CHAPTER 3.3.5.

AVIAN MYCOPLASMOSIS
(*Mycoplasma gallisepticum, M. synoviae*)

SUMMARY

Definition of the disease: *Avian mycoplasmosis is caused by several pathogenic mycoplasmas of which* Mycoplasma gallisepticum (*MG*) *and* M. synoviae (*MS*) *are the most important; they are the only ones listed by the OIE.*

Description of the disease: *MG causes chronic respiratory disease of domestic poultry, especially in the presence of management stresses and/or other respiratory pathogens. Disease is characterised by coryza, conjunctivitis, sneezing, and by sinusitis, particularly in turkeys and game birds. It can result in loss of production and downgrading of meat-type birds, and loss of egg production. MS may cause respiratory disease, synovitis, or may result in a silent infection. MG and MS strains vary in infectivity and virulence, and infections may sometimes be unapparent.*

Identification of the agent: *MG and MS can be identified by immunological methods after isolation in mycoplasma media or by detection of their DNA in field samples or cultures.*

Samples for isolation can be swabs of organs or tissues, exudates, diluted tissue homogenates, aspirates from the infraorbital sinuses or joint cavities, or material from egg yolk or embryos. Clinical signs and lesions will influence the sample selection. Broth and agar are used for isolation, but it is normally necessary to obtain mycoplasma colonies on agar before attempting identification. Basic biochemical tests can be helpful in preliminary classification of isolates but final identification is by immunological tests, the most satisfactory being fluorescent antibody and immunoperoxidase tests.

DNA detection methods based on the polymerase chain reaction are used in specialised laboratories. Once validated, they can be used on swab material or cultures.

Serological tests: *Several serological tests are used to detect MG or MS antibodies, but due to variations in specificity and sensitivity, they are recommended for flock screening rather than for testing individuals.*

The most commonly used are the rapid serum agglutination (RSA) test, the enzyme-linked immunosorbent assay (ELISA) and the haemagglutination inhibition (HI) test. In the RSA test, sera are mixed with commercially produced stained antigen and sera that react within 2 minutes are heated at 56°C for 30 minutes and retested. Sera that still react, especially when diluted, are considered positive and are tested by either ELISA or HI for confirmation. Several commercial MG and MS antibody ELISA kits are available.

Requirements for vaccines and diagnostic biologicals: *Although the preferred method of control is maintenance of MG- and MS-free flocks, both live and inactivated vaccines are used in chickens. Vaccination should be considered only on multi-age sites where infection is inevitable. The normal use is to prevent egg-production losses in commercial layers, although vaccines may also be used to reduce egg transmission in breeding stock or to aid MG eradication on multi-age sites. It is important to vaccinate before field challenge occurs.*

Available live vaccines for MG are produced from the F strain, and, more recently, strains ts-11 and 6/85, which are apathogenic strains with improved safety characteristics. Administration of the F strain by the intranasal or eyedrop route is preferred, but aerosol or drinking water administration may be used. The eyedrop method is recommended for ts-11, and a fine spray for 6/85. Pullets are generally vaccinated between 12 and 16 weeks of age. One dose is sufficient and vaccinated birds

remain permanent carriers. Long-term use of the F strain on multi-age sites results in displacement of field strains. The ts-11 strain has been successfully used to eradicate F strain in multi-age commercial layers. A live MS vaccine has been produced from the MS-H strain and should be administered by eyedrop.

Bacterins consist of a concentrated suspension of MG organisms in an oil emulsion. They are administered parenterally to pullets at 12–16 weeks of age, usually subcutaneously in the neck. Two doses are desirable. Bacterins are effective in preventing egg-production losses and respiratory disease, but they do not prevent infection with wild-type MG. A similar bacterin has been licensed in the United States of America for MS, but it is not widely used.

A. INTRODUCTION

Mycoplasma gallisepticum (MG) and *M. synoviae* (MS) belong to the class *Mollicutes*, order *Mycoplasmatales*, family *Mycoplasmataceae*. It should be noted, however, that *M. meleagridis* and *M. iowae* can also cause disease in poultry, but MG and MS are considered to be the most important of the pathogenic mycoplasmas, and both occur world-wide.

MG infection is particularly important in chickens and turkeys as a cause of respiratory disease and decreased meat and egg production (Bradbury, 2001; Ley, 2003). It can also cause upper respiratory disease in game birds. More recently MG has been recognised in North America in house finches as a cause of conjunctivitis (Luttrell *et al*., 1996). In poultry the infection is spread vertically through infected eggs and horizontally by close contact; the MG nucleic acid has been identified in environmental samples (Marois *et al*., 2002). Other methods of spread are less well documented.

The clinical signs of MG in infected poultry can vary from subclinical to obvious respiratory signs including coryza, conjunctivitis, coughing and sneezing. Nasal exudate, rales and breathing through the partially open beak may occur. Unilateral or bilateral sinusitis may also be a feature, particularly in turkeys and game birds and the infraorbital sinuses may become so swollen that the eyelids are closed. Conjunctivitis, with frothy ocular exudate is also a common feature in turkeys and game birds, and sometimes in chickens. In turkeys there is often soiling of the wing feathers as the result of attempts to remove exudate from the eyes. Infected finches may reveal ocular and nasal discharge and swollen eyelids in addition to the conjunctivitis.

Mycoplasma gallisepticum may be associated with acute respiratory disease in chickens and turkeys, especially in young birds, with the turkey being more susceptible. The severity of the disease is greatly affected by the degree of secondary infection with viruses such as Newcastle disease and infectious bronchitis, and/or bacteria such as *Escherichia coli*. In turkeys there is synergism with avian pneumovirus infection. A more chronic form of the disease may occur and can cause reduced egg production in breeders and layers.

Lesions of the respiratory tract take the form initially of excess mucous exudate followed by catarrhal and caseous exudate, which may form amorphous masses in the air sacs. In turkeys and game birds the swollen infraorbital sinuses contain mucoid to caseous exudate.

MG or MS disease in chickens may superficially resemble respiratory disease caused by other pathogens such as mild strains of Newcastle disease (Chapter 3.3.14) and avian infectious bronchitis (Chapter 3.3.2). These may be present in mixed infection with MG or MS. Infections with *Haemophilus paragallinarum* (now *Avibacterium paragallinarum*), and *Pasteurella multocida*, should also be ruled out. MG in turkeys may be confused with avian pneumovirus infections and the presence of sinusitis may also suggest infection with *Pasteurella multocida*, *Chlamydia* (Chapter 3.3.1) or MS. Infectious synovitis caused by MS should be differentiated from *Staphylococcus aureus* infection and from infectious tenosynovitis caused by reovirus.

Chickens with infectious synovitis may exhibit pale combs, lameness and retarded growth. Swellings may occur around joints. Greenish droppings containing large amounts of urates are commonly seen. Joints may contain a viscous, creamy to grey exudate in the joint and along tendon sheaths, along with hepatosplenomegaly and mottled, swollen kidneys (Kleven, 2003). Respiratory signs and lesions are similar to those observed with MG, except that they are generally milder, and, as with MG, there is a synergistic effect with other respiratory agents (Kleven *et al*., 1972). MS strains exhibit significant variability with respect to their virulence and tissue tropism (Kleven *et al*., 1973; Landman & Feberwee, 2004; Lockaby *et al*., 1999).

