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RESEARCH ARTICLE

Molecular Confirmation and Immunological Cross Reactivity among *Mycoplasma gallisepticum* Isolates Recovered from Broiler Chicken in Khyber Pakhtunkhwa, Pakistan

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A B S T R A C T

Mycoplasma gallisepticum (MG) is responsible for serious economic losses in poultry industry in Pakistan. The present study was designed to evaluate the molecular and immunological cross reactivity among MG isolates recovered from broiler birds. For this purpose a total of four hundred and thirty-four (n=434) specimen samples were taken from suspected broilers as well as environmental samples of forty commercial poultry (broiler) farms from DIK, Tank, Peshawar, Abbottabad and Mansehra districts of Khyber Pakhtunkhwa. These samples were inoculated and serially passage into Modified Frey's broth. One hundred and nine (25.1%) samples showed turbidity and were further processed through biochemical tests (Catalase production, Tetrazolium reduction, Phosphatase production, Film and spot Assay and Glucose fermentation). Typical fried egg colonies on Frey's agar were purified. Biochemical tests indicated Mycoplasma species as 2.5% of total specimen samples. The PCR results confirmed (185 bp) three MG isolates (MG-I, MG-II and MG-III) with 0.7% prevalence in total specimen samples. All three antigens (Formalin inactivated and Montanide adjuvanted) were subcutaneously inoculated @ 0.5 ml into rabbits and booster on 7 days post first inoculation. Sera samples collected after 14 days post booster shot, were processed for antibody titration using Haemagglutination inhibition (HI) test with homologous and heterologous antigens. The immunological cross reactivity indicated all three MG isolates as distinctly separate from each other. The immunological cross reactivity was existing with 66 to 87.18% among all three isolates. The results showed high homologous GMT titers as compared to heterologous for each MG isolate.

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INTRODUCTION

Mycoplasma gallisepticum (MG) is wall less, small and slow growing bacterium (Zakeri, 2016). MG is responsible for many respiratory ailments in commercial poultry, turkeys, backyard chicken and wild birds. Bacterial culturing is a laborious and time consuming procedure as Mycoplasma are comparatively slow growing organism **(**Kleven, 2008) and bacterial growth can be contaminated by non-pathogenic species (Zhao and Yamamoto, 1993). Culturing technique showed lower sensitivity than Polymerase Chain Reaction (PCR) to detect Mycoplasma species in medicated bird (Gondal *et al.,* 2015).MG causes a very lethal disease in poultry named as chronic respiratory disease (CRD) (Ley, 2013; Fujiswa *et al.,* 2019) which is accountable for huge economic losses in poultry industry worldwide (Liu *et al.*, 2001). The clinical symptoms take a long time to develop so that CRD is persistent infection (Ley, 2008). Decreased productivity, lower carcass value, vaccination and

treatment costs are major causes of economic losses (Raviv and Ley, 2013). MG along with other microorganisms like infectious bronchitis virus, Newcastle disease vaccinal strains and *E. coli* result into sever conjunctivitis and air sacculitis (Farouk *et al.*, 2017; Younis *et al*., 2018).

The Antigenic dissimilarity is common among all mycoplasmas (Wise, 1993). In case of MG strains, the antigenic variation is reported by (Lin and Kleven, 1981). MG isolates of apparently low virulence differs serologically from others MG isolates (Lin and Kleven, 1981). Vardaman and Yoder (1969) while using MG and MS HI antigens, documented that HI titers of the sera were found higher in case of homologous HA antigen than the heterologous. Solsona *et al.* (1996) recorded that the antigenic difference among different isolates of Mycoplasma is based on presence of the epitopes on different sized proteins (in different types of isolates) at the same time. Same phenomena have also been reported for MG (Avakian *et al.*, 1991; Panangala *et al.,* 1992) and *Mycoplasma bovis* (Rosengarten *et al.*, 1994).

