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Sixth Report

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JOINT FAO/WHO EXPERT COMMITTEE ON BRUCELLOSIS

Geneva, 12-19 November 1985

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JOINT FAO/WHO EXPERT COMMITTEE ON BRUCELLOSIS

Sixth Report

1. INTRODUCTION

The Joint FAO/WHO Expert Committee on Brucellosis met in Geneva from 12 to 19 November 1985. The meeting was opened on behalf of the Directors-General of the Food and Agriculture Organization of the United Nations and the World Health Organization by Dr. S.K. Litvinov, Assistant Director-General of the World Health Organization.

Although the support given jointly by WHO and FAO to the recommendations of the Joint Expert Committee since its first meeting in 19501 had stimulated progress in the control and local eradication of brucellosis in several parts of the world, brucellosis remains a major public health hazard and is an ever-increasing cause of concern in many countries. Expansion of animal industries and urbanization, particularly in developing countries, and the lack of appropriate hygienic measures in animal husbandry and in food handling may partly account for this. The expansion of international travel is also thought to be a contributory factor. Foreign travel stimulates the taste for exotic dairy goods, such as fresh cheeses, that may be infected, and these are being imported into brucellosis-free regions to satisfy the demand. In addition, many travellers become infected while abroad and develop symptoms after returning home. Consequently, it is important that general medical practitioners throughout the world should be alert to the possibility of encountering cases of brucellosis.

In view of the social and economic impact of brucellosis on animal production and rural development, community-based approaches are proposed in this report for the prevention and control of the disease. Emphasis is placed on the development of intersectoral cooperation in support of primary health care approaches. In this way, the control of a specific zoonosis may

1 WHO Technical Report Series, No. 37, 1951 (Joint FAO/WHO Expert Committee on Brucellosis: report of the first session.)
contribute to the development of appropriate infrastructures in areas of animal production, food hygiene, and health care. On the other hand, the prevention and control of brucellosis needs supportive action from various sectors, including those responsible for food safety and consumer education.

While there is still a need for technical advances in some areas, for example, more widely applicable vaccines, the basic scientific information and methods required for the control and local eradication of brucellosis in ruminants are at hand. The Committee noted, however, that, in many parts of the world, the control of Brucella infection in animals is today in need of improvement from the point of view of national administrative measures for the application of existing knowledge. For this reason, the Committee chose, for the first time, to devote a part of its report to describing the administrative, social, and anthropological aspects that must be taken into account if a national programme for brucellosis control is to be effective.

Application of new biological procedures and managerial approaches that are now available should reduce the impact of brucellosis as a world problem and make a major contribution to achieving WHO's goal of health for all by the year 2000.

2. BACTERIOLOGY

2.1 Safety aspects

Brucella organisms present a very serious risk of infection to laboratory workers. The risk is greatest to those handling cultures or heavily infected samples such as abortion material. The examination of serum samples presents a minimal hazard and precautions in excess of those normally required by good laboratory practice are unnecessary.

It is strongly recommended that the examination of heavily infected materials or cultures should be carried out in safety cabinets of a type that permits complete containment of infectious material and aerosols. These should be located away from accommodation used for other purposes and work should be done by adequately trained staff. Suitable protective clothing should be worn and facilities provided for its disinfection and for the safe decontamination of apparatus, discarded cultures, and other
hazardous material. Specific recommendations have been made for the safety precautions to be observed with Brucella infected materials;\textsuperscript{1, 2} further details are given in Annex 1.

2.2 Classification and typing of the genus Brucella: the current position

The present system of taxonomy for the genus Brucella is based on recommendations made by the Subcommittee on Taxonomy of Brucella of the International Committee on Bacteriological Nomenclature in 1963 and subsequently extended in later reports.\textsuperscript{3, 4, 5} This scheme was devised to eliminate problems that arose in the identification of the original species, B. melitensis, B. abortus, and B. suis, when these were typed by the conventional procedures involving examination of CO\textsubscript{2} requirement, H\textsubscript{2}S production, dye sensitivity, and agglutination reactions with monospecific antisera. The introduction of tests for sensitivity to lysis by phages and measurement of oxidative metabolism with selected substrates resolved these problems and enabled a system of species identification to be achieved that was consistent with the epidemiological evidence. Currently, phage lysis and/or oxidative metabolism tests are used for the differentiation of the species.

Attention has also been given to criteria to be applied for recognition at the genus level. These are included among recommendations by the Subcommittee on Taxonomy of Brucella of the International Committee on Systemic Bacteriology. Identification is achieved in the first instance by examination of morphological, cultural, metabolic, and serological properties. Confirmatory evidence may be provided in the case of atypical isolates by examination of DNA purine–pyrimidine base


composition, DNA nucleotide sequence (usually indirectly, by DNA–DNA hybridization), electrophoretic separation patterns produced by phenol–acetic acid–water-soluble proteins, absorption spectrum of cytochrome a and c bands, and demonstration of the presence of intracellular antigens shared with Brucella reference strains. Gas-liquid chromatography of the fatty acid methyl esters is also a useful supplementary procedure as the structural fatty acids of Brucella strains produce a characteristic elution profile.

The phage-lysis pattern and oxidative-metabolism profile are used primarily for species identification, but the phage tests are also useful for confirmation of genus identity as they are specific for Brucella. The conventional tests, including examination of CO₂ requirement, dye sensitivity, H₂S production, urease activity, and agglutination reactions with monospecific antisera are of supplementary value for species identification and are also used to differentiate the biovars of the major species, B. abortus, B. melitensis, and B. suis.

Oxidative-metabolism tests performed by manometric methods are not suitable for the routine identification of cultures by nonspecialist laboratories. They require expensive apparatus and specially trained staff, are hazardous and time-consuming to perform, and need expert interpretation. Their performance is best left to reference laboratories with experience in these methods. Simpler methods for the semiquantitative determination of oxidative-metabolism activity using thin-layer chromatography have been described. These are safer and easier to perform than manometric techniques and require only simple apparatus. Oxidative-metabolic tests are invaluable for the identification of atypical cultures—for example, the rare phage-resistant smooth isolate or, more commonly, non-smooth variants of the normally smooth species. The invariably non-smooth species, B. ovis and B. canis, are now amenable to identification by phage typing and conventional tests. Wherever possible, identification should be carried out down to the biovar level as the biovars often follow a particular geographical distribution and can provide epidemiologically important information.

It is recommended that national brucellosis centres should carefully examine cultures isolated in their own country, by phage and conventional tests. Those that differ from established biovars or that have been isolated from unusual hosts should be preserved by deep-freezing, freeze-drying, or vacuum-drying as soon as possible.
after isolation. When a number of similar cultures have been isolated and the type appears to be of epidemiological significance or of unusual interest, they should be sent to an FAO/WHO or WHO collaborating centre (see Annex 7), but only after the centre has been contacted with regard to the regulations to be observed for the transport of cultures across national boundaries. Full information on preliminary typing results, source, and history should accompany the cultures.

The differential characteristics of the species and biovars of the genus *Brucella* are given in Annex 2, Tables 1–5.

### 2.3 Genetics of Brucella

Attempts to demonstrate genetic transformation and conjugation in *Brucella* have proved unsuccessful so far, although some evidence of transduction has been reported recently. Similarly, examination of many strains has also failed to reveal plasmids or other extrachromosomal forms of DNA. Indirect evidence of the occurrence of plasmids, such as antibiotic resistance, has not been obtained. Nevertheless, genetically determined variations in the properties of *Brucella* cultures occur quite frequently. These include changes from the smooth to the rough colonial phase, loss of CO₂ requirement or loss of H₂S production, changes in sensitivity to lysis by phage, or resistance to dyes and antibiotics. The mechanisms whereby such changes occur remain to be elucidated.

#### 2.3.1 Deoxyribonucleic acid (DNA) homology

The DNA prepared from *B. abortus*, *B. melitensis*, *B. suis*, *B. neotomae*, *B. ovis*, and *B. canis* contains 56–58 mol% G + C.¹

The ability of single stranded DNA to hybridize with single-stranded DNA from a heterologous source provides a measure of genetic relatedness among organisms. DNA homology studies have shown that members of the genus *Brucella* lack homology with other microorganisms having similar guanine-plus-cytosine ratios (*Serratia marcescens*, *Escherichia coli*, *Agrobacterium tumefaciens*) and the phenotypically similar species *Francisella tularensis* and *Bordetella bronchiseptica*. The polynucleotide sequences of the DNA in organisms of the genus *Brucella* show marked similarities. The DNA of *B. abortus*, *B. melitensis*, *B. suis*, *B. neotomae*, *B. ovis*, and

¹ Mole percent guanine plus cytosine.
*B. canis* is completely homologous within the limitations of the techniques used. The DNA of *B. ovis* was formerly reported to lack some of the polynucleotide sequences present in the other species, but this was not confirmed in a more recent study.

The degree of genetic relatedness indicated by DNA hybridization studies is consistent with the existence of a single species within the genus *Brucella*. This may lead in the future to changes in nomenclature to conform with taxonomic conventions.

2.3.2 *Dissociation of Brucella: S–R variation*

Smooth or S forms produce round, translucent colonies with an entire edge and a smooth glistening surface, giving a slight bluish-white opalescence in reflected light, although they are a translucent pale yellow in transmitted light.

Non-smooth forms, which may be rough (R) or mucoid (M), produce colonies that are often slightly larger than smooth colonies and have a more granular or slimy surface. The colour or these non-smooth forms varies from off-white to brown in transmitted or reflected light. The colonies are usually translucent, but may be opaque. Forms intermediate between S and R types (SI and I forms) can also occur.

Examination of colonial morphology alone may not be sufficient to indicate the dissociation status of a culture. Additional properties that should be examined include the appearance in reflected light after crystal violet staining and the agglutination reaction with acriflavine solutions or with antiserum to the R antigen of rough strains.

It is essential to define the dissociation status of cultures for purposes of identification, as the serological properties, phage sensitivity, and virulence are usually drastically altered in the M and R phases.

2.3.3 *Brucella phages*

Since 1970, the situation with respect to *Brucella* phages has changed substantially. It was apparent from many of the earlier reports that the host range of the first *Brucella* phage isolates was

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very limited and that most, if not all, of the phage strains then
available were very similar to the Tbilisi (Tb) reference phage. In
other words, they were capable of efficient replication in smooth
cultures of *B. abortus* and replication at low efficiency in
*B. neotomae*, but did not replicate in the other *Brucella* species as
defined by oxidative-metabolism tests, although producing lysis-
from-without¹ on smooth *B. suis* cultures when applied at high
concentration. The homogeneity of these isolates was further
confirmed by morphological examination, cross-neutralization tests,
and DNA base-ratio analysis.

Over the past 15 years the host range of the *Brucella* phages has
been widened considerably by the isolation of new phage strains.
These now bring the whole genus within the scope of phage typing.
As these phages have not been shown to lyse bacteria of other
genera, they are useful for identification at both genus and species
level.

On the basis of host range, the *Brucella* phages have been
classified into six groups. Those in group 1, typified by the Tb strain,
are capable of replication only in cells of *B. abortus* in the S, SI, or
I phase. Limited replication occurs in S, SI, or I cells of *B. neotomae*,
but the efficiency of replication is low. At high concentrations, these
phages produce lysis-from-without of S, SI, or I cultures of *B. suis*.
Cultures of *B. melitensis*, *B. ovis*, and *B. canis* and M and R phase
cultures of *B. abortus*, *B. neotomae*, and *B. suis* are not lysed.

The phages of group 2, typified by Firenze (Fi) strain 75/13,
replicate in S, SI or I cultures of *B. abortus*, *B. neotomae*, and *B. suis*.
Plaque formation is more efficient on *B. abortus* than on *B. neotomae*
and *B. suis* biovar 4 and much higher on these than on *B. suis* biovars
1, 2, and 3. It should be noted that the Fi phage strains other than
75/13 belong to group 1.

Phage group 3 is typified by the Weybridge (Wb) strain. Phages
of this group replicate in and lyse S, SI and I cultures of *B. abortus*,
*B. neotomae*, and *B. suis*. The efficiency of plating of the phages on
these species varies, but not sufficiently to produce major differences
in lysis patterns with standardized preparations. Some of these
phages have been reported to form plaques on some *B. melitensis*
cultures but the efficiency of plating is low. Phages of this group do
not lyse M and R strains of any *Brucella* species, including *B. canis*
and *B. ovis*.

¹ Enzyme lysis, but without penetration and replication.
Phage group 4 comprises the Berkeley phages BK₀, BK₁, and BK₂. The BK₂ strain is the most useful for typing purposes and this is lytic for S cultures of B. abortus, B. melitensis, B. neotomae, and B. suis. It is not lytic for non-smooth cultures of any Brucella species and its lytic activity for smooth strains declines rapidly as these undergo dissociation to I, R, or M forms. B. melitensis strains from different geographical sources appear to vary in susceptibility to BK₂ phage.

Phage group 5 includes those phages lytic for non-smooth Brucella cultures and derived from phage R. Phage R is lytic for non-smooth cultures of B. abortus but not other species. Phage R/O is lytic for non-smooth cultures of B. abortus and those of B. ovis. Both these phage strains are genetically unstable and produce mutants lytic for smooth Brucella cultures. Phage R/C is much more stable and is lytic for B. ovis, B. canis, and most non-smooth cultures of B. abortus. It shows negligible lytic activity for smooth Brucella cultures.

Phage group 6 comprises the Iztatnagar (Iz) phages typified by the strain Iz₁. This is lytic for S, SI, and I cultures of B. abortus, B. melitensis, B. neotomae, and B. suis. It is also lytic for R and M cultures of B. melitensis and B. suis. It shows minimal lytic activity towards cultures of B. ovis and R cultures of B. abortus and even less towards those of B. canis.

All of the Brucella phages belong to the same phage species. They are morphologically similar, having a hexagonal head about 60 nm wide with a tubular tail about 25 nm long. Attachment to the host cell is effected via short fibrous structures forming the distal part of the tail. Recommended propagating strains for Brucella phages are given in Annex 2, Table 7.

The phages are stable to anionic and non-ionic detergents and to common organic solvents, with the exception of chloroform. They are inactivated by heat, cationic detergents, and oxidizing agents. Their stability to proteolytic enzymes and reducing agents varies between strains. Divalent cations are not essential for phage attachment. As far as is known, all contain DNA as their genetic material and for those of group 1 this has a mol% G + C of 45.3–46.7 (Tₘ).¹ The base composition of the other phage groups has not been reported.

¹ Tₘ = melting temperature.
The phage susceptibility pattern correlates closely with the oxidative metabolic activity towards selected carbohydrate and amino acid substrates and is of major importance in defining the species of Brucella.

Strong circumstantial evidence indicates that lysogeny occurs among Brucella cultures and that phage infection can modify the phenotypic properties of cultures.

2.4 L-forms of Brucella

Brucella may undergo L-transformation when exposed to antibiotics and chemical preparations under experimental conditions or during the treatment of brucellosis patients.

The L-forms of Brucella so far described are aerobic and grow on semi-solid 15 g/litre agar, which for some must contain 200–250 μl of normal horse serum per litre. The colonies vary in form and size from those of the Mycoplasma type, with solid centres and lacy edges, to minute colonies (200 μm diameter) of typical Brucella type. The morphology of the cells changes during the process of L-transformation and is characterized by heteromorphic forms, spheroplasts of 1–15 μm, globular cells of 2–7 μm, large bodies of up to 50 μm, filamentous structures and elementary bodies. L-forms can be stained by fluorescein-labelled antisera to S and R forms of Brucella. They are often auto-agglutinable and agglutination reactions cannot be performed with specific antisera. Species differentiation of L-forms can in some cases be carried out by means of cultural tests and by phage adsorption and lysis tests. The virulence of L-forms declines during the process of L-transformation. L-forms are usually in the smooth phase, are deficient in lipopolysaccharide-endotoxin, and have fewer high relative molecular mass components than cells with intact outer membranes.

Reversion from the L-phase can sometimes occur following passage in vitro or in vivo. Following reversion, the antigenic structure, virulence, and chemical composition are not usually fully restored, when compared with the parent strain.
2.5 Cultivation of *Brucella*

2.5.1 Media for primary isolation, cultivation, and preservation

*(a) Solid media.* Direct inoculation of the specimen on to appropriate solid media is generally the most satisfactory method of isolating *Brucella*; with foods or other heavily contaminated material, however, animal inoculation may be necessary. The use of solid rather than liquid media for primary isolation is usually to be recommended because such media facilitate the recognition and isolation of the developing colonies and limit the establishment of non-smooth mutants. Liquid media are widely used for primary isolation from blood and other body fluids. It is essential to make subcultures from liquid to solid media early, in order to detect growth and because dissociation of organisms occurs with continued growth in liquid media. Although most recoveries from animal sources are made within 7–14 days, the inoculated media should be kept for at least 35 days before being discarded as negative particularly in the case of human blood specimens.

Specimens should be cultured on basal (non-selective) media enriched by the addition of 50–100 ml of serum per litre, as well as on selective media, if available. Duplicate plates should be made and incubated in an atmosphere containing 5–10% added CO₂ where the presence of *B. abortus* or *B. ovis* is possible. The existence of dye-sensitive and fastidious biovars of *Brucella* underlines the necessity for the routine testing of each new batch of medium for its ability to support the growth of any *Brucella* biovar likely to be encountered. The reference strain of *B. abortus* biovar 2 is useful for this purpose. Attention is drawn to the acidity produced by certain contaminating bacteria in non-buffered media containing serum; this acidity inhibits the growth of *Brucella* organisms.

The choice of basal medium frequently depends on considerations of cost and availability. A basal medium can be considered as satisfactory only if it is able to support the growth of the great majority of *Brucella* strains from small inocula. It is recommended, therefore, that laboratories check their basal media of choice for ability to support the growth of the fastidious, serum-requiring *B. abortus* biovar 2 from small inocula (5–10 cells). The addition of serum free of *Brucella* antibodies enhances the ability of the media to support the growth of *Brucella*, especially from small inocula.

The following media are recommended for primary isolation:
— serum-dextrose agar (SDA) (50–100 ml of serum and 10 g of dextrose added to sufficient basal medium to give a total volume of 1000 ml);
— commercial media (trypsinase soy, tryptose agar, or any good quality blood agar base supplemented with serum);
— blood agar (50 ml of sheep blood added to sufficient basal agar medium to give a total volume of 1000 ml).

(b) Liquid media. Either trypticase soy broth, tryptose broth, or any of the commercial Brucella broth preparations are suitable liquid media. For the isolation of Brucella from blood or other body fluids using the Castañeda technique (see section 7.1), any of these media may be used, the solid phase being prepared by adding agar.

2.5.2 Selective media

The addition of appropriate antibiotics to media allows growth of Brucella while suppressing growth of contaminants. The addition of ethyl violet aids in suppressing contaminants but inhibits growth of dye-sensitive Brucella biovars. It may be used when the more satisfactory antibiotic agents are not obtainable. The choice of selective media will depend, therefore, upon the nature and purpose of the work. Generally, selective media should not be used if uncontaminated samples are to be cultured.

The surface of the medium should be dried before use, preferably by leaving the plates unopened and inverted in the 37°C incubator overnight. They are best used fresh, i.e., the day after preparation, but may be stored in the refrigerator for up to 2–3 weeks if necessary.

Farrell's modified SDA is prepared from SDA basal medium by the addition of bacitracin, polymyxin B, cycloheximide, vancomycin, nalidixic acid, and nystatin. Ready mixed formulations of the antibiotic supplement are available commercially. Details of the method of preparation of Farrell's medium have been described by Corbel et al.,1 and the less inhibitory Kudzas & Morse SDA antibiotic medium, together with a selective medium for the culture of B. ovis, have been described by Alton et al. (1975).2

2.5.3 Media for enrichment

For the isolation of *Brucella* from material likely to contain only small numbers of organisms, enrichment culture is advised. For uncontaminated samples, such as human blood or tissues, the Castañeda procedure can be used. The liquid phase should be one of the media listed in Section 2.5.1. If whole blood is to be cultured, the anticoagulant trisodium citrate should be added to give a final concentration of 20 g/l. Freeze-thawing of blood or buffy coat samples prior to culture is reported to increase the isolation rate of *Brucella*.

For the isolation of *Brucella* from contaminated material containing small numbers of organisms, such as macerated tissues, milk, etc., enrichment should be performed in a biphasic medium, such as that used in the Castañeda method, with the addition of the following antibiotics to the liquid phase: amphotericin B, 1 mg/l; bacitracin, 25 mg/l; cycloheximide, 100 mg/l; D-cycloserine, 100 mg/l; nalidixic acid, 5 mg/l; polymyxin B, 6 mg/l; and vancomycin, 20 mg/l.

It is recommended that tissue suspensions, milk, etc., should be cultured on one Petri plate containing solid selective medium and in 2 bottles or flasks of the biphasic medium, the first bottle receiving 1 ml and the second 2 ml of the specimen. The cultures are incubated in an appropriate atmosphere, the bottles being examined and tilted every few days to allow the liquid to flow over the solid phase of the medium. Bottles containing biphasic medium should be incubated for at least 35 days before being discarded as negative.

This medium is not recommended for the isolation of *B. ovis* or other fastidious strains. Each batch should be checked for the ability to grow a reference strain from a small inoculum.

Tissue samples for enrichment should be homogenized—either by blending or by maceration in a Colworth-type stomacher with a small volume of liquid medium.

For further cultivation any of the basal media recommended in section 2.5.1 can be used, except blood agar, on which colonial morphology is not distinctive.

2.5.4 Media for dye sensitivity tests

Except for blood agar, any of the transparent basal media recommended in section 2.5.1 above, containing the appropriate dilution of the dyes, can be used. Since SDA will support the growth
of all *Brucella* species and biovars, it is especially recommended as basal medium. The inhibitory activity of each dye to be used varies with its source and batch and also with the basal medium employed. Each new batch of dye must be tested to determine the optimal dye concentration required to inhibit *Brucella* reference strains growing on the basal medium used.

2.5.5 *Medium for the examination of cultures for dissociation*

A nutrient basal agar medium with added glycerol and dextrose (2 g glycerol and 1 g dextrose per 100 ml) is recommended.

2.5.6 *Medium for preservation of Brucella strains in liquid nitrogen*

Glutamate medium is recommended for suspending *Brucella* strains for preservation at liquid nitrogen temperature. It is prepared by dissolving 25 g of hydrolysed casein in 1 litre of sterile distilled water and autoclaving at 115°C for 20 minutes. Then, 50 g of sucrose and 10 g of monosodium glutamate are added to the solution and dissolved by steaming for 10 minutes. The medium is pumped through a Seitz EKS or equivalent filter into sterile flasks and finally autoclaved at 106°C for 15 minutes.

Other cryoprotective media may also be suitable for *Brucella*.

2.5.7 *Large-scale cultivation of Brucella*

Control and eradication campaigns against animal brucellosis have led to demands for large quantities of *Brucella* cells, both for vaccine production and for diagnostic antigens for use in an increasing array of serological tests. The two main methods in use are culture on a solid medium in Roux flasks and culture in a liquid medium. The handling of large volumes of cultures presents the following hazards: (a) the risk of contaminating the cells during inoculation, harvesting, and bottling; (b) the potential risk to staff, who may become infected or sensitized by contact with *Brucella* cells.

Since brucellosis is an easily acquired laboratory infection, great care should always be taken in handling *Brucella* cultures, especially in bulk, and precautions should always be taken when inoculating, harvesting, bottling and freeze-drying *Brucella*. Periodic serological and health checks should be made on personnel involved, since the
sensitization effects of constant exposure to avirulent *Brucella* are not clearly understood. For a further discussion of the precautions to be taken when handling *Brucella* cultures, see Annex 1.

The choice of method for producing cells will depend on the quantity of cells required and on the facilities available; whichever method is used, great care should be exercised (a) in maintaining cultures for seed material in the correct colonial phase and (b) in the choice of medium used. When new batches of ingredients are used for making up medium, checks should be made to ensure not only that the medium adequately supports growth but also that it does not lead to colonial dissociation.

Seed cultures should be carefully selected, and preserved by drying, freeze-drying, or storage at low temperature, e.g., in liquid nitrogen. A fresh ampoule must be used for the preparation of each batch of material, to avoid the risk of possible antigenic change arising from repeated subculture.

2.5.7.1 *Use of liquid medium.* When large volumes of cells are required for vaccine or antigen production, the use of liquid medium has the advantage over propagation in Roux flasks that the laborious and costly process of handling large numbers of individual flasks is eliminated and the attendant risk of contamination is reduced. Since the growth of *Brucella* in liquid media leads to an increase in pH, either the medium must be adequately buffered or the pH must be controlled by constant addition of the appropriate buffer. In order to promote growth and prevent dissociation, sterile air must be continuously added and the medium constantly agitated. It is essential, therefore, that the equipment be correctly designed to prevent any risk of contamination and to allow for the safe addition of the seed material and other ingredients and the collection and handling of the harvested culture. Arrangements for the provision of large volumes of sterile medium are essential, especially for a continuous culture process.

2.5.7.2 *Batch process.* The batch process is simpler to operate than continuous culture and is performed in a large vessel fitted with an impeller, air inlet and outlet, and a means of temperature control. After inoculation, incubation is allowed to proceed for approximately 48 hours, after which the growth is harvested and the cells are recovered by centrifugation or by precipitation with
carboxymethyl cellulose. Details of the method of propagation by the batch process are available.¹

2.5.7.3 Continuous culture method. This method has been used successfully for the large-scale culture of *B. abortus* cells both for vaccine and for antigen production. Very large volumes of sterile medium are required before a cycle can be started and constant checks must be made to ensure freedom from contamination. Checks should also be made to ensure that the cells obtained throughout the culture cycle are of the required standard as regards dissociation, antigenicity, agglutinability, and immunogenicity. Tests performed in one laboratory showed that *B. abortus* strain 19 cells obtained at the end of an 11-day culture cycle (the longest period used) did not differ in immunogenicity or other properties from cells obtained at the beginning of the culture cycle.

2.6 Antigenic structure of *Brucella*

Since the discovery of A and M epitopes on smooth cells, numerous studies based on immunolectrophoretic and immunodiffusion analysis of soluble extracts of smooth (S) and rough (R) cells have extended knowledge of the antigenic structure of *Brucella*. The main antigens so far identified include the smooth and rough lipopolysaccharide complexes (S-LPS and R-LPS) and two related polysaccharides: native hapten (NH) and B polysaccharide (poly B), and at least 20 protein or glycoprotein antigens. The LPS antigens are located on the surface, whereas most of the protein antigens occur within the *Brucella* cell. The LPS and some protein antigens are involved in diagnostic tests and in the protective activity of vaccines.