B. DIAGNOSTIC TECHNIQUES

The presence of MG or MS can be confirmed by isolating the organism in a cell-free medium or by detecting its DNA directly in infected tissues or swab samples. Serological tests are also widely used for diagnosis. When results are equivocal the birds are usually resampled, although chicken embryos or chickens may be inoculated with suspect material.

1. Identification of the agent

1.1. Culture

Samples are taken from live birds, fresh carcasses or the carcasses of birds that have been frozen when fresh. From live birds, swabs may be taken from the choanal cleft, oropharynx, oesophagus, trachea, eyes, cloaca and phallus. In the case of dead birds, samples may be taken from the nasal cavity, infraorbital sinus, trachea, or air sacs. Exudates can be aspirated from the infraorbital sinuses and joint cavities.

Samples may also be collected from dead-in-shell embryos or chickens or poults that have broken the shell but failed to hatch. Samples can be taken from the inner surface of the vitelline membrane, and from the oropharynx and air sacs of the embryo.

All samples should be examined as soon as possible after collection. If transportation is necessary, small pieces of tissue should be placed in mycoplasma broth, or swabs should be vigorously agitated in 1–2 ml of mycoplasma broth and then discarded. Alternatively, the swabs can be dipped in mycoplasma broth before the specimens are taken (Zain & Bradbury, 1996) and then replaced in the swab holders for transportation. An ice pack or some other means of chilling should be included as MG and MS die rapidly at room temperature. Serial dilutions of specimens in mycoplasma broth may be of value because the presence of specific antibodies or antibiotics or inhibitory substances in tissues may inhibit mycoplasma growth unless they are diluted out.

Several suitable culture media have been formulated (Freundt, 1983) and those suitable for isolation of avian mycoplasmas can be purchased from Mycoplasma Experience, Reigate, Surrey, United Kingdom. Mycoplasma media generally contain a protein digest and a meat-infusion base supplemented with serum or a serum fraction, yeast factors, glucose and bacterial inhibitors. It is important that each new batch of medium be tested with recently isolated MG cultures of low *in-vitro* passage because some components, especially the yeast extract and the serum may vary in their ability to support growth.

The medium developed by Frey *et al.* is widely used in the United States of America (USA) and other countries for isolation of MG and MS (Frey *et al*., 1968; USDA, 2004). Nicotinamide adenine dinucleotide (NAD) is a growth requirement for the primary isolation of MS, but it may be omitted from the medium for the cultivation of MG.

The following broth and agar media are also satisfactory:

i) Part A: Pleuropneumonia-like organism (PPLO) broth base without crystal violet (Difco) (14.7 g); distilled or deionised water (700 ml).

ii) Part B: Pig serum (heated at 56°C for 1 hour) (150 ml): 25% (w/v) fresh yeast extract (100 ml); 10% (w/v) glucose solution (10 ml); 5% (w/v) thallous acetate (10 ml); 200,000 International Units (IU)/ml penicillin G (5 ml); and 0.1% (w/v) phenol red solution (20 ml). Thallous acetate can be toxic to humans and the precautions for its use should be followed. The pH is adjusted to 7.8. Pig serum may be replaced by horse serum, but it is important to ascertain that it supports the growth of MG.

Part A is autoclaved at 121°C, at 1 atmospheric pressure for 15 minutes and, after cooling, is added to Part B, which has previously been sterilised by filtration.

For the corresponding solid medium, 10 g of purified agar, known to support the growth of MG, is added to part A above. The mixture is autoclaved as before and kept in a water bath at 56°C. The constituents of part B, omitting the phenol red, are mixed separately and then incubated at 56°C. Parts A and B are mixed carefully to avoid the production of air bubbles, and are dispensed into 50 mm dishes using 7–9 ml/dish. Excess surface moisture can be removed by a short incubation at 37°C. Plates are stored in an airtight container at approximately 4°C for up to 2 weeks.

Fresh yeast extract is available commercially, although it is preferable to prepare it 'in-house' by taking active dry baker's yeast (250 g) and suspending it in distilled water (1 litre). This is heated to boiling point, cooled and then centrifuged for 20 minutes at 3000 ***g***. The supernatant fluid is decanted and adjusted to pH 8.0 with 0.1 M NaOH. This is clarified by centrifugation or by filtration, and then sterilised by filtration. The extract is stored at –20°C. Reagent grade glucose (10 g) is dissolved in distilled or deionised water (100 ml) and adjusted to pH 7.8–8.0 with 0.1 M NaOH. It is sterilised by filtration and stored at 4°C. Reagent grade thallous acetate is dissolved (5 g) in distilled or deionised water (100 ml), filter-sterilised and stored at –20°C. Penicillin solution (10^6 IU benzyl penicillin in 5 ml distilled water) is stored at 4°C, and has a shelf life of 1 week. For isolation from heavily contaminated samples, penicillin concentration can be increased to 2000 units/ml or ampicillin, 0.5–1.0 mg/ml, maybe used instead. Phenol red (0.1 g) is ground in 0.1 M NaOH (2.8 ml), and then made up to 100 ml in sterile distilled water and autoclaved at 115°C at 1 atmosphere for 30 minutes. It is stored at 4°C. (Note: Thallous acetate is highly toxic and care should be taken, especially when preparing the stock solution.)

Specimens are inoculated on to mycoplasma agar and into broth. Solid medium may help detection of slow-growing mycoplasma colonies, which can be overgrown by saprophytes in broth. It may be necessary to make serial dilutions up to 10^{-3} for successful isolation. Inoculated plates are incubated at 37°C in sealed containers. Increased humidity and CO_2 tension in the atmosphere have been reported to enhance growth; these conditions may be obtained by the inclusion of damp paper or cotton wool, and by flushing the container with 5–10% CO_2 in nitrogen, by placing a lighted candle in the container, or by using a CO_2 incubator or suitable gas-generating system.

The caps of liquid medium containers should be tightly sealed before incubation at 37°C to avoid spurious changes in pH. For the first few days, the plates are examined daily for colonies with a stereoscopic microscope; after that they are examined less frequently. Cultures from field material should not be discarded as negative for at least 20 days.

Broth medium should be examined daily for acidity, indicated by a change from red to orange or yellow in the indicator. Any observable growth is subcultured on to solid medium immediately. Even if no colour change occurs, subculture on to solid medium should be made after 7–10 days or earlier as the presence of an arginine-hydrolysing (alkali-producing) mycoplasma species may mask the acid colour change produced by MG.

Mycoplasma colonies on solid medium can usually be recognised, although they may not have the typical 'fried egg' appearance. Bacterial colonies may appear on the first passage, but they are often more pigmented and fail to passage on mycoplasma media.

Biochemical reactions (e.g. fermentation of glucose and failure to hydrolyse arginine) can assist in identification, but they are not specific for MG or MS and necessitate purification of the culture by cloning.

