WHO Technical Report Series
910

WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Fifty-first Report

World Health Organization
Geneva

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WHO Library Cataloguing-in-Publication Data

Typeset in Hong Kong
Printed in Singapore
2001/14230 — SNPBest-set/SNP — 6500

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Geneva, 30 October–3 November 2000

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Introduction

The WHO Expert Committee on Biological Standardization met in Geneva from 30 October to 3 November 2000. The meeting was opened on behalf of the Director-General by Dr Y. Suzuki, Executive Director, Health Technology and Pharmaceuticals.

Dr Suzuki emphasized the contribution of the work of the Committee to global standardization and control of biological substances used in medicine. He also drew attention to the importance of promoting the role of WHO in setting recommendations.

Dr Suzuki noted that WHO is emerging from a period of considerable restructuring aimed at fostering better coordination and cooperation of activities at all levels of the Organization and minimizing duplication of effort. In the case of WHO's Biologicals unit, a further driver for change has been the Report of the Independent Review of the Remit and Activities of WHO in the Biological Field and the Biologicals Unit submitted to WHO in November 1998. In line with the recommendations of the Review, the Biologicals unit has been renamed Quality Assurance and Safety: Biologicals (QSB) to describe more accurately its responsibilities and has been located in the Department of Vaccines and other Biologicals. The Quality and Safety of Plasma Derivatives and Related Substances (QSD) unit has been placed in the Department of Blood Safety and Clinical Technology to improve its visibility and funding.

QSB and QSD continue to work closely together to fulfil the normative functions of WHO and have special status as an intra-cluster biologicals team acting as a clear focus for biological activities within WHO, as recommended by the Review, as well as having the degree of independence necessary for the standard-setting process.

Dr Suzuki also noted that recent scientific and technical developments have led to a rapid expansion in the number and complexity of biologicals, with new products and new biotechnologies posing new challenges for standardization, quality control and safety. The independent review of biologicals had recognized the need to strengthen WHO's activities in biological standardization to meet these challenges. The shortfall in staffing and funding of biologicals that has occurred in recent years is now being made up. Dr Suzuki acknowledged the generous support of the Government of the United Kingdom, through staff secondment from the National Institute for Biological Standards and Control, Potters Bar, and thanked the French Government for its support for a new post in QSD. Although the regular budget had not increased, more financial resources are

becoming available for biologicals through funding from various sources.

Another recommendation of the independent review of biologicals was that special consideration be given to the area of International Biological Standards for in vitro diagnostic procedures. It had suggested that high priority be given to the detection of microbial agents which could be transmitted to humans and recommended that WHO should consult international scientific organizations working in this field and seek advice on priorities in the development of appropriate biological reference materials with relevance to global public health. Such a consultation had been arranged recently by the Secretariat and good progress had been made in identifying and discussing issues related to a global approach to the development of International Biological Standards for in vitro diagnostic procedures.

Finally Dr Suzuki noted that the heavy agenda once again reflected the increasing complexity and sophistication of the biologicals field and its growth in volume. He commented that this presented a considerable challenge, especially for developing countries, which can expect to benefit greatly from the use of new biologicals such as vaccines. Some are developing their own capacity in this area and the need for a coordinated international approach to assure the quality and safety of biologicals cannot be overemphasized.

General

Developments in biological standardization

The Committee was informed of a number of developments that did not feature elsewhere on the agenda.

Once again, there had been delays in publication of the report of the previous two years' Committee meetings. However, summary reports of the major decisions and the changes in the list of WHO standards had been published in *WHO Drug Information* and in an appropriate scientific journal. The Committee expressed concern that the publication of detailed information on requirements and other matters of importance to control authorities and manufacturers was taking so long.

The Committee was informed of a meeting planned in 2001 to review the standardization and control of anti-venoms because of global problems relating to production, standardization and regulatory issues. Another meeting is planned in collaboration with the WHO–United Nations Programme on HIV/AIDS (UNAIDS) HIV

Vaccine Initiative to review scientific and regulatory issues relevant to the evaluation of HIV vaccines. The Committee welcomed these initiatives.

Comparability of biotechnology products

A meeting organized by the Center for Biologics Evaluation and Research of the United States Food and Drug Administration, the International Association for Biologicals and the United States Pharmacopeia had been held in June 2000 to consider the amount of clinical data required to establish comparability with respect to safety and efficacy for new sources of biotechnology products. This was of increasing importance on public health and regulatory grounds because of the expiry of patents and the development, production and use of biotechnology products in many developing countries.

Although much can be achieved using sophisticated physico–chemical analysis and by paying careful attention to issues of viral safety and validation studies, the meeting had concluded that a move towards "generic biologicals" was not possible with the present level of scientific development. The Committee asked the Secretariat to monitor developments in this field and consider whether WHO guidelines would be useful.

DNA vaccines

The Committee was informed that the WHO International Working Group on Standardization and Control of DNA Vaccines had met in March 2000 and reviewed special safety concerns in the light of several years' research and development. Early reports of plasmid integration had been negative or showed at most an extremely low rate of integration. However, more recent data showed co-inoculation of plasmid encoding granulocyte-macrophage colony stimulating factor (GMCSF) with the DNA vaccine, or electrostimulation, may lead to enhanced integration but the maximum levels detected were still below the background mutation rate. It now seems likely that the previous absence of evidence for integration after intramuscular inoculation of plasmid DNA was due to the inefficiency of this route of administration. The Committee endorsed the conclusions that assays for plasmid integration should continue; that such assays may be unnecessary if sufficient information is available on the behaviour of similar plasmids; and that integration levels should be reassessed if there is a significant change in the method of delivery, especially if this may lead to an increase in the ability of the plasmid to enter the nucleus. There had also been concern that DNA vaccines using

bacterial plasmids may induce autoimmune disease, through production of IgG anti-DNA antibodies. Low levels of antibodies to DNA had been detected, but their levels were much lower than those associated with autoimmune disease. However, it had been concluded that production of such antibodies should continue to be monitored following DNA vaccination. Although the use of plasmids encoding cytokines as molecular adjuvants offers advantages over recombinant cytokines, all molecular adjuvants are likely to have their own specific safety issues and each system needs to be carefully monitored for deleterious effects.

Working reference materials

The Committee noted the essential role that WHO International Biological Reference Materials play in ensuring the quality and safety of many biological products. It is intended that national, regional and manufacturers' working reference materials for routine use are calibrated against the WHO preparations. It is now clear, however, that many national control laboratories lack the resources and expertise to prepare suitable working standards and that the lack of these may severely compromise the quality and safety of vaccines and other biologicals of critical importance for public health.

The Committee was pleased to learn of proposals to overcome the problem by supply of working reference materials from the WHO International Laboratory at the National Institute for Biological Standards and Control, Potters Bar. A new production plant is under construction that will provide greater capacity for filling reference materials, larger batch sizes, sterile facilities and provisions for the safe handling of pathogenic materials. Cell culture facilities are also being developed. The Committee gratefully acknowledged the unique and sustained contribution of the National Institute for Biological Standards and Control to the WHO programme on biological standardization, and in particular the initiative to obtain resources to develop the new unit, and the high level of support provided by the Government of the United Kingdom over a long period to this end. In the light of these developments, the Committee considered it important to ensure consistency of approach to the production and use of both international and working reference materials. Guidance could be incorporated into the ongoing revision of the Guidelines for the Preparation, Characterization and Establishment of International and other Standards and Reference Reagents for Biological Substances (WHO Technical Report Series, No. 800, 1990, Annex 4). The Committee also recommended that the revision should also give consideration to the nomenclature of international

reference materials, as well as to further review of stability assessment of reference preparations, which in selected cases might need to involve real-time studies.

Smallpox

The Committee was informed that, following a decision of the World Health Assembly, the known remaining stocks of smallpox virus had not been destroyed in 1999 as scheduled and that stocks of vaccine are being kept for special use. However, in view of the age of these vaccine stocks, now more than 30 years old, the potency of some vaccine batches is declining to unacceptable levels. Also, the Committee was reminded of its decision taken at the fiftieth meeting (WHO Technical Report Series, No. 904, 2002, p. 10) not to discontinue the Requirements for Smallpox Vaccine. In view of these developments, the Committee recommended that a special Working Group be established to consider what action to take.

Stability testing of vaccines

The Committee reminded the Secretariat of a proposal made at its forty-eighth meeting (WHO Technical Report Series, No. 889, 1999, pp. 11–12) to provide more detailed guidance on stability testing procedures for biological products. In particular, there was an urgent need for more guidance on the stability testing of vaccines. The Committee requested that this proposal be taken forward by the Secretariat.

WHO Consultation on International Biological Standards for in vitro Diagnostic Procedures

The Committee was informed of discussions that had taken place at a WHO Consultation held in Geneva in September 2000. International biological standardization is becoming increasingly important for the regulation of clinical diagnostic procedures and the Consultation was the first occasion when participants from various disciplines had met together to discuss issues associated with the provision of international biological standards in this area.

The Committee noted that in the field of clinical chemistry, and as proposed by a draft International Standards Organization (ISO) standard (ISO 17511), the standardization of diagnostic procedures is based on a reference system concept which seeks to provide:

— traceability of unitage to previous standards;
— knowledge of the level of uncertainty attached to unitage assignments;

— commutability (an estimate of the equivalence between the content of the standard and of test results derived from clinical samples).

The Committee agreed that procedures for establishing biological standards used in diagnostic tests might benefit from further consideration of such principles. However, it noted that significant differences exist between small completely defined analytes and complex biological macromolecules, whether well characterized at the molecular level or not. These include the difficulty in establishing reference methods to quantify a biological substance in matrices of biological fluids and the lack of traceability to SI units. The Committee therefore considered that the draft ISO standard 17511 as presently written may not be applicable to the measurement of complex biological substances in clinical samples. It recommended that WHO collaborate closely with the ISO and other scientific bodies with an interest in in vitro diagnostics to resolve these issues so that the principles of ISO 17511 are maintained as far as possible, but the distinct characteristics and difficulties of biologicals are clearly recognized. It also recommended that the Secretariat works with other parts of WHO to clarify issues of terminology and nomenclature, and to improve communication so that the respective responsibilities towards the medical, academic research and diagnostic communities are understood and recognized.

International guidelines, recommendations and other matters related to the manufacture and quality control of biologicals

Poliomyelitis vaccines

The Committee noted the report of a WHO Working Group on Transgenic Mice as an Animal Model for Neurovirulence Testing of Oral Poliomyelitis Vaccine (BS/00.1924 and Add.1), together with the statistical analysis of phases 4 and 5 of the collaborative study of the application of this test to types 1 and 2 oral poliovirus vaccines. At its fiftieth meeting in 1999 (WHO Technical Report Series, No. 904, 2002, p. 6), the Committee had agreed that the use of transgenic mice provided a suitable alternative to monkeys for neurovirulence testing of the type 3 vaccine.

The Working Group had reviewed all the data obtained in a collaborative study involving five laboratories for poliovirus type 1 vaccine and six laboratories for type 2 vaccine. It had concluded that the

mouse model provided a sound basis for testing both types of poliomyelitis vaccine. However, it would be necessary for laboratories intending to use the test to follow a standard procedure to implement it (BS/00.1923). This would be necessary for each serotype of vaccine and would involve a blinded evaluation of a panel of six vaccines for each. The laboratories would be able to use the accumulated data as described in the Standard Operating Procedure (SOP) for the method to determine their own values for acceptance and rejection of subsequently tested vaccine batches. In order to achieve all this, a programme of training and staged implementation would be necessary. The Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, had offered to provide such training and the National Institute for Biological Standards and Control, Potters Bar, to coordinate the implementation process.

The Committee reviewed the recommendations and proposals in these documents. It expressed its gratitude to the supplier of the transgenic mice in Japan for the generous donation of animals to permit the collaborative studies to be performed. Steps to make supplies of the transgenic mice more widely available offered the possibility of increased use of the alternative procedure. It was recognized that implementation of the mouse procedure has economic implications and that this procedure would not necessarily be adopted universally. However, it would be important that all laboratories using the procedure should provide reports to WHO, through the Center for Biologics Evaluation, Bethesda, and the National Institute for Biological Standards and Control, Potters Bar, so that the introduction and performance of the test could be monitored closely. It would be essential to have a guaranteed supply of mice and the Committee was pleased to learn that WHO was actively working to ensure sources of supply in different regions. However, there would have to be adequate documentation to ensure that mice of the specified quality are supplied because of requirements for good manufacturing practice. Industry was supportive of the achievements of the Working Group.

The Committee urged that a workshop be organized involving all interested parties to discuss the implications of implementation of the Working Group's recommendations.

Recommendations for the production and control of poliomyelitis vaccine (oral)

In view of the above discussion, the Committee noted that consequential changes would be necessary to the Recommendations for Poliomyelitis Vaccine (Oral). These had been revised in 1999 and an addendum addressing changes with respect to the neurovirulence

testing in transgenic mice of types 1 and 2 oral poliomyelitis vaccines had been drafted (BS/00.1922). The Committee reviewed the draft document and, after making some modifications, adopted the text as Recommendations for the Production and Control of Poliomyelitis Vaccine (Oral) (Addendum 2000), and agreed that it should be annexed to its report (Annex 1).

Recommendations for the production and control of poliomyelitis vaccine (inactivated)

The Committee also noted a need for revision of the Recommendations for the Production and Control of Poliomyelitis Vaccine (Inactivated). The Recommendations (formerly Requirements) for inactivated poliomyelitis vaccine had not been revised since 1985 and there had been several advances in vaccine production technology and quality control since that time that made a further revision necessary. Furthermore, as the goal of eradicating poliomyelitis due to wild-type polioviruses is in sight, laboratories that use wild polioviruses will become an important potential source of accidental reintroduction of wild virus into the community. To minimize this risk, WHO has developed a global action plan that requires increased biosafety containment of wild polioviruses. This applies to parts of the production process for inactivated polio vaccine where wild poliomyelitis viruses are used. A first draft revision of the recommendations for inactivated poliomyelitis vaccine was developed by the Secretariat and reviewed at an informal WHO Consultation held in October 2000 in Bethesda, USA and comments noted. The Committee reviewed a revised draft, which took into account comments received by the Secretariat, and after making some further modifications, adopted the text as Recommendations for the Production and Control of Poliomyelitis Vaccine (Inactivated) and agreed that it should be annexed to its report (Annex 2).

The Committee also recommended that further consideration be given to the advice concerning the use of assays for potency measurement of inactivated poliomyelitis vaccines. The recommendations adopted above specify that both an in vitro and an in vivo potency assay should be used. The Committee considered it useful to develop criteria for replacing the in vivo test by the in vitro assay.

Guidelines for the production and control of Japanese encephalitis vaccine (live) for human use

Japanese encephalitis is a major public health problem in Asia and two vaccines, one inactivated and one live attenuated, are in use. The Committee noted that draft Guidelines for the Production and Con-

trol of Japanese Encephalitis Vaccine (Live) for Human Use (BS/00.1925) had been developed in response to issues raised by the Committee at its fiftieth meeting in 1999 (WHO Technical Report Series, No. 904, 2002, p. 7) when an earlier draft had been reviewed, and in the light of comments received at an informal WHO working group held in Beijing, China. The document provides information and guidance concerning the history, characteristics, production and control of live attenuated Japanese encephalitis vaccine and is designed to facilitate progress towards the eventual international licensure of the vaccine. As recommended by the Committee at its fiftieth meeting (WHO Technical Report Series, No. 904, 2002, p. 7), the text is written in the form of guidelines instead of recommendations in view of the fact that further work is needed to develop and standardize appropriate methods and criteria for certain tests, such as the neurovirulence test. In reviewing the responses to the concerns raised at its previous meeting, the Committee was reassured by the evidence indicating a very low incidence of vaccine-associated disease and a high level of immunogenicity and efficacy. It recognized the need to demonstrate that the neurovirulence test will reliably discriminate suitable from unsuitable materials. Participation in collaborative studies and inclusion of a common neurovirulence reference preparation that is tested in parallel using the same procedures would be extremely useful and was strongly encouraged by the Committee. It welcomed the offer from the National Institute for Control of Pharmaceutical and Biological Products, Beijing, to provide a candidate reference preparation.

Following further changes to the draft made by the Committee, the text was adopted as Guidelines for the Production and Control of Japanese Encephalitis Vaccine (Live) for Human use and it was agreed that it should be annexed to the Committee's report (Annex 3).

Guidelines for the production and control of inactivated oral cholera vaccines

The Committee was informed that an Informal WHO Consultation had been held in July 2000 to consider what action should be taken in view of the development of new oral cholera vaccines and the need perceived by WHO to stockpile vaccines for use in emergencies. The Committee was reminded of its decision at the forty-ninth meeting (WHO Technical Report Series, No. 897, 2000) to discontinue the Requirements for Cholera Vaccines first adopted in 1959. Following the Consultation, draft guidelines were developed, which were intended to reflect the needs for production and control of the new inactivated oral vaccines, even though further development work

would be required on testing procedures. The Committee recognized that this is a rapidly evolving field of great importance in developing countries. It welcomed the action taken by the Secretariat and endorsed the steps to develop new and appropriate guidelines.

Requirements for diphtheria, tetanus, pertussis and combined vaccines

At its fiftieth meeting in 1999 (WHO Technical Report Series, No. 904, 2002), the Committee had welcomed the report of a Working Group on the harmonization of antigen content and potency measurement of diphtheria and tetanus vaccines and endorsed the recommendation that WHO establish a small group of experts to coordinate the development of a simple, robust and standardized assay suitable for demonstrating consistency of immunological characteristics for lot release of vaccines containing diphtheria and/or tetanus toxoid. At its present meeting, the Committee was reminded of the diversity of the diphtheria and tetanus vaccines now available and discussed a proposal to amend the present Requirements for Diphtheria, Tetanus, Pertussis and Combined Vaccines to permit the use of a test procedure based on serological assay. The proposed simplified assay for routine lot release had been circulated to interested parties and comments had been received. The Committee noted the principles on which the proposed amendment was based and that the need to work towards a common model was accepted by all parties provided that new problems were not created.

The Committee welcomed the steps taken to resolve the current problems, recognized that the proposal was only a first stage requiring further study and discussion before it could be implemented, and encouraged those involved to continue their work. The Committee also noted that if the proposed amendment to the Requirements is to be adopted, there will be a need for clear guidance for national control authorities as to when such a simplified lot release assay could be introduced. In the light of these developments, the Committee requested the Secretariat to review also the nature and suitability of existing antitoxin reference materials, some of which are old and possibly not appropriate for future needs, with a view to their replacement.

Mouse protection models for acellular pertussis vaccines

In the case of acellular pertussis vaccines, a range of single component and combined vaccines is currently available. Earlier meetings of the Committee (i.e. forty-seventh and fiftieth) had identified a need for a suitable animal model to evaluate vaccine potency given that immuno-

genicity of the vaccines does not necessarily reflect clinical efficacy and that ethical considerations prevent clinical protection studies on new vaccines or vaccine combinations. The Committee was informed that a meeting of a WHO Ad Hoc Working Group on Acellular Pertussis Vaccines had been held in July 2000. The Working Group had reviewed data obtained in a collaborative study of models to assess the protective activity of acellular pertussis vaccines. It had concluded that a respiratory challenge model, already being evaluated by two manufacturers, offered a good prospect for product characterization. The criteria for a suitable model had been agreed and a further collaborative study of both an intranasal and aerosol assay would be performed. In addition, it had been agreed that more data were needed on the modified intracerebral challenge assay used for routine lot release in Japan and some other countries and a further collaborative study of this model was also being planned. The Committee suggested a number of modifications to the design of the proposed studies and looked forward to receiving the results in due course.

The Committee noted that these developments would be discussed further in a forthcoming meeting to be held at the Center for Biologics Evaluation and Research, Bethesda, MD. It expressed its thanks to Dr Y. Sato, formerly of the National Institute of Infectious Diseases, Tokyo, who had generously made available panels of relevant monoclonal antibodies and other reference reagents which are proving valuable in aiding research and development in this area. These materials are now held at the National Institute for Biological Standards and Control, Potters Bar.

Guidelines on evaluation of preclinical and clinical testing of vaccines

The Committee was informed that a number of guidelines are available concerning clinical testing in general, but that there was a need for specific guidelines to cover preclinical and clinical testing of vaccines as part of the regulatory overview. The Committee's advice was sought on how to proceed now that resources are available to allow this project to go ahead. The Committee supported this initiative and agreed that such guidelines would be of benefit in support of national regulations and as guidance for control authorities and industry, especially in developing countries, and for ethical committees. Their use in developing a common format for the information to be supplied in support of licensing would help assessment by national authorities. However, the Committee counselled against repetition of existing guidance and recommended that emphasis should be placed on critical issues. The specific problems presented by combined vaccines

should be addressed. It also suggested that consideration should be given to dividing the guidelines into two, dealing with preclinical and clinical aspects separately, since the considerations and expertise involved are different. It emphasized that the preclinical section should not consist merely of a checklist.

Meningococcal vaccines

Serogroup B meningococcal protein-based vaccines

The Committee was informed of the proceedings of a meeting held in May 2000 under the auspices of WHO and the Pan American Health Organization (PAHO) to consider standardization and control of protein-based vaccines against serogroup B meningococcal disease. This disease is of major public health importance globally and can give rise to long-lasting epidemics with serious consequences, particularly in developing countries. A number of vaccines against serogroup B organisms were being developed, the most advanced being those based on the outer membrane vesicles (OMV). Phase III trials of these vaccines had been conducted in a number of countries, including Brazil, Cuba and Norway. The OMV vaccines had proved to be safe and immunogenic, although protection seemed to be only short term, of doubtful efficacy in infants and specific to serogroup subtypes. The development of "designer OMV vaccines" to control clonal epidemics is currently being evaluated.

