

Detection of *Mycoplasma synoviae* antibodies in day-old chicks using rapid serum agglutination assay and enzyme-linked immunosorbent assay

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Abstract

Mycoplasma synoviae antibodies detection using rapid serum agglutination (RSA) assay and enzyme-linked immunosorbent assay (ELISA) is considered as the suitable screening tool in chicken flocks. Both RSA assay and ELISA have performed an ability to detect an antibody formation during mycoplasma infection due to their sensitivity in detecting both IgM and IgY. However, the IgY is the major antibody isotype transferred from the hen to the newly hatched broiler chick via egg yolk. The amount of maternal IgY circulating in the chicks' serum is approximately 30% of the IgY level present in the hen's bloodstream. This study was conducted to evaluate performance of serological assays; RSA assay and ELISA; for detecting the *M. synoviae* antibodies in day-old chicks which reflect maternal antibodies. RSA assay was tested using a commercial *M. synoviae* antigen (Nisseiken Co., Ltd., Japan) and ELISA was performed by using the commercial *M. synoviae* indirect kit (ProFlock, Synbiotics Corporation, USA). Serum samples of day-old-chicks, originating from MS-H vaccinated parent flocks, were 97.5% sero-positive for *M. synoviae* antibodies examined by RSA assay and ELISA, with a mean ELISA S/P ratio of 1.589. The result demonstrated that both RSA assay and ELISA are reliable serological tools for detecting maternal-derived antibodies (IgY) in the serum of day-old chicks which could indicate the serological status of *M. synoviae* in the parent flocks.

Keywords: *Mycoplasma synoviae*, Chicken, Antibodies, Rapid serum agglutination assay, Enzyme-linked immunosorbent assay

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Introduction

M. synoviae infection causes extensive economic losses in the poultry industry due to its ability to induce airsacculitis, synovitis, and salpingitis (Kleven and Ferguson-Noel, 2008; Buim *et al.*, 2009; Pakpinyo *et al.*, 2009; Feberwee and Landman, 2010; Seifi and Shirzad, 2012; Landman, 2014; Limpavithayakul *et al.*, 2016). In addition to vertical transmission during first few weeks post-infection (Roberts and McDaniel, 1967), 1-day-old chicks originated from *M. synoviae* infected parent flocks could be the important source of horizontal transmission predisposing *M. synoviae* problem in commercial progeny farms because the horizontal transmission could occur anytime during the chronic phase (Fahey and Crawley, 1954; Feberwee *et al.*, 2008; Buim *et al.*, 2009; Pourbakhsh *et al.*, 2010; Seifi and Shirzad, 2012). Therefore medication and vaccination in breeder flocks have been suggested as alternative practices with an attractive cost-benefit relation and proven efficacy in reducing clinical signs or improving production performance in the poultry industry (Stipkovits and Kempf, 1996; Markham *et al.*, 1998; Fiorentin *et al.*, 2003; Kleven and Ferguson-Noel, 2008; Ley, 2008; Feberwee *et al.*, 2009; Landman, 2014; Hong *et al.*, 2015; Kreizinger *et al.*, 2017). However, successful control strategies require early disease detection and accurate risks assessments in poultry farms (Stipkovits and Kempf, 1996). Due to the seroconversion gap period after infection, serological assays including rapid serum agglutination (RSA) assay and enzyme-linked immunosorbent assay (ELISA), are conventional diagnostic tools for monitoring *M. synoviae* infection, especially during early infection (Kleven, 1975; Hong *et al.*, 2004). In vaccinated chickens, the serological response could be typically detected at 2 weeks post vaccination, and protective immunity can be developed within 7-14 days thereafter (Jones *et al.*, 2006b). Conversely, antibody detection from natural infection is usually presented within 1-3 weeks post infection (Kleven, 1975). Besides, serological response and protective immunity in sero-positive chickens are maintained for at least 40 weeks after immunization (Jones *et al.*, 2006a). RSA assay has been reported to perform an ability to detect antibody formation from early mycoplasma infection; 3-5 days after infection; due to its sensitivity in detecting IgM rather than IgY which is the antibody isotype transferred from the hen to the newly hatched broiler chick via egg yolk (Hamal *et al.*, 2006; Luciano *et al.*, 2011; Wanasawaeng *et al.*, 2015). Since the amount of maternal IgY circulating in the chick's serum is approximately 30% of the IgY level in the hen's blood circulation (Hamal *et al.*, 2006), this study aims to evaluate the performance of both serological assays; RSA assay using a commercial *M. synoviae* antigen (Nisseiken Co., Ltd., Japan) and ELISA using a commercial kit (ProFlock, Synbiotics Corporation, USA); for detecting *M. synoviae* antibodies in day-old chicks which could reflect the level of maternal antibodies and indicate the serological status of *M. synoviae* in the parent flocks.

