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Original Article

Comparison of Mycoplasma gallisepticum Infection in Different Samples and Ages of Chicken Breeder Flocks

■Author(s)

Demirbilek SK[I] https://orcid.org/0000-0001-6138-7163
Ardicli Ö[I] https://orcid.org/0000-0001-6077-0478
Carli KT[I] https://orcid.org/0000-0001-6045-8644

[I] Uludag University Faculty of Veterinary Medicine Microbiology Department, Bursa, Turkey.

■Mail Address

Corresponding author e-mail address
Serpil Kahya Demirbil
Uludag University Faculty of Veterinary Medicine Microbiology Department, Bursa, Nilüfer, 16080, Turkey.
Phone: 0090 224 294 08 54
Email: serpilkahya@uludag.edu.tr

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ABSTRACT

This study aimed to compare method-based and newly developed sample-based methods for *Mycoplasma gallisepticum* (MG) detection in different samples of breeder flocks suffering from respiratory disease problems by using culture, real-time PCR (rPCR) and ELISA from chicks and embryonated eggs. Overall, 450 samples of 19-day-old chicken embryo's trachea, 450 samples of 8-day-old chicken tracheal swabs and 900 blood samples of 20-, 27-, 34-, 40- and 46-week-old breeder chickens from 5 flocks were sampled for 26 weeks, and were all tested for MG by culture, MG-rPCR and MG-ELISA. Culturing assays and rPCR were applied to 450 mixture samples from 19-day-old chicken embryo's trachea and 450 tracheal swab samples (each pooled into groups of 3) from 8-day-old chicks from the same flocks. Also, 900 blood samples from the same 5 breeder flocks suffering from respiratory disease problems were tested by MG-ELISA.

In individual sample-based analyses, 55 (18.3%) of the 300 pooled swab samples were positive for MG using culture methods, and 106 (35.3%) of the same samples were found positive by rPCR (sensitivity, specificity). The ELISAs indicated that 252 (28%) of the 900 breeding blood samples were MG seropositive. Using age-based analyses, the most positive period was 46 weeks, followed by 40 weeks, 34 weeks, 27 weeks and at least 20 weeks, in order of decreasing seropositivity. When comparing the culture and rPCR results of the two different sampling methods, chicken embryo's trachea yielded more positive results than did tracheal swabs from the same flocks. In conclusion, rPCR is a highly specific, sensitive and reliable method for MG identification.

INTRODUCTION

Mycoplasma gallisepticum (MG) represents an infectious agent of chronic respiratory diseases in chickens. MG infections are of great concern in the poultry sector because they cause high economic losses in poultry production. MG also causes important primary and secondary bacterial poultry diseases. These losses occur as a result of decreased hatchability and egg production, reduced quality of day-old chicks, increased mortality of chicks, reduced growth rate, increased costs of eradication procedures, and costly monitoring programmes. Due to the persistent nature of *Mycoplasma* infection and its vertical mode of transmission, monitoring and eradication is a preferable strategy for the achievement of a long-lasting *Mycoplasma*-free poultry stock (Raviv & Kleven, 2009). The reported rates of transovarian transmission of MG after the experimental infection vary, with peak egg transmission rates ranging from 14% to 53% between 3 and 8 weeks after infection, as reported in different studies with the R strain of MG (Ortiz *et al*., 1995).

There are several diagnostic methods for MG detection including culture and MG-polymerase chain reaction (PCR). Serology testing has been used in most MG laboratories (Ley, 2008). The diagnosis of Avian Mycoplasmosis is typically based on serological assays to detect antibody production and/or on isolation and identification of the organism. Cultivation techniques are laborious, slow, time-consuming (require 4-30 days), expensive and require sterile conditions. Other problems experienced with culture methods include overgrowth by faster-growing *Mycoplasma* species or other organisms, which also give low serological responses or no growth in subculture (Khalda *et al*., 2013). Serology including ELISA is much faster than culturing, but nonspecific reactions and cross-reactions between bacterial species, misinterpretations due to recent vaccination, and high cost are all disadvantages of serology. MG strains with low virulence typically produce a poor antibody response, and isolation from clinical specimens may be difficult (Kahya *et al*., 2014). Recently, the Office International Epizootie (OIE) and National Poultry Improvement Plan (NPIP) recommended PCR as a reliable test for the detection of MG infections.

