).

4.1 Acknowledgement of the role of the NRA in the national pandemic influenza preparedness plan

The national pandemic influenza preparedness plan should be established and endorsed before a pandemic arises and should include acknowledgement of the roles and responsibilities of the NRA in regulatory oversight of vaccines (9, 13, 17). The majority of WHO Member States developed and published their national pandemic influenza preparedness plans in 2005 and 2006 and updated them after the 2009 H1N1 influenza pandemic (9).

4.2 Considerations for national regulatory preparedness

During the interpandemic phase the NRA should be responsible for developing the following procedures and plans to support the national pandemic influenza preparedness plan and vaccine deployment plan (12):

- suitable regulatory pathways for pandemic influenza vaccines during the emergency;
- appropriate vaccine lot release procedures for emergency use;
- post-marketing safety surveillance plans.
It is recommended that the NRA's preparedness procedures for facilitating the rapid availability of pandemic influenza vaccines should include:

- an NRA contact point for communications with WHO and other stakeholders on public health/regulatory issues;
- allocation of resources to be used when a pandemic alert has been declared by WHO (note that the national declaration of a pandemic emergency would be made by the responsible national authority following the declaration by WHO);
- a public risk-communications plan summarizing the basis for decision-making;
- procedures for the timely appointment of an emergency evaluation task team for pandemic influenza vaccines (and medicines) that will:
  - (a) include appropriate regulatory and programmatic expertise;
  - (b) prepare procedures for evaluation of applications for pandemic influenza vaccine;
  - (c) define the dossier and supporting documents needed for NRA evaluation;
  - (d) evaluate and recommend marketing authorization of suitable vaccines to the NRA; and
  - (e) allow, during the interpandemic phase, for the regular review of task team appointments and procedures;
- procedures for interactions (including discussion of options for appropriate sources of vaccine) with the public health agencies that will procure, deploy and administer the vaccines;
- a system to accelerate the licensure and lot release of pandemic influenza vaccine including recognition of the decisions, or reliance upon the expertise, of supporting NRAs, and the optimizing of available resources in response to the pandemic;
- procedures and requirements for lot release of pandemic influenza vaccines by the NRA during the pandemic phase (or emergency situation).

The following steps should be included in the regulatory preparedness procedures:

- a working procedure for marketing authorization of the seasonal influenza vaccine annual virus strain change (this may be used where the pandemic influenza vaccine involves a strain change from a licensed seasonal influenza vaccine);
WHO Expert Committee on Biological Standardization   Sixty-seventh report

- preparation of a template emergency risk–benefit consideration and assessment report;
- a procedure for emergency approval of the NRA recommendation, as appropriate;
- a process to expedite marketing authorization through the WHO collaborative procedure for prequalified vaccines, when appropriate;
- preparation of an outline post-marketing surveillance plan which should include special provisions for post-marketing surveillance of the pandemic influenza vaccine in use.

A checklist of regulatory actions for pandemic influenza preparedness and response is provided in Appendix 1.

4.3  **Reliance on the decisions and expertise of other regulatory authorities**

In the event of a pandemic emergency, the NRA of a non-vaccine-producing country should consider reliance on the product evaluation decisions made by other NRAs in vaccine-producing countries. Non-vaccine-producing countries may select, and where possible establish links with, suitable supporting NRAs during the interpandemic period. Reliance on the decisions or expertise of supporting NRAs is highly encouraged.

The NRA of the non-vaccine-producing country should establish mechanisms and procedures for recognizing the marketing authorization decisions of the NRA of the country producing the vaccine, or of other supporting NRAs as appropriate, when considering the licensing of a pandemic influenza vaccine. Mechanisms and procedures may include the establishment during the interpandemic phase of a memorandum of understanding or recognition, including an information-sharing agreement between receiving and selected supporting NRAs in the event of a pandemic.

The assessment reports (summary basis for decision) from other NRAs may provide valuable information and insight into the decision-making processes of these NRAs but may not be readily available in a public health emergency. In this case communication with the relevant NRA regarding the licensure is strongly encouraged.

In addition, a procedure for joint review of a pandemic influenza vaccine dossier with neighbouring and supporting NRAs may be considered. This could be facilitated by WHO.

The WHO collaborative procedure for marketing authorization of prequalified vaccines (18, 19) could be used as a model.
It should be noted that both joint reviews and the WHO collaborative procedure require advance planning so that agreements are brought into effect at the earliest opportunity and that the vaccine product is already identified.

It is expected that future pandemic influenza vaccines prequalified by WHO will include a summary assessment report outlining the basis for prequalification that will be available to countries intending to import, grant marketing authorization for and use these vaccines to mitigate an influenza pandemic. Requests for more detailed information regarding prequalification of a particular pandemic influenza vaccine should be addressed to the WHO prequalification programme.

The NRAs of some vaccine-producing countries with considerable experience in the evaluation of seasonal and pandemic influenza vaccines supported WHO in expediting the prequalification of pandemic influenza vaccines during the 2009 H1N1 influenza pandemic, and are encouraged to support the NRAs of non-vaccine-producing countries in regulatory decision-making and marketing authorization of pandemic influenza vaccines.

4.4 **Seasonal influenza vaccines and pandemic preparedness influenza vaccines**

Seasonal influenza vaccines present many production and regulatory challenges similar to those of pandemic influenza vaccines due to the need for an annual change in formulation to reflect currently circulating virus strains, and very short development timelines. Many countries have established accelerated regulatory procedures for licensing seasonal influenza vaccines. Some non-vaccine-producing countries may also have provisions in place for accelerated regulatory approval of annual influenza virus strain changes in a seasonal vaccine formulation. In all cases, the WHO recommendations on seasonal influenza vaccine strain composition\(^1\) should be followed (8).

In appropriate circumstances, the NRA may decide that the procedure for an annual seasonal vaccine strain change can be adapted to authorize pandemic influenza vaccines. The combination of circumstances under which the strain-change procedure can be adapted to license pandemic influenza vaccines are:

- the candidate monovalent pandemic influenza vaccine has an antigen content similar to that of the corresponding single component in a licensed trivalent or tetravalent seasonal influenza vaccine containing the same subtype; and

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- the excipients in the candidate vaccine are the same as those in the licensed vaccine; and
- the manufacturing technology (for example, eggs, inactivant, purification process) and controls are the same as those of the licensed vaccine.

Pandemic preparedness influenza vaccines are vaccines that have been prepared using strains of influenza viruses that are considered to have pandemic potential. These vaccines may be novel in formulation, antigen content and/or adjuvant. Influenza vaccine manufacturers have been encouraged to develop pandemic preparedness influenza vaccines and to conduct suitable nonclinical and clinical testing to demonstrate their safety and immunogenicity.

The rationale for the decision to review pandemic preparedness influenza vaccines should be made publicly available (10, 11). Some countries may choose to make specific provisions for evaluating pandemic preparedness influenza vaccine as a precautionary step so that the strain-change policy and procedures used for seasonal influenza vaccine can be adapted for suitable pandemic influenza vaccine applications. Once the pandemic preparedness influenza vaccine has been evaluated and approved (although not marketed for sale), the change to an appropriate pandemic virus strain – when identified and formulated into a pandemic influenza vaccine – can be approved using similar criteria to those used for an annual seasonal vaccine strain change. This procedure may be implemented in countries with adequate regulatory expertise and resources.

Some pandemic influenza vaccines or pandemic preparedness influenza vaccines may be novel constructs or formulations requiring expert regulatory evaluation. NRAs of non-vaccine-producing countries may request assistance in such evaluations from WHO or other NRAs more experienced in the regulation of both seasonal and pandemic influenza vaccines (see section 4.3 above).

### 5. Regulatory evaluation processes

The following elements are necessary to ensure an orderly and legal regulatory marketing authorization or emergency approval and lot release of a pandemic influenza vaccine in an emergency situation in the shortest possible time:

- an NRA or a regulatory system;
- a national pandemic preparedness plan that acknowledges that pandemic influenza vaccines that are used shall be formally licensed or granted emergency approval by the NRA and released onto the market;
NRA policies and procedures for:
(a) NRA evaluation of pandemic influenza vaccine applications;
(b) procedures and criteria for rapid identification of suitable experts for regulatory evaluation of pandemic influenza vaccine applications (task team);
(c) consideration of a joint review with neighbouring or supporting NRAs; and
(d) recognition of the marketing authorization decisions of other NRAs and the WHO prequalification decision;

- a procedure for emergency approval of the NRA’s pandemic influenza vaccine recommendations (where higher authority ratification is required);
- a collaborative procedure for expedited marketing authorization of prequalified vaccines, when appropriate;
- a situation analysis of possible procedures for marketing authorization of vaccines received through self-procurement, donations and/or United Nations supply. The situation should also be recognized whereby a pandemic preparedness influenza vaccine has been evaluated and approved during the interpandemic period and where the application can subsequently be approved for pandemic use on the basis of the national seasonal influenza vaccine strain-change procedure;
- recognition of lot release certificate of other responsible NRAs;
- plan for post-marketing surveillance of the pandemic influenza vaccine in use.

Depending on the pandemic phase and the source of the vaccine, the following regulatory approaches could be followed by an NRA (see section 5.2 below):

- Full review – a standard review process to authorize a product licensure that can include fast-track review.
- Fast-track review of basic documentation – a fast-track review process based on basic available information for emergency authorization.
- Reliance – a process to review the marketing authorization report/decision issued by a supporting NRA or WHO prequalification (19).
- Recognition – recognition of the marketing authorization decision of another NRA or WHO prequalification without further evaluation.
Strain-change procedure: a procedure for authorizing a seasonal strain change for influenza vaccines:

(a) a procedure for the evaluation and approval of seasonal influenza virus strain changes in an approved seasonal influenza vaccine (see Appendix 2);

(b) the approved procedure to be used for pandemic preparedness influenza vaccine evaluation and marketing authorization following inclusion of the identified pandemic virus strain.

5.1 Expected basic documentation according to the source of pandemic influenza vaccine

Non-vaccine-producing countries can access pandemic influenza vaccine from different sources, including a United Nations agency, a donation from a company or other source, or through national self-procurement. In general, full dossiers are required for evaluation of the quality, safety and efficacy of vaccines – however, in an emergency situation the accompanying documentation dossier may be provided in sections as it becomes available.

Under these circumstances, at least the following documents should be made available for evaluation to ensure the quality, safety and efficacy of vaccines from each source:

United Nations agency supply (WHO-prequalified vaccines)

- Evidence/certificate of WHO prequalification with assessment report (18, 19).

Donation from a company or other source

- Information on strain change of a licensed seasonal influenza vaccine or pandemic preparedness influenza vaccine (if applicable).
- If the vaccine has been prequalified by WHO the Common Technical Document (CTD) Module-2 and prequalification assessment report should be provided.
- If the vaccine has been licensed by a supporting NRA the CTD Module-2 and assessment report by the NRA, if available, should be provided.
- Where the vaccine has been licensed by an NRA other than a supporting NRA the full dossier for marketing authorization and the assessment report by the NRA, if available, should be provided.
- In the case of a vaccine that has not previously been licensed a full dossier for marketing authorization should be provided by
the manufacturer. The procedures and requirements in the WHO Guidelines on regulatory preparedness for human pandemic influenza vaccines should be followed (1).

National guidelines on donations of medicines should be followed. If these do not exist the recommendations in the WHO Guidelines for medicine donations (20) should be followed.

**National self-procurement**

- Information on strain change of a licensed seasonal influenza vaccine or pandemic preparedness influenza vaccine (if applicable) should be provided.
- If the vaccine has been prequalified by WHO the CTD Module-2 and prequalification assessment report should be provided.
- If the vaccine has been licensed by a supporting NRA the CTD Module-2 and assessment report by the NRA, if available, should be provided.
- Where the vaccine has been licensed by an NRA other than a supporting NRA the full dossier for marketing authorization and the assessment report by the NRA, if available, should be provided.
- In the case of a vaccine that has not previously been licensed a full dossier for marketing authorization should be provided by the manufacturer. The procedures and requirements in the WHO Guidelines on regulatory preparedness for human pandemic influenza vaccines should be followed (1). Seeking support from the NRA of the producing country is strongly encouraged.

### 5.2 Possible regulatory review processes in a pandemic emergency

Even in the midst of a pandemic emergency the NRA should conduct an appropriate review of the documentation submitted that covers the components set out below, and should document the extent of the available evidence on which the recommendation to authorize, approve or reject had been based.

In a pandemic emergency it is possible that not all documentation for a vaccine will be available at the time of application, and many NRAs have accepted that applicants will submit the evidence as it becomes available. This approach is generally known as a “rolling review” (21). It would be expected that the sections on manufacturing, specifications and controls would be available, together with evidence of consistency of manufacture. For nonclinical safety studies, preliminary results should be available. The results of stability studies would be delayed as would any results from clinical studies.
Where possible the NRA could make arrangements for the joint review of pandemic influenza vaccine dossiers with neighbouring and/or supporting NRAs. The possible parties involved in such an arrangement should establish this agreement during the interpandemic phase.

Depending on the pandemic phase and the source of the vaccine, review activities may include one or more of the following procedures (see also Fig A7.1):

- full review;
- fast-track review of basic documentation;
- reliance;
- recognition;
- strain-change procedure.

5.2.1 Full review

This is the standard process of review of the full dossier in a fast-track review process (as normally conducted in that country) for vaccines that are new applications or previously licensed by NRAs other than a supporting NRA.

- Available documentation: the documentation should be complete, as legally required in each country.

Applicability: this procedure would apply to licensed vaccines in the interpandemic phase.

This would require evaluation of the documentation of product quality and of the results of nonclinical and clinical studies to demonstrate safety and efficacy in the target population. The documentation should be as legally required in each country.

During the interpandemic phase the NRA of a non-vaccine-producing country may conduct a full pandemic preparedness influenza vaccine dossier review to ensure familiarity with the characteristics of such vaccines.

5.2.2 Fast-track review of basic documentation

This is a fast-track review process in which marketing authorization is based upon the information available at the time. In the event that a fast-track review is deemed appropriate (as defined in the approved NRA pandemic emergency procedures) the following documents from the manufacturer and the responsible NRA and/or WHO should be reviewed. The full application dossier may be provided when available.

- Available documentation:
  a) assessment reports of the responsible NRA;
b) evidence of quality (certificate of analysis or lot release) and good manufacturing practices (GMP) compliance (GMP certificate);
c) CTD Module-2 quality, nonclinical and clinical overviews (if available).

**Applicability**: this procedure would apply during the pandemic alert phase and transition phase for a pandemic influenza vaccine licensed by an NRA other than a supporting NRA.

### 5.2.3 Reliance

This is the process of reviewing the decisions of other competent NRAs with which there has been an agreement for support. Where it has been agreed (as defined in the approved NRA pandemic emergency procedures) that the decision of another NRA can be considered and used as the basis of a recommendation for marketing authorization, this approach would involve acceptance on the basis of the already agreed conditions and limitations on the use of the vaccine, and would require the following *available documentation*:

- certificate of the responsible NRA’s marketing authorization decision;
- assessment reports of the responsible NRA.

**Applicability**: this procedure would apply during the pandemic alert phase, pandemic phase and transition phase for a pandemic influenza vaccine licensed by an NRA other than a supporting NRA.

### 5.2.4 Recognition

This is the process of recognizing the WHO prequalification decision or the decision of a supporting NRA. Where it has been agreed (as defined in the approved NRA pandemic emergency procedures) that the decision of a supporting NRA can be used as the basis for a recommendation for marketing authorization, this approach would involve acceptance on the basis of the already agreed conditions and limitations on the use of the vaccine, and would require the following *available documentation*:

- certificate of the responsible NRA’s marketing authorization decision or WHO prequalification assessment report.

**Applicability**: this procedure would apply during the pandemic alert phase, pandemic phase and transition phase for a pandemic influenza vaccine licensed by a supporting NRA or prequalified by WHO. It may also apply during the pandemic phase for a pandemic influenza vaccine licensed by an NRA other than a supporting NRA.
5.2.5 **Strain-change procedure**

This is a procedure for addressing a strain change in a licensed seasonal influenza vaccine. Where it has been agreed (as defined in the approved NRA pandemic emergency procedures) the dossier of a pandemic preparedness influenza vaccine may be evaluated in this way, following the criteria set out for an annual strain change, as applicable for pandemic use.

- Available documentation: as for an annual strain change.

The approved conditions and limitations on the use of the vaccine should be accepted.

*Applicability:* this procedure would only apply to a pandemic influenza vaccine licensed by a strain-change approach from a pandemic preparedness influenza vaccine or seasonal influenza vaccine by the NRA of the producing country.

If a pandemic influenza vaccine is licensed by a strain-change approach from a pandemic preparedness influenza vaccine or seasonal influenza vaccine then a receiving country NRA could use the strain-change procedure (or other appropriate approach based on the source of vaccine).

If a vaccine has not been licensed by any NRA then the guidance provided in the WHO Guidelines on regulatory preparedness for human pandemic influenza vaccines should be followed (1).

5.3 **WHO collaborative procedure for prequalified vaccines**

Apart from the regulatory procedures for marketing authorization of pandemic influenza vaccines, expedited licensure through the WHO collaborative procedure for prequalified vaccines (18) may also be used for suitable pandemic influenza vaccines as appropriate. An information-sharing agreement between WHO, the receiving NRA and the manufacturer should be signed in the interpandemic phase – particularly given that, during an emergency, time may not allow for this step to occur prior to the decision to use the vaccine. For this procedure the WHO prequalification assessment report should be provided to the receiving NRA. The full dossier in the format of the CTD could also be provided to the NRA.

It would be expected that, following the pandemic phase, the full dossier as required by the relevant non-vaccine-producing country would be completed and submitted for evaluation.
Fig. A7.1
Illustrative chart of regulatory approaches relative to the status of the vaccine and the continuum of pandemic phases

Licensed vaccines from any source

- Prequalified
- Licensed by supporting NRA
- Licensed by other NRA

Recognition procedure in all phases
Recognition in the pandemic phase OR Reliance or Fast-track procedure in the alert or transition phases

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5.4 Final evaluation

Before a regulatory decision to recommend marketing authorization of a pandemic influenza vaccine is taken a final evaluation of the available documentation should be conducted to ensure that the pandemic influenza vaccine presentation is suitable for use in the country (22, 23).

Provided the necessary procedures are in place this final evaluation can be conducted rapidly (for example, in as little as one day depending on circumstances and pandemic influenza vaccine marketing authorization status) with a risk–benefit consideration and recommendation for marketing authorization.

The NRA should ensure that the following conditions are met:

- An adequate document package is provided. A post-marketing commitment by the manufacturer to provide any outstanding information should be considered.

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a Interpandemic phase, alert phase, pandemic phase or transition phase as defined by WHO (see section 3, Terminology above).

b Any NRA selected by the NRA of the receiving country as suitable in supporting pandemic influenza vaccine licensing decisions; the eligibility of supporting NRAs could be decided upon after consultation with WHO.

c Any NRA not designated as a "supporting NRA" by the NRA of the receiving country.
- There is a local agency responsible for supply of the product (that is, an “applicant” or state body that is a defined responsible legal entity).
- Packaging, label and package insert are nationally acceptable.
- The vaccine is compatible with the national pandemic influenza preparedness plan.
- The vaccine is indicated for the circulating strain(s).

This evaluation may need to be based upon minimal and incomplete documentation, and this should be acknowledged in the recommendation. An evaluation report should be produced by the NRA.

5.5 Emergency approval

In some countries the NRA may have the authority to approve use of a medicine or vaccine without reference to another authority, while in other countries a final approval or directive is required. Thus reference can be made to either an “approval” or “recommendation” process.

During the pandemic period, emergency approval procedures may be used. Approval may be based upon limited clinical data or quality data (for example, on stability) and upon expedited evaluation of the available evidence. Therefore, the approval may include one or more special conditions for use. These can include post-marketing safety reporting requirements, and limitations such as:

- use only during the pandemic period
- use only by certain agencies
- use only in certain listed groups at high risk
- special conditions for post-marketing safety reporting.

5.6 Post-marketing risk management and surveillance

Each country should include post-marketing surveillance of adverse events in the pandemic vaccine deployment plan. This should follow the WHO Guidelines on regulatory preparedness for human pandemic influenza vaccines (1) and the WHO Global manual on surveillance of adverse events following immunization (16). The risk-management plan for pandemic influenza should be monitored by the NRA and national immunization programme with input from the vaccine manufacturer.

National systems for post-marketing surveillance and reporting of adverse events following immunization should not be compromised by the implementation of a pandemic influenza vaccination campaign.
6. Quality control preparedness

Lot release and quality control of pandemic influenza vaccines by the NRAs and/or national control laboratories (NCLs) of non-vaccine-producing countries should follow the guidance set out in relevant WHO documents (17, 18, 20, 22–24).

Vaccines received by procuring countries should be produced in compliance with GMP, and tested for quality and safety by the vaccine manufacturer. Typically, such vaccines should also be subjected to independent quality control testing and released by the responsible NRA/NCL in accordance with the WHO Guidelines for independent lot release of vaccines by regulatory authorities (17). For vaccines supplied through United Nations agencies it is recommended that further release by the NRA/NCL of receiving countries should not be performed because such products are prequalified by WHO and released by the responsible NRA/NCL. Likewise, self-procured WHO-prequalified vaccines are normally released by the responsible NRA/NCL and, if so, should not be subjected to further lot release by the importing country in the event of an influenza pandemic. Recognition of the lot release certificate of the responsible NRA/NCL of the producing country is recommended by WHO (17).

For self-procured non-WHO-prequalified pandemic influenza vaccines the NRA/NCL of the procuring country may, in the event of an influenza pandemic emergency, conduct lot release through review of the summary lot protocol. Further laboratory testing by the NRA/NCL of the receiving country may not be necessary, based on risk assessment. Part F of the WHO Guidelines on regulatory preparedness for human pandemic influenza vaccines (1) should be consulted.

The procedures adopted should ensure the deployment of vaccines without undue delay.

Authors and acknowledgements

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Bhutan, Bhutan; Mr M. Eisenhawer, WHO Regional Office for South-East Asia, India; Dr L. Elmgren, Health Canada, Canada; Dr O.G. Engelhardt, National Institute for Biological Standards and Control, England; Dr E. Griffiths, Consultant, Kingston-upon-Thames, England; Mr S. Hiem, Registration Bureau of Department of Drugs and Food, Cambodia; Mrs T. Jivapainsarnpong, Ministry of Public Health, Thailand; Dr H. Langar, WHO Regional Office for the Eastern Mediterranean, Egypt; Dr I.B. Mansour, Laboratoire National Contrôle Médicaments, Tunisia; Ms M.L.L. Mendez, Comisión Federal para la Protección contra Riesgos Sanitarios, Mexico; Ms E. Nantongo, National Drug Authority, Uganda; Dr L. Oueslati, Laboratoire National Contrôle Médicaments, Tunisia; Dr P. Palihawadana, Ministry of Healthcare and Indigenous Medicine, Sri Lanka; Dr M. Pfleiderer, Paul-Ehrlich-Institut, Germany; Mr A.R.A. Rauf, Drug Regulatory Authority of Pakistan, Pakistan; Ms J. Rodgers, Food and Drugs Authority Ghana, Ghana; Dr S. Sebai, Laboratoire National Contrôle Médicaments, Tunisia; Dr S.F. Shah, Consultant, WHO Regional Office for the Western Pacific, Philippines; Dr J. Southern, Adviser to the Medicines Control Council of South Africa, South Africa; Dr I. Tebib, Laboratoire National Contrôle Médicaments, Tunisia; Ms E. Yonis, Food, Medicines and Health Care Administration and Control Authority, Ethiopia; and Dr C.P. Alfonso, Ms D. Decina, Dr R.O.A. Dehaghi, Ms L. Hedman, Dr D. Lei, Ms C.A. Rodriguez-Hernandez and Dr T. Zhou, World Health Organization, Switzerland.

