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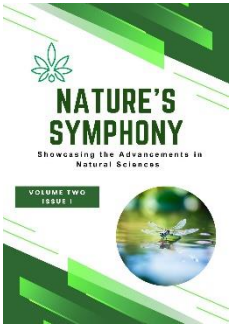

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Research Article

Surveillance of *Mycoplasma gallisepticum* in Bird's Retail Stalls in Quetta District

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ABSTRACT

One of the most economically significant and contagious Mycoplasma species is *Mycoplasma gallisepticum*, a significant pathogen of poultry production facilities causing high economic losses in Pakistan. Most countries rely on *M. gallisepticum* control efforts to maintain disease-free commercial breeding stock. Molecular biology techniques have been applied for the quick diagnosis of these infections and the implementation of disease control measures. A rapid and precise alternative to traditional culture techniques is the polymerase chain reaction (PCR). A single tube reaction was devised for the entire assay (Multiplex MG), which was designed with primers and probes unique for each pathogen. The aim of this project was to assess the molecular technique for *Mycoplasma gallisepticum* detection in bird trachea. 22 tracheal samples in total were processed using the PCR method of detection to examine the disease's spread inside the sample. 10 out of the 22 tracheal samples that were examined by PCR were positive and 12 were negative. The findings of this study showed that presence of *Mycoplasma gallisepticum* were detected in the samples. Additionally, this investigation demonstrated that *Mycoplasma gallisepticum* is still present in commercial poultry and should be controlled by novel preventative measures based on drugs and immunizations, likely employing new vaccines depending on the local *Mycoplasma gallisepticum* strain's characteristics.

1. INTRODUCTION

Mycoplasma gallisepticum (MG) belongs to the class mollicutes and the family Mycoplasmataceae. *M. gallisepticum* is the causative agent of mycoplasmosis, a dangerous bacterial infection that regularly strikes flocks of chickens. Watery eyes, tracheal rales, nasal discharge, and coughing are signs of natural infections. These are exceedingly small organisms that resemble bacteria but differ from other bacteria in having a thinner cell wall. *M. gallisepticum* can cause respiratory illnesses in chickens, turkeys, and other avian species. Morbidity is frequently severe and mortality is typically low in affected flocks; weight loss stems from reduced feed consumption. Adult laying birds often experience a decrease in egg output or an end to egg production altogether (Marn *et al.*, 2022). Young birds usually have more serious infections than older or adult birds. The chicken industry suffers enormous financial losses due to *M. gallisepticum* infection, including decreased egg output, decreased hatchability, and poor meat quality. Superoxide radicals, one of the extra virulence agents produced by *M. gallisepticum*, disrupt the immune system. Mycoplasmosis is influenced by parameters such as the method of exposure,

the microbe infection dose, environmental stressors such as temperature and ammonia content, age, and bird species (Ferguson-Noel *et al.*, 2020).

Additionally, even without visible clinical signs, *M. gallisepticum* infection may result in a significant decrease in feed consumption efficiency with substantial economic consequences. *M. gallisepticum* infection can also lead to output loss in laying birds, especially in egg sheets infected at the peak of lay. *M. gallisepticum* transmits to ova, as do all pathogenic avian mycoplasmas. Due to the proximity of the abdominal air sacs to the oviduct, an acute respiratory infection is likely the cause of infection of the embryo and transmission to the progeny (egg transmission). When *M. gallisepticum* levels in the respiratory tract are at their peak, the highest frequency of transmission is observed during the acute phase of the illness (Ayim-Akonor *et al.*, 2018).

