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Comparison of Detection Methods for Mycoplasmas of Significance to the Poultry Industry

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1. Introduction

1.1 Description of *Mycoplasma*

Mycoplasmas sp. are prokaryote pseudo bacteria that lack a cell wall but have a cell membrane. The name *Mycoplasma* is derived from this characteristic, molli meaning “soft” and cute meaning “skin”. *Mycoplasmas* are taxonomically placed in the Class Mollicutes, Order Mycoplasmatales, and Family Mycoplasmataceae. This genus is distinguished from the other genera in the family by a growth requirement for cholesterol and an inability to hydrolyze urea. Members of the genus have a small genome (580 to 1350 Kb) and relatively low G+C % content (Papaszi et al. 2003). The small genome size is clearly reflected by the reduced metabolic capabilities of *Mycoplasmas*. *Mycoplasmas* lack pathways for cell wall production and biosynthesis of purines and also lack a functional tricarboxylic acid (TCA) cycle and a cytochromemediated electron transport-chain system. These organisms must obtain many of the necessary nutrients needed to sustain the organism from the environment. For this reason, *Mycoplasmas* are obligate parasites. This characteristic is also reflected in the ideal culturing temperature (37°C) the same body temperature as that of humans and many animals.

Mycoplasmas are the smallest self-replicating organisms. They were discovered in the late 1800's after being isolated from blood serum that had been enriched with cholesterol. In the 1950's Klinenberrger discovered a loss of the cell wall in the organism when she noticed that the *Mycoplasmas* were still able to divide even after being treated with antibiotics specific for inhibition of cell wall production. Currently, there are more than 120 named *Mycoplasma* species (<http://www.ncbi.nlm.nih.gov/>).

1.2 Mechanisms of pathogenesis

Mycoplasmas have a variety of animal hosts including humans and are capable of producing disease in many of these hosts. Of the 120 named species, 20 infect poultry with *Mycoplasma gallisepticum* and *Mycoplasma synoviae* being most commonly isolated from chickens (Kleven

2008). *Mycoplasmas* typically cause respiratory diseases in their host and in chickens the disease is characterized by coughing, nasal discharge, and air sac lesions, but in some infections no clinical symptoms appear (Feberwee et al. 2005a).

Although *Mycoplasmas* are typically isolated from the respiratory tract, they have also been isolated from the reproductive organs, brain and eyes of poultry. Once infected, *Mycoplasmas* must adhere to the surfaces of epithelial cells for successful colonization. The molecular mechanisms of pathogenesis have been investigated and along with whole genome sequencing, much of the disease process has been described (Papazisi et al., 2002; Papazisi et al., 2003).

Research into the molecular mechanisms of *M. gallisepticum* attachment and subsequent virulence has identified a specialized terminal organelle, or bleb-like structure, that serves as an attachment tip (Papazisi et al., 2002). Other potential adhesion structures include surface proteins containing highly reiterated domains. These proteins are members of large gene families, and individual members often undergo high-frequency phase variation which is thought to promote evasion of the host immune system (Dybvig and Voelker 1996).

Current theory argues that *Mycoplasmas* remain attached to the surface of epithelial cells and invasion is either not likely or does not occur significantly (King 1993). During attachment, damage to host tissues takes place releasing nutrients that can be utilized. *Mycoplasmas* primarily infect the respiratory tract causing damage to the ciliated epithelial cells lining the trachea. Ciliostasis results and mucus is not moved upwards out of the trachea which also prevents the organism from being removed.

During attachment of *Mycoplasmas* to the surface of host cells, interference with membrane receptors or altered transport mechanisms of the host cell can occur. Although no known toxins have been described, *Mycoplasmas* can produce metabolites and enzymes that are toxic to the epithelial cells. *Mycoplasmas* may also hydrolyze phospholipids utilizing phospholipases which compromises the host cell membrane. In addition, the host cell membrane is also vulnerable to peroxide and superoxide radicals (Amikan et al. 1984).

