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Diagnosis of *Mycoplasma gallisepticum* from a Broiler Breeder Flock: Comparison of Three Diagnostics Methods

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Abstract: NPIP-mandated serological screening of a multiplier breeder flock detected possible *Mycoplasma gallisepticum* (MG) exposure. The flock was quarantined and further samples including blood and choanal swabs were collected and sent to a research facility for independent testing and confirmation. Subsequent analyses included diagnosis by Serum Plate Agglutination (SPA), MG-specific Polymerase Chain Reaction (PCR) and culture identification. Results of the various diagnostic tests were compared. The findings of the various diagnostic tests were in agreement and confirmed MG infection of the breeder flock. Time requirements of the various diagnostic procedures were recorded and were 1 h, 27 h and 30 days for SPA, MG-specific PCR and culture identification, respectively. The results affirm the validity of the diagnostic procedures and emphasize the importance of timely screening and diagnostic procedures for control of MG.

Key words: *Mycoplasma gallisepticum*, diagnostics, chronic respiratory disease, poultry, broiler breeders, NPIP

INTRODUCTION

Mycoplasma gallisepticum (MG) is a major and economically significant pathogen of avian species. In chickens, MG is the etiological agent of Chronic Respiratory Disease (CRD) and may be vertically or horizontally transmitted (Stipkovits and Kempf, 1996). Chickens infected with MG exhibit reductions in egg production, hatchability and feed efficiency and increases in mortality, carcass condemnation and infection-associated medication costs (Ley and Yoder, 1997; Stipkovits and Kempf, 1996). Cumulatively, these potential performance and production losses necessitate the stringent control of MG among all sectors of the poultry industry.

Within the broiler industry, MG control has been largely realized due to intense biosecurity and the “all in-all out” nature of this sector. Further, broiler breeders have successfully cooperated in a voluntary breeder biosecurity and biosurveillance program, the National Poultry Improvement Plan (NPIP). NPIP compliance minimizes exposure risks to MG and monitors flock status reducing MG breaks with this sector. Guidelines set forth by the NPIP program describe biosecurity practices, testing protocols, individualized sampling schedules and result interpretations. NPIP-approved testing protocols for MG diagnostics include culture, Serum Plate Agglutination (SPA), Hemagglutination Inhibition (HI) test, Enzyme-linked Immunosorbent Assay (ELISA) and MG-specific Polymerase Chain Reaction (PCR) (NPIP, 2009). These MG diagnostics are performed at a variety of publicly- or privately-owned facilities, though the individual tests used to detect MG infection may vary among diagnostic laboratories.

The report herein describes the MG diagnosis of broiler breeders via three NPIP-approved methods and compares method-associated results. In addition, a method-associated timeline is presented comparing time requirements for these diagnostic means.

Case history: Compliant to NPIP-mandated guidelines (NPIP, 2009), a multiplier broiler breeder flock was diagnosed for MG exposure. The flock consisted of 28 week of age (w.o.a.) Ross X Ross breeders housed in three adjacent breeding houses with approximately 15,000 birds per house. The flock originated from U.S. MG Clean sources and demonstrated no clinical signs of MG infection. To assess MG-status, blood samples were collected from the wing vein of 150 breeders by an agent of the state veterinary agency (Mississippi Board of Animal Health). Samples were then processed by the state laboratory (Mississippi Diagnostic Laboratory, Jackson, MS) and tested serologically via ELISA. ELISA results indicated that 10 samples were serologically positive for MG exposure. Follow-up analyses of the ELISA-positive samples by HI tests concluded that 9 of the 10 serum samples had titers between 1:80 and 1:160. With positive ELISA titers and HI titers of 1:80 or greater and in accordance with the NPIP guidelines, the flock was presumed to be MG-infected and quarantined under supervision of the Mississippi Board of Animal Health awaiting confirmation of the MG status by further sampling and testing (NPIP, 2009).

MATERIALS AND METHODS

Serology: Subjects were bled from the cutanea ulnae (wing) vein. Samples were chilled and transported to the research facility (Poultry Research Unit, Mississippi State, MS). Serum components were separated by sedimentation of the red blood cells and serum samples were tested for antibodies to MG by SPA analysis adapted from Yoder (1975). Briefly, 25 µL of antigen were mixed with 25 µL of serum on a ruled glass plate. The mixture was rotated for approximately 3 min. Agglutination determination was as outlined by Stanley *et al*. (2001).

