CHAPTER 3.3.5.

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

SUMMARY

Description of the disease: *Avian mycoplasmosis is caused by several pathogenic mycoplasmas among which* Mycoplasma gallisepticum (*MG*) *and* M. synoviae (*MS*) *are considered the most important. MG causes chronic respiratory disease of domestic poultry, especially when flocks are stressed and/or other respiratory pathogens are present. The disease is characterised by coryza, conjunctivitis, sneezing, and sinusitis, particularly in turkeys and game birds. It can result in significant production losses and downgrading of meat-type birds, and loss of egg production. MS may cause respiratory disease, synovitis, eggshell alteration, loss of egg production and carcass downgrading or it may result in a silent infection. MG and MS strains vary in infectivity and virulence, and infections may sometimes be inapparent.*

Detection 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, material from egg yolk or embryos. Clinical signs and lesions will influence the sample selection. Broth and agar combined with basic biochemical tests are used for isolation and first recognition of the Mycoplasma*, but identification of the genus and species is made through immunological tests* (*e.g. fluorescent antibody or immunoperoxidase tests*) *and/or biomolecular tests.*

DNA detection methods based on the polymerase chain reaction are commonly used in several specialised laboratories.

Serological tests: *Several serological tests are used to detect MG or MS antibodies, but due to variations in test specificity and sensitivity, they are recommended only 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: *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 in specific cases on the basis of the epidemiological situation in the area or in farms 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 for chickens are produced from the F strain and 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 9 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 can result in displacement of field strains. The ts-11 strain has been successfully used to eradicate F strain in multi-age commercial layers. In the past few years, two live MS vaccines produced from the MS-H strain and MS1 have been licensed in several countries. Eyedrop administration is advised for the MS-H strain whereas fine aerosol is recommended for MS1. The birds should be vaccinated by 5 weeks of age. New MG vaccine candidates are under study, some of them are attenuated strains and others are based on vectored-virus technology.

Inactivated vaccines consisting of concentrated suspension of MG or MS organisms in an oil emulsion are licensed in several countries. they should be administered parenterally to pullets at 12–16 weeks of age. Two doses are desirable. MG bacterins are effective in preventing egg-production losses and respiratory disease, but they do not prevent infection with wild-type MG. MS inactivated vaccine are not in common use.

A. INTRODUCTION

1. Description and impact of the disease

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 avian 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 (Raviv & Ley, 2013). It can also cause upper respiratory disease in game birds. Some strains of MG have been recognised in North America in house finches as a cause of conjunctivitis with important impacts in wild bird populations such as inability to find food, starvation or death, but they do not appear to be pathogenic for poultry. 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. Recently it was shown that MG and MS may survive up to 9 days in synthetic fibres though less in human hair (Abolnik *et al.,* 2014) showing a predisposition of these mycoplasmas for attachment to surfaces. 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 seen in turkeys, 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.

MG 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 influenced 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 metapneumovirus infection. A more chronic form of the disease may occur resulting in reduced egg production in breeders and layers.

Lesions of the respiratory tract initially present as 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.

MS may be associated in chickens with infectious synovitis; the birds 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 (Ferguson-Noel & Noormohammadi, 2013). 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. MS strains exhibit significant variability with respect to their virulence and tissue tropism (Catania *et al.,* 2016a; Landman, 2014; Landman & Feberwee, 2004). Recently a new clinical form resulting in high shell breakage and low egg production in chicken layers was reported initially in Europe (Catania *et al.,* 2010; Feberwee *et al.*, 2009a; 2009b) and then reported worldwide. The lesions appear to be confined to the apex of the shell and consist of rough dark areas of 2 cm in diameter with clear edges; in addition, a decrease in egg production is reported (Catania *et al.,* 2010; 2016a).