The confirmatory identification of these pathogens is based on molecular characterization through PCR (Raviv & Kleven, 2009; Marouf *et al.,* 2020). Molecular confirmation of MG (71.19%) was carried out through species specific PCR by Abbas *et al.* (2018) in poultry and Pheasants in Northern areas of Khyber Pakhtunkhwa (KP). Seroprevalance of MG (35.03%) and *Mycoplasma synoviae* (16.03%) in Broilers and Backyard Poultry (80% MG & %50% MS) was studied by Rehman *et al.* (2018) using Serum plate agglutination (SPA) test in Five Districts of KP. but appropriate cited work about immunological cross reactivity is very limited. Therefore, the present study was intended to isolate and characterize MG isolates through cultural, biochemical and molecular techniques and to further resolve immunological cross reactivity among recovered MG isolates.

MATERIALS AND METHODS

Sample size: For an unknown population with 20% prevalence having 95% confidence interval and 5% an absolute precision (Thrusfield, 1997), 150 specimen (cloacal, tracheal, choanal, synovial and nasal) samples were collected from the 40 broiler farms in five districts (Abbottabad (n=30), Dera Ismail Khan (n=32), Mansehra (n=28), Peshawar (n=30) and Tank (n=30)) of Khyber Pakhtunkhwa. About 264 environmental samples (Droppings, Drinkers, Feeder, Feathers and Dust) were also collected. In district Dera Ismail Khan (D.I. Khan) and Tank from backyard poultry meat shops 20 specimen samples (10 from each district) were also collected. Number of farms selected for specimen selection were distributed as Abbottabad (n=4 closed shed), D.I. Khan (n=12 open shed), Mansehra (n=4 closed shed), Peshawar (n=10 open shed) and Tank (n=10 open shed). Birds with respiratory symptoms were selected for sample collection and different types of specimen samples were taken from different birds. A sterilized swab was used to collect each sample separately. Cotton end of the swab impregnated with sample was then dipped into broth test tube, tightly screwed, placed in ice box and transported to Institute of Microbiology, University of Agriculture, Faisalabad Pakistan.

Frey's broth medium was made with slight modification as described by Frey *et al*. (1968) and Kleven (1998). The NAD 1% solution and 1% cysteine hydrochloride were mixed 1:1 and 20 ml of the mixture was added/1000 ml of medium. Thallium acetate was mixed into distilled water to prevent protein precipitation. Supernatant was poured out and pH (7.8-8.0) was maintained with 1N NaOH. The specimen samples showing turbidity in Frey's broth were then shifted to Frey's agar. After the appearance of typical MG colonies the biochemical tests (Catalase, Phosphatase Production, Tetrazolium Reduction, Film & Spot Assay and Glucose fermentation) were performed to differentiate among avian mycoplasma species.

Mycoplasma growth with specific biochemical characteristics was then verified through molecular study. Following the instructions of the TIANamp Genomic DNA extraction kit TIANGEN, China), the DNA of MG was extracted (Cheng *et al.,* 2014). Amplification (185 bp) of MG was completed by using the following 16sRNA gene specific primers as documented by Gharibi *et al*. (2018)

F: 5′-AGCTAATCTGTAAAGTTGGTC –3′

R: 5′- GCTTCCTTGCGGTTAGCAAC - 3′.

In PCR tubes 25µl of master mix was taken and 3µl of DNA template was mixed thoroughly. About 1µl forward and reverse primers were mixed till the total volume was made equal to 50µl with 20µl of water (nuclease free water). After short-term mixing, PCR was done according to the instructions prescribed by (Gharibi *et al*., 2018). The amplified PCR product was then stained with ethidium bromide and visualized through gel electrophoresis in 1% agarose gel using UV illuminator (Sajid *et al.,* 2020). The PCR product of MG samples were unidirectional sequenced by Advanced Bioscience Internationals, Singapore, using BLAST homology between amino acid and nucleotide sequences of MG. Mega X software was used to construct Phylogenetic tree.

