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PREVALENCE AND PHYLOGENETIC ANALYSIS OF MYCOPLASMA GALLISEPTICUM ISOLATES IN BROILERS AND LAYERS

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Abstract

Mycoplasma gallisepticum *(M. gallisepticum*) is a bacterial pathogen and causes economic losses in poultry. It is responsible for acute respiratory disease which stay without clinical signs but the infected birds become prone to secondary infections. The study was aimed for molecular detection and phylogenetic analysis of *M. gallisepticum* collected from commercial poultry birds (broilers and layers). Total 400 swab samples were collected and analyzed through serum plate agglutination test for seroprevalence followed by culturing on Frey's medium. Culture positive samples were furthered processed by PCR and sequenced for phylogenetic study. From seroprevalence results, it was found that the positive samples showed agglutination in serum plate agglutination test. The seroprevalence results of *M. gallisepticum* indicated that layers are more positive (31.9%, n=210) than broilers (24.7%, n=190). In culture identification, positive isolates depicted typical fried egg-shaped colonies of *M. gallisepticum* on modified Frey's agar medium. Based on colony morphology and biochemical characterization, 15.24% of layer samples were positive while broiler samples showed low positivity rate (9.47%). Through PCR, culture positive isolates indicated the product of 185bp, confirming the specie of *M. gallisepticum*. In comparative prevalence of PCR positive samples, it was found that layer samples were more positive (20.48%) than broiler samples (13.16%). Samples collected from various organs of broilers and layers were processed separately through culturing and PCR. It was found that tracheal samples showed higher prevalence than other organ samples of broilers. While the same trend was observed in layer samples collected from various organs. In phylogenetic analysis, sequences with more than eighty percent similarity were analyzed by MEGA X for evolutionary history. A total of 871 positions were observed in the final data which was submitted to GenBank. The phylogenetic analysis of *M. gallisepticum* from the present study (MZ373234.1, MZ373235.1, and MZ424735.1) showed genetic sequence mimicry to the other isolates. In conclusion, layers could be the reservoir for *M. gallisepticum* infection and new genetic methods are needed for confirmation and differentiation of new emerging strains.

Keywords: Prevalence, Phylogenetic Analysis, Genetic Methods

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1. INTRODUCTION

In Pakistan, the poultry sector has a considerable role in bridging the gap between demand and supply for protein. *M. gallisepticum* is an emerging bacterium causing serious problems in the poultry industry of Pakistan. It causes chronic respiratory disease in chickens and this disease remains asymptomatic resulting in considerable economic losses. Typical signs of the disease include coughing, nasal discharge, abnormal feathers, ocular discharge, decreased egg production, and low productivity (1, 2). Previously, various diagnostic methods have been reported and applied for the detection of *M. gallisepticum* infection. The reported assays are dependent upon cultural and serological identification, and the use of molecular technique like PCR, are referred to as 'gold standard' methods. The molecular method, PCR, is advantageous over others due to its sensitivity and quickness. Moreover, it is a beneficial assay and helpful in epidemiological studies for the determination of infection source and the strains causing the infections (3).

Implementation of strict biosecurity measures may break the chain of *M. gallisepticum* infection with possible reduced losses. Through genome sequencing, it has been declared that new field strains of *M. gallisepticum* are emerging which may hamper the control of infection and the development of a vaccine (4, 5). In poultry, for efficient management and removal of *M. gallisepticum* infection, there is a dire need for the development of a vaccine as well as diagnostic antigens based on local isolates. Due to financial issues, most of the poultry farmers are using antibiotics for controlling *M. gallisepticum* infection which results in the development of antibiotic resistance while a few poultry farmers are using vaccines for the same purpose (6). The current study aimed to determine the seroprevalence of *M. gallisepticum* in layer and broiler flocks. The study was further extended for molecular detection and phylogenetic analysis using positive serum samples.

2. MATERIALS AND METHODS

The present research was conducted at the Institute of Microbiology, University of Agriculture Faisalabad (UAF) following the Institutional Bioethics Committee (IBC) guidelines.

