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Evaluation of serological tests for the diagnosis of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infections in broiler chickens with respiratory ailments

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Abstract

The pathogenic avian mycoplasmas, such as Mycoplasma gallisepticum (MG) and Mycoplasma synoviae (MS) are responsible for causing significant economic losses to the poultry industry. The present study was aimed at serodiagnosis of MG and MS infection in broiler chicken flocks using serum plate agglutination test (SPAT), indirect-enzyme linked immunosorbent assay (i-ELISA) and Dot-ELISA, followed by measuring the relative diagnostic efficacy of SPAT and Dot-ELISA in reference to i-ELISA. The blood samples (n=68) collected from the broiler chicken aging 4–6 weeks affected with respiratory tract infection from poultry farms located in Mathura district of Uttar Pradesh, India were used in the study. Initially, SPAT was used to detect MG and MS antibodies in collected sera. In SPAT, 36.76% and 72.06% sera were found positive for MG and MS antibodies, respectively. Later, the samples were screened using laboratory standardized i-ELISA and Dot-ELISA. The culture of MG (NCBI accession no. KX759104.1) and MS (NCBI accession no. KY486506.1) isolates were used to prepare antigens to *standardize i-ELISA and Dot-ELISA. Using i-ELISA, the seropositivity of MG and MS antibodies in collected sera was found to be 85.29% and 80.88%, respectively. However, seropositivity of MG and MS antibodies using Dot-ELISA was found to be 66.18% and 77.94%, respectively. The i-ELISA was used as gold standard test to compare the diagnostic efficacy of SPAT and Dot-ELISA. The relative diagnostic sensitivity, specificity and kappa values for the detection of MG and MS using SPAT was found to be 43.10%, 100%, 0.1822 and 86.09%, 100%, 0.7574, respectively. However, the relative diagnostic sensitivity, specificity and kappa values for the detection of MG and MS using Dot-ELISA was found to be 77.59%, 100%, 0.2524 and 96.36%, 100%, 0.9102, respectively. The present study reports that i-ELISA and Dot-ELISA should be used along with SPAT for accurate detection of avian mycoplasmas infection in poultry flocks affected with respiratory tract infection.*

Keywords: *Avian mycoplasmas, broiler chicken, Dot-ELISA, indirect-ELISA, standard plate agglutination test*

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Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are responsible for causing severe economic losses to the poultry industry (Zhang *et al*., 2015; Umar *et al*., 2017). Avian mycoplasmosis caused by MG and MS are responsible for 10-20% reduction in productivity, a 5–10% increase in embryo mortality, as well as the cost involved in the prevention and control of disease in poultry flocks (Kleven, 1990; Nascimento *et al*., 2005). The infection is widely reported from different geographical regions of India with a prevalence rate of 10-27% and 20.1-33% for MG and MS, respectively (Yadav *et al*., 2022). The MG is responsible for the causation of chronic respiratory disease (CRD) in chickens (Liu *et al*., 2001; Sprygin *et al*., 2010; Yadav *et al*., 2025). However, MS mainly causes infectious synovitis, airsacculitis, arthritis, mild upper respiratory tract infections, with or without symptoms of septicemia (Kleven, 2008; Yadav *et al*., 2021; Liu *et al*., 2025). Avian Mycoplasma infection in chicken triggers coinfection of other important poultry pathogens, such as *Escherichia coli*, infectious bronchitis virus and Newcastle disease virus, which may further enhance the economic losses (Bolha *et al*., 2013; Yadav *et al*., 2023). Therefore, it is important to establish a diagnostic facility for early detection of pathogens and to improve the disease surveillance to prevent major outbreaks (Alam *et al*., 2012; Liu *et al*., 2025). Serodiagnosis is considered to be the first-line diagnostic tool for the detection of avian Mycoplasma infection in a poultry flock (Mardassi *et al*., 2008). Various serological tests such as serum plate agglutination (SPA), enzyme linked immunosorbent assay (ELISA) and haemagglutination inhibition (HI) are commonly used for screening flocks for MG and MS infections, which are later confirmed by culture and polymerase chain reaction (PCR) based assays (Kleven, 1998; Nascimento *et al*., 2005; Sprygin *et al*., 2010; Yadav *et al*., 2021).

