AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

STANDING COMMITTEE ON AGRICULTURE AND RESOURCE MANAGEMENT

ANIMAL HEALTH COMMITTEE

SUB-COMMITTEE ON ANIMAL HEALTH LABORATORY STANDARDS

Avian Mycoplasmosis

Bacteriology

K. G. Whithear

School of Veterinary Science, The University of Melbourne, Princes Highway, Werribee, Vic. 3030, Australia.

Contents

First published as: *Avian Mycoplasmosis*, by D. Eggleton, P. F. Lewis and W. T. K. Hall, by the Australian Bureau of Animal Health (1976).

1. Introduction

Mycoplasmas are a diverse group of small prokaryotes which lack the capacity to produce a cell wall. The trivial name mycoplasma is used to refer to all organisms belonging to the class *Mollicutes*. Because of their small genome and cell size, mycoplasmas have limited biosynthetic capacity and they require rich and complex media to sustain growth. Mycoplasmas (except *Acholeplasma* sp.) are unique among the prokaryotes in requiring sterol as a growth factor. The absence of a cell wall is responsible for many of the peculiar properties of mycoplasmas, including their filterability through pore sizes smaller than their cell diameter, their 'fried egg' colony shape and their resistance to penicillins. It is also the basis for their incorporation in a separate class *Mollicutes* (mollis, soft; cutis, skin), of the *Procaryotae*.

About 20 named mycoplasmas (belonging to the genera *Mycoplasma*, *Acholeplasma* and *Ureaplasma*) have been isolated from birds (Table 1), and at least 9 of these have been identified in Australia (Table 2). However, the laboratory diagnosis of avian mycoplasmosis in Australia is primarily concerned with identification of *Mycoplasma gallisepticum* and *M. synoviae* infection in chickens, and to a lesser extent, turkey flocks. The major companies controlling the breeding of commercial poultry in Australia have adopted a policy of attempting to maintain *M. gallisepticum*-free and *M. synoviae*-free breeder flocks. The most frequently used laboratory procedures involve monitoring the infectious status of these flocks. This is usually done by flock serological testing, with culture being used for confirmation. However, laboratories offering an avian mycoplasma diagnostic service may be required to process specimens from a variety of sources. This chapter provides guidance about the diagnosis of avian mycoplasmosis as it is likely to be broadly presented to diagnostic laboratories within Australia. More information can be obtained by consulting the references.

Table 2. Mycoplasmas isolated from birds in Australia

Mycoplasma	*Bird*
M. gallisepticum	Chicken, turkey, partridge, pheasant, quail
M. gallinarum	Chicken
M. pullorum	Chicken
M. gallinaceum	Chicken
M. meleagridis	Turkey
M. synoviae	Chicken, turkey, pigeon
M. anatis	Duck
M. columbinum	Pigeon
M. columborale	Pigeon

Information provided by G. Cottew, L. Ireland, C. Morrow and K. Whithear.
A number of other isolates have not been positively identified or were untypable.

2. Clinical Aspects

M. gallisepticum, *M. synoviae*, *M. meleagridis*, and possibly *M. iowae* are potentially pathogenic avian *Mycoplasma* sp., although subclinical infections are common and there may be considerable strain variation in virulence. *M. gallisepticum* is the most economically significant, causing respiratory disease, reduced growth rate, and lowered egg production and hatchability in chickens, turkeys and some other avian species (Yoder, 1991). Respiratory signs include coughing, rales and severe airsacculitis. Swollen sinuses

Table 1. Usual host and biochemical reactions of avian mycoplasmas

Aer = Aerobic growth; Anaer = Anaerobic growth; w = weak reaction; d = variable reaction

Species	*Usual host*	*Glucose fermentation*	*Arginine hydrolysis*	*Phosphatase activity*	*Tetrazolium reduction* Aer/Anaer	*Film and spots*
Acholeplasma laidlawii [1]	Various	+	–	–	w/+	–
Mycoplasma anatis	Duck	+	–	+	–/+	+
M. anseris	Goose	–	+	–		
M. cloacale	Turkey	–	+	–		
M. columbinasale	Pigeon	–	+	+	–/–	+
M. columbinum	Pigeon	–	+	–	–/+	+
M. columborale	Pigeon	+	–	–	–/+	–
M. gallinaceum	Chicken	+	–	–	–/–	–
M. gallinarum	Chicken	–	+	–	+/+	+
M. gallisepticum	Chicken/turkey	+	–	–	+/+	–
M. gallopavonis	Turkey	+	–	–	d/d	–
M. glycophilum	Chicken	+	–	d		
M. iners	Chicken	–	+	–	–/–	+
M. iowae	Turkey	+	+	–	+/+	–
M. lipofaciens	Chicken	+	+.			
M. meleagridis	Turkey	–	+	+	–/–	
M. pullorum	Chicken	+	–	–	–/–	–
M. synoviae	Chicken/turkey	+	–	–	–/w	+
Ureaplasma gallorale[2]	Chicken/turkey	–	–			

[1] Do not require sterol for growth; [2] Require urea for growth.

are frequently seen in turkeys. *M. synoviae* causes synovitis and respiratory disease in chickens, turkeys and other species (Kleven *et al.*, 1991). *M. meleagridis* has been isolated only from turkeys. It causes respiratory disease in young birds and has been associated with stunting, skeletal abnormalities and poor feathering (Yamamoto, 1991). *M. iowae* has been reported overseas as a cause of late embryonic mortality and reduced hatchability in turkeys (Kleven, 1991). There have been no confirmed isolations of *M. iowae* in Australia, although it would be surprising if it were not present.

All of the above species are transmitted laterally by contact or infectious aerosol, and vertically through the egg. Venereal infection is particularly important in the case of *M. meleagridis*. Most of the non-pathogenic species also occur in the respiratory tract so it is likely that they are transmitted laterally in a similar manner to the pathogens. *M. gallinarum* and *M. gallinaceum* have been isolated from the oviduct of chickens (Wang *et al.*, 1990), suggesting that egg transmission of these species is possible.

3. Collection of Specimens for Culture

3.1. Sites for Culture

3.1.1. Culture Sites at Post Mortem

Direct swabs are taken of exudate from nasal sinuses, trachea, air sacs or joints. When no gross lesions are present, swabs of the mucosae of the nasal cavity (in the region of the turbinates), upper trachea or oviduct can be taken.

