⟨63⟩ MYCOPLASMA TESTS

INTRODUCTION

The genus Mycoplasma represents a group of minute bacteria which have no cell walls. The genus comprises more than 120 species. They are the smallest self-replicating prokaryotic organisms. The cells vary in size and morphology and cannot be Gram stained, but impressions of colonies on solid agar can be stained with methylene blue or equivalent stain. Mycoplasma are parasites and commensals, and some may be pathogenic to a variety of animal and plant hosts. In humans, Mycoplasma are usually surface parasites that colonize the epithelial lining of the respiratory and urogenital tracts. Mycoplasma are common and may cause serious contamination in cell and/or tissue cultures used to generate compendial articles. They may also cause contamination of filtered sterilized soybean casein digest broth. A cell culture infection may persist for an extended period of time without causing apparent cell damage. Infection of cells in a culture can affect nearly every pathway of cell metabolism, including alteration of the cells' phenotypical characteristics and normal growth. The presence of Mycoplasma species does not always result in turbid growth in cultures or visible alteration of the cells.

Testing for Mycoplasma is a necessary quality control requirement to assure reliably pure biotechnological products and allied materials used to generate these products. This general test chapter describes two methods required to detect Mycoplasma contamination of test articles, tissues and/or cell cultures used to produce test articles, digest broth, or any other material in which Mycoplasma contamination is suspected. These are: (A) the agar and broth media procedure and (B) the indicator cell culture procedure. These tests require careful aseptic technique and suitable laboratory conditions. In order to ensure appropriate testing and interpretation of results, personnel should be properly trained and qualified. A validated nucleic acid amplification technique (NAT) or an enzymatic activity based method may be used to detect Mycoplasma, provided such a method is shown to be comparable to both methods (A) and (B). Alternative methods must be suitably validated. Validation requirements for alternate methods will not be addressed in this chapter.

CULTURE METHOD

Choice of Media

The test is carried out using a sufficient number of both solid and liquid media to ensure growth in the chosen incubation conditions of small numbers (approximately 100 colony-forming units, cfu; or 100 color-changing units, ccu) of Mycoplasmas that may be present in the test article/material. Liquid media must contain phenol red. The range of media chosen is shown to have satisfactory nutritive properties for at least the microorganisms shown in *Quality Control Test Strain Organisms* (below). The nutritive properties of each new batch of medium are verified for the appropriate microorganisms in the list. When testing for Mycoplasmas include in each test at least two known Mycoplasma species or strains (listed in *Quality Control Test Strain Organisms*) as positive controls, one of which should be a dextrose fermenter (i.e., *M. pneumoniae* or equivalent species and strain) and one of which should be an arginine hydrolyzer (i.e., *M. orale* or equivalent species and strain). Only when testing insect cell lines should one include a *Spiroplasma* control strain (e.g., *S. citri* ATCC 29747, *S. melliferum* ATCC 29416, or equivalent species and strains). Additionally, these strains may be a little more fastidious in their nutritional requirements. They require lower incubation temperatures (as do insect cell lines).

Quality Control Test Strain Organisms

Positive control cultures should be not more than 15 passages from isolation. Mycoplasma species or strains suitable for use are listed below:

- *Acholeplasma laidlawii* (vaccines and/or cell-derived materials/cultures for human and veterinary use when an antibiotic has been used during production)
- *M. gallisepticum* (when avian material has been used during production or when the vaccine or cell culture is intended for use in poultry)
- *M. hyorhinis* (nonavian veterinary vaccines or cell cultures)
- *M. orale* (vaccines for human and veterinary use)
- *M. pneumoniae* (vaccines or cell banks for human use) or another suitable species of D-glucose fermenter such as *M. fermentans*
- *M. synoviae* (when avian material has been used during production or when the vaccine or cell bank is intended for use in poultry)

The test strains may be field isolates that have undergone a limited number of subcultures (not more than 15), are stored frozen (−20° or lower) or freeze-dried, and are identified as being of the required species by comparison with type cultures, for example, those shown in *Table 1*.

