

Development of ELISA and Serum Plate Agglutination for Detecting Antibodies of *Mycoplasma gallisepticum* using Strain of Thai Isolate

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Abstract

The aim of the present study was to develop enzyme-linked immunosorbent assay (ELISA) and serum plate agglutination (SPA) for detecting antibodies of *Mycoplasma gallisepticum* (MG), using a Thai isolated field strain. MG isolation and identification was performed using conventional culture method, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The growth characteristics of each strain were analyzed to determine the most appropriate conditions for the cultivation of MG. The results indicated that the Thai isolated field strain gave the highest growth rate of MG yield with a peak concentration of 1.4×10^8 cfu/ml at 36-48 hr post inoculation. The in-house agglutination antigen was evaluated for freedom of contamination and sterility, specificity and potency tests were conducted. All prepared antigens met all the quality criteria. The in-house serum plate agglutination antigen using a Thai isolated field strain gave 99% sensitivity and 100% specificity compared with a commercial agglutination antigen. The indirect ELISA test using a field strain was also developed with the purified antigen, and its potential for the detection of antibodies was compared with S6 and F strains. The in-house ELISA using the Thai isolated field strain provided 67% sensitivity and 95% relative specificity compared with the commercial ELISA test.

Keywords: ELISA, F strain, *Mycoplasma gallisepticum*, SPA, S6 strain

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Introduction

Mycoplasma gallisepticum (MG) belongs to the class Mollicutes, order Mycoplasmatales and family Mycoplasmataceae (OIE, 2008). It is an important avian pathogen associated with chronic respiratory disease (CRD) in chickens resulting in considerable economic loss to the poultry industry in Thailand and worldwide (Wanasawaeng et al., 2006). The characterization of MG infection includes respiratory rales, coughing, nasal discharge and conjunctivitis. Clinical signs usually develop slowly and the disease may be chronic (Ley, 2008). Stress and concurrent bacterial or viral infections are exacerbating factors for the disease in natural by affected farms (Sprygin et al., 2011), particularly the field use of mixed live vaccine in flocks infected with MG may predispose to *Escherichia coli* septicemia with consequent mortality (Nakamura et al., 1994). In European legislation, MG is in the Council Directive of 90/539/EEC on animal health conditions governing intra-community trade in and imports from third party countries for poultry and hatching eggs with conditions concerning trade with the European Community and imports from third party countries of poultry and hatching eggs.

There are various serological tests for monitoring MG status such as serum plate agglutination (SPA), the haemagglutination (HI) test and enzyme-linked immunosorbent assay (ELISA) (OIE, 2008). SPA is a rapid screening method. Its sensitivity is superior to ELISA and HI tests in its ability to detect antibody formation from early MG infection (Kempf et al., 1994). However, SPA is prone to give false positive reactions and different sources of antigen may differ in sensitivity and specificity with the variations between batches (Kleven, 1998), according to Noormohammadi et al. (2002) who suggested that strain variation in the MG major membrane antigen has been responsible for the poor reactivity of antibodies to a heterologous strain. In fact, plate agglutination detects IgM, while HI and ELISA detect IgG. Thus, SPA can detect seroconversion a few days earlier than HI and ELISA. ELISA assays have a number of widely accepted advantages as serological assay systems, since they are 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 use of autologous antigens in serological diagnosis may increase the sensitivity of the tests for mycoplasma antibodies (Noormohammadi et al., 2002). Hence, the ELISA test, using a local strain, is of considerable interest.

In Thailand, most laboratories use SPA and ELISA to determine antibody levels in chicken sera. OIE (2008) suggested that suitable crystal violet-stained antigens may be prepared in-house, using culture methods. The current study describes the preparation of SPA and indirect ELISA to measure MG-antibody levels in chicken sera using various MG strains including the Thai isolate field strain.

