Journal of Agriculture and Veterinary Science
ISSN: 2959-1198 (Print), 2959-1201 (Online)

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Research Article

Development of Serum Plate Agglutination Antigen using *Mycoplasma gallisepticum* Field Isolate

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ABSTRACT

Mycoplasma gallisepticum is an important pathogen responsible for respiratory tract infections in poultry. Clinical manifestation of disease varies from mild respiratory infection to chronic respiratory disease (CRD) in case of co-infections with other viral or bacterial pathogens. Mycoplasma infections cause huge economic losses due to reduction in hatchability and egg production as well as due to increased morbidity and mortality in a flock. Early screening of infection is done by using serological assays including Serum Plate Agglutination Assay (SPA) and Enzyme Linked Immunsorbent Assay (ELISA). The present study was aimed to prepare SPA antigen using *M. gallisepticum* isolate recovered from the field. In house developed antigen was compared with commercially available antigen using sera collected from the suspected flocks. Results revealed 205/300 (68.35%) positive reactions using locally developed antigen and 198/300 (66%) positive reactions while using commercially available imported antigen. After statistical analysis by using Fisher's exact test, it was inferred that difference between detection rate of both antigens was non-significant (P =1.0000). Local antigen was assessed for cross reactivity and it gave suitable results till four months. Local antigen appears to provide a cheaper and easy method for initial screening of *M. gallisepticum* infection.

Keywords: Serum Plate Agglutination Assay, In-house Antigen, Non-specific Reaction, Sensitivity, Specificity.

OPEN ACCESS

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Article History
Received: September 29, 2024
Accepted: November 12, 2024
Published: December 03, 2024

INTRODUCTION

From past few decades the development of commercial poultry sector is facing challenges of infectious diseases in the form of viral and bacterial infections. *Mycoplasma gallisepticum* infections are of foremost importance in poultry industry and are oftenly expressed as secondary infections resulting in Chronic respiratory disease (CRD) (Raviv and Ley, 2013). CRD has slow rate of spread and persistent nature. Transmission of infection in chickens from *M. gallisepticum* infected parent to the offspring through egg (vertical transmission) is a major problem. In addition, infection can also spread by contact or by airborne dust or droplets (horizontal transmission). Losses attributed to mycoplasmosis, mainly *M. gallisepticum* infection, are due to decrease in egg production and egg quality, poor hatchability (high rate of embryonic mortality and culling of day-old birds), poor feed efficiency, increase in mortality and carcass condemnations, besides medication costs (Feizi et al., 2013; Stipkovits and Kempf, 1996; WOAH, 2018)). Diagnosis of infection in layer and breeder flocks is important to reduce economic losses.

Main approaches to diagnose avian mycoplasma infections are based on isolation of organism, detection of immune response and molecular detection of the organism's nucleic acid by polymerase chain reaction (PCR) (Raviv and Kleven, 2009). Isolation of organism is laborious, so emphasis is laid on serological screening of potentially infected flock. In addition, vaccine response is also assessed by serological testing. Serological techniques used for screening includes Serum Plate Agglutination (SPA) test and Enzyme Linked Immunosorbent Assay (ELISA). SPA test is advantageous in its sensitivity, and rapid detection. Screening tests are used to assess the probability of disease, in asymptomatic population. Such tests are not used to diagnose the disease, further confirmation by defined diagnostic tests is required (Maxim et al., 2014; WOAH, 2018).

Seromonitoring data collected from the field indicated that prevalence of *M. gallisepticum* may range from 44%-76% among layers and breeding stocks in Pakistan. In broilers recorded seroprevalence is 7.14%-37.23%, in broiler breeders it is 59.6%, whereas in layers the rate is 44.9% (Khatoon et al., 2018; Mukhtar et al., 2012; Shoaib et al., 2020; Siddique et al., 2012). Moderate to high level of seroprevalence in commercial poultry has been reported in Algeria, Bangladesh, Belgium, India, Iran and Kuwait (Ali et al., 2015; Feizi et al., 2013; Heleili et al., 2011; Michiels et al., 2016; Qasem et al., 2015; Rachida et al., 2013; Rajkumar et al., 2018).

SPA test and ELISA can detect positive antibodies in experimental infections as earlier as 10 days post infection. SPA detects significantly more positive samples than ELISA during initial infection (Kempf and Gesbert, 1998). Among different serological techniques for early, rapid and reliable detection of *M. gallisepticum* infection immune response, SPA test is reported to be more sensitive and less time consuming than ELISA and Heamagglutination Inhibition assay (HI) (Asif et al., 2015; Hanif and Najeeb, 2007). In Pakistan, commercially available SPA antigens used in different laboratories include Nobilis *M. gallisepticum* antigen S6 strain and Charles River *M. gallisepticum* antigen based on *M. gallisepticum* A5969 strain. Differences exists in sensitivity of two SPA antigens used for the detection of serological response against *M. gallisepticum* strains K503 and K730 having low virulence. Highest sensitivity was observed against homologous antigen. Whereas, among heterologous antigens S6 was more sensitive in assessment of serological response than A5969. Infection of *M. gallisepticum* strains R, F and S6 was equally well detected by using any SPA antigen e.i A5969, S6, K503 and K730 (Lin and Kleven, 1982).

