Current Knowledge of Avian Mycoplasmosis

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Abstract

Mycoplasma gallisepticum (MG) is an avian pathogen involved in chronic respiratory disease (CRD) in chickens. The characterized of MG infection compose with respiratory rales, coughing, nasal discharge and conjunctivitis including a reduction in egg production. Initiation of avian mycoplasmosis in poultry may be due to vertical or horizontal transmission. MG infection predisposes the affected birds to action of other secondary bacterial infection or viral infection complicate and cause "air sac disease". Diagnostic method of MG infection compose with several techniques: the gold standard method is bacterial isolation from tracheal or cloanal cleft swab sample defined by the character of "fried egg" colony and supplemented by biochemical properties, and by various measures of serological relatedness including serum plate agglutination (SPA), hemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) method. Besides that polymerase chain reaction (PCR) method, histopathological appearances and bio-assay are other methods for MG detection. Recently, PCR has become a rapid and sensitive method for direct detection of the MG nucleic acid, and there are several available molecular biology techniques for the differentiation of MG strains. Measurement of MG control has been centered on maintenance of mycoplasma-free breeding stock and keeping parent and production flocks free of MG by utilizing single-age, all-in-all-out farms with good biosecurity. A strict biosecurity and slaughter program remains the best method but it is not possible under commercial conditions. MG vaccination may be used to control MG in broiler breeders based on theory that live vaccines provide reduction on clinical signs and have been shown to replace indigenous strains when used in a consistency program for several placements.

Keywords: *Mycoplasma gallisepticum*, avian, diagnostic, control

Introduction

Mycoplasma gallisepticum (MG) is an avian pathogen involved in chronic respiratory disease (CRD) in chickens resulting in considerable economic losses in poultry production industries worldwide. Economic losses particularly air sacculitis, causes increased condemnation at processing plant or downgrading of carcasses, in addition with reduced feed and egg production, increased medication costs, and additional costs from prevention and control programs which may include surveillance (serology, isolation and identification) (Ley, 2003). Actually, avian mycoplasmosis is caused by several pathogenic mycoplasmas, however MG is the most important and the only one that causes an OIE notifiable disease (OIE, 2004). In European legislation, MG is also included in Council Directive on animal health conditions governing intra-community trade in, and imports from third countries of, poultry and hatching eggs (90/539/EEC) with conditions concerning trade within European Community and imports from third country of poultry and hatching eggs.

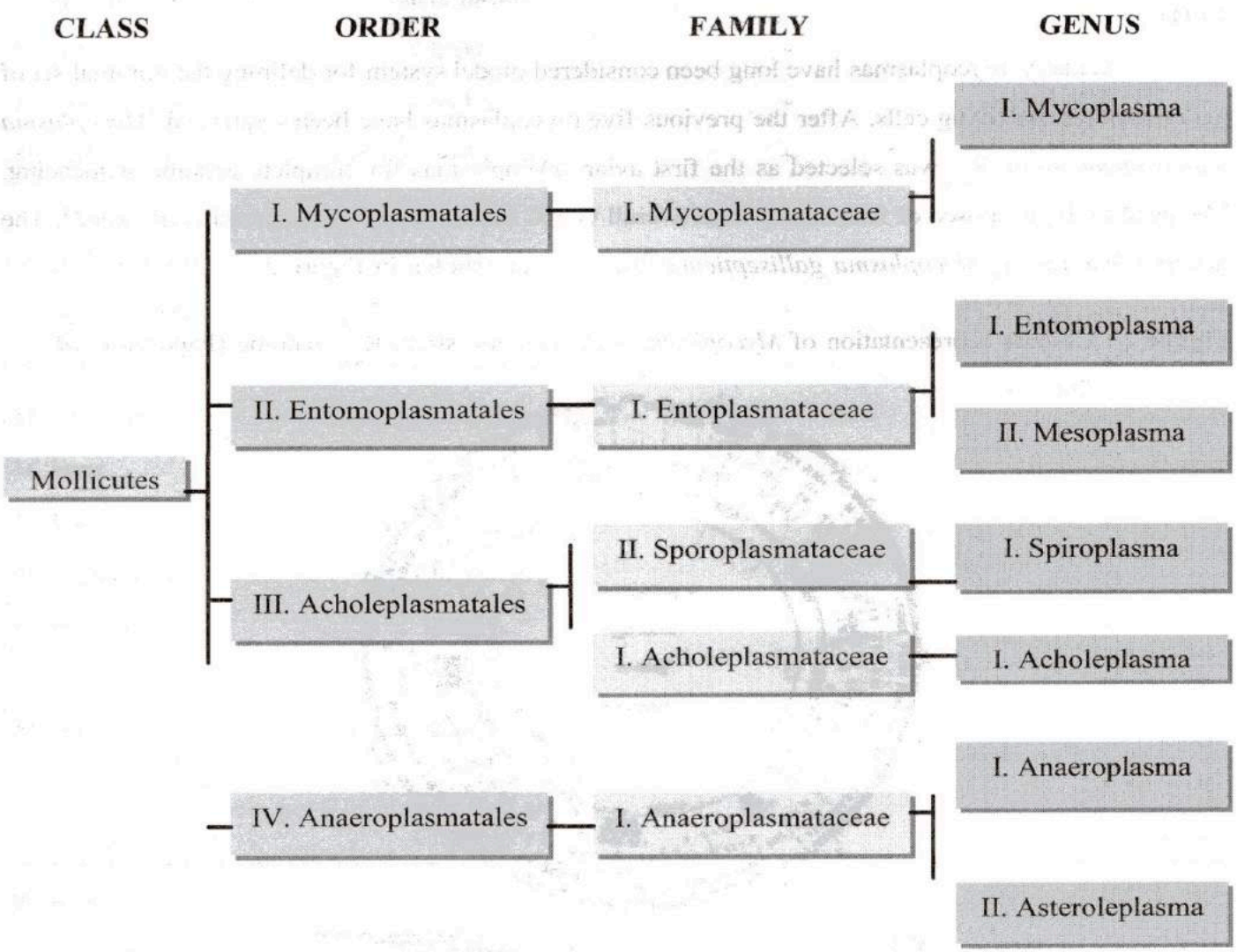
MG is characterized by respiratory rales, coughing, nasal discharge, and conjunctivitis. Clinical manifestations are usually slow to develop, and the infection or disease may have a long course (Ley, 2003) establish chronic infections and are difficult to control with antimicrobial therapy. MG infection results in numerous clinical signs including a reduction in egg production in laying chickens (Evans *et al*., 2002). In recent years, the world-wide industry is growing rapidly with huge populations of poultry in small geographic areas particularly multi-age population sites are becoming more common, making control utilizing biosecurity more difficult (Kleven, 1997).