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Further changes were subsequently made to document WHO/BS/2016.2289 by the WHO Expert Committee on Biological Standardization.

References


Appendix 1

Checklist of regulatory actions for pandemic influenza preparedness and response

It is important to ensure that regulatory legislation is in place to enable the various approaches listed below to be applied as needed in preparation for, or during, a pandemic.

1. Prepare regulatory preparedness procedures compatible with the national pandemic influenza preparedness plan during the interpandemic phase.

2. Appoint and maintain a pandemic task team (with staff, training, budget and annual review).

3. In the interpandemic phase (provisionally) grant marketing authorization to pandemic preparedness influenza vaccines.

4. Liaise with other national agencies on pandemic preparedness procedures.

5. Develop memoranda of understanding with potential supporting NRAs.

6. In the pandemic alert phase (or earlier, if possible):
   (a) determine which vaccines may be sourced by national agencies;
   (b) request data packages from potential vaccine suppliers;
   (c) decide on appropriate evaluation procedures and evaluators;
   (d) prepare a format for an assessment report and post-marketing surveillance plan;
   (e) make a recommendation for licensure or rejection that includes the assessment report; and
   (f) alert the national control laboratory regarding potential vaccines that may be granted marketing authorization and imported.

7. In the pandemic phase:
   (a) complete the activities from the alert phase;
   (b) conduct vaccine lot release procedures or, where appropriate, recognize the lot release certificate issued by the national regulatory authority/national control laboratory of the producing country;
   (c) where possible, keep records of the vaccine lot deployment (consider that there may be more than one vaccine approved for use);
(d) implement the national post-marketing surveillance plan;
(e) continue to update the data packages from the vaccine supplier(s); and
(f) conduct regular reviews of activities and optimize where possible.

8. In the pandemic transition phase:
   (a) complete the data package for the emergency-approved vaccine(s);
   (b) collate and analyse the data from post-marketing surveillance activities;
   (c) withdraw the licence of the emergency-approved pandemic influenza vaccine(s) if appropriate;
   (d) review the activities of the pandemic task team and propose improvements; and
   (e) review the reports from the pandemic surveillance plan.
Appendix 2

Examples of information and documentation that may be required for the evaluation of a seasonal influenza vaccine annual virus strain change

1. WHO-recommended strain list for the relevant hemisphere.
2. Manufacturer’s choice of strains for inclusion.
3. Details of manufacturing procedure (declaration if unchanged).
4. Validation of the inactivation and fragmentation.
5. Source, history and master/working seed characterization of each strain included.
6. Egg or cell culture: safety specifications and tests (declaration if unchanged).
7. Qualification of potency test (single radial immunodiffusion – SRID) reagents.
8. Final product release specifications and results (this must include endotoxin release limit).
9. Retrospective data on the “efficacy or performance” of influenza vaccines (preceding year or season).
10. Stability data (accelerated or from the most recent, or most similar, batch of approved vaccine).
11. Copy of the approved package insert.
12. Copy of the proposed package insert, indicating:
   (a) the year/season for which the vaccine will be used;
   (b) WHO-recommended strains; and
   (c) a statement that the vaccine complies with WHO recommendations (southern or northern hemisphere) for the year/season.
13. Copy of the approved patient information leaflet.
14. Copy of the proposed patient information leaflet, indicating:
   (a) the year/season for which the vaccine will be used; and
   (b) WHO-recommended strains.

15. All labels and inner and outer containers must prominently indicate the
    year/season for which the vaccine will be used, and a facsimile must be
    submitted as proof.

16. International core data sheet or summary of product characteristics.
Annex 8

Labelling information of inactivated influenza vaccines for use in pregnant women

Addendum to Annex 3 of WHO Technical Report Series, No. 927

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Guidance documents published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products.
**Abbreviations**

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<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
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<tr>
<td>GACVS</td>
<td>WHO Global Advisory Committee on Vaccine Safety</td>
</tr>
<tr>
<td>IFPMA</td>
<td>International Federation of Pharmaceutical Manufacturers &amp; Associations</td>
</tr>
<tr>
<td>IIV</td>
<td>inactivated influenza vaccine</td>
</tr>
<tr>
<td>NITAG</td>
<td>national immunization technical advisory group</td>
</tr>
<tr>
<td>NRA</td>
<td>national regulatory authority</td>
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<tr>
<td>SAGE</td>
<td>WHO Strategic Advisory Group of Experts</td>
</tr>
<tr>
<td>SmPC</td>
<td>summary of product characteristics</td>
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</table>
1. Introduction

Rates of morbidity and mortality resulting from seasonal influenza virus infection are considered to be substantial worldwide (1, 2). Pregnant women are especially vulnerable and have an increased risk of severe disease and death from influenza. The infection may also lead to fetal complications such as stillbirth, neonatal death, preterm delivery and decreased birthweight (3, 4). For these reasons, the 2012 WHO position paper on vaccines against influenza (3) – endorsed by the WHO Strategic Advisory Group of Experts on Immunization (SAGE) – recommended the immunization of pregnant women with trivalent inactivated influenza vaccine (IIV) at any stage of pregnancy. SAGE also recommended that pregnant women should be given the highest priority in countries considering the initiation or expansion of immunization programmes for seasonal influenza vaccination (3, 5, 6). This recommendation is based on evidence of a substantial risk of severe disease in this population group and on evidence that the use of seasonal influenza vaccine is both safe throughout pregnancy and effective in preventing influenza in women as well as in their young infants in whom the disease burden is also high (3, 5). After careful analysis of data worldwide, the WHO Global Advisory Committee on Vaccine Safety (GACVS) concluded that there was no evidence of adverse pregnancy outcomes associated with the vaccination of pregnant women with several inactivated viral or bacterial vaccines, including IIVs (5, 6). However, for various reasons, the implementation of influenza immunization during pregnancy remains suboptimal (4). One reason for this has been the perceived risk of administering influenza vaccine, or indeed any vaccine, to this population group, particularly due to the precautionary language used in some product labels and the likelihood of misinterpretation (7).

The development of this explanatory addendum arises from the recommendations of SAGE regarding the immunization of pregnant women with IIV and the resulting discussions at several WHO consultations (3, 6, 8–10), as well as discussions held during the 2015 meeting of the WHO Expert Committee on Biological Standardization (11).

2. Background

Enhancing the uptake of vaccines during pregnancy is an important element of WHO’s ongoing work to improve maternal and child health. As part of this work, WHO held a consultation in July 2014 on influenza vaccines for pregnant and lactating women which focused on the clinical data requirements for product labelling information (8). The consultation was organized by the WHO Technologies, Standards and Norms team and the WHO Initiative for Vaccine
Research and brought together regulators, manufacturers and researchers with experience in vaccines. A further consultation was organized by WHO in 2015 to review existing guiding principles and regulations relating to product package information for IIVs, and to explore the possibility of developing an addendum to the existing WHO Recommendations for the production and control of influenza vaccine (inactivated) (12) with the aim of clarifying and interpreting the labelling information subsections to facilitate the appropriate use of IIVs in pregnancy (9). Regulatory policy and requirements regarding permitted text in the pregnancy and lactation subsections of product inserts were reviewed from selected developed and developing countries (Brazil, Canada, Ghana, India, Indonesia, Thailand and the United States of America) and from the European Union. Also presented were the results of a 2014 Developing Country Vaccine Regulators’ Network survey regarding regulatory policies and the interpretation of the wording in the pregnancy and other subsections of IIV labelling. The diversity of approach and understanding in different countries and regions was evident. It also became clear that, in countries that import IIVs, the format, data and language included in the product insert usually reflected the text approved by the national regulatory authority (NRA) in the respective country of licensing. Some developing countries require additional language that makes the perceived cautionary message for use in pregnant women even stronger. The regulatory position is based on the fact that licensing is product-specific, and reliant upon data generated during the clinical evaluation of the vaccine and submitted by the manufacturer. The European Medicines Agency (EMA) implemented a policy based on an evaluation of all available evidence on the safety and effectiveness of IIVs and expected all IIV licence holders in the European Union to amend the pregnancy subsection of the labelling to include advice that IIVs can be used during all stages of pregnancy (8, 13). However, this policy has recently changed and the new guideline on influenza vaccines (clinical module) clarifies that a core summary of product characteristics (SmPC) for IIV is no longer maintained but individual SmPCs should be tailored to product-specific data (10, 14).

Some NRAs include recommendations made by national public health advisory bodies on the use of IIVs in pregnancy to protect mother and infant against influenza (15, 16), thus adding to the confusion regarding the meaning of labelling information. Even though the wording in the package insert for IIVs does not represent a contraindication to the use of the vaccines in pregnancy, the particular wording employed is often misinterpreted to imply a contraindication. Consequently it is interpreted as differing from statements made by advisory bodies such as national immunization technical advisory groups (NITAGs) and SAGE that recommend the use of IIVs in pregnancy to protect mother and infant from the disease. Thus there is a perceived contradiction between the statements of advisory bodies and the position of the NRA.
NITAG recommendations on the use of vaccines in pregnancy are made on the basis that the benefit of vaccination in pregnant women usually outweighs the risk of potential adverse effects in the mother or developing offspring when: (a) the risk of disease exposure is high; (b) the infection poses a special risk to mother and fetus; and (c) the vaccine is unlikely to cause harm. In contrast, any statement in the “Indications and Usage” section of the labelling that specifically addresses the use of the product in pregnancy can be approved by an NRA only when supporting data from adequate and well-controlled studies in pregnant women are available. As pregnant women are usually excluded from clinical studies during vaccine development, licensure dossiers generally do not include information on the safety and efficacy of a particular vaccine in pregnant women. In the absence of such data, therefore, the “Indications and Usage” section of the labelling will lack a statement that specifically describes the use of the product in pregnancy.

3. Purpose and scope

The aim of this addendum is to provide clarification and interpretation of the labelling information provided in the product insert of IIVs in order to facilitate maternal immunization programmes. It is also intended to raise awareness of the convergence of regulatory positions in spite of differing approaches to labelling and regulatory language regarding the use of these vaccines in pregnant women. On the basis of current evidence, the use of IIV in pregnant women is not contraindicated.

This addendum applies to inactivated trivalent and quadrivalent (tetravalent) influenza vaccines for which sufficient safety data are available. It is intended for NRAs, manufacturers, end-user programme managers and NITAGs. Liability issues are beyond the scope of this document.

4. Terminology

The definitions given below apply to the terms as used in this WHO guidance document. These terms may have different meanings in other contexts.

Label(ling): all forms of product information – that is, container label, SmPC, product/package insert, package leaflet and prescribing information.

Maternal immunization: frequently used to refer to vaccination prior to, during or after pregnancy. For the purposes of this document the term refers specifically to vaccination during pregnancy.

National Immunization Technical Advisory Group (NITAG): a national expert advisory group that evaluates the available evidence on national disease incidence, and available vaccines, in order to provide advice to the health ministry
on national immunization programme policies, and on priority vaccines and target populations.

Summary of product characteristics (SmPC): the SmPC is the basis of information for health-care professionals on how to use the medicinal product safely and effectively. Product labelling should be drawn up in accordance with the SmPC.

5. Labelling information

As with all prescription drugs and biological products, IIVs must be accompanied by labelling that summarizes the scientific information concerning their safe and effective use.

Labelling includes the package insert, which is also referred to as prescribing information or the SmPC (17). This component of labelling is the primary mechanism through which regulatory agencies and vaccine manufacturers communicate essential, science-based information. This information is then used by health-care professionals to make prescribing decisions and to counsel patients about the risks and benefits of a product. The content and format requirements for labelling are prescribed by regulations specific to the country where the vaccine is licensed and may differ between countries (17, 18). Nevertheless, common principles include that prescribing information should be based on available data, that it must not be misleading and that it must not contain implied claims or uses for which there is inadequate evidence of safety or effectiveness (19).

The labelling sections relevant to the use of vaccines in pregnancy – namely, “Indications and Usage”, “Warnings and Precautions”, “Contraindications”, and “Use in Specific Populations” – are described below. Countries have information on vaccination in pregnancy under various sections. Information regarding the use of an IIV in pregnancy is typically found under the “Use in Specific Populations” section. However, in some countries the NRA has required that precautionary statements about the use of an IIV in pregnancy should be included under the “Warnings and Precautions” and “Contraindications” sections because safety data on use of the vaccine in pregnancy may be unavailable or insufficient.

5.1 Indications and Usage

The “Indications and Usage” section of the product labelling communicates a product’s approved indication(s) and should clearly convey the use(s) for which the product has been shown to be safe and effective. Although pre-licensure clinical trials are generally conducted in carefully selected populations, the “Indications and Usage” statement(s) often reflect a broader population and take
into consideration the generalizability of the evidence. Typically, for preventive vaccines, the “Indications and Usage” statement(s) state the disease being prevented and the age range for which use is approved.

Specific regulatory requirements and standards for demonstrating that a vaccine is safe and effective may vary between NRAs. However, in general, the standards for demonstrating the safety of a vaccine for its intended indication take into consideration the condition of the recipients and the characteristics of the product. It is expected that pre-licensure data demonstrating that a vaccine is effective for the intended indication and use are derived from adequate and well-controlled clinical studies. Data from pregnancy exposure registries, epidemiological studies and case-series are typically collected in the post-marketing period and are used to inform the “Use in Specific Populations” section of the labelling (see section 5.4 below).

While data from related, similar vaccines may be supportive of an indication for use, it is typically expected that the specific vaccine is evaluated for safe and effective use in the intended population. For most IIVs that are currently licensed, data from adequate and well-controlled studies demonstrating that vaccination during pregnancy is safe and effective for the pregnant woman or newborn infant may not be available to support an indication in the labelling. Data from studies published in the literature on the use of IIV in pregnancy may not have been submitted to NRAs or may not meet regulatory requirements. In such cases, product- (brand)-specific data demonstrating that the vaccine is safe and effective may not be available. Consequently, the prescribing information for IIVs will not include an “Indications and Usage” statement that specifically addresses use in pregnancy. This does not mean, however, that IIVs are contraindicated for use in pregnancy. IIVs are licensed for use in an age range that includes women of childbearing age. In the absence of evidence that the risk of use in pregnancy clearly outweighs any possible benefit, there is no specific contraindication for use in pregnancy and, consequently, IIVs may be administered to pregnant women. Available data specific to the use of IIVs in pregnancy will be included in the “Use in Specific Populations” section of the labelling (see section 5.4 below).

5.2 **Warnings and Precautions**

The “Warnings and Precautions” section of the product labelling is intended to include, but is not limited to, a description of adverse events that are serious or otherwise clinically significant because they have implications for prescribing decisions or for patient management. For an adverse event to be included in this section there should be reasonable evidence of a causal association between the adverse event and the drug or biological product.
Clinically significant adverse reactions that have not been observed following use of the specific drug or biological product, but which are anticipated on the basis of data on another drug in the same class, or from animal data, should be included under “Warnings and Precautions”. In addition, any clinically significant interference with laboratory tests, clinically significant drug interactions, and any special care or monitoring required to ensure safe use, should also be included under “Warnings and Precautions”. The description of each adverse reaction or topic included under “Warnings and Precautions” is cross-referenced to a more detailed discussion of the risk elsewhere in the labelling (for example, in “Adverse Reactions” and “Use in Specific Populations”). Some NRAs require the inclusion of information on use of IIVs during pregnancy in the “Warnings and Precautions” section of the labelling.

5.3 Contraindications
Although the specific wording used in the “Contraindications” section of the product labelling may depend on the requirements of the NRA where the vaccine has been licensed, there is a common requirement that drugs or biological products, including vaccines, should be contraindicated only in those situations where the known risk from use clearly outweighs any possible benefit. Only known hazards, not theoretical possibilities, should be the basis for contraindication. As an example relating to vaccine use in pregnant women, evidence in humans or animals that a vaccine poses a serious risk of developmental toxicity during pregnancy would usually warrant a contraindication for use during pregnancy. However, for IIVs, if available animal or human data do not indicate a risk in pregnancy that clearly outweighs benefit, or if data are unavailable to inform risk in pregnancy, there should not be a contraindication for use during pregnancy.

5.4 Use in Specific Populations
The “Use in Specific Populations” section of the product labelling summarizes important differences in the response to the product, or in recommendations for use, in specific populations. Information relevant to the use of a product during pregnancy is generally found under this section and is sometimes referred to as the “pregnancy subsection” of product labelling. However, depending on the labelling requirements of the NRA where the vaccine was licensed, information regarding the use of IIV in pregnancy may also be found in other sections of product labelling, such as the “Warnings and Precautions” section (9).

The pregnancy subsection of the product labelling includes data, when available, from reproductive-toxicity studies conducted in animal models to assess the potential developmental and reproductive risks of the product.
Data that may be available concerning the safety of the product in pregnant women are also described in this section. Sources of human data may include pregnancy registries, pre-licensure clinical trials in which pregnant women were inadvertently exposed to the product, large-scale epidemiological studies and case-series reporting rare adverse events. In general, information regarding use of IIVs during pregnancy is derived from post-marketing studies (for example, via registries and/or from maternal immunization studies published in the literature). The quality and quantity of data from specific sources will be evaluated by the NRA to determine whether the data are scientifically acceptable for inclusion in the pregnancy subsection of the product labelling. In some countries, the NITAG recommendations are included in this section.

As with other sections of the product labelling, country-specific requirements prescribe the information, and frequently the specific wording, to be included in the pregnancy subsection in relation to what is known about the risks of using the product in pregnancy.

WHO's prequalification evaluation of the prescribing information is evidence-based and takes into consideration the prescribing information approved by the NRA of record for prequalification (generally the NRA in the country of manufacture).

Required statements included in the pregnancy subsection of the product labelling have often been precautionary (for example, Should be used only following advice of a health-care professional; If pregnant, please inform your doctor or pharmacist; Use only if clearly needed). The rationale for requiring such language has largely stemmed from a lack of data from well-controlled clinical trials rather than evidence suggesting specific risks of vaccination during pregnancy. Such precautionary language has sometimes been misinterpreted to mean that pregnancy is a contraindication for use.

Whereas many NRAs require that labelling includes such precautionary language regarding use in pregnancy, some countries are considering ways to improve the clarity of the information included in the pregnancy subsection of the product labelling. For example, the United States Food and Drug Administration recently revised its labelling regulations so that they no longer require such precautionary language (18, 20). With the implementation of the Pregnancy and Lactation Labeling Rule in the United States of America in June 2015 (18), the revised regulations now require that the pregnancy subsection of product labelling includes narrative summaries of the risks of a product during pregnancy and discussions of the data supporting those summaries. Under the revised regulations, labelling will include relevant available clinical information arising from the use of the product in pregnant and lactating women, as well as relevant available animal and pharmacological data, to help inform prescribing decisions and the counselling of women on the use of the product during pregnancy and lactation.
6. Summary

IIVs are not contraindicated for use in pregnancy. The “Indications and Usage” section for these vaccines specifies an age range that includes women of childbearing age. Consequently, the lack of an “Indications and Usage” statement specifically addressing use in pregnant women does not preclude use of these vaccines during pregnancy. Certain countries include information on the use of IIV in pregnancy under the “Warnings and Precautions” or “Contraindications” sections of product labelling. However, this does not reflect a known or suspected safety issue relating to the use of these vaccines during pregnancy but rather a precautionary approach taken by certain NRAs. Programmatic recommendations (such as those from SAGE and some NITAGs) on the use of IIVs during pregnancy are consistent with labelling.

Authors and acknowledgements

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References


Annex 9

Guidelines on clinical evaluation of vaccines: regulatory expectations

Replacement of Annex 1 of WHO Technical Report Series, No. 924

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Guidelines published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these WHO Guidelines are made only on condition that such modifications ensure that a vaccine is at least as safe and efficacious as one evaluated in accordance with the guidance set out below.
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AE</td>
<td>adverse event</td>
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<tr>
<td>AEFI</td>
<td>adverse event following immunization</td>
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<tr>
<td>AESI</td>
<td>adverse event of special interest</td>
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<td>AR</td>
<td>attack rate</td>
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<tr>
<td>ARU</td>
<td>attack rate in unvaccinated (control group)</td>
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<td>ARV</td>
<td>attack rate in vaccinated group</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>GCP</td>
<td>good clinical practice</td>
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<td>GMC</td>
<td>geometric mean concentration</td>
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<tr>
<td>GMP</td>
<td>good manufacturing practice</td>
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<tr>
<td>GMT</td>
<td>geometric mean titre</td>
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<tr>
<td>HPV</td>
<td>human papillomavirus</td>
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<tr>
<td>ICH</td>
<td>International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use</td>
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<tr>
<td>ICP</td>
<td>immune correlate of protection</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>LLOD</td>
<td>lower limit of detection</td>
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<tr>
<td>LLOQ</td>
<td>lower limit of quantification</td>
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<tr>
<td>NRA</td>
<td>national regulatory authority</td>
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<tr>
<td>OPA</td>
<td>opsonophagocytic antibody</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RR</td>
<td>relative risk</td>
</tr>
<tr>
<td>SAE</td>
<td>serious adverse event</td>
</tr>
<tr>
<td>SBA</td>
<td>serum bactericidal antibody</td>
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</tbody>
</table>
1. Introduction

These WHO Guidelines are intended to replace the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations, which were adopted by the Expert Committee on Biological Standardization in 2001 (1). The document of 2001 provided guidance on the clinical evaluation of vaccines as well as on WHO vaccine prequalification.

Since 2001, more than 20 vaccine-specific documents (each including a section on clinical evaluation) have been adopted by the Committee. Originally intended to be read in conjunction with the 2001 document, these documents provide guidance on both oral and inactivated polio vaccines, whole cell pertussis and acellular pertussis vaccines, meningococcal conjugate vaccines for serotypes A and C, and pneumococcal conjugate vaccines, as well as on vaccines intended to prevent diseases caused by rotaviruses, dengue viruses, human papillomaviruses (HPVs) and malaria parasites.

These revised WHO Guidelines have been prepared to reflect the scientific and regulatory experience that has been gained from vaccine clinical development programmes since the adoption of the 2001 version. They are intended for use by national regulatory authorities (NRAs), companies developing and holding licences for vaccines, clinical researchers and investigators. The document takes into account the content of clinical development programmes, clinical trial designs, the interpretation of trial results and post-licensing activities.

The main content changes (modification or expansion of previous text and additional issues covered) include, but are not limited to, the following:

**Immunogenicity**

- general principles for comparative immunogenicity studies, including selection of the comparators, end-points and acceptance criteria for concluding non-inferiority or superiority of immune responses;
- situations in which age de-escalation studies are not necessary;
- assessment of the need for and timing of post-primary doses;
- use of different vaccines for priming and boosting;
- assessment of the ability of vaccines to elicit immune memory or to cause hyporesponsiveness;
- use of immunogenicity data to predict vaccine efficacy, with or without bridging to efficacy data;
- the derivation and uses of immune correlates of protection (ICPs);
- vaccination of pregnant women to protect them and/or their infants.
Efficacy and effectiveness

- the need for, and feasibility of, conducting vaccine efficacy studies;
- selection of appropriate control groups in different circumstances;
- comparison of new and licensed vaccines containing antigens from different numbers of types or subtypes of the same organism;
- prediction of vaccine efficacy when there is no ICP and vaccine efficacy studies are not feasible;
- preliminary and pivotal vaccine efficacy studies and their design;
- vaccines with modest efficacy and/or that provide a short duration of protection;
- extrapolation of data between geographically or genetically diverse populations;
- the role and potential value of human challenge studies;
- the role of sponsors and public health authorities in generating vaccine-effectiveness data.

Safety

- detailed consideration of the collection and analysis of safety data from clinical trials;
- consideration of size of the pre-licensure database by type of vaccine and its novelty;
- consideration of the safety database by population subgroup;
- special safety considerations by vaccine construct;
- circumstances of limited pre-licensure safety data;
- use of registries;
- issues regarding vaccine pharmacovigilance activities.