MG isolation and culture: Thirty birds (10 birds/house) were swabbed (choanal cleft/palatine fissure) and resulting swabs were individually used to inoculate 3 mL of modified Frey's broth media (Frey *et al*., 1968) containing 3,000,000 U/L Pen G, 35 g/L thallium acetate and the color indicator phenol red (Branton *et al*., 1984). Inoculated broth cultures were subsequently chilled and transported to the research facility (Poultry Research Unit, Mississippi State, MS). The broth cultures (n = 30) were then incubated at 37°C. At 24 h, a 1 mL aliquot of each culture was collected for use in PCR identification. The remaining culture volumes (.2 mL) were returned to 37°C until sufficient microbial growth had incurred as predicted by color indicator change due to culture pH shift. Upon color indicator change, .10 µL was struck on individual Frey's agar plates. Inoculated plates were incubated at 37°C until colonies were visible.

MG identification/diagnostics: MG colonies on Frey's agar plates were identified by immunofluorescence, as described by (Kleven, 1981; Baas and Jasper, 1972).

For PCR identification, genomic DNA was extracted using the InstaGene Purification Matrix (Bio-Rad Laboratories, Hercules, CA). Briefly, cell pellets resulting from 1 mL of 24 h culture were washed in 150 mM (pH 7.2) Phosphate Buffered Saline (PBS) (Sigma Aldrich, St. Louis, MO) and resuspended in 200 µL InstaGene matrix. Following a 30 min incubation at 56°C, cell suspensions were boiled (100°C) for 10 min, centrifuged (20,000x g, 5 min) and resulting supernatants were used directly for PCR. Control DNAs were similarly isolated from 1 mL individual overnight cultures derived from laboratory stocks of strain 6/85 and the F strain of MG.

MG-specific PCR was adapted from Lauerman (1998). Briefly, the 50 µL PCR mixture for each reaction contained 19.6 µL of H_2O, 1 µL of 10 mM dNTP solution (Sigma Aldrich, St. Louis, MO), 5 µL of 10X buffer (Promega, Madison, WI), 3 µL of 25 mM $MgCl_2$ solution, 0.5 µL of each Lauerman primer (MG-13R and MG-14F) solution (50 µM), 0.4 µL (2 U) of *Taq* DNA polymerase (Promega, Madison, WI) and 20 µL of template solution. PCR reactions were carried out on a DNA Engine PTC-200 (Bio-Rad Laboratories, Hercules, CA) thermocycler. The reaction program included an initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min and concluded with a 5 min 72°C extension step. PCR products were determined via gel electrophoresis on a 1.0% agarose gel containing 0.5 µg/mL ethidium bromide and visualized on a ChemiDoc system (Bio-Rad Laboratories, Hercules, CA).

RESULTS

Upon receipt of the clinical samples, blood samples were immediately processed and SPA analyses performed. MG antibodies were detected in 100% (30/30) of the clinical samples with an agglutination grade range from 1-4 and a mean grade of 2.17. Separation of blood serum components and subsequent SPA analysis required approximately 1.0 h (Table 1).

Swab-inoculated broth cultures were incubated at 37°C for 24 h prior to removal of 1 mL aliquots from each sample for MG-specific PCR analysis. Results of MG-specific PCR are shown as Fig. 1. Variable levels of product were detected in 36.7% (11/30) of the clinical samples and these products corresponded in size with MG control DNA's indicating the presence of MG genomic DNA in these samples. Sample preparation for MG-specific PCR required 1.0 h, completion of the PCR reaction required approximately 1 h and electrophoresis of PCR products required 1 h (Table 1). Cumulatively, MG-specific PCR required .27 h.

Further incubation of inoculated broth cultures at 37°C resulted in color indicator change in 14 of 30 cultures of swab-inoculated cultures. These cultures were streaked individually on Frey's agar plates and incubated at 37°C until isolated colonies were visible. Plates exhibiting the earliest bacterial colonies were used for identification of MG via immunofluorescence (Kleven, 1981; Baas and Jasper, 1972). Multiple colonies were tested on each of these plates and were identified as MG (data not shown). Total time required for identification via immunofluorescence was .30 days (Table 1).

DISCUSSION

Within the broiler industry, diseases such as MG have been minimized through the National Poultry Improvement Plan surveillance program. Broiler flocks originate from monitored MG-free breeders and are reared physically isolated from sources of MG infection. NPIP guidelines outline biosecurity and biosurveillance practices to protect broiler and broiler breeder flocks from MG infection. However, MG breaks sporadically occur and immediate steps including flock quarantine or eradication are taken to limit further transmission and risk to neighboring flocks. Subsequently, accurate and timely diagnoses of MG breaks are critical for control of

the avian pathogen.