Table 1. Type Cultures for Identifying Field Isolates Used as Test Strains

Test Organism	NCTC Number	CIP Number	ATCC Number
A. laidlawii	NCTC 10116	CIP 75.27	ATCC 23206
M. gallisepticum	NCTC 10115	CIP 104967	ATCC 19610

Table 1. Type Cultures for Identifying Field Isolates Used as Test Strains *(continued)*

Test Organism	NCTC Number	CIP Number	ATCC Number
M. fermentans	NCTC 10117	CIP 105680	ATCC 19989
M. hyorhinis	NCTC 10130	CIP 104968	ATCC 17981
M. orale	NCTC 10112	CIP 104969	ATCC 23714
M. pneumoniae	NCTC 10119	CIP 103766	ATCC 15531
M. synoviae	NCTC 10124	CIP 104970	ATCC 25204

Incubation Conditions

Incubate liquid media in tightly stoppered containers at 36 ± 1°. Incubate solid media in microaerophilic conditions (hydrogen atmosphere containing < 0.5% oxygen and/or nitrogen containing 5%–10% carbon dioxide in nitrogen). Sufficient humidity should be available to prevent desiccation of the agar surface at 36 ± 1°.

Nutritive Properties

Carry out the test for nutritive properties for each new batch of medium. Inoculate the chosen media with the appropriate test microorganisms; use not more than 100 cfu per plate containing at least 9 mL of solid media and per 100-mL container of liquid medium; use a separate plate and container for each species of microorganism. Incubate the media and make subcultures from 0.2 mL of liquid medium to solid medium at the specified intervals (see below under *Test for Mycoplasma in the Test Article/Material*). The solid medium complies with the test if a count within a 0.5-log unit range of the inoculate amount is found for each test microorganism. The liquid medium complies with the test if growth is found on agar plates subcultured from the broth, for at least 1 subculture for each test microorganism. The use of a microscope at 100× or greater may be helpful.

Inhibitory Substances

The test for inhibitory substances is carried out once for a given product and is repeated whenever there is a change in production method that may affect the detection of Mycoplasma. To demonstrate absence of inhibitory substances, carry out the test for nutritive properties in the presence and absence of the test article/material. If growth of a test microorganism occurs more than 1 subculture sooner in the absence of the test article/material than in its presence, inhibitory substances are present. The same is true if plates directly inoculated with the test article/material are not within a 0.5-log unit range of the number of colonies of those inoculated without the test article/material. In both cases, inhibitory substances must be neutralized or their effect otherwise countered, by an appropriate method, for example, by passage in substrates not containing inhibitors or dilution in a larger volume of medium, before the test. If dilution is used, larger medium volumes may be used or the inoculums' volume may be divided among several 100-mL flasks. The effectiveness of the neutralization or other process is checked by repeating the test for inhibitory substances after neutralization.

Test for Mycoplasma in the Test Article/Material

Inoculate no less than 10 mL of the test article/material per 100 mL of each liquid medium. If a significant pH change occurs upon the addition of the test article/material, the liquid medium is restored to its original pH value by the addition of a sterile solution of either sodium hydroxide or hydrochloric acid. Inoculate 0.2 mL of the test article/material on each plate of each solid medium. Incubate liquid media for 20–21 days. Incubate solid media for not less than 14 days, except those plates corresponding to the 20–21 day subculture, which are incubated for 7 days. Concurrently, incubate an uninoculated 100-mL portion of each liquid medium and agar plate, as a negative control. On days 2–4 after inoculation, subculture each liquid medium by inoculating 0.2 mL on at least 1 plate of each solid medium. Repeat the procedure between days 6 and 8, again between days 13 and 15, and again between days 19 and 21 of the test. Observe the liquid media every 2 or 3 days and if a color change occurs, subculture. If a liquid medium shows bacterial or fungal contamination, the test is invalid. The test is valid if at least 1 plate per medium and per inoculation day can be read. Include in the test positive controls prepared by inoculation of not more than 100 cfu of at least 1 test microorganism on agar medium or into broth medium. Where the test for Mycoplasmas is carried out regularly, it is recommended to use the test microorganisms in regular rotation. The test microorganisms used are those listed under *Choice of Media*. Incubate broths and plates in a humidified atmosphere with microaerophilic conditions (5%–10% CO_2).

