

Research Article

Comparative Study of Serological Tests for *Mycoplasma synoviae* Diagnosis in Commercial Poultry Breeders

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Received 30 September 2010; Revised 17 December 2010; Accepted 7 February 2011

Academic Editor: Daral J. Jackwood

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Avian mycoplasmosis causes great economic losses to the poultry industry, and one of the major agents involved is *Mycoplasma synoviae* (MS). Serum from commercial poultry breeders ($n = 2781$) was tested for MS by serum plate agglutination (SPA), hemagglutination inhibition (HI), and enzyme-linked immunosorbent assay (ELISA). From 2,781 samples tested, 736 (26.46%) were positive in SPA. From 712 SPA-positive sera, 30 samples (4.21%) were positive in HI, and 150 samples (21.06%) were positive in ELISA. Copositivity between ELISA and HI was 90%, and conegativity was 82.0%. Agreement between HI and ELISA was rejected by McNemar's test ($P \leq .001$), and Kappa coefficient showed a weak correlation between the two techniques ($k = 0.25$; $0.21 \leq k < 0.40$). Weak statistical correlation was observed between all serological tests (SPA, HI, and ELISA), and they should only be used for initial screening for MS.

1. Introduction

Avian mycoplasmosis causes great losses to industrial poultry breeding [1–3]. *Mycoplasma synoviae* (MS) is one of the most important agents of this disease that may be presented as joint and/or respiratory condition. Although symptomatic animals show respiratory problems, cough, wheezing, aerosacculitis, impaired growth, sinusitis, and synovitis, chronic and asymptomatic infections are both more common and more important, because of the losses they cause [4–6]. Diagnosis of the disease is based on epidemiological data, clinical signs of the disease, analysis of macro- and microscopic lesions, and mycoplasma serology and/or isolation and identification. The agent may be detected in fragments of affected organs (trachea, air sacs, and lungs), as well as in infraorbital and ocular sinus and synovial exudate. Tracheal and cloacal swabs are used in the isolation of the agent by means of polymerase chain reaction (PCR) [7–10].

The most used serological tests are serum plate agglutination (SPA), hemagglutination inhibition (HI), and enzyme-

linked immunosorbent assay (ELISA) [11–19], followed by mycoplasma isolation and identification. SPA titers greater or equal to 1 : 10 are considered positive, 1 : 5 are suspicious, and titers lower than 1 : 5 are considered negative. In HI, titers equal to or greater than 1 : 80 are considered positive, between 1 : 20 and 1 : 40 are suspicious, and below 1 : 20 are considered negative [3].

The objective of this study was to compare the performance of these three serological tests (SPA, HI, and ELISA) used in the detection of antibodies against MS in commercial poultry breeder flocks of different ages.

2. Materials and Methods

2.1. Samples. A total of 2,781 serum samples were collected from 28 chicken breeder flocks of different lineages, 7 to 58 weeks old, and not vaccinated against *Mycoplasma gallisepticum* (MG) and MS. Blood samples were aseptically collected from the wing veins using 5-mL sterile disposable syringes and needles. Blood was allowed to clot in the syringe

and was kept for about 1 hour at room temperature. After this, serum of each sample was separated, centrifuged, and transferred to sterile microtubes kept at 4°C until the moment of use.

2.2. Serum Plate Agglutination (SPA) Test. All serum samples were inactivated by heating at 56°C/30 minutes to destroy nonspecific inhibitory substances and tested for MS by SPA, using a commercial antigen for the diagnosis of *Mycoplasma synoviae* by serum plate agglutination (Synovitest—Laboratório BioVet—Brazil), according to the manufacturer's instructions, with some adaptations. In short, 0.02 mL of the serum to be tested was mixed with 0.02 mL of the commercial antigen (1:1) in a glass plate. After that, the plate was placed under a light source, and samples that showed agglutination (presence of clots) were considered positive. Positive sera were diluted 1:5 and 1:10 with 0.5 M phosphate-buffered saline (PBS), pH 7.2. Both dilutions were tested again by SPA as described above. Sera were considered positive when clots were observed in dilutions up to 1:10.

