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# Molecular Epidemiology of *Mycoplasma gallisepticum* in Different Types of Chickens

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## Abstract

Molecular epidemiology of *Mycoplasma gallisepticum* in commercial and backyard poultry was conducted in different parts of Pakistan and Azad Jammu and Kashmir. Molecular identification of the isolates was made with the application of polymerase chain reaction (PCR). A total of 142 isolates of *M. gallisepticum* from 573 flocks were identified. The isolates were further characterized on the basis of SDS-PAGE. The cumulative prevalence of *M. gallisepticum* noted in broilers, broiler breeders, commercial layers and layer breeders including backyard/rural poultry was 14.62, 29.33, 23.16 and 42.85%, respectively indicating significant prevalence in all type of birds with Chi-square value of $X^2$=7.81. Significant seasonal variation ($X^2$=3.84) indicated the higher prevalence 56.34% in winter as compared to 43.66% in summer. The findings of SDS-PAGE indicated that 71.43% of the tested isolates proved pathogenic with the presence of 74 kDa adhesive protein bands representing *M. gallisepticum* F strain. The remaining isolates represented less virulent or variant stains. The high prevalence of *M. gallisepticum* in respiratory infection of poultry in Pakistan demands for framing national policy through surveillance and control of the disease. 

**Keywords:** Mycoplasma; Molecular epidemiology; Chicken

## Introduction

The *Mycoplasma gallisepticum* (MG) infections causing chronic respiratory disease (CRD) in association with other pathogens said to be very consistently inflicting economic losses to the poultry farming sector. The incurring losses constitute mortality, reduced feed efficiency, and decreased production of both meat and eggs along with condemnation of meat quality. The cost of preventive and therapeutic measures adds to the losses (Evans *et al.,* 2005). The transmissions of MG infections were reported both horizontally and vertically. Vertical transmission is either *in ovo* or transovarian and it has been established to occur through experimentally induced infections (Lin and Kleven, 1982; Glisson and Kleven, 1984; 1985; Ortiz *et al*., 1995; Siddique *et al*., 2012). By virtue of such potential threats MG amongst avian pathogenic Mycoplasmas, is the only pathogen that causes the infection an OIE notifiable disease (OIE, 2004).

Mycoplasmas in general are the small prokaryotes having 300–800 nm diameter and devoid of cell wall but bounded by a triple layered plasma membrane. Mycoplasmas have a genome size of 600 – 1350 Kb with DNA G + C content of 23 – 40%, and *in vitro* selective growth requirements makes them fastidious organisms (Razin *et al*., 1998; Mukhtar *et al.,* 2012; Mustafa *et al*., 2013). With discovering the new cell invasive character of pathogenic mycoplasmas, *M. gallisepticum*, phylogenetically belongs to *M. pneumoniae* cluster is also shown to invade Hela cells and chicken fibroblasts *in vitro* and other nonphagocytic cells. This cell invasive character plays the role in systemic spread of the infection (Citti *et al.,* 2005; Gunther *et al.,* 2008; Zhang *et al*., 2013). These findings provide novel insight into the patho-mechanism of MG and may have implications for developing of preventive strategy.

In Pakistan, clinically the respiratory disease problems are greatly prevailing posing great threats to the poultry sector but in view of very scanty information available regarding true prevalence of the diseases in the country there is a need to identify and differentiate MG infections amongst those respiratory diseases. In the current scenario nature of the pathogen demands for conducting large scale survey to ascertain the true prevalence of MG infection based upon more sensitive and specific molecular techniques prior to frame out the national policy for taking up the control measures.

## Material and Methods

### Clinical Examination and Recording of Case Information with the Collection of Samples

A total of 171 broiler flocks, 208 broiler breeder flocks, 145

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layer flocks and 49 flocks representing the layer breeders were investigated. The breeding stocks of Fayoumi, Rhode Ireland Red (RIR) and intercross of Fayoumi, RIR and undescriptive or desi birds meant for the replication of the rural poultry along with backyard poultry were categorized as layer breeding stocks during the study.