The Committee endorsed the recommendations arising from the meeting and in particular agreed that guidelines for OMV-based meningococcal vaccines should be developed and circulated to interested parties for comment. The Committee also recommended that another meeting should be held to review further evidence concerning immune responses to these vaccines and to consider how the assays could best be standardized and reliable immunological correlates of protection established. Finally it agreed that it would be useful for WHO to set up a working group to consider all aspects of clinical management of meningococcal disease and possibly draft suitable guidance.

Serogroup A, C meningococcal conjugate vaccines

In the United Kingdom in October 1999, a serogroup C conjugate vaccine became the first novel vaccine to be licensed for use in infants for which protective efficacy was not determined by a phase III study but inferred from immunogenicity data. Having set this precedent, a similar approach may be adopted for the licensure of new meningococcal conjugate vaccines being developed for the prevention of disease caused by other serogroups. Since decisions on the licensure of novel vaccines and the wider implementation of existing vaccines are

critically dependent upon serological data, it is essential to assess whether current serological assays provide adequate data and reliably reflect the comparability of different clinical studies.

The Committee was informed of a meeting of an expert panel set up to assess current methodologies and agreed with its recommendations that either human or rabbit serum could be used as the source of complement for the serum bactericidal assay used to assess immunological responses to serogroup C meningococcal conjugate vaccine. It also agreed with the criteria proposed to indicate protective human immune responses. However, the Committee emphasized the need to review these recommendations in the light of protection data now emerging from the United Kingdom following the introduction of the vaccine, especially in relation to the demonstration of immunological memory.

The Committee was also informed of the programme for the Epidemic Meningitis Vaccines for Africa project, launched recently. Meningitis caused by serogroup A is a serious public health problem in countries in the meningitis belt of Africa. It is expected that the introduction of conjugated vaccines in these countries could prevent and ultimately eliminate epidemics. At issue is whether the same approach could be taken to the introduction of these vaccines as that adopted by the United Kingdom with respect to the serogroup C meningococcal conjugate vaccine discussed above. The Committee supported in principle the concept that clinical protection studies might be unnecessary, but emphasized that many issues regarding standardization and validation of serological assays remain to be explored. WHO was encouraged to explore as a matter of urgency mechanisms for the licensing of vaccines for use in the affected countries that may not be licensed for use in the country where they were produced. The early consideration of standardization and control issues in the development and clinical testing of a new vaccine was welcomed and encouraged by the Committee since it allowed potential problems to be identified early and hopefully resolved, which in turn would facilitate and speed up the licensing process.

Standardization and validation of serological assays for the evaluation of immune responses to pneumococcal conjugate vaccines

The Committee was informed of the need for standardized IgG enzyme-linked immunosorbent assays (ELISAs) for the evaluation of the immunogenicity of pneumococcal vaccines. WHO had convened a panel of experts to reach a consensus on the use of a reference assay and assignment of values for the calibration of sera. The Committee

endorsed the recommendations made by the panel. It noted the problem of extrapolation of information concerning use of one vaccine to others without supporting clinical data. It advised that recommendations on the production and control of pneumococcal conjugate vaccines be developed.

Guidelines on viral inactivation procedures for plasma and plasma-derived medicinal products

The Committee was informed that the existing WHO Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives (WHO Technical Report Series, No. 786, 1989, Annex 4) did not adequately address issues of viral inactivation and removal. It reviewed draft guidelines on viral inactivation and removal procedures intended to assure the viral safety of plasma products and designed to overcome this deficiency. These guidelines are aimed at control authorities and manufacturers less familiar with viral decontamination procedures. The Committee learned that the draft had been circulated already to a number of national regulatory authorities in different regions and favourable comments had been received. The Committee considered that the draft guidelines would be very useful and recommended that they be circulated widely for further comment to ensure that they meet their intended purpose and that their structure and content are appropriate.

Good manufacturing practices for the collection of source materials for the production of plasma derivatives

In the light of the recommendations of the ninth International Conference of Drug Regulatory Authorities in 1999, the Committee was informed of the need for the development of good manufacturing practices (GMPs) for the collection of source materials for the production of plasma-derived medicinal products. This would form part of the global project for the quality assurance of plasma derivatives.

The Committee considered how best a GMP guide for blood centres prepared under the auspices of the Pharmaceutical Inspection Cooperation Scheme might be adapted to serve this need. It was recognized that blood centres do not fall within the scope of pharmaceutical legislation in all countries and that a document addressing more practical aspects would be more helpful. In addition, any WHO guidance would have to be applicable to a larger number of countries than the present document. After discussing various aspects of the document, the Committee concluded that it could form a basis for further development. It also concluded that basic GMP guidance is relevant in this context but that a specific document on good manufacturing practices

for the collection of source materials for the production of plasma derivatives would complement the two existing WHO guidelines in this area, "Good Manufacturing Practices for Pharmaceutical Products" (WHO Technical Report Series, No. 823, 1992, Annex 1) and "Good Manufacturing Practices for Biological Products" (WHO Technical Report Series, No. 822, 1992, Annex 1).

Aide memoire

The Committee took note of an aide memoire prepared by QSD summarizing the role and functions of WHO in relation to blood products and related biologicals and to national regulatory authorities and control laboratories. It recommended that the Secretariat consider whether similar aide memoires could be produced to cover all of the activities of WHO in the biologicals field.

Bovine spongiform encephalopathy and the safety of biologicals

The Committee attached considerable importance to measures to ensure the safety of vaccines and other biologicals with respect to transmissible spongiform encephalopathies (TSEs), including bovine spongiform encephalopathy (BSE). The Members were informed about the current situation regarding the sourcing of bovine serum and other bovine-derived materials used in the manufacture of vaccines and other biologicals.

The most recent risk assessment in the USA had been carried out jointly by the Vaccine and TSE Advisory Committees in July 2000 and it was concluded that any risk from vaccines was theoretical and negligible. Nevertheless, it had been recommended as a precautionary principle that, in addition to measures already in place for vaccines and other biological products, where the manufacturers' working cell banks or working bacterial or viral seeds had been constituted with bovine materials from countries with BSE, or when the source of bovine materials was unknown, these working banks and seeds should be replaced with those constituted using bovine materials from countries known to be free of BSE. Countries with BSE, or where it is considered that there is an undue risk of BSE, are defined in the USA by the United States Department of Agriculture (Code of Federal Register 9CFR 94.18). Master cell banks and master seeds constituted with bovine materials from countries with BSE would not need to be replaced because the risk associated with the use of bovine materials is theoretical and remote and changing to a new master cell or seed bank raises real and uncertain risks for vaccine safety and efficacy.

In the USA, information relating to BSE/TSEs and any perceived risks from vaccines and other biologicals is provided by the Food and

Drug Administration. The Center for Biologics Evaluation and Research, Rockville, MD, USA would be announcing such information on its web site.

In Europe, the European Medicines Evaluation Agency (EMEA) had also concluded that any risk associated with the use of bovine serum was negligible and was following a similar policy. Again there was no intention to recommend the reconstitution of existing master cell banks or master seed lots irrespective of the source of bovine materials used in their preparation. Where bovine materials from countries with BSE had been used for the production of working cell banks and working seeds, manufacturers would be given a time period within which to replace these banks. However, the definition of countries at risk from BSE in the European Union, as well as that of the Office International des Epizooties (OIE), followed by WHO, differs from that of the United States Department of Agriculture.

Manufacturers had agreed to take the necessary action to implement the recommendations. No retrospective withdrawal of vaccines had been undertaken by the USA or the European Union because of the high impact on vaccine programmes of withdrawal of vaccines when set against a negligible and theoretical risk in contrast to the great benefit of vaccination. Any residual amount of bovine material from the cell bank or seed in the final vaccine was so minute as to be irrelevant with respect to safety.

The Committee was informed that although bovine serum from animals with BSE had not been demonstrated to transmit disease, for added security careful attention was being given to the source of the bovine serum. Furthermore, rigorous attention was also being given to excluding materials known to carry BSE infectivity from the manufacturing processes for biological medicines. Categories of infectivity in bovine tissues and body fluids are listed in the report of a WHO Consultation on Medicinal and other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies (WHO/BLG/97.2). In addition, in relation to the safety of vaccines and other biologicals prepared in mammalian cells, there was no evidence that the transmissible agent of BSE was capable of amplification in cultures of the non-neuronal cells that are currently used as substrates for production. Furthermore, a recent publication had evaluated the age-related distribution of variant Creutzfeld–Jakob (vCJD) cases in the United Kingdom. The analysis suggested no evidence of an association with vaccination.

The safety of vaccines and other biologicals with respect to TSEs in general was considered to be assured by a combination of safe sourc-

ing of materials used in manufacture (i.e. only from safe sources with respect to country/herd/animal) and by using only tissues with no demonstrable infectivity. However, in view of the rapid developments in this area, the Committee recommended that WHO review the situation with regard to the risk of transmitting BSE/TSEs via medicinal products and update the recommendations made in the 1997 report (WHO/BLG/97.2). It also recognized the urgent need for research into the development of synthetic culture media.

International reference materials

Biological substances: international standards and reference reagents

The Committee was informed that the listing of international biological reference preparations, which is now available on the Internet, would be included in the report of its forty-ninth meeting (WHO Technical Report Series, No. 897, 2000, Annex 4). Annual publication of the list was not foreseen. A separate listing of the reference preparations for blood products and related substances had also been produced for easy reference. The Committee emphasized the need to ensure that the information available on the Internet is up to date and urged the Secretariat to move towards linking the information in the catalogues for the National Institute for Biological Standards and Control, Potters Bar, the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, and the Center for Biologics Evaluation and Research, Bethesda, which are also available on the Internet, to the WHO site.

The Committee was also informed of the continued demand for distribution of international reference materials by the National Institute for Biological Standards and Control, Potters Bar and the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam. It endorsed the programmes of work in progress and planned by the two International Laboratories and the WHO Collaborating Centre at the Center for Biologics Evaluation and Research, Bethesda.

The Committee reviewed proposals for the disestablishment of selected international biological reference preparations arising from the annual review of usage and stocks (BS/00.1931).

The Committee noted the decrease in use and the changed international requirements for assay of several antibiotics. The Committee requested the Secretariat to publish enquiries to determine whether there continues to be a need for the International Reference Preparations or International Standards for amikacin, capreomycin,

chlortetracycline, lymecycline, metacycline, novobiocin and paromomycin. The Committee also requested the Secretariat to publish enquiries to determine whether a need still exists for standards for porcine calcitonin for bioassay, porcine kininogenase, ovine prolactin for bioassay and bovine thyrotrophin for bioassay, in view of the low demand for these preparations and the availability of the corresponding human materials.

The Committee was informed that no response had been received to the enquiry about the continued need for the first International Reference Preparation for Protamine and therefore disestablished the preparation (Annex 5).

Because the stocks of the first International Standard for Anti-parvovirus B19 Serum (IgG), Human, and the second International Reference Preparation for Thyroid-stimulating Hormone (TSH), Human, for Immunoassay, are depleted, the Committee authorized the National Institute for Biological Standards and Control, Potters Bar to take steps to replace them. The Committee also authorized the National Institute for Biological Standards and Control, Potters Bar, to investigate reports that the second International Standard for *Clostridium botulinum* Type B Antitoxin, Equine, was unsuitable for measurements involving untreated equine serum and, if necessary, to take steps to replace it.

Review of stocks of oral poliovirus vaccine seeds and neurovirulence reference materials

The Committee was informed that a survey had been made of the stocks of original Sabin poliovirus, master seed stocks at one passage, and working seed lots at two passages, held at several centres on behalf of WHO. The information obtained revealed an uneven distribution. Not all holders had reported their storage conditions. The limited data on infectivity titres was reassuring about the stability of the stocks. The Committee recommended that sufficient stocks should be kept at the one passage level to ensure that further working seeds could be prepared. The Committee also requested the Secretariat to ascertain whether individual holders should store their stocks in more than one site for security and to consider whether a more even distribution would be appropriate.

WHO Working Group on International Reference Materials for Diagnosis and Study of Transmissible Spongiform Encephalopathies

The Committee strongly commended the action of the Secretariat in setting up this Working Group. The efforts of the Working Group

were aimed at developing and evaluating international reference materials which could be used for comparing potential diagnostic tests for TSEs.

The Committee noted the report of the second meeting of this Working Group which had been held in Geneva in May 2000. A panel of reagents had now been obtained for the diagnosis and study of TSEs and a collaborative study was planned involving the examination of homogenates of brain tissue from patients with variant CJD and sporadic CJD, and uninfected patients. The homogenates would be studied by in vivo and in vitro assay methods, including immunoblotting. Some assays were already sufficiently developed to allow comparison of their sensitivities. The Committee was pleased to note the good progress being made in this area.

Antigens

Tetanus toxoid

The Committee noted a proposal to establish a replacement reference material for tetanus toxoid, adsorbed, (BS/00.1932), based on a collaborative study performed by 27 laboratories in 19 countries. It was reminded of the difficulties associated with comparison of potency determinations of tetanus toxoid preparations in guinea-pigs and in mice. Evidence had been obtained some years ago to show that when the second (current) International Standard was assayed against the first International Standard in mice, its potency was half that observed in guinea-pigs. The present candidate preparation for the replacement standard had, however, given the same apparent potencies in guinea-pigs and mice as the second International Standard. Nevertheless, after considering all the evidence, including experimental data recently derived by several laboratories, the Committee decided to use only the calibration in guinea-pigs and not to pool all the assay data. The preparation showed adequate stability. On the basis of the results obtained, the Committee established the preparation, coded 98/552, as the third International Standard for Tetanus Toxoid, Adsorbed, and assigned a unitage to it of 469 International Units (IU) per ampoule based only on assays in guinea-pigs. Data derived from assays in guinea-pigs only had also been used for assignment of potency to the second International Standard.

The Committee noted that another preparation, coded C, had been included in the collaborative study with a view to its adoption as a regional standard. This preparation had behaved differently to the preparation adopted as the third International Standard both in the

collaborative study and in the subsequent supplementary studies that had been undertaken. The Committee noted that use of this material might give rise to difficulties in interpretation of results between manufacturers and control laboratories. In view of these difficulties and because of the known discrepancies in the assays, particularly the mouse potency assay, the Committee emphasized that studies should be continued urgently to move to a more suitable state-of-the-art assay to measure the potency of tetanus toxoid.

Diphtheria toxoid

The third International Standard for Diphtheria Toxoid, Adsorbed was established by the Committee at its fiftieth meeting (WHO Technical Report Series, No. 904, 2002, p. 19) on the basis of an in vivo challenge assay in guinea-pigs. At its present meeting, the Committee was informed that data had now been received from a collaborative study on the use of serological assays. The results showed that the assigned potency of the third International Standard is valid only for the guinea-pig challenge assay. The Committee was also informed of the need to obtain data relating to assays in mice and requested the Secretariat to take the necessary steps to obtain such data.

Blood products and related substances

Diagnostic kits for detecting hepatitis B surface antigen and antibodies to hepatitis C and human immunodeficiency virus in blood

The Committee noted the report of the third meeting of the WHO Working Group on the development of reference preparations for testing diagnostic kits for detecting hepatitis B surface antigen (HBsAg) and antibodies to hepatitis C (HCV) and HIV held in January 2000. It recognized the importance of having procedures available to distinguish between appropriate and inappropriate kits for detection of markers of infection. The Committee endorsed the recommendations of the Working Group with respect to the reference panels for HBsAg (a five-member panel), anti-HCV (inactivated material of six genotypes) and anti-HIV (seven anti-HIV-1 subtypes and one anti-HIV-2 preparation), and noted the priorities assigned.

Antibodies to hepatitis C virus, genotype 1

The Committee was informed of the results of a collaborative study to characterize candidate reference materials for anti-HCV genotype 1 (BS/00.1934) antibodies, performed by 15 laboratories in 13 countries using 25 different kits. The Committee also noted the proposal to establish one of the candidate preparations as an International Refer-

ence Reagent but without assignment of a unitage. After discussion, however, the Committee concluded that the main purpose of this material is to serve as an external positive run control for testing of the performance of diagnostic kits for the detection of HCV. Whilst the Committee agreed that such a preparation would be extremely useful, it noted that the preparation did not meet the criteria for a WHO International Reference Material. The Committee therefore requested the National Institute for Biological Standards and Control (NIBSC), Potters Bar, to distribute the preparation coded 99/608 as an NIBSC Reagent, together with a memorandum that reflects the Committee's discussion and the non-WHO status of the material.

Standardization of unfractionated heparin

The Committee was informed of a WHO Working Group that had met with the International Society on Thrombosis and Haemostasis (ISTH) in June 2000 to discuss the global harmonization of the biological measurement of unfractionated heparin in pharmaceutical preparations. A discrepancy of long standing exists between measurement of heparin in United States Pharmacopeia (USP) units and in IU. A globally harmonized assay method is an important step towards resolving this discrepancy. An opportunity for action had arisen with the proposed replacement of the USP reference material and the availability of the same material as used for establishing the International Standard. The Working Group had proposed that an anti-IIa chromogenic assay method be submitted to collaborative study. The Committee welcomed this initiative.

Tissue plasminogen activator, recombinant

At its fiftieth meeting (WHO Technical Report Series, No. 904, 2002, p. 22), the Committee had established a replacement for the second International Standard for Tissue Plasminogen Activator (t PA), a preparation derived from human melanoma cells, with a preparation of recombinant origin (BS/99.1913). A request had been received recently, from a participant in the collaborative study, to revise the assigned content of the new preparation from 10000 IU per ampoule to 9750 IU per ampoule. After reviewing the evidence provided in support of this proposal, the Committee decided to make no change to the assigned content and confirmed the assignment made in 1999. The Committee requested the Secretariat to seek clarification of the apparent problem that had given rise to the request and to determine whether any other action was necessary.

On the basis of certain precedents, the Committee had established the replacement preparation as the first International Standard for Tissue

Plasminogen Activator, Recombinant (Alteplase). The Committee had subsequently received a request from the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (BS.00/1935) that this name be changed and setting out the difficulties and potential confusion that this title would cause. It was proposed that the preparation be renamed the third International Standard for Tissue Plasminogen Activator, Recombinant and that the accompanying memorandum should state that it is a replacement for the second International Standard, derived from human melanoma cells. It was further proposed that the International Nonproprietary Name (INN), alteplase, should not be used. The Committee reconsidered the grounds for its previous decision on nomenclature. Since no stock remains of the second International Standard, future calibration of further standards against the human-derived material would not be possible. In addition, alteplase, unlike some other recombinant t PAs of modified structure, has the correct human sequence albeit with possible changes in glycosylation and assayed with parallelism against the second International Standard. Taking into account all the factors, the Committee agreed to rename the preparation as the third International Standard for Tissue Plasminogen Activator, Human, Recombinant. However, for clarity and to avoid any confusion in the future, the Committee directed that the identification of the material as alteplase be given in the leaflet accompanying the standard.

The Committee recommended that the Secretariat bring forward an item at the next meeting to discuss general principles and considerations on standardization with the intention of updating the guidelines for the preparation, characterization and establishment of international and other standards and reference reagents for biological substances (WHO Technical Report Series, No. 800, 1990, Annex 4). Nomenclature should be an important aspect, as should the evidence required to demonstrate the stability of reference materials at the time of establishment and during their lifetime.

Fibrinogen concentrate

The Committee noted the report on the collaborative study of two preparations of fibrinogen concentrate, carried out by 17 laboratories in seven countries (BS/00.1927). The Committee also noted that the aims of the collaborative study were to compare the two preparations by a variety of methods, and to calibrate them for total protein and clottable protein. Three methods were employed for the former measurement and five methods for the latter. In view of the results of the study, the Committee established the preparation coded 98/614,

as the first International Standard for Fibrinogen Concentrate with an assigned content of 15 mg total protein and 10.4 mg clottable protein per ampoule. This preparation had shown no detectable loss of total or clottable protein after storage at 45 °C for 19 months. The Committee requested that additional information about the source and analysis of the preparation of frozen fibrinogen concentrate used to prepare the standard be obtained from the supplier and provided to the Secretariat.

Blood coagulation factor VIII concentrate, human

The Committee was reminded that the sixth International Standard for Blood Coagulation Factor VIII Concentrate, Human established at its forty-ninth meeting (WHO Technical Report Series, No. 879, 2000, p. 21) consisted of recombinant material. Anecdotal evidence suggested that this behaved differently to the fifth International Standard (of plasma origin) when it was used to calibrate manufacturers' working standards, but investigation of these claims had not produced sufficient evidence to propose any changes. The Committee requested the National Institute for Biological Standards and Control, Potters Bar, to continue to collect data on this subject. The Committee was further informed that good progress is being made in the preparation of a large new batch of plasma-derived factor VIII, the so-called "Mega 2" batch, which would serve as a future standard. In the light of the project to develop a common assay for unfractionated heparin, the Committee recommended that similar action be taken by the International Society on Thrombosis and Haemostasis with factor VIII where two different assays are used in different regions.

Human parvovirus B19 DNA

The Committee was informed of developments in the standardization of genome amplification techniques (SoGAT) under the auspices of a WHO Working Group. Following establishment of the first International Standards for hepatitis B virus DNA, hepatitis C virus RNA and HIV-1 RNA, the establishment of an International Standard for human parvovirus B19 DNA had been identified as the next priority. The Committee commended the Working Group on its progress and supported continuation of its work.

The Committee noted a proposal to establish a reference material for human parvovirus B19 DNA (BS/00.1928), based on the results of a collaborative study performed by 26 laboratories in 14 countries using both qualitative and quantitative assays. Accelerated degradation studies (BS/00.1928 Add.1) had shown that the preparation has adequate stability. On the basis of the information provided, the

Committee established the preparation in vials coded 99/800 as the first International Standard for Human Parvovirus B19 DNA and assigned a potency of 500 000 IU per vial to it. The Committee noted that the intended use of the preparation is for calibration of assays based on nucleic acid technology and considered that it would serve an important and valuable role.

