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Efficacy and Safety of Different Live Mycoplasma gallisepticum Vaccines in Layer Chickens

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Original Article

Efficacy and Safety of Different Live *Mycoplasma gallisepticum* Vaccines in Layer Chickens

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Abstract

The efficacy and safety of different live *Mycoplasma gallisepticum* (MG) vaccines were determined. One hundred and fifty six-week-old female layer chickens were equally divided into 6 groups. Groups 1-6 received F (two groups), 6/85 and ts-11 strains, positive and negative control, respectively. At 8 weeks old, 5 birds/group were sacrificed and gross thoracic airsac lesion scores (GTALS) and microscopic tracheal lesion scores (MTLS) were blindly evaluated. At 12 weeks old, groups 1-5 were challenged through an eye dropping with approximately $1x10^6$ CFU of MG Thai isolated. At 13 and 14 weeks old, 10 birds/group were swabbed in the palatine fissure to isolate MG and further differentiate between the vaccine and MG Thai isolated by PCR and random amplified polymorphic DNA assays, respectively. At 14 and 16 weeks old, 10 birds/group were sacrificed and the degree of protection was evaluated by GTALS and MTLS. The results revealed that feed conversion rate during 6-12 and 12-14 weeks old ranged between 4.33-7.53 and 5.88-15.18, respectively. At 12 and 14 weeks old, the vaccinated groups had antibody determined by serum plate agglutination (SPA), only groups 1 and 2 being detected by ELISA. Only SPA could detect antibody response in group 5 at 14 weeks old. GTALS and MTLS at 8, 14 and 16 weeks old ranged from 0 and 0.05-1.80, 0-0.3 and 0.13-1.38, and 0.15-1.65 and 0.38-2.28, respectively. At 12 weeks old, the MG DNA of the vaccine strains was detected in groups 1, 2 and 4, whereas MG DNA of MG Thai isolated was detected in groups 3, 4 and 5 at 13 weeks old and in groups 2, 3, 4 and 5 at 14 weeks old. The F strain had good results in safety, growth performance, protection and re-isolation of the F strain from vaccinated birds followed by ts-11 and 6/85 strains, respectively.

Keywords: efficacy, layer chickens, live vaccines, *Mycoplasma gallisepticum*, protection, safety

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บทคัดย่อ

ประสิทธิภาพและความปลอดภัยของวัคซีนเชื้อเป็น *มัยโคพลาสมา กัลลิเซพติกุม* (เอ็มจี) ชนิดต่างๆ ในไก่ไข่

สมศักดิ์ ภัคภิญโญ [1] สุวรักษ์ วรรณรัตน์ [2] เกรียงไกร แสงทองแดง [3] มาร์ซีโล พาเนียโก [4]*