2.6.1 Surface antigens

As in other Gram-negative bacteria, the cell envelope of *Brucella* is composed of an inner cytoplasmic membrane surrounded by the rigid peptidoglycan layer associated with the outer membrane (OM), which contains mainly phospholipids, lipopolysaccharides, and proteins (outer membrane proteins: OMP).

¹ See footnote 2 on page 17.
(a) S-LPS and related haptens. Unlike the S-LPS of enteric bacteria, Brucella S-LPS is found in the phenol phase when cells are extracted by the hot phenol-water method. Such extracts contain S-LPS and also proteins (20–30%), nucleic acid (8%), and the native haptenic polysaccharide (NH). Further purification eliminates nucleic acid and reduces the protein content to below 6%. Carbohydrate components found in purified S-LPS preparations include glucose, mannose, glucosamine, quinovosamine, 4,6-dideoxy-4-formamido-d-mannose and 2-keto-3-deoxyoctonate (KDO). The S-LPS of B. abortus strain 1119-3 has been reported to comprise a lipid A fraction containing phospholipids, glucosamine, and fatty acids, linked via an acid-labile linkage probably involving KDO, to a core polysaccharide containing glucose, mannose, and quinovosamine. This is linked, to the O-specific chain, which is postulated to comprise an unbranched linear homopolymer of 96–100 residues of 4,6-dideoxy-4-formamido-d-mannopyranose linked through their 1,2 carbon atoms.

In contrast with the S-LPS of enteric bacteria, that of Brucella does not contain heptose. Similarly, the fatty acid composition of Brucella LPS comprises about 50% palmitic acid, about 10% stearic acid, and less than 5% hydroxylated fatty acids, β-hydroxyxymyristic acid being absent. In contrast, the S-LPS of Escherichia coli contains large amounts of lauric acid, myristic acid, and β-hydroxyxymyristic acid.

It should be pointed out that the characterization of B. abortus strain 1119-3 S-LPS was on protein-free preparations obtained following trypsin treatment. The possible role of protein residues in the serological specificity of Brucella S-LPS has yet to be established. The detailed structure of the O chains of M-antigen-dominant Brucella strains and R strains has not yet been determined.1

The S-LPS molecules carry the A and M epitopes, which have different quantitative distribution among the smooth Brucella biovars. Crude phenol-water, ether-water, and trichloroacetic acid endotoxic preparations contain the polysaccharide NH. This has no endotoxin activity and no fatty acids, KDO, or proteins. NH can also be obtained by autoclaving S cells followed by ethanol precipitation. It gives an immunological reaction of identity with the

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1 Editorial Note: Since the meeting, it has been established that the M antigen O chain is identical with that of the A antigen, except that every fifth glucose residue is linked through the 1,3 instead of the 1,2 carbon atoms.
degraded polysaccharide acetic acid hapten (AH) from hydrolysed S-LPS. However the quantitative differences found between the sugars of NH and those of AH suggest that these polysaccharides are similar but not identical. Another polysaccharide named poly B is present in the cytoplasm of *B. melitensis* B 115 and is extracted by trichloroacetic acid. Its identified components include mainly glucose (89%), a trace amount of quinovosamine, but no KDO. Poly B shows a reaction of identity with AH and NH, with some sera. Nevertheless, sera from infected cows and from rabbits injected with pure S-LPS give a partial identity reaction. Poly B has fewer antigenic determinants than AH; this is reflected in the chemical differences between poly B and AH and NH.

Electron micrographs of negatively stained S-LPS fractions show typical ribbons or vesicle structures with a trilamellar membrane appearance when observed with positive uranyl acetate staining.

(b) *R-LPS preparations*. R-LPS preparations are obtained by first treating the rough cells with a 25 g/l solution of NaCl in the cold for three days, which extracts proteins, and then by proceeding with the hot phenol–water method. R-LPS partitions into the aqueous phase, in contrast to S-LPS. This solubility in water could be attributed to its association with nucleic acid, as nuclease digestion makes R-LPS insoluble.

Pure R-LPS preparations contain no nucleic acid and little or no protein. Its fatty acids are identical with those of S-LPS. Sugars found in R-LPS include glucose, mannose (quantitatively less than in S-LPS), KDO, and glucosamine. R-LPS differs from S-LPS in lacking quinovosamine.

(c) *Outer membrane proteins (OMP)*. *Brucella* OMP have been isolated and partly characterized only recently. In strains of *B. abortus*, two major groups of proteins with relative molecular masses of 36–38K and 25–27K are present. One of these groups (36–38K) has been identified as a porin through functional assays, the other group (25–27K), which contains carbohydrates, may be the counterpart of OMPA of *E. coli*. Another major sodium dodecyl sulfate-soluble protein band of relative molecular mass 43K has been found by some workers. Three minor protein bands (88–94K, 31K, and 15K) are also present.

Strains of *B. melitensis*, *B. canis*, and *B. ovis* contain both major groups of OMP but the proportion of the 25–27K proteins to the
porins (36–38K) is much greater than in *B. abortus*. In separate studies of *B. melitensis*, one additional band above the 36–38K zone and another at 31K have been found. The OMP profiles of *B. suis* and *B. neotomae* have not yet been established.

*(d)* Peptidoglycan. The gross chemical composition of the *Brucella* peptidoglycan (PG) is similar to that of other Gram-negative bacteria, comprising glucosamine, muramic acid, alanine, glutamic acid, and diaminopimelic acid. Detailed information about the sequence and cross-linking of peptides is not available.

*Brucella* PG is present in two types of protective fraction: the phenol-insoluble fraction of cell walls and cells and the sodium dodecylsulfate-insoluble fraction of cell walls. The PG may enhance the immunological response by its adjuvant property.

### 2.6.2 Internal antigens

Immunoelectrophoretic analysis of soluble *Brucella* cell extracts obtained by various methods reveals at least 20 protein antigens mostly of intracellular origin and some external antigens, including S-LPS (or R-LPS) and NH, precipitating with high-titre antisera. When soluble extracts are tested against infected host sera in immunoprecipitation tests, the number of protein antigens identified is lower, i.e., from 1 to 9.

Protein extracts of *Brucella* have been employed as antigens in skin tests for delayed hypersensitivity. The saline extract of *B. melitensis* B 115 is the best-characterized intradermal allergen preparation. It contains, however, at least 20 molecular species, but is free of detectable LPS.

### 2.6.3 Role of individual antigens in diagnostic tests

Few antigens involved in diagnostic tests have been defined at the molecular level because of a lack of serious purification studies.

S-LPS is the major antigen involved in the standard tests: agglutination, complement-fixation, Rose Bengal plate, and milk ring tests. The NH and poly B haptens, which are S-LPS-related, are used in the radial-immunodiffusion test for differentiating infected from immunized animals. The intracellular A₂ antigen used in immunodiffusion and immunoelectrophoresis tests is also able to distinguish infected from immunized animals in some circumstances.
2.6.4 Protective antigens

Studies conducted in laboratory animals have shown that protective immunity can be stimulated by the PG fraction of *Brucella* cell walls. The active components include S-LPS, OMP, and possibly other components. Further studies are necessary to identify clearly the antigen(s) involved in protection of the natural host species against *Brucella*.

2.6.5 Cross-reactions

Serological cross-reactions occur between smooth *Brucella* species and *Escherichia coli* 0:116 and 0:157, *Francisella tularensis*, *Salmonella* group N(0:0) of Kaufmann-White, *Pseudomonas maltophilia*, *Vibrio cholerae*, and *Yersinia enterocolitica* 0:9. The exposure of the host to these organisms can provoke diagnostically significant titres of antibodies cross-reacting with cellular or S-LPS *Brucella* antigens used in diagnostic tests. These cross-reactions involve the carbohydrate component of S-LPS and have been attributed to the common presence of substituted 4,6-dideoxy-4-amino mannopyranosyl residues in the O chains. Agglutinating cross-reactions are reported between rough *B. canis* and *Actinobacillus equuli*, mucoid strains of *Pseudomonas aeruginosa*, and some *Pasteurella multocida* serotypes.

On the other hand, the study by means of immunoelectrophoresis of the antigenic relationship between *Brucella* protein antigens (mostly intracellular) and proteins from the genera *Bordetella*, *Haemophilus*, *Pasteurella*, *Yersinia*, *Escherichia*, *Salmonella*, *Klebsiella*, *Enterobacter*, and *Serratia*, has indicated that there are no common protein antigens, so that it can be accepted that antibodies against *Brucella* protein antigens are *Brucella*-specific. For that reason, immunoprecipitation tests with *Brucella* protein antigens can be useful in the differential serological diagnosis of brucellosis and other infections producing antibodies cross-reacting with *Brucella* S-LPS (yersiniosis, salmonellosis, tularemia, cholera vaccination).

2.7 Resistance and survival properties and disinfection

Under appropriate conditions *Brucella* organisms can survive in the environment for very long periods. Their ability to withstand
inactivation under natural conditions is relatively high compared with most other groups of non-sporing pathogenic bacteria.

2.7.1 Physical inactivation

*Brucella* organisms are readily killed by heat when in dilute suspension.

Pasteurization (high-temperature short-time or flash-methods) will kill *Brucella*. Very dense suspensions of organisms, such as those employed for antigen preparation or in some typing procedures, will not be completely inactivated by moderate heating and repeated heat treatment or exposure to boiling point temperatures may be required to render them safe.

The organisms are susceptible to normal sterilizing doses of ionizing radiation provided that complete exposure is ensured. *Brucella* survives drying, especially in protein-containing media, and will remain viable in dust or soil for up to 10 weeks. They can also survive in water for between 10 and 70 days, depending upon temperature. Survival is prolonged at low temperatures and the organisms will remain viable for many years in frozen tissues. They will survive in bovine faeces for at least 120 days, in aborted fetuses for at least 75 days, in uterine exudate for at least 200 days, and in liquid manure for up to 2½ years if the temperature is kept near 0°C.

2.7.2 Chemical inactivation

*Brucella* in aqueous suspensions are readily killed by most disinfectants. A 10 g/l solution of phenol will kill *Brucella* in water after less than 15 minutes' exposure at 37°C. The presence of organic matter drastically reduces the efficacy of most disinfectants. Formaldehyde solution is the most effective of the commonly available disinfectants, provided that the ambient temperature is above 15°C. Xylene at a concentration of 1 ml/litre has been found effective for destroying *Brucella* in liquid manure, however, exposure for at least 1 month may be required. Calcium cyanamide at 20 kg/m³ of liquid manure can be used as an alternative allowing an exposure time of at least two weeks.

Where possible, disinfection should be carried out by heat treatment rather than by the use of chemicals.
2.7.3 Selection of disinfectants for use on man and animals

For the decontamination of skin following accidental exposure to Brucella, solutions of substituted phenols are recommended. They should be used to wash off any gross contamination, after which more should be applied and allowed to dry on the skin. In their absence, ethanol, isopropanol, iodophors, or dilute hypochlorite solutions are effective alternatives. The alkyl quaternary ammonium compounds cannot be relied upon to kill Brucella.

Detailed recommendations on disinfection procedures for Brucella-contaminated materials and premises have been presented in a WHO unpublished document.¹

3. IMMUNOLOGY

3.1 The immune response

Infection with Brucella usually results in the induction of both humoral and cell-mediated immune responses. The magnitude and duration of these responses can be affected by many factors including virulence of the infecting strain, size of inoculum, age, sex, pregnancy, species, and immune status of the host. Both antibody- and cell-mediated immune responses are diagnostically useful, but the former have lent themselves most readily to quantitative measurement. The precise pattern of reactivity displayed in the variety of serological tests available for detecting antibodies to Brucella depends on the host species studied and on the distribution of antibody activity between the various immunoglobulin isotypes.

3.1.1 Anti-Brucella immunoglobulin isotypes

3.1.1.1 Human isotypes. Following natural infection in man, IgM appears early and for a few days is the only immunoglobulin present, as demonstrated by the reduction of agglutinating activity of serum after mercaptoethanol treatment. Later and in the subacute stage, IgG predominates. IgM and IgG are both agglutinating antibodies. IgG is also complement-fixing, while often IgM is not, so that in the

early stage of human brucellosis the complement-fixation reaction is frequently negative. This situation may be modified in cases of reinfection. Non-agglutinating IgA antibodies may be demonstrated by means of immunofluorescence, the Coombs antiglobulin test, or by enzyme-immunoassay or radioimmunoassay.

3.1.1.2 *Bovine isotypes.* The immunoglobulin isotypes present in serologically significant concentrations in bovine serum are IgG₁, IgG₂, IgM, and IgA. Similar isotypes at different relative concentrations occur in milk, although most of the IgA is present in the secretory form. IgA concentrations in bovine serum are usually very low and the role of this isotype in the various serological tests has not been clearly defined. Although secretory IgA in milk does play an important role in the milk ring test, IgM also participates in this reaction, whereas IgG₁ will produce an agglutinate at the bottom of the tube and may interfere with ring formation by the other isotypes.

Studies on the role played by the various isotypes in tests on serum have been conducted in a number of different centres using immunoglobulin preparations varying considerably in purity. The serological procedures and antigens used have also often varied and conflicting results have been reported in some cases. Nevertheless, for some serological procedures a clear pattern has emerged.

The first isotype produced after an initial heavy infection or strain 19 immunization is IgM. This can usually be detected in the first or second week following the initial antigenic stimulus, but is soon followed by IgG antibody. IgG₁ immunoglobulin is the most abundant in serum and exceeds the concentration of IgG₂. The magnitude and duration of the antibody response following immunization is directly related to the age at immunization and the number of organisms administered. Following immunization with the standard dose of strain 19 during calfhood, IgG antibody concentrations usually decline to diagnostically insignificant levels over 3–6 months. Residual antibody, if present, is usually predominantly of the IgM class.

Following exposure to virulent *B. abortus*, antibody may appear in 4–10 weeks or longer, depending on the size and route of entry of the inoculum and the stage of pregnancy of the animal, but even under controlled experimental conditions there is great variation in response from animal to animal. In infected environments, animals exposed to low doses may develop transient low antibody titres, but
show no clinical or bacteriological evidence of infection. A disturbing number of infected animals do not develop antibody of the IgG class until parturition, or 1–3 weeks after parturition. These animals may have low IgM titres a few weeks earlier, but in a vaccinated population they cannot be differentiated from non-infected vaccinated animals.

Antibodies of the IgA, IgM, IgG₁, and IgG₂ isotypes can all react in the tube-agglutination test, but those of the IgM class are by far the most efficient. Antibodies of the IgG₁ isotype produced in some sera, at least, have the capacity to block agglutination by other isotypes, particularly IgM. The agglutinating and precipitating activity of IgG₁ antibodies is enhanced at high salt concentrations or under acid conditions and this isotype is reactive in the card and Rose Bengal tests. The reactivity of IgM in this type of test is dependent on the precise method of preparation of the antigen and the procedures used. Conflicting results have been reported by different laboratories and the factors influencing the Rose Bengal plate test still require complete elucidation.

Treatment of serum with the acridine dye 6,9-diamino-2-ethoxyacridine lactate (ethacridine)¹ selectively precipitates out more IgM than IgG. Treatment of serum with sulphydryl reducing agents, such as 2-mercaptoethanol or dithiothreitol, dissociates the IgM pentamer and reduces its agglutinating activity without affecting that of the IgG isotypes.

When the complement-fixation test is performed by the warm fixation plate method, following serum inactivation at 58 °C for 30 minutes, effectively only antibodies of the IgG₁ isotype are detected. Using modifications of the test procedure, IgM antibodies may react in the complement-fixation test and this may account for differences reported between laboratories.

Probably the complement-fixation test measures IgM to different degrees in different laboratories, because of differences in the temperature and time of heating, the extent of any dilution of serum when it is heated, and the diluting buffer. If so, then the use of a system of international units alone will not be sufficient to make the results of complement-fixation tests internationally comparable.

It has been suggested that, as the relative complement-fixing efficiency of IgM compared with IgG is greater at 37 °C than at 4 °C, the complement-fixation test should be less sensitive to IgM with

¹ Marketed under various proprietary names, e.g., Rivanol and Ethodin.
cold fixation than with warm fixation. Observations from the field suggest that the reverse is the case.

The complement-fixation test fails to measure the non-complement-fixing isotypes IgG_2 and IgA. IgG_2 antibody can interfere with complement fixation by IgG_1 and, in sufficient proportion, causes prozones, atypical reactions, and false negatives. These effects depend on the antigen concentration used, and are greater with warm fixation than with cold fixation. Both the total concentration of IgG_2 immunoglobulin and the proportion of it that is directed specifically against \textit{B. abortus} can vary. Total IgG_2 concentrations vary greatly among cattle; they are affected by genetic factors and they increase with age and probably with the degree of total antigenic exposure. The ratio of IgG_2 to IgG_1 antibody rises around parturition when IgG_1 is selectively transferred into colostrum. The proportion of specific IgG_2 antibody appears to increase with multiple exposure to \textit{Brucella} antigen.

Antibodies of all isotypes except IgM may participate in the Coombs antiglobulin test but IgG_1 and IgG_2 are quantitatively the most important. Reactions in the enzyme immunoassay test depend upon the specificity of the enzyme-labelled antiglobulin reagent used for the secondary stage. However, because of the washing procedures involved, enzyme immunoassay tests tend to discriminate against antibodies of low avidity.

It is widely believed that sustained production of IgG_1 antibody is characteristic of chronic infection but that IgM antibody persists in animals immunized with strain 19. The complement-fixation test is superior to the tube agglutination test in detecting chronic infection, whereas the agglutination test is more subject to persistent reactions after strain 19 immunization. This is believed to be because the tube agglutination test is more sensitive to IgM than to other antibody isotypes, whereas the complement-fixation test is particularly sensitive to IgG_1.

3.1.1.3 Isotypes in other species. Immunoglobulins of sheep, goats, and pigs have a similar isotype classification to that of cattle. However, little information is available on the behaviour of these isotypes in relation to serological tests for brucellosis performed on the sera of these species.

3.1.1.4 Antigen specificity of \textit{Brucella} antibodies. In all the standard tests mentioned in the previous section, the serum antibody
measured is primarily that directed to the S-LPS antigen of *Brucella*. Infected cattle also produce antibody, predominantly IgG1, to NH or to polysaccharide B hapten. Precipitating antibody to these is only transiently produced by vaccinated cattle. It can be assayed by means of a rapid single radial immunodiffusion test and by enzyme immunoassay. Enzyme immunoassay and radioimmunoassay procedures can also be used to detect antibody responses to protein antigens, although when used with crude antigen preparations they usually detect antibodies directed mainly against the S-LPS antigen.

Immunodiffusion and immuno-electrophoretic procedures can be used to detect precipitating antibodies to a wide range of antigens and are effective in some cases for differentiating cross-reactions to *Brucella* S-LPS induced by other bacteria. Nevertheless, these procedures lack the sensitivity required to make them reliable for detecting infection.

3.1.1.5 Properties and significance of EDTA-labile agglutinins. Agglutinins for *Brucella* cells are sometimes encountered in the serum of animals that are known not to have been exposed to the organism. In such circumstances, agglutinating activity may arise from a number of sources. Thus, it may be attributable to antibodies produced in response to exposure to bacteria antigenically related to *Brucella*. In this case, the cross-reacting antibodies have properties typical of the isotype to which they belong.

In other instances, the agglutinating activity is associated with the so-called “natural antibodies”. These appear to be heterogeneous. Some may indeed be true antibodies, although the nature of the inciting antigen is unknown. Others do not behave as typical antibodies. These include heat- and acid-labile agglutinins associated mainly with the macroglobulin fraction. Such non-specific agglutinins occur quite frequently in the serum of cattle, pigs, and horses, less frequently in human, ovine, and feline serum, and rarely in rodent or lagomorph serum.

The *Brucella*-agglutinating activity present in the sera of a high proportion of uninfected cattle reacting to the tube agglutination test is labile in the presence of the chelating agent ethylenediaminetetraacetic (EDTA) sodium salt. These EDTA-labile agglutinins are associated mainly with IgM molecules, although, in a small proportion of animals, IgG may also be involved. These immunoglobulins attach to *Brucella* cells via the Fc region of their 7S subunits and not via the antibody-combining site.
located on their Fab portions. This interaction can be blocked by EDTA or by the structurally related ethylene glycolbis(β-aminoethyl)ether N,N,N',N'-tetraacetic acid (EGTA) but not by other dissimilar chelating agents. The action of EDTA or EGTA is not dependent upon the removal of divalent cations.

Agglutinins of this type may be involved in 70–80% of the agglutination reactions given by sera from cattle with negative complement-fixation reactions to Brucella. Cross-reacting antibodies probably account for most of the remainder of these reactions, as well as most of those produced by brucellosis-free animals giving positive complement-fixation reactions.

Differentiation of EDTA-labile agglutinins can be achieved by performing the standard tube agglutination test in a diluent composed of a 10 mmol/l solution of EDTA disodium salt in phosphate-buffered saline, pH 7.2, instead of phenol-saline. The incubation conditions and reading of the test are otherwise as for the standard procedure.

In sera in which the agglutinating activity is entirely attributable to EDTA-labile agglutinins, a complete, or almost complete, loss of titre will occur in the presence of the chelating agent. However, many sera will contain a mixture of EDTA-labile agglutinins and EDTA-stable antibodies. These will show only partial reduction in titre in the presence of EDTA. Such reactions have to be interpreted in the light of supporting evidence provided by the herd history and by other serological and cultural tests.

When the tube agglutination test is employed in testing animals for the purposes of international trade, it is recommended that agglutination reactions caused by EDTA-labile agglutinins should be excluded.

3.2 Cell-mediated immunity to Brucella

Brucella species are facultative intracellular pathogens. They are readily phagocytosed by macrophages and polymorphonuclear leucocytes and, in the case of virulent strains, are capable of surviving within these cells. Not all Brucella organisms in the host are to be found within cells, and phagocytosis is promoted by antibody. However, since virulent Brucella can survive within normal macrophages for long periods, recovery from infection is likely to be dependent upon the acquisition of increased bactericidal activity by these phagocytic cells.
Macrophage activation occurs when T lymphocytes of the appropriate subset are stimulated to release lymphokines (interleukins). The release of these activating factors is dependent upon recognition of the appropriate antigen by the T lymphocyte and is subject to regulation through the major histocompatibility complex. Live organisms capable of establishing persistent intracellular infection and certain types of antigen, with or without adjuvant, are the most effective inducers of cell-mediated immunity.

Cell-mediated immunity is associated with the delayed hypersensitivity reaction, which can often be elicited in infected animals by the intracutaneous injection of antigen. In experimental systems, antibacterial activity can be demonstrated at the site of a delayed hypersensitivity reaction that has been elicited by a quite unrelated antigen. Delayed hypersensitivity and increased bactericidal activity of the macrophages usually appear in parallel during infection, but the delayed hypersensitivity response can also occur in the absence of effective protective immunity. Thus, some Brucella adjuvant vaccines are effective inducers of delayed hypersensitivity to Brucella antigens, but this is not always accompanied by the development of protective immunity.

Pre-existing or passively transferred antibody can protect against subsequent infection with Brucella. However, in experimental infection in mice, antibody production can be artificially suppressed without affecting the outcome of infection, suggesting that cell-mediated immunity is of major importance in recovery. Nevertheless, Brucella organisms are less sensitive to killing by activated macrophages than Listeria monocytogenes, the facultative intracellular bacterium on which most of the early studies into the mechanisms of cell-mediated immunity were conducted. This relative resistance to killing of Brucella may contribute to the chronicity of infection.

The role of cytotoxic cells, including cytotoxic T lymphocytes, natural killer (NK), and killer (K) cells, in the cell-mediated immune response to Brucella has not been elucidated. Further studies are also needed to determine the basic processes underlying the development of protective immunity to Brucella in the natural host species.

Antigenic extracts containing outer membrane proteins (OMP) and the cell wall peptidoglycan can induce strong transferable cell-mediated immunity to Brucella, at least in mice. OMP in combination with an appropriate adjuvant may also stimulate cell-mediated responses in cattle and other domestic species.
Cell-mediated immunity has been assayed by a number of *in vivo* and *in vitro* tests. The delayed hypersensitivity reactions referred to previously, and elsewhere in this report in relation to intradermal allergic tests, come into this category. Tests that measure the inhibition of macrophage migration *in vitro* by *Brucella* antigens have received limited study but are difficult to standardize and have not been widely adopted. Measurements of lymphocyte stimulation in the presence of *Brucella* antigens have received much more attention. However, most studies have employed ill-defined antigen preparations and the problem of standardization has not been resolved. Furthermore, their requirement for viable lymphocytes limits their application under field conditions. At present, the wider adoption of these procedures for diagnostic purposes cannot be recommended.

3.3 Delayed hypersensitivity

Delayed hypersensitivity reactions specific to the genus *Brucella* may be induced by infection, by immunization with living vaccines (*B. abortus* strain 19 and *B. melitensis* strain Rev. 1), and by adjuvant vaccines such as H38 and 45/20.

A number of skin-test antigen preparations are in use. Those that are free of S-LPS, such as brucellin-INRA or the *Brucella* hydrolysates used in certain countries, should be used in preference to crude preparations that interfere with subsequent serological testing. The use of the skin test can help to resolve the ambiguities that occasionally arise when serological cross-reactions are produced by *Yersinia enterocolitica* serogroup 0:9 or other bacteria.

4. VACCINES

Both living vaccines of low virulence, such as *B. abortus* strain 19, *B. melitensis* Rev. 1, and *B. suis* strain 2, and killed vaccines in oily adjuvant, such as the 45/20 and H38 vaccines, are available. Each vaccine has its advantages and disadvantages.

4.1 *B. abortus* strain 19 vaccine

Strain 19 vaccine was developed for the immunization of cattle. Traditionally, the recommended immunization procedure has been
the administration of not less than $5 \times 10^{10}$ viable organisms subcutaneously to female calves at 4–8 months of age. More recently in the USA the dosage for calfhood immunization has been officially reduced to between $3 \times 10^8$ and $3 \times 10^9$ live organisms and the age limits have been adjusted to 4–12 months for both beef and dairy cattle. Age restrictions are imposed because the serological response to immunization is less prolonged in young animals and has time to disappear before they grow old enough to need testing. Most immunized female calves lose their serum antibody titres within 16–18 months, but their relative immunity to infection with virulent organisms has been considered to be lifelong. In the serological testing of animals immunized with strain 19 it is important to use a test such as the complement-fixation test that is relatively insensitive to vaccinal antibody.

Immunization of bulls with strain 19 vaccine is not usually recommended as serum agglutinins persist longer than in cows and localization of the vaccine strain in the genital tract of immunized bulls has been reported. It may be considered in heavily infected areas where bulls are used for natural service.