Lack of sensitivity, or poor reactivity of heterologous antigen used for detection can be due to variations in surface antigen among different *M. gallisepticum* strains (Kleven et al., 1988; Markham et al., 1992). Other studies reported inability of antigens used in *M. gallisepticum* serological assay to completely detect all types of antibodies in seroconverted chickens (Levisohn et al., 1995; Noormohammadi et al., 2002).

Keeping in view the variation among *M. gallisepticum* strains, it may be expected that such property may lead to incomplete detection of its antibodies upon using imported antigens as compared to the antigen prepared from indigenous isolate during SPA testing. So, to address this question, SPA test antigen was to be developed from local *M. gallisepticum* isolate followed by its standardization to detect early infection in the field.

MATERIALS AND METHODS

Isolation of *M. gallisepticum* from field

Field samples comprising of tracheal swabs were processed following the established protocols at National Reference Lab for Poultry Diseases (NRLPD), NARC, Islamabad, Pakistan. *M. gallisepticum* broth (Oxoid-CM0403) and agar (Oxoid-CM0401) were prepared with addition of Suplement G (Oxoid-SR0059). Processed samples were inoculated in the broth after filtration through 0.45 µ syringe filter and incubated at 37℃ 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 *M. gallisepticum* agar plates were incubated at 37℃ for 3-5 days in moisture rich environment, and observed daily under stereomicroscope (Labomed-CSM2). Positive sample was confirmed by PCR using *M. gallisepticum* specific primer as recommended by OIE. The primers consist of the following sequences: *M*G-14F: 5'GAGCTAATCTGTAAAGTTGGTC3', MG-13R: 5'GCTTCCTTGCGGTTAGCAAC-3' (WOAH, 2018).

DNA extraction was done by using FavorPrepTM Nucleic according to manufacturer's instructions (Favorgen, Taiwan). PCR was done by using Dream Taq Green PCR Master Mix (2X) (Cat. No. KF1081) according to manufacturer's instruction (Invitrogen) and recommended thermal profile. Negative and positive controls were included in PCR. Amplified product was visualized by gel electrophoresis, using 1% agarose gel (Ultra-pure Agarose Invitrogen-16500-500). DNA step ladder (Gene ruler Thermo Scientific SM0371) was used to analyze the band size.

Preparation of Mycoplasma gallisepticum antigen

Using field isolate labelled as ARL-1963 (Farooq et al., 2020), SPA test antigen was prepared by following a

published protocol (Arefin et al., 2011; Rasool et al., 2017) with slight modifications.

In this regard *M. gallisepticum* field isolate was inoculated in 5 ml of *M. gallisepticum* broth (Oxoid-CM0403) and incubated at 37 ℃ for 2 to 3 days. For culture enrichment,1.5 ml of log culture was then transferred to 5 ml of *M. gallisepticum* broth and incubated at 37℃ for 1-2 days. This culture was transferred to 90 ml of fresh *M. gallisepticum* broth (Oxoid-CM0403), incubated at 37℃ for 7 days. For inactivation of live culture, 0.5% phenol was added to *M. gallisepticum* broth, and kept for 2 hours. Inactivated culture was then centrifuged at 14000 rpm for 30 minutes. The supernatant was discarded and pellet was washed twice at 14000 rpm for 10 minutes using phosphate buffer saline (PBS). Pellet was suspended in PBS to get concentration of 100µg/ml. To the final suspension, 1% crystal violet was added. Thiomerosal sodium was added (0.01%) as preservative. Stained antigen was vortexed thoroughly for homogenization and stored at 4℃ till further use (Arefin et al., 2011).

Rapid serum agglutination antigen testing

In house developed SPA antigen and imported antigen were tested using serum from PCR positive cases of *M. gallisepticum*. Following OIE recommendations such agglutination tests were performed within 72 hours of serum collection. Test sera were added on the white ceramic tile in a quantity of 0.02 ml, followed by the addition of same amount of stained antigen. Antigen and test sera were mixed by sterile wooden stick, tile was swirled and results were recorded within 2 minutes at room temperature (3). Positive and negative controls were tested along with field sera. Agglutination is indicated by flocculation of the antigen within 2 minutes as shown in Figure 1. Same process was repeated using imported *M. gallisepticum* plate antigen (Charles River) (Arefin et al., 2011; Rasool et al., 2017; WOAH, 2018).

