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DEVELOPMENT OF INACTIVATED *MYCOPLASMA GALLISEPTICUM* VACCINE IN CHICKENS

Miss Arithat Limsatanun

A Dissertation Submitted in Partial Fulfillment of the Requirements
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*มัยโคพลาสมา กัลลิเซพติกุม*เป็นเชื้อแบคทีเรียที่ก่อโรคทางเดินหายใจ เรียกว่าโรคระบบทางเดินหายใจเรื้อรัง ผลกระทบสำคัญของเชื้อนี้คือ ด้านเศรษฐกิจต่ออุตสาหกรรมสัตว์ปีกเนื่องจากปริมาณผลผลิตไข่ลดลงและผลกระทบต่อคุณภาพของซากสัตว์ มีเทคนิคหลายเทคนิคที่ช่วยในการเฝ้าระวังเชื้อ *มัยโคพลาสมา กัลลิเซพติกุม* และวัคซีนทางการค้าหลายชนิดที่ใช้ป้องกันและควบคุมการติดเชื้อ *มัยโคพลาสมา กัลลิเซพติกุม* ในประเทศไทยการติดเชื้อ *มัยโคพลาสมา กัลลิเซพติกุม* ยังคงเป็นหนึ่งในปัญหาสำคัญในอุตสาหกรรมสัตว์ปีก จุดประสงค์ของงานวิจัยนี้คือ การทดสอบและเปรียบเทียบประสิทธิภาพของวิธีในระดับโมเลกุลที่ใช้ในการแยกสายพันธุ์ของเชื้อ*มัยโคพลาสมา กัลลิเซพติกุม* และการเตรียมวัคซีนเชื้อตายจาก *มัยโคพลาสมา กัลลิเซพติกุม* สายพันธุ์ในประเทศไทย การศึกษานี้แบ่งออกเป็น 3 การทดลอง การทดลองส่วนที่ 1 คือการศึกษาและประเมินประสิทธิภาพของ 2 วิธีในระดับโมเลกุล เชื้อ *มัยโคพลาสมา กัลลิเซพติกุม* สายพันธุ์ที่แยกได้ในประเทศไทยจำนวน 17 ตัวอย่างถูกนำมาวิเคราะห์โดยวิธี random amplified polymorphic DNA (RAPD) และ gene-targeted sequencing (GTS) ผลการศึกษาพบว่าทั้งสองวิธีสามารถจัดประเภทของเชื้อ *มัยโคพลาสมา กัลลิเซพติกุม* โดย RAPD สามารถจัดจำแนกเชื้อได้เป็น 3 กลุ่ม ในขณะที่ GTS สามารถจำแนกได้ 4 กลุ่มตามลำดับ เมื่อนำข้อมูลของลำดับเบสของจีน *mgc2* มาสร้างเป็นแผนภูมิวิวัฒนาการพบว่า เชื้อ*มัยโคพลาสมา กัลลิเซพติกุม* 11 สายพันธุ์ในไทยมีลำดับเบสของจีน *mgc2* คล้ายกับสายพันธุ์จากประเทศอินเดียและประเทศอิสราเอลถึง 100 % การทดลองที่สอง เชื้อ *มัยโคพลาสมา กัลลิเซพติกุม* สายพันธุ์ไทย AHRL 20/52 ถูกนำมาเตรียมเป็นวัคซีนเชื้อตายโดยใช้ไคโตซานเป็นสารเสริมวัคซีน และทดสอบประสิทธิภาพวัคซีนด้วยการให้เชื้อ *มัยโคพลาสมา กัลลิเซพติกุม*ไทยสายพันธุ์รุนแรง AHRL 58/46 การทดสอบประสิทธิภาพวัคซีนแบ่งออกเป็น 2 ส่วน เปรียบเทียบกับวัคซีนเชื้อตายที่มีขายในท้องตลาด ประสิทธิภาพของวัคซีนถูกประเมินจากรอยโรคที่ตำแหน่งที่ฉีดวัคซีน ระดับภูมิคุ้มกันแบบสารน้ำ คะแนนรอยโรคที่ท่อลมและคะแนนรอยโรคที่ถุงลมของช่องอก วัคซีนเชื้อตายที่เตรียมจากสายพันธุ์ไทยถูกเตรียมเป็น สามชนิดตามระดับความเข้มข้นของไคโตซาน (0.25, 0.5 และ 1%) และทำวัคซีนด้วยวิธีการฉีดเข้ากล้ามเนื้อ ในส่วนของการทดลองที่ 3 วัคซีนเชื้อตายที่เตรียมเองถูกทดสอบประสิทธิภาพจากวิธีการบริหารวัคซีน คือ ให้โดยการฉีดเข้ากล้ามเนื้อ และ/หรือการให้โดยวิธีการหยอดตา จากนั้นจึงใช้เทคนิค quantitative real-time PCR การให้คะแนนรอยโรคที่ท่อลมและคะแนนรอยโรคที่ถุงลมของช่องอก และผลทางซีรัมวิทยาเพื่อประเมินคุณภาพวัคซีน ผลการศึกษาในการทดลองครั้งที่ 2 และ 3 พบว่า วัคซีนเชื้อตายที่เตรียมเองพบรอยโรคเล็กน้อยที่ตำแหน่งที่ฉีดวัคซีนเมื่อเปรียบเทียบกับวัคซีนเชื้อตายที่จำหน่ายเชิงพาณิชย์ นอกจากนี้พบคะแนนรอยโรคที่ท่อลมต่ำกว่าอย่างมีนัยสำคัญ ($P < 0.05$). ขณะที่ไก่ที่ได้รับวัคซีนเชื้อตายที่เตรียมเองด้วยการฉีดเข้ากล้ามเนื้อที่อายุ 6 และ 10 สัปดาห์และ/หรือวัคซีนเชื้อตายที่เตรียมเองโดยการหยอดตาที่อายุ 6 และ 10 สัปดาห์สามารถลดความรุนแรงของรอยโรคที่ท่อลมได้อย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มที่ได้รับเชื้อแต่ไม่ได้รับวัคซีน สำหรับกลุ่มที่ได้รับวัคซีนเชื้อตายที่จำหน่ายเชิงพาณิชย์ด้วยการฉีดเข้าใต้ผิวหนังที่อายุ 6 สัปดาห์ และตามด้วยวัคซีนเชื้อตายที่เตรียมเองที่อายุ 10 สัปดาห์ พบว่าเป็นโปรแกรมวัคซีนที่ให้ผลดีที่สุดในการป้องกันโรคของไก่ทดลอง จากการศึกษาครั้งนี้ทำให้ได้ข้อมูลระดับโมเลกุลที่ใช้ในการแยกสายพันธุ์ของเชื้อ*มัยโคพลาสมา กัลลิเซพติกุม*ที่น่าสนใจและทราบผลของประสิทธิภาพของไคโตซานในฐานะเป็นสารเสริมวัคซีนซึ่งช่วยพัฒนาวัคซีนเชื้อตายสำหรับการป้องกันโรคติดเชื้อ*มัยโคพลาสมา กัลลิเซพติกุม* ในประเทศไทย

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สาขาวิชา อายุรศาสตร์สัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
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ARITHAT LIMSATANUN: DEVELOPMENT OF INACTIVATED *MYCOPLASMA GALLISEPTICUM* VACCINE IN CHICKENS. ADVISOR: ASSOC. PROF. SOMSAK PAKPINYO, D.V.M., Ph.D., D.T.B.V.M., CO-ADVISOR: PROF. JIROJ SASIPREEYAJAN, D.V.M., Ph.D., D.T.B.V.M., pp.

Mycoplasma gallisepticum (MG) is the respiratory pathogen causing chronic respiratory disease (CRD) in poultry. More importantly, MG infection affects the economic losses of poultry industry due to decrease egg production and carcass quality. There are several procedures for MG monitoring and several commercial vaccines to prevent and control the MG infection. In Thailand, MG infection has been the one of major problems of poultry industry. The objectives of this study were to investigate the different molecular techniques for MG monitoring in the poultry farms and to prepare the inactivated vaccine or bacterin with Thai MG strain. There were 3 experiments to accomplish in this study. The first experiment; the investigation and comparison of the virulent *mgc2* gene of MG outbreak in Thailand and in various countries were carried out. Targeted partial *mgc2* gene of 17 Thai MG strains were analyzed with 2 molecular techniques including random amplified polymorphic DNA (RAPD) and gene-targeted sequencing (GTS) assay. The results showed that RAPD and GTS assay could classify Thai MG strains into 3 and 4 groups, respectively. In addition, the phylogenetic tree which conducted from partial *mgc2* gene sequence showed that 11 Thai MG strains did not distinguish from Indian MG strains and Israel MG strain. The other studies; the bacterin preparing from Thai MG strain AHRL 20/52 and chitosan served as an adjuvant was determined against Thai MG strain AHRL 58/46. This study consisted of 2 experiments (the second and third experiment) to determine the safety and efficacy of chitosan-adjuvanted MG bacterin comparing with those of commercial MG bacterin. The second experiment was to investigate the local reaction at injection site, antibody responses, the histopathological tracheal lesion score and gross thoracic air sac lesion score. Chitosan-adjuvanted MG bacterin were prepared with different concentration of chitosan (0.25, 0.5 and 1%) and administered via intramuscular injection. The third experiment; chitosan-adjuvanted MG bacterin was determined based on the routes of vaccine administration including intraocular and/or intramuscular routes, and vaccine program. The serology, quantitative real-time PCR assay, and air sac and tracheal lesion scores were used to evaluate this experiment. The results showed that chitosan-adjuvanted MG bacterin caused milder tissue reaction at injection site than the commercial MG bacterin and provided the significantly effective protection on tracheal lesion ($P< 0.05$). In addition, birds vaccinated with either Chitosan-adjuvanted vaccine (IM) at 6 and 10 weeks of age or bird vaccinated with Chitosan-adjuvanted vaccine (IO) at 6 weeks of age and 10 weeks of age had the significantly lower mean tracheal lesion score than positive control group ($P< 0.05$). In addition, the commercial bacterin administered by intramuscular route followed by the chitosan adjuvant bacterin administered by intraocular route showed the best protection against the MG challenge. These results provided the interesting molecular technique and the potential adjuvant for preparation of MG bacterin. These data provided the useful knowledge to improve the monitoring, surveillance and protection program of MG in poultry industry in Thailand.

Department:	Veterinary Medicine	Student's Signature	
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CHAPTER 1

INTRODUCTION

1.1 Importance and rationale

Mycoplasma gallisepticum (MG) is the important pathogen in poultry industry in world wide. It is a causing agent of respiratory disease called a chronic respiratory disease (CRD) (Raviv and Ley, 2013). MG is classified in the Mollicutes family which lack of cell wall. MG colony is the fried egg in an appearance.

Infected chickens show clinical symptoms such as coughing, rale, sneezing, ocular and nasal discharge (Raviv and Ley, 2013). Most importantly, MG causes economic impacts as a result of decreasing egg production and carcass degradation. MG infection costs a lot of money for disease prevention, control and medication (Raviv and Ley, 2013). In Thailand, the surveillance of MG infection in mixed Thai native chickens showed that MG infection were found in all age groups (Pakpinyo et al., 2007).

To prevent the losses from MG infection, there are 3 types of MG commercial vaccines that have been available in poultry industry in Thailand including live vaccine, inactivated vaccine and recombinant vaccine. These vaccines have been widely used in breeders and layers. MG live commercial vaccine has 3 strains including F, ts-11 and

6/85 strains. Even though these vaccines show the effective to reduce production losses after challenge with field strain (Carpenter et al., 1981; Whithear et al., 1990; Evans and Hafez, 1992). The efficacy of protection and the pathogenicity of each strain are different. The disadvantages of F strain shows high pathogenicity and induces mild respiratory signs in turkeys and broilers compared to other live vaccine strains (Rodriguez and Kleven, 1980). However, ts-11 and 6/85 strains show less protection than F strain (Abd-El-Motelib and Kleven, 1993). Inactivated vaccine or bacterin is safe for using in turkeys and can prevent the egg production losses in MG infected pullets and turkeys (Hildebrand et al., 1983) but the inactivated vaccine is more expensive and does not protect respiratory infection (Panigraphy et al., 1981). For recombinant vaccine, the FP-MG is Fowl-Pox vaccine containing MG genes. The inserted Fowl Poxvirus will express the surface protein of MG and induces immune response. FP-MG is safe to vaccinate in chickens (Zhang et al., 2010) but is not effective against MG infection when compared to live or inactivated vaccine (Ferguson-Noel et al., 2012).

Vaccination program has been using in most farms in Thailand. The method which is capable to differentiate between vaccine strain and wild type strain is required. Randomly amplified polymorphic DNA (RAPD) assay is the one of common technique which has several disadvantages such as inconsistency of results, RAPD banding patterns standardization and inter-laboratory comparisons (Tyler et al., 1997). For long-term epidemiology studies and effective monitoring, the new technique which has high sensitive reproducible and will be able to compare between laboratories has been determined (Ferguson et al., 2005). Gene-targeted sequencing (GTS) analysis is the interesting molecular techniques using 4 MG genes to classify MG strain. Ferguson et al. (2005) determined 4 genes of MG; *gapA*, MGA_0319, *mgc2* and *pvpA*. The researchers revealed that GTS analysis has high discriminatory power to characterize MG strains and shows the genetic relation among MG strains. In this study, the GTS and RAPD assays were used to classify Thai MG strains. In addition, *mgc2* gene of MG was analyzed with GTS assay to investigate Thai MG strains, which were collected from different areas in Thailand and compared the results of MG strains to other countries.

However, MG infection is an economic impact on the poultry industry especially in Thailand. Although the live vaccines can protect chicken flocks against wild-type of MG infection but there is a chance that vaccine strains could delay onset of lay, decrease amount of egg production (Burnham et al., 2002), cause respiratory signs and spread to non-vaccinated flock (Evans and Hafez, 1992). While the MG inactivated vaccine is considered as safe and can induce high systemic immune response to decrease vertical transmission. Furthermore, the inactivated vaccine has been recommended to use in a long-term prevention and control program in multiple-age poultry farms (Levisohn and Kleven, 2000; Raviv and Ley, 2013).

There have been several studies on various adjuvants of MG inactivated vaccine (Barbour et al., 1987; Yagihashi et al., 1987; Barbour and Newman, 1989; Barbour and Newman, 1990; Elfaki et al., 1993). In addition, using MG surface proteins for construct subunit vaccines have been studied (Sundquist et al., 1996; Czifra et al., 2000; Yoshida et al., 2000).

To prepare inactivated vaccine, optimal adjuvants are required to enhance an immune response and strongly induce an innate immune response. The chitosan is the one of adjuvants that is a potential substance (Seferian and Martinez, 2000). Chitosan is a polysaccharide and is made from deacetylation of chitin. Chitosan has properties that are useful applications in many fields such as wound healing, antimicrobial activity, drug delivery and stimulation of immunity (Senel and McClure, 2004). Several studies found that chitosan could enhance humoral and cell-mediated immune responses in mice (Zaharoff et al., 2007) and chickens (Rauw et al., 2010a).

The aims of this study were to investigate the relation of Thai MG strains with RAPD and GTS assays. One of Thai MG strain was selected to prepare MG inactivated vaccine and to be compared the protective efficacy with the commercial MG inactivated vaccine. To determine protective efficacy, experimental birds were vaccinated with prepared MG inactivated vaccine or commercial MG inactivated vaccine and challenged with Thai virulent MG strains. This was the first study to investigate the genetic relationship of *mgc2* gene of Thai MG strains and to prepare MG inactivated vaccine with Thai MG strain.

1.2 Objectives of this study

1. To investigate genetic relationship between Thai MG strains and MG strains from different countries by using GTS assay.
2. To compare the discriminatory power of GTS and RAPD assays.
3. To prepare the inactivated vaccine of Thai MG strain by using the chitosan adjuvant.
4. To evaluate the protective efficacy of MG inactivated vaccine and MG commercial inactivated vaccine.in layer chickens.

1.3 Literature review.

Mycoplasma gallisepticum

There are several species of Mycoplasma, which affect the economic significance in poultry industry including *M. gallisepticum, M. synoviae, M. iowae* and *M. meleagridis* (Raviv and Ley, 2013). Only *M. gallisepticum*, *M. synoviae* and *M. iowae* cause disease in chickens. In poultry industry, MG infection has been considered as a cause of the economic losses (Raviv and Ley, 2013). MG organism is classified in class Mollicutes which has no cell wall but is bounded by plasma membrane (Razin et al., 1998). The colony morphology appears the fried egg under light microscopic observation. MG is a host specific pathogen which infects only avian host. No public health concern of MG has been reported (Raviv and Ley, 2013).