Healthy and morbid birds showing respiratory distress along with those showing sero-positive results were included in the study. Morbid and the dead birds were checked for clinical findings and the pathological lesions. The carcasses showing the typical signs, symptoms and lesions were included for further laboratory investigations with the record of all the relevant flock information.

The preferred sources of samples as referred by Kleven (1998) were harvested and preserved for further laboratory procedures. In acute cases choanal cleft, trachea and lungs were sampled. Whereas at chronic stages typical lesions of air sacculitis were included in the samples.

### Bacterial Isolation and Identification

For the isolation and preliminary identification the standard procedures were followed as detailed by Kleven (1998). During these procedures PPLO broth and agar were constituted according to the formulation of Frey *et al.* (1968). The isolates showing the microscopic colonies typical of *Mycoplasma* were subjected to biochemical reactions including glucose fermentation, phosphatase production, tetrazolium reduction and film and spot assay (Aloutta *et al.*, 1970; Thomas and Barber, 1971; Edward and Moore, 1975; Kreig and Holt, 1984). The isolates were identified on the basis of cultural, morphological, and biochemical parameters as reported by Kreig and Holt (1984) in comparison with the standard MG culture (Table 1).

### Molecular Identification

**DNA extracion and isolation:** DNA was extracted from *Mycoplasma* cultures through enzymatic/kit method. One ml of *Mycoplasma* culture was centrifuged at 13,000 × g for 6 min. The pellet was washed twice with 1 mL Phosphate Buffer Saline (PBS) and re-suspended in 100 µL PBS. After incubation at 95°C for 15 min the bacterial DNA extraction kit (Vivantis, S., Coast Highway Suite 1, Oceanside CA 92054, 1012, USA) was used for the purification of DNA through specified procedure (Dvorakova *et al.*, 2005).

**Primer selection for *M. gallisepticum:*** The primer pair sequences of MG were selected from the variable region. These parameters were prepared from Gene Link, (Hawthorne, NY., USA): MG FP 5'-CTTTCCCATCTCGACCAGGAGAAAA-3' the reverse primer MG RP sequence was: 5'-GGATCAATCAGTGAGTAACTGATGA-3' of 732 bp (Wang *et al.*, 1997).

**Amplification reaction for PCR:** The amplification reactions of the isolated DNA from specimen samples in 50 µL volumes were carried out. Each of the PCR mixtures contained master mix (Vivantis, S., Coast Highway Suite 1, Oceanside CA 92054, 1012, USA) 15 µL. Forward primer 1 µL (330 ng/µL (Gene Link NY, USA). Reverse primer 1 µL (330 ng/µL (Gene Link NY, USA). Ultra-pure water 28 µL. Different controls were also included such as: Primers without template DNA and template DNA without any primer.

DNA amplifications were performed in a thermal cycler (Model Peqlab 2s Primus, Germany). Following optimum running conditions as described by Wang *et al.* (1997) in the digital program of thermal cycler were adopted which included initial denaturation temperature for reaction mixture at 5 min. The numbers of PCR running cycles were 35 with the following setting at each cycle. Initial denaturation temperature = 94°C for 5 min, anealing at 50°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min.

**Gel-electrophoresis and visualization:** The amplified PCR product was subjected to agarose gel electrophoresis with 2% agarose, 1% TBE buffer and 0.5 µg/mL ethidium bromide. Each of the amplified products was loaded with 5 µL in the individual wells of the gel. In the first well row gene marker/ledder of 100 bp (Vivantis, S., Coast Highway Suite 1, Oceanside CA 92054, 1012, USA) was loaded. The electric current of 100 volts was applied for 30 min. The resulting bands were visualized under Ultra Violet trans-illuminator gel documentation system (WealTec, USA).

### Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Further characterization of the isolates was performed to resolve the variability of strains. All the culture suspensions standardized through prior protein estimation (Bradford, 1976) were subjected to SDS-PAGE (Laemmli, 1970). Pre-stained protein molecular weight markers ladder SM0441 (Fermentas) was used as standard in SDS – PAGE. The ladder consisted of 6 bands i.e., 20, 25, 35, 50, 85 and the last 120 kDa. The SDS gel was run at a constant voltage of 100 volts. The PAGE was stopped when tracking dye front reached at bottom of the gel. The gel was washed with water after staining in order to improve the intensity of the bands (Coughlan, 1988). The bands formed were visualized with naked eye and photographed.