ประสิทธิภาพและความปลอดภัยของวัคซีนเชื้อเป็น *มัยโคพลาสมา กัลลิเซพติกุม* (เอ็มจี) ชนิดต่าง ๆ ถูกนำมาศึกษาในไก่ไข่เพศเมีย อายุ 6 สัปดาห์ จำนวน 150 ตัว ซึ่งถูกแบ่งเป็น 6 กลุ่มเท่ากันดังนี้ กลุ่มที่ 1-6 คือ วัคซีนเสตรนเอฟ (2 กลุ่ม) 6/85 และ ts-11 กลุ่มควบคุมบวกและลบ ตามลำดับ พออายุ 8 สัปดาห์ ไก่กลุ่มละ 5 ตัวถูกนำมาผ่าซากเพื่อประเมินรอยโรคถุงลมช่องอก (จีทีเอแอลเอส) ทางมหพยาธิวิทยาและรอยโรคท่อลม (เอ็มทีแอลเอส) ทางจุลพยาธิวิทยา โดยที่ผู้ประเมินไม่ทราบกลุ่มมาก่อน พออายุ 12 สัปดาห์ กลุ่มที่ 1-5 ได้รับเชื้อพิษทับเอ็มจีที่แยกได้ในประเทศไทยตัวละประมาณ $1x10^6$ ซีเอฟยูด้วยการหยอดตา อายุ 13 และ 14 สัปดาห์ ไก่กลุ่มละ 10 ตัวถูกป้ายเชื้อที่ร่องเพดานปากด้านบนเพื่อแยกเชื้อเอ็มจี และแยกต่อไปว่าเป็นเชื้อจากวัคซีนหรือจากเชื้อพิษทับ ด้วยวิธีพีซีอาร์และแรนดอมแอมพลิฟายด์โพลีมอร์ฟิคดีเอ็นเอ พออายุ 14 และ 16 สัปดาห์ ไก่กลุ่มละ 10 ตัวถูกนำมาผ่าซากเพื่อศึกษาการป้องกันโรคโดยประเมินผลจากจีทีเอแอลเอสและเอ็มทีแอลเอสดังวิธีที่กล่าวมาข้างต้น จากการศึกษาพบว่า อัตราการแลกเนื้อระหว่าง 6-12 และ 12-14 สัปดาห์อยู่ระหว่าง 4.33-7.53 และ 5.88-15.18 ตามลำดับ เมื่ออายุ 12 และ 14 สัปดาห์ กลุ่มได้รับวัคซีนพบแอนติบอดีด้วยวิธีซีรัมเพลทแอกกลูติเนชัน (เอสพีเอ) และเฉพาะกลุ่มที่ 1 และ 2 พบแอนติบอดีด้วยวิธีอีไลซา กลุ่มที่ 5 พบแอนติบอดีเฉพาะวิธีเอสพีเอที่อายุ 14 สัปดาห์ ในไก่อายุ 8 14 และ 16 สัปดาห์ พบจีทีเอแอลเอสและเอ็มทีแอลเอส ระหว่าง 0 และ 0.05-1.80, 0-0.3 และ 0.13-1.38, และ0.15-1.65 และ 0.38-2.28 ตามลำดับ เมื่ออายุ 12 สัปดาห์ กลุ่มที่ 1 2 และ 4 พบดีเอ็นเอของเอ็มจีเสตรนวัคซีน ขณะที่พบดีเอ็นเอของเอ็มจีเชื้อพิษทับที่อายุ 13 สัปดาห์ ในกลุ่มที่ 3 4 และ 5 และที่อายุ 14 สัปดาห์ ในกลุ่มที่ 2 3 4 และ 5 วัคซีนเชื้อเป็นเอ็มจีเสตรนเอฟให้ผลที่ดีในเรื่องความปลอดภัย คุณลักษณะการเจริญเติบโต การป้องกันโรค และการแยกเชื้อจากไก่ที่ได้รับวัคซีน ตามมาด้วยเสตรน ts-11 และ 6/85 ตามลำดับ

คำสำคัญ: ประสิทธิภาพ ไก่ไข่ วัคซีนเชื้อเป็น *มัยโคพลาสมา กัลลิเซพติกุม* การป้องกันโรค ความปลอดภัย

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Introduction

Mycoplasma gallisepticum infections are commonly referred to as chronic respiratory disease (CRD) of chickens (Ley, 2008). MG disease can be transmitted by horizontal and/or vertical routes. Affected birds show respiratory signs including rales, sneeze, conjunctivitis, nasal and ocular discharges and even death (Pakpinyo and Sasipreeyajan, 2007). MG infection also has adverse effects on feed conversion rate, weight gain and egg production (Ley, 2008). Airsacculitis caused by MG infections results in an increase in condemnations at the processing plants. Various diagnostic methods such as serum plate agglutination, ELISA, microbial culture and polymerase chain reaction (PCR) have been used to identify the infection (Kleven, 1998; Lauerman, 1998).

Prevention and control are important procedures to manage MG infections. One appropriate way of controlling and reducing MG infections in areas that cannot depopulate flocks is vaccination (Ferguson-Noel et al., 2012). There are several types of vaccine including live, killed and a recombinant fowl pox-MG vaccine (Ley, 2008). Commercial live vaccines are 6/85, ts-11 and F strains (Ferguson-Noel et al., 2012). MG live vaccines have been investigated in different aspects including serologic response, egg production, blood characteristics and transmissibility (Ley et al., 1997; Peebles et al., 2009; Leigh et al., 2010; Purswell et al., 2011). However, the protection level of MG live vaccines depends on the virulence, infectivity and invasiveness of the strain of vaccine reviewed by Ferguson-Noel et al (2012). The 6/85 strain of vaccine, which originated in the United States, is of low virulence in chickens and turkeys, is rarely transmissible from bird to bird and is able to protect against challenge of virulent MG (Ley et al., 1997). The ts-11 strain, originating in Australia and selected by its temperature sensitivity, grows well at 33^0C, but poorly at 39.5^0C, and is therefore localized in the upper respiratory tract and stimulates long-term immune response. It shows low virulence in chickens and turkeys and a low transmissible rate from bird to bird, but it induces detectable systemic antibody response (Whithear, 1996).