Clinical sign

The disease of MG is known as a chronic respiratory disease (CRD) in chickens and an infectious sinusitis in turkeys. This pathogen can transmit through horizontal and vertical route. Clinical signs of MG infection are shown as respiratory symptoms such as nasal discharge, conjunctivitis, sneezing, rales and sinusitis. Moreover, MG also affects economic impacts due to increase feed conversion ratio (FCR), decrease egg production, increase mortality, poor hatchability and carcass degradation (Raviv and Ley, 2013).

Incubation period

From the MG experimental study in chickens and turkeys, the incubation period is 6 – 21 days depends on the strain, co-pathogen infection and environmental stress (Raviv and Ley, 2013).

Morbidity and mortality rate

In MG infected chickens, the mortality rate is low (Mohammed et al., 1987). But in broilers, the mortality rate can raise up to 30% in the cold season, predisposing infection or high virulence strain (Raviv and Ley, 2013).

Transmission and Carriers

MG can transmit through horizontal and vertical routes. The normal flocks expose MG pathogens via direct and indirect contact including aerosol or droplet containing MG organisms (Raviv and Ley, 2013). The entry routes of MG organisms are respiratory tract and/or conjunctiva. MG organisms can survive in feces for 1-3 days, on feathers for 2–4 days (Christensen et al., 1994), on clothes for 3 days at 20ºC and on rubber for 2 days (Chandiramani et al., 1966). MG organisms can be alive for 3 days on human body and 1 day in the human nasal passage (Christensen et al., 1994). However, the survival time depends on conditions of pH, temperature, and humidity. Infected hens can transfer MG organisms through their eggs. Some studies showed that the highest egg transmission rate was 25% of egg production at 4 weeks after MG challenge (Glisson and Kleven, 1985) and up to 50% of egg production at 3-6 weeks after MG challenge (Sasipreeyajan et al., 1987). Backyard flocks and some free-ranging songbirds are the important reservoir of MG organisms (McBride et al., 1991). Chickens infected with MG house finches isolate via intra-crop administration could show the serological response against and respiratory clinical signs (Dhondt et al., 2007; Grodio et al., 2008).

Pathogenicity

Pathogenesis of MG infection is that chickens inhale and take pathogen through upper respiratory tract or conjunctiva. MG will attach the glycoprotein of the tracheal epithelial cell and induce ciliostasis, erosion of surface and infiltration of inflammatory cells (Lam, 2003). Moreover, MG organisms can express various their surface epitopes with motility and cytadhesion abilities (Shimizu and Miyata, 2002). This is possible mechanism of MG organisms to enter the host cells and affects the host immune response (Garcia et al., 1994; Chen et al., 2011). The virulence of MG also depends on genotypic and phenotypic characterization of each strain (Raviv and Ley, 2013).

Pathology

Mucosal congestion and exudate can be found in nostrils, trachea bronchi and air sacs. Sinusitis with exudate is the common lesion in turkeys. In severe case of infected chickens and turkeys may have caseous airsacculitis. In addition, MG organisms can cause keratoconjunctivitis and corneal opacity (Nunoya et al., 1995). In microscopic observation, the infiltration of mononuclear cells and mucous glands enlargement resulting in the thickness of mucous membranes are observed (Dykstra et al., 1985). The swollen of respiratory epithelial also causes cilia erosion of tracheal epithelium (Dykstra et al., 1985).

Economic impacts

In 1987, economic impacts caused by MG infection were investigated in commercial layer flocks in Southern California. Commercial layer producers had been found losing about 127 million eggs and about $7 million for MG prevention (Mohammed et al., 1987). Another example, the National Poultry Improvement Plan reported that there was a broiler farm in North Carolina lost approximately $500,000 to $750,000 for control MG infection in 1999 and until now. Presently, the United States of America still deals with poultry economic losses due to MG infection (Evans et al., 2005). In Thailand, Pakpinyo et al. (2007) reported MG infection in mixed Thai native chickens that the older flock had the higher the number of positive reactors of ELISA antibody titer, serum plate agglutination test (SPA) and polymerase chain reaction (PCR).

Host immunity against MG infection

There are several studies showed that MG specific antibody in tracheal washes from infected chickens was related to the decrease of MG organisms and tracheal lesion scores (Chhabra and Goel, 1981; Yagihashi and Tajima, 1986). Secretory antibodies of the recovered chickens play as the role of MG organism clearance, lower tracheal lesion score and prevention of MG organism to attach the tracheal epithelial cells in re-exposure birds with MG organisms (Yagihashi and Tajima, 1986; Yagihashi et al., 1992). In addition, natural killer and cytotoxic T cell are significantly responsible for MG infection (Gaunson et al., 2006).

Diagnosis

Isolation and identification is the gold standard technique for MG diagnosis; however, this technique is time consuming because MG colony may take up to 3 weeks to be observed. Exudates from tracheal, choanal cleft (palatine fissure), air sac and lungs can be inoculated into mycoplasma broth or agar medium (Zain and Bradbury, 1995; Kleven, 2008). The formulation of mycoplasma culture broth was modified and called

Frey's broth medium (Raviv and Ley, 2013). To identify mycoplasma species, cultured agar is imprinted and tested by direct or indirect immunofluorescence test (Gardella et al., 1983). Polymerase chain reaction (PCR) becomes common techniques in all diagnostic laboratories. PCR is the efficient technique which is simple procedure and more rapid, high sensitivity and high specific (Silveira et al., 1996; Salisch et al., 1998). A quantitative real-time PCR is another technique which is suitable for DNA quantitation and can express as colony-forming unit (CFU) equivalents per milliliter (Mekkes and Feberwee, 2005).

Serology

Serological test is a useful procedure to monitor MG status in farm. Serum plate agglutination (SPA) is the technique to detect antibodies against MG infection. The SPA test is a commercial available and widely used as a screening test. This test is fast, inexpensive and sensitive technique (Kleven, 2008) but it causes nonspecific reactors due to cross-reactive MS antigens (Avakian and Kleven, 1990). The hemagglutination inhibition (HI) test is another technique to screen MG exposure but it is time consuming technique, not commercially available reagents and low sensitivity (Kleven et al., 1988; Kleven, 2008). The enzyme-linked immunosorbent assays (ELISA) has less sensitivity and more specificity than SPA test (Avakian et al., 1988; Czifra et al., 1993). Presently, commercial ELISA test kits are generally used in monitoring program in poultry industry.

Prevention and control

For control MG infection, replacement stock must obtain from free MG breeding farm and manage farm with proper biosecurity system and surveillance program (Raviv and Ley, 2013).

Treatment

The antimicrobials for treatment of MG infected chicken can decrease number of MG organisms in trachea during the initial phase of outbreak (Cummings et al., 1986). Because MG organism is lacking cell wall bacteria; therefore, the ß-lactamase antibiotics including penicillin which inhibit the final step in cell wall biosynthesis cannot kill the MG organism. Some antimicrobials have been used to control MG infection including macrolides, tiamulin and fluoroquinolones which can reduce egg production losses and MG transmission (Ortiz et al., 1995). Presently, MG organisms have gradually increased the resistance against several antimicrobials such as fluoroquinolones, macrolides and pleuromutilins (Bradbury et al., 1993; Gautier-Bouchardon et al., 2002). To reduce the incidence of egg transmission, egg injection or dipping were the methods which being used in the past. Egg injection is to inject antimicrobials into hatching eggs by ovo transmission while egg dipping is to dip fertilized eggs in antibiotic solution (Hall et al., 1963; Stuart and Bruins, 1963). These methods could reduce MG transmission from hens to their eggs, however it affected on hatchability rate and bacterial contamination (Hall et al., 1963).

Vaccines

Vaccination programs are used in poultry industry to prevent the egg production losses and the respiratory clinical signs. There are 3 types of commercial vaccines: live vaccines, inactivated vaccines and recombinant fowlpox vaccine (Raviv and Ley, 2013). There are 3 strains of live vaccines including F, ts-11 and 6/85 strains. F strain was isolated by Adler and Yamamoto and originated from the Connecticut F strain (Adler et al., 1957). F strain showed the protection against airsacculitis after virulent strain challenge (Levisohn and Dykstra, 1987) and provided protection against egg production losses (Branton and Deaton, 1985; Branton et al., 1988). However, the F strain was too virulent for broilers (Rodriguez and Kleven, 1980) and it was not recommended to vaccinate in turkeys (Lin and Kleven, 1982). The F strain vaccine is able to colonize in the upper respiratory tract and to displace MG field strain. After vaccination, F strain can persist in farm for several years (Kleven et al., 1990).

The 6/85 strain vaccine was a non-virulent MG strain. It was developed as a live vaccine which could protect chicken from challenge and did not transmit to unvaccinated bird. However, the 6/85 strain showed low or no detectable serologic response after vaccination in birds and took 4 to 8 weeks after vaccination to colonize in the upper respiratory tract (Evans and Hafez, 1992). Feberwee et al. (2006) found that 6/85 strain could not stop spreading of virulent MG strain in vaccinated chickens. The ts-11 strain vaccine is a temperature-sensitive mutant of Australian MG isolate by chemical mutagenesis method and can grow well at 33ºC. The ts-11 vaccine developed slow and low systemic immune response (Whithear et al., 1990). MG inactivated vaccine was initially produced in late 1970s with oil emulsion (Raviv and Ley, 2013). MG inactivated vaccine could protect against egg drop production in layers and against airsacculitis in broilers after challenge with virulent MG organisms (Yoder and Hopkins, 1985). Some studies showed that inactivated vaccine reduced the number of field MG colonization. However, inactivated vaccine could not eliminate field MG strain from tracheal epithelial cells (Yoder et al., 1984; Kleven, 1985; Yoder and Hopkins, 1985). The inactivated vaccine is considered as an economic vaccine for long-term control in the multiple-age flocks (Levisohn and Kleven, 2000), and is safe compared to live vaccines. The inactivated vaccine cannot transmit to unvaccinated flock and cannot cause clinical signs. However, the optimal protection requires at least two injections of vaccine (Raviv and Ley, 2013). In addition, the inactivated vaccine is more expensive and may cause inflammation at the injection area (Droual et al., 1993).

From several studies, the several adjuvants of MG inactivated vaccines such as liposomes, iota-carrageenan, formaldehyde and saponin have been determined (Barbour et al., 1987; Yagihashi et al., 1987; Barbour and Newman, 1989; Elfaki et al., 1993). In addition, the MG subunit vaccines have been explored (Sundquist et al., 1996; Czifra et al., 2000; Yoshida et al., 2000).

Another commercial vaccine of MG was recombinant fowl pox-MG (FP-MG) vaccine, developed by using MG genes encoding surface protein in recombinant fowl pox virus. There was report showed that FP-MG vaccine was safe for chicken vaccination (Zhang et al., 2010). Ferguson-Noel et al. (2012) showed that FP-MG vaccine could induce the lower systemic immune response and had lower efficacy to protect chickens against the virulent MG strain on the air sac and trachea lesion scores compared to the MG inactivated vaccine or F strain live vaccine.

Random amplified polymorphic DNA (RAPD) and Gene-targeted sequencing (GTS) assays

There are several molecular techniques are developed to differentiate MG strain and monitor MG outbreaks. The most common technique which have been using until nowadays is RAPD assay. RAPD assay has very efficiency and very useful for epidemiological studies and can differentiate between vaccine and field strains (Fan et al., 1995; Charlton et al., 1999). However, this assay has some disadvantages. The visual band pattern standardization is somehow difficult to interpret (Raviv and Ley, 2013). Another disadvantage is the gel electrophoresis process which is time consuming and costly (Marois et al., 2001; Mettifogo et al., 2006). In addition, the result of RAPD assay cannot be used to compare among laboratories (Tyler et al., 1997).

GTS is the molecular technique described by Ferguson et al. (2005). This technique used 4 sequence virulence MG genes including *pvpA*, *gapA*, *mgc2* and MGA_0319 genes to identify MG strains. In this study, 67 MG strains from the USA, Israel and Australia and reference strains were obtained and characterized by using GTS assay. The results showed that GTS assay had more discriminatory power than RAPD assay to identify MG pattern types. This technique could compare MG strains between laboratories. Virulent MG genes were used in this study encoding the surface membrane proteins. The surface membrane proteins play as a role in the pathogenicity, antigenic variation and immune invasion (Raviv and Ley, 2013). *GapA* gene encodes GapA cytadhesin protein which is a primary cytadhesin relating to cell attachment (Goh et al., 1998). GapA protein is required *CrmA* gene encoding protein for MG cytadherence and pathogenesis (Papazisi et al., 2002). *Mgc2* gene encodes MGC2 protein which is an organelle structure for cell surface attachment (Boguslavsky et al., 2000). *PvpA* gene encodes variable size of antigenic proteins leading to antigenic variation of MG. PvpA protein is one of immunogenic protein (Boguslavsky et al., 2000; Jiang et al., 2009). PMGA gene family is responsible for immune evasion and chronic infection (Markham et al., 1993; Markham et al., 1998). MG specific lipoprotein A gene (MslA) encodes protein which involves in the virulence of MG organisms. The MG attenuated F strain expressed the low level of MslA protein (Szczepanek et al., 2010). MG organisms also express osmotically inducible protein C (OsmC)-like adhesion protein. This protein helps MG organisms to resist the hydrogen peroxide (H_2O_2) resulting that MG organisms can survive and remain virulence (Jenkins et al., 2008). VlhA protein encoded by *vlhA genes* plays as a role in hemagglutination and antigenic variation of MG organisms. *vlhA* gene family also has been found in *M. synoviae* organisms (Levisohn et al., 1995).

Thimerosal compound and chitosan

In the present study, MG can be inactivated by thimerosal compound which is a derivative of mercury. The thimerosal compound is used for antiseptic and antifungal agents. There are several studies that have used thimerosal compound to inactivate and preserve inactivated vaccines (Blackall and Reid, 1987; Fernandez et al., 2005). The mechanism of thimerosal compound is to kill bacteria by breaking down and releasing ethyl-mercury. Ethyl-mercury can penetrate the bacterial cell and inhibit intracellular enzyme synthesis of bacteria. As a result, bacteria die because of biological malfunction (Elferink, 1999).

Unlike other inactivating agents including formalin and glutaraldehyde, the thimerosal compound does not break bacterial protein membranes. Therefore, the surface proteins of bacteria which play as a role in immune response are not damaged (Fitzgerald and Welter, 1994). This is the promising advantage of thimerosal compound to be chosen as inactivating agent (Blackall and Reid, 1987; Gupta et al., 1987; Pope and Johnson, 1987). In the present study, chitosan was used and served as an adjuvant. Chitosan is an acetylated chitin and is obtained from aquatic animal (Paul and Garside, 2000). It was widely used in many fields including cosmetics and feed additives

(Rinaudo, 2007). In addition, chitosan could enhance humoral and cell mediated immune response in mice (Zaharoff et al., 2007) and chickens (Rauw et al., 2010b).

Chitosan is also called mucosal adjuvant because of mucoadhesive properties (Hea et al., 1998). The positive charge of amino group in chitosan molecule interacts with the negative charge on mucosal cell surface resulting in the opening of tight junction. After that, the chitosan-antigen complex can pass through this junction (Artursson et al., 1994). Several studies showed that chitosan worked as a practically mucosal vaccine adjuvant (Zaharoff et al., 2007; Ghendon et al., 2009; Sui et al., 2010). Sui et al. (2010) found that chitosan adjuvant increased both IgA and IgG antibody and showed 100% survival rate in mice after vaccination with 100 µg M1 protein of influenza virus.

In addition, chitosan could trap and slowly release the antigen at the site of injection resulting that the immune response was consistently stimulated leading to the high antibody titer production (Zaharoff et al., 2007). Moreover, chitosan sustained more than 60% of protein antigen at injection site for 7 days due to the high viscosity (Zaharoff et al., 2007).

CHAPTER 2

The efficacy of chitosan-adjuvanted, *Mycoplasma gallisepticum* bacterin in chickens

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The efficacy of chitosan-adjuvanted, *Mycoplasma gallisepticum* bacterin in chickens

Abstract

Mycoplasma gallisepticum (MG) is one of the major pathogens that cause respiratory signs in poultry industry. To control MG infection, vaccination is the useful procedure. In this study, MG vaccine was developed by using the local Thai MG isolate (AHRL 20/52). Chitosan, a polysaccharide adjuvant derived from crustaceans, has been successfully used in various vaccines. The objectives of this study were to prepare inactivated MG vaccine by using chitosan served as an adjuvant, to determine protection against the field Thai MG isolate and to evaluate tissue reaction at injection site. Six groups of 6-weeks old layers (20 or 29 birds/group) were intramuscularly vaccinated with inactivated vaccines containing various concentrations of chitosan (0.25, 0.5 and 1%), a commercially inactivated MG vaccine, sham-negative and sham-positive controls, respectively. Six weeks post vaccination, all groups excluding the negative control were intratracheally challenged with 100 µl of 10^8CFU Thai MG isolate (AHRL 58/46). At 1, 2, 3 and 4 weeks post challenge, five birds each group were euthanized and necropsied to blindly determine the gross air sac lesion and histopathologically tracheal lesion. For tissue reaction study, three groups of 9 birds each; sham negative control, 0.5% chitosan and commercial vaccine were given as previously described. At 1, 2 and 3 weeks post vaccination, three birds each group were randomly selected to euthanize and necropsy to determine gross and histopathological lesion. The results demonstrated that prepared-inactivated vaccines induced less antibody responses compared with the commercial vaccine but groups receiving inactivated vaccine containing 0.5 and 1 % chitosan exhibited significantly lower tracheal lesion than the positive control and commercial vaccine groups ($p < 0.05$). Chitosan formulation caused less tissue reaction than the commercial vaccine. These results demonstrated that the prepared-inactivated MG vaccines could effectively reduce MG-induced pathological lesions and chitosan could be used as adjuvant in inactivated MG vaccine.

