

CHAPTER 1.1.9.

TESTS FOR STERILITY AND FREEDOM FROM CONTAMINATION OF BIOLOGICAL MATERIALS INTENDED FOR VETERINARY USE

INTRODUCTION

The international trade-related movements of biological materials intended for veterinary use are subject to restrictions imposed to minimise the spread of animal and human pathogens. Countries may impose requirements for proof-of-freedom testing before allowing the regulated importation of materials of animal derivation and substances containing such derivatives. Where chemical or physical treatments are inappropriate or inefficient, or where evidence of the effectiveness of the treatment is lacking, there may be general or specific testing requirements imposed by authorities of countries receiving such materials. This chapter provides guidance on the approach to such regulated testing, particularly as might be applied to the movement of vaccine master seed and master cell stocks, and to related biological materials used in manufacturing processes. The term seed stocks is used when testing live products, for killed products the preferred reference is master cell stocks. While the onus for ensuring safety of a product remains with the manufacturer and may be regulated by therapeutic guidelines, this chapter provides procedures that are designed in particular to minimise the risk of undetected contaminants in veterinary therapeutics and biological reagents causing the cross-border spread of agents of concern to particular importing countries. Control of contamination with transmissible spongiform encephalopathy (TSE) agents is not covered in this chapter because testing and physical treatments cannot be used to ensure freedom from these agents.

Sterility is defined as the absence of viable microorganisms, which for the purpose of this chapter, includes viruses. It should be achieved using aseptic techniques and validated sterilisation methods, including heating, filtration, chemical treatments, and irradiation that fits the intended purpose. Freedom from contamination is defined as the absence of specified viable microorganisms. This may be achieved by selecting materials from sources shown to be free from specified microorganisms and by conducting subsequent procedures aseptically. Adequate assurance of sterility and freedom from contaminating microorganisms can only be achieved by proper control of the primary materials used and their subsequent processing. Tests on intermediate products are necessary throughout the production process to check that this control has been achieved.

Biological materials subject to contamination that cannot be sterilised before or during use in vaccine production, such as ingredients of animal origin, e.g. serum and trypsin, primary and continuous cells and cell lines, and viral or bacterial seed stocks, etc., should be tested for viable extraneous agents before use. Assays to detect viral contaminants, if present, can be achieved by culture methods supported by cytopathic effects (CPE) detection, fluorescent antibody techniques and other suitable methods such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). As is explained in more detail in this chapter care must be taken when using PCR and ELISA techniques for detection as such tests do not distinguish viable from non-viable agent detection.

Avian materials and vaccines are required to be inoculated on to primary avian cell cultures or eggs for the detection of avian viruses. A combination of general tests, for example to detect haemadsorbing, haemagglutinating and CPE-causing viruses and specific procedures aimed at the growth and detection of specific viruses is recommended to increase the probability of detection. Assays to detect other contaminants, such as bacteria, fungi, protozoa, rickettsia and mycoplasma are also described.

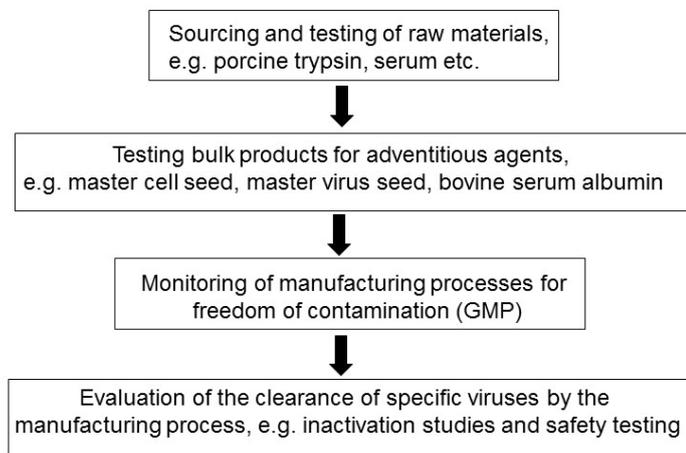
Procedures applied should be validated and found to be “fit for purpose” following Chapter 1.1.6 Validation of diagnostic assays for infectious diseases of terrestrial animals, where possible.