Materials and Methods

Chickens and experimental design: Forty 1-day-old healthy chicks (n= 40) of the brown egg layer (ISA Babcock B-380 breed) were obtained from a commercial hatchery and immediately equally divided into four, each comprising 10 chickens. The chicks were placed in wired cages in separate rooms at the chicken experimental unit of the Veterinary Student Training Center, Faculty of Veterinary Science, Chulalongkorn University, Nakhon Pathom, Thailand. The experimental chickens were reared under full-day lighting management with *ad libitum* access to feed and water. The use of experimental animals was approved by the Chulalongkorn University Animal Care and Use Committee (IACUC; protocol No.1931051). All methods were performed following the relevant guidelines and regulations, with a focus on minimizing discomfort and improving the well-being of the chickens.

Each chicks was bled for 1 ml via the jugular vein at 1 day and 5 weeks. Serum samples were separated and immediately examined using the rapid serum agglutination (RSA) assay. The remaining serum samples were stored at -20°C for subsequent determination of *M. synoviae* antibody levels using the enzyme-linked immunosorbent assay (ELISA).

Palatine cleft swab samples were also collected from 10 chickens in each group at 1 day old and 5 weeks old. Ten swab samples in each group were pooled for further determining the presence of *M. synoviae* antigen. The pooled samples were inoculated into 2 ml of Frey's broth medium supplemented with 15% swine serum (FMS) and examined using *M. synoviae*-specific PCR and MSH-specific PCR-RFLP assays after incubating at 37°C in a humidified chamber for 5 to 7 days.

Serology: Serum samples obtained from individual forty chickens at 1 day old (n= 40) and 5 weeks old (n= 40) were examined using serological assays according to the manufacturer's instructions. Besides, reference antisera (Royal GD, Deventer, the Netherlands) including Mg antiserum specific to *Mycoplasma gallisepticum* strain S6 and Ms antiserum specific to *Mycoplasma synoviae* strain ATCC-25204; were also used as negative and positive controls in both RSA assay and ELISA.

Fresh serum samples were immediately tested using the RSA assay with a commercial *M. synoviae* antigen (Nisseiken Co., Ltd., Japan). The serum was serially diluted two-fold to 1:8 in sterile PBS, and then 30 µl of undiluted and 1:8 diluted serum samples were tested with the same volume of RSA *M. synoviae* antigen to determine the presence of *M. synoviae*-specific antibodies, which performed agglutination at dilution of 1:8 or higher (Feberwee *et al.*, 2005).