Materials and Methods

MG isolation and identification: Strains of MG used in this study included F (Schering Plough, USA), S6

(Charles River Laboratory, USA), ts-11 (Fort Dodge Animal Health, USA) (Whithear et al., 1990) and 6/85 (Intervet, USA). The field strains of mycoplasma cultures were taken from tracheal swabs of broiler chickens with chronic respiratory disease (CRD) complex. MG isolation was modified from Kleven (2008) using Frey's medium. The swabs were incubated in 3 ml of Mycoplasma broth at 37°C for 3-5 days. When the media color changed to orange or yellow the culture was transferred to a new Mycoplasma broth and was incubated at 37°C for 3-5 days. Typical mycoplasma colonies were observed under light microscope and selected for further identification using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP).

Extraction of DNA was performed by DNA Trap II (DNA Technology Laboratory, BIOTEC, Thailand), according to the manufacturer's instructions. Specific primers (pvpA1F and pvpA2R) for phase variable protein (PvpA) modified from Liu et al. (2001) were used for amplification. The amplifications were carried out in a total volume of 20 µl using, 3 µl template DNA, 1 µl of each primer (20 µM) (Sigma-Aldrich, Singapore), 2 µl of 10X PCR buffer (Fermentas, USA), 1.2 µl of 25 mM MgCl₂ (Fermentas, USA), 0.4 µl of 10 mM dNTPs (Fermentas, USA), 0.25 µl of 5 U/µl recombinant Taq polymerase (Fermentas, USA) and nucleus free water to 20 µl. The PCR reaction conditions included a 3 min denaturation at 94°C, 40 cycles of 0.3 min denaturation at 94°C, 0.3 min annealing at 55°C, 1 min extension at 72°C and a final additional 10 min at 72°C.

The PCR products were purified with Wizard® SV gel and PCR clean up system (Promega corporation, USA) according to the manufacturer's instruction. RFLP reaction was carried out using *AccI* and *ScrFI* (Fermentas, USA). The purified PCR products were incubated with each restriction enzyme at 37°C for 1 hr.

Growth of the MG in broth: Three different strains of MG including F, S6 and the field strain were inoculated into 3 ml of Frey's medium. The cultures were incubated at 37°C for 3 days, then 1 ml of each culture was transferred to 9 ml of Frey's medium and incubated at 37°C for 3 days. Finally, the cultures were transferred to 90 ml of Frey's medium and incubated at 37°C for 3 more days before harvesting and transferring to Mycoplasma agar. Mycoplasma yields were determined by counting numbers of colonies and measuring pH at 0, 12, 24, 36, 48, 60, 72, 84, 96 and 108 hr of incubation.

Protein determinations and analysis: Protein concentrations of each MG strain were measured with Quick Start™ (Bio-Rad Laboratories, USA) protein assay according to the manufacturer's instructions before use for SPA and ELISA preparation. The protein concentration was determined using the method of Bradford with Quick start™ (Bio-Rad Laboratories, USA). Samples containing MG were analyzed using SDS-PAGE by electrophoresis in gels containing 12.5 % polyacrylamide as described by Laemmli (1970). A full-range rainbow molecular weight marker 12-225 KDa (GE Healthcare Bio-Science, Sweden) was included in

the analysis. Gels were stained with Coomassie blue R for 1 hr and washed with destaining solution.

Serum collection: The selection criterion for MG-positive sera from broiler breeders in this study was strictly those affected by respiratory disorders with positive SPA test results (Intervet Shering Plough Animal Health, Netherlands). MG-negative sera were collected from healthy flocks with negative SPA test results. All of these birds were from farms that did not have a history of MG vaccine application. Fifteen serum samples were collected from 10 MG-positive and 10 MG-negative flocks each. Reference sera against infectious bronchitis virus (IBV), infectious bursal disease (IBD), REO virus, infectious laryngotracheitis virus (ILT), *Pasteurella multocida* (PM) and *Mycoplasma synoviae* (MS) (Synbiotics Corporation, USA) were used for cross-reactivity test.

