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EPIDEMIOLOGICAL AND PATHOLOGICAL STATUS OF *Mycoplasma gallisepticum* IN LAYER CHICKS AT FAISALABAD, PAKISTAN

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Avian mycoplasmosis is an infectious and contagious disease that mainly infects chickens and turkeys. Due to the presence of limited information on incidence of *Mycoplasma gallisepticum* in Faisalabad, Pakistan and the importance of the poultry industry stimulated to study very pathogenic *M. gallisepticum* in layer chickens in the Faisalabad area. Diagnosis of this disease in layers was done on overall history, clinical signs, post-mortem examination, serological and molecular methods. In the current study, a total of 92 samples of blood, liver, spleen, lungs, trachea and air sacs were collected from layer chicks. The serological screening was done by using the serum plate agglutination test (SPAT) and for further confirmation at the molecular level polymerase chain reaction (PCR) was performed. Out of the total, 20.65 and 15.22% samples were tested positive for *M. gallisepticum* by SPAT and PCR, respectively. More positive results by SPAT could be due to false-positive findings. So, PCR is regarded as a confirmed and reliable technique for the detection of the disease. This study successfully described that PCR is a more specific and reliable method for the detection of *M. gallisepticum* as compared to other technique like SPAT. The current study also showed that a single test or technique is not considered as a confirmatory tool for the detection of any pathogen like *M. gallisepticum*.

Keywords: *Mycoplasma gallisepticum*, Layer, chicks, Mycoplasmosis, Epidemiology, Pathology.

INTRODUCTION

The poultry industry is one of the most energetic domains of livestock in Pakistan that is providing sources of likelihood to about 1.5 million people in Pakistan (Hasni *et al*., 2020). Poultry meat production in Pakistan is contributing about 30% of total meat production (Hussain *et al*., 2015; Ghonaim *et al*., 2020; Yasmin *et al*., 2020). Among emerging diseases, one of the major diseases in the poultry industry is mycoplasmosis (Farooq *et al*., 2020). Infection with *M. gallisepticum* causes major economic losses to the poultry industry around the world; even infection can be severe enough that the whole flock can be destroyed to prevent further transmission (Hennigan *et al*., 2012; El-Yazid *et al*., 2019; Shoaib *et al*., 2020). *Mycoplasma* is considered as the simplest and the smallest prokaryote which lacks a cell wall and resides in the class Mollicutes having the genus *Mycoplasma*. Taxonomic characterization can be done on basis of serology, phenotype and sequencing of 16sRNA (Baksi *et al*., 2016; Yi *et al*., 2020).

Roughly, 120 species of *Mycoplasma* have been isolated, but only 20 species are pathogenic to birds (Fraga *et al*., 2013). Avian mycoplasmosis includes many pathogenic *Mycoplasma* in which *M. gallisepticum* and *Mycoplasma synoviae* are of utmost importance. *M. gallisepticum* is also recognized as chronic respiratory disease (CRD) affecting chicken and turkeys, but some domestic birds can also get the infection. Young birds are more susceptible to avian mycoplasmosis as compared to adult and mature birds (Ali *et al*., 2015). *Mycoplasma* forms very typical colonies having fried egg appearance. The predilection sites of *Mycoplasma* are mucosal membranes of the respiratory tract, eyes, urogenital tract and joints (Ahmed *et al*., 2015). This disease is characterized by conjunctivitis, sinusitis, and sneezing in the turkeys. It results in decreased egg production and low graded meat production in meat-type birds. *M. gallisepticum* and *M. synoviae* differ in infectivity and virulence and sometimes infection becomes unobvious (OIE, 2008).