Cytokines, growth factors and endocrinological substances

Biological therapeutics

The Committee was informed of the discussions at the sixth WHO Informal Consultation on Standards for Cytokines, Growth Factors and Endocrinological Substances. The remit of this group had expanded over time so that it can serve as an expert advisory group to the Committee. The short time between the group meetings and those of the Committee had caused difficulties in that documents prepared for the Committee had sometimes needed revision following consideration by the consultative group, leading to duplication and unnecessary circulation of reports. After considering the issues involved, the Committee recommended that the time for submission of documents to the Secretariat for its meetings be moved forward by 3 months. This would probably lead to delay in submission of reports in the first year but would then allow due consideration by the consultative group in May or June, revision if necessary, and submission to WHO in good time before the meeting of the Committee. It would be critical to inform all those involved in the organization and analysis of collaborative studies of the change now adopted.

The Committee noted and endorsed a request from the consultative group to strengthen communication between QSB and the Programme on International Nonpropretary Names.

The Committee also noted the outcome of the review of the work programme of the consultative group on cytokines and other substances and confirmed the need for a more focused effort to prepare a reference material for interferon beta.

Other matters drawn to the Committee's attention included the eventual replacement of preparations of natural pituitary hormones by recombinant materials; the value of including reference back to relevant decisions of the Expert Committee when officially proposing the establishment of reference preparations; the desirability of clarification of the position of the Committee with regard to reference materials for recombinant enzymes, scheduled for consideration in

2001; and the need for adequate information about the component materials used in International Standards of Reference Reagents to be held at the National Institute for Biological Standards and Control, Potters Bar, and preferably also at WHO even if such information is not provided in officially circulated documents or memoranda because of confidentiality considerations.

Finally, the Committee was informed of the need for standards and reference methods to study iatrogenic antibodies and for guidance on assay procedures. The Committee was pleased to learn of a proposed meeting organized jointly by the International Association of Biological Standardization, Paris and the Center for Biologics Evaluation and Research, Bethesda, on this topic. Similar considerations apply to the measurement of antibodies induced by vaccine antigens which could be the subject of a separate workshop.

Follicle-stimulating hormone and luteinizing hormone, human, urinary

The Committee noted a proposal to establish a replacement reference material for follicle-stimulating hormone and luteinizing hormone human, urinary (BS/00.1926 and Rev. 1), based on a collaborative study performed by 10 laboratories in nine countries. It was informed that, despite the increasing availability of recombinant glycoprotein preparations, there remains a need for a preparation of natural human origin. The candidate preparation is enriched but poorly characterized. Relatively few laboratories have the capability of performing the bioassays and the study did not involve multiple methods. The preparation exhibited adequate stability for both hormone activities. On the basis of the results obtained, the Committee established the preparation, coded 98/704, as the fourth International Standard for Follicle-stimulating Hormone and Luteinizing Hormone, Human, Urinary and assigned a unitage to it of 72 IU of follicle-stimulating hormone and 70 IU of luteinizing hormone per ampoule.

Somatropin

The Committee noted a proposal to establish a replacement reference material for somatropin (rDNA-derived human growth hormone) (BS/00.1929 and Rev. 1), based on a collaborative study performed by 16 laboratories in nine countries. It was informed that the aims of the study were to determine the suitability of the preparation based on extensive physico–chemical examination, to assign a content using the current non-biological method of assay, and to confirm the specific biological activity and stability of the preparation. The assigned specific activity of 3.0 IU per mg was confirmed by the study.

The preparation exhibits degradation at elevated temperatures that fits with the Arrhenius equation and appears to be stable. The stability will continue to be monitored in real time. On the basis of the results obtained, the Committee established the preparation, coded 98/574, as the second International Standard for Somatropin with an assigned content of 1.95 mg of somatropin and related proteins per ampoule. The Committee agreed that full details of the high-performance liquid chromatography (HPLC) assay method used should be attached to the memorandum supplied with the International Standard.

Inhibin B

The Committee noted a proposal to establish a reference material for inhibin B of human origin (BS/00.1930 and Rev. 1), based on a collaborative study performed by six laboratories in five countries. The candidate preparation had been purified by affinity chromatography. It had been examined by bioassay and immunoassay and the Committee noted that the units for the two types of assay are not interconvertible. The study had also shown that recombinant inhibin B does not behave in the same way as the natural material and that the presence of a non-ionic surfactant in the preparation may affect the performance of some bioassays. The preparation exhibited adequate stability. On the basis of the limited results obtained, the Committee established the preparation, coded 96/784, as the first Reference Reagent for Inhibin B, Human, and assigned a unitage to it of 12 Units of inhibin B per ampoule for bioassay and 12 ng of inhibin B per ampoule for immunoassay. The Committee emphasized that these assignments cannot be assumed to be interconvertible.

In order to avoid any confusion between the new preparation and the first International Standard for Inhibin, Human, Recombinant, the Committee renamed the latter preparation as the first International Standard for Inhibin A, Human, Recombinant.

Insulin-like growth factor I

The first International Reference Reagent for Insulin-like Growth Factor I, for Immunoassay had been discontinued by the Committee at its forty-fifth meeting in 1996 (WHO Technical Report Series, No. 858, 1998, p. 17) when the first International Standard for Insulin-like Growth Factor I was established, because the latter preparation appeared to be suitable for use in both bioassays and immunoassays. Since that time, the International Standard has not been widely used for immunoassay because it is calibrated in IU not micrograms, there was no formal continuity of unitage for immunoassay between the two preparations and the amount of material in the International

Standard is too great for immunoassay purposes. The discontinued preparation has continued to be used unofficially for calibration of immunoassays. The Committee therefore accepted a proposal (BS/00.1933) to rectify this situation. The Committee re-established the preparation coded 87/518 as the first International Reference Reagent for Insulin-like Growth Factor I, for Immunoassay, with an assigned content of 3.1 µg per ampoule, and renamed the preparation coded 91/544 as the first International Standard for Insulin-like Growth Factor I, for Bioassay, with an assigned content of 150 IU per ampoule.

WHO Technical Report Series, No. 910, 2002

Annex 1
Recommendations for the production and control of poliomyelitis vaccine (oral) (Addendum 2000)

Introduction

The Recommendations (formerly Requirements) for Poliomyelitis Vaccine (Oral) were last revised in full in 1999 (*1*). At that time, new quality control tests were introduced for the vaccine. One such test was a neurovirulence test for poliovirus vaccine in the TgPVR21 transgenic mouse line. The test in TgPVR21 mice was shown in WHO-supported studies to be a suitable alternative to the monkey neurovirulence test for poliovirus type 3. At its fiftieth meeting, in 1999, the Committee was informed of the excellent progress with TgPVR21 neurovirulence tests for poliovirus types l and 2 and encouraged completion of these studies as soon as possible.

The studies with poliovirus types l and 2 in TgPVR21 mice were completed by June 2000 and a Working Group met to review the data. The Working Group concluded that the data validated the neurovirulence test in TgPVR21 mice for poliovirus types l and 2. They advised that the Recommendations for the Production and Control of Poliomyelitis Vaccine (Oral) be amended to include the neurovirulence test in TgPVR21 mice as an alternative to the neurovirulence test in monkeys for all three poliovirus serotypes.

The 1999 Recommendations state that to qualify as competent to perform the mouse neurovirulence test, laboratories should complete a standard implementation process. The Working Group defined the details of a standard implementation process. This is incorporated into a revision of the Standard Operating Procedure for the mouse neurovirulence procedure.

The report of the Working Group is available as a separate document from WHO.[1]

The proposed amendments to the 1999 Recommendations are given below:

A.4.4.5.3 Neurovirulence test in transgenic (TgPVR21) mice for poliovirus type 1, 2 or 3

The TgPVR21 transgenic mouse model provides a suitable alternative to the monkey neurovirulence test for the neurovirulence testing

[1] Coordinator, Quality Assurance & Safety: Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

of type l, 2 or 3 vaccines once a laboratory qualifies as competent to perform the test as specified below and experience is gained to the satisfaction of the national control authority. Experience in a national control laboratory should be assessed by WHO. The test should be performed according to the standard operating procedure, "WHO neurovirulence test of type l, 2 or 3 live poliomyelitis vaccines (oral) in transgenic mice susceptible to poliovirus", available from WHO.[1]

Although the murine model may be used for neurovirulence testing of filtered bulk suspensions, the monkey neurovirulence test should remain as the definitive reference test to requalify vaccine production, for example to evaluate any new virus seed materials or vaccines produced on a new substrate and lots prepared to establish consistency from the new seed or substrate.

To qualify as competent to perform the mouse neurovirulence test, laboratories should complete a standard implementation process. Details of the process are included in the standard operating procedure "WHO neurovirulence test of type l, 2 or 3 live poliomyelitis vaccines (oral) in transgenic mice susceptible to poliovirus", available from WHO.[1] The process should be fully documented. Once qualified as competent, each laboratory should continue to monitor their competence to perform the test. Laboratories are requested to submit the results of their tests both during the implementation phase and subsequent routine use to WHO. These data should be evaluated by WHO in order to monitor globally how the new test performs in practice.

(l) TgPVR21 mice

Mice used for the neurovirulence test must be aged 6–8 weeks at the time of inoculation and should be from a source defined in the standard operating procedure "WHO neurovirulence test of type l, 2 or 3 live poliomyelitis vaccine (oral) in transgenic mice susceptible to poliovirus". Mice should be allowed to recover from shipping for at least 7 days before inoculation. Procedures and standards for maintenance of TgPVR21 mice should follow the WHO Guidelines (*2*).

(2) Number of mice

A vaccine and appropriate homotypic reference virus should be tested concurrently. Equal numbers of animals, with equal numbers of males and females, should be inoculated with the reference virus and the vaccine being tested. Mice should be allocated

[1] Coordinator, Quality Assurance & Safety: Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

to vaccine or reference virus and to particular cages using a randomization procedure (defined in the standard operating procedure); 32 mice per test dose should be used for evaluation of the vaccine and 32 mice per test dose for evaluation of the reference. More than one vaccine may be tested with the same homotypic reference virus at the same time. If a test is done on two working days, equal numbers of mice should be inoculated with the vaccine and the reference virus on each working day.

(3) Virus content of vaccines and reference virus inoculated

The virus content of the vaccines and reference preparations should be determined with a precision of $\pm 0.3 \log_{10}$ cell culture infections doses 50 ($CCID_{50}$) or better, and normalized against titration standards, as described in the standard operating procedure. Groups of mice should be inoculated with two test doses of vaccine and reference. For poliovirus type 1 vaccines tested against the WHO(SO+2)/I reference virus, the doses are 1.75 and $2.75 \log_{10} CCID_{50}$ in 5 µl. For poliovirus type 2 vaccines tested against the WHO (SO+2)/II reference virus, the doses are 5.0 and $6.0 \log_{10} CCID_{50}$ in 5 µl. For poliovirus type 3 vaccines tested against the WHO(SO+2)/III reference virus, the doses are 3.5 and $4.5 \log_{10} CCID_{50}$ in 5 µl.

If other reference viruses are used, the doses should be determined by the paralysis proportions of the reference: at the high dose <0.95, at the low dose >0.05.

Mice should be sedated appropriately and inoculated into the lumbar region of the spinal cord as described in the standard operating procedure.

(4) Observation of mice

Mice should be observed for occurrence of paresis or paralysis daily for 2 weeks after inoculation. Paralysed mice should be humanely killed as soon as paralysis is confirmed. Other mice should be humanely killed on day 14 after inoculation. Data should be recorded on a standard form (see the standard operating procedure). Mice with traumatic paralysis (appearing 24 hours or less after inoculation and not progressing) and those that die from causes other than poliomyelitis should be excluded from evaluation.

(5) Evaluation of the neurovirulence test

Comparison of the virus neurovirulence activity in the vaccine(s) and reference preparations should be based on the numbers of animals with paresis or paralysed animals in both groups of mice,

inoculated with two test doses of the vaccine and reference preparation. Validity criteria for each test must be met and are specified in the standard operating procedure.

The filtered bulk suspension passes the test if the numbers of animals with paresis or paralysed mice in the groups inoculated with vaccine are not significantly greater than the numbers in the groups inoculated with the reference material. Detailed statistical criteria for acceptance of vaccines after neurovirulence testing in TgPVR21 mice are given in the standard operating procedure.

References

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WHO Technical Report Series, No. 910, 2002

Annex 2
Recommendations for the production and control of poliomyelitis vaccine (inactivated)[1]

Recommendations published by WHO are intended to be scientific and advisory. Each of the following sections constitutes guidance for national control authorities and for the manufacturers of biological products. If a national control authority so desires, these Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by a national control authority. It is recommended that modifications to these Recommendations be made only on condition that the modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below. The parts of each section printed in small type are comments for additional guidance intended for manufacturers and national control authorities, which may benefit from those details.

In these Recommendations, Part A describes the general provisions for the production of poliomyelitis vaccine (inactivated) (IPV) and is applicable to production of vaccine in all permissible cell substrates, including the use of a cell bank. Part B describes national control requirements. The terms "national control authority" and "national control laboratory", as used in these Recommendations, always refer to the country in which the vaccine is manufactured.

To facilitate the licensing and international distribution of IPV made in accordance with these Recommendations, a summary protocol for recording the results of tests is provided in the Appendix.

[1] Replaces the 1982 Requirements for Poliomyelitis Vaccine (Inactivated) and Addendum 1987.

General considerations

Since the Recommendations (formerly Requirements) for Poliomyelitis Vaccine (Inactivated) were last revised in 1981 (*1*) and amended in 1985 (*2*), there have been several advances in technology in vaccine production and control that make a further revision of the Recommendations necessary.

At the time of the last revision, the introduction of continuous cells for manufacture of poliomyelitis vaccine (inactivated) (IPV) was a novel development. Since then, the control of products manufactured in continuous cells has been standardized, as reflected in these Recommendations.

The section on production of IPV in primary monkey kidney cells has been deleted in favour of production in continuous cells (Vero), secondary or tertiary monkey kidney cells, or human diploid cells. For vaccines produced in secondary or tertiary monkey kidney cells, a recommendation to use monkeys bred in captivity has been introduced. For these vaccines antibody tests are introduced to exclude animals with antibodies to cercopithecid herpesvirus 1 (B virus), simian virus 40 (SV40), foamy viruses and simian immunodeficiency virus (SIV) from the production process, and a test for retroviruses is introduced for monovalent pools.

Among the most significant changes in production has been the increasing use of IPV in combination with other vaccines and this introduces considerations that do not apply when IPV is used alone, such as interaction of the poliovirus antigens with other antigens and/or adjuvants. These considerations are not dealt with in the present Recommendations. However, to provide further guidance for control of the vaccine, key tests that may be influenced by other antigens and/or adjuvants in combined vaccines are identified.

As the goal of eradicating poliomyelitis due to wild-type polioviruses is in sight (*3*), laboratories that use wild-type polioviruses will become an important potential source of accidental reintroduction of such viruses into a community. To minimize this risk, WHO has developed a Global Action Plan that requires increased biosafety containment of wild-type polioviruses (*4*). In line with the Global Action Plan, these Recommendations introduce increased biosafety containment 1 year after the last wild-type poliovirus is detected, both at the level of production of vaccines using wild-type strains and for the control of such vaccines. It will be important to ensure that the increased biosafety containment levels are applied to all laboratories that need to work with live polioviruses. For example, the tests for bacterial and mycotic sterility on live virus harvests will require the transfer of samples containing live virus from a virology to a bacteriology laboratory. This process should be carried out in such a way as to prevent the escape of live wild-type polioviruses.[1]

As an alternative to the use of wild-type polioviruses for production of IPV, recent evidence suggests that production of IPV from the attenuated Sabin poliovirus seed viruses is technically feasible (*5–7*). New manufacturers are encouraged to explore the production of IPV from the live attenuated polioviruses developed by Sabin as alternative and safer seed viruses than the currently used wild-type viruses. IPV manufacturers that currently use wild-type strains are encouraged to evaluate the potential offered by a Sabin-based IPV versus upgrading production and control facilities to meet the enhanced biosafety requirements.

An in vivo potency test is described in these Recommendations in which neutralizing antibodies to each of the three poliovirus types are assayed. The neutralizing antibody test for poliovirus requires the use of live poliovirus. For historical reasons, many laboratories use wild-type strains of poliovirus. The attenuated Sabin vaccine strains of poliovirus, on the other hand, will not require increased laboratory containment until vaccination ceases completely. Therefore, the use

[1] Further detailed guidance is in preparation and will be available from WHO.

of Sabin strains of poliovirus to assay neutralizing antibodies in the in vivo test, shown to be suitable for this purpose by a collaborative study (*8*), is specified. Validation of the use of the Sabin strains by each manufacturer should be provided. It is probable that immunization with poliomyelitis vaccine (oral) should cease at some point in the future, once the disease has been eradicated. After that time the containment levels for use of the Sabin strains for laboratory work will be reviewed. Laboratories are thus encouraged to investigate the use of alternatives to live viruses for assay of poliovirus neutralizing antibodies.

The present Recommendations have been updated with respect to reduction of risk from transmissible spongiform encephalopathies (TSEs).

The tests in animals have been replaced by in vitro alternatives wherever possible. For example, the test in monkeys for the detection of virulent virus that may have escaped inactivation and the part it may play in assuring the safety of the vaccine was given as an optional test in the previous Requirements. This has been deleted and replaced by the more sensitive test in cell culture. The cell culture systems that are recommended are considered the gold standard for detection of residual live virus during the inactivation process and laboratories wishing to use other cell cultures will have to demonstrate equivalence with the recommended cells. The use of rabbits for testing for cercopithecid herpesvirus 1 is replaced by a more sensitive tissue culture test. The general safety (innocuity) test may be deleted for products where validated by historical data and experience, and with the agreement of the national control authority.

In recent investigations the in vivo potency assay in rats has been standardized and shown to have advantages over previously described in vivo tests for IPV (*8*). The assay in rats is therefore described in detail and the in vivo tests in guinea-pigs and chicks are deleted. The in vivo assay should be used to characterize the vaccine after changes in the manufacturing process that may influence the quality of the vaccine, for stability studies of the vaccine, and to establish consistency of production. Some national control authorities may require the potency of each batch of vaccine to be determined by an in vivo test. This should be performed at the level of the final bulk.

The development of transgenic mice that express the human poliovirus receptor (TgPVR mice) (*9, 10*) has led to the development of an immunization/challenge model (*11*) that may be useful for assessment of vaccine efficacy for new strains. This test is not proposed for lot release. Any work with transgenic mice should comply with WHO guidelines (*12*).

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 *International name and proper name*

The international name should be "Vaccinum poliomyelitidis inactivatum". The proper name should be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 *Descriptive definition*

Vaccinum poliomyelitidis inactivatum should consist of an aqueous suspension of poliovirus types 1–3 grown in cell cultures, concentrated, purified and inactivated. The preparation should satisfy all the recommendations formulated below.

A.1.3 *International standards or reference preparations and International Units*

The first International Reference Preparation of Poliomyelitis Vaccine (Inactivated) (established in 1994) is stored frozen in ampoules containing 1 ml of trivalent inactivated poliomyelitis vaccine (*13*). This reference preparation is intended for the calibration of secondary reference preparations of poliomyelitis vaccines (inactivated), which in turn are used for determination of relative potencies. The preparation may be used in both D antigen and immunogenicity assays.

The second International Standard for Anti-poliovirus Serum (types 1, 2, 3) (established in 1991) is stored in ampoules, each containing dried human serum with antibodies to all three poliovirus serotypes (*14*). This standard is intended for calibration of national standards for anti-poliovirus sera.

The above standard and reference preparation are available from the National Institute for Biological Standards and Control, Potters Bar, Herts., England. Samples are distributed free of charge on request to national control laboratories.

A.1.4 *Terminology*

The following definitions are given for the purposes of these recommendations only.

Cell bank: A collection of ampoules containing material of uniform composition derived from a single pool of cells and stored under defined conditions.

Cell seed: A quantity of well-characterized cells derived from a single tissue or cell of human or animal origin and stored frozen in liquid nitrogen in aliquots of uniform composition, one or more of which may be used for the production of a master cell bank.

Master cell bank: A quantity of fully characterized cells of human or animal origin derived from the cell seed and stored frozen in liquid nitrogen in aliquots of uniform composition, one or more of which may be used for the production of a manufacturer's working cell bank. The testing performed on a replacement master cell bank (derived from the same clone or from an existing master or working cell bank) is the same as for the initial master cell bank, unless a justified exception is made.

Manufacturer's working cell bank (MWCB): A quantity of cells of uniform composition derived from one or more ampoules of the master cell bank, which may be used for the production cell culture.

> In normal practice, a cell bank is expanded by serial subculture up to a passage number (or population doubling, as appropriate) selected by the manufacturer, at which point the cells are combined to give a single pool and preserved cryogenically to form the MWCB. One or more of the ampoules from such a pool may be used for the production cell culture.

Production cell culture: A cell culture derived from one or more ampoules of the MWCB or kidney tissue used for the production of IPV.

Adventitious agents: Contaminating microorganisms of the cell substrate or materials used in its culture, including bacteria, fungi, mycoplasmas, and endogenous and exogenous viruses.

Original seed viruses: The monovalent seed viruses from which a vaccine was prepared according to defined specifications and which, after inactivation, was shown on administration to humans in field trials to be suitably safe and immunogenic by induction of neutralizing antibodies.

Virus master seed lot: A quantity of virus of uniform composition derived from the original seed virus processed at one time and passaged for a number of times that does not exceed the maximum approved by the national control authority.

