

VICH DISCUSSION DOCUMENT

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Topic: Rewritten Mycoplasma Procedure after November 1999 WG Discussion

1. INTRODUCTION

1.1. Objective of the guideline

It is important that biological products for veterinary use are free of contamination with Mycoplasma. Potential sources of contamination are the master seed viruses (MSV), the master cell stocks (MCS), starting materials of animal origin, and the cell production lots of viral biological products. Therefore it is necessary to demonstrate through testing that Mycoplasma are not present in the final product, working cell stock and those starting materials such as the MSV, MCS, and ingredients of animal origin. This guideline establishes the accepted test procedures and sampling procedures to assure the absence of Mycoplasma contamination.

1.2. Background

The present methods for testing for Mycoplasma contamination are described in the Japanese "Minimum requirements of biological products for animal use", the European Pharmacopoeia (Supplement 1998, 2.6.7), and the United States Title 9 Code of Federal Regulations, 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 viral biological products for veterinary use, which will be shipped between countries, shall be tested for Mycoplasma contamination. These tests shall be applied to finished live viral products, and MSV, MCS, working seeds, working cells of all viral products.

1.4. General principles

All viral master and working seeds, all master cell cultures, and cell production lots, all ingredients of animal origin used in the production of viral vaccines, and all final production lots of live viral biologics must be thoroughly tested for Mycoplasma contamination. The broth and agar test procedure specified by this guideline must be used to test MSV, MSC, working seeds and cells, ingredients of animal origin and all final live virus production lots for Mycoplasma contamination. Additional supplemental test procedures outlined in this guideline shall be used to test, MSV, MCS, and working seeds and cells for Mycoplasma contamination non-cultivable on broth and agar.

2. GUIDELINE FOR TESTING FOR MYCOPLASMA CONTAMINATION

2.1. Samples

Tests for Mycoplasma contamination shall be done on each final lot (batch) of live viral vaccine and whenever a test for mycoplasmas is prescribed for a virus harvest or a bulk vaccine. Each lot of master

seed virus (MSV), each lot of primary and master cell stock (MCS), and each working cell bank shall be tested for the Mycoplasmas contamination.

2.2. 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 final batches of vaccine. MSV, MCS, and working cell lots shall be tested using both a solid and liquid media culture method and either an indicator cell culture method or a polymerase chain reaction (PCR) detection method. When Mycoplasma contamination is detected using an indicator cell culture method the contamination must be confirmed by either colonies on agar or PCR. When Mycoplasma contamination is detected by a PCR method the contamination must be confirmed by DNA stained growth in an indicator cell culture or by colonies on agar.

2.3. Culture method

2.1.1. Laboratory validation for the culture method

The culture method should be carried out in a sufficient number of both solid and liquid media to insure the growth of a predetermined titer of the following 4 strains of mycoplasmas. (These species were chosen on the basis of their; likelihood of being a contaminant, sensitivity to antibiotics, fastidiousness, and pathogenicity. The number of species was based on practicality.)

<i>Acholeplasma laidlawii</i>	Passage 1-15
<i>Mycoplasma hyorhinis</i>	Passage 1-15
<i>Mycoplasma orale</i>	Passage 1-15
<i>Mycoplasma synoviae</i>	Passage 1-15

These strains will be isolated by labs of the European Pharmacopoeia who will produce a sufficient quantity of these Reference Strain Master Preparations to be distributed to the 3 regional government laboratories (Japan, Europe, and US) of this VICH Mycoplasma working group. A group of 3 government (1 from each member group) and 3 biologic firm laboratories (1 from each member group) will standardize and determine the variability of the titer of each of the 4 strains of Mycoplasma. These strains will then be distributed to government and biologics industry laboratories who wish to be validated as capable to detect Mycoplasma contamination. Those laboratories testing only mammalian products with no significant risk of exposure to avian origin ingredients will be exempt from testing.