Because a separate document on the nonclinical evaluation of vaccines was established in 2003 (2), the corresponding section in the 2001 Guidelines has been removed. Furthermore, the structure of the document has changed, with a number of methodological considerations now incorporated into the relevant sections and subsections rather than being described in a separate section. In line with all the changes made in the document, the terminology and references have been updated.

WHO has also made available several guidelines, manuals and reports relevant to vaccine clinical development programmes. These should be consulted as appropriate, and include:
Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (3);

WHO good manufacturing practices for pharmaceutical products: main principles (4);

WHO good manufacturing practices for biological products (5);

Guidelines on nonclinical evaluation of vaccines (2);

Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines (6);

Guidelines on procedures and data requirements for changes to approved vaccines (7);

Guidelines for independent lot release of vaccines by regulatory authorities (8);

Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (9);

Clinical considerations for evaluation of vaccines for prequalification (10);

The WHO manual Immunization in practice: a practical guide for health staff (11);

Expert consultation on the use of placebos in vaccine trials (12).

Furthermore, guidance on various aspects of pre-licensure clinical development programmes for vaccines and on post-licensure assessment is also available from several other bodies, such as the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), the European Medicines Agency (EMA), the United States Food and Drug Administration and the United Kingdom Medical Research Council. These WHO Guidelines are intended to complement these other documents.

2. Purpose and scope

These WHO Guidelines consider clinical development programmes for vaccines that are intended to prevent clinical disease in humans by eliciting protective immune responses. The protective immune response to vaccination may be directed against one or more specific antigenic components of microorganisms or against substances produced and secreted by them (for example, toxins) that are responsible for clinical disease. The clinical disease prevented by vaccination
may be an acute infectious disease and/or a disease that results from chronic infection with an infectious agent.

These Guidelines are applicable to the clinical development of:

- new candidate vaccines;
- licensed vaccines;
- vaccines that are given by any route of administration;
- vaccines that may be given before exposure or shortly after known or presumed exposure to an infectious agent to prevent the onset of clinical disease.

The Guidelines are further applicable to vaccines that contain one or more of the following:

- microorganisms that have been inactivated by chemical and/or physical means;
- live microorganisms that are not virulent in humans as a result of attenuation processes or specific genetic modification;
- antigenic substances that have been derived from microorganisms (these may be purified from microorganisms and used in their natural state, or they may be modified, for example, detoxified by chemical or physical means, aggregated or polymerized);
- antigens that have been manufactured by synthetic processes or produced by live organisms using recombinant RNA or DNA technology;
- antigens (however manufactured) that have been chemically conjugated to a carrier molecule to modify the interaction of the antigen with the host immune system;
- antigens that are expressed by another microorganism which itself does not cause clinical disease but acts as a live vector (for example, live viral vectored vaccines and live-attenuated chimeric vaccines).

In addition, although naked DNA vaccines are not specifically discussed, the principles and development programmes outlined are broadly applicable. These Guidelines do not apply to:

- therapeutic vaccines (that is, those intended for treatment of disease);
- vaccines intended for any purpose other than the prevention of clinical disease caused by infectious agents.
3. Terminology

The definitions given below apply to the terms as used in these WHO Guidelines. These terms may have different meanings in other contexts.

**Adverse event (AE):** any untoward medical occurrence in a participant in a clinical trial. An AE does not necessarily have a causal relationship with the vaccine.

**Adverse event following immunization (AEFI):** any untoward medical occurrence that follows immunization and which does not necessarily have a causal relationship with the use of the vaccine. The AEFI may be any unfavourable or unintended sign, abnormal laboratory finding, symptom or disease. In clinical trial documentation AEFI may often be shortened to AE.

**Adverse event of special interest (AESI):** a clinically important untoward medical occurrence that is either known to occur following administration of the type of vaccine under study (for example, hypotonic-hyporesponsive episodes or febrile convulsions) or is considered to be a possible risk on the basis of knowledge of the content of the vaccine and/or its interaction with the host immune system (for example, autoimmune disease or antibody-dependent enhanced clinical disease).

**Attack rate (AR):** the proportion of the population exposed to an infectious agent that goes on to develop clinically manifest disease.

**Blinding:** a procedure by which one or more parties involved in a clinical trial are kept unaware of the randomized intervention.

**Booster dose:** a dose that is given at a certain interval after completion of the primary series that is intended to boost immunity to, and therefore prolong protection against, the disease that is to be prevented.

**Case ascertainment:** the method adopted for detecting cases of the disease targeted for prevention by vaccination in a vaccine efficacy trial or in a study of vaccine effectiveness.

**Case definition:** the predefined clinical and/or laboratory criteria that must be fulfilled to confirm a case of a clinically manifest disease in a vaccine efficacy trial or in a study of vaccine effectiveness.

**Cluster randomization:** randomization of subjects by group (for example, by household or by community) as opposed to randomization of individual subjects within a clinical trial.

**Geometric mean concentration (GMC):** the average antibody concentration for a group of subjects calculated by multiplying all values and taking the nth root of this number, where n is the number of subjects with available data.

**Geometric mean titre (GMT):** the average antibody titre for a group of subjects calculated by multiplying all values and taking the nth root of this number, where n is the number of subjects with available data.
**Good clinical practice (GCP):** GCP is a process that incorporates established ethical and scientific quality standards for the design, conduct, recording and reporting of clinical research that involves the participation of human subjects. Compliance with GCP provides public assurance that the rights, safety and well-being of research subjects are protected and respected, consistent with the principles enunciated in the Declaration of Helsinki and other internationally recognized ethical guidelines, and also ensures the integrity of clinical research data.

**Good manufacturing practice (GMP):** GMP is the aspect of quality assurance that ensures that medicinal products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the product specification.

**Immune correlate of protection (ICP):** an ICP is most commonly defined as a type and amount of immunological response that correlates with vaccine-induced protection against an infectious disease and that is considered predictive of clinical efficacy (13).

**Immune memory:** an immunological phenomenon in which the primary contact between the host immune system and an antigen results in a T-cell-dependent immune response, often referred to as priming of the immune system. Effective priming results in the development of antigen-specific memory B-cells and an anamnestic (memory) immune response to post-primary doses, which are commonly referred to as booster doses.

**Immunogenicity:** the capacity of a vaccine to elicit a measurable immune response.

**New candidate vaccine:** a new candidate vaccine is a vaccine that is regarded in national regulations as separate and distinct from other candidate and licensed vaccines. Examples of new candidate vaccines include but are not limited to:

- a vaccine that contains a new antigenic component (that is, one not previously used in a licensed vaccine);
- a vaccine that contains a new adjuvant;
- a vaccine that contains antigen(s) ± adjuvant(s) not previously combined together in a vaccine;
- a vaccine with the same antigenic component(s) ± adjuvant as a licensed vaccine that is produced by a different manufacturer (including situations in which seed lots or bulk antigenic components used to make a licensed vaccine are supplied to other manufacturers for their own vaccine production).

**Non-inferiority trial:** non-inferiority trials aim to demonstrate that the test intervention is not worse than the reference intervention by more than
a small pre-specified amount known as the non-inferiority margin. In non-
inferiority trials it is assumed that the reference intervention has been established
to have a significant clinical effect (against placebo).

**Pharmacovigilance**: pharmacovigilance encompasses the science and
activities relating to the detection, assessment, understanding and prevention of
adverse effects or any other possible drug-related problems (14).

**Pivotal trials**: pivotal clinical trials provide the major evidence in
support of licensure.

**Posology**: the vaccine posology for a specific route of administration
and target population includes:

- the dose content and volume delivered per dose;
- the dose regimen (that is, the number of doses to be given in the
  primary series and, if applicable, after the primary series);
- the dose schedule (that is, the dose intervals to be adhered to
  within the primary series and between the primary series and any
  further doses).

**Post-licensure safety surveillance**: a system for monitoring AEFIs in the
post-licensure period.

**Post-primary doses**: additional doses of vaccine given after a time
interval following the primary series of vaccination.

**Preliminary trial**: a clinical trial that is not intended to serve as a pivotal
trial. Preliminary trials are usually conducted to obtain information on the
safety and immunogenicity of candidate vaccine formulations and to select the
formulation(s) and regimen(s) for evaluation in pivotal trials. Preliminary trials
may also serve to inform the design of pivotal trials (for example, by identifying
the most appropriate populations and end-points for further study). On occasion,
a preliminary trial may provide an initial evaluation of vaccine efficacy.

**Primary vaccination**: the first vaccination or the initial series of
vaccinations intended to establish clinical protection.

**Protocol**: a document that states the background, rationale and objectives
of the clinical trial and describes its design, methodology and organization,
including statistical considerations and the conditions under which it is to
be performed and managed. The protocol should be signed and dated by the
investigator, the institution involved and the sponsor.

**Randomization**: in its simplest form, randomization is a process by
which \( n \) individuals are assigned to test \( (n_T) \) or control \( (n_C) \) treatment(s) so
that all possible groups of size \( n = n_T + n_C \) have equal probability of occurring.
Thus, randomization avoids systematic bias in the assignment of treatment.

**Responder**: a trial subject who develops an immune response (humoral
or cellular) that meets or exceeds a predefined threshold value using a specific
assay. This term may be applied whether or not there is an established ICP and when the clinical relevance of achieving or exceeding the predefined response is unknown.

**Responder rate:** the responder rate is the percentage of subjects in a treatment group with immune responses that meet (or exceed) a predefined immune response.

**Serious adverse event (SAE):** an AE is serious when it results in: (a) death, admission to hospital, prolongation of a hospital stay, persistent or significant disability or incapacity; (b) is otherwise life-threatening; or (c) results in a congenital abnormality or birth defect. Some NRAs may have additional or alternative criteria for defining SAEs.

**Seroconversion:** a predefined increase in serum antibody concentration or titre. In subjects with no detectable antibody – below the lower limit of detection (LLOD) – or no quantifiable antibody – below the lower limit of quantification (LLOQ) – prior to vaccination, seroconversion is usually defined as achieving a quantifiable antibody level post-vaccination. In subjects with quantifiable antibody prior to vaccination, seroconversion is commonly defined by a predefined fold-increase from pre- to post-vaccination.

**Sponsor:** the individual, company, institution or organization that takes responsibility for the initiation, management and conduct of a clinical trial. The sponsor of a clinical trial may not be the entity that applies for a licence to place the same product on the market or the entity that holds the licence (that is, is responsible for post-licensing safety reporting) in any one jurisdiction.

**Superiority trial:** a trial with the primary objective of demonstrating that a test group is superior to a reference group on the basis of the primary end-point. In the context of vaccine development the primary end-point may be a safety parameter (for example, occurrence of a specific type of AE), a clinical condition (for example, occurrence of a specific infectious disease) or an immunological parameter (for example, a measure of the immune response to one or more antigenic components of the vaccine).

**Vaccine efficacy:** vaccine efficacy measures direct protection (that is, protection induced by vaccination in the vaccinated population sample). Vaccine efficacy is most commonly a measure of the proportionate reduction in disease attack rate (AR) between the control group that did not receive vaccination against the infectious disease under study (ARU) and the vaccinated (ARV) group(s). Vaccine efficacy can be calculated from the relative risk (RR) of disease among the vaccinated group as \( (\text{ARU} - \text{ARV}/\text{ARU}) \times 100 \) and \( (1 - \text{RR}) \times 100 \). This estimate may be referred to as absolute vaccine efficacy. Alternatively, vaccine efficacy may be defined as a measure of the proportionate reduction in disease AR between a control group that is vaccinated against the infectious disease under study and the group vaccinated with the candidate vaccine. This estimate may be referred to as relative vaccine efficacy.
Vaccine effectiveness: vaccine effectiveness is an estimate of the protection conferred by vaccination. It is usually obtained by monitoring the disease to be prevented by the vaccine during routine use in a specific population. Vaccine effectiveness measures both direct and indirect protection (that is, the estimate may in part reflect protection of unvaccinated people secondary to the effect of use of the vaccine in the vaccinated population).

Vaccine vector: a vaccine vector is a genetically engineered microorganism (which may be replication competent or incompetent) that expresses one or more foreign antigen(s) (for example, antigens derived from a different microorganism).

4. Vaccine clinical development programmes

4.1 General considerations

4.1.1 Consultation with national regulatory authorities

It is strongly recommended that dialogue with the appropriate NRAs occurs at regular intervals during the pre-licensure clinical development programme to allow for agreement to be reached on the content and extent of the application dossier. This is especially important in the following cases:

- The clinical programme proposes a novel approach to any aspect of development for which there is no precedent or guidance available.
- The proposed programme conflicts with existing guidance to which the NRAs involved would usually refer when considering programme suitability.
- Particular difficulties are foreseen in providing evidence to support an expectation of vaccine efficacy (that is, there is no ICP and a vaccine efficacy study is not feasible).
- There are other special considerations for the total content of the pre-licensure programme (for example, when different vaccine constructs are to be used for priming and boosting).

Appropriate NRAs should also be consulted when planning clinical trials that are intended to support a revision of the prescribing information. In addition, changes to the manufacturing process of a vaccine before or after licensure should be discussed with NRAs to establish whether or not clinical trials are required. When issues of vaccine safety or effectiveness arise in the post-licensure period, consultation with NRAs is essential to determine any actions that are needed.
4.1.2 Use of independent monitoring committees

The members of an independent monitoring committee should not include persons who are employed by the sponsor of the clinical trial. The responsibilities of an independent monitoring committee may include one or more of the following:

- ongoing review of safety data;
- oversight of planned interim analyses of safety and/or efficacy, and recommending to the sponsor that a trial is terminated early in accordance with predefined stopping rules;
- determination of the eligibility of individual subjects for inclusion in the primary analysis population or other analysis population(s), as defined in the protocol;
- adjudication to determine whether cases of clinically apparent infections meet the predefined case definition for inclusion in analyses of efficacy;
- adjudication to determine whether reports of AEs meet the criteria for specified types of AEs and AESIs and/or to determine causality.

The same or different independent monitoring committees may be appointed to oversee one or more aspects of a clinical trial. Depending on their role(s), independent monitoring committees may be referred to by specific terms (for example, Data Monitoring Committee, Safety Data Monitoring Committee and Independent Data Adjudication Committee).

4.1.3 Registering and reporting clinical trials

Before any clinical trial is initiated (that is, before the first subject receives the first medical intervention in the trial) the details of the trial must be registered in a clinical trial registry so that the information is publicly available, free to access and can be searched. The registry should comply with individual NRA requirements and, as a minimum, should comply with the WHO internationally agreed standards.

The entry into the clinical trial registry site should be updated as necessary to include final enrolment numbers achieved and the date of actual study completion. A definition of study completion for this purpose should be included in the protocol. For example, this may be defined as the point in time when data analyses have been completed to address the major study objectives. If a clinical trial is terminated prematurely the entry should be updated to reflect this with a report of the numbers enrolled up to the point of termination.
The key outcomes of a clinical trial should be posted in the results section of the entry in the clinical trial registry and/or posted on a publicly available, free to access and searchable website (for example, that of the trial sponsor or principal investigator). It is recommended that posting of these results should usually occur within 12 months of completion or termination of the study, or in accordance with the relevant NRA requirements.

Depending on individual NRA requirements, some or all regulatory submissions may need to include a listing of all completed and ongoing trials conducted with the vaccine by the sponsor. It is recommended that any trials that are known to the sponsor (for example, from searching registries or from publications) that were initiated by entities other than the sponsor (for example, by a public health body, academic institution or another company that used the product as a comparator) should be included.

4.2 Pre-licensure clinical development programmes
The main objective of the pre-licensure clinical development programme is to accumulate adequate data to support licensure. The main elements of the programme are:

- to describe the interaction between the vaccine and the host immune response (see section 5 below);
- to identify safe and effective dose regimens and schedules (see sections 5 and 6);
- to estimate vaccine efficacy by directly measuring efficacy and/or to provide evidence of vaccine efficacy based on immune responses (see sections 5 and 6);
- to describe the safety profile (see section 7);
- to assess co-administration with other vaccines if this is relevant (see section 5.6.3).

Consideration of the content of pre-licensure clinical development programmes is undertaken on a product-specific basis. Requirements may differ depending on the type of vaccine, its manufacturing process, its mechanism of action, the disease to be prevented and the target population.

4.2.1 Preliminary trials
The clinical programme for new candidate vaccines usually commences with an exploration of the safety of different amounts of the antigen(s) in each dose of candidate vaccine formulations, with or without an adjuvant. It is usual that immune responses to the antigenic components are also explored. These are commonly referred to as Phase I trials. In most cases the first clinical trials
are conducted in healthy adults. It may be appropriate, if feasible, that the first trials are confined to subjects who have no history of previous exposure to the organism(s) against which the candidate vaccine is intended to protect.

Further safety and immunogenicity trials that are conducted to build on the Phase I trial results are commonly referred to as Phase II trials. In most cases these trials are conducted in subjects who are representative of the intended target population for the vaccine at the time of licensure. For vaccines intended for a broad age range it may not be necessary in all instances to apply an age de-escalation approach (for example, to move from adults to adolescents, then to children aged 6–12 years, followed by younger children, toddlers and finally infants) to sequential trials or to groups within trials. For example, if a vaccine has negligible potential benefit for older children it may be acceptable in some cases to proceed directly from trials in adults to trials in younger children, including infants and toddlers.

These trials are usually designed to provide sufficient safety and immunogenicity data to support the selection of one or more candidate formulations for evaluation in pivotal trials (that is, to select the amount(s) of antigenic component(s) and, where applicable, adjuvant in each dose).

### 4.2.2 Pivotal trials

Pivotal trials are intended to provide robust clinical evidence in support of licensure. They are commonly referred to as Phase III trials. There may be exceptional cases in which licensure is based on a Phase II trial that has been designed to provide robust statistical conclusions. It is usual that the investigational formulations used in pivotal trials are manufactured using validated processes and undergo lot release in the same way as intended for the commercial product.

Pivotal trials may be designed to provide an estimate of vaccine efficacy or to provide an indication of the ability of the vaccine to prevent clinical disease on the basis of immunogenicity data (see section 6.1 below). On occasion, an assessment of a specific safety aspect may be the primary (or a co-primary) objective in a pivotal trial (see section 7.2.1 below).

### 4.3 Post-licensure clinical evaluations

After licensure:

- It is essential to monitor vaccine safety in routine use (see section 7 below).
- Studies designed to address specific safety issues that were identified as potential concerns from pre-licensure trials may need to be conducted.
It may be appropriate to conduct studies specifically intended to estimate vaccine effectiveness (see section 6.4 below).

Sponsors may choose to conduct additional trials that are intended to extend or to otherwise modify the use of the vaccine through revision of the prescribing information. In some jurisdictions, conducting one or more trials after licensure to address specific issues may be a formal requirement.

5. Immunogenicity

5.1 General considerations

Immunogenicity trials are conducted at all stages of pre-licensure vaccine development and additional trials may be conducted in the post-licensure period. The evaluation of immune responses relies upon the collection of adequate specimens at appropriate time intervals and the measurement of immune parameters most relevant to the vaccine.

Pre-licensure and post-licensure clinical trials commonly evaluate and compare immune responses between trial groups to address a range of objectives. In trials that are primarily intended to estimate vaccine efficacy and/or safety, assessment of the immune response is usually a secondary objective but it is important that data on immune responses are collected to support analyses of the relationship between immunogenicity and efficacy, which may lead to the identification of ICPs.

5.2 Characterization of the immune response

The appropriate range of investigations to be conducted should be discussed with NRAs. As a general rule, for vaccines that contain microorganisms and antigens that have not been used previously in human vaccines a thorough investigation of their interaction with the human immune system should usually be conducted as part of the overall clinical development programme. For microorganisms and antigens that are already in licensed vaccines, it is not usually necessary to repeat these types of investigations but consideration should be given to conducting at least some trials in certain circumstances (for example, when a new adjuvant is to be added to known antigens, a different method of attenuation is used, a different carrier protein is used for antigen conjugation or an antigen previously obtained by purification from cultures is to be manufactured using recombinant technology).

In general the clinical development programme should include a description of the magnitude of the immune response, including an assessment of functional antibody (for example, antibody that neutralizes viruses or toxins, or antibody that mediates bactericidal activity or opsonophagocytosis) if this can
be measured. Decisions on the range of additional investigations that may be appropriate should take into account what is known about the immune response resulting from natural exposure and whether or not this provides partial or complete protection and, if so, whether it is temporary or lifelong. The range of investigations chosen should also reflect the characteristics of the infecting microorganism (for example, whether there are multiple subtypes that cause human disease) and the content of the vaccine (15).

On a case-by-case basis, other investigations of the immune response could possibly include some of the following:

- assessment of the ability of the vaccine to elicit a T-cell-dependent primary immune response, with induction of immune memory (that is, priming of the immune system) giving rise to anamnestic responses to: (a) natural exposure following vaccination; (b) further doses of the same vaccine; and/or (c) further doses of a vaccine that contains closely related but non-identical microorganisms or antigens (that is, cross-priming);
- assessment of the specificity and cross-reactivity of the immune response;
- assessment of changes in antibody avidity with sequential doses, which may be useful when investigating priming;
- evaluation of factors that could influence the immune responses, such as the effect of maternal antibody on the infant immune response to some antigens, pre-existing immunity to the same or very similar organisms, and natural or vaccine-elicited antibody against a live viral vector.

5.3 Measuring the immune response

5.3.1 Collection of specimens

Immune responses to vaccination are routinely measured in serum (humoral immune responses) and blood (cellular immune responses). For some vaccines it may be of interest to explore immune responses in other body fluids relevant to the site at which the target microorganism infects and/or replicates (for example, in nasal washes or cervical mucus), especially if it is known or suspected that the systemic immune response does not show a strong correlation with protective efficacy for the type of vaccine under trial (for example, intranasal vaccination against influenza). Nevertheless, specimens other than sera have not to date provided data that have been pivotal in regulatory decision-making processes and have not resulted in the identification of ICPs. Therefore the rest of this section focuses on the collection of blood samples.
Pre-vaccination samples should be collected from all subjects in early preliminary immunogenicity trials, after which it may be justifiable to omit these samples or to obtain them from subsets (for example, if antibody is rarely detectable or quantifiable prior to vaccination in the target population). Pre-vaccination sampling remains essential if it is expected that the target population will have some degree of pre-existing immunity due to natural exposure and/or vaccination history, since the assessment of the immune response will need to take into account seroconversion rates and increments in geometric mean titres (GMTs) or geometric mean concentrations (GMCs) from pre- to post-vaccination. Pre-vaccination sampling is also necessary if it is known or suspected that pre-existing immune status may have an impact on the magnitude of the immune response to vaccination that is positive (for example, because pre-existing antibody reflects past priming) or negative (for example, due to maternal antibody interfering with primary vaccination with certain antigens in infants).

The timing of post-vaccination sampling should be based on what is already known about the peak immune response after the first and, if applicable, sequential doses (for example, for vaccines that elicit priming, the rise in antibody after a booster dose is usually much more rapid than the rise after earlier doses). For antigens not previously used in human vaccines, sampling times may be based on nonclinical data and then adjusted when data that are specific to the antigen(s) under trial have been generated. As information is accumulated, the number and volume of samples taken from individual subjects may be reduced to the minimum considered necessary to meet the trial objectives.

5.3.2 Immunological parameters

Immunological parameters are measures that describe the humoral immune response (for example, antibody concentrations or antibody titres, depending on the assay output) or the cell-mediated immune response (for example, percentages of sensitized T-cells). To date, immunological parameters other than those that measure the humoral immune response have not played a pivotal or major role in vaccine licensure, so the focus is usually on determination of antibody levels.

