Original Article

Laboratory Diagnosis of *Mycoplasma gallisepticum* (MG) Infection in Experimental Layer Chicken Receiving MG Vaccines and MG Organisms

Somsak Pakpinyo* Pinyo Pitayachamrat Seubchat Saccavadit Than Santaswang Achara Tawatsin Jiroj Sasipreeyajan

Abstract

This experiment was designed to study the results of laboratory diagnosis of *Mycoplasma gallisepticum* (MG) infection in layer chicken receiving MG vaccines and MG organisms. One hundred and twenty 1-day-old-male-layer chickens were raised in separate isolation rooms and equally allocated into 4 separated groups. When 4 weeks old, groups 1, 2, 3 and 4 served as a negative control, a MG commercially inactivated vaccine group, a MG commercially live vaccine group and a MG infected group, respectively. In each group, ten birds were swabbed and bled when 4, 5, 6, 7, 8, 9 and 11 weeks old. Swab samples were tested for MG Infection by culture and polymerase chain reaction (PCR) procedures. Sera were determined for MG antibody responses by a serum plate agglutination (SPA) test and by 2 commercial enzyme-linked immunosorbant assay (ELISA) test kits, IDEXX® and Synbiotics®. Results revealed that MG organisms were only found in groups 3 and 4 using culture and isolation when 5 and 11 weeks old, and by PCR during the 5 to 9 week old period. The SPA results showed positive reactors in groups 3 and 4 during the 7 to 11 week old period. The positive reactors detected by both commercial ELISA test kits were similar, detecting MG antibody responses in groups 3 and 4 during the 7 to 11 weeks old period. However, only group 2 found positive reactors by the Synbiotic® ELISA test kits, during the 9 to 11 week old period.

Keywords : *Mycoplasma gallisepticum*, SPA, ELISA, culture and isolation, PCR.

Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand.
***Corresponding author**

บทคัดย่อ

การวินิจฉัยโรคทางห้องปฏิบัติการ โรคติดเชื้อ*มัยโคพลาสม่า กัลลิเซพติกุ่ม* (MG) ในไก่ไข่ทดลองที่ได้รับวัคซีนป้องกันโรคติดเชื้อMG และเชื้อMG

สมศักดิ์ ภัคภิญโญ* ภิญโญ พิทยจำรัส สืบชาติ สัจจาวาทิต ธัญ สันตสว่าง อัจฉรา ธวัชสิน จิโรจ ศศิปรียจันทร์

วัตถุประสงค์เพื่อศึกษาถึงผลการวินิจฉัยทางห้องปฏิบัติการ โรคติด*เชื้อมัยโคพลาสม่า กัลลิเซพติกุ่ม* (เอ็มจี) ในไก่ไข่ทดลองที่ได้รับวัคซีนป้องกันโรคติดเชื้อเอ็มจี และเชื้อเอ็มจี ใช้ลูกไก่ไข่เพศผู้อายุ 1 วัน จำนวน 120 ตัว เลี้ยงจนถึงอายุ 4 สัปดาห์ ทำการแบ่งไก่ออกเป็น 4 กลุ่ม กลุ่มที่ 1 กลุ่มควบคุม กลุ่มที่ 2 3 และ 4 ได้รับวัคซีนเอ็มจีเชื้อตาย วัคซีนเอ็มจีเชื้อเป็น และเชื้อเอ็มจีตามลำดับ ทำการป้ายเชื้อจากร่องเพดานปากและเก็บเลือดไก่ทุกกลุ่มเมื่ออายุ 4 5 6 7 8 9 และ 11 สัปดาห์ ตามลำดับ ตัวอย่างที่ได้จากการป้ายเชื้อจะนำไปตรวจหาเชื้อเอ็มจีด้วยวิธีเพาะแยกเชื้อ และวิธีพีซีอาร์เพื่อหาสารพันธุกรรม ต่อเชื้อเอ็มจี นำซีรั่มตรวจหาแอนติบอดีต่อเชื้อเอ็มจีด้วยวิธีเอสพีเอ และวิธีอีไลซ่า โดยใช้ ชุดทดสอบอีไลซ่าสำเร็จรูปของ 2 บริษัท (IDEXX® และ Synbiotics®) ผลพบว่าวิธีการเพาะแยกเชื้อสามารถตรวจพบเชื้อเอ็มจี เฉพาะกลุ่มที่ 3 และ 4 ระหว่างอายุ 5 - 11 สัปดาห์ และวิธีพีซีอาร์พบสารพันธุกรรมของเชื้อเอ็มจีระหว่างอายุ 5 - 9 สัปดาห์ พบการตอบสนองของแอนติบอดีต่อเชื้อเอ็มจีด้วยวิธีเอสพีเอเฉพาะกลุ่มที่ 3 และ 4 ระหว่างอายุ 7 - 11 สัปดาห์ จากการตรวจด้วยวิธีอีไลซ่าของทั้ง 2 บริษัท ให้จำนวนผลบวกใกล้เคียงกัน พบการตอบสนองของแอนติบอดีต่อเชื้อเอ็มจี ของกลุ่มที่ 3 และ 4 ระหว่างอายุ 7 สัปดาห์ ถึง 11 สัปดาห์ ส่วนกลุ่มที่ 2 พบผลบวกเฉพาะชุดทดสอบสำเร็จรูปของ Synbiotics ระหว่าง 9 - 11 สัปดาห์

