



RESEARCH ARTICLE

Biological Characterization of Locally Circulating *Mycoplasma gallisepticum* in Poultry

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ABSTRACT

Mycoplasma gallisepticum (MG) is a pathogen of concern for poultry. Present study was conducted to determine the biological characteristics of a field isolate of MG, recovered from an MG-affected flock. This isolation was made through conventional method of MG cultivation, using modified Frey's media after confirming the isolate by polymerase chain reaction (PCR). A total of 48 birds were segregated into experimental group (32 birds) and the control group (16 birds). To appraise primary site of infection, MG broth propagated culture containing 1×10^6 CFU/ml was inoculated intratracheally to each bird in the experimental group, whereas the control group was sham inoculated by uninoculated broth. The clinical signs and symptoms were recorded daily from day 1 to 21 post-infection (p.i.). Seroconversion monitoring was carried out, at day 5, 10, 15, 20 p.i. by Serum Plate Agglutination test (SPA) and Enzyme Linked Immunosorbent Assay (ELISA). To determine the dissemination pattern of MG, birds were sacrificed according to plan, swabbed from various organs and subjected to MG-specific PCR. Tracheal lesions and air sac lesions were scored after necropsy. Clinically, mild signs of respiratory discomfort were observed on day 5 p.i., which intensified on day 9 to 21 p.i. in the experimental group. PCR of tracheal swab samples was positive from day 7 to 21 p.i., and the swabs collected from lungs were positive for MG from day 9 to 21 p.i. The study concluded that, MG isolate from field showed limited dissemination pattern and is restricted to respiratory tract.

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INTRODUCTION

Among various poultry pathogens responsible for respiratory tract infections, *Mycoplasma gallisepticum* (MG) is incriminated as an organism having the ability to persist for long duration once it has infected the host.

Infections caused by MG ranges from mild respiratory illness to chronic respiratory disease when complicated with other co-infecting pathogens (Levisohn and Kleven, 2000). Host and environmental factors play an important role in regulating pathogenesis of the disease. Exposition of invasion process of MG in non-phagocytic cells including chicken embryo fibroblast and HeLa-229 and ability to survive in intracellular spaces, provided an insight about the mechanism of evasion of host defenses, limited effect of anti-mycoplasma therapy and potential to cause systemic infection (Winner *et al.*, 2000; Fürnkranz *et al.*, 2013).

Respiratory tract infections caused by MG involves colonization of upper respiratory tract, which can further lead to inflammation of trachea and air sacculitis. Earlier studies reporting arthritis, salpingitis, conjunctivitis, meningoencephalopathy in chicken and turkeys suggested that the organism is not restricted to respiratory tract only. Experimental infection by pathogenic MG R strain provided evidence of systemic spread to the heart, brain, liver, spleen, and kidneys, unveiling the potential of MG to cross mucosal barrier of respiratory tract and dissemination to internal body organs (Much *et al.*, 2002; Vogl *et al.*, 2008; Ramadan, 2019).

Marked differences have been observed in infectivity potential of different strains of MG. In case of experimental infections, it varies with route of inoculation, type of MG strain and number of passages of the strains used for challenge (Levisohn and Kleven, 2000).

In Pakistan, some selected studies have been reported about MG infection and its seroprevalence in layers and breeding stocks (Haque, 2010; Siddique *et al.*, 2012; Khatoon *et al.*, 2018; Shoaib *et al.*, 2019; Qadir *et al.*, 2020). Experimental co-infection of MG and Low Pathogenic Avian Influenza Virus (LPAIV) H9N2 exaggerated disease outcome (Subtain *et al.*, 2016).

Persistent nature of the organism and potential of vertical as well as horizontal transmission render MG infections as one of the most important infections of poultry, causing significant economic losses. Even in the absence of apparent clinical infection of MG, co-infecting bacterial or viral pathogens can exacerbate disease condition. Pakistani MG isolates have not been studied in terms of infectivity and predilection sites of infection. The present study was designed to assess preferred sites of colonization and infection of an MG isolate recovered from MG-vaccinated flock.

