# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLE OF CONTENTS</td>
<td>1</td>
</tr>
<tr>
<td>BIOLOGY AND TAXONOMY</td>
<td>2</td>
</tr>
<tr>
<td>SAMPLING AND PRESERVATION OF SPECIMENS</td>
<td>2</td>
</tr>
<tr>
<td>ISOLATION OF MYCOPLASMAS FROM CLINICAL SPECIMENS</td>
<td>3</td>
</tr>
<tr>
<td>OBTAINING PURE CULTURES</td>
<td>4</td>
</tr>
<tr>
<td>IDENTIFICATION OF MYCOPLASMAS ISOLATED FROM ANIMALS</td>
<td>4</td>
</tr>
<tr>
<td>Microscopic identification</td>
<td>4</td>
</tr>
<tr>
<td>Identification</td>
<td>4</td>
</tr>
<tr>
<td>Biochemical Identification Methods</td>
<td>5</td>
</tr>
<tr>
<td>Serological Identification Methods</td>
<td>5</td>
</tr>
<tr>
<td>CONTAGIOUS BOVINE PLEUROPNEUMONIA DIAGNOSIS</td>
<td>8</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>9</td>
</tr>
<tr>
<td>MYCOPLASMAS CAUSING DISEASE IN ANIMAL AND THEIR DIAGNOSTIC INDEX</td>
<td>9</td>
</tr>
<tr>
<td>APPENDIX: MEDIA FORMULATIONS AND TECHNIQUE</td>
<td>12</td>
</tr>
<tr>
<td>Biochemical Tests</td>
<td>13</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>20</td>
</tr>
</tbody>
</table>
BIOLOGY AND TAXONOMY

Mycoplasmas are the smallest known bacteria and lack several of the capabilities expressed by other bacteria. The absence of a cell wall is the primary basis for inclusion of an organism in the class Mollicutes. The organism is bound by a single trilaminar membrane which is the reason for the "fried egg" appearance that is characteristic of colonies growing on solid media. Lack of a cell wall renders the organism resistant to antibiotics (such as penicillins and cephalosporins) that interact with bacterial cell wall-associated proteins.

All mycoplasmas are included under the class *Mollicutes* (mollis, soft; cutis, skin), order *Mycoplasmatales*. Subdivision into families is based on habitat, sterol requirement for growth, genome size, oxygen requirement and the mechanism used to obtain energy. *Mycoplasma* species are defined by the above criteria, supplemented by additional biochemical properties, and by serological relatedness.

Class: *Mollicutes*

Order: *Mycoplasmatales*

Family I: *Mycoplasmataceae*

Genus I: *Mycoplasma*:

5 species: Human and animal habitat; Cholesterol required for growth

Genus II: *Ureaplasma*:

5 species: Urea hydrolysis; Human and animal habitat; Cholesterol required for growth

Order: *Acholeplasmatales*

Family I: *Acholeplasmataceae*

Genus I: *Acholeplasma*:

9 species described; Animals, some plants and insects as habitat; No cholesterol requirement

The characteristics of small size, close association to mammalian cells, and resistance to penicillin are used to design strategies for the isolation of mycoplasmas from clinical specimens. Thus complex media is required and must contain adequate levels of protein to supply the necessary osmolarity, and it must be prepared free of contaminants (due to poor quality laboratory water or reagents). Animal serum is added to the medium because many mycoplasmas require an external source of sterols and fatty acids.

SAMPLING AND PRESERVATION OF SPECIMENS

Samples from live animals are limited to body secretions and accessible sites that are amenable to swabbing. Joint aspirates, transtracheal washes, endometrial lavages or biopsies, and cystocentesis of the urinary bladder require specialized invasive methods to obtain samples for mycoplasma isolation.
In dead animals and fetuses that are in a good state of preservation, samples of affected tissue with visible lesions are preferred for culture.

Swabs used to collect specimens for culture must always be placed in an appropriate transport medium, preferably in a small aliquot of mycoplasma broth, but Amies' (without charcoal) or Stuart's medium can also be used. Dry swabs are useless if not cultured immediately after sample collection. To ensure maximal survival of mycoplasmas in samples, these three cardinal rules apply: (1) keep it moist, (2) keep it cool, and (3) move fast. Biological samples for isolation of mycoplasmas should be submitted moist and refrigerated and processed promptly for maximal recovery. Bacterial and fungal overgrowth is usually a much more serious problem than reduced mycoplasma survival on samples. Environmental extremes resulting in excessive heat, desiccation, etc., must also be avoided.

If a delay of a day or more is anticipated before inoculation and incubation of the specimens, or if a sample needs to be preserved for future culturing, it should be placed in liquid nitrogen or in an ultra-cold (< -70°C) freezer. Dry ice, although preferable to wet ice, is less effective in preserving mycoplasmas than liquid nitrogen.

**ISOLATION OF MYCOPLASMAS FROM CLINICAL SPECIMENS**

Isolation of mycoplasmas is maximized if samples are placed on media within a few hours of collection. The samples are inoculated directly on the appropriate agar-based media (usually dispensed in 50 ml Petri dishes to conserve media). To increase the probability of isolation, part of the sample can be plated directly while part is incubated 2-3 days in broth medium before being incubated. Broth-to-broth passages may also increase results.

Tissue samples may be cultured by pushing a piece of aseptically collected tissue across the surface of an agar plate. Large tissue specimens may be seared with a hot spatula to reduce surface contamination, cut open with a sterile scalpel blade and the inner surface swabbed. Alternatively, the tissue may be dipped in alcohol, flamed, and minced, macerated or ground with appropriate mycoplasma broth or phosphate-buffered saline (PBS pH 7.4. However, tissue homogenates may release mycoplasmacidal substances. Homogenized tissues are titrated using serial 10-fold dilutions in broth medium, to remove mycoplasma inhibitors inherently present in tissues. Each of the dilutions is then directly, and after 3 days of incubation in the broth, inoculated onto agar to detect the presence of mycoplasmas.