The initiation of avian mycoplasmosis in poultry may be due to vertical or horizontal transmission (Marois *et al*., 2002). At first control of MG primarily as response to a high condemnation rate of air sacculitis at the post mortem inspection of poultry (Kleven, 1997). MG is frequently present as one of major agents in multi-factorial disease complex. MG infection predisposes the affected birds to action of *Escherichia coli*, *Haemophilus paragallinarum*, vaccine strains of Newcastle disease or infectious bronchitis and infectious laryngotracheitis viruses to various stress factors of environment particularly increased or decreased temperature, ammonia and dust in air (Stipkovits, 2001). Ley (2003) gave a definition of "air sac disease" as a severe air sacculitis that is the result of MG or *Mycoplasma synoviae* (MS) infection complicated by a respiratory virus infection e.g. infectious bronchitis virus or Newcastle disease virus and *Escherichia coli*. Their appearances are usually the most familiar pictures which are threatening poultry industries worldwide.

Biology and Taxonomy of the Mycoplasmas

Mycoplasmas are very small prokaryotic organisms devoid of cell walls bounded by a plasma membrane (Kleven, 1997). *Mycoplasma gallisepticum* (MG) belongs to the class Mollicutes (*mollis*, soft; *cutis*, skin), order Mycoplasmatales, family Mycoplasmataceae and genus *Mycoplasma* (OIE, 2004). Families subdivision is based on habitat, sterol requirement for growth, genome size, and oxygen tolerance, further genera differentiation takes into account the mechanism used by the organism to obtain energy such as glucose, arginine, or urea fermentation. *Mycoplasma* species are defined by the above criteria, supplemented by additional biochemical properties, and by various measures of serological relatedness (Rosenbusch, 1994).

Figure 1: Taxonomy of avian mycoplasmas modified from Rosenbusch (1994).



The primary basis for inclusion of an organism in the class Mollicutes is the absence of cell wall which can be demonstration by a single trilaminar membrane bounds the organism. Lack of cell wall is also the underlying reason for the "fried egg" colonial morphology that is characteristic of growth on solid media. In addition, the absence of cell wall material and cell wall associated proteins renders mycoplasmas resistant to the action of antibiotics that interact with these proteins (Rosenbusch, 1994). There are approximately twenty-five mycoplasmas belonging to the genera *Mycoplasma*, *Acholeplasma* and *Ureaplasma* have been isolated from avian species (Whithear, 1996). The list of avian mycoplasma species is found in Table 1.

Generally, mycoplasmas have correspondingly small genomes size with 600 000 to 1 350 000 bp only one-sixth the size of the *Escherichia coli* genome, and their G + C content is very low 23-40 %, so that only a part of the total genome is presumable used for expression of genetic information. As a consequence of this limited genetic potential, mycoplasmas usually require intimate association with mammalian cell surfaces and manifest complex nutritional requirements for in vitro growth (Rosenbusch, 1994).

Actually, mycoplasmas have long been considered model system for defining the minimal set of genes required for living cells. After the previous five mycoplasmas have been sequenced, *Mycoplasma gallisepticum* strain R_{low} was selected as the first avian mycoplasmas for complete genome sequencing. The genome is composed of 996 422 bp with overall G + C content of 31 % (Papazisi *et al.*, 2003). The genome features of *Mycoplasma gallisepticum* strain R_{low} are shown in Figure 2.

Figure 2 : Circular representation of *Mycoplasma gallisepticum* strain R_{low} genome (Papazisi *et al.*, 2003).

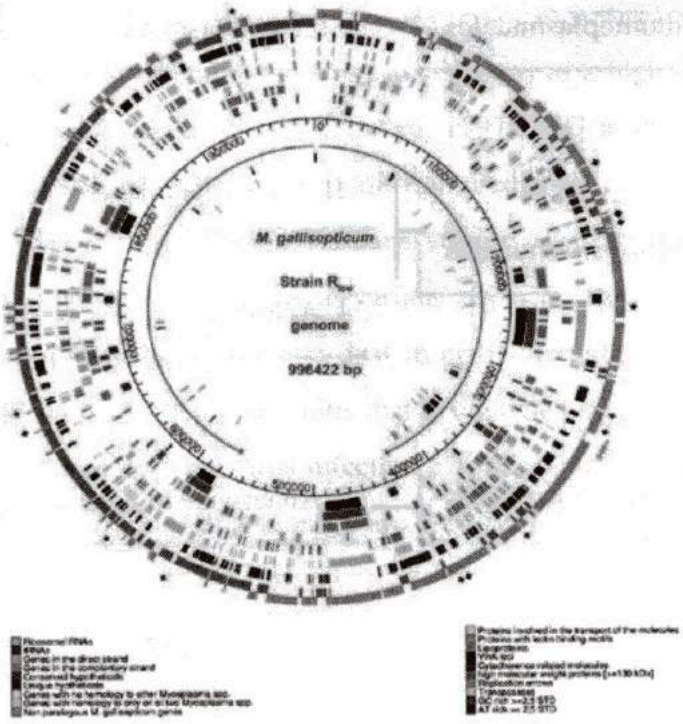


Table 1: Characteristics of avian mycoplasmas (Kleven, 2003).

Species	Usual host	Glucose fermentation	Arginine hydrolysis
A. laidlawii[A]	Various	+	-
M. anatis	Duck	+	-
M. anseris	Goose	-	+
M. buteonis	Buteo hawk	+	-
M. cloacale	Turkey, goose	-	+
M. columbinasale	Pigeon	-	+
M. columbinum	Pigeon	-	+
M. columborale	Pigeon	+	-
M. corogypsi	Black vulture	+	-
M. falconis	Saker falcon	-	+
M. gallinarum	Chicken	-	+
M. gallinaceum	Chicken	+	-
M. gallisepticum	Chicken, turkey, house finch, other	+	-
M. gallopavonsis	Turkey	+	-
M. glycophillum	Chicken	+	-
M. gypsi	Griffon vulture	-	+
M. imintans	Duck, goose, partridge	+	-
M. iners	Chicken	-	+
M. iowae	Turkey	+	+
M. lipofaciens	Chicken	+	+
M. meleagridis	Turkey	-	+
M. pullorum	Chicken	+	-
M. sturni	European starling	+	-
M. synoviae	Chicken, turkey	+	-
U. gallorale[B]	Chicken	-	-

Remarks:

A: Acholeplasma species do not require sterols for growth.

