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Comments should be provided using this template. The completed comments form should be sent to vet-guidelines@ema.europa.eu

[1] Draft VICH GL34 was published for consultation first in 2002. Following a 12 months it was agreed to suspend the consultation to wait for the testing of reference strains. The consultation was re-opened to allow submission of any further comments.

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VICH International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products

VICH GL34 (BIOLOGICALS: MYCOPLASMA)
November 2011
For consultation at Step 4 - Draft 2

TESTING FOR THE DETECTION OF MYCOPLASMA CONTAMINATION

Recommended for Consultation
at Step 4 of the VICH Process
on 17 November 2011
by the VICH Steering Committee

THIS GUIDELINE HAS BEEN DEVELOPED BY THE APPROPRIATE VICH EXPERT WORKING GROUP AND IS SUBJECT TO CONSULTATION BY THE PARTIES, IN ACCORDANCE WITH THE VICH PROCESS. AT STEP 7 OF THE PROCESS THE FINAL DRAFT WILL BE RECOMMENDED FOR ADOPTION TO THE REGULATORY BODIES OF THE EUROPEAN UNION, JAPAN AND THE USA.

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1. INTRODUCTION

1.1. Objective of the guideline

This VICH (International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products) guideline is intended to facilitate the harmonized licensing of new products for veterinary use. It is important that biological products for veterinary use are free of contamination with Mycoplasmas to help assure consistency of production and final product safety. Mycoplasma contaminants may be introduced into cell culture and *in ovo* origin biological products through the master seeds, the master cell seed (stock), starting materials of animal origin, and in processing of biological materials during passage and product assembly. Therefore it is necessary to demonstrate through testing that Mycoplasmas are not present, within the limits of the test, in the final product, working seeds and cells and harvests, and starting materials such as the master seed, master cell seed, and ingredients of animal origin. This guideline establishes stages of manufacture to be tested and test procedures to detect the presence of Mycoplasma contamination. It will provide a unified standard that will facilitate the mutual acceptance of test data by the relevant regulatory authorities. Methods proven equivalent to the guideline method by scientifically accepted criteria could also be acceptable.

1.2. Background

The present methods for testing for Mycoplasma contamination are described in the Japanese “Minimum requirements of biological products for animal use (2002)”, the European Pharmacopoeia (7th Edition, 2011, 2.6.7), and the United States Code of Federal Regulations, Title 9, 113.28. These requirements are all similar in that they require testing for Mycoplasma contamination using a broth and agar technique. The requirements do however differ in the specifics of these broth and agar tests as well as other alternative test methods that are required or approved for use in detecting Mycoplasma contamination.

1.3. Scope of guideline

This guideline describes the manner in which tests conducted to detect the presence of Mycoplasma contamination in cell culture and *in ovo* origin biological products for veterinary use shall be done to assure the absence of Mycoplasma contamination. Tests on master seeds, master cell seeds (stocks), working seeds and cells, ingredients of animal origin, harvests and live final vaccine and harvests for killed products are included. Bacterial products which grow in the mycoplasma test media and products for which mycoplasma contamination risk has been addressed through a validated mycoplasma inactivation procedure will be considered outside this guideline. The absence of mycoplasma contamination in eggs used for production is controlled by appropriate testing of the flock, which is not covered by this guideline.

1.4. Test Methods

The guideline describes two test methods: 1) expansion in broth culture and detection by colony formation on nutrient agar plates; and 2) expansion in cell culture and characteristic fluorescent staining of DNA (a technique capable of detecting non-cultivatable strains).

A third test methodology, nucleic acid amplification (NAT), is acknowledged, but is not included in this guideline. The use of validated NAT techniques is currently approved or under consideration by regulatory authorities for more rapid detection confirmation,

and strain identification. Appropriately validated NAT techniques may be used as an alternative to the broth/agar culture method and/or the indicator cell culture method provided the NAT test is shown to be at least equivalent in detection limit to the test methods in this guideline. A sample that tests positive by NAT may be directly considered unsuitable for use. If confirmation of the presence of living mycoplasma in the material under test is needed the broth/agar culture method or the indicator cell culture method should be used. Evaluation of NAT method use is encouraged in parallel testing to further develop, compare, and refine the technique for possible inclusion in future versions of this guideline.