- For known microorganisms or antigens in a candidate vaccine the range of parameters to be measured in clinical trials is usually selected on the basis of prior experience and whether or not there is an established ICP.
- For microorganisms or antigens not previously included in human vaccines the selection of parameters to be measured should take into account what is known about natural immunity. For some infectious diseases the nature of the immune response to infection in animal models may also be useful for parameter selection.
### 5.3.2.1 Humoral immune response

The humoral immune response is assessed from the post-vaccination appearance of, or increase after vaccination in, antibody directed at specific microorganisms or antigens in the vaccine.

- If data are available, most weight is usually placed on functional antibody responses – for example, serum bactericidal antibody (SBA), toxin- or virus-neutralizing antibody or opsonophagocytic antibody (OPA). In some cases an appropriate assay for functional antibody may not be available (for example, for typhoid vaccines based on the Vi polysaccharide) or the only available assay may have low feasibility for application to large numbers of samples (for example, because it is very labour-intensive or requires high-level biocontainment facilities).

- Alternatively, or in addition to the determination of functional antibody, the immune response may be assessed by measuring total antibody – for example, total immunoglobulin G (IgG) measured by enzyme-linked immunosorbent assay (ELISA) that binds to selected antigens (or, on occasion, to specific epitopes). Only a proportion of the total antibody detected may be functional.

The following should be taken into consideration when deciding how to measure the humoral immune response:

- If a correlation has already been established between total and functional antibody responses to a specific microorganism or antigen it may be acceptable to measure only total IgG in further trials (for example, antibody to tetanus toxin). However, determination of functional immune responses might be important for specific age groups or target populations where it is known or suspected that the binding and functional capacity of the antibodies elicited differs (for example, pneumococcal conjugate vaccines in older people).

- For antigens for which there is an established ICP it may suffice to measure only the relevant functional antibody (for example, SBA for meningococcal vaccines) or total IgG (for example, for antibody to tetanus toxin) response.

- If the ICP is based on total IgG there may be instances where there is still merit in measuring functional antibody (for example, for antibody to diphtheria toxin for which a microneutralization assay is available).
If there is no ICP the functional antibody response should be measured if this is feasible.

Occasionally there may be more than one immunological parameter that can measure functional antibody but one is considered to be a more definitive measure than the other (for example, neutralizing antibody to influenza virus versus antibody that inhibits haemagglutination). In this case the more definitive parameter may be determined, at least in a subset.

For some vaccines against certain viruses there is a possibility that some of the total antibody detected has no protective effect (for example, is non-neutralizing) but could enhance cellular infection by wild-type virus and result in an increased risk of severe disease after vaccination (for example, this may apply to dengue vaccines). To assess this possibility, the routine measurement of total antibody to assess the humoral immune response to vaccination should be supported by other detailed investigations.

5.3.2.2 Cell-mediated immune response

For some types of infectious disease (such as tuberculosis) assessment of the cell-mediated immune response may have a role to play in the assessment of the interaction between the vaccine and the human immune system. In other cases, evaluation of the cellular immune response may serve to support findings based on the humoral immune response (for example, when assessing the benefit of adding an adjuvant or when evaluating the degree of cross-priming elicited by a vaccine).

The cell-mediated immune response is most commonly assessed by detecting and quantifying sensitized T-cells in blood from trial subjects. These investigations may also serve to characterize the predominant cytokines released and to detect differences in sensitization between T-cell subpopulations. Several methods may be used. These are typically based on measuring the production of a range of cytokines following in vitro stimulation of T-cells with individual or pooled antigens.

The results may provide useful comparisons between treatment groups within any one study (for example, they could describe the effect, if any, of an adjuvant). If there are marked discrepancies in the patterns of responses observed between cell-mediated and humoral responses (for example, if adding an adjuvant has a major effect on antibody levels but does not increase the percentages of sensitized cells in one or more T-cell subsets) the findings should be carefully considered and discussed.
5.3.3 **Assays**

Assays of functional or total antibody that are used to report immune responses to vaccination (whether to the candidate vaccine or to co-administered vaccines) in trials intended to support licensure (that is, in pivotal trials) should be acceptable to the relevant NRAs. They may be:

- commercially available assays specifically designed and intended for quantification of antibody (that is, assays that have undergone a robust regulatory review);
- assays that are not commercially available but have been validated according to principles similar to those recommended for quantitative lot release assays in the ICH Q2 (R1) document *Validation of analytical procedures: text and methodology* (16);
- assays that are not commercially available but have been shown to be comparable to a reference assay (for example, to an assay established in a WHO reference laboratory or to an assay that is established in a recognized public health laboratory and has been used previously to support clinical trials that were pivotal for licensure).

It is expected that, if these exist, WHO International Standards and Reference Reagents will be used in assay runs. Any omission of their use should be adequately justified.

Clinical trial protocols should specify which assays will be used. Clinical trial reports should include a summary of the assay methodology and its commercial or other validation status. For assays that are not commercially available any available validation reports should be provided.

The same assays should preferably be used in the same laboratories throughout the clinical development programme (including pre- and post-licensure trials) for an individual vaccine. It is also preferable that each assay (whether it measures the response to the candidate vaccine or to a concomitant vaccine) is run by one central laboratory. If this is not possible (for example, because different laboratories have to be used, assays change over time, or a switch is made to an improved and/or more suitable assay) the new and original assays should be shown to give the same result or interpretation, or the impact of any differences should be evaluated and the use of a new assay justified. It is recommended that, as a minimum, a selection of stored sera (for example, covering a range of low to high results when using the previous assay) should be re-run using the previous and new assays in parallel. The number of sera retested should be sufficient to support a statistical assessment of assay comparability and/or reproducibility.
The microorganisms (for example, in assays of SBA, OPA and virus neutralization) and antigens (for example, in ELISAs and for in vitro stimulation of sensitized T-cells) used in the assay may affect both the result and the interpretation of the result. For example:

- It is important to use purified antigen to avoid the possibility that the assay detects and measures antibody to any extraneous antigenic substances that may be in the vaccine.
- For vaccines that contain antigens from multiple strains of the same pathogen (for example, multiple bacterial capsular types) the assays selected (whether separate or multiplex) should determine the immune response to each antigen.
- Although it is usually acceptable to conduct routine testing using the same microorganisms or antigens as those present in the vaccine, it may be very informative to perform additional testing, at least in subsets of samples, using circulating wild-type organisms or antigens derived from them in the assay. It is not expected that these additional assays will necessarily be validated since they are exploratory in nature. The results of additional testing can provide an indication as to whether the results of routine testing could represent an overestimate of the immune response to circulating strains. This additional testing can also provide an assessment of the cross-reactivity of the immune responses elicited by the vaccine to other organisms of the same genus or species (for example, to different flaviviruses, different clades of influenza virus or different HPV types) and can guide decisions on the need to replace or add strains or antigens in a vaccine to improve or maintain its protective effect.

5.4 Identification and use of immune correlates of protection

5.4.1 Immune correlates of protection and their uses

All established ICPs are based on humoral immune response parameters that measure functional or total IgG antibody. Some examples of well-established ICPs include those for antibody to diphtheria and tetanus toxoids, polioviruses, hepatitis B virus and *Haemophilus influenzae* type b capsular polysaccharide (17). In most cases established ICPs have been shown to correlate with prevention of clinically apparent infectious disease, but for some pathogens the ICP correlates with prevention of documented infection (for example, hepatitis A and hepatitis B).

Sections 5.5.2 and 5.5.3 below consider trial end-points and the approach to analysis and interpretation of immunogenicity data in the presence or absence of an ICP.
5.4.2 Establishing an ICP

Documentation of the immune response to natural infection, the duration of protection after clinically apparent infection (that is, whether natural protection is lifelong (solid immunity), temporary or absent) and the specificity of protection (that is, whether the individual is protected only against specific subtypes of a microorganism) should be taken into account when attempting to establish an ICP from clinical data. For example, to date, widely accepted clinical ICPs have been established on the basis of one or more of the following:

- serosurveillance and disease prevalence in specific populations;
- passive protection using antibody derived from immune humans or manufactured using recombinant technology;
- efficacy trials;
- effectiveness trials;
- investigation of vaccine failure in immunosuppressed populations.

In the majority of cases clinical ICPs have been determined from vaccine efficacy trials that were initiated pre-licensure, often with long-term follow-up of subjects that extended into the post-licensure period. Efficacy trial protocols should plan to collect sufficient information to allow for analyses of the relationship between immune parameters and protection against clinically apparent disease. At the minimum this requires the collection of post-vaccination samples from all, or from a substantial subset of, the vaccinated and control groups. Serial collection of samples over the longer term, along with follow-up surveillance for vaccine breakthrough cases, has also served to support identification of ICPs.

To investigate the predictive capacity of a putative ICP, protocols should predefine the assessments to be applied to all cases of the disease to be prevented that occur in the vaccinated and control groups. These assessments should include investigation of the immune status of subjects as well as microbiological studies with the infecting microorganisms whenever these have been recovered. For breakthrough cases from which both post-vaccination sera and organisms have been recovered it is recommended that, whenever feasible, functional antibody (or, if not possible, total antibody) should be determined for individuals against their own pathogen. An exploration of vaccine-elicited cell-mediated responses in individuals against their own pathogen may also be useful and, for some types of infectious disease (such as tuberculosis), may be very important for further understanding vaccine-associated protection. These data may be very important for investigating the broad applicability of the ICP, depending on host and organism factors.

A single clinical ICP identified from a vaccine efficacy trial in a defined population may not necessarily be applicable to other vaccine constructs.
intended to prevent the same infectious disease. In addition, an ICP may not be applicable to other populations and disease settings. For example, putative ICPs have sometimes differed between populations of different ethnicities with variable natural exposure histories for subtypes of a single microorganism. Thus, the reliance that is placed on a clinical ICP, even if regarded as well supported by the evidence, should take into account details of the efficacy trials from which it was derived.

Clinical ICPs have also been derived from, or further supported by, data collected during use of a vaccine to control an outbreak and from analyses of effectiveness data. The methods used to derive ICPs from these types of data have been very variable. The estimates may in part reflect the type of immunization programme put in place and the extent to which the protection of individuals relies on herd immunity rather than the initial and persisting immune response in the individual. Therefore the wider applicability of ICPs derived from interventional or routine use should be viewed in the light of how and in what setting the estimates were obtained.

If it is not possible to derive a clinical ICP the interpretation of the human immune response data may take into account what is known about immunological parameters that correlate with protection in relevant animal models and any nonclinical ICPs that have been identified (for example, from trials that assess passive protection and active immunization). This approach may be the only option available for interpreting immune responses to some new candidate vaccines. Nevertheless, ICPs derived wholly from nonclinical data should be viewed with caution and attempts should be made to obtain a clinical ICP whenever the opportunity arises (for example, when the vaccine is used in the context of an outbreak).

If conducted, human challenge trials may also provide preliminary evidence supporting an ICP. If a human challenge trial suggests a correlation between a specific immunological parameter and protection, this may be further investigated during the clinical development programme.

5.5 Immunogenicity trials

5.5.1 Objectives
The objectives of immunogenicity trials include, but are not limited to, the following:

- to select vaccine formulations and posologies (including primary and booster doses) (see section 5.6.1 below);
- to compare immune responses documented in a specific population and, using one vaccine formulation and posology, to immune responses to the same vaccine when used in other settings (for example, different populations) or with alternative posologies, or to
a different vaccine intended to protect against the same infectious disease(s) (see section 5.6.2);
- to support co-administration with other vaccines (see section 5.6.3);
- to support maternal immunization (see section 5.6.4);
- to support major changes to the manufacturing process (see section 5.6.5);
- to assess lot-to-lot consistency (7) (see section 5.6.6).

5.5.2 General considerations for trial designs

Immunogenicity trials are almost without exception comparative trials. For candidate vaccines containing antigens for which there are well-established ICPs that can be applied to interpret the results sponsors may sometimes question the value of including a comparative arm. Nevertheless, there is great value in conducting a randomized controlled trial. For example, the inclusion of a control group that receives a licensed vaccine provides assurance of the adequacy of the trial procedures and methods, including the assays, and facilitates interpretation of data in circumstances in which unexpected results (for example, low immune response to one or more antigens, high rates of specific AEs or unexpected AEs) are observed.

Comparative trials include those in which all subjects receive the same vaccine formulation but there are differences between groups in terms of how or to whom the vaccine is administered (for example, using a different dose or dose interval, or administering the vaccine to different age groups) as well as trials in which one or more group(s) receive an alternative treatment, which may be placebo and/or another licensed vaccine.

The design of comparative immunogenicity trials is driven by the characteristics of the vaccine, the trial objectives, the stage of clinical development, the trial population, the availability and acceptability of suitable comparators, and what is known about immune parameters that correlate with protection (including whether or not there is an established ICP).

In comparative immunogenicity trials, subjects should be randomized to one of the trial groups at enrolment. This also applies to trials that enrol sequential cohorts of subjects (as in ascending dose trials in which at least some subjects are assigned to receive placebo or another vaccine). In some cases it may be appropriate that subjects who meet certain criteria (for example, completed all assigned doses in the initial part of the trial) are re-randomized at a later stage of the trial to receive a further dose of a test or control treatment.

In all comparative trials the assays should be performed by laboratory staff unaware of the treatment assignment. Whenever possible, comparative immunogenicity trials should be of double-blind design. If the vaccines to be compared are visually distinguishable it is preferable that designated individuals
at each trial site who are not otherwise involved in the trial should administer
the products. If this is not feasible, or if the vaccines to be compared are
given by different routes or according to different schedules, attempts should
be made to maintain blinding of the trial site staff conducting the study visits
and assessments.

In trials intended to provide only descriptive analyses of the
immunogenicity data the trial sample size is usually based on considerations
of feasibility and collection of sufficient safety data to support the design of
sequential trials. Trials that aim to assess superiority or non-inferiority between
vaccine groups should be sized according to the intended power and the
predefined margins. It is recommended that protocols and statistical analysis
plans for each trial should be developed in conjunction with an appropriately
experienced statistician.

5.5.2.1 End-points

The choice of the primary trial end-point and the range of other end-points for
immunogenicity trials should take into account sections 5.2, 5.3 and 5.4 above.
Protocols should predefine the primary, co-primary, secondary and any other
end-points (which may be designated tertiary or exploratory). Co-primary end-
points may be appropriate in some cases, namely:

- The vaccine is intended to protect against multiple subtypes of the
  same microorganism (for example, HPV vaccines or pneumococcal
  conjugate vaccines).
- The vaccine contains multiple microorganisms (such as measles,
  mumps and rubella vaccine) or multiple antigens (such as
  combination vaccines used for the primary immunization series
  in infants).

The following should be taken into consideration when selecting the
primary end-point(s) following primary vaccination:

- When an ICP has been established the primary end-point is usually
  the percentage of subjects that achieves an antibody level at or above
  the ICP, which is sometimes referred to as the seroprotection rate.
- When there is no established ICP the primary end-point or the
  co-primary end-points is/are usually based on a measure of the
  humoral immune response.

  (a) In some instances there may be evidence to support the
      application of a threshold value (that is, the primary end-point
      may be the percentage of subjects that achieves antibody levels
      at or above the threshold value).
(b) If there is no threshold value that can be applied it may be appropriate to base the primary end-point on the seroconversion rate or on some other definition of the magnitude of the immune response that differentiates responders from non-responders. Comparisons of post-vaccination seropositivity rates may also be informative if pre-vaccination rates are very low.

An anamnestic (memory) immune response is anticipated following administration of a vaccine to subjects who are already primed (by natural exposure or prior vaccination) against one or more microorganisms or antigens in the vaccine. Thus the seroprotection, seroconversion (fold-rise from pre-boost to post-boost) and seropositivity rates after the booster dose are likely to be very high. In these cases, and in other situations in which post-vaccination seroprotection and/or seroconversion rates are expected to be very high (that is, the vaccine is very immunogenic) the most sensitive immunological parameter for detecting differences between groups may be the GMC or GMT.

After primary vaccination and after any additional doses the results for all measured immunological parameters should be presented in the clinical trial report.

5.5.2.2 Trials designed to demonstrate superiority

Trials may assess whether a specific candidate vaccine formulation elicits superior immune responses compared to no vaccination against the disease to be prevented. In some cases trials may also assess whether immune responses elicited by a specific formulation of a candidate vaccine are superior to those elicited by other formulations.

An assessment of superiority may also be applicable when an adjuvant is proposed for inclusion in the vaccine (for example, to demonstrate that the immune response to at least one of the antigenic components in an adjuvanted formulation is superior to the response in the absence of the adjuvant).

Protocols should predefine the magnitude of the difference between vaccine groups or between vaccine and control groups that will be regarded as evidence of superiority. This difference should be defined in such a way that it provides some evidence of a potential clinical advantage.

5.5.2.3 Trials designed to demonstrate non-inferiority

Most comparative immunogenicity trials are intended to show that the test vaccinated groups achieve comparable immune responses to the selected reference groups. If these trials are intended to be pivotal they should be designed and powered to demonstrate non-inferiority using a predefined and justifiable non-inferiority margin.
Factors to consider with regard to the stringency of the non-inferiority margin include the clinical relevance of the end-point, seriousness of the disease to be prevented, vulnerability of the target population, availability of a well-established ICP and the performance characteristics of the assay(s). A more stringent margin may be appropriate when the vaccine is intended to prevent severe or life-threatening diseases and/or will be used in particularly vulnerable populations (for example, infants and pregnant women). A more stringent margin could also be considered when there is potential for a downward drift in immunogenicity such as that which could occur when a new candidate vaccine can be compared only with vaccines that were approved on the basis of non-inferiority trials. In contrast, if a new candidate vaccine is known to offer substantial benefits in terms of safety or improved coverage then margins that are less stringent may be considered. As a result of such considerations it is possible that different non-inferiority margins may be considered appropriate in different settings.

When it is proposed to demonstrate non-inferiority between vaccine groups based on GMT or GMC ratios for antibody titres or concentrations it is suggested that the lower bound of the 95% confidence interval around the ratio (test versus reference vaccine) should not fall below 0.67. Under certain circumstances NRAs may consider allowing a lower bound (for example, 0.5) or alternative criteria. The selection of a criterion should take into account whether or not an ICP has been identified. In addition, any marked separations between the reverse cumulative distributions of antibody titres or concentrations should be discussed in terms of potential clinical implications, including those which occur at the lower or upper ends of the curves.

5.5.3 Analysis and interpretation

A statistical analysis plan should be finalized before closing the trial database and unblinding treatment assignments (if these were blinded). This should include any planned interim analyses, which should be adequately addressed in terms of purpose, timing and any statistical adjustments required.

The immunogenicity data from all subjects with at least one result for any immunological parameter measured in the trial should be included in the clinical trial report. The analysis of the immune response based on any one parameter is commonly restricted to all subjects with a pre-vaccination measurement (if this is to be obtained from all subjects) and at least one post-vaccination measurement. Protocols may also restrict the primary analysis population to subjects with pre- and post-vaccination results, or to those with post-vaccination results who received all the assigned doses within predefined windows of the intended schedule and had no other major protocol violations. Other analysis populations of interest may be predefined in accordance with the primary or secondary objectives (for example, age subgroups or pre-vaccination serostatus
subgroups). Whatever the predefined primary analysis population, all available immunogenicity data should be presented in the clinical trial report.

If a trial fails to meet the predefined criteria for superiority and/or non-inferiority with respect to any of the antigenic components, the possible reasons for the result and the clinical implications of it should be carefully considered before proceeding with clinical development or licensure. The considerations may take into account: (a) the basis for setting the predefined criteria (for example, does failure to meet the criteria strongly imply that lower efficacy may result?); (b) the comparisons made for all other immune parameters measured (for example, were criteria not met for only one or several of many antigenic components of the vaccine?); (c) any differences in composition between the test and comparator vaccines that could explain the result; (d) the severity of the disease(s) to be prevented; and (e) the overall anticipated benefits of the vaccine, including its safety profile (17). Section 5.6 below provides further examples and issues for consideration.

If additional analyses of the data that were not pre-specified in the protocol and/or the statistical analysis plan (that is, post hoc analyses) are conducted, they should usually be viewed with caution.

5.6 Specific uses of immunogenicity trials

5.6.1 Selection of formulation and posology

The vaccine formulation is determined by the numbers of microorganisms or amounts of antigens and, if applicable, the amount of adjuvant that is to be delivered in each dose, as well as by the route of administration.

The vaccine posology for a specific route of administration includes:

- the antigen content (as for formulation) and volume delivered per dose;
- the dose regimen (number of doses to be given in the primary series and, if applicable, after the primary series);
- the dose schedule (dose intervals within the primary series and between the primary series and any further doses).

The posology for any one vaccine may vary between target populations (for example, between age groups or according to prior vaccination history) in one or more aspects (content, regimen or schedule).

The following sections outline the immunogenicity data that are usually generated to support vaccine formulation and posology, and to assess the need for, and immune response to, additional doses of the vaccine after completion of the primary series. Section 7 below then addresses the importance of the safety profile when selecting vaccine formulations and posologies.
5.6.1.1 Selecting the formulation and posology

The vaccine formulation and posology should be supported by safety and immunogenicity data, with or without efficacy data, collected throughout the pre-licensure clinical development programme. At the time of licensure the data should at least support the formulation and posology for the primary series, which may consist of one or more doses.

Depending on the intended formulation of the new candidate vaccine, the following considerations may apply:

1. When a new candidate vaccine contains any microorganisms or antigens not previously used in human vaccines, with or without others already used in human vaccines, the preliminary trials may explore the immune responses to different amounts of each of the new microorganisms or antigens when given alone to non-immune healthy adult subjects. These trials can be used to describe the dose–response curve and may indicate a plateau for the immune responses above a certain dose level. The next trials usually evaluate immune responses to further doses at various dose intervals in order to evaluate the kinetics of the immune response and any increment in immune response achieved by further doses. The transition from trials in healthy adults to trials in subjects in the target age range at the time of licensure should occur as soon as this can be supported, taking into account the safety profile.

However, evaluating the immune response to each of the new microorganisms or antigens alone may not be a feasible undertaking. For example, if the vaccine construct is manufactured in such a way that production of individual antigens is not feasible then evaluation of the appropriate vaccine dose may be based solely on studies with the entire construct. Another example concerns vaccines intended to protect against multiple subtypes of an organism. In this case, the use of microorganisms or antigens that could be regarded as broadly representative in the first trials may provide some idea of the likely response to other subtypes. Further trials may then explore formulations that contain increasing numbers of the subtypes with the objective of assessing the effect on the immune response of combining them into a single product.

2. For new candidate vaccines that contain known antigenic components not previously combined in a single vaccine, the preliminary trials are usually conducted in subjects within the age ranges approved for licensed vaccines that contain some or all of the same antigenic components. The aim is to demonstrate non-
inferiority of immune responses to each of the intended antigenic components when combined in a candidate formulation compared with co-administration of licensed vaccines that together provide all of the same antigenic components. The same approach applies whenever the antigenic components are not combined into a single formulation but the contents of more than one product have to be mixed immediately before administration to avoid a detrimental physicochemical interaction.

3. For new candidate vaccines that contain both known and one or more new antigenic components the preliminary trials may aim to demonstrate non-inferiority of immune responses to each of the known antigenic components when combined into a candidate formulation compared with the separate administrations of known and new antigenic components. It may also be informative to include a control group that receives co-administration of the known and new antigenic components. The exact trial design will depend upon the availability of a single licensed vaccine that contains the known antigenic components and whether more than one licensed vaccine has to be given.

4. For vaccine formulations to which an adjuvant is to be added there should be adequate data already available (known adjuvants) or data should be generated (new adjuvants or when using any adjuvant with a new antigenic component) to describe the effect of the adjuvant on the immune responses. Some, or a major part, of the evidence supporting the addition of an adjuvant may come from nonclinical studies. The addition of an adjuvant, which may or may not elicit superior immune responses to one or more antigens, should not have a potentially detrimental effect on the responses to any antigenic components. Addition of an adjuvant may allow for the use of a much lower dose of an antigenic component to achieve the desired level of immune response, and it may also broaden the immune response (for example, it may result in higher immune responses to antigens closely related to those in the vaccine). Trials should evaluate a sufficient range of combinations of antigenic components and adjuvants to support the final selected formulation (that is, the ratio of adjuvant to antigenic components).