2. Zoonotic potential and biosafety and biosecurity requirements

There have been no reports of MG or MS infection in humans. Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

3. Differential diagnosis

MG or MS disease in chickens may superficially resemble respiratory disease caused by other pathogens such as mild strains of Newcastle disease virus (Chapter 3.3.14) and avian infectious bronchitis virus (Chapter 3.3.2). These may be also present in mixed infections with MG or MS. Infections with *Avibacterium paragallinarum* and *Pasteurella multocida* should also be ruled out. In broilers, the co-infection with avian metapneumovirus and *E. coli* could show similar features. MG in turkeys may be confused with avian metapneumovirus infections and the presence of sinusitis may also suggest infection with *Bordetella avium, Pasteurella multocida*, *Chlamydia* (Chapter 3.3.1) or MS. Infectious synovitis caused by MS should be differentiated from *Staphylococcus aureus, Riemerella anatipestifer, Ornithobacterium rhinotracheale* and *Enterococcus* joint infections and, in chicken, from infectious tenosynovitis caused by avian orthoreoviruses.

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.

Table 1. *Test methods available for diagnosis of avian mycoplasmosis* (Mycoplasma gallisepticum, M. synoviae) *and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[1]						
Isolation on culture media	+[a]	–	+	+	–	–
Conventional PCR	++[a]	++[a]	++	+++	++	–
Real-time PCR	+++[a]	+++[a]	+++	+++	+++	–
PCR-DGGE[b]	+	–	+	+	–	–

1 A combination of agent identification methods applied on the same clinical sample is recommended.

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
HI	++[c]	–	+	++[e]	++	+
RSA	+[d]	–	+	+[e]	+	+
ELISA	++[c]	–	++	++[e]	++	++[f]

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
[a]Not suitable for day old birds;
[b]applied in culture medium, isolated colonies;
[c]suitable for ensuring lack of infections dating back more than 2–3 weeks;
[d]suitable for ensuring lack of infections dating back more than 5–8 days;
[e]suitable provided paired samples collected a few weeks apart can be analysed;
[f]suitable only for the group vaccinated with killed vaccine, F strain and by temperature sensitive vaccines.
PCR: polymerase chain reaction; DGGE = denaturing gradient gel electrophoresis; HI = hemagglutination inhibition;
RSA = rapid serum agglutination; ELISA = enzyme-linked immunosorbent assay.

1. Detection of the agent

1.1. *In-vitro* culture

Samples are taken from live birds, fresh carcasses or the carcasses of birds that have been frozen quickly after death. The tracheal swab is considered to be the best sample in live animals for most mycoplasma species. In addition, for isolation purposes, it is possible to collect swabs from the choanal cleft. When dead birds are available, mycoplasma isolation can be performed with swabs from different tissues or organs such as the upper and/or mid-trachea, lungs, air sacs, oviduct and joints.

Swabs of the yolk sac should be collected during the last third of the egg incubation period where decreased hatchability of embryonated eggs has occurred (i.e. after the 15th and the 20th day of incubation in chickens and in turkeys, respectively).

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 tested as soon as possible after collection. If transportation is necessary, collected swabs should be vigorously agitated in 1–2 ml of mycoplasma broth and then discarded; tissues or organs should be frozen. An ice pack or some other means of chilling should be included as MG and MS die rapidly at room temperature. Serial dilutions of samples should be made in mycoplasma broth because the presence of specific antibodies, antibiotics or inhibitory substances in tissues may inhibit mycoplasma growth.

Several suitable culture media have been formulated to support the growth of avian mycoplasma; in addition, several commercial media are also available. mycoplasma media generally contain a digested protein 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). Nicotinamide adenine dinucleotide (NAD) is a growth requirement for the primary isolation of MS, but it may be omitted in 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 (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) thallium acetate (10 ml); 200,000 International Units (IU)/ml penicillin G (5 ml); and 0.1% (w/v) phenol red solution (20 ml). Thallium 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. For primary isolation of MS in this medium, a mix of 10% (v/v) NAD solution (1 ml) and 10% (v/v) cysteine solution (1 ml) is also added.