Clinical signs of *M. gallisepticum* are greatly variable in the birds causing sub-clinical infection to clear respiratory signs including coryza, sneezing, and coughing. Other symptoms include, difficult breathing, tracheal rales, and nasal exudate oozing out through incomplete open beak. Occasionally, conjunctivitis with frothy ocular exudate can also be seen in the chicken (Sun *et al*., 2014; Mehmood *et al*., 2020). This disease is highly variable depending upon age, sex, season, production status, flock size and strain of the infecting bacteria (Islam *et al*., 2015). National Poultry Improvement Plan (NPIP) proposed three basic control measures to prevent *Mycoplasma* infections such as high level of security

measures, serological monitoring, and immediate culling of infected birds (Levisohn and Kleven, 2000). *M. gallisepticum* is known to be vulnerable to many antibiotics like tetracycline, quinolones, and macrolides, but it is resistant to penicillin. The use of antimicrobials decreases clinical signs and trans-ovarian transmission. At present, live attenuated and inactivated vaccines are commonly used in layer flocks to prevent infection but these vaccines are not recommended for breeder flock because these disturb the monitoring and diagnosis of *M. gallisepticum* in parent flock (Nascimento *et al*., 2005).

Detection of *M. gallisepticum* in the birds has been done by using some serological tests like SPAT, hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA). For the conformation of *M. gallisepticum*, PCR is regarded as the best technique (OIE, 2008). Due to the false-positive results, serological testing is not a favorable method in the monitoring of the disease (Wanasawaeng *et al*., 2015).

The current study was designed to check the *M. gallisepticum* status in layer flocks in Faisalabad, Pakistan. Gross and histopathological lesions, SPAT and PCR techniques were used for the diagnosis of *M. gallisepticum* in layer chicks.

MATERIALS AND METHODS

***Samples Collection*:** Diseased birds from Faisalabad region were presented to the Diagnostic Laboratory, Department of Pathology, University of Agriculture, Faisalabad, Pakistan. Farmers brought live along with dead birds to this laboratory with the complaint of respiratory signs. Live birds were used for the current study. A convenient method of sampling was done to collect the samples. Samples were collected from October, 2016 to April 2017. The overall condition of each bird was recorded, the live birds of above 17 weeks of age were sacrificed humanely and 92 blood samples were collected. A postmortem examination was done to observe the gross lesions. Tissues (liver, spleen, lungs, trachea and air sacs) were collected for DNA isolation and histopathology. The collected tissue samples were preserved in formalin for histopathology (Itoo *et al*., 2014), and for DNA isolation stored at -20°C (Rauf *et al*., 2013). The serum was extracted from blood samples and used for the serological test.

***Gross Histopathological lesions*:** Gross lesions were observed after conduction of postmortem examination. Organs including liver, spleen, lungs, trachea and air sacs were collected from the layer birds showing the lesions of *M. gallisepticum*. Lungs and trachea were used for histopathological study. Firstly, tissue samples were preserved in 10% neutral buffered formaldehyde. Later on, dehydration, clearing and embedding were performed. Transverse section of 5 µm thickness were cut, fixed on the slide and stained with hematoxylin and eosin (H and E stain) (Itoo *et al*., 2014). The tissue sections were examined under microscope coupled with a Microcomputer integrated digital imaging analysis system (Nikon Eclipse 80i, Nikon Co., Tokyo, Japan).

***Serum plate agglutination test (SPAT)*:** Blood samples were collected from the wing vein before humanely slaughtering. Serum was extracted. The test was carried out at room temperature (20–25°C) within 72 hours of serum collection and the reagent was also maintained at room temperature. The commercially available antigen of *M. gallisepticum* was used for SPAT (Atique *et al*., 2012). On a glass slide 3-5 µL serum was placed and the same amount of *M. gallisepticum* antigen was poured and gently mixed it for 30-40 seconds and agglutination was noted within 40-80 seconds before the serum and antigen mixture became dry.