Figure 1. Serum plate agglutination test using imported and in-house antigen.

Testing plan for field samples

In house developed antigen along with commercially available antigen was tested against 300 sera collected from the suspected flocks which were initially confirmed by PCR during a routine flock monitoring activity taking place at 6-week interval in the flocks registered for this study. Sera were first tested undiluted, positive sera were retested after being heated at 56℃ and diluted 1/4 with phosphate-buffered saline pH 7. Sera that still reacted when diluted were considered positive. Sera that reacted only when undiluted were recorded as suspicious (Kempf and Gesbert, 1998). Percentage of positive and negative sera was calculated. Statistical analysis was carried out by using Fisher's exact test (Rasool et al., 2017).

Cross reactivity assay

In house developed antigen was used to check cross reactivity against positive sera of *Salmonella pullorum*, *Pasteurella multocida* (PM) and *Mycoplasma synoviae*. In addition, reference sera against various respiratory viruses such as infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV), Reovirus, infectious laryngotracheitis virus (ILTV) (Synbiotics Corporation, USA) were used to check cross reactivity. Using the same testing protocol described for SPA assay, 15 positive sera of each bacterial and viral pathogen were used to assess cross reactivity (Wanasawaeng et al., 2015).

Shelf life Confirmation

For shelf life confirmation, *M. gallisepticum* positive sera were tested using in house antigen after 14 days interval for 4 months.

RESULTS

M. gallisepticum field strain was isolated using *M. gallisepticum* broth and agar. Characteristic fried egg-shaped

colonies were observed on *M. gallisepticum* agar after 3-5 days incubation. It was confirmed by PCR using *M. gallisepticum* specific OIE recommended primers. The field isolate yielded amplified product of 185 bp observed upon conducting agarose gel electrophoresis.

Comparative evaluation of local vs imported antigen

Among 300 total sera 227(75.6%) were found positive upon testing with in-house developed antigen and 210 (70%) sera were positive when tested using commercially available antigen. Positive sera were retested using both antigens after heating and serial dilution. Samples giving positive reaction after heat inactivated and diluted revealed 205/227 (68.3%) positive with in-house antigen and 198/210 (66%) when tested with commercially available antigen Table 1. Non-significant difference was observed (P=1.0000) in detection of anti-*M. gallisepticum* antibodies by local and imported antigen by Fisher's exact test Table 2.

Cross reactivity

No cross reaction was observed with sera against respiratory pathogens including *Salmonella pullorum*, *Pasteurella multocida* (PM) and *Mycoplasma synoviae*, infectious bronchitis virus (IBV), infectious bursal disease (IBD), Reovirus, infectious laryngotracheitis virus (ILTV).

Table 1. Comparative positivity between in-house and imported antigen.

Sera Samples	Total Number	In House *M. gallisepticum* Ag		
		Positive	Negative	Suspicious
Pre Heating	300	227 (75.6%)	73 (24%)	0
Post Heating	227	205 (68%)	06 (2%)	16 (5%)
		Imported *M. gallisepticum* Ag		
Pre Heating	300	210 (70%)	90 (30%)	0
Post Heating	210	198 (66%)	02 (0.9%)	10 (4.7%)

Table 2. Statistical difference between in-house and imported antigen.

Antigen	No. of Sera Samples	Post-heat treatment		P value
		Positive	Negative	
In House *M. gallisepticum* Ag	300	205	79	
Imported *M. gallisepticum* Ag	300	198	92	1.00

Table 3: Cross Reactivity of In-house *M. gallisepticum* Ag.

Sera tested against In-house *M. gallisepticum* Ag		Total Number	Positive	Negative
Bacterial pathogens	*Salmonella pullorum*	15	Nil	15
	Pasteurella multocida	15	Nil	15
	Mycoplasma synoviae	15	Nil	15
Viral pathogens	Infectious bronchitis virus (IBV)	15	Nil	15
	Infectious bursal disease (IBD)	15	Nil	15
	Reovirus	15	Nil	15
	Infectious laryngotracheitis virus (ILTV)	15	Nil	15

Shelf life

For shelf life confirmation, known positive sera of *M. gallisepticum* were tested using in house antigen after 14 days interval for 4 months. Antigen stably detected agglutinating antibodies against *M. gallisepticum* throughout the 4 month period.

Table 4. Shelf-life evaluation of In-house *M. gallisepticum* Ag.