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 mycoplasma, 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 4 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 thallium 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: thallium acetate is highly toxic and care should be taken, especially when preparing the stock solution.)

Specimens are inoculated on to both mycoplasma agar and into broth. Solid medium may help detection of slow-growing mycoplasmas, 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; flushing the container with 5–10% CO_2 in nitrogen, 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 be kept for at least 20 days before discarding.

Broth cultures should be examined daily for changes in colour and/or in turbidity. Most mycoplasmas, including MG and MS, metabolise sugar-producing acid causing a change in the pH of the medium from red/orange to yellow. Other mycoplasmas hydrolyse arginine creating alkaline conditions causing a change in pH and consequentially of broth colour from red/orange to strong red or fuchsia. Any observable growth in broth is subcultured onto solid medium immediately. If there is no colour change after 7–14 days, the broth should be subcultured onto solid medium. This should be done because the presence of an arginine-hydrolysing (alkali-producing) mycoplasma species may mask the acid colour change produced by MG or MS, or because there might be mycoplasma strains with less active metabolism.

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 growth inhibition (GI); and metabolism inhibition (MI) indirect fluorescent antibody (IFA) and immunoperoxidase (IP) tests, the latter two can be considered simple, sensitive and specific. Purified cultures (produced by one colony) 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 only the colonies specific for the antiserum will react. However, *M. imitans*, a mycoplasma species that is serologically and biochemically similar to MG 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 specific biomolecular methods. 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 polymerase chain reaction (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 have largely 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 thallium 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- to 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.

1.2. Antigen detection

Immunofluorescence and IP procedures for diagnosis are generally applied to suspect 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 corresponding negative and positive control samples are 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 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 demonstrate variable expression of their surface epitopes and an MAb may fail to recognise the target microorganism. 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 unknown mycoplasma colonies 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 gently for 10 minutes on a rotary mixer, then rewash as before, 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 must give the correct reactions and are essential.

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. 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/immunobinding test

The indirect IP test follows 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 visualised 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).

1.3. Molecular methods – detection of nucleic acids

PCR assays are routinely used in many laboratories and are characterised by good sensitivity. These methods represent a good alternative to *in-vitro* culture of mycoplasmas because they are based on the detection of specific DNA sequences of the pathogen directly on clinical samples or isolates grown *in vitro*. MG or MS DNA are amplified by PCR using species-specific primers. The real-time PCR with fluorescent labelled probes is becoming increasingly used, shortening the detection time compared with conventional PCR. Great care has to be taken to avoid contamination of samples with MG or MS DNA from nearby necropsy rooms, mycoplasma culture laboratories or from previous PCR runs (see Chapter 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases* and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*). Several commercial kits for PCR and real-time PCR of both MG and MS are available and several in-house procedures have also been published (Dijkman *et al.,* 2017; Raviv & Kleven, 2009).

A conventional PCR and a real-time PCR for MG and MS are described below. Within the PCR-based methods, the denaturing gradient gel electrophoresis (DGGE) technique could be applied for the identification of most avian mycoplasmas, including MG and MS, but this test is validated only on isolates from mycoplasma culture. This method is described in Section B.1.3.2.

Genotyping techniques based on the analysis of the *mgc2, pvpA* and *vlh*A genes are currently widely applied for the classification of MG (Armour *et al.,* 2015; Garcia *et al.,* 2005) and MS isolates, respectively (Hammond *et al.,* 2008). Moreover, a core genome multilocus sequence typing (MLST) scheme for *M. gallisepticum* and two *M. synoviae* MLST schemes have been published (Dijkman *et al.,* 2016; El-Gazzar *et al.,* 2017; Ghanem *et al.,* 2017) and are likely to become globally applied epidemiological tools.