Immunological and DNA detection methods can be used to identify mycoplasma isolates. They include the indirect fluorescent antibody (IFA) and immunoperoxidase (IP) tests, both of which are simple, sensitive, specific and rapid to perform; growth inhibition (GI); and metabolism inhibition (MI). Purified (cloned) cultures are required for the GI and MI tests, but not for the IFA or IP test. IFA and IP can detect the presence of more than one species of mycoplasma, as the colonies specific for the antiserum will react while the others will not. However, *M. imitans*, a mycoplasma species that is serologically related to MG and that presents the same biochemical properties has been isolated from ducks, geese and sometimes from other nondomestic bird species in some countries. It may be distinguished from MG by use of a PCR-RFLP (polymerase chain reaction/restriction fragment length polymorphism), as described by Kempf (1998). Alternatively, colonies of the isolate can be examined by immunofluorescence using serial dilutions of antisera to MG and *M. imitans* in parallel. The homologous antiserum should have a considerably higher titre.

DNA detection methods for identifying MG or MS directly in tissues or for identifying laboratory isolates are discussed below and are usually based on the PCR.

In certain circumstances where results of the above methods are not conclusive, inoculation of chick embryos or bioassays in live chicks may be appropriate. However these techniques are time-consuming and costly and tend to have been replaced by PCR technology, although they remain a useful research tool. The specimens required for inoculation of chicken embryos are the same as those used for the inoculation of artificial media. They are prepared in broth from which thallous acetate is omitted, incubated for 30–60 minutes at 37°C, and then a 0.05–0.1 ml aliquot is inoculated into the yolk sac of several 6–8-day-old chicken embryos derived from mycoplasma-free flocks. The eggs are candled daily and embryos that die within 24 hours of inoculation are discarded. Any further dead

embryos are kept refrigerated until cultured and those surviving after 5 days are placed at 4°C for 4 hours to kill them and to reduce haemorrhages on opening. The yolk is subcultured into broth and on to agar. Yolk lipid tends to obscure colonies so it is essential to streak the yolk thinly or, preferably, to dilute it first in mycoplasma broth.

Bioassays may be performed by the inoculation of a homogenate of suspect material into at least four 8–16 week-old susceptible mycoplasma-free chickens. Diagnosis is confirmed by the recovery of the mycoplasma from these birds, demonstration of its DNA and/or the demonstration of specific antibodies (Mallinson *et al*., 1981).

1.2. Immunological methods

Immunofluorescence and IP procedures for diagnosis are generally applied to suspect laboratory isolates rather than directly to infected exudates or tissues. This is because the organisms are too small to recognise conclusively under the light microscope and because the corresponding negative and positive control exudate/tissue is unlikely to be readily available.

1.2.1. Indirect fluorescent antibody test

The recommended technique for the IFA test (Rosendal & Black, 1972) requires an agar culture of the unknown isolate, consisting of numerous small discrete colonies, a known MG or MS culture as a positive control, and a culture of a another mycoplasma species, such as *M. gallinaceum* or *M. gallinarum* as a negative control. Also required are polyclonal rabbit anti-MG or MS serum, a normal rabbit serum and an anti-rabbit immunoglobulin fluorochrome-conjugated serum. Sera may be prepared in species other than rabbits, but monoclonal antibodies (MAbs) should not be used because MG or MS demonstrates variable expression of its surface epitopes and an MAB may fail to recognise the organism. Suitable working dilutions in sterile phosphate buffered saline (PBS; 0.01 M, pH 7.2) of the anti-MG or MS serum and the conjugate are first determined by cross-titration, and are selected for use at two-to-four-fold dilutions less than the actual end-points. These are applied to the colonies of mycoplasmas to be identified that have been previously grown on agar plates as indicated below.

1.2.1.1. Test procedure

i) From colony-bearing agar plates, cut blocks of about 1.0 × 0.5 cm and place them on to labelled microscope slides with the colonies uppermost.

ii) To make subsequent orientation possible, cut off the lower right hand corner of the blocks. One block with the unknown isolate, a block with the known MG culture, a block with the known MS culture and a block with a different but known mycoplasma culture are placed on one slide. A block of the unknown isolate is placed on another slide.

iii) Add a drop of suitably diluted MG (or MS) antiserum to the surface of each block of the first slide and add normal rabbit serum to the single block on the second slide.

iv) Incubate all blocks for 30 minutes at room temperature in a humid atmosphere.

v) Place each block in a labelled tube containing PBS, pH 7.2 and wash for 10 minutes in a rotary mixer, then similarly rewash, and finally return the blocks to the original microscope slides.

vi) Blot excess moisture from the sides of the blocks. Add one drop of the diluted conjugate to each block, and incubate and wash as before.

vii) Return the blocks to their original slides, and examine the colonies by incident light using fluorescence microscopy.

Interpretation of the results is subjective and requires some expertise; comparisons with the controls are essential, and they must give the correct reactions.

Some laboratories use fluorescein-conjugated antiserum in a direct fluorescent antibody test (DFA). A technique that is widely used for DFA is one in which the reagents are applied successively within stainless steel cylinders placed on the original mycoplasma agar plate (Talkington & Kleven, 1983). Although this is quick and easy to perform, the results obtained are less specific than using the indirect method, which is therefore preferred.

1.2.2. Indirect immunoperoxidase test

This involves a similar principle to the IFA test except that the binding of specific antibodies to colonies *in situ* is detected by adding an anti-rabbit immunoglobulin that has been conjugated to the enzyme peroxidase. A positive reaction is then developed by adding an appropriate substrate which, on oxidation, produces coloured colonies. An immunobinding procedure can also be used in which the test colonies are blotted on to nitrocellulose (Kotani & McGarrity, 1985) and then reacted in a similar manner. As with IFA, polyclonal sera should be used for serotyping isolates by IP. The advantage of the IP test over immunofluorescence is that the IP test does not require an expensive fluorescence microscope.

1.2.3. Growth inhibition test

In the GI test, the growth of mycoplasmas is inhibited by specific antiserum, enabling species to be identified. It is relatively insensitive and sera must be high-titred, monospecific and prepared in mammalian hosts as poultry sera do not always inhibit mycoplasma growth efficiently. The organism under test must be in pure culture (cloned) and several dilutions should be tested; a concentration of 10^4 colony-forming units (CFU/ml) is optimal. The rate of growth of the organism may influence growth inhibition, and it is helpful to retard growth initially by incubating at 27°C for 24 hours, followed by incubation at 37°C thereafter. Details of the test and its interpretation are published elsewhere (Clyde, 1983).

2. Nucleic acid detection methods

An alternative to conventional culture and identification is the use of specific DNA detection methods. MG or MS may be detected by hybridisation with DNA probes, but now it is much more common to use the PCR to amplify specific portions of DNA in the test material. At least one commercial MG DNA test kit uses a PCR directly on material extracted from swabs. One commercial company produces a kit to detect MG field strains and one that identifies the vaccine F strain. Several 'in-house' PCR-based tests have also been published for MG including a multiplex PCR, which is designed to detect all four avian mycoplasma pathogens (Wang *et al*., 1997), but which has not been validated with clinical samples. Several methods are cited by Kempf (1998) and, in addition, a manual published by Lauerman (1998) contains a validated PCR assay for MG, MS, and other avian mycoplasmas based on unique sequences contained in the 16S rRNA gene. This method for MG is presented below. In the USA, a PCR based on the *mgc2* gene of MG (García *et al*., 2005) or the *vlhA* gene of MS (Hong *et al*., 2004) is becoming more widely used, because preliminary strain identification can be made by sequencing of the PCR product; it must be remembered that unrelated strains may occasionally share the same sequence.

