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Short Communication

Pathology of *Mycoplasma gallisepticum* in Naturally Infected Broilers and its Diagnosis through PCR

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ABSTRACT

This study documented the involvement of *Mycoplasma gallisepticum* (MG) in the respiratory diseases of commercial broilers. Birds from 48 commercial broiler farms with respiratory problem were visited in Lahore district. Variable clinical signs, gross pathological lesions and histopathological changes were recorded, which led to the placement of bird in four distinct groups. The polymerase chain reaction (PCR) detected MG infection in birds from 22/48 (46%) farms. These results showed that MG is a noxious bacterium affecting the broilers health and productivity, by weakening their immune system.

Key Words: Pathology of *Mycoplasma gallisepticum*; Pakistan; PCR; Broilers

INTRODUCTION

Among other factors, infectious diseases are the real threat to poultry industry in Pakistan. Of the various infectious diseases, Chronic Respiratory Disease (CRD) is of prime importance (Bajwa *et al*., 1992; Ehtisham *et al*., 2011). CRD is caused by *Mycoplasma gallisepticum (MG)*, a cell-wall less prokaryote that is widely prevalent in nature and causes disease in poultry and wild birds having predilection for respiratory tract (Vogl *et al*., 2008; Hafez, 2011). It causes reduced feed conversion and downgrading quality of carcasses at slaughter resulting high economic losses through mortality and loss of weight. Transmission can occur through eggs or by inhalation of air borne droplets, resulting in rapid disease transmission throughout the flock. Infection often remain asymptomatic, however, clinical signs associated with MG infection in broiler birds include respiratory rales, coughing, sneezing, nasal discharge and frequently sinusitis and open mouth breathing (Saif *et al*., 2003).

Traditionally, the diagnosis of MG was based on serological assays to detect antibody and/or isolation and identification of the organism. However, it is difficult to diagnose MG infection in poultry on the basis of clinical signs. Some of the disadvantages of serological methods are false positive and false negative reactions due to non specific reactions (Yamamoto, 1991; Yoder, 1991; Kempf, 1998). In vitro, isolation is usually done to confirm the serological results. Cultivation techniques are laborious, expensive and require sterile conditions moreover; there are again chances of false positive and false negative results while confirming the isolates (Fan *et al*., 1995; Kempf, 1998). Recently, these techniques have been replaced by Polymerase Chain Reaction (PCR) technology for diagnosis of MG. PCR test can be performed on clinical samples without the need of isolation. Its high sensitivity and fast turn around time is making it most frequently used test to monitor MG infection (Gharaibeh and Al Roussan, 2008).
The present project, therefore, is designed to standardize PCR for the rapid diagnosis of MG in naturally infected broiler birds and investigate the gross and histopathological changes produced by MG infection.

MATERIALS AND METHODS

During the period of four months, November 2008–February 2009, 48 commercial broiler farms (located in Lahore district) with respiratory symptoms were examined. A detailed study of clinical signs and gross lesions was recorded on examination of suspected birds of each flock. Furthermore, tissues samples of trachea and lungs were collected from each flock and were kept at –80°C until they were tested through PCR. Similarly, morbid tissues of trachea, lung, liver and heart were also collected from MG infected birds for histopathological studies. These tissues were fixed in natural buffered formalin and then processed by using paraffin embedding technique for preparation of sections and stained with haematoxylin and eosin (H & E) for observing microscopic changes caused by M. gallisepticum (Anjum, 1980; Bancroft & Marilyn, 2002).

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DNA extraction: was performed from tissues using Fermentas life sciences Pure Extreme ™, EU DNA purification kit. Twenty mg of grinded lungs and trachea were collected in 1.5 mL Eppendorf tubes separately. A 200 µL of TE buffer was poured in each tube and mixed by vortex for few seconds. Tubes were incubated at 65°C for 20 minutes in thermostatically controlled water bath. A 400 µL of cell lysis solution was added and mixed with the help of the tip of pipette. Samples were then incubated at 65°C in water bath for over night followed by addition of 600 µL of chloroform in each tube and was mixed with several inversions and by vortex for a minutes. Spinning was done at 13,000 rpm for 10 min at 4°C. The aqueous phase of supernatant was carefully collected containing DNA in new eppendorf tubes and 800 µL of precipitation solution were added in each tube and were vortexed, centrifuged at 13000 rpm for 10 min at 4°C. Supernatant was removed carefully and DNA pellet was dissolved in 100 µL of 1.2 % NaCl solution by vortexing, afterward added 300 µL of cold absolute ethanol and the DNA was allowed to precipitate for 10 min at -20°C, followed by centrifugation at 13000 rpm for 3-5 min and pouring off ethanol. Final wash with 70% cold ethanol was given to DNA and dissolved in 30 µL sterile deionized water. The DNA was used as template in PCR reaction. For detection of MG, specific primer pair was used. The sequence of the forward primer MG-14F: 5´-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3´ the sequence of the reverse primer was MG-13R: 5´-GCT-TCC-TTG-CGG-TTA-GCA-AC-3´ (Lauerman, 1998).

The amplification reaction was performed in total volume of 25 µL containing 2.5 µL of 10 X PCR buffer (Fermentas), 2 µL of 1.5 mM $MgCl_2$ (Fermentas), 2.5 uL of 2.5 mM dNTPs, 0.3 µL of 2.5 U/µL AmpliTaq DNA polymerase (Fermentas), 1 µL of 10 pM/µL each primer, ,4 µL of DNA template and 11.7µL of molecular grade water to complete the final volume. DNA amplification was performed in thermal cycler (Master Cycler Eppendorf) that was programmed to heat 94ºC for 4 min followed by 35 cycles of denaturation at 94ºC for 45 sec, annealing at 58ºC for 45 sec and extension at 72ºC for 45 sec and a final extension at 72ºC for 10 min and soak at 4°C.