The remaining serum samples were later examined for *M. synoviae* antibody levels using a commercial indirect ELISA test kit (ProFlock, Synbiotics Corporation, USA). Serum samples, positive control solution, and normal control solutions were diluted 1:100 before adding to the *M. synoviae*-coated ELISA microplates and incubated 30 min at 21-24 °C. The ELISA microplates were washed 3 times

with 300 µl of washing buffer, added 100 µl of 1X conjugate solution, and incubated 30 min at 21-24 °C. After wash procedure, 100 µl of substrate was added and incubated for 15 min at 21-24 °C. Finally, 100 µl of 1X stop solution was added and the ELISA plates were read and recorded for optical density (OD) value at 450 nm using an absorbance reader (BioTek Model EL800, USA). The sample-to-positive (S/P) ratio was calculated by subtracting the average negative control OD from each sample OD and dividing the difference by the corrected positive control OD. All S/P values ≥ 0.6 were considered positive, and S/P values ≤ 0.6 were negative for the presence of *M. synoviae* antibodies.

***M. synoviae* vaccine, reference and field strains:** Live *M. synoviae* vaccine, Vaxsafe MS® (Bioproperties, Australia), is a temperature-sensitive MS-H strain that was developed in Australia in 1996 and has been used in Thailand since 2012. The reference strains, *M. synoviae* WVU 1853 strain (ATCC 25204), and *M. gallisepticum* S6 strain (ATCC 15302) were used as positive and negative controls in PCR and PCR-RFLP assays, respectively. Thai *M. synoviae* field isolates, identified as types C, E, and L (Limpavithayakul *et al.*, 2016), were also used in this study. An appropriate risk assessment was approved by the Institution Biosafety Committee (CU-VET-BC), protocol No. IBC1931023.

DNA template preparation: DNA was extracted from the FMS broth samples using the modified rapid boiling DNA extraction method (Ley *et al.*, 1997). The broth samples were centrifuged at 16,000× g for 6 min, washed twice with sterile phosphate-buffered saline (PBS), and resuspended in 50 µl of sterile PBS. The suspended cells were boiled at 100°C for 10 min, placed on ice for 10 min, and centrifuged at 16,000× g for 6 min. The supernatant containing the DNA template was collected and stored at -20°C until used. Concentration of the DNA template was determined using a NanoDrop™ Spectrophotometer.

***M. synoviae*-specific PCR assay:** DNA templates were examined by the Lauerma 16S rRNA gene-based PCR assay (Lauerma *et al.*, 1993). The PCR mixture of 50 µl contained 35 µl of nuclease-free distilled water, 5 µl of 5× Green GoTaq® Flexi Buffer (Promega, Madison, WI, USA), 2.5 µl of 25 mM MgCl₂, 1 µl of 10 mM dNTP (Fermentas, Leon-Rot, Germany), 0.5 µl of each 10 µM primer MSL-1 (5'-GAA GCA AAA TAG TGA TAT CA-3') and primer MSL-2 (5'-GTC GTC TCC GAA GTT AAC AA-3') (Qiagen®, Valencia, CA, USA), 0.5 µl of 5 U/µl GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA), and 5 µl of DNA template 100–200 ng. *M. gallisepticum* S6 strain and *M. synoviae* WVU 1853 strain were used as negative and positive controls, respectively. The PCR mixtures were amplified in a DNA thermal cycler (Life express, BIOER®, ROC) starting at 94°C for 5 min and 40 cycles of 94°C for 1 min, 5°C for 1 min, and 72°C for 2 min, followed by 72°C for 5 min as the final extension. The PCR products were analyzed using gel electrophoresis.

MSH-specific PCR-RFLP assay based on partial *vlhA* gene: The *vlhA* gene fragment of *M. synoviae* positive samples was amplified using the revised Hammond

vlhA gene-targeted PCR assay (Wetzel *et al.*, 2010). The 50 µl PCR mixture contained 34 µl of nuclease-free distilled water, 5 µl of 5× Green GoTaq® Flexi Buffer (Promega, Madison, WI, USA), 2.5 µl of 25 mM MgCl₂, 1 µl of 10 mM dNTP (Fermentas, Leon-Rot, Germany), 1 µl of each 10 µM primer MSRH-1 (5'-GGC CAT TGC TCC TRC TGT TAT -3') and primer MSRH-2 (5'-AGT AAC CGA TCC GCT TAA TGC -3') (Qiagen®, Valencia, CA, USA), 0.5 µl of 5 U/µl GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA), and 5 µl of DNA template 100–200 ng. *M. gallisepticum* S6 strain and *M. synoviae* WVU 1853 strain were used as negative and positive controls, respectively. The PCR mixtures were amplified in a DNA thermal cycler (Life express, BIOER®, ROC) starting at 95°C for 3 min and 40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, followed by 72°C for 5 min as the final extension. The PCR products were analyzed using gel electrophoresis.