1.3.1. Conventional polymerase chain reaction

The assay described is a validated PCR for MG and MS detection based on a 16S rRNA fragment amplification (Lauerman, 1998). Another widely used method based on *mgc2* gene for MG detection (García *et al.,* 2005) is reported. It must be remembered that unrelated strains may

occasionally share DNA sequences and give DNA amplification bands in different laboratory conditions. All new PCRs require validation using criterion in Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases* and Chapter 2.2.3 *Development and optimisation of nucleic acid detection assays*. The conventional PCR for MS reported in this chapter can also detect *M. bovirhinis* DNA.

1.3.1.1. DNA extraction

DNA is extracted from swab samples (up to 10 swabs may be pooled) suspended in PCR-grade PBS in a 1.5 ml snap-cap Eppendorf tube. Several commercial extraction kits based on the spin column are available for DNA extraction from swabs, tissues, etc. Automated extraction of *Mycoplasma* DNA is possible with specific commercial kits. The appropriate kit for the type of sample should be selected and the manufacturer's protocol for DNA extraction should be followed.

1.3.1.2. Primers

PCR method	PCR primer sequences	Expected amplicon
16S rRNA for MG	MG-14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3'	183 bp
	MG-13R: 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3	
*mgc*2 for MG	MG-1: 5'-CGC-AAT-TTG-GTC-CTN-ATC-CCC-AAC-A-3	236–302 bp
	MG-2: 5'-TAA-ACC-CRC-CTC-CAG-CTT-TAT-TTC-C-3'	
16S rRNA for MS	MS–F:5'-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A-3'	211 bp
	MS–R:5'-CAG-TCG-TCT-CCG-AAG-TTA-ACA-A-3'	

1.3.1.3. Polymerase chain reaction

i) The reaction mixture should be prepared, according to the manufacturer's instruction, in a separate clean area using a set of dedicated pipettes. For each sample, dispense into a PCR tube 45 µl volume of mix containing 0.4 µM of each primer for 16S rRNA MS and *mgc*2 MG, or 0.2 µM for 16S rRNA MG. An internal amplification control (IAC) could be included as a commercial exogenous kit or using designed primers for endogenous sequence (e.g. 18S rRNA for eukaryotic derived samples) amplifying at same PCR conditions. 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 taken to another clean area where the appropriate extracted DNA sample (5 µl) is added to each tube. Positive and negative control DNA should be run in each assay. 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.

ii) For amplification of 16s rRNA MS and *mgc*2 MG using a hot start Taq polymerase, run the thermocycler at the following profile:

Polymerase activation	95°C	10 minutes	
40 cycles	95°C	45 seconds	
	54°C	60 seconds	
	72°C	60 seconds	
1 cycle (final extension)	72°C	7 minutes	soak at 4°C

1.3.1.4. Electrophoresis

PCR amplification products are detected by conventional gel electrophoresis, incorporating appropriate size markers. The stained products are visualised under UV light or with silver nitrate under a hazardous chemical hood. Alternatively, the amplified product can be run in a capillary electrophoresis machine loaded with appropriate size markers. The base-pair dimension of the amplified fragments could be statistically different by about 10% than expected using this method. Examination of the PCR products should be carried out in a laboratory area, well separated from places where other steps in the PCR procedure are performed.

1.3.2. 16s-rDNA-PCR and denaturing gradient gel electrophoresis

The 16s-rDNA-PCR-DGGE technique is a method that can be applied for the identification of mycoplasma isolates (McAuliffe *et al.,* 2005), including avian mycoplasma from cultivated broths or agar colonies. It can also be used on DNA extracts from clinical specimens. The genetic target is the V3 region of the 16s gene, which is amplified by the combination of a mycoplasma-specific primer (reverse primer) and a universal bacterial one (forward primer) containing a GC-clamp (40 repeated GC).

The method is based on the migration of DNA fragments following strand separation caused by chemical denaturants in the gel. It is capable of detecting single-base mutations in DNA.

After migration on the denaturing gel, the pattern of the band(s) produced by the unknown samples is compared with positive avian controls, which are run in parallel.