Swabs of the yolk sac endothelium are used to isolate egg transmitted mycoplasma from pipped embryos. The embryos should be examined for evidence of caseous plaques on the thoracic air sacs. If such lesions are present there is a high likelihood that mycoplasmas will be isolated. However, it is also possible to isolate pathogenic mycoplasmas from the yolk sac of embryos showing no obvious air sac lesions. Culture of the oesophagus of pipped embryos or one-day-old poults is recommended for detecting egg transmitted *M. iowae* (Kleven and Yoder, 1989).

3.1.2. Cultures from Live Birds

Swabs are collected from the choanal cleft or trachea to isolate *M. gallisepticum* or *M. synoviae*. Swabs can be taken directly from the phallus, oviduct and semen for isolation of *M. meleagridis* from mature turkeys.

3.2. Number of Samples

Pathogenic mycoplasmas can much more easily be isolated from lesions during the acute phase of the disease. They tend to disappear from air sac and synovial exudates as the lesion becomes chronic (two to three weeks). *M. gallisepticum* and *M. synoviae* will persist in the upper respiratory tract for much longer. Nevertheless it is important to have some idea about how long a flock has been infected when deciding on the number of samples required to make a cultural diagnosis. The number of organisms in the upper respiratory tract and the incidence of infected birds in a flock is much higher during the acute phase of infection (up to three months after introduction). Culture of 5–10 birds from such flocks is usually sufficient to make a diagnosis. In chronically infected flocks, or in flocks infected with slow spreading strains of *M. gallisepticum*, 30 or more birds may need to be cultured to establish a diagnosis.

3.3. Choice of Swabs

Care should be taken with the choice of swabs, as some cotton tipped swabs contain fatty acids toxic to mycoplasmas. Calcium alginate, dacron or polyester swabs are preferred.

3.4. Transport of Samples to the Laboratory

Inhibitors present in tissues and exudates may suppress the growth of fastidious mycoplasmas. The effect of inhibitors is potentiated if the tissue specimen is allowed to stand at room temperature or at 4°C for 24–72 hours before culture. Specimens should, therefore, be processed as quickly as possible.

Swabs collected in the field should be placed in broth medium (see 10.1.2.) and transported at room temperature to the laboratory for processing. Tissues such as tracheas, air sacs or tissue exudates should preferably be frozen immediately on sufficient dry ice to get them to the laboratory frozen. Specimens held at 4°C are less satisfactory, especially if they cannot be handled within 24 hours of collection.

4. Isolation and Propagation of Avian Mycoplasmas

4.1. Principles of Mycoplasma Nutrition

The biological features of mycoplasmas dictate the requirements necessary for their successful cultivation. The medium must:

(a) supply all essential nutrients in an assimable form; and,
(b) avoid factors which may be inhibitory or lethal such as low moisture content, unsuitable osmotic conditions, surface active agents, salts of heavy metals and other toxic compounds.

4.1.1. Components of Mycoplasma Media

For sources of media ingredients see 10.1.

4.1.1.1. Basal medium

The basal medium used for the cultivation of mycoplasmas consists of beef heart infusion and/or high quality peptones derived from enzymatic digests of meat or some other protein source. Sodium chloride and sometimes other

mineral salts are also added. Some commercially available media are further enriched with extracts of meat or yeast. Quality of agar is important, because some agars inhibit mycoplasma growth. Special Noble Agar or agarose are recommended when solidification of a broth base is required.

4.1.1.2. Serum

Sterile serum must be added to the basal medium in order to provide cholesterol and essential fatty acids in an assimable and non-toxic form. Heat inactivated swine serum at concentrations from 10 to 20% v/v is most commonly used for the isolation of avian mycoplasmas. However, other sera such as horse, chicken, turkey or foetal calf may also be suitable. Horse serum is preferred for the isolation of *M. meleagridis*. The quality of the serum supplement should be assessed before its use, because different batches of serum can vary markedly in their growth promoting properties.

4.1.1.3. Other enrichments

Growth of mycoplasma may be stimulated by supplementing media with fresh yeast autolysate, glucose and/or arginine, DNA, L-cysteine, nicotinamide adenine dinucleotide (NAD) (a growth factor required by *M. synoviae*) and various vitamins.

4.1.1.4. Inhibitors

A broad spectrum penicillin such as amoxycillin up to 1 mg/mL, and thallous acetate up to 0.01% w/v are used to inhibit bacterial growth. Actidione may be added to the medium when growth of mycelial fungi is a problem.

4.2. Media Used for the Isolation of Avian Mycoplasmas

4.2.1. Formulations

Several different media formulations have been successfully used to grow avian mycoplasmas, although none of these should be regarded as optimal for the isolation and propagation of all species. Media may be used in broth form or may be solidified with agar. Some workers prefer an overlay medium for primary isolation. The problem with broth and overlay media is that if non-pathogenic mycoplasmas are present in a specimen, they tend to overgrow the slower growing pathogenic species. The medium devised by Frey *et al.* (1968) and its various modifications has gained acceptance as a suitable medium for the isolation of pathogenic avian mycoplasmas. The formulation of this medium and the preparation of its ingredients are given in 10.1.

4.2.2. Quality control

Proper quality control of media is essential and should preferably be done using clinical material known to contain pathogenic mycoplasmas. Failing this, cultures which have been passaged no more than twice should be used. Laboratory adapted cultures may grow on suboptimal medium which fails to support the growth of primary isolates. Three criteria should be used to quality control media — the number of colonies present (plating efficiency), the rapidity of growth, and the amount of growth (size of colonies on agar or density of cells in broth). It is essential to determine whether the growth promoting properties of new batches of serum and basal medium are satisfactory before they are incorporated in media used for the isolation of avian mycoplasma.

4.3. Inoculation of Media

On agar medium the swab is wiped over part or all of the agar surface. In the former case the specimen is spread over the remainder of the agar surface with a platinum loop, using the streak plate technique. This assists in providing well isolated colonies required for cloning. Swabs can be inoculated directly into broth, which is generally a more sensitive primary isolation medium than agar culture, although *M. meleagridis* grows better on agar than in broth medium. Alternatively, the same swab may be used to inoculate both agar and broth. The swab should be removed from the broth medium prior to incubation to reduce the risk of bacterial contamination. The chances of successful isolation of *M. gallisepticum* from chronically infected flocks are increased if both agar and broth media are inoculated.