Preparation of MG SPA test: Three different types of agglutination antigens were prepared including F, S6 and the Field strain. The S6 was chosen because it is used commercially for SPA antigen production. Each antigen contained 100 µg in 1 ml of distilled water with 1% crystal violet (Sigma, USA). Before use, in these studies, all antigens were evaluated for quality by contamination, sterility, specificity and potency tests according to Sangsuwan et al. (1994). Sera were dropped on to a clean white tile followed by stained MG antigen. Then, a stirring rod was used to spread the mixture over a circular area of approximately 1.5 cm diameter and the tile rocked for 2 min. Agglutination was read by flocculation of the antigen within 2 min (OIE, 2008). In-house SPA reagent was compared with a commercial reagent (Intervet Shering Plough Animal Health, Netherlands).

Indirect ELISA: For checkerboard titration, ninety-six well immunosorbent plates were coated with varying dilutions of 100 µl of MG antigen at concentrations of 2, 4, 8 and 16 µg/mL of 0.2 M sodium carbonate-bicarbonate buffer, pH 9.4 (Sigma, CA) overnight. These were washed 3 times with 300 µl of washing buffer and 300 µl of blocking solution (BSA, Sigma, USA) added, then incubated for 1 hr. MG positive and negative controls, which were purchased from Synbiotics Corporation (USA), were diluted at 1:1000 before adding to the MG-coated plates and incubated

for 30 min. These were washed 3 times with 300 µl of washing buffer and varying dilutions of 100 µl of goat anti-chicken IgG horseradish peroxidase (Synbiotics Corporation, USA) added at concentrations of 1:100, 1:500, 1:1000, 1:2000 and 1:4000, and incubated for 1 hr. One hundred µl of ABTS hydrogen peroxidase (Synbiotics Corporation, USA) was added and incubated for 15 min. Finally, 100 µl of stop solution was added and the 450 nm absorbance determined with ELISA reader (Biotek Instrument, USA). Commercial ELISA using a ProFlock MG ELISA kit (Synbiotics Corporation, USA) was conducted following the manufacturer's instructions.

Determination of positive/negative cut-off and tested serum sample: A mean of sample-to-positive (SP) ratio value plus 2 standard deviations (SD) of the control sera was calculated according to the formulation below as describe by Crowther (2002).

$$\text{Cut off} = (\bar{X} + 2SD (\text{Negative})) - (\bar{X} - 2SD (\text{Positive})) / 2$$

Results

MG isolation and identification: Field, S6 and F strains of MG were cultured using Frey's medium. The agar plate was examined for Mycoplasma colonies under low magnification using a light microscope with the light intensity reduced. The morphology of the isolated field strain showed tiny, smooth colonies with dense, elevated centers (Fig 1), which according to OIE (2008), describing that Mycoplasma colonies on solid medium can usually be recognized by typical fried egg appearance.

Then, the DNA detection method was used to identify the Mycoplasma isolates and to distinguish between MG strains using RFLP. The PCR using pvpA1F and pvpA2R, amplified the product of the F strain giving a single band of 450 bp that differed from other strains (Fig 2). Additional RFLP using *AccI* gave a prominent band of 350 bp from ts-11 to differentiate from other strains. Moreover, RFLP using *ScrF1* provided differentiation between the S6 and 6/85 strains of MG. In this study, our isolated MG had a remarkable DNA pattern that differed from other vaccinated and reference MG. Therefore, this isolated MG was, uniquely a field strain of Thailand.