***PCR*:** PCR was done for the detection of *M. gallisepticum* from the DNA extracted from different organs (liver, lungs, spleen and air sacs) of the morbid birds as defined by Santha *et al*. (1990). Briefly, DNA was extracted from tissue swabs by heating (for 10 minutes at 95°C in the water bath) and cold (for 10 minutes at -20°C) shocks. These swabs were dipped in phosphate buffer saline (PBS), centrifuged for 20 minutes at 13,000 rpm and the pellet was washed in PBS two times. The supernatant containing the DNA was collected in 1.5 ml Eppendorf tube. Forward and reverse primers for *M. gallisepticum* consisted of the following sequences (Osman *et al*., 2009): MG-14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3'; MG-13R: 5'-GCT-TCC-TTG-CGG-TTA-GCA-AC-3'. In short, the reaction mixture was attained through (Taq Buffer-2.5µl, dNTPs Mix.-2.5µl, $MgCl_2$-2µl, Taq Polymerase-0.3µl, DNA-4µl, DNAase free deionized water-11.7µl, Primer F-1µl and Primer R-1µl) for amplification of DNA. Shortly, initial denaturation for four minutes at 94°C followed by 35 cycles of denaturation at 94°C, annealing at 58°C and extension for 45 seconds at 72°C and final extension for 10 minutes at 72°C in a thermal cycler (Eppendorf master cycler) was done. Amplified DNA fragments were studied by Agarose gel (1.2%) electrophoresis using the DNA ladder (1kbp, Viventes) as standard. Agarose gel was put in an electrophoresis tank having 1X TAE running buffer by giving 110 volts for 45 minutes (Rauf *et al*., 2013). The gel was seen under UV trans-illuminator and image clicked by the gel documentation system.

***Statistical analysis*:** Epidemiological data of SPAT and PCR were analyzed using percentage method of analysis (positive or negative samples/total samples ×100) of data for respective techniques.

RESULTS

***Gross lesions*:** On postmortem examination, it was observed that many of the chicks did not show gross lesions, however, some birds showed sinusitis (inflammation of sinuses), conjunctivitis (inflammation of conjunctiva), tracheitis