2.1. DNA isolation

DNA is extracted from swab samples (three–five may be pooled) suspended in 1 ml of PCR-grade PBS in a 1.5 ml snap-cap Eppendorf tube. The suspension is centrifuged for 30 minutes at 14,000 ***g*** at 4°C. The supernatant is carefully removed with a Pasteur pipette and the pellet is suspended in 25 µl PCR-grade water. The tube and the contents are boiled for 10 minutes and then placed on ice for 10 minutes before centrifugation at 14,000 ***g*** for 5 minutes. The DNA is in the supernatant.

2.2. Primers

The MG primers consist of the following sequences.

MG-14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3'
MG-13R: 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3'

For MS, the following primers are used:

MS–F: 5'-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A-3'
MS–R: 5'-CAG-TCG-TCT-CCG-AAG-TTA-ACA-A-3'

2.3. Polymerase chain reaction

The reaction mixture should be prepared in a separate clean area using a set of dedicated pipettes. For one 50 µl PCR reaction the mixture is as follows:

H_2O Ultra-pure	35.75 µl
10 × PCR Buffer	5.00 µl
dNTP(l0 mM)	1.00 µl
F Primer (20 pmole/µl)	0.50 µl
R Primer (20 pmole/µl)	0.50 µl
Taq (5 U/µl)	0.25 µl
$MgCl_2$ (50 mM)	2.00 µl

A 45 µl volume of the reaction mixture is dispensed into each PCR tube. The reaction mixture should be overlaid with a few drops of light weight mineral oil unless the thermocyler is equipped with a heated lid. The tubes are then taken to another clean area where the appropriate DNA sample (5 µl) is added to each tube. Positive and negative controls should be used in each run.

The tubes are then placed in a thermal cycler for the following cycles: 40 cycles: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 1 cycle (final extension): 72°C for 5 minutes and soak at 4°C.

2.4. Electrophoresis

PCR products are detected by conventional 2% agarose gel electrophoresis, incorporating appropriate size markers, followed by examination under UV light. The PCR product for MG is 185 bp. Visualisation of the PCR products should be carried out in a separate laboratory area, well separated from all other steps in the PCR procedure.

PCR tests still tend to be carried out by specialist laboratories and should probably be regarded as useful adjuncts to the present diagnostic methods once their validity is firmly established. Great care needs to be taken to avoid contamination of samples with MG or MS DNA from nearby post-mortem rooms, culture laboratories or from positive amplificates from previous PCR runs (see Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases*, for appropriate safeguards). However one commercial kit referred to above is now licensed by the United States Department of Agriculture (USDA) as a diagnostic method and approved for use in the National Poultry Improvement Plan (NPIP). It should be noted that PCR tests are not validated for testing day-old birds for accurate detection of infection.

Molecular methods are also available for differentiation of MG and MS strains (Kempf, 1998), but their use tends to be restricted at present to specialist laboratories. A rapid and accurate method for DNA fingerprinting uses arbitrary primed PCR or random amplified polymorphic DNA (RAPD). This technique uses short, arbitrary PCR primers, which generate reproducible patterns in agarose gels (Fan *et al*., 1995). This method is rapid and simple, and has proven to be very useful for rapid identification of strains of MG for epidemiological studies. However, there may be problems with reproducibility, so strains to be compared must be run on the same gel. Also, interpretation of banding patterns which appear to be similar can be difficult.

Gene-targeted sequencing (GTS), using PCR primers for the *mgc2, gapA, pvpA*, and MGA_0309 genes of MG can be used to provide an accurate and reproducible method of typing of strains, which will allow rapid global comparisons between laboratories (Ferguson *et al*., 2005). Preliminary strain identification with diagnostic PCR primers for the *mgc2* gene of MG or the *vlhA* gene of MS by sequencing of the PCR product allows for preliminary strain identification without the need for prior isolation of the organism (Hong *et al*., 2004; 2005). However, unrelated strains sometimes have identical sequences using these primers, and further characterisation may be necessary.

3. Serological tests

The serological tests in common use may lack specificity and/or sensitivity; their use is strongly recommended for monitoring flocks rather than for testing individual birds. Diagnosticians wishing to use such tests are advised to establish the test sensitivity and specificity (Chapter 1.1.6) under their own laboratory conditions. It should also be noted that these tests have not been validated for use with sera from day-old birds or from game birds (Bradbury, 2005).

The most commonly used tests are RSA, ELISA and HI although several others have been described such as radioimmunoassay, microimmunofluorescence and IP assay. The number of sera to be tested within a flock depends on the level of detection and the confidence limits required. Minimal requirements may be laid down for international trade and the frequency of testing may also be stipulated as, for example, in the European Communities Council Directive 90/539/EEC. Minimal requirements and approved tests are also set out for members of the NPIP of the USA.

Poultry companies using ELISA technology for screening large numbers of sera for virus antibodies may find this type of assay convenient also for mycoplasma testing. The ELISA technology will not be described in detail here because several MG kits are available commercially. Instead, the details of the HI test are provided as the reagents needed for this test are not widely available commercially.

3.1. Rapid serum agglutination test

Sera are collected from a sample of the flock and, if not tested immediately, are stored at 4°C and not frozen. The test should be carried out at room temperature (20–25°C) within 72 hours of serum collection and the reagents should also be at room temperature. Prior centrifugation will reduce nonspecific reactions. The RSA antigens are available commercially, but they may vary in specificity and sensitivity from different manufacturers and from batch to batch. They must be stored according to the manufacturer's instructions. Suitable RSA-stained antigens may also be prepared 'in-house' using culture methods as described in Section B.1.; these are then stained with crystal violet dye. Quality control standards for mycoplasma antigens for serological tests are described below.

3.1.1. Test procedure (Allan & Gough, 1974)

i) Drop one volume (approximately 0.02 ml) of serum on to a clean white tile or glass plate followed by one volume of stained MG or MS antigen. Do not allow the serum to dry out before addition of the antigen. It is important to shake the antigen bottle vigorously and frequently during use to keep the correct amount of antigen in suspension.

ii) Use a stirring rod to spread the mixture over a circular area of approximately 1.5 cm diameter. Rock the tile or plate for 2 minutes. Agglutination is indicated by flocculation of the antigen within 2 minutes.

iii) Include known positive and negative controls in the test.

iv) Retest serial dilutions of any sera that agglutinate after heating at 56°C for 30 minutes. If they still react strongly, they are considered to be positive on dilution (1/4 or more).

In the USA, MG and MS positive reference antisera can be obtained from the USDA National Veterinary Services Laboratories (NVSL), and in Europe from AFSSA Ploufragan[1], France. MG and MS and control sera produced in chickens or in turkeys and with a range of titres can be purchased. Sets of antisera can be purchased also from the University of Georgia Department's of Avian Medicine, subject to availability.

There are no international standards for interpreting these tests, but a high proportion of positive sera in a flock (10% or more) indicates MG infection, especially if confirmed by HI test or ELISA. For further confirmation, the flock should be retested within a month. Inconclusive results make it necessary to attempt to isolate the organism or to demonstrate the presence of its DNA. Doubtful results for MG or MS should be investigated by performing tests with MS antigen (and *vice versa*) as infection with these organisms sometimes causes cross-reactions.