B: Ureaplasma species are characterized by splitting of urea.

One useful characteristic of MG and MS is hemagglutination of erythrocytes from chickens which is used for hemagglutination inhibition serological test for these two pathogenic species (Kleven, 2003).

Pathobiology of MG

MG usually colonizes along the respiratory system of chickens. Isolates and strains of MG vary widely in their relative pathogenicity, depending on the genotypic and phenotypic characteristics of the isolates, method of propagation, the number of passages through which they have been maintained, and challenge route and dosage. Yolk-passaged organisms from MG inoculated embryonated chicken eggs were often considered to be more infective than broth-passaged organisms (Ley, 2003).

Normally, the primary habitats of mycoplasmas are the mucosal membranes of the respiratory tract, and/or the urogenital tract, eyes and joints. Most mycoplasmas are considered surface parasites, rarely invading tissues. Adhesion of mycoplasmas to host cells is a prerequisite for successful colonization, and ensuing pathogenesis. MG is one of the species of mycoplasma that, as primary pathogens, can cause acute and chronic diseases at multiple sites, with wide-ranging complications (Levisohn and Kleven 2000).

Infection with MG has a wide variety of clinical manifestations, but even in the absence of overt clinical signs, the economic impact may be significant. The most dramatic disease presentation of MG is chronic respiratory disease in meat-type birds, often as one of several etiological agents in a multi-factorial disease complex. MG infection alone may cause respiratory disease in turkey that is most often mild or sub-clinical in chickens. However, MG is frequently present as one several etiological agents in a multi-factorial disease complex. The interactions among MG, various respiratory viruses, *Escherichia coli*, *Haemophilus paragallinarum*, and others have been documented. Vaccine strains of Newcastle disease virus or infectious bronchitis virus may give marked respiratory reactions in MG-infected birds. Moreover, infection with MG may cause a marked reduction in feed consumption efficacy with a significant economic impact, even in the absence of overt clinical signs (Levisohn and Kleven 2000).

MG infection can spread vertically from hens to progeny and horizontal by direct contact of susceptible chickens with infected carriers or by airborne route and by indirect contact by contaminated environment (Stipkovits, 2001).

One of virulence factors associated with MG is cytadhesion; the ability to vary surface components, which may function in immune evasion and/or adaptation to host environment and possibly the ability to invade cells as described by Papazisi *et al.* (2003), cytadherence to the epithelial surfaces of the host tissues is a requirement for successful colonization. One of the prominent features of MG is a lymphoproliferative response in the respiratory tract (Gaunson *et al.*, 2000). Guanson *et al.* (2000)

revealed that the mucosal thickness increased significantly at 1 to 2 weeks after MG infection followed with significant decrease between 2 to 3 weeks after MG infection. Meanwhile MG genome titer continual decreased 1 to 3 weeks after MG infection therefore, MG multiplication peaked prior to 2 weeks after MG infection but the microscopic lesions of tracheal mucosal thickness development peaked at 2 weeks after MG infection. MG caused a significant decrease in chemotactic response which may play a pivotal role in the progression and maintenance of MG in chickens (Lam, 2004). Presence of a high degree of variability in expression of surface antigens among strains of MG might play a role in pathogenesis particularly evasion of the immune systems of the host (Kleven, 1997). The chronic nature of mycoplasma infections demonstrates a failure of the host immune system to deal effectively with these organisms (Papazisi *et al.*, 2003). Antigen variation of MG strains has been demonstrated by monoclonal antibodies after in vivo passage which might indicate an immune evasion mechanism (Garcia and Kleven, 1994).

Contaminated feathers are the greatest risk to a mycoplasma free poultry environment particularly if they become disseminated on the farms, or are not properly cleaned from a contaminated shed prior to restocking. Survival time up to 2 days on rubber such as boots and cotton such as clothes is causing the importance of using disinfectant footbaths and of changing overalls by personnel moving between flocks. The nasal mucosa seems to be a favorable site of mycoplasmas than the skin and the ear; this may be because of its escape from normal washing and showering routine (Christensen *et al.*, 1994).

Serological Tests

Systemic antibody response is widely used as a diagnostic and epidemiologic tool in the poultry industry to identify or determine the prevalence of infectious agents (Noormohammadi *et al.*, 2002). Serological test is an extremely useful tool for monitoring and diagnosis of MG in flock control program (Ley *et al.*, 1997). It is extremely important for producers to be able to detect MG-infected birds early in the course of infection (Kempf *et al.*, 1994).

Infections by mycoplasma can be diagnosed by serological test such as serum plate agglutination (SPA) test, enzyme-linked immunosorbent assay (ELISA) test, hemagglutination inhibition (HI) test (OIE, 2004; Moscoso *et al.*, 2004) although there is a lack of relationship between levels of circulating antibody and protection against MG (Whithear, 1996).

Positive serological test according with flock history and typical clinical signs of MG, allows a presumptive diagnosis pending the further isolation and/or identification of the organisms (Ley, 2003). Kleven (1994) suggested 10 % of the flock or a minimum of 300 birds are tested before the onset of egg production, and approximately 30 birds per flock are tested every 60-90 days thereafter. Ley (2003) suggested the serological monitoring of breeder flocks at very short intervals for chickens every 2 weeks could optimize the ability to detect and prevent egg transmission. However, serological tests are often

hampered by interspecies cross-reactions and non-specific reactions (Fan *et al.*, 1995). Detection of tracheal-washing antibodies give a more accuracy indication of protective immunity but this technique is presently used only in experimental studies (Whithear, 1996).

Serum plate agglutination (SPA) Test

During the 1960s the serum plate agglutination test displaced the tube agglutination test to become the most frequently used procedure for monitoring flocks for MG antibody status (Collet *et al.*, 2005). Because the SPA test is rapid, cheap and highly sensitive therefore it has been widely used as an initial screening test for flock monitoring and sero diagnosis.

SPA antigen is commercial available including Intervet (Boxmeer, Holland) (Ley, 2003). Sensitivity of SPA is superior to ELISA and HI tests in the ability to detect antibodies formed in early response to MG infection, this can attribute to the SPA test's ability to detect IgM antibody which is the first class of immunoglobulin produced in response to infection (Kempf *et al.*, 1994) and antigens obtained from different sources may differ in sensitivity and specificity, and the variations also exist between batches (Kleven, 1998a). The SPA test has diagnostic value if the number of positive reactors is more than 30 %, in case of lower number of reactors, it is recommended to retest 2 weeks later. Increase number of reactors indicates acute infection, no changing or slightly decrease of number of reactors indicates late phase of infection or presence of variant strains infection (Stipkovitz, 2001).

