Open Veterinary Journal, (2023), Vol. 13(10): 1299–1307
ISSN: 2226-4485 (Print)
ISSN: 2218-6050 (Online)
Original Research
DOI: *10.5455/OVJ.2023.v13.i10.9*

Submitted: 14/07/2023 Accepted: 18/09/2023 Published: 31/10/2023

Molecular identification and cross-immunogenic study on two field isolates of *Mycoplasma synoviae* isolated from broilers in five districts of Khyber Pakhtunkhwa

Atta Ur Rehman[1], Abdul Haleem Shah[2], Sajjad Ur Rahman[3], Saifur Rehman[1,4], Muhammad Kamal shah[1], Widya Paramita Lokapirnasari[5*], Imdadullah Khan[1], Muhammad Inamullah Malik[1] and Andreas Berny Yulianto[6]

[1]*Faculty of Veterinary and Animal Sciences, Gomal University, Dera Ismail Khan, Pakistan*
[2]*Institute of Biological Sciences, Gomal University, Dera Ismail Khan, Pakistan*
[3]*Institute of Microbiology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan*
[4]*Division of Veterinary Public Health, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia*
[5]*Division of Animal Husbandry, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia*
[6]*Faculty of Veterinary Medicine, University of Wijaya Kusma, Surabaya, Indonesia*

Abstract

Background: *Mycoplasma synoviae* (MS) is an important poultry pathogen causing heavy economic losses Worldwide. Subclinical persistence of this pathogen is the major issue to control its prevalence.

Aim: This study aimed to determine the molecular and cross-immunogenicity of MS among broilers in five Districts of Khyber Pakhtunkhwa (KP).

Methods: This study was conducted by collecting 434 specimen samples from 40 broiler farms and desi poultry in five districts of KP. Specimen samples from the broiler birds (n = 150), broiler farm environment (n = 264), and desi poultry birds (n = 20) were aseptically collected and serially passaged in Modified Frey's broth. The homologous and heterologous antibody reactions were studied in rabbits. Before inoculation into rabbits, the MS isolates were inactivated by formalin and adjuvanted with Montanide.

Results: The overall turbidity prevalence in Frey's broth was observed as 109/434 (25.11%) samples, and these turbidity-positive samples were shifted on Frey's agar. After the appearance of classic fried egg colonies, the Biochemical confirmation was supported by the production of catalase and phosphatase, reduction of tetrazolium, film and spot assay, and fermentation of glucose for species differentiation in avian mycoplasma. The MS prevalence percentage was recorded as 2% (9/434) through biochemical tests. The PCR results showed 0.5% MS prevalence with two field isolates (named MS-1 and MS-2). Both MS-1 and MS-2 field isolates showed similar values (42.2) of homologous geometric mean titer (GMT). While the heterologous GMT for MS-1 serum against MS-2 isolate was lower (27.9) as compared to MS-2 serum against MS1 isolate (38.9). No titer was detected in the control group (Group-III).

Conclusion: In conclusion, the results indicated the existence of MS in broiler birds and high homologous titers recorded between field isolates, which is a perpetual menace to poultry.

Keywords: *Mycoplasma synoviae*, PCR characterization, Homologous and heterologous antigenic responses.

Introduction

Genus Mycoplasma contains a lot of vital poultry pathogens, which are responsible for heavy economic losses all over the World (Dufour-Gesbert *et al*., 2006). Among these avian Mycoplasma spp: *Mycoplasma synoviae* (MS) and *Mycoplasma gallisepticum* are known as the worst of all the avian species (Buim *et al*., 2009). MS is a wall-less bacteria having a high number of sterols in the cell membrane (Kleven, 2008). MS has the smallest genome with 23%–40% G+C contents (Nicholas and Ayling, 2003). The main target of MS is the nose, throat, pharynx, trachea, and bronchi, resulting in subclinical upper respiratory disease. When MS is accompanied by MG, it causes air sacculitis (Silva *et al*., 2008). Horizontal as well as vertical modes of transmission are found in MS. From infected birds, MS can easily be transferred through aerosols generated by diseased birds. Direct contact as well as contaminated fomites can also be a source of infection (Marois *et al*., 2005). Immunocompromised

***Corresponding Author:** Widya Paramita Lokapirnasari. Division of Animal Husbandry, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia. Email: *widya-p-l@fkh.unair.ac.id*

birds are more affected by MS (Dhondt *et al*., 2007). Infectious synovitis, respiratory sickness, lowered egg yield, miss shaped eggs, and slow weight gain are the main clinical infections caused by MS. Death rates are very low due to these infections, but their economic impact is long-lasting in the poultry birds all over the Globe (Kursa *et al*., 2019).