Maintaining flocks that are free of MG infection first depends on the early and reliable detection of infected birds. Because of vertical transmission, breeder flocks are an important growing type in poultry. The sample type, sampling timing, sterile samples, antibiotic therapy, faster-growing *Mycoplasma* species, etc. are important factors for *Mycoplasma* detection. In this study, we compared the sample type and sample timing according to culture, PCR and serology results.

MATERIALS AND METHODS

Standard MG Strains

The MG S6 strain was kindly provided by the *Mycoplasma* Laboratory, Pendik Veterinary Research Institute in Istanbul, Turkey, and was used as a positive control.

Mixed Chicken Embryo's Trachea, Tracheal Swab and Blood Samples

During the 26-week trial period (including one spring and one autumn season), a total of 450 live chicken embryo's trachea and 450 tracheal swab samples, comprising 900 individual samples (Table 1), were each pooled into 3, from 5 breeder flocks. All flocks were from the same genetic background, and they were reared under the same environmental condition. Whole flocks had respiratory symptoms and did not receive antibiotic treatment. MG samples were transferred to the laboratory in dry ice within 6 h of collection for culture, real-time PCR (rPCR) and ELISA tests. Analysis of all samples was initiated immediately after the transfer to the laboratory. Egg trachea samples were pooled into one microcentrifuge tube and vortexed (mixture). The mixture was diluted to 10^{-4} with sterile phosphate buffered saline. Diluted samples were used in culture and as PCR templates. Tracheal swabs were vortexed in Frey's broth, and these suspensions were used for both culture and rPCR. Concurrently, 900 blood samples from the same chickens were collected and tested by MG-specific ELISA. All flocks had respiratory symptoms, and tracheal swab samples were taken from breeder flock embryos with marked respiratory symptoms.

MG Culture

Sampling from trachea of the chicken embryo was developed as a mixture, as mentioned above, sampling from trachea of the birds was performed (Zain & Bradbury, 1995) and the isolation of MG from these mixtures and tracheal swabs were done following the standard culture method described previously (Kleven, 2008). Briefly, the mix and tracheal swabs were first streaked onto Frey's agar, and the plates were incubated at 37°C with 5% CO_2 at high humidity. A 100 µl aliquot from the vortexed 500 ml sterile physiological saline containing the tracheal swab was transferred into Frey's broth and incubated at 37°C with 5% CO_2 at high humidity. A colour change in Frey's broth was observed daily, and the change in colour from pink to orange-yellow was considered positive. One week after incubation, cultured broths with unchanged colours were transferred into new Frey's broth, followed by one more passage if the colour was unchanged after a 1-week incubation. Positive broths were streaked onto Frey's agar and incubated at 37°C with 5% CO_2 at high humidity. Plates were checked for typical colonies under an inverted microscope for at least 2 weeks. Possible *Mycoplasma* colonies on plates were confirmed as MG by colony PCR. MG S6 culture was used as a positive control during culturing to assure the testing efficacy of the media used.

DNA Extraction

DNA was extracted from the pooled mix and tracheal swab samples and then suspended and vortexed in 1 ml of sterile PBS in 1.5 µl tubes. The suspension was centrifuged for 30 min at 14,000 x g at 4°C, and the swabs were discarded. The supernatant was carefully removed, and the pellet was resuspended in 25 µl of

sterile deionised water. Then, this mix was boiled at 95-100°C for 30 min and kept on ice for 10 min before centrifugation at 14,000 x g for 5 min. The supernatant was used as a template in rPCR analysis.

MG Primers

Forward and reverse PCR primers, MG1 (GAGCTAATCTGTAAAGTTGGTC) and MG2 (GCTTCCTTGCGGTTAGCAAC), were selected from a region within the sequence of MG 16S rRNA gene partial codons; this primer set had a previously determined sensitivity and specificity for MG (Kahya *et al.*, 2015), and the expected amplicon size was 185 bp.