Tests can be conducted on yolk as well as sera although the yolk must first be diluted or extracted.

3.2. Haemagglutination inhibition test

MG and MS are capable of haemagglutinating avian red blood cells (RBCs), and specific antibodies in sera cause inhibition. A strain should be selected that grows well and haemagglutinates reliably. The HI test requires a satisfactory haemagglutinating MG and MS antigen, washed fresh chicken or turkey RBCs, as appropriate, and the test sera. The antigen can be either a fresh broth culture or a concentrated washed suspension of the mycoplasma cells in PBS. It may be difficult to sustain a supply of high-titred broth culture antigen; however, the use of concentrated antigen (usually containing 25–50% glycerol and stored at –70°C), increases the likelihood of nonspecific reactions. In the USA, MG and MS haemagglutination (HA) antigen can be purchased from the NVSL.

The HI test follows well-known procedures (Allan & Gough, 1974). The HA titre of the antigen is first determined in doubling dilutions, the HA unit being defined as the least amount of antigen giving complete HA in the test system employed. The HI test should be performed using 4 HA units by the following method or a method having equivalent sensitivity as determined by tests with known positive sera.

All HA titrations and HI tests are best performed in multiwell plastic plates with V-shaped wells and using constant volumes of 50 µl. A positive and a negative control serum are incorporated into each test. One row of eight wells is required for each serum under test.

1 Agence française de sécurité sanitaire des aliments (AFFSA) Ploufragan, Mycoplasmology Bacteriology Unit, 22440 Ploufragan, France.

3.2.1. Test procedure

i) Add 50 µl of PBS to the first well in each row.

ii) Add 8 HA units of antigen in 50 µl volumes to the second well in each row and add 50 µl of 4 HA units of antigen to each of wells 3 to 8.

iii) Add 50 µl of a previously-prepared 1/5 dilution of the serum under test to the first well, mix, and transfer 50 µl to the second well, and so on, and discard 50 µl from the last well. The first well is the serum control well.

iv) Six wells are required for the antigen control. Add 50 µl of PBS to wells 2 to 6, inclusive, and add 50 µl of the 8 HA unit antigen to wells 1 and 2. Mix the contents of well 2 and transfer 50 µl to well 3, mix and repeat up to well 6, and discard 50 µl.

v) Two wells are required for the RBC control. Add 50 µl of PBS to each of these.

vi) Add 50 µl of a 0.5% suspension of RBCs (chicken cells for chicken serum and turkey for turkey serum) to all wells.

vii) Shake the plate lightly to ensure thorough mixing of the well contents, and read after standing for approximately 50 minutes at room temperature or when the antigen titration is reading 4 HA units. For reading, the plate should be tilted and only those wells in which the RBCs 'stream' at the same time as those in the RBC control wells should be considered to be inhibited. The serum control should show a clear button of RBCs and the positive and negative controls should react as expected. The HI titre is the highest serum dilution exhibiting complete inhibition of HA.

Sera giving nonspecific HA must be adsorbed to remove all nonspecific haemagglutinins so that a clear button is obtained in the control well without HA antigen. The adsorption is carried out by incubating 1 ml of the serum dilution with 6–8 drops of packed washed chicken or turkey RBCs. The cells are removed after incubation at 37°C for 10 minutes, and the supernatant is tested for haemagglutinating activity.

There is no official definition of positive and negative results for international trade but the NPIP of the USA states that titres of 1/80 or above are considered to be positive and titres of 1/40 are strongly suspicious.

3.3. Enzyme-linked immunosorbent assay

Several commercial MG and MS antibody ELISA kits are marketed. The sensitivity is determined to some extent by the manufacturer's recommendations for the cut-off levels for positive and suspicious reactions. Sensitivity may sometimes be 'damped down', to avoid the well-known cross-reaction between MG and MS. One ELISA uses an MAb that recognises an epitope on a 56 kDa polypeptide of MG (Czifra *et al*., 1993). In this system, ELISA plates are coated with whole cell MG antigen and the sera under test are added as in the conventional indirect ELISA, but the reaction is assessed by the extent of blocking that occurs when the conjugated MAb is added. A similar ELISA has also been marketed for MS. One advantage is that the system can be used for sera from any avian species without adaptation.

3.3.1. Quality control of *Mycoplasma gallisepticum* and *M. synoviae* antigens

i) *Mycoplasma gallisepticum* antigens

Antigens are usually prepared from the S6 strain or the A5969 strain of MG. Antigens prepared from other strains may also be used when necessary.

MG antigen for the RSA test: The methods of quality control described below apply solely to suspensions of MG stained with a suitable dye and containing preservative and intended for use in the rapid plate agglutination test with serum. Such antigens are available commercially.

On microscopic examination, the antigen should appear as a homogeneous suspension without floccules or precipitates and the suspending liquid should be free from residual dye. It must be free from contamination with bacteria and fungi. The pH must be between 6.5 and 7. It must be stored at 5±3°C and be warmed to room temperature before use.

The sensitivity and specificity of the antigen is determined with respect to its reaction with known positive sera of high and low titre and known negative sera. A positive reaction is recognised by the formation of coloured floccules and the clearing of the suspending medium. The criteria described above continue to apply until the expiry date declared by the manufacturer.

MG antigen for the HI test: The test is preferably performed with live, actively growing cultures. The antigen must be free from contamination with bacteria and fungi.

MG antigen for the ELISA: It may be difficult to prepare satisfactory antigen for use in the indirect ELISA without considerable prior experimentation and confirmation of sensitivity and specificity. Use of a reliable commercial kit is probably the best approach for most diagnostic laboratories. Some kits are now USDA-licensed and approved for use in the NPIP in the USA.

ii) *Mycoplasma synoviae* antigens

Antigens prepared from the WVU 1853 strain or other suitable strains should be used.

Mycoplasma synoviae *antigen for the RSA test*: the specifications apply as for MG antigen for the RSA test.

Mycoplasma synoviae *antigen for the HI test:* the same specifications apply as for MG antigen for the HI test.

iii) Additional comments

Sera giving nonspecific reactions to the RSA test do not usually give a positive reaction in the HI test using live HA antigen. Positive RSA reactions can be confirmed by the HI test with sera taken after the first 2–3 weeks of infection (the time taken for HI antibodies to develop). However, the HI test tends to be strain specific (Kleven *et al*., 1988) and therefore may lack sensitivity. ELISA may be a useful alternative.

Samples of serum should not be frozen before use in RSA tests. They should be free from haemolysis and contamination to avoid nonspecific reactions. The use of inactivated vaccines for other diseases may result in nonspecific reactions. Samples should be tested as soon as possible (within 72 hours) because mycoplasma antibodies may deteriorate on storage. Sera may be inactivated in a water bath at 56°C for 30 minutes.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The preferred method of control is to maintain MG- and MS-free flocks. Vaccination should be considered only in situations where field exposure is inevitable, such as on multi-age sites. Potential exposure of neighbouring poultry flocks should also be carefully considered.