2.3. Hemagglutination Inhibition (HI) Test. Serum samples that were positive in SPA were also tested by HI using MS ATCC strain as the antigen, standardized at four hemagglutinating units. HI was performed as described elsewhere [20]. Titer was the highest serum dilution that showed complete inhibition of agglutination. Titers of 1:80 or greater were considered positive [14, 20, 21].

2.4. Enzyme-Linked Immunosorbent Assay (ELISA). Sera that were positive in SPA were analyzed for antibodies against MS using a commercially available ELISA antibody test kit (*Mycoplasma synoviae* antibody Test Kit—Idexx Laboratories, Inc., Maine, USA) according to the manufacturer's instructions. Briefly, samples were diluted five-hundredfold (1:500) with the diluent, and 0.1 mL of each sample was dispensed in a well of a plate previously coated with MS antigen. Plates were incubated for about 30 minutes at room temperature. After that, plates were washed with deionized water, and 0.1 mL of the conjugate was placed in each well (Goat antichick: horseradish peroxidase conjugate HRPO). Plates were incubated for about 30 minutes and washed again. Finally, 0.1 mL of the substrate solution (tetramethylbenzidine or TMB) was dispensed into each well and incubated for 15 minutes at room temperature. The reaction was blocked with 0.1 mL of stop solution. Absorbance was measured at 650 nm. Results were expressed as serum-to-positive ratios (S/P ratios) relative to a standard positive control. Serum samples, with S/P ratios greater than 0.5 (titers greater than 1,076) were considered positive.

2.5. Statistical Analysis. Results were analyzed using Kappa Index and McNemar's paired chi-square. Values of conegativity (similar to specificity) and copositivity (similar to sensitivity) were calculated as described elsewhere [12, 13, 22, 23].

TABLE 1: Frequency of anti-MS antibodies using SPA, HI, and ELISA in *Mycoplasma synoviae* diagnosis in commercial poultry breeder flocks^a.

TEST	Number of positive results/Total of samples ^a	Frequency (%)
SPA	736/2781	26.46
ELISA	150/712 ^b	21.06
HI	30/712 ^b	4.21

^aResults are expressed as number of positive birds/total number of birds in each test.

^bMaterial collected in 24 samples was not sufficient to be tested by HI and ELISA.

3. Results

3.1. Serological Tests (SPA, HI, and ELISA). Table 1 shows the frequency of anti-MS antibodies detected by SPA, HI, and ELISA. Analysis showed that 26.46% (736/2,781) of the samples were positive in SPA. As material collected in 24 samples was not sufficient to be used in the three tests, only 712 samples were tested by ELISA and HI. ELISA detected 21.06% of positive samples whereas HI showed positive titers (equal to or greater than 1:80) in only 4.21% of the samples tested.

Comparison of the diagnostic methods is presented in Table 2, which also shows the flocks, age of the birds (in weeks), and results of SPA, ELISA, and HI both in numbers and percentage of positive samples. It was not possible to draw any correlation between the age of the birds and occurrence of *Mycoplasma synoviae*. Flocks of younger birds (between 7 and 23 weeks old) were negative in ELISA and HI and so were flocks of older birds (28, 32, 42, 50, and 54 weeks old). From the 28 flocks analyzed, 13 were negative in ELISA and HI (46.43%). In the 15 flocks positive in ELISA, 11 (73.34%) showed antibodies against MS in HI. Only 5 flocks were positive in HI.

Results of the comparison between HI and ELISA showed that from the 712 sera analyzed, 27 samples (3.79%) were positive in HI and ELISA, whereas 559 samples (78.51%) were negative in these tests.

Agreement between serological methods used in MS diagnosis is presented in Table 3. From the 28 flocks analyzed, 12 were negative (42.86%) and 16 were positive (57.14%) in ELISA, whereas 23 flocks were negative (82.14%) and just 5 were positive (17.86%) in HI. Results of the two tests (HI and ELISA) showed agreement in 17 flocks (60.71%). Twelve (70.59%) of them showed negative agreement, that is, they were negative in both HI and ELISA. Agreement was positive (HI and ELISA positive) in 5 (29.41%) flocks.