MG Antigen preparation: Equal quantity of MG suspension and 0.3% formaldehyde were mixed for 15 minutes and then kept in refrigerator for 6 hours. Then this formalin inactivated antigen and Montanide adjuvant (SEPPIC, France) were mixed in 1:1 ratio (Ibrahim *et al.,* 2018). For the immunological analysis the production of antigen of each mycoplasma strain was carried out from stock cultures in broth medium as documented by (Lind *et al.*, 1984). The sterility and safety tests were performed as documented in OIE, 2018. A total of twenty (20) rabbits were distributed into 4 groups. These groups were designated as Group 1, Group 2, Group 3 and Group 4. Five rabbits were placed in each group and Group 4 was kept as control group. In first three groups all the rabbits were injected through subcutaneous (s/c) route with 0.5 ml of adjuvanted MG-I , MG-II and MG-III isolates as documented by Lind *et al.* (1984) and Abdelmoumen & Roy (1995). After 7 days 0.5 ml of the each isolate was given s/c to their respective group as booster dose, keeping 4th group un-inoculated. The blood samples were taken from the rabbits of all corresponding groups 14 days post booster shot and Haemagglutination Inhibition (HI)

test as documented in (OIE, 2018). Homologous GMT value against respective isolate was taken as 100% and heterologous GMT values were compared with it on the basis of percentage differences.

RESULTS

Prevalence of MG in Poultry birds: All specimen samples (n=434) were inoculated into Modified Frey's broth and 109 samples (25.1 %) showed turbidity. In Mansehra the prevalence percentage was found as (57.1 %) which was the highest among all the districts. The lowest prevalence percentage was documented in Tank (43.3%) as shown in Table 1. In Abbottabad, Dera Ismail Khan and Peshawar the prevalence percentage was recorded as (53.3%), (47%) and (46.7%) respectively.

Out of 264 environmental samples only 30 (11.4 %) samples showed turbidity and the highest prevalence percentage was recorded in droppings (19.6%) followed by feathers (17.7%), feeder (10.5%), drinker (5.6 %) and dust (4%) as mentioned in Table 2. In Dera Ismail Khan, 3/10 samples showed turbidity (30 %) and in Tank the percentage of turbidity positive samples was lower (20%) as indicated in Table 3.

Cultural and Biochemical characterization: The highest MG prevalence percentage on the basis of biochemical tests was recorded in Dera Ismail Khan as 9.4% followed by Mansehra 7.1% and the lowest MG prevalence was recorded as 6.7% (Abbottabad, Tank and Peshawar) as shown in Table 1.

Molecular confirmation and Phylogenetic analysis: The PCR assay confirmed three MG isolates from the specimen samples collected from the poultry birds and these isolates were symbolized as MG-I, MG-II and MG-III. The overall percentage based on biochemical tests showed 2.5% MG prevalence. The percentage of MG occurrence confirmed by PCR assay was recorded as 0.7% in Table 1.

The MG amplified products of PCR were then referred to NCBI for further analysis. The accession MW397012 was allotted to ours query. According to phylogenetic tree of 16S rRNA, it is quite clear that the isolate DQ677303.1 is quite analogous to the *M. gallisepticum* isolate Fig. 2.

Immunological cross Reactivity: The homologous antibody titer in Group 1 rabbit's serum against MG-I isolate indicated a range (1:16 to 1:64) of titers while heterologous response of MG I serum against MG-II isolate and MG-III isolate varied from 1:16 to 1:32 in both cases as shown in Table 4. Likewise the homologous antibody titer in case of Group 2 rabbit's serum against MG-II isolate varied from 1:16 to 1:64 and the heterologous antibody response of Group 2 rabbit's serum against both the MG-1 and MG-III isolate indicated analogous response of 1:16 to 1:64. The serum of Group 3 rabbits indicated a homologous antibody response of 1:16 to 1:64 against MG-III isolate and the heterologous response of 1:16 to 1:32 and 1:16 to 1:64 against MG-I and MG-II isolates respectively as indicated in Table 4. In control group (Group 4) no titer was recorded. The most suitable technique to calculate the antigenic cross reactivity documented in local MG isolates was applied. The homologous antibody titer raised against MG-I was (GMT 32.0) and it was (GMT36.8) against both MG-II and MG-III isolates as indicated in Fig 3. The heterologous titers were fluctuating from 24.3 and 27.9 GMT indicating the lesser values as compared to homologous titer (Fig. 3). These heterologous GMT values indicated that MG isolates had strong cross immunity with each other.

