Short Communication

Simultaneous Detection of *Mycoplasma* Infections in Clinically Sick and Apparently Healthy Broilers

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Abstract | *Mycoplasma* infections in gallinaceous birds are reported throughout the world and are caused by either *Mycoplasma gallisepticum* or *Mycoplasma synoviae*. Hence, in the present study we have detected *Mycoplasma spp.* from tissue samples collected from clinically sick (60 samples) and apparently healthy broiler birds (30 samples) in a commercial farm, using classical isolation and compared with molecular identification of *Mycoplasma* infections. A total of 96 tissue samples from trachea, air sacs, lungs and oral swabs along with blood from each bird were collected and initially subjected to isolation of *Mycoplasma spp.* using brain heart infusion broth supplemented with horse serum. The blood was used for serum plate agglutination assay. Thereafter, the samples were subjected to genomic DNA extraction to detect *Mycoplasma spp.* using multiplex-PCR. Isolation and identification data showed that a total of 58/96 samples (60.41%) were positive for *Mycoplasma* infections whereas a total of 61/96 blood samples were also found positive using serum plate agglutination. However, the multiplex-PCR detected *Mycoplasma* in all of the tissue samples, either one of the species or simultaneous infection. *M. gallisepticum* was found in 39/96 samples (40.62%) and *M. synoviae* in 32/96 samples (33.34%), whereas 25/96 samples (26.04%) were detected with simultaneous infection with both species. Altogether, it has been observed that multiplex-PCR is a sensitive tool for early and accurate diagnosis of *Mycoplasma* infections from samples collected from clinically sick and apparently healthy birds. In this way, current approach could be contributed well towards elimination and reduction of losses due to *Mycoplasma* infections in commercial poultry.

Editor | Muhammad Abubakar, National Veterinary Laboratories, Park Road, Islamabad, Pakistan.
Received | February 28, 2017; **Accepted** | April 17, 2017; **Published** | April 25, 2017
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Citation | Zahoor, M.A., Z. Nawaz, A.B. Siddique, S.U. Rahman and S. Ali. 2017. Simultaneous detection of *Mycoplasma* infections in clinically sick and apparently healthy broilers. *Veterinary Sciences: Research and Reviews*, 3(2): 34-37.
DOI | http://dx.doi.org/10.17582/journal.vsrr/2017.3.2.34.37
Key words: Microbiology, Broilers, DNA, Disease, Genome

Introduction

The rearing and housing of gallinaceous birds at commercial levels has emerged as an alternative to beef and mutton throughout the world. These birds are prone to various types of infectious diseases. Among these *Mycoplasma* infections are of paramount importance causing huge economic losses to commercial poultry. *Mycoplasma species* are mainly responsible for respiratory distress, air sacculitis and synovitis. Almost 120 species of *Mycoplasma* have been identified, however only few are known to cause infections in

gallinaceous birds. Among these species, *M. gallisepticum* and *M. synoviae* are major pathogens associated with respiratory and joint disease and cause huge economic losses worldwide (Bradbury, 2005; Chanie, 2009).

For the diagnosis of Mycoplasma infections; isolation/identification, serological assays and molecular identification have been reported previously (Stanley et al., 2001; Hess et al., 2007; Ehtisham et al., 2011; Siddique et al., 2012). Some of the previous studies descried molecular identification of *Mycoplasma spp.* with PCR as an efficient and accurate diagnostic tool (Nascimento et al., 1991; Lauerman et al., 1995; García et al., 2005; Hess et al., 2007). Similarly, comparison of different laboratory conditions for optimized protocols of PCR and simultaneous detection of bacterial pathogens has been described using multiplex-PCR (Wang et al., 1997; Hess et al., 2007; Siddique et al., 2012). The prevalence of *Mycoplasma* infections are reported as 30% in Pakistan (Ehtisham et al., 2011). Therefore, in an extension to the previously published data and research methodologies, we have designed the present study to optimize and validate the multiplex-PCR in our lab conditions to establish an accurate diagnostic technique for *Mycoplasma* infections.