Keywords: Bacterin, Chickens, Chitosan, *Mycoplasma gallisepticum*

2.1 Introduction

In poultry industry, *Mycoplasma gallisepticum* (MG) is the important respiratory pathogen worldwide. MG causes of the chronic respiratory disease (CRD). Infected chickens show respiratory signs such as rales, coughing, sneezing, ocular and nasal discharge. Economic impacts of MG infection are decreasing egg production and quality carcass degradation (Raviv and Ley, 2013).

To prevent economic losses due to MG infection, the vaccination has been widely used in poultry industry. Currently modified live, inactivated and recombinant fowl pox-MG (rFP-MG) vaccines are commercially available. The modified live vaccines were derived from the 3 MG strains including F, ts-11 and 6/85 strains (Raviv and Ley, 2013). Each strain has different efficacies on the protection and pathogenicity (Ferguson-Noel et al., 2012). The inactivated vaccine can reduce egg production losses in pullets after challenge with MG virulent strain. However, inactivated vaccine is more expensive than other commercial vaccines (Raviv and Ley, 2013). The rFP-MG is constructed by insertion partial MG surface protein genes in fowlpox virus vector. The rFP-MG is safe for chickens (Zhang et al., 2010). However, Ferguson-Noel et al. (2012) found that the rFP-MG vaccine had lower efficacy on air sac and trachea lesion than the inactivated vaccine and live F strain vaccine.

In poultry industry, the use of live vaccines has been concerned. MG live vaccines can turn more virulent after vaccination. Live vaccine can transmit from vaccinated chickens to non-vaccinated chickens and induce respiratory clinical signs. Therefore, the MG inactivated vaccine has been used in the long-term vaccination at multiple age production sites due to its safety (Raviv and Ley, 2013).

To improve efficacy of MG inactivated vaccine, various adjuvants have been explored (Barbour et al., 1987; Yagihashi et al., 1987; Barbour and Newman, 1989; Barbour and Newman, 1990; Elfaki et al., 1993). Optimal adjuvants require both immunostimulant and antigen delivery properties. In recent years, there have been several studies emphasized on the adjuvant effect of chitosan. Chitosan is a deacyltylation form of chitin, a component of crustaceans shell such as shell, crab or shrimp (Paul and Garside, 2000). In a medical field, chitosan has several medical properties including enhancing wound healing, antimicrobial activity, drug carrier and immunostimulant (Rinaudo, 2007). A few studies found that chitosan could be used as an adjuvant and induced humoral and cellular immunity in mice and chickens (Zaharoff et al., 2007; Rauw et al., 2010a). Moreover, chitosan is known as a mucosal adjuvant due to its effective absorption following oral or intranasal administration (Hea et al., 1998). In previous studies, chitosan adjuvant could increase local and systemic immune response after different routes of immunization (Zaharoff et al., 2007; Ghendon et al., 2009; Sui et al., 2010). The objectives of the present study were to explore the use chitosan as an adjuvant for MG inactivated vaccine. The safety of vaccine was determined from local tissue reaction at the injection site. Antibody titers, air sac and tracheal lesion scores in chicken receiving the prepared inactivated vaccine were compared with the commercially available inactivated vaccine in a vaccinated-challenged layer chicken model.

2.2 Materials and methods

2.2.1 Preparation of MG inactivated vaccines

The various concentrations of chitosan of an inactivated vaccine were prepared from MG strain AHRL20/52 which was provided by Dr. SomsakPakpinyo (Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University). The MG strain AHRL20/52 were propagated in 50 ml Frey's broth supplemented with 15% swine serum (FMS) (Kleven, 2008) and then incubated at 37 °C until the color of broth changed from pink to orange-yellow color. After propagation, the cultured broth containing 1x 10^9colony forming unit (CFU)/ml of MG were prepared followed described protocol (Stone et al., 1978). The cultured broth was centrifuged at 12,000xg at 4^{o}C for 30 min and supernatant was discarded. MG cells were washed with phosphate-buffered saline (PBS) and centrifuged at 12,000xg for 30 min for three times and finally inactivated by 0.01% thimerosal in PBS (Sigma-Aldrich Corp., USA). The treated MG cells were incubated at 4°C for 7 days (6) and confirmed inactivation by MG culture method (Chukiatsiri et al., 2009) and confirmed inactivation by MG culture method (Kleven, 2008). Treated MG cells were washed with phosphate-buffered saline (PBS) and diluted with 0.01% thimerosal in PBS (Chukiatsiri et al., 2009). Chitosan (Sigma-Aldrich Corp., USA) was added in 1% acetic acid solution (pH 5.0) 100 ml (Ghendon et al., 2009).). Chitosan solution was added to the prepared MG organisms as a final concentration of 0.25%, 0.5% and 1% chitosan solution and stored at 4°C until use.

2.2.2 Viscosity and stability test.

The viscosity test was determined the time of dropping vaccine from 0.4 - 0.0 ml (second) at room temperature. MG inactivated vaccines and MG commercially inactivated vaccine were placed in 1ml disposable Pasteur pipette Corning Costar® (Sigma-Aldrich Corp., USA) and set vertically. The time was measured when vaccine dropped for 0.4 ml at room temperature (Stone et al., 1978; Stone and Xie, 1990) and repeated for 3 times.

The stability test was observed including color changing. Five ml of all MG inactivated vaccines was kept in closed glass tube wrapping with the aluminum foil at 4°C, room temperature and 37°C and observed weekly for 4 months.

2.2.3 Local tissue reaction

Forty-five, 6-weeks-old commercial layer birds, Isa Brown breed, were used in this study. All birds were equally divided into 3groups as following Group 1 was received 0.5% chitosan solution serving as a sham negative control. Group 2 and 3 was received 0.5% chitosan inactivated vaccine and commercially inactivated MG vaccine, respectively. All birds were injected with 0.5 ml/bird into the left side of deep pectoral muscle. At 1, 2 and 3 weeks post-vaccination, five birds of each group were randomly selected, euthanized, necropsied and blindly evaluated the grossly tissue reaction at the injection area including muscle swelling, cystic and granulomatous formation described by Droual et al. (1993).

2.2.4 Experimental designs

To evaluate the efficacy of chitosan serving as an adjuvant of inactivated vaccines, one hundred and forty-seven, one-day-old commercial layer chicken, Isa brown breed, were obtained from free of *M. gallisepticum* and *M. synoviae* breeder farm, raised on wired cages in an isolated room and given feed and drinking water *ad lib*. At

6 weeks of age, 30 birds were randomly bled for MG serology and then divided into 6 groups, 20 or 29 birds each. Each group was designed as shown in Table 1. All birds were injected with 0.5 ml/bird into the left side of deep pectoral muscle. At 1, 2 and 3 weeks post-vaccination, 3 birds of groups 1, 4 and 6 were randomly selected, euthanized, necropsied and evaluated the histopathology of the deep pectoral muscle at the injection area. At 12 weeks of age, all birds were bled for MG serology and challenged with 0.1 ml FMS or 0.1 ml MG cultured broth (MG strain AHRL 58/46) containing approximately 1.0x10^8 CFU/ml by intratracheal route. At 13, 14 and 15 weeks of age, all remaining birds were bled for MG serology, swabbed from choanal cleft into FMS broth for PCR and 5 birds of each group were randomly selected, euthanized, necropsied and blindly evaluated the gross air sac lesion scores and histopathologic tracheal lesion scores. Each trachea was cut into 4 sections as follows 1 upper proximal end, 2 middle sections and 1 lower proximal end, and submitted for histopathology. This study was approved by IACUC No. 13310081.

2.2.5 MG serology

2.2.5.1 Serum plate agglutination (SPA) test

The commercial MG antigen Soleil® (Biovac Animal Health, France) was used in this study. This test was followed the procedure described as manufactures' instructions. Briefly, 30 µl of serum was mixed with 30 µl of MG antigen on glass slide and left for 2 min at room temperature. The positive reaction shows the clumping reaction. If the tested serum showed positive reaction, this serum was diluted in PBS as 1:8 dilutions then re-tested as previously described.

2.2.5.2 Enzyme-linked immunosorbent assay (ELISA)

The commercial test kit, ProFLOK® (Synbiotics Corporation, USA) was used to detect antibody titers and followed the manufacturers' instructions. Briefly, diluted serum was placed onto a MG antigen-coated plate, incubated, washed and added horseradish peroxidase-labeled anti-chicken immunoglobulin G. After 30 min incubation period, the plate was washed extensively then adding a substrate and the stop solution. The plate was read in ELISA reader. The result was considered as negative, suspicious and positive when antibody titer ranges 0-148, 149-743 and greater than 744, respectively.

2.2.6 Polymerase chain reaction (PCR) assay

MG samples from choanal cleft were extracted for DNA template. DNA was amplified by polymerase chain reaction (PCR) follow the protocol described by (Lauerman, 1998). Briefly, the cultured broth was centrifuged at 15,000xg for 6 minutes. MG pellets were washed with distilled water and centrifuged for 2 times. MG pellets were diluted with distilled water approximately 30 µl depending on the size of pellets. After diluting, the samples were boiled for 10 minutes. The samples were immediately placed on ice 5 minutes and centrifuged 15,000xg for 2 minutes. The supernatant containing the DNA template were collected and keep used for PCR assay. The PCR mixture were prepared as 25 µl volume containingKCl 500 mM, Tris-HCl (pH 8.3) 100 mM, dNTP (Fermentas) 1 mM, primer F (5'GAGCTAATCTGTAAAGTTGGTC3') (Qiagen) and primer R (5'GCTTCCTTGCGGTTAGCAAC3') (Qiagen) 10 pmole each, Taq polymerase (Fermentas) 1.25 U, $MgCl_2$ 1.25 mM and DNA template 2.5 µl (250 ng). MG strain F (ATCC 15302) was used as positive controls. PCR mixtures were be amplified in a DNA thermal cycler, PCR Sprint® (Thermo Electron Corporation, USA) with 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec for 40 cycles and followed by 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 UV transilluminator, and photographed.

2.2.7 Evaluation of lesion scores

Grossly air sac lesion score was described by Kleven et al. (1972). 0: no air sac lesion is found; 1: lymphofollicular lesions or slight cloudiness of air sac membrane are found; 2: air sac membrane is slightly thick and usually presents small accumulations of cheesy exudates; 3: air sac membrane is obviously thick and meaty in consistency, with large accumulations of cheesy exudates in one air sac; 4: lesions are observed the same as 3, but 2 or more air sacs are found.

Histopathologically tracheal lesion score were blindly evaluated following Yagihashi et al. (1987) as follows. 0: no significant changes are observed; 1: small aggregate of cells (mainly lymphocytes) is found; 2: moderate thickening of the wall due to the cell infiltration, and edema commonly accompanied with epithelial degeneration and exudation is present; 3: extensive thickening of the wall due to the cell infiltration with or without exudation is determined.

2.2.8 Statistical analysis

The grossly thoracic air sac lesion scores and histopathologically trachea lesion scores were analyzed by using Kruskal-Wallis test and Mann-Whitney U test at 95% confidence level ($P<0.05$). SPSS® version 22 programwas used to determine statistical analysis.

2.3 Results

2.3.1 Viscosity and stability test

The group 6 (commercial MG vaccine) had the significant highest viscosity (1.788±0.075 sec) comparing with group 3 (0.25%chitosan) (1.103±0.089 sec), group 4 (0.5% chitosan) (1.241±0.119 sec) and group 5 (1% chitosan) (1.401±0.106 sec) ($P<0.05$). All chitosan vaccines stored at 4°C, 25°C (room temperature) and 37°C for 16 weeks was still stable including none of color change or separation of vaccine solution.

2.3.2 Local tissue lesions

At 1-week post vaccination, the carcasses of chickens of group 6 had the most severely gross inflammation. All birds of group 6 had abscess in the pectoral muscle, whereas birds in all chitosan MG vaccinated groups did not show any gross inflammation. At 2 and 3 weeks post vaccination, the inflammation of pectoral muscle of group 6 was gradual decrease and the inflammation was not observed at 3 weeks post vaccination (Fig. 1).

Histopathology of tissue sections was not remarkable lesions in all chitosan MG vaccinated groups (Fig. 1A). Group 6 had myositis due to infiltration of lymphocyte and heterophils (Fig. 1B).

2.3.3 Clinical signs

After challenge, the respiratory signs were not observed in all groups during experimental period except the group 2 (positive control). Chickens of group 2 showed mild respiratory signs including sneezing and respiratory rales for 3 days. No chicken death of all groups was observed throughout after challenge.

2.3.4 Serology

2.3.4.1 SPA test

At 12 weeks of age or 6 weeks post vaccination, the SPA test could detect the positive reactors only in vaccinated groups ranging 11 – 20 out of 20 birds. At 13, 14, 15 and 16 weeks of age, all samples of all vaccinated groups were positive reactors against MG antibody determined by the SPA test (Table 2).

2.3.4.2 ELISA

At 6 weeks of age, no MG antibody in all groups was found (data not shown). At 12 weeks of age, only groups 4, 5 and 6 were positive reactors ranging 5 –20 out of 20 birds. At 14 and 15 weeks of age, all samples of groups 3, 4, 5 and 6 were positive results, while only 4 and 1 positive samples of group 2 were found, respectively. At 16 weeks of age, all samples of all groups were found positive results excluding samples of group 1 (Table 2).

2.3.5 Lesion score evaluation

2.3.5.1 Gross air sac lesion scores

At 13, 14, 15 and 16 weeks of age, the mean air sac lesion scores of all birds were ranging 0.2-0.8, 0.1-0.5, 0-0.5 and 0-0.2, respectively. No significant differences were observed among groups at the same age ($P>0.05$) (Table 3).

2.3.5.2 Histopathological tracheal lesion scores

At13, 14, 15 and 16 weeks of age, the mean tracheal lesion scores of all birds were ranging 0.8-2.7, 0.6-2.7, 0.3-3.0 and 0.5-3.0, respectively. No significant differences were observed between group 2 and 3 at the same age. The mean tracheal lesion scores of group 1 were lower than those of other groups during the entire experimental period ($P<0.05$). At 13 and 14 weeks of age, the mean tracheal lesion scores of group 5 had significantly lower than those of group 6. At 15 weeks of age, the mean tracheal lesion scores of group 4 were significantly lower than those of group 6. At 16 weeks of age, there were no significant difference among groups 4, 5 and 6 (Table 3).

2.3.6 Polymerase change reaction (PCR) assay

After challenge, the PCR assay could detect the MG DNA in the positive control and all vaccinated groups ranging 3-5 out of 5 birds (Table. 4).

2.4 Discussion

The viscosity test showed that chitosan MG bacterin had lower viscosity. Decreased viscosity is useful for vaccine management due to easier vaccine preparation and administration (O'Hagan and De Gregorio, 2009). In stability test, chitosan MG could store at 4˚C to 37 ˚C at least 16 weeks without color changes, suggesting that chitosan MG vaccine is easier for vaccine administration and they could store at high ambient temperature. These properties are appropriate for vaccine management in hot climate countries like Thailand (Wirkas et al., 2007). Unfortunately, the present study did not determine the efficacy of the stored chitosan MG vaccine in chickens.