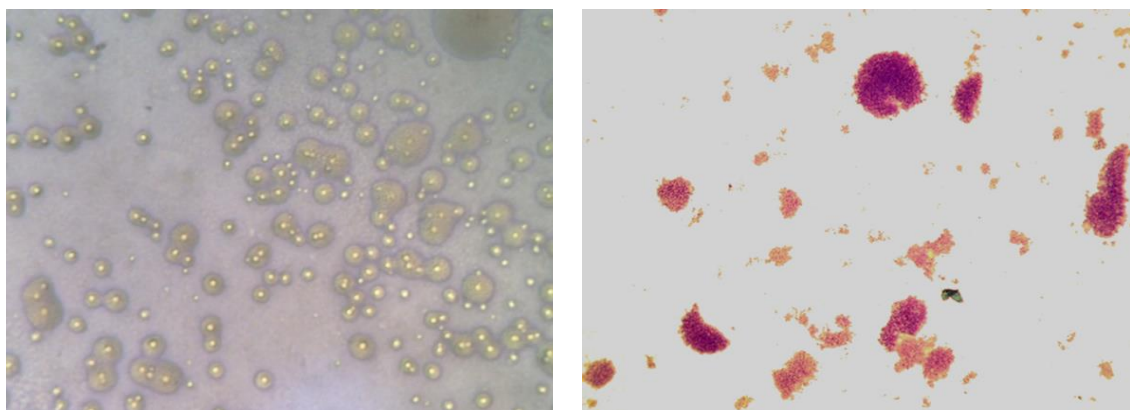


Figure 1 The morphology of a field strain of mycoplasma colonies on the Frey's mycoplasma agar (left, 10X magnifications) and the Gram's stain mycoplasma organism (right, 40X magnifications).

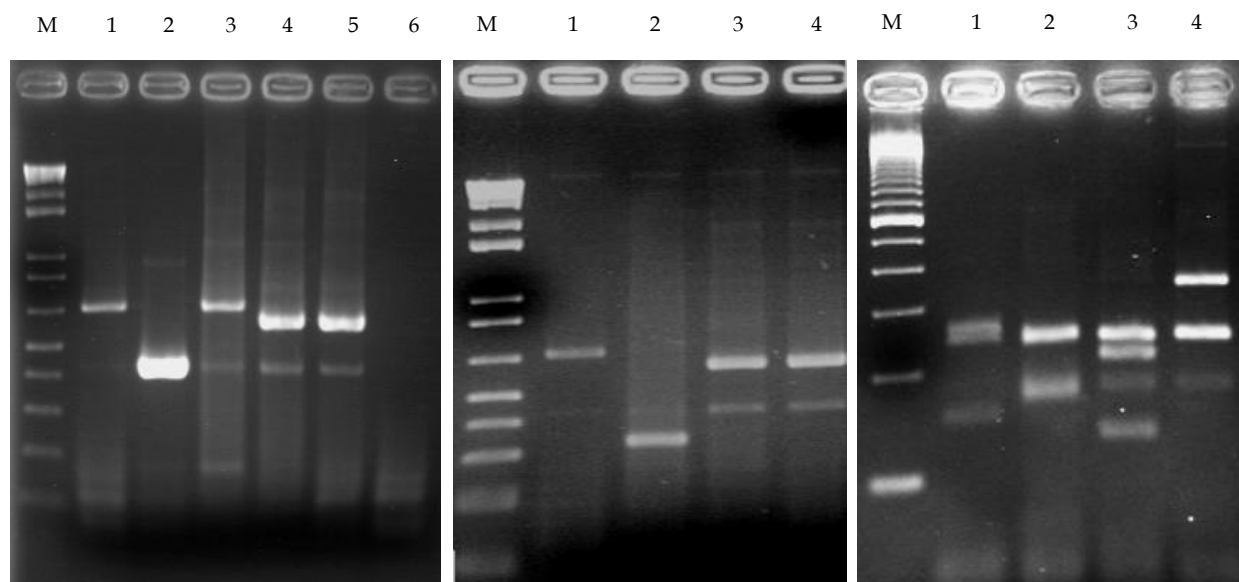
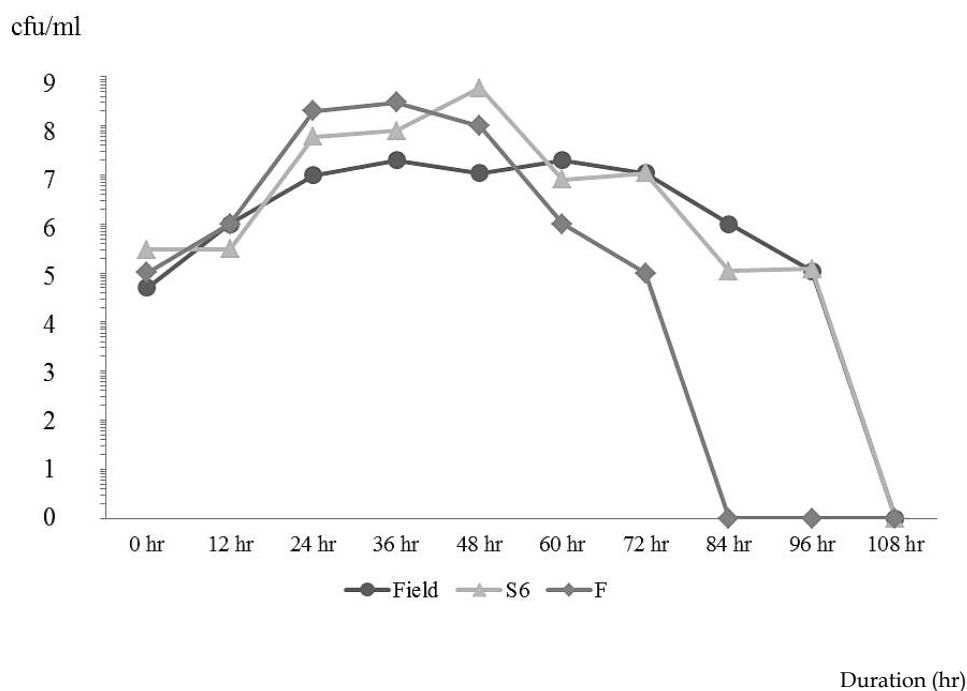