Infected birds may positive as early as 7-10 days after infection (Kleven, 1994). According with Guanson *et al.* (2000) has been found that antibodies against MG could detectable with strong SPA reactions at 2 and 3 weeks after infection. The SPA reaction will prolong for about 2-3 months, at flock level for 6 months (Stipkovitz, 2001). However, the SPA has disadvantage of producing false positive reactions and the ELISA and HI is regarded as a less positive but more specific assay than SPA (Kempf *et al.*, 1994). In addition, there are also batch-to-batch variations. Non SPA specific or false positive reactions occur in flocks with Mycoplasma synoviae (MS) due to cross reactive antigens (Kempf *et al.*, 1994), or those recently vaccinated with oil emulsion vaccines especially if there are remnants of serum in the vaccine (Glisson *et al.*, 1984; Kleven, 1998a) and/or some tissue-culture origin vaccines such as infectious bursal disease (Ross *et al.*, 1990). Non specific SPA reaction in proportion of about 10-30 % can occur after application of inactivated oil emulsion vaccine against Newcastle disease, infectious bronchitis and infectious laryngotracheitis. These reactions will disappear after 4-6 weeks post vaccination (Stipkovitz, 2001). In some laboratories, certain non specific SPA reaction is reduced by two-fold dilution of the serum in buffer such as phosphate buffered saline and those that still reacted are considered positive (Kempf *et al.*, 1994). Antigen manufacturer instruction suggested that the dilution of the test sera may indicate whether a positive reaction should be considered as false positive. Real positive reactions will be

countered even if the test sera are diluted > 1:8.

Fresh serum should be used for serum plate agglutination tests, since nonspecific agglutination may be a problem with frozen sera. Either fresh or frozen serum is suitable for other serologic tests and flocks with SPA reactors should be confirmed as positive or negative with the hemagglutination inhibition (HI) test or other acceptable serologic test, or the agent can be detected by culture or PCR (Kleven, 1998a).

ELISA Test

ELISA test systems have been developed through various commercial ELISA test kits including IDEXX (IDEXX Laboratories, Westbrook, Maine, USA), Synbiotics (Synbiotics Corporation, 11011 Via Frontera, San Diego, California, USA), MyGa-Tests (Diagnosticum, 1124 Budapest, Hungary) are now commonly used for flock monitoring and sero diagnosis. In many production companies and diagnostic laboratories, ELISA is the test of choice for MG serology (Ley, 2003). In general, ELISA tests are slightly less sensitive but more specificity than SPA test and less specific but more sensitive than HI test (Kempf *et al.*, 1994). However, lack of specificity and/or sensitivity of ELISA in the acute phase of infection have been reported (Feberwee *et al.*, 2004). An egg sampling is the alternative method to serum sampling, in which yolk serve as a source of antibodies which is an easy matter for the flock owner to collect a sampling of eggs; it is inexpensive, since all cull eggs may be used; and the procedure could avoid the negative aspects of blood collection such as stressed the birds with adversely affect on egg production and increase the incidence of cracked eggs (Mohammed *et al.*, 1985). Kempf *et al.* (1998) suggested that, when it is not possible to sample breeders, the method of choice to detect a vertical MG infection in hatching eggs, because of its easiness and precocity, seem to be ELISA detection on yolks from non incubated eggs. However, MG antibody in one-day-old chicks can be detected by both ELISA and SPA tests.

Hemagglutination inhibition (HI) Test

Hemagglutination inhibition test has been commonly used to confirm reactors detected by SPA or ELISA and considered to be highly specific (Ley, 2003). However, the HI test is time consuming, the reagents are not commercially available and the test may lack sensitivity (Kempf *et al.*, 1994). Generally, HI titer of 1:40 to 1:80 or greater are considered positive, but results must be interpreted on a flock basis (Kleven, 1994). The HI test detected IgG antibodies which becomes positive 3 weeks post challenge (Stipkovits, 2001).

Disadvantage of HI test is considered to be less sensitive than other serological tests; infected birds may not test positive until 3 weeks or longer after infection. In addition, there is antigenic variation among MG strains as measured by HI (Kleven, 1994).

According to OIE (2004), high proportion of positive sera in a flock with higher than 10 % indicates MG infection, especially when the sera confirmed by HI test or ELISA. However inclusive results make it necessary to attempt to isolate the organism or demonstrate the presence of its DNA.

Isolation and Identification of MG

The gold standard for MG diagnosis is isolation and/or identification of the organism (Ley, 2003), it is the strongest evidence of infection available and collection of mycoplasma field strains allows study and comparison of properties such as pathogenicity or antibiotic sensitivity (Kempf *et al.*, 1994) but MG is a slow-growing, relative fastidious organism that requires one or more weeks for growth and identification. Isolation of MG has often unsuccessful as a result of the culture overgrowth of saprophytic mycoplasmas that inhibit the upper respiratory tract, particular in older chickens (Garcia *et al.*, 2005).

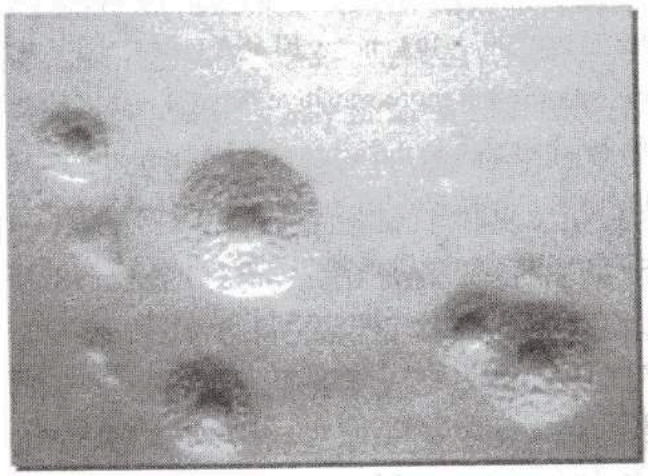


Figure 3: Typical colonies of Mycoplasma gallisepticum on Frey's agar medium.