In this study, the local tissue reaction after receiving commercial inactivated MG vaccine (MG-bac®) showed the most severe lesions at injection site. According to previous researches, birds vaccinated with oil-adjuvanted MG bacterin were observed the severe lesions including cysts and cellulitis in muscle after evisceration (Lauerman, 1998). The researchers also found that 12 weeks old intramuscularly vaccinated chicken at leg muscle with oil adjuvant MG bacterin had lameness and granulomatous cellulitis at the lower and upper parts of legs. In another previous study, Droual et al. (1990) investigated 6 carcasses of broiler breeder at 100 - 110 weeks of age vaccinated with multiple oil-adjuvant vaccines at breast area. One of 6 carcasses vaccinated with a MG bacterin at 14-16 weeks of age. In the carcass which vaccinated with MG bacterin found the cystic granulomatous lesion. The cysts found that contained dark pigmented and a cellular debris in pathological view. The present study also determined the histopathological changes at injection site to support the gross severe tissue lesions. Inflammation due to lymphocytic cell infiltration was found in the pectoral muscle area of birds vaccinated with commercial inactivated MG vaccine. Furthermore, the inflammation observed in the first three weeks post vaccination could cause the discomfort of chickens including pain, difficult movement, and loss of appetite. From these results, the commercial vaccine induced more severe tissue reaction comparing with chitosan MG vaccine. This would be the disadvantage of oil-adjuvanted MG bacterin. Because the chitosan could be used as an adjuvant of vaccine and the mild tissue reaction of using chitosan in the present study; therefore, chitosan could be useful to improve or develop other inactivated vaccines in the future.

Serology responses were performed by SPA and ELISA tests. The results indicated that chitosan adjuvant could induce systemic immune response although antibody levels of birds vaccinated with chitosan vaccine were lower than those of birds vaccinated with commercial vaccine. These results are inconsistent with several previous studies. Seferian and Martinez (2000) vaccinated BALB/c mice with formulation of recombinant β, human chorionic gonadotropin and chitosan by intraperitoneal administration. The results showed that chitosan enhanced high systemic immune response. Another study, Ghendon et al. (2009) inoculated H5 inactivated influenza vaccine with chitosan as an adjuvant in mice by intramuscular administration. After 4 weeks of first vaccination or 10 days after second vaccination, the researchers found that vaccine could induce antibody titer. In addition, chitosan was used to enhance the efficacy of recombinant turkey herpesvirus expressing the fusion (F) gene of Newcastle disease virus (rHVT-NDV) vaccinated at 1-day old White Leghorn chicken (Rauw et al., 2010a). The lower antibody titers might be due to the mucoadhesive properties of chitosan (Hea et al., 1998). The mechanism of increasing adaptive immune response was described that chitosan can open the endothelial cell junction and pass through the junction after binding with an antigen and taken up by lymphoid tissue (Artursson et al., 1994; Schipper et al., 1997; van der Lubben et al., 2001). It is possible that the routes of vaccine administration might affect the levels of immune response. The chitosan has been used as an adjuvant for mucosal vaccination and increase adaptive immune response after intranasal administration in several studies (Illum et al., 2001; Moschos et al., 2004; Sui et al., 2010; Wang et al., 2012).

Interestingly, the tracheal lesion scores of birds received with 0.5% and 1% chitosan MG vaccine were significantly lower than birds received commercial MG vaccine after MG challenge suggesting that prepared chitosan vaccines could effectively reduce tracheal lesions. Cell mediated immunity and secretary IgA are responsible for MG pathogenesis protection (Elfaki et al., 1993; Reddy et al., 1998; Gaunson et al., 2000; Papazisi et al., 2002). In previous study, chitosan adjuvant could induce both humoral and cellular immunity after vaccination via subcutaneous route (Zaharoff et al., 2007). However, there is no evidence that chitosan adjuvant could induce secretory IgA after subcutaneous administration (van der Lubben et al., 2001). This study did not determine cell mediated immunity, but it is possible that chitosan MG vaccine induced cell mediated immunity resulting in reduced trachea lesion scores after MG challenge. In addition, the MG strain prepared as chitosan MG vaccines ofthe present study was heterologous strain with MG challenged strainsuggesting that the chitosan might induce antibody response across MG strains.

From PCR results, the numbers of positive samples were lower in 0.5, 1% chitosan MG vaccine and commercial MG vaccine groups at 13 weeks of age. However, the differences were not significant.

In summary, these results indicated that MG inactivated vaccine prepared with 0.5 or 1% concentration of chitosan served as an adjuvant could induce moderate systemic immune response after vaccination and decrease tracheal lesion scores against virulence Thai MG challenge. In addition, the results also demonstrated that chitosan is safe and effective and can be used as an adjuvant of inactivated vaccine. Preparation vaccine with chitosan adjuvant is a promising vaccination strategy.

2.5 Acknowledgments

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Tables & Figure

Table 1. Experimental designs of chickens in each group at 6 and 12 weeks old (n = 20).

Group	**Vaccination (intramuscular route) (6 weeks old)**	**Challenge (intratracheal route) (12 weeks old)**
1	0.5% chitosan	FMS brothpe
2	0.5% chitosan	MG strain AHRL 58/46
3	MG bacterin with 0.25% chitosan	MG strain AHRL 58/46
4	MG bacterin with 0.5% chitosan	MG strain AHRL 58/46
5	MG bacterin with 1% chitosan	MG strain AHRL 58/46
6	MG-Bac® (Zoetis)	MG strain AHRL 58/46

Table 2. Serology of birds of each group at different ages. Challenged MG organisms by intratracheal route at 12 weeks old excluding group 1 (challenged with FMS)

Group	**SPA**					**ELISA**				
	Age (weeks)									
	12	13	14	15	16	12	13	14	15	16
1	0/20[A]	0/20	0/15	0/10	0/5	0/20	0/20	0/15	0/10	0/5
2	0/20	20/20	15/15	10/10	5/5	0/20	0/20	4/15	1/10	5/5
3	11/20	20/20	15/15	10/10	5/5	0/20	20/20	15/15	10/10	5/5
4	16/20	20/20	15/15	10/10	5/5	6/20	19/20	15/15	10/10	5/5
5	11/20	20/20	15/15	10/10	5/5	5/20	20/20	15/15	10/10	5/5
6	20/20	20/20	15/15	10/10	5/5	20/20	20/20	15/15	10/10	5/5

Group 1 = negative control, group 2 = positive control, group 3 = 0.25% chitosan bacterin, group 4 = 0.5% chitosan bacterin, group 5 = 1% chitosan bacterin and group 6 = commercial bacterin

[A] Number of MG positive samples/ total tested samples

Table 3. Air sac and trachea lesion scores of birds of each group at different ages (mean ± standard deviation) (n=5). Challenged MG organisms by intratracheal route at 12 weeks old excluding group 1 challenged with FMS

Group	**Air sac lesion scores**				**Trachea lesion scores**			
	Age (weeks)							
	13	14	15	16	13	14	15	16
1	0.2±0.3	0.1±0.2	0±0	0±0	0.8±0.6^{a}	0.60±0.3^{a}	0.3±0.3^{a}	0.5±0.5^{a}
2	0.3±0.5	0.1±0.2	0.3±0.5	0.1±0.2	2.4±0.6^{b}	2.7±0.3^{b}	2.9±0.1^{b}	3.0± 0.0^{b}
3	0.8±0.3	0.5±0.5	0.2±0.3	0.1±0.2	2.6±0.5^{b}	2.6±0.4^{b,c}	2.8±0.2^{b}	2.6±0.1^{b,d}
4	0.3±0.5	0.2±0.3	0.5±0.4	0.2±0.3	2.1±0.8^{b,c}	2.2±1.0^{c,d}	1.7±0.6^{c}	2.2±1.0^{c,d}
5	0.3±0.5	0.2±0.3	0.3±0.4	0±0	1.8±0.4^{c}	1.5±0.2^{d}	2.3±0.5^{b,c,d}	1.9±0.7^{c}
6	0.3±0.5	0.2±0.3	0.2±0.3	0.2±0.3	2.5±0.3^{b}	2.2±0.3^{c}	2.4±0.2^{d}	2.3±0.5^{c,d}

Group 1 = negative control, group 2 = positive control, group 3 = 0.25% chitosan bacterin, group 4 = 0.5% chitosan bacterin, group 5 = 1% chitosan bacterin and group 6 = commercial bacterin

^{a,b,c,d} Different superscripts in the same column means statistically significant difference ($p<0.05$)

Table 4. MG DNA positive samples reisolated from of tracheal swabs of each group at different ages

Group	**Age (weeks)**			
	13	14	15	16
1	0/5[A]	0/5	0/5	0/5
2	5/5	5/5	5/5	4/5
3	5/5	5/5	5/5	3/5
4	4/5	5/5	5/5	3/5
5	4/5	5/5	5/5	4/5
6	3/5	5/5	4/5	4/5

[A] Number of MG DNA positive samples/ total tested samples

Figure

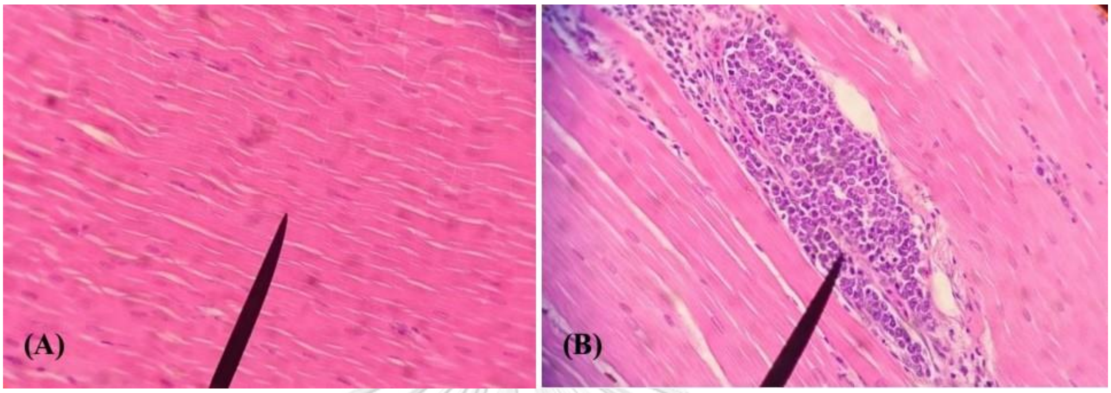

Fig. 1. Histologic view of pectoral muscle of vaccinated birds at 7 wk old (1 wk post vaccination). (A) No lymphocytic cell infiltration was observed in the pectoral muscle of birds in Group 5 (arrow). (B) Severe multifocal lymphocytic cell infiltration of pectoral muscle of a bird in Group 6 (arrow). H&E. 340.

CHAPTER 3

Chitosan-adjuvanted *Mycoplasma gallisepticum* bacterin via intraocular administration enhances *Mycoplasma gallisepticum* protection in commercial layers

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Chitosan-adjuvanted *Mycoplasma gallisepticum* bacterin via intraocular administration enhances *Mycoplasma gallisepticum* protection in commercial layers

Abstract

Mycoplasma gallisepticum (MG) causes respiratory signs and economic losses in the poultry industry. MG vaccination is one of the effective prevention and control measures that have been used around the world. Our previous study demonstrated that chitosan-adjuvanted MG bacterin could effectively reduce pathological lesions induced by MG and that chitosan could be used as an adjuvant in MG bacterin. The present study determined the efficacy of MG bacterins against the Thai MG strain was based on vaccine programs. Seven groups (25 layers/group) were intramuscular (IM) or intraocular (IO) with MG bacterins containing 0.5% chitosan or a commercial bacterin at 6 and 10 weeks of age. Sham-negative and sham-positive controls were groups 1 and 2, respectively. Group 3: IM route of chitosan bacterin followed by IM route of chitosan bacterin, group 4; commercial bacterin via IM route followed by chitosan bacterin via IO route, group 5; commercial bacterin via IM route followed by commercial bacterin via IM route, group 6; chitosan bacterin via IM followed by chitosan bacterin via IO route and group 7, chitosan bacterin via IO route followed by chitosan bacterin via IO route were determined. At sixteen weeks of age, all groups excluding group 1 were challenged intratracheally with 0.1 mL containing Thai MG strain 10^7 colonies forming unit. At 17, 18 and 20 weeks of age, five birds in each group were bled for serological testing and swabbed at the choanal cleft for the quantitative real-time PCR assay, euthanized and necropsied. The results showed that birds vaccinated with a commercial intramuscular bacterin followed by an intraocularly chitosan adjuvant bacterin showed the best protection against the MG challenge. The study indicated that chitosan could be the effective mucosal adjuvant to increase the effectiveness of MG bacterin.

Key words: *Mycoplasma gallisepticum,* chitosan-adjuvanted bacterin, commercial bacterin, layer, mucosal adjuvant

3.1 Introduction

Mycoplasma gallisepticum (MG) is one of the important pathogens of poultry around the world. MG causes chronic respiratory disease (CRD) leading to respiratory symptoms including rales, coughs, sneezing, ocular discharge and nasal discharge. In addition, MG infection increases condemnation in processing plants and degradation of carcasses, causing economic losses in the poultry industry (Raviv and Ley, 2013). To reduce the economic impact of MG infection in chickens, there are 3 commercial vaccines, which have been used for the prevention program. Live, inactivated, and recombinant MG poultry (rFP-MG) vaccines are commonly used in the poultry industry. The efficacy of each type of vaccine in the protection of clinical signs and induction of the systemic immune response is different; however, these vaccines can reduce post-challenge production losses with the MG field strain (Carpenter et al., 1981; Whithear et al., 1990; Evans and Hafez, 1992; Zhang et al., 2010). Commercial MG vaccines are strains; F, ts-11 and 6/85. F strain vaccine is more virulent compared to other live vaccines (Raviv and Ley, 2013). It may induce mild respiratory signs in turkeys and broilers (Rodriguez and Kleven, 1980). Other vaccine strains including ts-11 and 6/85 showed less protection against the MG challenge compared to the F strain (Abd-El-Motelib and Kleven, 1993). Although the inactivated vaccine is safe for use in turkeys and may reduce production losses in chickens and turkeys, the inactivated vaccine is more expensive and cannot protect MG infection in the respiratory tract (Panigraphy et al., 1981). For the recombinant vaccine, rFP-MG, the MG surface protein is encoded by the recombinant avian poxvirus (Vectormune® FP-MG vaccine, CEVA Santé Animale). Although FP-MG is safe to vaccinate in chickens (Zhang et al., 2010), it did not show good protection against the MG challenge (Ferguson-Noel et al., 2012). Recently, the novel vaccine adjuvant has been developed to improve the efficacy of the vaccine. Recently, the novel vaccine adjuvant has been developed to improve the efficacy of the vaccine. Chitosan is one of the potential adjuvants for the inactivated vaccine and is a polysaccharide made from deacetylation of chitin. Several studies found that chitosan could enhance the humoral and cell-mediated immune response in mice (Zaharoff et al., 2007) and chickens (Rauw et al., 2010a). From our previous study, chitosan could be used as an adjuvant for MG bacterin (Limsatanun et al., 2016). The chitosan-adjuvanted MG bacterin provided better protection of the tracheal lesion and caused less tissue reaction at the site of injection, compared to the commercial bacterin MG. Despite the fact that it has produced a low level of systemic immune response. Experimental groups receiving bacterin with chitosan adjuvant had lower numbers of antibody-positive birds compared to commercial MG bacterin group (Limsatanun et al., 2016). However, the efficacy of the vaccine from the previous study was determined only by intramuscular route, which could provoke tissue reaction and low immune response to the mucosa for vaccination with MG from poultry. To improve MG-induced immunity, different administrations including intraocular routes and vaccination protocols, which included only MG bacterin adjuvanted with chitosan and/or commercial MG bacterin, were explored in the present study.

3.2 Materials and methods

3.2.1 Vaccine

MG bacterin with chitosan adjuvant was prepared and described by Limsatanun et al. (2016). Briefly, Thai MG strain AHRL 20/52 was propagated in Frey broth supplemented with 15% porcine serum (FMS) (Kleven, 2008) and incubated at 37 ° C until the color of broth changed from pink to yellow-orange color or phase log. The culture broth was centrifuged at 12,000 xg at 4 ° C for 30 min to collect MG cells. MG cells were inactivated with 0.01% thimerosal (Sigma-Aldrich Corp., St. Louis, MO) in phosphate buffered saline (PBS) and incubated at 4 ° C for 7 days (Chukiatsiri et al., 2009). Chitosan solution (Sigma-Aldrich Corp., St. Louis, MO) was added to the MG cells to a final 0.5% (w/v) concentration. Two concentrations of MG organisms were determined in the bacterin with chitosan adjuvant. Bacteria were used with chitosan adjuvant containing approximately 5 x 10^8 MG microorganisms and 5 x 10^{11} colony forming units (CFU)/mL for intramuscular and intraocular administration, respectively. All the chitosan-adjuvanted MG bacterins were stored at 4 ° C until use.