Less effectivity of many antibiotics against various Mycoplasma spp: has increased the interest in the prevention of the diseases caused by these bacteria. However, very few effective vaccines against mycoplasma are available to provide long-lasting immunity (Nicholas and Ayling, 2003). The production of more efficient vaccines is mandatory to control the diseases caused by avian mycoplasma. The variations in the antigenicity were witnessed in many species of mycoplasma (Wise, 1993) as documented by 2 (Lin and Kleven, 1982) in MG strains. Virulence of these MG isolates is also associated with antigenicity (Lin and Kleven, 1982). Homologous and heterologous titers were studied by Vardaman and Yoder (1969) using HI antigens (*M. gallisepticum and* MS). The researchers found higher homologous HI titers than the heterologous titers. Rehman *et al.* (2022) also found high homologous GMT values among *M. gallisepticum* field isolates in KP (Rehman *et al*., 2022).

Quick and timely diagnosis of MS is imperative to avert MS spread to minimize the economic losses in commercial broilers. There are three approaches to diagnose mycoplasma infection: identification and isolation of the bacterium by culture recognition of its DNA. Serological methods like ELISA, serum plate agglutination test, and Haemagglutination inhibition tests (Dufour-Gesbert *et al*., 2006; Abbas *et al*., 2018) are used to detect the specific antibodies. VlhA gene was found more satisfactory in MS strain differentiation (Beylefeld, 2018). Hemagglutinin and pro-lipoprotein are present on *VlhA* gene (Dijkman *et al*., 2014). The antigenic and phase differentiation are expressed by hemagglutinin (Matucci *et al*., 2020). The precise and fast characterization of MS is mostly based on the amplification (Diene *et al*., 2016) of the conserved region of the vlhA gene using the most familiar conventional PCR-based technique. Due to a lack of applicable written data about MS isolation, PCR conformation, and antigenic cross immunity in various districts of Khyber Pakhtunkhwa (KP), the current study was planned to characterize MS isolates through various tools, including PCR.

Materials and Methods

A total of 434 specimens as well as environmental samples, were collected for an unknown population with a 95% confidence interval from the forty commercial poultry (broiler) farmhouses of various localities (districts) of KP. Eight (n = 8) closed sheds were selected from Abbottabad and Mansehra 4 from each district. Thirty-two (n = 32) open broiler sheds were nominated from D.I. Khan, Peshawar, and Tank as 12, 10, and 10, respectively. Specimen samples were collected aseptically from the birds with clinical symptoms of respiratory distress. Only one specific specimen sample was collected from individual birds using a separate, properly disinfected cotton bud. The detail of specimen samples (n = 150) collected from broiler birds is mentioned below in Table 1.

Two hundred and sixty-four (264) samples from housing premises including: droppings (n = 51), drinkers (n = 54), feeder (n = 57), feathers (n = 51), and dust (n = 51) were taken from the forty (n = 40) selected broiler farmhouses in various localities of KP. Only 20 tissue samples from desi (local) poultry were also collected from various desi poultry processing points in the District Dera Ismail Khan (n = 10) and Tank (n = 10). For further processing, every sample was aseptically put into a sealed test tube filled with Modified Frey's broth (Kleven, 1998) in an iced container and transported to the University of Agriculture Faisalabad.

The Biochemical characterization was completed by performing some specific tests such as film and spot assay, catalase test, tetrazolium reduction test, phosphatase test, and glucose test to segregate different species of Genus Mycoplasma.