Although, HI test might be used as a confirmatory test, it has several drawbacks such as being time-consuming, less sensitive and requires reagents that are not commercially available. Also, the result varies between different diagnostic laboratories (Mardassi *et al*., 2008; Feberwee *et al*., 2022). In routine, the primary screening of flocks for avian mycoplasmosis is usually done by serum plate agglutination test (SPAT) and/or ELISA. SPAT mainly detects the early stage of infection by detecting IgM antibodies; however, ELISA is used for the detection of chronic infection by measuring IgG antibodies (Wanasawaeng *et al*., 2015). The ELISA is more specific than SPA and HI tests and more sensitive than the HI (Hagan *et al*., 2004; Feberwee *et al*., 2005). Dot-ELISA is considered to be a simple, reliable and cost-effective technique compared to ELISA and was used earlier for the detection of important poultry diseases (Manoharan *et al*., 2004; He *et al*., 2010; Alam *et al*., 2012). The present study aimed to detect MG and MS infection in broiler chicken flocks affected with respiratory tract infections using SPAT, i-ELISA and Dot-ELISA, followed by measuring the relative diagnostic efficacy of SPAT and Dot-ELISA in reference to i-ELISA.

Material and methods

Sample collection and areas of study

The blood samples (n= 68) were collected from the broiler chicken aged 4–6 weeks affected with respiratory tract infections from poultry farms located in Mathura district of Uttar Pradesh, India (Table 1). The respiratory tract infections have been seen in chicken as they grow older in flocks (age group 4-6 weeks). Blood sample from each bird was collected aseptically in 5 mL capacity tube (BD Vacutainer® SST II Advance) for serum separation. In order to separate sera, the clot activator tubes containing blood samples were kept overnight at 4°C and then centrifuged at 2500 x g for 10 min. The separated sera were kept at -20°C for further use.

Serum plate agglutination test

The serum plate agglutination test was performed with coloured antigens of MG and MS (Soleil Biovac, Animal health, France) as per earlier described method (Shadmanesh and Mokhtari, 2013). The test was conducted by placing equal volume (25 µL) of test serum and MG/MS coloured antigen on a grease free glass slide, mixed using micropipette tips and rotated clock wise and counter-clock wise direction. The presence of clear blue violet agglutinates (clumps) within two minutes was considered positive, whereas absence of agglutination was considered negative for avian mycoplasmosis. The MG and MS commercial test sera received from Soleil, BioVac Animal Health, France were used as positive and negative test controls to perform the test.

Indirect-Enzyme Linked Immunosorbent Assay

The indirect-Enzyme Linked Immunosorbent Assay (iELISA) was standardised as per the earlier described protocol (Muhammad *et al*., 2018), with some modifications (Yadav *et al*., 2021). In brief, 50 µL sonicated protein antigens of MG (70 µg/mL; NCBI accession no. KX759104.1) and MS (40 µg/mL; NCBI accession no. KY486506.1) field isolates, prepared in carbonate/bicarbonate buffer (50 mM, pH 9.6), was used for coating the 96 wells microtitre plate and incubated overnight at 4°C. Next day, the plates were washed three times for a period of five minutes each with washing buffer [PBST (phosphate buffer saline {pH 7.2}, containing 0.05% Tween-20)], to remove the unadsorbed antigen. After that, 100 µL blocking agent (2% BSA in PBST) was added to each well and incubated for 1 hour at 37°C to block the residual binding sites in the plate. The plates were washed three times with washing buffer. Serial 10-fold dilutions of test sera (1:10 to 1:1280) were made and 50 µL of dilution of each sample was added into wells of microtitre plate and incubated for 1 hour at 37°C. Plates were washed three times with washing buffer followed by addition of 50 µL of 1:10,000 diluted conjugate (Rabbit anti-chicken