3.2. Statistical Analysis. Agreement index between ELISA and HI was calculated, considering HI as the reference test because of its lower inconsistency with SPA and ELISA [22]. Copositivity between HI and ELISA was 90%, and conegativity was 82.0%.

McNemar's test rejected the hypothesis of agreement between HI and ELISA ($P \leq .001$). Kappa coefficient, used

TABLE 2: Comparison of SPA, HI, ELISA for *Mycoplasma synoviae* diagnosis in commercial poultry breeders by flock and age^a.

Flock identification	Age (weeks)	SPA		ELISA		HI	
		Positive/total	%	Positive/total	%	Positive/total	%
44205 1	07	17/100	17.00	0/17	0	0/17	0
43857 2	08	11/100	11.00	0/11	0	0/11	0
43856 3	09	5/100	5.00	0/5	0	0/5	0
44080 4	12	3/99	3.03	0/3	0	0/3	0
44263 5	12	6/100	6.00	0/6	0	0/6	0
44216 6	22	16/64	25.00	0/16	0	0/16	0
44079 7	23	47/100	47.00	0/47	0	0/47	0
44078 8	23	45/100	45.00	0/45	0	0/45	0
44077 9	26	14/95	14.74	5/14	35.71	0/14	0
44232 10	27	40/100	40.00	2/39 ^b	5.12	0/39 ^b	0
44076 11	28	10/100	10.00	0/10	0	0/10	0
44208 12	28	21/100	21.00	1/21	4.76	0/21	0
44204 13	30	39/100	39.00	3/39	7.69	0/39	0
43859 14	30	11/100	11.00	2/11	18.18	0/11	0
44203 15	31	42/100	42.00	2/42	4.76	0/42	0
44241 16	32	17/100	17.00	0/17	0	0/17	0
44075 17	35	4/96	4.17	3/4	75.00	2/4	50.00
44074 18	37	78/100	78.00	29/75	38.66	7/75 ^c	9.33
43858 19	42	38/100	38.00	0/38	0	0/38	0
44202 20	44	13/100	13.00	2/13	15.38	0/13	0
44231 21	48	55/142	38.73	43/51 ^d	84.31	0/51 ^d	0
44207 22	48	24/100	24.00	4/24	16.66	0/24	0
44206 23	48	18/100	18.00	5/18	27.77	0/18	0
44071 24	49	46/100	46.00	2/46	4.34	0/46	0
44072 25	49	27/100	27.00	4/27	14.81	1/27	3.70
43536 26	50	11/100	11.00	0/11	0	0/11	0
44070 27	54	32/96	33.33	22/22 ^e	100.00	19/22 ^e	86.36
44230 28	58	46/89	51.69	21/40 ^f	52.50	01/40 ^f	2.50

^aResults are expressed as number of positive birds/total number of birds in each test.

^bMaterial collected in 1 sample was not sufficient to be tested by HI and ELISA.

^cMaterial collected in 3 samples was not sufficient to be tested by HI and ELISA.

^dMaterial collected in 4 samples was not sufficient to be tested by HI and ELISA.

^eMaterial collected in 10 samples was not sufficient to be tested by HI and ELISA.

^fMaterial collected in 6 samples was not sufficient to be tested by HI and ELISA.

^gMaterial collected in 24 samples was not sufficient to be tested by HI and ELISA.

to analyze agreement between the two techniques, was equal to 0.25 considered to be a weak correlation ($0.21 \leq k \leq 0.40$).

4. Discussion

Low agreement index between the techniques analyzed in the study was also reported by other authors. Feberwee et al. [20] carried out a comparative study between several diagnostic methods (culture, PCR, SPA, HI, and ELISA) for *Mycoplasma gallisepticum* and *M. synoviae* and demonstrated a high number of false positive results in ELISA and SPA. The authors considered that these results may have been due to cross reactions, lack of inactivation, age of the flock, and use of inactivated vaccines that may affect the results, findings that are similar to those of the present study. From 712 sera positive in SPA, only 4.21% were positive in HI.

Results of the statistical analysis demonstrated lack of agreement (Kappa index) or weak agreement (McNemar's paired chi-square) between HI and ELISA. Chirinos et al. [11] showed similar data in the comparison of HI and ELISA for *Mycoplasma gallisepticum* and *M. synoviae* diagnosis: lack of agreement between the two techniques and conflicting results between HI and ELISA, as determined by Kappa index analysis.