Interpretation of Results

At the end of the prescribed incubation period, examine all inoculated solid media for the presence of Mycoplasma colonies. The product complies with the test if growth of typical Mycoplasma colonies has not occurred. The product does not comply with the test if growth of typical Mycoplasma colonies has occurred on any of the solid media. The test is invalid if 1 or more of the positive controls do not show growth of Mycoplasmas on at least 1 subculture plate. The test is invalid if 1 or more of the negative controls show growth of Mycoplasmas. If suspect colonies are observed, use a suitable validated method to determine whether they are due to Mycoplasmas.

Recommended Solutions and Media for the Culture Method

[NOTE—This section is provided for information.]

SOLUTIONS

Beef Heart Infusion Broth

Beef heart (for preparation of the infusion)	500 g
Peptone	10 g
Sodium chloride	5 g
Distilled water	to 1000 mL

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
Riboflavine	10 mg
Thiamine hydrochloride	100 mg
Distilled water	to 1000 mL

Agar, Purified

A highly refined agar for use in microbiology and immunology, prepared by an ion-exchange procedure that results in a product having superior purity, clarity, and gel strength. It contains the following ingredients:	
Water	12.2%
Ash	1.5%
Acid-insoluble ash	0.2%
Chlorine	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%

Hanks' 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
Distilled water	to 800 mL

Brain Heart Infusion

Calf-brain infusion	200 g
Beef-heart infusion	250 g
Proteose peptone	10 g
Glucose monohydrate	2 g
Sodium chloride	5 g

Brain Heart Infusion *(continued)*

Disodium hydrogen phosphate, anhydrous	2.5 g
Distilled water	to 1000 mL

PPLO Broth

Beef-heart infusion	50 g
Peptone	10 g
Sodium chloride	5 g
Distilled water	to 1000 mL

MEDIA

The following media are recommended. Other media may be used, provided they meet the criteria given in the sections *Choice of Culture Media, Incubation Conditions, Nutritive Properties,* and *Inhibitory Substances.*

Hayflick Media (Recommended for the general detection of Mycoplasmas)

Liquid Medium	
Beef heart infusion broth	90.0 mL
Horse serum (unheated)	20.0 mL
Yeast extract (250 g/L) (fresh yeast extract is recommended)	10.0 mL
Phenol red (0.6 g/L solution)	5.0 mL
Penicillin (20,000 IU/mL)	0.25 mL
Deoxyribonucleic acid (2 g/L solution)	1.2 mL
Adjust to a pH of 7.8	
Solid Medium	
Prepare as described above replacing beef heart infusion broth by beef heart infusion agar containing 15 g/L of agar.	

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

Liquid Medium	
Beef heart infusion broth	90.0 mL
Essential vitamins	0.025 mL
Glucose monohydrate (500 g/L solution)	2.0 mL
Swine serum (inactivated at 56° for 30 min)	12.0 mL
β-Nicotinamide adenine dinucleotide (10 g/L solution)	1.0 mL
Cysteine hydrochloride (10 g/L solution)	1.0 mL
Phenol red (0.6 g/L solution)	5.0 mL
Penicillin (20,000 IU/mL)	0.25 mL
Mix the solutions of β-nicotinamide adenine dinucleotide and cysteine hydrochloride and after 10 min add to the other ingredients. Adjust to a pH of 7.8.	
Solid Medium	
Beef heart infusion broth	90.0 mL
Agar, purified	1.4 g
Adjust to pH 7.8, sterilize by autoclaving then add:	
Essential vitamins	0.025 mL
Glucose monohydrate (500 g/L solution)	2.0 mL
Swine serum (unheated)	12.0 mL
β-Nicotinamide adenine dinucleotide (10 g/L solution)	1.0 mL
Cysteine hydrochloride (10 g/L solution)	1.0 mL
Phenol red (0.6 g/L solution)	5.0 mL
Penicillin (20,000 IU/mL)	0.25 mL

Friis Media (Recommended for the detection of nonavian Mycoplasmas)

Liquid Medium	
Hanks' balanced salt solution (modified)	800 mL
Distilled water	67 mL
Brain heart infusion	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 to a pH of 7.40–7.45	
Solid Medium	
Hanks' balanced salt solution (modified)	200 mL
DEAE-dextran	200 mg
Agar, purified	15.65 g
Mix well and sterilize by autoclaving. Cool to 100°. Add to 1740 mL of *Liquid Medium* as described above.	