5. The total data generated should be explored to identify the criteria that should be applied to the release and stability specifications, and to the determination of an appropriate shelf-life for the vaccine. This is usually of particular importance for vaccines that contain
live microorganisms. Depending on data already generated, it may be necessary to conduct additional trials with formulations known to contain a range of microorganism numbers or antigen doses in order to identify appropriate limits at the end of the shelf-life.

6. Comparative immunogenicity trials may be needed to determine schedules that are appropriate for specific target populations, taking into account the urgency to achieve protective immunity (that is, trials based on diseases to be prevented and their epidemiology). The data generated across all the trials should determine the minimum period that should elapse between doses, as well as the effects of delaying doses to support acceptable windows around scheduled doses. Additionally, for some vaccines it may be useful to explore the shortest time frame within which doses may be completed without a detrimental effect on the final immune response (for example, for vaccines for travellers who may need to depart at short notice or for vaccines intended to provide post-exposure prophylaxis).

Assessment of the effects of dose interval and the total time taken to complete the primary series is a particular issue for vaccines intended for use in infants as there is a very wide range of schedules in use in different countries (for example, 3-dose schedules include 6–10–14 weeks and 2–4–6 months). In general, experience indicates that the magnitude of the post-primary series immune responses broadly correlates with the age of infants at the time of the final dose.

7. All data generated in accordance with points 1–6 above should be taken into account when selecting the final formulation and posology or posologies. The selection process is more straightforward if there are established ICPs that can be applied to the interpretation of the results for at least some of the antigenic components. In the absence of an ICP the posology may be selected on the basis of consideration of any plateau effects that are observed and on the safety profile of various doses and regimens.

It is not unusual for the final selected formulation and posology to represent, at least to some extent, a compromise between immunogenicity and safety or, for combination vaccines, a compromise between the potential benefits of a vaccine that can protect against multiple types of infectious disease and some negative effects on immune response that may occur. These negative effects may result from a physicochemical interaction between vaccine components and/or a negative immune interference
effect of some antigenic components. Such negative effects may be accompanied by enhanced immune responses to other vaccine components. The rationale for the final selection should be carefully discussed in the application dossier.

5.6.2 Amending or adding posologies

Clinical trials may be considered necessary to address one or more of the following situations:

- **Change to the number of doses or dose intervals** – in this case the control group could be vaccinated using the licensed posology and the trial could be conducted in a population for which the vaccine is already licensed.

- **Use of the licensed posology in a new population** (for example, in subjects who are younger or older than the currently licensed age group, or in subjects with specific underlying conditions, such as immunosuppression) – in this case the trial could compare use of the licensed posology in the new target population with use in the population for which the vaccine is already licensed.

- **Use of an alternative to the licensed posology in a new population** – in this case the trial could compare the alternative posology administered to the new population with the licensed posology in the population for which the vaccine is already licensed.

- **Support for alternative routes of administration for the licensed formulation** (for example, adding subcutaneous or intradermal injection to intramuscular use).

Post-licensure clinical trials may also be conducted to support changes in formulation. Formulation changes other than adding or removing a preservative or removing thiomersal from the manufacturing process may or may not result in a modified product that is considered to be a new candidate vaccine from a regulatory standpoint (that is, would require a new application dossier and adequate trials to support separate licensure).

5.6.3 Post-primary doses

5.6.3.1 Need for post-primary doses

The need to administer additional doses, and the timing of these doses, may be determined before and/or after first licensure.

There may be experience with other similar vaccines indicating that additional doses of a new candidate vaccine will be needed after completion of the primary series (for example, after infant immunization with *H. influenzae*...
type b and Neisseria meningitidis group C vaccines). In such cases the clinical development programme should usually incorporate an assessment of immune responses to a post-primary dose.

If it is not known whether post-primary doses of a new candidate vaccine will be needed to maintain protection, it is preferable that this should be determined from long-term follow-up of subjects who were enrolled in efficacy trials and/or from post-licensure effectiveness studies. Although the long-term monitoring of antibody persistence is important, these data alone cannot determine if another dose is needed unless there is evidence, or a strong reason to expect, that failure to maintain circulating antibody above a certain level (for example, above the ICP if there is one) is associated with a risk of breakthrough disease.

If it is unclear whether additional doses are needed it is prudent to plan to obtain data on the immune response to doses administered at different intervals after the last dose of the primary series so that such data are available should it become clear that a further dose is required.

5.6.1.3.2 Assessment of prior priming

It is not always necessary to assess whether or not a vaccine elicits a T-cell-dependent immune response that results in priming of the immune system and an anamnestic (memory) response to further doses. However, for some new candidate vaccines (for example, polysaccharide-protein conjugate vaccines in which the polysaccharide and/or conjugate protein have not previously been included in a licensed vaccine) there may be considerable interest in understanding the ability of the vaccine to prime the immune system.

When assessing the immune response to additional doses and determining whether or not the primary series elicited immune memory, the following should be taken into account:

- Trials in which additional doses are administered may be extension phases of primary series trials or new trials in subjects with documented vaccine histories.
- When assessing whether the primary series elicited immune memory the optimal design is to compare subjects who previously completed a full primary series of the candidate vaccine with a control group consisting of subjects not previously vaccinated. Control subjects should be matched for age and for any host or demographic factors that might have an impact on their immune response (for example, they should be resident in similar areas so that any natural exposure is likely to be similar).
- If the new candidate vaccine elicited immune memory in the primary series the immune response to the additional (that is, booster) dose
should usually be superior (on the basis of comparisons of the GMCs or GMTs of antibody) to that observed in individuals who have not been vaccinated against the disease to be prevented. The percentages that achieve seropositivity or seroprotection (as defined) may not differ between the two groups if a single dose of the vaccine is highly immunogenic even in unprimed individuals.

- The immune response to the additional dose in primed and unprimed subjects may also be differentiated on the basis of the rapidity of the rise in antibody levels (faster in primed) and in terms of antibody avidity (greater in primed). Note that not all primed individuals (whether priming results from natural exposure or from previous vaccination) have detectable humoral immunity against the relevant organism or the toxin that causes clinical disease.

- If the immune response as measured by geometric mean antibody concentrations or titres in the vaccine-primed group is not superior to that in controls this does not always mean that the primary series did not elicit immune memory. For example, the immune response in the vaccinated group may not be superior to the immune response in the control group when natural priming has occurred in a substantial proportion of subjects not previously vaccinated against the disease to be prevented – in which case the rapidity of response and measurements of avidity may also not be distinguishable between groups. If natural priming has occurred it may or may not be detectable from pre-vaccination antibody levels in the control group.

- If an immune memory response is elicited in the primary series it may be possible to achieve a robust anamnestic response using a much lower dose of an antigenic component compared to the primary series. A lower boosting dose may also provide a better safety profile (for example, as occurs with diphtheria toxoid).

- For polysaccharide-protein conjugate vaccines that elicit immune memory it may be informative to compare boosting with the same type of conjugate used for priming with an alternative conjugate (for example, to prime with a tetanus toxoid conjugate and boost with a CRM197 conjugate and vice versa).

- It may also be informative to assess the ability of a candidate vaccine to achieve cross-priming by using heterologous antigenic components for priming and boosting. This may be assessed by comparing boosting with the same vaccine used to prime with administration of a formulation (which may be a licensed vaccine
or an unlicensed product manufactured specifically for the trial) containing a different microorganism or antigen that is known to be closely related but not identical to that in the vaccine (for example, material derived from an influenza virus of a different clade).

- Elicitation of an immune memory response to a vector for an antigen after the first dose(s) may sometimes interfere with or wholly prevent the immune response to the antigen after subsequent doses (for example, this may be observed when using certain adenoviruses capable of infecting humans as live viral vectors). It is essential to understand whether or not this occurs since it may necessitate the use of a different vector for the antigen or an entirely different vaccine construct to deliver subsequent doses.

- Some antigens elicit immune hyporesponsiveness to further doses. The best known examples are some of the unconjugated meningococcal and pneumococcal polysaccharides (18, 19). In the past these were sometimes administered to assess whether corresponding conjugated polysaccharides had elicited immune memory in the primary series, based on the premise that this would better mimic the immune response to natural exposure compared to administration of a further dose of the conjugate. This practice is not recommended since it is possible that a dose of unconjugated polysaccharide could result in blunted immune responses to further doses of the conjugate.

- Studies of cell-mediated immunity may provide supportive evidence that the primary series elicited immune memory and may be particularly useful for assessing cross-priming.

5.6.2 Using immunogenicity data to predict efficacy

5.6.2.1 Bridging to efficacy data

Immunogenicity data may be used to provide evidence of efficacy when:

- there is a well-established ICP that can be used to interpret the immune responses to a specific antigenic component;
- it is possible to use immune responses to bridge to estimates of vaccine efficacy obtained from prior well-designed clinical trials (that is, to conduct bridging trials).

The following two main situations should be considered when using immunogenicity data to bridge to estimates of vaccine efficacy obtained in prior clinical trials. In both cases comparative immunogenicity trials designed to
demonstrate non-inferiority are recommended. The choice of comparator is a critical factor in the interpretation of the results.

5.6.2.1 Modifying the use of the vaccine for which efficacy has been estimated

As described in section 6 below, vaccine efficacy trials are usually conducted in specific target populations – characterized by factors such as age, region (which may define the endemcity of some infectious diseases) and health status – using the intended final vaccine posology. Before or after licensure, trials may be conducted with the aim of extending the use of the vaccine to other populations and/or to support alternative posologies.

When a different age group or posology is proposed it is usually very clear that a bridging trial is necessary. A bridging trial may be required if there are compelling scientific reasons to expect that the immune response to the vaccine, and therefore its efficacy, could be significantly different to that documented in a prior efficacy trial because of host factors (such as common underlying conditions that may affect immune responses) and/or geographical factors (such as distribution of subtypes of organisms and levels of natural exposure). In infants there is also the possibility that very different levels of maternal antibody could occur in different regions, resulting in variable interference with infant immune responses to the primary series.

The trial design may involve a direct comparison between: (a) the new posology and that used in the efficacy trial; or (b) the new intended population and a control group consisting of subjects who are representative of the prior efficacy trial population. It may also be acceptable to make an indirect (cross-trial) comparison with the immunogenicity data that were obtained during the efficacy trial.

The vaccine formulation and assay used should be the same as those used in the efficacy trial whenever possible:

- If the exact vaccine used in the efficacy trial is no longer available the comparator should be as similar as possible to the original vaccine that was evaluated. Over time, it may be that the only bridge back to the efficacy data is via a comparison with a licensed vaccine that was itself licensed on the basis of a bridging efficacy trial. As the number of bridging steps that have occurred between the original efficacy data and the licensed comparator vaccine increases, the reliance that may be placed on a demonstration of non-inferiority to predict efficacy is weakened. This consideration also applies when the vaccine for which efficacy was estimated contained a certain number of subtypes but was later replaced by a vaccine containing a larger number of subtypes on the basis of comparing immune responses to the shared subtypes.
If the assay has changed and has not been, or cannot be, directly compared to the original assay used during the efficacy trial it may be possible to re-assay stored sera collected during the prior efficacy trial in parallel with the sera from the new trial population.

If it remains unknown which immunological parameter best correlates with efficacy it is preferable that the primary comparison between vaccines is based on functional antibody whenever this is feasible.

5.6.2.1.2 Inferring the efficacy of a new candidate vaccine

In this case the main evidence of efficacy for licensure comes from one or more bridging efficacy trials. The same considerations described above regarding primary comparison, choice of comparative vaccine and assay apply.

If the new candidate vaccine contains additional subtypes of an organism compared to licensed products and/or it contains subtypes of an organism that have not previously been included in any licensed vaccine then interpretation of the immune responses to the added or new subtypes is not straightforward. Approaches that could be considered include comparing immune responses to each added or new subtype with the mean immune response to all subtypes or with the lowest immune response to any individual subtype included in a vaccine for which efficacy was demonstrated. Although these approaches may provide a route to licensure, the limitations of these comparisons in predicting efficacy should be taken into account when considering the overall risk–benefit relationship for the new vaccine.

5.6.2.2 Other approaches

When there is no ICP and it is not possible to bridge to a prior demonstration of efficacy the evidence that may be provided to support likely vaccine efficacy must be considered and discussed with NRAs on a case-by-case basis. In each case the strength of evidence that may be provided should be weighed against the advantages of having a licensed vaccine – one that has been subjected to a full review of quality and nonclinical data, and for which it is considered that there are adequate clinical safety and immunogenicity data – available for use when needed.

Potential approaches may include establishing a nonclinical model of efficacy that is thought to be relevant to the human infection and identifying which immunological parameter best correlates with protection (and, if possible, a putative ICP). Data on immune responses that occur in response to natural infection and the resulting protection against further disease may be useful, as may any passive protection data that are available from nonclinical or clinical trials.
5.6.3 **Co-administration trials**

Comparative immunogenicity trials that are intended to support co-administration of a vaccine with one or more other vaccines should demonstrate non-inferiority for immune responses to each of the co-administered antigenic components in the group that receives co-administered vaccines compared with the groups that receive each vaccine given alone.

When multiple licensed products contain the same antigenic components that could be co-administered with the vaccine under trial (for example, combination vaccines intended for the routine infant primary immunization series) it is not feasible, nor is it usually necessary, to assess co-administration with each licensed product.

A particular issue arises when there are several different types of polysaccharide-protein conjugate vaccines available that may be co-administered with the vaccine under trial. When the vaccine under trial contains protein that is the same as, or similar to, that in available conjugate vaccines it is important to appreciate that the results obtained with any one conjugate may not be applicable to other types of conjugate (for example, lack of immune interference with a tetanus toxoid conjugate does not rule out the possibility that this could occur when a different protein is used in the conjugate). Therefore, if co-administration with several different conjugate vaccines is foreseen the effects of representative vaccines that contain different conjugative proteins should be evaluated.

If multiple doses of the co-administered vaccines are needed then it is usual to make the comparison between groups only after completion of all doses. The schedule at which the vaccines are co-administered may also be a consideration if there are several possibilities (for example, as in the case of vaccines for the primary immunization series in infants or for vaccines against hepatitis A and B). Consideration may be given to using a schedule that is most likely to detect an effect of co-administration on immune responses if there is one.

Trials that assess the effects of co-administration may randomize subjects to receive only one or all of the vaccines proposed for co-administration. Alternatively, all subjects may receive all vaccines proposed for co-administration but are randomized to staggered administration or co-administration. Staggered administration is necessary when it is not possible to withhold any antigenic components to be co-administered (for example, during the infant primary schedule). In staggered administration trials the final dose and post-dose sampling occur later compared to the co-administration group, which in infants could have some impact on the magnitude of the immune response.

5.6.4 **Immunization of pregnant women**

Whenever the target population for a vaccine includes women of childbearing age there is a need to consider the importance of generating data in pregnant women. These considerations should take into account the nature of the vaccine
construct (for example, whether the vaccine contains a live organism that is replication competent), whether pregnant women can reasonably avoid exposure to an infectious agent (for example, by not travelling) and whether they may have the same risk of exposure but a greater risk of experiencing severe disease compared to non-pregnant women of the same age.

Not all vaccines are, or need to be, evaluated in trials in pregnant women. If there is no or very limited experience of the use of a vaccine in pregnant women, NRAs may consider whether nonclinical data and any data available from the clinical use of the vaccine and very similar vaccines could be provided in the prescribing information.

5.6.4.1 Aims of immunization during pregnancy

The immunization of women during pregnancy may benefit the mother and, in some cases, may also result in benefit to the infant for a limited postnatal period by means of placental transfer of maternal antibody (for example, influenza, acellular pertussis and tetanus vaccines). In other cases the immunization of women during pregnancy may provide some benefit to the infant with no or negligible benefit to the mother (for example, respiratory syncytial virus vaccine).

It is also possible that immunization during pregnancy could prevent an infection occurring in the mother and so protect the fetus from the consequences of infection in utero.

5.6.4.2 Safety and immunogenicity in pregnancy

Before conducting trials in pregnant women, safety and immunogenicity data should be available from clinical trials conducted in non-pregnant women of childbearing age (20). Once there are adequate relevant nonclinical data with satisfactory findings and some clinical data on safety and immune responses in non-pregnant women, data may be obtained from pregnant women covering a representative age range, so that the effects of pregnancy on the immune response can be evaluated. The doses tested in pregnant women should be based on the non-pregnant adult data but may need to be adjusted (in terms of antigen dose or dose regimen) if the results indicate an effect of pregnancy on the immune response.

In all trials conducted in pregnant women adequate mechanisms should be in place to document the outcome of the pregnancy, including the duration of gestation at time of delivery, the condition of the infant at birth and the presence of any congenital conditions (see section 7.4 below).

5.6.4.3 Passive protection of infants

If there is already evidence of humoral immunity in a substantial proportion of pregnant women against the infectious disease to be prevented, such that that
the aim of vaccination during pregnancy is to increase the amount of antibody transferred to the fetus, then the trials in pregnant women may need to include exploration of maternal immune responses to vaccination in both seropositive and seronegative subjects.

Dose-finding trials in pregnant women should include measurement of antibody levels in cord blood samples taken at delivery. The number of samples obtained should be sufficient to provide an estimate of inter-individual variability. Additional investigations may include the collection of cord blood covering a range of times between maternal vaccination and delivery. Cord blood antibody levels in infants born to vaccinated mothers who received the final selected vaccine posology should be superior to those in infants born to mothers who were not vaccinated. Secondary analyses could examine whether this finding also applies within subsets of mothers who were seronegative or seropositive prior to vaccination.

To avoid multiple bleeds in individual infants when evaluating the duration of detectable maternal antibody, mothers may be randomized so that their infants are sampled once or a few times at defined intervals. The total data collected can be used to describe the antibody decay curve. These data are particularly important when it is planned that passive protection via maternal antibody will be followed by active vaccination of infants against the same antigen(s) because of the possibility that high levels of maternal antibody may interfere with the infant immune response.

If an ICP is established for the infectious disease to be prevented then the aim of the immunogenicity trials should be to identify a maternal vaccination regimen that results in cord blood antibody levels that exceed the ICP in a high proportion of newborn infants. If no ICP exists there should be discussion with NRAs regarding whether vaccine efficacy should be estimated in a pre-licensure efficacy trial or whether an evaluation of vaccine effectiveness may suffice.

5.6.5 Changes to the manufacturing process

Changes made to product composition (for example, adding, removing or changing a preservative) or to product manufacture (such as changes to process, site or scale of manufacture) during the pre-licensure clinical development programme or after licensure do not always need to be supported by comparative clinical immunogenicity trials between the prior and newer products.

For example, although it is common for the scale of manufacture to change during the pre-licensure development programme, this step alone may not necessarily have a clinically significant effect in the absence of other changes. To avoid the need for additional clinical trials to address manufacturing changes the pivotal trials should whenever possible be conducted using vaccine made according to the final process. If this is not the case, and for all changes that are
made post-licensure, consideration must be given to whether a clinical trial is required to compare vaccines manufactured using the prior and new processes. This decision must be taken on a case-by-case basis after a full evaluation of the in vitro data, and of any nonclinical in vivo data describing and supporting the change. Although a single lot of vaccine made using each process may typically be sufficient for the comparison, data may on occasion be required from multiple lots.

In the post-licensure period there may be many changes to the manufacturing process over time. Whereas each one of these changes may be considered too minor to merit the conducting of a clinical trial, the product that results from multiple minor changes could be substantially different from that which was first licensed. Therefore, when considering the merit of a clinical trial, it may be important to consider the full history of changes that have been allowed without clinical data and whether the sum total of these changes could have a clinical impact. In this situation, when many years have passed, a clinical trial of the current vaccine compared to the original licensed vaccine will not be possible. However, if disease surveillance suggests that there could be a problem with vaccine effectiveness, a clinical trial that compares the current vaccine against another licensed vaccine may be considered useful.

5.6.6 Clinical lot-to-lot consistency trials

Clinical lot-to-lot consistency trials are conducted to provide an assessment of manufacturing consistency in addition to the information provided on the manufacturing process. Clinical lot-to-lot consistency trials may or may not be considered necessary. Such trials may be considered particularly useful for certain types of vaccines where there is inherent variability in the manufacture of the product or when manufacturing consistency cannot be characterized adequately by bio-physicochemical methods.

If a clinical lot-to-lot consistency trial is conducted then the usual expectation is that the 95% confidence interval around each pair-wise comparison of the post-vaccination geometric mean antibody concentrations/titres falls within predefined limits. The clinical implications of results that show that one or more comparisons do not meet the predefined criteria set around the ratios should be considered in light of all available clinical immune response data and relevant product-characterization data.

Whether or not a clinical lot-to-lot consistency trial is conducted, the consistency of manufacturing for the vaccine lots used in clinical trials should be both demonstrated and well documented. The lots used in clinical trials should also be adequately representative of the formulation intended for marketing.
6. Efficacy and effectiveness

6.1 General considerations for efficacy trials

The need for, and feasibility of, evaluating the protective efficacy of a candidate vaccine should be considered at an early stage of vaccine development because the decision made will determine the overall content of the pre-licensure clinical programme and will impact on its duration. In all application dossiers that do not include an evaluation of vaccine efficacy the sponsor should provide sound justification for the lack of such data, taking into account the points raised in the following sections 6.1.1–6.1.3.

6.1.1 Efficacy data are not required

Vaccine efficacy trials are not necessary if it is established that clinical immunological data can be used to predict protection against disease. For example, if there is an established ICP against a specific disease (for example, antitoxin levels against diphtheria and tetanus toxins, or antibody against hepatitis B surface antigen) the candidate vaccine should be shown to elicit satisfactory responses based on the relevant correlate(s).

6.1.2 Efficacy data are usually required

Vaccine efficacy trials are usually required whenever a new candidate vaccine is developed with intent to protect against an infectious disease and one or more of the following apply:

- There is no established ICP that could be used to predict the efficacy of the new candidate vaccine.
- There is no existing licensed vaccine with documented efficacy against a specific infectious disease to allow for bridging to a new candidate vaccine.
- Use of immune responses to bridge the documented efficacy of a licensed vaccine to a new candidate vaccine is not considered to be possible. For example, because there is no known relationship between specific immune response parameters and efficacy or because the new candidate vaccine does not elicit immune responses to the same antigen(s) as the licensed vaccine.
- There are sound scientific reasons to expect that the efficacy of a vaccine cannot be assumed to be similar between the population(s) included in the prior efficacy trial(s) and one or more other populations.
It cannot be assumed that the vaccine efficacy demonstrated against disease due to specific strains of a pathogen (for example, serotypes or subtypes) would apply to other strains.

6.1.3 **Efficacy data cannot be provided**

It may not be feasible to conduct efficacy trials. For example, if the new candidate vaccine is intended to prevent an infectious disease that:

- does not currently occur (for example, smallpox);
- occurs in unpredictable and short-lived outbreaks that do not allow enough time for the conducting of appropriately designed trials to provide a robust estimation of vaccine efficacy (for example, some viral haemorrhagic fevers);
- occurs at a rate that is too low for vaccine efficacy to be evaluated in a reasonably sized trial population and period of time. This situation may apply:
  (a) because of natural rarity of the infectious disease (for example, plague, anthrax and meningitis due to *N. meningitidis* group B);
  (b) because of rarity of the disease resulting from the widespread use of effective vaccines.