Various factors such as introduction of the disease into previously free areas, changing husbandry and marketing practices, revised government control programmes, etc. have necessitated the immunization of mature females in some areas. This practice can cause occasional abortions when pregnant heifers are immunized, but there is no evidence of spread of infection. It is recommended that immunized adults be permanently identified as such. When full doses are used, persistent and high antibody titres interfere with diagnostic testing. Doses of between $3 \times 10^8$ and $10 \times 10^8$ live organisms when given subcutaneously are protective for at least a year and antibody titres, as detected by complement-fixation or diaminoethoxyacridine tests, generally drop below diagnostic levels after 6–8 months.

However, observations both in the laboratory and in the field indicate that 90–95% of cattle immunized with a low dose ($3-10 \times 10^9$) viable organisms, lose their complement-fixation titres in less than 6 months, but the other 5–10% retain positive, sometimes very high, titres for 8–12 months or longer. Strain 19 can be isolated from a substantial proportion of these persistent reactors.

An alternative method of immunization is by giving $5-10 \times 10^9$ viable strain 19 cells by the conjunctival route. Under experimental conditions two doses administered at an interval of 4–8 months
provided good protection and almost no serological reactions occurred. This method is, therefore, fully compatible with a test-and-slaughter control programme and can be advantageously used in infected areas, even on adult cows.

Strain 19 induces a relatively good immunity. The Committee considered that the question of duration of immunity needs further study. As a living vaccine, strain 19 must be handled carefully to preserve its viability and quality. Standards for the production of strain 19 vaccine have been recommended by the WHO Expert Committee on Biological Standardization.\(^1\)

It is recommended that reconstituted freeze-dried strain 19 vaccine should be used on the day of preparation. Some deterioration may nevertheless occur. There is a need to develop better methods for maintaining the viability of strain 19 vaccine.

4.2 B. melitensis strain Rev. 1 vaccine

Rev. 1 vaccine was developed for the immunization of sheep and goats. The information available on this live attenuated vaccine up to 1980 has been summarized in two reports.\(^2,3\) The specific requirements for the vaccine recommended by the WHO Expert Committee on Biological Standardization were published in 1977.\(^4\)

B. melitensis strain Rev. 1 was isolated as a non-streptomycin-dependent revertant from a population of streptomycin-dependent cells that were, in turn, derived from a virulent strain of B. melitensis 6056. Since 1955 it has been shown to be of low virulence and to produce good immunity, but to give rise to serum antibodies that persist for various periods of time.

The stability of attenuation of strain Rev. 1 has been amply demonstrated in guinea-pigs, sheep, and goats. Occasionally, an animal will excrete the vaccine strain in the milk for some time, but there is no known public health risk.

In goats and sheep immunization has usually been performed at the age of 4–6 months with the recommended subcutaneous dose of

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10⁹ cells. The duration of protection for goats is at least 4.5 years. In lambs and ewes protection has lasted up to the second or third pregnancies. It has been suggested that a lower dose of Rev. 1 vaccine (5–10 × 10⁴ cells) given to adult sheep and goats might play a useful role at the beginning of a campaign where some of the animals in a herd may be pregnant and where kidhood immunization is under way.

Immunization of lambs by conjunctival instillation of a single dose of 10⁹ cells of Rev. 1 in 0.1 ml of vaccine induces protection similar to that given by subcutaneous inoculation, but with a very weak and short-lasting serological response.

Wide variations have been reported by various authors in the serological response in young goats and lambs after injection of a full dose of Rev. 1 vaccine. These apparent discrepancies may be due to differences between breeds or in the type of test and interpretation used. The reported duration of response varied from 4 to 20 months.

When a test-and-slaughter control programme is to be applied after or simultaneously with an immunization programme, highly sensitive methods of diagnosis are required. Hence, there could be some difficulties with both programmes, unless a sufficient time is allowed to elapse between them or, alternatively, a low-dose method, employing either subcutaneous inoculation or conjunctival instillation, is used.

It is now well established that the Rev. 1 vaccine will protect rams against B. ovis infection. Rev. 1 vaccine can also be used with good results in cattle, but it should be restricted to areas where B. melitensis is prevalent in small ruminants.¹

Workers using Rev. 1 and strain 19 vaccines should exercise care to prevent their accidental injection. Similar caution should be exercised with non-living preparations, especially those containing oily adjuvant.

### 4.3 B. suis strain 2 vaccine

This vaccine strain was developed in China where it has been used very extensively for the control of brucellosis in sheep and goats. The vaccine strain is an attenuated smooth strain of B. suis biovar 1 and

¹ Seed cultures of the Rev. 1 strain are available for distribution to official laboratories, WHO collaborating centres, and regional zoonosis centres. Requests should be sent to Professor M. Plommet, Director. Reproductive Pathology Unit, National Institute of Agronomical Research, Nouzilly, 37380, France.
is typical of this in its general properties. Its pathogenicity for guinea-pigs is of the same order as that of the *B. abortus* strain 19 vaccine. It does not produce abortion in pregnant cattle, sheep, goats, or pigs, nor does it persist in the tissues of immunized animals.

In sheep and goats, *B. suis* strain 2 vaccine gives better protection against virulent *B. melitensis* than does *B. abortus* strain 19 vaccine. It also confers significant protection against infection with virulent *B. suis* strains in pigs challenged by a variety of routes, but not in those exposed through natural service.

The vaccine is normally given by the oral route in drinking-water. Doses of $10^{10}$ live organisms produce good immunity against *B. melitensis* in sheep and goats. A dose of $5 \times 10^9$ live organisms is recommended for cattle, while for pigs two doses of $2 \times 10^{10}$ live cells given 2–3 months apart are advised.

The protection given by the vaccine is effective for up to 4–5 years in sheep and goats. Serological responses are reported to be low and transient, particularly if immunization is restricted to young animals.

Encouraging results with this vaccine have been reported from China. The Committee recommended that the efficacy and safety of this vaccine should be studied under different ecological conditions and in different breeds of livestock, since a non-parenteral vaccine giving long-lasting immunity would be ideal for mass immunization in many countries.

4.4 *B. abortus* strain 45/20 vaccines

These vaccines are prepared from killed rough *B. abortus* strain 45/20 in oily adjuvant. Since a rough strain lacks the smooth LPS antigens that cause post-immunization serological reactions, animals immunized with these vaccines do not usually react to serological tests with smooth antigens. They do, however, react to tests with rough antigens.

Because there are many commercially available 45/20 vaccines, differing greatly in several aspects, and because there is no agreement on a quality control method such as that given in Annex 3, very different results have been reported in the literature. Some authors have reported an immunity similar to that given by strain 19. Others have reported very poor immunity. Generally, in controlled field experiments, poor protection has been reported. However, 45/20 vaccines can be used on animals of any age, whether pregnant or not.
They can be successfully used as an aid to the diagnosis of latent infection in the anamnestic test.

Usually, two doses of 45/20 vaccines 6–12 weeks apart are considered to be necessary to induce a good immunity, and an annual booster is generally recommended. However, recent investigations in two countries have suggested that a single dose may give protection. The duration of immunity given by 45/20 vaccines has not yet been established for either single-dose or two-dose schedules.

Since there are still many uncertainties associated with manufacture, standardization, and conservation of immunogenic activity, 45/20 vaccines cannot be recommended unless effective quality control, as described in Annex 5, can be applied. If so, this type of vaccine could be very useful in certain circumstances—for example, in range areas (Australia) or in countries that must comply with import regulations.

4.5 B. melitensis strain H38 vaccine

H38 vaccine provides effective immunity and can be given to animals of all ages and at any stage of pregnancy. It can be used for lambs and adults, allowing the population to be immunized immediately and thus reducing the number of abortions and the spread of infection.

However, it has the disadvantages that it produces severe local lesions and induces serological reactions of long duration; it is therefore incompatible with test-and-slaughter control programmes, which will make it unacceptable for most countries.

4.6 Vaccines for use in man

4.6.1 Live vaccines

In the USSR, since 1952, certain groups of workers exposed to B. melitensis infection have been successfully immunized using a live vaccine known as B. abortus 19-BA, derived originally from B. abortus strain 19. It is administered by intradermal injection or by skin scarification. The effective dose is 10⁵ cells. It is used only when there is a high risk of exposure and immunization is repeated with half the initial dose after 10–12 months. To avoid sensitization, the vaccine is administered only when intradermal and serological tests are negative.
A live vaccine has also been in use in China among similar groups of persons. The vaccine was derived from \textit{B. abortus} strain 104M, a strain notably more virulent than 19-BA. It is given by placing a drop containing $7 \times 10^9$ cells on the skin and scarifying the area under the drop, taking care to avoid any possibility of subcutaneous inoculation.

4.6.2 \textit{Non-living vaccines}

In the USSR a protein-polysaccharide complex derived from the cell wall of \textit{Brucella} S-forms by acetic acid hydrolysis has been tested in a controlled trial. It is as effective as live \textit{B. abortus} 19-BA and is used for primary immunization, as well as to protect individuals previously immunized with live vaccine and returning to an occupation involving a high risk of exposure.

In France, an immunogenic phenol-insoluble fraction obtained by phenol extraction of delipidated \textit{Brucella} cells has been tried. This vaccine has been shown to be innocuous in non-sensitized humans. It can be used to protect exposed people, but the efficiency and the duration of the resulting immunity have not yet been well established.

4.7 \textit{Methods of conservation of seed strains and vaccines}

Strains of \textit{Brucella} should be preserved in such a way as to minimize the risk of the variation that can occur when cultures are maintained for long periods or when media are stored at room temperature. Reference and vaccine strains and live vaccines should be preserved by drying, freeze-drying, or storage at or below $-65^\circ\text{C}$.

A simple technique for drying \textit{Brucella} cultures has been described.\textsuperscript{1}

5. EPIDEMIOLOGY

5.1 \textbf{Bovine brucellosis}

Bovine brucellosis is distributed throughout the world except for the growing number of countries where eradication has been achieved.

\textsuperscript{1} See footnote 2 on page 17.
In most developing countries, resources have not been sufficient to control brucellosis. Although information on prevalence is inadequate, there are indications of a very high incidence in many areas, particularly in the tropics, and in countries that can least afford the loss in milk production and animal protein that accompanies this disease.

Limited surveys, as well as officially reported figures, indicate a striking similarity in the factors that influence the incidence of bovine brucellosis in different tropical countries. Incidence levels tend to be relatively high on organized farms, whether these have indigenous cattle or introduced breeds. Both in Africa and in Indo-Pakistan, *B. abortus* biovar 1 is most frequent in the type of herd found on these farms and the disease presents the classical signs of abortion as described in the temperate zones. Under traditional systems of husbandry, with both small dispersed herds of village cattle and nomadic or seminomadic cattle herds, *B. abortus* biovar 3 is most frequently isolated. However, in some areas, abortion is relatively uncommon in such herds, and in some parts of Africa, hygromas and abscesses are the major clinical sign. The incidence and the abortion rate is apparently more related to rainfall than to herd size in both organized and traditional systems of husbandry.

Infection of cattle with *B. melitensis* occurs in regions where *B. melitensis* infection is enzootic in sheep and goats. Here cattle shedding *B. melitensis* in the milk constitute a serious public health problem.

5.1.1 *Pathogenesis and transmission*

The major route of infection appears to be through the mucous membranes of the oropharynx, upper respiratory tract, and the conjunctiva. Very large numbers of organisms are shed at the time of parturition. The system of husbandry as well as the environmental conditions will greatly influence the spread of infection. Thus, calving in dark, crowded enclosures is more favourable to spread than calving in the open air in a dry environment.

Animals generally abort only once, although reinvasion of the uterus occurs in subsequent pregnancies and *Brucella* organisms are shed with the membranes and fluids. Non-pregnant animals exposed to small numbers of organisms may develop self-limiting, immunizing infections or they may become latent carriers.
Persistent infection of the mammary glands and supramammary lymph nodes is common, with constant or intermittent shedding of the organisms in the milk in succeeding lactations. The inflammatory changes in the infected mammary gland reduce milk production by an estimated 10%.

In the male, localization in the testis, epididymis, and accessory sex organs is common, and organisms may be shed in the semen. This may result in infertility, but is not believed to be a major mode of transmission to cows.

Some viable calves born to infected dams have infections that may persist in the lungs and regional lymph nodes. Those fed infected milk may have infections of the lymph nodes draining the gastrointestinal tract and may shed *Brucella* organisms in the faeces while receiving infected milk. Most recover from these infections and are fully susceptible when they reach sexual maturity. About 5% of calves born to infected dams remain latently infected, with the clinical signs and the serological response becoming apparent only at abortion or parturition in their first pregnancy. Other sites of localization include the carpal and other bursae where hygromas containing large populations of *Brucella* may be formed.

Experimental work with cattle has demonstrated that embryo transfer is likely to be a useful method of preserving valuable genetic lines provided that recommended embryo washing and other hygienic measures are followed in moving embryos from infected donors to healthy recipients. It is important to monitor the health of recipient animals to ensure the effectiveness of these techniques in preventing disease transmission.

5.2 *B. melitensis* infection in sheep and goats

In areas where *B. melitensis* is endemic, notably on the Mediterranean littoral, in South-West Asia, and in parts of Latin America, it constitutes a serious zoonosis. In some countries, there has been an increase in prevalence following intensive development of sheep farming.

Pathologically and epidemiologically *B. melitensis* infection in sheep and goats is very similar to *B. abortus* infection in cattle. Most breeds of goat are fully susceptible to infection, but there is great variation in the susceptibility of different breeds of sheep. For example, Maltese sheep are very resistant, whereas the fat-tailed sheep of South-West Asia are very susceptible and form a reservoir
of infection that gives rise to widespread brucellosis outbreaks in man. In most countries bordering the Mediterranean and in South-West Asia, the brucellosis problem largely centres on sheep, while in Latin America goats are chiefly involved. Although a variety of symptoms have been described in artificially infected goats, abortion in females and orchitis in males appear to be the main symptoms seen in natural cases of brucellosis in small ruminants.

5.2.1 Pathogenesis and transmission

Generally, pathogenesis and transmission operate in the same way in sheep and goats as they do in cattle, materials excreted from the female genital tract forming the main supply of material for transmission to other animals. In goats, excretion of the organisms from the vagina is prolonged and copious. In both sheep and goats the persistent excretion of *B. melitensis* in the milk provides an important source of infection for man.

5.3 *B. ovis* infection of sheep

*B. ovis* has greater affinity for the reproductive tract in the male than in the female. Epididymitis and sterility are the most frequent and serious consequences of infection with *B. ovis*.

5.3.1 Pathogenesis and transmission

In rams, under conditions of natural infection, there is a relatively long period (6–17 weeks) between exposure and the development of lesions, during which time the animals are asymptomatic. The organism remains confined to the exposure site and regional lymph nodes for 10–14 days before the infection advances to the bacteraemic stage with a more generalized infection that involves the spleen, kidney, and lymph nodes distant from the exposure site. During the latter part of the bacteraemic stage, the infection begins to localize in the genital organs. The localized lesions appear in the seminal vesicles, ampullae, testes, tail of the epididymis, and, less frequently, the head of the epididymis.

During the bacteraemic stage, the organism may become localized in the kidney and some rams become urinary shedders of *B. ovis*. In fact, this organism can be a cause of chronic interstitial nephritis in sheep.
In ewes, when the infection does progress, the regional and bacteraemic stages of the disease are quite different from those in rams. Under conditions simulating natural infection, it was found that organisms disappear from the local site shortly after exposure, bacteraemia is prolonged, and the organisms reappear in the genital tract at approximately the third month of gestation. For abortion to occur, there has to be a sufficient accumulation of bacteria and exudate to cause necrosis of the placenta and separation from the caruncles. The slow progress of the infection probably accounts for the frequent finding of positive complement-fixation titres but infrequent occurrence of abortion, either by experimental or natural exposure. Fetal lambs can survive long periods in utero in the presence of infection and the primary consequence of the infection is usually not abortion but a placentitis that interferes with fetal nutrition and results in lambs with lowered birth weights.

Rams are more susceptible to infection than are ewes, they are more actively responsible for the transmission of the organisms, and are the reservoir animal for the maintenance of the disease. The source of the organism is contaminated semen secreted by infected rams.

In breeding flocks, the organisms are spread from infected rams to non-infected rams by passive venereal transmission. Passive transmission to a non-infected ram, via a common ewe, usually requires both rams to mate within the same estrous cycle. Ram-to-ram transmission can also occur by direct contact or by non-infected rams being housed in barns and sheds previously occupied by infected rams.

Young rams also are susceptible to infection with *B. ovis* and, in some flocks, up to 80% of unmated young rams have been found to have clinical and serological evidence of infection. Sodomy has been suspected as the main method of spread in young rams; experimental transmission via the rectum has been produced. Infection via the nasopharynx has also been thought to be important, since rams often nuzzle the genital organs of other rams.

Compared with rams, ewes are relatively resistant to infection. Ewes mated with infected rams rarely become actively infected but often develop positive complement-fixation titres. Direct contact during abortion transmits the disease from ewe to ewe only rarely. The carry-over of infection by the ewe from one breeding season to the next is not a means of maintaining infection in the flock. However, following abortion or parturition an infected ewe may
excrete *B. ovis* for approximately 10 days and could transmit infection if she comes into estrus during this period. Occasionally ewes inseminated with infected semen develop a transitory vaginocervicitis, which may recur at the next estrus and even after parturition. Clearly ewes with this condition could be transmitters.

*B. ovis* infection in rams has a deleterious effect on their semen quality, breeding efficiency, and breeding capacity. Semen of infected rams, even in the absence of observable lesions, contains desquamated epithelial cells, sperm debris, and frequently *B. ovis*. Lower fertility and seminal degeneration almost always precede the appearance of lesions.

In flocks where abortions have occurred, lamb yields have been known to drop from 100% to 25%. Additionally, up to 20% of the ewes remain barren and up to 16% of the lambs born alive, die before reaching six weeks of age. A prolongation of the lambing period occurs as a result of interference with normal breeding operations.

### 5.4 *B. suis* infection in pigs

Biovars 1, 2, and 3 of *B. suis* affect swine. Biovar 2 is mainly confined to Europe and is peculiar in that the European hare (*Lepus europaeus*) and the domestic dog are involved in its epidemiology. It is not known to infect man. Biovar 3 is endemic in North America and South China and has only rarely been reported from other areas. It resembles biovar 1 in its epidemiology.

Biovar 1 is the predominant biovar epidemiologically and economically. Many strains of this biovar are slightly atypical in their growth characteristics on media containing the dyes thionin and basic fuchsin. It is widely distributed and especially prevalent in South-East Asia, the islands of the Pacific, and parts of Latin America. In some of these areas, action to expand and improve the pig industry has led to the importation of highly susceptible breeding stock, overcrowding, and a serious brucellosis problem. *B. suis* infection has also been found prevalent in feral swine in some areas, e.g., Queensland, Australia, and Florida, USA.

The disease usually runs an acute course when first introduced into a herd of swine. In some herds, particularly small ones, the disease may disappear or cease to be an important problem because some infected animals recover and most are removed for slaughter in the course of normal farming practice. In the larger breeding
herds, the infection is apt to persist as a chronic disease in many animals, and to appear again as an acute condition in the next generation.

5.4.1 Pathogenesis and transmission

Swine brucellosis affects all types of swine, including boars, castrated males, and females, whether breeding or fattening. The principal route of transmission is by ingestion of contaminated materials. Pigs will readily consume aborted materials. Venereal transmission also occurs readily.

In an infected herd the owner may not detect any signs. The classical manifestations of swine brucellosis are abortion, infertility, orchitis, posterior paralysis, and lameness. Abortion may occur at any time during pregnancy and aborted materials and subsequent excretion provide large numbers of bacteria to contaminate the environment.

The pathogenesis of \textit{B. suis} biovars 1, 2, and 3 is very similar, with the exception that miliary brucellosis of the uterus has been reported only in animals infected with biovar 2. Invasion of the animal body by \textit{B. suis} follows the usual course of acute brucellosis. In the early stages, bacteria are found only in the lymphatic system, bacteraemia follows, and later there is localization in the genital organs, udder, and joints, in addition to the lymph nodes and occasionally other organs. Many infected swine recover spontaneously.

5.5 Brucellosis in dogs

It has long been recognized that the dog can act as a mechanical and biological vector of \textit{B. abortus}, \textit{B. suis}, and \textit{B. melitensis}. In nearly all cases, the source of the canine infection can be traced to the consumption of materials from infected domestic and wild animals. Wild carnivora, such as foxes and wolves, have also been shown to be liable to infection with these \textit{Brucella} species.

Brucellosis in dogs caused by \textit{B. canis} was initially described in the USA in 1966. The disease has subsequently been diagnosed in many other countries, often associated with outbreaks of abortion and infertility in females and epididymitis in male dogs. Although \textit{B. canis} was initially recovered from widespread outbreaks in beagles there is no breed preference. Prevalence rates vary, depending on the area studied, type of diagnostic test, incidence and
intensity of breeding, and the extent to which stray dogs are permitted to roam. Commercial breeding kennels seem particularly at risk, especially those that raise beagles. Studies in Mexico and Argentina have found high seroprevalence rates in dogs and man. In contrast, surveys for *B. canis* antibodies in the Federal Republic of Germany and in the USA have found a low rate. Human infections have been diagnosed, but the disease is generally less severe than that caused by the classical species (*B. abortus*, *B. melitensis*, and *B. suis*).

Natural spread of *B. canis* appears to occur only in the canine species.

5.5.1 **Pathogenesis and transmission**

The clinical manifestations of canine brucellosis in dogs vary, and inapparent infections are frequent, but abortions and infertility in bitches and epididymitis with scrotal dermatitis in males are common signs. Lymph node enlargement may be present. Abortions are most common between the 45th and 55th day of gestation, but they may occur earlier. There is a prolonged period of bacteraemia that may persist for 2 years, or longer. The bacteraemia may be intermittent, especially during the chronic stages. Semen from infected males is usually markedly abnormal, containing leucocytes and aggregated spermatozoa. Another reported manifestation of canine brucellosis is discospondylitis of the lumbar vertebrae. Recurrent uveitis has been reported in both experimental and field cases.

The most important mode of *B. canis* transmission is via placental tissue and vaginal discharges following an abortion. Organisms may be shed in vaginal fluid in great numbers for several weeks after an apparent or inapparent abortion. Venereal transmission occurs because males harbour the bacteria for long periods in the epididymides and prostate gland. Seminal excretion of *Brucella* is unpredictable, since organisms may be isolated consistently from ejaculated semen for only 1–2 months after initial infection. Intermittent shedding has been observed for as long as 60 weeks, and *Brucella* organisms have been cultured from the prostate gland and epididymal tissues for periods longer than 2 months after the bacteraemia has ceased. Carrier males may appear normal; however, examinations of semen will generally reveal abnormalities. Transmission was not observed when infected non-pregnant and
uninfected females, or infected and uninfected males were housed together in isolation units for several months.

5.6 Brucellosis in other species

5.6.1 Brucellosis in horses

Horses may become infected with *B. abortus* and *B. suis*. The infection may remain asymptomatic in some animals; others may suffer from osteoarthritis and osteomyelitis, bursitis (fistulous withers, poll evil), tenosynovitis, abortion, and infertility.

The tube agglutination, complement-fixation, and buffered *Brucella* antigen tests are all used in the diagnosis of equine brucellosis. Current information indicates that the degree of hazard presented to other susceptible animals by *Brucella*-infected horses is slight.

5.6.2 Brucellosis in camels

In Mongolia, a high rate of brucellosis infection occurs in the two-humped camel (*Camelus bactrianus*), especially when it is in contact with infected large and small ruminants. Brucellosis has also been reported in the one-humped camel (*Camelus dromedarius*). There may be no clinical symptoms, but abortions are observed sporadically. *B. abortus* has been isolated from aborted fetuses, genital discharges, urine, and milk. Infection can be transmitted to man by the consumption of unheated milk. Serological studies have shown that the complement-fixation test is more sensitive than the agglutination test in revealing reactors. Inactivation at 60–62°C for 30 minutes was found to be optimal for camel sera. The milk-ring test can be used for screening herds.

Control of brucellosis on camel farms has been limited to serological tests once a year and the isolation of reactors. Control of the disease among camels was more successful when reactors were slaughtered and other species, such as cattle, yaks, sheep, and goats were immunized each year.

More information is required on the mode of transmission within the camel herd and on the relationship between brucellosis in camels and in other ruminants.

*Brucellosis* also occurs in llamas and other small camelids in some South American countries.
5.6.3 Brucellosis in reindeer and other Cervidae

Brucellosis is endemic in many herds of caribou and domesticated reindeer (Rangifer tarandus) in the Arctic areas of Alaska, Canada, and the USSR. In these areas, reindeer are the major farm animal.

The causal organism of brucellosis in these hosts is B. suis biovar 4. Curiously, this is the only ruminant animal that serves as a reservoir for any member of the species B. suis. B. melitensis occurs in reindeer in China. The manifestations of the disease under natural conditions of exposure include abortion, retained placentas accompanied frequently by vaginal exudation and bleeding, metritis with abscesses, orchitis and epididymitis, bursitis, synovitis, and abscesses of the lymph nodes, spleen, liver, and mammary glands.

Extensive field studies have shown that B. suis biovar 4 is transmitted by the same mechanisms as other Brucella. Since abortion followed by vaginal exudation and bleeding is one of the manifestations of the disease in females, and reindeer have a strong herd instinct, direct contact with infected animals and exposure to environmental contamination are certainly two mechanisms of spread. Additionally it is very probable that males transmit the organism venereally. Chronic orchitis and epididymitis are frequent manifestations of infection in males, and it has been demonstrated experimentally that females can readily be infected via the vagina.

Brucellosis of reindeer is more easily diagnosed on a herd basis than on an individual basis. In fact, short of isolating the organism, it can be difficult or impossible to ascertain the infection status of an individual animal within a herd. Reindeer that are actively infected, and chronically so, may still have no detectable serum antibody response. Thus, a negative serological test is not a reliable indicator of freedom from infection.

Natural infections of Brucella abortus in elk (Alces alces) have occurred in herds in the USA. Abortions and other clinical symptoms have been reported.

5.6.4 Brucellosis in buffalo, saigas, and yaks

(a) Buffalo

Brucellosis has been observed in the domestic buffalo (Bubalus bubalis) and appears to follow the same pattern as in cattle. The B. abortus biovar that is indigenous to the cattle population of an area is usually the same biovar that is found in buffalo in that area,
c.g., biovar 3 in the Eastern Mediterranean area, although in Italy infections in cattle are caused by *B. abortus* biovar 6 whereas biovar 1 occurs in buffalo. The same serological methods are usually employed as in the diagnosis of bovine brucellosis. Some difficulty may be encountered with the milk-ring test because of the high fat content of buffalo milk, unless it is diluted (1:1) in 100 g/l saline solution.

*B. abortus* biovar 3 has been isolated from the enlarged testis of an African buffalo (*Syncerus caffer*).