2.1 Sampling

In total, four hundred serum and swab samples were collected from 16 flocks of commercial poultry (broilers & layers). The birds with suspected infection of *M. gallisepticum* showing clinical signs of nasal discharge, beaks with feed, and with postmortem signs like air sacculitis, lungs, and abdomen filled with caseous pus material along with pericarditis were selected and samples were collected from the trachea, choanal cleft, lung, liver and air sacs. All the collected samples were transferred to the laboratory immediately.

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2.2 Serological identification

For serological screening, a serum plate agglutination test was performed. A volume of 0.02 mL of antigen (Ceva Biovac MG RPA-Test, Product Code AS9) was taken and mixed with 0.02 mL of serum on a glass slide followed by a reading of reaction after two minutes under a white background (7).

2.3 Culture identification and Biochemical characterization

For each tested sample (positive by serum plate agglutination test), samples were enriched in PPLO broth, and colonies were isolated on modified Frey's solid medium (8, 9). The suspected positive isolates were further identified by biochemical tests including glucose fermentation, reduction of 2, 3, 5-triphenyltetrazolium chloride, and phosphatase production (10, 11).

2.4 DNA extraction and PCR identification

For molecular confirmation of suspected positive isolates of *M. gallisepticum* culture, DNA was extracted using the TIANamp Bacterial DNA kit (DP302) using the protocol described. The extracted DNA was used further for amplification using a thermal cycler (Aeris-BG096, Esco Lifesciences) with a specific set of primers; MG-14F: 5'-GAGCTAATCTGTAAAGTTGGTC-3' MG-13R: 5'-GCTTCCTTGCGGTTAGCAAC-3' targeting 16SrRNA gene (Pakpinyo and Sasipreeyajan, 2007). The thermal cycle included three steps as follows: Primary denaturation was performed at 94 ˚C for 3 min as the first step. In the second step, 40 cycles each included three sections; denaturation at 94 ˚C for 30 sec, annealing at 55 ˚C for 30 sec, and extension at 72 ˚C for 60 sec was performed. Eventually, the final extension was conducted at 72 ˚C for 5 min as the third step. The PCR products were electrophoresed on 2% agarose gel for 45 minutes at 120 V and visualized by staining with ethidium bromide (7, 12, 13, 14).

2.5 Gene sequencing and Phylogenetic analysis

The amplicons of the selected DNA were sequenced with the forward and reverse PCR primers and sequences were determined in a 3500 Genetic Analyzer (Applied Biosystems). The sequences were analyzed using Bio-edit software. The bases having low efficiency were cut off followed by checking the efficiency of the chromatogram for accurate results. For consensus sequence, both reverse and forward sequence of every sample was arranged and blasted using a tool NCBI BLAST (www.ncbi/BLAST)). This yielded resemblances of the tested samples with other found samples. The study proceeded using sequences with > 80% resemblance with present queries. Phylogenetic analysis was performed using MEGA X. Maximum likelihood method was used for determining the evolutionary history of tested strains while the evolutionary distance was interpreted *via* the Jukes-Cantor model (14, 15, 16).

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3. RESULTS

3.1 Serological screening and phenotypic identification of *M. gallisepticum*

For serological screening of *M. gallisepticum* in broiler and layer birds, a serum plate agglutination test was performed. After 2 minutes, agglutination was observed showing positivity of *M. gallisepticum* (Figure 1). The seroprevalence results of *M. gallisepticum* indicated that layers positivity (31.9%, n=210) is higher than broilers (24.7%, n=190).

In culture identification, swab samples streaked on modified Frey's agar medium depicted typical fried egg-shaped colonies of *M. gallisepticum* (Figure 2). After cultural isolation, the suspected samples of Mycoplasma spp. were further processed for biochemical tests. The suspected culture-positive samples showed positive results for glucose fermentation with a change in color from red to yellow and reduction of 2, 3, 5-triphenyltetrazolium chloride test with the appearance of red color while negative for phosphatase production test. Overall, samples collected from layers showed high positivity rate than broilers samples (for oral swabs, trachea, lungs, and air sac samples) while none of the liver samples were found positive both for layers and broilers (Table 1). Based on colony morphology and biochemical characterization, 15.24% of layer samples were positive while broiler samples showed a low positivity rate (9.47%).