2. GUIDELINE FOR TESTING FOR MYCOPLASMA CONTAMINATION

2.1. General test procedures for detecting Mycoplasma contamination

The culture method using broth and agar is the fundamental method of Mycoplasma detection. A solid and liquid media culture method shall be used to test harvests or final batches of vaccine, and ingredients of animal origin. Master seed, master cell seed (stock), and working seed and cell lots shall be tested using both a solid and liquid media culture method and an indicator cell culture method with DNA stain. Should either method result in a positive test for mycoplasma the sample is considered positive and is unsuitable for use.

Material	Broth & Agar Culture	DNA Stain
Master Seed & Master Cell Seed	Required	Required
Working Seed & Working Cell Seed	Required	Required
Ingredient of Animal Origin[1,2]	Required	
Harvest	When testing required[3]	
Final Product	When testing required[3]	

[1] Excluding eggs
[2] Unless a validated mycoplasma inactivation procedure has been applied
[3] The competent authorities require testing of different combinations of harvests and final product.

2.2. Culture test system validation

The culture method should be carried out to validate the detection limit of a laboratory's mycoplasma detection method. A sufficient number of both solid and liquid media shall be used to insure the growth of a low level of the following 5 strains of mycoplasmas.

Acholeplasma laidlawii
Mycoplasma hyorhinis
Mycoplasma orale
Mycoplasma synoviae
Mycoplasma fermentans

The species were selected to reflect a range (within a practical number) of antibiotic sensitivity (to detect inhibition of mycoplasma growth in the assay), fastidiousness, rapidity of growth, likelihood of being a contaminant, and pathogenicity in avian or mammalian target species. *Acholeplasma laidlawii* is a common cell culture contaminant of animal and possibly environmental origin. *Mycoplasma hyorhinis* is fastidious, a common cell culture contaminant of animal origin, and a mammalian pathogen. *Mycoplasma orale* is antibiotic sensitive and is a common cell culture

contaminant of human origin. *Mycoplasma synoviae* is fastidious (having a nicotinamide-adenine-dinucleotide [DPN, NAD] and cysteine requirement) and is an avian pathogen. *Mycoplasma fermentans* is a slow-growing organism and a common cell culture contaminant of human origin.

References of the strains used to validate the laboratory mycoplasma contamination culture test system should be of low passage level (15 or less), and identified relative to type culture isolates, (see Appendix 3.2 for further information on reference strains). The reference strains used to validate the culture test system will be appropriate to the products tested (see table). Validation for *M. synoviae* is required when materials of avian origin are used at any stage in development and production. Validation for *M. hyorhinis* and *A. laidlawii* is required when materials of mammalian origin are used at any stage in development and production. Validation for *M. orale* is required when an antibiotic has been used at any stage in development and production. Reference Preparations shall be used to validate each production lot of broth and agar. At least one reference strain must be used as a control with each test.

Required Reference Organisms by: product type; test method, and presence of antibiotics

Vaccine type Antibiotic content Test Method	*A. laidlawii*	*M. orale*	*M. hyorhinis*	*M. synoviae*	*M. fermentans*
Avian *in ovo* origin vaccine Without Antibiotics Broth/Agar Method				X	X
Avian *in ovo* origin vaccine With Antibiotics Broth/Agar Method		X		X	X
Avian cell culture origin vaccine Without Antibiotics Broth/Agar Method	X			X	X
Avian cell culture origin vaccine With Antibiotics Broth/Agar Method	X	X		X	X
Mammalian cell culture origin vaccine Without Antibiotics Broth/Agar Method	X		X		X
Mammalian cell culture origin vaccine With Antibiotics Broth/Agar Method	X	X	X		X
Vaccine Without Antibiotics DNA Staining Method		X	X		
Vaccine With Antibiotics DNA Staining Method		X	X		

2.3. Culture Method

2.3.1. Incubation conditions

Incubate the broth culture medium or media in tightly stoppered containers in air. Incubate all agar plates under microaerophilic conditions (nitrogen containing 5-10% CO_2). For the solid medium or media, maintain an atmosphere of adequate humidity to prevent desiccation of the agar surface.