It is the responsibility of the submitter to ensure a representative selection and number of items to be tested. The principles of Appendix 1.1.2.1 Epidemiological approaches to sampling: sample size calculations of Chapter 1.1.2 Collection, submission and storage of diagnostic specimens apply. Adequate transportation is described in Chapter 1.1.2 and Chapter 1.1.3 Transport of biological materials.

A. AN OVERVIEW OF TESTING APPROACHES

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 (Figure 1).
2. Materials that are not sterilised and those that are to be processed further after sterilisation must be handled aseptically. Such materials will require further assessment of freedom of contaminants at certain stages of production to assure freedom of adventitious agents.
3. Materials that can be sterilised without their biological activities being affected unduly must be sterilised by a method effective for the pathogens of concern. The method must reduce the level of contamination to be undetectable, as determined by an appropriate sterility test study. (See Section D.1. below). If a sterilisation process is used, it shall be validated to demonstrate that it is fit for purpose. Suitable controls will be included in each sterilisation process to monitor efficiency.
4. The environment in which any aseptic handling is carried out must be maintained in a clean state, protected from external sources of contamination, and controlled to prevent internal contamination. Rules governing aseptic preparation of vaccines are documented in Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility*.

Figure 1. Testing algorithm for vaccine production.



Some procedures have been properly validated and found to be “fit for purpose”, whilst others may have undergone only limited validation studies. For example, methods for bacterial and fungal sterility have not been formally validated although they have been used for many years. In particular, the *in-vivo* and cell culture methods have essentially unknown sensitivity and specificity (Sheets *et al.*, 2012) though there is an accepted theoretical sensitivity of 1 colony-forming unit (CFU). For example, an evaluation of methods to detect bovine and porcine viruses in serum and trypsin based on United States (of America) Code of Federal Regulations, Title 9 (9CFR) revealed gaps in sensitivity, even within virus families (Marcus-Secura *et al.*, 2011). It is therefore important to interpret results in the light of specific conditions of cultures employed and considering sensitivity and specificity of detection systems.

Newer, more sensitive methods such as molecular assays may afford the ability to detect contaminants, which may not be successfully amplified in traditional culturing systems. The detection range can be broadened by using

family specific primers and probes if designed appropriately. However, most, if not all such new tests are also able to detect evidence for non-infectious contaminants, such as traces of nucleic acid from inactivated contaminants. Follow-up testing would be required to determine the nature of the contaminant, for example, non-infectious nucleic acid or infectious virus. Attempts at virus isolation or sequencing may remedy this. Note: molecular assays if not designed as fit for purpose may miss detection of contaminating agents or lack sensitivity to do so (Hodinka, 2013).

More recently metagenomic high throughput sequencing (HTS) workflows have shown potential for quality control of biological products (van Borm *et al.*, 2013) and vaccines (Baylis *et al.*, 2011; Farsang & Kulcsar, 2012; Neverov & Chumakov, 2010; Onions & Kolman, 2010; Victoria *et al.*, 2010) in particular for the identification and characterisation of unexpected highly divergent pathogen variants (Miller *et al.*, 2010; Rosseel *et al.*, 2011) that may remain undetected using targeted diagnostic tests. Nevertheless, targeted assays, e.g. amplification in cell culture followed by polymerase chain reaction (PCR) may be superior to HTS for specific agent detection (Wang *et al.*, 2014) due to lack of sensitivity of HTS at this time. Similarly, recent improvements in protein and peptide separation efficiencies and highly accurate mass spectrometry have promoted the identification and quantification of proteins in a given sample. Most of these new technologies are broad screening tools, limited by the fact that they cannot distinguish between viable and non-viable organisms.

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 by means of extraneous agents testing.
2. Seed lots of virus, any continuous cell line and biologicals used for virus growth shall be shown to be free from viable bacteria, fungi, mycoplasmas, protozoa, rickettsia, 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 vaccines in embryonated chicken eggs and the quality control procedures for these vaccines, it is recommended (required in many countries) that eggs from specific pathogen-free birds should be used.