Testing Period	Total Number	Month 1		Month 2		Month 3		Month 4	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Day 1	12	12	Nil	12	Nil	12	Nil	12	Nil
Day 15	12	12	Nil	12	Nil	12	Nil	11	01

DISCUSSION

Mycoplasma gallisepticum infections in commercial poultry cause substantial economic losses worldwide and in Pakistan. Unique characteristic of *M. gallisepticum* infection is persistence in laying and breeding flocks and vertical transmission to progeny. Once the infection is introduced in the flock, due to high transmissibility it spreads rapidly with in the flock. In spite of the use of anti-mycoplasma treatment, infections are still reported in commercial poultry (Qadir et al., 2021; Umar et al., 2017).

Continuous flock monitoring is a pre-requisite for effective control program. In the presence of clinical signs of infection, serological assays may provide preliminary diagnosis, which can be further confirmed by isolating the infectious agent or by its molecular detection in a sofisticated laborator setting. Most common screening test employed for sero-monitoring in Pakistan is SPA assay. It is rapid, cheaper than ELISA and easy to perform than HI in the absence of trained or skilled labour. Here detection of early seroconversion is achievable by testing the presence of IgM antibody by using SPA, which is detectable even in the absence of apparent disease symptoms in suspected flocks. On the other hand, for more specific detection of MG infection, the detection of IgG antibodies has been recommended by using an ELISA or HI based tests (Ahmad et al., 2008; Atique et al., 2012; Hanif and Najeeb, 2007; Rasool et al., 2017). However, lack of specificity can be a matter of concern in conducting a reliable SPA assay, which would require the inclusion of multiple controls while setting up a SPA assay.

In the present study, conventional method of isolation of *M. gallisepticum* was used, coupled with molecular detection. Although molecular assays have replaced the need to isolate organism from infected flock, isolation of *M. gallisepticum* is still considered a gold standard in diagnosis of infection. Field isolate of *M. gallisepticum* was used to develop SPA in-house antigen as recommended by OIE, 2018. Development of local antigen using field isolates is already reported for *M. gallisepticum* in different countries and for *Mycoplasma synoviae* in Pakistan (Arefin et al., 2011; Rasool et al., 2017; Wanasawaeng et al., 2015).

To be used for diagnostic purposes, optimization of locally developed antigen was done by assessing its sensitivity, specificity, cross reactivity and shelf life. Sera for antigen testing were collected from the flocks suspectd of *M. gallisepticum* infection located in Islamabad region. The present study reports that no significant difference was found between sensitivity and specificity of locally developed antigen and imported *M. gallisepticum* antigen (Charles River, USA) with comparable detection rate of 68.5% and 66%, respectively. Non-specific reactors in the sera limit the specificity of assay. These can be either due to cross reacting proteins or use of inactivated vaccines. To avoid false positive results, it is recommended to pre-heat the sera. Alternatively, non-specific reactions can be avoided by diluting test sera. Sample reacting at 1: 8 dilution are considered positive. This is used to differentiate specific and nonspecific reactions (Raviv and Kleven, 2009; WOAH, 2018).

Variation in sensitivity and specificity of antigen may lead to false positive results (Kleven et al., 1988). Sensitivity and specificity of immunological assays depends largely on the strain infecting the flock and the strain used as an antigen to detect related antibodies. In this regard, earlier studies have reported lack of sensitivity of A5969 MG strain used as antigen in HI and SPA. Similarly, variation in antigenic profile was evident in detection of antibody response after vaccination with *M. Gallisepticum* ts-11 strain. It is therefore recommended to improve sensitivity of serodiagnosis by using homologous antigen instead of heterologous antigen (Raviv and Ley, 2013).

For evaluating the cross reactivity of MG antigen, standard antisera of *Salmonella pullorum*, *Pasteurella multocida* and *M. synoviae*, infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV), Reovirus and infectious laryngotracheitis virus (ILTV) has been previously reported (Lin, 1982). Upon following the same pattern of testing the newly prepared MG antigen, no cross reactivity with the listed antisera of bacterial or viral pathogens was observed in this study. In addition to this the developed antigen was repeatedly assessed for determining its shelf life. This in-house prepared antigen was found to be compatible to the imported antigen for upto 4 months testing period.

Optimization of SPA using local antigen provided an opportunity to replace imported antigen for early screening of *M. gallisepticum* infections in local flocks. Similar studies have already been reported elsewhere (Atique et al., 2012; Hanif and Najeeb, 2007).

CONCLUSION AND RECOMMENDATIONS

In conclusion, development and standardization of SPA antigen using local field isolate of *M. gallisepticum* may provide an opportunity to replace imported antigen, and facilitate in the provision of cheap antigen for initial screening of MG infection in poultry flocks. Furthermore, the closer compatibility of such antigen prepared from locally prevalent strain of MG would result in offering low probability of giving false negative reactions in SPA assay.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this research.

COMPETING OF INTEREST

The authors declare no competing interests.

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