Table 1: District wise culture, biochemical and molecular characterization of specimen samples taken from five districts of Khyber Pakhtunkhwa

Name of District	Samples (*n*)	Turbidity positive samples n (%)	Biochemical Characterization (*n*) Mycoplasma spp:	Percentage	PCR Confirmation (*n*) MG	Percentage MG
Abbottabad	30	16 (53.3)	2	6.7	1	3.3
Dera Ismail Khan	32	15 (47)	3	9.4	1	3.12
Mansehra	28	16 (57.1)	2	7.1	-	-
Peshawar	30	14 (46.7)	2	6.7	-	-
Tank	30	13 (43.3)	2	6.7	1	3.3
Backyard poultry	20	5 (25)	-	-	-	-
Environmental samples	264	30 (11.4)	-	-	-	
Total	434	109 (25.1)	11	2.5	3	0.7

Table 2: Distribution and turbidity based results of specimen samples taken from housing environment of various broiler farms in five major districts of Khyber Pakhtunkhwa

Environmental sample source	Number of samples	Samples showing Turbidity in Frey's Broth	Percentage	Samples without Turbidity in Frey's Broth	Percentage
Dust	51	2	4.0	49	96.0
Drinker	54	3	5.6	51	94.4
Feeder	57	6	10.5	51	89.5
Feather	51	9	17.7	42	82.3
Droppings	51	10	19.6	41	80.4
Total	264	30	11.4	234	88.6

Table 3: Distribution and Turbidity based results of Backyard poultry specimen samples collected from Dera Ismail Khan and Tank

Backyard poultry samples	Number of samples	Samples showing Turbidity in Frey's Broth	Percentage	Samples without Turbidity in Frey's Broth	Percentage
Dera Ismail Khan	10	3	30	7	70
Tank	10	2	20	8	80
Total	20	5	25	15	75

Table 4: The Haemagglutination inhibition titers recorded in rabbits after experimental exposure to adjuvanted *Mycoplasma gallisepticum* isolates

		HI Titer		
Group wise Rabbit sera	Rabbits (R) (n)	MG-I Isolate	MG-II Isolate	MG-III Isolate
Group 1	1	(1: 32)	(1: 16)	(1: 16)
	2	(1: 16)	(1: 16)	(1: 32)
	3	(1: 32)	(1: 32)	(1: 32)
	4	(1: 64)	(1: 32)	(1: 32)
	5	(1: 32)	(1: 32)	(1: 32)
Group 2	1	(1: 32)	(1: 32)	(1: 16)
	2	(1: 32)	(1: 32)	(1: 16)
	3	(1: 64)	((1: 64)	(1: 32)
	4	(1: 32)	(1: 64)	(1: 64)
	5	(1: 16)	(1: 16)	(1: 32)
Group 3	1	(1: 32)	(1: 32)	(1: 32)
	2	(1: 32)	(1: 16)	(1: 64)
	3	(1: 32)	(1: 32)	(1: 64)
	4	(1: 16)	(1: 64)	(1: 32)
	5	(1: 16)	(1: 16)	(1: 16)
Group 4 (Control) group) Serum	1	---	---	---
	2	---	---	---
	3	---	---	---
	4	---	---	---
	5	---	---	---

--- = No titer.

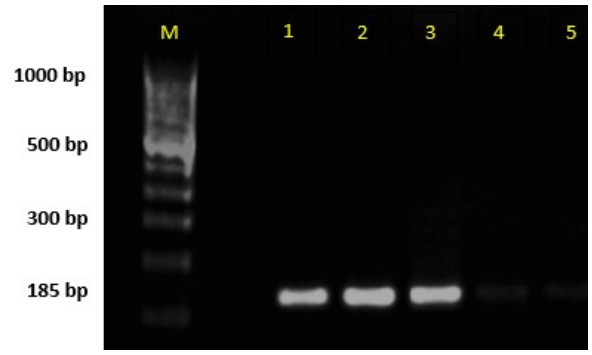

Fig. 1 M lane indicates the test markers (1000bp) and Lane 1- 5 signifies the samples.

