CHAPTER 1.1.9.

TESTS OF BIOLOGICAL MATERIALS FOR STERILITY AND FREEDOM FROM CONTAMINATION

INTRODUCTION

Sterility is defined as the absence of living organisms. It is achieved by heating, by filtration, by treatment with ethylene oxide or by ionising irradiation, and by conducting any subsequent processes aseptically. Freedom from contamination is defined as the absence of specified living organisms. This may be achieved by selecting materials from sources shown to be free from the specified organisms and by conducting subsequent procedures aseptically. Adequate assurance of sterility and freedom from contamination can only be achieved by proper control of the primary materials used and their subsequent processing and storage. Tests on the product are necessary to check that this control has been achieved.

A. GENERAL PROCEDURES

1. Primary materials must be collected from sources shown to be free from contamination and handled in such a way as to minimise contamination and the opportunities for any contaminants to multiply.

2. Materials that can be sterilised without their biological activities being affected unduly must be sterilised by a method effective for the materials concerned. The method must reduce the level of contamination to be undetectable, as determined by an appropriate sterility test (see paragraph B.3 below).

3. If a sterilisation process is used, it shall be validated to demonstrate its suitability and adequately controlled to show that it has functioned properly on each occasion.

4. Materials that are not sterilised and those that are to be processed further after sterilisation must be handled aseptically.

5. The environment in which any aseptic handling is carried out must be maintained in a clean state and protected from external sources of contamination, and must be controlled to prevent internal contamination.

B. LIVING VIRAL VACCINES FOR ADMINISTRATION BY INJECTION

1 Materials of animal origin shall be (a) sterilised, or (b) obtained from healthy animals that, in so far as is possible, should be shown to be free from pathogens that can be transmitted from the species of origin to the species to be vaccinated, or any species in contact with them, or (c) the material shall be shown to be free from such pathogens.

2. Seed lots of virus and of any continuous cell line used for virus growth shall be shown to be free from bacteria, fungi, mycoplasmas, extraneous viruses and other pathogens that can be transmitted from the species of origin to the species to be vaccinated or any species in contact with them. For the production of avian vaccines and the quality control procedures for these vaccines, it is recommended that specific pathogen-free embryonated chicken eggs be used.

3. Each batch of vaccine shall pass a test for sterility that is similar to published methods (Code of Federal Regulations, 2007; European Pharmacopoeia, 2012; European Union, 1999; WHO, 1998).

4. Each batch of vaccine shall pass tests appropriate to prove that the vaccine is free from extraneous viruses. (Such tests include tests in cell cultures susceptible to viruses of the species to be vaccinated, tests in embryonated eggs, and, where necessary, tests in animals.)

5. Some countries require that each batch of vaccine pass a test for freedom from mycoplasma. Suitable test methods have been published (Code of Federal Regulations, 2007; European Pharmacopoeia, 2012; WHO, 1998).

6. Tests for freedom from certain specific bacteria may be required, e.g. tests for *Salmonella*, *Mycobacterium tuberculosis* and *M. paratuberculosis*, *Brucella* spp. and *Leptospira* spp. (Code of Federal Regulations, 2007; European Pharmacopoeia, 2012).

C. LIVING VIRAL VACCINES FOR ADMINISTRATION THROUGH DRINKING WATER, SPRAY, OR SKIN SCARIFICATION

1. Paragraphs B.1, 2, 4, 5, and 6 apply.

2. A limited number of contaminating, nonpathogenic bacteria and fungi may be permitted (see Section J.2.5).

D. INACTIVATED VIRAL VACCINES

1. Paragraphs B.1, 2 and 3 apply.

2. Each batch of vaccine shall pass a test for inactivation of the vaccinal virus. This is done before addition of preservative. The inactivation process and the tests used to detect live virus after inactivation must be validated and shown to be suitable for their intended purpose.

3. Demonstration that the method of inactivation also inactivates representative pathogens may be required unless the vaccine satisfies the conditions of paragraphs B.4 and B.5.

E. LIVING BACTERIAL VACCINES

1. Paragraphs B.1 applies.

2. Seed lots of bacteria shall be shown to be free from other bacteria as well as fungi and mycoplasmas.

3. Each batch of vaccine shall pass a test for purity carried out using solid media and ignoring the growth of the vaccinal bacterium.