Most mycoplasmas grow poorly in ordinary aerobic incubators, as they require lowered oxygen tension and high humidity for growth. The ideal is an incubator set at 36-37°C with a 5-10% CO₂ atmosphere that is bubbled through water to maintain high humidity. Candle jars are not acceptable because there are probably some toxic products generated by the burning candle that inhibit growth of mycoplasmas. Once inoculated, mycoplasma colonies are usually seen after at least 48-72 hours incubation, however, they may require incubation up to 10-14 days for growth to be observed. Mycoplasma colonies are easily observed with the aid of an inverted compound microscope equipped with a 10X objective. A stereo dissecting microscope may also be used.
OBTAINING PURE CULTURES

A pure culture of mycoplasma is essential before any biochemical study can be performed. The recommended procedure is to clone 3 times by gentle filtration of a broth culture through a membrane filter of 0.22-0.45μm. The ability to pass through a filter that would normally retain bacteria is a typical characteristic of mycoplasma. Dilutions of the filtrate (10-fold) are then placed onto agar to obtain single colonies. Each colony should represent the progeny of a single cell that passed through the filter and is therefore a clone. Clones are picked and transferred to broth. The procedure is repeated at least two more times.

IDENTIFICATION OF MYCOPLASMAS ISOLATED FROM ANIMALS

Microscopic identification

Electron microscopy will give clear evidence for the absence of a cell wall, but it is not practical for most laboratories. An alternate procedure is to examine a broth culture by phase-contrast or dark field microscopy to demonstrate pleomorphism. Mycoplasmas have a variety of sizes and shapes, from small coccoid bodies to large aggregates to fine filaments.

Distinguishing mycoplasmas from walled bacteria and bacterial L-forms

Most mycoplasma colonies have a “fried egg” morphology on agar media. A dense central core grows down into the medium whereas the periphery is primarily surface growth. An inoculating loop will easily remove the periphery, but the central core will remain embedded in the medium. Mycoplasma colonies can be differentiated from bacterial colonies by a variety of criteria. They are usually smaller (50-500 μm in diameter), have the “fried egg” morphology, and retain the Dienes’ stain. A 1 cm x 1 cm plug of agar is excised and placed colony-side-up on a glass microscope slide. A cover slip that has been smeared with Dienes’ stain is placed on the agar plug. Mycoplasma colonies stain blue and will retain the stain while most bacterial colonies will remain unstained. The final determination can be made within 30 minutes.

To exclude bacterial L-forms, broth cultures or colonies are subcultured five consecutive times in an inhibitor-free (lacking penicillin) broth medium, followed by subculture to an inhibitor-free agar medium to test for reversion to a wall-covered bacterial form. Alternatively, colonies on an agar medium can be transferred directly from inhibitor-free agar plate to plate by friction smears. Bacterial L-forms are also excluded if the cultures are shown both biochemically and serologically to be a known Mycoplasma species.

Identification

Tests to detect specific enzymatic and nutritional requirements are used to differentiate mycoplasmas at the family and genus level. The requirement for sterols separates the family Mycoplasmataceae from the family Acholeplasmataceae, whereas the hydrolysis of urea differentiates the genus Ureaplasma from the genus Mycoplasma. Biochemical tests are useful at the species level to characterize an isolate and narrow the choice of specific antisera or reagents needed for the final serological identification.
Speciation of mycoplasmas is based on serological tests involving membrane antigens. These tests include growth inhibition (GI), metabolic inhibition (MI), and immunofluorescence (IF), which can vary in their sensitivity, specificity and ease of performance.

**Digitonin: an indirect test for sterol requirement**

The requirement for sterol separates mycoplasmas (sterol-dependent) from acholeplasmas (non-sterol dependent). A direct test measures the growth response of the organism to increasing levels of cholesterol. An indirect test is growth inhibition by digitonin. This test is carried out on a solid medium that will support growth of the organism. The surface of a plate is flooded with a broth culture and the excess inoculum removed to produce a uniform lawn of growth upon incubation. A digitonin disk is placed on the surface of the medium after it has dried. The disks can be prepared by impregnating 6mm paper disks with 25µl of a 1,5% solution of digitonin in ethanol. They can be used fresh or allowed to dry and then stored at 4°C. Mycoplasmas are sensitive to digitonin (4-5mm clear zone of inhibition) and acholeplasmas are resistant (no or a 1-2mm zone of inhibition).

**Biochemical Identification Methods**

The following biochemical tests have proven adequate to characterize most *Mycoplasma* isolates prior to serological identification.

1. **Glucose fermentation**
   
The simplest test is based on the determination of a decrease in pH of the growth medium. This test is carried out in a basal broth medium with added glucose (1%) and 0,5 ml of a 1% phenol red solution per 100 ml. Appropriate controls are used to make a valid interpretation of this test. These are an (a) inoculated and (b) an uninoculated test tube of the basal medium with the test substrate and (c) an inoculated test tube of basal medium without the test substrate. The inoculum should be 1 ml of a triple cloned culture actively growing in basal medium. Media can be incubated up to 2 weeks.

2. **Arginine and urea hydrolysis**
   
The procedure for glucose fermentation should be followed with these two modifications: replace glucose with arginine (0,2%) or urea (1%), and adjust the pH of the medium to 7,0. The use of appropriate controls and quality control checks, as for glucose fermentation, will largely overcome problems involved with the interpretation of these tests.

3. **Phosphatase activity**
   
Petri plates (50 mm diameter) are inoculated in triplicate and incubated at 37°C along with 3 uninoculated control plates. On days 3, 7 and 14 post- inoculation, one test plate and one control plate are flooded with 5N NaOH. A positive test is indicated by the immediate appearance of red in the medium. A negative test is indicated by little or no color change.

**Serological Identification Methods**

The most commonly serological tests used for identification of mycoplasmas are the growth inhibition (GI), metabolic inhibition (MI) and immunofluorescence (IF) tests.
1. Growth inhibition (GI) test

Specific antisera will inhibit the growth of homologous mycoplasmas. Inhibition of growth can occur either on solid or in liquid media. The result of the test on solid media can be observed directly or microscopically. The results in liquid media can be determined indirectly by means of a metabolic substrate. The growth inhibition test on solid medium is a species-specific test that is economical to perform, rapid to set up, and requires only potent antisera, appropriate growth medium, and a stereo or low-power light microscope.

The GI test is performed on a solid medium using 6 mm filter paper disks impregnated with 25μl antiserum. High-titred, monospecific antiserum should be used and preservatives that might inhibit mycoplasma growth should be avoided. Serum can be applied to the disks immediately before use, or the disks can be made up in quantity, dried, and stored at -20°C for later use.

The test organism should be a pure culture, i.e., triply cloned through membrane filters. Mixed cultures can lead to faulty conclusions because a zone of inhibition could be obscured by the growth of an uninhibited organism. The inoculum titer should be in the range 10^3 to 10^4 CFU/ml. A high inoculum will decrease the size of the zone whereas a small one will give an inconclusive result.