M. gallisepticum can spread vertically through specific eggs from infected breeders to offspring as well as horizontally by contaminated feed, water, and the environment. It can also be spread by people working on farms (shoes, equipment, etc.). Horizontal transmission via aerosols and the respiratory route may occur swiftly in stressed birds. As a result, the flock was infected and developed clinical illness. Some birds' infections may go undetected for days or even months. Once individuals or flocks are infected, they live the remainder of their lives carrying the infection or acting as a reservoir for it (Levison, 2016). Transmission from flock to flock can happen either directly or indirectly when diseased birds, people, or other fomites migrate between susceptible flocks. Conjunctiva, nasal passages, sinuses, and the epithelium of the trachea are the areas where initial colonization and infection are most likely to occur; but, in situations of severe, acute disease, infection may also damage the bronchi, air sacs, and rarely the lungs. Those infected birds might always carry the illness. There is a clear interaction (polymicrobial illness) between respiratory viruses, *Escherichia coli*, and *M. gallisepticum* in the onset and severity of chronic respiratory disease.

All species of birds are susceptible to this disease, but young birds are more vulnerable than adult birds. As early as 1964, there was serological evidence of avian mycoplasmosis in Pakistan, and reports from various locations throughout the nation consistently showed the presence of *M. gallisepticum* infection (Rafique *et al.*, 2024).

The study aimed to detect *M. gallisepticum* in a bird's trachea and to estimate the prevalence of *M. gallisepticum* infection at retail stalls in Quetta district using molecular methods.

2. MATERIAL AND METHODS

2.1 Study Area Description and Sample Collection

The study area covered the live poultry retail stalls in Quetta district. It was a cross-sectional survey of live poultry retail stalls (LPRSs) in Quetta district and was conducted during the month of March–April 2022. Quetta city is the largest city in Baluchistan province. Oropharyngeal swabs were collected from each chicken as shown in figure 1. A total of 22 samples were collected from healthy chickens, samples were kept at lower temperatures and brought to Microbiology laboratory BUITEMS, Quetta till samples were further studied. The swab samples were kept at -20^{o}C for further use.

2.2 Isolation of Bacterial Colonies

PPLO (Pleuro-pneumonia) medium was prepared by adding horse serum 15 ml, for 22 petri plates. The media was prepared and autoclaved at 121^{o}C for 40 minutes using the standard autoclave procedure. After this, the

Figure 1. Sampling Strategy using swabs

medium was poured into autoclaved petri plates aseptically. After solidification, swabbing was done by wearing a head cover, and latex gloves. After swabbing the plates were kept in incubation for 7 days at 37 °C and samples were stored in the refrigerator again.

2.3 Antibiotic Sensitivity Test

A sterile cotton swab was used to pick up a small amount of bacterial culture from a fresh agar plate or broth culture and streaked evenly across the surface of a Mueller-Hinton agar plate. The plate was allowed to dry for a few minutes with the lid partially open. Antibiotic discs i.e., imipenem, tulathromycin, cephalothin, bacitracin, and optochin were placed and incubated for 16-18 hours at usually 35-37°C. After incubation, the diameter of zones of inhibition were recorded.

2.4 DNA Isolation and Gel Electrophoresis

A QIAGEN DNA isolation kit was used for DNA isolation. The isolation was followed by the detection of DNA via gel electrophoresis.

2.5 Polymerase Chain Reaction (PCR)

The PCR reaction contained 8.5 µl water, 12.5 µl 2X PCR master mix, and 2µl of each forward and reverse primers. The forward primer sequence was 5'-GTTGCAAATCCGTAAGGTGG - 3' whereas the reverse primer sequence was 5' – TTAGCAACACGGTTTTAGAT – 3' to amplify the 16S rRNA gene. The master mix tubes were vortexed thoroughly. 5µl of DNA sample was added to each PCR tube. The tubes were caped, and the contents were mixed. The PCR tubes containing the master mix and DNA were placed in the thermal cycler, the lid of the thermal cycler was closed. The PCR program was having the initial denaturation temperature set at 95^{o}C for 5 min and final extension at 72^{o}C for 15 min, with 30 cycles of denaturation at 94^{o}C for 3 min, annealing at 54^{o}C for 45 seconds, and extension at 72^{o}C for 1 min.

