

Short Communication

Diagnosis of Avian Mycoplasmas: A Comparison between PCR and Culture Technique

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ABSTRACT

Mycoplasma gallisepticum and *Mycoplasma synoviae* are the causative agents of avian mycoplasmosis in commercial poultry. Among the available tools, polymerase chain reaction (PCR) and culture are confirmatory tools for the diagnosis of mycoplasmosis after the initial serological screening of suspected birds. Overall, 181 samples were analyzed, 152 (84%) and 103 (57%) of which were found positive by PCR and culture, respectively. Further, 54 (92%) broiler samples were found positive for general avian mycoplasma. Among the total positive samples, MS positivity was as high as 72 (47%) by PCR, while it was 45 (44%) by culture. MG positivity was 23% and 25% in PCR- and culture-positive samples. MG grows more easily compared to MS. The agreement value between the tests was 67%. Overall, flock wise prevalence was not much varied. The prevalence of mycoplasmosis was higher during winter. Our study confirmed that PCR is the most sensitive and reliable tool for the diagnosis of avian mycoplasmosis in field samples.

Keywords: Avian mycoplasmosis, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, PCR, culture

Diagnostic des Mycoplasmes Aviaires: Comparaison entre la PCR et la Technique de Culture

Résumé: *Mycoplasma gallisepticum* et *Mycoplasma synoviae* sont les agents responsables de la mycoplasmosé aviaire chez les volailles commerciales. Parmi les outils disponibles, l'amplification en chaîne par polymérase (PCR) et la culture représentent des méthodes de confirmation pour le diagnostic de la mycoplasmosé après le dépistage sérologique initial des oiseaux suspectés. Au total, 181 échantillons ont été analysés, 152 (84%) et 103 (57%) se sont révélés positifs par ACP et culture, respectivement. De plus, 54 (92%) des échantillons de poulet de chair étaient positifs pour les mycoplasmes aviaires communs. Parmi les échantillons positifs détectés par PCR, 72 étaient positifs pour MS (47%), alors que ce nombre était réduit 45 (44%) pour les échantillons analysés par culture. La positivité du MG était respectivement de 23% et 25% dans les échantillons analysés par PCR et culture. MG semblait proliférer plus facilement que MS. La valeur de l'accord entre les tests était de 67%. Globalement, la prévalence évaluée dans les différents troupeaux n'était pas très variable. La prévalence de la mycoplasmosé était plus élevée en hiver. Notre étude a confirmé que la PCR est l'outil le plus sensible et le plus fiable pour le diagnostic de la mycoplasmosé aviaire dans les relevés de terrain.

Mots-clés: Mycoplasmosé Aviaire, *Mycoplasma gallispticum*, *Mycoplasma synoviae*, ACP, Culture

INTRODUCTION

Avian mycoplasmas remain to be a significant threat to the poultry industry causing respiratory diseases worldwide. It leads to poor quality meat, low egg production, and decreased feed conversion rate with high rate of carcasses condemnation resulting in heavy economic losses (Mohammad et al., 1987). The organism is devoid of cell wall and is characterized by phenotypes, serology, and 16s rRNA sequences (Brown et al., 2007). There are more than 120 species, some of which are involved in mild to chronic poultry diseases, among these *M. gallisepticum* and *M. synoviae* are sporadic and endemic in certain parts of the world (Stipkovits and Kemp, 1996). Early laboratory diagnosis is an important factor in controlling an infectious and contagious disease with isolation, identification of the causative agent, and evaluating seroconversion. The serodiagnostic methods are mainly employed for presumptive control measures. The World Organization for Animal Health has recommended serological tests for screening of flocks (Luciano, 2012). Nonetheless, pathogen isolation is always the gold standard (Kleven, 2008) followed by the detection of its unique sequence through PCR (Luciano, 2012). Being a slow grower in nature and having fastidious nutrient requirements, culture method is less effective for the isolation and diagnosis of mycoplasma. Additionally, commensals and fast-growing species of the Mycoplasma genus (i.e., *M. gallinarium*, *M. gallinaceum*, and *Acholeplasma* spp.) hamper the growth of *M. gallisepticum* and *M. synoviae* (Kleven, 2008). Therefore, PCR has become the alternative test for the direct detection of pathogens in clinical samples (Lauerman et al., 1993; Gracia et al., 2005). A few molecular and cultural studies have been carried out to determine the prevalence of avian mycoplasmas in commercial chicken industry of Pakistan (Arshad et al., 2013). Although mycoplasmosis incidence occurs throughout the year, the incidence is much higher during winter (Ferberwee et al., 2008; Buim et al., 2009; Sun et al., 2014; Kahya et al., 2015). Therefore, this study was designed for the