4. Some countries require that each batch of bacterial vaccine passes a test for freedom from mycoplasmas. Suitable test methods have been published (WHO, 1998, and, for avian mycoplasmas: European Pharmacopoeia, 2012).

F. INACTIVATED BACTERIAL VACCINES

1. Paragraphs B.1, B.3, and E.2 apply.

2. Each batch of vaccine shall pass a test for inactivation of the vaccinal bacterium. If appropriate, the test for sterility may be used for this purpose.

G. SERA FOR ADMINISTRATION TO ANIMALS

1. Paragraph B.1 applies. Some countries require quarantine, health certification, and specific disease tests be completed for all serum donor animals (Code of Federal Regulations, 2007).

2. Paragraph B.2 or E.2 applies, as appropriate, if a virus or a bacterium is used in serum production.

3. Each batch of serum shall pass a test for sterility. Suitable test methods have been published (Code of Federal Regulations, 2007; European Pharmacopoeia, 2012).

4. Each batch of serum shall pass tests appropriate to prove that the serum is free from extraneous viruses. (Such tests include tests in cell cultures susceptible to viruses of the species to be treated, tests in embryonated eggs and, where necessary, tests in animals.)

5. Some countries require that each batch of serum passes a test for freedom from mycoplasmas. Suitable test methods have been published (WHO, 1998, and, for avian mycoplasmas: European Pharmacopoeia, 2012).

H. DIAGNOSTIC AGENTS FOR ADMINISTRATION TO ANIMALS

1. Paragraphs B.1 and 3 apply.

2. Paragraphs B.2 and D.2 apply if a virus is used in the production of the diagnostic agent; E.2 and F.2 apply if a bacterium is used.

I. EMBRYOS, OVA, AND SEMEN

Special precautions must be taken with relation to the use of embryos, ova and semen (Hare, 1985).

J. PROTOCOL EXAMPLES

1. General procedures

Materials used in the production of biological products should be sterilised and/or tested to ensure freedom from contaminants before being used. Samples of the finished biological product should also be tested for bacterial, fungal, or mycoplasmal contaminants.

The assays for bacteria, mycoplasma, fungi, and viruses described here are derived from various sources and they are given as examples of methods that can be used with confidence.

2. Detection of bacteria and fungi

These assays describe the materials and methods that are used for the detection of bacteria and fungi by either the membrane filtration method, or the direct inoculation of fluid media method used for materials that are unsuitable for membrane filtration.

2.1. General procedure for detecting viable bacteria and fungi

Standard tests for detecting extraneous bacteria and fungi in raw materials, seed stocks, or final product are: the membrane filtration test or the direct inoculation sterility test.

For the membrane filtration technique, a filter having a nominal pore size not greater than 0.45 µm and a diameter of at least 47 mm should be used. Cellulose nitrate filters should be used if the material is aqueous or oily; cellulose acetate filters should be used if the material is strongly alcoholic, oily or oil-adjuvanted. Immediately before the contents of the container or containers to be tested are filtered, the filter is moistened with 20–25 ml of Diluent A or B.

Diluent A – for aqueous products or materials: Dissolve 1 g peptic digest of animal tissue in water to make 1 litre, filter or centrifuge to clarify, adjust the pH to 7.1 ± 0.2, dispense into containers in 100 ml quantities, and sterilise by steam.

Diluent B – for oil-adjuvanted products or materials: Add 1 ml polysorbate 80 to 1 litre Diluent A, adjust the pH to 7.1 ± 0.2, dispense into containers in 100 ml quantities, and sterilise by steam.

If the biological being tested has antimicrobial properties, the membrane is washed three times after sample application with approximately 100 ml of the appropriate diluent (A or B). The membrane is then transferred whole to culture media, aseptically cut into equal parts and placed in media, or the media is transferred to the membrane in the filter apparatus. If the test sample contains merthiolate as a preservative, fluid

thioglycollate medium (FTM) is used and the membranes are incubated at both 30–35°C and 20–25°C. If the test sample is a killed biological without merthiolate preservative, FTM is used at 30–35°C and soybean casein digest medium (SCDM) at 20–25°C. If the sample tested is a live viral biological, SCDM is used at both incubation temperatures. Recently, it has been suggested that sulfite-polymyxin-sulfadiazine agar be used to enhance the detection of *Clostridium* spp. when the membrane filtration technique is used (Tellez *et al*., 2005).