(b) *Saigas*

In the south-eastern USSR, saigas (*Saiga* spp.) are evidently infected from sheep as a result of contacts on pastures or near watering places. However, the high susceptibility of saigas to brucellosis, the prolonged excretion of *Brucella* by the infected animal, and the ecology of the animal all suggest that prolonged and independent circulation of *Brucella* can take place in saiga populations.

(c) *Yaks*

The yak (*Bos grunniens*), like cattle, is highly susceptible to *B. abortus* infection. In Mongolia, brucellosis in yaks follows a regular course, characterized by infection of the animals at an early age when they are kept at pasture to suckle their dams. Calves are kept separately for some time after weaning, but when they reach sexual maturity they again join the herds of adult yaks and are subject to mass infection. Many abort during their first pregnancy and sometimes also during their second. If the herd is self-contained, the infection begins to die down after a year, and in time some of the animals recover.

When young animals are added to the herd after another year, as is the usual practice, the infection again flares up and is accompanied by a large number of abortions, particularly amongst heifers.

Early in the course of infection, most infected animals can be detected by means of the agglutination test. As the disease becomes chronic, infection can more often be detected by the complement-fixation test.

The immunization of young calves with strain 19 vaccine reduces the incidence of brucellosis and the number of abortions.
5.6.5 Brucellosis in other Bovidae

*B. abortus* is endemic in some herds of North American bison (*Bison bison*), the biovars involved being the same as those found in cattle in the region.

*B. abortus* biovars 1 and 3 have been isolated from the chamois (*Rupicapra rupicapra*), in which clinical symptoms of orchitis have been observed.

*B. abortus* biovar 1 has been isolated from the placenta of a water buck (*Kobus ellipsiprymnus*). Brucella antibodies have been detected in sera of other African Bovidae, such as eland, impalas, gazelles, topi, and wildebeests, but isolation of the organism has not been reported. Among these Bovidae, the Serengeti wildebeests have the highest incidence of reactors, about 10%.

On a herd basis, the diagnosis can be made from a composite of the serological test results on the whole herd or a representative portion thereof, and from observations for clinical signs of the disease.

5.7 Wild animal reservoirs and localization in nature

There are two principal epidemiological situations in which *Brucella* infection may be prevalent in wildlife.

The first situation involves various species of wild and domestic animals, birds, insects, and ticks to which the infection is transmitted from the principal carriers of *Brucella* (farm animals), disappearing again after eradication of the foci of infection among the latter. As a rule, strains of *Brucella* isolated from the members of this group are identical with strains distributed among the principal carriers in the given neighbourhood. Examples of this group include dogs, coyotes, opossums, raccoons, rats, and fur-bearing animals fed meat that has not been properly cooked. Some of these animals, especially dogs, are capable of transmitting infection to other animal species. Infected fur-bearing animals may also be a danger to man.

The second situation involves a number of animal species among which brucellosis exists independently, e.g., hares infected with *B. suis* biovar 2, wild reindeer, bison, saigas, feral swine, and certain species of rodent. Members of this group constitute a source of danger to man when hunted for food but are capable of transmitting the infection to domestic animals only when direct contact occurs, as is sometimes the case with feral swine and hares. Rodents may
harbour a variety of strains of *Brucella*, some of which have so far not been classified.

5.8 Brucellosis in man

Sheep, goats, cattle, water buffalo, and swine are common sources of human infection. It can also be acquired from reindeer, caribou, camels, yaks, and dogs. Transmission from secondary hosts, for example, mink and fox reared on fur farms, has also been described.

The modes of infection are ingestion, direct contact, inhalation, and accidental inoculation. The likeliest source of infection by ingestion is milk or its derivatives, especially cream and in many countries fresh cheese, which may be the cheapest and most readily available source of protein. Raw meat containing remnants of lymphatic tissue and blood from infected carcasses can also contain viable organisms. Gastric hydrochloric acid gives some protection, so that patients with achlorhydria or under treatment with antacids are more at risk.

Contact with animals or their products is a cause of infection in veterinarians, abattoir workers, farmers, and others who work with animals and their products.

The incidence of human brucellosis can sometimes be related to climatic conditions, if animals are brought close to, or within, the home environment for protection. In man, *Brucella* organisms can enter through any small skin abrasion but there is added risk for workers using saws, cleavers, and other sharp implements. Agricultural engineers may be infected while repairing contaminated farm machinery and tractors. Manure, soil, and pastures can be a source of viable organisms for several months after contamination. Infection readily follows inhalation or conjunctival contamination. Laboratory workers are at risk from direct contact, inhalation, and accidental inoculation. In veterinary surgeons, a severe systemic or local reaction can follow accidental self-inoculation with live or dead *Brucella* vaccines.

Children are susceptible to *Brucella* infection and, as in the case of adults, are commonly infected by consuming raw milk or fresh cheese or by direct contact with animals.

In rural areas the incidence of brucellosis in man reflects the incidence of animal disease, which is greater during the season of parturition when there is the added risk of exposure to contaminated products of conception.
*Brucella* organisms in milk can be killed by boiling or pasteurization. Fewer organisms are present in milk allowed to sour for several days and the numbers present in cheese gradually decrease during maturation, although it may be some months before hard, fermented cheeses are safe. Butter from well soured as opposed to fresh milk is an unlikely source of infection.

The transmission of *Brucella* infection to man and its prevalence in different parts of the world depends upon local food habits, methods of processing milk for cream, butter, and cheese, social customs, types of animal husbandry, species of *Brucella* prevalent in the region, climatic conditions, and standards of personal and environmental hygiene.

Environmental sanitation, which is particularly important in preventing airborne and contact infection, requires that the surroundings be kept as free as possible of contamination.

6. **DIAGNOSIS AND TREATMENT OF HUMAN BRUCELLOSIS**

6.1 Clinical aspects

*B. melitensis* and *B. suis* often give rise to a more severe form of infection than does *B. abortus*. However, *B. abortus* infection can also be severe and life-threatening, especially when resistance is low owing to pre-existing disease or malnutrition. The few reported cases of *B. canis* infection, mostly in laboratory workers, dog handlers, and dog owners, have been brief and uncomplicated.

6.1.1 The acute form

The incubation period may be as short as 1–3 weeks, although after long-standing exposure, as in occupational brucellosis, it usually cannot be determined. The illness may be mild and self-limiting or severe and prolonged with toxaemia.

The symptoms include lassitude, headache, and muscular or articular pain. Drenching sweats, especially at night, are characteristic. They are preceded or accompanied by shaking chills and, when severe, may be followed by extreme prostration. Splenomegaly is usual, the liver may be palpable, but significant lymphadenopathy is an inconstant sign. An evening rise in temperature is observed.
In an illness of average severity, natural recovery usually occurs in 1–3 months, although lassitude may persist for very much longer. In prolonged illness, the temperature chart may show an undulating pattern, hence the epithet "undulant fever".

In acute brucellosis, death may occur as the result of extreme toxæmia, thrombopenia, endocarditis, or another of its more serious complications.

6.1.2 The chronic form

The illness is labelled "chronic" when it persists or recurs over a period of 6 months or more. The onset may be insidious or it may follow an acute attack. Symptoms are often attributed to recurring influenza and, as in acute brucellosis, the commonest are lassitude, headache, pain, and sweats. Anxiety and depression are common in long-standing, undiagnosed infection, especially when the patient is obliged to remain at work and has little opportunity to rest. Severe, suicidal depression, however, is rare. The only noteworthy abnormal sign is a palpable spleen found in a minority of patients.

6.1.3 Complications

Some patients present with complications, others develop complications while under surveillance with diagnosed acute or chronic disease.

The commonest complications are thrombophlebitis, epididymo-orchitis, spondylitis, and peripheral arthritis, especially of the hip, knee, and shoulder, but all systems can be involved.

6.1.4 So-called allergic Brucella dermatitis in veterinarians

Papular and pustular rashes on the arms of veterinary surgeons after obstetric procedures have previously been attributed to Brucella allergy, but transmissible follicular infection with other serious bovine pathogens, including Salmonella dublin, Salmonella typhimurium, and Listeria monocytogenes, must be carefully excluded before this diagnosis is made.
6.2 Diagnosis

Diagnosis depends on the interpretation of both clinical and laboratory findings. Moderate leukopenia is common in acute brucellosis. Thrombopenia or pancytopenia is rare.

6.2.1 Bacteriological tests

Attempts to isolate *B. abortus* from blood usually fail. In *B. melitensis* and *B. suis* infection, confirmation by blood culture is often achieved. For all species, culture of marrow aspirated from the iliac crest or sternum is more likely to be successful.

6.2.2 Serological tests

These tests indicate the specific antibody status of each patient. Four tests commonly used are: the standard, tube agglutination test; the standard, tube agglutination test with added 2-mercaptoethanol; the Coombs antihuman globulin test; and the complement-fixation test.

Most patients with acute brucellosis show positive reactions to all four tests. Usually, the tube agglutination test is the first to become positive. Rarely, the Coombs antiglobulin test and the complement-fixation test may become positive before the agglutination test. Exceptionally, all serological tests remain negative, even in patients with bacteriologically confirmed infection.

Circulating *Brucella* antibodies can also be demonstrated by the plate agglutination test with Rose Bengal stained antigen, by the passive haemagglutination test, by immunodiffusion, or by the indirect immunofluorescence test.

It should be emphasized that, in individuals repeatedly exposed to *Brucella* antigen—for example, veterinary surgeons—serological tests are often strongly positive regardless of symptoms. In occupational brucellosis, therefore, they are of limited value in diagnosis. In all serological tests, standard antigen suspensions should be used whenever available. Occasionally, false positive reactions to serological tests are produced by antibodies cross-reacting with *Francisella tularensis*, some *Escherichia coli* and *Salmonella* serogroups, *Yersinia enterocolitica* serotype 0:9, or *Vibrio cholerae* vaccine.

Tests that specifically detect *Brucella* antibodies of the IgG and IgM isotypes include radioimmunoassay and enzyme-linked
immunosorbent assay. These have proved useful in some patients and their value in clinical practice and in epidemiological surveys is at present under assessment.

6.2.3 Intradermal test

As a rule, this test using conventional antigen preparations gives no information that cannot be obtained from serological tests; by stimulating an antibody response or reinforcing an established antibody response it is often misleading. In certain countries, however, reliance is placed on the intradermal test during clinical surveillance and epidemiological surveys. Information on brucellosis is given in Annex 4.

6.2.4 Leucocyte lysis test

Adding Brucella antigen in vitro to a suspension of leucocytes from sensitized patients results in lysis. It has been shown that, by using a protein extract, brucellosis can be differentiated from yersiniosis.

6.3 Treatment

Severe acute brucellosis is an indication for hospital admission, when circumstances allow, but patients with milder forms of infection can be treated at home. It is a popular misconception that the disease is incurable; consequently, patients and their families must be firmly reassured that recovery is the rule. When malnutrition is suspected, attention must also be given to protein, energy, and vitamin requirements in order to facilitate recovery.

6.3.1 Antibiotic treatment for acute brucellosis

Previously, oral tetracycline was given every 6 hours for 6 weeks in 500-mg doses, together with intramuscular streptomycin in a dosage of 1.0 g daily for the first 3 weeks. This regimen has now been superseded as the treatment of choice in acute brucellosis. Better results are achieved when rifampicin in a dosage of 600–900 mg daily is combined with doxycycline at 200 mg daily. Both drugs are given in the morning as a single dose and relapse is unusual after a course of treatment continued for at least 6 weeks. Patients are more likely
to comply with one daily doxycycline dose than with tetracycline four times daily. A claim that doxycycline is more apt to cause photosensitization is unproven.

Co-trimoxazole is also effective, but when this preparation is prescribed alone, relapse is common, even after a prolonged course of treatment. Co-trimoxazole (trimethoprim, 160 mg plus sulfamethoxazole, 800 mg) is given 3 times daily for 2 weeks and subsequently twice daily. Undesirable side-effects are an intensely irritating rash and dyspepsia. Blood dyscrasias due to co-trimoxazole are rare.

Brucellosis can cause fetal death at any stage of pregnancy, whether maternal infection is mild or severe. Therefore, treatment must not be withheld. The drug of choice is rifampicin. Co-trimoxazole or tetracycline should be given only if rifampicin is unavailable, streptomycin being contraindicated.

Other therapeutic agents are rarely needed in the management of patients with brucellosis. A Herxheimer reaction at the start of antibiotic treatment may warrant intravenous cortisol. Together with other measures, cortisol may be life-saving when thrombopenia is severe or there is evidence of disseminated intravascular coagulation. Significant thrombophlebitis and pulmonary embolism are indications for anticoagulants. Certain complications demand urgent surgical intervention, e.g., spondylitis, when subdural suppurative threatens spinal compression. Endocarditis, if untreated, is invariably fatal and medical treatment alone seldom gives a satisfactory outcome. Destruction of the aortic or mitral valve is sometimes associated with subendocardial abscess formation, and perforation of the heart has been described. Transfer to a cardiothoracic unit should be arranged in order to anticipate fulminating heart failure; cure can then be achieved by replacing the natural or prosthetic valve under continued antibiotic cover.

*B. suis* infection can present acutely as intra-abdominal suppuration. Also, when radiographs show left hypochondrial calcification long after assumed recovery from *B. suis* infection, splenectomy is often advised.

### 6.3.2 Chronic brucellosis

Several courses of treatment may be needed before cure is achieved. When the response even to prolonged treatment is poor,
a chronic focus of infection must be sought—especially in *B. melitensis* and *B. suis* infection. Patients are often depressed and in need of constant reassurance, especially when the illness is of long duration. The management of those who continue to complain of symptoms despite prolonged antibiotic treatment and in whom a complicating lesion and other organic diseases have been excluded can prove difficult. The demonstration of circulating antibodies is not proof of a persisting endogenous focus of infection. The role of hypersensitivity in the causation of symptoms has been invoked as an explanation in some cases, but the Committee found this question to be unresolved. Vaccines and other agents that can modify the immune response are in use in some parts of the world.

6.3.3 *Treatment after accidental exposure*

Following accidental contamination with *Brucella* organisms, including live vaccines, antibiotic treatment should be started immediately, its duration depending on the degree of exposure and the results of serial antibody tests.

7. **DIAGNOSIS OF ANIMAL BRUCELLOSIS**

All persons handling *Brucella*-contaminated materials should be warned of the dangers of self-infection. A description of the safety precautions required will be found in Annex 1.

7.1 **Bovine brucellosis**

7.1.1 *Demonstration by microscopic examination*

This is a useful procedure for the examination of abortion material. Smears of placental cotyledon, fetal stomach contents, or uterine exudate should be heat-fixed and stained by a differential method such as Köster’s, Macchiavello’s, or Stamp’s modification of the Ziehl-Neelsen stain. *Brucella* organisms resist decolorization by weak acids and a diagnosis can often be based solely on microscopic examination. The method will not differentiate between *Brucella* and *Coxiella burnetii* or *Chlamydia*, however. The fluorescent antibody method has been advocated to increase specificity. In
practice, interpretation of the results of this test is difficult and it offers few advantages over the modified acid-fast stains.

7.1.2 Isolation of Brucella

(a) From milk and other liquids. Udder secretions are good sources for isolating Brucella. Solid and biphasic selective media are used (see section 2.5.2). Secretion is collected from each teat (about 20 ml), centrifuged at 3000 g for 10 minutes, and a mixture of cream and sediment is cultured. Samples that are likely to be heavily contaminated can be inoculated into guinea-pigs.

Other liquids, such as fetal stomach contents, semen, and fluid from hygromas, etc., may be cultured directly or after centrifugation where appropriate.

(b) From tissues. The specimen is sectioned with sterile instruments and macerated with a stomacher or tissue grinder with a small amount of diluent before being streaked on the surface of agar medium or added to biphasic medium. If apparatus for macerating the tissues is not available, they may be minced with scissors. If tissues cannot be obtained aseptically, the surface should be seared in a flame before being processed for culture. Culture in selective biphasic medium will improve the isolation rate from lightly infected samples. Fetal membranes are often grossly contaminated and a fragment may be washed in successive quantities of sterile saline before being processed for culture. The wash liquid must be carefully disposed of.

(c) From genital discharges. A vaginal swab taken after parturition or abortion is an excellent source for the recovery of Brucella in cows, sheep, and goats; the use of a solid selective medium is recommended.

(d) Guinea-pig inoculation. This technique has value for the isolation of Brucella when specimens are derived from potentially contaminated sources, such as milk, cheese, semen, or genital discharges. Direct culture techniques are superior for uncontaminated materials. Solid specimens need to be homogenized before injection. Inoculations should be made subcutaneously and two guinea-pigs used per sample. With milk samples, all the cream-sediment mixture obtained from a 20-ml sample should be used, one
half being injected into each guinea-pig. For heavily contaminated material not suitable for parenteral injection, infection of guinea-pigs can be achieved by oral application.

One guinea-pig is killed 3 weeks, and the second 6 weeks, after inoculation. A blood sample for serological examination is taken at the time of killing; macroscopic lesions are recorded and the spleen, is cultured. Either a positive serological result or the isolation of *Brucella* warrants a diagnosis of brucellosis.

*(e) Mouse inoculation.* For this technique, specimens, such as tissue, placenta, or milk, should be washed in sterile saline solution and ground to make a homogeneous suspension. The inoculum should be injected intravenously (0.1 ml), or subcutaneously if the material is heavily contaminated, into 2–4 mice. The mice are killed 7 days after inoculation and the spleen and liver removed for culture on nutrient medium.

### 7.1.3 Serological tests

Unequivocal diagnosis of *Brucella* infections can be made only by isolation and identification of *Brucella* using some of the culture methods just described. There are, however, many situations where bacteriological diagnosis is not practicable and diagnosis has to be based on serological methods, e.g., in surveys or eradication programmes.

### 7.1.4 Herd surveillance tests

The milk-ring test is the most practical and economical method for locating infected dairy herds and for surveillance of brucellosis-free herds. If performed on pooled milk 3 or 4 times a year on each herd, it will detect the majority of infected herds. Modifications to the original procedure are now available to increase the sensitivity of the tests for use on large herds. It can also be used to detect herd infection in nomadic or seminomadic herds. Herds with a positive milk-ring test can then be examined by individual serum or milk tests to identify the infected individuals. Milk from individual animals can be serially diluted in *Brucella*-free milk to determine the end-titre of the milk-ring reaction. Titres above 1:10 are suggestive of infection. Enzyme immunoassay has been suggested as a more sensitive and specific alternative for detecting *Brucella* antibody in milk but requires further evaluation.

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Surveys can be made on serum samples taken from animals at slaughter, when assembled at markets, or for periodic veterinary attention such as dipping or immunization programmes. Rapid tests make it possible to identify suspect animals so that they can be traced to the area or herd of origin.

Information on the brucillin intradermal test is given in Annex 4.

7.1.5 Tests for individual diagnosis

(a) Tube agglutination test. The agglutination test performed by the tube method is the most widely used procedure for the measurement of anti-Brucella antibodies for purposes of international trade. A B. abortus antigen is used and the results expressed in international units (I.U.). The procedures vary but are usually the United States Department of Agriculture (USDA) or European methods.¹

The tube test measures the total quantity of agglutinating antibodies and has the disadvantage of reacting to post-immunization agglutinins and sometimes to those caused by heterospecific antigens. Many studies have reported that other tests are more sensitive and specific for diagnosing infected cattle. A modification of this test has involved the introduction of EDTA, which reduces non-specific reactions as discussed in section 3.1.1.5.

(b) Plate agglutination test. This is a modification of the USDA tube test adapted for detection of rapid agglutination on a glass plate. The techniques and interpretations have been described by Alton et al.² It has the advantages of being simple and more rapid than the tube agglutination test but is affected by environmental conditions. The sensitivity and specificity are similar to those of the tube test and other tests are often superior. The use of the plate agglutination test should be discouraged, except when serum quality is inferior and its use is essential.

(c) Buffered Brucella antigen tests. These are simple spot agglutination tests using stained antigens and buffered to a low pH, usually 3.65 or 4.0. The card and Rose Bengal tests are best known; the antigen is stained with Rose Bengal. More recently the buffered

¹ See footnote 2 on page 17.
² See footnote 2 on page 17.
plate agglutination test has been introduced in North America; here the antigen is stained with brilliant green and gentian violet.

The Rose Bengal test has found wide application as a screening test for individual diagnosis in herds of cattle. It is generally considered to be oversensitive, especially in cattle immunized with strain 19. For this reason sera positive in the Rose Bengal test are usually retested by a definitive test, such as the complement-fixation test. The Rose Bengal test has also been used in surveys and surveillance. The buffered plate agglutination test has a similar role to the Rose Bengal test.

(d) The complement-fixation test. The complement-fixation test is recognized as the most reliable diagnostic test now in routine use for individual animals. It is relatively insensitive to antibody resulting from strain 19 immunization.

The work load resulting from the technical complexity of the complement-fixation test can be greatly reduced by using it only as a definitive test on samples that have been found positive in a preliminary screening test, usually one of the buffered Brucella antigen tests.

Either warm or cold fixation may be used for the reaction between test serum, antigen, and complement. In warm fixation the mixture is held at 37 °C for half an hour. In cold fixation the mixture is held at approximately 4 °C for 14–18 hours. A number of factors affect the choice of method:

1. Anticomplementary activity in serum samples of poor quality is more evident with cold fixation.
2. Fixation at 37°C increases the frequency and intensity of prozones and several dilutions must be tested for each sample.
3. Fixation in the cold produces higher titres in positive sera.
4. With cold (overnight) fixation the working week is reduced by one day.

When the complement-fixation test has been used as the principal definitive diagnostic test in eradication campaigns, warm fixation has generally been used. Bovine serum is usually inactivated at 58 °C for 30 minutes. Higher temperatures reduce anticomplementary activity but also reduce the complement-fixing activity of IgM.

The classical complement-fixation procedure in tubes with a total volume of 1 or 2 ml is practical only for testing a few individual samples, e.g., in the diagnosis of human brucellosis. In veterinary
practice, the large numbers of samples involved in eradication programmes necessitate some degree of automation, which may be based either on continuous flow or micro-methods.

Micro-methods are generally preferred. They represent the classical technique reduced to small volume and allow various degrees of automation to be applied, such as mechanical dilution of serum and addition of reagents. The continuous flow method, on the other hand, is entirely automatic.

**Standardization of the complement-fixation test.** A unitage system has been recommended based on the second International Standard for Anti-Brucella abortus Serum (ISABS). The second ISABS is taken to contain 1000 international complement-fixation test units (ICFTU) and if this serum is tested in a given method and gives a titre of say 500, then the factor for an unknown serum tested by that method can be found from the formula:

\[
\frac{1000}{500} \times \text{titre of test serum} = \text{no. of ICFTU of antibody in the test serum}
\]

The European Economic Community (EEC) has adopted this unit as the EEC Unit. The ISABS contains only IgG; national standard sera should also depend on this isotype for their specific complement-fixing activity.

Difficulties in standardization arise because different techniques selectively favour complement-fixation by different immunoglobulin isotypes. It is recommended that any country using the complement-fixation test on a national scale should obtain agreement between the different laboratories performing the test by a standard method. This allows the same level of sensitivity to be obtained. The periodic distribution of a collection of sera can then be used to monitor the level of agreement between laboratories.

**Interpretation of test results.** The fifth report of the Joint FAO/WHO Expert Committee on Brucellosis¹ deals at length with the wide differences of opinion that exist as to the titre that ought to be taken as positive. Disagreements persist to this day, e.g., at one extreme a titre of 2/2 is judged to be indefinite and 2/4 as positive, whereas at the other extreme a titre of 1:40 is regarded as the minimum positive. This situation reflects to a considerable extent the different levels of sensitivity of the techniques used.

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7.1.6 Supplementary tests

(a) Ethacridine agglutination test. In the USA, the ethacridine test (see p. 29) is often performed on sera that are positive in screening procedures, such as the buffered antigen tests. Equal quantities of serum and ethacridine solution are mixed in a tube. A precipitate is formed after which charcoal is added and the tube is centrifuged. The supernatant is then tested with ethacridine plate test antigen. Different dilutions are tested on a glass plate. The principle of the test is to eliminate the reactions caused by IgG antibodies that persist following strain 19 immunization.¹

The ethacridine agglutination test has been extensively evaluated and can often replace more difficult procedures, such as the complement-fixation test.

(b) Enzyme immunoassay. The enzyme immunoassay has been extensively investigated as a definitive test for the detection of antibody to Brucella in bovine sera. The test shows great promise of increased sensitivity and specificity. Both whole-cell and purified lipopolysaccharide antigens have been used and a variety of antiglobulin conjugates and substrates. A great deal of work is still required on standardization of reagents.

Enzyme immunoassay is a valuable research tool that can employ purified Brucella antigens and specific and sensitive anti-immunoglobulin reagents, thus permitting the measurement of the immunoglobulin subclass of Brucella antibody to defined antigens. These methods promise to provide a basis for the development of a new generation of simple tests that can be conducted with inexpensive equipment and reagents, for example as dot immunoassays using antigen adsorbed to paper.

The Committee noted that Australia and Canada have adopted enzyme immunoassays for use in their national brucellosis programmes.

Various other serological tests have been used experimentally without so far being accepted for routine use. These tests include the indirect haemolysis test, a sensitive and specific procedure that gives little reaction with antibody resulting from strain 19 immunization. The haemolysis-in-gel test is a similar procedure in gel, but is

¹ The procedures for preparation of reagents and performance of the test may be obtained from: United States Department of Agriculture, P.O. Box 70, Ames, IA 50011, USA.
considerably more sensitive to vaccinal antibody than the indirect haemolysis test. The radial immunodiffusion test is a simple gel-diffusion test utilizing a polysaccharide (Poly B) hapten. It is especially insensitive to vaccinal antibody, and detects a high proportion of cattle excreting B. abortus in the milk.

(c) The anamnestic test. This test has been developed to detect latent infection in cattle in eradication programmes. It is based on the principle that it is possible to distinguish serologically between a primary and a secondary response to the injection of a Brucella antigen. In practice, the cattle are tested by the routine serological method, usually the complement-fixation test, and reactors are removed. The negative cattle are immunized, usually with B. abortus 45/20 vaccine. Approximately 6 weeks later they are again tested and any further reactors removed. The anamnestic test cannot be used on cattle previously immunized with strain 19. This test has been used with success to establish brucellosis-free groups of weaned heifers in range cattle that cannot be mustered regularly for serological testing.

It is necessary to ensure that the batch of vaccine to be used does not produce positive complement-fixation reactions in negative animals.

(d) Monitoring serological reactions by culture. The carcasses of reacting cattle slaughtered during an eradication campaign are often available for the collection of specimens, chiefly lymph nodes, but sometimes udders and uteri, for bacteriological examination. Such examinations are often of value in resolving the status of problem herds and for evaluating serological tests. The identification of isolates to biovar level can also provide epidemiologically useful information.