The plate is flooded with the inoculum and the excess fluid removed. The surface of the plate should be dry before applying the serum disk. Allow 2cm² of surface area for each disk. It is useful to incubate at 30°C overnight for rapid growing organisms before transferring to 37°C. The reason for this is that rapid growers can occasionally yield smaller inhibition zones when incubated directly at 37°C. Cutting wells in the agar and filling them with antiserum will also enhance results and is the method of choice. Zones up to 2 mm should be considered equivocal.

2. Metabolic inhibition (MI) test.

The MI test is a GI technique carried out in liquid medium. It has the specificity of the GI test but is more sensitive and can be used for the measurement of antibodies to mycoplasmas. The technique is based upon the fact that mycoplasmas multiplying in a liquid medium containing a specific substrate will metabolize the substrate with a pH shift in the medium as indicated by a color change of an appropriate pH indicator. The inhibitory activity of homologous antiserum to the mycoplasma under test will decrease the cell metabolism and therefore prevent the color change. A microtiter system is used to determine the amount of inhibition of glucose fermentation, arginine hydrolysis or urea hydrolysis. The titer of a test serum is the highest dilution of serum that prevents a change in color of the medium. The test can be used to identify isolates with known, titered antisera, or to evaluate the potency of a test serum with known cultures.

A variation of the metabolic inhibition test is the tetrazolium reduction inhibition test. This test is based on the observation that certain mycoplasmas will reduce colorless 2,3,5-triphenyltetrazolium chloride to its brick-red formazin. The titer of a test serum is the highest dilution of test serum that prevents the color change. This test is useful if an organism is unable to utilize either arginine or glucose, but can reduce tetrazolium.

The MI test is highly specific and can be used for mycoplasma speciation. Unlike the GI test, it is highly sensitive. Results are more reproducible if cultures are divided into aliquots, stored at -70°C, and a thawed aliquot is used for each test. Filtering the culture through a 0.45μm membrane just before
freezing to obtain a suspension of single cells is recommended, especially for rapidly growing cultures. Antiserum will inhibit the multiplication of individual cells more effectively than that of clumps of organisms. The thawed aliquot must contain 100-1000 color changing units (CCU). The test organism should multiply easily in the test medium and produce an appropriate pH shift or reduction of tetrazolium. The serum and other liquid components of the test must be sterile and free of inhibitors.

Controls should include a medium control (specific medium minus mycoplasma and test serum), a mycoplasma control (specific medium plus mycoplasma minus test serum), and an endpoint medium control (specific medium with pH adjusted to desired endpoint, i.e., 0.5 pH units higher or lower than the medium control). The test is read when the medium in the mycoplasma controls is the same color as the endpoint medium controls. Tetrazolium reduction readings are made when mycoplasma controls have a red precipitate.

3. Immunofluorescence (IF)

Immunofluorescence staining is an easy and practical technique for the identification of mycoplasmas. The most widespread application is the IF staining of colonies on agar medium with evaluation by epifluorescence. IF used for the identification of direct agar isolations is valuable for mycoplasmas that are difficult to subculture. Each mycoplasma colony can be stained to give a representative view of the mycoplasma population of the sample. The purity of cultures can be evaluated, and mixed cultures can be easily detected.

IF is a species-specific test like the GI and MI tests and, like the MI test, is highly sensitive. A microscope attached with an attachment for an incident illuminator is required. Identification can be made within a few hours. Cloning of the isolates is not a requirement, and the reading of the test is not influenced by inoculum size as long as colonies are not confluent. This is in contrast to the GI and MI tests that require quantified pure cultures and may take days to yield a result. Both the direct and indirect IF staining of colonies are of value for mycoplasma identification. The direct test requires a conjugated antisera specific for each mycoplasma to be tested. Conjugating large numbers of antisera can be time-consuming. The indirect test often requires only one conjugated reagent, an anti-rabbit globulin, since most antisera are produced in rabbits. This conjugate is available commercially. Both tests are specific, but the indirect test is more sensitive and tends to give less non-specific background fluorescence that might interfere with the interpretation of the test.

Working dilutions of antisera and conjugate must be determined before performing the tests. The highest dilution of conjugate giving maximum fluorescence against the homologous strain in a direct test often is not the best working dilution. The homologous fluorescence titer may be one or two dilutions lower since some strains require a concentration of antibody that is needed by the strain that is used in the production of the antisera (4X higher is usually adequate). The working dilution of the fluorescein-conjugated antiglobulin is determined by checker box titration using a potent positive rabbit antiserum. The endpoint is the highest dilution of conjugate that give maximum fluorescence against the highest dilution of serum. This endpoint titer is then used to determine the optimal dilution of other antisera.

Excised agar blocks (about 1 cm x 1 cm) are transferred to a moist chamber for staining.
4. Immunoblotting for speciation of mycoplasma

- Nitrocellulose membranes (NCM) (Bio-Rad #162-0116) cut into 2.5 cm² sheets and placed in 60 x 15 mm dishes.
- Assay done at room temperature with agitation.
- Optimal dilutions of primary and secondary antisera must be individually determined.
- Can use colonies up to 2 months old.
- Each NC impression can be cut into 4 or 5 smaller pieces.
- NC impressions can be refrigerated several months before the assay is performed.

1. Wash the NCM in 5 ml. Distilled water. Aspirate the water off the sheets and dry.
2. Dot 10 μL of broth culture on sheets and dry OR place NC sheet on agar colonies, press lightly with a hockey stick, remove with tweezers and place in 60 x 15 dish. (can make 5 impressions on the same agar colonies)
3. 10% buffered formalin (Sigma #HT50-1-128) added to sheets for 10 min. to fix. Wash with Tris-buffered saline (TB) for 5 min. (you need not do this washing step)
4. 0.3% hydrogen peroxide in TBS is added for 10 min. Wash with TBS for 5 min (you need not do this washing step).
5. Block for 30 min. with 10% horse serum and 0.1-0.2% Tween 20 in TBS.
6. Dilute antisera (rabbit polyclonal or mouse monoclonal) with blocking solution Add to NCM and incubate for 30 min. Wash 3 times each for a period of 3 min. with TBS.
7. Add anti rabbit IgG-HRP (for polyclonal) or anti mouse IgG-HRP (for monoclonal) diluted in blocking solution, incubate for 30 min. Wash 3 times each for a period of 3 min. with TBS.
8. Developing solution added. Specific reaction = development of purple dots 5-10 sec (may take a little longer). Immediately wash 3x with distilled water (before background development occurs) and dry.