The pathogenic avian mycoplasmas persist for long periods in the upper respiratory tract, tracheas or palatine clefts are preferred sites for culture, and however tissues showing lesions such as air sacs should be cultured as well (Kleven, 1994). Furthermore the small pieces of air sac, trachea, or other tissue may be inoculated directly into broth medium for culture also (Kleven, 1998a). The reason for considers to collect the trachea for isolation because the upper respiratory tract is generally accepted to be the portal of entry in natural infection by MG and the trachea also appears to be the preferential site of infection, although disease signs are usually manifested in other parts of the respiratory system (Levisohn and Kleven, 2000). In many cases the organisms are cleared from lesions but may persist in the upper respiratory tract (Kleven, 1994). During the acute stages of infection (up to 60-90 days post infection), the population of organisms in the upper respiratory tract and the incidence of infection in the flock are high. In such cases 5-10 cultures from the trachea or choanal cleft are often sufficient. However, in chronic cases or when infection with mycoplasma strains of low pathogenicity is suspected, 30-100 individuals should be cultured (Kleven, 1998a).

Inoculated broth cultures are viable for several days at room temperature, although they should be incubated as soon as possible. Incubation is at 37°C. Ordinarily, aerobic incubation is sufficient, some laboratories may prefer to incubate under CO_2, cultures are incubated until the phenol red indicator changes in color from red to orange or yellow, and then plates and fresh tubes of broth are inoculated. Plates are incubated in a closed jar to prevent dehydration. The pathogenic species, colonies are usually not observed until 4-5 days of incubation (Kleven, 1998a).

MG cultivation requires a rather complex medium usually enriched with 10-15 % heat inactivated swine serum (Ley, 2003). Because of MG lack of cell wall, the media used must contain adequate levels of protein to supply the necessary osmolarity (Rosenbusch, 1994). Mycoplasmas are resistant to penicillin because lack of a cell wall and relatively resistant to thallium, therefore mycoplasmas media contain these components to avoid bacterial and mycotic contamination (Kleven, 1998).

Under ordinary light microscope with the light intensity reduced, tiny, smooth, circular, translucent masses 0.1-1 mm in diameter with dense, elevated centers ("fried egg appearance") are suggestive of mycoplasma species (Ley, 2003; Kleven, 1998). They rarely are more than 0.2-0.3 mm in diameter and frequently occur in ridges along the streak line, because closely adjacent colonies readily coalesce (Ley, 2003). However, 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 (OIE, 2004). MG stains well with Giemsa and is weakly gram negative (Ley, 2003) and the characterization of individual cells by light microscopy vary from 0.2-0.5 um and are basically coccoid to coccobacilliform, but slender rods, filaments and ring forms have been described (Kleven, 2003).

The isolates should be identified by biochemical test such as glucose fermentation, arginine hydrolysis and serological tests such as growth inhibition, indirect fluorescent antibody test or indirect immunoperoxidase test (OIE, 2004; Stipkovitz, 2001). Furthermore, recent nucleic acid recognition method particularly PCR represent rapid and sensitive method, which is able to provide accuracy results in the presence of mixed mycoplasma infection and bacterial contamination or inhibition of growth by antibiotics, antibodies and other host factors (Stipkovitz, 2001).

Two well-known methods for quantitative studies of the mycoplasma are color changing unit (CCU) method and colony-forming units (CFU) method. The CCU method could be applied more easily to the large number of samples than the conventional colony counting method, however the samples should be sterility or require the additional step of growing the samples on mycoplasma agar and blood agar to demonstrate that color changes in the broth were actually due to mycoplasma and not bacteria (Christensen *et al.*, 1994).

Histopathological appearances

Study by Guanson *et al.* (2000) showed a prominent feature of disease induced by MG is a lymphoproliferative response in the respiratory tract, and MG multiplication peaked in the trachea prior to 2 wks postinfection (PI) thus the titer of mycoplasma genomes in the trachea decreased significantly from 1 to 2 weeks, but the lesion of mucosal thickness development peaked at 2 wks PI then decreased from 2 to 3 weeks.

Bio assay

Some situations where flocks have exhibited a low level of serological response, low percentage of PCR reactions and failed to culture the organism, it has been possible to transfer such reactivity by placing SPF chickens in contact with the principals. There may be atypical strains which have been undetectable with traditional diagnostic methods (Kleven, 1997). Ley (2003) suggested yolk-passaged organisms from MG-inoculated embryonated chicken eggs were often considered to be more infective than broth-passaged organisms.

Nucleic Acid Recognition Methods

In early MG infection or during a carrier stage in which traditional serologic and cultural procedures may be unsuccessful, a sensitive method for detection of the organism would facilitate diagnosis (Nascimento *et al.*, 1991).

Early development of MG molecular approaches via various probes, were not satisfied sensitive although its specificity is very high. The minimum of MG DNA was generally 1 ng, equivalent to 10^5-10^6 cfu. More recently, polymerase chain reaction (PCR) has been becoming a standard technique for early detection of MG (Moscoso *et al.*, 2004). Nascimento *et al.* (1991) developed the PCR method which was able to detect only 10^{-6} pg of MG DNA; a fraction of the total chromosomal contents of one mycoplasma cell according to Kempf *et al.* (1993) could detect MG DNA from the samples contained less than 1 colony forming unit in 0.1 ml only.

Polymerase chain reaction (PCR) has become a valuable supplemental test for diagnosis of *Mycoplasma gallisepticum* (MG) infection due to a rapid and sensitive method of direct detection of the organism nucleic acid in tracheal swabs compared with isolation techniques, which are time consuming and may be problematic (Garcia *et al.*, 2005; Kleven, 1997; Fan *et al.*, 1995). In addition, these molecular tools are also more sensitive than culture to detect MG in the environment (Marois *et al.*, 2002) and specimens (Kempf *et al.*, 1993). However, bacterial or yeast contamination and the presence of non-viable mycoplasmas could be one reason for the low number of positive cultures compare with PCR. Only viable mycoplasmas should be concerned as a potential source of infection therefore, Marois *et al.* (2002)

suggested extracting and analyzing RNA by reverse transcription-PCR because of the short half life of RNA, its detection can indicates that cells are still viable.

Two of the most commonly available PCR procedures are used in the United States, namely the MG 16S rRNA gene-target PCR and the commercial IDEXX Flock Chek kit (Garcia *et al.*, 2005). In addition, new PCR procedures amplify a region of the mgc2, mgc1 and surface lipoprotein (LP) (Garcia *et al.*, 2005).

Table 2: Primer location in target genes and amplicon size.