MATERIALS AND METHODS

Isolation of *Mycoplasma gallisepticum*: MG broth (Oxoid) and MG agar (Oxoid) were prepared with addition of Supplement G (Oxoid) as recommended by manufacturer. Processed samples were inoculated in the broth after filtration through 0.45 µm syringe filter and incubated at 37°C for 7 days. Samples were observed daily for change in colour of the broth from red to orange yellow and subsequently inoculated on agar. Inoculated MG agar plates were incubated at 37°C for 3-5 days in moisture rich environment, and observed daily under stereomicroscope (Labomed-CSM2) and further confirmed by PCR.

Experimental design: To determine the predilection sites of MG, 48 day-old chicks were reared at animal house facility of National Reference Lab of Poultry Diseases (NRLPD), National Agricultural Research Centre, Islamabad, Pakistan.

At the age of 10 days, birds were randomly divided in experimental and control groups, each having 32 and 16 birds, respectively. Experimental group was inoculated intratracheally with 0.5ml of MG culture (1×10^6 CFU/ml) using hypodermic needle. Control group was sham inoculated with sterile MG broth intratracheally. Both groups were separately placed in glove port chicken isolator chambers (Alternative Design Manufacturing). Blood samples were withdrawn from each group randomly at day 5, 10, 15, 20 post-infection (p.i.). At day 3, 5, 7, 9, 11, 15, 19, 21 p.i., 4 experimental birds and 2 control birds were sacrificed.

Clinical and pathological examination: Experimental and control groups were subjected to daily observations for development of respiratory signs and symptoms. Trachea of each necropsied bird was examined for development of lesions as described by Machado *et al.*, (2016). Air sacs of each sacrificed bird were examined for gross lesions and scored as described by (Much *et al.*, 2002; Gaunson *et al.*, 2006).

Statistical analysis: Tracheal lesion scores and air sac lesions scores were compared between the birds

necropsied at different d.p.i. by using Kruskal–Wallis test. A P value ≤ 0.05 was considered significant.

Serology: Serum samples were subjected to SPA test and indirect ELISA. SPA test was performed using commercially available SPA test antigen (Charles River Laboratories) according to manufacturer's instructions. ELISA was performed using commercially available MG ELISA kit (IDEXX Laboratories, USA).

Molecular detection: Swab samples collected from different organs were subjected to polymerase chain reaction (PCR). Here DNA extraction from the sample material was carried out by using FavorPrep™ Viral Nucleic Acid kit according to manufacturer's instructions (Favorgen). PCR was performed using Dream Taq Green PCR Master Mix (2X) (Invitrogen) following the recommended protocol. PCR was carried out in a thermal-cycler (Eppendorf, Germany) using the profile described in OIE, 2018. Amplified product was visualised by gel electrophoresis, using 1% agarose gel. Briefly, 1 gm of Invitrogen Ultra-pure Agarose (16500-500) was dissolved in 100 ml of 1X TBE buffer and boiled till clear. Afterwards, 6 µl of ethidium bromide (Vivantis) was added and mixed. The mixture was poured in casting tray with comb inserted in it and allowed to solidify. Upon solidification, 10µl of amplified PCR product was loaded along with DNA step ladder (Gene ruler Thermo Scientific). The gel was run for 40 mins at 170V and viewed under UV light (Rafique, 2018).

RESULTS

M. gallisepticum recovered from the field sample was labelled as ARL-1963 and cultured in MG broth followed by its inoculation onto MG agar. Fried egg-shaped colonies of MG were observed under stereomicroscope (Fig. 1). PCR confirmation of colonies yielded amplified product of 185 bp using MG14F+ MG13R primers (Fig. 2) (OIE, 2018).

Clinical and Pathological Observations: Respiratory distress was observed in experimental group from day 6 p.i. onwards. Signs and symptoms included tracheal rales and sneezing, which intensified from day 9 till day 21 p.i. No such signs were apparent in sham inoculated group.