CONTAGIOUS BOVINE PLEUROPNEUMONIA DIAGNOSIS

Contagious bovine pleuropneumonia (CBPP) is a highly infectious respiratory disease of cattle. It is characterised by pneumonia, oedema of interlobular lung areas and a sero-fibrinous pleuritis and is caused by *Mycoplasma mycoides* subsp mycoides Small colony (SC). The disease usually spreads among susceptible animals by inhalation of aerosols. The incubation period in the acute stage is usually 3 -6 weeks, but may be as long as 3 months or longer under natural conditions. Isolation of *M. mycoides* mycoides SC is relatively easy as it grows well on most mycoplasma isolation media. Broth cultures may require 7 days to obtain optimal growth. A 10% suspension of each tissue should be made in broth medium, and five 10-fold serial dilutions prepared. 0,1 ml of each dilution is spread over the surface of an agar plate. Broth cultures will appear cloudy in 3 to 7 days and should be plated onto agar for positive identification of colonies.

Identification is carried out by GI, MI and IF techniques.
REFERENCES


MYCOPLASMAS CAUSING DISEASE IN ANIMAL AND THEIR DIAGNOSTIC INDEX

The identification of common mycoplasmas affecting animals are in Tables 1, 2, 3 and 4.

Table 1: Avian mycoplasmas

<table>
<thead>
<tr>
<th>Identification</th>
<th>Mycoplasma</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose positive, arginine positive; phosphatase negative:</td>
<td>M. iowae</td>
<td>Embryo mortality in turkeys</td>
</tr>
<tr>
<td>Glucose negative, arginine positive; phosphatase negative:</td>
<td>M. columbinum</td>
<td>Respiratory disease in chickens</td>
</tr>
<tr>
<td>M. gallinarum</td>
<td>M. iners</td>
<td></td>
</tr>
<tr>
<td>M. cumbinisale</td>
<td>M. meleagrisid</td>
<td>Synovitis in turkeys</td>
</tr>
<tr>
<td>Glucose positive, arginine negative; phosphatase negative:</td>
<td>M. columborale</td>
<td>Respiratory disease in chickens</td>
</tr>
<tr>
<td>M. gallinaceum</td>
<td>M. gallisepticum</td>
<td></td>
</tr>
<tr>
<td>M. gallopavonus</td>
<td>M. pullorum</td>
<td></td>
</tr>
<tr>
<td>M. synoviae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose negative, arginine negative; phosphatase negative:</td>
<td>M. cumbornale</td>
<td>Synovitis in poultry</td>
</tr>
<tr>
<td>M. gallinarum</td>
<td>M. gatae</td>
<td></td>
</tr>
<tr>
<td>M. gallisepticum</td>
<td>M. mycoides</td>
<td></td>
</tr>
<tr>
<td>M. mycoides</td>
<td>Group 7 ( PG 50)</td>
<td></td>
</tr>
<tr>
<td>M. pullorum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. synoviae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose negative, arginine negative; phosphatase positive:</td>
<td>M. anatis</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 2: Bovine mycoplasmas

<table>
<thead>
<tr>
<th>Identification</th>
<th>Mycoplasma</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose positive, arginine positive; phosphatase variable:</td>
<td>M. alvi</td>
<td></td>
</tr>
<tr>
<td>Glucose negative, arginine positive; phosphatase negative:</td>
<td>M. arginini</td>
<td>Infertility &amp; mastitis</td>
</tr>
<tr>
<td>M. canadense</td>
<td>M. equirhinis</td>
<td></td>
</tr>
<tr>
<td>M. gallinarum</td>
<td>M. gatae</td>
<td></td>
</tr>
<tr>
<td>Glucose positive, arginine negative; phosphatase positive:</td>
<td>M. canadense</td>
<td>Infertility and mastitis</td>
</tr>
<tr>
<td>M. bovinrini</td>
<td>M. bovoculii</td>
<td></td>
</tr>
<tr>
<td>M. conjunctivae</td>
<td>M. dispar</td>
<td></td>
</tr>
<tr>
<td>M. gallisepticum</td>
<td>M. mycoides</td>
<td></td>
</tr>
<tr>
<td>M. mycoides ss mycoides Group 7 ( PG 50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose negative, arginine negative; phosphatase negative:</td>
<td>M. bovigenitalium</td>
<td>Pneumonia, mastitis &amp; infertility</td>
</tr>
<tr>
<td>Glucose negative, arginine negative; phosphatase positive:</td>
<td>M. bovigenitalium</td>
<td>Pneumonia, mastitis &amp; infertility</td>
</tr>
<tr>
<td>M. bovis</td>
<td>M. californicum</td>
<td>Pneumonia, mastitis &amp; infertility</td>
</tr>
<tr>
<td>M. verecundum</td>
<td></td>
<td>Mastitis</td>
</tr>
</tbody>
</table>

Table 1: Canine and feline mycoplasmas

<table>
<thead>
<tr>
<th>Identification</th>
<th>Mycoplasma</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose positive, arginine positive; phosphatase negative:</td>
<td>M. arginini</td>
<td>None</td>
</tr>
</tbody>
</table>
Identification of Mycoplasma species

<table>
<thead>
<tr>
<th>Glucose, arginine</th>
<th>Mycoplasma</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>M. gatae</td>
<td>Feline arthritis</td>
</tr>
<tr>
<td>Negative</td>
<td>M. maculosum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. opaescens</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. spumans</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glucose, arginine</th>
<th>Mycoplasma</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>M. canis</td>
<td>Canine infertility</td>
</tr>
<tr>
<td></td>
<td>M. edwardii</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. feliminutum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. molare</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>M. cynos</td>
<td>Canine pneumonia</td>
</tr>
<tr>
<td></td>
<td>M. felis</td>
<td>Feline conjunctivitis &amp; equine pneumonia</td>
</tr>
<tr>
<td></td>
<td>HRC689</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glucose, arginine</th>
<th>Mycoplasma</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>M. canis</td>
<td>Canine infertility</td>
</tr>
<tr>
<td></td>
<td>M. edwardii</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. feliminutum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. molare</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>M. cynos</td>
<td>Canine pneumonia</td>
</tr>
<tr>
<td></td>
<td>M. felis</td>
<td>Feline conjunctivitis &amp; equine pneumonia</td>
</tr>
<tr>
<td></td>
<td>HRC689</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Equine mycoplasmas