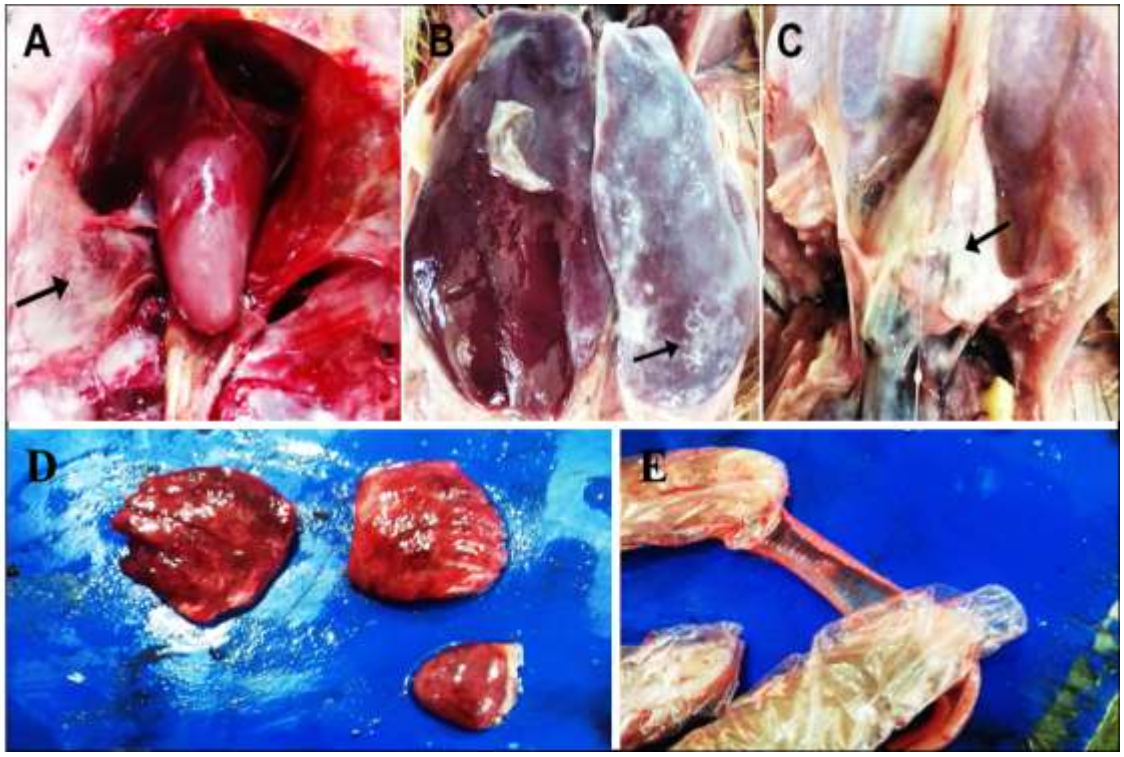

Figure 1. A: Photograph of airsacculitis (arrow), B: Perihepatitis (arrow), C: Pericarditis (arrow), D: Congested lungs and spleen, and E: Congested trachea from *M. gallisepticum* infected birds.

(inflammation of trachea) with lot of mucus, airsacculitis (inflammation of air sacs, Fig. 1: A), pneumonia, synovitis (inflammation of synovial joint), osteomyelitis (inflammation of joints), perihepatitis (Fig. 1: B), pericarditis (Fig. 1: C), congested and swollen lungs (Fig. 1: D), congested trachea (Fig. 1: E) and salpingitis (inflammation of fallopian tube). Airsacculitis is considered as the characteristic lesion of *M. gallisepticum*. Postmortem lesions revealed that respiratory system, reproductive system and synovial membrane are target organs in the *M. gallisepticum* infected birds. Some typical gross lesions observed in *M. gallisepticum* infected birds has been shown in Table 1.

Table 1. Gross lesions observed in *M. gallisepticum* infected layer chicks.

Gross Lesions	Morbid chicks	%
Tracheitis	3	3.20
Trachea with mucous	0	0.00
Airsacculitis	4	4.30
Fibrinous pericarditis	3	3.20
Fibrinous perihepatitis	4	4.30
Edematous lungs	16	17.39
Total birds = 92	30	32.60

***Histopathological changes in Trachea*:** The trachea is bounded by a pseudo-stratified ciliated columnar epithelium, hyaline cartilage, and tracheal lumen in healthy chicks but the histological structure of (morbid birds) trachea was indicating a moderate degree of epithelial degeneration and complete loss of cilia (Fig. 2; A-A2). Sub-epithelial region was normal. There was a mild degree of lymphocytic infiltration in the epithelial region. Mild to moderate degenerative changes were observed with the loss of cilia. A mild degree of epithelial hyperplasia with epithelium disruption was seen. There was a mild to moderate degree of congestion in the epithelial region of the trachea.

***Lungs*:** The moderate degree of congestion was present in the lungs. Severe congestion was observed at the bronchiole and alveoli level.

Alveolar septa had a thick and mild degree of fibrosis. Congestion was seen throughout the alveolar parenchyma. There was a mild to moderate degree of necrotic changes with congestion and inflammatory cells. Red hepatization was also observed in which lungs look like the liver (Fig. 2; B-B2). Interalveolar septa became thickened with emphysematous areas at some places.