IgG HRP-Sigma, USA; PBST dilution) into each well. The plates were incubated for 1 hour at 37°C. After incubation and washing, 50 µL of substrate [OPD (Sigma, USA) with H_2O_2] was added to each well and plates were incubated for 10 minutes for colour development (yellow to orange). The reaction was stopped by adding 50 µL of 4M H_2SO_4 and optical density (OD) was measured at 492 nm in ELISA reader (Synergy™ 2, BioTek). The known positive and negative MG and MS commercial test sera received from Soleil, BioVac Animal Health, France was used to standardize the assay. The cross reactivity of sonicated MG and MS antigens was checked against each other and other commonly occurring poultry *Mycoplasma* species [*M. gallinarum*, *M. gallinacium* and *Acholeplasma laidlawii*) and found to be specific for MG and MS, respectively. The maximum optical density (OD) value in ELISA reading at 492 nm wavelength for known negative serum was considered as cut-off value. Test serum having OD value more than maximum OD value of known negative serum sample {cut-off value (amount of the analyte being tested, it varies in each ELISA plate reading)} and titre more than 1:10 was considered as positive for the respective organism (MG/MS) (Bansal *et al*., 2002).

Dot-ELISA

The Dot-ELISA was standardised as per the method described earlier (Alam *et a*l., 2012), with some modifications (Yadav *et al*., 2021). The 2.0 µL sonicated protein antigens of MG (70 µg/mL; NCBI accession no. KX759104.1) and MS (40 µg/mL; NCBI accession no. KY486506.1), were used to develop Dot-ELISA. The 2 µL antigen of each MG and MS was spotted onto nitrocellulose membrane strips (6x75 mm, 0.45µ; Advanced Microdevices PVT. LTD.), and allowed to dry at 37°C for 10-15 minutes. The strips were rinsed briefly in washing buffer [PBST (phosphate buffer saline {pH 7.2}, containing 0.05% Tween-20)], and incubated overnight at 4°C in 3% BSA (PBST dilution) to block the residual binding sites. After that, the strips were rinsed three times for a period of five minutes each in washing buffer and then incubated with 1:20 diluted test sera in PBST for 1 hour at 37°C. After incubation, the strips were rinsed three times in washing buffer and further incubated for 30 minutes at 37°C with 1:5000 PBST dilution of rabbit anti-chicken IgG HRP conjugate (Sigma, USA). After that, the strips were thoroughly rinsed three times in washing buffer and then the substrate diaminobenzedine (50 mg DAB/100 ml of 50 mM Tris, pH 7.4 + 25 µL of 30% H_2O_2; Sigma, USA) was added to develop the coloured dots. The development of brown colored spot was indicative of a positive reaction. The test was standardised by using known positive and negative MG and MS commercial test sera received from Soleil, BioVac Animal Health, France. The cross reactivity of sonicated MG and MS antigens was checked against each other and other commonly occurring poultry *Mycoplasma* species [*M. gallinarum*, *M. gallinacium* and *Acholeplasma laidlawii*) and found to be specific for MG and MS.

Statistical analysis

The data obtained by all the three serological tests (SPAT, i-ELISA and Dot-ELISA) were statistically analysed using Epitools Epidemiological Calculators (Rogan and Gladen, 1978).

Results and discussion

A total of 68 serum samples from broiler chicken affected with respiratory tract infection were collected from poultry farms located in Mathura district of Uttar Pradesh, India and screened for the serodetection of avian mycoplasmas (MG and MS) infection using SPAT, i-ELISA and Dot-ELISA. The apparent prevalence of MG and MS using SPAT was found to be 36.76% (95% CI; 26.30-48.64) and 72.06% (95% CI; 60.44–81.32), respectively (Table 1; supplementary table 1). However, apparent seropositivity of MG and MS antibodies using i-ELISA was found to be 85.29% (95% CI; 75.0–91.81) and 80.88% (95% CI; 69.99–88.47), respectively. In Dot-ELISA, the apparent seropositivity of MG and MS antibodies were found to be 66.18% (95% CI; 54.34–76.29) and 77.94% (95% CI; 66.74-86.15), respectively. The relative diagnostic efficacy (relative sensitivity, specificity and kappa value) of SPAT and Dot-ELISA was measured in reference to i-ELISA (Table 2). Cohen's Kappa values between 0.10-0.20, 0.21-0.40, 0.41-0.60, 0.61-0.80 and 0.80-0.99 indicate slight agreement, fair agreement, moderate agreement, substantial agreement and near perfect agreement, respectively.