<table>
<thead>
<tr>
<th>Identification</th>
<th>Mycoplasma</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose positive, arginine positive</td>
<td>M. arginini</td>
<td>None</td>
</tr>
<tr>
<td>Glucose negative, arginine positive</td>
<td>M. equirhinis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. salivarum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. subdolum</td>
<td></td>
</tr>
<tr>
<td>Glucose positive, arginine negative</td>
<td>M. fastidiosum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. feliminutum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. mycoides (related)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. pulmonis (related)</td>
<td></td>
</tr>
<tr>
<td>Glucose negative, arginine negative</td>
<td>M. equigenitalium</td>
<td>Pleuropneumonia</td>
</tr>
<tr>
<td></td>
<td>M. felis (related)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Caprine and ovine mycoplasmas

<table>
<thead>
<tr>
<th>Identification</th>
<th>Mycoplasma</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose positive, arginine positive</td>
<td>M. arginini</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>M. gallinarum</td>
<td></td>
</tr>
<tr>
<td>Glucose negative, arginine positive</td>
<td>M. capricolum</td>
<td>Ccpp</td>
</tr>
<tr>
<td>Glucose positive, arginine negative</td>
<td>M. capricolum</td>
<td>Infectious keratoconjunctivitis</td>
</tr>
<tr>
<td></td>
<td>M. conjunctivae</td>
<td>Polyarthritis &amp; septicemia</td>
</tr>
<tr>
<td></td>
<td>M. mycoides ss capri</td>
<td></td>
</tr>
<tr>
<td>Glucose negative, arginine negative</td>
<td>Group 7 (A1343)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. agalactiae</td>
<td>Pizzle rot</td>
</tr>
<tr>
<td></td>
<td>M. bovis</td>
<td>Pneumonia</td>
</tr>
<tr>
<td></td>
<td>Group 11 (2-D)</td>
<td>Ccpp</td>
</tr>
<tr>
<td></td>
<td>M. putrefaciens</td>
<td>Mastitis-agalactiae</td>
</tr>
</tbody>
</table>

Table 6: Porcine mycoplasmas

<table>
<thead>
<tr>
<th>Identification</th>
<th>Mycoplasma</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose positive, arginine positive; phophatase variable</td>
<td>M. sualvi</td>
<td></td>
</tr>
<tr>
<td>Glucose negative, arginine positive</td>
<td>M. arginini</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. buccale</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. hyopharyngis</td>
<td></td>
</tr>
<tr>
<td>Glucose positive, arginine negative</td>
<td>M. flocculare</td>
<td>M. hyopneumoniae</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>--------------</td>
<td>------------------</td>
</tr>
<tr>
<td>phosphatase negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>phosphatase positive</td>
<td>M. hyorhinis</td>
<td></td>
</tr>
<tr>
<td>Glucose negative, arginine negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>phosphatase positive</td>
<td>M. bovigenitalium</td>
<td></td>
</tr>
</tbody>
</table>
## APPENDIX: MEDIA FORMULATIONS AND TECHNIQUE

### Media

#### Mycoplasma Medium (Hayflick's)³

**Broth Base**

- PPLO broth (Difco laboratories, 21g
  Detroit, MI)
- Distilled, deionized water 1000 ml
- Phenol red, 1% solution 2 ml
  - Adjust pH to 7.4.
  - Autoclave 121°C 20 minutes
  - Store 4°C.

**Complete Broth**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broth base</td>
<td>75 ml</td>
</tr>
<tr>
<td>Unheated horse serum</td>
<td>15 ml</td>
</tr>
<tr>
<td>OR Inactivated swine serum</td>
<td>20 ml</td>
</tr>
<tr>
<td>Fresh yeast extract, 25% solution</td>
<td>10 ml</td>
</tr>
<tr>
<td>Glucose, 50% solution</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Penicillin, 200,000 units/ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Thallium acetate, 10% solution</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>

**Complete Agar**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPLO broth powder</td>
<td>1.47g</td>
</tr>
<tr>
<td>Distilled, deionized water</td>
<td>70 ml</td>
</tr>
<tr>
<td>Oxoid purified agar</td>
<td>0.8g</td>
</tr>
<tr>
<td>Autoclave at 121°C.</td>
<td>Cool to 56°C.</td>
</tr>
<tr>
<td>ADD</td>
<td>Unheated horse serum 15 ml</td>
</tr>
</tbody>
</table>

### OR

**Inactivated swine serum** 20 ml

- Fresh yeast extract, 25% solution 10 ml
- Glucose, 50% solution 0.2 ml
- Penicillin, 200,000 units/ml 0.5 ml
- Thallium acetate, 10% solution 0.2 ml

#### Ureaplasma Medium¹¹,¹²

**Broth Base**

- PPLO broth (Difco) 21g
- Double distilled, deionized water 1000 ml
- Phenol red, 1% solution 2 ml
  - Adjust pH to 6 with 1N HCl.
  - Dispense 70-ml volumes.
  - Autoclave 121°C 15 minutes.
  - Store at 4°C.

**Complete Broth**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broth base</td>
<td>70 ml</td>
</tr>
<tr>
<td>Unheated horse serum</td>
<td>20 ml</td>
</tr>
<tr>
<td>Fresh yeast extract, 25% solution</td>
<td>10 ml</td>
</tr>
<tr>
<td>Urea, 10% solution</td>
<td>1 ml</td>
</tr>
<tr>
<td>Penicillin G potassium, 200,000 units/ml</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>
  - Dispense in 3-ml volumes in sterile disposable plastic tubes.
  - Store at -20°C.

**Agar Base**
Identification of *Mycoplasma* species

**Trypticase Soy broth (BBL, Becton Dickinson, Cockeysville, MD)**
- 2.1g
- Double distilled, deionized water 70 ml
- Phenol red, 1% solution 0.2 ml
  - Adjust pH to 5.5 with 1N HCl.
  - Oxoid purified agar 0.8g
  - Autoclave 121°C 10 minutes.
  - Cool to 56°C.

**Complete Agar**
- Agar base 70 ml
- Unheated horse serum 20 ml
- Fresh yeast extract, 25% solution 10 ml
- Urea, 10% solution 1 ml
- Penicillin, 200,000 units/ml 0.5 ml
- MnSO4, 3% solution 0.5 ml
  - Dispense in plates.
  - Store in closed containers, 4-10°C.
  - Use within 7-10 days.