MG rPCR

rPCR reactions were performed with the same conditions and cycling parameters by a LightCycler™ 2.0 system (Roche Diagnostics, Mannheim, Germany) using FastStart DNA Master SYBR Green I (SGI) PCR mix and reagents (Roche, catalogue No: 03 003 230 001) (Kahya *et al*., 2015). Each reaction was performed in 20 µl volumes, including 18 µl of reaction mixture containing 2 µl of 1X LC FastStart DNA SGI Master Mix, 4 mM $MgCl_2$, 0.5 mM of each primer and 2 µl of template DNA. The cycling parameters were as follows: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 50°C for 10 s and extension at 72°C for 20 s.

MG ELISA

ELISA was performed using a *Mycoplasma gallisepticum* antibody test kit (Synbiotics, catalogue No: AUCMG900, Zoetis) following the instructions described by the manufacturer.

Statistical analyses

Agreement analyses between rPCR and culture, different ages and different samples were performed in SPSS 17.00 by applying the McNemar Test.

RESULTS

MG Culture

In individual sample-based analyses, 55 (18.3%) of the 300 pooled swab samples were positive for MG using culture methods.

Specificity of rPCR

rPCR yielded specific amplification and T_m peaks of 78.39–79.96 °C with all standard MG strains tested.

MG rPCR

In individual sample-based analyses, and 106 (35.3%) of the 300 pooled swab samples were found positive by rPCR.

MG ELISA

The ELISAs indicated that 252 (28%) of the 900 breeding blood samples were MG seropositive.

Sample-based evaluation of serology, culture and rPCR results

On individual sample-based analyses, MG was detected in 55 (18.3%) of the 300 pooled swab samples by using culturing methods, and 106 (35.3%) of the same samples by rPCR. By ELISA, 252 (28%) of 900 breeding blood samples were MG seropositive (Table 1 and 2).

Table 1 – Comparison of embryo trachea and tracheal swab samples by 2 methods according to weeks.

	MG Recovery					
	From embryo trachea			From tracheal swabs		
Weeks/Pooled samples	Number of samples	Culture (%)	rPCR (%)	Number of samples	Culture (%)	rPCR (%)
20/180	30	4 (13.3)	5 (16.6)	30	5 (16.6)	7 (23.3)
27/180	30	3 (10)	7 (23.3)	30	4 (13.3)	8 (26.6)
34/180	30	5 (16.6)	9 (30)	30	4 (13.3)	7 (23.3)
40/180	30	6 (20)	13 (43.3)	30	7 (23.3)	17
46/180	30	7 (23.3)	18 (60)	30	10 (33.3)	15 (50)
Total/Average %		25 (16.6)	52 (34.6)		30 (19.9)	54 (35.9)

Table 2 – Serology, culture and rPCR results from flocks tested.

Flock no	Age of flock (week)	No. Culture positive/no tested	No. ELISA positive/no tested	No. rPCR positive/no tested
1	20	9/30	13/180	12/60
2	27	7/30	12/180	15/60
3	34	9/30	31/180	16/60
4	40	13/30	56/180	30/60
5	46	17/30	140/180	33/60
Total		55/150	252/900	106/300

Age-based assessment of serology, culture and rPCR results

In age-based analyses, the most positive period was 46 weeks, followed by 40 weeks, 34 weeks, 27 weeks and 20 weeks, in that order (Table 2).

Sample type's results

MG was detected 25 (16.6%) cultures, 52 (34.6%) rPCR samples from embryo trachea, 30 (19.9%) cultures, 54 (35.9%) rPCR samples from tracheal swabs (Table 1).

DISCUSSION

Mycoplasma gallisepticum is prevalent in chicken flocks, breeding flocks and their eggs and can be controlled periodically. Trachea are routinely used to isolate MG, as this is reported to be the predilection site for its multiplication and has been widely used for isolation purposes (Muhammed *et al*., 2012). Because of contamination problem with tracheal swabs by reason of normal microflora, we decided that embryonated eggs are a better sample for the detection of *Mycoplasma* spp. than are tracheal swabs. Therefore, egg samples can be a good alternative for the control of MG.

When we looked at previous embryonated egg studies, egg yolks were frequently investigated for MG; for example, in general, egg yolk antibodies were examined and compared to those in serum samples by ELISA (Hagan *et al*., 2004; Brown *et al*., 1991).