If direct inoculation of culture media is chosen, a sterile pipette or syringe and needle are used to aseptically transfer the biological material directly into liquid media. If the biological being tested has antimicrobial properties, the ratio of the inoculum to the volume of culture medium must be determined before the test is started. To determine the correct medium volume to negate antimicrobial activity, 100 colony-forming units (CFU) of the control microorganisms listed in Table 1 are used. If the test sample contains merthiolate as a preservative, FTM is used in test vessels incubated at both 30–35°C and 20–25°C. Growth should be clearly visible after an appropriate incubation time (see Section J.2.2). If the test sample is a killed biological without merthiolate, or a live bacterial biological, FTM is used at 30–35°C and SCDM at 20–25°C. If the test sample is a live viral biological, SCDM is used at both incubation temperatures. If the inactivated bacterial vaccine is a clostridial biological, or contains a clostridial component, the use of FTM with 0.5% added beef extract (FTMB) in place of FTM is preferred. It may also be desirable to use both FTM and SCDM for all tests.

Table 1*. Some American type culture collection*[1] *strains with their respective medium and incubation conditions*

		Incubation	
Medium	**Test microorganism**	**Temperature (°C)**	**Conditions**
FTM	*Bacillus subtilis* ATCC # 6633	30–35	Aerobic
FTM	*Candida krusei* ATCC # 6258	20–25	Aerobic
SCDM	*Bacillus subtilis* ATCC # 6633	30–35	Aerobic
SCDM	*Candida kursei* ATCC # 6258	20–25	Aerobic
FTMB	*Clostridium sporogenes* ATCC # 11437	30–35	Anaerobic
FTMB	*Staphylococcus aureus* ATCC #6538	30–35	Aerobic

For both membrane filtration and direct inoculation sterility tests, all media are incubated for no fewer than 14 days. At intervals during incubation, and after 14 days' incubation, the test vessels are examined for evidence of microbial growth. Microbial growth should be confirmed by subculture and Gram stain.

2.2. Growth promotion and test interference

The sterility of the media should be confirmed by incubating representative containers at the appropriate temperature for the length of time specified for each test.

The ability of the culture media to support growth in the presence and absence of product, product components, cells, seeds, or other test material should be validated for each product to be tested, and for each new batch or lot of culture media.

To test for ability to support growth in the absence of the test material, media should be inoculated with 10–100 viable control organisms of the suggested American Type Culture Collection (ATCC) strains listed in Table 1 and incubated according to the conditions specified.

To test for ability of the culture media to support growth in the presence of the test material, containers should be inoculated simultaneously with both the test material (see Section J.2.3) and 10–100 viable control organisms. The number of containers used should be at least one-half the number used to test the product or product component. The test media are satisfactory if clear evidence of growth of the control organisms appears in all inoculated media containers within 7 days. In the event that growth is evident, the organism should be identified to confirm that it is the organism originally added to the medium. The sterility test is considered invalid if any of the media show inadequate growth response, or if the organism recovered is not the organism used to inoculate the material.

2.3. Number of items to be tested

1 American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA.

The number of items in a batch determines the number of containers that should be tested for sterility. If the batch size is not more than 100, then 10% or four containers, whichever is the greater, should be tested. If the batch contains between 100 and 500 containers, then ten containers should be tested. If the batch has more than 500 containers, then 2% or 20 containers, whichever is the lesser, should be tested. An alternative is to test a maximum of 10 containers for all serials other than autogenous products.

The amount of sterility test inoculum is dependent on the quantity of biological in each container. If the quantity is less than 1 ml, then the entire contents are used for each medium. If the quantity is from 1 to 4 ml, then half the contents are used in each medium. If the quantity is from 4 to 20 ml, then 2 ml inoculum per medium is used. If the quantity in each container is from 20 to 100 ml, then 10% of the contents are used per medium. If the quantity per container is greater than 100 ml, then 10% or 50 ml, whichever is the greater, is used to inoculate each medium.