Inhibitors present in tissues and exudates may be overcome by making serial 10-fold dilutions of the specimen in broth prior to inoculation. Subculture of the various broth dilutions back onto agar prior to incubation may also be done.

4.4. Incubation Conditions

Agar plates should be incubated at 37°C in a humidified atmosphere (sealed container with moistened paper towel). Use of a candle jar to provide additional carbon dioxide may aid primary isolation. Some *Mycoplasma* sp. appear to grow better in a mixture of 5–10% carbon dioxide in nitrogen. However, this does not seem to be necessary for avian *Mycoplasma* sp. Overlay and broth media should be incubated in screw top containers at 37°C.

4.5. Examination of Inoculated Media

Agar media should be examined daily for the first few days of incubation using a stereoscopic dissecting microscope at 10–40x magnification. However, colonies can also be observed with an ordinary light microscope with the iris diaphragm partially closed and the condenser racked down to improve contrast. Colonies of non-pathogenic species such as *M. gallinaceum* and *M. gallinarum* may be visible after 24 hours

incubation. The pathogenic species *M. gallisepticum, M. synoviae* and *M. meleagridis* usually do not produce obvious colonies until four to five days incubation, although occasionally they will be visible as soon as three days, and sometimes they may require more prolonged incubation. In the case of field material, plates should not be discarded as negative before 21 days of incubation.

Mycoplasma colonies vary from 50 µm to >1 mm diameter. Typical colonies have a fried egg shape with an opaque granular centre embedded in the agar, surrounded by a flat translucent peripheral zone on the agar surface. Colonies of *M. gallisepticum* frequently lack the typical fried egg appearance on primary isolation. Colony appearance is also affected by the medium and the conditions of culture. If these are suboptimal, colonies may lack the peripheral zone of growth.

Broth media should be examined daily for a colour change or slight turbidity. With phenol red indicator, an orange–yellow colour may indicate growth of glucose fermenters, and a pink–purple colour may indicate organisms that utilise arginine. When signs of growth appear, the broth should be subcultured onto agar medium. It is important not to incubate glucose containing broth for too long (until phenol red indicator goes bright yellow) otherwise the culture will lose viability. This is particularly important in the case of *M. synoviae* which has a very short stationary phase of growth before an exponential decline in viability. If no signs of growth appear after about seven days incubation, the broth can be blind passaged into fresh broth or onto agar medium.

4.6. Possible Causes of Confusion When Examining Mycoplasma Media

The L-phase variants of bacteria possess a fried egg colony shape, but they are usually coarser in texture than mycoplasma colonies. L-phase variants usually revert to their bacterial colony form if grown on penicillin-free media. Epithelial cells will survive and even grow on mycoplasma media and their nuclei surrounded by cytoplasm can be mistaken for small mycoplasma colonies. Pseudocolonies are structures with a dense granular central core surrounded by swirls of fibrillar chains which occur particularly after prolonged incubation. Air bubbles in the media may also cause confusion, although they are usually obvious because of their highly refractile appearance.

In broth medium an orange–yellow colour change in the indicator may be produced by the metabolism of tissue cells or by bacterial contamination. Bacterial growth usually produces much more turbidity of the medium than mycoplasma growth.

4.7. Subculture

Routine subculture on agar is achieved by removing with a sterile scalpel blade a block of agar containing colonies. The agar block is transferred to broth or inverted and spread over the surface of a fresh agar plate (push-block). Broth cultures are passaged by transfer of one or more drops to a fresh broth medium using a Pasteur pipette or other sterile dropper. Broth cultures can be transferred to agar by drop, or by streaking a swab or a loop full of culture over the agar surface.

5. Identification of Cultures

Colonies on primary plates may be identified by immunofluorescence. Alternatively, a pure culture must be obtained and identified using biochemical and serological methods.

5.1. Immunofluorescence to Rapidly Identify Mycoplasma Colonies on Agar

Immunofluorescence is a rapid, species specific method for identification of mycoplasma colonies. Mixed cultures can be readily detected, and the relative proportions of different *Mycoplasma* present on the plate determined. Colonies (preferably in areas of moderately heavy but not confluent growth) are located with a low power objective using white light and are subsequently checked for specific fluorescence with a x40 objective using ultraviolet (UV) light. Either transmitted light fluorescent microscopes or epi-illuminated fluorescent microscopes can be used.

If a broth culture containing glucose and a pH indicator has been inoculated it should provide evidence of whether the organism is fermentative or not. This information assists in selecting the range of typing antisera which may be required for identification.

Two methods can be used for immunofluorescence — direct and indirect. For direct immunofluorescence it is necessary to conjugate a fluorescent dye such as fluorescein isothiocyanate to immunoglobulins prepared against each *Mycoplasma* sp. For laboratories doing a lot of mycoplasma diagnostic work, or interested only in the identification of one or two *Mycoplasma* sp., the direct technique has the advantage that one of the staining, incubation and washing steps is eliminated. Indirect immunofluorescence requires a supply of unconjugated antisera prepared in rabbits against the required range of avian mycoplasmas. Specific antigen–antibody complexes can then be detected using goat- or sheep-antirabbit fluorescein conjugates. The latter are readily available commercially.

5.1.1. Direct Method

An area on an agar plate where colonies are relatively numerous but well separated is selected. The following method is adapted from Baas and Jasper (1972) and Kleven and Yoder (1989).

A plastic cylinder (15 mm long x 13 mm diameter) is pressed through the agar so that the colonies are within a reservoir formed by the cylinder and agar. Two to three drops of the appropriately diluted specific conjugate are carefully placed into the reservoir, and the plate incubated at 37°C for 20–30 min with the lid on. Removal of unbound conjugate is achieved by carefully filling the reservoir with phosphate buffered saline [PBS; NaCl (0.14 mol/L), KCl (2.7 mmol/L), potassium dihydrogen phosphate (KH_2PO_4, 1.5 mmol/L), disodium hydrogen phosphate (Na_2HPO_4, 8.1 mmol/L)] and then removing with a Pasteur pipette.

The plastic cylinder is removed, leaving a circular disc in the agar. A drop of mounting solution (50–90% glycerol in PBS) is put on a clean slide, and the agar disc carefully placed over the drop of mounting fluid with colonies toward the glass (for conventional microscope). For a microscope with epi-illumination, colonies should be mounted right side up. Up to four agar discs can be placed on one slide. The agar discs are moistened with PBS and a cover slip is placed over the surface.