M. synoviae as a reference organism. Those laboratories testing only avian products with no significant risk of exposure to mammalian origin ingredients will be exempt from testing *M. hyorhinis* as a control organism. Those laboratories testing products and ingredients which are free of antibiotics and preservatives will be exempt from testing *A. laidlawii* as a control organism. For validation each laboratory will be sent 3 vials of each reference strain for which they are required to determine a titer. A different production lot of the media or medias shall be used for each vial of a reference strain. After the laboratory completes the testing they shall report the colony forming units (CFU) of all 3 vials for each reference strain tested to the regional government laboratory supplying the reference vials. The regional government laboratory will then inform the laboratory whether their CFUs are within the determined variability limits and whether the laboratory is validated as capable of detecting Mycoplasma contamination. Each laboratory must prepare Reference Strain Working Preparations at the time when the first vial of each Reference Strain Master Preparations are tested. These Working Preparations should be dispensed into multiple vials and stored frozen. The working preparation of each species of master preparation will be used

to validate each production lot of broth and agar or whenever new lots of media ingredients are incorporated. The titer and variation of the working preparations shall be established with the same media lots used to test the 2nd and 3rd vials of the master preparations. Whenever a laboratory deems it necessary to change a media composition or method of preparation, they must request new vials of master reference from the regional government laboratory in order to revalidate their laboratory. At least one working reference strain must be used as a control with each test assay or test day. Each master reference will have its passage level marked on the vial and the passage level of each working reference should not exceed 15 passages with the master references passages included. (EXAMPLE; a laboratory testing for Mycoplasma contamination in mammalian products, containing antibiotics, would receive 3 vials each of *M. hyorhinis*, *M. orale*, and *A. laidlawii* master references from which they would produce working references and then determine the CFUs per ml of both the master and working references. The 3 working references would then be used to test each newly prepared lot of broth and agar. One of these working references would be used as a control on each test assay or test day.)

2.1.2. NUTRITIVE PROPERTIES OF NEW BATCHES OF MEDIA

Each new lot (batch) of media must be tested for the nutritive properties of working references determined above. Each testing laboratory must determine the inoculum, for each of their frozen working references, which will contain not more than 100 CFUs. Inoculate the solid media with not more than 100 CFUs per 60 mm plate and per 100 ml container of broth media; use a separate agar plate and broth container for each working reference. Incubate the agar and broth media and make subcultures from the broth onto agar at the specified intervals. The agar media batch complies with the test for nutritive properties if the titer of the CFU on the plates is as expected within the variability range. The broth complies if Mycoplasma growth on those agar plates sub-cultured from the broth is within the established variability range for each working reference.

2.1.3. INCUBATION CONDITIONS

Incubate the broth culture media in air. Incubate all agar plates under microaerophilic conditions (nitrogen containing 5-10% CO₂). For solid media maintain an atmosphere of adequate humidity to prevent desiccation of the surface.

2.1.4. INHIBITORY SUBSTANCES

Carry out the test for nutritive properties in the presence of the product to be examined. If growth of the working references are notably less (using the variability range) than that found in the absence of the product to be examined, the latter contains inhibitory substances which must be neutralized or their effect otherwise countered, for example; by dilution in a larger volume of media, before the test for mycoplasma contamination is carried out. The effectiveness of the neutralization or other process is checked by repeating the test for inhibitory substances after neutralization or dilution. These tests for inhibitory substances should be carried out during prelicense testing, and any additional neutralization or dilution required should be included in the product's outline for production. If the inhibitory testing was not carried out during the prelicense process then this testing should be conducted before the next batch of product is tested for mycoplasma contamination.