**Ureaplasma Medium U4 Broth (Buffered)**
- Hanks' Balanced Salt Solution, 10 x concentrate 4 ml
- Hartley Digest Broth (Oxoid), or Brain heart infusion (Difco or Accumedia, Baltimore, MD) 20 ml
- Fetal bovine serum 15 ml
- Fresh yeast extract, 25% solution 10 ml
- Phenol red, 1% solution 0.2 ml
- Urea, 10% solution 0.5 ml
- Magnesium sulphate, 250 μg/ml 1.0 ml

**Biochemical Tests**

**Digitonin Sensitivity**
- Digitonin 15 mg
- Ethanol 10 ml
  - Dispense on blank discs, .025 ml each
  - Dry overnight at 37°C
  - Store at 4-10°C (Stable for several months)

**Interpretation**

- Zone of inhibition: *Mycoplasma* species
- No zone of inhibition: *Acholeplasma* species

**Controls:**
- For mycoplasma - *M. arginini* or *M. bovis*
**Applied Veterinary Bacteriology and Mycology: Mycoplasmas**

**Identification of Mycoplasma species**

**For acholeplasmas - A. laidlawii**

Note: Digitonin is practically insoluble in H2O; 1g dissolves in 57 ml absolute alcohol or in 220 ml 95% alcohol.

### Fermentation of Glucose

<table>
<thead>
<tr>
<th>A</th>
<th>PPLO broth 70 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh yeast extract, 25% solution 10 ml</td>
</tr>
<tr>
<td></td>
<td>Inactivated serum, pig or</td>
</tr>
<tr>
<td></td>
<td>horse 20 ml</td>
</tr>
<tr>
<td></td>
<td>Glucose, 50% solution. 1 ml</td>
</tr>
<tr>
<td></td>
<td>Penicillin, 200,000 units/ml 0.5 ml</td>
</tr>
<tr>
<td></td>
<td>Phenol red, 1% solution 0.4 ml</td>
</tr>
</tbody>
</table>

| B | As above but omit glucose |

Adjust pH to 7.6

Dispense in 5-ml volumes

**Method:**

- Prepare 24-hour broth culture (10% serum, if it will grow)
- Inoculate approximately 0.05 ml/tube of
  - A. broth with glucose
  - B. broth without glucose
- Incubate at 37°C
- Subculture after 24 and 48 hrs to confirm growth
- Continue incubation up to 14 days if necessary
- Observe and record any changes in pH; acid (yellow) is positive
- Incubate controls:
  1. uninoculated broth with glucose
  2. uninoculated broth without glucose
- If organism is anaerobic, overlay with 1.5 ml sterile vaseline, paraffin mixture.

### Hydrolysis of Arginine

<table>
<thead>
<tr>
<th>A</th>
<th>PPLO broth, pH = 7.0 70 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh yeast extract, 25% solution 10 ml</td>
</tr>
<tr>
<td></td>
<td>Inactivated serum pig or horse 20 ml</td>
</tr>
<tr>
<td></td>
<td>L-arginine HCl 10% solution 5 ml</td>
</tr>
<tr>
<td></td>
<td>Penicillin, 200,000 units/ml 0.5 ml</td>
</tr>
<tr>
<td></td>
<td>Phenol red, 1% solution 0.4 ml</td>
</tr>
</tbody>
</table>

| B | As above but omit arginine |

Adjust pH to 6.8

Dispense in 5-ml volumes.

**Method:**

- Prepare 24-hour broth culture
- Inoculate 0.05 ml/tube of
  - A. broth with arginine
  - B. broth without arginine
- Incubate at 37°C
- Subculture to plates after 24 and 48 hours to confirm growth
- Continue incubation up to 14 days
- Record changes in pH; alkaline (dark red) is positive
- Incubate controls: 1
  1. uninoculated broth with arginine
  2. uninoculated broth without arginine

### Phosphatase Activity

| PPLO or heart infusion broth 74 ml |
| Purified agar (Oxoid) 0.8g |

- Autoclave.
Cool to 56°C.
Inactivated serum, horse or 20 ml swine.
Fresh yeast extract, 25% 10 ml solution.
Sodium phenolphthalein 1.0 diphosphate, 1% w/v solution.
Penicillin, 200,000 units/ml 0.2 ml

Method:
- Inoculate two plates. Inoculate only on one half of each plate either by push block or running drop. Leave the other side uninoculated as a control.
- After incubation for 3 and 5 days, test with 5N NaOH. Run a drop across the control side and then the inoculated side. Appearance of a red color in 30 seconds is positive.
- If uninoculated side turns red in this time, it means that the serum was not sufficiently inactivated or that the plates are too old.

Interpretation:
Positive control: M. bovis
Negative control: M. arginini

Milk Agar for Casein Hydrolysis³
Skim milk powder 32g
Distilled, deionized water 100 ml
Sterilize 10 minutes 112-115°C
Prepare Hayflick's agar with 20% HS, 10% yeast etc.
Cool to 56°C
Add 1 part skim milk to 7 parts medium
Pour plates

Interpretation:
Positive control: M. mycoides subsp. mycoides, LC-type, e.g., strain Y3343; positive reaction is a clear zone around colonies.
Negative control: M. alkalescens

IMMUNOLOGICAL TESTS
Indirect Fluorescent Antibody Test (FA)¹,¹⁰
1. You need colonies small and close together, but separated. Large colonies sometimes give equivocal results.
2. Plates ready for FA can be kept in the refrigerator for 2-4 weeks in plastic bags or boxes to keep them from drying out.
3. Cut strips of agar that contain colonies and place on microscope slide. Cut strips into approximately 5 mm squares. Place the blocks far enough apart so that the sera will not run together.
4. Fix to the slide by embedding squares in a mixture of 65% paraffin and 35% Vaseline. Six to eight squares can be placed on one slide (the wax should not be higher than the agar).
5. Place a drop of buffer on each square while you prepare the antiserum dilutions. This helps to rehydrate the agar so the blocks will not absorb too much serum.
6. Prepare serum dilutions in buffer, pH 7.2-7.4. Do not use serum undiluted or 1/10; you may get false negatives.
7. Remove excess buffer from surface of each block with tissue. Do not blot the top surface.
8. Place one drop of diluted antiserum on each block.
9. Incubate at room temperature 30 minutes in moist chamber.
10. Wash 2 x 10 minutes in phosphate buffer.
11. Remove excess buffer from each block.
12. Place one drop of fluorescein-conjugated anti-rabbit globulin on each block.
13. Predetermine the dilution of each batch of conjugate, usually 1/20 or 1/30.
14. Incubate at room temperature 30 minutes in moist chamber.
15. Wash 2 x 10 minutes in phosphate buffer.
16. Read with epi-fluorescent illumination.