Figure 2 Gel electrophoresis of amplified DNA of various MG strains including field, F, ts-11, S6, 6/85, respectively (left). Restricted products using *AccI* (middle) and restricted products using *ScrF1* (right).
Left: M represents 1 Kb Plus DNA ladder (Invitrogen, USA); lane 1, field; lane 2, F; lane 3, ts-11; lane 4, S6; lane 5, 6/85; lane 6, distilled water.
Middle and right: M represents 1 Kb Plus DNA ladder (Invitrogen, USA) (middle) and 100 bp DNA ladder (Promega, USA) (right); lane 1, Field strain; lane 2, ts-11; lane 3, S6; lane 4, 6/85.



pH changing during cultivation at hr post inoculation										
	7	7	7	7	7	6	6	6	6	6
Field	7	7	7	7	7	6	6	6	6	6
S6	7	6	6	6	6	6	6	6	6	6
F	7	7	6	6	6	6	6	6	6	6

Figure 3 Growth of the MG in broth and pH changing during cultivation. Three different kinds of MG including field, S6 and F strains were inoculated in 3 ml of Frey's medium. Field (●), S6 (▲) and F (◆).

Growth of the MG in broth: To define the optimal conditions for MG cultivation, growth of 3 different kinds of MG including field, S6 and F strains was carried out. The results indicated that the F strain gave the highest growth rate of MG yield with a peak

concentration of 2.4×10^8 cfu/ml at 36 hr post inoculation. Secondly, the field strain gave a peak concentration of 1.4×10^8 cfu/ml at 36-48 hr post inoculation. Finally, S6 had a peak concentration of 2.4×10^8 cfu/ml at 48 hr post inoculation. A more rapid

and greater decline was observed with F compared with others. The growth of MG strains did not correlate with the pH change of the culture medium (Fig 3). Therefore, this study harvested the organism at the incubation period giving a peak concentration for each strain of MG.

Protein analysis using the SDS-PAGE of various MG strains including S6, F and the field strains compared with porcine serum, indicated that there was some porcine serum's protein remaining in the antigen before preparing the SPA and ELISA tests (Fig 4).