The PCR-RFLP assay was developed to reveal the restriction enzyme *TasI* (Thermo Fisher Scientific, San Jose, CA, USA) as the suitable restriction enzyme, cutting best at 65°C in B buffer, which could digest *vlhA* gene-targeted PCR products of *M. synoviae* positive samples (Limpavithayakul *et al.*, 2023). Briefly, 20 µl of the *vlhA* gene-based PCR product containing DNA of at least 0.05 µg/µl was added to 23 µl of *TasI* mixture containing 18 µl of nuclease-free distilled water and 4 µl of 10× Buffer B, 1 µl of *TasI* (10 U/µl; Thermo Fisher Scientific, San Jose, CA, USA). *M. gallisepticum* S6 strain and *M. synoviae* WVU 1853 strain were used as negative and positive controls, respectively. After incubation at 65°C for 2 hrs, the digested PCR products were separated and analyzed using gel electrophoresis.

Gel electrophoresis: The PCR products from the PCR and PCR-RFLP assays were analyzed in 2% agarose gel (Vivantis Technologies, Malaysia) in 1× TBE buffer at 100 volts for 35 min, pre-stained with MaestroSafe™ dye (Maestrogen, Las Vegas, NV, USA), visualized by a UV transilluminator, and photographed. The amplicon size was compared to the standard 100 bp DNA ladder (New England Biolab, UK).

Statistical analysis: Serological results from RSA assay and ELISA were used to calculate sensitivity and specificity to determine the agreement between the two assays. Co-negativity (similar to specificity) and co-positivity (similar to sensitivity) values were determined as previously described (Ewing *et al.*, 1996a).

Results

Evidence of *M. synoviae* detection: The *M. synoviae* MS-H vaccine strain and non-vaccine isolates, including *M. synoviae* WVU 1853 strain and Thai *M. synoviae* field isolates identified as types C, E, and L, were not detected by *M. synoviae* specific PCR and PCR-RFLP assays in any palatine cleft swab samples, which obtained from chickens at 1 day and 5 weeks (Table 1).

Serological responses: Serological results characterized by RSA assay and ELISA were presented in Tables 1

and 2. At 1 day old, 97.5% of serum samples were seropositive, as examined by RSA assay and ELISA. Serum samples from day-old-chicks exhibiting a mean ELISA S/P ratio of 1.589 (0.524 - 2.598) showed 100% of sensitivity and specificity by both RSA assay and ELISA. Meanwhile, seronegative of all serum samples from uninfected 5-week-old chickens tested by both

RSA assay and ELISA showed a specificity of 100%. The 5-week-old-chickens presented mean ELISA S/P ratio of 0.030, with values ranging between 0.002 and 0.084. The seropositive and seronegative results from RSA assay and ELISA performed good agreement between the two assays, with similar sensitivity and specificity.

Table 1 The percentage of positive sample examined by *M. synoviae* specific assay, RSA assay, and ELISA

Age	Laboratory assays				
	<i>M. synoviae</i> specific assays ¹		RSA assay ³		ELISA
	MS-PCR	PCR-RFLP	Undiluted	Diluted	S/P $\geq$ 0.6
1 day	Negative (0/4) ²	Negative (0/4)	100 (40/40) ⁴	97.5 (39/40)	97.5 (39/40) ⁴
5 weeks	Negative (0/4)	Negative (0/4)	0 (0/40)	0 (0/40)	0 (0/40)

¹Palatine cleft swab samples from four group were examined the presence of *M. synoviae* organisms

²Four palatine cleft swab samples consist of four pooled swab samples collected from 10 chickens in each four group at 1 day old and 5 weeks old.