3. RESULTS

3.1 Enrichment, Isolation, and Identification

Unique fried egg-shaped colonies (figure 2) on agar plates were noted and those distinct colonies were picked and streaked on each individual solidified PPLO media plate under a laminar flow hood, there were a total of 32 distinct colonies, and the plates were kept in the incubator for 7 days at 37 °C after standard streak plate method.

Figure 2. Mycobacterium cultures obtained in this study

3.2 Antibiotic Sensitivity Test

All the samples showed resistance to tulathromycin. While 91.66% of isolates were susceptible to imipenem. A similar percentage of 91.66% showed resistant isolates against cephalothin, bacitracin, and optochin.

3.3 DNA Extraction and PCR

The QIAGEN kit method was used to isolate DNA, which was detected via agarose gel electrophoresis. Figure 3 shows the presence of illuminated DNA bands on the gel in all the wells. The DNA samples were then subjected to PCR for the amplification of the 16S rRNA gene. The results obtained are shown in Figure 4.

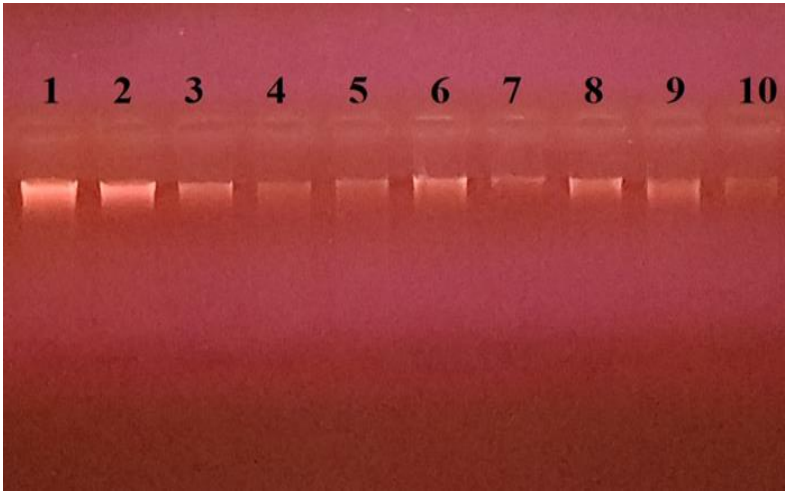

Figure 3. Agarose Gel Electrophoresis of DNA extracted from bacterial strains

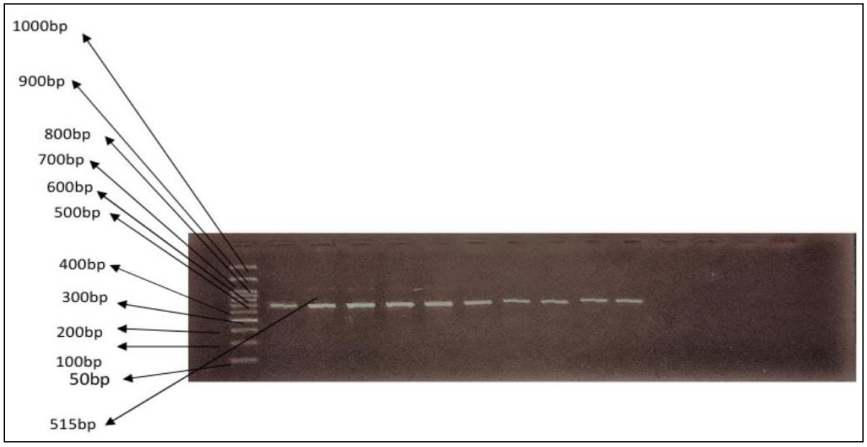

Figure 4. Polymerase chain reaction and Gel Electrophoresis

4. DISCUSSION

The poultry industry of Pakistan is the one of most significant agricultural sectors in poultry farming, which supplies the majority of the nation's animal protein needs (white meats and eggs). Animal protein for human consumption is a product of the chicken industry, which is linked to other sectors like animal feed, pharmaceuticals, and veterinary inputs. The global chicken business faces a danger from respiratory illnesses. In the genus Mycoplasma of the family Mycoplasmataceae, *Mycoplasma gallisepticum* is the most virulent species, and it is destructive to poultry (Marouf *et al.*, 2022). The expanding chicken sector in Pakistan has a persistent challenge from chronic respiratory disease (CDR). A PCR test revealed that it can be used to diagnose *M. gallisepticum* and isolate *M. gallisepticum* from hens. Serological approaches are still employed as standard screening tests for mycoplasma infections, which cause financial losses in poultry flocks and require early diagnosis (Umar *et al.*, 2017).

Difficulties were faced in isolating and identification. Although this method is labor-intensive, expensive, and prone to overgrowth due to saprophytic mycoplasmas and other bacterial infections, the gold standard for detecting MG pathogen culture limits its use. Due to its meticulous nature, it takes one week or even longer for them to grow and be identified in pleuropneumonia-like organism broth or/and agar media. Horse serum should be added to the media as a supplement. Swabs from the choanal cleft, cloaca, and phallus of live fowl are utilized to isolate the infection (Wakenell, 2016).