DNA extraction of MS

After biochemical conformation, the molecular characterization was done by extracting the DNA from the recovered Mycoplasma cells as initially characterized on the basis of biochemical tests. The DNA extraction kit (TIANGEN, China) was used

Table 1. District wise and organ-based distribution of specimen samples from 40 commercial broiler farms.

Specimen	Abbottabad (n)	D.I.Khan (n)	Mansehra (n)	Peshawar (n)	Tank (n)	(N)
Trachea	6	7	6	6	6	30
Cloaca	6	7	6	6	6	30
Choanal cleft	6	6	6	6	6	30
Synovial fluid	6	6	5	6	6	30
Nasal cavity	6	6	5	6	6	30
Total	30	32	28	30	30	150

Table 2. PCR protocols to amplify MS DNA.

Conditions	Initial Denaturation	Denaturation	Annealing	Extension	Final extension
Temperature	94°C	96°C	54°C	68°C	72°C
Time	**2 minutes**	15 seconds	15 seconds	20 seconds	5 minutes
Cycles	—	35 cycles	35 cycles	35 cycles	—

to extract MS DNA, succeeding the procedure as instructed by Cheng *et al*. (2014).

MS DNA amplification

Amplification of MS *vlhA* gene was performed using the below primer sequence;

- Forward primer: 5′-TACTATTAGCAGCTAGTGC-3′
- Reverse primer: 5′-AGTAACCGATCCGCTTAAT-3′

A 25 µL of master mix and 3 **µ**L of MS DNA template were put into a PCR tube along with 1 µL of forward and reverse primers, 1 µL Taq DNA polymerase was mixed with 20 µL of nuclease-free water. Following the instructions as documented by Jeffery *et al*. (2007), the extracted DNA of MS was intensified in a thermal cycler, as illustrated in Table 2.

After thermal cycling, the amplified product was processed for visualization. The process of electrophoresis was completed by making one percent agarose solutions, and Ethidium bromide was applied to stain the product of PCR. The stained product was finally pictured under an ultraviolet source (Sajid *et al*., 2020).

MS antigen preparation

To study the immunogenic cross reactivity between the MS isolates recovered in this research, the Haemagglutination antigen was prepared as used by Ibrahim *et al*. (2018). The recovered isolates were dipped into Frey's broth (Douma *et al*., 1989) along with phosphate buffer saline to maintain pH at 7.2. The suspension was then centrifuged three times, and the final dilution was prepared with a protein concentration of 1 mg per ml and kept in a freezer (-20°C) for future use. A 0.3 percent solution of formaldehyde and antigen suspension were assorted with each other for 15 minutes and placed in the refrigerator for six hours. After formaldehyde treatment, the inactivated MS antigen was then mixed with Montanide (SEPPIC, France) adjuvant and kept in a refrigerator for future use (Lind *et al.*, 1984). The safety and sterility tests were completed before being administered to rabbits.

Relative study on cross antigenic titers

A total of fifteen rabbits were distributed in 3 groups (Group I, Group II, and Group III). Five rabbits were allotted to every group. All the rabbits of Group I and Group II were injected ½ ml of inactivated adjuvanted MS-1 and MS-2 antigen using subcutaneous route, respectively, as an initial dose and booster dose (0.5 ml) was given after seven days of the initial dose. The Group III was left un-inoculated (control). This practice of rising antiserum against whole MS cells in rabbits was also documented by Poveda *et al*. (1990). Fourteen days after the second (booster) dose, the sera samples were obtained from the rabbits of Group I, Group II, and Group III. Fresh poultry blood was taken in a sterilized test tube with EDTA as an anticoagulant to prepare a 1% solution of three times washed RBCs for the HI test. The highest dilution of the serum completely preventing Haemagglutination, was taken as the HI titer (Allan and Gough, 1974).

Results

In environmental samples, the overall percentage of turbidity positive is 11.36% (30/264). The droppings were found with the highest prevalence (19.6%), then feathers (17.64%), feeders (10.52%), drinkers (5.56%), and dust (4%), as indicated in Table 3. Out of 20 tissue samples collected from Desi (Local) poultry in Tank, the turbidity positive prevalence was high (30%) as compared to DIK (20%), as shown in Table 3. Broiler specimen samples (n = 150), 74 samples (49.3%) showed turbidity out of the total samples. The highest percentage of positive samples was recorded in Mansehra (57.3%), then Abbottabad (53.3%), DIKhan (47%), Peshawar (46.7%) and Tank (43.3%) as shown in Table 3.