The F strain of MG vaccine originated in the United States and is more virulent compared with

6/85 and ts-11 strains (Whithear, 1996), persists for a long time in the upper respiratory tract in chickens, is able to displace infection from the MG challenge strain (Kleven, 1998) and can decrease egg production loss in MG infected flocks.

Poultry industry in Thailand has been concerned with the prevention and control of MG infection in chickens especially in breeder flocks. In this type of bird, antimicrobials have been used to prevent and control economic loss associated with this disease (Pakpinyo and Sasipreeyajan, 2007). Nevertheless, the use of antimicrobials is expensive and has to be repeated frequently and, most importantly, the possibility of drug-resistance is of real concern for the industry. For egg producers, residues of drugs in eggs can raise food safety issues. Therefore, vaccination is one of the effective prevention and control procedures in combination with appropriate biosecurity in breeder and layer farms.

Nowadays, live and inactivated oil emulsion vaccines are widely used in commercial layers, broiler breeders and layer breeders. Recently, a new commercial F strain of MG vaccine was introduced in Thailand (Ceva Animal Health (Thailand) Ltd). The objective of this study was to compare the safety and efficacy of the two commercially available F strain-, 6/85 and ts-11 strains of MG vaccine based on the efficacy, serological immune response, histopathologic tracheal lesion scores, gross thoracic airsac lesion scores and recovery of the MG DNA between vaccine and challenge strains.

Materials and Methods

Chickens: One hundred and fifty, one-day-old commercial female layer chickens, Isa Brown breed, free of *Mycoplasma gallisepticum* and *M. synoviae* (MS), were obtained from commercial layer breeders. At 21 days of age, all pullets were treated with Tilmicosin (Plumotil®, Elanco Animal Health) 100 mg/l of drinking water for 5 days. At 6 weeks of age, swab samples were taken from the palatine fissure of thirty randomly selected pullets to determine whether they were MG or MS negatives by MG and MS polymerase chain reaction (PCR). All pullets were raised in a wired cage in isolated rooms and provided with feed and water *ad libitum*. All handling procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Chulalongkorn University, Thailand number 1031064.

***Live vaccines*:** MG vaccines commercially available in Thailand were obtained from local distributors. Cevac® MG-F (Ceva Santé Animale, France), F Vax MG® (MSD Animal Health, USA), Nobilis® MG6/85 (MSD Animal Health, USA) and Vaxsafe® MG ts-11 (Bioproperties, Australia) were determined in this study.

***Media*:** Frey's broth medium (GIBCO Diagnostics, Madison, Wisconsin) (Kleven, 1998) was used in this study. The sterile broth was supplemented with 15% swine serum, dextrose, cysteine, nicotinamide adenine dinucleotide, penicillin, thallium acetate and phenol red and referred to as Frey's broth medium supplemented with swine serum (FMS). Agar medium was prepared by addition of 1% agar medium (Noble agar, Difco® Becton Dickinson, MD, USA) to the FMS with the exception of phenol red.

***MG challenge organism*:** The MG challenge organism used in this study was isolated by culling broiler breeder hens in the central part of Thailand in 2003 (AHRU 54/46 of Dr. Somsak Pakpinyo, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand). The affected broiler breeder hens aged 50 weeks old showed respiratory signs including rales, sneeze, conjunctivitis and ocular discharge and were submitted to Faculty of Veterinary Science, Chulalongkorn University, Thailand for diagnosis. Airsacculitis at the thoracic airsacs was observed, swabbed and placed in FMS broth. MG was isolated and identified by direct immunofluorescent assay (Kleven, 1998) using fluorescein-conjugated rabbit antiserum provided by S.H. Kleven (Department of Avian Medicine, University of Georgia, Athens, GA) and PCR assay (Lauerman, 1998). The MG isolate, named as AHRU 54/46, was propagated, aliquoted and stored at -70^{0}C to be used in MG experimental studies.