3.2.2 Experimental Design and Sample Collection Protocol

One hundred and seventy-five, a one-day-old commercial female layer chickens, Isa brown breed were obtained from MG and MS free breeder farm and raised in wire cages in the isolated room. All birds received food and drinking water ad lib. At 6 weeks of age, 30 birds were bled at random for MG antibody titers and swabbed at the choanal cleft. Each cotton swab was placed in 2 mL of Frey medium supplemented with 15% porcine serum (FMS) broth for standard PCR to confirm free MG. All birds were divided into 7 groups, 25 birds each. Groups 1 and 2 served as sham negative control and sham positive control, respectively. Groups 3 and 6 received 0.5 mL of adjuvant bacterin with 0.5% chitosan containing approximately 2.5 x 10^8 CFU MG organisms via the intramuscular route. Groups 4 and 5 received commercial MG bacterin (MG-Bac®, Zoetis, Florham Park, NJ) intramuscularly. Group 7 received 0.1 mL of bacterin with 0.5% chitosan containing approximately 5 x 10^{10} CFU MG organisms, which were divided into both eyes intraocularly. At 10 weeks of age, groups 3 and 5 received 0.5 mL of 0.5% chitosan-adjuvanted bacterin containing approximately 2.5 x 10^8 CFU MG microorganisms and commercial MG bacterin intramuscularly, respectively. Groups 4, 6 and 7 received 0.1 mL of adjuvant bacterin with 0.5% chitosan containing approximately 5 x 10^{10} CFU MG microorganisms as described above (Table 1). Clinical signs, including nasal and ocular discharges, were observed in the groups receiving intraocular administration. At 16 weeks of age, all birds were bled for MG serology by means of the serum plate agglutination (SPA) test and ELISA and then inoculated with 0.1 mL of FMS or 0.1 mL of Thai MG organisms strain AHRL 58/46 containing approximately 1x10^7 CFU intratracheally. At 1, 2 and 4 weeks after inoculation, for MG serology as previously described. Five birds in each group at 1, 2 and 4 weeks post inoculation were randomly selected, swabbed at the choanal cleft, euthanized and assayed to blindly assess the gross thoracic air sacs and histopathological tracheal lesions. The cotton swab was placed in 1 mL of PBS for the quantitative real-time PCR assay. For histopathological tracheal lesion scores, each trachea preserved in 10% formalin was cut into 4 sections as follows: 1 proximal end, 2 middle sections and 1 distal end, and subjected to histopathological examination. The protocol for the use of animals in this study was approved by Institutional Animal Care and Use (IACUC), Faculty of Veterinary Sciences, Chulalongkorn University (Protocol No. 13310081).

3.2.3 MG Serology

3.2.3.1 Serum Plate Agglutination (SPA) Test

In this study, the commercial MG antigen, Soleil® (Biovac Animal Health, Beaucouze, France) was used. The test was followed by the procedure described in the manufacturer's instructions. Briefly, 30 µL of serum was mixed with 30 µL of MG antigen on a glass slide at room temperature for 2 min. The positive reaction was identified by clumping reaction. If the serum tested showed a positive reaction, the sera were diluted to a 1:8 dilutions with PBS and then re-analyzed as described above.

3.2.3.2 Enzyme Linked Immunosorbent Assay (ELISA)

MG-ELISA test kits (Synbiotics Corporation, Kansas City, MO, USA) were used to detect MG-specific antibody titers following the manufacturer's instructions. Briefly, the diluted serum was placed on the MG antigen-coated plate, incubated, washed, followed by addition of anti-chicken immunoglobulin G labeled with horseradish peroxidase. After 30 min of the incubation period, the plate was washed extensively, solution. The plate was read in an ELISA reader at the optical density (OD) at 450 nm. The result was considered negative, suspect and positive when the antibody titers ranged from 0-148, 149-743 and above 744, respectively3.2.4 Polymerase Chain 3.2.4 Reaction (PCR) Assay.

The FMS broth samples were incubated at 37 ° C for 2 hours and then subjected to DNA extraction. Target DNA amplified by polymerase chain reaction (PCR) followed the protocol described by (Lauerman, 1998). Briefly, the culture broth was centrifuged at 15,000 xg for 6 min. The MG pellets were washed with distilled water and centrifuged twice. The pellets were re-suspended with approximately 30 μL of distilled water, depending on the size of the pellets. Samples were boiled for 10 min, then immediately placed on ice for 5 min and centrifuged at 15,000 xg for 2 min. The supernatant containing the DNA template was collected and maintained at -20 ° C until use. The PCR mixture was prepared at 25 μL volume containing 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 1.25 mM $MgCl_2$, 1 mM dNTP (Thermo Scientific, Vilnius, Lithuania) 10 pmole each of primer F 5'-GAGCTAATCTGTAAAGTTGGTC-3' and primer R 5'-GCTTCCTTGCGGTTAGCAAC-3') (Qiagen®, Valencia, CA, USA), 1.25 U Taq polymerase (Promega, Madison, WI, USA) and 2.5 μL (250 ng) of the DNA template. MG S6 strain (ATCC 15302) was used as the positive control. PCR mixtures were amplified in a DNA thermal cycler, Sprint® PCR (Thermo Electron Corporation, USA) At 94 ° C for 30 secs, 55 ° C for 30 s and 72 ° C for 60 sec for 40 cycles and followed by 72 ° C for 5 min. The PCR product was analyzed by 2% agarose gel electrophoresis (Pharmacia Biotech AB, Uppsala, Sweden), stained with 0.20 μg/mL ethidium bromide and visualized by the E-BOX VXII UV transilluminator (Vilber Lourmat, Eberhardzell, Germany).

3.2.5 Quantitative Real-time PCR Assay

3.2.5.1 DNA Extraction

The DNA samples were extracted using the QIAamp DNA Mini Kit (Qiagen®, Valencia, CA, USA) according to the manufacturer's instructions. After elution, the genomic DNA was collected and stored at -20 ° C until use.

3.2.5.2 Preparation of Standard DNA and Standard Curve

The standard DNA was prepared from the DNA of the partial *mgc2* gene from Thai MG strain AHRL58/46, which was amplified by PCR as previously described (Ferguson et al., 2005). The amplified MG DNA was gel purified using a gel extraction kit and a PCR cleaning kit (GenepHlow ™ Gel, Geneaid, Taipei, Taiwan) according to the manufacturer's instructions. The purified DNA was measured at 260 nm using the nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., MA, USA) and stored at -80 ° C until use. The purified *mgc2* gene was diluted in 10-fold dilution to perform real-time PCR and constructed a standard curve of Ct value versus copies/µL.

3.2.5.3 Primer and Probe Design

Probes for the *mgc2* gene of MG consisted of oligonucleotides with the 5 '6-carboxyfluorescein (FAM) reporter dye and the 3' quencher dye Black Hole Quencher-1 (BHQ1). The real-time TaqMan probe sequence of the *mgc2* gene was 5'6-FAMTGATGATCCAAGAACGTGAAGAACACC3'- BHQ1 (Integrated DNA Technologies, Coralville, IA, USA). The primers of the *mgc2* gene sequences were followed; forward 5'- ATCGGCAGAAGGGGCAAAGTAG- 3; reverse 5' -GCAACGCAGACTTCTCATCTTCAAG-3' (Raviv and Kleven, 2009). The reaction was followed the previously described protocol (Raviv and Kleven, 2009). The 25 μL PCR mixture contained 12.5 μL 2X QuantiFast Probe PCR Mix without ROX dye (Qiagen®, Valencia, CA, USA). The final concentration probe was 1.25 μL of each primer (0.5 μM), 0.5 μL of the probe (0.2 μM), 4.5 μL of nuclease-free water and 5 μL of DNA template solution. The PCR reaction was performed using Rotor-Gene®RG-3000 (Corbett Research, Sydney, Australia) under the following conditions: 95 ° C for 3 min and 40 denaturation cycles at 94 ° C for 3 s and annealing at 60 ° C for 30 s (Raviv and Kleven, 2009). Each amplification, fluorescence emission was measured at 518 nm for the FAM indicator dye and 534 nm for the quencher BHQ-1 dye. All data were analyzed using the Rotor-Gene software, v.6.0.19. The sample with a CT value less than or equal to 40 was considered positive.

3.2.6 Evaluation of Lesion Scores

The score of the gross thoracic injury was determined as described by (Kleven et al., 1972). Scores include: 0: no injury; 1: slight cloudiness of the air sac membrane; 2: slightly thicker air sac membrane with small accumulations of cheese exudates; 3: air sac membrane obviously thick and fleshy, with large accumulations of cheese exudates in an air sac; 4: same as 3, but 2 or more air sacs were involved.

The histopathological lesion score of the trachea was evaluated after the previously described (Yagihashi and Tajima, 1986) as followed; 0: no significant changes; 1: small aggregate of cells (mainly lymphocytes); 2: moderate thickening of the wall due to cellular infiltration and edema commonly accompanied by epithelial degeneration and exudation; 3: extensive thickening of the wall due to cellular infiltration with or without exudation.

3.2.7 Statistical Analysis

Gross air sac lesion scores and histopathological lesions of the trachea were analyzed using the Kruskal-Wallis test and the Mann-Whitney U test with a 95% confidence level ($P < 0.05$). Analysis of unidirectional variance (ANOVA) was performed on antibody titers in order to identify statistical significance at a 95% confidence level ($P < 0.05$). SPSS® version 22 was used for statistical analysis.

3.3 Results

3.3.1 Clinical Signs

After receiving the intraocular vaccination, all birds were normal, and no inflammatory reaction or eye irritation was observed. After the challenge, respiratory signs were not observed in all groups excluding group 2 (positive control). Some birds in group 2 showed mild respiratory signs, including sneezing and respiratory rales for 3-7 days. There was no death in all groups.

3.3.2 Serology

3.3.2.1 SPA Test

At 6 weeks of age, all birds were seronegative. At 16 weeks of age, seroconversions were observed in groups receiving chitosan-agglutinated MG bacterin and/or commercial MG bacterin including groups 3, 4, 5, 6 and 7 ranging from 4-20 birds of 24-25 birds; however, group 7 had the lowest number of positive birds. One week after the challenge, 15-20 of 19-20 birds in only MG challenge groups were positive. At 2 and 4 weeks post challenge, all birds in the MG challenge groups were positive. Group 1 did not show positive birds at any age (Table 2).

3.3.2.2 ELISA

At 6 weeks of age, all birds were seronegative. At 16 weeks of age, the antibody titer of birds in groups 3, 4, 5 and 6 ranged from 91-4717 (n = 24 or 25), while group 7 received only MG bacterin with the chitosan-adjuvant intraocular route at 6 and 10 weeks of age were seronegative. The highest number of bird antibodies was found in groups 4 and 5 (receiving commercial MG bacterin) and group 5 had the highest antibody titers ($P < 0.05$). At 1 week after exposure, the antibody titer of groups 3,4, 6 and 7 were found in the range 303-5277 (n = 19 or 20); however, the antibody titer of group 5 was greater than that of groups 3, 4, 6 and 7. After 2 and 4 weeks after stimulation, the antibody titer of groups 2, 3, 4, 5, 6 and 7 ranging from 774 -7774 (n = 14 or 15) and 2266-9787 (n = 9 or 10), respectively. Group 2 (positive control) had significantly lower antibody titer at 2 and 4 weeks post challenge, respectively ($P < 0.05$). Group 1 was seronegative at all ages (Table 2).

3.3.3 Lesion Score Evaluation

3.3.3.1 Gross Thoracic Air Sac Lesion Scores

At 16 weeks of age and 1, 2 and 4 weeks after inoculation, mean lesion scores of the gross thoracic air sacs of 5 birds ranged from 0-0.7. At 2 weeks after inoculation, group 2 (positive control) showed higher air sac lesion scores than the other groups. There were no significant differences between groups of the same age ($P > 0.05$) (Table 3).

3.3.3.2 Histopathological Tracheal Lesion Scores

At 16 weeks of age and 1, 2 and 4 weeks after inoculation, the histopathological scores of the tracheal lesions of 5 birds ranged from 0-1.7. No significant differences were observed in all groups at 16 weeks of age. After the inoculation, the mean histopathological scores of the tracheal lesion of group 1 (negative control) were significantly lower than the other groups ($P < 0.05$). At 1 week after the inoculation, the histopathological scores of mean tracheal lesion of groups 2 and 5 were significantly higher than those of groups 3, 4 and 6 ($P < 0.05$). At 4 weeks after inoculation, the mean histopathological scores of tracheal lesion of group 4 (which received commercial MG bacterin and chitosan-adjuvanted MG bacterin) were significantly lower than those of other inoculated groups ($P < 0.05$).

3.3.4 Conventional PCR and Quantitative Real-time PCR Assays

At 6 weeks of age (prior to vaccination), no MG DNA was detected in all FMS broth samples (data not shown). For the quantitative real-time PCR assay, the result was obtained from the standard curve between the number of copies and the cycle threshold. The linear equation of the standard curve was $y = -2.789X + 38.916$ with $R^2 = 0.998$. At 4 weeks after inoculation, birds in groups 4 and 6 (receiving chitosan-adjuvanted MG bacterin or MG commercially injected intramuscularly followed by chitosan-adjuvanted MG bacterin via intraocular administration) gave fewer copy numbers of DNA of the *mgc2* gene, while birds in groups 3 and 5 were the second groups that had low DNA numbers. MG DNA indicating positive samples was found in almost groups excluding group 1, ranging from 4-5 samples from 5 samples (Table 4).

3.5 Discussion

Chitosan is the potential mucosal adjuvant (Hea et al., 1998). There were several studies demonstrated that chitosan is the effective adjuvant, which can stimulate local immunity through mucosal immunization (Zaharoff et al., 2007; Ghendon et al., 2009; Sui et al., 2010). From the previous study, chitosan-adjuvanted MG bacterin could reduce the tracheal lesion score, while the antibody response was lower compared to the commercial MG bacterin (Limsatanun et al., 2016). To improve the efficacy of the vaccine, the different administrations and protocols of MG bacterin vaccination with chitosan adjuvant alone and/or commercial MG bacterin were explored in the present study.

Birds that received commercial MG bacterin showed significantly higher antibody titer similar to the previous study bacterin (Limsatanun et al., 2016). The MG bacterin with chitosan adjuvant did not improve systemic antibody response as compared to commercial MG bacterin, which was possibly caused by the efficacy of chitosan following intramuscular vaccination. As chitosan showed the prominent effect as a mucosal adjuvant (Illum et al., 2001; Moschos et al., 2005; Wang et al., 2012). The results of the present study are incompatible with other studies. Chitosan is the potential substance to use as mucosal and humoral immunostimulation (Jabbal-Gill et al., 1998; Sui et al., 2010). Jabbal-Gill et al. (1998) demonstrated that mice vaccinated with recombinant toxin containing chitosan as an adjuvant via intranasal route could stimulate a higher level of serum IgG and IgA secreting against Bordetella pertussis than those receiving only recombinant toxin. In other studies, the M2 influenza subunit vaccine using chitosan as an adjuvant could increase the higher systemic IgG and secretory IgA titers in mice after intranasal immunization compared to mice receiving the M2 subunit alone (Carpenter et al., 1981).). In contrast, the live mixture of Newcastle disease vaccine in the chitosan-PBS diluent, which was vaccinated in birds via the oculonasal route, slightly stimulated the systemic and local immune system, but effectively measured the cellular immune response (Rauw et al., 2010a). The low concentration of pathogenic cells in a distinct animal model probably caused the low level of systemic immune response (Rauw et al., 2010a).

Taking into account lesion scores, birds receiving intramuscular commercial bacterin or chitosan-adjuvanted bacterin and following with intraocular chitosan adjuvant showed better protection against tracheal lesion score than that of other groups. In particular, birds immunized with commercial bacterin at 6 weeks of age and chitosan-adjuvanted bacterin via the intraocular route at 10 weeks of age had significantly lower mean tracheal lesion score at 20 weeks of age. This study suggested that the chitosan-adjuvanted bacterin could enhance the local immunity of the birds to protect the tracheal lesion against MG challenge. In addition, the result of the real-time PCR assay suggested that the chitosan-adjuvanted bacterin reduced the number of MG microorganisms colonizing the trachea after administration via intramuscular route following with intraocular route. Although the mucosal immune response was not investigated in this study, there is strong evidence from several studies that chitosan adjuvant vaccines promote the mucosal immune response (McNeela et al., 2000; Rauw et al., 2010b; Svindland et al., 2012; Wang et al., 2012). In a previous study, intranasal administration of live influenza vaccine with chitosan adjuvant in mice stimulated IgA, IgG antibody, and virus-specific CD4$^+$ T cells, producing cytokines that received the adjuvant influenza subunit vaccine (Wang et al., 2012). In some studies, chitosan increased the immune response mediated by the nasal passages, especially IgA after mucosal administration of the subunit vaccine in mice (van der Lubben et al., 2001). However, the thoracic air sac lesion scores were fairly low in the MG inoculated groups possibly that the number of microorganisms in the MG inoculum was quite low or the MG strain used in this study was not virulent.