If it is not feasible to perform vaccine efficacy trials and there is no ICP it may be possible to obtain evidence in support of vaccine efficacy and/or to derive an immunological marker of protection from one or more of the following:

- Nonclinical efficacy trials.
- Passive protection trials – that is, nonclinical or clinical trials which assess the effects of administering normal or hyperimmune human gamma globulin or convalescent sera. The results may point to the sufficiency of humoral immunity for the prevention of clinical disease and may suggest a minimum protective antibody level that could be used to interpret data obtained in clinical trials with candidate vaccines.
- Comparison of immunological responses with those seen in past trials of similar vaccines with proven protective efficacy even if the relationship between immune responses to one or more antigenic components and efficacy remains unknown.
- Human challenge trials.
6.2 **Types of efficacy trials**

6.2.1 **Human challenge trials**

Human challenge trials, in which subjects are deliberately exposed to an infectious agent in a controlled setting, are not always feasible or appropriate. However, in some settings it may be useful and appropriate to obtain an assessment of vaccine efficacy from human challenge trials. If conducted, human challenge trials may be of particular use:

- when there is no appropriate nonclinical model (for example, when a candidate vaccine is intended to protect against an infectious disease that is confined to humans);
- when there is no known ICP;
- when vaccine efficacy trials are not feasible.

6.2.2 **Preliminary efficacy trials**

If conducted, preliminary vaccine efficacy trials may provide an estimate of the magnitude of protection that can be achieved by the new candidate vaccine. However, preliminary efficacy trials are not usually designed and powered to provide robust estimates of vaccine efficacy. These trials may be used to inform the design of pivotal trials. For example:

- by evaluating the efficacy of different doses and dose regimens;
- by estimating efficacy on the basis of a range of efficacy variables;
- by analysing efficacy on the basis of various case definitions in order to identify or refine the most appropriate case definition;
- by exploring efficacy in specific subgroups in order to decide if there is a need to design pivotal trials specifically to further evaluate efficacy in such subgroups;
- by assessing the method of case ascertainment for feasibility in larger and more geographically diverse trials;
- by using immunogenicity and efficacy data to support a provisional assessment of potential ICPs.

If the candidate vaccine is intended to prevent a severe and/or life-threatening infectious disease for which there is no vaccine, or no satisfactory vaccine, already available then individual NRAs may agree to accept an application for licensure based on one or more preliminary efficacy trial(s). In these cases it is essential that sponsors and NRAs should discuss and agree
upon the main features of the design of the trials before initiation (including the sample size) so that, subject to promising results, the data may be considered robust and sufficient.

The availability of a licensed vaccine has potentially important implications for the acceptability and feasibility of initiating or completing additional efficacy trials that include a control group that does not receive active vaccination. These issues should be discussed between NRAs and sponsors so that expectations for the completion of additional efficacy trials are agreed upon prior to the start of trials that could potentially support licensure.

6.2.3 Pivotal efficacy trials

Pivotal vaccine efficacy trials are designed and powered to provide statistically robust estimates of vaccine efficacy to support licensure. Pivotal efficacy trials may evaluate one or more vaccination regimen(s), and may or may not include evaluations of efficacy before and after booster doses.

6.3 Design and conduct of efficacy trials

The protective efficacy of a vaccine against a specific infectious disease is usually determined in randomized trials that compare the incidence of disease after vaccination relative to the incidence of disease in the control group that has not been vaccinated. Less frequently, vaccine efficacy may be determined in a prospective randomized trial which compares the incidence of disease after vaccination between the group that received the new candidate vaccine and a control group that received a licensed vaccine intended to prevent the same infectious disease.

The following sections (6.3.1–6.3.9) are applicable to both types of trial. As the details of statistical methodologies are beyond the scope of these WHO Guidelines only broad principles are described. It is recommended that an appropriately experienced statistician should be consulted.

6.3.1 Selection of trial sites

Vaccine efficacy trials require the presence of a sufficient burden of clinical disease to enable estimates to be obtained from feasible numbers of subjects within a reasonable time frame. The infectious disease to be prevented may occur at sufficiently high rates to enable efficacy trials to be conducted only in certain geographical areas. Even when the disease to be prevented is more widespread it may be necessary to confine efficacy trials to specific areas for reasons that may include feasibility, the need to ensure adequacy of monitoring, and a desire to accumulate representative numbers of cases due to specific serotypes or subtypes of the relevant pathogen.
If adequate data are not already available from public health authorities then sponsors may have to conduct feasibility assessments in order to accurately ascertain the clinical disease rates in various age subgroups of populations before selecting trial sites. Any nationally recommended non-vaccine-related preventive measures that are in place (for example, prophylactic drug therapy in high-risk settings or in individuals at high risk, or the use of insect repellents and bednets) should be identified. Trials are usually conducted against a background of such measures.

Trial sites need to be sufficiently accessible to allow regular visits for monitoring. Prior to initiation of the trial, sponsors may have to engage in site capacity-building exercises, including training of study personnel, and may need to provide essential infrastructure to support the trial (for example, adequate blood-collection and processing facilities, refrigeration facilities suitable for the vaccine and/or sera, access to competent laboratories, data-handling capacity and communication methods to allow for electronic randomization schemes, rapid reporting of safety data and other trial issues to the sponsor).

6.3.2 **Candidate (test) vaccine group(s)**

If previous data do not support selection of a single dose level or regimen of the candidate vaccine for assessment of efficacy then trials may include one or more groups in which subjects receive the candidate vaccine (for example, more than one dose or schedule may be evaluated). In some cases one or more placebo doses may need to be interspersed with candidate vaccine doses to enable the matching of all regimens under trial in a double-blind design (for example, if two or three doses of the candidate vaccine are to be compared with the control group).

6.3.3 **Control (reference) group(s)**

Control groups comprise all subjects who do not receive the candidate vaccine. Usually only one control group is enrolled in any one trial. Sometimes it may be important to include more than one of the possible types of control groups discussed below.

6.3.3.1 **Control groups not vaccinated against the infectious disease to be prevented**

Following consultation between the sponsor, NRA, ethics committees, local public health authorities and investigators it may be appropriate to use a control group that is not vaccinated against the disease to be prevented by the new candidate vaccine. For example, this may be the case when the trial is to be conducted in countries in which:

- no vaccine is yet licensed for prevention of the disease in question; and/or
- no such vaccine is included in the routine immunization schedule; and/or
- there are sound reasons to believe that no licensed vaccine is likely to provide useful efficacy (for example, because the licensed vaccine does not cover, or is known/expected to have poor efficacy against, the pathogen types that are most prevalent in a specific region).

In these cases the control group may receive:

- A true placebo (that is, material without any pharmacological activity, such as normal saline). This has the advantage of providing safety data against a control that has no pharmacologically active components. The use of an injectable placebo may not be acceptable to all NRAs, ethics committees, investigators, trial subjects or their caregivers in some age groups (for example, particular objections may be raised against true placebo injections in infants). In contrast, there is usually no objection to the use of a true placebo when the candidate vaccine is administered orally or by nasal instillation.

- A licensed vaccine that does not prevent the infectious disease under study but may have some benefit for recipients. In some cases both licensed vaccine and placebo doses may have to be administered to the control group to match the candidate vaccine regimen in order to maintain blinding.

If there are major objections to the use of placebo injections but no potentially beneficial licensed vaccine would be suitable for the target age group, the control group may be randomized to receive no injection. This is an undesirable situation and should be regarded as a last resort since it precludes the blinding of trial personnel or subjects/caregivers.

### 6.3.3.2 Control groups vaccinated against the infectious disease to be prevented

In this case the control group receives a vaccine that is already licensed to prevent the same infectious disease as the candidate vaccine.

In some instances the control group may receive a licensed vaccine that prevents infectious disease due to some, but not all, types of the pathogen responsible for the disease that is to be prevented – in which case the group that receives the licensed vaccine may be regarded as an unvaccinated control group for the types found only in the candidate vaccine.

It is important that selection of the control vaccine takes into account the available evidence supporting its efficacy and, if relevant, whether it appears to have similar efficacy against all types of the pathogen involved. When there is more than one available licensed control vaccine, or the selected control vaccine
is unlicensed or is not the product in routine use in a particular jurisdiction(s), sponsors are advised to discuss selection of the comparator with the relevant NRA(s). If it is not possible to reach agreement on the use of the same control vaccine in all regions where efficacy is to be evaluated, consideration should be given to conducting more than one efficacy trial with a different vaccine used in the control group in each trial.

6.3.4 Trial designs

6.3.4.1 Randomization

The unit of randomization is most usually the individual. Alternatives include the household or the cluster under trial (for example, a school population or a local community). Randomization of groups or clusters, rather than individuals, may be preferred when it is logistically much easier to administer the vaccine to groups than to individuals and when estimates of the indirect effects of vaccination (for example, herd immunity) are of interest. When the trial aims to vaccinate pregnant women to protect the infant during the early months of life then the unit of randomization is the mother.

6.3.4.2 Types of trial design

The simplest design involves randomization of equal numbers of subjects to the candidate vaccine and control groups (that is, 1:1). In trials that employ a control group that is not vaccinated against the disease to be prevented, but some clinical data are available to support the likely efficacy of the candidate vaccine, it may be appropriate (subject to statistical considerations and an assessment of the impact on the total trial sample size) to use unbalanced randomization (for example, 2:1 or 3:1) to reduce the chance that individual subjects will be randomized to the control group, thus ensuring that the majority of trial subjects receive the candidate vaccine.

Trials may be planned to follow trial subjects for a fixed period after the last dose of the primary series. The time at which the primary analysis is conducted should take into account the anticipated rates of the disease under study in each treatment group, including the unvaccinated control group if applicable. Other considerations regarding the timing of the primary analysis may include the possible importance of having some information on the duration of protection before licensure occurs, the feasibility of following up subjects for prolonged periods, and whether or not the vaccine could address a pressing unmet need (for example, in an outbreak situation where there is no approved vaccine to prevent the disease).

Alternatively, a case-driven approach may be taken based on the anticipated rates of the primary efficacy end-point in the control group and the expected or minimum desirable level of efficacy of the candidate vaccine.
In this design the primary analysis is conducted once a pre-specified total number of cases has been detected – based, in a double-blind setting, on the anticipated numbers in test and control groups required to demonstrate the projected vaccine effect.

Alternative designs that allow for comparison with a control group that is not vaccinated against the disease to be prevented may, at least in the short term, include the following:

- In a randomized stepped wedge trial, the candidate vaccine is administered to predefined groups in a sequential fashion. Each predefined group is a unit of randomization. These may be geographical groups or groups defined by host factors (for example, age) or other factors (for example, attendance at a specific school or residence within a specific health-care facility catchment area). Such a design may be chosen when there is good evidence to indicate that the vaccine will do more good than harm (affecting the equipoise associated with randomization to a control group that is not vaccinated against the disease to be prevented) and/or when it is impossible to deliver the intervention to all trial participants within a short time frame.

- In a ring vaccination trial, the direct contacts (and sometimes secondary contacts) of a case may be randomized to vaccine or control or may be randomized to receive immediate vaccination or vaccination after a period of delay (21). This type of post-exposure cohort trial usually requires smaller sample sizes than prospective randomized controlled trials.

  Ring vaccination trials may be particularly applicable when the infectious disease to be prevented is associated with a relatively high incidence of secondary cases in susceptible populations. Therefore the use of this trial design requires prior knowledge of the infectivity of the infectious agent and of the proportion of infections that are clinically apparent, as well as of the general susceptibility of the trial population.

  Ring vaccination trials may not be appropriate if the vaccination regimen requires multiple doses over an extended period to induce a protective immune response.

  The follow-up period for subjects after contact with the index case should extend to the upper limit of the incubation period, taking into account both the period during which the index cases were infectious and the contact period. The inclusion period for new cases and controls and their contacts following the detection of the first
case should be stated in the protocol. The duration of the inclusion period should take into account the potential for introducing bias if the disease incidence changes over time.

6.3.5 **Clinical end-points**

6.3.5.1 **Primary end-points**

The primary end-point(s) in preliminary trials may be different from the primary end-point(s) used in the pivotal trial(s).

In most cases the focus of vaccine efficacy trials is the prevention of clinically apparent infections that fit the primary case definition based on clinical and laboratory criteria.

If an organism causes a range of disease manifestations (for example, from life-threatening invasive disease to disease that is not serious if adequately treated or is self-limiting) the primary end-point in any one trial should be carefully selected in accordance with the proposed indication(s) for use.

A candidate vaccine may contain antigens derived from one or several types (serotypes, subtypes or genotypes) of the same organism. There may also be some potential for cross-protection against types not included in the vaccine (for example, as observed with rotavirus vaccines and HPV vaccines). In such cases it is usual for the primary end-point to comprise cases due to any of the types included in the vaccine, and the trial is powered for this composite end-point. It is not usually possible to power the trial to assess efficacy against individual types in the vaccine or to assess cross-protection against types not in the vaccine.

Alternative primary end-points may include:

- clinical manifestations of reactivated latent infection (for example, herpes zoster);
- established chronic infections that may be asymptomatic but predispose to infection-related disease later in life (for example, chronic hepatitis B infection or persistent infection with HPV);
- other markers that predict progression to clinically apparent disease (for example, histological changes associated with HPV infection that are established precursors of malignant neoplasia).

6.3.5.2 **Secondary end-points**

As applicable to the individual candidate vaccine, other important end-points may include:

- cases that occur after each dose, when the vaccine schedule includes multiple doses and/or a booster;
- cases due to each of the individual types of the organism included in the vaccine;
- cases due to the organism, regardless of whether the cases are caused by types that are or are not included in the candidate vaccine;
- cases due to non-vaccine types;
- cases occurring in groups with host factors of interest (for example, age or region);
- cases meeting criteria for disease severity – if available, validated measures of criteria for severity should be used to facilitate interpretation of the results;
- duration and/or severity of the illness, which may include clinical measurements (for example, duration of fever or rash) and laboratory measurements (for example, duration of shedding).

Eradication of carriage and/or reduction in disease transmission that is not directly linked to, and/or accompanied by, a clinical benefit of vaccination to the individual are not usually considered to be sufficient to support licensure. Sponsors contemplating trials with these as primary end-points are advised to consult widely with NRAs.

6.3.6 **Case definition**

As part of the predefined primary efficacy end-point, the protocol should describe the clinical and laboratory criteria that must be met to define a case.

- If an end-point is defined as the occurrence of an acute infectious disease then the case definition should include the core clinical features as well as laboratory confirmation of the presence of the target pathogen.
- If the end-point is defined as a consequence of a persistent infection then details of sampling (frequency and method) and grading (if applicable) should be described.

All laboratory assays used to define a case should be validated to the satisfaction of relevant NRAs prior to initiating pivotal clinical trials. Adequate case definitions should also be provided for secondary end-points.

6.3.7 **Case ascertainment**

It is critical that the same methodology for case detection should be applied consistently at all clinical sites throughout the duration of the trial. Active case ascertainment usually requires frequent monitoring and contact with trial
subjects/caregivers. Passive case ascertainment is usually based on trial subjects/caregivers presenting to or otherwise contacting a local health-care facility due to the onset of specific symptoms. In this case, contact is commonly triggered by one or more of a list of signs or symptoms given to trial subjects/caregivers at the time of randomization, when they may also have been instructed to contact a specific health-care facility. Alternatively, or in parallel, cases may be detected by monitoring all local clinics and hospitals.

For efficacy end-points based on clinically apparent disease the possible range of clinical presentations will determine the mode of case ascertainment. For example, this may be hospital based for cases of life-threatening infections, or community-based for less severe infections. If community-based, case detection may depend on family practitioners and on initial suspicion of infection by vaccinated subjects or their caregivers. It is critically important that the individuals who are most likely to initiate detection of a possible case should have clear instructions. These may need to cover issues such as the criteria for initiating contact with designated health-care professionals, telephone contacts, first investigations and further investigations once a case is confirmed.

For efficacy end-points other than clinically apparent disease it is essential for subjects to be monitored at regular intervals to detect clinically non-apparent infections or changes in other selected markers (for example, the appearance of histological changes). The frequency of these visits, and acceptable windows around the visits, should be stated in the trial protocol and carefully justified.

The appropriate period of case ascertainment during a trial should be determined mainly by the characteristics of the disease to be prevented and the claim of protection that is sought at the time of licensure. For infectious diseases that have marked seasonality, at least in some geographical locations (for example, influenza and respiratory syncytial virus), it is usual to follow subjects through one or more seasons to accumulate sufficient cases to conduct the primary analysis. In these settings it is usual to conduct an enrolment campaign over a short period just before the expected onset of each season.

6.3.8 Duration of follow-up

At the time of conducting the primary analysis for the purposes of obtaining licensure the duration of follow-up in vaccine efficacy trials may be relatively short (for example, 6–12 months) and may be insufficient to detect waning protection, if this occurs. If feasible, case ascertainment may continue in vaccine efficacy trials with maintenance of the randomized populations for a sufficient duration to assess waning protection over time. Alternatively, or in addition, waning protection may be assessed during the post-licensure period. These data may serve both to indicate the need for, and optimal timing of, booster doses and to estimate efficacy after booster doses.
6.3.9 **Analysis of efficacy**

Detailed plans for the analysis of efficacy, including any interim analyses and/or plans to adjust the sample size during the study on the basis of specific criteria, should be developed in conjunction with appropriately experienced statisticians, and should be discussed with the NRA(s) before the protocol is finalized (and/or during the conducting of the study, as necessary).

6.3.9.1 **Sample size calculation**

The trial sample size should be calculated on the basis of:

- the selected primary efficacy end-point, which could be a composite of cases due to any of the organism types included in the candidate vaccine;
- the primary analysis population (see below);
- the primary hypothesis (that is, superiority or non-inferiority and the predefined success criteria).

If the primary analysis population represents a subset of the total randomized population then the sample size calculation should include an adequate estimation of numbers likely to be excluded from the primary analysis for various reasons. In addition, a blinded review (for example, using an independent data adjudication committee) of total numbers of subjects enrolled who are eligible for the primary analysis population may be conducted after randomization of a predefined number so that the trial sample size can be adjusted accordingly.

6.3.9.2 **Analysis populations**

Clinical efficacy is usually assessed in the total randomized trial population (that is, those who are assigned to receive vaccine and/or control) and in predefined subsets of the randomized population.

The predefined trial populations should include as a minimum:

- all randomized subjects (that is, the full analysis set);
- all vaccinated subjects regardless of the numbers of assigned doses actually received and whether or not doses were administered within predefined windows;
- subjects who have generally complied with the protocol and have received all assigned doses within predefined windows.
Other populations may be appropriate for some predefined secondary or exploratory analyses. These may include, for example:

- those who completed specific numbers of assigned doses or received all doses within predefined windows around the scheduled trial visits (that is, analyses of efficacy according to adherence to the vaccination regimen);
- subsets of all vaccinated subjects separated according to baseline seropositivity versus seronegativity;
- subgroups defined by demographic factors known or postulated to have an impact on vaccine efficacy.

6.3.9.3 Primary analysis

The primary analysis may sometimes be based on estimating efficacy in the “per protocol” population and on rates of true vaccine failures. In this case, the calculation of efficacy takes into account only those cases with onset after a minimum time has elapsed following completion of the assigned doses. For example, depending on knowledge of the kinetics of the immune response, true vaccine failures may be limited to cases with onset more than a specified number of days or weeks after the final dose of the primary series. In addition, for a vaccine that contains antigens from only certain serotypes or subtypes the primary analysis may be based on cases due to vaccine types only. Alternative primary analysis populations that may be preferred by NRAs in some cases include the all-randomized or the all-treated populations.

In trials that compare a candidate vaccine group with a group that is not vaccinated against the disease to be prevented, the aim is to demonstrate that the lower bound of the 95% confidence interval around the estimate of vaccine efficacy is above a predefined percentage (which will always be above zero). The predefined percentage should be selected on the basis of the expectation of the point estimate of vaccine efficacy, taking into account what might be seen as the minimum level of efficacy that could be considered clinically important. The sample size calculation is based on this objective.

In trials that compare a candidate vaccine with an active control the aim is usually to demonstrate non-inferiority of the candidate vaccine against a control vaccine with demonstrated efficacy. This requires a predefined non-inferiority margin, which should be justified in accordance with prior estimates of vaccine efficacy for the disease to be prevented and the level of alpha on which the sample size calculation depends. If the sponsor also intends to assess superiority of the candidate vaccine over the active control the statistical analysis plan should predefine a hierarchical assessment so that superiority is assessed only after establishing that non-inferiority has been demonstrated.
6.3.9.4 Other analyses

The full range of secondary and exploratory analyses will depend on the predefined end-points. Some of these analyses may be conducted in specific predefined trial populations. For example, important sensitivity analyses for supporting the primary analysis include those based on all proven cases whenever they occurred after randomization and in each analysis population. If the schedule includes more than one dose, analyses should be conducted to count cases from the time of each dose or from a specified number of days after each dose for all subjects who were dosed up to that point.

Other analyses may be based on cases that meet some but not all of the case definition criteria, cases that are severe and cases that require a medical consultation or hospitalization.

6.3.9.5 Other issues

6.3.9.5.1 Vaccines that contain antigens derived from several serotypes, subtypes or genotypes

If the primary analysis was confined to cases due to organism types included in the vaccine then additional analyses should be conducted to evaluate efficacy on the basis of all cases, regardless of the organism type responsible. If there are sufficient numbers of cases due to organism types not included in the vaccine these analyses may provide some indication of cross-protection.

If the data suggest unusually low efficacy against one or more organism types in the vaccine it may be necessary to explore this issue in further trials.

6.3.9.5.2 Magnitude of vaccine efficacy

The point estimate of vaccine efficacy and 95% confidence intervals that are obtained may indicate that a relatively modest proportion of cases can be prevented. This fact alone does not preclude licensure provided that the sponsor can provide evidence that the vaccine efficacy observed represents an important clinical benefit (for example, if the vaccine prevents life-threatening infections for which there is no very effective specific therapy and for which no vaccine is available).

6.4 Approaches to determination of effectiveness

Vaccine effectiveness reflects direct (vaccine-induced) and indirect (population-related) protection during routine use. The information gained from assessments of vaccine effectiveness may be particularly important to further knowledge on the most appropriate mode of use of a vaccine (for example, the need for booster doses to maintain adequate protection over time). Vaccine effectiveness is influenced by a number of factors, including:

- vaccination coverage of the population;
- pre-existing immune status of the population;
- differences between organism types included in a vaccine and the predominant circulating types;
- changes in circulating predominant types over time;
- transmissibility of the pathogen and any effect that the introduction of routine vaccination may have had on transmission rates.

Vaccine effectiveness may be estimated in several ways, namely:

- In observational cohort studies that describe the occurrence of the disease to be prevented in the target population over time. However, there is no randomization step and there is a potential for considerable biases to be introduced.
- During phased introduction (for example, in sequential age or risk groups) of the vaccine into the target population in which the groups might form the units of randomization (that is, using a stepped wedge design).
- Using other designs such as a case test-negative study design. In this modification of a case control study, subjects with symptoms suggesting the infectious disease under trial and seeking medical care are tested for the infectious agent of interest. The cases are those who are positive and controls are those who are negative for the pathogen of interest. Bias may occur if vaccinated cases are less or more severely ill and seek care at different rates compared to cases that occur in individuals who are not vaccinated against the disease to be prevented (22).

It may not be possible or appropriate for sponsors to conduct studies to estimate vaccine effectiveness themselves. For reasons of feasibility it may be necessary to collect the data via regional or national networks. For some types of disease the use of data collected by means of national or international registries may be appropriate. In addition, in some jurisdictions the estimation of vaccine effectiveness in the post-licensure period is not considered to fall within the remit of the licence holder.

Whatever the local requirements and arrangements, sponsors should discuss arrangements for ongoing disease surveillance and the potential for estimating effectiveness with the public health authorities in countries where the vaccine is to be used and where appropriate surveillance systems are in place. The plans for estimation of effectiveness should also be agreed with NRAs at the time of licensure and the requirements for reporting effectiveness data to the NRA, either via the sponsor or directly from a public health authority, should be clarified.
It may be that reliable estimates of effectiveness can be obtained only in certain countries in which vaccination campaigns are initiated and where there is already a suitable infrastructure in place to identify cases. In addition, it would likely be inappropriate to extrapolate any estimates of effectiveness that are obtained to other modes of use (such as introducing the same vaccine to different or highly selected sectors of the population).