detection of avian mycoplasmas by PCR and comparison of PCR with culture technique.

MATERIALS AND METHODS

Sampling. The present study was conducted during December 2015-February 2016. Tracheal samples of birds with postmortem lesions suspected for chronic respiratory disease were obtained from commercial diagnostic laboratories. The following parameters were considered such as dyspnea, pronounced respiratory rales with sneezing, coughing, nasal discharge, low egg production, and downgraded body weight. All the (n=181) clinical samples were analyzed for the presence of mycoplasmas by culture and PCR simultaneously.

Culture of Mycoplasmas. The samples were swabbed with a wet wooden-stick cotton swab and inoculated into 2.0 ml of modified Frey broth (Frey, 1968). The swabs were vortexed after 60-90 min of incubation and discarded. Then, 1 ml of broth sample was separated for PCR, and the rest was incubated at 37 °C until turbidity was achieved and then streaked on agar for colony development. Cultures were identified by their growth pattern and biochemical reactions (Bradbury et al., 1993) and then confirmed by PCR using species- and genus-specific primers.

Polymerase chain reaction (PCR). DNA was extracted for PCR by simple heating block method. Briefly, 1.0 ml of broth sample was centrifuged at 15000 x g for 5 min and washed twice with phosphate buffered saline (PBS) (pH 7.2). The pellet was resuspended in 25 µl of sterile demineralized water and heated up to 100 °C in heating block for 10 min, then it was shifted to ice for another 5 min. The supernatant was collected after centrifugation and stored at -20 °C until use. The amplification mixture contained 0.2 mM of dNTPs, 1.5 mM of MgCl₂, 0.2 µM of each primer, and 0.5 U of Taq Polymerase (Promega). Finally, 2 µl of template was added to the final 20 µl of master mix. The amplified products were visualized in 1.5% agarose gel stained with ethidium bromide (0.5 µg/mL) after electrophoresis in TBE buffer. For MG and MS,

species-specific primers were used (Table 1). Those mycoplasma cultures that were not identified through species-specific primers were amplified by genus-specific primer (Table 1) based on 16s rDNA (unpublished data). The PCR products were sequenced, and BLAST ([https:// blast. ncbi. nlm.nih.gov /Blast](https://blast.ncbi.nlm.nih.gov/Blast)) was used for the identification of the mycoplasma species.

RESULTS AND DISCUSSION

Most of the *M. synoviae* cultures were isolated as pure, while *M. gallisepticum* was generally isolated as mixed culture. The non-pathogenic mycoplasma colonies appeared within 18-24 h of incubation, while *M. gallisepticum* took 48-96 h to fully develop typically-shaped colonies. *M. synoviae* was detected in broilers more than *M. gallisepticum*. Upon comparison of flock wise distribution of avian mycoplasmas (Table 2), most broiler samples (92%) were found positive for mycoplasmas, especially *M. synoviae* (52% by PCR). Moreover, *M. synoviae* positivity was highest (47%) by PCR, while it was 43% by culture. Among the 181 samples, 84% (152) were identified to contain mycoplasmas by PCR, while 57% (103) of PCR-positive