1.3 Costs for poultry production

M. gallisepticum and *M. synoviae* are the most common poultry pathogens and can impact breeder, broiler, and egg laying production. For layer operations, reductions in egg production are estimated at \$140 million annually (Peebles et al. 2006). In broilers, a reduced feed conversion efficiency, depressed growth rate, and condemnation of carcasses can be economically devastating. Losses as high as \$750,000 have been reported from a single outbreak of *M. gallisepticum* (Evans et al. 2005).

Economic burdens of *M. gallisepticum* and *M. synoviae* also include the cost of monitoring and detection. Culturing is a time consuming and lengthy process requiring multiple types of media and regular man hours. Serology is more rapid, but costs are also high for this method. Molecular based approaches are less costly however the initial investment in equipment can be expensive. For some producers, especially breeders, the choice may be to utilize a combination of all three for confirmation and assured detection. This approach can be quite costly, but may be worth the investment considering the cost of a loss of a breeder flock.

2. Detection methods

Antibiotics can be used to treat poultry for a *Mycoplasma* infection, but may not be fully effective at clearing the infection (Gautier-Bouchardon et al. 2002; Reinhardt et al. 2005). In most instances, it is necessary to eradicate the entire flock. Because *Mycoplasma* infection may not result in outward symptoms, a stringent biosecurity and biosurveillance practice which can facilitate early intervention strategies are necessary to control *Mycoplasma* infections. Currently, methods for detecting *Mycoplasma* infection that are typically used include culture, serology or molecular assays. Traditional culturing is not commonly utilized because the method is time consuming, the organism is slow growing, and some fastidious strains may not be detected (Dewitt 2000). Serology is much faster than culturing, but disadvantages of serology include non-specific reactions and cross-reactions between species, misinterpretations due to recent vaccination for *Mycoplasma*, and cost are all disadvantages (Feberwee et al. 2005b). Furthermore, antibodies to *M. gallisepticum* and *M. synoviae* may not be detected until 1 to 3 weeks post-infection (Kleven 1975). The following sections will describe these three techniques for detection and give advantages and disadvantages for each method.

2.1 Culturing

As discussed in the earlier sections, culturing of *Mycoplasma* can be quite difficult due to the fastidious nature of the organism. Typically tissue samples are acquired from the respiratory tract such as the lungs, air sacs, or trachea. If whole organs such as lungs are utilized, a lavage can be performed with phosphate buffer saline (PBS). Inhibitors may be released from the host tissues during isolation if tissues are ground, but this problem can be overcome with the addition of chemicals or antibodies or by diluting the sample.

The samples are typically enriched in a broth medium with a meat-infusion base prior to plating. *M. gallisepticum* and *M. synoviae* require cholesterol and other fatty acids as a nutrient source. Supplemental antibiotics are also added to inhibit competing organisms. Frey et al. (1968) developed a culture medium that is widely used in the United States of America (USA) and other countries for isolation of *M. gallisepticum* and *M. synoviae*. Nicotinamide adenine dinucleotide (NAD) is added for the isolation of MS, but it may be omitted for the cultivation of *M. gallisepticum*. A soft agar is typically utilized (6-8%) with a neutral pH (7.4 to 7.6) and plates incubated at 37°C in a moist environment.

Colonies display a fried egg shape on agar. For confirmation, commercially available antibodies specific for *Mycoplasma* with fluorescent tags can be used as well as growth tests utilizing antiserum. Preservation of cultures is similar to preservation of most bacteria. Freezing at lower temperatures will preserve the cultures for an extended period of time and adding a cryoprotecting reagent can also extend the life of the culture.