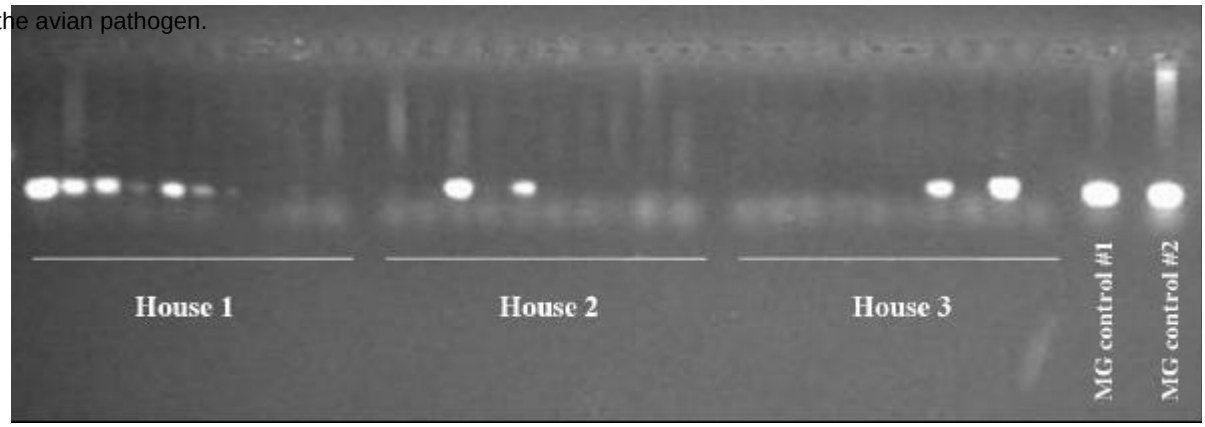

Fig. 1: Electrophoresis of MG-specific PCR products on a 1% agarose gel. Gel depicts PCR products from 10 randomly selected birds from three neighboring houses (house 1, house 2 and house 3) and 2 positive laboratory controls.

Table 1: Time requirements for MG diagnostic procedures

Diagnostic Test	Time Required
SPA	1 h
MG-Specific PCR	27 h
MG Culture/Immunofluorescence	30 days

In the present case, initial testing of a flock of broiler breeders by the state veterinary agency (Mississippi Board of Animal Health) yielded positive ELISA titers and HI titers of 1:80. The flock was presumed infected and subsequently choanal cleft swabs and blood samples were promptly collected from 30 randomly selected birds for SPA, MG-specific PCR and culture analyses. Upon receipt at the laboratory, each test was immediately initiated and run simultaneously and continuously through completion. Collectively, the three MG diagnostics means (SPA analysis, MG-specific PCR and MG culture) were in agreement and indicated MG infection confirming earlier findings by the state veterinary agency (Mississippi Board of Animal Health). The concurring results also further validate the selected diagnostic protocols and procedures. Supportive to the findings, further serological sampling and testing of the flock by the state veterinary agency (Mississippi Board of Animal Health/ Mississippi Diagnostic Laboratory) yielded MG positive titers.

As previously mentioned, timely diagnosis of MG infection is vital for control of MG. In our experience, SPA analysis, which detects the presence of MG antibodies in poultry sera is the most time-efficient diagnostic means, requiring less than 1 h upon receipt to complete. Although this method can be used for flock screening, the utilization of SPA towards MG diagnosis is limited by its reduced specificity and the associated high incidence of false positives necessitating confirmation by alternative diagnostic means (Avakian *et al*., 1988). NPIP-approved methods of confirmation of serological-based diagnoses include cultivation or a PCR-based procedure (NPIP, 2009). MG-specific PCR has been demonstrated as a simple, rapid and accurate means of MG diagnosis (Silveira *et al*., 1996). Confirmation by MG-specific PCR in our laboratory required approximately 27 h, 24 h of which were utilized for broth enrichment. While this enrichment is not required, its inclusion does increase the sensitivity of this diagnostic method. Cultivation and subsequent identification of MG has historically been the primary means of confirming diagnoses. However, its application is limited by associated time requirements. MG isolates are normally slow growing in laboratory media and the NPIP guidelines require at least a 21-day incubation of inoculated broth cultures before a MG-negative diagnosis can be made. Further, tubes indicating microbial growth are then transferred to solid media and allowed to incubate for 5 additional days before final MG determination (NPIP, 2009). In this study, microbial growth and subsequent MG identification required 30 days demonstrating the significant time requirements of this method.

In summary, the accurate and timely diagnosis of MG breaks is essential for control of the avian pathogen. The report herein describes the utilization of three NPIP-approved diagnostic means to confirm MG exposure of a broiler breeder flock and compares their associated findings and the time requirements associated with the techniques. Further, this report affirms the need for timely screening and diagnostic procedures to control the spread of the avian pathogen.

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Abbreviations: PCR = polymerase chain reaction; HI = hemagglutination inhibition; ELISA = enzyme-linked immunosorbent assay; SPA = serum plate agglutination; MG = *Mycoplasma gallisepticum*, NPIP = National Poultry Improvement Plan