Preparation of MG DNA for PCR and randomly amplified polymorphic DNA (RAPD): The preparation of MG DNA was done using previously described protocol (Fan et al., 1995) with some adaptations. Briefly, 2 ml of FMS broth swabbed from palatine fissure were incubated at 37^{0}C for 2 hours. After incubation, the cultured broth was equally divided into 2 1.5-ml-tubes; the first tube was determined for PCR assay and the remaining tube was stored at -70^{0}C until used. The cultured broth of the first tube was centrifuged to collect the pellet, washed twice with phosphate-buffered saline (PBS) and suspended with 25 µl of PBS. The pellet was then boiled for 10 min, placed on ice for 5 min and centrifuged. The supernatant containing the MG DNA was collected and stored at 4^{0}C for further PCR assay. For RAPD assay, only positive sample by PCR assay was further process as follows. The cultured broth of the second tube stored at -70^{o}C was thawed and 200 µl was transferred into the 1.8 ml of sterile FMS broth then incubated at 37^{0}C until the MG cultured broth changed from pink to orange-yellow in color. All 2 ml of MG isolated broth culture was used to prepare as MG DNA as previously described.

PCR assay: Amplified reaction was performed in a 25 µl volume using modified described protocol (Lauerman, 1998), each PCR mixture consisted of 2.5 mM $MgCl_2$, 1 mMdNTP (Fermentas, USA), 10 pmole primer MG 13F (5′ GAGCTAATCTGTAAAGTTG GTC 3′), 10 pmole primer MG 14R (5′ GCTTCCTTGCGGTTAGCAAC 3′) for MG PCR or10 pmole primer MSL-1 (5'-GAAGCAAAATAGTGAT ATCA-3') and 10 pmole primer MSL-2 (5'-GTCGTCTCCGAAGTTAACAA-3') for MS PCR (Qiagen, Germany), 1.25 U of Taq polymerase (Promega, USA), and 2.5 µl of MG DNA. Each reaction was performed concurrently with the S6 strain (ATCC 15302) as a positive control and distilled

water as a negative control. The amplification conditions were 94^{0}C for 30 sec, 55^{0}C for 30 sec, and 72^{0}C for 1 min for 40 cycles. The final extension cycles were 72^{0}C for 5 min. The amplification product was 185 base pairs (bps).

RAPD assay: The primer set for RAPD analyses was modified from Ley et al. (1997). Briefly, Geary primer set (Geary et al., 1994) was performed in a 50 µl volume and each RAPD mixture consisted of 2 mM $MgCl_2$, 1 mM dNTP (Fermentas, USA), 500 ng primer 1254 (5′ CCGCAGCCAA 3′) (Qiagen, Germany), 2.5 U of Taq polymerase (Promega, USA) and 1 µl of MG DNA containing approximately 50 to 100 ng DNA. The amplification conditions were performed starting with four cycles of 94^{0}C for 5 min, 36^{0}C for 5 min, and 72^{0}C for 5 min, ending with 30 cycles of 94^{0}C for 1 min, 36^{0}C for 1 min, and 72^{0}C for 1 min and finally a cycle of 72^{0}C for 10 min. The PCR banding pattern was analyzed by agarose gel electrophoresis.

Gel electrophoresis: A volume of 10 µl of the amplified DNA products was loaded into 2 % agarose (Pharmacia Biotech AB, Uppsala, Sweden) gels, and separated by agarose gel electrophoresis. The gel was stained with 0.5 µg/ml ethidium bromide and analyzed by a gel documentation system (Vilber Lourmat, France).

Serum plate agglutination (SPA): Fresh sera were tested against 3 commercial MG antigens (Flockscreen™ *Mycoplasma gallisepticum* Stained Antigen Kit, X-Ovo Ltd, UK; Nobilis® MG Antigen, MSD Animal Health, USA; MG RPA-Test, Soleil Sarl, France), following the manufacturers' 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 determined in each test.

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

Airsac lesion score: Left and right thoracic airsac lesion scores were grossly evaluated according to the following criteria (Kleven et al., 1972): 0 : No airsac lesion was observed, 1 : Slight cloudiness of the air sac membrane were found, 2 : Air sac membrane was slightly thick and usually presented small accumulations of cheesy exudates, 3 : Airsac membrane was obviously thick and meaty in consistency, with large accumulations of cheesy exudates in one air sac, 4 : Lesions were observed the same as 3, but 2 or more air sacs were found. The average of the left and right thoracic air sac lesion scores was recorded.

Tracheal lesion score: Trachea of each chicken was collected and divided into 4 parts; the proximal, 2 middle and the distal part. Each part of the trachea was microscopically evaluated for its lesion score according to the following criteria (Yagihashi and Tajima, 1986): 0 : No significant changes were observed, 1 : Small aggregate of cells (mainly lymphocytes) was found, 2 : Moderate thickening of the wall due to cell infiltration, and edema commonly accompanied with epithelial degeneration and exudation was present, 3 : Extensive thickening of the wall due to cell infiltration with or without exudation was determined. The average of each part of tracheal lesion score was recorded.