On the basis of biochemical characterization, the MS positivity percentage was documented as 6.7% in Peshawar, Tank, and Abbottabad, as indicated in Table 4. The overall MS prevalence percentage based on biochemical tests was recorded as 2.07%. PCR confirmed two MS isolates from the specimen samples with 0.5% as the overall percentage. These MS isolates were, named MS-1 and MS-2, that were only detected positive for MS by PCR.

The PCR amplicon of 392 bp was recorded as a positive sample in Lane 2 and Lane 3, as indicated in Figure 1. Lane 1 showed a negative sample. After amplification, the product amplified product was processed for visualization. For the purpose of electrophoresis, one percent agarose solutions were prepared, and the molecular product was pictured under an ultraviolet source after staining with Ethidium bromide (Sajid *et al*., 2020), as shown in Figure 1. The recovered MS sequence was submitted to National Centre for Biotechnology Information (NCBI) under accession number MW397013 and on the basis of *vlhA* gene HQ326483.1, was found to be similar to MS isolates as shown in Figure 2. The molecular product was sent to Advanced Bioscience Internationals, Singapore, for

Table 4. Biochemical and molecular confirmation of specimen samples collected from various localities (Districts) of KP.

Name of district	Samples (*n*)	Biochemical characterization (*n*) MS	% MS	PCR confirmation (*n*) MS	%
Abbottabad	30	2	6.7	-	-
Dera Ismail Khan	32	2	6.2	-	
Mansehra	28	1	3.6	1	3.6
Peshawar	30	2	6.7	1	3.3
Tank	30	2	6.7	-	-
Backyard Poultry	20	-		-	-
Environmental samples	264	-		-	-
Total	434	9	2.07	2	0.5

Table 3. Distribution and Turbidity based results of specimen samples taken from Broiler Farm environment, local poultry and broiler birds of various broiler farms in five major districts of Khyber Pakhtunkhwa.

Sample type	Subtype	Number of samples	Turbidity +	%	Turbidity-	%
Environmental Samples	Dust	51	2	4	49	96
	Drinker	54	3	5.56	51	94.44
	Feeder	57	6	10.52	51	89.47
	Feather	51	9	17.64	42	80.4
	Droppings	51	10	19.6	41	80.4
	Total	**264**	**30**	**11.36**	**234**	**88.63**
Local Poultry	DIK	10	2	20	8	80
	Tank	10	3	30	7	70
	Total	**20**	**5**	**25**	**15**	**75**
Broiler bird specimen samples	Abbottabad	30	16	53.3	14	46.7
	DIK	32	15	47	17	53
	Mansehra	28	16	57.1	12	42.9
	Peshawar	30	14	46.7	16	53.3
	Tank	30	13	43.3	17	56.7
	Total	**150**	**74**	**49.3**	**76**	**50.7**
Grand total		**434**	**109**	**25.11**	**325**	**74.88**

sequencing through BLAST. The Phylogenetic analysis was performed using Mega X software, as indicated in Figure 2.

Immunogenic cross reactivity

The minimum homologous antibody titer of the serum of Group I MS (rabt) against MS-1 was recorded as1:32 and the maximum titer was 1:64. The maximum heterologous HI titers of Group I MS rabbit's serum against MS-2 isolate antigen ranged as (1:16 and 1:32) as shown in Table 5. Whereas the homologous and heterologous HI titers of Group II MS rabbit's serum against MS-2 isolate antigen were recorded as 1:16–1:64 and 1:32–1:64, respectively, as indicated in Table 5. Null titer was recorded in Group III as it was denoted as the control group. In both groups of experimental rabbits, the homologous GMT value (42.2) of MS-1 and MS-2 isolates was similar and recorded as (100%). However, the heterologous GMT value in the serum of MS I against MS 2 isolate was lower (27.9% or 66%), as shown in Figure 3. Likewise, the heterologous GMT for MS-2 serum against MS-1 isolate was recorded as (38.9% or 92.2%) GMT.