Examine with x10 objective, using a UV light source with a BG-12 exciter filter and proper barrier filter. A dry dark field condenser may also used. This allows location of colonies by darkfield microscopy before UV examination.

Known positive and negative controls should be used.

5.1.2. Indirect Method

The procedure is as described for the direct method except that two to three drops of an appropriately diluted specific antiserum (prepared in rabbits) is first placed in the reservoir. After washing the colonies are stained with anti-rabbit IgG fluorescein conjugate (Wellcome Australia). A final washing step is required to remove unbound conjugate.

5.2. Isolation of Pure Cultures

There are some circumstances which justify the more time consuming (than immunofluorescence) process of obtaining and identifying pure cultures of mycoplasma. These include instances where immunofluorescence gives equivocal results, or where it is necessary to obtain a pure culture for antibiotic sensitivity testing or strain identification. The enthusiast may also wish to categorise a previously undescribed mycoplasma isolate. The complex series of steps required for acceptance of a new species have been outlined (Subcommittee, 1979). For confirming a diagnosis, or for obtaining a pure culture for antibiotic sensitivity testing, a much simplified procedure is used. Well-isolated colonies are selected and picked up using negative pressure with a fine Pasteur pipette and a rubber bulb. The plug of agar containing the colony is aspirated into broth and incubated. Up to five colonies are usually selected from each plate. When the broth culture shows signs of growth it is ready for use in biochemical and serological tests. Carefully done, this procedure is generally satisfactory for diagnostic purposes. However, it does not guarantee a pure culture. For critical work the cloning procedure should be repeated three times and should include filtration through a 450 nm membrane filter at each stage.

5.3. Biochemical Tests Used in the Identification of Avian Mycoplasmas

A large number of biochemical and other laboratory tests have been used to help identify mycoplasma cultures. The most commonly used are probably glucose fermentation, arginine hydrolysis, phosphatase activity, tetrazolium reduction and film and spots phenomenon. Results for these tests given by avian mycoplasmas are listed in Table 1.

5.3.1. Digitonin Sensitivity

5.3.1.1. Preparation of digitonin discs

Add 75 mg digitonin (BDH) to 5 mL 95% ethanol (C_2H_5OH). Heat to boiling to dissolve digitonin completely. Store stock solutions at 4°C. Inoculate 6 mm filter paper discs (Schleicher and Schuell) with 25 µL of digitonin solution. Dry overnight at 37°C. Store in a sealed container at 4°C.

5.3.1.2. Method

Dry agar plate in an inverted position with lid off at 37°C for 20 min. Inoculate plate with broth culture containing about 10^5 colony forming units (CFU)/mL. When the culture has absorbed into the agar, carefully place a paper disc containing 25 µL of a 1.5% solution of digitonin on the agar surface. Incubate as usual.

5.3.1.3. Interpretation

Zone of inhibition around disc — *Mycoplasma* sp. No zone of inhibition around disc — *Acholeplasma* sp.

5.3.2. Carbohydrate Fermentation

5.3.2.1. Method

Use broth medium containing up to 1% w/v glucose or other carbohydrate and phenol red indicator pH 7.8. Inoculate one broth with the test organism, and one broth each with known positive and negative control cultures. One broth is left as a non-inoculated control. In addition, broths containing no carbohydrate source should be inoculated with the test culture and with the known carbohydrate fermenting and non-fermenting control cultures. Broths are incubated at 37°C for up to 14 days.

5.3.2.2. Interpretation

A positive reaction is one where the pH of the test culture and the positive control culture in carbohydrate containing medium are *lower* by greater than 0.5 of a pH unit than each of the negative control cultures.

5.3.3. Arginine Hydrolysis

5.3.3.1. Method

Use broth medium containing 0.2% arginine and phenol red indicator pH 7.0. Inoculation is as described for glucose fermentation.

5.3.3.2. Interpretation

A positive reaction is one where the pH of the test culture in arginine containing medium is *higher* by greater than 0.5 of a pH unit than each of the negative control cultures.

5.3.4. Phosphatase Activity

5.3.4.1. Method

Agar containing 0.1% w/v sodium phenothalien diphosphate (Selby Anax) is inoculated with the test culture. Incubate test culture and non-inoculated control at 37°C for seven days, then flood plates with 5 mol/L sodium hydroxide.

5.3.4.3. Interpretation

A pink–red change around the colonies occurring 30 s after the addition of sodium hydroxide is indicative of a positive reaction. No change should occur on the non-inoculated plate.

5.3.5. Tetrazolium Reduction

5.3.5.1. Method

Broth medium (without phenol red) containing 55 mg/L of 2, 3, 5- triphenyltetrazolium chloride (Sigma) is inoculated with the test culture. Incubate at 37°C and observe daily for up to 14 days.

5.3.5.2. Interpretation

A red insoluble formazan forming in the medium indicates a positive reaction.

5.3.6. Film and Spots

5.3.6.1. Method

Agar medium supplemented with 10% Egg Yolk Emulsion (Oxoid Australia) is inoculated with the test culture for up to 14 days.

5.3.6.2. Interpretation

A wrinkled, pearly film on the medium with black spots beneath and around colonies indicates a positive reaction.

5.4. Serological Tests

The ultimate identification of a mycoplasma isolate depends on serology. Many tests, e.g. immunofluorescence, growth inhibition, metabolic inhibition, have been used for this purpose. Besides immunofluorescence (which has been described previously), the growth inhibition test is probably most widely used. The growth inhibition test relies on mycoplasma growth being inhibited by its homologous antibody. The test is highly specific, although rather insensitive. Monospecific, hyperimmune sera are required. Details of the test are given in 5.4.1. Typing antisera can be purchased from VETLAB in South Australia (see 10.2.).

An alternative is agar gel immunodiffusion where lysed mycoplasma cells are placed in wells opposite known hyperimmune antiserum. One or more precipitin lines showing identity with antigen from a known mycoplasma species will confirm the identification.

5.4.1. Growth Inhibition Test

Knowledge of the avian species involved and whether the *Mycoplasma* sp. is a glucose fermenter or not, assists in the selection of the appropriate antisera to be used.