Tracheal lesions and air sac lesions were recorded macroscopically after necropsy. Statistical analysis revealed no significant difference among tracheal lesions recorded on day 7, 9 and 21 p.i. Similarly, no significant difference was found in tracheal lesions recorded on day 11, 15 and 19 p.i. Tracheal lesion scores recorded on day 11, 15 and 19 p.i. differed significantly from those recorded on days 7, 9 and 21 p.i. Air sac lesion scores recorded on day 11 and 15 p.i. differed significantly from the air sac lesions recorded on day 3, 5, 7, 9, 19 and 21 p.i. No birds in control group develop tracheal or air sac lesions (Table 1).

Serological Evaluation: Seroconversion was observed from day 5 p.i. by SPA test (Table 2). Number of positive samples remained low as 2 out of 5 (2/5) and 3 out of 5 (3/5) on day 10 and 15 p.i. By day 20 p.i. all samples were

positive for SPA test. For detection of IgG, ELISA was conducted. On day 10, 15, 20 p.i., 2/5, 4/5, 3/4 samples were positive showing antibody titre range of 223-1509, 114-5860, and 628-6192, respectively. No seroconversion was observed in control group either by SPA or ELISA.

Detection of *Mycoplasma gallisepticum* by PCR: Swabs from organs of necropsied birds were collected including trachea, lungs, liver/spleen and cloaca (Table 3). PCR was

done to detect MG in swab samples collected from designated organs. Tracheal swabs were positive by PCR from day 7 to 21 p.i. On 7, 9, 19 and 21 d.p.i. 2/4 i.e. 50% of tracheal swabs were positive. On day 11 and 15 p.i., 4/4 i.e. 100% of tracheal swabs were positive for MG. Swabs collected from lungs were positive for MG on day 9 till 21 p.i. (Table 3). No swab sample collected from liver/spleen and cloaca was positive. In control group all samples were negative for MG (Table 3).

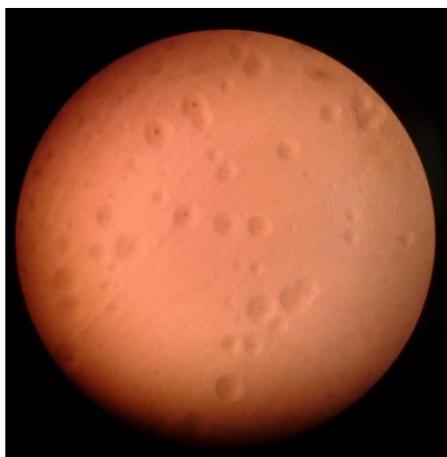


Fig. 1: *Mycoplasma gallisepticum* colonies under stereomicroscope.

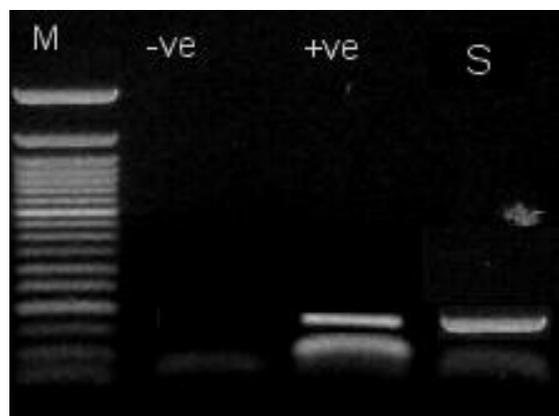


Fig. 2: Gel electrophoresis of MG PCR products, lane 1; DNA step ladder, lane 2; negative control, lane 3; positive control of MG, lane 4; MG field isolate.



Fig. 3: Tracheal lesions of experimentally infected chickens. a) Control group with no tracheal lesions and no mucous accumulation. b) Hyperemic trachea with presence of mucous. c) Hyperemic trachea with petechial haemorrhages and considerable mucous.