Authors	Target gene	Location Gen Bank sequence	PCR product
Lauerman (1998)	16S rRNA	1,249-1,434	183
Hnatow *et al.* (1998)	mgc2	637-937	236-302
Keeler *et al.* (1996)	mgc1	3,696-4,200	332
Nascimento *et al.* (1998)	LP	1-732	349

Garcia *et al.* (2005) suggested that the mgc2 PCR is the method of choice for MG in the field.

The sensitivity of PCR method could be significantly improved after hybridization with a digoxigenin-labeled probe and chemiluminnescent detection (Kempf *et al.*, 1993).

MG strain differentiation

Rapid and accuracy differentiation of MG strains are fundamental to epidemiologic investigations of outbreaks and trace the point sources of infection, and design relevant controlling strategies (Yong *et al.*, 2005; Ley *et al.*, 1997). In addition to, molecular biology techniques are advantages than other cellular MG detection but identification of mycoplasma isolates is also useful for many laboratories.

Several techniques are available for the differentiation of MG strains including southern blot analysis using a ribosomal RNA gene probe, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of mycoplasmal proteins for direct comparison of protein banding patterns (Kleven *et al.*, 1990; Ley, 2003), restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD), MG strain/species-specific probe in dot blot assays (Kleven *et al.*, 1990), DNA sequence analysis on genes encoding immunogenic surface proteins (Hong *et al.*, 2005) and physical chromosomal mapping have also been used to characterize MG intraspecies (strain) differences (Ley, 2003), and the recently molecular typing method amplified fragment length polymorphism (AFLP) has been developed and have proven efficient. (Hong *et al.*, 2005).

Fan *et al.* (1995) and Lauerman *et al.* (1995) developed PCR/ RFLP (Restriction Fragment Length Polymorphism) techniques to detect conserved genes of 16s rRNA and 16s/23s rRNA of avian mycoplasmas and differentiate various mycoplasma species. However RFLP techniques is time consuming and laborious, making identification of a specific strains a tedious procedure, therefore recently Arbitrary-Primed PCR (AP-PCR) or Random Amplified Polymorphic DNA (RAPD) has been developed for identifying specific strains. These techniques are very simple and rapid, and have provided a routine procedure for the rapid identification of MG strains and very useful for epidemiological studies and for identification of specific MG strains in field outbreaks (Kleven, 2003; Kleven, 1998b; Fan *et al.*, 1995; Kleven, 1997). According to Thorne Steinlage *et al.* (2003) and Stipkovitz (2001) mentioned that RAPD has been shown to be a reliable method of distinguishing different MG field isolates with unique strain fingerprint, however multiple primer sets may be needed to detect subtle differences. However, there are intrinsic problems such as poor discriminatory power, inconsistency results for duplicated runs or an inability to maintain a database of RAPD patterns (Hong *et al.*, 2005).

Fan *et al.* (1995b) described the advantages of RAPD for identifying strains and isolates:

1. No requirement for extensive knowledge of the biochemistry or molecular biology of the species under study
2. No requirement for functional selection based on a specific target gene
3. Allow to examine the organisms in short time

However, some disadvantages have been encountered using RAPD:

1. Pure cultures are required.
2. The banding patterns may be different from one laboratory to another owing to slight differences in technique.
3. Different thermal cyclers may result in different banding patterns of the amplified DNAs.

Unfortunately, RAPD technique is lack of reproducibility, therefore it is necessary to run the strains by comparing side by side on the same gel. Also, unlike sequence analysis, RAPD requires pure cultures, which are some cases are difficult to obtain (Hong *et al.*, 2005).

Regarding to currently use of live F-strain as a vaccine for MG eradication, MG-PCR together with F-vaccine strain specific PCR is a simple procedure that efficiently direct-detected the MG F-vaccine strain, should enhance the control of MG infection (Nascimento *et al.*, 1993).

AFLP (Amplified Fragment Length Polymorphism) is the other DNA typing method, which the reproducibility and discriminatory ability between closely strains are very high and also makes it possible

to developed a sizable database of the AFLP patterns of all previously analyzed strains, standardization the procedures, compare and share various AFLP profiles among different laboratories, thus leading to an expanding database of profiles. Unfortunately, AFLP procedures, although not complicated, require pure cultures and more time consuming than RAPD analysis. In addition, a DNA sequencer is needed, and the data analysis software is still expensive (Hong *et al.*, 2005).

Recently, Moscoso *et al.* (2004) suggested FTA® filter paper which is a cotton-based cellulose membrane containing lyophilized chemicals that lyses most types of bacteria and viruses for collecting and simultaneously inactivating mycoplasma. Furthermore, it offers the convenience of storage and transport of DNA in cost-effective manner and long term DNA stability at a wide range of temperatures for further molecular analysis such as restriction enzyme length polymorphism and nucleotide sequencing. All above these make the FTA® cards as a good alternative for collecting and storing mycoplasma DNA.

Measurement of MG Control considered on Laboratory Diagnosis

In most modern poultry producing areas of the world, the emphasis on the control of MG has been centered on maintenance of mycoplasma-free breeding stock and keeping parent and production flocks free of MG by utilizing single-age, all-in-all-out farms with good biosecurity (Kleven, 1997).

The main route of spread of MG infection is egg transmission, therefore it is the reason for highly desirable to eliminate breeder flocks (Kemp *et al.*, 1998). Control of mycoplasmosis depends on biosecurity and early detection to achieve total eradication (Moscoso *et al.*, 2004; Kleven, 1997). A strict biosecurity and slaughter program remains the best method of MG control for breeder flocks but it is not possible under commercial conditions (Collet *et al.*, 2005).

Cited by Kleven (1994), in the United States, MG control programs administered under the National Poultry Improvement Plan (NPIP). Breeder replacements are tested and certified as clean after negative serological tests. Outbreaks of MG in broiler breeding stocks are sporadic. When grand parent breeder flocks or genetic lines become infected, the flock is slaughtered. Kleven (1998) mentioned that majority of broilers and turkeys are free of MG infection while commercial layer production is threatened with MG.

MG vaccination may be used, particularly outside of the United States to control MG in broiler breeders, and it complicates the interpretation of SPA test results (Collet *et al.*, 2005).

Commercial available vaccines

Whithear (1996) suggested the ideal mycoplasma vaccine, should be safe to use and consistently cost-effective. The first commercial available vaccine was bacterin. Bacterins provide little value in MG eradication (Kleven, 1997; Kleven *et al.*, 1990). In addition, safety is a significant issue with bacterins

Animal welfare and food quality issue related to adjuvant used in bacterins are also important concerns (Whithear, 1996).