2.3.2. Nutritive properties of a new batch of medium

Each new lot (batch) of medium must be tested for the nutritive properties using references specified above in **Section 2.2**. Each testing laboratory must determine the inoculum for each of their references that will contain a low level (not more than 100 CFU). Inoculate the solid medium with a low level (not more than 100 CFU) per 60 mm plate and per 100 ml container of broth medium. Use at least one agar plate and broth container for each reference. Incubate the agar and broth media and make subcultures from the broth onto agar at the specified intervals. The agar medium batch complies with the test for nutritive properties if for all the references specified, growth obtained does not differ by a factor greater than 5 from the value calculated with respect to the inoculum. The broth complies if Mycoplasma growth on those agar plates subcultured from the broth is achieved for each reference specified. Media formulations found effective are included in **Appendix 3.1** of this guideline.

2.3.3. Inhibitory substances

Carry out the test for nutritive properties in the presence and absence of the material to be tested at the time of prelicense and whenever there is a change in the production method that may affect the detection of mycoplasmas. If growth of the references occurs more than one sub-culture sooner without the test material than with the test material, or if plates directly inoculated with the test material have less than one-fifth of the colonies of those directly inoculated without the test material, the test material contains inhibitory substances. These substances must be neutralized or their effect otherwise countered, e.g., through passage in substrates not containing inhibitors or dilution in a larger volume of medium, before the test for mycoplasma contamination is carried out. For the dilution technique, larger medium volumes may be used or the inoculum volume may be divided among multiple 100 ml flasks. The effectiveness of the neutralization or other process is confirmed by repeating the test for inhibitory substances after neutralization.

2.3.4. Test method

2.3.4.1. The amount of inoculum for each plate of solid medium is 0.2 ml of product to be examined. When an assay for mycoplasma concerns master and working seeds, master and working cells, and ingredients of animal origin a volume of not less than 10 ml of undiluted sample shall be tested in each liquid medium. The volume of final product to be tested in each liquid medium shall be as required by the regulatory authority issuing the marketing authorization. These are currently not less than 1 ml in Japan and the US and not less than 10 ml in the EU. Incubate the agar plates at 35ºC to 38ºC, microaerophilically, for 10-14 days in an atmosphere of adequate humidity to prevent desiccation of the surface. Incubate the liquid media at 35º C to 38º C in tightly stoppered containers in air for 20-21 days. At the same time incubate

an uninoculated 100 ml portion of each liquid medium and agar plates as a negative control. If any significant pH change occurs upon the addition of the product to be examined (this should be determined at the time of prelicense), the liquid medium shall be restored to its original pH value by the addition of a solution of either sodium hydroxide or hydrochloric acid. Between the 2nd and 4th day after inoculation, subculture each liquid culture by inoculating at least 1 plate of each solid medium with 0.2 ml and incubate them at 35ºC to 38ºC microaerophilically for 10-14 days. Repeat the procedure between the 6th and 8th day, again between the 13th and 15th day, and again between the 19th and 21st day of the test. Incubate those agar plates inoculated on day 19, 20, or 21 for 7 days. Observe the liquid medium or media every 2 or 3 days and if a color change occurs, subculture. Color change detection requires the addition of phenol red to the media.

2.3.4.2. If the liquid medium or media shows bacterial or fungal contamination, repeat the test. If it is not possible to read at least one plate per inoculation day, the test must be repeated.

2.3.4.3. Include in the test, positive controls prepared by inoculating a low level (not more than 100 CFU) of at least one of the reference species onto the agar plates and into the broth medium or media. If the test is run on a routine basis, the control species should be rotated on a regular basis. This control shall be used in each test conducted with a medium that has been validated for nutritive properties using references determined by the types of products being tested as specified in **Section 2.2** of this guideline.

2.3.5. Judgment of the culture method

At the end of the incubation period, examine all the inoculated solid media microscopically for the presence of mycoplasma colonies. The product is negative for Mycoplasma contamination if the growth of typical Mycoplasma colonies has not occurred on any of the inoculated solid media. If growth of typical Mycoplasma colonies has occurred on any of the solid media, the test and sample tested are considered positive for Mycoplasma contamination. The test is invalid if the positive controls do not show growth of mycoplasma on at least one subculture plate or the negative controls are positive for mycoplasma contamination. If either of the controls is invalid the test must be repeated. If suspect colonies are observed, confirmation of mycoplasma contamination may be accomplished using an appropriate and validated method.