3.2 Molecular confirmation of *M. gallisepticum* from poultry

Swab and tissue samples collected separately from broilers and layers were further processed for molecular confirmation of Mycoplasma spp. using PCR. Through PCR, positive isolates indicated the product of 185bp, confirming the species of *M. gallisepticum* (Figures 3, 4 & 5).

3.3 Prevalence of *M. gallisepticum* in poultry birds

A total of 400 swab samples (190 from broilers & 210 from layer flocks) were collected and processed for serological, cultural, and molecular identification of *M. gallisepticum*. Samples (n=190) from 7-broiler flocks having respiratory distress signs & symptoms were collected and out of them, 47 were found positive, showing 24.74% positivity/seroprevalence and 18 (9.47%) through cultural identification and 25 (13.16%) PCR confirmation in broiler flocks. Similarly, 210 serum samples were collected from 9-layer flocks having respiratory distress signs & symptoms and postmortem lesions, 67 were found positive, showing 31.90% seroprevalence and 32 (15.24%), 43 (20.48%) cultural and molecular prevalence respectively in layer flocks. In comparative prevalence, it was observed that samples collected from layer flocks showed high prevalence than broiler flock samples (Figure 6).

Samples collected from various organs of broilers and layers were processed separately through culturing and PCR. There was no detection of *M. gallisepticum* in liver samples in broilers while in layer flocks PCR testing showed 6.67% prevalence/detection in a liver sample. It was found that tracheal samples showed a higher prevalence/detection than

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other organ samples of broilers. While the same trend was also observed in layer samples (Figure 7).

3.4 Phylogenetic analysis of *M. gallisepticum* from poultry

In phylogenetic studies, the evolutionary analysis was conducted in MEGA X. This analysis involved 15 nucleotide sequences. The phylogenetic analysis of *M. gallisepticum* from the present study (MZ373234.1, MZ373235.1, and MZ424735.1) showed genetic sequence mimicry to the other isolates reported from the USA (KC995337.1, KC995288.1, KC995289.1, KC995296.1, and KC995330.1), India (MN069580.1, MN069558.1, MN069583.1, MW876250.1, DQ677303.1, and MW517331.1) and Pakistan (MW397012.1) (Figure 8). These data suggest that the Mycoplasma strains circulating globally.

4. DISCUSSION

Mycoplasma gallisepticum is an important infectious pathogen that has adverse effects on commercial poultry production worldwide. The infection is characterized by respiratory rales, coughing, nasal discharge, conjunctivitis, etc. *M. gallisepticum* is mostly considered to occur as a subclinical upper respiratory infection, which can progress to respiratory disease with air sac lesions when combined with Newcastle disease or infectious bronchitis, and to infectious synovitis when it becomes systemic (17).

In the present study, seroprevalence studies indicated that layer samples are more positive than broilers. In culture testing, the same trend of prevalence was observed (15.24% of layer samples and 9.47% of broiler samples). Diagnosis of Mycoplasma through the conventional method of culturing is a “Gold Standard” but this technique is very laborious, expensive, time-consuming due to slow growth, and requires very skilled staff. These things may lead to variations in the results of conventional methods (18**)**. Results of the current study from the same samples showed similar variations in three tests (serum plate agglutination test, culture, and PCR). The swab samples tested through PCR showed the highest positivity for *M. gallisepticum* than other methods. Moreover, layer samples depicted a higher prevalence rate than broilers.

The high positivity rate through PCR may be due to the slow growth rate of *M. gallisepticum*. As per World Organization for Animal Health (OIE) standard, as an alternative to culturing, the PCR is a test of choice for diagnosis of *M. gallisepticum* in the poultry industry due to its high sensitivity and rapid diagnosis. Identification of *M. gallisepticum* through PCR resulted in a product length of 185bp. Similar results were found in previous studies (19, 20).