### Statistical Analysis

The data was subjected to statistical analysis applying Chi-Square test (SPSS for windows, version 16). $P<0.05$ was accepted as statistically significant or dependent and $P>0.05$ was considered as insignificant or independent.

## Results

### Clinical Findings

The usual features included rales, nasal discharge, sneezing,

coughing and swollen eyes with the variable mortality maximum up to 50% depending upon the complexity of the case along with reduced weight gain and production. Ruffled feathers were more commonly observed in younger birds. The most prominent lesions in acute phase included catarrhal exudation in air passages like trachea and bronchi with congestion and hemorrhages in tracheal and bronchial walls. In subacute and chronic cases caseous beads in the affected air passages were the frequent findings. The complicated cases were commonly accompanied by the association of *E. coli* infection frequently exhibiting airsacculitis. Accumulation of caseous material in the thoracic air sacs and the lungs was the peculiarity of chronic phase of the disease.

**Isolation, Identification and Biochemical Characterization**

The initial cultures giving typical reactions of changing the color of PPLO broth in inoculated tubes into turbid and yellow within specified duration, were selected for further procedures starting with PPLO agar plate inoculation. The rest were discarded. The typical appearance on the incubated PPLO agar plates with the tiny translucent colonies were taken into account. The colonies giving fried egg appearance when examined microscopically under 10X were suggestive of mycoplasma growth. A total of 142 culture isolates were further identified in comparison with the standard MG culture on the basis of biochemical reactions profile harvested from different organs showing typical clinical and pathological exhibitions including 45 from trachea, 38 from lungs, 24 from air sacs, and 35 from oral swabs in different types of birds (Table 1).

**Polymerase Chain Reaction (PCR)**

The samples identified as MG through growth and biochemical characters were subjected to PCR and the results were recorded after electrophoresis of amplified PCR products under Ultra-Violet (UV) light Trans-illuminator. A 732 bp product was obtained after PCR (Plate 1).

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

Most of the MG isolates (71.43%) identified through PCR when subjected to SDS-PAGE showed uniformity in the exhibition of protein bands. Remaining of the tested isolates (28.57%) differed in lacking in the protein band of 74 kilo-Dalton (kDa) (Plate 2).

**Prevalence of *M. gallisepticum* Infection**

The respective cumulative prevalence of *M. gallisepticum* in broilers, broiler breeders, commercial layers and layer breeders was recorded as 14.62%, 29.32%, 23.17% and 42.86% with the overall prevalence as 24.78% (Fig. 1). Seasonal prevalence of MG noted during the study was 56.34% in winter as compare to 43.66% in summer season (Fig. 2). Statistical analysis revealed that MG infection is significantly ($p<0.05$) prevailing in all types of chicken included in the study with variable figures. Further it was found that MG infection is significantly ($p<0.05$) associated with the season. In broilers age wise prevalence of MG was recorded the highest 27.3% during the age of 14-21 days followed by 18.5%, 18% and 16.7% in the age groups of 29-36 days, 35-43 days and 22-28 days, respectively. In broiler breeders age wise prevalence of MG was recorded the highest 37.50% during the age of 9-13 weeks followed by 36.4% each during the age of 4-8 weeks and 41-60 weeks and 33.3%, 31.8%, 27.8%, 23.8% and 6.3% in the age groups of 14-20 weeks, >60 weeks, 21-30 weeks, 31-40 weeks, and 1-3 weeks, respectively. In commercial layer the highest prevalence of MG was recorded 30% during the age of 4-8 weeks followed by 28.6%, 25%, 23.6%, 20% and 16.7% in the age groups of 14-20 weeks, 21-30 weeks, 31-40 weeks, 41-60 weeks and 9-13 weeks, respectively. In layer breeder flocks 60% was the highest prevalence of MG recorded during the age of 4-8 weeks, followed by 57.9%, 50% and 25% in the age groups of 41-60 weeks, 14-30 weeks and > 60 weeks, respectively.