2.4. Indicator cell culture method

Cell cultures are stained with a fluorescent dye that binds to DNA. Mycoplasmas are detected by their characteristic particulate or filamentous pattern of fluorescence on the cell surface, and if contamination is heavy, in the surrounding areas. Mitochondria in the cytoplasm may be stained, but may be differentiated from mycoplasma.

2.4.1. Validation of the indicator cell culture method

Using a VERO or other equivalent in efficiency indicator cell culture substrate, validate the procedure using an inoculum not more than 100 CFU or CFU-like micro-organisms of appropriate references of *M. hyorhinis* and *M. orale.* Both

references must be positive when stained with the DNA stain at the end of the test.

If for viral, etc., suspensions the interpretation of results is affected by cytopathic effects, the virus may be neutralized using a specific antiserum that has no inhibitory effects on mycoplasmas, or an alternative cell culture substrate that does not allow the growth of the virus may be used. To demonstrate the absence of inhibitory effects of serum, carry out the positive control tests in the presence of neutralizing antiserum. Antiserum lots may be qualified once rather than at use.

2.4.2. Test method

2.4.2.1. Seed the indicator cell culture at a suitable density that will yield confluence of the cells after 3 days of growth (example: 2×10^4 to 2×10^5 cells per ml, 4×10^3 to 2.5×10^4 cells/cm^2) in a cell culture vessel of not less than 25 cm^2. The indicator cell culture should be sub-cultured without antibiotic prior to use. Inoculate 1 ml of the sample to be examined into the cell culture vessel and incubate at 35º C to 38º C.

2.4.2.2. After at least 3 days of incubation and the cells have grown to confluence, make a subculture onto cover slips in suitable containers or on some other surface (chambered slides) suitable for the test procedure. Seed the cells in the second subculture at a low density so that they reach only 50% confluence after 3-5 days of incubation. Complete confluence must be avoided because it impairs visualization of mycoplasmas after staining.

2.4.2.3. Remove medium from cover slips or chambered slides. Rinse the monolayer of indicator cells with phosphate buffered saline (PBS) and then fix with glacial acetic acid/methanol (1 to 3) or some other suitable fixing solution.

2.4.2.4. Remove the fixing solution and discard. Wash the fixing solution with sterile water and dry slides completely if they are to be stained more than one hour later.

2.4.2.5. Add a suitable fluorescent dye that binds to DNA such as bisbenzimide stain (Hoechst compound 33258, bisbenzimidazole, 5 ug/L) and allow to stain for a suitable time.

2.4.2.6. Remove the stain and rinse the monolayer with water. Mount the cover slips if applicable and examine the slides by fluorescence (for bisbenzimide stain use a 330 nm/380 nm excitation filter, LP 440 nm barrier filter) at 400 X magnification or greater.

2.4.2.7. Compare the microscopic appearance of the test cultures with that of the negative and reference controls, examining for extranuclear fluorescence. Mycoplasmas produce pinpoints or filaments over the indicator cell's cytoplasm. They may also produce pinpoints and filaments in the intercellular spaces. Multiple microscopic fields as validated should be examined.

2.4.3. Judgment of the indicator cell culture method

The product being examined is negative for Mycoplasma contamination if there is no evidence of pinpoints or filaments of extranuclear fluorescence. If the slides inoculated with the product contain evidence of pinpoints or extranuclear fluorescence indicative of Mycoplasma the test and sample tested are considered positive for Mycoplasma contamination. The test is invalid if the positive controls do not show the presence of the appropriate extranuclear fluorescence of the reference organisms or the negative cell controls contain extranuclear fluorescence. If either of the controls is invalid the test must be repeated.

3. APPENDICES

3.1. Regional examples of suitable broth and agar formulations

9 CFR Mycoplasma Broth

Heart Infusion Broth	62.5 g
Proteose Peptone #3	25.0 g
Yeast Extract	12.5 ml
1 % Thallium Acetate	62.5 ml
1 % Tetrazolium Chloride	13.75 ml
Penicillin (100,000 units/ml)	12.5 ml
Heat inactivated Horse Serum	250 ml
H_2O	2425 ml

Mix all ingredients well and adjust pH to 7.9 with 10 Normal NaOH.
Filter sterilize through a 0.2 µ filter. Dispense into sterile test vessels.
Add DPN/L-Cysteine solution before use, 2 ml/100 ml of broth.