Culturing is considered the gold standard. Isolating these organisms can be very useful for further diagnostic and future epidemiological studies. Pure cultures can be characterized phenotypically and genotypically which makes culturing advantageous over serology and molecular based detection techniques. However, due to the sensitive nature of this bacterium, culturing can be labor intensive and unsuccessful. For example, Jarquin et al. (2009) compared isolation techniques and found culturing produced the greatest number of false positives when compared with serology and molecular detection techniques. The authors suggested that the time gap from sample collection to processing may have resulted

in loss of cultures. In addition, the study pointed out that freezing the tissue samples may have also affected culture recovery.

2.2 Serology

Serological based assays utilized in poultry are aimed at detecting any antibodies produced by the host in response to *Mycoplasma* infection. Blood is collected from the birds and the collected sample is allowed to separate. The serum then can be used in an antibody based assay. Assays are usually in one of three formats: plate agglutination, hemagglutination inhibition (HI), or ELISA (enzyme labeled immunosorbent assay). Plate agglutination detects IgM, while HI and ELISA detect IgG.

Plate agglutination is a very simple assay in which serum is mixed with *Mycoplasma* antigens on a glass slide and positive results can be rapidly visualized by clumping due to the antibody binding with the antigens. Plate agglutination detects IgM antibodies which are pentamers and thus, bind well to antigens. The general term agglutinin is used to describe antibodies that agglutinate to antigens. When the antigen is an erythrocyte the term hemagglutination is used. For *Mycoplasma* specifically, the plate agglutination is an assay where serum is mixed with antigens specific for *M. gallisepticum* and *M. synoviae*.

Because hemagglutination inhibition (HI) detects IgG, infection cannot be detected as early as with HI compared to plate agglutination. The assay is performed in a microtiter plate composed of 96 wells. Like plate agglutination, positive results are visualized as a cloud (inhibition of agglutination of erythrocytes) due to the antibody-antigen binding. A microtiter plate can be used where each well has a varying concentration of antibody-antigen. In this way, it is possible to quantify the amount of antibodies present in the serum sample. There are false negative results from plate agglutination and HI for two reasons: 1) early during the infection, not enough antibodies have been produced for the test to detect them (lack of sensitivity), and 2) the quality of the HI and plate agglutination antigens will impact the assay as insufficient titer of antigen will produce false negatives. These serum antigens vary considerably in titers and quality. Hence the need for internal quantitative controls is necessary to make sure each new bottle of antigen has the same or similar titer as the previous one.

Plate agglutination and HI assays are both prone to false positives. Several factors can lead to false positives but the primary contributor is vaccination with *mycoplasma* vaccines. Vaccination simulates the production of antibodies that can circulate for 2 to 5 weeks. Contaminated serum, frozen and thawed serum, and cross-reactions to other antibodies can also cause false positives. False positive reactions can be reduced by heating serum to 56°C for 30 minutes or by diluting serum (Butcher 2007). Typically, plate agglutination assays are more sensitive, but HI assays are more specific.

ELISA is the third type of antibody detecting assay. In this assay, antibodies or antigens are bound to the wells of a microtiter plate. The wells then are filled with diluted serum and given time for the binding reaction to occur. The wells are washed and a secondary antibody or antigen that is tagged with an enzyme-labeled anti-species conjugate. The addition of the enzyme chromogen reagent causes the color to develop. The amount of bound antibody or antigen is directly proportional to the intensity of the color developed. Thus, positive reactions can be visualized by noting a color change. The level of antibody present in the

sample can be quantified by measuring the color intensity by spectrophotometry and extrapolating the value from a standard curve.

HI and ELISA are typically used as conformational assays for the simple plate agglutination assay. HI and ELISA are comparatively more labor intensive and thus, not utilized as a primary method. These two methods also take more time than simple plate agglutination.