5.4.1.1. Preparation of antiserum discs

Paper discs (6 mm diameter) are loaded with 25 µL of the appropriate antiserum. They may be dried overnight at 37°C and stored in sealed containers at 4°C until required for use.

5.4.1.2. Method

The surface of an agar plate is dried by inverting the plate with the lid off for 20 min at 37°C. A 25 µL drop of diluted culture containing about 10^5 CFU/mL is placed on the agar surface and allowed to run across the plate. A late log phase culture usually contains about 10^8–10^9 organisms/mL so dilutions of 10^{-3} and 10^{-4} are generally satisfactory.

N.B. The viability of mycoplasma cultures is often short once growth becomes unbalanced. Up to four separate drops of culture can be run across the agar in a 90 mm Petri dish. Thus dilutions of the same culture or different cultures can be tested on the same plate. Once the inoculum has absorbed into the medium the appropriate antiserum discs are carefully placed at 15–20 mm intervals along the line of the running drop. Alternatively the entire agar surface is flooded with culture. Incubate plates for the appropriate period and examine.

5.4.1.3. Interpretation

Record results by measuring the width of the zone of inhibition from the edge of the paper disc to the nearest colonies. Inhibition should be produced only by a single antiserum. Sometimes there may not be complete inhibition of colony formation. However colonies appearing within the zone of inhibition have a stunted deformed appearance. At times, circular precipitin bands may also be seen where diffusing antiserum and soluble mycoplasma antigens have met at optimum concentrations.

5.5. Newer Methods for Identifying Mycoplasmas

Analysis of mycoplasma proteins by polyacrylamide gel electrophoresis Khan *et al.*, 1987) and DNA by restriction endonuclease analysis or Southern hybridisation with DNA probes (Kleven *et al.*, 1988) are now used in research laboratories for the characterisation of mycoplasmas. These techniques are particularly useful for identifying strains in epidemiological studies. A

DNA hybridisation probe kit is available in other countries for identification of *M. gallisepticum*. *M. gallisepticum* strain specific DNA probes have also been developed. The highly sensitive polymerase chain reaction is also being developed for the identification of avian mycoplasmas *in situ*.

6. Antibiotic Sensitivity Testing of Cultures

Because of their relatively slow growth, mycoplasmas cannot be tested using standard disc diffusion tests. A method for a miniaturised broth dilution procedure (Whithear *et al.*, 1983) for testing antibiotic sensitivity is described below. Antibiotics which should be considered for antibiotic sensitivity testing of avian mycoplasmas include erthromycin base (Abbott Australasia), tylosin tartrate (Elanco Products Company), tiamulin hydrogen fumarate (Squibb), tetracycline hydrochloride (Squibb), lincomycin hydrochloride (Upjohn) and spectinomycin sulfate (Upjohn).

6.1. Microbroth Antibiotic Sensitivity Test (from Whithear et al., 1983)

6.1.1. Preparation of Antibiotics

Water-soluble antibiotics are dissolved in double-glass-distilled water. Tetracycline and erythromycin can be dissolved in 0.01 mol/L hydrochloric acid (HCl) and 7% ethanol, respectively. Each antibiotic solution should be passed through a 450 nm membrane filter and appropriate dilutions made in mycoplasma broth medium (see 10.2.) to the concentration required for the test.

6.1.2. Method

A 50 µL volume of broth (see 10.1.) is added to all wells of a sterile tissue-culture-quality 96-well U-bottomed microtitration plate. Three antibiotics can be tested on each plate. Duplicate doubling dilutions of each antibiotic are made down the 12 wells of the long axis of the plate (rows AB, DE and GH). The range of concentrations of active compound used for testing the sensitivity of avian mycoplasmas to antibiotics are: erythromycin, tetracycline, tiamulin and tylosin from 2.5 to 0.00125 µg/mL (for strains resistant to erythromycin concentrations from 80.0 to 0.04 µg/mL should be used), lincomycin, spectinomycin, and a 1:2 combination of lincomycin and spectinomycin from 80.0 to 0.04 µg/mL.

The third and sixth rows (C and F) contain no antibiotic and serve as culture controls. A 150 µL volume of broth containing the appropriate concentration of organisms (usually a 10^{-3}–10^{-4} dilution of a late-logarithmic-phase broth culture) is added to each well. The plates are covered with Mylar tape (ICN Biomedicals Australasia) and incubated at 37°C in a stationary position.

Control of inoculum density is achieved by doing a viable count. A standard culture should be included with each batch of tests as a control of reproducibility.

6.1.3. Reading and Interpretation of the Test

Tests are read when the phenol red indicator in the culture control turns orange-yellow (about pH 7). The minimum inhibitory concentration (MIC) is the lowest concentration of antibiotic that completely prevents colour change in the medium.

Inoculum densities in the range of 10^3–10^6 viable organisms/mL are considered acceptable.

A MIC value for the standard control culture that is within ± one doubling dilution of the most common value for each test antibiotic is regarded as an acceptable level of reproducibility.

7. Flock Serological Testing

Serological testing is used to confirm the status of *M. gallisepticum*-free and *M. synoviae*-free breeder flocks and also to assist with laboratory diagnosis when an outbreak of mycoplasmosis is suspected. The rapid serum agglutination (RSA) test is most widely used. Other tests include haemagglutination inhibition (HI, see 7.3.3.) and enzyme-linked immunoassay (ELISA). Breeder flocks often show no obvious clinical respiratory signs although birds develop serum agglutinins to the mycoplasma within two weeks of infection. Infected flocks pose a risk to other mycoplasma-free flocks and, if in lay, will egg transmit pathogenic mycoplasmas to their progeny. Infected flocks must be identified quickly so that appropriate quarantine and control measures can be taken. This is achieved by regular serological testing.

7.1. Time of Collection and Number of Samples

Deciding on number of serum samples to test, the age of the flock when samples should be first collected and the time intervals between testing depends on a number of factors, not the least of which is cost. The veterinarian will need to make a judgement about what is most appropriate according to particular circumstances. For example, on farms that are well isolated and where there is no history of introduction of infection in previous flocks, testing before point-of-lay and thereafter at about three-month intervals should be adequate. When the risk of introduction of infection is perceived to be higher, more regular testing will be required. Because of their importance as a source of mycoplasma-free genetic stock, primary breeder flocks and grandparent flocks will be tested more frequently than parent flocks.