9 CFR Mycoplasma Agar

Heart Infusion Agar	25 g
Heart Infusion Broth	10 g
Proteose Peptone #3	10 g
1% Thallium Acetate	25 ml
H_2O	995 ml
Heat Inactivated Horse Serum	126 ml
Yeast Extract	5 ml
Penicillin (100,000 units/ml)	5.2 ml
DPN/L-Cysteine	21 ml

Combine heart infusion agar, heart infusion broth, proteose peptone #3, Thallium acetate, and H_2O.
Mix and bring to boil, then cool. Adjust the pH to 7.9 with 10 Normal NaOH.
Autoclave 20 min. at 121° C. Cool in water bath to 56° C.
Aseptically add: horse serum, yeast extract, Penicillin, and DPN/L-Cysteine.
Dispense 12 ml into each 15 X 60 mm petri dish.

DPN/L-Cysteine solution

Nicotiamide-adenine-dinucleotide (DPN, NAD)	5 g
Q.S. with H_2O to	500 ml
L-Cysteine	5 g
Q.S. with H_2O to	500 ml

Mix each chemical separately until dissolved.
Mix the two solutions and filter sterilize.

Japanese Liquid Medium for Mycoplasma

Basal Medium

50 % w/v Bovine Cardiac Muscle Extract	100 ml
Meat Peptone	10 g

Sodium Chloride	5 g
Glucose	1 g
Sodium L-glutamate	0.1 g
L-arginine hydrochloride	1 g
H_2O	QS to 1000 ml

Filter sterilize through 0.22 µ membrane filter or sterilize at 121º C for 15 min.
Adjust the pH of the medium after sterilization to 7.2-7.4.

Additives for 77 ml of the Basal medium;

Horse Serum	10 ml
Inactivated Porcine Serum	5 ml
25 % w/v Fresh Yeast Extract	5 ml
1 % w/v β-NAD (oxidized)	1 ml
1 % w/v L-cysteine HCL (1 H_2O)	1 ml
0.2 % w/v phenol red	1 ml

Previously filter sterilize the additives and aseptically add to the sterilized basal medium. The additives which can be sterilized by high pressure can be autoclaved. Penicillin G potassium, 500 units/ml of the medium, or Thallium acetate, 0.02 % w/v, can be added.

Japanese Agar Medium for Mycoplasma

Basal Medium	78 ml
Agar	1 g

Sterilize by autoclaving 121ºC for 15 min.

Additives:

Horse Serum	10 ml
Inactivated Porcine Serum	5 ml
25 % w/v fresh yeast extract	5 ml
1 % w/v β-NAD (oxidized)	1 ml
1 % w/v L-cysteine HCl (1 H_2O)	1 ml

Penicillin G potassium, 500 units per ml of medium, or thallium acetate, 0.02 % w/v can be added.
Add the additives to basal/agar medium which has been liquefied by heating, and divide into sterile petri dishes, 45-55 mm. Cool and allow to solidify.

EP Hayflick media (Recommended media for the general detection of *mycoplasmas)*

Liquid Medium:

Beef Heart Infusion Broth (1)	90 ml
Horse Serum (unheated)	20 ml
Yeast Extract (250 g/L)	10 ml
Phenol Red (0.6 g/L solution)	5 ml
Penicillin (20,000 I.U. per ml)	0.25 ml
Deoxyribonucleic acid (2 g/L solution)	1.2 ml

Adjust to pH 7.8.

Solid Medium:

Prepare as described for the liquid medium above but replace beef heart infusion broth with beef heart infusion agar containing 15 g/L of agar.

EP Frey media (Recommended Media for the detection of *M. synoviae)*

Liquid Medium:

Beef Heart Infusion Broth (1)	90 ml
Essential Vitamins (2)	0.025 ml
Glucose monohydrate (500 g/L solution)	2 ml
Swine serum (inactivated at 56ºC for 30 min.)	12 ml
β-Nicotinamide adenine dinucleotide (10 g/L solution)	1 ml
Cysteine hydrochloride (10 g/L solution)	1 ml
Phenol Red (0.6 g/L solution)	5 ml
Penicillin (20,000 I.U. per ml)	0.25 ml

Mix the solutions of β-nicotinamide adenine dinucleotide and cysteine hydrochloride and after 10 minutes, add the other ingredients. Adjust pH to 7.8.