Muktar *et al*. (2012) isolated MG from different organs including trachea, air sacs and lungs of commercial layer chickens. Out of all the organs examined, 18.51% of trachea (n = 5/27) and 7.41% of air sacs (n = 2/27) were positive for MG, whereas no isolation was made from the lungs. Thus, the trachea is an important organ for MG detection. However, when chicken embryo hatch, the immune system will be established by commensal microorganisms. Obtaining a sterile sample from trachea becomes increasingly difficult as the organism develops. Thus, we investigated and compared these different sample types at different times to detect *Mycoplasma*.

The cultivation techniques of *Mycoplasma* are laborious, slow, expensive, time-consuming (require 4-30 days) and are complicated by overgrowth by faster-growing *Mycoplasma* species or other organisms, which also give low serological response or no growth in subculture (Khalda *et al.,* 2013). Sterile samples can be obtained, but it is almost impossible, especially in later years of the animal's life. *Mycoplasma* species are very fastidious organisms and require special growth media supplemented with activated amino acids, coenzymes and vitamins. MG is a nutritionally deficient organism that requires a complex medium enriched with 10-15% heat-inactivated swine, avian and horse serum as a source of cholesterol (Muhammed *et al*., 2012). Contamination can also be a problem for molecular tests, especially PCR, which is mostly used and independent of the viability of the organisms. PCR contamination can lead to false-negative or false-positive results. Contamination is as important for serological tests as it is for culture and molecular techniques. Serologically false-negative and false-positive results can be observed due to cross-contamination.

Out of 300 pooled swab samples, 55 (18.3%) were detected positive by using MG culture, and 106 (35.3%) of the same samples were found positive by rPCR. Kahya *et al.* (2010) also found a relatively low sensitivity of culture and rPCR methods for MG detection (50% and 80%, respectively), compared to that of serology, for determining MG-seropositive flocks. Although serology was positive in the majority of the flocks, the detection rate of microorganisms or DNA was low. When evaluated with clinical findings, the results may indicate the presence of chronic or vertical infection in these flocks. In the diagnosis of MG infection of chickens, Kesler *et al.* (2012) reported that serological tests are not sufficient alone because antibodies remain for a long time after infection. The comparison between PCR and culture from a common tracheal swab wash suspension has been reported by Kempf *et al*. (1993, 1994) for MG. The culture was positive for 49 of 73 swabs (67%), while 70 of 72 (97%) of the samples were found to be positive by the MG DNA probe test.

To resolve this contamination problem, we tried to use different samples from the same flock to detect MG (Table 2). We found that 25 (16.6%) cultures and 52 (34.6%) PCRs were positive for embryonic trachea; 30 (19.9%) cultures and 54 (35.9%) PCRs were positive from the tracheal swabs. Accordingly, our hypothesis did not appear to be suitable. We detected more positive results from tracheal swabs than those from trachea samples using both culture methods and PCR. This result could be due to the combination of vertical and horizontal transmission of MG; i.e., some chicks had vertical infection, and other chicks had gained horizontal infection from the environment until sampled. Unlike our results, Cengiz

et al. (2011) collected trachea and tracheal swab samples from various broiler flocks and found that 14 of 26 investigated flocks (53.8%) were MG positive from trachea, 5 (19.2%) flocks were considered to be positive from tracheal swabs. Their result showed that MG infections can be diagnosed rapidly and more specifically and that more positive results can be achieved in tracheal organ samples than those in tracheal swabs.

Different from our study Sasipreeyajan *et al.* (1987) compared MG detection from eggs and 18-day-old embryos, and MG was isolated at a higher rate from the yolk sac of 18-day-old embryos than it was from the air sac or oral cavity of the same embryos. MG was isolated at a significantly higher rate from eggs than from 18-day-old embryos. MG was isolated at a high rate from the yolk sac of 18-day-old embryos than from the air sacs or oral cavity of the same embryos.

It can be concluded that embryo trachea and tracheal swabs can be used in laboratories for the rapid and accurate detection of MG. Vertical transmission is important for MG along with lateral transmission in later weeks when the MG infection rate increases. We found a relatively low sensitivity of culture and rPCR of 18.3% and 35.3%, respectively, compared to that of serology for determining MG-seropositive results. Thus, we recommend the rPCR test for MG diagnosis for MG-seropositive flocks. The correct detection method and correct time of sampling can be adapted to protect chickens and breeder flocks.

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