คำสำคัญ: *มัยโคพลาสม่า กัลลิเซพติกุ่ม* เอสพีเอ อีไลซ่า การเพาะเชื้อและแยกเชื้อ พีซีอาร์

ภาควิชาอายุรศาสตร์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330
*** ผู้รับผิดชอบบทความ**

Introduction

Mycoplasma gallisepticum (MG) infection is a chronic respiratory disease (CRD) in avian species (Ley, 2003). Chickens, turkeys, quails, parrots, pheasants, pigeons, and peacocks are the natural hosts of MG infection (reviewed by Ley, 2003). The mortality rate is low unless a secondary microorganism infection is present. MG infection causes sneezing, conjunctivitis, airsacculitis, and decreased egg production in affected birds. The MG organisms in infected birds can be transmitted to the other birds via direct contact; that is, horizontal transmission. In addition, affected breeders can spread MG organisms through their progeny which is called "vertical transmission" (Ley, 2003).

The economic losses due to decreased in egg production in breeders have been estimated at about 21 eggs/bird, or over US$ 100 millions per year for the US poultry industry (Mohammed et al., 1987). Furthermore, their progeny show decreased feed efficiency, high feed conversion rate, poor carcass quality, and losses due to prevention and treatment costs (Ley, 2003).

For prevention, two types of vaccines; live and inactivated which have been widely used in many countries, including Thailand (Ley, 2003). Strains of

6/85, ts-11, and F are commercially available, live, MG vaccines. Because each strain has a different degree of virulence, the use of live vaccines should be carefully considered and the manufacturers' instructions followed (Kleven, 1998, Ley, 2003). Inactivated MG vaccine is safer but has the disadvantage of requiring 2 doses for optimal protection, plus the cost of individual bird injection and a reduced ability to control long-term infection in multiple age production units (Ley, 2003).

There are 2 major diagnostic methods, MG detection and MG serology, both of which are widely used (Kleven, 1998). MG detection including MG culture and isolation, and MG polymerase chain reaction (PCR) testing have been used in most MG laboratories. However, the gold standard for MG diagnosis is MG culture and isolation but it requires MG antibody and a fluorescent microscope (Ley, 2003). MG serology, such as serum plate agglutination (SPA), enzyme linked immunosorbent assay (ELISA) and hemagglutination inhibition (HI) tests are widely used in the laboratory; however, each method is limited by sensitivity and specificity (Kleven, 1998). SPA is used as the screening test because it's rapid, has high sensitivity, and low specificity, as well as being inexpensive. ELISA has been proved to have good sensitivity and more specificity compared to SPA (Kleven, 1998). HI has high specificity but the disadvantages are low sensitivity and it is not commercially available (Kleven, 1998).

In Thailand, there are few laboratories which can culture and isolate MG. Most laboratories diagnose MG by SPA and/or ELISA methods, which are convenient, inexpensive, and not time consuming, compared to culture and isolation, or PCR. However, no fundamental data on the SPA, ELISA, or MG detection methods have been reported by Thai laboratories.

The purpose of this study was to determine the results of MG diagnosis using SPA, ELISA, culture and isolation and PCR methods, in layer chicken receiving MG live vaccine (strain F), killed vaccine, and MG organisms (S6 strain). The results of this study should be useful for MG prevention and control in Thailand's poultry industries.

Materials and Methods

Layer chickens, MG vaccines and MG organisms.