Statistical analysis: Evaluation of lesion scores was analyzed statistically using nonparametric Kruskal-Wallis test and Mann Whitney U test. Data were analyzed using SPSS Statistics 17.0. Statistical significance was determined as $p < 0.05$.

Experimental designs: At 6 weeks of age, all birds were weighed and divided depending on their average weight into 6 groups, 25 birds in each group, raised separately in isolated rooms and designed as follows. Groups 1 to 4 were vaccinated with one full dose by the eye drop route with the following vaccines; Group 1: F strain (Cevac® MG F), Group 2: F strain (F Vax® MG), Group 3: 6/85 strain (Nobilis®) and Group 4: ts-11 strain (Vaxsafe®). Groups 5 and 6 did not receive any vaccine and served as positive and negative controls, respectively. After vaccination, post vaccination reactions including ocular discharge, conjunctivitis and sneeze were observed daily in all groups for two weeks. The feed consumption (Kg) and body weight gain (Kg) were recorded during a 6–12 weeks period to determine feed conversion rate (FCR). Twenty blood samples from each group were taken from the pullets at 6, 12 and 14 weeks of age. All sera were collected and properly identified. Fresh sera were used to detect antibodies against MG using serum plate agglutination (SPA). Subsequently, the serum samples were frozen to be further tested at once for MG antibodies by ELISA. At 8 weeks of age (2 weeks post vaccination), 5 pullets from each group were sacrificed and the thoracic air sac and tracheal lesion scores were blindly evaluated as previously described. At 12 weeks of age (6 weeks post vaccination), all pullets of groups 1 to 5 were challenged via the right eye dropping with 50 µl of MG Thai isolated AHRU 54/46 consisting of approximately 1 x 10^6 colony forming unit (CFU). All pullets of group 6 were inoculated with 50 µl of the Frey's broth medium through the right eye dropping route. At 13 and 14 weeks of age (1 and 2 weeks post challenge), 10 swab samples from each group were taken from the palatine fissure. These swabs were used to evaluate the recovery of the MG DNA and differentiate between vaccine strains and challenge isolate by PCR and RAPD assays, respectively. At 14 and 16 weeks of age, ten pullets per group were sacrificed, and the protection was evaluated by

scoring the gross thoracic airsac lesions and microscopic tracheal lesions according to the score system as previously described.

Results

During the assessment of the post vaccination reactions, one bird from groups 2 and 4 showed a very mild degree of respiratory signs including mild serous ocular discharge for 1-2 weeks post vaccination but this disappeared within a week. The feed consumption, body weight gain and FCR during 6-12 weeks old ranged between 43.8 and 53.4, 5.82 and 10.32 and 4.33 and 7.53, respectively (Table 1). After challenge, severe clinical signs including ocular discharge, conjunctivitis and sneeze were observed in 2 birds from group 6, whereas 1 or 2 birds from groups 1, 2 and 4 showed mild degrees of clinical signs. The feed consumption, body weight gain and FCR during 12-14 weeks old ranged from 28.9-33.1, 2.18-5.49 and 5.88-15.18, respectively. The highest body weight gain and lowest FCR was observed in group 6, whereas group 5 had the lowest body weight gain and highest FCR (Table 1).

Serology was determined by SPA test and ELISA. At 6 weeks old (vaccination day), all serological results were negative against MG by SPA test and ELISA and for MS by ELISA.

At 12 weeks old, 6 weeks after vaccination and immediately before challenge, groups 1, 2, 3 and 4 showed 100%, 100%, 15% and 95% of positive results by SPA test for the three different antigens used in this experiment, respectively. Groups 5 and 6, which did not receive any MG live vaccine, remained negative during this period. With reference to MG ELISA test results, groups 1 and 2 showed 33% and 40% of positivity, respectively, while groups 3 and 4 were completely negative (Table 2).