Virus working seed lot: A quantity of virus of uniform composition derived from the master seed by not more than two passages by a method approved by the national control authority.

Single harvest: A virus suspension of one virus type harvested from cell cultures prepared from a single production run.

Monovalent pool: A virus suspension of a single virus type processed at the same time.

Purified monovalent pool: A concentrated and purified virus suspension of a single virus type processed at the same time.

Trivalent bulk: A pool of a number of inactivated monovalent pools and containing all three virus types.

Final bulk: The finished biological preparation present in the container from which the final containers are filled.

Filling lot (final lot): A collection of sealed final containers of liquid vaccine that are homogeneous with respect to the risk of contamination during the filling process or the preparation of the finished vaccine. A filling lot must therefore have been filled or prepared in one working session.

Cell-culture infective dose 50% ($CCID_{50}$): The quantity of a virus suspension that will infect 50% of cell cultures.

Closed colony: A group of animals sharing a common environment and having their own caretakers who have no contact with other animal colonies. Each animal is tested repeatedly to ensure freedom from specified pathogens and their antibodies. New animals are not admitted to the colony until they have been shown to be free from these specified pathogens.

A.2 General manufacturing recommendations

The general manufacturing requirements contained in Good Manufacturing Practices for Biological Products (*15*) should apply to establishments manufacturing IPV, with the addition of the following:

Production establishments that manufacture IPV should comply with the current version of the WHO Global Action Plan for Laboratory Containment of Wild Polioviruses (*4*) in both the production and quality control departments.

> Recent evidence suggests that production of IPV from the attenuated Sabin poliovirus seed viruses is technically feasible (*5–7*). New manufacturers are encouraged to explore the production of IPV from the live attenuated polioviruses developed by Sabin as alternative and safer seed viruses than the currently used wild-type viruses. IPV manufacturers that currently use wild-type strains are encouraged to evaluate the potential offered by a Sabin-based IPV versus upgrading production and control facilities to meet enhanced biosafety requirements.

The staff involved in the production and quality control of IPV should be shown to be immune to all three poliomyelitis viruses.

> Consideration should be given to regular assessments of serum and mucosal immunity, and freedom from shedding of polioviruses, in all staff involved in the production and quality control of IPV.

A.3 Control of vaccine production[1]

A.3.1 *Control of source materials*

A.3.1.1 Virus strains and seed lot system

Strains of poliovirus used in the production of IPV should be identified by historical records, which should include information on their origin, by infectivity tests and by immunological methods. Only virus strains that are approved by the national control authority and that yield a vaccine meeting the Recommendations set out in the present document should be used.

Vaccine production should be based on the virus seed lot system. The virus working seed lot used for the production of vaccine should not have passed more than 10 subcultures, counted from the original seed virus approved by the national control authority and on which the original laboratory and field tests were done.

Each virus working seed lot used for the production of vaccine batches should be subjected to all tests applicable to a single harvest (in section A.3.2 and certain tests in section A.3.4 (A.3.4.1, A.3.4.2, A.3.4.4 and A.3.4.5)) plus the test for sterility according to the requirements given in sections A.5.1–A.5.3 of the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*16*). The working seed lot should also be tested in human diploid cells after neutralization with serum (as described in section A.3.4.1) in accordance with the 1998 Requirements for the use of cell substrates (*17*). The virus working seed lot used for the production of vaccine batches should be free from detectable SV40 sequences as determined by a validated nucleic acid amplification test.

> Sequences of SV40 are widely used as molecular biological reagents, and contamination of polymerase chain reaction (PCR) assays is potentially a major problem. One approach is to identify separate genomic regions for amplification, and to use one for screening purposes and the other for the confirmation of repeatedly positive samples. It is useful if the second genomic region used for confirmation varies between isolates from different sources, as it is then possible to show that it has a unique sequence and that positive results are not due to contamination with laboratory strains of SV40. The sensitivity of the PCR assays for the genomic regions used should be established.

Each virus working seed lot should have been derived from materials that comply with the Recommendations made in sections A.3.1.2–A.3.1.4 and should be approved by the national control authority.

A.3.1.2 Master cell bank and manufacturer's working cell bank

The use of a cell line for the manufacture of IPV should be based on the cell bank system. The master cell bank should be approved by

[1] Information on the production and testing of poliomyelitis vaccine (inactivated) should be summarized in a protocol (an example is given in the Appendix).

the national control authority. The maximum number of passages (or population doublings) by which the MWCB is derived from the master cell bank and the maximum number of passages of the production cultures should be established by the national control authority.

> WHO has established a cell bank of Vero cells characterized in accordance with the requirements in the forty-seventh report of the WHO Expert Committee on Biological Standardization (*17*).[1]

A.3.1.2.1 Identity test

The master cell bank should be characterized according to the requirements for continuous cell lines used for production of biologicals or those relating to human diploid cells (*17*), as appropriate.

The MWCB should be identified by means, inter alia, of biochemical (e.g. isoenzyme analysis), immunological and cytogenetic marker tests, approved by the national control authority.

A.3.1.3 Monkey cell cultures for virus production

If virus is produced in monkey kidney-cell cultures, animals of a species approved by the national control authority, in good health and not previously used for experimental purposes, should be used. The animals should be bred in captivity.

All procedures on monkeys should be carried out in accordance with relevant national animal care regulations.

The monkeys should be kept in well-constructed and adequately ventilated animal rooms in cages spaced as far apart as possible. Adequate precautions should be taken to prevent cross-infection between cages. Not more than two monkeys should be housed per cage, and cage-mates should not be interchanged. After the last monkey of a group has been taken, the room that housed the group should be thoroughly cleaned and decontaminated before being used for a fresh group.

All actions taken by working personnel should be based on the assumption that a great potential hazard exists at all times in the quarantine area. Personnel should be provided with protective clothing, including gloves, footwear and masks or visors. Street clothes should not be permitted in the animal rooms. Smoking, eating and drinking should be forbidden while personnel are in the animal rooms.

A supervisor should be made responsible for reporting illnesses among employees and for ensuring that all injuries are properly treated. No worker who has cuts or abrasions on exposed areas of the

[1] Available to manufacturers on application to the Coordinator, Quality Assurance and Safety: Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

body should enter the animal area. Any unexplained febrile illness, even while off duty, should be considered as potentially related to the employee's occupation.

The monkeys should be shown to be free from antibodies to cercopithecid herpesvirus 1 (B virus), SV40 virus, foamy viruses and SIV.

> It is desirable that kidney-cell cultures are derived from monkeys shown to be free from antibodies to a range of other potential adventitious agents of monkeys. In one country, monkeys are tested for antibodies to monkey polyoma virus, measles, mumps and rubella.

Monkeys from which kidneys are to be removed should be thoroughly examined at necropsy, particularly for evidence of tuberculosis and cercopithecid herpesvirus 1 (B virus) infection.

If a monkey shows any pathological lesion relevant to the use of its kidneys in the preparation of a seed lot or vaccine, it should not be used, nor should any of the remaining monkeys of the quarantine group concerned be used unless it is evident that their use will not impair the safety of the product.

All the operations described in this section should be conducted outside the areas where vaccine is made.

The monkey kidney cells are passaged in series. The national control authority should establish the number of cell doublings permitted before the cell cultures are used for the growth of the poliomyelitis virus.

> It has been shown that a "primary" cell culture may have undergone about five cell doublings, "secondary" cells about 10 cell doublings, and "tertiary" cells about 15 doublings. Records of the cell doublings should be kept.
>
> Experience with tertiary monkey kidney cells shows that the cells at the production level do not exhibit evidence of tumorigenicity. A test for tumorigenicity (*17*) is not therefore required for previously validated manufacturing processes unless significant changes are made to the cell culture procedures.
>
> In some countries karyology is also required. The extent of such tests should be determined by the national control authority.

A.3.1.4 Cell culture medium

Serum used for the propagation of cells should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to the requirements given in sections A.5.2 and A.5.3 of the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*16*), and from infectious viruses. Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of the Recommendations for the Production and Control of Poliomyelitis Vaccine (Oral) (*18*).

Where appropriate, more sensitive tests for bovine viruses may be used.

As an additional monitor of quality, sera may be examined for freedom from phage, endotoxin and poliovirus inhibitors.

Irradiation may be used to inactivate potential contaminant viruses.

The source(s) of serum of bovine origin should be approved by the national control authority. The serum should comply with current guidelines in relation to animal transmissible spongiform encephalopathies given in the report of a WHO Consultation on Medicinal and other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies (*19*).

Human serum should not be used. If human albumin is used, it should meet the 1992 Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives (*20*), as well as current guidelines in relation to human transmissible encephalopathies (*19*).

Manufacturers are encouraged to explore the possibilities of using serum-free media for production of IPV.

Penicillin and other β-lactams should not be used at any stage of manufacture.

Other antibiotics may be used at any stage of manufacture, provided that the quantity present in the final product is acceptable to the national control authority. Nontoxic pH indicators may be added, e.g. phenol red at a concentration of 0.002%. Only substances that have been approved by the national control authority may be added.

Trypsin used for preparing cell cultures should be tested and found free of cultivable bacteria, fungi, mycoplasmas and infectious viruses, especially bovine or porcine parvoviruses appropriate to the species of animals used. The methods used to ensure this should be approved by the national control authority.

The source(s) of trypsin of bovine origin, if used, should be approved by the national control authority. Bovine trypsin, if used, should comply with current guidelines in relation to animal transmissible spongiform encephalopathies (*19*).

A.3.2 *Control cell cultures*

A cell sample equivalent to at least 500 ml of the cell suspension, at the concentration employed for seeding vaccine production cultures, should be used to prepare control cultures.

In countries with the technology for large-scale production of vaccine, the national control authority should determine the size of the cell sample to be examined, the time at which it should be taken from the production culture, and the appropriate control vessels.

These control cell cultures should be incubated under similar conditions to the inoculated cultures for at least 2 weeks, and should be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cell cultures should have been discarded for nonspecific, accidental reasons.

At the end of the observation period, the control cell cultures should be examined for evidence of degeneration caused by an extraneous agent. If continuous cell lines or human diploid cells are used, the control cells should also be tested as described in sections A.3.2.1, A.3.2.2 and A.3.2.5. If subcultured monkey cells are used, the control cells and the supernatant fluid from such cells should also be tested for extraneous agents by the tests described in sections A.3.2.1–A.3.2.4. If the examination for extraneous agents, or any of the tests specified in this section, shows evidence of the presence in a control culture of any adventitious agent, the poliovirus grown in the corresponding inoculated cultures should not be used for vaccine production.

Samples not tested immediately should be stored at –60 °C or below.

A.3.2.1 Tests for haemadsorbing viruses

At the end of the observation period or at the time the virus is harvested from the production cultures, whichever is the later, at least 25% of the control cells should be tested for the presence of haemadsorbing viruses using guinea-pig red blood cells. If the latter cells have been stored, the duration of storage should not have exceeded 7 days and the storage temperature should have been in the range of 2–8 °C. In tests for haemadsorbing viruses, calcium and magnesium ions should be absent from the medium.

> Some national control authorities require, as an additional test for haemadsorbing viruses, that other types of red cells, including cells from humans (blood group IV O), monkeys and chickens (or other avian species) should be used in addition to guinea-pig cells.

A reading should be taken after 30 minutes' incubation at 0–4 °C and again after a further incubation for 30 minutes at 20–25 °C.

> If a test with monkey red cells is performed, readings should also be taken after a final incubation for 30 minutes at 34–37 °C.
>
> In some countries the sensitivity of each new batch of red blood cells is demonstrated by titration against a haemagglutin antigen before use in the test for haemadsorbing viruses.

A.3.2.2 Tests for other adventitious agents

At the end of the observation period a sample of the pooled supernatant fluid from each group of control cultures should be tested for other adventitious agents. For this purpose, at least 10 ml of each pool should be tested in the same cells, but not the same batch of cells, as

those used for the production of virus, and additional samples of at least 10 ml of each pool should be tested in both human and simian cells.

The pooled fluid should be inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet should be at least 3 cm^2 per ml of pooled fluid. At least one bottle of each kind of cell culture should remain uninoculated and should serve as a control.

The inoculated and control cultures should be incubated at a temperature of 35–37 °C and should be observed for a period of at least 2 weeks.

A.3.2.3 Tests in rabbit kidney cell cultures

A sample of at least 10 ml of the pooled supernatant fluid from each group of control cultures should be tested for the presence of cercopithecid herpesvirus 1 (B virus) and other viruses in rabbit kidney cell cultures. Serum used in the nutrient medium of these cultures should have been shown to be free from B virus inhibitors using herpes simplex virus as an indicator virus. The pooled fluid should be inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet should be at least 3 cm^2 per ml of pooled fluid. At least one bottle of each kind of cell culture should remain uninoculated and should serve as a control.

The inoculated and control cultures should be incubated at a temperature of 37 °C and should be observed for a period of at least 2 weeks.

For the test to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test period. The sensitivity of each batch of rabbit kidney cells should be demonstrated by challenge with a validated amount of herpes simplex virus. The challenge test should be approved by the national control authority.

If the presence of B virus is demonstrated, the manufacture of IPV should be discontinued and the national control authority should be informed. Manufacturing should not be resumed until a thorough investigation has been completed and precautions have been taken against reappearance of the infection, and then only with the approval of the national control authority.

A.3.2.4 Test in Cercopithecus kidney cell cultures

A sample of at least 10 ml of the pooled supernatant fluid from each group of control cultures should be tested for the presence of SV40

virus and other extraneous agents by inoculation on to cell cultures prepared from the kidneys of *Cercopithecus* monkeys by the method described in section A.3.2.3.

The inoculated cultures should be incubated at 37 °C and observed for a period of at least 4 weeks. A subculture should be made after 2 weeks and observed for 2 weeks.

A cell culture shown to be equally sensitive to SV40 virus may be used.

For the test to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the observation period. The sensitivity of each batch of *Cercopithecus* kidney cells used should be demonstrated by challenge with a validated amount of SV40 virus. The challenge test should be approved by the national control authority.

If there is any cytopathogenic effect attributable to the supernatant fluid, the virus grown on the same batch of cells should not be used for vaccine production.

A.3.2.5 Identity tests

For vaccines produced in continuous cells or in human diploid cells, the control cells should be identified by means of tests approved by the national control authority.

Suitable tests are isoenzyme analysis and immunological and cytogenetic marker tests.

A.3.3 ***Production precautions***

The general production precautions called for by Good Manufacturing Practices for Biological Products (*15*) should apply to the manufacture of IPV. For vaccines prepared using wild-type poliovirus, the current version of the WHO Guidelines for the Safe Production and Quality Control of IPV manufactured from wild polioviruses should also be implemented.[1]

If animal serum is used for the growth of cell cultures, the serum protein concentration in the final vaccine should be no more than 50 ng/ml. The serum protein concentration should be reduced to this level by rinsing the cell cultures with serum-free medium and/or purification of the virus harvests.

In some countries, control tests are carried out to detect the residual animal serum content in the final vaccine (see section A.5.5).

[1] Editorial Note: These Guidelines were established by the 2003 Expert Committee on Biological Standardisation and are available from WHO.

A.3.4 *Control of single harvests and monovalent pools*

Samples that are not tested immediately should be stored at –60 °C or below.

A.3.4.1 Test in Cercopithecus kidney cell cultures

Applies to all seeds, irrespective of the cells in which they were prepared, and to all harvests grown in monkey kidney cells, except for continuous cell lines. A sample of at least 40 ml of each single harvest produced in tertiary monkey kidney-cell cultures should be tested for the presence of SV40 virus or other adventitious agents. The single harvest should be neutralized by a high-titred antiserum against the specific type of poliovirus.

> The Sabin strains may be used as immunizing antigen. The immunizing antigen used for the preparation of the antiserum should not be the same as the production seed.
>
> The immunizing antigen should be shown to be free from extraneous agents and grown in cell cultures free from extraneous microbial agents that might elicit antibodies that could inhibit the growth of any adventitious agents present in the single harvest.

The sample should be tested in primary *Cercopithecus* kidney cell cultures or cells that have been demonstrated to be of equal susceptibility to SV40 virus. The tissue cultures should be incubated at 37 °C and observed for 2 weeks. At the end of this observation period, at least one subculture of supernatant fluid should be made in the same tissue culture system. The sample should be inoculated in such a way that the dilution of the supernatant fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet should be at least 3 cm^2 per ml of supernatant fluid. At least one bottle of the cell cultures should remain uninoculated and should serve as a control.

The inoculated and control cultures should be incubated at 37 °C and observed for an additional 2 weeks.

> If necessary, serum may be added to the primary cultures at this stage, provided that the serum does not contain SV40 antibody or other inhibitors.

The single harvest passes the test if there is no evidence of the presence of SV40 virus or other adventitious agents attributable to the single harvest. For the test to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the observation period.

A.3.4.2 Test in primary rabbit kidney cells

Applies to all seeds, irrespective of the cells in which they were prepared, and to all harvests grown in monkey kidney cells, except for continuous cell lines. A sample of at least 40 ml of each single harvest should be tested in primary rabbit kidney cells as described in section A.3.2.3.

A.3.4.3 Sterility test

Each single harvest should be tested for sterility according to the requirements given in sections A.5.1–A.5.3 of the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*16*).

If this test is done outside the production facilities, then adequate containment procedures (*4*) should be used if wild-type polioviruses are used to produce the vaccine.

A.3.4.4 Purification of monovalent pools

Each monovalent pool of virus should be purified before inactivation.

> An acceptable method is to clarify the virus suspension by filtration, to concentrate the virus by ultrafiltration and, thereafter, collect the virus peak after passing it through a gel-filtration column. Further purification is achieved by passing the virus through an ion-exchange column. Other purification procedures, such as passing the preparation through an immobilized DNA-ase column, may be used.

The purified monovalent pool should be shown to contain not more than 0.1 µg of protein per D-antigen unit of poliomyelitis virus.

For viruses grown in continuous cells the purified monovalent pool should be tested for residual cellular DNA. By calculation the purification process should be shown to reduce consistently the level of cellular DNA to less than 10 ng per human dose. This test may be omitted, with the agreement of the national control authority, if the manufacturing process is validated to achieve this specification.

A.3.4.5 Filtration before inactivation

Each purified monovalent pool should be filtered before inactivation.

> The importance of filtration or clarification of the crude virus suspensions as a means of improving the regularity of the inactivation process has been clearly established. Generally, filters are used in series or filtration is performed stepwise through filters of decreasing porosity. Satisfactory results have been reported with several filter types but a final filtration using a 0.22-µm filter should be used.
>
> Filters containing asbestos should not be used.

Inactivation should be initiated as soon as possible and not later than 72 h after filtration.

> It is preferable to start inactivation within 24 h of filtration. Since the purpose of the filtration step is to remove particulate matter and other interfering substances that may diminish the effectiveness of the inactivation process, and since aggregates tend to increase on standing after filtration, efforts should be made to keep within this time limit.

A.3.4.6 Identity test

The poliovirus in the filtered purified monovalent pool should be tested for identity by neutralization with specific antiserum.

Care should be taken to ensure that the sera used are monospecific by titrating them against homotypic and heterotypic viruses of known virus titre. Monoclonal antibodies may be useful in this test.

A.3.4.7 Virus titration

Before any inactivating agent is added, a sample should be taken of each filtered purified monovalent pool for titration of infective poliovirus using tissue culture methods. This titration should be carried out in not more than 10-fold dilution steps and using 10 cultures per dilution, or any other arrangement yielding equal precision.

Each filtered purified monovalent pool should show a titre of not less than that known to yield inactivated vaccine of suitable potency.

The use of Hep-2C cells in microtitre plates is suitable for this purpose (*18*). The same cells should be used for virus titrations before and after the inactivation process.

The main purposes of determining the titre of virus pools destined for inactivation are to provide the starting titre to monitor the kinetics of inactivation and to select pools that can be expected to meet potency requirements after inactivation.

A.3.4.8 Test for retroviruses

For vaccines produced in secondary or tertiary monkey kidney cells, test samples from the filtered purified monovalent pool should be examined for the presence of retroviruses by an assay for reverse transcriptase (RTase) acceptable to the national control authority.

Recently developed highly sensitive assays for RTase may be considered (*21*), but the results need to be interpreted with caution because RTase activity may be derived from sources other than retroviruses, such as retrovirus-like elements which do not represent a complete genome. Nucleic acid amplification tests for retroviruses may also be used.

The test for retroviruses is not required if the closed monkey colony used as the source of kidneys is certified to be free of retroviruses.

A.3.4.9 Inactivation procedure

The virus in the filtered purified monovalent pools should be inactivated through the use of a method approved by the national control authority. The method of inactivation should be shown to give consistent inactivation for the production of acceptable vaccine. A record of consistency should be established by the production of five consecutive lots and if broken a further five monovalent lots should be prepared and shown to be satisfactory for re-establishing this record.

The progress of inactivation should be followed by suitably spaced determinations of virus titres. The inactivation period should exceed the time taken to reduce the titre of live virus to undetectable amounts by a factor of at least 2.

Formaldehyde has been used for over 40 years as the inactivating agent in the production of IPV. Most manufacturers have encountered some

irregularities in the inactivation process that have not been fully explained. On the basis of the observation that a decrease in the concentration of free formaldehyde occurs in the course of the process, it has been recommended that tests for free formaldehyde should be performed at intervals and the concentration maintained at the desired level by, if necessary, intermittent readjustments.

A second filtration during the process of inactivation should be made.

This step is made after the virus titre has fallen below detectable levels but before the first sample for the safety test is taken.