Avian respiratory tract infection, caused by pathogenic avian mycoplasmas (mainly MG and MS), adversely affects the poultry industry (Roussan *et al*., 2008; Sprygin *et al*., 2010). Early detection of these organisms in the poultry flock becomes important to initiate the effective control measures to prevent further spread of infection (Yadav *et al*., 2022). Isolation of the pathogen is considered to be the most confirmatory test for detection of avian mycoplasmas; however, this technique is laborious, time-consuming, expensive and faces over growth problem of secondary bacterial infection (Sprygin *et al*., 2010). Molecular tests are widely used for rapid and accurate detection of avian mycoplasmas (Wu *et al*., 2019; Yadav *et al*., 2024). However, it requires sophisticated laboratory facilities, skilled man power, and high cost involved in screening of samples for targeted pathogens; which hamper its wide application in resource poor laboratories, mostly in developing countries (Ahmed *et al*., 2015). Serological techniques could be useful in early diagnosis of infectious diseases by detection of specific antibodies for a particular pathogen, which helps to take necessary prophylactic measures to control the infection (Alam *et al*., 2012). In the present study, serum samples collected from broiler chicken flocks affected with respiratory tract infection were used for serodetection of MG and MS antibodies using SPAT, i-ELISA and Dot-ELISA, followed by measuring the relative diagnostic

Table 1: Statistical analysis of SPAT, i-ELISA and Dot-ELISA for serodiagnosis of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infection in broiler chicken

Variables	Diagnostic tests					
	SPAT		i-ELISA		Dot-ELISA	
	MG	MS	MG	MS	MG	MS
Apparent prevalence [% (95% CI)]	36.76 (26.3-48.64)	72.06 (60.44-8132)	85.29 (75.0-91.81)	80.88 (69.99-88.47)	66.18 (54.34-76.29)	77.94 (66.74-86.15)
True prevalence [% (95% CI)]	40.19 (28.43-53.53)	79.84 (66.78-90.25)	90.71 (83.15-102.04)	89.76 (77.52-98.28)	73.23 (59.93-84.59)	86.45 (73.87-95.68)
PPV	0.9837	0.9972	0.9994	0.9987	0.996	0.9983
NPV	0.9365	0.7143	0.356	0.5305	0.7835	0.6081
LR (+ve)	90	90	90	90	90	90
LR (-ve)	0.101	0.101	0.101	0.101	0.101	0.101

efficacy of SPAT and Dot-ELISA in reference to i-ELISA.

SPAT- Serum plate agglutination test; i-ELISA- Indirect-enzyme linked immunosorbent assay; MG- *Mycoplasma gallisepticum*; MS- *Mycoplasma synoviae;* PPV- positive predictive value (proportion of individuals with a positive test result who actually have the disease); NPV- negative predictive value (proportion of individuals with a negative test result who actually free from the disease); LR- Likelihood ratio (LR (+ve)- indicates how much more likely a positive test result is in individual with the disease compared to those without it; LR (-ve)- indicates how much less likely a positive test result is in individual with the disease compared to those without it. LR+ > 1 suggests the presence of disease is more likely with a positive result [sensitivity/(1-specificity]; LR- > 1 suggests the presence of disease is less likely with a negative result [(1-sensitivity)/specificity].