³Serum samples, undiluted and 1:8 diluted serum, were tested with MS antigen.

⁴Forty serum samples collected from forty chickens at 1 day old and 5 weeks old.

Table 2 Serological results of 1-day-old and 5-week-old determined by RSA assay and ELISA

Samples ¹	1-day-old				5-week-old			
	RSA assay ²		ELISA		RSA assay ²		ELISA	
	UD	D	S/P	+/-	UD	D	S/P	+/-
1	+	+	2.598	+	-	-	0.019	-
2	+	+	2.527	+	-	-	0.033	-
3	+	+	2.452	+	-	-	0.054	-
4	+	+	2.352	+	-	-	0.033	-
5	+	+	2.215	+	-	-	0.017	-
6	+	+	2.122	+	-	-	0.070	-
7	+	+	2.036	+	-	-	0.035	-
8	+	+	2.019	+	-	-	0.002	-
9	+	+	2.013	+	-	-	0.012	-
10	+	+	1.981	+	-	-	0.035	-
11	+	+	1.958	+	-	-	0.040	-
12	+	+	1.953	+	-	-	0.045	-
13	+	+	1.885	+	-	-	0.077	-
14	+	+	1.851	+	-	-	0.023	-
15	+	+	1.810	+	-	-	0.031	-
16	+	+	1.784	+	-	-	0.023	-
17	+	+	1.752	+	-	-	0.010	-
18	+	+	1.736	+	-	-	0.005	-
19	+	+	1.69	+	-	-	0.010	-
20	+	+	1.66	+	-	-	0.003	-
21	+	+	1.598	+	-	-	0.073	-
22	+	+	1.530	+	-	-	0.016	-
23	+	+	1.520	+	-	-	0.084	-
24	+	+	1.417	+	-	-	0.030	-
25	+	+	1.405	+	-	-	0.019	-
26	+	+	1.345	+	-	-	0.038	-
27	+	+	1.326	+	-	-	0.070	-
28	+	+	1.326	+	-	-	0.012	-
29	+	+	1.326	+	-	-	0.003	-
30	+	+	1.271	+	-	-	0.042	-
31	+	+	1.266	+	-	-	0.009	-
32	+	+	1.193	+	-	-	0.042	-
33	+	+	1.019	+	-	-	0.014	-
34	+	+	0.982	+	-	-	0.017	-
35	+	+	0.925	+	-	-	0.063	-
36	+	+	0.891	+	-	-	0.009	-
37	+	+	0.823	+	-	-	0.021	-
38	+	+	0.793	+	-	-	0.031	-
39	+	+	0.692	+	-	-	0.033	-
40	+	-	0.524	-	-	-	0.012	-

¹ Serum samples collected from 40 chickens.

² Serum samples, undiluted (UD) and 1:8 diluted (D) serum, were tested with MS antigen.

Discussion

This study was conducted to determine the serological assays for detecting *M. synoviae* antibodies in day-old chicks, which reflect maternal antibodies. In chickens, IgY is the principal antibody isotype transferred from the hen to the egg and subsequently to the offspring (Brambell, 1970; Hamal *et al.*, 2006). Although the half-life of maternal IgY was estimated to be 3 days, the amount of IgY deposited in the egg and transferred to the offspring was directly related to the circulating levels of IgY in the hen (Patterson *et al.*, 1962; Brambell, 1970). Besides, IgY level in the blood of 3-day-old chicks was approximately 30-40% of the hen's plasma IgY level (Patterson *et al.*, 1962; Hamal *et al.*, 2006). On the other hand, although IgA and IgM could be found in the egg yolk at significantly lower levels than IgY, maternal transfer of IgA and IgM was estimated to be less than 1% (Hamal *et al.*, 2006).