A.3.4.10 Test for effective inactivation

Two samples of a volume equivalent to at least 1500 human doses of each monovalent pool should be taken and, after removal or neutralization of the inactivating agent, should be tested by inoculation into tissue cultures for the absence of infective poliovirus. Kidney cells from some monkey species, for instance those of the genera *Macaca*, *Cercopithecus* and *Papio*, appear to be more sensitive than others. If other tissue culture systems are used, they should have been shown to possess at least the same sensitivity as those specified above.

The kinetics of inactivation should be established by each manufacturer and approved by the national control authority. One sample should be taken at the end of the inactivation period and the other not later than three-quarters of the way through this period. When primary monkey kidney cells are used for this test, the two samples should be inoculated into bottles of tissue cultures derived from different batches of cells. The dilution of the sample in the nutrient fluid should not exceed 1 in 4 and the area of the cell sheet should be at least 3 cm^2 per ml of sample. One or more bottles of each batch of cultures should be set aside to serve as uninoculated control bottles with the same medium.

The formaldehyde in samples of vaccine for tissue culture tests is generally neutralized at the time of sampling by the addition of bisulfite. Usually, the samples are subsequently dialysed.

It is possible to conduct tissue culture tests on nondialysed material; however, this is often found to be toxic to cells, even with a dilution of 1 in 4. If in such tests nonspecific degeneration of cells occurs, or if the sensitivity of the tissue culture system is reduced, the test should be repeated on dialysed material.

In some countries this test is performed using tissue cultures that are also sensitive to SV40 virus, as an additional measure for detecting this extraneous agent.

The tissue culture bottles should be observed for at least 3 weeks. Not less than two subcultures should be made from each original bottle, one at the end of the observation period and the other 1 week earlier. The subcultures should be observed for at least 2 weeks.

If infectious poliovirus is isolated, the monovalent pool should not be used. The isolation of active poliovirus from a monovalent pool must be regarded as a break in the consistency record.

If primary monkey kidney cells are used in this test, they may contain adventitious agents that could interfere with the test result. It is important to demonstrate that each test retains sensitivity to detect partially inactivated polioviruses.

At the end of the observation period, the cell culture used for the detection of residual live virus should be challenged with a validated amount of live Sabin virus of the same type as that of the monovalent pool. The details of the challenge procedure should be approved by the national control authority.

> The problem of detecting residual active poliovirus in a vaccine is not the same as that of measuring infective virus in untreated suspensions. Poliovirus that has been exposed to the action of formaldehyde without becoming inactivated has been shown to require a much longer time to produce cytopathogenic changes than does untreated virus. For this reason it is desirable that tissue cultures in tests for the presence of residual active virus be observed for as long a time as is technically possible. A satisfactory tissue culture system for this purpose therefore depends not only on the sensitivity of the cells used for the preparation of the cultures but also on the nutrient fluid.
>
> The serum added to the nutrient fluid should be tested for inhibitors to poliovirus. Only serum free from inhibitors to all three types of poliovirus should be used.
>
> Maintenance of the cultures in good condition may require frequent changes of culture medium. However, it should be borne in mind that by early changes of fluid unadsorbed virus might be removed and the validity of the test thus impaired; therefore, the fluid should be changed no earlier than 5–7 days after inoculation.

A.3.4.11 Sterility test

Each purified monovalent pool should be tested for sterility after inactivation, according to the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*16*).

A.3.5 ***Control of trivalent bulk***

Only those inactivated monovalent pools that have been shown to be satisfactory should be blended to form a trivalent bulk.

A.3.5.1 Test for absence of infective poliovirus

A sample of at least 1500 ml or, if purified and concentrated vaccine is prepared, the equivalent of at least 1500 doses of each trivalent bulk should be tested in cell cultures for the absence of infective poliovirus by the procedure described in section A.3.4.10 of these Recommendations. If infective poliovirus is isolated, this batch of trivalent bulk product should not be used.

A.3.5.2 Sterility test

The trivalent bulk should be tested for sterility according to the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*16*).

A.3.5.3 Residual formaldehyde

The content of free residual formaldehyde in the trivalent bulk should be determined by a method approved by the national control authority. The limits should be approved by the national control authority.

A.3.6 ***Control of final bulk***

Preservatives or other substances that might be added to or combined with the trivalent bulk to form the final bulk should have been shown to have no deleterious effect on the immunizing potency of the poliovirus antigens.

A.3.6.1 Sterility test

The final bulk should be tested for sterility according to the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*16*).

A.3.6.2 Potency tests

Each final bulk should be tested for immunizing potency by tests approved by the national control authority. Such tests should include an in vitro assay for antigen content and may include an in vivo assay for immune response. In both tests the results obtained with the test sample should be compared with those obtained with a reference preparation calibrated by comparison with the WHO International Reference Preparation (see section A.1.3).

> The in vitro assay that has been found most suitable for measuring the antigen content is the D-antigen enzyme-linked immunosorbent assay (ELISA). Although this assay is widely used, particular attention is required for its standardization. Some national control authorities accept the use of polyclonal antisera whereas others accept the use of monoclonal antibodies in the test. The D-antigen specificity of the antibodies should be demonstrated. Whichever types of antisera are used, the validation studies should show that the assay can determine consistency of production.
>
> If IPV is formulated with other antigens into a combination vaccine, then the suitability of performing the D-antigen ELISA on the final bulk will have to be determined. If the D-antigen ELISA is not suitable for a particular combination, an in vivo assay should be used.
>
> A suitable in vivo assay method consists of intramuscular injection into the hind limb(s) of rats of four dilutions of the vaccine to be examined and a reference vaccine, using for each dilution a group of not fewer than 10 rats of a suitable strain, and which are specific pathogen-free. The number of dilutions used and the number of animals used may be different from that specified here, provided that any alternative scheme gives the same sensitivity in the test. The weight of the individual animals should not vary by

more than 20% from the group mean. An inoculum of 0.5 ml is used per rat. The dose range is chosen such that a dose response to all three poliovirus types is obtained. The animals are bled after 20–22 days. Neutralizing titres against all three poliovirus types are measured separately using 100 $CCID_{50}$ of the Sabin strains as challenge viruses, Vero or Hep-2C as indicator cells, and neutralization conditions of 3 h at 35–37 °C followed by 18 h at 2–8 °C. Results should be read after fixation and staining after 7 days of incubation at 35 °C. For the antibody assay to be valid, the titre of each challenge virus must be shown to be within the range of 30–300 $CCID_{50}$ and the neutralizing antibody titre of a control serum must be within two 2-fold dilutions of the geometric mean titre of the serum. The potency is calculated by comparison of the proportion of animals defined as responders to the test vaccine and the reference vaccine by the probit method. To define an animal as a responder, it is necessary to establish a cut-off neutralizing antibody titre for each poliovirus type. Owing to between-laboratory variation, it is not possible to define cut-off values that could be applied by all laboratories. Rather, the cut-off values should be determined by each laboratory, based on a minimum series of three tests with the reference vaccine. The mid-point on a $\log_2$ scale of the minimum and maximum geometric mean titres of the series of three or more tests is used as the cut-off value. For each of the three poliovirus types, the potency of the vaccine should not be statistically significantly less than that of the reference preparation. The test is not valid unless:

— the median effective dose (ED_{50}) for both the test and reference vaccines lies between the smallest and the largest doses given to the animals;
— the statistical analysis shows no significant deviation from linearity or parallelism;
— the fiducial limits of the estimated relative potency fall between 25% and 400% of the estimated potency.

Laboratories that have established the parallel line method of analysis of antibody titres for the rat test may use it instead of converting titres to proportions of responders as in the probit method of analysis.

Laboratories are encouraged to validate alternatives to the neutralizing antibody test to reduce the use of live polioviruses in laboratories.

If IPV is formulated with other antigens into a combination vaccine, then the suitability of performing the rat immunogenicity test will have to be determined.

If the immunogenicity test is performed, the potency of the final bulk for each virus type should be approved by the national control authority.

A.3.6.3 Preservative content

If preservative is added, the content in the final bulk (or final lot) should be determined by a method approved by the national control authority. The preservative used and content permitted should be approved by the national control authority.

A.3.6.4 Endotoxin content

The endotoxin content in the final bulk (or final lot) should be determined by a method approved by the national control authority. The endotoxin limit should be approved by the national control authority.

A.4 Filling and containers

The requirements concerning filling and containers given in Good Manufacturing Practices for Biological Products (*15*) should apply.

Single- and multiple-dose containers may be used.

A.5 Control tests on final product[1]

A.5.1 *Identity test*

An identity test should be done on at least one labelled container from each filling lot by an appropriate method.

The potency test described in section A.5.4 of these Recommendations may serve as the identity test.

A.5.2 *Sterility test*

Each filling lot should be tested for sterility according to the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*16*).

A.5.3 *General safety (innocuity) test*

Each filling lot should be tested for unexpected toxicity (sometimes called abnormal toxicity) using a general safety (innocuity) test approved by the national control authority.

This test may be omitted for routine release of filling lots if consistency of production has been well established to the satisfaction of the national control authority and good manufacturing practices are in place. Each lot, if tested, should pass a test for abnormal toxicity.

A.5.4 *Potency test*

Each filling lot should be tested by an in vitro assay for antigen content. The results obtained with the filling lot should be compared with those obtained with a reference vaccine calibrated by comparison with the WHO International Reference Preparation (see section A.1.3).

The D-antigen ELISA is sufficiently sensitive to measure the antigen content of the final vaccine. Although this assay is widely used, particular attention is required for its standardization. Some national control authorities accept the use of polyclonal antisera, whereas others accept the use of monoclonal antibodies in the test. The D-antigen specificity of the antibodies should be demonstrated. Whichever types of antisera are used, the validation studies should show that the assay can determine consistency of production. For D-antigen ELISAs to be valid, they should comply with specified criteria of linearity and parallelism. The effect of a change in the method of calculation of the D-antigen content on registered specifications should also be taken into account.

---

[1] Information on the production and testing of poliomyelitis vaccine (inactivated) should be summarized in a protocol (an example is given in the Appendix).

In general, vaccines manufactured from wild-type poliovirus strains that have been formulated to contain 40, 8 and 32 D-antigen units or more per dose for types 1, 2 and 3, respectively, are effective (*22*). Vaccines with lower D-antigen contents may be acceptable, where supported by clinical data. Vaccines produced from other seed viruses (e.g. Sabin viruses) may also be licensed with a different antigenic composition.

If IPV is formulated with other antigens into a combination vaccine, then the suitability of performing the D-antigen ELISA on the final lot will have to be determined. If the D-antigen ELISA is not suitable for a particular combination, an in vivo assay such as that described in A.3.6.2 should be used.

The potency of the vaccines for each virus type should be approved by the national control authority.

A.5.5 *Protein content*

Poliomyelitis vaccine (inactivated) should not contain more than 10 µg of protein per human dose.

In some countries in vitro tests for residual serum proteins are included.

A.5.6 *Preservative content*

Where appropriate, the preservative content of each filling lot should be determined by a method approved by the national control authority, if this has not been done for the final bulk. The method used and content permitted should be approved by the national control authority.

A.5.7 *Endotoxin content*

The endotoxin content of each filling lot should be determined by a method approved by the national control authority, if this has not been done for the final bulk. The content permitted should be approved by the national control authority.

A.5.8 *Test for residual formaldehyde*

The content of free residual formaldehyde in each filling lot should be determined by a method approved by the national control authority, if this has not been done for the trivalent bulk. The limit should be approved by the national control authority.

A.5.9 *Test for pH*

The pH of each filling lot should be determined and be within limits approved by the national control authority.

A.6 Records

The requirements given in Good Manufacturing Practices for Biological Products (*15*) should apply.

A.7 Samples

The requirements given in Good Manufacturing Practices for Biological Products (*15*) should apply.

A.8 Labelling

The requirements given in Good Manufacturing Practices for Biological Products (*15*) should apply, with the addition of the following.

The label on the container or package should include the following information:

— the designation(s) of the strain(s) of poliovirus contained in the vaccine;
— the cell substrate used for the preparation of vaccine;
— the D-antigen content of each poliovirus type;
— the method used for inactivating the virus;
— the nature and amount of any stabilizer and preservative present in the vaccine.

A.9 Distribution and shipping

The requirements given in Good Manufacturing Practices for Biological Products (*15*) should apply.

A.10 Stability testing, storage and expiry date

A.10.1 *Stability testing*

Adequate stability studies form an essential part of vaccine development. The stability of the vaccine in its final containers, maintained at the recommended storage temperature up to the expiry date, should be demonstrated to the satisfaction of the national control authority.

> As a guide, containers from at least three consecutive final lots, and derived from different antigen production lots, may be tested.

A.10.2 *Storage and expiry date*

The statements concerning storage temperature and expiry date appearing on the label and the leaflet, as recommended in Good Manufacturing Practices for Biological Products (*15*), should be based on experimental evidence and should be submitted for approval to the national control authority.

> Poliomyelitis vaccine (inactivated) should be stored at all times at a temperature between 2 °C and 8 °C.

The maximum duration of storage should be fixed with the approval of the national control authority and should be such as to ensure that the approved antigenic content of each virus type specified on the

label of the container (or package) will still be maintained after release by the manufacturing establishment until the end of the shelf-life, if the conditions under which the vaccine is stored are in accordance with what is stated on the label. The maximum duration of storage at 2–8 °C should be specified.

The date of the potency test may be used to determine the start of the shelf-life period. Where an in vivo potency test is used, the date of the potency test is the date on which the test animals were inoculated with the final bulk. Other defined events may be used to specify the start of the shelf-life period with the approval of the national control authority.

Part B. Recommendations for national control authorities

B.1 General

The general requirements for control laboratories given in Guidelines for National Authorities on Quality Assurance for Biological Products (*23*) should apply, with the addition of the following:

- The national control authority should approve the strains used.
- The national control authority should approve the cell substrate used.
- The national control authority should specify the potency requirements.
- The national control authority should approve the challenge tests used to validate the sensitivity of tests for adventitious agents and to detect residual live virus in inactivated samples.

The national control authority should be satisfied that the results of all tests, including those done on monovalent pools during the process of manufacture, are satisfactory and that consistency has been established.

National control authorities should progressively move towards implementation of a quality system so that, in time, all tests carried out by the national control authority are performed under a suitable quality assurance system that is based on ISO 17025 and is fully documented and maintained.

B.2 Release and certification

A vaccine lot should be released only if it fulfils Part A of the present Recommendations.

A statement signed by the appropriate official of the national control laboratory should be provided if requested by a manufacturing establishment, and should certify whether or not the vaccine lot in question meets all national requirements as well as Part A of the present

Recommendations. The certificate should further state the date of the last satisfactory potency test, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document should be attached.

The purpose of the certificate is to facilitate the exchange of IPV between countries.

Acknowledgements

The first draft of these revised Recommendations was prepared by the following WHO consultants:

Dr K. Chumakov, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, USA; Dr N. Elzinga, National Institute of Public Health and the Environment, Bilthoven, Netherlands; Dr D.J. Wood, National Institute for Biological Standards and Control, Potters Bar, Herts., England.

The draft was reviewed by the following participants at an Informal WHO Consultation held at the Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, USA, from 5 to 6 October 2000:

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Ms G. Stawski, Statens Seruminstitut, Copenhagen, Denmark; Dr L. Vujcic, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, USA; Dr D.J. Wood, Quality Assurance and Safety: Biologicals, World Health Organization, Geneva, Switzerland.

For their comments and advice on the first draft of these revised Recommendations, acknowledgements are also due to:

Dr F. Fuchs, Agence Française de Securité Sanitaire des Produits de Santé, Lyon, France; Dr V. Grachev, Institute of Poliomyelitis and Encephalitides, Moscow, Russian Federation.

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Appendix

Summary protocol for the production and testing of poliomyelitis vaccine (inactivated)

Identification of final lot

Name and address of manufacturer _____

_____

Lot no. of final product _____

Date of manufacture of final lot _____
(namely, date of last potency test) _____

Expiry date _____

Total volume of final lot _____

Information on source materials

Virus seed lots

Type (1, 2 or 3)[a] _____

Strain used _____

Origin and source of strain _____

Authority that approved virus seed _____

No. of subcultures between primary seed lot and production _____

Cell cultures (complete only the relevant part, i.e. A, B or C)

Type of cell cultures used _____

A. Continuous cell cultures

Origin and short history of cell seed _____

Authority that approved cell seed _____

Amount of cell culture inoculated _____

Amount of control cell culture investigated _____

Tests performed on control cultures _____

Result _____

[a] A separate protocol should be completed for each type.

B. Monkey secondary or tertiary cell cultures

Type of cells (kidney, fetal kidney, etc.) _____

Species of monkey from which the cells originated _____

Controls performed on animals from which the cells originated _____

No. of cell doublings _____

Result _____

Amount of cell culture inoculated _____

Amount of control cell culture investigated _____

Tests performed on control cultures _____

Results _____

C. Human diploid cell cultures

Origin and short history of cell seed _____

Authority that approved cell seed _____

_____

Amount of cell culture inoculated _____

Amount of control cell culture investigated _____

Tests performed on control cultures _____

Results _____

Serum for cell cultures

Origin of serum used _____

Tests performed on serum _____

Results _____

Information on production

Virus type (1, 2 or 3)[a] _____

Single harvests and monovalent pools

Type of cells used _____

Date of inoculation of cells with virus _____

[a] A separate protocol should be completed for each type.

Date of virus harvest _____

Special observations on single harvests _____

Date of pooling _____

Tests on pooled single harvests

Tests in *Cercopithecus* cell cultures:

Method used _____

Results _____

Test in rabbit kidney cell cultures:

No. of cell cultures _____

Total volume inoculated _____

Period of observation _____

Result _____

Sterility test

Method used _____

Result _____

Tests on control cells

Test for extraneous agents:

Method used _____

Result _____

Test for haemadsorbing agents:

Method used _____

Result _____

Tests in *Cercopithecus* cell cultures (if applicable):

Method used _____

Results _____

Test in rabbit kidney cell cultures (if applicable):

No. of cell cultures _____

Total volume inoculated _____

Period of observation _____

Result _____

Monovalent product before inactivation:

Details of filtration and/or clarification and/or purification (if applied) _____

Date _____

Tests on monovalent pool

Identity test:

Method _____

Result _____

Virus titration:

$TCID_{50}$ per ml _____

Details on filtration and/or clarification and/or purification (if applied) _____

Date(s) _____

Test for retroviruses (if applicable):

Method _____

Result _____

Inactivation of monovalent product:

Agent(s) and concentration _____

Date of start of inactivation _____

Date of taking first sample _____

Date of completion of inactivation _____

Test for effective inactivation (after removal/neutralization of inactivating agent):

Sample size tested _____

Date of first sample _____

Date of second sample _____

Details of testing procedure _____

Period of observation of cell cultures _____

Period of observation of subcultures _____

Result _____

Result of challenge of used culture with live virus _____

Sterility tests:

Method (media) _____

Results _____

Trivalent bulk product (monovalent pools incorporated)

Date of preparation _____

Preservative (if added, type and concentration) _____

Tests on trivalent bulk

Test for absence of infective poliovirus:

Sample size tested _____

Details of testing procedure _____

Period of observation of cell cultures _____

Period of observation of subcultures _____

Result _____

Sterility test:

Method (media) _____

Result _____

Tests on final product

Identity test:

Method used _____

Result _____

Sterility test:

Method (media) _____

Result _____

Innocuity test (if applicable):

No. of animals _____

Dose _____

Period of observation _____

Result _____

Potency test:

Results (and date) of in vitro tests (D-antigen) _____

Results (and date) of in vivo tests, (in rats) if performed _____

Protein content:

Content of protein in μg per human dose _____

Serum protein tests (if applicable):

Result _____

Signature of head of laboratory _____

Certification by person taking overall responsibility for production of the vaccine.

I certify that lot no. _____ of trivalent poliomyelitis vaccine (inactivated) satisfied Part A of the WHO Requirements for Poliomyelitis Vaccine (Inactivated).

Date _____

Signature _____

Name typed _____

The protocol must be accompanied by a sample of the vaccine and a copy of the patient information leaflet.

WHO Technical Report Series, No. 910, 2002

Annex 3
Guidelines for the production and control of Japanese encephalitis vaccine (live) for human use

This document provides information and guidance concerning the history, characteristics, production and control of live attenuated Japanese encephalitis (JE) vaccine to facilitate progress towards the eventual international licensure of the vaccine. The text is therefore written in the form of guidelines instead of Recommendations in view of the fact that further work is needed to develop and standardize appropriate methods and criteria for certain tests, such as the neurovirulence test. Guidelines allow greater flexibility than Recommendations with respect to expected future developments in the field.

1. General considerations[1]

1.1 Japanese encephalitis vaccine

Japanese encephalitis (JE) virus is the most important cause of viral encephalitis in the Asia–Pacific region, accounting for more than 16 000 reported cases and 5000 deaths annually. JE virus is a mosquito-borne flaviviral infection. In the last 25 years, JE virus transmission has intensified in certain countries and the disease has extended its geographical range to previously unaffected areas of Asia and to northern Australia. The high fatality rate and frequent residual neuropsychiatric sequelae in survivors make JE a considerable health problem. For example, in a placebo-controlled study in Thailand in which patients with JE received a high standard of supportive care that included treatment with dexamethasone, 25% of the patients died and 45% demonstrated neurological sequelae 3 months after diagnosis. Furthermore, in the best-documented study of long-term disability due to JE, which was conducted 10 years after the 1947 outbreak of the disease in Guam, neurological sequelae were reported in 40% of surviving patients, 11% of which were considered severe. Neither of these studies evaluated the proportion of cases with psychomotor retardation, fine motor deficits or behavioural disorders.