Table 1 Feed consumption (kg), body weight gain (kg) and feed conversion rate (FCR) during 6-12 weeks old and 12-14 weeks old (2 weeks post challenge) of each group

Group	6-12 weeks old			12-14 weeks old		
	Feed	BW gain	FCR	Feed	BW gain	FCR
1	50	8.25[a]	6.06	30	4.17	7.19
2	53.4	9.74	5.48	32.1	4.49	7.15
3	47	7.28	6.46	31.1	4.94	6.3
4	43.8	5.82[b]	7.53	28.9	4.84	5.97
5	44.7	10.32	4.33	33.1	2.18	15.18
6	48.1	7.62	6.31	32.3	5.49	5.88

Group 1: F strain (Cevac® MG F), Group 2 : F strain (F Vax® MG), Group 3 : 6/85 strain, Group 4 : ts-11 strain, Group 5 : positive control and Group 6 : negative control.
[a] One bird was culled at 10 weeks old due to cannibalism.
[b] Two and one bird were culled at 7 and 8 weeks old, respectively, due to cannibalism.

At 14 weeks old, 2 weeks after challenge, only the vaccinated group showed positive against MG serology by SPA test, whereas only groups 1 and 2 showed a positive antibody response against MG serology by ELISA found. For the positive group, only the SPA test could detect antibody response at 14 weeks old. With the SPA test, the MG antigen of Nobilis® could detect the highest numbers of positive samples followed by Soleil Sarl and Flockscreen™, respectively (Table 2).

At 14 and 16 weeks old, 2 and 4 weeks after challenge, respectively, the SPA results from groups 1 and 2 showed very high rate of positivity with the exception of group 1 at 14 weeks assessed by antigen FlockScreen™ and group 2 which had 78% of positive result.

The blind gross thoracic airsac lesion scores showed that no lesions were observed at 8 weeks old (2 weeks after vaccination). At 14 weeks old (2 weeks after challenge), low scores without significant difference were observed. Finally, at 16 weeks old (4 weeks post challenge), the thoracic airsac lesion scores were observed to have significant differences ranging between 1.05-1.65 ($p < 0.05$); the highest thoracic airsac lesion score was found in group 5 followed by groups 1, 4, 2, 3 and 6, respectively (Table 3).

The blind histopathological tracheal lesion scores of 8 weeks old ranged between 0.05-1.80 with significant difference ($p < 0.05$). At 14 and 16 weeks old, the lesion score ranged between 0.13–1.38 and 0.38-2.28, respectively ($p < 0.05$). The highest lesion scores were observed in group 5 at 14 and 16 weeks old (Table 4).

Table 2 Serology at 12, 14 and 16 weeks old (4 weeks post challenge) (Numbers of positive samples/total tested samples)

Group	12 wks old				14 wks old				16 wks			
	F	N	S	ELISA	F	N	S	ELISA	F	N	S	ELISA
1	18/18	18/18	18/18	6/18*	13/18	18/18	18/18	3/18*	10/10	10/10	10/10	2/10*
2	20/20	20/20	20/20	8/20*	20/20	20/20	17/20	11/20*	10/10	10/10	10/10	6/10*
3	3/20	3/20	3/20	0/20	2/20	14/20	10/20	0/20	3/10	9/10	3/10	0/10
4	19/20	19/20	19/20	0/20*	10/20	17/20	14/20	0/20*	5/10	8/10	5/10	0/10*
5	0/20	0/20	0/20	0/20	4/20	10/20	7/20	0/20*	5/10	6/10	5/10	0/10*
6	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/10	0/10	0/10	0/10

Group 1: F strain (Cevac® MG F), Group 2 : F strain (F Vax® MG), Group 3 : 6/85 strain, Group 4 : ts-11 strain, Group 5 : positive control and Group 6 : negative control, F : Flockscreen™, N : Nobilis®, S : Soleil Sarl
* Remaining samples were classified as suspected group

Table 3 Grossly thoracic airsac lesion scores at 8, 14 and 16 weeks old (Mean±SD).

Group	8 wks old (n = 5)	14 wks old (n = 10)	16 wks old (n = 10)
1	0 ± 0	0.06 ± 0.18^{**,a}	1.05 ± 0.50^{b}
2	0 ± 0	0.10 ± 0.32^{a}	0.40 ± 0.46^{a,d}
3	0 ± 0	0.30 ± 0.48^{a}	0.40 ± 0.46^{a,d}
4	0 ± 0*	0 ± 0^{a}	0.75 ± 0.59^{b,d}
5	0 ± 0	0 ± 0^{a}	1.65 ± 0.53^{c}
6	0 ± 0	0.10 ± 0.32^{a}	0.15 ± 0.34^{a}

Group 1: F strain (Cevac® MG F), Group 2 : F strain (F Vax® MG), Group 3 : 6/85 strain, Group 4 : ts-11 strain, Group 5 : positive control and Group 6 : negative control.
* n = 2, ** n = 8, Different superscripts in the same column mean significantly different ($p < 0.05$)

Table 4 Histopathological tracheal lesion scores at 8, 14 and 16 weeks old (Mean±SD).