Currently, there are three commercial live vaccines are available in Thailand: F-strain from Schering Plough, Union, New Jersey, ts-11 strain developed in Australia and licensed by Select Laboratories, Inc., Gainesville, Georgia and 6/85 strain from Intervet America, Millsboro, Delaware. The ideal mycoplasma live vaccine should not cause disease in the vaccinated animals and should not inadvertently spread to, and certainly not cause disease in neighboring flocks. Attenuated strains should not revert to a virulent form (Whithear, 1996).

Live vaccines provide reduction on clinical signs and have been shown to replace indigenous strains when used in a consistency program for several placements (Evans *et al.*, 2002). MG vaccine strains 6/85 and ts-11 produce little or no vaccine reaction in chickens and turkeys (Ley *et al.*, 1997). One of the disadvantages of currently available live vaccine is that there is no convenient serological technique to distinguish between vaccinated or naturally infected flocks which Whithear (1996) mentioned for the future direction of mycoplasma vaccination that developing of specific gene-deleted vaccine and serological test based on the deleted gene could allow this distinction will be made. Only animals exposed to the wild-type pathogen would develop antibody to the antigen. The details of live vaccines that are available in Thailand are following.

1. *F MG live vaccine:* The F strain is a naturally occurring strain of moderate virulence to chickens and high virulence to turkeys (Whithear, 1996), therefore F strain has been implicated in field infections in commercial turkeys (Kleven, 1997). They may be also preferred in situations where inadvertent exposure of neighboring poultry flocks is of concern. F strain vaccine administration could be performedvia intranasal, intraocular, per oral in the drinking water and aerosol administration. A respiratory reaction should be expected at approximately 5-7 days after vaccination via aerosol administration. Vaccinated flocks should be tested with the SPA approximately 3-4 weeks post-vaccination to be sure that all birds are properly vaccinated (OIE, 2004). Based on field trials, Kleven *et al.* (1990) suggested that long term use of an F strain live vaccine on multiple ages, commercial layer farms resulted in eventual displacement of original field strain with the vaccine strain. However, F strain vaccine can affect the egg production, since F strain vaccinates had significantly lower egg production 5.76-5.80 % than controls (Branton *et al.*, 1988). This is one of the major concerns for layer or breeder producers, when they decide to select the F-strain vaccine in their flocks, other than the strong antibody response which interfere the monitoring interpretation of MG.

2. Ts-11 MG live vaccine: Strain ts-11 should be administered by eye drop (OIE, 2004). The ts-11 vaccine had three different sources of manufacturers with different minimum release titer requirements for vaccine production including in France (Merial France), in Australia (Bioproperties Australia Ptv.,

Victoria) and in the United States (Select Laboratories, Inc., Gainsville, GA). Cited by Collett *et al.* (2005) mentioned the manufacturer instruction, vaccine with a titer of $10^{8.6}$ cfu/dose stimulate a seroconversion of 70-80% of the flock, whereas with a titer of $10^{8.1}$ cfu/dose was associated with a 30% seroconversion rate. By nature of ts-11 MG live vaccine, serological monitoring of vaccinated flocks has generally been difficult because of the low level of antibodies detected by current assays, in particular the SPA test, thus lack of a strong serum antibody response after vaccination may be beneficial in differentiating vaccinated flocks from those exposed to wild-type MG where high levels of antibodies are present (Noormohammadi *et al.*, 2002). The age of the chicken is crucial for successful ts-11 vaccination, and vaccination at less than 4 wk of age may fail to induce a satisfactory level of immunity. In Australia, poultry industry, vaccinate ts-11 vaccine at 5 wk of age (Noormohammadi *et al.*, 2002).

The lateral spread of the ts-11 strain vaccine occurred over short distances but may be enhanced by flock size and density since in larger flocks; lateral transmission is facilitated by proximity of birds at high density (Collett *et al.*, 2005) according with Ley *et al.* (1997) found that ts-11 is transmissible from vaccinated when birds are in direct contact with each other and/or share a common feeder and waterer, but transmission is unlikely with indirect contact and reaffirmed by Turner and Kleven (1998) field studies of multiage commercial layer farm. Ts-11 strain vaccine may survive longer in the upper trachea than in the cloanal cleft (Collett *et al.*, 2005). In Australia, ts-11 vaccine is being extensively used in broiler breeder pullets as well as in commercial layers (OIE, 2004). In the United States, MG ts-11 vaccine has been used primarily for prevention of egg production losses in commercial table egg layers. The vaccine (MG ts-11, Select Laboratories, Inc., Guinesville, Georgia) is formulated as a frozen (-40^{0}c) suspension and administered by the eye drop route as a single dose to growing pullets 9 weeks of age or older, at least 3 weeks before expected exposure to field challenge (Ley, 2003).

In 1981, a multiage commercial layer operation in South Carolina decided to use a live F-strain MG vaccination program with the objective of displacing the field MG strain. In 1994, the live ts-11 strain was introduced on the farm which each new placement flock was vaccinated with ts-11 for one production cycle, and then all subsequent placement flocks were left unvaccinated. After the last vaccinated flock was terminated, no MG was detected on the farm (Turner and Kleven, 1998).

3. 6/85 MG live vaccine: The 6/85 vaccine should be given as a fine spray (OIE, 2004). It has been shown to have low potential for transmission from vaccinated chickens to non-vaccinated chickens (Ley *et al.*, 1997). Throne Steinlage *et al.* (2003) described the case report of 83-wk-old commercial table egg layers with swollen sinuses and a history of increased mortality may have a pathogenic potential of live 6/85 strain MG vaccine which spread into susceptible chickens.

Comparison of these three available live vaccines, F strain can induce a stronger antibody response and immunity on challenge, than 6/85, whereas ts-11 induced an intermediate response.

Furthermore, F strain and ts-11 colonized on trachea efficiently, whereas isolation of 6/85 after vaccination was inconsistency (OIE, 2004; Kleven, 1998b). In pen trial studies of Kleven (1998b) have shown that F strain had completely displaced the R strain, on the other hand, ts-11 and 6/85 strains exhibited no ability to displace. Field experiences utilizing live vaccines have generally been very favorable in commercial layers and multiage broiler breeders by displace challenge strains with F strains after then displace F strains with ts-11 strain and finally withdrawal ts-11 to resume the flock to be MG-free (Kleven , 1997). Kleven (1998b) suggested MG eradication program by using live vaccine based on his long time trials both field and experiment that on multiple-age production sites, it may be important to vaccinate initially with F strain to displace the field strain present on the site. Switching to the less virulent and safer 6/85 or ts-11 vaccine strain after the F strain has already displaced the original field strain, estimate to require at least 1 year or one production cycle.