samples were found cultivable. This indicates that PCR is a more powerful tool for the detection of infection. The agreement value between the two diagnostic tests was 67%. *M. gallisepticum* was found more culturable as 74% (26) of *M. gallisepticums* were positive in PCR as compared to *M. synoviae*, where 62% (45) of PCR-positive samples of *M. synoviae* were culturable (as shown in Figure 1). The economic significance of *M. gallisepticum* is much higher than *M. synoviae* (Mohammad et.al., 1987). However, the economic impact of *M. synoviae* is now increasing worldwide by affecting egg shell quality and egg production with the recent emergence of arthropathic and amyloidogenic strains. Reduced body weight and poor feed conversion in broiler birds has long been reported in *M. synoviae* -infected broilers (King et al, 1973). Moreover, the seroprevalence of *M. synoviae* compared with *M. gallisepticum* was found higher in poultry industry (Landman, 2014). Synergism with other avian pathogens such as New castle disease virus and infectious bronchitis virus (Feberwee et al., 2009) further increases its significance in commercial poultry industry. Our findings were not in alignment with those of Marois et al. (2002) since *M. gallisepticum* was

Table 1. Polymerase chain reaction primer sequence used for avian mycoplasmas identification

Targeted species	Primer Sequences	Target gene	Cycles	PCR product size
<i>Mycoplasma</i> -genus specific	F-5'AATACATAGGTTGCAAGCGTTATC3' R-5'CCCCACGTCTCTCGTAGG3'	16S rRNA	95 °C –5min 95 °C – 30 sec 55 °C – 30sec 72 °C – 1min	980 bp
<i>M. gallisepticum</i> (Gracia et al., 2005)	F-5'AGGCAGCAGTAGGGAAT3' R-5'CGTTCTCGGGTCTTGTA3'	<i>mgc 2</i>	94 °C – 5min 94 °C – 30 sec 58 °C – 30 sec 72 °C – 1min	236-302 bp
<i>M. synoviae</i> (Leuerman et al., 1993)	F-5'GAGAAGCAAATAGTGATATCA3' R-5'CAGTCGTCTCCGAAGTTAACAA3'	16s rRNA	94 °C – 5min 94 °C – 30 sec 55 °C – 30 sec 72 °C – 1min	207 bp

Table 2. Flock wise distribution of avian mycoplasmas

Flock types	PCR				Culture			
	Myco	MG	MS	NP	Myco	MG	MS	NP
Broiler (n=59)	54 (92%)	9	28	15	29 (49%)	5	12	12
Layer (n=101)	82 (81%)	22	34	28	58 (57%)	17	23	18
Breeder (n=21)	16 (76%)	4	10	2	16 (76%)	4	10	2
Total (n=181)	152 (84%)	35	72	45	103(57%)	26	45	32

cultured more easily than *M. synoviae* from field samples during our study. *M. synoviae* is commonly isolated from synovial membrane and the upper respiratory system in serologically and clinically healthy birds (Feberwee et al., 2008). The results of PCR and culture did not match as most of the samples were collected from dead birds, sometimes after postmortem examination and the samples were not properly stored at low temperatures for culture. This delay usually results in the death of mycoplasma, however, dead organisms can be detected by PCR. However, culture technique depends on proper sample collection and storage. The careful handling of sample is very necessary for live microorganisms because mycoplasmas are very fragile and die fast with prolonged inoculation into the medium. Similar studies were carried out by Elbehiry et al. (2016), which found 70-75% agreement value of detection between PCR and culture for different respiratory tissues collected from suspected birds. The highest positivity of culture was found 55.8% from the trachea, as well as 20.7% and 16.9% from the air sac and the lungs, respectively. However, PCR positivity was 79.4%, 28.3%, and 22.6% for the trachea, air sac, and lungs, respectively. The overall positivity was 37.1% and 51.9% by culture and PCR, respectively. In this study, no effect was observed on samples when processed by PCR either directly or after incubation in broth for either *M. gallisepticum* or *M. synoviae*. However, it has been found that positive cases increased when the same samples were incubated into broth for 48 h prior to PCR. Direct PCR detection from respiratory tissues showed 65% results, while after 48 h of incubation in broth medium, 70.9% of the samples produced positive results. Overall, 57.14% of the culture samples produced fried egg colonies, whereas only 17.66% were confirmed as *M. gallisepticum*. The highest percentage of *M. gallisepticum* infection (77.64%) was