Four isolated rooms were cleaned, and disinfected for 10 days prior to being restocked with layer chicken. One hundred and fifty, day-old-male-layer birds were received from a MG-clean, integrated breeder farm and were raised on a wire floor cage in four isolated rooms. Thirty birds were bled from the jugular vein for MG serology, and swabbed from the choanal cleft, for MG culture and isolation (3 birds pooled as 1 sample), and the polymerase chain reaction (PCR) test. When 3, and 4 week old, thirty birds were selected at random, bled from the brachial vein for MG serology, and swabbed from choanal cleft for MG culture and PCR. At 4 weeks of age, one hundred and twenty birds were equally allocated into 4 groups, identified by a bird number from 1 to 30 in each group, and raised in separate, isolated rooms as follows. Group 1 served as a negative control, groups 2, 3, and 4 were given a commercially killed MG vaccine containing 1 x 10^9 CFU/bird, by subcutaneous injection, a live MG strain F vaccine containing 1 x 10^9 CFU/bird via a nasal drop, and MG strain S6 (ATCC 15302) organisms containing 1 x 10^7 CFU/bird via a nasal drop, respectively. When 5, 6, 7, 8, 9, and 11 weeks old or 1, 2, 3, 4, 5, and 7 weeks post vaccination or inoculation, the birds numbered 1 - 10 were bled for SPA and ELISA, and 1 - 30 swabbed (3 birds pooled as 1 sample) for MG isolation and culture, as well as for PCR.

MG serology.

Serum plate agglutination (SPA). Fresh sera were tested against MG antigen (Nobilis®, Intervet

International B.V., Holland), following the manufacturer's instructions. Briefly, thirty µl of serum were mixed with thirty µl of antigen and then incubated at room temperature for 1-2 min before the result was read. Negative and positive sera were included in each test.

Enzyme linked immunosorbent assay (ELISA): Sera were tested with 2 commercial test kits, ProFLOK® (Synbiotics Corporation, USA) and FlockChek® (IDEXX Laboratories, Inc., USA), following the manufacturers' directions. Briefly, diluted sera were added onto a MG antigen-coated plate, incubated, washed, and peroxidase-labeled, anti-chicken antibody (conjugated antibody) was added. After incubation, the plate was again washed before adding a substrate, and adding the stop solution. The plate was read in a ELISA reader, manufactured by Labsystems Multiskan MS Type 352, Finland. The optical density of the negative, positive controls, and samples were calculated and interpreted according to the manufacturers' recommendations. For the interpretation of ProFLOK® ELISA, titre levels 0 - 148, 149 - 743, and equal or higher than 744 were negative, suspicious, or positive reactors, respectively. For the interpretation of FlockChek® ELISA, titre levels 0 - 1075, and levels equal or higher than 1076 were negative, or positive, respectively.

MG detection.

Culture and isolation: The procedure was described by Ley et al. (1997). Cotton swabs were transferred into 4 ml of Frey's medium, containing 15% swine serum (FMS) broth and then divided equally into 2 parts, for culture and isolation, and for the polymerase chain reaction (PCR) test. The broth was incubated at 37° C with humidity, and observed until the color changed from pink to orange-yellow. One week after incubation, the unchanged cultured broth was passaged to a new FMS broth, followed by one more passage if the color was unchanged. For the changed cultured broth, the broth was streaked onto FMS agar containing 15% swine serum, then incubated at 37°C with humidity, and observed for colonies using an inverted microscope, for at least 2 weeks. The mycoplasma colonies were confirmed as MG by a direct immunofluorescence test (Kleven, 1998). For the direct immunofluorescence test, mycoplasma colonies on the agar plate were given MG fluorescein-conjugated rabbit antiserum (provided by S.H. Kleven, Department of Avian Medicine, University of Georgia, USA) and then incubated at 37°C, while being observed under an ultraviolet microscope.

Polymerase chain reaction (PCR) : The broth samples numbered 1 - 3 (from bird numbers 1 - 9, 3 birds pooled as 1 sample) were investigated in this study. This method was described by Lauerman (1998). The broth was extracted for the DNA template by centrifugation at 15,000 xg, washing with distilled water, following by diluting the pellet with distilled water, boiling for 10 min, keeping at -20°C for 10 min, centrifugation, and collecting the supernatant at -20°C until use. The PCR mixture, in a 50 µl volume, contained KCl 500 mM, Tris-HCl (pH 8.3) 100 mM, dNTP (Fermentas) 1 mM, primer F (5'GAGCTAATCTGTAAAGTTGG TC3') and primer R (5'GCTTCCTTGCGGTTA GCAAC3') (Qiagen) 10 pmole each, Taq polymerase (Fermentas) 1.25 U and the DNA template, 5 µl (250 ng). The MG strain S6 (ATCC 15302), was used as a positive control. PCR mixtures were amplified in a DNA thermal cycler (PCR Sprint, Thermo Electron Corporation, Milford, MA) at 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec over 40 cycles, followed by maintaining 72°C for 5 min. The PCR product was analyzed in 2% agarose gel (Pharmacia Biotech AB, Uppsala, Sweden), stained with ethidium bromide, visualized by an UV transilluminator, and photographed.