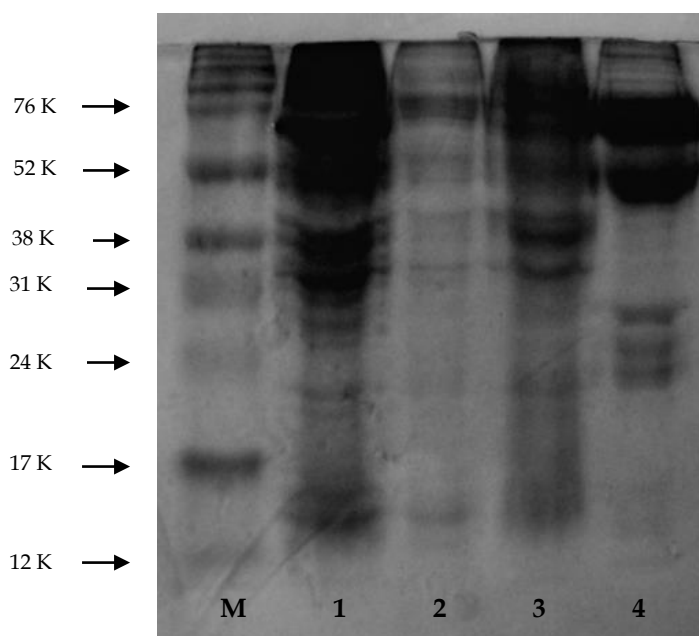


Figure 4 SDS-PAGE of various MG strains including S6, F and field strains compared with porcine serum. M represents Full-range rainbow molecular weight marker 12-225 KDa; lane 1, S6; lane 2, F; lane 3, Field strain; and lane 4, porcine serum.

Preparation of MG antigen for SPA test: In the production of MG antigen for SPA, three strains of MG including field, S6 and F were grown in broth culture and harvested between 36-48 hr post-inoculation as describe previously. After evaluation of the antigen quality by contamination, sterility, specificity and potency tests, all prepared antigen met all those quality criteria. Under microscopic examination of both Gram's and Giemsa's stain, there was no other bacterial contamination. Bacterial and fungal contamination was not present after 5 days of incubation. One hundred μ g of MG including 1% crystal violet staining revealed well-homogenized suspension without auto agglutination, when tested with 0.85% NaCl. The positive reaction was recognized by the formation of colored floccules and the clearing of the suspending medium. Cross-reaction was also evaluated using MS positive sera (Synbiotics Corporation, USA) and no cross reactivity was observed. The potency of the MG antigen was established using 10 MG-SPA positive sera and 10 MG-SPA negative sera.

Preparation of MG ELISA: Checkerboard titration. The optimal concentration of conjugate and antigen to be used for in-house ELISA was determined by matrix titration using MG-antibody positive and negative sera (Synbiotics Corporation, USA) with a protein content ranging from 0.5 to 16 μ g/ml and conjugate dilution ranging from 1:100 to 1:4000. The results were evaluated for the maximal positive-to-negative (S/N) ratio between the positive and negative sera. At 1:500 of conjugate dilution and 1 μ g/ml of protein content chosen throughout the studies according to a subsequent dilution indicated a satisfactory difference between references of positive and negative sera (Table 2).

Determination of positive/negative cut-off. A cut-off value, calculated by the means of sample-to-positive (SP) ratio value and standard deviation (SD) of the control sera, was set at 0.172. There was no cross reaction of in-house ELISA test to 6 avian pathogens including IBV, IBD, REO, ILT, PM and MS (Table 3). The result indicates that the in-house ELISA was specific for MG.

Table 1 Comparison of relative sensitivity, specificity, accuracy, false positive and negative of various in-house MG SPA using field strain, S6 and F strains with commercial reagent (Intervet Shering Plough Animal Health, Netherlands).

In-house SPA	Sensitivity	Specificity	Accuracy	False positive	False negative
Field	99 %	100 %	100 %	0 %	1 %
S6	100 %	100 %	100 %	0 %	0 %
F	99 %	100 %	100 %	0 %	1 %

Table 2 Checkerboard titration of in-house MG ELISA using field strain. To optimize between protein content from 0.5 to 16 µg/ml and conjugate dilution from 1:100 to 1:4000.