2.1.5. TEST METHOD

2.1.5.1 Inoculate each plate of each solid media with 0.2 ml of product to be examined and inoculate 10 ml per 100 ml of each liquid media. Incubate the agar plates at 35°C to 38°C, microaerophilically, for 14 days in an atmosphere of adequate humidity to prevent desiccation of the

surface. Incubate the liquid media at 35° C to 38° C in air for 14 days. At the same time incubate an uninoculated 100 ml portion of each liquid media and agar plates as a negative control. If any significant pH change occurs on addition of the product to be examined, restore the liquid media to its original pH value by the addition of a solution of either sodium hydroxide or hydrochloric acid. On day 2 or 3 after inoculation, subculture each liquid culture by inoculating 1 plate of each solid media with 0.2 ml and incubate them at 35°C to 38°C microaerophilically for not less than 14 days. Repeat the procedure on the 6th or 7th day and again on the 13th or 14th day of the test. Observe the liquid media every 2 or 3 days and if a color change occurs, subculture immediately. Observe solid media once per week.

2.1.5.2 If the liquid media shows bacterial or fungal contamination, repeat the test. If any of the plates inoculated before the 7th day are broken or contaminated then the test must be repeated. As long as one of the plates inoculated on the 7th or 14th day, can be read for mycoplasma contamination the test is considered valid and the bacteria or fungi contamination is ignored. Read all plates that can be read.

2.1.5.3 Include in the test, positive controls prepared by inoculating not more than 100 colony forming units of one of the working reference species onto the agar plates and into the broth medias. This control shall be used on each test session conducted with a media that has been validated for nutritive properties using working references determined by the types of products being tested as explained in section 2.1.1 of this guideline.

2.1.6 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 Mycoplasma colonies has not occurred on any of the inoculated solid media. If growth of Mycoplasma colonies has occurred on any of the solid media, the test may be repeated once (2^d test) with the original technique. If the retest is not performed the sample is considered contaminated. If retesting is done and the retest (2^d test) is positive for Mycoplasma. colonies then the sample is considered contaminated with Mycoplasma. If the retest (2^d test) is negative for Mycoplasma colonies, another retest (3rd test) must be performed. Laboratories may run two retests (2^d and 3^d tests) simultaneously to save time. If two or more of the three tests are positive for Mycoplasma colonies, the product is considered contaminated with Mycoplasma. The test is invalid if the positive controls do not show growth of the relevant working reference organisms.

2.2 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.

2.2.1 VALIDATION OF THE INDICATOR CELL METHOD

Using an indicator cell culture substrate which has a discrete nucleus and no extra nuclear chromatin DNA (e.g., VERO or 3T6 cells), validate the procedure using an inoculum of not more than 100 CFU of the working references determined by the types of products being tested, as explained in section 2.1.1. Working references used to test batches of the laboratories broth and agar shall be used to test 3 different passages of indicator cells to determine the method's ability to isolate Mycoplasma at least as well as the validated broth and agar method.

2.2.2 TEST METHOD

Take not less than 1 ml of the product to be examined and use it to inoculate, as described in the Procedure (Section 2.2.3), indicator cell cultures representing not less than 25 cm² of cell culture confluence. Include in the test a negative (non-infected) control and 1 working reference Mycoplasma control. Use an inoculum of not more than 100 CFU for the working reference control.

If for viral 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 a 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 and absence of neutralizing antiserum.

2.2.3 RECOMMENDED PROCEDURE

2.2.3.1 Seed indicator cell culture at a regular density (2 X 10⁴ to 2 X 10⁵ cells per ml, 4 X 10³ to 2.5 X 10⁴ cells/cm²) and incubate at 35° C to 38° C for at least 2 days. Inoculate the product to be examined and incubate for at least 2 days; make no fewer than 1 subculture. Grow the last subculture on cover slips in suitable containers or on some other surface (chambered slides) suitable for the test procedure. Do not allow the last subculture to reach confluence since this would inhibit staining and impair visualization of mycoplasmas.

2.2.3.2 Remove and discard 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.

2.2.3.3 Remove the fixing solution and discard. Wash off the fixing solution with sterile water and dry slides completely if they are to be stained later.

2.2.3.4 Add a suitable fluorescent dye that binds to DNA such as bisbenzamide stain (Hoechst) and allow to stand for a suitable time.