This technique is capable of detecting single avian *Mycoplasma* spp., infection and co-infections, in a single sample (Catania *et al.,* 2014; 2016b).

i) Primers

The following primers are used:

R543: 5'-ACC-TAT-GTA-TTA-CCG-CG-3
GC 341 F: 5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GCC-TAC-GGG-AGG-CAG-CAG-3'

ii) Polymerase chain reaction

The PCR reaction mixture should be prepared in a separate clean area as follows (final volume: 25 µl):

H_2O Ultra-pure	12.5 µl
5 × PCR buffer	5.00 µl
dNTP (l0 mM)	1.00 µl
F Primer (50 µM)	0.25 µl
R Primer (50 µM)	0.25 µl
Taq (5 U/µl)	0.25 µl
DMSO (1%)	0.25 µl
$MgCl_2$ (25 mM)	4.00 µl

A 23.5 µl master mix is dispensed into the tubes and 1.5 µl of nuclease free water/sample/ control DNA is then added.

Tubes are then placed in a thermocycler at the following profile:

35 cycles:	95°C	5 minutes	
	95°C	1 minute	
	58°C	45 seconds	
	72°C	60 seconds	
1 cycle (final extension)	72°C	20 minutes	soak at 4°C

iii) Electrophoresis

20 µl of each PCR product is loaded in 10% polyacrylamide/bis (37.5:1) gels, with denaturing gradients from 30 to 60% (where 100% is 7 M urea and 40% [v/v] deionised formamide) in 1× TAE buffer. The electrophoresis is performed at 100 V, 300 mA at a temperature of 60°C for 18 hours, the run time could change based on the DGGE device and on the gel dimension. Gels are then stained with a suitable DNA stain for 30 minutes (5 µl in 50 ml of 1× TAE buffer) and visualised under UV illumination.

1.3.3. Real-time PCR

Species-specific real-time PCRs were developed to increase the throughput of diagnostic samples (Raviv & Kleven, 2009). This method uses specific fluorescent probes that increase the

cost of specific analysis, but avoid potential contamination post-amplification. MG gene amplification is targeted to *mgc2* gene and MS amplification on 16S–23S ISR. The assay is conducted as a duplex amplification that includes an internal amplification control (IAC). A detection limit of 10 copies of MS DNA per reaction and 1 copy of MG DNA is determined.

i) Primers

For	Primers and probes
MG	MGFrt F 5'-TTG-GGT-TTA-GGG-ATT-GGG-ATT-3'
	MGRrtr 5'-CCA-AGG-GAT-TCA-ACC-ATC-3'
	MGPrt 5'-Texas Red-TGA-TGA-TCC-AAG-AAC-GTG-AAG-AAC-ACC-BHQ1-3'
MS	MSFrt 5'-CCT-CCT-TTC-TTA-CGG-AGT-ACA-3'
	MSRrt 5'-CTA-AAT-ACA-ATA-GCC-CAA-GGC-AA-3'
	MSPrt 5'-FAM-ATT-CTA-AAA-GCG-GTT-GTG-TAT-CGC-T-BHQ1-3

A commercial IAC DNA kit could be included into the amplification mix to reduce the likelihood of false negatives results.

ii) Polymerase chain reaction

The amplification is conducted in a 96-well real-time thermocycler. A tube reaction is prepared for each sample to be tested for MG or MS. Each reaction contains a total of 25 µl comprising 5 µl of target DNA, 12.5 µl of 2× of universal real-time PCR master mix, 5 µM of each final primer and 0.2 µM final probe. An IAC reagent could be added in each reaction following manufacturer's instructions.

A common amplification protocol is used:

1 cycle	95°C	10 minutes	(single denaturation step)
45 cycles:	95°C	15 seconds	
	60°C	30 seconds	

Fluorescence signal for specific *Mycoplasma* probe and IAC is acquired in the appropriate detector channel during the extension step.