Isolates and strains vary in their pathogenicity pattern with differentiation in phenotypic and genotypic characteristics (21). For a detailed study, we need some genetic study for confirmation/ similarity determination of species of *M. gallisepticum* with previously known strains (22). PCR method can be used as a reference for molecular based identification of *M. gallisepticum* (23). The sequences of the partial 16SrRNA gene of *M.*

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gallisepticum isolates in the current study were compared with already reported and available sequences of the same gene on NCBI GenBank using nucleotide BLAST analysis.

The phylogenetic tree represented that the sequences of *M. gallisepticum* from this study clustered with previously reported sequences. The nucleotide sequences of currently characterized isolates showed 98% similarity with worldwide *M. gallisepticum* isolates. All sequences isolated from this study are available at NCBI GenBank with accession numbers: MZ373234.1, MZ373235.1, and MZ424735.1. Similar reports have also been published in the USA, Israel, and Australia (24). Our results were closely matched with others (25, 26).

5. CONCLUSION

Our serological, molecular, and phylogenetic based data evidenced that *M. gallisepticum* is circulating in the poultry flocks. A control strategy suggested to contain *M. gallisepticum* infection at the breeder, layer, and broiler flocks by vaccination and biosecurity.

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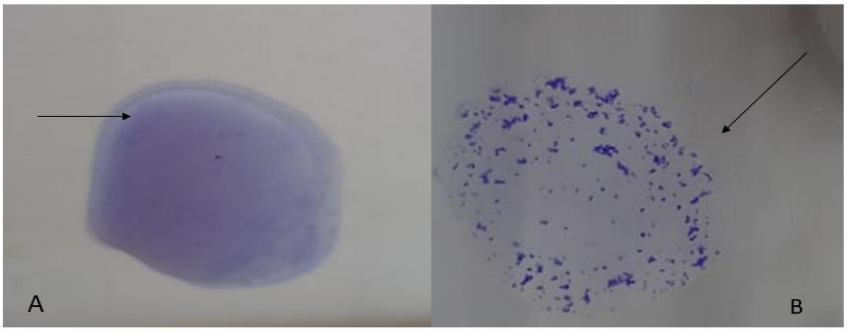
Figure 1: Serum Plate Agglutination Test



(A) No agglutination (B) Positive agglutination test

Figure 2: Typical Fried egg colonies of *M. gallisepticum* observed on modified Frey's agar medium (~1000X)

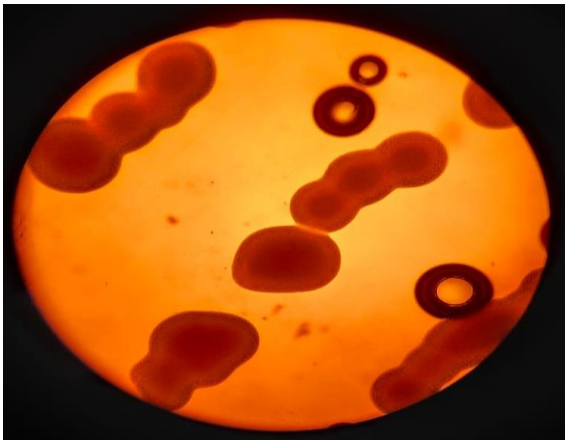


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Table 1: Results of biochemical characterization of avian Mycoplasma species after culture isolation (Exceptional)

Types of Samples	No.of Positive Samples	Glucose Fermentation	Tetrazolium Reduction test (Ae/An)	Phosphatase Production test	Probable identification of Mycoplasma Species
Oral Swabs	Broiler-18 Layer -32	+	+/+	-	*M. gallisepticum*
Trachea	Broiler-2 Layer -4	+	+/+	-	*M. gallisepticum*
Liver	Broiler-0 Layer -0	-	-/-	-	-
Lungs	Broiler-1 Layer -3	+	+/+	-	*M. gallisepticum*
Air Sacs	Broiler-1 Layer -3	+	+/+	-	*M. gallisepticum*