2.4. Interpretation of sterility test results

If growth is found in any medium but it can be demonstrated by controls that the media or technique were faulty, then the first test is declared invalid and may be repeated. If microbial growth is found in any of the test vessels of the first test but there is no evidence invalidating it, then a retest may be conducted. The minimum number of biological containers, test vessels, and membrane filters in a retest is double the number used in the first test. If no growth is found in the first test or retest, the biological meets the requirements of the test and is considered satisfactory for sterility. If microbial growth is found in any of the retest vessels, the biological is considered unsatisfactory for sterility. If, however, it can be demonstrated by controls that the media or technique of the retest were faulty, then the retest may be repeated.

2.5. General procedure for testing live viral vaccines produced in eggs and administered through drinking water, spray, or skin scarification for the presence of bacteria and fungi

Each batch of final container biological should have an average contamination of not more than one bacterial or fungal colony per dose for vaccines recommended for poultry, or ten colonies per dose for other animals (see Section J.2.3 above to determine the number of samples to test). From each container sample, each of two Petri dishes are inoculated with vaccine equal to ten doses if the vaccine is recommended for poultry, or one dose if recommended for other animals. To each plate add 20 ml of brain–heart infusion agar containing 0.007 IU (International Units) of penicillinase per ml. One plate should be incubated at 30–35°C for 7 days and the other at 20–25°C for 14 days. Colony counts are made at the end of each incubation period. An average colony count of all the plates representing a batch should be made for each incubation condition. If the average count at either incubation condition exceeds one colony per dose for vaccines recommended for poultry, or ten colonies per dose for vaccines recommended for other animals in the initial test, one retest to rule out faulty technique may be conducted using double the number of unopened final containers. If the average count at either incubation condition of the final test for a batch exceeds one colony per dose for vaccines recommended for poultry, or ten colonies per dose for vaccines recommended for other animals, the batch of vaccine should be considered unsatisfactory.

2.6. General procedure for testing seed lots of bacteria and live bacterial biologicals for purity

Each seed lot of bacteria or batch of live bacterial biological should be tested for purity by inoculation of SCDM, which is incubated at 20–25°C for 14 days, and FTM, which is incubated at 30–35°C for 14 days (see Section J.2.3 above to determine the number of samples to be tested and the amount of test inoculum to be used). A sterile pipette or syringe and needle is used to aseptically transfer the quantity of biological directly into the two types of culture medium. The minimum ratio of inoculum to culture medium is 1/15.

If the inoculum or growth of the bacterial vaccine renders the medium turbid so that the absence of atypical microbial growth cannot be determined by visual examination, subcultures should be made from all turbid tubes on day 3 through to day 11. Subculturing is done by transferring 0.1–1.0 ml to differential broths and agar and incubating for the balance of the 14-day period. Microscopic examination by Gram stain should also be done.

If no atypical growth is found in any of the test vessels when compared with a positive control included in the test, the lot of biological may be considered satisfactory for purity. If atypical growth is found but it can be demonstrated by control that the media or technique were faulty, then the first test may be repeated. If atypical growth is found but there is no evidence invalidating the test, then a retest may be conducted. Twice the number of biological containers and test vessels of the first test are used in the retest. If no atypical growth is found in the retest, the biological is considered to be satisfactory for purity. If atypical growth is found in any of the retest vessels, the biological is considered to be unsatisfactory for purity. If, however, it can be demonstrated by controls that the media or technique of the retest were faulty, then the retest may be repeated.

3. Detection of *Mycoplasma* contamination

3.1. General procedure for detecting *Mycoplasma* contamination

Each batch of live viral vaccine, each lot of master seed virus (MSV), each lot of primary and master cell stock (MCS), and all ingredients of animal origin not steam sterilised should be tested for the absence of mycoplasmas. Solid and liquid media that will support the growth of small numbers of test organisms, such as typical contaminating organisms *Acholeplasma laidlawii*, *Mycoplasma arginini*, *M. fermentans*, *M. hyorhinis*, *M. orale*, and *M. synoviae* should be used. The nutritive properties of the solid medium should be such that no fewer than 100 CFU should occur with each test organism when approximately 100–200 CFUs are inoculated per plate. An appropriate colour change should occur in the liquid media when approximately 20–40 CFUs of each test organism are inoculated. The ability of the culture media to support growth in the presence of product should be validated for each product to be tested, and for each new batch or lot of culture media.