This method allows you to identify more than one species when the culture is mixed. Stained blocks can be stored at 4-10°C in a moist chamber for several days if desired.

**Precipitation of Serum for Indirect FA**

- 5 ml saturated (NH₄)₂SO₄
- 10 ml serum-add slowly, drop-wise, to (NH₄)₂SO₄ with continuous stirring; stir overnight at 4°C
- Centrifuge at 6000 rpm, 10 minutes, 4-10°C (Sorvall)
- Resuspend precipitate in 10 ml distilled water
- Add to 5 ml (NH₄)₂SO₄ as above
- Stir 1½ hrs at room temperature
- Centrifuge at 6000 rpm, 10 minutes, 4-10°C
- Resuspend in 3.3 ml distilled water
- Dialyze overnight with saline
- Measure volume and bring up to original volume with PBS, pH 7.5
- Store -20°C
- Use for indirect FA method

Precipitated serum reduces background staining in the indirect method.

**Conjugation of Serum with Fluorescein Isothiocyanate (FITC)**

1. Precipitate globulin by 1/3 saturation with ammonium sulfate; e.g., add drop-wise 10 ml serum to 5 ml saturated ammonium sulphate with continuous stirring. Stir overnight at 4°C.
2. Centrifuge at 6000 rpm, 10 minutes, 4-10°C (Sorvall).
3. Redissolve precipitate in distilled water equal to 1/3 of original serum volume.
4. Dialyze against saline at 4°C with stirring. Change saline 3-4 times during day and continue dialysis overnight.
5. Remove from casing and measure volume.
6. Add PBS pH 7.5 to bring total volume to twice the original serum volume.
7. Determine protein content of solution by spectrophotometer at 280 nm.
   \[ \text{O.D.} \times \text{dilution factor} \times 10 = \text{mg protein/ml} \]
   x vol. = total protein
8. Prepare fresh carbonate-bicarbonate buffer: 0.5 M pH 9.0
   - Sodium bicarbonate 0.37g
   - Sodium carbonate NaCO₃ 0.06g
   Dissolve and bring to 10 ml with distilled water
10. Weigh FITC in an amount equal to 1/60 of the total protein.
12. Add drop by drop VERY SLOWLY to protein solution on stirrer.
13. React overnight at 4°C with continuous stirring, or 3 hours at room temperature.
14. Prepare Sephadex 025 or 050 column, 12 mm diameter.
    Pack to height of 3.5 cm/ml of serum to be used.
    Equilibrate and run with Tris-HCl buffer 0.1M pH 8.7.
15. Apply protein-FITC solution to column. Elute with Tris-HCl pH 8.7 and collect first peak.
(Steps 16 and 17 are optional.)
16. Apply this fraction to a DEAE-cellulose column equilibrated with Tris-HCl buffer. Adjust flow rate to 0.7-1.0 ml/minute Elute with Tris-HCl 0.1M pH 8.7, then Tris-HCl
0.1M pH 8.7 + 0.1M NaCl then Tris-HCl
0.1M pH 8.7 + 0.2M NaCl
17. Concentrate fractions if necessary.
18. Test conjugate with homologous organism.
19. Store in small volumes in sealed ampoules in -70°C.

Tris-HCl Buffer 0.1M pH 8.7
Tris hydroxymethyl aminomethane M.W. = 121.4 6.
• Dissolve 48.46 9 in distilled water
• Bring to 3990 ml
• Adjust pH to 8.7 with HCl
• Bring volume to 4000 ml

Antigen Preparation for Immunization
1. For mycoplasmas: Prepare 500-1000 ml broth. Use 1% serum fraction or rabbit serum in place of horse serum. For ureaplasmas: Prepare 1000 ml Howard's U4 broth with buffer.
2. Centrifuge broth at 15,000 g for 1 hour. Filter through 0.22 μm membrane filter to sterilize.
3. Inoculate with culture. Incubate appropriate time.
4. Centrifuge 15,000 g, 4°C, 1 hour. Wash sediment twice in PBS pH 7.2- 7.5.
5. For mycoplasmas, resuspend sediment in 15-20 ml PBS. For ureaplasmas, resuspend in 10 ml. Plate on mycoplasma or ureaplasma agar to check growth. Plate drops on blood agar to check purity.
6. Store in 1 ml volumes at -70°C.

Rabbit Immunization Procedure for Mycoplasmas/ Ureaplasmas

Immunization Schedule
Pre-bleed and test serum: for mycoplasmas -by growth inhibition and FA and for ureaplasmas -by metabolic inhibition and FA

Day 0
Emulsify 2 ml antigen suspension with 2 ml Freund's complete adjuvant.
• Emulsion is ready when a drop on the surface of water does not spread but remains intact.
• Amount recovered from this mixture is usually about 3 ml.
• Inject 0.2 ml sub-cut. into 8 sites along back.
• Inject 0.5 ml intramuscularly into each hip.

Day 21
• Inject 1 ml antigen (without adjuvant) intravenously 3 times per week for 6 injections.

OR
• Repeat schedule for day 0.

Day 40
• Test blood.
• Test for growth or metabolic inhibition and FA.

Day 43
• If serum satisfactory, bleed rabbit out.
• If serum unsatisfactory repeat IV injections, as on day 21.

OR
• Give a single 3-ml dose IV.
• Test-bleed 7 days after last injection.
• Bleed out no later than 10 days after last injection.
• If serum is still unsatisfactory, it is probably best to immunize another rabbit.

Semen Culture

A. Raw Semen
1. Plate 2 x 0.01 ml drops of undiluted semen onto:
   a) mycoplasma (Hayflick's) agar with porcine serum
   b) Ureaplasma agar.
2. Make 4 10-fold dilutions in ureaplasma broth.
4. Plate 2 x 0.01 ml drops of 10⁻¹ dilutions onto corresponding plates.
5. Subculture from broth to plates on day 2, and again on day 4 (or 5).
6. Incubate plates for 10 days before discarding as negative. Incubate broths aerobically. Incubate mycoplasma plates in 5-7% CO₂. Incubate ureaplasma plates anaerobically (we use the H₂+CO₂ system).
7. Observe ureaplasma broths daily for color change, and subculture to agar as soon as color changes to light pink.
8. Read plates every 2 days for mycoplasma and ureaplasma colonies.
9. Incubate plates for 10 days before discarding as negative. Incubate broth to agar. Some arginine hydrolyzing mycoplasmas and some diphtheroids can cause a color change without turbidity.