JE virus is amplified in nature in a cycle involving *Culex* mosquitoes and vertebrate animals, especially pigs. Humans of all ages are susceptible unless immunized by natural infection or vaccination. Evidence shows that effective vaccines will protect animals and humans against illness and will remove the vaccinated animals from the pool of potential amplifying hosts of the virus. Although the control of mosquitoes and the vaccination of pigs are effective in certain circumstances, these measures are not practical as a means of preventing human illness. It is also important to recognize that humans are incidental hosts, and that, for vaccination to be effective, coverage must be maintained indefinitely in all persons who may be exposed to the virus.

The virus replicates in a variety of cultured cells of vertebrate and arthropod origin. Since the 1960s, both live and inactivated vaccines have been developed that provide active immunity against JE virus. The development of these vaccines represents a major advance in the ability to control JE virus infection and reduce the burden of disease. Viruses isolated from human patients in Japan in 1935 and in China in 1949 provided the prototype Nakayama and Beijing and P3 strains, respectively, that are in principal use in the production of inactivated

[1] Parts of the text of this section are abstracted from the minutes of a WHO/(Children's Vaccine Initiative (CVI)) meeting on new initiatives for the control of Japanese encephalitis by vaccination, held in Bangkok, Thailand, 13–15 October 1998 (*1*).

JE vaccine today. National vaccination programmes in China (Province of Taiwan), Japan and the Republic of Korea, using an inactivated mouse-brain-derived vaccine that meets international requirements (*2*), have controlled the disease to the point of elimination, but in other countries, the expense and complexity of producing the vaccine and the need for repeated doses have limited vaccine use. In addition to the problems posed by multiple doses, use of the vaccine to protect travellers has led to hypersensitivity events, including demyelination sequelae, such events have been reported in North America, Australia and Europe.

As an alternative to inactivated vaccines, a live-attenuated JE vaccine (the SA 14-14-2-PHK strain) was developed in China. Since its licensure in China in 1988, more than 300 million doses of JE vaccine (live) have been produced for administration to children in annual vaccination programmes. The vaccine is of considerable interest to countries where JE virus is endemic but, as of the year 2000, is not yet licensed elsewhere.

1.2 History of development of the live attenuated SA 14-14-2 vaccine and characteristics of the vaccine strain[1]

The wild-type parental virus, SA 14, was isolated from a pool of *Culex pipiens* larvae from Xian, China, by 11 passages in mouse brain. The derivation of the SA 14-14-2 strain was through an empirical process of serial passage, principally in primary hamster kidney (PHK) cells, and demonstrated that a fine balance exists between safety through stable neuroattenuation and immunogenicity, with sufficient viral replication to stimulate immunity. Properties of viral clones in the pedigree from which the vaccine strain was eventually derived illustrate the inherent subtleties of JE viral attenuation. The early 12-1-7 strain exhibited a low degree of pathogenicity; however, it was unstable and reverted to neurovirulence after one passage in mouse brain or several passages in PHK cells. The 9-7 strain derived from the 12-1-7 clone was attenuated in mice and monkeys and did not revert with passage in either mouse brain or PHK cells, but it produced seroconversions in less than 10% of vaccinated children. To increase its immunogenicity, the strain was passaged orally in hamsters and plaque purified in primary chick embryo (PCE) cells to derive the 5-3 strain. This clone was attenuated, but its immunogenicity was only 65% of vaccinated children. Further passages in suckling mice and plaque purifications in PHK cells led to the 14-14-2 strain, which was stably attenuated and immunogenic in 85–100% of vaccinated children.

[1] Further details have been published (*1*).

In contrast to its parent strain, the SA 14-14-2 strain is avirulent when administered by the intracerebral and intraperitoneal routes in weanling mice, Syrian hamsters and in mice given immunosuppressive treatment with cytoxan. The virus is virulent for nu/nu mice only when administered by intracerebral inoculation, but with a longer incubation period than its parent. Monkeys inoculated by both the intrathalamic and intraspinal routes develop asymptomatic infections and, on neuropathological examination, exhibit a minor degree of inflammatory reaction only along the needle track, with minimal neuronal infection or neuronal death. Small plaque morphology and neuroattenuation in mice is retained through at least 23 further PHK cell passages, using conditions of infection (e.g. multiplicity of infection and incubation temperature) identical to those employed in production. Molecular studies indicated that the sequence of the neurovirulent parent SA 14 differs from that of SA 14-14-2 in 57 nucleotides and results in 24 amino acid changes. Studies also showed that eight amino acid substitutions in the E protein genome of SA 14-14-2 vaccine virus were unchanged after additional passage in PHK cells. The points mentioned above provide strong evidence for the genetic stability of the SA 14-14-2 vaccine virus in the PHK cell substrate.

An initial attempt to adapt the SA 14-14-2 strain to primary dog kidney cells found that only nine additional passages led to further attenuation and a reduction in its immunogenicity to only 40% of vaccinated children.

The complete passage history of the SA 14-14-2 strain is given in Appendix 1. Because the passage history of this strain included passage in both hamsters and mice, special attention is required to demonstrate the absence of detectable adventitious agents of these two species in the seed stocks of the vaccine. As with any vaccine, tests for adventitious agents may need to be extended as new infectious agents or test procedures become known and available.

The SA 14-14-2 strain grows to a titre of $>10^7$ in PHK cells and produces a cytopathic effect and small plaques under overlay. The use of PHK cells for routine production of JE vaccine (live) is clearly effective, but raises particular cell substrate issues (see section 1.4).

An alternative strategy to the current vaccine production system, which includes extensive testing as described below, is to re-derive a virus master seed from the current seed. This approach would require considerable effort to characterize the new seed and to assess its clinical safety and efficacy, but would result in a significant reduction in testing during manufacture.

1.3 Preclinical studies

In guinea-pigs, a single dose of SA 14-14-2 virus elicits immunity that significantly reduces viraemia levels produced by viral challenge. Either spleen cells or serum could passively transfer immunity. Evidence that the live vaccine elicited a stronger cellular immune response than inactivated vaccine was seen in challenge experiments comparing mice immunized with the respective vaccines. Despite equal titres of circulating neutralizing antibody, survival after intracerebral challenge was significantly higher in animals previously immunized with live vaccine than in those immunized with inactivated vaccine. Cytoxan immunosuppression of mice vaccinated with SA 14-14-2 did not alter their resistance to lethal viral challenge in contrast to mice vaccinated with inactivated vaccine, in whom survival was reduced by 90% after immunosuppression.

In vaccination/challenge studies, mouse survival was significantly greater after vaccination with one dose of live vaccine compared with two doses of inactivated vaccine derived from mouse brain or PHK cells, and challenge with the P3, Nakayama or 12 field strains isolated in China. Experiments also showed that one dose of the live vaccine protected 90–100% of mice against challenge with field strains isolated in countries such as India, Indonesia, Japan, the Philippines, Thailand and Viet Nam (*3*).

Although the growth of SA 14-14-2 virus in *Culex tritaeniorhynchus* has not so far been evaluated, the attenuated SA 14-1-8 clone, derived from the same pedigree with a similar phenotype, showed no transmission in experimental studies (*4*). In contrast, the SA 14 parent was transmitted in mosquitoes at rates of 75–78%. Recent studies to assess the extent of detectable viraemia following vaccination with the SA 14-14-2 strain showed that JE virus is undetectable from the time of inoculation through day 9. These results are consistent with previous reports showing that wild-type JE virus is undetectable in infected symptomatic persons and that there is no amplification of JE virus in humans. In view of the above, and the estimated requirement for 100 000–1 000 000 virions per ml of plasma for mosquitoes to access sufficient virus from a human to transmit to others (*5*), it is highly unlikely that the SA 14-14-2 strain would be transmitted via a mosquito bite of a recent vaccinee. Nevertheless, further consideration should be given to additional transmission studies with the SA 14-14-2 vaccine strain.

1.4 Cell substrate issues

Prevention of transmission of adventitious infections from the virus seed, the cell substrate, and the serum or trypsin used in the manufac-

turing process, is a general concern with all live-virus vaccines. For the SA 14-14-2 vaccine, the lack of precedence for a PHK cell substrate in live attenuated vaccine is a special issue. However, PHK is recognized as an acceptable substrate for inactivated JE vaccine and hantavirus vaccine (Haemorhogic Fever with Renal Syndrome (HFRS) vaccine). Current controls cover a broad range of potential rodent virus contaminants. State-of-the-art developments should be introduced as appropriate. Validation issues with respect to the assays used, e.g. the application of assays for retroviruses to PHK cells, need to be considered. In addition, the principle of reducing the risk of adventitious agents entering the manufacturing process should be encouraged by using healthy animals, preferably from a closed specific pathogen-free colony that is monitored regularly, as a source material for preparation of PHK cells. In common with all live-virus vaccines, steps to exclude potential contaminants of serum and trypsin employed in manufacturing, including specific bovine and porcine viruses and transmissible spongiform encephalopathy agents, would be expected.

The demonstration of consistency of vaccine production in PHK cell culture is also important. As of 2000, three manufacturers in China have 12, 6 and 1 years of experience, respectively, of vaccine production in PHK cells. Criteria that may be monitored include aspects of cell growth such as morphological characteristics and days-to-plating confluency. Consistency of viral yield is evaluated by precise and reproducible titration procedures and possibly by monitoring genotypic and phenotypic markers. For new manufacturers, consistency measures under conditions of scale-up and large-scale production are required.

The creation of a well-characterized master PHK cell bank at the secondary or tertiary level is a potential way to improve lot-to-lot consistency and simplify quality control. Preliminary experience at one manufacturer showed that plating efficiency declined by 40% after primary cells were frozen and thawed, but this does not mean that this approach would not be practicable in the future. Attempts to adapt the SA 14-14-2 strain to other cell cultures would be expected to result in fundamental changes in biological characteristics, and attempts using MRC-5, primary chick and duck embryo cells, and primary dog kidney (PDK) cells were unsuccessful (see above). Preliminary research indicates that the strain can be adapted to Vero cells with the preservation of neuroattenuation in mice but preclinical and clinical equivalence with the PHK-produced SA 14-14-2 vaccine remains to be proven.

1.5 Neurovirulence testing

During development of the SA 14-14-2 strain, it was demonstrated that attenuation and a lack of reversion to neurovirulence of some JE

virus clones could be achieved. This was on the basis of extensive testing in mice, using three different types of test: a test for neurovirulence, a test for reversion to neurovirulence; and a test for neurotropism. It is therefore necessary to demonstrate that master and working seeds, and vaccine lots, have retained the characteristics of attenuation of the original SA 14-14-2 strain. The tests that were used in the development of the vaccine are described later in these Guidelines. It will be necessary to demonstrate that the tests will reliably discriminate suitable from unsuitable materials. Participation in collaborative studies and inclusion of a common reference preparation that is tested in parallel using the same procedures would be extremely useful and is strongly encouraged.

1.6 Clinical safety[1]

Vaccine safety has been evaluated in several small-scale studies and in two large-scale postmarketing studies in China. Studies of 588 512 children aged between 1 and 15 years inoculated with vaccine from one manufacturer and of 60 000 children given vaccine from another manufacturer reported no cases of temporally associated encephalitis. The most common adverse effect associated with vaccination was fever, which was reported in less than 0.2% of vaccinated children, with lower rates for rash and other systemic symptoms.

Daily examination of 867 vaccinated children for fever (>38 °C) disclosed low rates with onset distributed evenly over the 21-day observation period, without clustering as might have been expected if onset were associated with a specific incubation period. Temperature elevations were limited to a single day in most cases. In uncontrolled observations of 1946 children vaccinated between the ages of 1 and 6 years, short-term fever was reported in only two, who had concurrent respiratory infections. Local reactions, occurring in 6.2% of vaccinees overall, were more common in older children who had received previous doses of inactivated JE vaccine.

One longer-term study followed approximately 150 000 children vaccinated between 1 and 6 years of age for 5 years and found no potential late-onset complications such as optic atrophy (*6*). Another recent study (7) included 230 children aged 13–15 years who were evaluated clinically and serologically at 1, 2, 2.5 and 3 months after vaccination. No neurological signs or symptoms, or other significant medical sequelae were found.

The best-characterized study, conducted under the auspices of the Rockefeller Foundation in Chengdu, southwestern China, compared

[1] Further details have been published (*1*).

hospitalizations and specific illnesses and symptoms in block randomized cohorts of more than 13 000 vaccinated children and more than 12 500 children in whom routine JE vaccination was deferred for 1 month (*8*). The 1–2-year-olds were followed prospectively for 1 month after their initial visit for vaccination, and on their return, parents were questioned about hospitalizations and the occurrence of specific illnesses in the intervening month, including encephalitis and meningitis, and the new onset of seizures. No illnesses involving the central nervous system were reported in either group, and the rates of occurrence of recent onset of seizures, hospitalizations, fever lasting >3 days, and various other illnesses such as diarrhoea, respiratory infections and reactions consistent with anaphylaxis were similar in the two groups.

A subset of 266 vaccinated children was examined prospectively for side-effects 1, 2, 3 and 7 days after the vaccination visit. Various minor local and systemic symptoms were observed, but all at a low rate. Fever occurred in 5% of vaccinees. This study provides convincing evidence of the short-term safety of the SA14-14-2 vaccine during the first 30 days after immunization.

Concern that a live vaccine derived from an encephalitogenic virus might lead to vaccine-associated encephalitis could not be addressed to a high level of sensitivity, even with a study of 26 000 children. From the observation of no cases in the month after immunization, an interval expected to encompass the incubation period of infectious encephalitis, the upper 95% confidence interval indicates the rate of encephalitis following vaccination with SA 14-14-2 vaccine is unlikely to exceed 2.3 cases per 10 000.

Since the introduction of SA 14-14-2 vaccine in China in 1988, over 300 million doses have been given to an estimated 120 million children. All doses were administered on a campaign basis in March and April of each year. Vaccine-associated cases of encephalitis would be expected to occur in April and May, thus creating a shoulder on the normal seasonal curve of hospitalization due to JE. No such shoulder has been observed in a preliminary examination of the data. Further monitoring and refinement of these data are continuing in China, and are encouraged. Other epidemiological opportunities to evaluate the safety of the vaccine should also be considered.

An assessment of the benefits and risks related to SA 14-14-2 vaccine is an important element in determining the appropriate use of the vaccine, and should include an evaluation of the health burden attributable to JE before the introduction of the vaccine in another country.

1.7 Immunogenicity in children[1]

Immunogenicity in children follows a dose–response gradient, with antibody responses in >92% of vaccinees receiving 1 000 000 viral plaque-forming units (PFU) from SA 14-14-2 vaccines produced in China after 1988. Antibody responses are seen in all subjects given a second dose. Among the responders to primary immmunization, all retained measurable neutralizing antibodies for at least 3 years. On the basis of data on the immunogenicity and efficacy of the vaccine in children in various ethnic groups in China as well as in children in Nepal and the Republic of Korea, the immune response does not appear to be influenced by the ethnic background of vaccinees.

1.8 Efficacy in clinical studies[1]

The SA 14-14-2 vaccine was shown to provide protection in over 300 000 children aged between 1 and 10 years in four field trials. The efficacy of one dose was >95% in every trial and, in one location where the vaccinated children were followed through five transmission seasons, the efficacy was >98% throughout this interval. In 1999, the SA 14-14-2 vaccine was given as a single dose to over 220 000 residents of the Terai region of Nepal in an effort to reduce the impact of an emerging epidemic of JE. A case–control study demonstrated 99.12% efficacy (*9*).

The above-mentioned trials were conducted in such a way that may have introduced biases in risk of exposure in vaccinated and unvaccinated subjects. However, when a fully effective vaccine is licensed and in actual use, it is not ethically permissible to re-study vaccine efficacy using a placebo-controlled field trial. A reliable, sensitive and accurate alternative that takes advantage of inadvertent vaccine failures, usually due to missed immunizations, is a case–control study in which the vaccination histories of cases and matched controls are compared. A study conducted under the auspices of the Rockefeller Foundation in Sichuan province, China, in which all village children of the same age as serologically confirmed JE cases were selected as controls, found an effectiveness of 98% with two doses and 80% after one dose (95% confidence interval (CI), 44–93%). The broad confidence interval of the latter estimate reflects the small number of subjects in that category. The results of this study support the previously cited studies of the effectiveness of SA 14-14-2 vaccine while extending those observations relating to its effectiveness under field conditions of delivery, storage and administration.

[1] Further details have been published (*1*).

2. Scope

These Guidelines relate to the production of JE vaccine (live) in PHK cells using the SA 14-14-2 strain of virus. No provision has been made for vaccines produced with other viral strains or cell lines.

The Guidelines cover control of the following three areas:

— the starting materials;
— the manufacturing process;
— the final product.

The general manufacturing requirements contained in Good Manufacturing Practices for Pharmaceutical (*10*) and Biological (*11*) Products should apply to establishments manufacturing live JE vaccine, with the addition of the following:

All staff directly involved in the production and testing of live JE vaccine should be shown to be immune to JE by appropriate haemagglutination inhibition or neutralizing antibody tests.

Written descriptions of the standard operating procedures used for the preparation and testing of live JE vaccine, together with evidence of appropriate validation of each production step, should be submitted for approval to the national control authority as part of the licensing application. Proposals for any modifications of the manufacturing and/or control methods should be submitted for approval to the national control authority before they are implemented.

2.1 Control of starting materials

2.1.1 *Animals*

Syrian hamsters aged between 10 and 14 days may be used as the source of kidneys for cell culture. Only hamster stock approved by the national control authority and derived from a closed, healthy colony should be used as the source of tissue. A closed colony is a group of animals sharing a common environment and having their own caretakers who have no contact with other animal colonies. The animals should be tested according to a defined programme to ensure freedom from specified pathogens and antibodies to those pathogens. At the time the colony is established, all animals should be tested to show freedom from antibodies to Hantaan virus, Kilham rat virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse hepatitis virus, mouse poliovirus, pneumonia virus of mice, respiratory enteric orphan (REO) virus 3, Sendai virus (parainfluenza virus 1), Simian virus 5 and Toolans H-a virus. Tests for production of hamster, mouse and rat antibodies should also be performed. A test for retroviruses using a sensitive polymerase chain reaction (PCR)-based reverse

transcriptase (Rtase) assay should also be included. The results of such assays may need to be interpreted with caution because Rtase activity is not unique to retroviruses and may be derived from other sources, such as retrovirus-like elements that do not encode a complete genome (*12*). Nucleic acid amplification tests for retroviruses may also be used. A PCR test for hamster polyoma virus should be used on a selected number of hamster tissues, especially kidneys, to qualify the colony, and should be repeated at intervals thereafter. When new animals are introduced into the colony, they should be maintained in quarantine in vermin-proof quarters for a minimum of 2 months and shown to be free from these specified pathogens.

The parents of animals to be used as a source of tissue should be maintained in vermin-proof quarters. Neither the parent hamsters nor their progeny should previously have been used for experimental purposes, especially those involving infectious agents. The colony should be monitored for zoonotic viruses and markers of contamination according to a defined programme at regular intervals.

Once the colony is established, it should be monitored by testing a representative group of animals consisting of at least 5% of the animals that are bled at intervals acceptable to the national control authority. For example, birds used in the production of chick embryo fibroblast cells for measles vaccine (*13*) are bled at monthly intervals. In addition, the colony should be screened for pathogenic bacteria, including mycobacteria, fungi and mycoplasmas, as agreed with the national control authority. The screening programme should test 100% of the animals over a defined period of time, as agreed with the national control authority.

Any animal that dies should be investigated to determine the cause of death. If the presence of an infectious agent is demonstrated in the colony, the national control authority should be informed and the manufacture of live JE vaccine may be discontinued. In this case, manufacture should not be resumed until a thorough investigation has been completed and precautions have been taken against the infectious agent being present in the product, and then only with the approval of the national control authority.

At the time of harvest of the kidneys, the animals should be examined for the presence of any abnormalities. If any kidney abnormalities or other evidence of pathology is found, those animals should not be used for JE vaccine production.

Each group of control cultures derived from a single group of animals used to produce a single virus harvest should remain identifiable as such until all testing, especially for adventitious agents, is completed.

2.1.2 *Cell cultures for virus propagation*

2.1.2.1 Primary hamster kidney cells

Kidneys derived from animals which comply with section 2.1.1 should be dissected and homogenized under conditions approved by the national control authority. A primary cell suspension is obtained after digestion with trypsin and this is distributed, together with growth medium, into cell culture vessels.

Penicillin and other β-lactam antibiotics should not be used at any stage of manufacture.

Minimal concentrations of suitable antibiotics such as kanamycin may be used if approved by the national control authority.

2.1.2.2 Serum used in cell culture medium

Serum used for the propagation of cells for JE vaccine production should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to the requirements given in Part A, sections A.5.2 and A.5.3 of the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*14*), and to demonstrate freedom from infectious viruses.

> In some countries, sera used for other vaccines are also examined for freedom from certain phages.

Serum of bovine origin should be approved by the national control authority and should come from countries or herds certified to be free of bovine spongiform encephalopathy and should comply with the requirements given in the Requirements for the Use of Animal Cells as in vitro Substrates for the Production of Biologicals (*15*) and the Report of a WHO Consultation on Medicinal and other Products in Relation to Human and Animal Transmissible Encephalopathies (*16*).

> For other vaccines, some countries require that bovine serum should come from herds that have not been given feed derived from ruminant protein.

Suitable tests for detecting bovine viruses in serum are given in Appendix 1 of the 1999 Recommendations for the Production and Control of Poliomyelitis Vaccine (Oral) (*17*).

If human albumin is used, it should meet the 1992 Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives (*18*), as well as current guidelines in relation to human transmissible encephalopathies (*16*). In addition, human albumin, if used, should be tested by PCR to demonstrate freedom from human immunodeficiency virus (HIV) and hepatitis C virus nucleic acids.