Results

This study showed that antibodies to SPA were not found in 1 and 28 day old birds, and 5 to 6 week old birds. The positive SPA reactors were observed at 7, 8, 9, and 11 weeks old, in groups 3 and 4, and the number of positive SPA reactors ranged from 2 - 7 birds in each 10 birds sampled. (Table 1).

For ProFlok® ELISA, positive reactors were found in 20 birds out of 30 birds when 1 day old (data not shown). However, no positive reactors were observed at 4, 5 and 6 weeks of age. At 7, 8, 9 and 11 weeks of age in groups 3 and 4, birds that were detected as positive and suspicious reactors, ranged from 0 - 8 and 2 - 10 out of 10, respectively. Group 2 showed positive reactors when 5 and 11 weeks old, ranging from 0 - 3 out of 10. ProFlok® ELISA results at age 4 - 11 weeks are summarized in Table 2.

For IDEXX® ELISA, positive reactors were detected in 24 birds out of 30 birds when 1 day old (not shown in Table 3). No positive reactors were observed at 4, 5 and 6 weeks of age. The number of positive reactors ranged from 1 - 10 out of 10, at 7, 8, 9 and 11 weeks of age, in groups 3 and 4. However, The IDEXX® ELISA test on birds in group 2 showed negative reactors when 5 - 11 weeks old (Table 3).

Choanal cleft cultures for mycoplasma did not show any growth on FMS agar when birds were 1, 21 and 28 days old, nor at 5 - 11 weeks old, in groups 1 and 2. When 5 - 11 weeks old, the numbers of positive MG samples in groups 3 and 4 were 2 - 6 and 5 - 8 out of 10 samples, respectively (Table 4).

Table 1 SPA results of serum from various chicken groups at different ages (weeks old) (numbers of positive samples/ total tested samples).

Group	Age (weeks old)						
	4	5	6	7	8	9	11
1	0/30	0/10	0/10	0/10	0/10	0/10	0/10
2		0/10	0/10	0/10	0/10	0/10	0/10
3		0/10	0/10	2/10	5/10	7/10	3/10
4		0/10	0/10	3/10	4/10	7/10	5/10

Table 2 ELISA (ProFlok®) results on serum from various chicken groups at different ages (weeks old) (numbers of positive samples/total tested samples).

Group	Age (weeks old)						
	4	5	6	7	8	9	11
1	0/30	0/10	0/10	0/10	0/10	0/10	0/10
2		0/10	0/10	0/10	0/10	3/10	2/103/8*
3		0/10	0/104/10*	0/109/10*	0/1010/10*	5/105/5*	8/102/2*
4		0/10	0/104/10*	1/107/9*	7/103/3*	5/104/5*	1/108/9*

* suspicious reactor samples/total samples

Table 3 ELISA (IDEXX®) results on serum from various chicken groups at different ages (weeks old) (numbers of positive samples/total tested samples).

Group	Age (weeks old)						
	4	5	6	7	8	9	11
1	0/30	0/10	0/10	0/10	0/10	0/10	0/10
2		0/10	0/10	0/10	0/10	0/10	0/10
3		0/10	0/10	1/10	4/10	10/10	10/10
4		0/10	0/10	1/10	7/10	9/10	10/10

Table 4 Detection of MG by culture and isolation procedures (numbers of positive samples/total cultured samples).

Group	Age (weeks old)						
	4	5	6	7	8	9	11
1	0/10	0/10	0/10	0/10	0/10	0/10	0/10
2		0/10	0/10	0/10	0/10	0/10	0/10
3		2/10	5/10	6/10	5/10	4/10	4/10
4		5/10	8/10	8/10	8/10	6/10	5/10

Table 5 Detection of MG by the PCR procedure (numbers of positive samples/total cultured samples).

Group	Age (weeks old)						
	4	5	6	7	8	9	11
1	0/10	0/3	0/3	0/3	0/3	0/3	0/3
2		0/3	0/3	0/3	0/3	0/3	0/3
3		1/3	3/3	2/3	2/3	0/3	0/3
4		0/3	3/3	3/3	3/3	1/3	0/3

Choanal cleft swabs, sampled at 1, 21 and 28 days old, and all samples from groups 1 and 2 could not detect MG DNA. MG DNA was observed in group 3 when 5, 6, 7 and 8 weeks old, whereas MG DNA was observed in group 4 when 6, 7, 8 and 9 weeks old. The number of positive MG DNA samples in group 4 were more than in group 3. In addition, 6, 7 and 8 weeks old birds detected more MG DNA than other ages (Table 5).