Group	8 wks old (n = 5)	14 wks old (n = 10)	16 wks old (n = 10)
1	1.40 ± 0.38^{a,c}	0.63 ± 0.47^{**,b}	1.33 ± 0.39^{b,d}
2	1.80 ± 0.11^{b}	0.90 ± 0.28^{b,c}	1.58 ± 0.49^{d}
3	1.08 ± 0.54^{a,d}	1.00 ± 0.29^{b,c}	1.13 ± 0.25^{b,d}
4	1.0 ± 0*^{a}	0.98 ± 0.25^{b,c}	0.93 ± 0.38^{b}
5	0.50 ± 0.71^{a,e}	1.38 ± 0.33^{c}	2.28 ± 0.28^{c}
6	0.05 ± 0.11^{e}	0.13 ± 0.27^{a}	0.38 ± 0.55^{a}

Group 1: F strain (Cevac® MG F), Group 2 : F strain (F Vax® MG), Group 3 : 6/85 strain, Group 4 : ts-11 strain, Group 5 : positive control and Group 6 : negative control.
* n = 2, ** n = 8, Different superscripts in the same column mean significantly different ($p < 0.05$)

Table 5 MG DNA of vaccine strains or challenged isolate at 12, 13 and 14 weeks old by PCR RAPD (Numbers of positive MG DNA/total samples)

Group	12 wks old (n = 10)	13 wks old (n = 10)	14 wks old (n = 10)
1	10/10*	10/10*	10/10*
2	10/10*	10/10*	8/10* (1/10)** (1/10)***
3	0/10	10/10**	9/10** (1/10)***
4	10/10*	7/10** (3/10)***	4/10** (6/10)***
5	0/10	10/10**	10/10**
6	0/10	0/10	0/10

Group 1: F strain (Cevac® MG F), Group 2 : F strain (F Vax® MG), Group 3 : 6/85 strain, Group 4 : ts-11 strain, Group 5 : positive control and Group 6 : negative control.
* : Numbers of positive MG DNA of vaccine strain/total samples
** : Numbers of positive MG DNA of challenged isolate/total samples
( )*** : Remaining samples could not be identified.

The PCR assay of all 30 birds (pooled as 10 samples) at 6 weeks old (prior to vaccination) were negative for MG and MS. At 12 weeks old, the MG DNA of the vaccine strains of groups 1, 2 and 4 could be detected in all birds. In group 3, no vaccine strain was detected in the sampled birds. At 13 weeks old, one week after challenge, only the MG DNA of the vaccine strain was detected in groups 1 and 2. In groups 3, 4 and 5, the MG DNA of the challenge isolate (AHRU 54/46) was detected. Finally, at 14 weeks old, two weeks after challenge, the MG DNA of the vaccine strain was detected in all sampled birds in group 1 and in 8 out of 10 birds from group 2. In groups 3 and 4, no MG DNA of the vaccine strain was detected (Table 5).

Discussion

This study used commercial layer chickens instead of specific pathogen free chickens because no experimental studies had determined the efficacy and safety of commercial live vaccine especially the F strain vaccines produced by different sources in layers. The results can be directly applied to layer chicken growers or other types of chicken growers.

The purpose of using live vaccines was that MG organisms of the live vaccines can grow in the bird to provide an appropriately long period of protective immunity but one that is not adequate to cause disease or be transmitted to other susceptible birds; that live vaccines should replace the MG field strain and respiratory immune responses including antibodies and cell-mediated immunity is important (Gaunson et al., 2006).

In this study, a mild degree of post vaccine respiratory reaction was observed and disappeared within a week. MG 6/85 and ts-11 strains are non-virulent compared with F strain. Nevertheless, the F strain used in group 1 seemed to be less virulent than the F strain applied to group 2. As for performance, the FCR data of the vaccinated groups were higher than those of the unvaccinated groups possibly because of heat stress. Simmons and Branton (1988) found that layer chickens inoculated with MG F strain had an increased rectal temperature than control layer chickens. Heat stress results in decreasing body weight gain and growth performance (Mashaly et al., 2004). Furthermore, the higher microscopic tracheal lesion scores of the vaccinated groups during 2 weeks post vaccination resulted in lower body weight gain; this finding accord with Grgíc et al. (2008).