Standard plate agglutination test is a cheaper, rapid and sensitive serodiagnostic test for primary screening of avian mycoplasmas infection in poultry by detection of IgM antibodies (Wanasawaeng *et al*., 2015; Tomar *et al*., 2017). It can detect seroconversion a few days earlier than HI and ELISA. However, this test has several limitations such as, different sources of antigen may differ in sensitivity and specificity with the variations between batches, nonspecific reactions due to bad quality or freezing of the sera, properties of the antigen preparation, and cross-reactions based on the antigenic relationship between MG, MS, and *Mycoplasma imitans* (Kleven, 1998; Feberwee et al., 2005). In the present study, through SPAT, the seropositivity of MG and MS antibodies was found to be 36.76% and 72.06%, respectively. Earlier studies from India reported lower seropositivity of MG (22.4%) and MS (18.3-51.1%) infection in chicken using SPAT (Sumitha and Sukumar, 2017; Tomar *et al*., 2017). Higher seropositivity of MG and MS in the present study may be due to all screened birds were affected with respiratory tract infections. Higher seropositivity of MG and MS in chicken was also reported in previous studies using SPAT. Aimeur *et al*. (2010) reported 43.3% seropositivity of MG in chicken at Eastern, Algeria. Heleili *et al*. (2012) reported 69.9% and 66.3% seropositivity of MG and MS in chicken at Batna commercial farms in Algeria. Shadmanesh and Mokhtari (2013) reported a very high prevalence of MG (85%) in native hens of Eghlid, Iran. Nouzha *et al*. (2013) reported 61.1% seropositivity of MG in chicken at Batna Governorate, Algeria. Amer *et al*. (2019) reported 35.0% and 87.5% seropositivity of MG and MS in chicken flock located in Dakahlia, Egypt. Shoaib *et al.* (2020) reported 45.9% and 40.4% seropositivity of MG and MS in chicken from the poultry farms located in the different regions of Rawalpindi, Pakistan. The differences in seropositivity of MG and MS in different studies may also be due to difference in health status of birds, season of studies and different geographical areas.

Table 2: Diagnostic efficacy of SPAT and Dot-ELISA in reference to i-ELISA for serodiagnosis of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infection in broiler chicken

Diagnostic efficacy of test	SPAT		Dot-ELISA	
• SPAT *vs* i-ELISA (reference test) • Dot-ELISA *vs* i-ELISA (reference test)	MG	MS	MG	MS
Relative sensitivity [% (95% CI)]	43.10 (30.16-56.77)	89.09 (77.75-95.89)	77.59 (64.73-87.49)	96.36 (87.47-99.56)
Relative specificity [% (95% CI)]	100 (69.15-100)	100 (75.29-100)	100 (69.15-100)	100 (75.29-100)
Kappa value	0.1822	0.7574	0.2524	0.9102

Supplementary Table 1: Comparative analysis on SPAT, i-ELISA and Dot-ELISA for sero-diagnosis of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infection in broiler chicken