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 surrounding areas. Mitochondria in the cytoplasm may be stained but are readily distinguished from Mycoplasmas. For viral suspensions, if the interpretation of results is affected by marked cytopathic effects, neutralize the virus using a specific antiserum that has no inhibitory effects on Mycoplasmas, or use a cell culture substrate that does not allow growth of the virus. To demonstrate the absence of inhibitory effects of serum, carry out the positive control tests in the presence and absence of the antiserum.

Verification of the Substrate

Use Vero cells or equivalent cell culture (for example, the production cell line) that is equivalent in effectiveness for detecting Mycoplasmas. Test the effectiveness of the cells to be used by applying the procedure shown below and inoculating not more than 100 cfu or ccu microorganisms of suitable reference strains of *M. hyorhinis* and *M. orale.* The cells are suitable if both reference strains are detected. The indicator cells must be subcultured without an antibiotic before use in the test.

Test Method

[NOTE—The following is provided for information.]

SOLUTIONS

Phosphate Buffered Saline

2.0 M Monobasic Potassium Phosphate—Dissolve 13.61 g of anhydrous monobasic potassium phosphate in 50 mL of water.

2.0 M Dibasic Potassium Phosphate—Dissolve 17.42 g of anhydrous dibasic potassium phosphate in 50 mL of water.

Phosphate Buffered Saline Solution (pH 7.4)—Combine 3.6 mL of *2.0 M Monobasic Potassium Phosphate,* 16.4 mL of *2.0 M Dibasic Potassium Phosphate,* 8 g of sodium chloride, and 1 L of water. Mix thoroughly. Adjust the pH if necessary.

Bisbenzimide Stock Solution—Dissolve 5 mg of bisbenzimide in water, and dilute with the same solvent to 100 mL. Store in the dark.

Bisbenzimide Working Solution—Immediately before use, dilute 100 µL of *Bisbenzimide Stock Solution* with *Phosphate Buffered Saline Solution (pH 7.4)* to 100 mL.

Phosphate-Citrate Buffer Solution pH 5.5—Mix 56.85 mL of a 28.4-g/L solution of anhydrous disodium hydrogen phosphate and 43.15 mL of a 21-g/L solution of citric acid.

METHOD

1. Seed the indicator cell culture at a suitable density (for example, 2×10^4 to 2×10^5 cells/mL, 4×10^3 to 2.5×10^4 cells/cm^2) that will yield confluence after 3 days of growth. Inoculate 1 mL of the product to be examined into the cell culture vessel, and incubate at 36 ± 1°.

2. After at least 3 days of incubation, when the cells have grown to confluence, make a subculture on cover slips in suitable containers or on some other surface (for example, chambered slides) suitable for the test procedure. Seed the cells at low density so that they reach 50% confluence after 3–5 days of incubation. Complete confluence impairs visualization of Mycoplasmas after staining and must be avoided.
3. Remove the medium and rinse the indicator cells with phosphate buffered saline, pH 7.4, then add a suitable fixing solution (a freshly prepared mixture of 1 volume of acetic acid, glacial, TS and 3 volumes of methanol, is suitable when bisbenzimide is used for staining).
4. Remove the fixing solution and wash the cells with sterile Purified Water. Dry the slides completely if they are to be stained more than 1 hour later (particular care is needed for staining of slides after drying owing to artifacts that may be produced).
5. Add a suitable DNA stain and allow standing for a suitable time (bisbenzimide working solution and a standing time of 10 minutes are suitable).
6. Remove the stain and rinse the monolayer with Purified Water.
7. Mount each coverslip, where applicable (a mixture of equal volumes of glycerol and *Phosphate-Citrate Buffer Solution pH 5.5* is suitable for mounting). Examine by fluorescence (for bisbenzimide stain a 330 nm/380 nm excitation filter and an LP 440 nm barrier filter are suitable) at 400× magnification or greater.
8. Compare the microscopic appearance of the test cultures with that of the negative and positive controls, examining for extranuclear fluorescence. Mycoplasmas produce pinpoints or filaments over the indicator cell cytoplasm. They may also produce pinpoints and filaments in the intercellular spaces. Multiple microscopic fields are examined according to the protocol established during validation.

Interpretation of Results

The product to be examined complies with the test if fluorescence typical of Mycoplasmas is not present. The test is invalid if the positive controls do not show fluorescence typical of Mycoplasmas. The test is invalid if the negative controls show fluorescence typical of Mycoplasmas.