## Discussion

The study confirms the high prevalence ($X^2=7.81$) of MG infection in commercial poultry sector previously reported by Ahmed (1998). Further the present study included first time the broiler flocks and the rural/backyard poultry sector for ascertaining the prevalence of MG infection in Pakistan.

In broilers, the highest prevalence of MG is shown during the age of 14-21 days without a single isolation during first 2 weeks of age. This indicates the transovarian infection caused by considerably high prevalence of MG in breeder flocks. Meanwhile the reason of no isolation of MG during first two weeks of age could be the day old broiler chicks are marketed consciously with prior injectable antibiotic therapy resulting the mycoplasma infection is temporarily suppressed. The complex problem in the form of CRD with overwhelming association of *E. coli* is quite significant and a usual exhibition in broilers. The later trend with bit lower prevalence of MG in broilers shows the therapeutic measures taken at farm level including the intensive use of antibiotics, meanwhile with poor biosecurity status. Regarding the cumulative figures of MG in different types of chicken the lowest prevalence as recorded in broiler is endorsed by Osman *et al.* (2009) and others. The reason being the broilers exist in the field for a smallest period of time of all the categories of birds and accordingly challenge duration of MG for broiler is quite low. Further the stocking density is an important epidemiological variable affecting the MG prevalence rate (McMartin *et al.,* 1987) as the broiler flocks in open

**Table 1:** Tentative identification of Mycoplasma *gallisepticum* recovered from different tissues

| Type of Samples | Total Samples | Glucose Fermentation | Phosphatase Production | Tetrazolium Reduction | Film Spot Assey |
|---|---|---|---|---|---|
| Trachea | 45 | + | - | + | - |
| Lungs | 38 | + | - | + | - |
| Air Sacs | 24 | + | - | + | - |
| Oral swabs | 35 | + | - | + | - |
| Standard MG culture | 1 | + | - | + | - |
| Total. | 143 | + | - | + | - |

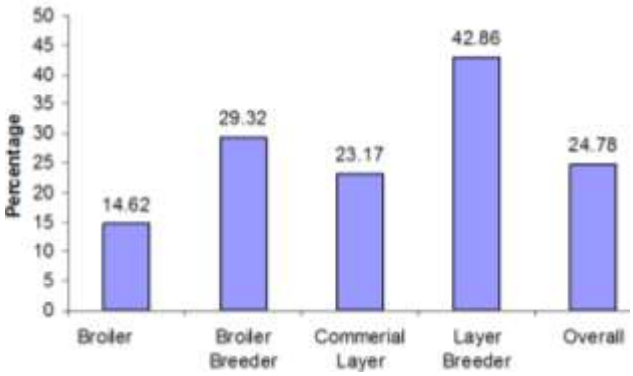


**Fig. 1:** Cumulative prevalence of *Mycoplasma gallisepticum* infection in different types of birds. (Chi-Square value of $X^2$=7.81 at 5%)

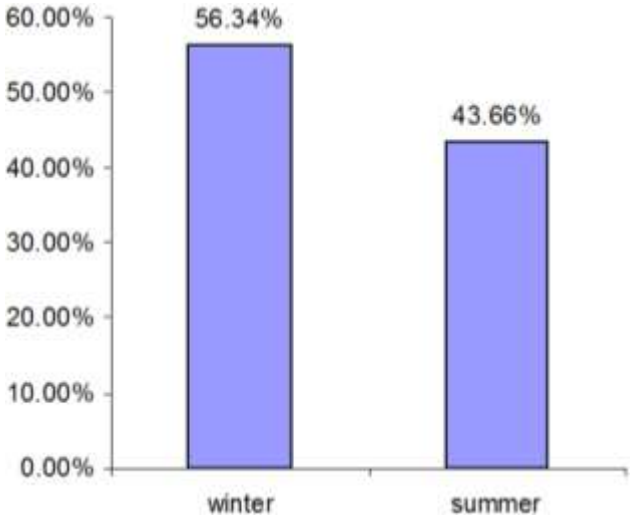


**Fig. 2:** Seasonal Prevalence of *Mycoplasma gallisepticum* including all types of birds with Chi-Square value of $X^2$=3.84 at 5%

environmental conditions are reared in lower birds' density as compare to the laying flocks.