2.3 Molecular

Molecular based techniques have become increasingly popular. Polymerase chain reaction (PCR) assays which target and detect specific nucleic acid sequences, can give results in less than 24 hrs. Real-time PCR also detects specific nucleic acid sequences but utilizes a fluorescent based system so the amplification of the target can be monitored during the reaction. Real-Time PCR has additional advantages over traditional PCR including: 1) real time is more rapid and can be accomplished in as little as 40 minutes; 2) no post amplification processes are required which decreases total detection time, cost in terms of materials, and hazardous waste; 3) are more sensitive - some real time assays can detect as few as 10 template copies per 5µl sample; 4) questionable results can be confirmed using melting curves.

Most PCR based methods require the sample be suspended in a non-nutrient medium. Specific to poultry, cleft palatine swabs are usually performed and the swab is then suspended in nuclease free water to release the sample from the swab. Samples are subsequently heated to boiling which lyses the cells and releases the nucleic acids. Centrifugation of this preparation collects debris in the pellet while target nucleic acids remain in the supernatant.

There are several molecular assays available for detection of *M. gallisepticum* and *M. synoviae*. Jarquin et al. (2009) and Hess (2007) utilized primers that targeted the 16S ribosomal subunit. Carli and Eygor (2003) performed detection of *M. gallisepticum* with primers that were specific for a lipoprotein gene. Hammond et al. (2009) designed their primer set to target the *vhlA* gene. The *vhlA* gene is typically utilized for genotyping and differentiating strains (Hong et al. 2004). Thus, the authors were able to detect and sequence the PCR product which facilitated epidemiological tracking efforts. Ramirez et al. (2006) targeted the interspacer region (ISR) between the 16S and 23S rRNA genes to detect and distinguish *M. synoviae* from 22 other poultry *Mycoplasmas*. Raviv et al. (2007) used the same approach for *M. gallisepticum*. All of these different primer sets have not been compared therefore it is not known whether one primer set is more accurate or sensitive than another.

3. Intervention

As discussed earlier, intervention measures are typically not performed for infected birds. A constant monitoring program is a key to early intervention. In addition, a strict biosecurity protocol is also very helpful for preventing infections with *M. gallisepticum* and *M. synoviae*. Entire flocks can become infected in 2 to 10 days (Feberwee et al. 2005a) and given that antibiotics may take 3 days to be effective, the infection can be difficult to control once it has begun. Thus, the course of action is dependent on many factors including the type of birds that are being produced. The next section will discuss three types of production operations and how *M. gallisepticum* and *M. synoviae* are controlled in these operations.

3.1 Breeders

Primary breeder operations are by far the most expensive of all three types of operations. In these system, genetic lines of birds are well established and specific traits are maintained through genetic selection. Operations typically utilize farms for production however the farms are state of the art and kept extremely clean. The cost of one bird can be as great as \$5,000 and thus much time and effort is invested into maintaining a healthy population.

Primary breeders operate under the National Poultry Improvement Plan (NPIP; USDA 2009). The NPIP was formed in 1935 to target *Salmonella gallinarum* and *S. pullorum*. At this time, these bacteria were economically devastating to producers. Through cooperative vaccination and biosecurity, *S. gallinarum* and *S. pullorum* were eradicated from the U.S. Currently, *M. gallisepticum* and *M. synoviae* are a main focus of this program. Primary Breeders operating under the NPIP must comply with the program regulations that include the vending of *M. gallisepticum* and *M. synoviae* free birds.

Due to the high cost of primary breeder birds, infection with *M. gallisepticum* and *M. synoviae* are monitored frequently. Although the cost of monitoring can be expensive, given the cost of primary breeder birds, the investment in diagnostic assays is relatively low compared the potential cost of a loss of a flock. To control infection, breeders typically destroy entire flocks if *M. gallisepticum* or *M. synoviae* outbreaks occur. Since vending infected birds is not allowed under the NPIP program, eradication is the only solution.