3. Each batch of vaccine shall pass tests for freedom from extraneous agents that are consistent with the country's requirements for accepting the vaccine for use. Published methods that document acceptable testing procedures in various countries include: (US) Code of Federal Regulations (2015); European Pharmacopoeia (2014); European Commission (2006); World Health Organization (WHO) (1998; 2012) and Department of Agriculture (of Australia) (2013).
4. Tests for sterility shall be appropriate to prove that the vaccine is free from extraneous viruses, bacteria including rickettsia and mycoplasmas, fungi, and protozoa. Each country will have particular requirements as to what agents are necessary to exclude for and what procedures are acceptable. Such tests will include amplification of viable extraneous agents using cell culture that is susceptible to particular viruses of the species of concern, tests in embryonated eggs, bacterial, mycoplasma and fungal culturing techniques and, where necessary and possible, tests involving animal inoculation. PCR, fluorescence antibody test (FAT), presence of colonies or cytopathic effects (CPE) and enzyme-linked immunosorbent assay (ELISA) will be used for detection purposes after amplification using culturing techniques. If *in-vitro* or *in-vivo* amplification of the target agent is not possible, direct PCR may be useful if validated for this purpose.

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

1. Section B applies.
2. A limited number of contaminating, non-pathogenic bacteria and fungi may be permitted (see Section 1.2.2 *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*).

D. INACTIVATED VIRAL VACCINES

1. Each batch of vaccine shall pass a test for inactivation of the vaccinal virus and should include inactivation studies on representative extraneous agents if the virus seed has not already been tested and shown to be free from extraneous agents. An example of a simple inactivation study could include assessment of the titre of live vaccine before and after inactivation and assessing the log₁₀ drop in titre during the inactivation process. This would give an indication of the efficacy of the inactivation process. There is evidence that virus titration tests may not have sufficient sensitivity to ensure complete inactivation. In these circumstances, a specific innocuity test would need to be developed and validated to be fit for increased sensitivity. To increase sensitivity more than one passage would be required depending on the virus of concern. An example of this approach can be found at:
https://www.aphis.usda.gov/animal_health/vet_biologics/publications/memo_800_117.pdf.
2. If studies on representative extraneous agents are required, then spiking inactivated vaccine with live representative agents and following the example of an inactivation study as in D.1 above would be useful. 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.

In addition, each country may have particular requirements for sourcing or tests for sterility as detailed in Section B above.

E. LIVING BACTERIAL VACCINES

1. Section B applies.
2. Seed lots of bacteria shall be shown to be free from other bacteria as well as fungi and mycoplasmas, protozoa, rickettsia, and extraneous viruses. Agents required for exclusion will be dependent on the country accepting the vaccine for use. Use of antibiotics to 'inactivate' the living bacterial seed or vaccine prior to exclusion of viruses and fungi is recommended to ensure testing in culture is sensitive. Interference testing is recommended to ensure that the antibiotics used do not affect the growth of the extraneous virus or fungi that is being excluded.
3. Due to the difficulties and reduced sensitivity in exclusion of extraneous bacteria and some mycoplasma, protozoa, and rickettsia from high-titred seed lots of bacteria, the use of narrow-range antibiotics aimed specifically at reducing seed lot bacteria is recommended if antibiotics do not affect the growth of bacteria being excluded. The optimal concentration of antibiotics can be determined in a dilution experiment such as documented in 9CFR Section 113.25(d). Other methods of exclusion of extraneous bacteria from bacterial seeds may include filtering for size exclusion such as removing bacteria seed to look for mycoplasma contamination and use of selective culturing media. Such processes would require validation to ensure the process does not affect the sensitivity of exclusion of extraneous agents of concern.
4. Direct PCR techniques may be useful when culturing processes fail to be sensitive in detecting extraneous bacteria from live bacterial seeds or vaccines.

F. INACTIVATED BACTERIAL VACCINES

1. Section D applies. It should not be necessary to test for extraneous viruses that would not grow in bacteriological culture media as long as freedom from contamination of all starting materials can be assured. Complete inactivation of the vaccinal bacteria should be demonstrated by means of titration and innocuity tests – in some cases general bacterial sterility testing (Section 1.2.1) may suffice.