2.1.2.3 Trypsin used for preparing cell cultures

Trypsin used for preparing cell cultures should be tested and found free from cultivable bacteria, fungi, mycoplasmas and infectious viruses, especially bovine or porcine parvoviruses, as appropriate. The methods used to ensure this should be approved by the national control authority.

The source(s) of bovine trypsin, if used, should be approved by the national control authority. Bovine trypsin, if used, should comply with the Requirements for the Use of Animal Cells as in vitro Substrates for the Production of Biologicals (*15*) and the Report of a WHO Consultation on Medicinal and other Products in Relation to Human and Animal Transmissible Encephalopathies (*16*).

2.1.3 ***Virus seeds***

The virus master and working seed lots used to produce JE vaccine (live) should be shown to be safe and immunogenic by appropriate laboratory tests (sections 2.1.3.2.1, 2.1.3.2.2 and 2.2).

2.1.3.1 Virus strain certification

Only SA 14-14-2 PHK strains of JE virus that are approved by the national control authority should be used in the production of vaccine. Each strain should be identified by historical records, which should include information on its origin, its method of attenuation, and the passage level(s) at which attenuation, immunogenicity, safety and efficacy were demonstrated by clinical studies.

2.1.3.2 Virus seed lot system

The production of vaccine should be based on the virus seed lot system that includes a master seed and a working seed. All virus seed lots should be stored in a suitable manner.

2.1.3.2.1 Tests on virus master seed lots

The virus master seed should be produced under the conditions described in section 2.2, and should be free from detectable adventitious agents.

(1) Identity test

Each virus master seed lot should be identified as JE virus by serological methods approved by the national control authority.

Appropriate serological methods include enzyme immunoassays using a JE virus-specific monoclonal antibody, immunofluoresence, or JE neutralization assays using a reference serum or monoclonal antibody specific to JE virus. A test for genetic identity should also be performed.

(2) Virus titration and infectivity

The infectivity of each virus master seed lot should be established in an assay approved by the national control authority, such as the PFU method in the baby hamster kidney (BHK)-21 cell line. Alternative cell lines may also be acceptable to the national control authority. Manufacturers should determine the appropriate titre to reliably produce vaccine.

In the PFU method in BHK-21 cells used in some laboratories, samples from the virus suspension are diluted 10-fold serially, and the titre is estimated after plaques have developed.

The titration should be made in parallel with a reference vaccine that is approved by the national control authority.

Alternative assays such as that to determine the cell-culture infective dose 50% ($CCID_{50}$) may be used with the approval of the national control authority, provided they have been calibrated against the PFU assay.

(3) Freedom from bacteria, fungi and mycoplasmas

Each virus master seed lot should be shown to be free from bacterial, mycotic and mycoplasmal contamination by appropriate tests as specified the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*14*).

(4) Tests for adventitious agents

Each virus master seed lot should be tested in cell cultures for adventitious agents appropriate to the passage history of the seed virus. Neutralization of JE virus is necessary for many tests because the virus is generally cytopathogenic. JE virus immune serum, produced in a heterologous system, or a JE virus-specific monoclonal antibody, should itself be shown to be free of adventitious agents.

A volume of each virus master seed lot of at least 10 ml should be tested for adventitious agents by inoculation onto cultures of human cells, mouse cells, simian cells, mosquito cells (e.g. C6/36), BHK-21 cells and PHK cells. The cell culture should not be from the same batch as that used in the preparation of the virus seed. Uninoculated control cell cultures should be included in the tests. All cell cultures (except mosquito cells) should be incubated at 35–37 °C and observed for at least 14 days. At least one subculture of one cell culture fluid should be made and observed for 14 days in order to enhance the opportunity to detect adventitious agents.

The cells should be observed microscopically for cytopathic changes. At the end of the observation period, the cells or fluids should be tested for haemadsorbing viruses as specified in section 2.2.1.1. For the test to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test period. For the seed virus to be satisfactory, no cytopathic changes or adventitious agents should be detected.

Tests for the following agents should be conducted on the virus master seed lot to rule out the presence of adventitious agents associated with any primary cell cultures that were used in the adaptation of the JE virus. Such agents include mouse viruses using the mouse antibody production (MAP) assay; hamster viruses using the hamster antibody production (HAP) assay; human, porcine and bovine viruses by PCR; endogenous hamster, avian and other retroviruses using a sensitive assay such as product-enhanced reverse transcriptase (PERT) (*12*).

Consideration should be given to electron microscopic investigation using the negative stain technique in order to obtain additional information on extraneous agents that may not be detected by other methods.

(5) Tests for attenuation

Neurovirulence tests in monkeys
New virus master seed lots should be tested for neurovirulence in monkeys (see Appendix 2). To avoid the unnecessary use of monkeys, virus master seed lots should be prepared in large quantities. A reference preparation should be included in each test. An alternative test in monkeys may be used, with agreement of the national control authority, if equal or greater sensitivity has been demonstrated.

Neurovirulence tests in mice
The virus master seed lot should be shown to be free from neurovirulence by a test in mice. A reference preparation should be prepared and included as a positive control to validate each test. The selection of one or more reference preparations is a matter of high priority and should be made in consultation with experts in neurovirulence testing who also should advise on the development and implementation of a collaborative study to validate the ability of the test system to reliably distinguish suitable from unsuitable vaccine preparations.

During development of the vaccine the following test was used:

Each of at least 10 mice of the Kunming strain of Swiss mice, 17–19 days old, were inoculated intracerebrally with 0.03 ml of the undiluted virus master seed lot. Mice were observed daily for 14 days after intracerebral inoculation. Mice that died within 3 days of inoculation were considered to have died from brain trauma, and were not included in the evaluation of the test. If more than 20% of mice died within 3 days, the test was considered invalid. From any mouse that showed signs of any illness more than 3 days after inoculation, the brain was removed immediately for further testing. An approximately 10% mouse brain suspension was made and 0.03 ml of –1, –2, –3 and –4 log dilutions were each separately inoculated into four 17–19-day-old mice (Kunming strain of Swiss mice). If the intracerebral titre of any mouse brain suspension exceeded 3.0 log LD_{50}/0.03 ml in 17–19-day-old mice the test material was considered unsuitable.

Modifications to this test may be used to evaluate a new virus master seed lot if approved by the national control authority.

Female ICR mice aged between 28 and 32 days provide a neurovirulence test system for laboratory strains of SA 14-14-2 virus, and may be further evaluated and considered as an alternative to Kunming Swiss mice (*19*). NIH mice have also been used in one country as an alternative to Kunming Swiss mice.

Test for reversion to neurovirulence in mice

The virus master seed lot should be shown to be free from reversion to neurovirulence by a test in mice that will reliably distinguish suitable from unsuitable preparations. A reference vaccine should be included to validate each test.

During development of the vaccine the following test was used:

Each of at least 10 suckling mice of the Kunming strain of Swiss mice 3–5 days old were inoculated intracerebrally with 0.02 ml of the undiluted virus master seed lot. Experience had shown that most mice showed signs of illness after 6–8 days. The brains of 3–5 of the mice with the most severe signs (of convulsions and irritation) were removed and then emulsified together into a 10% suspension. The suspension was assessed in two ways. Firstly by the intracerebral inoculation of 17–19-day-old mice (Kunming strain of Swiss mice) with suspension prepared as follows. Serial 10-fold dilutions of the 10% suspension, from –1 to –4 inclusive, were made in medium that contained 0.5% lactalbumin hydrolysate and 2% calf serum and 0.03 ml of each dilution was inoculated into four mice. If the intracerebral LD_{50} titre exceeded 3.0 log LD_{50}/0.03 ml the preparation was considered unsuitable.

The parental SA14 virus had an LD_{50}/0.03 ml of 8.5 log in this test. In the second assessment the 10% brain suspension was inoculated by the subcutaneous route into at least ten 15–17-day-old mice (Kunming strain of Swiss mice) (0.1 ml per mouse). If any mouse showed signs of JE viral infection (such as convulsions or irritation) during the 14-day observation period the preparation was considered unsuitable. The parental SA14 strain had an LD_{50}/0.1 ml of 5.0 log in this test.

Alternative strains of mice may also be suitable for this test and the incubation period may be extended beyond 14 days.

Modifications to this test may be used to evaluate a new virus master seed lot if approved by the national control authority.

Test for neurotropism in mice
During development of the vaccine the following test was used:

Each of at least 10 mice of the Kunming strain of Swiss mice 15–17 days old were conditioned by injecting a sterile needle into the head to destroy the blood–brain barrier, and were then inoculated subcutaneously with 0.1ml of the master virus seed. If any mice showed signs of infection with JE virus (such as convulsions or irritability) during the 14-day observation period, the preparation was considered unsuitable.

Omission of this test on a new virus master seed lot may be considered with the approval of the national control authority.

2.1.3.2.2 Tests on virus working seed lots

The virus working seed lot used for the production of vaccine batches should be prepared from a qualified virus master seed lot and by a method approved by the national control authority. The virus working seed lot should be limited to three passages in PHK cells beyond the master seed lot. If the virus working seed is in liquid form, it should be stored at –60 °C or below for a period agreed with the national control authority. If the virus working seed is in freeze–dried form, it should be stored at –20 °C or below for a period agreed with the national control authority.

(1) Identity

Each virus working seed lot should be identified as JE virus by serological methods approved by the national control authority. Appropriate serological methods include enzyme immunoassays using a JE virus-specific monoclonal antibody, immunofluorescence, or neutralization assays using a reference serum or monoclonal antibody specific to JE virus. A test for genetic identity

should also be performed, such as determination of the sequence of the E protein.

(2) Virus titration and infectivity

The infectivity of each working seed lot should be established in an assay approved by the national control authority, such as the PFU method in the BHK-21 cell line. Alternative cell lines may also be acceptable to the national control authority. Manufacturers should determine the appropriate titre to reliably produce vaccine.

In the PFU method in BHK-21 cells used in some laboratories, samples from the virus suspension are 10-fold serially diluted and the titre is estimated after plaques have developed.

The titration should be made in parallel with a reference vaccine that is approved by the national control authority.

Alternative assays such as those to determine the $CCID_{50}$, may be used with the approval of the national control authority.

(3) Freedom from bacteria, fungi and mycoplasmas

Each virus working seed lot should be tested for bacterial, fungal and mycoplasmal sterility according to the requirements given in Part A, sections A.5.2 and A.5.3 of the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*14*).

(4) Tests for adventitious agents

Each virus working seed lot should be tested in cell cultures for adventitious agents. Neutralization of JE virus is necessary for most tests because the virus is generally cytopathogenic.

A volume of each seed lot of at least 10 ml should be tested for adventitious agents by inoculation into a cercopithecid cell culture. Similar volumes should be tested in cultures of human cells, BHK-21 cells and cells of the same type but not the same batch as those used in the preparation of the virus seed. Uninoculated control cell cultures should be included in the tests. All cell cultures should be incubated at 35–37 °C and should be observed for at least 14 days.

The cells should be examined for cytopathic changes during the observation period. At the end of the observation period, the cell cultures should be tested for haemadsorbing viruses and other adventitious agents as specified in sections 2.2.1.1.1 and 2.2.1.1.2, respectively. One subpassage of one cell culture per cell line should be done and the tests in sections 2.2.1.1.1 and 2.2.1.1.2

repeated. For a test to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test period. For the seed virus to be satisfactory, no cytopathic changes or adventitious agents should be detected.

(5) Test for neurovirulence in mice

Comparative experimental studies of 17–19-day-old mice and rhesus monkeys have shown that mice infected by intracerebral inoculation are a more sensitive system to detect JE viral neurovirulence than are intracerebrally inoculated monkeys (*20*).

The virus working seed lot should be shown to be free from neurovirulence by the test for neurovirulence in mice (section 2.1.3.2.1).

2.2 Control of the manufacturing process

2.2.1 *Control of vaccine production*

2.2.1.1 Control cell cultures

At least 5% of the total volume or 500 ml, whichever is greater, of the cell suspension should be used to prepare control cultures. This approach is similar to that taken for measles vaccine produced in chick embryo fibroblast cells (*13*).

The control cells should be maintained under similar conditions of time, temperature and media as the infected cells. They should be observed for cytopathic effects for 14 days from the time of seeding the cells, which may include a subpassage and observation for a further 14 days (i.e. a total of 28 days). At the end of the observation period, the cells should be checked for haemadsorbing viruses as described in section 2.2.1.1.1.

Samples that are not tested immediately should be stored at –60 °C or below.

If any such tests show evidence of the presence in control cultures of any adventitious agents, the harvest of virus should not be used for vaccine production.

For the test to be valid, not more than 20% of the control culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test period.

2.2.1.1.1 Test for haemadsorbing viruses

At the end of the observation period, 25% of the control cells should be tested for the presence of haemadsorbing viruses, using guinea-pig red blood cells. If the red cells have been stored, the duration of

storage should not have exceeded 7 days, and the temperature of storage should have been in the range of 2–8 °C.

In some countries, the national control authority requires that the control cell cultures described above should be tested for the presence of haemadsorbing viruses at the end of the production culture incubation period(s). If this is the case, the test for haemadsorbing viruses described here may be deleted.

In some countries, the national control authority requires that other types of red cells, including cells from humans (blood group O), monkeys and chickens (or other avian species) should be used in addition to guinea-pig cells. In all tests, readings should be taken after incubation for 30 minutes at 0–4 °C, and again after a further incubation for 30 minutes at 20–25 °C. For the test with monkey red cells, readings should also be taken after a final incubation for 30 minutes at 34–37 °C.

For the tests to be valid, not more than 20% of the control culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test period.

2.2.1.1.2 Tests for other adventitious agents

At the time of harvest, a sample of 10 ml of the pooled fluid from each group of control cultures should be taken and tested in the same type of cell culture, but not the same batch, as that used for virus production. The test should be performed in both human and cercopithecid cell cultures.

Each sample should be inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet should be at least 3 cm^2 per ml of pooled fluid. At least one bottle of each kind of cell culture should remain uninoculated and should serve as a control.

The cultures should be incubated at a temperature of 35–37 °C and should be observed for a period of at least 14 days. During this observation period the cultures should be examined at least every third day for cytopathic changes.

The tests are satisfactory if no cytopathic changes attributable to adventitious agents in the test sample are detected.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test period.

2.2.2 *Virus inoculation and incubation*

Cell cultures with a dense single layer should be selected and washed thoroughly, after which a suitable volume of maintenance medium

should be added. The virus working seed inoculum should achieve a multiplicity of infection of approximately 0.001 (some manufacturers use a final titre in the culture vessel of 2.7–3.7 log PFU per ml). The inoculated cultures should be incubated at 35–36 °C, usually for 3 days, but not more than 4 days.

2.2.3 *Control of single virus harvests*

2.2.3.1 Harvest of vaccine virus

The vaccine virus fluid should be harvested when the cytopathic effect (CPE) becomes obvious. This is usually after 3 days, and should not be more than 4 days. The harvests should be stored at an appropriate temperature, as agreed with the national control authority.

2.2.3.2 Tests for sterility

A sample of each single harvest or virus culture supernatant should be tested for bacterial, fungal and mycoplasmal sterility according to the requirements given in the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*14*). If contamination is detected, the harvest should be discarded.

2.2.3.3 Virus content

The virus content of each single harvest should be tested using an infectivity assay approved by the national control authority, such as the PFU method in the BHK-21 cell line to determine the acceptability of the material for further processing and to confirm consistency of production. Tests using other cell lines may also be acceptable to the national control authority.

> In the PFU method in the BHK-21 cell line used in some laboratories, samples from the virus suspension are diluted 10-fold serially, and the titre is estimated after plaques have developed.
>
> In one country the virus titre should not be less than 7.2 log PFU per ml.
>
> The titration should be made in parallel with a reference vaccine that is approved by the national control authority.

Alternative assays, such as those to determine the $CCID_{50}$, may be used with the approval of the national control authority.

2.2.3.4 Test for identity

A test for identity (see section 2.1.3.2.2 of these Guidelines) should be performed if not done on the virus pool. It is not necessary to perform a test for genetic identity on single virus harvests.

2.2.3.5 Test for adventitious agents

If the single virus harvests are not pooled on the same day, a test for adventitious agents should be performed on each single virus harvest (see section 2.1.3.2.2).

2.2.4 *Preparation and control of virus pool*

2.2.4.1 Pooling of single virus harvests

Only virus harvests meeting the recommendations for sterility and virus content in sections 2.2.3.2 and 2.2.3.3 of these Guidelines should be pooled.

2.2.4.2 Clarification of vaccine virus pool

The vaccine virus pool should be clarified or filtered by a method that maximizes removal of cells and cell debris.

Samples of the clarified bulk suspension should be taken immediately after clarification to ensure that no microscopically observable cells or cell particles remain. Samples should also be taken to confirm the identity and determine the infectious virus content of the pool. If not tested immediately for virus content, the samples should be stored below –60 °C until testing is done.

2.2.4.3 Virus content

The virus content of the virus pool should be assayed by titration in cell culture against a reference preparation of live JE vaccine as described in section 2.2.3.3 of these Guidelines, and must be approved by the national control authority.

2.2.4.4 Test for identity

A test for identity (see section 2.1.3.2.2 of these Guidelines) should be used if not done on the single virus harvest. However, it is not necessary to perform the genetic identity test on the vaccine virus pool.

2.2.4.5 Tests for sterility

After clarification the virus pool should be tested for bacterial, fungal and mycoplasmal sterility according to the requirements given in the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*14*), or by a method approved by the national control authority.

2.2.4.6 Test for adventitious agents

A test for adventitious agents (see section 2.1.3.2.2 of these Guidelines) should be performed if not done on the single virus harvests.

2.2.4.7 Test for residual materials

Each manufacturer should demonstrate, by testing each virus pool, or by validation of the manufacturing process, that any residual materials used in manufacture, such as animal sera, are consistently reduced to a level acceptable to the national control authority.

2.2.4.8 Tests for retroviruses

Samples from the filtered virus pool should be examined for the presence of retroviruses by an assay for reverse transcriptase (Rtase) acceptable to the national control authority. Confirmation that the assays used will detect retroviruses potentially present in PHK cells should be presented.

Recently developed highly sensitive PCR-based assays for Rtase may be considered, but the results need to be interpreted with caution because Rtase activity is not unique to retroviruses and may be derived from other sources, such as retrovirus-like elements that do not encode a complete genome (*12*). Nucleic acid amplification tests for retroviruses may also be used.

2.3 Preparation and control of the final bulk

2.3.1 *Pooling bulk material*

More than one virus pool satisfying the control tests in section 2.2.4 of these Guidelines may be pooled and diluted to form the final bulk.

2.3.2 *Added substances*

In the preparation of the final bulk, only substances such as diluents or stabilizers approved by the national control authority should be added. The concentration of such substances should be approved by the national control authority.

Such substances should have been shown by appropriate tests not to impair the safety or effectiveness of the vaccine.

2.3.3 *Tests for sterility*

Each final bulk should be tested for bacterial, fungal and mycoplasmal sterility according to the requirements given in the 1973 General Requirements for the Sterility of Biological Substances as amended in 1995 (*14*), or by a method approved by the national control authority.

2.3.4 *Virus content*

The live virus content of the virus pool should be assayed by titration in cell culture against a reference preparation of live JE vaccine as described in section 2.2.3.3 of these Guidelines, and must be approved by the national control authority.

2.3.5 *Test for neurovirulence in mice*

Each final bulk should be tested for neurovirulence in mice (see section 2.1.3.2.1 of these Guidelines).

The test may be performed before or after the addition of other substances, such as stabilizers, as approved by the national control authority.

2.4 Control of the final lot

The requirements concerning filling and containers in Good Manufacturing Practices for Biological Products (*11*) should apply.

2.4.1 *Control tests on the final lot*

2.4.1.1 Identity test

An identity test, as described in section 2.1.3.2.1 of these Guidelines, should be performed on at least one final labelled container from each filling lot after reconstitution of the vaccine according to the instructions of the manufacturer for preparing the vaccine for human administration. However, it is not necessary to perform the genetic identity test on the final lot.

2.4.1.2 Virus content

The virus content in each of at least three containers selected at random from the final lot should be determined individually against a reference preparation of live JE vaccine (using the method described in section 2.2.3.3 of these Guidelines). The virus content should be determined after reconstitution of the freeze–dried product. Limits for accuracy and precision of the virus titration should be agreed with the national control authority. Since no international reference materials have been established for live JE vaccine, no recommendations for potency based on such materials can be formulated. The national control authority should provide or approve a reference preparation of live JE vaccine for use in tests to determine virus concentration.

The national control authority should specify the minimum amount of vaccine virus that one human dose should contain.

> In one country, the minimum amount of vaccine virus in one human dose is 5.4 log PFU per 0.5 ml at the time of release.

2.4.1.3 Test for residual animal serum protein

A sample of the final lot should be tested to verify that the level of serum in the final reconstituted vaccine is less than 50 ng per human dose. Alternatively, this test may be performed on the clarified virus pool or on the final bulk.

2.4.1.4 Thermostability test

An accelerated stability test should be performed. The detailed procedures for carrying out this test and for interpreting the results

should be approved by the national control authority, which also should specify the acceptable confidence limits.

In one country, samples are incubated at 37 °C for 7 days and the loss of titre should not be more than 1.0 log.

2.4.1.5 Sterility test

Each final lot should be tested for bacterial, mycotic and mycoplasmal sterility according to the requirements given in the 1973 General Requirements for the Sterility of Biological Substances, as amended in 1995 (*14*).

2.4.1.6 General safety (innocuity) tests

If a general safety (innocuity) test is required by the national control authority, each final lot should be tested for the absence of abnormal toxicity by appropriate tests in mice and guinea-pigs, using parenteral injections. The test procedures should be approved by the national control authority.

2.4.1.7 Residual moisture tests on freeze–dried vaccine

The residual moisture in a representative sample of each freeze–dried lot should be determined by a method approved by the national control authority. The upper limit for the moisture content should be approved by the national control authority.