Discussion

MG diagnosis can be carried out by various techniques using necropsy to observe gross and microscopic lesions, serology to determine the immune response, including SPA and ELISA tests, and MG detection to find either the organisms or their DNA , using culture and isolation and PCR procedures (Ley, 2003). The results of this study showed that the SPA test could detect positive reactors only in live vaccinated and inoculated groups

when 7, 8, 9 and 11 weeks old or 3, 4, 5 and 7 weeks post vaccination or inoculation. The SPA test can be used as a screening test for MG infection and generally shows a positive reactor about 7 - 10 days post vaccination or after inoculation, because SPA detects immunoglobulin (Ig) M, which is the first immunoglobulin to be formed after infection (Kleven, 1975; Kleven, 1998). These differences may be due to impairment of the birds' immune status, causing a late immune response or perhaps a loss of antigenicity of the MG antigen, caused by long term storage. However, positive and negative sera were included as positive and negative controls in each test, so antigenicity should not be a problem.

ELISA test kits, ProFlok® and IDEXX®, are presently used for flock monitoring and serodiagnosis (Ley, 2003). Due to differences in the test kits, interpretation may also be different, including the cut off points of the tests, as positive, suspicious and negative is used for ProFlok®, and only positive and negative for IDEXX®. Both commercial test kits showed similar results for the numbers of positive reactors in day old birds, which was an indication of maternal derived antibody. Maternal derived antibody could not be detected at 4 weeks of age and no evidence of MG DNA was found in these birds. This suggests that these birds were free of MG infection prior to vaccination and/or inoculation. The different numbers of positive reactors when using both test kits were observed in groups 3 and 4, 3 weeks after vaccination or inoculation. IDEXX® detected more positive reactors than ProFlok®, possibly caused by differences in the cut off point. Positive reactors were detected 5 weeks post vaccination and 3 weeks post inoculation, which were similar to the results of Kleven (1998) who showed that infected birds tested positive no earlier than 3 weeks after infection. However, the inoculated birds showed positivereactors 2 weeks earlier than the vaccinated birds, possibly because the MG S6 inoculum originated from a pathogenic isolate (Zander, 1961). Interestingly, the numbers of positive reactors in the birds vaccinated with live vaccine dramatically increased after 5 weeks, the opposite to that of the inoculated birds. A possible reason was that the MG organisms of the F strain vaccine are maintained in the upper respiratory tract over the life time of the birds (Kleven, 1981); therefore, as antigens were released they were immunized many times. Unfortunately, this study was terminated 7 weeks post inoculum, and consequent results from vaccinated and inoculated birds were not determined. The birds given killed vaccine apparently gave later immune responses compared to live vaccinated ones. The late immune responses of killed vaccinated birds in this study were similar to the results of Avakian et al. (1988). Avakian et al. (1988) suggested that the release of IgG from immunization with killed vaccine, may take longer than 3 weeks.

Due to the lack of differences in colony morphology of mycoplasma species, immunofluorescence has been used for the species identification of avian mycoplasmas (Kleven, 1998). Isolation and/or identification of the organisms are the gold standard for MG diagnosis but this procedure is time consuming, laborious and some field isolates take longer and require a few serial passages (Ley, 2003). In addition, detection of MG using PCR has been proven as a rapid, sensitive, and specific diagnosis (Nascimento et al., 1991). In this study, only in groups 3 and 4 could be MG detected by culture, isolation, and PCR procedures, but the culture and isolation procedure took 2 weeks longer than the PCR procedure. The latter might be explained because of low numbers of MG organisms in the cultured broth, no multiplication growth or an incubation failure of the cultured broth for the PCR procedure. Regarding detection sensitivity, at least 100 or 250 colony forming units/ml of MG, allowed detection by

the PCR procedure (Slavik et al., 1993; Mardassi et al., 2005). Salisch et al. (1998) concluded in their study that a commercial PCR-based test kit for the detection of MG is specific and at least as sensitive as culture because of the few samples detected by culture.

Conclusions

Our results showed that chickens receiving live MG vaccine or MG organisms produced immune responses, which could be detected by serological procedures including SPA, and ELISA. SPA and ELISA could detect positive reactors only from the groups receiving live MG vaccine or MG organisms. However, the group given killed vaccine showed positive reactors using the ELISA test. Culture, isolation and PCR confirmed the seropositive results in the killed vaccine group. The PCR procedure was rapid, specific and at least as sensitive as the culture and isolation procedures, which takes a much longer time for mycoplasma growth.

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