For guidance on the sample size required to detect different levels of infection in flocks of various sizes, Australian Bureau of Animal

Health (1982) should be consulted. The most usual observation under Australian conditions is that *M. gallisepticum* and *M. synoviae* spread relatively rapidly when introduced into a susceptible flock. It is normal practice, therefore, to collect enough samples to detect a 10% prevalence of infection at a 95% level of certainty. In a flock of 10 000 this amounts to about 30 samples. It is preferable to test this number of samples on a regular basis (say monthly) than to test a larger number of samples at more infrequent intervals. It is important to test flocks in all sheds on any particular farm, since the movement of pathogenic mycoplasmas between sheds may occur relatively slowly and inconsistently.

7.2. Handling of Serum Samples

Blood samples are ideally placed in siliconised 'clotting' tubes that allow retraction of the clot from the sides of the tube. The tubes can be placed in polystyrene racks designed to allow multiple serum samples to be picked up simultaneously using a multichannel dispenser. Serum samples to be used in the RSA test should not be frozen as this may lead to non-specific reactions. If they cannot be tested on the day of collection then sera should be stored overnight at 4°C.

7.3. Rapid Serum Agglutination Test

7.3.1. Method

Stained *M. gallisepticum* (Antigen Nobilis) and *M. synoviae* (Antigen Nobilis) antigens are marketed in Australia by Intervet. Commercial *M. meleagridis* antigens are not currently available in Australia. The RSA test is very sensitive and is capable of detecting antibody as soon as 7–10 days after infection. The basic procedure as recommended by the manufacturer involves mixing a drop of undiluted serum with an equal volume of antigen on a glass plate at 20–25°C. The dropper supplied with the antigen delivers about 40 µL. The plate is rotated for two minutes, and the test is then read for evidence of macroscopic clumping of antigen. The reactions are often graded (Fig. 1) from 0 (no clumping) to 4+ (large clumps with complete clearing of the antigen suspension). According to the instructions supplied by the manufacturer, clumping that occurs after two minutes should be interpreted as a doubtful reaction.

Some laboratories have modified the procedure to facilitate the testing of large numbers of samples. This involves the use of multichannel dispensers to pick up sera which are then placed in perspex World Health Organisation (WHO) trays or in flat-bottomed plastic microtrays. Use of 25 µL volumes of serum and antigen allows convenient visualisation of the reaction as well as conservation of antigen. The trays are tapped to mix antigen and serum, then rotated for two minutes on a rotary agglutinator and read immediately over a radiograph examination light. Duplicate samples of each serum may be placed in adjoining rows and tested with both *M. gallisepticum* and *M. synoviae* antigens. Because of the very high cost of *M. synoviae* antigen, this test is sometimes performed using antigen diluted 1/3 in PBS. When making modifications to expedite or reduce the cost of testing the modified procedure must be properly validated before it is accepted, and appropriate positive and negative controls always used.

7.3.2. Problems with the Rapid Serum Agglutination Test

The major disadvantage of the RSA test is its propensity to give non-specific reactions. These may be due to a variety of known and unknown causes. The known causes include reactions with serum proteins adsorbed onto the mycoplasma antigen from the growth medium, and cross-reaction with antigens shared with other mycoplasmas and bacteria.

False positive reactions with both *M. gallisepticum* and *M. synoviae* antigens are often seen in flocks that have been vaccinated with inactivated oil emulsion vaccines against other infectious agents, especially if the vaccine contains serum proteins from the medium used to grow the agent. False positive reactions may persist for up to four to eight weeks or longer after vaccination. There are several cross-reacting antigens between *M. gallisepticum* and *M. synoviae* and these may cause problems in interpretation of the RSA test.

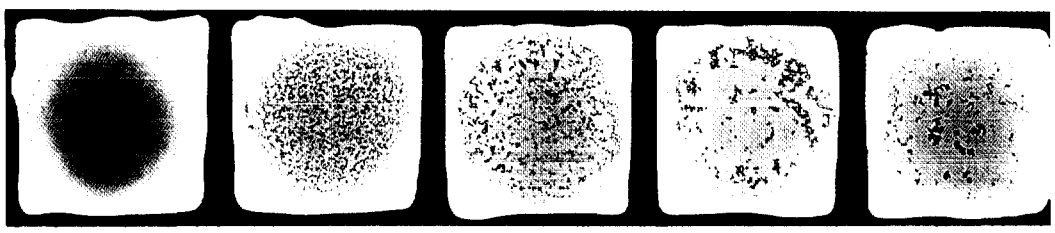

Figure 1. Interpretation of Agglutination Reactions. Stained antigen was mixed in equal volumes with five different chicken sera. The plate was then rotated for two minutes prior to photography. The serum shown in the left panel was from a specific-pathogen-free chicken. The antigen serum mixture forms a homogeneous blue suspension with no evidence of antigen clumping. Such a serum would be scored as zero or negative. The other panels (from left to right) show increasing clumping of antigen. The serum–antigen reactions would be scored 1+ (second from the left) through to 4+ (right hand panel).

7.3.3. Confirming the Specificity of Rapid Serum Agglutination Reactions

Some laboratories attempt to confirm the specificity of reacting sera by testing them in doubling dilutions in PBS to a dilution of 1:10. Sera that react at ≥1:10 are considered positive. However, sometimes false positive reactions may still occur at serum dilutions ≥1:10 while specific weak reactions may have a titre of ≤1:10.

Another possible solution is to rebleed the flock two to three weeks later, particularly pens or groups of birds whose sera gave RSA reactions. One would expect an increase in intensity and incidence of reactions if a specific *M. gallisepticum* or *M. synoviae* infection was occurring. However, this is also not fool proof because sometimes the infection can spread very slowly, particularly with low virulence strains of *M. gallisepticum*.

In the United States of America, RSA reactors are often confirmed using the HI test (Kleven and Yoder, 1989). This is regarded as a highly specific although rather insensitive test. It is not currently used widely in Australia and no commercial antigens are available. ELISA has been used in Australia in an attempt to provide a specific and sensitive serological test (Higgins and Whithear, 1986). Unfortunately it also gave an unacceptably high level of non-specific reactions with field sera. Final confirmation of a diagnosis of avian mycoplasmosis in Australia relies on culture of the suspected organism from the flock.

8. References

Australian Bureau of Animal Health (1982). 'Livestock disease surveys: A field manual for veterinarians.' (Australian Bureau of Animal Health: Canberra.)