7. Safety

7.1 General considerations
All clinical trials that are conducted pre-licensure or post-licensure should include an exploration of safety.

The assessment of safety may be the primary objective, a co-primary objective or a secondary objective in a clinical trial. Since the methods for collection, analysis and interpretation of safety data during clinical trials contrast with those applicable to post-licensure routine safety surveillance they are considered separately below.

In principle, many of the approaches to documenting and reporting safety data during vaccine clinical trials and conducting vaccine pharmacovigilance activities are similar to those used for all medicinal products. The sections that follow should be read in conjunction with the extensive guidance that is available from numerous publications, and on the websites of WHO, the Council for International Organizations of Medical Sciences (CIOMS), ICH and individual regulatory bodies. The focus of the following sections is thus on a number of methods and practices that are different for vaccines compared to other medicinal products, and on issues that may need to be addressed because of vaccine composition.

7.2 Assessment of safety in clinical trials

7.2.1 Safety outcomes as primary or secondary end-points

7.2.1.1 Safety outcomes as primary end-points
When the assessment of safety is a primary objective of a clinical trial it is usual for the primary analysis to be based on a specific safety end-point (for example, rates of a certain AE or rates of AEs that may be part of a clinical syndrome of interest). The trial may or may not be powered to address the pre-specified hypothesis.

7.2.1.2 Safety outcomes as secondary end-points
When the assessment of safety or specific aspects of the safety profile is a secondary objective, trials are not usually powered a priori to support statistical
analyses of end-points such as rates of all, or of specific, AEs. Descriptive comparisons are commonly used to screen for any differences in AE rates between treatment groups. If statistical analyses of AE rates are conducted they should be pre-specified in the protocol and in the statistical analysis plan. If any findings indicate statistically significant differences in rates of AEs (overall or for specific AEs) between treatments then they should be interpreted with caution unless the trial was primarily designed to address pre-specified hypotheses regarding safety end-points. The biological plausibility that AEs that occur more frequently in the new candidate vaccine group may be related to vaccination should be taken into consideration when deciding on the need for further pre- or post-licensure clinical trials to investigate and quantify the potential risks.

7.2.2 Recording and reporting adverse events

7.2.2.1 Methods

AEs should be reported and recorded by investigators and sponsors according to detailed procedures described in the trial protocol. AEs should be classified according to a standardized terminology (such as ICH MedDRA) to enable their categorization by System Organ Class (SOC) and Preferred Term (PT). If the classification terminology is updated while the trial is being conducted then the clinical trial report should indicate how the changes affect the tabulations.

Expedited reporting of AEs that meet specific criteria should take place in accordance with the requirements of individual NRAs relevant to the location of the trial sites.

It is standard practice for vaccinees to be observed immediately after each dose (for example, for a defined period – commonly 20–60 minutes) for any severe immediate reactions (for example, severe hypersensitivity reactions requiring immediate medical attention).

It is usually expected that all AEs are collected from all randomized subjects for defined periods after each dose:

- Solicited signs and symptoms are usually recorded daily for at least 4–7 days after each dose (see section 7.2.2.2 below). Longer periods (for example, 10–14 days) may be appropriate for certain vaccines, such as those that replicate in recipients.
- Unsolicited AE reports are usually collected for the entire period between each dose or, for single doses or final doses of regimens, for approximately 4 weeks post-dose (see section 7.2.2.3 below).
- Reports of serious adverse events (SAEs) and any pre-specified AEs of special interest (AESIs) should be collected from all trial subjects for at least 6 months after the last dose of assigned treatment.
For vaccines that contain new adjuvants it is recommended that there should be follow-up for at least 12 months after the last dose to allow for the documentation of any autoimmune diseases or other immune-mediated AEs.

In trials involving large numbers of subjects (for example, vaccine efficacy trials) it may be acceptable for reports of non-serious AEs to be collected from a representative (and preferably randomized) subset or, occasionally, not at all, taking into account the safety profile observed in the previous trials and the number of subjects from which detailed safety data have already been obtained. In this case, reports of all SAEs and any pre-specified AESIs should be collected from all randomized subjects. It may be acceptable that only SAE and AESI reports are collected during long-term safety follow-up.

7.2.2.2 Solicited signs and symptoms

In most trials it is common practice for certain local and systemic AEs to be documented for a predefined period after each dose of a vaccine or placebo. The recording of AEs may be facilitated by the use of diary cards or other methods to ensure that the information is captured. If diary cards are used they may be completed by vaccinees, caregivers or by study staff who have questioned the vaccinees or their caregivers. These AEs are commonly referred to as “solicited signs and symptoms” since information on their occurrence is actively sought and they should be listed in the trial protocol.

For injectable vaccines the local signs and symptoms to be documented usually include, as a minimum, pain, redness and swelling at the injection site in all age groups. Pain should be graded according to a scoring system and preferably one that has been validated. Measuring devices of various types may be used to record the extent of redness and swelling.

Consideration should be given to assessing whether reports of pain are associated with immediate pain during and just after the injection or whether the pain is of later onset. If there is frequent reporting of pain at or around the injection site during the hours or days following vaccination this may suggest that the overall tolerability of the vaccine could negatively impact on vaccine uptake in routine immunization programmes. In these circumstances it may be appropriate to consider whether an attempt should be made to reformulate the vaccine to improve local tolerability.

When two or more vaccines are given by injection at the same time, the diary card should ensure that separate data are recorded for the new candidate vaccine injection site.

The systemic signs and symptoms to be collected and documented are determined by the age range in the trial (for example, those appropriate for
infants will not be wholly applicable to toddlers and older subjects) and by the route of administration (for example, nausea and vomiting could be solicited symptoms for vaccines given orally). Fever should be documented using digital thermometers and should be determined at a specific site (for example, rectal or axillary in infants). Recordings of fever should be made at predefined times and for a specified number of days after each dose. For subjective symptoms (for example, fatigue and myalgia) a simple scoring system should be included in the diaries to allow for the grading of severity.

Any self-administered treatments used to address signs or symptoms (such as antipyretic and analgesic medicines) and any contact with – or treatment administered by – a health-care professional should be captured. Instructions on the use of antipyretics and analgesics should be stated in the clinical trial protocol. If at the time of each dose a supply of a specific antipyretic or analgesic was provided for use as needed, or as instructed in accordance with the protocol, the post-dose usage recorded should be checked against returned supplies. If prior safety data suggest that pre-vaccination antipyretic use is appropriate then this can be administered and recorded by trial staff at the vaccination visit.

At each trial visit, whether involving face-to-face or telephone contact between the trial subject/caregiver and site staff, all diary cards completed by vaccinees or caregivers should be checked for level of completion and further instructions given as needed to improve data recording after the next dose is given. At face-to-face visits the prior vaccination site(s) should be inspected for any remaining signs such as induration. Trial subjects or caregivers should also be asked about the maximum extent of signs (for example, to determine whether whole limb swelling occurred). Any unresolved local or systemic signs and symptoms should be recorded and action taken as appropriate.

7.2.2.3 Unsolicited adverse events

Trial subjects/caregivers should be questioned at each visit on the occurrence of any AEs since the last visit or for predefined periods following the last dose. For each AE, the timing of onset in relation to vaccination should be captured, as should any consultation with a health-care professional, whether hospitalization occurred and any treatment that was given (prescribed or non-prescribed). If the AE is not already resolved there should be further follow-up to document the outcome.

It may be useful to pose specific questions to trial subjects/caregivers at each visit to ensure that certain AEs or AESIs are captured in a systematic fashion – for example, to determine whether persistent inconsolable crying or hypotonic-hyporesponsive episodes occurred in infants. Where well-established and widely applied definitions of these and other AEs are available, they should be included in the protocol.
For all AEs that meet the criteria for classification as SAEs there should be careful documentation of dates of onset, underlying conditions and concomitant medications, and adequate follow-up to record the outcomes.

7.2.2.4 Other investigations

The collection of data on routine laboratory tests (haematology, chemistry and urine analysis) is not necessary in many clinical trials of vaccines. If the sponsor or NRA considers that there is a good rationale for obtaining such data then the protocol should specify the tests to be performed at certain time points. The tests should be conducted in appropriately certified laboratories and results reported using well-established grading scales for laboratory abnormalities.

For vaccines that contain live organisms (including attenuated wild-types, organisms that have been genetically engineered to render them non-virulent and/or non-replicative, and live viral vector vaccines) additional investigations related to safety may include the detection of viraemia and assessments of shedding (quantity and duration) unless the omission of such studies can be justified (for example, on the basis of prior experience with the same or very similar strains and/or nonclinical data). Organisms recovered from vaccinees may also be subject to genetic analyses to determine any instances of recombination with wild-types and reversion to virulence and/or replication competency.

The release specifications for vaccines should take into account the safety profile documented for the highest amount(s) of antigen(s) that have been administered in the clinical trials. It may be necessary to support the final proposed release specification by conducting a trial with the primary objective of comparing safety between formulations that contain different numbers of live organisms or amounts of antigen(s).

7.2.3 Categorization of adverse events

7.2.3.1 Causality

Section 8.5 of the WHO Global manual on surveillance of adverse events following immunization (23) recommends that in clinical trials the investigator should make a judgement on relatedness to vaccination for all solicited signs and symptoms, and unsolicited AEs. The sponsor may have access to additional information that is not available to investigators and should assess causality for all SAEs. The assessment of relatedness to vaccination should take into account factors such as:

- plausibility of relatedness, taking into account the vaccine construct (for example, live-attenuated vaccines may be associated with modified manifestations of natural infection, such as rashes);
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■ timing in relation to dosing (while most vaccine-related AEs occur within 1–2 weeks of the dose, there may reasons to suspect that illnesses with onset many months after the last dose could be related to prior vaccination);
■ concurrent illnesses, vaccines or other medications;
■ the frequency with which any one AE occurred in groups that received the candidate vaccine compared to groups that received another vaccine or placebo;
■ any correlation between rates of any one AE and dose of antigenic components;
■ changes in rates of any one AE with sequential doses;
■ the results of medical investigations (for example, diagnostic tests for concurrent illnesses) and of autopsies (for example, in cases of sudden infant death).

7.2.3.2 Severity

Sufficient data should be collected for each solicited sign and symptom and unsolicited AE in order to assess severity. Wherever possible, widely used grading scales (including scales that may be age specific) should be used. The same scales should be applied throughout the clinical development programme.

7.2.3.3 Other categorization

The classification of AEs as serious and the categorization of frequencies (that is, very common, common, uncommon, rare and very rare) should follow internationally accepted conventions, as described in section 3.1.2 of the WHO Global manual on surveillance of adverse events following immunization (23). Frequencies of solicited signs and symptoms by subject and of AEs in each treatment group should be calculated on the basis of the denominator of all vaccinated subjects in that group. Calculation of the frequencies of solicited signs and symptoms after each dose should use as the denominator the number of subjects who received each dose.

7.2.4 Adverse event reporting rates within and between trials

During any clinical development programme the reporting rates in clinical trials for all AEs and/or for specific types of AEs, whether solicited or unsolicited, may demonstrate the following:

■ Differences between candidate vaccines and control groups within a clinical trial. For example, differences in AE rates may be anticipated between a candidate vaccine group and a placebo group or a
that receives a licensed vaccine that does not have a similar composition to the candidate vaccine. Any marked differences between a candidate vaccine and a licensed vaccine that has the same or very similar composition are generally not anticipated and may require further investigation.

- Differences between clinical trials that may be observed in one or both of the candidate vaccine and control groups for total or specific AE reporting rates. It is important to consider possible explanations, taking into account whether or not the same effect on the pattern of reporting rates was observed in groups that received candidate vaccines and licensed vaccines and whether the study was double-blind or open-label. There may be real and anticipated differences in vaccine reactogenicity between trial populations (for example, age-related differences for specific AEs, such as higher fever rates in trials conducted in infants and toddlers compared to trials in older children and adults). When there is no clear explanation for the differences observed, further investigation is merited. For example, there may have been incomplete reporting of AEs or data-entry errors, as well as cultural factors that lead to a greater reluctance to report side-effects in some regions.

7.3 Size of the pre-licensure safety database

The size of the pre-licensure safety database must be considered on a case-by-case basis and agreed with relevant NRAs. It is not possible to predefine a minimum number of exposed subjects (usually confined to the number exposed to the final dose and regimen appropriate for their age group and who received the final vaccine formulation) that can be generally applied across vaccine development programmes.

When considering the pre-licensure safety database the need for a sufficient sample size to estimate AE rates with precision is an important factor. For example, a total database of 3000 subjects across all trials and populations provides a 95% chance of observing one instance of an AE that occurs on average in 1 in 1000 subjects.¹ Nevertheless, this figure should not be assumed to be appropriate in all settings. In particular, this figure should not be applied to application dossiers for any type of new candidate vaccine without further consideration. When considering the size of the pre-licensure safety database, factors to take into account include, but are not limited to, the following:

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¹ The number that would provide a 95% chance of observing one instance of an AE that occurs on average in 1 in 10 000 subjects is 30 000.
Fewer than 3000 subjects may be acceptable if the new candidate vaccine consists only of antigenic components that are already licensed in other vaccines with which there is considerable experience in routine use. The method of manufacture should also be taken into account.

For specific types of vaccines (for example, new constructs or new adjuvants) or specific modes of use (for example, in a population considered to be vulnerable or otherwise at high risk that could predispose it to certain AEs) individual NRAs may require that considerably more than 3000 subjects are exposed prior to licensure.

Additional considerations may apply to vaccines that contain antigenic components not previously used in human vaccines but for which efficacy trials are not possible. For example, the safety profile documented in the preliminary trials may lead to reluctance to expose large numbers of subjects unnecessarily in the absence of an immediate threat and/or to expose large numbers in particular population subsets.

The acceptable size of the pre-licensure safety database should take into account the actual safety profile observed in the clinical trials. If there is concern regarding the occurrence and/or severity of a particular AE and the available safety data do not allow for a clear assessment of risk then, depending on the perceived benefit of the vaccine, it may be appropriate to conduct further pre-licensure trials and/or to conduct a post-licensure safety study to better estimate the risk.

The total number of subjects exposed in clinical trials may cover many age subgroups, or a single age group may predominate. In general there should be adequate representation of all target age groups in the total safety database. In some cases, and depending on the actual safety profile, it may be acceptable for the majority of subjects included in the safety database to come from a specific age range.

7.4 Post-licensure safety surveillance

The main purpose of post-licensure safety surveillance is to detect AEs that occur too infrequently for detection in pre-licensure clinical trials.

The requirements of individual NRAs for reporting safety data collected from post-licensure safety surveillance activities should be consulted along with other guidance such as ICH E2E. NRAs should provide publicly available guidance regarding their requirements for the content and timing of periodic reports of safety data and for any expedited reporting considered necessary.
Licence holders should demonstrate that they have adequate capability and appropriate staff to collect, interpret and act upon the safety data received. It is important that efforts are made to accurately identify the vaccine(s) and lot number(s) associated with each AEFI report.

It has become routine at the time of licensure for detailed proposals to be in place for post-licensure safety surveillance activities, often in the form of risk-management plans. These documents and proposals are then routinely updated at intervals in line with additional data that become available. The plans usually outline the safety specification for the vaccine on the basis of all available safety data at the time of submitting each version of the plan, along with details of routine and proposed additional pharmacovigilance and risk-minimization activities.

When planning pharmacovigilance activities for a vaccine it is important to take into account that, in addition to routine pharmacovigilance (that is, passive surveillance), important information may come from other sources, namely:

- Data from active safety surveillance, which may be put in place by public health bodies when a vaccine is introduced into a national routine immunization programme, or when the use of a vaccine within a programme changes significantly (for example, an entirely different age group is vaccinated for the first time). Active surveillance seeks to ascertain completely the number of AEs in persons given a dose of a vaccine using a pre-organized process. It may involve reviewing medical records or interviewing patients and/ or physicians in a sample of sentinel sites to ensure that complete and accurate data are collected on reported AEs from those sites.

- Large databases that link information on vaccination history in patient records with the occurrence of specific types of illness. These databases can be searched to explore links between specific vaccines and safety issues in the short and longer term.

- Various types of registries intended to capture details of vaccine use in specific populations. For example, some registries collect information on exposure of pregnant women to various types of vaccines and indicate the outcome of the pregnancy (including rates of spontaneous abortion, premature delivery and congenital malformations in infants).

The limitations of each of these approaches are well known. Furthermore, access to information from these other sources varies greatly between countries. These factors underline the need to consider safety data from all sources along with data that may come from post-licensure trials.
An additional consideration for vaccines is that when a safety signal is identified for any one vaccine it may or may not be possible to ascribe the AEFIs observed to any one antigenic component of the vaccine or to an adjuvant. Furthermore, if there was concomitant administration of vaccines in some or all cases generating the signal, it may not be possible to ascribe the AEFI to only one of the products co-administered. The same or very similar antigenic component(s) or adjuvant in the vaccine(s) from which the signal arose may be present in several other licensed products marketed worldwide. Ultimately, several different licence holders and NRAs without established data-sharing agreements may need to be involved. As a result, the actions taken, if any, and the speed at which action is taken are sometimes very variable between countries. Such issues underscore the need for the efficient use of electronic databases to facilitate rapid data sharing.

Authors and acknowledgements

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The draft document was then posted on the WHO Biologicals website for a second round of public consultation from 1 February to 15 March 2016. Comments were received from: Dr B. Brock, Sanofi Pasteur, the USA (provided the consolidated comments of the International Federation of Pharmaceutical Manufacturers & Associations (IFPMA)); Dr K. Farizo, United States Food and Drug Administration, Center for Biologics Evaluation and Research, the USA; Dr C. Meric, Lausanne University Hospital, Switzerland; Mr J.F. Modlin, Bill & Melinda Gates Foundation, the USA; Dr D. Pratt, United States Food and Drug Administration, Center for Biologics Evaluation and Research, the USA; Dr A. Rinfret, Health Canada, Canada; and Dr K. Sohn, Ministry of Food and Drug Safety, Republic of Korea.

A WHO meeting of the Working Group on clinical evaluation of vaccines was then held in Geneva, Switzerland, 3 May 2016 and was attended by the following participants: Dr G. Coleman, Health Canada, Canada; Dr M. Darko, Food and Drugs Authority, Ghana; Dr D. Etuko, National Drug Authority, Uganda; Dr E. Griffiths, Consultant, Kingston-upon-Thames, England; Dr S. Kennedy, University of Liberia, Liberia; Dr J. McEwen, Therapeutic Goods Administration, Australia; Dr M. Powell, Medicines and Healthcare products Regulatory Agency, England; Dr R. Sheets, Consultant, Silver Spring (MD), the USA; Dr J. Southern, Medicines Control Council, South Africa; Dr Y. Sun, Paul-Ehrlich-Institut, Germany; Dr K. Zoon, National Institutes of Health, the USA; and Dr I. Knezevic, World Health Organization, Switzerland.

Based on the comments received during the public consultation and on the discussions of the above Working Group meeting, the document WHO/BS/2016.2287 was prepared by the above-mentioned WHO drafting group.
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References


Annex 10

Human challenge trials for vaccine development: regulatory considerations

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Guidance documents published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products.
Abbreviations

GCP           good clinical practice
GMO           genetically modified organism
ICP           immune correlate of protection
NRA           national regulatory authority
1. Introduction

Infectious human challenge trials involve the deliberate exposure of human volunteers to infectious agents. Trial participants are intentionally challenged (whether or not they have been vaccinated) with an infectious disease organism. This challenge organism may be close to wild-type and pathogenic, adapted and/or attenuated from wild-type with less or no pathogenicity, or genetically modified in some manner.

Human challenge trials have been conducted over hundreds of years and have contributed vital scientific knowledge that has led to advances in the development of drugs and vaccines. Nevertheless, such research can appear to be in conflict with the guiding principle in medicine to do no harm. A number of well-documented historical examples of human exposure studies would be considered unethical by current standards. It is essential that challenge trials be conducted within an ethical framework in which truly informed consent is given. When conducted, human challenge trials should be undertaken with abundant forethought, caution and oversight. The value of the information to be gained should clearly justify the risks to human subjects.

Although human challenge trials are not a required element of every vaccine development programme, there are many reasons why a developer may ask to conduct a “challenge-protection” study with humans, which might normally be conducted in animals. Animal models are often quite imprecise in reflecting human disease, and many infectious organisms against which a developer might wish to develop a vaccine are species-specific for humans. Human challenge trials may be safely and ethically performed in some cases, if properly designed and conducted. Considerable insight can then be gained into the mode of action and potential benefit of drugs and vaccines in humans. However, there are also limitations on what challenge trials may be able to ascertain because, as with animal-model challenge-protection studies, a human challenge trial represents a model system. Nevertheless, because there are often such significant limitations to animal models, the model system of a human challenge trial may significantly advance, streamline and/or accelerate vaccine development (1).

It is important to note that not all diseases for which vaccines might be developed are suitable for conducting human challenge trials. In many cases, human challenge with a virulent or even attenuated organism would not be considered ethical or safe. For example, if an organism causes a disease with a high case-fatality rate (or there is a long and uncertain latency period) and there are no existing therapies to prevent or ameliorate disease and preclude death, then it would not be appropriate to consider human challenge trials with such an organism. However, a human challenge trial might be considered when the
disease an organism causes has an acute onset, can be readily and objectively detected, and existing efficacious treatments (whether curative or palliative) can be administered at an appropriate juncture in disease development to prevent significant morbidity and eliminate mortality.

It will also be important to consider the regulatory framework in which the human challenge trial may be conducted. In some countries, challenge stocks are expected to be regulated in the same manner as vaccines, and are expected to be studied with authorization in accordance with clinical trial regulations, whether or not an investigational vaccine is to be used in the same clinical investigation protocol. For example, a challenge trial might be conducted to titrate the challenge organism in humans (before using the challenge in a vaccine study) in order to determine the proper dose of the challenge organism to administer, and to characterize the symptoms, kinetics, shedding and transmissibility to be expected from the challenge. The dose of challenge organism is usually titrated to induce a relatively high attack rate while limiting disease severity. In cases where the challenge should be studied in compliance with clinical trial regulations there is greater clarity about regulatory expectations, including the quality of the challenge stock to be used, because the clinical trial regulations or requirements would apply. However, in many countries, because the challenge stock is not itself considered to be a medicinal product, such characterization/model development studies would not come under national regulatory authority (NRA) review and authorization. Thus, much less clarity would exist on regulatory expectations and issues of quality in such cases.

It should be understood that a pathogenic challenge strain will not have the “safety” of an intended safe candidate vaccine. However, its quality should be comparable to a candidate vaccine at the same clinical trial phase. Ideally, a human challenge trial to establish the challenge model (that is, without use of an investigational medicinal product) should also match the expectations for conducting a vaccine study – that is, compliance with good clinical practice (GCP) and subject to approval or concurrence under a Clinical Trial Authorization by NRAs and ethics committees on the basis of requirements appropriate for this type of study. If such a framework does not exist, countries are encouraged to establish an appropriate regulatory and ethical framework for challenge trials. However, there may be no regulatory framework to promulgate such expectations in the country where the challenge study is to be conducted. Trial sponsors, vaccine developers, researchers and other involved parties should determine what regulatory expectations the relevant NRA may have when clarity does not exist and when the human challenge study is intended to support the development of a vaccine candidate they would ultimately like to license (that is, obtain marketing authorization or registration).
2. Background

In July 2014, WHO held a consultation on Clinical evaluation of vaccines: regulatory expectations (2). One area that was considered to be an important element in facilitating vaccine development was human challenge trials. It was recognized that the regulation of such trials needed to be well-defined by NRAs and that vaccine developers and manufacturers needed to be aware of regulatory expectations in this area.