Figure 3: **Agarose gel electrophoresis of PCR-amplified product at 185bp band confirming *M. gallisepticum* from culture +ve swab samples of broiler flocks** (M-100bp plus ladder, L1, L2 are negative controls and L3 to L7 are positive samples)

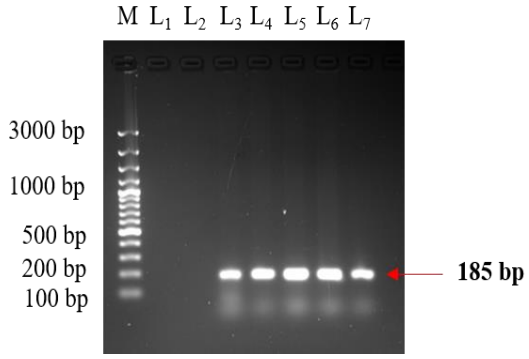

Figure 4: **Agarose gel electrophoresis of PCR-amplified product at 185bp band confirming *M. gallisepticum* from culture +ve swab samples of layer flocks** (M-100bp plus ladder, L1 to L8 are positive samples and L9, L10 are negative controls)

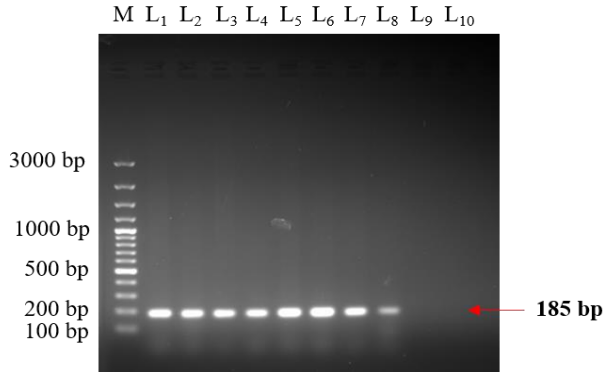

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Figure 5: **Agarose gel electrophoresis of PCR-amplified product at 185bp band confirming *M. gallisepticum* from culture +ve tissue samples of broiler & layer Birds** (M-100bp plus ladder, L1 to L6 are positive samples and L7, L8 are negative controls)

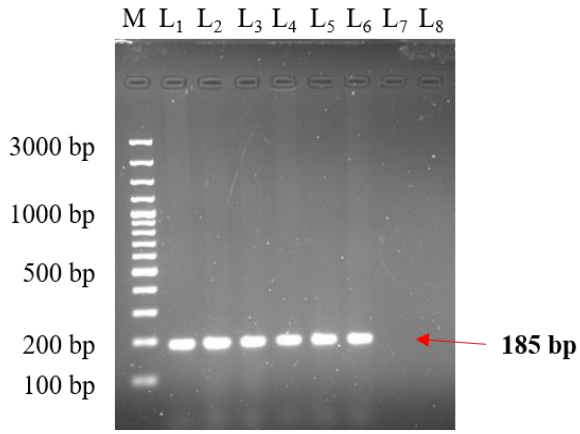

Figure 6: Comparative prevalence of *M. gallisepticum* in broiler & layer flocks through SPA, Culture, and PCR based results