However, the number of MG microorganisms prepared in the bacterin with chitosan adjuvant for intraocular administration was greater than that of intramuscular administration in the present study. Because the volume of intraocular administration was lower than that of intramuscular administration. Although the largest number of MG microorganisms were used in the intraocular bacterial with chitosan adjuvant, no eye, and nasal irritation were observed in the vaccinated birds, suggesting that the chitosan adjuvant bacterin was safe and non-toxic for chickens.

In conclusion, chitosan is one of the potent mucosal adjuvants. The results suggested that chitosan-adjuvanted bacterin could be used as a mucosal adjuvant. Chitosan showed the potential to improve protection against MG infection through the mucosal route. Chitosan could be the promising promoter to improve the MG vaccination program.

3.6 Acknowledgements

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Tables

Table 1. Experimental designs of chickens of each group at 6, 10 and 16 weeks of age (n=25)

Group	**6 weeks of age**	**10 weeks of age**	**16 weeks of age**[1]
1	0.5% chitosan (IM)[2]	0.5% chitosan (IM)	FMS broth
2	0.5% chitosan (IM)	0.5% chitosan (IM)	MG strain AHRL 58/46
3	Chitosan-adjuvanted vaccine (IM)	Chitosan-adjuvanted vaccine (IM)	MG strain AHRL 58/46
4	MG-Bac® (IM)	Chitosan-adjuvanted vaccine (IO)	MG strain AHRL 58/46
5	MG-Bac® (IM)	MG-Bac® (IM)	MG strain AHRL 58/46
6	Chitosan-adjuvanted vaccine (IM)	Chitosan-adjuvanted vaccine (IO)	MG strain AHRL 58/46
7	Chitosan-adjuvanted vaccine (IO)[3]	Chitosan-adjuvanted vaccine (IO)	MG strain AHRL 58/46

[1] Challenged by intratracheal route

[2] (IM) = intramuscular route

[3] (IO) = intraocular route

Table 2. Serology determined by SPA and ELISA antibody titers of each group at 16, 17, 18 and 20 weeks of age.

Group[1]	**SPA**[2]				**ELISA antibody titers**[3]			
	16	**17**	**18**	**20**	**16**	**17**	**18**	**20**
1	0/25	0/20	0/15	0/10	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}
2	0/25	20/20	15/15	10/10	0±0^{a}	0±0^{a}	774±568^{a}	2266± 1528^{a}
3	17/25	19/20	15/15	10/10	680±620^{a,b}	1533±1166^{a,b}	6484±1636^{b}	6947±2170^{b,c}
4	16/25	15/19[4]	14/14	9/9	1606±151^{b}	1951±1901^{b}	7774±1354^{b}	9072±957^{b}
5	20/24[4]	18/19	14/14	9/9	4717±276^{c}	5277±3706^{c}	7691±2343^{b}	9532±1300^{b}
6	18/25	17/20	15/15	10/10	91±343^{a}	1187±1672^{a,b}	6295±2117^{b}	9787±2385^{b,c}
7	4/25	19/20	14/14[4]	9/9	0±0^{a}	303±854^{a,b}	4246±1773^{c}	5895±3055^{c}

[1] Group 1, negative control; group 2, positive control; group 3, bird vaccinated with Chitosan-adjuvanted vaccine (IM) at 6 and 10 weeks of age; group 4, bird vaccinated with MG-Bac® (IM) at 6 weeks of age and chitosan-adjuvanted vaccine (IO) at 10 weeks of age; group 5, bird vaccinated with MG-Bac® (IM) at 6 and 10 weeks of age; group 6, bird vaccinated with Chitosan-adjuvanted vaccine (IM) at 6 weeks of age and chitosan-adjuvanted vaccine (IO) at 10 weeks of age; group 7, bird vaccinated with Chitosan-adjuvanted vaccine (IO) at 6 weeks of age and Chitosan-adjuvanted vaccine (IO) at 10 weeks of age.

[2] Number of MG positive birds/ total tested birds

[3] Expressed as mean ± SD. The number of tested birds were same as that of tested birds of SPA

[4] One bird died during this week; no remarkable lesion was observed.

[a,b,c] Different superscripts in the same column mean statistically significant difference ($P < 0.05$).

Table 3. Air sac and tracheal lesion scores of each group at 16, 17, 18 and 20 weeks of age.

Group[1]	**Air sac lesion scores**[2]				**Tracheal lesion scores**[2]			
	16	**17**	**18**	**20**	**16**	**17**	**18**	**20**
1	0±0	0.1±0.2	0±0	0.1±0.2	0.3±0.3	0.1 ± 0.2^{a}	0.4 ± 0.2^{a}	0.1 ± 0.1^{a}
2	0±0	0.4±0.5	0.7±0.6	0.2± 0.3	0.2±0.1	1.7 ± 0.6^{c}	1.0 ± 0.5^{b}	1.4 ± 0.7^{b}
3	0±0	0.2±0.3	0.2±0.3	0±0	0±0.1	1.0 ± 0.4^{b}	1.4 ± 0.4^{b}	1.5 ± 0.5^{b}
4	0±0	0.1±0.2	0.1±0.2	0.2±0.4	0±0	1.0 ± 0.2^{b}	1.0 ± 0.9^{b}	0.8 ± 0.3^{c}
5	0±0	0±0	0.4±0.4	0.2±0.4	0±0.1	1.7 ± 0.4^{c}	1.4 ± 0.4^{b}	1.4 ± 0.6^{b}
6	0±0	0.1±0.2	0.4±0.4	0.1±0.2	0.1±0.1	1.0 ± 0.4^{b}	1.0 ± 0.5^{b}	1.3 ± 0.3^{b}
7	0±0	0.5±0.4	0.3±0.4	0.1±0.2	0.1±0.1	$1.3\pm0.5^{b,C}$	1.5 ± 0.6^{b}	1.5 ± 0.5^{b}

[1] Group 1, negative control; group 2, positive control; group 3, bird vaccinated with Chitosan-adjuvanted vaccine (IM) at 6 and 10 weeks of age; group 4, bird vaccinated with MG-Bac® (IM) at 6 weeks of age and chitosan-adjuvanted vaccine (IO) at 10 weeks of age; group 5, bird vaccinated with MG-Bac® (IM) at 6 and 10 weeks of age; group 6, bird vaccinated with Chitosan-adjuvanted vaccine (IM) at 6 weeks of age and chitosan-adjuvanted vaccine (IO) at 10 weeks of age; group 7, bird vaccinated with Chitosan-adjuvanted vaccine (IO) at 6 weeks of age and Chitosan-adjuvanted vaccine (IO) at 10 weeks of age.

[2] Expressed as mean ± SD and n=5.

[a,b,c] Different superscripts in the same column mean statistically significant difference ($P < 0.05$).

Table 4. MG DNA from choanal cleft samples expressed as DNA load and number of MG DNA positive samples at 17, 18 and 20 weeks of age.

Group[1]	**DNA load and number of MG DNA positive samples**[2]		
	17	**18**	**20**
1	0 (0/5)	0 (0/5)	0 (0/5)
2	932.0±0.1 (5/5)	678.0±3.9 (4/5)	398.0±3.2 (5/5)
3	1179.0±2.0 (5/5)	49.0±3.0 (5/5)	38.0±2.2 (5/5)
4	411.0±2.3 (5/5)	504.0±0.8 (5/5)	1.1±0.4 (4/5)
5	812.0±4.4 (5/5)	267.0±0.6 (5/5)	31.2±1.3 (5/5)
6	1448.0±2.3 (5/5)	595.0±3.4 (4/5)	5.0±0.2 (4/5)
7	469.0±3.5 (4/5)	131.0±2.8 (5/5)	204.0±2.6 (5/5)

[1] Group 1, negative control; group 2, positive control; group 3, bird vaccinated with Chitosan-adjuvanted vaccine (IM) at 6 and 10 weeks of age; group 4, bird vaccinated with MG-Bac® (IM) at 6 weeks of age and chitosan-adjuvanted vaccine (IO) at 10 weeks of age; group 5, bird vaccinated with MG-Bac® (IM) at 6 and 10 weeks of age; group 6, bird vaccinated with Chitosan-adjuvanted vaccine (IM) at 6 weeks of age and chitosan-adjuvanted vaccine (IO) at 10 weeks of age; group 7, bird vaccinated with Chitosan-adjuvanted vaccine (IO) at 6 weeks of age and Chitosan-adjuvanted vaccine (IO) at 10 weeks of age.

[2] DNA load (copies/µL) and expressed as mean ± SD and n=5, (Number of MG DNA positive samples/ total tested samples). No significant difference was observed in the same age ($P > 0.05$)

CHAPTER 4

Characterization of Thai *Mycoplasma gallisepticum* strains by sequence analysis of partial mgc2 gene

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Characterization of Thai *Mycoplasma gallisepticum* strains by sequence analysis of partial mgc2 gene

Abstract

Mycoplasma gallisepticum (MG) is one of the important pathogen which causes economic impact of poultry industry worldwide. Molecular characterization is the effective method to study the relation among international strains and epidemiology of MG transmission. In this study, 17 Thai MG strains were characterized by using 2 molecular techniques including random amplified polymorphic DNA (RAPD) and gene-targeted sequencing (GTS). The results showed that RAPD could distinguish among Thai MG isolates into 3 patterns. Most of MG isolates obtained from the same area were in the same pattern. While the partial *mgc2* gene was used to distinguish between Thai MG strains and MG strains from various countries. The phylogenetic tree of nucleotide sequence of *mgc2* gene showed that 11 Thai MG strains had 100% similarity sequence to Indian MG strains and one strain was 100% similar to Israel MG strain. Targeted partial *mgc2* gene could characterize Thai MG strains into 4 groups while RAPD pattern classified Thai MG isolates to 3 groups. These results gave us the interesting data about the relation of Thai MG strains and MG from other countries. GTS might be the new optional technique to monitor MG outbreak in Thailand

Keywords: Gene-targeted sequencing (GTS), *mgc2* gene, *Mycoplasma gallisepticum*, random amplified polymorphic DNA (RAPD), Thailand

4.1 Introduction

Mycoplasma gallisepticum (MG) is still being the one of significant pathogens in poultry industry in Thailand. MG causes chronic respiratory disease in chicken, dropping in eggs production and carcasses degradation (Raviv and Ley, 2013). MG is the important respiratory pathogen worldwide. It can transmit through egg from infected hen and contaminate in aerosol (Raviv and Ley, 2013). Eradication of MG from infected farm is the best prevention strategy. However, the eradication is impractical to do in Thailand. Therefore, biosecurity, surveillance and vaccination become the important management of MG prevention. There are 3 available commercial types of MG vaccines, which have been used including MG bacterin, attenuated live MG vaccines (F, 6/85 and ts-11 strains) and recombinant fowlpox-MG vaccine (rFP-MG) (Carpenter et al., 1981; Whithear et al., 1990; Evans and Hafez, 1992; Zhang et al., 2010). Because of the increase using of attenuated live MG vaccine, the effective techniques to differentiate MG vaccine strain and field strain are required. The common method to differentiate is the random amplification of polymorphic DNA (RAPD) assay (Geary et al., 1994; Fan et al., 1995). However, RAPD assay has several disadvantages including low reproducibility and difficult to compare results among laboratories (Tyler et al., 1997; Mettifogo et al., 2006). Ferguson et al. (2005) introduced the new molecular technique known as gene-targeted sequencing (GTS) assay to differentiate MG strains. The GTS assay has high reproducibility and can compare results among laboratories; however, this assay is time consuming and more expensive comparing to RAPD assay. The *mgc2* gene is the one of targeted genes, which have been used in several epidemiological studies of MG (Gharaibeh and Roussan, 2008; Sprygin et al., 2011; Moretti et al., 2013; Khalifa et al., 2014). This gene encodes Mgc2 protein that works with GapA protein relating to cell attachment of MG (Boguslavsky et al., 2000). The purposes of this study were to determine the RAPD assay of Thai MG strains and *mgc2* gene of Thai MG strains. In addition, the *mgc2* gene of Thai MG strains were analysed and compared to that of other strains from various countries.

4.2 Materials and Methods

4.2.1 Sample collection

Seventeen of MG samples were collected from different regions in Thailand (Provided by Somsak Pakpinyo, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University). These samples were obtained from broiler breeder, broiler and layer farms, which were not vaccinated with attenuated live MG vaccines, located in the central, eastern and western parts of Thailand during 2003-2009 (Table 1). The samples were taken from choanal cleft and confirmed as pure MG isolates by MG PCR and direct immunofluorescent assays. All MG cultures were stored in Frey’s broth medium at -80°C. All MG samples were thawed and re-propagated in 2 ml Frey’s medium until broth color changed to orange and were re-confirmed MG positive and MS negative results by PCR (Lauerman, 1998). All samples were prepared for the *mgc2* gene-targeted PCR and RAPD assays (Table 1).

Four reference strains were used in this study. F strain is vaccine strain which was provided by local distributor (MSD, Thailand) and S6 strain was obtained from the ATCC (15302). The ts-11 and R strain sequences were retrieved from Genbank data (Table 1).

For MG differentiation analysis, eighteen *mgc2* gene sequences retrieved from 7 countries were used to compare alignments of *mgc2* gene sequences (Table 2). All *mgc2* gene sequence were obtained from Genbank data.

For MG differentiation analysis, eighteen *mgc2* gene sequences retrieved from 7 countries were used to compare alignments of *mgc2* gene sequences (Table 2). All *mgc2* gene sequence were obtained from Genbank data.

4.2.2 Polymerase chain reaction (PCR) assay

Briefly, the cultured broth was centrifuged at 15,000xg for 6 min. MG pellets were washed with distilled water and centrifuged twice. The pellets were re-suspended with approximately 30 µl of distilled water, depending on the size of pellets. The samples were boiled for 10 min, then immediately placed on ice for 5 min and centrifuged 15,000xg for 2 min. The supernatant containing the DNA template were collected and kept at -20°C until use. The PCR mixture were prepared at 25 µl volume containing 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 1.25 mM $MgCl_2$, 1 mM dNTP (Thermo Scientific, Vilnius, Lithuania) 10 pmole each of primer F (5'-GAGCTAATCTGTAAAGTTGGTC-3) and primer R (5'-GCTTCCTTGCGGTTAGCAAC-3') (Qiagen®, Valencia, CA, USA), 1.25 U Taq polymerase (Promega, Madison, WI, USA) and 2.5 µl (250 ng) of the DNA template. MG S6 strain (ATCC 15302) was used as a positive control. PCR mixtures were amplified in a DNA thermal cycler, PCR Sprint® (Thermo Electron Corporation, USA) with 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec for 40 cycles and followed by 72°C for 5 min. The PCR product was analyzed by 2% agarose gel electrophoresis (Pharmacia Biotech AB, Uppsala, Sweden), stained with ethidium bromide 0.20 µg/ml, and visualized by E-BOX VXII UV transilluminator (Vilber Lourmat, Eberhardzell, Germany).

4.2.3 RAPD analysis

RAPD assay was modified from (Ley et al., 1997). Briefly, PCR reaction was performed using a 25 µl volume of PCR mixture. Each RAPD mixture consisted of 2 mM $MgCl_2$, 1 mM dNTP (Fermentas, USA), 500 ng Geary primer set (Geary et al., 1994) (5'-CCGCAGCCAA-3') (Qiagen, Germany), 2.5 U of Taq polymerase (Promega, USA), and 0.5 µl of MG DNA containing 50 ng DNA. The amplification reactions were performed four cycles of 94˚C for 5 min, 36˚C for 5 min, and 72˚C for 5 min, ending with 30 cycles of 94˚C for 1 min, 36˚C for 1 min, and 72˚C for 1 min, and final elongation of 72˚C for 10 min. The PCR banding pattern or genotypic profile was analyzed by agarose gel electrophoresis. Samples were interpreted as indistinguishable when no major band differences were found (Fig 1).

4.2.4 PCR amplification of mgc2 gene

Genomic DNA was extracted individual sample in FMS broth by using the QIAamp DNA Mini Kit (Qiagen®; Valencia, CA) according to the manufacturer's recommendations. To amplify *mgc2* gene, the primers and reaction were conducted following the previously described protocol (Ferguson et al., 2005). Briefly, the 25 µl of PCR mixture contained 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 1.25 mM $MgCl_2$, 1 mM dNTP (Thermo Scientific, Vilnius, Lithuania) 10 pmole each of primer F (5-GCTTTGTGTTCTCGGGTGCTA- 3) and primer R (5' -

CGGTGGAAAACCAGCTCTTG-3') (Qiagen®, Valencia, CA, USA), 1.25 µl of Taq polymerase (Promega, Madison, WI, USA) and 2.5 µl (125 ng) of the DNA template. The amplification reaction was performed in a DNA thermal cycler, PCR Sprint® (Thermo Electron Corporation, USA) with condition at 94 C for 3 min, and 40 cycles of 94 ˚C for 20 s, 58 ˚C for 40 s, 72 ˚C for 60 s, and 72 ˚C for 5 min. The PCR product was analyzed by 2% agarose gel electrophoresis (Pharmacia Biotech AB, Uppsala, Sweden), stained with ethidium bromide 0.20 µg/ml, and visualized by E-BOX VXII UV transilluminator (Vilber Lourmat, Eberhardzell, Germany). The amplified *mgc2* gene product was 824 base pairs.