Baas, E.J., and Jasper, D.E. (1972). Agar block technique for identification of mycoplasmas by use of fluorescent antibody. *Applied Microbiology* **23**, 1097–1100.

Frey, M.L., Hanson, R.P., and Anderson, D.P. (1968). A medium for the isolation of avian mycoplasmas. *American Journal of Veterinary Research* **29**, 2163–171.

Higgins, P.A., and Whithear, K.G. (1986). Detection and differentiation of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* antibodies in chicken serum using ELISA. *Avian Diseases* **30**, 160–8.

Khan, M.I., Lam, H.M., and Yamamoto, R. (1987). Mycoplasma gallisepticum strain variations detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Avian Diseases **31**, 315–20.

Kleven, S.H. (1991). Other mycoplasma infections. *In* 'Diseases of Poultry'. (Eds B.W. Calneck, H.J. Barnes, C.W. Beard, W.M. Reid, and H.W. Yoder.) pp. 231–5. Ninth Edn. (Iowa State University Press: Ames, Iowa.)

Kleven, S.H., Browning, GF., Bulach, D.M., Giocas, E., Morrow, S.J., and Whitear, K.G. (1988). Examination of *Mycoplasma gallisepticum* strains using restriction endonuclease DNA analysis and DNA–DNA hybridization. *Avian Pathology* **17**, 559–70.

Kleven, S.H., Rowland, G.N., and Olson, N.O. (1991). *Mycoplasma synoviae* infection. *In* 'Diseases of Poultry'. (Eds B.W. Calneck, H.J. Barnes, C.W. Beard, W.M. Reid, and H.W. Yoder.) pp. 223–31. Ninth Edn. (Iowa State University Press: Ames, Iowa.)

Kleven, S.H., and Yoder, H.W. (1989). Mycoplasmosis. *In* 'A Laboratory Manual for the Isolation and Identification of Avian Pathogens.' (Eds H.G. Purchase, L.H. Arp, C.H. Domermuth, and J.E. Pearson.) pp. 57–62. 3rd Edn. (Kendall/Hunt Publishing: Dubuque.)

Subcommittee (1979). Proposal of minimal standards for descriptions of new species of the class Mollicutes. *International Journal of Systematic Bacteriology* **29**, 172–80.

Wang, Y., Whithear, K.G., and Ghiocas, E. (1990). Isolation of *Mycoplasma gallinarum* and *M. gallinaceum* from the reproductive tract of hens. *Australian Veterinary Journal* **67**, 990.

Whithear, K.G., Bowtell, D.D., Ghiocas, E., and Hughes, K.L. (1983). Evaluation and use of a micro broth dilution procedure for testing sensitivity of fermentative avian mycoplasmas to antibiotics. *Avian Diseases* **27**, 937–49.

Yamamoto, R. (1991). *Mycoplasma meleagridis* infection. *In* 'Diseases of Poultry.' (Eds B.W. Calneck, H.J. Barnes, C.W. Beard, W.M. Reid and H.W. Yoder.) Ninth Edn. pp. 212–23. (Iowa State University Press: Ames, Iowa.)

Yoder, H.W. (1991). *Mycoplasma gallisepticum* infection. *In* 'Diseases of Poultry.' (Eds B.W. Calneck, H.J. Barnes, C.W. Beard, W.M. Reid and H.W. Yoder.) Ninth Edn. pp. 198–212. (Iowa State University Press: Ames, Iowa.)

8.1. Further Reading

Jordan, F.T.W. (1979). Avian mycoplasmas. *In* 'The Mycoplasmas. Vol II. Human and Animal Mycoplasmas.' (Eds J.G. Tully and R.F. Whitcomb.) pp. 1–48. (Academic Press: New York.)

Razin, S., and J.G. Tully (Eds). (1983). 'Methods in Mycoplasmology Vol I, Characterization of mycoplasmas.' (Academic Press: New York.)

Tully J.G., and S. Razin (Eds). (1983). 'Methods in Mycoplasmology. Vol II. Diagnostic Mycoplasmology.' (Academic Press: New York.)

9. Acknowledgments

Information kindly provided by Geoff Cottew, Peter Curtin, Stan Kleven, Lyn Ireland, Philip Markham and Chris Morrow was used in the preparation of this document.

10. Appendixes

10.1. Appendix 1 — Media for the Isolation of Avian Mycoplasmas

Many different media formulations have been described for the isolation of avian mycoplasmas. Perhaps the most widely used are media modified from the formulation originally described by Frey *et al.* (1968). Cysteine HCl and NAD are included when attempting to isolate *M. synoviae*. Horse serum is preferred to swine serum for the isolation of *M. meleagridis*.

10.1.1. Mycoplasma Agar (modified Frey *et al.*, 1968)

BBL Mycoplasma broth base (Becton Dickinson)	22.5 g
Special Noble Agar (Difco)	10.0 g
Cysteine hydrochloric acid (Ajax)	50 mg
Thallous acetate (Sigma) (10% w/v)	2.5–10 mL
Double glass distilled water (varies with concn of serum)	750–850 mL

Adjust pH to 7.7–7.8 with 5 mol/L sodium hydroxide. Autoclave 121°C for 15 min. Cool to 50°C and then aseptically add the following (warmed to 50°C) sterile enrichments:

Swine serum (inactivated at 56°C for 30 min)	100–200 mL
Fresh yeast autolysate (see 10.1.1.1.)	10 mL
DNA (Koch-Light) (0.2% w/v)	10 mL
NAD (Sigma) (1.0% w/v)	10 mL
Amoxycillin (Beecham)	1.0 g

Mix well and pour aseptically into sterile Petri dishes to a depth of about 5 mm.

N.B. All enrichments (except serum) are prepared in double glass distilled water and sterilised by filtration through 220 nm membrane filters. They are stored in convenient sized aliquots at -20°C.

10.1.1.1. Fresh Yeast Autolysate

Fresh yeast autolysate is prepared as follows. Fresh Baker's Yeast (1 kg, Mauri Foods) is added to 500 mL double glass distilled water, kneaded into a paste, and a further 500 mL water added. pH is adjusted to 4.5 (with about 6.5 mL of 1 mol/L hydrochloric acid). Hold at 80°C for 20 min. Allow cells to settle or centrifuge, decant supernatant and filter through Whatman filter paper to clarify. Filter through Seitz Z-9 (Industrial Equipment) filter. Check for sterility and dispense in convenient aliquots. Store at -20°C.