Protein content (µg/ml)	Conjugate dilution				
	1:100	1:500	1:1000	1:2000	1:4000
0.5	10.82	14.44	13.02	10.32	7.06
1	10.65	16.11*	14.13	10.17	7.23
2	10.57	13.23	12.84	10.39	7.00
4	10.03	13.95	12.55	10.00	7.57
8	8.80	13.77	12.37	9.65	8.41
16	7.62	12.22	12.39	10.55	8.46

Remarks: *represent the highest S/N ratio from this study.

Table 3 Mean O.D. of MG ELISA using field strain. Reference serum against various diseases including IBV, IBD, REO, ILT, PM and MS was evaluated.

Reference sera	Optical density (O.D.) ± Standard Deviation
IBV	0.083 ± 0.001
IBD	0.080 ± 0.004
REO	0.078 ± 0.001
ILT	0.095 ± 0.014
PM	0.099 ± 0.007
MS	0.080 ± 0.003

A total of 300 samples originated from 10 MG-positive and 10 MG-negative flocks (15 samples per flock). The criterion for MG-positive and MG-negative flocks from broiler breeders in this study was based on a history of respiratory disorders and SPA test. The reliability of newly developed MG ELISA was examined and simultaneously compared with a commercial ELISA test, ProFlock MG ELISA kit (Synbiotics Corporation, USA). All samples were tested according to the protocol with antigen and conjugate dilution according to checkerboard titration or the instructions of the manufacturer. In terms of relative sensitivity, specificity and accuracy, the in-house MG ELISA using S6 antigen had the highest sensitivity (96%), specificity (95%) and accuracy (95%), while the in-house ELISA using field and F strain had a relatively high specificity (95 and 94%, respectively), but low sensitivity (68 and 57%, respectively) and accuracy (83 and 77 %, respectively).

Discussion

MG is a major cause of chronic respiratory disease in commercial broiler production, especially

with the presence of poor management and/or other respiratory pathogens. It can result in loss of production and the downgrading of carcasses (OIE, 2008).

In these studies, the Thai field MG strain was isolated, morphologically characterized and identified by PCR and RFLP. Identification of PCR and RFLP from our isolated MG indicated that it was a unique field strain of Thailand that differed from other reference strains. This isolate was used for the entire study. To culture MG, a previous report had suggested that the most sensitive antigens for SPA/ELISA could be prepared by harvesting organisms early in culture (Loughnane *et al.*, 1993). The peak concentration of MG in our study was seen around 36 to 48 hr post-inoculation, which was later than Loughnane *et al.* (1993), who suggested that MG ELISA antigens should be harvested between 18-24 hr or while the pH of the medium is 6.5 or above. Hence, collection of the organism prior to this point may obtain the highest concentrations of MG antigen and the most appropriate antigens for SPA and ELISA as described.

Table 4 Comparison of relative sensitivity, specificity, accuracy, false positive and negative of various in-house MG ELISA using field strain, S6 and F strains with commercial ELISA (Synbiotics Corporation, USA).

MG strains	Sensitivity	Specificity	Accuracy	False positive	False negative
Field	67 %	95 %	83 %	5 %	33 %
S6	96 %	95 %	95 %	5 %	4 %
F	57 %	94 %	77 %	6 %	43 %

Before SPA/ELISA test preparation, the purification of the antigen was analyzed. SDS-PAGE of MG strains of S6, F and field revealed that there was some porcine serum protein remaining. Thus, some nonspecific reactions may have occurred from the factors involved the purification of the antigen (Li et al., 2010). However, the SPA result related to cross-reaction and the specificity test in this study were quite specific for the detection of MG antibody. The relative sensitivity, specificity and accuracy for all strains of field, S6 and F were almost 100 %. Regarding OIE recommendations (OIE, 2008), SPA-positive sera should be confirmed by ELISA. Therefore, the in-house ELISA was also developed.