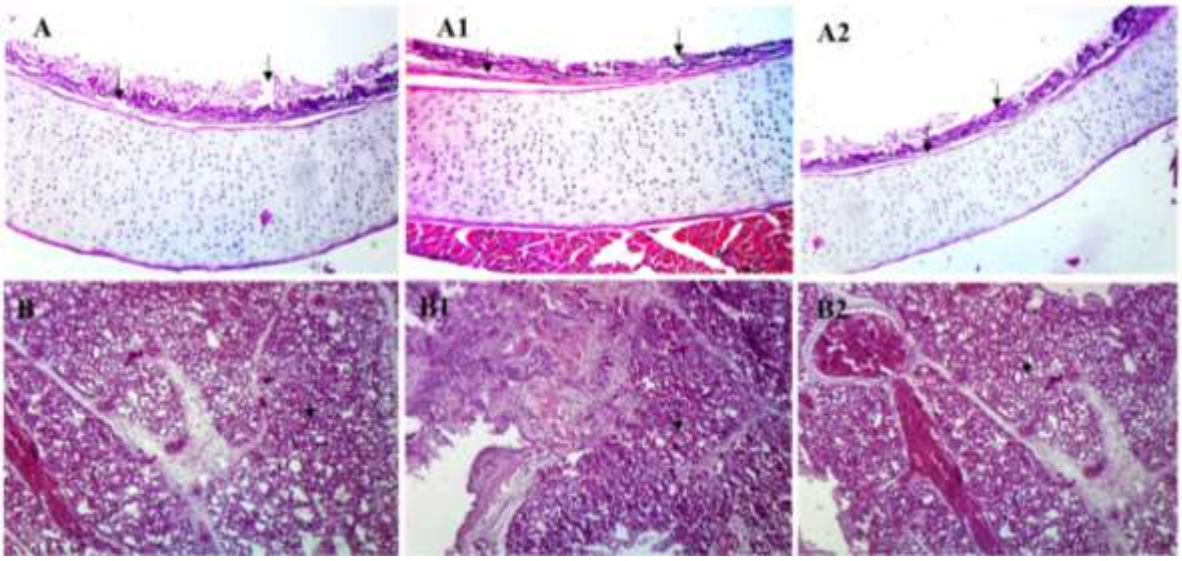

Figure 2. A-A2: Photomicrograph of trachea from *M. gallisepticum* infected birds showing loss of cilia with congestion in epithelial region (arrows) (H & E Staining 200x). B-B2: Photomicrograph of lungs from *M. gallisepticum* infected birds showing the red hepatization of lungs in which lungs appearance becomes like liver and severe congestion at bronchiole and alveoli level with some necrotic changes at many places (arrow) (H & E Staining 200x).

***M. gallisepticum* status by SPAT:** A total of 92 samples (blood) were collected for SPAT. Serum was separated and mixed with commercially available *M. gallisepticum* antigen at room temperature, to check the agglutination of serum and antigen. The agglutination was observed in 19 (20.65%) samples that were positive and the rest of the samples were negative for *M. gallisepticum* (Table 2).

Table 2. Positive and negative *M. gallisepticum* samples by SPAT and PCR.

Tests	Total Samples	Type of samples	Positive samples		Negative samples	
			No.	%	No.	%
SPAT	92	Serum samples	19	20.65	73	79.35
PCR	92	DNA template was isolated from organs (liver, spleen, air sacs and lungs)	14	15.22	78	84.78

***M. gallisepticum* status by PCR:** A total of 92 samples (liver, spleen, lungs and air sacs) were collected and amplicon PCR product having 183 base pairs visualized in agarose gel electrophoresis revealed that general *M. gallisepticum* primers had successfully targeted the respective pathogen or disease findings. Results were noted for the *M. gallisepticum* positive and negative samples. Out of total samples, 14 (15.22%) samples were positive for *M. gallisepticum*. The rest of the samples had not showed PCR amplicon product base pair (without PCR bands), hence termed as negative samples for *M. gallisepticum* (Table 2; Fig. 3).

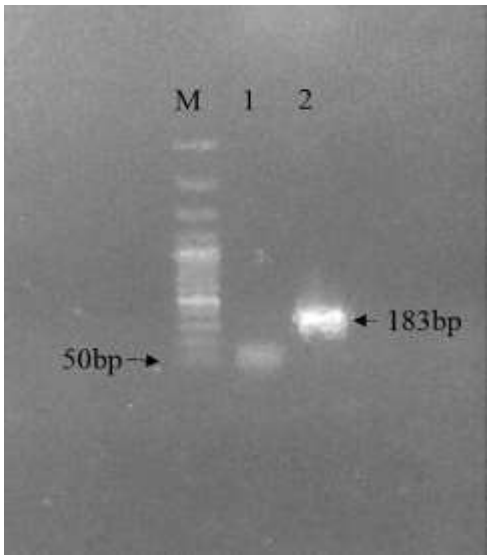

Figure 3. DNA based detection of *M. gallisepticum.* M: marker, Lane 1: Negative control, Lane 2: *M. gallisepticum* infected bird's sample showing positive band (183 bp).