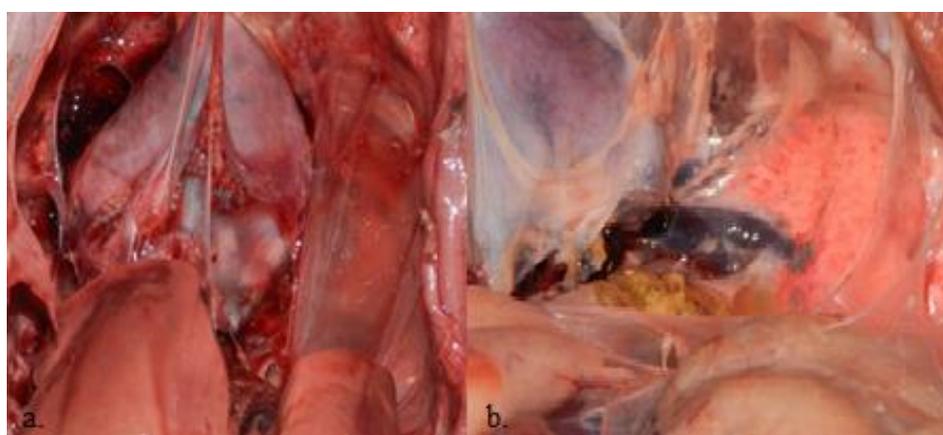


Fig. 4: a) Thoracic air sacs showing thin walls with glossy appearance in control group. b) Thoracic air sacs showing thickened and hazy appearance with flacks of pus in experimentally infected birds.

Table 1: Post inoculation observations of pathological lesions in Trachea and Lungs of Experimental and Control group

	Day 3	Day 5	Day 7	Day 9	Day 11	Day 15	Day 19	Day 21
	Experimental group							
Tracheal lesions ^{ab}	0/4(0.0) ^A	0/4(0.0) ^A	3/4(0.75) ^B	3/4(0.75) ^B	4/4(2.0) ^C	4/4(2.0) ^C	3/4(1.5) ^C	2/4(0.5) ^B
Air sac lesions ^{ac}	0/4(0.0) ^A	0/4(0.0) ^A	0/4(0.0) ^A	1/4(0.2) ^A	2/4(0.5) ^B	2/4(0.5) ^B	1/4(0.0) ^A	1/4(0.0) ^A
	Control group							
Tracheal lesions	0/2(0.0) ^A	0/2(0.0) ^A	0/2(0.0) ^A	0/2(0.0) ^A	0/2(0.0) ^A	0/2(0.0) ^A	0/2(0.0) ^A	0/2(0.0) ^A
Air sac lesions	0/2(0.0) ^A	0/2(0.0) ^A	0/2(0.0) ^A	0/2(0.0) ^A	0/2(0.0) ^A	0/2(0.0) ^A	0/2(0.0) ^A	0/2(0.0) ^A

Values within a row with a different uppercase, superscripted letter are significantly different ($P \leq 0.05$). ^aNo. of positive samples/No. of tested samples.

^bMean tracheal lesion score (macroscopically scored from 0 to 4). ^cMean air sac lesion score (macroscopically scored from 0 to 4).

Table 3: Post Infection detection of *Mycoplasma gallisepticum* from different organs of Experimental group by PCR

Organs	Day 3	Day 5	Day 7	Day 9	Day 11	Day 15	Day 19	Day 21	Total PCR positive
Trachea	0/4	0/4	2/4	2/4	4/4	4/4	2/4	2/4	16/32
Lungs	0/4	0/4	0/4	1/4	2/4	2/4	2/4	1/4	08/32
Liver/ Spleen	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/32
Cloaca	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/32

Table 2: Anti-*Mycoplasma gallisepticum* antibody detection by SPA test and ELISA

Post infection	Day 5	Day 10	Day 15	Day 20
Experimental Group				
ELISA	0/5	2/5+ve	4/5+ve	3/4+ve
Titre Range		223-1509	114-5860	628-6192
SPA	2/5+ve	3/5+ve	3/5+ve	4/4+ve
Control Group				
ELISA	0/5	0/5	0/5	0/4
SPA	0/5	0/5	0/5	0/4

DISCUSSION

Currently, in Pakistan *M. gallisepticum* infection has been on the rise in commercial poultry, despite the fact that number of drugs are used to control such infections along with using MG vaccines. This study was designed to evaluate pathogenesis of field isolate of MG by studying its predilection sites in commercial chickens. For this purpose, MG field isolate was recovered from MG-suspected, serologically positive breeder flock. Since MG is transmitted via horizontal as well as vertical route, assessment of locally circulating field isolate with regard to its localization to upper respiratory tract and further dissemination to other organs and persistence in any specific organ was considered for evaluation.