7.1.7 Significance of serological cross-reactions

Serological cross-reactions have been demonstrated between smooth Brucella species and Escherichia coli serotypes 0:116 and 0:157, Salmonella serotypes of Kaufmann-White group N (0:30 antigen), Pseudomonas maltophilia, and Yersinia enterocolitica serotype 0:9. Parenteral or oral exposure of cattle or swine to these organisms can provoke diagnostically significant titres of antibodies cross-reacting with Brucella in agglutination, complement-fixation,
indirect fluorescence, precipitation, and antiglobulin tests. Differentiation of cross-reacting antibodies can be difficult, but immunodiffusion with internal antigens, immunoelectrophoresis, and competitive antibody binding tests (enzyme immunoassay, radioimmunoassay) are useful.

In relation to the control of brucellosis by eradication procedures, serological cross-reactions produced by other organisms tend to be of little significance until the prevalence of the disease has fallen to a very low level. At this stage, it becomes much more important to identify correctly the status of animals reacting to serological tests for brucellosis. The incorrect attribution of such reactions to factors other than *Brucella* infection is likely to result in herd breakdowns and failure to control the disease. On the other hand, the misinterpretation of cross-reactions as evidence of brucellosis results in the imposition of unnecessary restrictions and waste of resources. Furthermore, such reactions are likely to occur for an indefinite period, even in the absence of brucellosis, and may unnecessarily delay the recognition of an area as brucellosis-free. Similar considerations apply to the sporadic occurrence of cross-reactions in a population already established as brucellosis-free. Thus, it is clearly desirable to develop reliable methods for the differentiation of cross-reacting antibodies from those resulting from brucellosis. However, in applying these, the possibility of concurrent infection by *Brucella* and cross-reacting organisms must always be borne in mind. The emphasis should always be placed on establishing the absence of brucellosis rather than the presence of cross-reactions.

7.2 Sheep and goats (*B. melitensis* infection)

The cultural and microscopical methods described for cattle are equally applicable to the diagnosis of *B. melitensis* infection in sheep and goats.

7.2.1 Detecting infected herds

The milk-ring test is not considered effective as a herd test.

A more promising method for detecting infected herds is the allergic test. It requires neither individual animal identification nor bleeding, involves only a minimum of handling of the animals, and the result is demonstrable to the owner. However, the use of this test does open up the possibility of positive animals being hidden. The
Committee considered that the allergic test has great merit as a screening procedure, but did not recommend it for diagnosis in individual animals. More information is given in Annex 4.

7.2.2 Diagnosis in individual animals

(a) The Rose Bengal plate test. This is a simple procedure that would be extremely useful if it could be proved to be reliable for use in sheep and goats, even as a screening test that needed to be supported by a definitive test. The published information on its efficiency is conflicting.

In one study, the test was found to be more efficient than either the serum agglutination or the complement-fixation test in detecting infected sheep, but only when the cell concentration of the Rose Bengal antigen had been reduced to 50 g/litre. Another group of investigators found the Rose Bengal test to be less reliable than the complement-fixation test for detecting brucellosis in 942 sheep. However, eradication was achieved in a newly, lightly infected flock of 1000 sheep using the Rose Bengal test for diagnostic purposes.

The potential usefulness of the Rose Bengal test in developing methods for the eradication of brucellosis in sheep and goats warrants a comprehensive evaluation of its merit as either a screening test or a definitive test. The Committee recommended that WHO seek one or more laboratories willing to undertake such an evaluation. It may be that the cell concentration of the antigen (80 g/l of buffered saline solution) that has proved so useful in cattle needs modifying to produce optimum results in sheep and goats. The desirability of using a B. melitensis antigen in some areas may be worthy of consideration. The reason for the persistence of a positive reaction in immunized animals needs further clarification.

(b) The complement-fixation test. There is no doubt that, of the established procedures, this is the most effective test for diagnosing brucellosis in individual sheep and goats. However, as with all serological tests, negative results are occasionally found in infected animals. The same alternative technical choices are available as when the complement-fixation test is used in other species. Many laboratories inactivate serum at 62°C since anticomplementary reactions may be more evident with sheep serum than, for example, with cattle serum.
(c) The serum agglutination test. Even when the test is done in
50 g/l saline, which improves its efficiency, the serum agglutination
test is both less sensitive and less specific than the complement-
fixation test in the diagnosis of brucellosis in sheep and goats,
especially in immunized animals.

(d) Enzyme immunoassay. There appears to have been little
evaluation of enzyme immunoassay in the diagnosis of brucellosis
in small ruminants. The Committee considered that its potential
merits investigation in depth.

7.3 Sheep (B. ovis infection)

7.3.1 Diagnosis

Diagnosis depends on a combination of clinical examination,
bacteriological examination, and serology. Clinically the disease is
diagnosed by palpation of the scrotum.

Semen may be examined by staining with one of the methods
described in section 7.1.1. Fluorescent antibody has also been used
for demonstrating B. ovis in smears. Semen may be cultured on B.
ovid selective medium. It should be remembered that B. ovis requires
50–100 ml of serum per litre of medium and an added 5–10% CO₂
in the atmosphere for growth (see section 2.5.1 (a)), and that the
organism occurs only in the rough phase.

Materials that can be cultured from ewes include the vaginal
discharges, milk, fetal membranes, and fetus. B. ovis can sometimes
be recovered for up to 10 days in the vaginal discharges of ewes that
have aborted or delivered live lambs prematurely. It can also usually
be isolated from udder secretions for up to two weeks. Aborted
fetuses are not always infected. The best materials for culture are the
abdominal contents, lungs, spleen, and lymph nodes.

Infertility in sheep has many causes other than infection with B.
ovid. In making a differential diagnosis, other organisms that must
also be considered are Actinobacillus seminis, B. abortus, B.
melitensis, Campylobacter, Corynebacteria, Chlamydia, Listeria, and
various virus infections.

As with other forms of brucellosis, serological tests are widely
used in diagnosis. Complement-fixation, gel diffusion, agglutination,
passive haemagglutination, and more recently antiglobulin tests and
enzyme immunoassay have been used.
The complement-fixation test is considered a specific and sensitive test for the detection of specific antibody to *B. ovis*, although a few rams will be found that are positive on semen culture and negative serologically. A polysaccharide antigen prepared by heating saline suspension of *B. ovis* is used; it is available commercially. There is evidence to show that fixation in the cold is more effective than warm fixation.

It has been claimed that the gel-diffusion test, using the same antigen, gives similar results to the complement-fixation test but is much simpler. In practice, its sensitivity is lower than that of the complement-fixation test and it is best used as a flock test.

The enzyme immunoassay shows promise of proving to be more sensitive and specific than either the complement-fixation test or gel-diffusion. The antiglobulin test has also shown greater sensitivity than the complement-fixation test in experimentally infected rams.

### 7.4 Swine

Serological diagnosis presents some special problems in swine. Some non-infected swine exhibit agglutinating antibody, usually ascribed to heterospecific IgM, and infected swine may be negative serologically.

The buffered *Brucella* antigen tests (card and Rose Bengal tests) are considered the most practical serological procedures for use in the diagnosis of brucellosis in swine. The allergic skin test is also widely used in many countries and shows high specificity.

Results obtained with the tube agglutination test have been variable and the test has largely been superseded by the buffered *Brucella* antigen tests, which appear to give similar results to the complement-fixation test.

An enzyme immunoassay has been adapted for the diagnosis of brucellosis in individual swine and also for the screening of large numbers of breeding and slaughter swine. This test shows promise of providing a more sensitive and specific serological method for the diagnosis of swine brucellosis.

### 7.5 Brucellosis in dogs

Clinical signs are not adequate for the diagnosis of canine brucellosis. Infections in dogs can be caused by *B. abortus*, *B. melitensis*, or *B. suis*, as well as by *B. canis*. The methods for
diagnosing infection caused by the smooth species are as described for other animals. Specific methods are indicated for *B. canis* infection, however.

The only certain method of diagnosis is by isolation of the organism. Cultures of blood should be performed in association with serological procedures. However, infected dogs usually remain serologically positive for several months after the bacteremia has ceased. As dogs recover, often after a 1–4 year period of bacteremia, titres decline over a period of 4–5 months and become negative to standard tests.

Four serological tests employing non-smooth antigen preparations are available for the diagnosis of canine brucellosis: the rapid slide agglutination test, the tube agglutination test, the modified mercaptoethanol tube agglutination test, and the immunodiffusion test. A titre of 1:200 is generally considered presumptive evidence of infection in both slide and tube tests, but false-positive and false-negative reactions occur. The slide agglutination test was developed to provide presumptive diagnostic information in a short period of time. The agglutination test has been modified to include an additional step involving a brief reaction of undiluted sera with 2-mercaptoethanol (0.2 mol/l) before adding the test antigen (a Rose Bengal-stained standardized suspension of *B. ovis*). The modified test procedure does not sacrifice sensitivity, but specificity is greatly improved. Presumptive results, if positive, should always be followed by more specific serological procedures and by bacteriological examinations.

The tube agglutination test and its modification are widely used procedures. The immunodiffusion test using *B. canis* or *B. ovis* antigen has also been employed and found to detect antibodies persisting in infected dog sera. It is stressed that antigens prepared from non-smooth *Brucella* strains must be used in these tests. Alternative procedures for non-smooth antigen preparation are listed in Annex 5.
8. PREVENTION AND CONTROL OF BRUCELLOSIS
IN ANIMALS

8.1 Control in cattle

8.1.1 Elimination by test-and-slaughter

Successful campaigns have been carried out in several countries. Eradication has been successful in countries where the farms are small and the cattle under close control. Under these conditions, the main procedures have become more or less standardized, as follows:

1. All cattle eligible for testing, i.e., females of 1 year old and above and bulls, are permanently identified, usually by an ear tag.
2. All eligible cattle are bled and the serum is tested serologically. Any reactors found are removed and the herd retested after 30–60 days. Where no reactors are found, the herd is retested after 6 months and if no reactors are found on the second occasion this may be accepted as evidence that the herd is brucellosis-free. In certain circumstances, another retest after a further period of 6 months may be considered advisable before a herd can be certified as free from brucellosis.
3. The Rose Bengal test is recommended as a screen test, with positive samples being retested by the complement-fixation test (see section 7.1.5 for details).
4. Reactors should be removed from the herd as soon as possible and slaughtered.
5. Adequate compensation should be paid to farmers for any animals slaughtered.
6. The sale of female cattle over 1 year old from infected herds, i.e., those that have not yet reached a brucellosis-free status, should be prohibited unless they are to be sent direct for slaughter. The exhibition of such cattle at shows should also be prohibited.
7. Regulations to prevent the introduction of infection to brucellosis-free premises are also required.
8. When eradication is progressing favourably in a region, immunization should be stopped, since occasional persistent vaccinal reactors cause problems. The appropriate moment will depend on circumstances but, as a guide, it has been suggested that immunization with strain 19 vaccine should be stopped once the prevalence of infected animals falls to 0.2% or below.
9. Where the cattle have contact with sheep and goats infected with \textit{B. melitensis}, the latter will also need to be eliminated, since \textit{B. melitensis} is readily transmitted to cattle.

10. Problem herds, i.e., those continuing to have reactors after a number of herd tests, will inevitably be found from time to time. These should be recognized as quickly as possible and given special attention. This problem is more likely to arise in large herds subject to intensive husbandry. Additional serological tests, such as enzyme immunoassay, may be tried. In the absence of strain 19 immunization, the anamnestic test (see section 7.1.6 (c)) has been used successfully. In some circumstances, slaughter of the whole herd is advisable.

11. Adequate arrangements must be made to monitor the brucellosis status of free herds. With dairy cattle, this can be done by use of the milk-ring test. With beef cattle, in some cases examination of blood from animals slaughtered at abattoirs can be satisfactory. Alternatively, periodic herd tests can be arranged. Where practicable, reporting the occurrence of abortion should be made compulsory. In non-immunized brucellosis-free herds, the level of susceptibility of the cattle becomes very high and surveillance systems must be designed to detect recurrence of infection at the earliest possible moment. The frequency of surveillance tests can be reduced when a region or country has remained brucellosis-free for some years.

In extensive pastoral areas, and where herds are large, it may be impossible to apply the regimen for test-and-slaughter outlined above. Conditions of terrain and climate may prevent the mustering of cattle at regular intervals for testing.

In some parts of the world, to overcome such problems, a method developed in Australia and called the heifer-segregation plan may be applied. In this method, heifers are tested at weaning and reactors slaughtered. The negative heifers are retested using the anamnestic test with 45/20 vaccine. Any further reactors are slaughtered and those that pass the test are raised apart from untested cattle.\footnote{\textsc{Corner}, L. A. \textit{et al.}, An evaluation of the anamnestic test for brucellosis in cattle of the northern pastoral area. \textit{Australian veterinary journal}, 60: 1 (1983).}

8.1.2 Immunization

Mass immunization is, at present, the only way to reduce the rate of brucellosis infection in high-prevalence areas. Live attenuated
vaccines should be used where their full potency can be assured by use of a cold-chain. Inactivated vaccines are more stable but generally of lower potency, so that inoculations are required more frequently. The costs of the cold-chain needed for live vaccine administration may be much lower than the vaccine delivery costs for repeated administration of inactivated vaccine over the life-span of the animals.

*B. abortus* strain 19 vaccine has been widely used to reduce the incidence of bovine brucellosis. It cannot be expected to eradicate the disease from a herd but can be expected to provide some protection, especially in reducing the number of abortions and limiting the spread of infection. When calfhood immunization is applied systematically in a region, and a coverage of 80% or more achieved, a gradual reduction in the incidence to a low level can be expected. Usually, the immunization is restricted to calves between the ages of 3 and 6 months. In some cases, the upper limit has been extended to 8 months. This age restriction results in a minimal antibody response, which has time to subside before the animal becomes old enough to need testing. However, a small proportion of persistent reactors is always found. The dose for this age group has been 50–120 × 10⁹ viable bacteria, but recently it has been demonstrated that a dose of 3–10 × 10⁹ viable bacteria in a 2-ml volume stimulates similar protection.

Circumstances arise where the immunization of adult cattle may be advisable, e.g., in some very large dairy herds where eradication by test-and-slaughter has failed, or where it is necessary to introduce cattle that have not been immunized as calves. Adult cattle can be immunized with a reduced dose of 1 × 10⁹ viable cells of strain 19 given subcutaneously. If pregnant cattle are immunized in this way there is a slight risk of abortion occurring and a few immunized cattle may excrete strain 19 organisms in the milk, but this does not cause a public health problem. Alternatively, strain 19 vaccine can be given conjunctivally in a dose of 5–10 × 10⁹ viable cells. In either case, serological testing can be resumed after 6 months, although a small proportion of persistent reactors will have been created (see section 4). Immunized adults should be permanently marked.

In the Republic of Ireland, *B. abortus* strain 45/20 vaccine has been used with success; being killed, and giving rise to only minimal antibody response, this vaccine can be given to cattle 9 months old or above, whether pregnant or not. The advantages of inactivated vaccines (higher stability, safety, as well as applicability in pregnant
cows) and their disadvantages are described in section 4.4 and section 4.5.

Reports from China show that the live vaccine strain 2 may also be suitable for the control of brucellosis in cattle.

8.2 Control in sheep and goats

8.2.1 Eradication by test-and-slaughter

On a national scale, eradication has so far been achieved only by identifying infected herds and slaughtering all animals in the herd. This procedure is normally only feasible where \( B. melitensis \) infection has been newly introduced into a previously non-infected region or where the prevalence is very low.

Where \( B. melitensis \) infection is endemic and widespread in a population of small ruminants, control by immunization is recommended, at least as a preliminary step in most situations.

Only a few countries in which \( B. melitensis \) infection is present have the resources needed for eradication. The Committee considered that, when countries possess such resources, the technical methods available will, under most circumstances, prove adequate to achieve this objective. The eradication of \( B. melitensis \) from goat herds by test-and-slaughter has been shown to be feasible in a trial in 50 herds in Malta using the complement-fixation test. Eradication on a larger scale has been attempted in Cyprus using the allergic test as a herd test, and the complement-fixation test as the definitive test for individual animals. The feasibility of eradication by test-and-slaughter in small ruminants appears to depend largely on the conditions under which they are kept. The chances of success are greatest in small isolated flocks kept under close control and least in large flocks, especially when they intermingle with other flocks and/or are herded in large numbers in enclosures at night. The risks involved in the system of transhumance also need special attention.

Little experience is available to assist in planning a campaign for the eradication of \( B. melitensis \) infection in sheep and goats. The allergic test appears to be effective in detecting infected herds, but the milk-ring test is not reliable with the milk of sheep and goats. Otherwise, the epidemiology and the planning requirements are similar to those for cattle. The Committee strongly recommends that any country planning the eradication of \( B. melitensis \) infection from
sheep and goat herds should carry out a pilot project before finalizing plans.

The measures outlined in section 8.1 for the eradication of brucellosis in cattle can be applied to the eradication of *B. melitensis* infection in sheep and goats, with the following exceptions:

1. In the absence of an effective milk-ring test, infected and uninfected flocks can be identified by the use of the allergic test or serological test.

2. All individual animals in the infected flock should be bled in the same way as cattle. In non-immunized flocks the Rose Bengal test may be used to identify infected individuals.

3. In flocks immunized with Rev. 1 vaccine, sheep and goats can be tested 1 year or more after immunization provided that the complement-fixation test is used.

4. Tests on flocks should include all animals that the flock is likely to come in contact with, e.g., the whole population of sheep and goats in villages where communal grazing is practised.

5. Surveillance in areas that have been declared free from brucellosis can be achieved by carrying out periodic allergic palpebral tests or serological tests on all sheep and goats in the area. Otherwise, it will be necessary to bleed flocks at intervals and test them serologically. Compulsory reporting of abortion is very useful.

8.2.2 *Immunization of sheep and goats*

In most countries where *B. melitensis* infection is endemic, immunization will be the most appropriate first step, either as a preliminary to eradication or as the sole measure.

Amongst live vaccines, strain Rev. 1 has been most thoroughly tried and tested in field use and its characteristics are fully described in section 4.2. The characteristics of this vaccine when used to immunize sheep and goats against *B. melitensis* infection are similar to those of strain 19 used in cattle. To avoid stimulating serological reactions that will interfere with subsequent diagnosis, immunization is usually restricted to lambs and kids aged between 3 and 8 months. This restriction results in immunization having little immediate impact on the prevalence of the disease. To overcome this difficulty, in the first year of an immunization programme adult sheep and goats may be immunized with a reduced dose (5–10 × 10⁴ viable cells) of Rev. 1. The use of Rev. 1 in adults is best confined to the season when few if any of the animals are pregnant. Lambs
and kids immunized with Rev. 1 can, when adult, be tested serologically for eradication purposes, provided that the complement-fixation test is used. In view of the great variation in the susceptibility of different breeds of sheep to *B. melitensis* infection, immunization of adult sheep with Rev. 1 should be preceded by a trial immunization of a representative sample of animals to prove its suitability.

In China, a living attenuated vaccine using *B. melitensis* strain M5, given either by aerosol or in the animal’s drinking water, has been used extensively for the immunization of sheep and goats against *B. melitensis* infection. Also in China, a live attenuated strain of *B. suis* (strain 2) has been used for this purpose.

The only non-living vaccine to be used routinely in the field is the H38 vaccine, which consists of a killed virulent strain of *B. melitensis* in an oily adjuvant. This vaccine produces a good immunity but gives rise to a prolonged serological response and cannot be used in situations where immunized animals might later need to be subjected to serological diagnosis.

8.2.3 *Control and elimination of B. ovis infection in sheep*

Control of *B. ovis* infection depends upon effective action to deal with the disease in rams. Under normal conditions ewes are not capable of maintaining the disease in a flock in the absence of infected rams. Control in rams depends on immunization and/or test-and-slaughter, though certain management procedures facilitate control.

*B. melitensis* strain Rev. 1 vaccine is effective in preventing *B. ovis* infection in rams when administered at weaning age. One advantage is that the smooth-antigen specific antibody induced by Rev. 1 immunization can be distinguished from the rough-antigen specific antibody of *B. ovis* infection.

Eradication of *B. ovis* infection may be achieved by a combination of testing by the complement-fixation test and clinical examination, with the elimination of positive animals, aided where feasible by management practices such as isolation of immature rams and restricting the mating period to two months, which reduces the risk of mating a ram to a recently aborted ewe.
8.3 Control in swine

At present there is no recommendable vaccine for the prevention of swine brucellosis. Control depends, therefore, on eradication by slaughter or on general hygienic methods to limit spread.

In planning eradication it must be recognized that diagnosis of brucellosis in individual pigs is less satisfactory than in the other domesticated species. Swine brucellosis has been eradicated (e.g., in Denmark) by testing herds serologically and killing all the pigs in herds found infected. An eradication programme now in progress in the USA makes use of three alternative strategies: (1) Slaughter of the whole herd; this is by far the most successful and the most economical in the long run. (2) Slaughter of the adult swine and retention of the weaning pigs for breeding stock. This plan is designed to save valuable blood lines but it is not always successful and requires considerable isolation and testing facilities. (3) Removing serological reactors and retesting the herd as often as necessary; this plan is rarely successful where there is active infection, but is the plan of choice where there are very few reactors and where there is reasonable doubt as to whether brucellosis exists in the herd. The introduction of breeding stock from herds of unknown status should be avoided, as should the use of communal boars in infected areas.

Measures to limit the spread of infection within the herd include the provision of separate farrowing pens and proper disposal of birth materials. Sows that abort or exhibit fertility problems should be sent for slaughter as should boars that are known to be, or suspected of being, infected. Where dairy cattle are kept on the same premises, care should be taken to avoid carrying *B. suis* infection to the cows’ udders on the hands of workers.

Since *B. suis* is readily transmitted to man and causes a very serious illness, precautions to protect personnel on infected pig farms are very important.

8.4 Control and treatment in dogs

There is no vaccine for *B. canis* infection, although several attempts have been made to develop one.

Control measures are similar to those with other *Brucella* infections. All bitches that abort or fail to conceive after successive matings, and males with genital disease should be considered as suspect and subjected to diagnostic tests, including blood cultures.
Except in special circumstances, dogs found positive should be destroyed. A kennel should not be considered free from infection until results of at least three tests at monthly intervals of all dogs have been declared negative. All dogs scheduled to be introduced into a kennel, especially pregnant females and animals to be used for breeding, should be admitted only after two negative tests at 30-day intervals.

In those areas where B. canis has been shown to be locally endemic (e.g., Mexico and Argentina), canine brucellosis constitutes an additional reason for control of stray dogs. Males may shed organisms in the semen for long periods after the bacteraemia has ceased, often when antibody levels have fallen below diagnostically significant levels.

Several treatment regimens for dogs have been investigated, but only those using tetracyclines combined with streptomycin have shown any success. The most successful experimental treatment regimen used minocycline (27.5 mg/kg twice daily); 15 out of 18 dogs were bacteriologically negative for B. canis at necropsy 6–28 weeks after therapy was discontinued.

Animals that respond successfully to antibiotic therapy, i.e., with cessation of bacteraemia, and then return to a seronegative status, may be found fully susceptible to reinfection by the oral route in contrast to dogs that spontaneously “recover” and have proved subsequently immune to reinfection.

8.5 General non-specific control measures

Whether immunization and/or eradication programmes are being undertaken or not, certain general non-specific control measures help to reduce the spread of infection.

1. Isolation at parturition has been shown to be effective in aiding control. This requires the provision of separate calving or lambing boxes and also presupposes that the attendant is capable of recognizing impending abortion.

2. In an infected herd, all parturitions should be regarded as potential sources of infection and the non-living products should be incinerated, where possible, or else buried deeply. Heavy agricultural plastic sacks are useful for transporting potentially contaminated materials. Contaminated areas should be disinfected before being swept or hosed down.
8.6 Health education and training

Control of brucellosis should be an attractive economic proposition to farmers and others engaged in animal production. Unfortunately, in practice, the prospects of immediate losses through elimination of infected animals and the inconvenience caused by repeated testing and immunization may outweigh in the mind of the farmer the long-term advantages of control at a remote date in the future. Thus, it is necessary to explain to all concerned the rationale and the advantages of the control programme, especially the continuing economic benefits and the elimination of the serious risk to human health, including the health of the farmer himself, of his family, and of other farm workers. This is the main task of health education in brucellosis control programmes.

Other important health education tasks include:

—dissemination of information about various phases of the programme and about the current operations;
—motivation of animal owners, animal attendants, workers in relevant food industries, and the general public to participate in appropriate parts of the programme;
—informing exposed persons and visitors to endemic areas about the measures they should take to protect themselves;
—informing government authorities, politicians, and other leading personalities about the programme in order to secure and maintain their support;
—ensuring that all concerned remain vigilant after the desired level of control or elimination has been achieved in order to guard against a return of the infection.

Thus, health education is intimately linked with all the various phases of the control programme and should not be regarded as a separate activity.

In addition to the specific measures described above, and especially where there is no national programme, certain measures can be taken aimed at motivating people to control brucellosis in their animals and to protect themselves against infection. First, various groups in the community, veterinarians, animal health workers, artificial inseminators, medical laboratory workers, dairy employees, abattoir workers and animal attendants on infected premises are all considered to be occupationally exposed. Second, information must be made available about the methods of spread of
Brucella infection; contact with infected animals and their birth products and consumption of untreated dairy products are the main sources of human infection. Third, people must be made aware of the means of control in animals, i.e., the fact that immunization is available for cattle, sheep, and goats, and that the disease can be eradicated by a planned programme of testing and eliminating infected animals.

Details of the planning and operation of health education of the public in programmes for prevention and control of brucellosis, and the methods that can be employed, have previously been described.  