DISCUSSION

M. gallisepticum is responsible for causing CRD and sinusitis in chickens. Respiratory signs in CRD are sneezing, coughing, and apnea (Prajapati *et al*., 2018). Various methods are in use to diagnose *M. gallisepticum* infection, such as SPAT, HI test, ELISA, and PCR. SPAT and HI test are

considered as quick or easy techniques for the detection of *M. gallisepticum*. Detection of *M. gallisepticum* in the flocks has been done by using some serological tests including the SPAT, HI test, and ELISA. For the conformation of *M. gallisepticum,* PCR is regarded as the best technique (OIE, 2008). In the current study, SPAT and PCR technique were used for the detection of *M. gallisepticum*. According to the farmer's history, the majority of young birds seemed to be healthy but some of them were emaciated, depressed and stunted growth appearance. Similar sings were observed and reported by the other scientists (Islam *et al*., 2015; Li *et al*., 2020). The majority of the young birds did not show the gross lesions but in few birds, lungs were swollen and the trachea was congested whereas, pericarditis, perihepatitis and airsacculitis were observed, while airsacculitis is considered as the characteristic lesion of *M. gallisepticum*. Postmortem lesions revealed that respiratory system, reproductive system and synovial membrane are target organs in the *M. gallisepticum* infected birds. Histological examination of the trachea showed a moderate degree of epithelial degeneration with loss of cilia. There may be a presence of a mild degree of lymphocytic infiltration in the epithelial region. A mild degree of disruption can be seen. Mild to moderate degree of congestion in the epithelial region of the trachea can be seen (Itoo *et al*., 2014). A mild to moderate degree of congestion can be seen in the lungs. Congestion can be observed at the bronchiole and alveoli level. Mild to moderate degree of necrotic changes with congestion can be observed (Garmyn *et al*., 2004). In the current study, the SPAT was performed which showed 20.65% positive results for *M. gallisepticum*. Positive (17%) flocks observed for *M. gallisepticum* in the present study was very close to the results of Hanif *et al.* (2007). 11.2% and 10.47% positive flocks were seen for *M. gallisepticum* in layer chicks by using the SPAT which showed less positive results than the current study in other findings (Hossain *et al.*, 2010; Atique *et al*., 2012). This low percentage might be due to low poultry farming in that area. These results showed that the SPAT is used for quick screening of *M. gallisepticum* in birds or it can be said that the SPAT is helpful for early diagnosis of *M. gallisepticum* in the poultry sector but a single test is not considered a reliable method for detection of any kind of pathogen like *M. gallisepticum*. So, after SPAT, PCR was done as a confirmatory test to detect the *M. gallisepticum* in layer birds and 15.22% positive results were observed in layer chicks which were similar to other reported results, i.e., 16.7% (Hossain *et al.*, 2010). In other research, *M. gallisepticum* infection in layer chicks was seen to be 21.84% (Rauf *et al*., 2013) that was much higher than the results of the present study. Other researchers (Nouzha *et al*., 2013) detected a very high (63.63%) prevalence of *M. gallisepticum* in poultry birds that was much more than that of the current study, which might be due to high poultry farming in that area. These drastic variations in results indicates that there may be a poor hygienic environment at the farm level including poor ventilation system, overcrowding, and low feed quality (Sevostyanova *et al*., 2020).

***Conclusions*:** It is concluded that the infection might be distributed at the hatchery level (transmitted from parent flock/ vertical transmission) but also horizontal at the farm level due to poor managemental or biosecurity practices. This infection causes swelling of infra-orbital sinuses and closure of eyelids. Serological screening should be done on a routine basis to control the disease at early stages but the confirmatory diagnosis is only possible by molecular methods. Thus productive efforts should be done to educate the poultry farmers regarding management practices, *M. gallisepticum* threats and preventive measures. However, more epidemiological studies are required to access the disease incidence in other areas of Pakistan for routine monitoring to reduce the economic losses occurring due to *M. gallisepticum* infection.

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