For SPA test and ELISA test kits to detect the MG antibodies at 12 weeks of age, groups 1 (Cevac® MG F), 2 (F Vax® MG), 3 (6/85) and 4 (ts-11) showed positive results by SPA test for the three different antigens. With reference to MG ELISA test results, groups 1 and 2 showed positive results, while groups 3 and 4 were negative. These findings are in agreement with previously published data that show that the F strain could stimulate birds to elicit the circulatory antibody response greater than ts-11 and 6/85 did (Abd-El-Motelib et al., 1993; Ley et al., 1997; Levisohn and Kleven, 2000). However, according to Abd-El-Motelib and Kleven (1993), apparently there is no clear correlation between circulatory antibodies and protection. This suggests that the local antibody may be responsible for resisting and controlling MG infection (Gaunson et al., 2006).

At 14 and 16 weeks old, the numbers of antibody responses were apparently similar to those at 12 weeks old with the exception that group 3 had higher numbers of positive samples detected by all SPA tests of the MG challenged birds. Interestingly, ELISA detected the numbers of positive results as only 17-60% in groups 1 and 2 even though receiving F strain vaccination and/or MG challenge. In Thailand, many chicken production companies have

found similar results of low numbers of positive results detected by commercial ELISA test kits (personnel contact); therefore, the monitoring of vaccinated flocks with F strain has been delayed until at least 8 weeks post vaccination and/or SPA test has been used instead of ELISA even though SPA test is less specific than ELISA (Avakian et al., 1988; Kempf et al., 1994). The present study also compared the results of all three commercial MG antigens suggesting that Nobilis® was revealed to be more sensitive than Flockscreen™ and Soleil Sarl. Furthermore, distinctive agglutination has been observed in MG antigen of Nobilis®. Unfortunately, the Nobilis® MG antigen is not on the market anymore.

For the gross airsac lesion scores, at 16 weeks of age, remarkable lesion scores were observed in birds from group 5 (positive control). Based on criterion, group 5 had significantly the highest airsac lesion score followed by groups 1, 4, 2, 3 and 6, respectively. The results of this study were different from those previously described (Abd-El-Motelib et al., 1993), which revealed that chickens receiving F strain vaccine following MG aerosol challenge showed better protection against airsacculitis compared with those receiving ts-11 and 6/85 strains. The different results are possibly caused by the route of MG challenge, time of post challenge and/or vaccine preparations. In the present study, all MG challenged groups were inoculated by eye dropping, whereas the previous study was determined by aerosol route (Abd-El-Motelib et al., 1993). The aerosol route can induce more airsacculitis compared with the eye dropping route (Lin and Kleven, 1984). In addition, the time of post challenge of the previous study was determined every 30 days until 90 days while the present study was observed for only 4 weeks. The longer period for determination possibly induces the more airsac lesions in challenged birds. Both route of challenge and time of post challenge influence the severity of airsac lesion scores. Finally, the vaccine preparation of this study used the lyophilized F-strain instead of the fresh broth culture F-strain, which may be more effective in colonizing in the upper respiratory tract than the lyophilized F-strain.

The detection of the MG DNA after challenge showed that both F strains could be re-isolated. Contrastly, for the groups receiving 6/85 and ts-11 strains, MG DNA from the challenge isolate was detected. The present study suggests that the F strain succeed in preventing the colonization of the upper respiratory tract (palatine fissure). Published data also indicate that the F strain vaccine can reduce, protect and displace the MG challenge strain in the upper respiratory tract (Kleven et al., 1998). Furthermore, there has been a report that the F strain can colonize at the upper respiratory tract of chickens for long life. However, 6/85 and ts-11 strains could not be re-isolated from the birds after challenge, but the lesion scores of the thoracic airsacs and tracheas were significantly lower than those of the positive control birds, suggesting that these vaccines could protect birds from MG challenge isolate (Gaunson et al., 2006).

In conclusion, live MG vaccines in layer chickens are safe; there was no vaccine reaction or effects on FCR compared with the negative control birds. For protection against Thai MG isolate challenge, all live vaccines had lower lesion scores of the thoracic airsacs and tracheas than the positive control birds. After MG challenge, only F and ts-11 strains could be re-isolated from the palatine fissure of the vaccinated birds. However, the F strain had more prevention of colonization of MG challenge isolate than ts-11 strain. This data will be useful to the poultry industry for the control and prevention in MG endemic areas. However, appropriate biosecurity must be taken into account concurrent with using the vaccines.

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