RSA assay and ELISA have been conventionally used for detecting seroconversion in surveillance of *M. synoviae* infection, especially during early infection (Kleven, 1975; Hong *et al.*, 2004). Antibody detection from natural infection typically occurs within 1-3 weeks post-infection (Kleven, 1975), while in vaccinated flocks, serological response is initially detected at 2 weeks post-vaccination, followed by the development of protective immunity within 7-14 days (Jones *et al.*, 2006b). Although the RSA assay has been reported an ability to detect antibody formation from early mycoplasma infection (3 to 5 days after infection) due to its sensitivity in detecting IgM rather than IgY, which is the antibody isotype transferred from the hen to the newly hatched broiler chick via egg yolk (Hamal *et al.*, 2006; Luciano *et al.*, 2011; Wanasawaeng *et al.*, 2015), this study demonstrated that both RSA assay and ELISA are reliable laboratory tests for detecting maternal-derived antibodies (IgY) in the serum of day-old chicks, which could represent the serological status of *M. synoviae* in the parent flocks. However, RSA assay is mostly applied as a rapid screening method due to its sensitivity in detecting antibodies formation from early infection or to detecting seroconversion a few days earlier than ELISA (Kleven, 1998; Jones *et al.*, 2006b; Wanasawaeng *et al.*, 2015).

Because RSA assay is likely to present false positive reactions, ELISA has been developed and widely used due to its advantages in being rapid, convenient, and capable of screening large numbers of sera (Kleven, 1998; Miao *et al.*, 2000; Wanasawaeng *et al.*, 2015). Although RSA assay was verified to have 100% specificity by using the 1:8 diluted serum without heat inactivation, denaturation of non-specific components in serological assays by heat inactivation has been suggested to reduce false positive reactions (Ross *et al.*, 1990; Feberwee *et al.*, 2005).

Serological assays are suitably applied as screening tools in surveillance or monitoring programs of *M. synoviae* infection in poultry flocks (Kleven, 1975; Hong *et al.*, 2004). However, positive serological samples should be further confirmed using culture method and molecular assays (Ewing *et al.*, 1996b; Luciano *et al.*, 2011). In this study, palatine cleft swab samples obtained from chickens at 1 day and 5 weeks were tested using both *M. synoviae*-specific PCR assay

and MSH-specific PCR-RFLP assay to confirm the *M. synoviae*-free condition during the study, where the chickens were reared in experimental units with strict biosecurity and biosafety procedures. Therefore, the seropositive result at 1 day old represented the normal passive immunity from their commercial parent layer flock, which was immunized with the available live MS-H vaccine and was regularly monitored for the occurrence of *M. synoviae* organisms in the flock.

M. synoviae organisms could be isolated from 1-day-old chickens, originated from infected parent flocks, approximately 6.09% in egg yolks and 10.5% in trachea (Vardaman, 1976) because of vertical or egg transmission occurring in first 1-4 weeks post infection (Roberts and McDaniel, 1967). However, horizontal transmission could occur anytime during the chronic phase (Fahey and Crawley, 1954). Therefore, serological assays including RSA assay and ELISA should be applied for routine monitoring both early and late stages of *M. synoviae* infection.

In conclusion, in addition to being the routine monitoring assays of *M. synoviae* infection in breeder and layer flocks, this study also demonstrated that both RSA assay and ELISA are reliable serological tools for detecting maternal-derived antibodies (IgY) in the serum of day-old chicks, showing 100% of sensitivity and specificity, which could indicate the serological status of *M. synoviae* in the parent flocks.

RSA assay and ELISA are reliable serological tools for detecting maternal-derived antibodies (IgY) in the serum of day-old chicks, showing 100% of sensitivity and specificity, which could indicate the serological status of *M. synoviae* in the parent flocks. Furthermore, both serological assays are suitable for routine monitoring *M. synoviae* infection in breeder and layer flocks.

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