Table 1 gives examples of topics that might form the basis of health education activities among different population groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Topic</th>
<th>Expected conduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Livestock breeders</td>
<td>• Concept of brucellosis</td>
<td>Collaboration with the measures of prevention and control of brucellosis carried out by the services of public health and animal health</td>
</tr>
<tr>
<td></td>
<td>• Characteristics of the disease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Damage done to human health</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Damage done to animal production</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Legislation backing the measures taken by the control agencies</td>
<td></td>
</tr>
<tr>
<td>Personnel that work in direct contact with</td>
<td>• Concept of brucellosis</td>
<td>Application of the recommended measures in order to prevent the disease</td>
</tr>
<tr>
<td>animals (shepherds, milkers)</td>
<td>• Characteristics of the disease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Damage done to human health</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Affected species</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Means of transmission to man</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Preventive measures:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>— use of protective clothing</td>
<td></td>
</tr>
<tr>
<td></td>
<td>— medical supervision</td>
<td></td>
</tr>
<tr>
<td></td>
<td>— handling of live and dead animals</td>
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</tr>
<tr>
<td></td>
<td>— personal hygiene</td>
<td></td>
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<tr>
<td></td>
<td>— environmental health</td>
<td></td>
</tr>
<tr>
<td>Population</td>
<td>• Concept of brucellosis and its importance as a zoonosis</td>
<td>Positive attitude with respect to the care of their own health and acknowledgement of brucellosis as a human disease</td>
</tr>
<tr>
<td></td>
<td>• Ways of transmission to man</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Symptomatology in man</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Methods of prevention, especially related to milk or fresh cheese</td>
<td></td>
</tr>
<tr>
<td></td>
<td>consumption</td>
<td></td>
</tr>
</tbody>
</table>

The report of a WHO Expert Committee on Training of Health Personnel in Health Education of the Public\textsuperscript{1} also provides very valuable guidance on the subject of training of health workers.

8.7 Control in countries lacking developed veterinary and laboratory services

In some countries that lack the facilities for a national eradication programme, there may be individual farms (e.g., government farms providing breeding stock) or even specific areas where eradication is feasible and highly desirable and where the methods described in the previous section are applicable. On the other hand, some countries with developed veterinary services may include remote areas where these services do not operate, and in such areas sheep, goat, and/or cattle raising may be important.

In countries where \textit{B. melitensis} infection is common in the sheep and goat population there may be a serious public health problem that needs urgent attention. Information and control programmes should be targeted to these and other regions where brucellosis is known to be giving rise to a public health problem.

8.7.1 Mobilizing action to control brucellosis in developing regions

In regions where veterinary services are poorly developed, the principal means of control will be immunization of the affected animal population.

The impetus for action may originate at community, regional, national, or international level. In any case, it is essential for the authorities at all levels to cooperate in dealing with the problem. There may be legal requirements to consider, as materials, equipment, vehicles, vaccinators, drugs, etc., will usually need to be provided from national or international funds. It is, however, extremely important to consider local conditions of animal husbandry when formulating plans.

The principal elements of the programme are: (1) establishing a diagnosis and defining the geographical extent of the problem; (2) informing the community and motivating those involved in control action; (3) the provision of vaccine; (4) organizing immunization, including the provision of vaccinators, equipment, and vehicles; (5)

\textsuperscript{1} WHO Technical Report Series, No. 156, 1958 (\textit{Training of Health Personnel in Health Education of the Public: report of the Expert Committee}).

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training; and (6) protecting the public against infection and arranging for the treatment of human cases.

(1) Establishing a diagnosis and defining the geographical extent of the problem. When a number of people develop fever, headache, muscular pains, and other suggestive symptoms, brucellosis should be suspected and action taken to establish a diagnosis, including isolation and identification of the causal organism. Identification of the causal bacterium will indicate the reservoir animal host, i.e., *B. melitensis* implicates sheep and goats, *B. abortus* cattle, and *B. suis* pigs. The occurrence of abortion in animals and, in some areas, hygromas in cattle will also suggest the presence of brucellosis.

In defining the extent of the problem in animals, milk-ring tests are valuable in dairy cattle; these can be done at dairies, milk cooperatives, etc. Allergic skin tests are effective herd tests for sheep and goats; in these animals the milk-ring test is not sufficiently accurate. In all species, the Rose Bengal test is valuable for diagnosis where blood samples can be obtained, either by bleeding representative samples of the animal population or obtaining blood from abattoirs. This test has the advantage that it can be done without electricity. In areas where sheep and goat owners are reluctant to have their animals bled, it may be possible to organize agglutination tests on samples of whey, which are easily obtained from milk. Where no veterinary laboratory exists, medical laboratories will generally make bacteriological examinations, e.g., on samples of milk or aborted materials.

Medical and veterinary cooperation is important in establishing the epidemiology, especially when human cases are occurring in the area.

(2) Informing the community. Action is required at various levels.

(a) International level. The Joint Expert Committee recommended that FAO, and WHO as well as the International Office of Epizootics (OIE) and other interested international organizations, should take a more active part in disseminating information on brucellosis control. The information should be understandable by laymen so that it can be distributed at both regional and community levels. There are two zoonosis centres and several cooperating centres within the United Nations system that provide excellent scientific assistance. Perhaps some of these institutions could direct part of their work towards providing more information and advice
on control methods appropriate to the type of brucellosis and
husbandry practices in their regions.
Programmes and centres responsible for international
surveillance of brucellosis and information exchange are listed in
Annex 6. Addresses of international institutions that provide
technical cooperation for brucellosis control are given in Annex 7.
(b) National level. Information on the characteristics of the
disease and the action proposed to deal with it should be prepared
in language understandable by the target population. National
meetings should be arranged with community administrators from
the regions involved.
(c) Community level. The Committee recommended that adequate
attention should be given to community participation in efforts to
control and eliminate brucellosis. Action at the community level
should be assisted by the following groups of people: members of
community councils, village headmen, local health committees,
public health and social workers, religious bodies, schoolteachers,
dairy and abattoir managers, meat inspectors, farmers, etc. The
community participation programme should be planned by the
community council and endorsed by participating groups in the
community, and one of its main aims should be to disseminate basic
information about brucellosis (see section 8.6).

(3) Provision of vaccine. Vaccines should be obtained from
reputable suppliers who are known to submit their products to
official biological control. Suitable means must be provided for
distributing and storing vaccines and diluents, bearing in mind that
live vaccines need to be stored in a refrigerator or suitable cold-chain
equipment. Countries wishing to produce vaccine locally should
consult sources of technical information of vaccine production (see
section 4).

(4) Organizing immunization. Departments of veterinary and
public health will normally be responsible for organizing
immunization programmes. These may be nationwide or cover one
or more provinces or districts, depending on the epidemiology of the
disease. Planning will normally be at the national level in
consultation with those charged with the day-to-day organization.
It would be an advantage if the central planners were trained in
management and organizational techniques. Immunization may be
compulsory or voluntary. As a rule, voluntary immunization
succeeds only when supported by public opinion and animal owners often prefer programmes to be made compulsory. Plans will need to be made for the provision of vaccine, its distribution and storage, transport for personnel, including off-the-road vehicles; “trail bikes” are especially useful and economical in many rugged situations. An adequate supply of suitable syringes, needles, etc. is required and, unless disposable types are to be used, some means of sterilization will be necessary.

Procedural plans need to make provision for prior education of the animal owners, notice of impending immunization, immunizing the animals, and the keeping of adequate records. The convenience of the animal owner should be carefully considered when immunizations are being planned.

It is desirable that immunized animals should be identified, either by ear punch, tattoo, or ear-tag, if possible with the consent of the owner.

(5) *Training*. It is preferable to select for training as vaccinators people who have a secondary education, a rural background, and some knowledge of the conditions under which they will have to operate. A formal course of training should be organized to include the following topics: (a) the characteristics of the disease, including its zoonotic aspects; (b) relevant animal husbandry practices; (c) the control measures to be practised; (d) the benefits of the programme; (e) the characteristics of the vaccine; (f) the technique of immunization; (g) the care and handling of the vaccine and disposal of surplus vaccine; (h) the care of syringes and sterilization; (i) the dangers faced by vaccinators (including those of self-immunization and natural brucellosis) and remedial action to be taken; (j) protection against brucellosis for exposed personnel, their families, and consumers of their dairy products.

(6) *Action to protect the people against infection*. Effective immunization of the animal population that constitutes the reservoir of infection for human beings should greatly reduce the incidence of human brucellosis. Other measures that can be taken include health education of people at risk (see section 8.6).
8.7.2 Special considerations for controlling bovine brucellosis

Brucellosis seldom presents a problem in village cattle. However, in parts of Africa, herds of indigenous cattle may be seriously affected as may commercial dairy farms, especially where highly susceptible imported cattle, which may not have been immunized, come in contact with local stock.

Before remedial measures are planned it is essential to establish a diagnosis, either by serology or bacteriology. Once a diagnosis has been made, and a significant problem has been shown to exist, immunization with strain 19 should be organized. In China, strain 2 live vaccine has been administered orally in such situations.

Newly introduced breeding stock should also be immunized before being allowed into a Brucella-infected environment.

8.7.3 Special considerations for controlling B. melitensis infection in sheep and goats

Serious brucellosis problems arise where herds of sheep and goats are kept for the production of milk and, especially, fresh cheeses.

The Committee considered that in countries where B. melitensis infection occurs, human brucellosis should be a notifiable disease. Notice of outbreaks should be communicated to the relevant veterinary authorities and other interested groups, thus alerting them to the existence of animal brucellosis.

In the absence of established veterinary or laboratory services, the only specific measure for the control of brucellosis in small ruminants is immunization. The recommended vaccine is strain Rev. 1, which is fully discussed in section 4.2 and its application to the control of sheep and goat brucellosis has already been discussed in section 8.2.2.

8.7.4 Special considerations for controlling brucellosis in pigs

This infection is expected to become more widely recognized as an important zoonosis in some regions, especially South-East Asia. Unfortunately, there is, as yet, no recommendable vaccine available for pigs and no serological test effective for the diagnosis of infection in individual animals. The Rose Bengal test is effective as a herd test and encouraging progress has been made in developing an enzyme immunoassay. General preventive measures can be applied, as previously discussed in section 8.3.
9. PLANNING AND MANAGEMENT OF BRUCELLOSIS CONTROL

9.1 Strategy selection

The Committee recognized that different countries and ecologically distinct areas within countries require different strategies for the prevention and control of brucellosis in the population of the various animal species, depending on the prevailing epidemiological and socioeconomic conditions. Decisions as to the appropriate strategy for the control and/or elimination of brucellosis are usually a national responsibility, though in some large countries this may be delegated to states or provinces or made applicable to individual islands or communities.

A computer model may be of value in planning the detailed strategy of control and eradication programmes.\(^1\)\(^-\)\(^2\) The model may be expected to predict how various conditions, such as herd size, management systems, calving conditions, breeding patterns, immunization status, and other factors, can influence the choice of the most effective course of action. Predictive modelling is seen as being complementary to practical field knowledge rather than a substitute for it. Techniques are available for cost–benefit analysis in brucellosis and may be of considerable assistance in deciding the best approach to its control under a given set of circumstances.

A selection may be made from the following five strategies, with the understanding that they are not necessarily mutually exclusive:

1. Prevention of spread between animals and monitoring of brucellosis-free herds and zones. In areas where the brucellosis-free status has been established or where such a status is suspected on the basis of prevailing ecological conditions, the movement of potentially infected animals into such areas must be prohibited or importation permitted only from certified brucellosis-free farms or areas. This applies to the transport of animals and of certain animal products within countries as well as between countries, following the

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\(^1\) HUGH-JONES, M. E. ET al. \textit{An assessment of the eradication of bovine brucellosis in England and Wales}. Unpublished document (1975); available from Department of Agriculture and Horticulture, University of Reading, Reading, England.

\(^2\) \textit{Australian brucellosis and tuberculosis information system} (1976). Third report to the Australian Bureau of Animal Health from the Veterinary Epidemiology and Preventive Medicine Unit, University of Melbourne, Werribee, Victoria 3030, Australia.
principles and procedures specified by the International Zoo-sanitary Code of the International Office of Epizootics (OIE). This code also describes the essential testing of animals and quarantine measures.

The Committee drew attention to the fact that there have been incidents of the spread of brucellosis by serologically negative animals, particularly bulls and heifers, originating from inadequately certified and supervised sources.

2. Elimination of infected animals by test-and-slaughter to obtain brucellosis free herds and zones. According to present knowledge the elimination of brucellosis from livestock can be achieved only by systematic schemes of test-and-slaughter combined with strategy 1. In some countries, elimination follows several years of systematic mass immunization (see strategy 3). The Committee noted that there have been no reports indicating the elimination of brucellosis by immunization alone.

It is usually considered that a programme of elimination by test-and-slaughter is justified on economic grounds only when the prevalence of infected animals in an area is 2% or below. In areas where the prevalence is higher than 2%, a preliminary immunization campaign is recommended. The Committee recognized that religious beliefs in some countries and tribal customs in others do not allow the slaughter of cattle. Under these conditions, immunization seems the only option and eradication will be achieved only if immunization is applied exhaustively to all animals in the area for a sufficient time for those present at the beginning of the campaign to be eliminated by natural wastage.

An eradication plan can be either voluntary or compulsory. Voluntary schemes may be more appropriate in the early stages of eradication; they need to be supported by adequate incentives such as a bonus on the sale of milk from brucellosis-free herds or per capita payments. Compulsory eradication is required in the final stages and may be advisable from the start in many countries.

Complete elimination of bovine brucellosis has been achieved in a number of countries, generally after long and difficult campaigns and invariably under intensive farming conditions where the cattle are under close control. Before deciding on a programme of

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eradication it is necessary to ensure that the epidemiological situation is favourable and the necessary facilities and finance available, not only for eradication but also for continuing surveillance for a considerable period thereafter.

3. **Mass immunization to reduce the rate of infection in specified herds.** Mass immunization of cattle and small ruminants has been applied with success in many countries under different socioeconomic conditions. In general, mass immunization is indicated where the prevalence of infected animals is above 2%, except in certain situations, as mentioned in strategy 2 above. Mass immunization should be based on knowledge of the rate of infection in animals. However, the strategy depends on the continuous availability of vaccine.

4. **Non-specific measures to reduce the spread of infection.** In addition to strategies 2 and 3 above and in situations where conditions do not permit implementation of the above strategies, simple non-specific measures of prevention of the spread of the infection can still be applied in a systematic way (see section 8.5).

5. **Supporting education and training.** All the above strategies require adequate education of the public and training of professional staff (see section 8.6). Public education extends to farmers and communities as a whole. Professional training is essential for the implementation of the strategies by the appropriate national services. Special emphasis should be placed by international organizations on the management training of government officials and on all aspects of technical cooperation among developing countries. The training of the different groups in society will ensure that the right actions are taken and that resources are mobilized in all relevant sections.

### 9.2 Field methods

#### 9.2.1 Assessment of the epidemiological situation

An epidemiological surveillance system must be set up before starting any control programme in order:

(a) to determine the appropriate objective and hence the measures to be adopted;

(b) to follow the progress, or failure, of the programme and correct it;
(c) to delineate infection-free area(s) and herds from which uninfected (replacement) animals can be purchased.

Prevalence must be established by herd, by flock, or by groups of herds and flocks belonging to several owners but bred together. These groups may be described by village, or by common pasture, or by any other meaningful arrangement. Groups or herds must be surveyed by appropriate tests involving a significant number of samples, i.e., number of animals per group and number of groups in the area.

It is of paramount importance to delineate the main epidemiological traits of the disease in the area: animal species affected, domestic or wild; biovars of $B.\, abortus$, $B.\, melitensis$, and $B.\, suis$ involved; husbandry practices that may increase propagation and/or persistency of infections; movements of animals from and into the area: importation should be carefully considered and eventually properly controlled.

The recommended survey procedures to be used according to circumstances, are shown in Table 2. Although the occurrence of $Brucella$ infection in wildlife does not necessarily constitute a menace to livestock in the vicinity, epidemiological investigation is called for.

9.2.2 Selection of area of action

The limits of the area should be set by epidemiological or geographical evidence or from a preliminary survey. For example, if in a geographical area all herds are similarly managed, the area of action may include the whole area. On the contrary, different managerial habits, such as cohabitation of several animal species or use of common pastures, may require subdivision of the geographical area into several areas of action. A preliminary survey taking random samples from herds or flocks from different parts of the area(s) may suggest very different epidemiological situations not previously suspected and these may require careful attention.

9.2.3 Protection of low prevalence and brucellosis free areas

The Committee noted that detailed procedures for the protection of brucellosis-free areas are specified in the International Zoosanitary Code.\(^1\) It is recommended that these procedures be

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\(^1\) See footnote on page 87.
Table 2. Survey procedures recommended for the assessment of brucellosis epidemiology

<table>
<thead>
<tr>
<th>Animal</th>
<th>Survey procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle Dairy herds</td>
<td>Milk-ring tests to identify infected herds and establish the prevalence of infected herds in the various regions</td>
</tr>
<tr>
<td></td>
<td>Blood samples from positive herds to establish the prevalence of reactors in infected herds</td>
</tr>
<tr>
<td></td>
<td>Culture of milk from positive herds to support the serological data and identify the causal Brucella species and biotype</td>
</tr>
<tr>
<td></td>
<td>Culture of abortion material</td>
</tr>
<tr>
<td>Beef cattle</td>
<td>Serological tests on blood samples from breeding females sent to abattoirs, followed by identification of any infected farms</td>
</tr>
<tr>
<td></td>
<td>Blood samples from infected farms</td>
</tr>
<tr>
<td></td>
<td>Culture of lymph nodes and abortion material</td>
</tr>
<tr>
<td></td>
<td>Serological surveys on live animals on farms and at markets, shows, and fairs</td>
</tr>
<tr>
<td>Sheep and goats</td>
<td>Serological tests on blood taken at abattoirs, followed by identification of any infected flocks</td>
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<tr>
<td></td>
<td>Serological tests in flocks suspected of being infected</td>
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<tr>
<td></td>
<td>Survey tests using anergic tests on selected herds followed by blood samples from herds found positive</td>
</tr>
<tr>
<td></td>
<td>Culture of lymph nodes and abortion material and milk samples from positive herds</td>
</tr>
<tr>
<td>Pigs</td>
<td>Serological tests on blood taken at abattoirs, including non-breeding females and castrated males, followed by identification of the source of any infected animals</td>
</tr>
<tr>
<td></td>
<td>Blood tests in infected herds</td>
</tr>
<tr>
<td></td>
<td>Culture of lymph nodes collected at abattoirs</td>
</tr>
<tr>
<td></td>
<td>Culture of abortion material</td>
</tr>
<tr>
<td>Feral or wild-life species in contact with domestic animals</td>
<td>Capture or shooting and serological examination, supported by isolation and identification of the organism</td>
</tr>
</tbody>
</table>

followed in principle in order to protect brucellosis-free areas as well as areas of low prevalence.

In some regions, there has been an increase in the incidence of brucellosis as a result of countries importing animals to expand sections of their animal industries. Two situations need comment:

(1) Explosive outbreaks of brucellosis have occurred when highly susceptible breeding animals (cattle and pigs) from brucellosis-free countries have been imported into areas where brucellosis was endemic, usually at a low level, among the indigenous stock.
(2) Serious epidemics of brucellosis have occurred giving rise to many human cases when imported sheep and goats, some of them perhaps infected with brucellosis, have been kept under intensive conditions with or without mixing with indigenous animals.

The remedy to these types of problem lies in importing only brucellosis-free animals and in immunizing cattle, sheep, and goats if brucellosis is present in the new location. Imported pigs should be kept apart from local stock.

9.3 Implementation schemes at different levels

The Committee noted that tools and procedures for the planning of comprehensive national zoonoses control programmes had been developed and adopted by the WHO Expert Committee on Bacterial and Viral Zoonoses.1 The Committee adopted in principle these planning and management procedures. For the specific needs of brucellosis control programmes, the Committee recommended that the tree diagrams of objectives, and other management tools, be adapted to local conditions.

Brucellosis is one of the zoonotic diseases that can be prevented and brought under control with the active participation of the community. Where socioeconomic conditions do not permit the country-wide elimination of the disease, local control measures should at least be instituted and personal protection improved.

The various methods to be applied where resources are limited have been noted in section 8.5. The consequences of brucellosis for the economy as well as for health can be of such a magnitude that the community should take action without waiting for the implementation of comprehensive national plans. This should not mean that the community initiatives would not be helped by the government in a systematic way through the services for public health, food hygiene, animal health, animal production, health education, etc.

The communities should identify all available resources at different organizational and administrative levels for the prevention of brucellosis and for the protection of high-risk groups. Many of the measures have multiple beneficial effects and should therefore be seen as contributing to the general improvement of personal and community hygiene in livestock development and animal

production, processing, and consumption. Cooperative programmes of farmers in livestock development, dairy development, and meat production have been combined effectively with animal health schemes.

National services for diagnosis, mass immunization, and replacement of animals should be seen as activities supporting primary animal and human health care and organized animal health programmes at community level. However, such a concept calls for the training of livestock development officers and community health and animal health workers in new approaches to the health of man in his animal environment.

However, the Committee drew attention to the fact that brucellosis prevention and control can be initiated from different structures in the society. Special attention should be given to the following management approaches, which are not mutually exclusive:

1. Schemes initiated and sustained by the community

   (a) The community (a village or a group of villages, a milk-collecting area, a cooperative of farmers, etc.) should designate a responsible person for hygiene of animals and animal products. This person should cooperate closely with the primary health care workers, be able to immunize animals, diagnose major disease in animals, and know about general preventive measures, including aspects of meat and milk hygiene.

   (b) On the basis of simple guidelines approved by the community, the responsible community worker should locate the resources at community level and proceed according to a simple work plan. District officials should be informed or trained how to advise communities to proceed.

   (c) The community council should examine annually whether the planned activities have been implemented and how the measures can be improved and expanded.

   (d) When this basis of general measures has been achieved, the community may ask the next higher administrative service (district or province) to help with more specific measures. A plan of action for the epidemiological assessment and schemes of prevention and control should be prepared with the assistance of the responsible government service. This will include intersectoral cooperation between different national services.
2. Government-assisted community-initiated schemes

(a) The government elaborates guidelines in the form of a simple decision-making process that should be followed by the animal and human health specialist at an appropriate administrative level (e.g., the district veterinary or livestock development officer).

(b) Responsible officers at the appropriate levels (e.g., district) should organize a zoonoses control committee that can help communities, on request, to formulate local action plans. These may include (i) a simple epidemiological survey; (ii) education and information tools and procedures for personal hygiene; (iii) immunization programmes, and/or (iv) animal replacement schemes. Suggestions for cooperative projects among farmers concerning farm management, husbandry, milk and meat hygiene, and marketing, require the expertise of different disciplines and different administrative levels.

(c) Peripheral governmental services should be prepared to assist the communities or local cooperative projects in respect of (i) diagnostic services; (ii) vaccine provision; (iii) use of a cold chain; (iv) improvement of technologies in milk and meat hygiene and animal waste disposal or rendering, as well as in education and in adequate treatment of human patients.

Peripheral governmental services should report regularly to the central government about the progress of community initiatives. Moreover, these government services may elaborate suggestions for a country-wide programme of brucellosis control, including procedures for the control of the movement of animals and the provision of brucellosis-free animals for replacement of positive animals.

3. Comprehensive national programmes

The Committee recommended a gradual development of comprehensive national programmes for brucellosis control. This requires, in principle, the following stepwise development similar to that specified in the seventh report of the WHO Expert Committee on Rabies: 1

- Establishment of an interministerial committee.
- Designation of a national programme directory.

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• Preparation of guidelines for (a) community activities; (b) supporting services; and (c) a comprehensive national plan.
• Review and, if necessary, improvement of legal provisions.
• Formulation of the country-wide programme of brucellosis control, and endorsement (at cabinet level).
• Establishment of the institutional framework, if not already existing, including components of community-based activities.
• Resources mobilization.
• Programme implementation, harmonizing phases of development in respect of (a) geographical coverage; (b) technologies; and (c) manpower.
• Programme monitoring, periodic evaluation, and review.

An important task of a national programme director should be the elaboration of guidelines for community initiatives and for planning and management assistance by the technical services. Other activities concern the availability of diagnostic services, technological support, provision of vaccines, and educational schemes for those communities that are prepared and wish to take more specific measures of brucellosis assessment and control. If the national programme is to be effective it is essential that every group concerned with brucellosis control, from the ministerial level to that of the local farmer and his family, should understand and actively support the campaign.

10. RECOMMENDATIONS

10.1 Recommendations for research

10.1.1 Biology of the genus Brucella

(1) There is a need for research into the genetics and physiology of Brucella as a basis for the development of new strains and antigens for use in diagnosis or as vaccines.

(2) Manometric techniques for oxidative metabolic studies are laborious and dangerous. Thin-layer chromatography has been used as an alternative procedure, but there is scope for the development of simpler methods.

(3) Phages are now available for all smooth Brucella species. Further studies with rough phages are needed to develop a phage-typing system capable of differentiating various species in the rough
phase. This is particularly important since the wider use of liquid media in bacteriological diagnosis has resulted in the more frequent isolation of dissociated forms of species that normally exist in the smooth form. Phages applicable to identification at the strain level should be developed for epidemiological purposes.

(4) The use of monoclonal antibodies to identify epidemiological markers requires evaluation.

(5) The work in progress on the separation and characterization of the antigens of the Brucella cell wall should be continued since this work is likely to produce better antigens for use in serological and allergic tests and promises important advances in vaccine production.

10.1.2 Immunology

(1) The progress being made in the purification and characterization of antigens needs to be applied to the preparation of antigens for serological tests, especially with a view to distinguishing antibody responses to diagnostically significant antigens from those resulting from exposure to vaccinal antigens.

(2) Enzyme immunoassay is being studied in many laboratories and shows great promise. There is a need for a more closely defined antigen and for a standardization of test procedures. Evaluation of the test for species other than cattle is required, especially B. melitensis infection in sheep and goats and B. suis infection in pigs. Simplified versions of the technique for use under field conditions should be developed.

(3) The Rose Bengal test has proved very useful for the diagnosis of brucellosis in cattle and in pigs; it promises also to be useful in the diagnosis of human brucellosis and merits further evaluation in these and other species, such as sheep and goats.

(4) The merits of the newer serological tests, such as the indirect haemolysis test, radial immunodiffusion, and the haemolysis-in-gel test, need further evaluation.

(5) Studies are needed on the role of the immunoglobulin isotypes in protective immunity and on the mechanisms of immunity in acute and chronic forms of brucellosis. Immunoglobulin isotypes and lymphocyte subsets involved in protection and cure are of particular interest in animal models as well as in natural hosts.

(6) The serological diagnosis of chronic human infection needs further study. The development of improved antigenic materials to
define the degree of sensitization in brucellosis patients should be encouraged.

(7) There is a need for further research into serological cross-reactions, of which those due to *Yersinia* are the most difficult to resolve. There is still no satisfactory simple test for the differentiation of these cross-reactions.

10.1.3 Development of vaccines

(1) More satisfactory vaccines are needed for all species, especially man and swine. The characterization of vaccines should be followed up by study of their immunogenic properties.

(2) More knowledge of the fundamental mechanisms of resistance to *Brucella* infection is required, including investigations into the problems of extrapolation from animal models to the target species.

(3) Further research is required on *B. abortus* strain 19 vaccine to determine the optimum dose, route, and age at immunization and, where new methods are developed, the duration of immunity and the desirability of re-immunization.

(4) Information is required on the optimal dose for oral challenge of immunity in cattle.