Moisture levels of 3% or less are generally considered acceptable.

2.4.1.8 Test for pH values

When freeze–dried vaccine is dissolved by the approved diluent, the pH value should be approved by the national control authority, and be within the range of values found for vaccine lots shown to be clinically safe and effective.

2.4.2 ***Inspection of final containers***

Every container in each final lot should be inspected visually and those showing abnormalities should be discarded.

2.4.3 ***Records***

The recommendations given in section 8 of Good Manufacturing Practices for Biological Products (*11*) should apply.

2.4.4 ***Samples***

The recommendations given in section 9 of Good Manufacturing Practices for Biological Products (*11*) should apply.

2.4.5 ***Labelling***

The recommendations given in section 7 of Good Manufacturing Practices for Biological Products (*11*) should apply, with the addition of the following.

The leaflet accompanying the package should include the following information:

— the site of inoculation;
— the nature of the cell culture used;
— the strain of Japanese encephalitis virus used in the production of the vaccine;
— a statement that contact of the vaccine with disinfectants is to be avoided;
— the minimum amount of infectious virus contained in one recommended human dose;
— the age range for which the vaccine is recommended;
— a statement that the dose of vaccine is the same for all ages;
— the nature and amount of any residual antibiotic present in the vaccine;
— if the vaccine is in freeze–dried form, the volume and nature of the diluent to be added to reconstitute the vaccine;
— the recommended storage conditions for the vaccine, including after reconstitution;
— state that the reconstituted vaccine should be kept at 2–8 °C and in the dark, and should be used or discarded within 1 hour after the container is opened;
— any contraindications to the use of the vaccine.

2.4.6 *Distribution and shipping*

The recommendations given in section 8 of Good Manufacturing Practices for Biological Products (*11*) should apply.

2.5 Storage and expiry date

The statements concerning storage temperature and expiry date appearing on the label and in the leaflet, as recommended in Good Manufacturing Practices for Biological Products (*11*), should be based on experimental evidence and should be submitted for approval to the national control authority.

2.5.1 *Storage conditions*

JE vaccine (attenuated) should be stored at all times at a temperature between 2 °C and 8 °C. Alternative storage temperatures may be used only if justified and approved by the national control authority.

2.5.2 *Stability of vaccine and expiry date*

Adequate stability studies form an essential part of vaccine development. The stability of the vaccine in its final containers at the

recommended storage temperature should be established. As a guide, at least three consecutive final lots derived from different vaccine virus pools should be tested in the stability programme.

Accelerated degradation studies at 37 °C may provide useful additional information, but should not replace real-time studies at the recommended storage temperature.

The expiry date should be based on experimental evidence and should be submitted to the national control authority for approval.

2.6 Reference materials

No international reference materials for Japanese encephalitis vaccine are available currently. However, work has begun, or is planned, on the following materials:

- a condidate International Standard for Japanese Encephalitis Antisera.
- a condidate International Reference Preparation of Japanese Encephalitis Virus for Potency Testing. A national reference preparation for potency testing has been produced by one national control authority. This reference preparation of live attenuated JE virus should be tested further in an international collaborative study designed to establish an international reference preparation for validating tests to determine virus content.
- a condidate International Reference Preparation of Japanese encephalitis virus for neurovirulence testing. A reference preparation of live attenuated JE virus should be established for use in neurovirulence tests in mice.

2.7 Guidance for national control authorities

2.7.1 *General*

The general recommendations for control laboratories given in Guidelines for National Authorities on Quality Assurance for Biological Products (*21*), which specify that no new biological substance should be released until consistency of production has been established, should apply, with the addition of the following.

The national control authority should approve the virus strains and cell substrate to be used in the production of JE vaccine, and the potency specifiations. The national control authority should take into consideration information on the currently available strains before deciding on those to be permitted for vaccine production. The national control authority must be satisfied that the results of all tests,

including those done on virus pools during the process of manufacture, are satisfactory and that consistency has been established.

The national control authority should give directions to manufacturers concerning the recommended dose for humans.

In addition, the national control authority should provide or approve a reference preparation of live JE virus for tests for potency testing (see section 2.6) and should specify the virus content required to achieve adequate immunization of humans at the recommended dose.

2.7.2 *Release and certification*

A vaccine lot should be released only if it fulfils the national requirements.

A protocol based on the summary protocol for the lot release of virus vaccines (*22*), signed by the responsible official of the manufacturing establishment, should be prepared, and where appropriate, submitted to the national control authority in support of a request for release of JE vaccine for use.

At the request of the manufacturing establishment, the national control authority may provide a certificate that states whether the vaccine meets all national requirements.

The purpose of the certificate is to facilitate the exchange of JE vaccines among countries.

Acknowledgements

The first draft of these Guidelines was prepared by Dr Yu Yongxin, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China, and revised by the following: Dr V. Grachev, Chumakov Institute of Poliomyelitis and Viral Encephalitis, Moscow, Russian Federation; Dr S. Halstead, Office of Naval Research, Arlington, VA, USA; Dr J. Petricciani, International Association for Biologicals, Palm Springs, CA, USA; and Dr T. Tsai, Division of Vector-borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO, USA.

A second draft was formulated by the following participants at an Informal WHO Consultation held at the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China, from 7 to 9 July 1999:

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The second draft was reviewed by the WHO Expert Committee on Biological Standardization at its fiftieth meeting in 1999 (WHO Technical Report Series, 904, p. 7).

A third draft was prepared by Dr D. Wood, National Institute of Biological Standards and Control, Potters Bar, Herts., England, and reviewed by the following participants at on Informal WHO Meeting held at the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China, from 16 to 17 October 2000:

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For their comments and advice on these Guidelines acknowledgements are also due to:

Dr J. Furesz, Ottawa, Canada; Dr I. Levenbook, Chicago, IL, USA; Dr A. Lewis, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA; Dr L. Markoff, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA; Dr G. Stacey, National Institute of Biological Standards and Control, Potters Bar, Herts., England; Dr Y Yu, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China.

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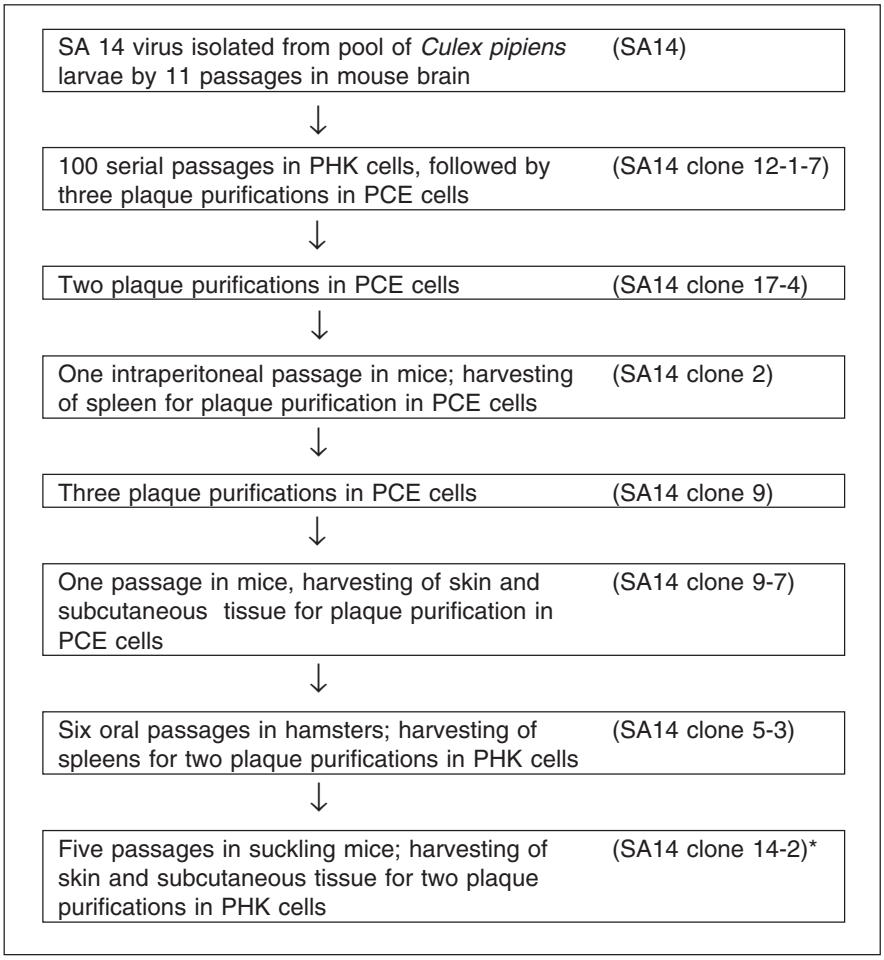
Appendix 1

Passage history of Japanese encephalitis SA 14-14-2 virus



PCE: primary chick embryo; PHK: primary hamster kidney.
* The notation SA14 clone 14-2 is abbreviated to SA14-14-2.

Appendix 2

Test for neurovirulence in monkeys

Each new master seed lot should be tested for neurovirulence in monkeys as described in the neurotropism test specified in the Requirements for Yellow Fever Vaccine (*1*), with the following modifications:

The monkeys should be *Macaca mulatta* (i.e. rhesus monkeys), as insufficient experience has been gained with the use of *Macaca fascicularis* (i.e. cynomolgus monkeys) for tests of live attenuated JE vaccines.

The animals shall have been demonstrated to be non-immune to JE virus immediately prior to inoculation of the samples.

The test dose should consist of the equivalent of not less than one human dose.

For the clinical criteria of the neurotropism test to be satisfied, the animals should not exhibit clinical signs of encephalitis and the clinical score of the monkeys injected with the virus being tested should not exceed the clinical score of those injected with reference virus.

Reference

1. Requirements for yellow fever vaccine. In: *WHO Expert Committee on Biological Standardization. Forty-sixth report.* Geneva, World Health Organization, 1998, Annex 2 (WHO Technical Report Series, No. 872).

WHO Technical Report Series, No. 910, 2002

Annex 4
Recommendations and guidelines for biological substances used in medicine and other documents[1]

The recommendations (previously called requirements) and guidelines published by the World Health Organization are scientific and advisory in nature but may be adopted by a national control authority as national requirements or used as the basis of such requirements.

These international recommendations are intended to provide guidance to those responsible for the production of biologicals as well as to others who may have to decide upon appropriate methods of assay and control in order to ensure that these products are safe, reliable and potent.

Recommendations concerned with biological substances used in medicine are formulated by international groups of experts and are published in the Technical Report Series of the World Health Organization,[2] as listed here. A historical list of requirements and other sets of recommendations is available on request from Quality Assurance and Safety: Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

Recommendations, Guidelines and other documents

Recommendations and Guidelines	Reference
Acellular pertussis component of monovalent or combined vaccines	Adopted 1996, TRS 878 (1998)
Animal Cells, use of, as in vitro Substrates for the Production of Biologicals	Revised 1996, TRS 878 (1998)
Anthrax Spore Vaccine (Live, for Veterinary Use)	Adopted 1966, TRS 361 (1967)
BCG Vaccine, Dried	Revised 1985, TRS 745 (1987); Amendment 1987, TRS 771 (1988)
Biological products prepared by recombinant DNA technology	Adopted 1990, TRS 814 (1991)
Brucella abortus Strain 19 Vaccine (Live, for Veterinary Use)	Adopted 1969, TRS 444 (1970); Addendum 1975, TRS 594 (1976)

[1] This annex was updated during the preparation of the report for publication.
[2] Abbreviated here as TRS.

Recommendations and Guidelines	Reference
Brucella melitensis Strain Rev. 1 Vaccine (Live, for Veterinary Use)	Adopted 1976, TRS 610 (1977)
Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives	Revised 1992, TRS 840 (1994)
Diphtheria, Tetanus, Pertussis and Combined Vaccines	Revised 1989, TRS 800 (1990)
DNA Vaccines	Adopted 1996, TRS 878 (1998)
Haemophilus influenzae Type b Conjugate Vaccines	Revised 1998, TRS 897 (2000)
Haemorrhagic Fever with Renal Syndrome (HFRS) Vaccine (Inactivated)	Adopted 1993, TRS 848 (1994)
Hepatitis B Vaccine prepared from Plasma	Revised 1987, TRS 771 (1988)
Hepatitis B Vaccines made by Recombinant DNA Techniques	Adopted 1988, TRS 786 (1989); Amendment 1997, TRS 889 (1999)
Human Interferons made by Recombinant DNA Techniques	Adopted 1987, TRS 771 (1988)
Human Interferons made from Lymphoblastoid Cells	Adopted 1988, TRS 786 (1989)
Immune Sera of Animal Origin	Adopted 1968, TRS 413 (1969)
Influenza Vaccine (Inactivated)	Revised 1990, TRS 814 (1991)
Influenza Vaccine (Live)	Adopted 1978, TRS 638 (1979)
Japanese Encephalitis Vaccine (Inactivated) for Human Use	Adopted 1987, TRS 771 (1988)
Japanese Encephalitis Vaccine (Live) for Human Use	Adopted 2000, TRS 910 (2002)
Louse-borne Human Typhus Vaccine (Live)	Adopted 1982, TRS 687 (1983)
Measles, Mumps and Rubella Vaccines and Combined Vaccine (Live)	Adopted 1992, TRS 840 (1994); Note TRS 848 (1994)
Meningococcal Polysaccharide Vaccine	Adopted 1975, TRS 594 (1976); Addendum 1980, TRS 658 (1981)
Monoclonal Antibodies	Adopted 1991, TRS 822 (1992)
Poliomyelitis Vaccine (Inactivated)	Revised 2000, TRS 910 (2002)
Poliomyelitis Vaccine, Oral	Revised 1999, TRS 904 (2002); Addendum 2000, TRS 910 (2002)

Recommendations and Guidelines	Reference
Rabies Vaccine (Inactivated) for Human Use, produced in Continuous Cell Lines	Adopted 1986, TRS 760 (1987); Amendment 1992, TRS 840 (1994)
Rabies Vaccine for Human Use	Revised 1980, TRS 658 (1981); Amendment 1992, TRS 840 (1994)
Rabies Vaccine for Veterinary Use	Adopted 1980, TRS 658 (1981); Amendment 1992, TRS 840 (1994)
Rift Valley Fever Vaccine	Adopted 1981, TRS 673 (1982)
Rift Valley Fever Vaccine (Live, Attenuated) for Veterinary Use	Adopted 1983, TRS 700 (1984)
Rinderpest Cell Culture Vaccine (Live) and Rinderpest Vaccine (Live)	Adopted 1969, TRS 444 (1970)
Smallpox Vaccine	Adopted 1966, TRS 323 (1966)
Snake Antivenins	Adopted 1970, TRS 463 (1971)
Sterility of Biological Substances	Revised 1973, TRS 530 (1973); Amendment 1995, TRS 872 (1998)
Synthetic Peptide Vaccines	Adopted 1997, TRS 889 (1999)
Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy	Revised 1997, TRS 889 (1999)
Tick-borne Encephalitis Vaccine (Inactivated)	Adopted 1997, TRS 889 (1999)
Tuberculins	Revised 1985, TRS 745 (1987)
Typhoid Vaccine	Adopted 1966, TRS 361 (1967)
Typhoid Vaccine (Live, Attenuated) for Veterinary Use	Adopted 1983, TRS 700 (1984)
Varicella Vaccine (Live)	Revised 1993, TRS 848 (1994)
Vi Polysaccharide Typhoid Vaccine	Adopted 1992, TRS 840 (1994)
Yellow Fever Vaccine	Revised 1995, TRS 872 (1998)

Other documents	Reference
A review of tests on virus vaccines	TRS 673 (1982)
Biological standardization and control: a scientific review commissioned by the UK National Biological Standards Board (1997)	Unpublished document WHO/BLG/97.1
Development of national assay services for hormones and other substances in community health care	TRS 565 (1975)
Good manufacturing practices for biological products	TRS 822 (1992)

Other documents	Reference
Guidelines for national authorities on quality assurance for biological products	TRS 822 (1992)
Guidelines for the preparation, characterization and establishment of international and other standards and reference reagents for biological substances	TRS 800 (1990)
Guidelines for quality assessment of antitumour antibiotics	TRS 658 (1981)
Laboratories approved by WHO for the production of yellow fever vaccine, revised 1995	TRS 872 (1998)
Procedure for approval by WHO of yellow fever vaccines in connection with the issue of international vaccination certificates	TRS 658 (1981)
Production and testing of WHO yellow fever virus primary seed lot 213–77 and reference batch 168–73	TRS 745 (1987)
Recommendations for the assessment of binding-assay systems (including immunoassay and receptor assay systems) for human hormones and their binding proteins (A guide to the formulation of requirements for reagents and assay kits for the above assays and notes on cytochemical bioassay systems)	TRS 565 (1975)
Regulation and licensing of biological products in countries with newly developing regulatory authorities	TRS 858 (1987)
Report of a WHO Consultation on Medicinal and other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies (1997)	Unpublished documents WHO/EMC/Z00/97.3 WHO/BLG/97.2
Report of a WHO Meeting on Hepatitis B Vaccines Produced by Recombinant DNA Techniques	TRS 760 (1987)
Report on the standardization and calibration of cytokine immunoassays	TRS 889 (1997)
Standardization of interferons (reports of WHO Informal Consultations)	TRS 687 (1983) TRS 725 (1985) TRS 771 (1988)
Summary protocol for the batch release of virus vaccines	TRS 822 (1992)

WHO Technical Report Series, No. 910, 2002

Annex 5
Biological substances: International Standards and Reference Reagents

A list of International Biological Standards, International Biological Reference Preparations and International Biological Reference Reagents was issued in WHO Technical Report Series, No. 897, 2000 (Annex 4) and is available on the Internet (http://www.who.int/biologicals). Copies may also be obtained from appointed sales agents for WHO publications or from Marketing and Dissemination, World Health Organization, 1211 Geneva 27, Switzerland.

The Expert Committee made the following changes to the previous list.

Additions

Antigens

Tetanus toxoid, adsorbed	469 IU/ampoule	Third International Standard 2000

These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts., EN6 3QG, England.

Blood products

Fibrinogen, concentrate, human	15 mg/ampoule for total protein and 10.4 mg/ampoule for clottable protein	First International Standard 2000
Parvovirus B19 DNA, human	500 000 IU/ampoule	First International Standard 2000

These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts., EN6 3QG, England.

Cytokines, growth factors and endocrinological substances

Follicle-stimulating hormone (FSH) and luteinizing, (LH) hormone, human urinary, for bioassay	72 IU of FSH and 70 IU of LH per ampoule	Fourth International Standard 2000
Somatropin (rDNA-derived human growth hormone)	1.95 mg protein/ampoule	Second International Standard 2000

Inhibin B	12 units/ampoule for bioassay and 12 ng/ampoule for immunoassay	First Reference Reagent 2000

These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts., EN6 3QG, England.

Re-establishment

Insulin-like growth factor I for immunoassay	3.1 µg/ampoule	First Reference Reagent 1988

This substance is held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts., EN6 3QG, England.

Change in name

Insulin-like growth factor I	First International Standard 1994
renamed:	
Insulin-like growth factor I for bioassay	First International Standard 1994
Inhibin, human recombinant	First International Standard 1994
renamed:	
Inhibin A, human recombinant	First International Standard 1994
Alteplase (recombinant tissue plasminogen activator)	First International Standard 1999
renamed:	
Tissue plasminogen activator (t-PA), human, recombinant	Third International Standard 1999

These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts., EN6 3QG, England.

Discontinuation

Protamine	First International Reference Reagent 1954

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 quality control of vaccines and other biologicals and the establishment of international biological reference materials. The report starts with a discussion of general issues brought to the attention of the Committee and provides information on issues relevant to international guidelines, recommendations and other matters related to the manufacture and quality control of biologicals. This is followed by information on the status and development of reference materials for bovine spongiform encephalopathy, various antigens, blood products, cytokines, growth factors and endocrinological substances. The second part of the report, of particular interest to manufacturers and national control authorities, contains sets of recommendations for the production and control of poliomyelitis vaccine (oral) and poliomyelitis vaccine (inactivated) and guidelines for the production and control of live attenuated Japanese encephalitis vaccine. Also included are lists of recommendations and guidelines for biological substances used in medicine, and other relevant documents.

ISBN 92 4 120910 0

SELECTED WHO PUBLICATIONS OF RELATED INTEREST

WHO Expert Committee on Biological Standardization.
Fiftieth report.
WHO Technical Report Series, No. 904, 2002 (107 pages)

WHO Expert Committee on Biological Standardization.
Forty-ninth report.
WHO Technical Report Series, No. 897, 2000 (106 pages)

WHO Expert Committee on Biological Standardization.
Forty-eighth report.
WHO Technical Report Series, No. 889, 1999 (111 pages)

WHO Expert Committee on Biological Standardization.
Forty-seventh report.
WHO Technical Report Series, No. 878, 1998 (101 pages)

WHO Expert Committee on Biological Standardization.
Forty-sixth report.
WHO Technical Report Series, No. 872, 1998 (90 pages)

WHO Expert Committee on Biological Standardization.
Forty-second report.
WHO Technical Report Series, No. 822, 1992 (84 pages)

The International Pharmacopoeia, 3rd ed.
Volume 1: general methods of analysis. 1979 (223 pages)
Volume 2: quality specifications. 1981 (342 pages)
Volume 3: quality specifications. 1988 (407 pages)
Volume 4: test methods and general requirements; quality specifications for pharmaceutical substances, excipients and dosage forms. 1994 (358 pages)

Further information on these and other WHO publications can be obtained from
Marketing and Dissemination, World Health Organization, 1211 Geneva 27, Switzerland