10.1.2. Mycoplasma Broth (modified Frey *et al.*, 1968)

BBL Mycoplasma broth base	22.5 g
Cysteine hydrochloric acid	50 mg
Double glass distilled water (varies with concentration of serum used)	765–865 mL
Phenol red (0.4% w/v)	5 mL
Thallous acetate (10% w/v)	2.5–10 mL
Swine serum (inactivated 56°C for 30 min)	100–200 mL
Fresh yeast autolysate	10 mL
DNA (0.2% w/v)	10 mL
NAD (1.0% w/v)	10 mL
Amoxycillin	1.0 g

Adjust pH to 7.7–7.8 with 5 mol/L sodium hydroxide. Sterilise by filtration through a 220 nm membrane filter.

A modification of the above medium uses 14.7 g/L PPLO broth without crystal violet (Difco) as a substitute for 22.5 g/L BBL Mycoplasma broth base.

10.2. Appendix 2 — Specialist Assistance

At the direction of the Subcommittee of Principal Laboratory Officers, the Mycoplasma Reference Collection formerly held at CSIRO Parkville, Vic. 3052, was transferred to VETLAB, GPO Box 1671, Adelaide, SA 5001. Reference cultures and typing antisera can be obtained from VETLAB on a user-pays basis. Contact Mrs Fay Frith on Tel. (08) 228 7216 or Fax (08) 228 7495.

The Victorian Institute of Animal Science (VIAS), 475 Mickleham Road, Attwood, Vic. 3049, has been officially recognised by the Subcommittee of Principal Laboratory Officers as the national Avian Mycoplasma Reference Laboratory. Services offered by VIAS include speciation of isolates and supply of media and reagents on a user-pays basis. Contact person is Dr Chris Morrow on Tel. (03) 217 4200 or Fax (03) 217 4299.

The School of Veterinary Science, The University of Melbourne, also offers assistance for the diagnosis of avian mycoplasma infections. Contact Dr Kevin Whithear on Tel. (03) 742 8273 or Fax (03) 741 0401.

10.3. Appendix 3 — Suppliers

Abbott Australasia Pty Ltd. PO Box 101, Cronulla, NSW 2230. Tel. (008) 225 311. Fax (02) 668 8459. Erythromycin base. Cat No. 6304.1.

Ajax Chemicals Ltd. 16–18 Hamlet Street, Cheltenham, Vic. 3192. Tel. (03) 584 2622. Fax (03) 584 8335. L-Cysteine HCl Labchem. Cat No. 37055 3M.

Beecham Research Laboratories. 300 Frankston Rd, Dandenong, Vic. 3175. Tel. (03) 797 6722. Amoxil. Cat. No. B0004.

BBL. Distributed in Australia by Becton Dickinson and Company, 80 Rushdale Street, Knoxfield, Vic. 3180. Tel. (03) 764 2444. Fax (03) 764 2550. Mycoplasma Broth Base (Frey). Cat. No. 12346.

BDH Chemicals Ltd. Distributed in Australia by FSE Scientific, Private Bag 42, Springvale, Vic. 3171. Tel. (03) 795 0077. Fax (03) 790 1900. Digitonin. Cat. No. 10095.

Difco Laboratories. Distributed in Australia by FSE Ltd, 47–49 Overseas Drive, Noble Park, Vic. 3174. Tel. (03) 795 0077, Fax (03) 795 3763. Agar Noble. Cat. No. 0142-08-1; crystal violet.

Elanco Products Company. 112 Wharf Road, West Ryde, NSW 2114. Tel. (02) 858 8792. Fax (02) 874 6396. Tylosin tartrate.

ICN Biomedicals Australasia Pty Ltd. PO Box 187, Seven Hills, NSW 2147. Tel. (008) 249 998. Fax (02) 838 7390.

Industrial Equipment. PO Box 230, Revesby, NSW 2212. Tel. (02) 774 4644. Fax (02) 792 1004. Ekwip Filter Pads.

Intervet Aust Pty Ltd. Private Bag 25, Lane Cove, NSW 2066. Tel. (02) 428 9446. Fax (02) 428 1732. *Mycoplasma gallisepticum* Antigen Nobilis; *Mycoplasma synoviae* Antigen Nobilis.

Koch-Light. Distributed in Australia by Laboratory Supply Pty Ltd, PO Box 203, Clayton, Vic. 3168. Tel. (03) 543 1155. Fax (03) 543 7246. DNA Na Salt ex Herring Sperm. Cat. No. 11495.

Mauri Foods. 5 Yarra Street, South Yarra, Vic. 3141. Tel. (03) 826 1254. Fresh Bakers Yeast. Cat. No. E10076.

Oxoid Australia Pty Ltd. 104 Northern Road, West Heidelberg, Vic. 3081. Tel. (03) 458 1311. Fax (03) 458 4759. Egg Yolk Emulsion. Cat. No. SR47.

Schleicher and Schuell. Distributed in Australia by Bartelt Instruments Pty Ltd, PO Box 237, Heidelberg West, Vic. 3081. Tel. (03) 459 8822. Fax (03) 457 5906. Antibiotica-Testblattchen. Cat. No. 321260.

Sigma. Distributed in Australia by Selby Anax, Private Bag 24, Mulgrave North, Vic. 3170. Tel. (03) 544 4844. Fax (03) 562 9840. Sodium phenothalein diphosphate. Cat. No. PD126; 2, 3, 5- triphenyltetrazolium chloride; thallium acetate Cat. No. T-8266; β-NAD. Cat. No. N-7381.

E.R. Squibb & Sons Pty Ltd. 556 Princes Highway, Noble Park, Vic. 3174. Tel. (03) 795 3722. Fax (03) 795 0968. Tiamulin hydrogen fumarate; tetracycline hydrochloride.

Upjohn Pty Ltd. PO Box 46, Rydalmere, NSW 2116. Tel. (008) 251 508. Fax (02) 684 2130. Lincomycin hydrochloride; spectinomycin sulfate.

Wellcome Australia Ltd. 53 Phillips Street, Cabarita, NSW 2137. Tel. (008) 22 5171. Fax (02) 743 6378. Sheep anti-rabbit IgG. Cat. No. MF02.