The cumulative prevalence of MG in commercial layer (23.17%) as recorded in 145 flocks during the study is quite unlike to the findings of Mukhtar *et al.* (2012) (8.64%) reported in only 18 layer flocks survey, whereas Gharaibeh and Al-Roussan (2008) and Feberwee *et al.* (2005) respectively report 31.6% and 33% prevalence of MG in layer flocks. Even the higher prevalence rate (62.96%) is

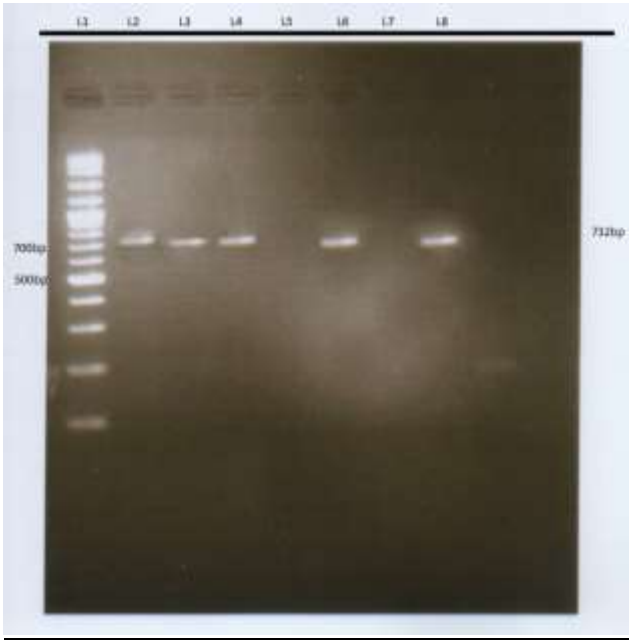

**Plate 1:** Thermal photograph showing detection of MG with 732 bps product through polymerase chain reaction (PCR)
Lane 1: 100 bP ladder
Lane 2, 3, 4 and 6 showing positive samples
Lane 8: showing control +iv MG
Lane 5 and 7: Control negative

reported by Heleili *et al*. (2011). But in all these references comparatively smaller number of birds were included in the studies. Some other factors like clinical health status related to MG infection of the flock along with sampling could also be playing role with variable results. Age wise prevalence of MG in broiler breeder and layer birds reflect high field challenges with more exposure time and poor biosecurity measures at farm level. Generally the highest prevalence at the age of 4-20 weeks speaks of some concurrent immunosuppressive factors when the birds are more prone to get the infection of Gumboro disease along with role of some mycotoxic levels in the feed. Coccidiosis is another immunosuppressive malady commonly prevailing particularly during that age period. Starting to lay age (18-20 weeks) itself is another stressing factor predisposing the birds to the field challenge of MG infection. Even the risk of transovarian infections cannot be ignored. Osman *et al.* (2009) confirms the overall prevalence of MG in broiler

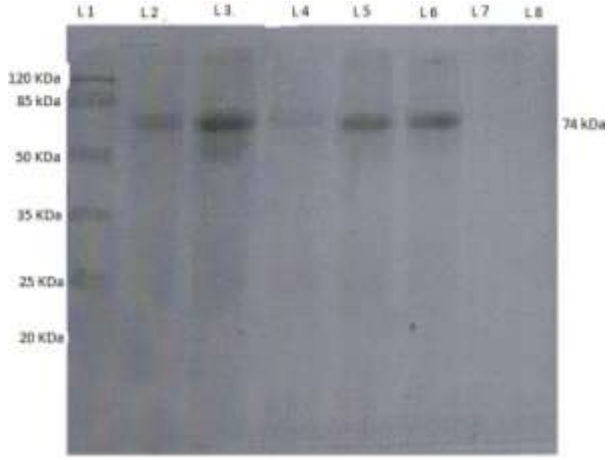


**Plate 2:** Polyacrylamide gel electrophoresis indicating L1 as molecular weight markers. Samples L2, L3, L4, L5 and L6 showing typical SDS-PAGE band product of 74 kDa indicating pathogenic isolates possessing adhesive bands or hemagglutinins. Samples L7 and L8 found negative for adhesive bands being nonpathogenic isolates of *Mycoplasma gallisepticum*