Sr. No.	Type of Sample	Collection place	ID given	SPAT		i-ELISA Titre		Dot-ELISA	
				MG	MS	MG	MS	MG	MS
1.	Poultry serum	Mathura	70134	+	+	1:80	1:40	+	+
2.	Poultry serum	Mathura	70136	-	+	1:40	1:40	+	+
3.	Poultry serum	Mathura	70137	-	-	1:10	1:10	-	-
4.	Poultry serum	Mathura	70138	-	-	1:20	N	-	-
5.	Poultry serum	Mathura	70140	-	-	N	1:20	-	-
6.	Poultry serum	Mathura	7012	+	-	1:160	1:80	+	+
7.	Poultry serum	Mathura	7014	-	-	1:40	1:10	-	-
8.	Poultry serum	Mathura	7015	+	+	1:160	1:40	+	-
9.	Poultry serum	Mathura	7016	+	+	1:160	1:40	+	+
10.	Poultry serum	Mathura	7041	+	+	1:160	1:20	+	+
11.	Poultry serum	Mathura	7042	-	-	1:40	N	+	-
12.	Poultry serum	Mathura	7044	-	-	1:40	1:10	+	-
13.	Poultry serum	Mathura	7045	+	+	1:160	1:80	+	+
14.	Poultry serum	Mathura	7047	-	-	1:40	1:10	+	-
15.	Poultry serum	Mathura	7048	+	+	1:160	1:20	+	+
16.	Poultry serum	Mathura	7071	-	-	1:40	1:10	+	-
17.	Poultry serum	Mathura	7074	-	-	1:40	N	+	-
18.	Poultry serum	Mathura	7079	-	+	1:40	1:20	+	+
19.	Poultry serum	Mathura	7101	+	+	1:160	1:20	+	+
20.	Poultry serum	Mathura	7103	+	+	1:80	1:40	+	+
21.	Poultry serum	Mathura	7105	+	-	1:80	1:10	+	-
22.	Poultry serum	Mathura	7106	-	+	1:80	1:80	+	+
23.	Poultry serum	Mathura	7107	+	+	1:80	1:80	+	+
24.	Poultry serum	Mathura	7108	+	+	1:80	1:40	+	+
25.	Poultry serum	Mathura	7138	+	+	1:160	1:80	+	+
26.	Poultry serum	Mathura	7140	+	+	1:160	1:80	+	+
27.	Poultry serum	Mathura	7163	-	+	1:40	1:40	+	+
28.	Poultry serum	Mathura	7164	-	+	1:20	1:80	-	+
29.	Poultry serum	Mathura	7165	-	+	1:40	1:80	+	+
30.	Poultry serum	Mathura	7166	+	+	1:160	1:40	-	+
31.	Poultry serum	Mathura	7168	-	+	1:40	1:80	+	+
32.	Poultry serum	Mathura	7193	-	+	1:10	1:80	-	+
33.	Poultry serum	Mathura	7194	+	+	1:160	1:160	+	+
34.	Poultry serum	Mathura	7195	+	+	1:80	1:80	+	+
35.	Poultry serum	Mathura	7196	-	-	1:10	1:10	-	-
36.	Poultry serum	Mathura	7199	-	+	1:40	1:80	+	+
37.	Poultry serum	Mathura	7200	-	+	1:20	1:40	+	+
38.	Poultry serum	Mathura	7221	-	+	1:40	1:80	-	+
39.	Poultry serum	Mathura	7223	+	+	1:160	1:80	-	+
40.	Poultry serum	Mathura	7224	-	+	1:40	1:40	+	+
41.	Poultry serum	Mathura	7225	-	+	1:20	1:40	-	+
42.	Poultry serum	Mathura	7226	-	+	1:10	1:40	-	+
43.	Poultry serum	Mathura	7228	-	+	1:20	1:80	-	+
44.	Poultry serum	Mathura	7252	-	+	N	1:40	-	+
45.	Poultry serum	Mathura	7253	-	+	1:10	1:20	-	+
46.	Poultry serum	Mathura	7257	-	+	1:20	1:80	-	+
47.	Poultry serum	Mathura	7258	+	+	1:160	1:80	-	+
48.	Poultry serum	Mathura	7260	-	+	1:20	1:80	+	+
49.	Poultry serum	Mathura	7281	-	+	1:40	1:40	-	+
50.	Poultry serum	Mathura	7282	-	+	1:20	1:80	-	+
51.	Poultry serum	Mathura	7284	-	+	1:40	1:40	+	+
52.	Poultry serum	Mathura	7287	+	+	1:80	1:40	+	+
53.	Poultry serum	Mathura	7288	+	-	1:80	1:20	+	+
54.	Poultry serum	Mathura	7389	-	-	1:10	1:10	-	-
55.	Poultry serum	Mathura	7290	+	-	1:80	1:20	+	+
56.	Poultry serum	Mathura	7312	-	-	1:20	1:20	+	+
57.	Poultry serum	Mathura	7313	+	+	1:80	1:40	+	+
58.	Poultry serum	Mathura	7314	+	+	1:80	1:80	+	+
59.	Poultry serum	Mathura	7315	+	+	1:80	1:40	+	+
60.	Poultry serum	Mathura	7318	-	-	1:10	1:20	-	+
61.	Poultry serum	Mathura	7320	-	-	1:40	1:10	+	-
62.	Poultry serum	Mathura	7345	-	+	1:40	1:160	+	+
63.	Poultry serum	Mathura	7347	-	+	1:20	1:40	+	+
64.	Poultry serum	Mathura	7375	-	+	1:40	1:40	+	+
65.	Poultry serum	Mathura	7377	-	+	1:40	1:40	+	+
66.	Poultry serum	Mathura	7378	-	+	1:40	1:40	+	+
67.	Poultry serum	Mathura	7379	-	+	1:20	1:40	-	+
68.	Poultry serum	Mathura	7380	-	-	1:10	N	-	-
Total				**25**	**49**	**58**	**55**	**45**	**53**