Materials and Methods

Sample collection and initial processing of samples

A total of 96 tissue samples including trachea (35), air sac exudates (28), lungs (15) and oral swabs (18) along with blood from each bird were collected and labeled properly from clinically sick (60 samples) and apparently healthy broiler birds (30 samples). The birds with tracheal rales, nasal discharge, sneezing and coughing were considered as clinically sick (Siddique et al., 2012). The tissue samples were directly inoculated into the Brain heart infusion broth supplemented with 5% horse serum and incubated at 37°C for 24-72 hours for initial screening and identification of *Mycoplasma* infections, whereas serum plate agglutination was performed for each blood sample (Branton et al., 1984; Siddique et al., 2012).

Molecular identification of *Mycoplasma* spp

The genomic DNA from all tissue samples was extracted using phenol chloroform method and the extracted DNA was used in multiplex-PCR. Amplification reaction was carried out in final volume of 50µl reaction mixture using the primers described in Table 1 (Wang et al., 1997; Ehtisham et al., 2011). Multiplex-PCR was performed with an initial de-naturation at 94°C for 5 minutes, followed by 35 cycles at melting temperature 94°C for 1 minute and to annealing temperature of 50°C for 1 minutes, and extension at 72°C for 2 minutes. The sample was then heated at 72°C for 10 min for final extension of the reaction mixture (Wang et al., 1997). The amplified product was visualized under UV light following 1.5% agarose gel electrophoresis.

Table 1: *Sequence of primers used for Mycoplasma gallisepticum and Mycoplasma synoviae*

Primer	Sequence
M. gallisepticum forward primer	5' CTTTCCCATCTCGACCAG-GAGAAAA-3
M. gallisepticum reverse primer	5' GGATCAATCAGTGAGTAACT-GATGA-3'
M. synoviae forward primer	5' GTCGTAAATAGTGATATCA-3'
M. synoviae reverse primer	5' GAAGCCTCGAAGTTAACAA-3'

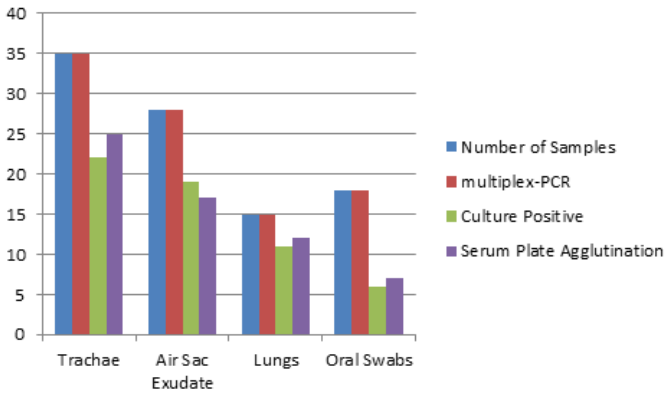

Figure 1: *Comparison of tissue and blood samples data for Mycoplasma spp. identification*

Results and Discussion

In the present study, a total of 96 tissue samples were processed for *Mycoplasma* isolation. Conventional isolation data showed that total of 58/96 samples (60.41%) were positive for *Mycoplasma* infections whereas 61/96 blood samples were also found positive using serum plate agglutination as shown in Figure 1.

All of the tissue samples (96) were found positive with multiplex-PCR for either one of the species *M. gallisepticum* or *M. synoviae* or simultaneous infection with both species. Altogether, all of 96 samples 39 (40.62%) samples were positive for *M. gallisepticum*,

32 samples (33.34%) were positive for *M. synoviae* and remaining 25 samples (26.04%) were positive for simultaneous infection as shown in Figure 1 and 2.