B. Processed Semen

1. Surface-sterilize semen straw with cotton gauze soaked in 70% alcohol and let air dry.
2. Aseptically remove semen from straw and place in sterile tube.
3. Plate 2 x 0.01-ml drops undiluted semen onto:
   a) mycoplasma (Hayflick's) agar with porcine serum
   b) Ureaplasma agar.
4. Make 1/10 dilution in mycoplasma broth base (without serum or yeast). PBS is used by some but can be inhibitory after 1 hour exposure.

   a) Plate 2 x 0.01-ml drops onto mycoplasma agar.
   b) Plate 2 x 0.01-ml drops onto ureaplasma agar.
5. Centrifuge 35,000 X a, 20 minutes, 4-10°C.
6. Resuspend sediment in 10 ml broth base.
7. Centrifuge 35,000 x a, 20 minutes, 4-10°C.
8. Repeat steps 6 and 7.
10. Resuspend sediment in 1 ml broth base.
11. Plate 2 x 0.01-ml running drops to mycoplasma plate.
12. Plate 2 x 0.01-ml running drops to ureaplasma plate.
14. Make one 10-fold dilution in mycoplasma broth.
15. Incubate all broths aerobically. Incubate mycoplasma plates in 5-7% CO₂. Incubate ureaplasma plates anaerobically.
16. Observe ureaplasma broths daily for color change, and subculture to agar as soon as color changes to light pink.
17. Read plates every 2 days for mycoplasma and ureaplasma colonies.
18. Subculture mycoplasma broth to plates at 2 and 4 (or 5) days.
19. Incubate plates for 10 days before discarding as negative.

Passive Hemagglutination Test

1. Phosphate-buffered glucose (PBG)

   Disodium phosphate 0.15M 76 ml
   Monopotassium phosphate 24 ml 0.15M
   Glucose 5.4% in distilled water 100 ml
   • pH should be 7.2.
   • Store in refrigerator.
2. **Red blood cells**
   - Collect sheep blood in Alsever’s solution.
   - Wash 4 times in PBG, 2000 x g, 4°C, 10 minutes each.
   - Make 20% suspension in PBG.

3. **Glutaraldehyde-fixed red cells**
   Mix equal volumes:
   - 20% sheep cell suspension
   - 0.2% Glutaraldehyde
   - Glutaraldehyde (EM) 25% aqueous solution
   - PBG

   - Incubate in 37°C water bath, 15 minutes mix occasionally.
   - Wash 5 times with 0.85% saline, 450 XG, 5°C, 10 minutes.
   - Make a 10% suspension in saline.
   - Add sodium azide to make 0.1% final concentration.

4. **Mycoplasma antigen**
   - Grow mycoplasmas in appropriate medium 2-3 days depending on rate of growth.
   - Centrifuge 14,000 x G, 1 hour, 5°C.
   - Wash twice with saline - 14,000XG, 30 minutes, 5°C.
   - Resuspend in saline, 5 ml/L of culture.
   - Reserve 0.5 ml for protein determination.
   - Store frozen at -70°C. Determine protein concentration by Lowry\(^6\) method or Bradford\(^7\) method (BioRad protein assay, BioRad Chemical Division, Richmond, CA).
   - Adjust volume to make a suspension of 10 mg protein/ml. 0.8 ml 99.2 ml

5. **Sensitization of glutaraldehyde-fixed red cells with mycoplasma antigen**
   - Centrifuge glutaraldehyde-fixed cells, 450xG, 10 minutes 5°C.
   - Wash twice with 0.01M PBS, same speed and time.
   - Make a 20% suspension in PBS.
   - To 5 ml of the 20% glutaraldehyde-fixed cells, add 2 ml antigen adjusted to 10 mg protein/ml. Add sodium azide to make a final concentration of 0.1%.
   - Mix thoroughly.
   - Incubate 37°C overnight (16-18 hrs) with occasional agitation.
   - Centrifuge 450xG, 10 minutes, 5°C.
   - Wash 3 times in PBS same speed and time.
   - Resuspend at 2% concentration in PBS.
   - Store at 5°C.

**Immunoblotting:**

**Tris-buffered Saline**

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>Tris-HCl (g)</th>
<th>NaCl (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>3.94</td>
<td>5.85</td>
</tr>
<tr>
<td>1 Liter</td>
<td>7.88</td>
<td>11.69</td>
</tr>
<tr>
<td>2 Liters</td>
<td>15.76</td>
<td>23.38</td>
</tr>
<tr>
<td>4 Liters</td>
<td>31.76</td>
<td>46.76</td>
</tr>
<tr>
<td>5 Liters</td>
<td>39.4</td>
<td>58-44</td>
</tr>
</tbody>
</table>

**Blocking Solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse serum</td>
<td>50 ml</td>
</tr>
<tr>
<td>Tween 20 or Tween 80</td>
<td>100 μl</td>
</tr>
<tr>
<td>TBS</td>
<td>Bring to 500ml</td>
</tr>
</tbody>
</table>

**Developing Solution**

15 mg. 4-chloro-l-naphthol (Sigma #C8890) dissolved in 5 ml methanol in one beaker
Applied Veterinary Bacteriology and Mycology: Mycoplasmas

Identification of Mycoplasma species

0.5 ml 3% H₂O₂ or 50 μl 30% H₂O₂ + 25 ml TBS in another beaker

Mix the two beakers immediately prior to use

O.3% H₂O₂ in TBS: 1 ml 300% H₂O₂ into 99 ml TBS

Antisera Dilutions

<table>
<thead>
<tr>
<th>Antiserum#</th>
<th>Volume Blocking Solution</th>
<th>Ab (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Sample</td>
<td>1, 2, 3</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>4, 5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

6, 7, 8 2.0 or 0.5 for 2, 3, 0.75 for 3

| Samples | 1, 2, 3                  | 5.0 ml | 2.0 |
| Samples | 4, 5                     | 6.0    | 0.4 |
| 6, 7, 8 | 4.0                      | 1.0    |

| Samples | 1, 2, 3                  | 7.5 ml | 3.0 |
| Samples | 4, 5                     | 6.0    | 0.4 |
| 6, 7, 8 | 8.0                      | 2.0    |

REFERENCES


Applied Veterinary Bacteriology and Mycology: Mycoplasmas → Identification of *Mycoplasma* species