G. SERA AND DIAGNOSTIC AGENTS FOR ADMINISTRATION TO ANIMALS

1. Section B.1 applies for non-inactivated sera.

2. Some countries require quarantine, health certification, and tests for specific diseases to be completed for all serum donor animals, for example, 9CFR (2015) and Australian Quarantine Policy and Requirements for the Importation of Live and Novel Veterinary Bulk and Finished Vaccines (1999).
3. It is recommended that each batch of non-inactivated serum be assessed for viable extraneous agents, including mycoplasma. Each batch of serum shall pass a test for freedom from extraneous agents. Suitable test methods have been published for various countries, for example, European Pharmacopoeia (2014); 9 CFR (2015) and Australian Quarantine Policy and Requirements for the Importation of Live and Novel Veterinary Bulk and Finished Vaccines (1999) and Department of Agriculture (of Australia) (2013).
4. Inactivated serum, Section D applies.
5. Section B or D may apply if a virus is used in the production of the diagnostic agent; Section E or F may apply if a bacterium is used.

H. EMBRYOS, OVA, SEMEN

Special precautions must be taken with relation to the use of embryos, ova, semen (Hare, 1985). Most countries will have regulatory guidelines for import of these biologicals for veterinary use. Such guidelines can be found at various websites such as the European Commission, FAO and though many such guidelines give more detail in regard to the food safety aspect.

I. PROTOCOL EXAMPLES

1. General procedures

In principle, proposed testing represents an attempted isolation of viable agents in culturing systems normally considered supportive of the growth of each specified agent or group of general agents. After amplification, potential pathogens can be detected further by sensitive and specific diagnostic tests such as FAT or PCR if required. General detection systems can include haemabsorbance and CPE by immunohistochemistry staining methods. The example procedures for sterility testing and general detection of viable bacteria, mycoplasma, fungi, and viruses described below are derived from standards such as the 9CFR (2015), European Pharmacopoeia (2014), European Commission (2006), WHO (1998; 2012).

Individual countries or regions should adopt a risk-based approach to determine the appropriate testing protocols based on their animal health status. As well as applying general testing procedures documented in national or regional standards as mentioned above, it may be necessary to apply rigorous exclusion testing for specific agents that are exotic to the particular country or region.

General procedures will not necessarily detect all extraneous agents that may be present in biological material; however, they are useful as screening tests. Some examples of agents that may require specific methods for detection in biologicals refer to Table 1 below. Procedures documented in the Review of Published Tests to Detect Pathogens in Veterinary Vaccines Intended for Importation into Australia (2013) available from the Department of Agriculture and Water Resources, Australia are able to address such agents in offering sensitive testing approaches based on reputable publications.

Exclusion of specific agents requires procedures that maximise sensitivity by providing ideal amplification and detection of the pathogen in question. Extraneous agents, such as Maedi Visna virus, bovine immunodeficiency virus, *Trypanosoma evansi* and porcine respiratory coronavirus are difficult to culture even using the most sensitive approaches. In these circumstances, application of molecular assays directly to the biological material in question to assess the presence of nucleic acid from adventitious agents offers an alternative. Refer to Table 1. Consideration must be noted as described in Section A.6 as non-viable agents may also be detected using this procedure.

Table 1 gives some examples of causative infectious agents that may be present in animal biologicals intended for veterinary use. This is not an exhaustive list of agents of concern or by any means required for exclusion by every country, they are just examples of infectious agents that are not culturable using general culturing procedures and require a more specific detection process by means of the indirect fluorescent antibody test, PCR or ELISA, where applicable. Notably, some subtypes of an agent type may be detectable by general methods, and some may require

specialised testing for detection. For example, bovine adenovirus subgroup 1 (serotypes 1, 2, 3 and 9) can be readily isolated using general methods (Vero cells) however bovine adenovirus subgroup 2 (serotypes 4, 5, 6, 7, 8 and 10) are not readily isolated and required specialised methods for isolation.

Table 1. Some examples of infectious agents of veterinary importance that require specialist culturing and detection techniques

Rotaviruses	Pestiviruses (non-CPE)	Turkey rhinotracheitis
Porcine epidemic diarrhoea virus	Bluetongue virus	<i>Brucella abortus</i>
Porcine circoviruses (PCV 1, 2)	Swine pox virus	Rickettsias
Swine/equine influenza, some strains	Some adenoviruses	Protozoa
Bovine respiratory syncytial virus	Rabies virus	Some fungi (e.g. <i>Histoplasma</i>)

2. Detection of bacteria and fungi

2.1. General procedure for assessing the sterility of viable bacteria and fungi

Standard tests for detecting extraneous bacteria and fungi (sterility testing) in raw materials, master cell 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.