Dot-ELISA is a simple, cheaper and reliable solid-phase immunoassay used for sero detection of important poultry diseases (Manoharan *et al*., 2004; He *et al*., 2010; Alam *et al*., 2012; Khanal *et al*., 2018). The advantage of Dot-ELISA is that single strip can be used for serodetection of more than one poultry pathogens at a time, hence considered to be more cost effective and reproducible as compare to liquid ELISA. However, Dot-ELISA is slightly less sensitive to ELISA, because more antibodies are required to give a visible reaction, but it is very specific, highly sensitive, less time-consuming, with fewer false positive results (Alam *et al*., 2012). It detects antibodies earlier than other more specific serological test *i.e*., haemagglutination inhibition (HI), and hence could be used directly at the farm level as an initial screening test for the detection of avian Mycoplasma infection (Folitse *et al*., 1998). The seropositivity of MG and MS antibodies in the present study using Dot-ELISA were found to be 66.18% and 77.94%, respectively. Earlier studies reported 54.7% and 58.7% seropositivity of MG using Dot-ELISA from Pakistan (Alam *et al*., 2012) and Nepal (Khanal *et al*., 2018), respectively, and 41.83% and 53.92% seropositivity of MG and MS antibodies, respectively from India (Yadav *et al*., 2021). The relative diagnostic sensitivity of the developed Dot-ELISA for the detection of MG and MS antibodies was found to be 77.59% and 96.36%; while, relative diagnostic specificity for the detection of MG and MS antibodies was found to be 100%. The earlier study from India also reported the relative diagnostic sensitivity of the developed DOT blot assay for the detection of MG and MS antibodies as 69.48% and 82.01%; while, relative diagnostic specificity was 86.18% and 91.45%, respectively as compare to i-ELISA (Yadav *et al*., 2021). Higher seropositivity of MG and MS antibodies in the present study using Dot-ELISA might be attributable to all the birds were clinically affected with respiratory tract infections and different geographical area of study.

ELISA is considered to be more sensitive and specific as compared to other serological tests for detection of avian mycoplasmas infection (Wanasawaeng *et al*., 2015; Muhammad *et al*., 2018). It has several widely accepted advantages, such as, it is rapid, capable of being used to screen large numbers of sera and use technology as microtiter plate readers that are now in wide-spread use in both developing and developed countries (*Miao et al.,* 2000). The present study reported seropositivity of MG and MS antibodies in collected sera using i-ELISA as 85.29% and 80.88%, respectively. Earlier studies carried out in different regions of India reported 16.3-62.1% (Sumitha *et al*., 2015; Udhayavel *et al*., 2016; Rajkumar *et al*., 2018) and 52.1-100% (Sumitha *et al*., 2015; Sumitha and Sukumar, 2018; Rajkumar *et al*., 2018), seropositivity of MG and MS, respectively using ELISA. However, at global level, 46.5–67.4% (Reda and Abd El-Samie, 2012; Sun *et al*., 2014; Abbas *et al*., 2018) and 46.1–100% (Nassik *et al*., 2014; Hong, 2018) seropositivity of MG and MS, respectively were reported using ELISA. The similar type of i-ELISA used for screening of serum samples in the present study was earlier validated on testing 566 serum samples of chickens collected from commercial poultry farms of Pakistan and reported seropositivity of 72% in clinically affected birds for the detection of MG antibodies (Muhammad *et al*., 2018). Similarly, the assay was earlier validated on testing 306 serum samples of chickens collected from different parts of Haryana, India and reported seropositivity of 50.32% and 61.76% for the detection of MG and MS antibodies, respectively (Yadav *et al*., 2021). The higher seropositivity of MG and MS in the present study might be attributable to all the birds were clinically affected with respiratory tract infections and different geographical area of study.

Conclusions

In the present study, the MG and MS infections in broiler chicken affected with respiratory tract infection was evaluated using SPAT, i-ELISA and Dot-ELISA, followed by measuring the relative diagnostic efficacy (relative sensitivity, specificity and kappa value) of SPAT and Dot-ELISA in reference to i-ELISA. The present study delineates the seropositivity of MG and MS infections in broiler chicken flock of studied area. On screening the serum samples, higher seropositivity of avian mycoplasmas was reported using i-ELISA, as compared to SPAT and Dot-ELISA indicating its higher sensitivity. The study suggests that ELISA based serological tests should be included along with SPAT for accurate detection of avian mycoplasmas in poultry flocks affected with respiratory tract infection.

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Declaration of Competing Interest

The authors have declared that there is no conflict of interest.

Data Availability

All the data generated are included in the manuscript and tables.

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