M. gallisepticum can now be diagnosed using PCR techniques. This method is utilized as a component of standard examinations to find *M. gallisepticum*. Because it can find microbial DNA in samples, this method is more sensitive than serology and culture. It is more likely than culture to produce favorable outcomes. It has frequently been observed that several techniques have been used to achieve *M. gallisepticum* distinction when cultivating layers (Rauf *et al.*, 2013). Winter has a higher incidence of *M. gallisepticum* than summer, and a similar report was published before.

Although *M. gallisepticum* is resistant to penicillin and other antibiotics that work by reducing cell wall production, it is known to be vulnerable to several antibiotics, including macrolides, tetracycline, fluoroquinolones, and others (Helmy *et al.*, 2020). *M. gallisepticum*

may become resistant to widely used antibiotics. Antibiotics have been used for treating respiratory diseases to prevent the reduction of egg formation (Collett *et al.*, 2020). Appropriate antibiotic treatment may not only considerably lower populations of *M. gallisepticum* in the respiratory tract, but it may also lessen the severity of clinical symptoms and lesions.

Although the clinical signs and symptoms of *M. gallisepticum* were varied, the respiratory systems of all birds and additionally the reproductive systems of coats and breeders were severely affected. Multiples, particularly in the lungs and trachea, cause chronic lesions such as those in the nasal and paranasal passages and trachea (Schmidt *et al.*, 2024).

The possibility exists that until and unless parent flocks are free of Mycoplasmosis, the disease will not be controlled by commercial flocks. Higher infection in young chickens may be caused by the vertical transfer of the organisms from the parent flock or by a lower immunity level. The highest infection incidence in larger flocks may be related to poor management and biosecurity measures in addition to the horizontal transfer of the organisms from one bird to another, it was determined that *M. gallisepticum* was particularly abundant in the flocks (Sawicka *et al.*, 2020).

5. CONCLUSION

This study demonstrated that *M. gallisepticum* is still present in commercial poultry and should be controlled by preventative measures based on drugs and immunizations, likely employing new vaccines depending on the local *M. gallisepticum* strain's characteristics. The techniques i.e., ELISA, PCR, and cell culture are highly beneficial in creating a baseline of information about the disease's prevalence in the poultry sector. Such data is required for upcoming regional and worldwide cooperation to locate the origin of the etiological agents, which will prevent their spread among the nation's farms. We suggest a large-scale study including large sample size and other geographical regions are recommended in future studies.

Conflict of Interest:

The authors declare no conflict of interest.

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