Poultry companies ELISA technology for screening large numbers of sera for *Mycoplasma* testing (OIE, 2008). In this study, we developed and validated an in-house ELISA for detecting antibodies against MG in chicken sera. Commercial MG ELISA plates are usually prepared from the whole cell S6 strain of MG (OIE, 2008). The cross-reaction to various avian pathogens indicates that the in-house ELISA is specific for MG, contrary to other results. It is noticeable that the in-house ELISA using S6 had the most satisfactory results in term of relative sensitivity, specificity and accuracy compared with F and the field strain. In poultry, a high specificity of serological tests is more important than high sensitivity since low sensitivity can be compensated for by using a greater number of chickens' sera (De Wit et al., 1997). Therefore, the in-house ELISA using the Thai isolated field strain may be applicable.

Overall, our study demonstrates that the SPA and ELISA tests using the Thai isolate developed in this study is much more affordable for the Thai poultry industry; thus it has great application potential for the long-term prevention and control of MG in Thailand, compared to similar commercial products available on the international market.

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บทคัดย่อ

การพัฒนาเทคนิค ELISA และ Serum plate agglutination สำหรับการทดสอบแอนติบอดีต่อเชื้อ *Mycoplasma gallisepticum* โดยใช้เชื้อสายพันธุ์ท้องถิ่นที่แยกได้จากประเทศไทย

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วัตถุประสงค์ของการศึกษาค้นคว้าครั้งนี้คือ การพัฒนาเทคนิค Enzyme-linked immunosorbent assay (ELISA) และ Serum plate agglutination (SPA) สำหรับการตรวจแอนติบอดีต่อเชื้อ *Mycoplasma gallisepticum* (MG) โดยใช้เชื้อที่แยกได้จากประเทศไทย การแยกและการจำแนกเชื้อ MG อาศัยวิธีการเพาะเชื้อ การทดสอบด้วยเทคนิคห่วงโซ่โพลีเมอเรส (PCR) การตรวจสอบความหลากหลายของเชื้อด้วยการตัดด้วยเอนไซม์ตัดเฉพาะ (RFLP) และการวิเคราะห์โปรตีนด้วยเทคนิค Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) วิเคราะห์รูปแบบการเจริญของเชื้อแต่ละสายพันธุ์เพื่อหาสภาวะที่เหมาะสมที่สุดสำหรับเพิ่มจำนวนเชื้อ MG ผลการทดลอง บ่งชี้ว่า เชื้อ MG สายพันธุ์ท้องถิ่นมีอัตราเจริญเติบโต และให้ปริมาณเชื้อสูงสุด ความเข้มข้นเป็น 2.4×10^8 cfu/ml ภายหลังการบ่มเป็นเวลา 36 ชั่วโมง การประเมินคุณภาพของแอนติเจนสำหรับใช้ทดสอบ SPA ที่เตรียมขึ้นใช้เองทั้งการทดสอบการปนเปื้อน การปลดเชื้อ ความจำเพาะ และประสิทธิภาพของวิธีการทดสอบ ผลการทดลองใช้แอนติเจน พบว่า มีความไวในการทดสอบ 99 เปอร์เซ็นต์ และความจำเพาะในการทดสอบ 100 เปอร์เซ็นต์เปรียบเทียบกับแอนติเจนที่มีการจำหน่ายเชิงพาณิชย์ การพัฒนาวิธีการทดสอบ Indirect ELISA โดยใช้เชื้อ MG สายพันธุ์ท้องถิ่นที่มีการเตรียมเชื้อให้บริสุทธิ์เปรียบเทียบกับสายพันธุ์ S6 และ F พบว่า ชุดทดสอบ ELISA ที่เตรียมจากเชื้อสายพันธุ์ S6 มีความไวในการทดสอบมากกว่าสายพันธุ์อื่นๆ โดยชุดทดสอบ ELISA ที่ใช้เชื้อ MG สายพันธุ์ท้องถิ่นมีความไวในการทดสอบเป็น 67 เปอร์เซ็นต์ และความจำเพาะในการทดสอบเป็น 95 เปอร์เซ็นต์เปรียบเทียบกับชุดทดสอบสำเร็จรูปที่มีการจำหน่ายเชิงพาณิชย์

คำสำคัญ: เทคนิค ELISA สายพันธุ์ F *Mycoplasma gallisepticum* เทคนิค SPA สายพันธุ์ S6

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