According to Ewing et al. [13], screening programs that are only based on seroconversion may be inadequate for mycoplasmosis diagnosis and control. Differences in the results of the tests used in this study (SPA, HI, and ELISA) confirm this information. These authors suggest the adoption of other techniques to confirm the presence of the agent (MS), such as culture and/or DNA detection by molecular assays (PCR).

TABLE 3: Agreement between serological methods used for *Mycoplasma synoviae* diagnosis in commercial poultry breeder flocks.

Flock identification	Results		Agreement
	ELISA	HI	
44205 1	–	–	YES
43857 2	–	–	YES
43856 3	–	–	YES
44080 4	–	–	YES
44263 5	–	–	YES
44216 6	–	–	YES
44079 7	–	–	YES
44078 8	–	–	YES
44077 9	+	–	NO
44232 10	+	–	NO
44076 11	–	–	YES
44208 12	+	–	NO
44204 13	+	–	NO
43859 14	+	–	NO
44203 15	+	–	NO
44241 16	–	–	YES
44075 17	+	+	YES
44074 18	+	+	YES
43858 19	–	–	YES
44202 20	+	–	NO
44231 21	+	–	NO
44207 22	+	–	NO
44206 23	+	–	NO
44071 24	+	–	NO
44072 25	+	+	YES
43536 26	–	–	YES
44070 27	+	+	YES
44230 28	+	+	YES

The World Organization for Animal Health (OIE) recommends the use of serological tests for avian mycoplasmosis only as screening tools in the diagnosis of flocks, not of individual birds. This recommendation is based on the presupposition that tests have different sensitivities and specificities [24]. Feberwee et al. [20] also recommend not relying on a single test because of the variation in the number of false positive results in serological tests.

Ewing et al. [12] evaluated procedures for MS detection and concluded that SPA should not be used without a confirmatory test and that HI was not adequate to confirm infective status of the flock. The author suggests the adoption of ELISA instead of SPA as a screening test, and the use of PCR as a confirmatory test.

Hagen et al. [25] suggest that positive results obtained in SPA should be confirmed by additional tests, such as HI, because of the lack of specificity and false positive results observed in SPA. In our study, 28 flocks were positive in SPA, 5 in HI, and 16 in ELISA. Based on this data, results would be very different according to the reference test used. If HI was

the reference test, only 5 flocks would be positive. However, if ELISA was the standard, 16 flocks would be positive for the disease. These findings show the need to use a confirmatory test based on the isolation of the agent (MS), either by conventional microbiological tests or molecular assays.

Butcher [26] considers that, as a screening test, SPA tends to show false positive results due to the use of inactivated or oily vaccines, contaminated sera, and cross reactions. However, this author points out that avirulent or atypical infections of low immunogenic potential may lead to false negative results. Fiorentin et al. [14] agree, and they suggest re-evaluating the practice of isolation only after positive serological results. In addition, the type of antibody detected by serological tests varies, while SPA detects IgM antibody found 3 to 5 days after infection, and which persists for 70–80 days—and HI and ELISA detect IgG antibody found 7 to 10 after infection, and which persists for up to 6 months [5].

5. Conclusions

SPA, HI, and ELISA showed weak statistical agreement in *Mycoplasma synoviae* diagnosis. McNemar's test rejected the hypothesis of agreement between HI and ELISA ($P \leq .001$).

Antibodies against MS were detected in flocks of different ages, and there was no correlation between the presence of MS antibodies and the age of the birds, demonstrating that the agent is disseminated among birds of different ages.

These diagnostic methods (SPA, HI, and ELISA) should be only used as screening tools in monitoring programs to detect avian mycoplasmosis in poultry breeder flocks. Positive results should be confirmed by isolation using traditional microbiological methods or biomolecular assays (PCR).

Acknowledgment

The authors are grateful to Professor Dr. Gener Tadeu Pereira, from the *Departamento de Ciências Exatas da Faculdade de Ciências Agrárias e Veterinárias (FCAV)—Universidade Estadual Paulista (UNESP)* for guiding them in the statistical analysis of the results.

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