The cycle threshold value (Ct = Cq quantification cycle) automatically calculated by the software should be used. Cq values of 35 or lower are considered as positive, IAC should amplify in negative samples with Cq between 30 and 40, otherwise it could be set as inhibited.

1.3.4. Molecular typing

Different molecular methods are also available for differentiation of MG and MS strains, but their use tends to be restricted at present to specialist laboratories. Sequence-based methods have been developed to identify circulating strains to understand better the epidemiology of mycoplasma and to support control measures. MS strains can be identified and classified with methods such as MLST (Dijkman *et al.,* 2016; El-Gazzar *et al.,* 2017), sequencing of *vlh*A 5' and number of proline-rich repeats (Hammond *et al.,* 2008), differentiation of MS-H with *obg* and *opp*F-1 mutations (Shahid *et al.,* 2013; Zhu *et al.,* 2017). Vaccine strains can be differentiated with DIVA (detection of infection in vaccinated animals) analysis (Dijkman *et al.,* 2017). MG and MS strains can be discriminated using core genome MLST (Ghanem *et al.,* 2017; Ghanem & El-Gazzar, 2018). Novel approaches such as MLVA (multilocus variable tandem array; Kreizinger *et al.,* 2018) and MAMA (mismatch amplification mutation assay) are very promising for discriminating field isolates from vaccine strains, but are not yet widely available.

These new methods are rapidly replacing the other molecular typing techniques, such as restriction endonuclease analysis, pulsed-field gel electrophoresis, amplified fragment length polymorphism and random amplified polymorphic DNA analysis because they are too labour intensive and costly for large-scale typing. Based on the high speed of improvement of the new analytical methods as well as the greater availability of increasingly sophisticated equipment, the methods currently available may be replaced in a short time. Furthermore, avian mycoplasmas

strain nomenclature shall be reviewed in the near future for a more usable classification of circulating mycoplasma strains and better of disease control policies.

2. Serological tests

The serological tests in common use lack specificity and/or sensitivity. It is strongly recommended that their use is limited to flock monitoring rather than 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. Several others have been described such as radioimmunoassay, micro-immunofluorescence, immunoblotting (Welchman *et al.,* 2013) and IP assay, but are rarely used. The number of sera to be tested within a flock depends on the level of detection and the confidence limits required. Minimal requirements, including frequency of testing for international trade within the European Union, have been described, for example for MG in Council Directive 2009/158/EC. Minimal requirements and approved tests are also set out for members of the National Poultry Improvement Plan (NPIP) of the USA.

Poultry companies using ELISA for screening large numbers of sera for virus antibodies may find this type of assay convenient also for mycoplasma testing. The ELISA will not be described in detail here because several MG and MS 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.

2.1. 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 using reagents kept at room temperature. Prior centrifugation of sera 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.

2.1.1. 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 Anses Ploufragan[2], France. MG, MS and control sera produced in chickens or in turkeys, with a range of titres, can be purchased. Sets of antisera can be purchased also from the Department of Avian Medicine, University of Georgia, 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) suggests 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.

2 Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (Anses) Ploufragan, Mycoplasmology Bacteriology Unit, 22440 Ploufragan, France.

2.2. Haemagglutination inhibition test

MG and MS are capable of haemagglutinating avian red blood cells (RBCs), which can be inhibited by specific antibodies in sera. A strain that grows well and reliably haemagglutinates should be selected. The HI test requires a satisfactory haemagglutinating MG and MS antigens, 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) antigens 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.

2.2.1. 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 recognised definition of positive and negative results for international trade.

2.3. Enzyme-linked immunosorbent assay

Several commercial MG and MS antibody ELISA kits are marketed and widely used in diagnostic laboratories. These ELISAs use different cut-offs and mathematical formulas to convert the ELISA result into a titre value. This means that every ELISA has its own interpretation and the titre results of different ELISAs on the same serum could differ.