One sample of each lot of vaccine, MSV, etc., should be tested. Inoculate each of four plates of solid medium with 0.25 ml of the sample being tested, and inoculate 100 ml of the liquid medium with 10 ml of the sample. An alternative is to inoculate each of the plates with 0.1 ml and to inoculate 100 ml of liquid medium with 1 ml of the sample being tested. Incubate two plates at 35–37°C aerobically (an atmosphere of air containing 5–10% CO_2 and adequate humidity) and two plates anaerobically (an atmosphere of nitrogen containing 5–10% CO_2 and adequate humidity) for 28 days. On day 3 or day 4 after inoculation, subculture 0.25 ml from the liquid media on to two plates of solid media. Incubate one plate aerobically and the second anaerobically at 35–37°C until day 28 of the test. Repeat the subculture procedure on day 6, 7, or 8 and again on day 13 or 14. An alternative method is to subculture on days 3, 5, 10, and 14 on to a plate of solid medium. All the subculture plates are incubated for 10 days except for the 14-day subculture, which is incubated for 14 days. Observe the liquid media every 2–3 days and, if any colour change occurs, subculture immediately.

3.2. Interpretation of *Mycoplasma* test results

At the end of the incubation period (day 28), examine all the inoculated solid media microscopically for the presence of mycoplasma colonies. The test sample passes the test if the growth of mycoplasma colonies has occurred on the positive controls, and if growth has not occurred on any of the solid media inoculated with the test material. If at any stage of the test, more than one plate is accidentally contaminated with bacteria or fungi, or is broken, the test is invalid and should be repeated. If mycoplasma colonies are found on any agar plate, the test should be repeated once to confirm the mycoplasma contamination. Twice the volume (0.5 ml) of biological material being tested may be used in the retest. If mycoplasma colonies are found on any of the agar plates of the retest, the test sample should be considered unsatisfactory because of mycoplasma contamination. Some mycoplasmas cannot be cultivated, in which case the MSV and MCS have to be tested using an indicator cell line (Vero cells), DNA staining, or polymerase chain reaction (PCR) methods.

4. Detection of *Salmonella* contamination

Each batch of live virus biological made in eggs should be free from contamination with *Salmonella*. This testing must be done before bacteriostatic or bactericidal agents are added. Five samples of each batch should be tested; 5 ml or one-half of the container contents, whichever is the lesser, of the sample should be used to inoculate 100 ml of tryptose broth and tetrathionate broth. The inoculated broths should be incubated for 18–24 hours at 35–37°C. Transfers from these broths should be made on to MacConkey and Salmonella–Shigella agar, incubated for 18–24 hours, and examined. If no growth typical of *Salmonella* is noted, the agar plates should be incubated an additional 18–24 hours and again examined. If colonies typical of *Salmonella* are observed, further subculture on to suitable differential media should be made for positive identification. If *Salmonella* is found, the batch of biological is unsatisfactory.

5. Detection of viruses in biological materials

Biological materials subject to viral contamination that cannot be sterilised before use, such as ingredients of animal origin (for example, serum), primary cells, line cells or viral seed stocks, should be tested before they are used. Assays are described to detect viral contaminants by cytopathic effects (CPE), haemadsorption, haemagglutination, fluorescent antibody techniques and other suitable methods, e.g. PCR and enzyme-linked immunosorbent assay. All biological materials should be specifically tested for pestiviruses. Avian materials and vaccines should be inoculated on to primary avian cell cultures, eggs and/or chicks for the detection of avian viruses. In addition to examining for CPE and cellular abnormalities in these inoculated cells/eggs/chicks, tests for haemadsorbing and haemagglutinating viruses should also be included.