breeders and commercial layer as recorded during the study. However the therapeutic measures in both broiler breeder and layer flocks may contribute in lowering the prevalence rate. Moreover, the present role of MG vaccines particularly in breeder flocks looks questionable and warrants further investigation to ascertain their role in controlling the problem of MG. Amazingly the rural poultry showed the highest prevalence of MG in poultry sector in Pakistan, no doubt smaller number of rural poultry stocks were included in the surveillance as required in the study. However the present MG status in different types of birds reflects that both commercial and rural poultry stocks are serving mutually as source of tramsmission of MG. Moreover, multiage layer and breeder flock kept in close contacts could be the foremost reason in the persistence of MG infection. The reports of Hoffman *et al*. (1997) and Jaganathan (2006) are also quite inline to the recorded prevalence of this study. Lack of disease controlling measures in the rural poultry sector particularly against MG may reflect such a significant prevalence of the problem in the rural sector.

Generally MG prevalence was noted higher in younger birds as compared to older ones. Many workers like Hossain *et al*. (2010) and Mukhtar *et al.* (2012) endorse this finding. Probably vertical transmission (Ortiz *et al*., 1995) and consequently therapeutic and control measures at farm level could be the reason in this age related variation in the prevalence. Further the smaller number of flocks aging 60 or more with very thin strength exiting in the field further explain this phenomenon.

Present study also indicated that MG infection prevailed to a significantly higher level ($X^2$=3.84) in winter as compare to summer in all type of birds. Seasonal stresses like extremely low environmental temperature and consequently poor temperature controlling measures (Shikra *et al*., 2005; Hossain *et al*., 2010; Heleili *et al.*, 2011) and other weak managemental practices may contribute to this seasonal difference (Ahmad, 1998; Mukhtar *et al*., 2012).

Widespread application of advanced molecular techniques like PCR in the diagnosis of avian pathogens has led to the development of commercial DNA based kits meanwhile increasing the cost of diagnosis. The reliability of PCR is established worldwide. During the study PCR proved really specific, sensitive and reliable as different studies endorse these findings as compared to conventional isolation and identification methods probably due to excessive usage of antibiotics in the commercial flocks causing temporary suppression leading to failure of the MG isolation (Jaganathan, 2006).

Molecular detection of pathogenic species of MG through SDS-PAGE was indicated by the presence of specific 74 kDa lipoprotein bands in 71.43% of the isolates, which is an important characteristic of MG F strain (Jaganathan, 2006). These have been described as immunodominant adhesions or hemagglutinins and considered important virulent factors and the antigens carrying such cytadhesion properties may play key role in the pathogenesis of the invading organism by attaching to receptor sites on host epithelial cells through sialic acid residues leading to colonization and infection thus initiating the immune response (Avakian and Ley, 1993). Membrane proteins and lipoproteins including PvpA and pMGA and three others are characterized as phase variant forms occurring at high frequency and confer antigenic variation and varying pathogenicity to MG (Boguslavsky *et al*., 2000; Papazisi *et al.*, 2003). Newly discovered host cell invasive character of MG may add to the complexity of disease causing ability of the pathogen (Citti *et al.*, 2005; Gunther *et al.*, 2008). The joint interplay of such factors may result into the emergence of chronic form of the disease (Benicina, 2002). It is further speculated that other less pathogenic or may be non pathogenic isolates of MG (28.57%) as detected during the study might be playing their role in hindering the proper results of serum plate agglutination tests (Jaganathan, 2006). However, they may attain the same disease causing nature in association with other pathogenic organisms like *E. coli,* Infectious bronchitis virus and Newcastle disease virus giving rise to the shape of Chronic respiratory disease (CRD). So there is a need to sort out those isolates in ascertaining their extent of role in disease causing process and the consequent incurring losses along with their reaction towards plate agglutination serology against the standard antigen.

In conclusion, high prevalence of MG infection was found in all types of birds in the poultry sector in Pakistan. PCR is an effective diagnostic tool for MG, which can be used for surveillance of this disease. Results also warrant formulation of a national MG monitoring policy especially in the wake of forthcoming recommendations of WTO.

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