Discussion

The molecular confirmatory study of MS was performed using PCR technique as documented by Jeffery *et al*. (2007). The present study was conducted on commercial poultry (broilers) to study the occurrence of MS, and it was found to be 0.5% with an amplicon of 392 base pairs, while (Gharibi *et al*.,

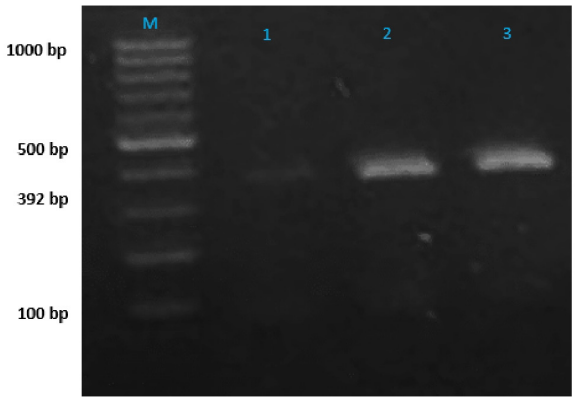

Fig. 1. Lane M shows PCR marker (1,000 base pairs) while Lane1-3 symbolize samples.

2018) found 0% MS occurrence in broilers. In Iranian broilers, 38% MS prevalence was confirmed (Ghaniei, 2016) through *vlhA* gene amplification. Amer *et al*. (2019) documented a high prevalence rate (100%) in Egyptian broiler breeders using *vlhA* gene primers. An amplicon of 396 bp was generated from all 12 joint samples. Rajkumar *et al.* (2018) carried out MS PCR through the amplification of *vlhA* gene, and a product of 392 bp was generated in most of the field isolates with 33.0% MS prevalence, which is higher than the current study. In contrast to our study, Tomar *et al*. (2017) also documented a higher (2.1%) MS prevalence in broiler birds in Haryana state of India. The differentiation study of Genus Mycoplasma, MG, and MS using distinct primers for each was carried out by Malekhoseini *et al*. (2017). The MG was identified by amplifying its 16S rRNA gene with an amplicon of (185 bp), and MS was recognized using vlhA gene (392 bp) with precise