Two types of vaccines are available for the control of MG. These are mild to avirulent MG strains used as live vaccines, or inactivated oil-emulsion bacterins. The subject of MG vaccination has been reviewed by Whithear (1996). Although there is antigenic variability among MG strains, it is thought that vaccination with a single strain is sufficient.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

C1. Live vaccines: methods of use

The use of live vaccines is equivalent to ‘controlled exposure’. The objective is to infect the flock with a mild, immunogenic MG strain at an age when little or no significant damage occurs. Such exposure results in resistance to challenge later in life, such as on multi-age commercial sites. Successfully vaccinated birds are resistant to respiratory disease, airsacculitis, and egg production drops caused by MG. Vaccination also results in reduced levels of egg transmission in breeders.

The F strain of MG has been the most commonly used vaccine strain (Carpenter *et al*., 1981). It is a naturally occurring strain of mild to moderate virulence for chickens, but it is virulent for turkeys. It ordinarily spreads slowly from bird to bird. When administered to healthy chickens via the upper respiratory tract, little or no respiratory reaction is observed. However, when administered by aerosol or in the presence of other respiratory disease agents, such as Newcastle disease or infectious bronchitis virus, respiratory signs and airsacculitis may result. Vaccinated chickens are permanent carriers, so a single dose is adequate. Use of F strain vaccine in each replacement flock on a multi-age site will eventually result in displacement of the field strain with the vaccine strain. Strains ts-11 and 6/85 are avirulent and spread to unvaccinated birds does not occur or occurs very poorly when birds are in very close contact (Ley *et al*., 1997).

Commercial pullets are usually vaccinated between 12 and 16 weeks of age, but vaccination of younger or older birds is permissible. It is essential that vaccination occurs before the flock is naturally infected. Vaccination in cases of probable early field exposure can be carried out in birds as young as 2–4 weeks of age. For the F strain, intranasal or eyedrop administration is preferred. Administration in the drinking water may result in some birds being missed unless the procedure is carried out properly. Aerosol administration should also be done carefully, so that all birds are exposed. A respiratory reaction should be expected at approximately 5–7 days after vaccination if aerosol administration is used. Vaccinated flocks should be tested with the agglutination test approximately 3–4 weeks post-vaccination to be sure that all birds were properly exposed. It is desirable that birds be vaccinated at an age when there is no reaction to other respiratory vaccines. Strain ts-11 should be administered by eyedrop, and 6/85 is given as a fine spray. Vaccination with ts-11 results in a low but distinctive serological response by serum plate agglutination, HI, and ELISA, but vaccination with 6/85 does not ordinarily result in a serological response. No post-vaccination reaction should be observed with 6/85 or ts-11. Flocks vaccinated with F strain or ts-11 are culture positive for the life of the flock, but 6/85 may be difficult to recover later than 4–6 weeks after vaccination.

Commercial live vaccines should be used within 1–2 hours after reconstitution. Lyophilised vaccine should be stored at 4°C. Some manufacturers supply the vaccine frozen. Such vaccine should be stored in liquid nitrogen, dry ice, or at –70°C or colder. Live MG vaccine is not stable for long periods at ordinary freezer temperatures. Storage for more than a few days at –20°C should be avoided.

Strains 6/85 and ts-11 are inherently safer than F strain, although the level of protection may be somewhat less, and may be useful as the primary vaccine strain on a multi-age site or as a 'second generation vaccine' on sites previously using F strain vaccine. They may also be preferred in situations where inadvertent exposure of neighbouring poultry flocks is of concern. F strain displaces wild-type MG more efficiently than either ts-11 or 6/85, but ts-11 has been used to eradicate F strain MG from a multi-age commercial egg-production site (Turner & Kleven, 1998). Multi-age sites where strain 6/85 is consistently used often test MG-negative, suggesting that it has displaced the wild-type strain.

Live vaccines have also been used in some countries in broiler breeder pullets. In Australia, ts-11 live vaccine is being extensively used in broiler breeder pullets as well as in commercial layers. F strain vaccine has been used in broiler breeder pullets raised under multi-age conditions in some Latin American countries for several years; more recently there has been limited use of strains ts-11 and 6/85. There has been limited use of the 6/85 strain as a vaccine for commercial turkeys in the USA, but no good data on its effectiveness are available. Generally, vaccination of turkeys with live vaccines is not recommended and vaccination of broilers with either live or inactivated vaccines has not been successful. None of the vaccines has been validated for use in game birds.

A live vaccine for MS is available in several countries for use in broiler breeder and layer chickens. It is produced from a temperature-sensitive mutant, MS-H (Markham *et al*., 1998). Its characteristics and method of use are similar to those for the MG vaccine, ts-11.

C2. Inactivated vaccines: method of use

MG bacterins are prepared from a concentrated suspension of whole cells that is emulsified into an oil adjuvant. A high antigen content is essential.

Bacterins are ordinarily used in commercial pullets to provide protection against egg-production drops that occur after MG exposure on multi-age layer sites (Hildebrand *et al*., 1983). They may also be used to reduce the level of egg transmission in breeder pullets. Use of bacterins in broilers is limited by the fact that birds vaccinated before 1–2 weeks of age are not protected. Although bacterins may provide protection against respiratory signs, airsacculitis, and egg-production losses, vaccinated flocks are readily infected. The duration of immunity is not known, but most flocks are exposed within 1–2 months after vaccination.

Administration is by the intramuscular or subcutaneous route, usually with a dose of 0.5 ml per bird. There is a risk that a persistent reaction at the site of vaccination will require trimming of carcasses of spent fowl vaccinated by the intramuscular route, so subcutaneous administration in the upper dorsal part of the neck is the most commonly used route. Two doses are preferred, but cost and labour considerations may dictate the use of a single dose, usually between 16 and 18 weeks of age for commercial pullets. A multidose syringe may be used. All equipment should be cleaned and sterilised between flocks, and vaccination crews should exercise proper methods of biosecurity when travelling between flocks. Vaccine should be stored at 2–8°C up to the time of use. It should not be frozen or exposed to strong light.

A similar bacterin for MS is also licensed in the USA, but it has received limited use.

1. Seed management

1.1. Characteristics of the seed

1.1.1. Live vaccine

The vaccine strain should be immunogenic, must readily colonise the upper respiratory tract, and cause minimal damage to the respiratory system. A strong antibody response does not necessarily correlate with immunity.

The seed culture should be free from all extraneous agents. The culture should be cloned to ensure purity. If desired, restriction endonuclease patterns of the mycoplasmal DNA on agarose gels can be run to be sure of the identity and purity of the strain.

The seed culture should be stable with no tendency to revert to virulence. This can be confirmed with ten back passages in susceptible chickens. Contact chickens can be introduced at weekly intervals. If necessary, tracheal swabs can be taken from infected chickens and can then be inserted into the trachea of contact chickens. Transmission of the organism should be proven. The resulting isolate can then be used to challenge susceptible chickens.

1.1.2. Killed vaccine

For killed vaccines the most important characteristics are high yield and good antigenicity. It is assumed, but not proven, that virulent strains are desirable. The seed culture should be free from all extraneous organisms.

1.2. Method of culture

The seed culture may be propagated in a medium similar to that described above (Section B.1) for live vaccines, the broth culture is lyophilised or frozen at –70°C or colder. For bacterins the culture must be concentrated and resuspended in a small volume of saline or PBS before the emulsion is prepared.