Cells shall be tested in the following manner. On day 0, primary or frozen cells to be tested are seeded on 75 cm^2 (or similar) flasks; 7 days later, at least two 75 cm^2 flasks are prepared. On day 14, one flask is used to test the cells for cytopathology, haemadsorption, and fluorescent antibody staining (procedures follow). The other flask is

passaged a second time, and on day 21 is subjected to three freeze–thaw cycles. An alternative method is to freeze–thaw the cells at 26 days instead of 21 days. After the third freeze–thaw cycle, the cells are centrifuged at 2000 ***g*** for 10 minutes, and the supernatant is used to inoculate appropriate virus-sensitive cells, i.e. cells susceptible to viruses that may be present in the species of animal from which the cells were obtained, cells susceptible to viruses that may occur in the animals in which the material is going to be used and cells susceptible to pestiviruses. These cells are then passed twice at 7-day intervals, and tested for cytopathology, haemadsorption and by fluorescent antibody staining.

Ingredients of animal origin are tested on both African green monkey kidney (Vero) cells and on a cell line or primary cells derived from the same species as the ingredient under test. Cells are inoculated using 75 cm^2 flasks with 3.75 ml of test material in 25 ml of media or 15% of the test material, whichever is the lesser. The cells are passaged two or three times at 7-day intervals, and tested for cytopathology, haemadsorption and fluorescent antibody staining. The cells should be observed for cytopathology every 2 to 3 days, and prior to each subculture, throughout the incubation period.

MSV are tested on Vero cells, cell lines or primary cells of the species for which the product is intended, and cell lines or primary cells of the species in which the product is prepared (if different from the intended species).

For each cell type required for testing, 1 ml of the test MSV is thawed or reconstituted and neutralised with the addition of 1 ml monospecific antiserum. The serum must be shown to be free from antibodies against any of the contaminants for which the test is intended. Antisera should also be tested for nonspecific inhibiting affects At least two cell types are always required, so a minimum of 2 ml of MSV and 2 ml of antiserum are required. The antiserum is allowed to neutralise the MSV at room temperature for 1 hour. Of the MSV/antiserum mixture, 2 ml is then inoculated on to a 75 cm^2 flask of the appropriate cells. If the MSV is known to be high-titred or is a difficult agent to neutralise, or if the blocking serum is known to be low-titred, the blocking antiserum can be added to the growth medium at a final concentration of 1–5%. The cells should be passaged at least twice over a 14-day period, and the final culture is examined for cytopathology, haemadsorption and by fluorescent antibody staining.

The May–Grünwald–Giemsa staining procedure is usually used to detect cytopathology caused by extraneous viruses. Monolayers are usually prepared on two-chambered tissue culture slides and incubated for 7 days. The plastic wells of the slides are removed leaving the rubber gasket attached to the slide. The slides are rinsed in warm Dulbecco's phosphate buffered saline (PBS), fixed in alcohol and placed on a staining rack. The slides are stained for 15 minutes at room temperature with May–Grünwald stain diluted 1/5 with absolute methanol. The May–Grünwald stain is removed by inverting the slides. The slides are then stained for 20 minutes with Giemsa stain diluted 1/15 in deionised water. The Giemsa stain is removed by inverting the slides and rinsing them in deionised water for 10–20 seconds. The slides are air-dried, and paraffin oil and a cover-slip are applied. The May–Grünwald–Giemsa stain will differentially stain DNA and RNA nucleoproteins. DNA nucleoproteins stain red-purple, while RNA nucleoproteins stain blue. The monolayers are examined with a conventional microscope for the presence of inclusion bodies, an abnormal number of giant cells, or other cytopathology attributable to a viral contaminant. The inoculated monolayers are compared with the noninoculated monolayers. If specific cytopathology attributable to an extraneous virus is found, the test material should be considered unsatisfactory.