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

2.4.1 *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.

i) MG antigen for the RSA test

The methods of quality control described below apply solely to suspensions of MG stained with a suitable dye 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 sterile with a pH of between 6.5 and 7.0 and stored at 5±3°C. It must 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 must continue to apply until the expiry date declared by the manufacturer.

ii) 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.

iii) 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 validated commercial kit is the best approach for most diagnostic laboratories.

2.4.2. *Mycoplasma synoviae* antigens

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

i) MS antigen for the RSA test

The specifications apply as for MG antigen for the RSA test.

ii) MS antigen for the HI test

The same specifications apply as for MG antigen for the HI test.

2.4.3. 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, to increase the biosecurity measures and to avoid or to limit mycoplasma production by the infected breeder groups, containing the spread of these pathogens through vertical transmission (Kleven, 2008). Vaccination should be considered only in situations where field exposure is inevitable, or in specific cases, such as on multi-age sites. Potential exposure of neighbouring poultry flocks should also be carefully considered.

Two types of vaccines, live and killed, are available for the control of MG and MS: mild to avirulent live MG or MS strains and inactivated oil-emulsion bacterins. Several scientific papers have been published on this issue, providing evidence of the effect of vaccination on reducing drop of egg production, on limiting respiratory signs, airsacculitis, and in reducing egg transmission.

Although antigenic variability is present among MG or MS strains, it is thought that vaccination with a single MG or MS strain is sufficient to achieve a good level of protection against the homologous species. Live vaccines can be considered a good and effective tool for the management and containment of mycoplasmoses. However, until recently, it was not easy to distinguish the vaccine strain from the wild strains using the available tests (Catania, 2016b). However, the newer biomolecular techniques, while not simple or easy, have been validated for differentiating the strains (Dijkman *et al.,* 2017; Kreizinger *et al.,* 2017), though they are usually based on single-point mutations.

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.

Bacterin-based vaccines are used to prepare the immune system for exposure to the disease to reduce the clinical signs, such us egg production drops that occur as a consequence of MG infection in layers. Their use at present is limited mainly to MG.

The use of live vaccines is equivalent to a 'controlled exposure'. The objective is to infect the flock with a mild, immunogenic MG or MS strain at an age when little or no significant damage occurs. Such exposure results in resistance to challenge later in life, particularly on multi-age commercial sites. Successfully vaccinated birds should be resistant to respiratory disease, airsacculitis, egg production drops or other specific lesions caused by MG or MS. Vaccination should also reduce transmission of the pathogen through the egg in breeders.

1. Outline of production and minimum requirements for vaccines

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, or other methods such as 16s PCR DGGE, 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).

1.4. Method of manufacture

1.4.1. Procedure

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 mycoplasma 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.

1.4.2. Requirements for ingredients

See Chapter 1.1.8 with special focus on products of biological origin originating from a country with negligible risk for transmissible spongiform encephalopathies.

1.4.3. In-process control

i) 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.

ii) 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.

iii) 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.

1.4.4. Batch control

i) Sterility

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

ii) Safety

a) Live vaccine safety test

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

b) Killed vaccine safety test

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

iii) Batch 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 by producers per dose per bird to last until the expiry date.

iv) 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.

1.5. Requirements for regulatory approval

1.5.1. Manufacturing process

For vaccine approval, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 and 2) should be submitted to the Authorities. Information should be provided from three consecutive vaccine batches to demonstrate consistency of production.

1.5.2. Safety requirements

i) 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.

1.5.3. Efficacy requirements

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection); each future commercial batch shall be tested before release to ensure it has the same potency value demonstrated by the batch(es) used for the efficacy test(s). Each batch of live vaccine should contain sufficient live mycoplasmas per dose per bird to last until the expiry date.

Vaccine efficacy (protection) should be estimated in vaccinated animals directly by evaluating their resistance to challenge.

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NB: There are OIE Reference Laboratories for avian mycoplasmosis (*Mycoplasma gallisepticum* and *M. synoviae*) (please consult the OIE Web site: https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
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 2021.