(5) More research is needed on the maintenance of virulence of challenge strains for vaccine potency tests.

(6) Investigation is needed into the possibility of developing vaccines for use in male breeding stock.

(7) Further studies are needed on killed vaccines, especially in relation to the application of improved adjuvants and immunostimulants.

(8) Further studies are required on the *B. suis* strain 2 live vaccine to assess its safety and efficacy under a variety of ecological and management conditions. In particular, its use as a vaccine against swine brucellosis deserves further evaluation.

(9) The requirements for efficient storage and transport of vaccines need investigating under different climatic conditions.

(10) The virulence of African strains of *B. abortus* for laboratory animals and cattle needs to be studied, especially in conjunction with studies on the efficacy of strain 19 vaccine against African strains.

(11) As live vaccines are usually both effective and cheap, they are of particular interest to developing countries. It would be desirable to explore the appropriateness and effectiveness of combining
certain live vaccines in one injection (as, for example, combined live vaccines for *Brucella, Chlamydia*, and *Salmonella* infections of ewes).

(12) A method for titration of the immunogenic activity of vaccines has been described using experimental infection of mice. This method deserves further evaluation.

10.1.4 *Epidemiology*

(1) More information is required on the significance of serological reactions in species other than the natural host. The epidemiological importance of such infections may be overestimated. Serological results in these species should be related to bacteriological and pathogenesis studies and, if possible, transmission experiments should be undertaken.

(2) The role of the fly (*Musca domestica*) and other insects in the transmission of bovine brucellosis, especially in warm climates, should be investigated.

(3) Field studies are required to compare different control and/or eradication strategies for bovine brucellosis from the point of view of economy and efficiency.

(4) There is a need to improve the methods for examination of foodstuffs, and especially fresh cheeses, for the presence of *Brucella*.

(5) The applications of computer technology, both for predictive modelling and for data handling to facilitate the execution of control and eradication programmes, should be explored, since relatively inexpensive equipment is now available.

(6) Further studies are required on the persistence of *Brucella* infections in sexually immature cattle, sheep, and goats.

10.1.5 *Chemotherapy*

The treatment of human brucellosis by antibiotics and/or immunostimulants should be evaluated by properly controlled clinical trials. Animal models of acute and chronic brucellosis can be very valuable for screening potential chemotherapeutic agents.

10.1.6 *Health services research*

(1) Comparative studies should be made of the efficiency of utilization of available resources by the health services engaged in brucellosis control programmes.
(2) Studies are needed on evaluation systems for brucellosis control programmes and on methods of cost-effectiveness analysis.

10.2 Other recommendations

(1) FAO and WHO, together with the other international agencies concerned and with Member States, should participate in developing a programme on the prevention and control of brucellosis in man and animals; the success of such a programme would depend, to a large extent, on the energetic application of primary health care practices. Activities at all levels should be organized systematically and made as effective as possible at the lowest cost feasible. Activities at the higher organizational levels should provide support to those at the primary health care level, and should be directed towards accelerating programme development, promoting the health status and productivity of people, and reducing morbidity and mortality.

(2) Biosafety programmes with regard to brucellosis still need to be improved upon, although great progress has been made in this area.

(3) Global surveillance should be strengthened for both human and animal brucellosis, with a view to providing valuable indicators that could assist the global and national programme.

(4) Prevention and control programmes should be strengthened in many countries, in particular in those where the incidence of brucellosis is quite high, or where its existence is persistent.

(5) Standardization and control of diagnostic reagents, antigens, and vaccines are required for the establishment of international standards. Biologicals for use in the field of brucellosis control produced by different manufacturers for export should be controlled according to the above standards by an independent control laboratory, possibly supervised by the state or by an international agency (e.g., FAO or WHO).

(6) WHO should consider issuing a statement that emphasizes the need for heat treatment of dairy products, especially milk.

(7) International cooperation, technology transfer, training and exchange of personnel should be continued and encouraged by all the international organizations concerned (e.g., FAO, OIE, and WHO) and by Member States.

(8) International agencies within the United Nations system, such as FAO and WHO, as well as other international organizations, such
as OIE, should take a more active part in disseminating information on brucellosis control as part of WHO’s efforts to achieve health for all by the year 2000. Such information should be understandable by laymen so as to be suitable for distribution at regional and community level. There are two zoonosis centres and several collaborating centres within the United Nations system, providing excellent scientific assistance. The work of at least some of these institutions could be directed towards providing more information and advice on control methods appropriate to the type of brucellosis and husbandry practices in their regions.

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Annex 1

SAFETY PRECAUTIONS REQUIRED WHEN HANDLING MATERIALS THAT MAY CONTAIN BRUCELLA

1. In the laboratory

1.1 Introduction

The WHO Laboratory biosafety manual\(^3\) classifies Brucella in Risk Group III. This indicates a high risk to the laboratory worker involved, but only a low risk to the community. Brucellosis is, in fact, one of the most easily acquired laboratory infections. Although some Brucella species, e.g., B. ovis, B. neotomae, and some individual biovars, like B. suis biovar 2, have not been reported as pathogenic for man, safety precautions should be observed when handling all Brucella species. The risk varies, not only with the virulence of the organism (B. melitensis and B. suis being the most dangerous for man) but also with the numbers of bacteria in the material being handled. Aborted materials (membranes, fetal tissues, and fluids) may contain up to \(10^{13}\) Brucella organisms per gram, and similar numbers may be encountered in handling cultures grown in the laboratory. Lymph nodes and cow’s milk present a lesser risk and it is unlikely that an infective dose would be acquired accidentally from clotted blood samples, especially those from cattle. Apart from good hygienic techniques and proper disposal of residues, special safety precautions are not required for personnel engaged in the routine serological diagnosis of brucellosis. Such work should be carried out in laboratory rooms separate from those where culture work is done and where infected or potentially infected materials are handled. In those laboratories where safety precautions are required, an officer responsible for safety should be appointed. This safety officer should be adequately trained and made responsible for the efficient working of safety precautions, including those against physical and chemical accidents not covered here. For a fuller treatment of the subject, the WHO Laboratory biosafety manual should be consulted. In addition to much valuable information, it contains a bibliography on safety in the laboratory.


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1.2 Physical requirements for a laboratory handling Brucella

A separate room is required with only one entrance; a biohazard notice prohibiting the entry of unauthorized persons should be prominently displayed at the entrance. The room should have a double-door entrance designed to provide an airlock. The ventilation should be so arranged as to maintain the pressure within the room at a slightly lower level than its surroundings. Air from the room should be discharged to the exterior, well away from air intakes. The walls should be impermeable and all windows sealed to allow disinfection and fumigation; it should be proof against infestation with rodents or insects. The room should have a properly installed and tested biohazard cabinet of Class II or Class III. The air exhaust from the cabinet should be so arranged as to avoid interference with the air balance in the room or within the cabinet when it is switched on. The room should have a sink, an autoclave, and enough incubator space for all culture requirements.

1.3 General precautions

Adequate accommodation, equipment, and hygienic facilities, together with good bacteriological techniques are necessary for safety. The officer in charge of the laboratory is responsible for this, but he should be advised and assisted by the safety officer, who is assumed to be familiar with all the necessary general measures. However, several points particularly relevant to a brucellosis laboratory need emphasis, whether the work is being done inside or outside a biohazard cabinet.

If potentially infectious materials or animals have to be handled outside a biohazard cabinet under circumstances in which a high risk is anticipated, the operator should wear impermeable clothing, an eye-shield, gloves (preferably disposable), and a respirator; a number of convenient types of respirator are now available.

The prohibition of mouth pipetting needs emphasis, since aspiration through pipettes is still a major cause of laboratory infection by bacteria.

The prevention of aerosols is particularly important. Processes that cause frothing must be avoided. Screw-capped bottles and tubes should be used wherever possible. Virulent suspensions should not be blown out of pipettes. After use, pipettes, swab sticks, etc., should be immersed horizontally in disinfectant in a metal container with a lid and autoclaved before being handled.
Other materials, such as Petri dishes and Roux bottles, should be placed in metal containers with lids after use. These should be sealed with autoclave tape to help ensure that they are not opened without having been autoclaved. Separate containers are necessary for disposable plastics, which melt in the autoclave.

Breakages, spills, leaks, etc. need to be dealt with immediately and effectively. This means that suitable disinfectant and cleaning materials should always be available. A register of accidents should be maintained.

Chlorine, in the form of sodium hypochlorite, and formaldehyde are very effective disinfectants. However, the former is corrosive for metals and the formaldehyde gas is irritant. Several proprietary brands of disinfectant are available, usually combining disinfectant and detergent properties. A swab moistened with 70% alcohol is useful in dealing with small spills. Further details on disinfectants for laboratory use are given in the WHO Laboratory biosafety manual.

Once diluted, disinfectants usually deteriorate rapidly, especially when mixed with macerated animal tissues or blood, and may eventually become nutrient media supporting the growth of the bacteria they are supposed to kill. Therefore, disinfectant solutions should be changed frequently. Whenever possible contaminated materials should be autoclaved.

1.4 Biohazard cabinets

There are three types of biohazard cabinet:

Class III. This assures complete physical separation of the operator from his work. High-efficiency particulate air (HEPA) filters are fitted to the air inlet and outlet, work is done through glove ports and materials are introduced and removed through a sealable opening. This is the only really satisfactory type of biohazard cabinet for high-risk work, but is expensive.

Class II. A laminar-flow cabinet with open access; “barrier air flow” provides protection for the laboratory worker at the work opening. A quantity of air equal to the barrier air is exhausted from the cabinet through a HEPA filter. Here the protection is less complete and, if the cabinet is carelessly used, cross-contamination is liable to occur. Nevertheless, this type of biohazard cabinet is more convenient to use than the Class III type.
Class I. This type can be recommended only on a "better-than-nothing" basis. It relies on a rapid stream of air entering the cabinet at the work opening and leaving through a HEPA filter.

Biohazard cabinets must be tested after installation and at regular intervals thereafter. They must be used strictly according to the manufacturer’s instructions.

All manipulations involving materials that may contain virulent *Brucella* organisms should, as far as possible, be done within the cabinet. When working in Class I or Class II cabinets the operator should wear long gloves. For Class III cabinets, gloves are an integral part of the unit.

In most biohazard cabinets there is rapid circulation of air. Bacteria on the outside of pipettes, drops on the end, or simply pouring a suspension from one container to another can produce aerosols that are capable of contaminating materials downwind in the air flow. Therefore, the cabinet needs to be emptied and thoroughly disinfected between each operation. Only materials required for the operation in progress should be in the cabinet and these should be placed at each end, away from the efferent air flow.

It is recommended that Bunsen burners should not be used in biohazard cabinets as they disrupt the air flow and there is a risk of damage to the filters. Loop micro-incinerators are more satisfactory, but disposable loops are preferable.

Pipette-fillers need special mention; malfunction, producing drops, is frequent. They need to be kept free from cotton strands. It is good practice when pipetting virulent suspensions to work over a cloth moistened in disinfectant that can be autoclaved after use.

Disinfection of the cabinet is best achieved by fumigation with formaldehyde or glutaraldehyde. Paraformaldehyde powder or tablets can be conveniently sublimed on a hot plate. Cabinets and rooms must be thoroughly sealed for fumigation, which requires at least 8 hours. All fumigant vapour must be removed before re-use.

1.5 Safety measures for specific laboratory processes

In the production of antigens, where possible, strains of low virulence, such as *B. abortus* strain 1119-3, strain 19, or strain 99, should be used, as they have the advantage that they do not require added CO₂ for growth. If a *B. melitensis* strain is required, Rev. 1 is suitable, and for *B. suis* the strain 2 vaccine strain may be used.
Virulent *Brucella* organisms should not be freeze-dried and sealed under vacuum in single containers without plugs because aerosols are released when the containers are opened. It is preferable to run dry nitrogen gas into the ampoules before sealing. A method for drying cultures in small tubes contained in larger tubes has been described.1

Centrifuges may cause dangerous aerosols, especially when tubes containing virulent material break. Glass tubes should not be used for virulent materials; polycarbonate tubes with tightly fitting screw-capped lids are recommended. If virulent material has to be centrifuged, the containers should be loaded and unloaded inside the biohazard cabinet and aerosol-proof centrifuge rotors used. The continuous-flow type of centrifuge should not be used for virulent strains. Electric homogenizers, stomachers, sonicators, and similar appliances should be used inside the cabinet.

The injection of virulent *Brucella* organisms into laboratory animals should be avoided where possible. It should also be remembered, however, that non-living material can cause hypersensitivity reactions in sensitized individuals. Only needles that lock to the barrel of the syringe should be used and the needle and syringe carefully disposed of. For the accommodation of animals it is possible to design cabinets that have negative pressure and filtered air outlets.

2. Field personnel

Aborted materials, infected birth products, uterine discharges, and objects contaminated by them provide the most dangerous materials for exposed personnel. Milk and milk products are also dangerous, especially when they contain *B. melitensis*. Exposed personnel, e.g., veterinarians engaged in pregnancy diagnosis, obstetrical, or immunization work, artificial inseminators, lay personnel collecting blood samples by tail-bleeding, and animal attendants working in infected or potentially infected herds, should take precautions. They should wear protective clothing, including gloves, and should carefully dispose of dangerous materials, such as birth products, and ensure the disinfection of contaminated areas. Protective clothing should be properly disinfected after use and washing facilities should be provided for personnel. The dangers of

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1 See footnote 2 on page 17.
eating, drinking, and smoking while working in an infected environment should be understood and effective measures taken to prevent infection by these means.

Full details of disinfection procedures for use in animal husbandry have been presented in an unpublished WHO document.¹

3. Packing and transport of specimens

This section applies to cultures and specimens for bacteriological examination, not to blood samples taken for serological diagnosis.

The person collecting the specimens should take precautions to protect himself and should dispose effectively of the remains of the specimen when all is not packaged for dispatch. Aseptic precautions should be taken and the instruments sterilized after use.

Packaging of infectious substances and specimens should be in three layers: (a) a primary water-tight receptacle containing the specimen; (b) a secondary water-tight receptacle enclosing enough absorptive material between it and the primary receptacle to absorb all of the fluid in the specimen in case of leakage; and (c) an outer package that is intended to protect the secondary package from outside influences, such as physical damage and water while in transit. It is important to tape securely on the outside of the secondary container one copy of the specimen data forms and other information that identifies or describes the specimen. The outside of the package should be labelled “Infectious substance”. A copy of the data form should be sent separately to the receiving laboratory.

Before dispatch of specimens across national boundaries, prior permission should be obtained from the receiving laboratory, together with any necessary documentation and instructions. The WHO Laboratory biosafety manual gives more details.

4. Abattoir workers

It is important for those concerned to understand the risk involved, in order that precautions may be concentrated where they are needed. The meat inspector can play an important role in this. Non-breeding cattle and sheep present little hazard. Brucella

reactors present a high risk and arrangements can usually be made
for them to be slaughtered after risk-free animals, preferably at the
end of the day; the area used can then be disinfected conveniently
before work begins again. Udders and uteri present the major risk;
they should not be opened or their contents spilled. The collection
of fetal calf serum can be highly dangerous and needs to be
effectively controlled.

When handling potentially infected animals, workers should wear
protective clothing, which is disinfected after use. More time should
be allowed for each carcass, and smoking and eating prohibited
during breaks. Adequate washing facilities are essential. Ideally the
abattoir should provide clean working clothing each day and
workers should take a shower before changing into home clothes.

In mechanized abattoirs handling Brucella-infected animals,
maintenance workers frequently become infected and preventive
measures are necessary, especially in relation to machinery, chutes,
etc., in contact with udders and uteri.

5. Health and medical surveillance

All personnel regularly exposed to Brucella infection should be
kept under close clinical and serological surveillance.

In spite of all precautions, there remain some situations, such as
handling domestic animals infected with B. melitensis or B. suis, that
are likely to present a serious hazard to the operator. In some
countries, prophylactic immunization is available to those at special
risk. This is discussed in the body of the report. People undergoing
treatment with immunosuppressive drugs should not be exposed to
the risk of infection.
Annex 2

DESCRIPTION OF THE GENUS *BRUCELLA*

*Brucella* is the generic name for a group of small non-motile Gram negative cocci, cocccobacilli, and short rods with straight or slightly convex sides and rounded ends 0.5–0.7 μm wide by 0.6–1.5 μm long. The microorganisms are usually arranged singly, or less commonly in pairs, short chains, or small clusters. They do not produce capsules, spores, or flagella. They do not usually show bipolar staining. They are not acid-fast but may resist decolorization by weak acids or alkalis as in the Macchiavello or modified Köster staining procedures.

Members of the genus *Brucella* respire aerobically, but strains of some species require supplementary CO₂ (5–10%) for growth, especially on primary isolation. They are chemo-organotrophic and have complex nutritional requirements. Growth requirements include: multiple amino acids, thiamin, nicotinamide, biotin, and magnesium. Some strains require serum or other colloid for growth but haemin (X factor) and nicotinamide-adenine dinucleotide (V factor) are not required. *Brucella* species are catalase positive and usually oxidase positive, but *B. neotomae, B. ovis*, and some strains of *B. abortus* are oxidase negative. They do not ferment carbohydrates in conventional media. *Brucella* oxidize various amino acids and carbohydrates, and they usually reduce nitrates to nitrites but *B. ovis* and some strains of *B. canis* do not. Urca is hydrolysed to a variable extent.

Production of H₂S varies between species and biovars. Citrate is not utilized by *Brucella* species as the sole carbon source, and indole is not produced. The methyl red test and Voges-Proskauer reactions are negative. Gelatin is not liquified and erythrocytes are not lysed. Litmus milk is either unchanged or rendered alkaline. The branched electron transport system of *Brucella* includes cytochromes a, a₃, b, c, and o.

The optimum temperature for *Brucella* species is 37 °C, with a range of 20–40 °C. The optimum pH is 6.6–7.4, and the optimum osmotic pressure 203–607 kPa (2–6 atmospheres) (0.05–0.15 mol/litre NaCl).

The species and biovars of *Brucella* are characterized in Annex 2, Tables 1, 2, and 3. Usually, the identity of typical strains can be
established by examination of the properties listed in Annex 2, Table 1. Where difficulty is encountered as in the case of rough or rare phage-resistant strains, oxidative metabolism tests performed either by manometric or chromatographic methods can be used for identification (Annex 2, Table 2).

Phage typing is applicable to non-smooth isolates (Annex 2, Table 3), although to achieve differentiation of *B. melitensis* and *B. suis* strains in the rough phase account has to be taken of properties listed in Annex 2, Table 1.

The properties that differentiate the live vaccine strains of *B. abortus* (strain 19) and *B. melitensis* (strain Rev. 1) from virulent strains of the biovars from which they are derived are listed in Annex 2, Tables 4 and 5. At present, the *B. suis* strain 2 live vaccine cannot be distinguished from field strains of *B. suis* biovar 1 on the basis of *in vitro* tests. *B. abortus* strain 45/20 has the properties of a rough strain derived from *B. abortus* biovar 1. *Brucella melitensis* strain H38 is a typical smooth virulent strain of *B. melitensis* biovar 1.

The FAO/WHO reference strains for *Brucella* species and biovars are listed in Annex 2, Table 6.

Susceptibility to lysis by genus-specific phages varies between species, and in some cases between strains. The recommended propagating strains for brucella-phages are indicated in Annex 2, Table 7.

Structural fatty acids produce characteristic elution profiles on gas–liquid chromatography (GLC) as methyl esters. Differentiation of *B. ovis* and *B. canis* from other species can also be achieved by GLC of fatty acid methyl esters.

*Brucella* species possess common structural proteins that are soluble in phenol acetic acid-water and that produce genus-specific patterns on disc electrophoresis. Major surface antigens differ between smooth and rough strains but some intracellular antigens are common to all strains and are genus-specific.

The G + C content of the DNA ranges from 56 to 58 mol% (buoyant density); DNA polynucleotide sequences show 90% or more homology in hybridization studies.
Annex 2. Table 1. Properties differentiating the species of the genus Brucella and their biovars

<table>
<thead>
<tr>
<th>Species</th>
<th>Biovar</th>
<th>CO₂ requirement</th>
<th>H₂S production</th>
<th>Growth on media (Thiostilbamidine 20 μg/ml)</th>
<th>Agglutination with monospecific antisera</th>
<th>Lysis by phage at RTD</th>
<th>Preferred natural host species</th>
<th>Pathogenicity for man</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. abortus</td>
<td>1</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>L, L, L, L</td>
<td>Cattle and other bovinae</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>Moderate, cases usually sporadic</td>
</tr>
<tr>
<td></td>
<td>3*</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>(+)</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6*</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. melitensis</td>
<td>1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NL, NL</td>
<td>Sheep, goats</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>High, cases can assume epidemic proportions</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. suis</td>
<td>1*</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>(−)</td>
<td>+</td>
<td>NL, L, L, PL</td>
<td>Swine</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NL, L, L, SW, L</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NL, NL, PL</td>
<td>Swine</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>(−)</td>
<td>+</td>
<td>NL, L, L, L, L</td>
<td>Reindeer, Moderate</td>
</tr>
<tr>
<td></td>
<td>5*</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>(−)</td>
<td>-</td>
<td>NL, L, L, PL</td>
<td>Murine and cricetine rodents</td>
</tr>
<tr>
<td>B. neotomae</td>
<td></td>
<td></td>
<td>+</td>
<td>−</td>
<td>(−)</td>
<td>+</td>
<td>PL, L, L</td>
<td>Desert wood rat (Neotoma lepida) Not reported</td>
</tr>
<tr>
<td>B. ovis</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>-</td>
<td>NL, NL, NL</td>
<td>Sheep</td>
</tr>
<tr>
<td>B. canis</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>NL, NL, NL, NL</td>
<td>Dogs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Low, clinical cases rare</td>
</tr>
</tbody>
</table>

*For more certain differentiation of B. abortus biovars 3 and 6, a growth medium containing 40 μg/ml of thiostilbamidine or basic fuchsin with the more concentrated thiostilbamidine growth media (40 μg/ml), all strains of biovar 3 are positive and all strains of biovar 6 are negative. However, all its most recent meeting, the ICSS Subcommittee on the Taxonomy of Brucella proposed that biovars 3 and 6 should be merged to form a single biovar 3/6, as many isolates do not conform precisely to the definitions of the original biovars. (International Journal of Systematic Bacteriology, 34: 366-367 (1984)).

*This biovar is typified by strains isolated from rodents in the USSR (International Journal of Systematic Bacteriology, 33: 399-400 (1983)). It should not be confused with the B. suis biovar 5 referred to in the fifth report of the Joint FAO/WHO Expert Committee on Brucellosis. (WHO Technical Reports Series, No. 454, 1971). The status of cultures isolated from rodents in Australia and Africa has not been resolved. Many other cultures are isolated from time to time that do not conform to the descriptions of the recognized species and biovars. Such cultures should be forwarded to a reference laboratory for further studies.

Agglutination sera: A = monospecific B. abortus antisera; M = monospecific B. melitensis antisera; R = anti-brucella serum.

(+ ) = most strains positive. (+ ) = most strains positive. − = all strains negative. (− ) = most strains negative. L = confluent lysis. Pl = partial lysis. NL = no lysis.
Annex 2. Table 2. Oxidative utilization of recommended substrates by the species of the genus *Brucella* and their biovars

<table>
<thead>
<tr>
<th>Species</th>
<th>Substrate</th>
<th>( L^-) alanine</th>
<th>( L^-) asparagine</th>
<th>( L^-) glutamic acid</th>
<th>( L^-) arginine</th>
<th>( L^-) citrulline</th>
<th>( \alpha^-) ornithine</th>
<th>( L^-) lysine</th>
<th>( D^-) ribose</th>
<th>( D^-) xylose</th>
<th>( D^-) galactose</th>
<th>( D^-) glucose</th>
<th>( L^-) erythritol</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. abortus</em></td>
<td>biovars 1–7, 9</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>B. melitensis</em></td>
<td>biovars 1, 2, 3</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. suis</em></td>
<td>biovar 1</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>biovar 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>biovar 3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>biovar 4</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>biovar 5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>B. neotomae</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. ovis</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. canis</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\*+* oxidized by all strains (rate of oxygen consumption greater than 50 μL/mg of cell nitrogen per hour).

\*-* not oxidized by any strain (rate of oxygen consumption less than 50 μL/mg of cell nitrogen per hour).

\(\frac{1}{2}\) oxidized by some strains.
### Annex 2. Table 3. Phage-typing reactions of non-smooth *Brucella* cultures

<table>
<thead>
<tr>
<th>Species</th>
<th>R</th>
<th>R/O</th>
<th>R/C</th>
<th>lz</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. abortus</em></td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>NL</td>
</tr>
<tr>
<td><em>B. melitensis</em></td>
<td>NL</td>
<td>NL</td>
<td>NL</td>
<td>L</td>
</tr>
<tr>
<td><em>B. suis</em></td>
<td>NL</td>
<td>NL</td>
<td>NL</td>
<td>L</td>
</tr>
<tr>
<td><em>B. neotomae</em></td>
<td>NL</td>
<td>NL</td>
<td>NL</td>
<td>NL</td>
</tr>
<tr>
<td><em>B. ovis</em></td>
<td>NL</td>
<td>L</td>
<td>L</td>
<td>PL</td>
</tr>
<tr>
<td><em>B. canis</em></td>
<td>NL</td>
<td>NL</td>
<td>L</td>
<td>NL</td>
</tr>
</tbody>
</table>

* Lysis detectable at very high phage concentrations (>10⁶ RTD).
* RTD = routine test dilution determined on the recommended propagating strain.
* L = lysis.
* PL = partial lysis.
* NL = no lysis.
* lz = Izatnagar.

### Annex 2. Table 4. Differential characters of *B. abortus* strain 19

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth on media</th>
<th>Growth in the presence of benzylpenicillin sensitivity discs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CO₂ requirement</td>
<td>Thionin blue</td>
</tr>
<tr>
<td>Typical biovar 1</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Variant biovar 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field strains</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>Strain 19*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Strain 19 derived from seed strains from different sources may vary somewhat in both in vitro and in vivo characteristics. Variation in sensitivity to i-erythritol is particularly common and resistant variants are easily selected during subculture or may be isolated from animals receiving the vaccine.

**Additional characteristics of strain 19**: Oxidative metabolic properties. Strain 19 oxidizes D-glutamate much more rapidly than other strains of *B. abortus*. The rate of oxygen consumption (OD₅₀₇₅) is normally greater than 500 µmol of cell nitrogen per hour. Strain 19 shows little or no tendency to oxidize i-erythritol.

**Virulence**: Strain 19 shows a low virulence for the guinea-pig and even large doses of organisms are rapidly cleared from the tissues. By the 11th day after intramuscular inoculation of doses of 5 x 10⁹ viable organisms/kg of tissue and the agglutination titre of the serum should not exceed 1:1000. Comparison should always be made with a strain of known virulence, for example, *B. abortus* strain 544.