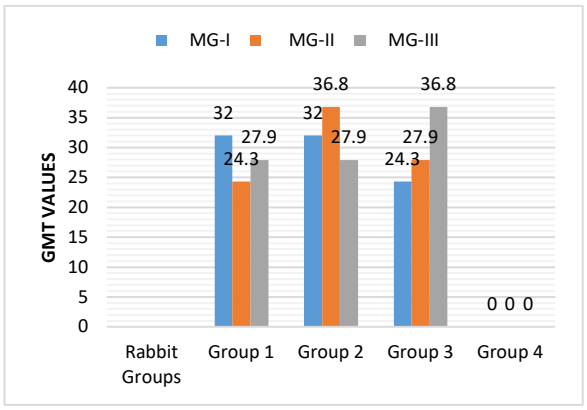

Fig. 3: Geometric Mean Titers of antibodies experimentally raised in rabbits against *Mycoplasma gallisepticum* field isolates.

DISCUSSION

The success rate of MG isolation in our study indicated as 25.1 and 2.5% based on culture and biochemical tests. Muhammad *et al.* (2021) documented the culture results of MG isolation in breeders, layers and broilers as 26.5 21 and 9% respectively. Closely related to current study Atique *et al.* (2017) recorded 23% culture isolation in layers in Baluchistan.

Molecular confirmatory characterization based upon PCR results, MG prevalence was reviled as 0.7% with 185bp in current study following the procedure adopted by Ghareibi *et al,* (2018) who documented 0% prevalence of MG specimen samples collected from broiler birds. In Pakistan the MG prevalence percentage was characterized by Hanif and Najeeb (2007) through PCR assay in broiler breeder birds. In Iranian broiler breeders Kaboli *et al*. (2013) also recorded the prevalence percentage of MG as 31.50% MG 16S rRNA PCR assay amplifying 185bp. Rajkumar *et al.* (2018) using PCR assay amplified an amplicon of 185 bp of 16S rRNA gene of MG and recorded a high prevalence of MG (33%) as compared to our results. In Indian broiler birds, Tomar *et al.* (2017) also documented a high MG prevalence (27%) as compared to current study. In Tamil Nadu, Manimaran *et al.* (2019) collected about 790 clinical samples of diseased birds and cultured on medium. After cultural isolation PCR technique was applied to amplify 16S rRNA gene and recorded a high MG prevalence (13.29%) as compared to cultural growth (11.51 %) in contrast to current study.

In Lahore- Pakistan, the detection of MG was completed by Islam *et al.* (2011) using species specific primers to amplify 16S rRNA gene, generating an amplicon of 185bp with a high MG prevalence (46%) in broiler as compared to present study. Similarly, in Egyptian poultry, Gamal *et al.* (2018) detected MG through PCR with an amplicon of 185bp of 16Sr RNA gene in 50% broiler samples, very high rate as compared to current study. It is pertinent that the success rate of culture isolation for MG is very low compared to molecular direct identification. However the PCR based technology remained valid to confirm very closely related Mycoplasma species which are otherwise not possible to resolve through biochemical characterization. In present study it was also reviled that the success rate of culture and biochemical tests based identification of MG was 25.1 and 2.5 respectively, whereas 0.7% of the culture isolate were confirmed as MG through PCR.

Many reports have been documented about the antigenic variation among different MG strains (Kleven, 1975). This difference in antigenic variation is evident in Haemagglutination inhibition (HI) test as the homologous titers are higher as compared to heterologous (Kleven, 1975). The high titers of homologous antigens indicated that these homologous antigens are more sensitive to their respective antibodies as compared to heterologous antibodies (Kleven, 1975; Newman, 1979). MG HI test is more strain specific as compared to Serum Plate Agglutination (SPA) test (Roberts, 1969).

The highest homologous Geometric Mean titers (GMT) indicated by MG-II and MG-III isolates was 36.8 and the lowest homologous GMT value (32.0) was exhibited by MG-I isolated. The heterologous GMT values varied from 24.3 to 27.9 in case of MG-I, 27.9 to 32.0 in case of MG-II and 24.3 to 27.9 in case of MG-III isolates. These values indicated that the homologous immune titers recorded in all the MG field isolates were comparatively higher than the heterologous titers. In the serum of Group 1 rabbits the overall heterologous