2.1.1. Diluent A

Diluent A is 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.

2.1.2. Diluent B

Diluent B is 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. 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, for example as explained in 9CFR 113.25(d) and detailed testing procedures can be found for example in supplemental assay method (SAM) 903. To determine the correct medium volume to negate antimicrobial activity, 100 CFU of the control microorganisms listed in Table 2 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 1.2.1.3 *Growth promotion and test interference*). 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 2. Some American Type Culture Collection¹ strains with their respective medium and incubation conditions

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

2.1.3. 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 for example as outlined in 9CFR 113.25(b). Detailed testing procedures can be found for example in SAMs 900-902.

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 ATCC strains listed in Table 2 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 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.

If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 ml) of the medium to fresh vessels of the same medium and then incubate the original and transfer vessels for not less than 4 days.

2.2. 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 veterinary vaccines. 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 20 ml of brain–heart infusion agar are added

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

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 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, the batch of vaccine should be considered unsatisfactory.

2.3. 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. 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 could be considered to be satisfactory for purity but the results from both the initial and retest should be reported for assessment by the individual countries relevant regulatory agency. 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.

2.4. An example of a procedure for exclusion where general testing is not sufficient for detection of *Brucella abortus*

It should be confirmed that each batch of culture medium supports the growth of *B. abortus* by inoculating plates and flasks of biphasic medium with a known number of cells (around 100) of the fastidious *B. abortus* biovar 2. If the media supports the growth of this biotype it will support all other biovars.

Inoculate 1.0 ml of prepared master or working viral or cell seed material (not containing antibiotics) by inoculating 50 µl of the test product into each of 10 flasks containing biphasic medium. At the same time 10 plates of serum dextrose agar (SDA) are inoculated with 50 µl of inoculum and spread with a sterile bent glass Pasteur pipette or hockey stick. An un-inoculated serum dextrose agar plate and a biphasic flask are also set up at the same time as negative controls.

For assessment of inhibitory substances 50 µl of previously prepared master or working viral or cell seed material and 10–100 CFU of *B. abortus* are inoculated on to duplicate SDA plates. Positive controls are prepared by inoculating 10–100 CFU of *B. abortus* on to duplicate SDA plates.

All plates and flasks are incubated at 37°C in a 5–10% CO₂ environment. Plates are incubated with the agar uppermost and flasks with the agar slope vertical. Flasks are incubated with the cap loose.

Plates are checked for growth of colonies at days 4 and 8 of incubation. The biphasic medium is examined every 4 to 7 days for 28 days. After each examination of the flasks, they are tilted so that the liquid phase runs over the solid phase, then righted and returned to the incubator.

During the incubation period, SDA plates with positive control and test material are visually compared with plates with the positive control only and if there is no inhibition of growth of the organism in the

presence of the test material, the interference testing test is successful and testing can be assured to be sensitive.

Any signs of growth of suspicious contaminating microorganisms on SDA plates, cloudiness or colonies in biphasic flasks require follow-up testing by PCR to confirm whether *B. abortus* is present.

2.5. An example of a general procedure for 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. Sensitive PCR tests are available for the detection of *Salmonella* spp. in cultured material. If *Salmonella* is detected, the batch is determined to be unsatisfactory.

3. Detection of *Mycoplasma* contamination

3.1. An example of a 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, e.g. MSV or MCS, should be tested. Four plates of solid medium are inoculated with 0.25 ml of the sample being tested, and 10 ml of the sample inoculated into 100 ml of the liquid medium. 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. Two plates are incubated at 35–37°C aerobically (an atmosphere of air containing 5–10% CO₂ and adequate humidity) and two plates are incubated anaerobically (an atmosphere of nitrogen containing 5–10% CO₂ and adequate humidity) for 14 days. On day 3 or day 4 after inoculation, 0.25 ml from the liquid media are subcultured on to two plates of solid media. One plate is incubated aerobically and the second anaerobically at 35–37°C for 14 days. The subculture procedure is repeated 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. Liquid media is observed every 2–3 days and, if any colour change occurs, has to be subcultured immediately.