PCR product of 185 bp was detected by agarose (1.5%) gel electrophoresis, 50 bp ladder (Fermentas) was used to accurately determine the size of PCR product, followed by viewing by using gel documentation system.

RESULTS AND DISCUSSION

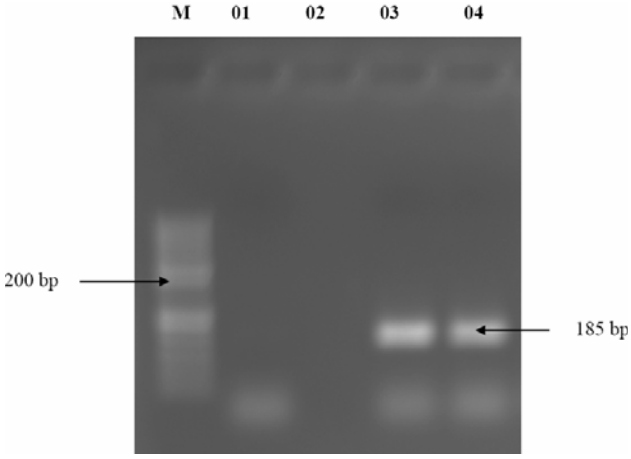
Clinical signs: The mycoplasma infected birds were dull, depressed with ruffled feather, coughing and sneezing with nasal discharges, open mouth breathing with moist rales and typical voices were recorded. Birds in complicated disease with other organism most commonly *E. coli,* were having the similar clinical signs but more goaded and severe with relatively high mortality and morbidity. Respiratory signs and poor conditions were the main clinical findings and the findings are consistent with (Saif *et al*., 2003).

Table I Prevalence of Mycoplasmosis in broilers suffering from respiratory illness. Total number of flock visited Positive by PCR Prevalence
48 22 46 %

Bird groups	Dull and depressed	Coughing and sneezing	Nasal discharge	Breathing with moist rales	Bird voice
1-12	+++	++	+++	++	Low
13-24	++	+++	+++	+++	Medium
25-36	+++	+++	++	++	Loud
37-48	++	+++	+	+	Low

Lesion scoring: +, mild; ++, moderate and +++, severe

Fig. 1: PCR detection of *Mycoplasma gallisepticum* infected broiler



All 50 samples were also subjected to PCR for diagnosis of Mycoplasmosis in broiler birds, 22 out of 48 (44%) were found positive. The high prevalence may be due to the screening of flocks having respiratory problems and the winter season of the study, hence, it is found that involvement of MG in respiratory problems is very high in winter season.

Gross pathology: Gross lesions were often confusing to suggest Mycoplasmosis as described by (Bajwa *et al*., 1992). There was serious involvement of trachea, lungs, air sacs, heart and liver as reported by (Bajwa *et al*., 1992; Saif *et at.,* 2003). Catarrhal exudates in nasal passages, tenacious, frothy exudates was adherent to the tracheal wall. The trachea showed the evidence of congestion and haemorrhages. Lung revealed dark red color appearance and showed congestion, light color foci and haemorrhages in complicated cases. Frothy exudate was present in some cases. Accumulation of caseous material in the bronchi and pneumonic areas in the lungs were observed. Air sacculitis was observed in air sacs, these become thickened and covered with caseous exudates and cloudiness was seen as mentioned by (Saif *et al.,* 2003; Gharaibeh & Al Roussan, 2008). Liver and heart was found clear in the mycoplasma infected birds. However, in complicated infections, heart showed some enlargement and covered with fibrinopurulent covering, while liver was

congested and showed haemorrhages. The color of the liver was slightly changed to pale and fibrinopurulent covering perihepatitis was observed (Ibragimov *et al.,* 1983; Bajwa *et al.,* 1992; Saif *et al.,* 2003).

Histopathological changes: In the present study, histopathological investigation of morbid tissues of positive cases conformed through PCR i.e., trachea, lungs, heart and liver were undertaken to record the changes at cellular level. Most consistent histopathological alterations were observed in trachea and lungs. The tracheal epithelium was necrosed and swollen. Epithelial cells and cilia in some areas were covered with thick layer of mucous. Epithelial and sub mucosal infiltration with leukocytes was observed. Hyperplasia of epithelial mucous glands, thickness was increased due to cellular infiltration and oedema. Sloughing of mucosa and hemorrhages of variable degree was also noted, which coincides with the findings of Gaunson *et al.* (2000).

Lungs showed congestion, haemorrhages, focal necrosis and leukocytic infiltration (lymphocytes & polymorphs). Emphysema and exudates in alveoli was also recorded. Grey hepatization in slides of some lungs was also observed. Giant cells, miliary granuloma in lungs were observed which were also recorded by Saif *et al.* (2003). In complicated cases, mostly with *E. coli*, liver and heart showed congestion and leukocytic infiltration (Lymphocytic & Polymorphonuclear). Haemorrhages, degeneration and necrosis of hepatocytes were recorded in some sections. Degeneration of cardiac muscles and pericarditis was also seen (Bajwa *et al.,* 1992).

CONCLUSION

PCR assay for detection of MG infection in field is a good and rapid technique for diagnosis. The prevalence of MG is a real threat to broiler farmers, especially in the winter season, since it weakens the immune system of birds, simultaneously injuring and exposing the body tissues to opportunist pathogens, like widespread prevalent *E. coli*, which causes mortality and aggravates morbidity resulting in great economic losses.

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