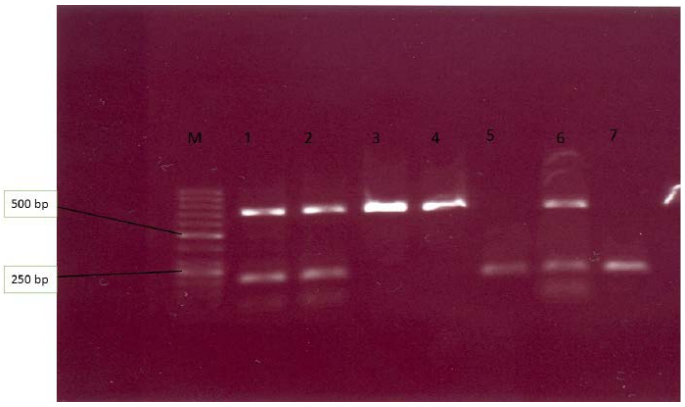

Figure 2: *Comparison of tissue and blood samples data for Mycoplasma spp. identification*

Huge economic losses have been reported from almost every part of the world in gallinaceous birds due to *Mycoplasma* infections which includes reduced egg production (10-20%), increased embryonic and chick mortality (5-10%) and reduced weight gain and feed conversion ratio (10-20%) as described by Kleven, 1990. *Mycoplasma* infections are caused by either *M. gallisepticum* or *M. synoviae* and are characterized by coughing, sneezing, tracheal rales and nasal discharge. *M. gallisepticum* usually causes subclinical to mild infection whereas the severity of disease increases with simultaneous infection of *M. synoviae* (Roussan et al., 2008). The diagnosis of *Mycoplasma* infections are based on serology and isolation/identification of *Mycoplasma*. Serological identification of *Mycoplasma* antigen is mainly considered nonspecific due to cross reactivity of *Mycoplasma spp*. and usually produces false positive results, however it is good screening tool at large scale or commercial poultry farms (Ehtisham et al., 2011, Hutton et al., 2017). Isolation and identification of *Mycoplasma* is gold standard for diagnosis, however it is time consuming as the micro organism showed slower growth patterns (Siddique et al., 2012). Therefore, in the present study multiplex-PCR was used to simultaneously detect *M. gallisepticum* and *M. synoviae* infections from clinically sick and apparently healthy broiler birds. Multiplex-PCR detection has increased diagnostic accuracy and reduced time consumption as compared to serology and isolation/identification of *Mycoplasma* infections.

In the present study, a total of 96 tissue samples were processed for *Mycoplasma* isolation. Conventional isolation data showed that 58/96 samples (60.41%) were positive whereas 61/96 blood samples were also found positive using serum plate agglutination. Previously published data also described the isolation/identification and serological assays for *Mycoplasma spp.* identification (Siddique et al., 2012; Ehtisham et al., 2011). However, in the present study all of the samples were found positive either with *M. gallisepticum, M. synoviae* or dual infection using multiplex PCR. has detected Mycoplasma.

Further, multiplex-PCR data showed absolute and accurate diagnosis including of simultaneous detection of *M. gallisepticum* and *M. synoviae* as compared to isolation/identification and serological data. The highest number of positive samples was *M. gallisepticum* 39/96 (40.62%), followed by *M. synoviae* 32/96 (33.34%), whereas simultaneous infection is reported among 25/96 (26.04%). The previously published data also showed a high efficacy and efficiency of molecular identification methods including PCR and multiplex-PCR from respiratory distress cases as compared to isolation/identification and serology (Hess et al., 2007; Siddique et al, 2011).

Conclusion

Finally, based on results of the present study, multiplex-PCR is an ideal approach to detect *Mycoplasma* infections including *M. gallisepticum* and *M. synoviae* or simultaneous infection with both species.

Authors' Contribution

MAZ and ZN prepared the manuscript, ABS and SA collected the samples and performed the experiments, whereas SUR designed and supervised the study.

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