+ = all strains positive
(+) = most strains positive
- = all strains negative
(-) = most strains negative

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Annex 2. Table 5. Differential characteristics of *Brucella melitensis* strain Rev. 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Colony size (mm)</th>
<th>Growth on media &lt;sup&gt;a&lt;/sup&gt;</th>
<th>Growth in the presence of sensitivity discs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Thionin 20 μg/ml</td>
<td>Fuchsain 20 μg/ml</td>
</tr>
<tr>
<td>Biovar 1 strain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 M</td>
<td>3-4</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Rev. 1</td>
<td>1-2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Serum dextrose agar medium; incubation in air without supplementary CO₂ for 4 days at 37°C.

<sup>b</sup>As in footnote (a) above but with incubation for 48 hours only.

*B. melitensis* strain Rev. 1 cannot be distinguished from the virulent strain (biovar 1 strain 16 M) of *B. melitensis* by means of the other tests used for routine identification. Strain Rev. 1 shows reduced virulence for guinea-pigs compared with field strains of *B. melitensis*. Doses of 10<sup>6</sup> are cleared from the tissues within 6 months of inoculation.

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Annex 2. Table 6. FAO/WHO species and biovar reference strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Biovar</th>
<th>NCTC</th>
<th>ATCC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. melitensis</em> 16M</td>
<td>1</td>
<td>10 094</td>
<td>23 456</td>
</tr>
<tr>
<td><em>B. melitensis</em> 63/9</td>
<td>2</td>
<td>10 508</td>
<td>23 457</td>
</tr>
<tr>
<td><em>B. melitensis</em> Ether</td>
<td>3</td>
<td>10 509</td>
<td>23 458</td>
</tr>
<tr>
<td><em>B. abortus</em> 544</td>
<td>1</td>
<td>10 093</td>
<td>23 448</td>
</tr>
<tr>
<td><em>B. abortus</em> 86/659</td>
<td>2</td>
<td>10 501</td>
<td>23 449</td>
</tr>
<tr>
<td><em>B. abortus</em> Tula</td>
<td>3</td>
<td>10 502</td>
<td>23 450</td>
</tr>
<tr>
<td><em>B. abortus</em> 292</td>
<td>4</td>
<td>10 503</td>
<td>23 451</td>
</tr>
<tr>
<td><em>B. abortus</em> B3196</td>
<td>5</td>
<td>10 504</td>
<td>23 452</td>
</tr>
<tr>
<td><em>B. abortus</em> B70</td>
<td>6</td>
<td>10 505</td>
<td>23 453</td>
</tr>
<tr>
<td><em>B. abortus</em> B7/75</td>
<td>7</td>
<td>10 506</td>
<td>23 454</td>
</tr>
<tr>
<td><em>B. abortus</em> C68</td>
<td>9</td>
<td>10 507</td>
<td>23 456</td>
</tr>
<tr>
<td><em>B. suis</em> 1330</td>
<td>1</td>
<td>10 315</td>
<td>23 444</td>
</tr>
<tr>
<td><em>B. suis</em> Thomasen</td>
<td>2</td>
<td>10 510</td>
<td>23 445</td>
</tr>
<tr>
<td><em>B. suis</em> C96</td>
<td>3</td>
<td>10 511</td>
<td>23 446</td>
</tr>
<tr>
<td><em>B. suis</em> 40</td>
<td>4</td>
<td>11 364</td>
<td>23 447</td>
</tr>
<tr>
<td><em>B. suis</em> 513</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>B. neotomae</em> 5K33</td>
<td>*</td>
<td>10 084</td>
<td>23 459</td>
</tr>
<tr>
<td><em>B. ovis</em> 63/290</td>
<td>*</td>
<td>10 512</td>
<td>25 840</td>
</tr>
<tr>
<td><em>B. canis</em> RM 5/66</td>
<td>*</td>
<td>10 854</td>
<td>23 365</td>
</tr>
</tbody>
</table>

* No biovars recognized.
NCTC = National Collection of Type Cultures (Great Britain).
ATCC = American Type Culture Collection.
Annex 2. Table 7. Recommended propagating strains for *Brucella* phages

<table>
<thead>
<tr>
<th>Phage strain</th>
<th>Group</th>
<th>Propagating culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tb</td>
<td>1</td>
<td><em>B. abortus</em> S19 (USSR) or <em>B. abortus</em> 544</td>
</tr>
<tr>
<td>Fi 75/13</td>
<td>2</td>
<td><em>B. abortus</em> S19 (USDA)</td>
</tr>
<tr>
<td>Wb</td>
<td>3</td>
<td><em>B. suis</em> 1330</td>
</tr>
<tr>
<td>M51*</td>
<td>3</td>
<td><em>B. suis</em> 1330</td>
</tr>
<tr>
<td>S708*</td>
<td>3</td>
<td><em>B. suis</em> 1330</td>
</tr>
<tr>
<td>MG 75</td>
<td>3</td>
<td><em>B. suis</em> 1330</td>
</tr>
<tr>
<td>D</td>
<td>3</td>
<td><em>B. suis</em> 1330</td>
</tr>
<tr>
<td>Bk4</td>
<td>4</td>
<td><em>B. melitensis</em> Isfahan</td>
</tr>
<tr>
<td>R</td>
<td>5</td>
<td><em>B. abortus</em> B1119R or <em>B. abortus</em> 45/20</td>
</tr>
<tr>
<td>R/O</td>
<td>5</td>
<td><em>B. ovis</em> 63/290</td>
</tr>
<tr>
<td>R/C</td>
<td>5</td>
<td><em>B. canis</em> Mex. 51</td>
</tr>
<tr>
<td>Iz2</td>
<td>6</td>
<td><em>B. melitensis</em> B115</td>
</tr>
</tbody>
</table>

* or M80.  
* or BM29.  
USDA = United States Department of Agriculture.  
Tb = Tbilisi.  
Fi = Firenze.  
Wb = Weybridge.  
Bk = Berkeley.  
Iz = Izanagar.
Annex 3

QUALITY CONTROL OF VACCINES

1. Live vaccines

Quality control methods for B. abortus strain 19 and for B. melitensis strain Rev. 1 vaccines have been described in reports of the WHO Expert Committee on Biological Standardization.1 The examinations required include: (i) verification of the identity of the strain; (ii) verification of the residual virulence; and (iii) estimation of immunogenicity. The first two points have been adequately covered. In contrast, the measurement of immunogenicity should be improved in order to avoid the distribution of strains that are poor in this respect. Improvement can be obtained by using either the guinea pig model, but with a statistically significant number of animals per group (about 30), or using the mouse model described below.

2. Killed vaccines and fractions

The control methods used so far have not been officially recognized or standardized. It is important, however, to standardize the measurement of immunogenicity so that all vaccines may be compared on the same basis.

The mouse method described below gives a statistical measurement of immunogenicity in vaccinal units (VU). For example, a bovine vaccinal dose should contain at least 10 000 VU. From published experimental data, it should be possible to devise a pass-or-fail test.2

Killed vaccines usually contain a large antigenic mass, i.e., 3–5 x 10¹¹ bacterial cells in oil adjuvant. The first control should verify the conformity of this mass with the weight of the residual bacterial cells after extraction of the adjuvant with acetone. If this point is in order, immunogenic activity can be measured and expressed in relation to the cell mass.

The immunogenic activity of bacterial cell-fraction vaccines can be checked directly since the total mass should be very small.

3. Measurement of immunogenic activity in mice

The level of splenic infection of mice challenged under standard conditions (including strain of mice, age, sex, inoculum, route of injection, delay between challenge and spleen count) reflects the degree of immunity. In addition, when killed vaccines have been used, the level of immunity depends on the dose of vaccine injected 1 month before challenge, up to a maximum level that cannot be exceeded even with larger doses. This level was chosen to define the vaccinal unit on a statistical basis.

A lyophilized, killed reference vaccine has been prepared and has to be used in each assay at a dose of 20 VU to give the normal maximum response. Control groups of unvaccinated mice are used to give the zero immunity response. The vaccine under test should naturally give a response between zero and the maximum value.

For live vaccines, a dose of $10^3$ bacteria is injected into 12 mice, in two groups of six. The two vaccinated groups and two similar non-vaccinated groups are then challenged. The immunogenicity of the live vaccine under test is regarded as satisfactory if it does not differ from that of the reference vaccine.

For killed vaccines, the test vaccine is first diluted in saline so that each mouse receives 20 VU. Usually four groups of mice should be used, but more may be used if higher accuracy is required. The immunogenicity is considered satisfactory if the difference between groups of mice that received the reference vaccine (20 VU) and those

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that received the test vaccine is not greater than prescribed statistical limits. Full details have been published by Bosseray et al. (see footnote 4 on page 116).
BRUCELLINS

Brucellins are allergens used for diagnosing animal and human brucellosis. Over the past half century many such preparations have been developed in a number of countries. Currently, two main types of brucellin are in use. These are the LPS-free protein preparations developed at the National Institute of Agronomical Research (INRA), Nouzilly, France, and the Brucella cell hydrolysates employed in a number of European countries and in China.

1. Brucellin INRA

A culture of B. melitensis B115 in the rough phase is prepared in a 20-litre fermentor in an Ames-USDA medium. The culture is interrupted at the beginning of the stationary phase by rapid cooling to 4°C, at which temperature the bacterial concentration is approximately $10^{11}$ per ml. The bacteria are harvested by centrifugation (9000 g for 15 min) at 4°C then washed in sterile distilled water and inactivated by dehydration with acetone. Eighteen litres of the culture yield 100–200 g of dry bacteria which are suspended in a sterile solution of NaCl (25 g/litre) by means of a homogenizer.

After agitation for three days at 4°C, the suspension is centrifuged as above. The supernatant is concentrated four times by ultrafiltration on a Diaflo PM 10 membrane. Three volumes of ethanol are then added slowly to one volume of the concentrate. After 24 hours the precipitate is harvested by centrifugation (11 500 g, 15 min, 4°C), dissolved in distilled water and ultracentrifuged (105 000 g, 6 hours, 4°C). The freeze-dried supernatant is the brucellin.

Physicochemical properties

Proteins, determined by the Lowry method, constitute 50–75% of the brucellin, and neutral monosaccharides (Orcinol method) make up 15–30%.

Analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate shows 30 bands, with the major bands at
70,000–72,000 relative molecular mass (RMM), 35,000–40,000 RMM, and 16,000–18,000 RMM. Analysis by isoelectric focusing discloses 20 bands.

**Immunological properties**

The serum of hyperimmunized rabbits\(^1\) yields: with simple immunoelectrophoresis, 8–10 immunoprecipitation arcs with 2-dimensional immunoelectrophoresis, 18–22 immunoprecipitation peaks. This serum produces no reaction in agglutination and complement-fixation tests with the S and R antigens used in routine serology.

**Biological properties**

The intradermal injection of brucellin produces neither a local skin reaction nor a general reaction in non-sensitized animals. It does, however, produce a delayed hypersensitivity skin reaction, which is specific to the *Brucella* genus, in animals sensitized by infection, vaccination, or the injection of brucellin with Freund's complete adjuvant. The reaction reaches a peak after 24 hours in guinea-pigs, 48 hours in sheep, goats, and swine, and 72 hours in cattle. This injection does not sensitize guinea-pigs or cattle and causes no increase in antibody titres in routine serological tests carried out on sheep and cattle, whether or not the animals are infected. It does not produce any immunity against brucellosis in mice or guinea-pigs that are subsequently challenged by experimental infection.

**Titration of biological activity**

Two methods of titration have been studied at INRA, Nouzilly. In the first, guinea-pigs were sensitized with the *B. abortus* 544 strain, and in the second with brucellin in Freund's complete adjuvant. The second method is the one recommended; injecting brucellin in Freund's complete adjuvant produces greater and more stable sensitivity than does *B. abortus* 544. Sensitization using brucellin in complete adjuvant lasts at least six months, thus enabling the same

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\(^1\) Three rabbits are given 5 consecutive injections at 1-week intervals of 5 mg of brucellin emulsified in 0.5 ml of Freund's incomplete adjuvant and are bled 1 week after the last injection.
animals to be used for a number of successive titrations. A calculation method can be used to reject products that do not conform with a reference preparation of brucellin.

**Utilization in diagnosing brucellosis**

The conditions in which brucellin can be used were studied at Nouzilly for sheep, goats, and cattle. In sheep, a solution of 50 μg of brucellin in buffered saline was injected into the lower eyelid, either subcutaneously (total volume 0.5 ml) or intradermally (total volume 0.1 ml) using a pneumatic syringe. In goats, a solution of 50 μg of brucellin was injected intradermally (total volume 0.1 ml) in the neck. In cattle, a solution of 100 μg of brucellin was injected intradermally (total volume 0.1 ml) in the neck.

**Efficacy of allergic diagnosis**

The efficacy of the allergic diagnosis was determined by comparing the results obtained by this method with those obtained from serological tests and bacteriological studies on herds of cattle and sheep. Allergic reactions were observed in herds in which either serological or bacteriological tests had shown some of the animals to be infected. No such reactions were observed in herds in which none of the animals was infected. Some animals showed an allergic reaction and positive serological results simultaneously and others showed either an allergic reaction without antibodies or vice versa. The allergic test was useful, therefore, with whole herds, but not as useful for testing individual animals.

In herds of cattle that had been recently infected, the allergic test seemed to give positive results earlier than the serological tests. The allergic test could thus be valuable in resolving the question of “problem herds”, and experience has shown that in cattle any skin reaction, no matter how slight, should be taken into account as reactions to brucellin are often less marked than those observed after injecting tuberculous subjects with tuberculin.

The allergic test is now recommended in France for diagnosing ovine brucellosis as part of the official prevention policy to compensate for the fact that very often sero-agglutination tests do not give significant results and because taking blood samples is a long and complicated operation. The absence of allergic reactions should be interpreted as signifying the absence of infection, while the
presence of a reaction means that the usual serological tests should be carried out to investigate for antibodies.

**Allergic diagnosis of human brucellosis**

In man, brucellin could be a valuable substitute for the unpurified allergens which are currently in use and which occasionally cause severe skin reactions. A number of hospitals are at present carrying out research into this use of brucellin. The doses administered are between 0.5 µg and 1 µg and are injected intradermally (0.1 ml) into the forearm. Readings must be taken after 24 and 48 hours. A reaction with a diameter of 5 mm or more should be considered as positive.

2. **Brucellin fraction F**

Acid hydrolysis has been used to split *Brucella* protein molecules into components of substantially reduced relative molecular mass, which do not stimulate antibody production.

One such preparation is the *Brucella* allergen fraction F, isolated from a hydrolysate of smooth *Brucella* cells (acid hydrolysis at pH 1.2–2.0 and 110°C). The fraction is a mixture of polypeptides (RMM 2000–3000) with some amino acids and polysaccharides. It does not contain lipids. The fraction is non-antigenic and it does not sensitize animals against repeated injections of the allergen, even at very high doses (1000 times that used for diagnosis). Immunologically, it behaves as a hapten; it is precipitated by positive sera, but does not fix complement. It is soluble at pH 6.8–7.0. The concentration required for diagnostic purposes is determined according to protein content and by titration in sensitized guinea pigs and sheep.

*Brucella* fraction F allergen provokes easily visible and palpable local reactions when injected intradermally in doses of 1–10 mg. In *Brucella*-free animals no reactions are observed.

The allergic skin test is officially approved for use in small ruminants and swine as a basic test in Czechoslovakia, but it is also used in other animal species for solving problematic cases (cross-reactions, etc.). The same preparation is also used for allergic diagnosis of human brucellosis.

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*Brucella* allergens of this type are routinely produced on a large scale in China, Czechoslovakia, Federal Republic of Germany, Greece, Mongolia, and the USSR.
PROCEDURES FOR THE SERUM AGGLUTINATION TEST WITH NON-SMOOTH BRUCELLA ANTIGENS

The two buffer systems recommended for preparing stable suspensions of non-smooth Brucella organisms are the Menzel's carbonate/bicarbonate buffered saline used by Shimizu & Shibata\(^1\) and a modification of the tris(hydroxymethyl)aminomethane-maleic acid (TM) buffer employed by George & Carmichael\(^2\) in the B. canis slide agglutination test (see below for details of these buffers). The antigen suspensions in each case are prepared by the following procedure:

1. The organisms are grown on Roux flasks of serum dextrose agar or other suitable medium incubated at 37°C in air (+ 100 ml of CO\(_2\) per litre if required) until satisfactory growth is produced. For most rough Brucella strains this is usually about two days but B. ovis cultures normally require 3–4 days' incubation. For cultures of B. canis or other mucoid strains, however, it is essential that incubation should not exceed 24 hours, and that the medium should be well buffered.

2. The organisms are harvested in sterile distilled water and immediately killed by heating with constant agitation at 60°C for 1 hour.

3. The killed suspension is strained through stainless steel gauze or muslin and the organisms washed by repeated centrifugation in sterile distilled water. The washed cells are finally deposited by centrifugation at 20,000 g for 10 minutes.

4. The deposited cells are resuspended either in Menzel's buffer, pH 8.9 or TM buffer, pH 8.9, and adjusted to an OD\(_{465}\) of 3.30. The buffered suspension is then titrated for sensitivity against a reference serum of known agglutinating activity.

All these operations should be performed as a single uninterrupted sequence.

5. Suspensions prepared in Menzel's buffer are stored in tightly closed containers kept in the dark at 4°C. They are discarded if not

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used within 4 weeks. Suspensions made in TM buffer may be stored under similar conditions but are best preserved by snap-frying small volumes in liquid nitrogen and drying from the frozen state. The freeze-dried antigen is then sealed in ampoules and will maintain its titre for at least 2 years if stored at 4°C. It is reconstituted by adding distilled water and making up to the original volume.

(6) The serum agglutination test is performed by making doubling dilutions of serum, in either Menzel's or TM buffer according to the antigen preparation used, in the range 1/10 to 1/5120. Unit volumes of 0.5 ml are used. An equal volume of standard antigen suspension in the corresponding buffer is then added and the test incubated at 37°C for 18-24 hours. The test is then read as for the standard tube agglutination test with smooth B. abortus antigen. Known positive and negative control sera are included with each batch of tests, together with opacity standards representing 5, 25, 50% and 75% agglutination.

Procedure for the Coombs antiglobulin test

(1) The tube agglutination test is performed as described.
(2) After reading the test, each tube is labelled with the serum reference number and the dilution number, and the antigen is centrifuged out of suspension (e.g., at 3000 g for 30 minutes). The supernatant is discarded.
(3) The deposited antigen is washed by resuspending in the buffer system used in the agglutination test and then redepositing by centrifugation. This process is repeated twice.
(4) Finally, the washed cells are resuspended in 0.5-ml volumes of the appropriate species-specific antiglobulin serum diluted to optimum titre in the corresponding buffer. The tubes are then reincubated at 37°C for 18-24 hours and read as for the tube agglutination test.

In general, results obtained with antigens made from the two buffer systems are comparable although the TM buffer produces a slightly more sensitive antigen. It is, however, also more prone to give non-specific reactions with certain sera. This tendency is much more pronounced if a lower pH is employed. Non-specific agglutination then becomes particularly troublesome with haemolyzed serum samples. Raising the pH of the buffer system to 8.9 also eliminates low-grade agglutinating activity towards rough Brucella organisms which is present in many serum samples. No
evidence has been obtained from studies with sera from experimentally infected animals to suggest that the high pH interferes with the detection of specific antibody.

Buffer composition

1. Menzel's carbonate/bicarbonate buffered saline

This is prepared from two stock solutions A and B.

A. 5 g of NaCl + 5 g of phenol dissolved in 1 litre of 0.5 mol/litre Na$_2$CO$_3$ solution
B. 5 g of NaCl + 5 g of phenol dissolved in 1 litre of 0.1 mol/litre NaHCO$_3$ solution

For use, 1.5 volumes of solution A and 8.5 volumes of solution B are mixed to give a solution of pH 8.9.

2. Tris(hydroxymethyl)aminomethane-maleic acid (TM) buffer

Two stock solutions are prepared:

A. Tris(hydroxymethyl)aminomethane
   Maleic acid
   Distilled water to
   242 g
   232 g
   1 litre

B. 5 mol/litre NaOH

The buffer is made by mixing 200 ml of solution A with 140 ml of solution B and making up to 1 litre with distilled water to give a pH of 8.9.
PROGRAMMES AND CENTRES RESPONSIBLE FOR INTERNATIONAL SURVEILLANCE AND INFORMATION EXCHANGE ON BRUCELLOSIS

The Expert Committee stressed that national authorities should be aware of the major regular surveillance activities carried out by international organizations and institutions concerning the occurrence and control of brucellosis. The following list of organizations and publications may not be complete, but includes some statistical reports as well as information exchange services dealing with scientific developments and epidemiological events.

1. World Health Organization (WHO)

1211 Geneva 27
Switzerland
Tel.: (022) 91 21 11
Telex: 27821
Cable: UNISANTE GENEVA

*Weekly epidemiological record*
*World health statistics annual*

2. Food and Agriculture Organization of the United Nations (FAO)

Via delle Terme
di Cassia
00100 Rome
Italy
Tel.: 57971
Telex: 610181
Cable: FOODAGRI ROME

*Animal health yearbook, FAO–WHO–OIE (annual)*

3. Pan American Health Organization
WHO Regional Office for the Americas (PAHO/WHO)

525, 23rd Street, N.W.
Washington, DC, 20037
USA
Tel.: (202) 861–3200
Telex: 248338
Cable: OFSANPAN WASHINGTON

*Epidemiological bulletin (bimonthly)*
Pan American Zoonoses Center (PAHO/WHO)
Casilla 3092
Correo Central
1000 Buenos Aires
Argentina
Tel.: 792 4047-48
Telex: 24577 CPZ ar
Cable: CEFANZO RAMOS MEJIA
(ARGENTINA)
Informative bulletin: Brucellosis in the Americas (irregular)

Caribbean Epidemiology Centre (CAREC – PAHO/WHO)
P.O. Box 164
Port-of-Spain
Trinidad
Tel.: 62-24745, 62-23277
Telex: 398
Cable: CAREC PORT OF SPAIN (TRINIDAD)
Carec surveillance report (monthly)
Review of communicable diseases in the Caribbean (annual)

4. International Office of Epizootics (OIE)
12, rue de Prony
75017 Paris
France
Tel.: 227 45 74
Telex: EPIZOTI 64285 F
Cable: INTEREPIZOOTIES PARIS
Statistiques O.I.E. (annual)
Bulletin of the International Office of Epizootics (monthly)

5. Mediterranean Zoonoses Control Centre
P.O. Box 3904
10210 Athens
Greece
Tel.: 6399.367 6380.163
Telex: 222670 MZCC GR
Information circular (quarterly)
Annex 7

ADDRESSES OF INTERNATIONAL INSTITUTIONS
FOR TECHNICAL COOPERATION
IN BRUCELLOSIS CONTROL

The following WHO services and centres, and other international organizations and institutions are ready to collaborate with national services on request:

1. Zoonoses centres

The Director
Pan American Zoonoses Center
Casilla 3092
Correo Central
1000 Buenos Aires
Argentina

The Director
Mediterranean Zoonoses Control Centre
P.O. Box 3904
10210 Athens
Greece

2. International centres for biological standards, reference preparations, and reference reagents

International Laboratory for Biological Standards
Central Veterinary Laboratory
Weybridge
Surrey KT15 3NB
England

International Laboratory for Biological Standards
Statens Serum Institut
80 Amager Boulevard
Copenhagen
Denmark

3. Collaborating and related reference centres

(a) Brucellosis

The Director
WHO Collaborating Centre for Research and Training
in Brucellosis and Leptospirosis
(b) Veterinary public health

The Director
WHO Collaborating Centre for Research and Training in
Veterinary Public Health
Veterinary Research Institute
Hudcova 71
62132 Brno 21
Czechoslovakia

The Director
WHO Collaborating Centre for Research and Training in
Veterinary Public Health
School of Veterinary Medicine
Bischofsholer Damm 15
3000 Hanover 1
Federal Republic of Germany

The Director
FAO/WHO Collaborating Centre for Research and Training in
Veterinary Public Health
Indian Veterinary Research Institute
Modular Laboratory Building
Izatnagar 243122
Bareilly U.P.
India
(c) Food Hygiene and Zoonoses

The Director
FAO/WHO Collaborating Centre for Research and Training
in Food Hygiene and Zoonoses
Institute of Veterinary Medicine
(Robert von Ostertag Institute)
Postfach 330013
Thielallee 88/92
D-1000 Berlin 33

The Director
WHO Collaborating Centre for Prevention and Control of Zoonoses
All-Union Institute for Experimental Veterinary Medicine
Kuzminky, VIEV
Moscow 109472
USSR

The Director
WHO Collaborating Centre for Veterinary Sanitation and Food Hygiene
All-Union Research Institute for Veterinary Sanitation
Zvenigorodskoe shosse 65
Moscow D-22
USSR

4. International organizations and services

Chief, Veterinary Public Health
Division of Communicable Diseases
World Health Organization
1211 Geneva 27
Switzerland

Regional Director
WHO Regional Office for Africa
PO Box No. 6
Brazzaville
Congo

Regional Director
WHO Regional Office for the Americas/Pan American Sanitary Bureau
525, 23rd Street NW
Washington, DC 20037
USA
Regional Director
WHO Regional Office for South-East Asia
World Health House
Indraprastha Estate
Mahatma Gandhi Road
New Delhi 110002
India

Regional Director
WHO Regional Office for Europe
8 Scherfigsvej
DK-2100 Copenhagen
Denmark

Regional Director
WHO Regional Office for the Eastern Mediterranean
PO Box 1517
Alexandria – 21511
Egypt

Regional Director
WHO Regional Office for the Western Pacific
PO Box 2932
Manila 2801
Philippines

Director
Animal Production and Health Division
Food and Agriculture Organization of the United Nations (FAO)
Via delle Terme di Caracalla
00100 Rome
Italy

Director-General
Office International des Epizooties (OIE)
12, rue de Prony
75017 Paris
France

Director
Inter-african Bureau for Animal Resources
Organization of African Unity (OAU)
P.O. Box 30786
Nairobi
Kenya

Director-General
Arab Organization for Agricultural Development
Sharia El Gamaa
Khartoum
Sudan
The President
Commission of the European Communities (CEC)
200, rue de la Loi
1049 Brussels
Belgium

5. Nongovernmental organizations

Secretary-General
International Council for Laboratory Animal Science (ICLAS)
University Laboratory of Physiology
Parks Road
Oxford OXI 3PT
England

World Wildlife Fund
Avenue du Mont-Blanc
1196 Giand
Switzerland