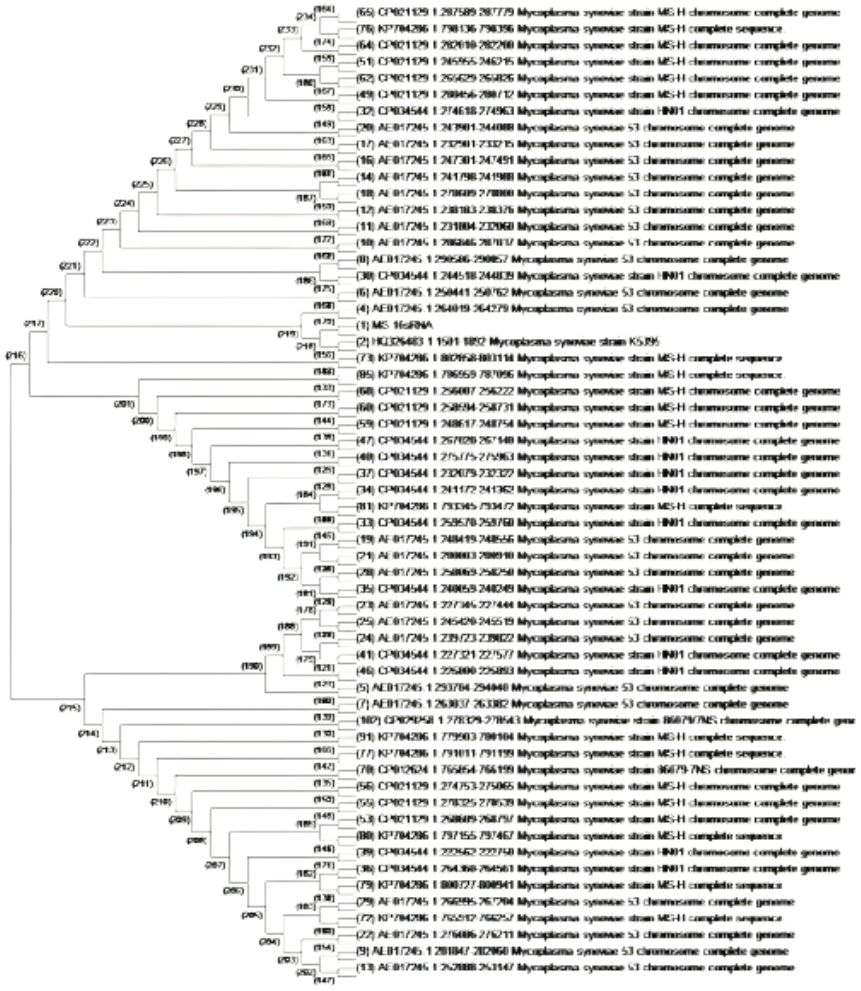

Fig. 2. MS with extreme resemblance by Phylogenetic study using Mega XI software (neighbor joining).

Table 5. The Haemagglutination inhibition titers documented among the MS field strains in rabbits.

Haemagglutination Inhibition Titers			
Groups	**Rabbits (Rabt)**	**MS-1 Isolate**	**MS-2 Isolate**
Group I Serum	Rabt 1	1: 32	1: 32
	Rabt 2	1: 32	1: 32
	Rabt 3	1: 64	1: 16
	Rabt 4	1: 64	1: 32
	Rabt 5	1: 32	1: 32
Group II serum	Rabt 1	1: 32	1: 32
	Rabt 2	1: 32	1: 64
	Rabt 3	1: 16	1: 32
	Rabt 4	1: 64	1: 32
	Rabt5	1: 64	1: 64
Group III (Control group)	Rabt 1	—	—
	Rabt 2	—	—
	Rabt 3	—	—
	Rabt 4	—	—
	Rabt 5	—	—

(Rabt): Rabbit.

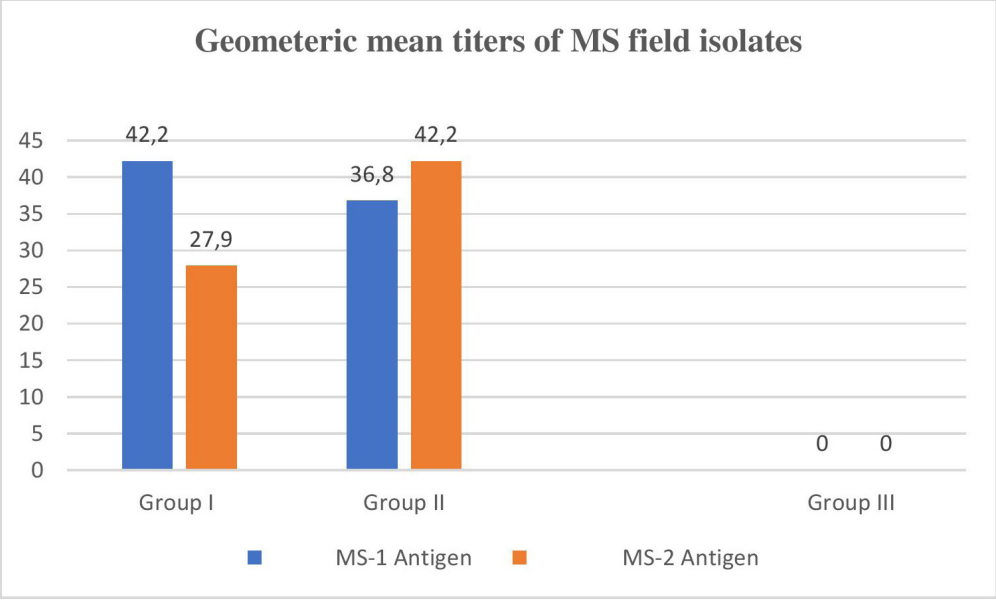

Fig. 3. Geometric mean titers of MS field isolates.

primers similar to the procedure applied in the current study.