Testing to detect extraneous viruses that produce haemadsorption in infected cells is usually carried out on monolayers of the second passage of test-material-inoculated cell cultures and noninoculated cell cultures. The monolayers are usually on 75 cm^2 plastic flasks. Guinea-pig, chicken, and any other blood for use in this assay is collected in an equal volume of Alsever's solution. The blood may be stored at 4°C for up to 7 days if it is washed several times in Alsever's solution before storage in an equal volume of Alsever's. Just prior to use, the stored erythrocytes are again washed by adding 5 ml of blood in Alsever's solution to 45 ml of calcium- and magnesium-free PBS and centrifuging in a 50 ml centrifuge tube at 500 ***g*** for 10 minutes. The supernatant is removed by suction and the erythrocytes are suspended in PBS and recentrifuged. This washing procedure is repeated at least twice until the supernatant is clear. Erythrocytes from each species are combined by adding 0.1 ml of each type of packed blood cells to 100 ml of PBS. The erythrocytes from different species may be kept separate or combined, as desired. To each flask, add 5 ml of the erythrocyte suspension, and incubate the flasks at 4°C for 30 minutes. The monolayers are washed twice with PBS and examined for haemadsorption. If no haemadsorption is apparent, 5 ml of the erythrocyte suspension is added to each flask, the flasks are incubated at 20–25°C for 30 minutes, rinsed as before, and examined for haemadsorption. Separate flasks may be used for each incubation temperature if desired. Monolayers are examined for the presence of haemadsorbtion both grossly (using an illuminated glovebox) and microscopically. It is important to compare the noninoculated monolayers with the test monolayers to detect nonspecific haemadsorption that may occur with some cell types. The use of calcium- and magnesium-free PBS and fresh erythrocytes should prevent most nonspecific haemadsorption from occurring. If specific haemadsorption attributable to an extraneous agent is found, the test material should be considered to be unsatisfactory.

Tests to detect extraneous viruses by fluorescent antibody usually use monolayers of the second passage of test-material-inoculated cell cultures and noninoculated cell cultures. The monolayers are usually on eight-chamber tissue culture slides. One positive control slide (consisting of eight monolayers) is made for each antiviral

conjugate by inoculating each monolayer with approximately 100 $TCID_{50}$ (50% tissue culture infective dose) of the appropriate virus. Three groups of monolayers are stained with each antiviral conjugate. They are Group 1 – the second passage of test-material-inoculated cell cultures; Group 2 – the second passage of the noninoculated cell cultures; and Group 3 – the second passage of noninoculated cell cultures (for the production of positive control cell cultures). At the time of staining, the plastic walls of the slides are removed, leaving the rubber gasket attached to the slide. The slides are rinsed in Dulbecco's PBS, fixed for at least 10 minutes in acetone at 4°C, and dried. Approximately 0.1 ml of each conjugate is placed on each well of one slide from Groups 1, 2, and the corresponding positive control slide from Group 3. The slides are incubated in a humidified chamber at 37°C for 30 minutes, rinsed once in Dulbecco's PBS, and placed in a container of Dulbecco's PBS for 10 minutes. The slides are rinsed thoroughly in deionised water and dried. All slides are examined for fluorescence attributable to each specific virus. The three slides from each group with the same conjugate are compared. If the slide prepared from cells inoculated with test material shows any evidence of specific viral fluorescence, the MSV should be considered unsatisfactory.

REFERENCES

CODE OF FEDERAL REGULATIONS (OF THE UNITED STATES OF AMERICA) (CFR) (2007). Animals and Animal Products. Title 9, Parts 1–199. US Government Printing Office, Washington DC, USA.

EUROPEAN PHARMACOPOEIA 7.0. (2012). European Directorate for the Quality of Medicines and HealthCare (EDQM), Council of Europe, Strasbourg, France.

EUROPEAN UNION (1999). The Rules Governing Medicinal Products in the European Union. Eudralex, Vols 4–9. Office Publications of the European Communities, Luxembourg.

HARE W.C.D. (1985). Diseases transmissible by semen and embryo transfer techniques. OIE Technical Series No. 4. OIE, Paris, France. ISBN 92-9044-164-1.

TELLEZ S., CASIMIRO R., VELA A.I., FERNANDEZ-GARAYZABAL J.F., EZQUERRA R., LATRE M.V., BRIONES V., GOYACHE J., BULLIDO R., ARBOIX M. & DOMINGUEZ L. (2005). Unexpected inefficiency of the European pharmacopoeia sterility test for detecting contamination in clostridial vaccines. *Vaccine,* **24**, 1710–1715.

WORLD HEALTH ORGANIZATION (WHO) (1998). WHO Expert Committee on Biological Standardization. World Health Organization Technical Report Series, Report No. 858. World Health Organization, Geneva, Switzerland. ISBN 92-4-120878-3.