In addition to live MG vaccine, there are MG bacterins available in Thailand. MG bacterins are prepared from a concentrated suspension of whole cells that is emulsified into an adjuvant. A high antigencontent is essential (OIE, 2004). MG bacterin is capable of protecting chickens from respiratory signs (Hildebrand *et al.*, 1983) and egg production losses from MG infection (Sasipreeyajan *et al.*, 1987). They may also be used to reduce the level of egg transmission in breeder pullets, however vaccinated flocks are readily infected and transmission of the disease (OIE, 2004). Furthermore, MG bacterins vaccinated flocks always given antibody response, which interfere the serological monitoring interpretation.

Medication

Chemotherapeutic control is one of a practical way to control the disease and has been used effectively to reduce the economic loss due to avian mycoplasmosis (Wang *et al.*, 2001). Therapeutic medication for mycoplasmosis approved for use in poultry includes tetracyclines and tylosin (Evans *et al.*,2002). Cerda *et al.* (2002) study about the minimum inhibitory concentrations (MICs) for 7 antibiotics (aivlosin, enrofloxacin, tylosin, tiamulin, kitasamycin, chlortetracycline and oxytetracycline) suggest that Aivlosin (3-acetyl-4"-isovaleryl tylosis tartrate), tylosin and tiamulin showed the lowest MICs with MIC_{90s} of 0.006, 0.012 and 0.05 ug/ml, respectively. In addition to Wang *et al.* (2001) mentioned that quinolones have also shown effectiveness against Mycoplasma species of veterinary importance including Fluoroquinolones, enrofloxacin and sarafloxacin are approved for the control of Escherichia coli-associated mortality.

Evans *et al.* (2002) suggested the alternative MG control by combination of 6/85 vaccination and sub therapeutic tylosin feed medication. In addition, these products are used to improve feed efficiency when fed at levels substantially less than the approved therapeutic level for mycoplasmosis.

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ความรู้ปัจจุบันของโรคมัยโคพลาสโมซิสในสัตว์ปีก

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บทคัดย่อ

เชื้อมัยโคพลาสมา กัลลิเซพติกุม (*Mycoplasma gallisepticum*) เป็นเชื้อก่อโรคในสัตว์ปีกที่ก่อให้เกิดปัญหาเกี่ยวกับระบบทางเดินหายใจแบบเรื้อรัง โดยสัตว์ปีกที่ติดเชื้อจะแสดงอาการทางระบบหายใจ โดยมีเสียงหายใจผิดปกติ ไอ มีน้ำมูก และเยื่อตาขาวอักเสบ รวมทั้งมีผลผลิตไข่ลดลง สำหรับการแพร่กระจายของเชื้อเข้าสู่ตัวไก่อาจจะเกิดจากการถ่ายทอดเชื้อผ่านทางแม่มาสู่ลูก หรือเป็นการติดเชื้อจากการสัมผัส ความสำคัญของเชื้อมัยโคพลาสมา กัลลิเซพติกุม คือสามารถเหนี่ยวนำให้สัตว์ปีกที่ติดเชื้อสามารถติดเชื้อแบคทีเรียหรือไวรัสที่เกี่ยวข้องกับระบบทางเดินหายใจอื่นๆ แทรกซ้อน ก่อให้เกิดปัญหาโรคถุงลม (air sac disease) ได้ วิธีการตรวจวินิจฉัยการติดเชื้อมัยโคพลาสมา กัลลิเซพติกุม ปัจจุบันมีหลายวิธี ประกอบด้วยวิธีที่ถือเป็นมาตรฐานสำหรับการตรวจวินิจฉัยคือการเพาะแยกเชื้อจากตัวอย่างที่เก็บด้วยการป้ายเชื้อจากส่วนท่อลมหรือส่วนช่องเพดานปาก ซึ่งลักษณะที่จำเพาะของเชื้อคือ ลักษณะโคโลนีที่มีลักษณะคล้ายไข่ดาว ตรวจยืนยันโดยใช้คุณสมบัติทางชีวเคมีของเชื้อ นอกจากนั้นคือวิธีการตรวจทางด้านซีรัมวิทยา ประกอบด้วยวิธี serum plate agglutination (SPA), hemagglutination inhibition (HI) และ enzyme-linked immunosorbent assay (ELISA) รวมทั้งวิธีทางอณูชีววิทยา ศึกษาการเปลี่ยนแปลงทางจุลพยาธิวิทยา หรือการฉีดตัวอย่างให้ไก่ที่ปลอดเชื้อ ปัจจุบันวิธีปฏิกิริยาลูกโซ่ (PCR) ถือเป็นวิธีการตรวจหากรดนิวคลีอิกของเชื้อโดยตรง ที่ให้ผลรวดเร็วและมีความไวในการตรวจพบสูง หลังจากตรวจพบเชื้อด้วยวิธีปฏิกิริยาลูกโซ่แล้วจะทำการแยกชนิดย่อยของเชื้อด้วยวิธีการทางอณูชีววิทยา ส่วนมาตรการควบคุมการติดเชื้อ มัยโคพลาสมา กัลลิเซพติกุม จะให้ความสำคัญในการคงสภาพการเป็นฝูงพ่อแม่พันธุ์ที่ปลอดเชื้อ และใช้มาตรการการเลี้ยงแบบอายุเดียว หรือมีการนำไก่เข้า-ออกพร้อมกัน ทั้งฟาร์ม ร่วมกับการมีความเข้มงวดของการจัดการความปลอดภัยทางชีวภาพ ซึ่งการทำลายไก่ที่ติดเชื้อถือเป็นวิธีการจัดการป้องกันโรคที่ดีที่สุด แต่มีโอกาสสำเร็จได้ยากในสภาพการเลี้ยงไก่ในเชิงพาณิชย์ การทำวัคซีนมัยโคพลาสมาอาจมีประโยชน์ในการควบคุมการติดเชื้อในพ่อแม่พันธุ์ไก่เนื้อ โดยอาศัยหลักการที่ว่าการใช้วัคซีนเชื้อเป็น สามารถช่วยลดอาการทางคลินิก และสามารถแทนที่ เชื้อมัยโคพลาสมาที่อยู่ในพื้นที่ เมื่อมีการจัดโปรแกรมวัคซีนอย่างต่อเนื่อง

คำสำคัญ : มัยโคพลาสมา กัลลิเซพติกุม สัตว์ปีก การวินิจฉัย การควบคุม