in breeder birds followed by broilers (70%) and layers (67%) (Hossam et al., 2016). *M. gallisepticum* was confirmed in 81.32% of tracheal samples followed by the air sac (61.54%) and the lungs (47.25%) after 48 h of incubation. Therefore, the trachea was found the most productive organ to be used for sampling purposes. We observed that the prevalence of avian mycoplasmosis is higher and the signs and symptoms are more noticeable during winter, particularly in temperate regions, which might be due to cold stress or poor ventilation, ammonia, and dust particles. We found the prevalence of mycoplasmosis during summer to be 5%. However, the incidence rate as assessed by PCR in commercial flocks was 72.7% and 73% in Brazil and the Netherlands, respectively (Feberwee et al. 2008; Buim et al. 2009). Kahya et al. (2015) reported that 33% of samples were positive for *M. gallisepticum* in winter, while zero positivity was observed in summer. Similarly, 8.1% of *M. synoviae* positive samples were detected in winter, while 0% were found in summer. The importance of *M. gallisepticum* and *M. synoviae* prevalence justifies the attention and interest of poultry industry and mandatory monitoring and surveillance system for its management. However, in cases where the elimination of *M. gallisepticum* and *M. synoviae*-infected flock is not economically feasible, treatment and vaccination remain the best choices. Although the complete elimination of mycoplasma does not occur with medications (Feberwee et al., 2008), *M. gallisepticum* vaccination is a common practice worldwide and has been reported successful in disease prevention, while the live *M. synoviae* vaccine has limited field application (Landman, 2014). PCR as a diagnostic tool for avian mycoplasmosis is an alternative or confirmatory parameter to culture and serology-based detection, which are labor-intensive, time consuming, and often non-conclusive.

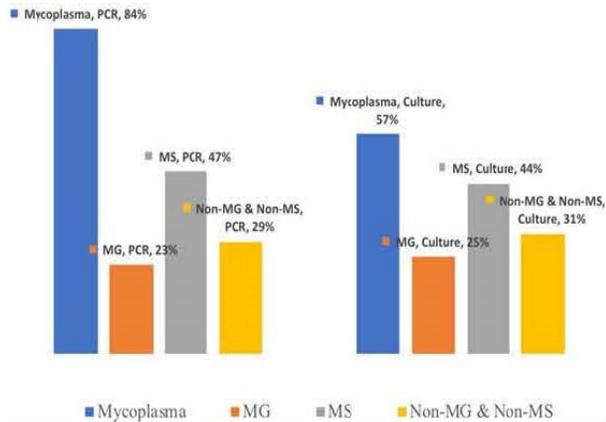


Figure 1. Comparison of avian mycoplasmas prevalence by polymerase chain reaction and culture

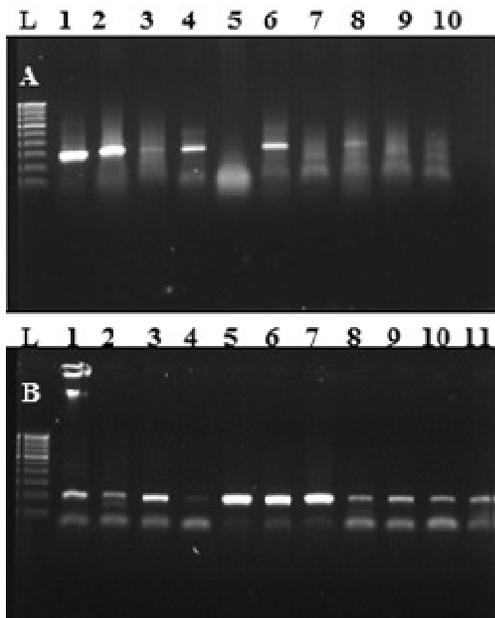


Figure 2. A: MG, L; Ladder (100 bp), 1: S6 (+ve Control), 2-10; Samples. B: L; Ladder, 1; WVU1853 (+ve Control), 2-11; samples

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Contribution of Authors

Faiz Muhammad and Taseer Ahmed Khan conceived, designed, executed, and wrote the study. Johur Hussain and Khurram Fareed performed field sampling. Shakeel Ahmed Khan performed a critical revision of the manuscript for important intellectual content. Aqeel Ahmad supervised the study.

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