3.2. Interpretation of *Mycoplasma* test results

At the end of the incubation period (total 28 days), 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 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, a suitable confirmatory test on the colonies should be conducted, such as PCR. Some mycoplasmas cannot be cultivated, in which case the MSV and MCS have to be tested using an indicator cell line such as Vero cells, DNA staining, or PCR methods.

Further detailed procedures can be found in Veterinary Medicinal Products, VICH GL34: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500140352.pdf

and USDA SAM 910: https://www.aphis.usda.gov/animal_health/vet_biologics/publications/910.pdf, (both accessed 4 July 2022.)

3.3. An example of a specific procedure for exclusion of *Mycoplasma mycoides* subsp. *mycoides* type from biologicals used in production of veterinary vaccines

Prior to beginning testing it is necessary to determine that each batch of media promotes the growth of *M. mycoides* subsp. *mycoides* (*Mmm*) type strain PG1. General mycoplasma broth and agar are used but contain porcine serum as a supplement. Each batch of broth and agar is inoculated with 10–100 CFU of *Mmm*. The solid medium is suitable if adequate growth of *Mmm* is found after 3–7 days' incubation at 37°C in 5–10% CO₂. The liquid medium is suitable if the growth on the agar plates subcultured from the broth is found by at least the first subculture. If reduced growth occurs another batch of media should be obtained and retested.

1 ml of cell or virus seed to be tested is inoculated into 9 ml of the liquid medium and 100 µl on to solid mycoplasma agar. The volume of the product is inoculated so that it is not more than 10% of the volume of the medium. The liquid medium is incubated at 37°C in 5–10% CO₂ and 100 µl of broth is subcultured on to agar at days 7, 14 and 21. The agar plates are incubated at 37°C in 5–10% CO₂ for no fewer than 14 days, except those corresponding to day 21 subculture, which are incubated for 7 days. An uninoculated mycoplasma broth and agar plate are incubated as negative controls. For assessment of inhibitory substances, inoculate 1 ml of sample to be tested into 9 ml of the liquid medium and 100 µl on to solid medium and add 10–100 CFU of *Mmm* to each. Prepare positive control by inoculating 9 ml of mycoplasma broth and a mycoplasma agar plate with 10–100 CFU of *Mmm*. Incubate as for samples and negative controls.

During incubation time, visually compare the broth of the positive control with sample present with the positive control broth and, if there is no inhibition of the organism either the product possesses no antimicrobial activity under the conditions of the test, or such activity has been satisfactorily eliminated by dilution. If no growth or reduced growth of *Mmm* is seen in the liquid and solid medium with test sample when compared with the positive control, the product possesses antimicrobial activity, and the test is not satisfactory. Modifications of the conditions to eliminate the antimicrobial activity and repeat test are required.

If antimicrobial activity is present it is necessary to dilute the test product further. Repeat the test above using 1.0 ml of sample in 39 ml of mycoplasma broth and then inoculate with 10–100 CFU of *Mmm* and incubate as above. All broths and plates are examined for obvious evidence of growth. Evidence of growth can be determined by comparing the test culture with the negative control, the positive control, and the inhibition control.

If evidence of microbial growth is found in the test samples the contaminating bacterium will be identified and confirmed as *Mmm* by PCR assay.

4. Detection of rickettsia and protozoa

There are no general test procedures for exclusion of rickettsia or protozoa. Procedures to exclude specific agents of concern such as *Coxiella burnetii* (Q fever), *Ehrlichia canis*, *Trypanosoma evansi* and *Babesia caballi* can be found for example, in the Review of Published Tests to detect pathogens in veterinary vaccines Intended for Importation into Australia (Department of Agriculture [of Australia] [2013]). The review is based on the reading and interpretation of applicable published papers from reputable journals and are regarded as examples of sensitive methods for detection of specified agents.

4.1. An example of protocol based on published methods for exclusion of *Babesia caballi* and *Theileria equi*

Babesia caballi and *Theileria equi* can be cultured *in vitro* in 10% equine red blood cells (RBC) in supportive medium supplemented with 40% horse serum and in a micro-aerophilic environment. Culture isolation of *T. equi* is more sensitive than for *B. caballi*. Giemsa-stained blood smears are prepared from cultures daily for 7 days (Avarzed *et al.*, 1997; Ikadai *et al.*, 2001). *Babesia caballi* is characterised by paired merozoites connected at one end. *Theileria equi* is characterised by a tetrad formation of merozoites or 'Maltese cross'. Confirmation of the diagnosis is by PCR (see Chapter 3.6.8 *Equine piroplasmosis*).