3.2 Broilers

Many large scale broiler operations house anywhere from 15,000 to 30,000 birds per house. Each bird is given approximately 1 sq. ft. of space. Due to the proximity of the birds, infection spreads rapidly. In a controlled setting, Feberwee et al. (2005b) designed a model to measure the rate of *M. gallisepticum* transmission. In this study, all birds were housed in separate cages that were 65 cm apart (approximately 2 feet). They found transmission occurred within 14 days from infected to uninfected birds. This study primarily focused on transmission via aerosols. However, in a broiler operation there are many other factors and modes of transmission including feed and water.

For broiler operations, the course of action a producer takes is dependent on the time of infection. Broilers are typically raised for a total of 42 days prior to slaughter. Infection of young birds can lead to large losses. Younger birds have an immature immune system and cannot clear the infection. Vaccination can be done at the hatchery but vaccination is not always fully effective at preventing infection. In addition to loss of birds due to death, producers may suffer economic losses because *M. gallisepticum* and *M. synoviae* infections can reduce production parameters, and cause plant condemnations due to airsacculitis. Thus, even if the infection can be treated, a reduced bird size at the end of the rearing period can occur. If infection occurs late in the production cycle, a producer may not suffer any losses and no course of action may be required. Control of *M. gallisepticum* and *M. synoviae* in broilers has been recently reviewed (Kleven 2008).

3.3 Layers

Egg laying production systems can also be impacted by *M. gallisepticum* and *M. synoviae*. A marked reduction in egg production may result from infection with *M. gallisepticum* and *M.*

synoviae. It has been reported that *M. gallisepticum* and *M. synoviae* can cause 20-30% reduction in egg production (North 1984). Furthermore, eggs with pimpled shells are also associated with *Mycoplasma* infections (Branton et al., 1995). Since egg laying hens have relatively longer periods of production compared to broilers (80 weeks or more), once infected it is nearly impossible to eliminate the infection and therefore, production can be affected for the life of the flock.

Vaccination of laying hens is performed at 12 weeks of age and delivered in the drinking water (Usman and Diarra 2008). However, *Mycoplasma* infection can be transmitted vertically. *Mycoplasma* vertical transmission can be controlled by incubating eggs at a relatively higher temperature (46°C). *Mycoplasmas* cannot survive this temperature, however a reduction in hatchability may result (Usman and Diarra 2008). Thus, like other production types rigid biosecurity and a constant monitoring system can reduce the risk of *Mycoplasma* infection.

4. Conclusions and future directions

Because *Mycoplasma* can be so economically devastating, control using a monitoring system and strict biosecurity are both necessary. The NPIP program has been successful in the past with eradication of other poultry significant pathogens. Whether or not *M. gallisepticum* and *M. synoviae* can be eradicated will be a matter of time. The program targets breeder operations and therefore uses a top down approach. By controlling *M. gallisepticum* and *M. synoviae* at the breeder level, it may be more effective in preventing dissemination to the production farms. One significant source of *M. gallisepticum* and *M. synoviae* is backyard flocks. These flocks are typically small and owned for personal use. These backyard chickens are exposed to more wild animals which may be sources of *M. gallisepticum* and *M. synoviae* and biosecurity is completely absent. Thus, backyard birds can serve as a potential reservoir for the pathogens.

Current research is exploring vaccines and alternatives to antibiotics. Antibiotic alternatives include treatments such as bacteriophage and recombinant vaccines. At this point, there are no treatments or preventive therapies that are 100% effective. Therefore, prevention through biosecurity and monitoring are the only options.

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Serological Diagnosis of Certain Human, Animal and Plant Diseases

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This book explains the concept of serological methods used in laboratory diagnoses of certain bacteria, mycoplasmas, viruses in humans, animals and plants, certain parasitic agents as well as autoimmune disease. The authors present up-to-date information concerning the serological methods in laboratory diagnosis of such infectious diseases. Section one deals with the serological methods for bacteria. Section 2 deals with serological methods in human, animal and plant viruses. Section 3 is concerned with the serological laboratory diagnosis of echinococcus and human toxocariasis agents. The last section deals with serological laboratory methods in the diagnosis of coeliac disease.

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