FURTHER READING

Details of methods and culture media will be found in the following books, and also in commercial catalogues.

BARROW G.I. & FELTHAM R.K.A., eds. (1993). Cowan and Steel's Manual for the Identification of Medical Bacteria, Third Edition. Cambridge University Press, Cambridge, UK.

CODE OF FEDERAL REGULATIONS (2003) Subchapter E. Viruses, serums, toxins, and analogous products; organisms and vectors. *In:* Code of Federal Regulation, Animals and Animal Products, Title 9, Parts 101–124. The Office of the Federal Register, National Archives and Records Administration. US Government Printing Office, Washington, DC, USA, 557–737.

COLLINS C.H., LYNE P.M. & GRANGE J.M., eds. (1995). Collins and Lyne's Microbiological Methods, Seventh Edition. Butterworth Heinemann, Oxford, UK.

MURRAY P.R., ed. (2003). Manual of Clinical Microbiology, Eighth Edition. American Society for Microbiology Press, Washington DC, USA.

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APPENDIX 1.1.9.1.

RISK ANALYSIS FOR BIOLOGICALS OTHER THAN VACCINES FOR VETERINARY USE

INTRODUCTION

For the purpose of this chapter, the term 'biologicals' means 'biologicals for veterinary use other than veterinary vaccines'.

CATEGORISATION OF BIOLOGICALS

Categorisation provides a means of facilitating risk analysis for the international trade in biologicals.

The categorisation system should take into account the source, the nature and the stated purpose of the biologicals. By conducting generic risk analyses, and by developing generic certification and quality assurance, continued supply of products can be made available without the need for repeated risk assessments that are expensive and consume significant resources. Once made, the risk assessment can be linked to appropriate manufacturing and testing parameters. Categories of biologicals for veterinary use into which generic risk assessments could apply may include (not in order of risk):

1. synthetic material;
2. amino acids, alcohols, esters, sugars and vitamins;
3. cosmetics;
4. plant extracts and processed biochemicals of plant origin;
5. products derived by microbial fermentation;
6. diagnostic, analytical and immunochemical kits for *in-vitro* use;
7. material of human origin;
8. therapeutics;
9. implantables of animal origin;
10. antibodies and immunoglobulins;
11. deoxyribonucleic acid (DNA), ribonucleic acid (RNA), restriction enzymes and other products of molecular biology;
12. cell-lines and hybridomas;
13. animal proteins, hormones, enzymes, albumins, tissue extracts and culture media containing animal material;
14. animal serum;
15. micro-organisms (conventional or genetically modified);
16. probiotics;
17. preserved specimens, microscope slides and smears.

All of these materials may contain pathogens depending on their source and processing procedures.

INFORMATION TO BE SUBMITTED WHEN APPLYING FOR AN IMPORT LICENCE

When undertaking risk analysis for biologicals, Veterinary Authorities should follow the *Terrestrial Manual*. The manufacturer or the Veterinary Authority of the exporting country should make available detailed information, in confidence if necessary, on the source of the materials used in the manufacture of the product (e.g. substrates). They should make available details of the method of manufacture (and where appropriate inactivation) of the substrates and component materials, the quality assurance procedures for each step in the process, final product testing regimes, and the pharmacopoeia with which the product must conform in the country of origin. They should also make available challenge organisms, their biotypes and reference sera, and other means of appropriate product testing.

RISK ANALYSIS PROCESS

Risk analysis should be as objective and transparent as possible and should be performed in accordance with Section 2 of the *Terrestrial Code*, and certification in line with Section 5 of the *Terrestrial Code*. Of necessity, assessment of the country and commodity factors and risk reduction measures will be based largely on manufacturers' data. These data depend on quality assurance at all stages of manufacture, rather than on testing of the final product alone.

Domestic exposure may be influenced by the approved usage of the product. Veterinary Authorities may place limits on usage of some products (e.g. restricting usage to institutions of appropriate biosecurity).

BIOCONTAINMENT

Suitable biocontainment may be necessary for many forms of biologicals. In particular, the importation of exotic micro-organisms should be carried out in accordance with Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

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