Molecular diagnosis is recommended for the testing of biological products that do not contain whole blood or organs. Molecular diagnosis by PCR or loop-mediated isothermal amplification (LAMP) assay are the most sensitive and specific testing methods for detection of the pathogens of equine piroplasmiasis (Alhassan *et al.*, 2007).

5. Detection of viruses in biological materials

In brief, general testing usually includes the use of continuous and primary cell lines of the source species, e.g.; cells of known susceptibility to the likely viral contaminants, which are inoculated for a period of up to 4 weeks with weekly subcultures. Virus seeds also require testing on a primary cell line of the species in which the final product is intended. At Day 21 or 28, assessment of the monolayers is done using H&E staining to assess CPE, and haemadsorption with guinea-pig and chicken RBC to assess the presence of haemadsorbing agents. Note that general testing is useful as a screening tool though not sufficiently sensitive enough to detect all viruses of concern.

Specific testing requires test material to be inoculated on to sensitive, susceptible cells lines for the virus to be excluded; the amplification process in cell culture is usually up to 28 days but depending on the virus, may require longer culturing times. Detection of specific viral contaminants is by recognition of CPE in conjunction with more sensitive antigen detection or molecular tests such as FAT and PCR and ELISA after the amplification process in cell culture.

All testing using cell lines to amplify for target viruses is contingent on the sensitivity of the cells for the target agent and the ability to recognise the presence of the agent in the cells. The quality, characteristics, and virus permissibility profile of cell lines in use should be determined as fit for purpose and appropriately maintained. Positive and negative controls should be used at all passages of cell culture to determine sensitivity and specificity. Interference testing should be performed at first pass to ensure that the test sample does not inhibit the growth of the virus being excluded for.

5.1. An example of general testing for the exclusion of viruses from virus and cell seed stocks used in production of veterinary vaccines

If the test virus inoculum is cytopathogenic, the effect must be specifically neutralised without affecting the likelihood of isolation of the target agent. For affected cell type, 1 ml of the test master (or working) virus seed (MVS) is thawed or reconstituted and neutralised with the addition of 1 ml mono-specific antiserum. The serum must be shown to be free from antibodies against any agents for which the test is intended to detect. Antiserum must be tested for nonspecific inhibiting affects. Serum should be of sufficiently high titre to neutralise the seed virus effectively with the use of an approximately equal volume or less of serum. A microplate titration is used to determine the titre of the antiserum required to neutralise the MVS of concern. The antiserum is allowed to neutralise the MVS at 37°C for 1 hour. The MVS and antiserum mixture is then inoculated on to a 75 cm² flask with appropriate cells. If the MVS is known to be high-titred or difficult to neutralise, the blocking antiserum can be added to the growth medium at a final concentration of 1–2%.

Master cell stocks do not require a neutralisation process.

The cells should be passaged weekly up to a 28-day period. Certain relevant viruses may be selected as indicators for sensitivity and interference (positive controls) but these will not provide validation for the broader range of agents targeted in general testing. The final culture is examined for cytopathology and haemadsorption.

May–Grünwald–Giemsa or H&E staining procedures are used to assess for cytopathological changes associated with virus growth. Monolayers must have a surface area of at least 6 cm² and can be prepared on 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 Dulbecco's phosphate buffered saline (PBS), fixed in acetone, methanol or formalin depending on the stain used and placed on a staining rack. For May–Grünwald–Giemsa staining: 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 mounted with a coverslip using paraffin oil. The May–Grünwald–Giemsa stain differentially stains ribonucleoprotein (RNP); DNA RNP stains red-purple, while RNA RNP

stains 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 of the test product. The inoculated monolayers are compared with suitable control non-inoculated monolayers. If specific cytopathology attributable to an extraneous virus is found, results are reported, and additional specific testing may be conducted.