4.2.4 Sequencing and Sequence Analysis of mgc2 gene

Products of amplified *mgc2* gene were subjected to sequence (Malaysia Genomics Resource Centre, Malaysia). All sequence data were analyzed with Editseq program (Lasergene, DNASTAR Inc., USA) and constructed the consensus with Seqman program (Lasergene, DNASTAR Inc., USA). Thai and reference gene sequence data were aligned to construct phylogenetic tree by clustal-W method with Molecular Evolutionary Genetic Analysis (MEGA 7) software (http://www.megasoftware.net).

4.3 Results

From RAPD analysis, the samples were divided into 3 different RAPD patterns based on major band presence (Table 1). AHRU/2002/CU0111.3, AHRU/2003/CU5113.2 and AHRU/2003/CU5311.2 MG strains showed same pattern of major band as F and S6 strains. Eleven Thai MG strains were categorized as the same group. Nine out of 11 strains were collected from the same geographical area.

The sequence of *mgc2* gene of Thai MG strains were compared with MG strains from various countries based on Maximum likelihood method of MEGA7 program. The data showed the sequenced similarity ranged from 93.85-100% (Fig. 2). The similarity of *mgc2* gene sequences of Thai strains and American strains ranged from 93.85-99.61%, while comparing with Australian strains showed ranged from 96.77-99.01%. There were 11 Thai MG strains, which showed 100% similarity to MGS1345 and MGS19B strains from India, and KS2 strain from Israel. When compared the sequence of Thai MG strains with vaccine strains, Thai strains showed 93.85-95.8% and 96.77-98.40% similarity to F and ts-11 strains, respectively.

4.4 Discussion

To control MG infection, attenuated live MG vaccines have been using worldwide. Therefore, technique which can identify between wild type and vaccine strains becomes more important. The effective technique will be useful to rapidly recognize and tracking the source of MG outbreak. In this study, RAPD assay and molecular techniques were used to differentiate Thai MG strains. RAPD procedure, which was introduced by Geary et al. (1994) was used in this study. All of Thai MG strains were categorized into 3 groups based on the visual band pattern. Three of Thai MG strains showed the same band pattern as F and S6 strains. Two strains were isolated from central part and another one was isolated from eastern part of Thailand. However, all Thai MG strain samples in the present study were obtained from unvaccinated poultry farms; therefore, these three strains could be exposed or contaminated with the F strain vaccinated flock. Most of MG isolates from the same area were grouped into the same group. Suggesting that, MG outbreak might take place in the same or nearby area and spread to other farms in other areas.

Considering the molecular technique result, *mgc*2 gene is one of the MG genes, which have been used in MG molecular characterization (Gharaibeh and Roussan, 2008; Sprygin et al., 2011; Moretti et al., 2013; Khalifa et al., 2014), and showed the high discriminatory power for differentiate MG strain. Armour et al. (2013) identified MG sequences with IGSR, *mgc2*, MGAL0319 and *gapA* genes. The result showed that *mgc2* gene was the best sequence to discriminate between MG isolates.

From GTS analysis, 11 out of 17 Thai MG strains had 100% of partial *mgc2* gene similarity to MG strains of India and Israel. AHRU/2003/CU5004.2, AHRU/2003/CU5113.2 and AHRU/2009/CU2006.1 were MG Thai strains, which showed the highest similarity of partial *mgc2* sequence to MG strains of USA (99.61%), Australia (99.01%), Egypt (99.21%) and S6 strain (99.21%). Comparing to vaccine strain, there were not any Thai MG strains shared 100% similarity with F and ts-11 strains. The results of GTS showed the inconsistent with RAPD results, there were 6 Thai MG strains were categorized into the different groups from both techniques. These inconsistent results might be explained by the error of visual band observation and there are more undetectable bands which might have different patterns of RAPD. In this study, we used partial *mgc2* gene to characterize all samples. It might have an error during the process due to shortness of sequencing reads. The advantage of RAPD assay is save the time and cost (Maurer et al., 1998), whereas the GTS assay is time consuming. However, the sequencing technique can present the global comparison of MG strain typing with high discriminatory power (Ferguson et al., 2005).

This comparison of Thai MG strains to reference, vaccine and MG strains from various countries showed the relation among them. However, the relationship among Thai MG strains and MG strains of India and Israel was not concluded in the present study. The possible explanation might be the migration of wild birds and trading route, which were similar to the avian influenza virus (AIV) spreading. Sequencing technique could track down the AIV infected migratory waterfowl at breeding site in China. H5N1 AIV genomes was collected from migratory birds showed that closely related to the originated virus in Hong Kong 2004 isolate and it caused the emerging disease in southeast Asia (Liu et al., 2005). In further study, the *pvpA*, *IGSR*, *MGAL0319* or *gapA* genes should be determined to provide more information compare to one target gene analysis.

In conclusion, *mgc2* gene sequence analysis showed that most of Thai MG strains had 100% similarity sequence to MG strains of India and Israel. Sequencing technique shows the potential in MG characterization. This technique allows us to understand better of MG epidemiological control. It will aid us to find the new affordable technique for MG surveillance in Thailand.

4.5 Acknowledgements

This study was supported by grants from the Thailand Research Fund through the Royal Golden Jubilee Ph. D (No.Ph.D./0047/2553) to Limsatanun and Pakpinyo, Ratchadaphiseksomphot 2013 Endowment Fund of Chulalongkorn University (CU-56-347-FW), the 90th Anniversary of the Ratchadaphiseksomphot Endorsement Fund, the

Microbial Food Safety and Prof. Dr. Alongkorn Amonsin (Senio Scholar Award RTA 6080012).

Tables & Figures

Table 1. Description of Thai MG isolates used in this study and RAPD type.

Isolate	Year	Part of Thailand	Genbank accession	RAPD type
AHRU/2002/CU0111.3	2002	Central part	KX268616	1
AHRU/2003/CU0103.3	2003	Central part	KX268617	2
AHRU/2003/CU0701.2	2003	Eastern part	KX268618	3
AHRU/2003/CU0802.2	2003	Eastern part	KX268619	3
AHRU/2003/CU3101.2	2003	Eastern part	KX268620	3
AHRU/2003/CU3215.1	2003	Eastern part	KX268621	3
AHRU/2003/CU3302.3	2003	Eastern part	KX268622	3
AHRU/2003/CU3401.1	2003	Eastern part	KX268623	2
AHRU/2003/CU5004.2	2003	Central part	KX268624	2
AHRU/2003/CU5113.2	2003	Central part	KX268625	1
AHRU/2003/CU5311.2	2003	Eastern part	KX268626	1
AHRU/2003/CU5415.2	2003	Eastern part	KX268627	3
AHRU/2003/CU5505.3	2003	Eastern part	KX268628	3
AHRU/2003/CU5507.3	2003	Eastern part	KX268629	3
AHRU/2003/CU5713.2	2003	Eastern part	KX268630	3

AHRU/2003/CU5808.2	2003	Central part	KX268631	3
AHRU/2009/CU2006.1	2009	Western part	KX268632	3

Table 2. Description of MG references isolates used in this study

Isolate	Country	Genbank accession	Type of sample
K5152ACK01	USA	AY556289	ND
K4669ATK98	USA	AY556303	ND
K4781ATK99	USA	AY556272	ND
K5033ATK00	USA	AY556278	ND
K5109BCK01	USA	AY556286	ND
K4705CK99	USA	AY556271	ND
K5120CK01	USA	AY556288	ND
K4902TK00	USA	AY556284	ND
Au94043CK94	Australia	AY556300	ND
Au96022 CK96	Australia	AY556301	ND
Au99169CK99	Australia	AY556304	ND
KS2	Israel	AY556293	Broiler breeders
SA1Y12	South Africa	KC130903	ND
ZM1Y12	Zimbabwe	KC130907	ND
Eis10-17	Egypt	KY421065	ND
MGS849	India	KP300756	Broiler breeders

MGS1345	India	KP300762	Broiler breeders
MGS19B	India	KP279743	Broiler breeders
F	Vaccine strain	KX268633	-
ts 11	Vaccine strain	JQ770175	-
S6	Laboratory strain	KX268634	-
Strain R	Laboratory strain	AY556228	-

ND, Not determined

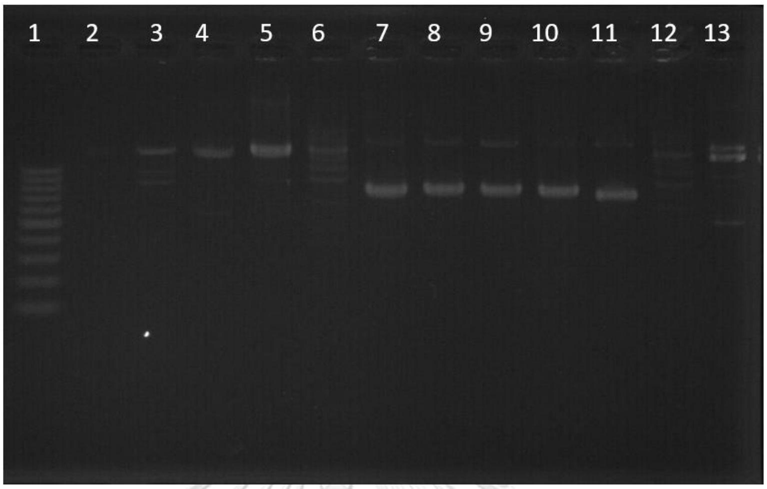


Fig. 1 RAPD analysis of Thai isolates MG with primer set described by Geary et al. (1994). Lane 1, molecular mass ladder; Lane 2, negative sample; Lane 3, F strain; Lane 4, S6 strain; Lane 5, AHRU/2003/CU5004.2; Lane 6, AHRU/2003/CU5113.2; Lane 7, AHRU/2003/CU5311.2; Lane 8, AHRU/2003/CU5415.2; Lane 9, AHRU/2003/CU5505.3; Lane 10, AHRU/2003/CU5507.3; Lane 11, AHRU/2003/CU5713.2; Lane 12, AHRU/2003/CU5808.2; Lane 13, AHRU/2009/CU2006.1.

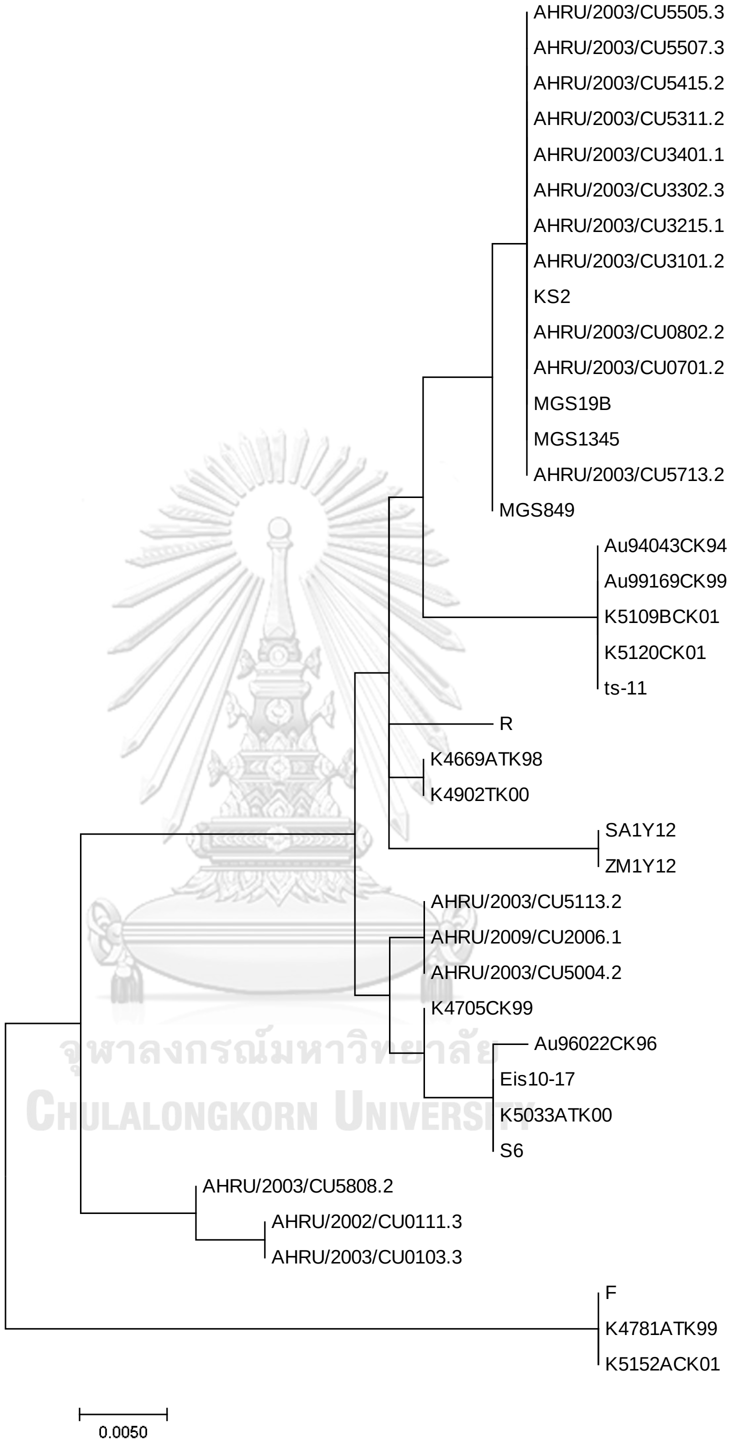

AHRU/2003/CU5505.3
AHRU/2003/CU5507.3
AHRU/2003/CU5415.2
AHRU/2003/CU5311.2
AHRU/2003/CU3401.1
AHRU/2003/CU3302.3
AHRU/2003/CU3215.1
AHRU/2003/CU3101.2
KS2
AHRU/2003/CU0802.2
AHRU/2003/CU0701.2
MGS19B
MGS1345
AHRU/2003/CU5713.2
MGS849
Au94043CK94
Au99169CK99
K5109BCK01
K5120CK01
ts-11
R
K4669ATK98
K4902TK00
SA1Y12
ZM1Y12
AHRU/2003/CU5113.2
AHRU/2009/CU2006.1
AHRU/2003/CU5004.2
K4705CK99
Au96022CK96
Eis10-17
K5033ATK00
S6
AHRU/2003/CU5808.2
AHRU/2002/CU0111.3
AHRU/2003/CU0103.3
F
K4781ATK99
K5152ACK01
0.0050

Fig 2. Phylogenetic tree of mgc2 gene constructed by Maximum Likelihood method using MEGA7 software. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 39 nucleotide sequences. There were a total of 509 positions in the final data set

CHAPTER 5

Conclusion

5.1 Research summary

Mycoplasma gallisepticum (MG) is still a problematic pathogen in poultry industry in Thailand causing the treatment costs and production losses. The main objective of this study is to investigate the safety and protective efficacy of chitosan-adjuvanted MG vaccine against the virulent MG strain. Chitosan-adjuvanted MG vaccine was prepared from Thai MG strain AHRL 20/52 and challenged with the virulent Thai MG strain AHRL 58/46. In the first study, the safety and protective efficacy of chitosan-adjuvanted MG vaccine were determined comparing with the commercial vaccine. From the result, chitosan-adjuvanted MG vaccine showed the milder inflammation tissue reaction at the injection area at 1, 2 and 3 weeks after vaccination. In addition, chitosan-adjuvanted MG vaccinated groups showed the significantly lower tracheal lesion score than commercial vaccinated group. However, chitosan-adjuvanted MG vaccinated groups had the lower antibody titers and number of positive antibody birds than group vaccinated with the commercial vaccine. In the second study, the experimental study was designed to determine the efficacy of chitosan-adjuvanted MG vaccine on vaccine program and administration routes. At 1 week after challenge, the bird vaccinated with chitosan-adjuvanted MG vaccine via either intramuscular (IM) route or intraocular (IO) route at 6 and 10 weeks of age showed the significantly lower tracheal lesion score than other groups. Interestingly, bird vaccinated with the commercial vaccine (IM) at 6 weeks of age and chitosan-adjuvanted vaccine (IO) at 10 weeks of age showed the lowest tracheal lesion score entire experimental period. In this study, discharge samples were swabbed from choanal cleft to determine the DNA load of MG organisms by using the Quantitative Real-Time PCR assay. All vaccinated groups could reduce the number of MG organisms in trachea of vaccinated birds. From the serology result, bird in experimental groups that received only chitosan-adjuvanted vaccines had the lower ELISA titers than the vaccinated groups with commercial vaccine. The results showed that birds vaccinated with a commercial vaccine (IM) followed by chitosan-adjuvanted vaccines (IO) showed the best protection against the MG challenge. Indicating that, chitosan is a promising adjuvant. Chitosan-adjuvanted vaccine had the low viscosity which is easily prepared the vaccine and easily administered to the birds. After MG challenge, the chitosan-adjuvanted vaccine showed milder tracheal and air sac lesions and could induce the moderate systemic immune response without severe inflammation at injection area. To improve the protective efficacy, chitosan-adjuvanted vaccine could be used as the second vaccination followed by commercial MG bacterin.