Solid Medium:

Beef Heart Infusion Broth (1)	90 ml
Ionagar (3)	1.4 g

Adjust pH to 7.8, and sterilize by autoclaving, then add:

Essential Vitamins (2)	0.025 ml
Glucose monohydrate (500 g/L solution)	2 ml
Swine serum (unheated)	12 ml
β-Nicotinamide adenine dinucleotide (10g/L solution)	1 ml
Cysteine hydrochloride (10 g/L solutions)	1 ml
Phenol Red (0.6 g/L solution)	5 ml
Penicillin (20,000 I.U. per ml)	0.25 ml

EP Friis media (Recommended Media for the Detection of Non-avian Mycoplasmas)

Liquid Medium:

Hank's Balanced Salt Solution (modified) (4)	800 ml
H_2O	67 ml
Brain Heart Infusion (5)	135 ml
PPLO Broth	248 ml
Yeast Extract (170 g/L)	60 ml
Bacitracin	250 mg
Meticillin	250 mg
Phenol Red (5 g/L)	4.5 ml
Horse Serum	165 ml
Swine Serum	165 ml

Adjust the pH to 7.40-7.45

Solid Medium:

Hank's Balanced Salt Solution (modified) (4)	200 ml
DEAE-dextran	200 mg
Ionagar (3)	15.65 g

Mix well and sterilize by autoclaving. Cool to 100º C. Add this to 1740 ml of the liquid medium described above.

EP Media Sub parts

(1) Beef Heart Infusion Broth

Beef Heart (for preparation of the infusion)	500 g
Peptone	10 g
Sodium Chloride	5 g
H_2O	QS to 1000 ml

Sterilize by autoclaving.

(2) Essential Vitamins

Biotin	100 mg
Calcium pantothenate	100 mg
Choline chloride	100 mg
Folic acid	100 mg
i-Inositol	200 mg
Nicotinamide	100 mg
Pyridoxal hydrochloride	100 mg
Riboflavin	10 mg
Thiamine hydrochloride	100 mg
H_2O	QS to1000 ml

(3) Ionagar

A highly refined agar for use in microbiology and immunology, prepared by an ion-exchange procedure which results in a product having superior purity, clarity, and gel strength.

It contains approximately:

H_2O	12.2 %
Ash	1.5 %
Acid insoluble ash	0.2 %
Chlorine	0.0 %
Phosphate (calculated as P_2O_5)	0.3 %
Total Nitrogen	0.3 %
Copper	8 ppm
Iron	170 ppm
Calcium	0.28 %
Magnesium	0.32 %

(4) Hank's Balanced Salt Solution (modified)

Sodium chloride	6.4 g
Potassium chloride	0.32 g
Magnesium sulphate heptahydrate	0.08 g
Magnesium chloride hexahydrate	0.08 g
Calcium chloride, anhydrous	0.112 g
Disodium hydrogen phosphate dihydrate	0.0596 g
Potassium dihydrogen phosphate, anhydrous	0.048 g
H_2O	QS to 800 ml

(5) Brain heart infusion

Calf brain infusion	200 g
Beef heart infusion	250 g
Proteose peptone	10 g
Glucose	2 g

Sodium chloride	5 g
Disodium hydrogen phosphate, anhydrous	2.5 g
H_2O	QS to 1000 ml

(6) PPLO broth

Beef heart infusion	50 g
Peptone	10 g
Sodium chloride	5 g
H_2O	QS to 1000 ml

Bisbenzimide stain solution for DNA Staining

Hoechst compound 33258 (bisbenzimidazole), 5 µg per liter of buffered aqueous solution.

Note: The solution should be protected from light.

3.2. Mycoplasma References

Standardization of testing between laboratories and between regions would be enhanced by use of references common within or between regions, This has been shown to be presently impractical due to the difficulty of producing consistent batches of lyophilized references and the shipping issues with frozen references. Therefore, regions or laboratories may use their own references, providing that they are of low passage level (15 or less), identified relative to type culture isolates, stable, and appropriately validated as suitable for use in the context of this guideline. It is strongly recommended to include in the validation of detection limit a comparison to the EDQM reference strains (described below) for international recognition. Regions or laboratories may produce their own validated references, or may acquire commonly available and appropriately validated references, such as the following produced by the EDQM.