Testing for haemadsorption uses 75 cm² area monolayers established in tissue culture flasks after the 28-day passage period described above. Guinea-pig, chicken, and any other blood for use in this assay is collected in an equal volume of Alsever's solution and may be stored at 4°C for up to 7 days. Immediately 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 (PBSA) and centrifuging in a 50 ml centrifuge tube at 500 *g* for 10 minutes. The supernatant is aspirated, and the erythrocytes are suspended in PBSA and re-centrifuged. 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 PBSA. 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 PBSA 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 (room temperature) 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 haemadsorption using an illuminated light box and microscopically. Non-inoculated monolayers are used as negative controls. The PBSA and fresh erythrocytes should prevent most nonspecific haemadsorption from occurring. If specific haemadsorption attributable to an extraneous agent is found, results are reported, and additional specific testing may be conducted.

Specific testing requires specialised test procedures that are sensitive to amplifying a particular agent in culture and then detection of that agent by means of fluorescence, antigen-capture ELISA or PCR; whichever is more sensitive. Specific testing is usually required when general procedures are not adequate for effective exclusion of more fastidious, viruses. Some examples are listed in Table 1.

5.2. An example of specific virus exclusion testing of biologicals used in the production of veterinary vaccines

5.2.1. Porcine epidemic diarrhoea virus (PEDV)

Trypsin is required at inoculation and in the culture medium for isolation of porcine epidemic diarrhoea virus (PEDV) in Vero cells (CCL81, ATCC). Just confluent monolayers (100%) are required; under confluent monolayers are more sensitive to the presence of trypsin and will be destroyed well before the 7 days required for each passage in culture. An over confluent or aging monolayer will not be sensitive for growth of PEDV. Maintenance media (MM) formulation consists of Earle's MEM (minimal essential medium) (with 5.6 M HEPES [N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid] and glutamine) + 0.3% Tryptose phosphate broth, 0.02% yeast extract and 4 µg/ml TPCK treated trypsin. The addition of the trypsin into the MM should occur on the day the media is to be used.

Prior to inoculation, confluent 75 cm² monolayers are washed twice with the MM (with trypsin added) to remove FCS. Virus or cell seed (1 ml) is added with 1 ml of MM to each monolayer; incubate at 37°C for 2 hours, then add 30 ml/flask of MM. Negative control monolayers of the same size are set up prior to inoculation of test material. Positive and interference controls are set up last in a separate laboratory area. Assessment for sensitivity and interfering substances requires assessment of PEDV of known titre. A control for interference using co-inoculation of test sample and PEDV needs only to be set up on the first pass. Positive controls must be set up at every pass to ensure each monolayer used gives expected sensitivity. PEDV virus is titrated in log dilutions starting at 10⁻¹ to 10⁻⁶ in MM (depending on the endpoint titre of reference virus) in duplicate rows of 6 wells of a 24-well tissue culture plate. For the interference test, PEDV is titrated in the same dilution series but using MM spiked with a 10% volume of test material. Decant off the growth media and discard. Wash plates to ensure no FCS is present. Two washes using approximately 400 µl/well MM (with trypsin added) are sufficient.

Add 100 µl of diluted virus on to each of two duplicate wells. Rock inoculated plates to distribute the inoculum evenly over the surface of the monolayer. Incubate at 37°C with 5% CO₂ for 2 hours then add 1 ml volumes/well of MM.

After 7 days, 75 cm² monolayers have cells disrupted using two freeze–thaw cycles at –80°C. Positive control plates are read for end-point titres, and these are compared with virus in the presence of test material to ensure titres are comparable and interference has not occurred. Freeze–thaw lysates are clarified at 2000 *g* for 5 minutes and re-passed on to newly formed monolayers as for the first passage. Passages are repeated until a total of four passages are completed at which point cell lysates are assessed by PCR for detection of PEDV and day 7 monolayers in 24-well plates are fixed and stained by IFA. If seed virus is to be tested and requires neutralisation using antiserum, extra care in the isolation of PEDV needs to be considered. Trypsin is rendered inactive in the presence of serum proteins and without trypsin present, PEDV is unable to grow in cell culture. Washing off the inoculum with two MM washes is required after an extended adsorption time of up to 4 hours to ensure acceptable sensitivity.

J. 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 as 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.

K. 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).

L. 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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FURTHER READING

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NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2017.