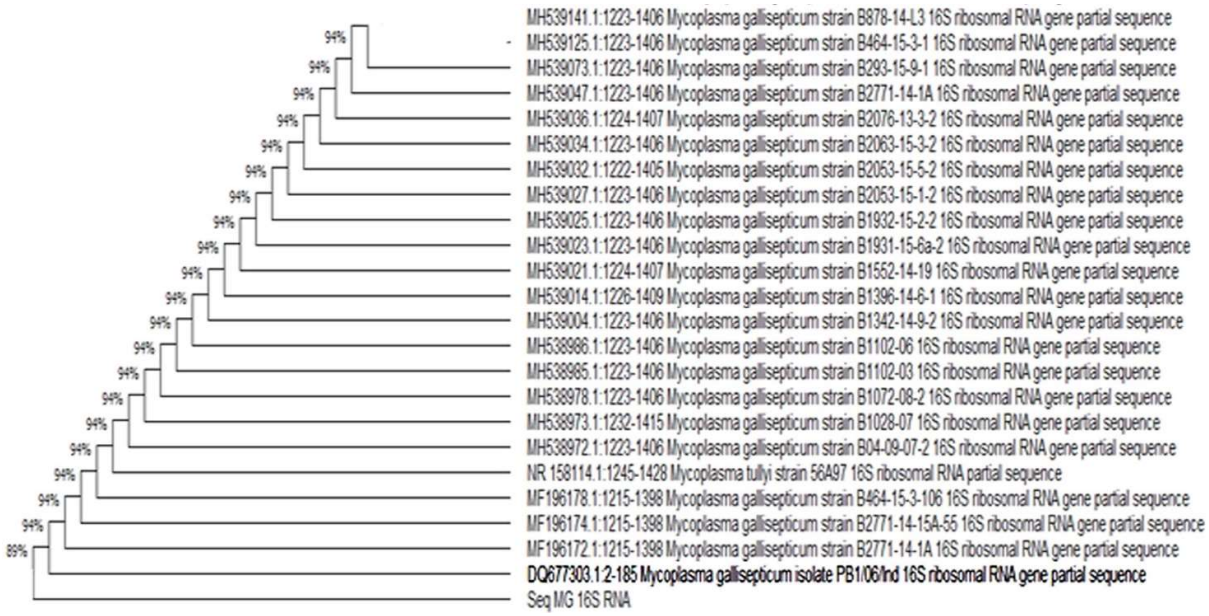

Fig. 2: The Phylogenetic analysis of MG indicating the similarity using neighbor joining analysis (Mega X software).

immune response titers were 76% against MG-II isolate and 87.18% against MG-III isolate as these rabbits were inoculated with MG-I isolate. The heterologous immune response against MG-1 isolate was (87% of homologous) and MG-III isolate (75% of homologous) in Group 2 rabbits which were inoculated with MG-II isolate. Similarly Group 3 serum showed a similarity of 66% (with MG-I isolate) and 75.81% (with MG-II isolate). Ferraz and Danelli (2003) recorded the strong cross reactions with in the MG strains as in the current study.

Lin and Kleven (1981) documented strong homologous and weak heterologous immune responses in five MG strains similar to our findings. In MG infected chicken and Turkeys the homologous and heterologous immune reactions were studied by (Kleven, 1975; Newman, 1979). Four MG strains were evaluated by (Timms and Gullen, 1972) in poultry birds for the assessment of the antibody titers (both homologous and heterologous) using Rapid plate Agglutination Test and Haemagglutination inhibition test and found the higher homologous HI titers similar to current study. Latest development in the invention of equipment and procedures to forecast cross-reactivity within the dissimilar pathogens as well as local isolates will provide better immunity titers if used in the preparation of vaccine (Agrawal, 2019).

Conclusions: Cultural, biochemical as well as molecular results confirmed the prevalence of MG in broiler birds in five districts of Khyber Pakhtunkhwa. All isolates of MG immunologically distinct 66 to 87.18%. The immunological cross reactivity results indicated high homologous GMT titers as compared to heterologous.

Authors contribution: Professor Dr. Abdul Haleem Shah designed the research study. Atta Ur Rehman collected the specimen samples and processed in the laboratory of Institute of Microbiology, University of agriculture Faisalabad under the supervision of Professor Dr. Sajjad Ur Rahman. Dr. Sanaullah Sajid. Dr. Imdadullah Khan, Muhammad Hashim Khan, Arshad Farid and Dr. Qudrat Ullah helped in the analysis of the data and proof writing of the manuscript.

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