Using the molecular method of MS characterization (Multiplex PCR) through the amplification of *vlhA* gene of MS,found a 25.3% prevalence of MS in Egyptian uninhabited birds. Rasool *et al*. (2018) also amplified and sequenced the 16 S r RNA (213 bp) and vlhA gene (375 bp) to differentiate MS *from* various field strains. Out of 40 Mycoplasma isolates, 20 (50%) were identified as MS through 16 S r RNA, and five (12.5%) were characterized as MS through fractional *vlhA* gene analysis.

Both MS field isolates (MS-1 and MS-2) showed the same homologous immunogenic titer of (42.2 GMT) as a result of the Haemagglutination Inhibition test. There were some variations in heterologous values.

The MS-2 isolate showed a lower (66%) heterologous immune response with MS-1 serum as compared to MS-1 isolate (87%) against MS-2 serum, as indicated in Figure 3. Similar findings were recorded by Weinack and Snoeyenbos (1976), who also elaborated on the immunogenic alterations amongst three stains of MS. The differential study using the haemagglutination inhibition test amongst the field isolates of MS and standard WVU 1853 MS strain was carried out by Vardaman and Drott (1980). Vardaman and Yoder also found increased levels of HI titers in homologous MG and MS antigens than the homologous HI levels (1969). A high level of homologous antigenic cross reactivity as compared to heterologous was also studied in different *M. gallisepticum* field strains isolated from commercial poultry (broiler) farmhouses in KP (Pakistan) by Rehman *et al*. (2022). A variety of serological tests were used to explore the existence of antigenic cross reactivity in different isolates of *M. pneumoniae* and *M. genitallium.* The study was carried out in the rabbits that were previously immunized with the different isolates of *M. pneumoniae* and *M. genitallium,* and antiserum was raised, as documented by Lind *et al*. (1984). Fakruddin *et al.* performed various serological techniques on food samples to study the cross reactions in different bacterial species and recorded robust immunological cross reactivity between Enterobacteriaceae and virulent bacteria present in various types of food stuff (2015). Current advancements in the invention of apparatuses and procedures to divine (Agrawal, 2019) cross immunity against various types of microorganisms will be helpful in the production of vaccines.

Conclusion

The presence of MS in broiler birds was confirmed by cultural, biochemical, and molecular tests in five districts of KP. The results of the immunological cross-reactivity showed that homologous GMT titers were high in comparison to heterologous GMT titers. Routine monitoring of the pathogen will be helpful to overcome the disease. The most authentic, easy, and reliable technique was PCR assay among all the techniques performed in this study.

Acknowledgments

The authors would like to express their sincere gratitude to Professor Sajjad Ur Rahman for his invaluable assistance and guidance during the duration of the study.

Conflict of interest

All authors declare that there is no conflict of interest.

Authors contributions

Conceptualization: Sajjad Ur Rahman and Abdul Haleem Shah. Data curation: Atta Ur Rehman Formal analysis Saifur Rehman, Kamal Shah, and Inamullah Malik, Funding acquisition: Widya Paramita Lokapirnasari and Atta Ur Rehman. Investigation: Sajjad Ur Rahman and Atta Ur Rehman. Methodology: Atta Ur Rehman, Sajjad Ur Rahman, and Abdul Haleem Shah. Project administration: Atta Ur Rehman; Imdadullah Khan, and Inamullah Malik Resources: Widya Paramita Lokapirnasari; Software: Atta Ur Rehman and Saifur Rehman Supervision: Sajjad Ur Rahman and Abdul Haleem Shah Validation: Atta Ur Rehman, Abdul Haleem Shah, Sajjad Ur Rahman, Saifur Rehman Muhammad Kamal shah, Widya Paramita Lokapirnasari, Imdadullah Khan, Inamullah Malik and Andreas Berny Yulianto, Writing - original draft: Attaur rehman; Saifur rehman; Writing - review and editing: All authors:

Funding

The Faculty of Veterinary Medicine (Fakultas Kedokteran Hewan), Universitas Airlangga, as indicated by document number (769/UN3.14/PT/2020), supported the APC.

Data availability

Additional data may be obtained from the primary author and corresponding author upon a reasonable request.

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