1.3. Validation as a vaccine

Data on efficacy should be obtained before bulk manufacture of vaccine begins. Chickens should be vaccinated by the same route that will be used in the field. Vaccinated birds should be challenged, and protection should be determined against respiratory signs, nasal discharge, and/or airsacculitis. Ideally, protection against egg-production losses should be evaluated, but such challenge trials are expensive and cumbersome.

Efficacy test: Groups of 20 specific pathogen free (SPF) chickens or at least mycoplasma-free chickens, 2 weeks of age or older, are vaccinated by eyedrop or other route of administration with one field dose of live vaccine, or subcutaneously or intramuscularly with one dose (usually 0.5 ml) of bacterin. A similar group of unvaccinated chickens is maintained separately as controls. All chickens should be challenged with a 24-hour broth culture of a virulent strain of MG, 2–3 weeks post-vaccination. A simple challenge method is inoculation of 0.1 ml of the challenge culture into the posterior thoracic air sac. All birds are necropsied 7–10 days post-challenge, and air sac lesions are scored. Alternative methods are to challenge by inoculating 0.1 ml into the infraorbital sinus and examining the birds for nasal discharge from 7 to 14 days post-challenge or to challenge by aerosol and measure the thickness of the tracheal mucosa on microscopic sections at four to six equidistant predetermined points (Whithear, 1996).

2. Method of manufacture

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry. Special care must be taken to avoid MG contamination of other products manufactured in the same facility.

Production of vaccine should be on a seed-lot system, using a suitable MG strain of known origin, passage history, and purity. The growth medium is similar to that given above. The serum used in the growth medium should be inactivated at 56°C for 1 hour to prevent contamination with any mycoplasmal organism that may be present, and filter sterilised. A source of SPF serum is desirable.

Broth medium is inoculated, with a rapidly growing inoculum, at a rate of approximately 5% (v/v). Incubation is at 37°C. Production can be in batches using large flasks or in a fermenter. In batch cultures, harvest is approximately 24 hours after inoculation. Live vaccines are preserved by lyophilisation or by freezing at –70°C, in liquid nitrogen, or on dry ice.

For bacterin production, the antigen must be concentrated, usually by centrifugation, ultrafiltration, or other suitable method. Bacterins are made as water-in-oil emulsions, typically 80% mineral oil, 20% aqueous, with suitable emulsifying agents.

3. In-process control

3.1. Antigen content

At harvest, the titre should be from 10^8 to 10^9 CFU/ml. The antigen concentration of bacterins is difficult to standardise but may be based on packed cell volume, which is typically 1% (v/v) packed cells in the final product.

3.2. Inactivation of killed vaccines

Inactivation is frequently done with either beta-propiolactone or formaldehyde. The inactivating agent and the inactivation procedure must be shown under the conditions of vaccine manufacture to inactivate the vaccine organism and potential contaminants.

Prior to inactivation, care should be taken to ensure a homogeneous suspension free from particles that may not be penetrated by the inactivating agent. A test for inactivation should be carried out by culture in mycoplasma broth on each batch of both the bulk harvest after inactivation and the final product. No evidence of growth of mycoplasma should be observed.

3.3. Sterility of killed vaccines

Oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are carried out on each batch of final vaccine as described, for example, in the British Pharmacopoeia (Veterinary) 1985.

4. Batch control

4.1. Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in Chapter 1.1.9.

4.2. Safety

4.2.1. Live vaccine safety test

The birds vaccinated in the efficacy test given above can be used to evaluate the safety of the vaccine.

4.2.2. Killed vaccine safety test

Birds vaccinated in the efficacy test described above may be observed for adverse local or systemic effects.

4.3. Potency

Potency tests for both live and killed vaccine can be conducted by the procedures given above for the efficacy test. The titre of live vaccines should be sufficient to induce infection by the route recommended; 10^5 CFU/dose is sufficient for eyedrop administration of live F strain vaccine. The recommended dose of ts-11 is ≥ $10^{7.7}$ colour changing units (CCU)/dose and for 6/85 a dose of 10^7–10^8 CFU was effective in challenge trials. For MS-H, doses of ≥ 4.8×10^5 were shown to be effective.

4.4. Duration of immunity (killed vaccine)

Because flocks are generally exposed within 1–2 months after vaccination, duration of immunity is not a primary consideration. After field challenge, resistance is considered to be permanent.

4.5. Stability

Evidence should be provided on three batches of vaccine to show that the vaccine passes the batch potency test at 3 months beyond the requested shelf life.

4.6. Preservatives

A preservative is normally required for vaccine in multidose containers. The concentration of the preservative in the final vaccine and its persistency throughout the shelf life should be checked.

A suitable preservative that has already been established for such purposes should be used. Mycoplasmas are susceptible to many antibacterials except for penicillins; such antibiotics should not be included as preservatives.

4.7. Precautions (hazards)

Oil-emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident, the person should go at once to a hospital, taking the vaccine package with him or her. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injection. Such wounds should be treated by the casualty doctor as a 'grease gun injury'.

Personnel vaccinating birds with live virus vaccines by aerosol spray should wear protective clothes and masks.

5. Tests on the final product

5.1. Safety

See Section C.4.2.

5.2. Potency

See Section C.4.3.

REFERENCES

ALLAN W.H. & GOUGH R.E. (1974). A standard haemagglutination test for Newcastle disease. 1. A comparison of macro and micro methods. *Vet. Rec.*, **95**, 120–123.

BRADBURY J.M. (2001). Avian mycoplasmas. *In*: Poultry Diseases, Fifth Edition, Jordan F., Pattison M., Alexander D. & Faragher T., eds. W.B. Saunders, London, UK, 178–193.

BRADBURY J.M. (2005). Workshop of European Mycoplasma Specialists. *World Poult. Sci. J.*, **61**, 355–357.

CARPENTER T.E., MALLINSON E.T., MILLER K.F., GENTRY R.F. & SCHWARTZ L.D. (1981). Vaccination with F-strain *Mycoplasma gallisepticum* to reduce production losses in layer chickens. *Avian Dis.*, **25**, 404–409.

CLYDE W.A., JR. (1983). Growth inhibition tests. *In*: Methods in Mycoplasmology, Vol. 1, Razin S. & Tully J.G., eds. Academic Press, New York, USA, and London, UK, 405–410.

CZIFRA G., SUNDQUIST B., TUBOLY T. & STIPKOVITS L. (1993). Evaluation of a monoclonal blocking enzyme-linked immunosorbent assay for the detection of *Mycoplasma gallisepticum*-specific antibodies. *Avian Dis.*, **37**, 680–688.

FAN H.H., KLEVEN S.H. & JACKWOOD M.W. (1995). Application of polymerase chain reaction with arbitrary primers to strain identification of *Mycoplasma gallisepticum*. *Avian Dis.*, **39**, 729–735.

FERGUSON N.M., HEPP D., SUN S., IKUTA N., LEVISOHN S., KLEVEN S.H. & GARCÍA M. (2005). Use of molecular diversity of *Mycoplasma gallisepticum* by gene-targeted sequencing (GTS) and random amplified polymorphic DNA (RAPD) analysis for epidemiological studies. *Microbiol.*, **151**, 1883–1893.

FREUNDT E.A. (1983). Culture media for classic mycoplasmas. *In*: The Mycoplasmas, Vol. 1, Razin S. & Tully J.G., eds. Academic Press, New York, USA and London, UK, 127–135.