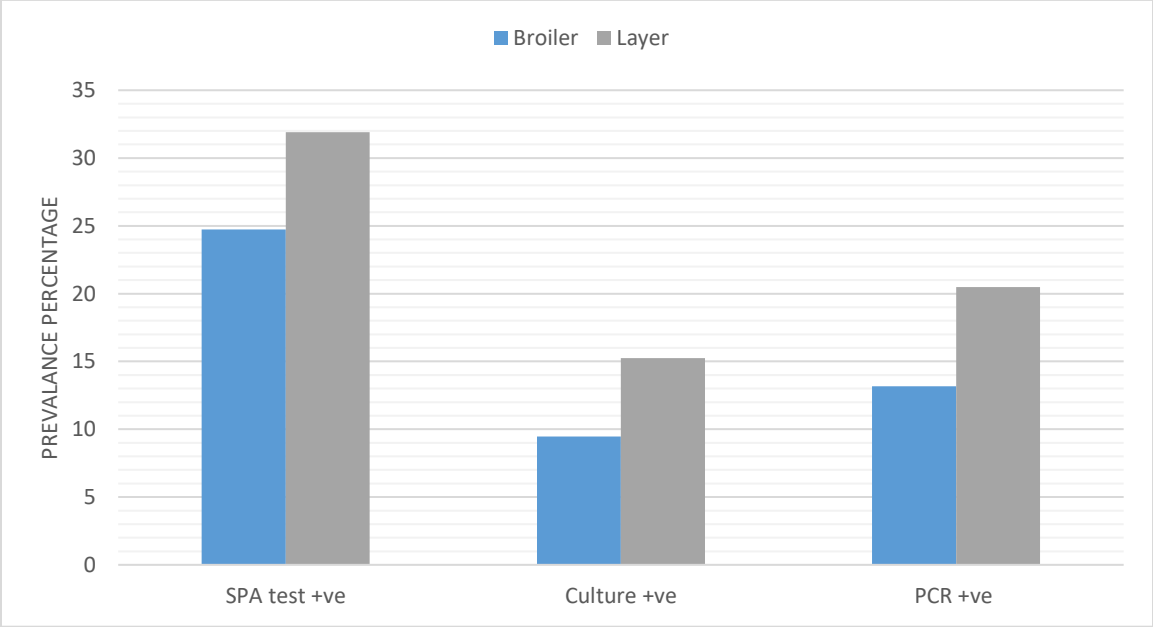

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Figure 7: Comparative Organ-wise detection of *M. gallisepticum* in broiler & layer flocks

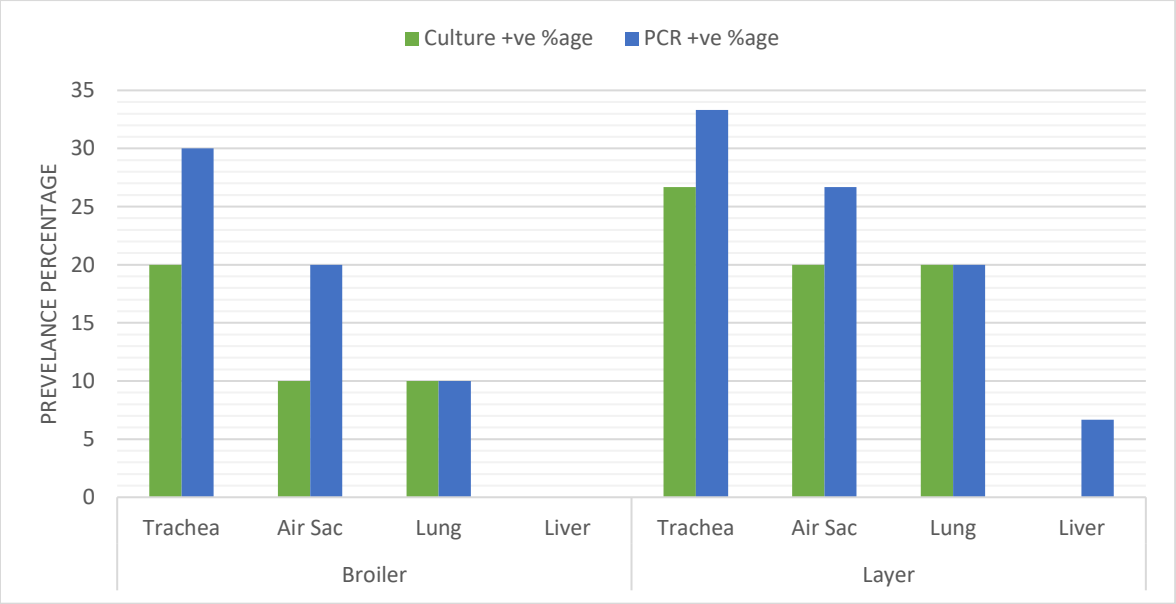

Figure 8: Phylogenetic Analysis of *M. gallisepticum* isolates from broiler & layer flocks