2.2.3.5 Remove the stain and rinse the monolayer with water. Mount the cover slips if applicable and examine the slides by epifluorescence (for bisbenzamide stain use a 330 nm/380 nm excitation filter, LP 440 nm barrier filter) at 100-400 X magnification or greater.

2.2.3.6 Compare the microscopic appearance of the test cultures with that of the negative and working reference controls, examining for extranuclear fluorescence. Mycoplasmas produce pinpoint or filaments over the indicator cell cytoplasm. They may also produce pinpoint and filaments in the intercellular spaces.

2.2.4 JUDGMENT OF THE INDICATOR CELL CULTURE METHOD

The product being examined is negative for Mycoplasma contamination if there is no evidence of pinpoint or filaments of extra-nuclear fluorescence. The test is invalid if the positive controls do not show the presence of the appropriate extra nuclear fluorescence of the test organisms. If there is evidence of pinpoint or extra-nuclear fluorescence indicative of Mycoplasma contamination it must be confirmed by either colonies on agar or by PCR.

2.3 PCR MYCOPLASMA DETECTION METHOD

..identify the PCR product by accepted relevant methodology (e.g. specific priming, size of amplicon, sequence, or probe hybridization).

The mycoplasma DNA is extracted from a broth or cell culture. The DNA extract is then gene amplified using a polymerase chain reaction (PCR). The gene amplification product is then identified by accepted relevant methodology (e.g., size of amplicon, probe hybridization, sequence). For example, the product may be processed by electrophoresis on an agarose gel. DNA bands on the agarose gel would then be determined to be specific for mycoplasma because of their size or specific staining.

2.3.1 VALIDATION OF THE PCR METHOD

Using a broth or cell culture substrate containing not more than 100 CFU of the working references determined by the types of products being tested, as explained in section 2.1.1. Working references used to test batches of the laboratories broth and agar shall be used to test 3 separate PCR method's ability to isolate Mycoplasma at least as well as the validated broth and agar method.

2.3.2 TEST METHOD

Take not less than 1 ml of the product to be examined and use it to inoculate 100 ml of broth culture substrate or cell cultures representing not less than 25 cm² of cell culture confluence. With each test include a negative (non-infected) control and 1 working reference control. Use an inoculum of not more than 100 CFU for the working reference control.

2.3.3 RECOMMENDED PROCEDURE

2.3.3.1 Inoculate 1ml of the product into 100 ml of broth or 25 cm² of cell culture confluence.

2.3.3.2 Incubate the broth for 7 days at 35°C to 38°C. Incubate the inoculated cells for at least 2 days at 35° C to 38° C and make at least 1 subculture, incubating it for at least 2 days.

2.3.3.3 Extract the DNA by centrifuging 0.5 ml of the broth or cell culture at 15,000 X g at 4°C for 20 min. Remove the substrate and react with 40 ul of a DNA extract solution for 20 min. at 60°C and then 10 min at 94°C.

2.3.3.4 Amplify the mycoplasma specific 16 S ribosomal RNA by adding 5 ul of the DNA extract to 45 ul of a polymerase chain react solution containing the primers: MP 1: 5'-GCTGCGGTGAATACGTTCT-3' MP2: 5'TCCCCACGTTCTCGTAGGG-3'

2.3.3.5 Achieve the amplification with 30 cycles of denaturation at 94°C for 30 sec., annealing at 50°C for 30 sec. and extension at 72°C for 5 min, followed by a final extension at 72°C for 1 min.

2.3.3.6 Identify the PCR product by accepted relevant methodology, e.g., size of amplicon, probe hybridization, or sequence.

2.3.4 JUDGMENT OF THE PCR METHOD

The product being examined is negative for Mycoplasma contamination when specific DNA amplification is detected. The test is invalid if the broth or cells inoculated with the working reference control do not contain DNA which is amplified to the specific 160 base pair product expected. When Mycoplasma contamination is detected by a PCR method the contamination must be confirmed by DNA stained growth in an indicator cell culture or by colonies on agar.