FREY M.L., HANSON R.P. & ANDERSON D.P. (1968). A medium for the isolation of avian Mycoplasmas. *Am. J. Vet. Res.*, **29**, 2163–2171.

GARCÍA M., IKUTA N., LEVISOHN S. & KLEVEN S.H. (2005). Evaluation and comparison of various PCR methods for detection of *Mycoplasma gallisepticum* infection in chickens. *Avian Dis.*, **49**, 125–132.

HILDEBRAND D.G., PAGE D.E. & BERG J.R. (1983). *Mycoplasma gallisepticum* (MG) – laboratory and field studies evaluating the safety and efficacy of an inactivated MG bacterin. *Avian Dis.*, **27**, 792–802.

HONG Y., GARCÍA M., LEITING L., BENCINA D., DUFOUR-ZAVALA L., ZAVALA G. & KLEVEN S.H. (2004). Specific detection and typing of *Mycoplasma synoviae* strains in poultry with PCR and DNA sequence analysis targeting the hemagglutinin encoding gene vlhA. *Avian Dis.*, **48**, 606–616.

HONG Y., GARCIA M., LEVISOHN S., SAVELKOUL P., LEITING V., LYSNYANSKY I., LEY D.H. & KLEVEN S.H. (2005). Differentiation of *Mycoplasma gallisepticum* strains using amplified fragment length polymorphism and other DNA-based typing methods. *Avian Dis.*, **49**, 43–49.

KEMPF I. (1998). DNA amplification methods for diagnosis and epidemiological investigations of avian mycoplasmosis. *Avian Pathol.*, **27**, 7–14.

KLEVEN S.H. (2003). *Mycoplasma synoviae* infection. *In*: Diseases of Poultry, Saif Y.M., Barnes H.J., Glisson J.R., Fadly A.M., McDougald L.R. & Swayne D.E., eds. Iowa State University Press, Ames, Iowa, USA, 756–766.

KLEVEN S.H., FLETCHER O.J. & DAVIS R.B. (1973). Variation of pathogenicity of isolates of *Mycoplasma synoviae* with respect to development of airsacculitis and synovitis in broilers. *Am. J. Vet. Res.*, **163**, 1196–1196.

KLEVEN S.H., KING D.D. & ANDERSON D.P. (1972). Airsacculitis in broilers from *Mycoplasma synoviae:* effect on air-sac lesions of vaccinating with infectious bronchitis and Newcastle virus. *Avian Dis.*, **16**, 915–924.

KLEVEN S.H., MORROW C.J. & WHITHEAR K.G. (1988). Comparison of *Mycoplasma gallisepticum* strains by hemagglutination-inhibition and restriction endonuclease analysis. *Avian Dis.*, **32**, 731–741.

KOTANI H. & MCGARRITY G.J. (1985). Rapid and simple identification of Mycoplasmas by immunobinding. *J. Immunol. Methods*, **85**, 257–267.

LANDMAN W.J.M. & FEBERWEE A. (2004). Aerosol-induced *Mycoplasma synoviae* arthritis: the synergistic effect of infectious bronchitis virus infection. *Avian Pathol.*, **33**, 591–598.

LAUERMAN L.H. (1998). Mycoplasma PCR Assays. *In*: Nucleic Amplification Assays for Diagnosis of Animal Diseases, Lauerman L.H., ed. American Association of Veterinary Laboratory Diagnosticians, Auburn, AL, USA, 41–52.

LEY D.H. (2003). *Mycoplasma gallisepticum* infection. *In*: Diseases of Poultry, Saif Y.M., Barnes H.J., Glisson J.R., Fadly A.M., McDougald L.R. & Swayne D.E., eds. Iowa State University Press, Ames, Iowa, USA, 722–744.

LEY D.H., MCLAREN J.M., MILES A.M., BARNES H.J., MILLER S.H. & FRANZ G. (1997). Transmissibility of live *Mycoplasma gallisepticum* vaccine strains ts-11 and 6/85 from vaccinated layer pullets to sentinel poultry. *Avian Dis.*, **41**, 187–194.

LOCKABY S.B., HOERR F.J., LAUERMAN L.H., SMITH B.F., SAMOYLOV A.M., TOIVIO-KINNUCAN M.A. & KLEVEN S.H. (1999). Factors associated with virulence of *Mycoplasma synoviae*. *Avian Dis.*, **43**, 251–261.

LUTTRELL M.P., FISCHER J.R., STALLKNECHT D.E. & KLEVEN S.H. (1996). Field investigation of *Mycoplasma gallisepticum* infections in house finches (*Carpodacus mexicanus*) from Maryland and Georgia. *Avian Dis.*, **40**, 335–341.

MALLINSON E.T., ECKROADE R.J. & KLEVEN S.H. (1981). *In vivo* bioassay and supplemental serologic techniques for the detection of *Mycoplasma* in suspect breeding chickens. *Avian Dis.*, **25**, 1077–1082.

MARKHAM J.F., MORROW C.J. & WHITHEAR K.G. (1998). Efficacy of a temperature-sensitive *Mycoplasma synoviae* live vaccine. *Avian Dis*., **42**, 671–676.

MAROIS C., DUFOUR-GESBERT F. & KEMPF I. (2002). Polymerase chain reaction for detection of *Mycoplasma gallisepticum* in environmental samples. *Avian Pathol.*, **31**, 163–168.

ROSENDAL S. & BLACK F.T. (1972). Direct and indirect immunoflurescence of unfixed and fixed mycoplasma colonies. *Acta Pathol. Microbiol. Scand.* [*B*], **80**, 615–622.

TALKINGTON F.D. & KLEVEN S.H. (1983). A classification of laboratory strains of avian *Mycoplasma* serotypes by direct immunofluorescence. *Avian Dis.*, **27**, 422–429.

TURNER K.S. & KLEVEN S.H. (1998). Eradication of live F strain *Mycoplasma gallisepticum* vaccine using live ts-11 on a multiage commercial layer farm. *Avian Dis.*, **42**, 404–407.

UNITED STATES DEPARTMENT OF AGRICULTURE (USDA) (2004). Animal and Plant Health Inspection Service (APHIS), National Poultry Improvement Plan and Auxiliary Provisions. APHIS Publication 91-55-063. APHIS, USDA, Riverdale, Maryland, USA, 97–100.

WANG H., FADL A.A. & KHAN M.I. (1997). Multiplex PCR for avian pathogenic mycoplasmas. *Molec. Cell. Probes*, **11**, 211–216.

WHITHEAR K.G. (1996). Control of avian mycoplasmoses by vaccination. *Rev. sci. tech. Off. int. Epiz.*, **15**, 1527–1553.

ZAIN M.Z. & BRADBURY J.M. (1996). Optimising the conditions for isolation of *Mycoplasma gallisepticum* collected on applicator swabs. *Vet. Microbiol.*, **49**, 45–57.

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NB: There is an OIE Reference Laboratory for Avian mycoplasmosis (*Mycoplasma gallisepticum* and *M. synoviae*) (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Avian mycoplasmosis (*Mycoplasma gallisepticum* and *M. synoviae*)

NB: FIRST ADOPTED IN 1991 AS MYCOPLASMOSIS (*MYCOPLASMA GALLISEPTICUM*); MOST RECENT UPDATES ADOPTED IN 2008.