The 5 strains of Mycoplasma listed in **Section 2.3** were isolated by laboratories of the European Union and donated to the European Directorate for the Quality of Medicines and HealthCare (EDQM). EDQM produced a sufficient quantity of these frozen references, and performed an intra-region EU validation/stability study (C. Milne, A. Daas. Establishment of European Pharmacopoeia Mycoplasma Reference Strains. *Pharmeuropa Bio* 2006(1):57-72). Completion of further validation studies by the regulatory agencies and the industries in Japan, USA, and Canada confirmed that the strains are very suitable for use in the context of this guideline. (VICH Collaborative Study on the Ph. Eur. Mycoplasma Reference Strains: EDQM Report Compiling and Analysing the Data Set for the VICH Collaborative Study on the European Pharmacopoeia Mycoplasma Reference Strains, EDQM Administrator Representative, C. Milne, 2010.) The BQMEWG commends the Staff at the EDQM for their efforts and perseverance in producing and validating these very excellent references.

For DNA staining validation, the following strains may also prove useful:
M. hyorhinis -- ATCC 29052
M. orale -- ATCC 23714

3.3. Glossary

Batch (lot, serial) of starting material of animal origin

The total quantity of homogenous material (e.g., cells, serum) identified by a unique serial number.

Cell-seed system

A system whereby successive final lots (batches) of a product are manufactured by culture in cells derived from the same master cell seed. A number of containers from the master cell seed are used to prepare a working cell seed.

Cell lines

Cultures of cells >10 passages or subcultures from the tissue of origin and having a high capacity for multiplication *in-vitro.*

Final product, batch, lot, or serial

A collection of closed, final containers or other final dosage units that are expected to be homogeneous and equivalent with respect to risk of contamination during filling or preparation of the final product. The dosage units are filled, or otherwise prepared, from the same final bulk product, freeze-dried together (if applicable) and closed in one continuous working session. They bear a distinctive number or code identifying the final lot (batch, serial). Where a final bulk product is filled and/or freeze-dried on several separate sessions, there results a related set of final lots (batches, serials) that are usually identified by the use of a common part in the distinctive number or code; these related final lots (batches, serials) are sometimes referred to as sub-batches, subserials, sub-lots or filling lots. For the purposes of mycoplasma testing, a single sub-batch may be considered representative of the batch.

Harvests

Material derived on one or more occasions from a single production culture inoculated with the same working seed lot (single harvest) or pooled material containing a single strain or type of micro-organism or antigen and derived from a number of eggs, cell culture containers, etc. that are processed at the same time (monovalent pooled harvest).

Master cell seed (stock)

A collection of aliquots of cells (primary or cell line) of a single passage level for use in the preparation of the product, distributed into containers in a single operation, processed together and stored in such a manner as to ensure uniformity and stability and to prevent contamination. Master cell seed is usually stored at temperatures of -70ºC or lower.

Master seed

A collection of closed containers of a culture of micro-organisms of a single passage level used for the production of all batches of a designated veterinary biological product, distributed from a single bulk into containers and processed together in a single operation in such a manner as to ensure uniformity and stability and to prevent contamination.

Microaerophilic condition

A nitrogen atmosphere containing 5-10% carbon dioxide and sufficient humidity to prevent drying of the agar plates.

Passage

One transfer of cells or microorganisms followed by the normally used incubation period for the cell or microorganism concerned.

Primary cell cultures

Primary cell cultures are cultures of cells essentially unchanged from those in the animal tissues from which they have been prepared and being no more than 10 *in-vitro* passages to the test level from the initial preparation from the animal tissue. The first *in-vitro* cultivation is regarded as the first passage of the cells

Seed-lot system:

A system in which successive batches of a product are derived from the same master seed virus. For routine production, a working seed virus may be prepared from the master seed virus.

Working cell seed (stock)

A collection of aliquots of cells derived from the master cell seed and at the passage level used in the preparation of production cell cultures. The working cell seed is distributed into containers, processed and stored as described for master cell seed. The term includes production cell seed.

Working References

A passage of the Reference strains of Mycoplasma produced in the testing laboratory for use as controls to satisfy the reference requirements specified in this document.

Working seed

A collection of aliquots of a microorganism derived from the master seed virus and at the passage level used in the preparation of product. Working seed virus is distributed into containers and stored as described for master seed virus. The term includes production seed.