This WHO guidance document on human challenge trials should be read in conjunction with the updated WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (3) which were adopted, along with the current document, by the WHO Expert Committee on Biological Standardization in October 2016.

3. Purpose and scope

The purpose of this document is to provide guidance to NRAs, manufacturers, vaccine developers, investigators and independent ethics committees – and potentially to biosafety committees and national agencies that regulate genetically modified organisms (GMOs) where separate from the NRA. The document only covers issues specifically relevant to the design and conduct of clinical trials that enrol healthy adult humans capable of truly informed consent, and that involve the intentional exposure to, and potential infection with, an infectious disease organism. All other issues common to the design, conduct and evaluation (assessment) of vaccine clinical trials may be found in the updated WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (3).

4. Purposes of human challenge trials in vaccine development

Human challenge trials are considered as a model by which challenge protection can be evaluated and represent one possible approach for vaccine development.

Therefore, all principles for the clinical evaluation of vaccines should apply, including the need for approval by the NRA and ethical committees as well as compliance to GCP.

A vaccine developer may conduct human challenge trials to accomplish one or more aims. The aims of the study determine the clinical phase in which the study is conducted. Human challenge trials are often a type of efficacy-indicating study, but most would not be considered to be pivotal efficacy studies. Almost all would be pilot in nature and performed to gain useful information to aid in the
development of a vaccine. Several challenge trials might be performed during the course of vaccine development.

Potential purposes of human challenge trials could include one or more of the following:

- characterization of the challenge stock and model system in terms of titration, symptoms, kinetics, shedding and transmissibility;
- clearer understanding of the pathogenesis of, and immunity to, the organism in order to guide decisions on what immune responses (type and/or quantity) a vaccine might need to elicit in order to protect against that disease as part of gaining insight into vaccine design – studies for this purpose may be referred to as experimental medicine studies;
- identification of potential immune correlates of protection (ICPs) which would then require validation in a traditional efficacy study;
- identification of the optimal design for traditional pivotal efficacy trial(s) – for example, case definitions, end-points and other study design aspects;
- generation of appropriate hypotheses to be formally tested in traditional efficacy trials;
- proof of concept as to whether a particular vaccine candidate might provide protection or not;
- down- or up-selection of various potential lead vaccine candidates to advance only the best to large pilot or pivotal efficacy trials and to eliminate those not worth advancement;
- de-risk or “left-shift”¹ risk of failure in a vaccine development programme;
- comparison of vaccine performance in endemic settings versus an efficacy trial population,² including evaluating the impact of prior immunity in the context of prevalent endemic diseases and conditions;

¹ When the timeline of vaccine development is viewed as a graph from early to the left to late to the right, shifting the risk of failure earlier (left) in the timeline could: (a) minimize risk to human subjects by avoiding large efficacy studies of vaccines that would not prove efficacious; (b) result in significant cost and resource savings; and (c) minimize lost opportunity costs by abandoning an unpromising candidate before committing greater expenditures to higher-phase clinical trials.

² The target population in a particular country may have a higher rate of individuals with, for example, sickle cell trait, poorer nutritional status or greater parasitic load in “normal” flora – any of which might affect immune responsiveness in the endemic setting and thus efficacy (benefit) compared to the efficacy trial population (ideal setting) or safety (greater risks). Either of these would have an impact on the risk–benefit decision-making.
- support for emergency use of an investigational vaccine (for example, during an influenza pandemic);
- provision of a basis for licensure (this purpose would be a rare exception rather than routine);
- post-licensure exploration of whether immunity following vaccination wanes, and if or when booster doses might be required for durable protection;\(^3\)
- others.

No single study could accomplish all of the above aims. For example, if the human challenge model system does not adequately mimic the wild-type disease and the actual situation in which a vaccine would need to provide protection, then a human challenge trial would not be usable as a primary basis for licensure.

5. Study design of human challenge trials

As in all studies, the aim(s) of the human challenge trial guides the study design. Consequently, even for the same disease, the challenge model may vary according to the purposes and design of the study to be conducted. In some cases (for example, to identify appropriate efficacy trial design and case definitions) the challenge model may need to mimic wild-type disease as closely as feasible. In other cases, consideration might be given to the use of an attenuated challenge organism (for example, a previous vaccine candidate) or to a model system in which objective early signs (for example, parasitaemia or viraemia) signal the onset of disease. These signals could then trigger initiation of treatment to prevent actual disease onset or morbidity. Such initiation of treatment should be based on criteria pre-specified in the study protocol.

Another important consideration for a human challenge model system would be its usefulness for positive or negative prediction. If used for down-selection, de-risking or to identify vaccine candidates that would not warrant advancement to large human efficacy studies, the degree of usefulness of the model system for negative prediction should be high. If intended to be used for evidence of vaccine efficacy, the degree of usefulness for positive prediction might need to be almost as compelling and credible as for a traditional pivotal efficacy trial. Whether the purpose of the study or studies is to provide supportive evidence for licensure or to help inform and design traditional efficacy studies or vaccine design, human challenge trials may contribute to the preponderance of

\(^3\) This might entail a challenge study in adults to extrapolate when children might need booster doses.
evidence upon which regulators could take a clinical trial or licensure decision. Thus, the purpose of the study would influence its design, which would in turn influence the conclusions and decisions that might be made by regulators following consideration of the study results.

6. Operational aspects

In addition to general principles for all clinical trials in human subjects there are some unique and important operational aspects to consider when conducting a human challenge trial. Human challenge trials should be undertaken in accordance with a protocol, and in special facilities that are designed and operated in a manner that prevents the spread of the challenge organism to people outside the study or to the environment. These clinical facilities should be capable of providing continuous monitoring and medical attention at the appropriate point(s) in time after the challenge is given. In addition to providing immediate access to appropriate medical care and treatment, the facilities should be designed to prevent the spread of disease, particularly when the challenge organism is a GMO or an organism that is not endemic to the locality. These facilities may need to be operated in a manner that permits all waste (including excrement) to be collected and decontaminated before release. All staff, including janitorial and administrative staff, might be required to work in personal protective equipment appropriate for the pathogenicity of the challenge organism and its potential hazard to the environment, and should be informed of the potential risks. It should be noted that not all human challenge trials require such a high level of control. When the challenge organism is attenuated and the wild-type organism is likely to be present in the locality anyway, it may be adequate to conduct human challenge trials in an outpatient setting or with appropriate procedures to prevent spread. Examples of such approaches and procedures include the use of BCG vaccine as a challenge organism, the use of bandaging to cover and prevent spread from an intramuscular injection (assuming the organism is not shed by other means) and the use of malaria challenge during winter months in a temperate region. There may be other circumstances in which a human challenge trial is undertaken, for example where the target organism of the vaccine to be developed is not present in the location where the target group for its indication lives (for example, in case of a traveller vaccine) – when the risk of spread of the organism is low, human challenge trials using appropriate procedures could be undertaken.

It may be necessary to ensure that controls and vaccinees are housed together if an objective of the human challenge trial is to identify the potential for transmissibility. In such a situation, only the vaccinees or unvaccinated participants would be challenged, and the controls (who were not challenged)
would be monitored for evidence of acquiring the challenge organism through contact with the challenged vaccinees. In this way, the transmissibility of the challenge organism from challenged vaccinees may be determined. In order to achieve the study objective of identifying transmissibility, it would be necessary to conduct the study in-house even if the challenge organism was attenuated and the wild-type organism was present in the locality.

It should be noted that human challenge trials have been, and can be, successfully conducted in low- and middle-income settings. The investigators need to be qualified, an independent ethics committee review is required, and assurance of compliance with NRA requirements and regulations is needed. If relevant, assurance of compliance with the national agency that regulates GMOs and/or with local biosafety committees may also be needed. If a controlled inpatient setting is required for the given study, this would also need to be in place.

7. Some key ethical considerations

Ethics in clinical trials include the precept of “minimizing risks to subjects and maximizing benefits” and clinical trials should be designed and conducted accordingly. Review of the proposed human challenge trial by an independent ethics committee is essential. By their nature (that is, intentional infection of humans with disease-causing organisms) human challenge trials would seem to contradict this basic precept. Consideration must therefore be given to both potential individual risks and benefits, as well as to potential societal risks and benefits, such as the release into the environment of a pathogen that might not otherwise be present. Provisions in clinical trial ethics are made for situations in which there may be greater than minimal risk but no (or little) potential for individual benefit when knowledge may be gained that benefits the larger societal population with whom the potential trial participant shares significant characteristics.

The ethical considerations concerning challenges in clinical trials should be thoroughly evaluated. During a WHO Expert Consultation held in January 2013 consideration was given to the way in which ethical principles should be applied to vaccine trials. The main consultation topic concerned the use of placebo in such trials, and a set of considerations for NRAs and ethics committees was provided in the meeting report (4) and subsequently published recommendations (5). Although specifically intended to facilitate review of the proposed use of placebo in vaccine trials on a case-by-case basis these considerations and recommendations are likely to have applicability to human challenge trials.

It has to be acknowledged that in reality some individuals are greater risk-takers than others, and that those who are risk-averse would be unlikely to
accept the risk of receiving a challenge. The key to asking individuals to accept
the risk from a challenge study (in which they have little potential to receive
individual benefit) lies in the element of informed consent. Healthy adults may
consent when they are well informed and understand what the risks are that
they are agreeing to take – even if those risks may be considerably greater than
minimal (for example, accepting that they will develop an acute, but manageable,
disease that will resolve but in the meantime may cause considerable morbidity,
such as severe diarrhoea managed with fluid and electrolyte replacement). There
could be some potential for direct benefit should the trial participant become
immune to the disease caused by the challenge (or wild-type) organism but,
conversely, pre-existing immunity upon exposure to the wild-type organism in
the future may be harmful. Thus, in appropriate situations, it may be considered
ethical to ask healthy and informed adults to consent to volunteer and participate
in a human challenge trial whether they will receive an investigational vaccine
that may or may not protect them from the challenge organism, a placebo that
will not protect them or only the challenge organism itself. However, it is an
absolute requirement that accepting such risks and providing voluntary consent
are based upon being truly informed. For this reason (the absolute requirement
for truly informed consent) it is not deemed acceptable at this time to consider
conducting human challenge trials in children, or in any other vulnerable
population with diminished capacity to give informed consent. One possible
exception to this principle that might be considered would be a challenge model
that used a licensed live-attenuated vaccine as the challenge organism.

The need to minimize the risks to subjects in clinical trials calls for
investigators to give due consideration to whether the challenge organism needs
be pathogenic or not, or to what degree. As noted above, the aim or purpose of
the study may drive decisions on pathogenicity or attenuation, but the ethical
precept of minimizing risks to human subjects – to the maximum extent feasible
within the framework of sound science – should be given due consideration.
Key to such considerations is the credibility of the data to support regulatory
decision-making, which also needs to be taken into account when deciding how
pathogenic or attenuated the challenge organism needs to be.

Authors and acknowledgements

The first draft of this WHO guidance document was prepared by Dr R. Sheets,
Consultant, Silver Spring (MD), the USA and Dr I. Knezevic, World Health
Organization, Switzerland, with inputs from: Dr J. McEwen, Therapeutic Goods
Administration, Australia; Dr M. Powell, Medicines and Healthcare products
Regulatory Agency, England; and Dr V. Moorthy, World Health Organization,
Switzerland. The authors would like to acknowledge the publications of Dr M.T.
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Grimalklin Partners, the USA; and particularly the report of the conference organized by the International Alliance for Biological Standardization on Human Challenge Trials in October 2014, which served as an important source of information during the preparation of this document.

The proposal to prepare WHO guidance on human challenge trials was developed during a WHO Consultation on clinical evaluation of vaccines held in Geneva, Switzerland, 17–18 July 2014, and attended by the following participants: Dr P. Annunziato, Merck & Co., the USA; Dr N. Bhat, Program for Appropriate Technology in Health, the USA; Dr A. Chatterjee, Biological E Ltd, India; Dr K. Chirgwin, Bill & Melinda Gates Foundation, the USA; Dr G. Coleman, Health Canada, Canada; Dr D. Tuan Dat, The Company for Vaccines and Biological Production No. 1 (Vabiotech), Viet Nam; Dr P.E. Fast, International AIDS Vaccine Initiative, the USA; Dr G. Foglia, Sanofi Pasteur, the USA; Dr M. Gruber, United States Food and Drug Administration, Center for Biologics Evaluation and Research, the USA; Dr P.M. Heaton, Bill & Melinda Gates Foundation, the USA; Dr D. Kaslow, Program for Appropriate Technology in Health, the USA; Dr Y.H. Lee, Ministry of Food and Drug Safety, Republic of Korea; Dr D.J.M. Lewis, University of Surrey, England; Dr A. Lommel, Paul-Ehrlich-Institut, Germany; Dr J. McEwen, Therapeutic Goods Administration, Australia; Dr P. Neels, Vaccine-Advice BVBA, Belgium; Dr M. Nijs, GlaxoSmithKline Biologicals, Belgium; Dr S.A. Nishioka, Ministry of Health, Brazil; Dr A. Podda, Novartis Vaccines Institute for Global Health, Italy; Dr M. Powell, Medicines and Healthcare products Regulatory Agency, England; Dr A. Ramkishan, Central Drugs Standard Control Organization, India; Dr R. Sheets, Consultant, Silver Spring (MD), the USA; Dr J. Shin, WHO Regional Office for the Western Pacific, Philippines; Dr P. Smith, London School of Hygiene and Tropical Medicine, England; Dr J. Southern, Medicines Control Council, South Africa; Dr Y. Sun, Paul-Ehrlich-Institut, Germany; Dr Z. Yang, Center for Drug Evaluation, China; and Dr U. Fruth, Dr I. Knezevic, Mr O.C. Lapujade, Dr V. Moorthy, Dr K. Vannice and Dr D. Wood, World Health Organization, Switzerland.

The resulting draft document was posted on the WHO Biologicals website (as an appendix to the WHO Guidelines on clinical evaluation of vaccines) for the first round of public consultation from 30 October to 30 November 2015.

The second draft was prepared by a WHO drafting group and posted on the WHO Biologicals website (as an appendix to the WHO Guidelines on clinical evaluation of vaccines) for a second round of public consultation from 1 February to 15 March 2016. Comments were received from: Dr B. Brock, Sanofi Pasteur, the USA (provided the consolidated comments of the International Federation of Pharmaceutical Manufacturers & Associations (IFPMA)); Dr K. Farizo, United States Food and Drug Administration, Center for Biologics Evaluation and Research, the USA; and Dr A. Rinfret, Health Canada, Canada.
At a WHO meeting of the Working Group on clinical evaluation of vaccines held in Geneva, Switzerland, 3 May 2016 it was concluded that this WHO guidance document should be provided as a separate document rather than as an appendix to the WHO Guidelines on clinical evaluation of vaccines. The meeting was attended by: Dr G. Coleman, Health Canada, Canada; Dr M. Darko, Food and Drugs Authority, Ghana; Dr D. Etuko, National Drug Authority, Uganda; Dr E. Griffiths, Consultant, Kingston-upon-Thames, England; Dr S. Kennedy, University of Liberia, Liberia; Dr J. McEwen, Therapeutic Goods Administration, Australia; Dr M. Powell, Medicines and Healthcare products Regulatory Agency, England; Dr R. Sheets, Consultant, Silver Spring (MD), the USA; Dr J. Southern, Medicines Control Council, South Africa; Dr Y. Sun, Paul-Ehrlich-Institut, Germany; Dr K. Zoon, National Institutes of Health, the USA; and Dr I. Knezevic, World Health Organization, Switzerland.

Based on the comments received during the public consultation and on the discussions of the above Working Group meeting, the document WHO/BS/2016.2288 was prepared by Dr R. Sheets and Dr I. Knezevic.

The document was then posted on the WHO Biologicals website for a third round of public consultation from 27 July to 16 September 2016 and comments received from: Dr J. Auerbach and Dr A. Podda, GSK Vaccines Institute for Global Health, Italy; Dr M. Gruber and Dr D. Pratt, United States Food and Drug Administration, Center for Biologics Evaluation and Research, the USA; Dr P. Njuguna, KEMRI Wellcome Trust Research Programme, Kenya; and Dr P. Smith, London School of Hygiene and Tropical Medicine, England.

Further changes were subsequently made to document WHO/BS/2016.2288 by the WHO Expert Committee on Biological Standardization.

References


Annex 11

Biological substances: WHO International Standards, Reference Reagents and Reference Panels

The provision of global measurement standards is a core normative WHO activity. WHO reference materials are widely used by manufacturers, regulatory authorities and academic researchers in the development and evaluation of biological products. The timely development of new reference materials is crucial in harnessing the benefits of scientific advances in new biologicals and in vitro diagnosis. At the same time, management of the existing inventory of reference preparations requires an active and carefully planned programme of work to replace established materials before existing stocks are exhausted.

The considerations and guiding principles used to assign priorities and develop the programme of work in this area have previously been set out as WHO Recommendations. In order to facilitate and improve transparency in the priority-setting process, a simple tool was developed as Appendix 1 of these WHO Recommendations. This tool describes the key considerations taken into account when assigning priorities, and allows stakeholders to review and comment on any new proposals being considered for endorsement by the WHO Expert Committee on Biological Standardization.

A list of current WHO International Standards, Reference Reagents and Reference Panels for biological substances is available at: http://www.who.int/biologicals.

At its meeting in October 2016, the WHO Expert Committee on Biological Standardization made the changes shown below to the previous list.

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## Additions

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood products and related substances</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ancrod</td>
<td>54 IU/ampoule</td>
<td>Second WHO International Standard</td>
</tr>
<tr>
<td>Batroxobin</td>
<td>50 U/ampoule</td>
<td>First WHO Reference Reagent</td>
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<tr>
<td>Blood coagulation factor XI (plasma, human)</td>
<td>FXI:C = 0.71 IU/ampoule, FXI:Ag = 0.78 IU/ampoule</td>
<td>Second WHO International Standard</td>
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<td>Thromboplastin (recombinant, human, plain)</td>
<td>1.11 IU/mL</td>
<td>Fifth WHO International Standard</td>
</tr>
<tr>
<td>Thromboplastin (rabbit, plain)</td>
<td>1.21 IU/mL</td>
<td>Fifth WHO International Standard</td>
</tr>
<tr>
<td><strong>In vitro diagnostics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zika virus RNA for NAT-based assays*</td>
<td>50 000 000 IU/mL</td>
<td>First WHO International Standard</td>
</tr>
<tr>
<td>Ebola virus VP40 antigen</td>
<td>Panel containing low- and medium-titre VP40 samples plus negative sample; no unitage assigned</td>
<td>First WHO Reference Panel</td>
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<tr>
<td>Dengue virus serotypes 1–4 RNA for NAT-based assays**</td>
<td>Four separate reference reagents with the following assigned values: DENV-1 RNA = 13 500 units/mL; DENV-2 RNA = 69 200 units/mL; DENV-3 RNA = 23 400 units/mL; DENV-4 RNA = 33 900 units/mL</td>
<td>First WHO reference reagents</td>
</tr>
<tr>
<td>Hepatitis B virus DNA for NAT-based assays</td>
<td>5.98 log&lt;sub&gt;10&lt;/sub&gt; IU/mL</td>
<td>Fourth WHO International Standard</td>
</tr>
</tbody>
</table>

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2 Unless otherwise indicated, all materials are held and distributed by the National Institute for Biological Standards and Control, Potters Bar, Herts, EN6 3QG, England. Materials identified by an * in the above list are held and distributed by the Paul-Ehrlich-Institut, 63225 Langen, Germany. Materials identified by an ** in the above list are held and distributed by the Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, MD 20993, the USA.
<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity</th>
<th>Status</th>
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<td>Prolactin (pituitary, human)</td>
<td>67 mIU/ampoule</td>
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<tr>
<td>Janus kinase 2 V617F gene mutation</td>
<td>Panel of JAK2 V617F DNA concentrations of 0, 0.03, 1.0, 10.8, 29.6, 89.5 and 100%</td>
<td>First WHO Reference Panel</td>
</tr>
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</table>
The World Health Organization was established in 1948 as a specialized agency of the United Nations serving as the directing and coordinating authority for international health matters and public health. One of WHO's constitutional functions is to provide objective and reliable information and advice in the field of human health, a responsibility that it fulfills in part through its extensive programme of publications.

The Organization seeks through its publications to support national health strategies and address the most pressing public health concerns of populations around the world. To respond to the needs of Member States at all levels of development, WHO publishes practical manuals, handbooks and training material for specific categories of health workers; internationally applicable guidelines and standards; reviews and analyses of health policies, programmes and research; and state-of-the-art consensus reports that offer technical advice and recommendations for decision-makers. These books are closely tied to the Organization's priority activities, encompassing disease prevention and control, the development of equitable health systems based on primary health care, and health promotion for individuals and communities. Progress towards better health for all also demands the global dissemination and exchange of information that draws on the knowledge and experience of all WHO's Member countries and the collaboration of world leaders in public health and the biomedical sciences.

To ensure the widest possible availability of authoritative information and guidance on health matters, WHO secures the broad international distribution of its publications and encourages their translation and adaptation. By helping to promote and protect health and prevent and control disease throughout the world, WHO's books contribute to achieving the Organization's principal objective – the attainment by all people of the highest possible level of health.

The WHO 'Technical Report Series' makes available the findings of various international groups of experts that provide WHO with the latest scientific and technical advice on a broad range of medical and public health subjects. Members of such expert groups serve without remuneration in their personal capacities rather than as representatives of governments or other bodies; their views do not necessarily reflect the decisions or the stated policy of WHO.

For further information, please contact: WHO Press, World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland (tel. +41 22 791 3264; fax: +41 22 791 4857; email: bookorders@who.int; order online: www.who.int/bookorders).

Further information on these and other WHO publications can be obtained from WHO Press, World Health Organization, 1211 Geneva 27, Switzerland (tel.: +41 22 791 3264; fax: + 41 22 791 4857; email: bookorders@who.int; order online: www.who.int/bookorders).
This report presents the recommendations of a WHO Expert Committee commissioned to coordinate activities leading to the adoption of international recommendations for the production and control of vaccines and other biological substances, and the establishment of international biological reference materials.

Following a brief introduction, the report summarizes a number of general issues brought to the attention of the Committee. The next part of the report, of particular relevance to manufacturers and national regulatory authorities, outlines the discussions held on the development and revision of WHO Guidelines for a number of vaccines, blood products and related substances. Specific discussion areas included WHO guidance on the production and evaluation of the quality, safety and efficacy of monoclonal antibodies as similar biotherapeutic products (SBPs); blood and blood components as essential medicines; estimation of residual risk of HIV, HBV or HCV infections via cellular blood components and plasma; snake antivenom immunoglobulins; human pandemic influenza vaccines in non-vaccine-producing countries; and clinical evaluation of vaccines: regulatory expectations. In addition, the following WHO guidance documents were also adopted: WHO manual for the preparation of secondary reference materials for in vitro diagnostic assays designed for infectious disease nucleic acid or antigen detection; calibration to WHO International Standards; and Human challenge trials for vaccine development: regulatory considerations. One WHO addendum document – Labelling information of inactivated influenza vaccines for use in pregnant women – was also adopted.

Subsequent sections of the report provide information on the current status, proposed development and establishment of international reference materials in the areas of: biotherapeutics other than blood products; blood products and related substances; cellular and gene therapies; in vitro diagnostics; and vaccines and related substances.

A series of annexes are then presented which include an updated list of all WHO Recommendations, Guidelines and other documents on biological substances used in medicine (Annex 1). The above nine WHO documents adopted on the advice of the Committee are then published as part of this report (Annexes 2–10). Finally, all additions and discontinuations made during the 2016 meeting to the list of International Standards, Reference Reagents and Reference Panels for biological substances maintained by WHO are summarized in Annex 11. The updated full catalogue of WHO International Reference Preparations is available at: http://www.who.int/bloodproducts/catalogue/en/.