In present study, intratracheal route of inoculation was used to reproduce infection under controlled conditions (Levisohn *et al.*, 1986). Development of symptoms of disease initiated on day 6 post infection (p.i.), which included slight sneezing and rales and intensified with nasal discharge and difficulty in breathing till day 21 p.i. It is earlier reported that MG infections in the field presents a wide spectrum of disease from mild infection in the presence of a single infectious agent to severe clinical disease complicated with other respiratory pathogens (Siddique *et al.*, 2012; Feizi *et al.*, 2013).

Seroconversion against field isolate of MG was detected by SPA test and ELISA. Results revealed positive SPA from day 5 till 20 p.i. Commercially available SPA test antigen prepared from MG strain A5969 was used here (Stipkovits and Kempf, 1996). On the other hand, indirect ELISA was conducted to assess IgG antibodies against MG. The results revealed development of moderate antibody titres from day 10 to 20 p.i. (Ahmad *et al.*, 2008). In the present study, development of mild respiratory symptoms coincided with the positive SPA test. This is in line with the earlier reported observation that serological response developed against MG is directly related to the degree of infectivity of corresponding strains (Levisohn and Kleven, 2000).

To evaluate infectivity of field isolate, birds from experimental and control groups were sacrificed according to plan and tracheal as well as air sac lesions were observed macroscopically. Tracheal lesion scores recorded on day 7, 9 and 21 p.i differed significantly from tracheal lesion scores recorded day 11, 15, and 19 p.i. with numerical values of former lower than those of latter. Our results vary from the previous study, which reported

development of severe tracheal lesions 2 to 3 week after challenge which eventually subsided slowly (Sanei *et al.*, 2007). Maximum severity observed in air sac lesions was from day 11 to day 19 p.i. which significantly differed from air sac lesions noted during study. As earlier reported by Majumder (2014), pathology of MG infection in chicken is based on inflammatory response in trachea, air sacs and lungs.

Persistence of MG in upper respiratory tract and dissemination of infection to internal body organs was detected by PCR through swabbing of organs after necropsy (Rauf *et al.*, 2013; Haque *et al.*, 2015; Spickler 2018). Tracheal swabs were positive from day 7 till 21 p.i. with 100% positivity on day 11 and 15 p.i. Swabs from lungs were positive from day 9 to 21 p.i. with 50% detection on day 11, 15 and 19 p.i. No detection by PCR was made from liver and cloacal swabs. Although moderate morbidity was observed, no mortality or severe infection occurred during this experiment. Lack of MG detection in the cloacal swabs could be due to poor potential of this isolate to persist and/or shed after infection. MG infections in the field are complicated by some co-infecting organisms as well as due to any environmental stress. Concurrent infection of LPAIV H3N8 and H9N2 with MG have been investigated previously and provided evidence of exaggerated disease condition than infections caused by a single pathogen (Sprygin *et al.*, 2011; Sid *et al.*, 2016; Subtain *et al.*, 2016; Canter, 2019). Different strains may differ in biological properties, including attachment and destruction of epithelial lining. Role of surface exposed cytoadhesin GapA and CrmA in effective colonization to upper and lower respiratory system but reduced dissemination potential to other body organs is well documented (Indiková *et al.*, 2013).

The study demonstrated upper respiratory tract as preferred site of infection of MG local isolate with moderate infection of lungs. There is high probability of MG strains circulating in Pakistan with diverse biological characteristics. Due to limitations, multiple MG isolates were not used in the study.

Conclusions: The present study provided an insight about predilection sites of MG isolate field. During the course of experiment, infection was limited to respiratory tract and no dissemination to internal organs was found. Appearance of symptoms of infection coincided with development of serological response. It is anticipated that such biological characterization of the local isolates would help in better understanding of circulating MG strains.

Authors contribution: SF and KN conceived this study. SF, SR and AS carried out bacterial culture propagation, serology and molecular detection. NS, SR and MAA helped in animal inoculation and necropsy. KN, MAA and SF took clinical and pathological observations. KN, AAS and SF interpreted the data. All the authors contributed in manuscript preparation and its final approval.

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