In the last study, the objective of this study was to determine the molecular technique called as gene-targeted sequencing (GTS) assay for Thai MG strain characterization. The discriminatory power of this assay was compared with RAPD assay. From the result, GTS and RAPD assays could distinguish Thai MG strains to 4 and 3 groups respectively. GTS assay was also used to differentiate Thai MG strains and MG strains from other countries by using partial *mgc2* gene sequence. The results showed that most of Thai MG strains had 100% similarity to Indian and Israel strains. GTS assay could provide the epidemiology and surveillance data of MG status in Thailand.

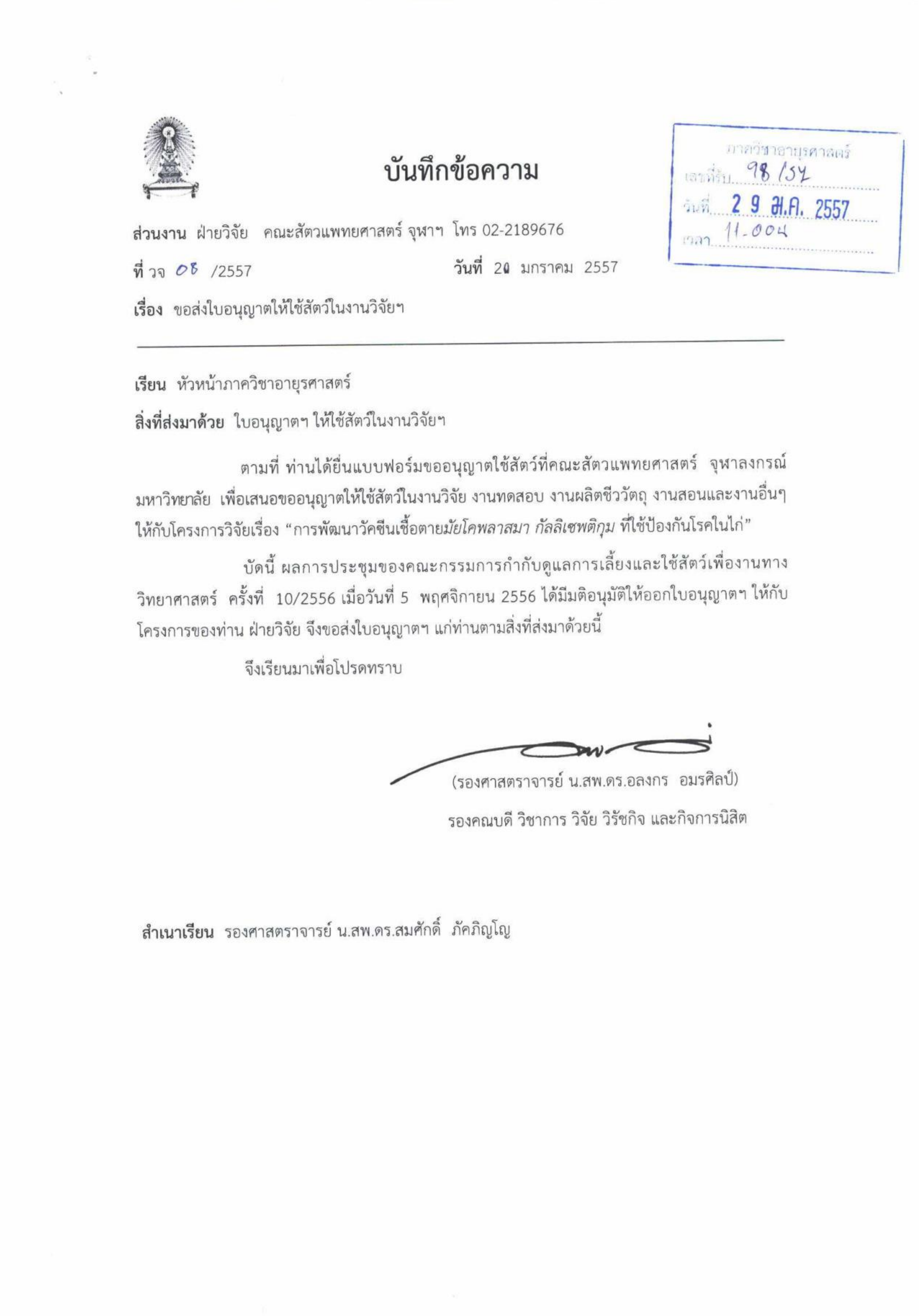
In summary, these studies provided the novel MG bacterin preparation and the use of GTS assay for MG surveillance in Thailand. In addition, these data will be useful for MG prevention and control program in poultry industry in Thailand.

5.2 Research limitation and further investigation

1. The concentration of MG cells in Chitosan-adjuvanted vaccine might not be enough to induce the proper systemic immunity.
2. The MG strain which was used in this study might not be virulent. Therefore, the severe lesions of challenged birds did not present.
3. GTS assay of multiple MG genes shows better discriminatory power than one gene analysis. In this study, only *mgc2* genes was used to determine GTS assay.

APPENDICES

Appendix A: The certification of Institutional Animal Care and Use Committee



ภาควิชาอายุรศาสตร์
เลขที่รับ 98/57
วันที่ 29 ม.ค. 2557
เวลา 11-00 น

บันทึกข้อความ

ส่วนงาน ฝ่ายวิจัย คณะสัตวแพทยศาสตร์ จุฬาฯ โทร 02-2189676

ที่ วจ 08 /2557 **วันที่** 20 มกราคม 2557

เรื่อง ขอส่งใบอนุญาตให้ใช้สัตว์ในงานวิจัยฯ

เรียน หัวหน้าภาควิชาอายุรศาสตร์

สิ่งที่ส่งมาด้วย ใบอนุญาตฯ ให้ใช้สัตว์ในงานวิจัยฯ

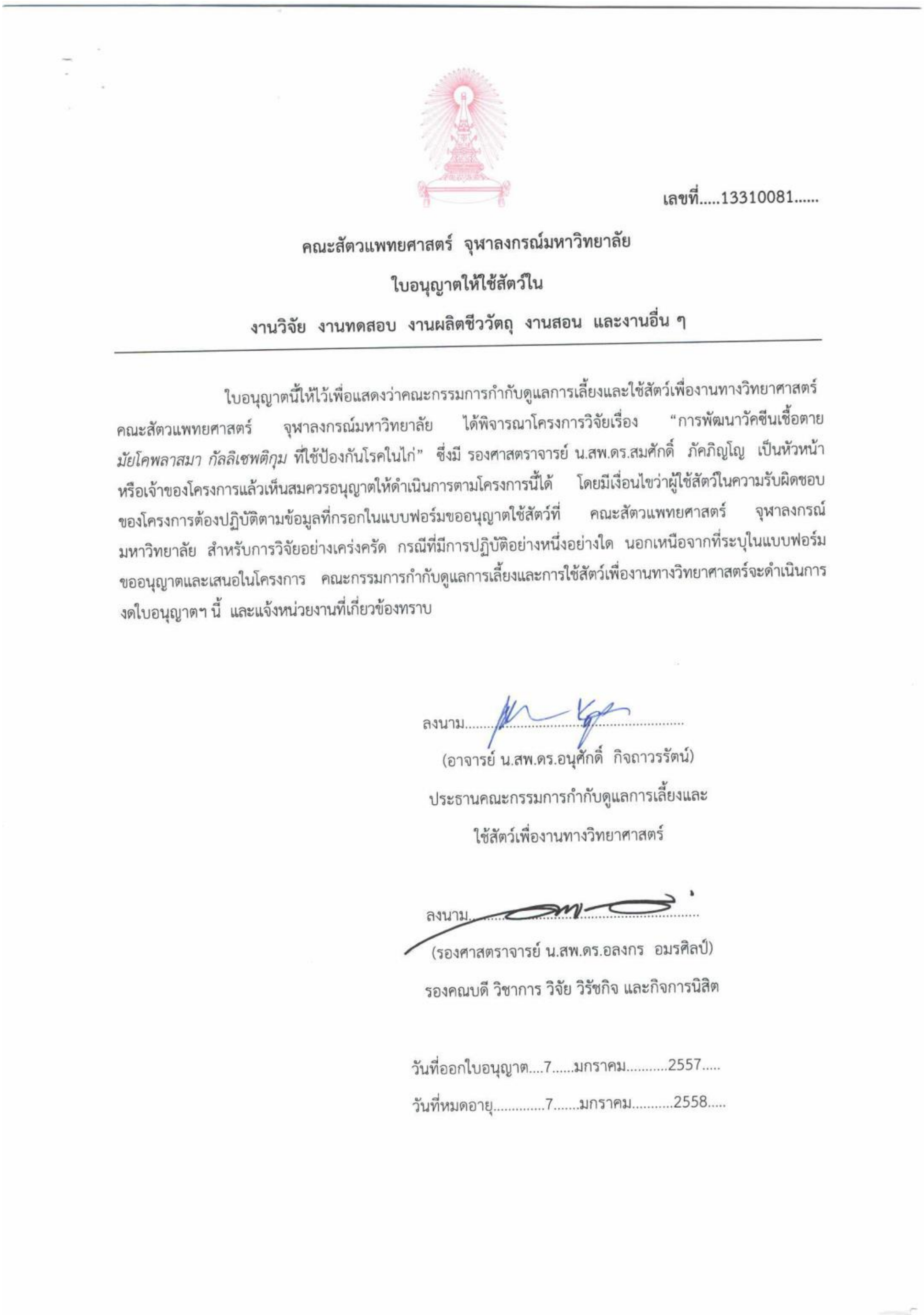
ตามที่ ท่านได้ยื่นแบบฟอร์มขออนุญาตใช้สัตว์ที่คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย เพื่อเสนอขออนุญาตให้ใช้สัตว์ในงานวิจัย งานทดสอบ งานผลิตชีววัตถุ งานสอนและงานอื่นๆ ให้กับโครงการวิจัยเรื่อง “การพัฒนาวัคซีนเชื้อตาย*มัยโคพลาสมา กัลลิเซพติกุม* ที่ใช้ป้องกันโรคในไก่”

บัดนี้ ผลการประชุมของคณะกรรมการกำกับดูแลการเลี้ยงและใช้สัตว์เพื่องานทางวิทยาศาสตร์ ครั้งที่ 10/2556 เมื่อวันที่ 5 พฤศจิกายน 2556 ได้มีมติอนุมัติให้ออกใบอนุญาตฯ ให้กับโครงการของท่าน ฝ่ายวิจัย จึงขอส่งใบอนุญาตฯ แก่ท่านตามสิ่งที่ส่งมาด้วยนี้

จึงเรียนมาเพื่อโปรดทราบ

(รองศาสตราจารย์ น.สพ.ดร.อลงกร อมรศิลป์)
รองคณบดี วิชาการ วิจัย วิรัชกิจ และกิจการนิสิต

สำเนาเรียน รองศาสตราจารย์ น.สพ.ดร.สมศักดิ์ ภัคภิญโญ



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คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ใบอนุญาตให้ใช้สัตว์ใน

งานวิจัย งานทดสอบ งานผลิตชีววัตถุ งานสอน และงานอื่น ๆ

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ลงนาม..
(อาจารย์ น.สพ.ดร.อนุศักดิ์ กิจถาวรรัตน์)
ประธานคณะกรรมการกำกับดูแลการเลี้ยงและ
ใช้สัตว์เพื่องานทางวิทยาศาสตร์

ลงนาม..
(รองศาสตราจารย์ น.สพ.ดร.อลงกร อมรศิลป์)
รองคณบดี วิชาการ วิจัย วิรัชกิจ และกิจการนิสิต

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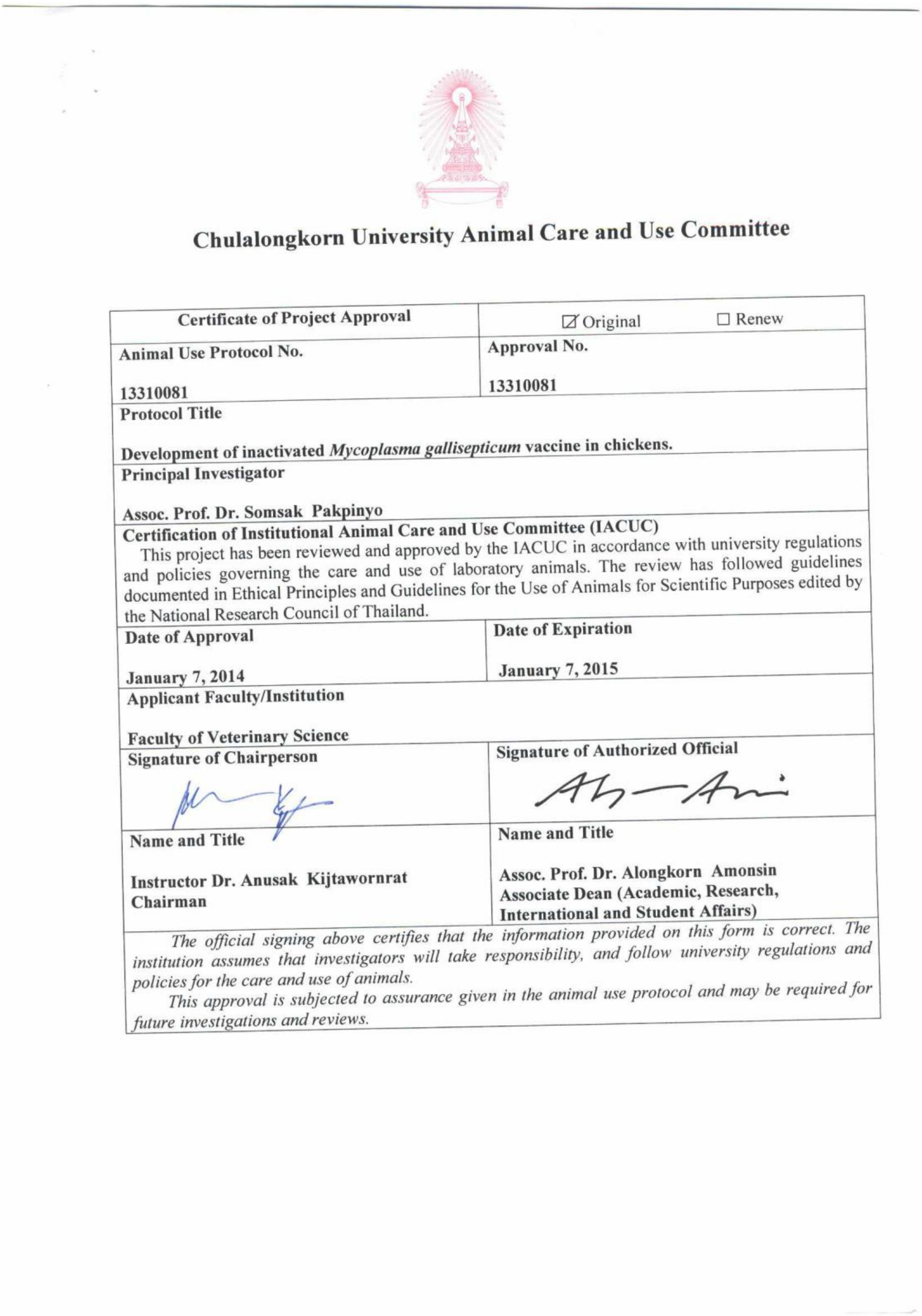
วันที่หมดอายุ.............7.......มกราคม...........2558.....



Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☑ Original ☐ Renew
Animal Use Protocol No. **13310081**	**Approval No.** **13310081**
Protocol Title **Development of inactivated *Mycoplasma gallisepticum* vaccine in chickens.**	
Principal Investigator **Assoc. Prof. Dr. Somsak Pakpinyo**	
Certification of Institutional Animal Care and Use Committee (IACUC) This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.	
Date of Approval **January 7, 2014**	**Date of Expiration** **January 7, 2015**
Applicant Faculty/Institution **Faculty of Veterinary Science**	
Signature of Chairperson	**Signature of Authorized Official**
Name and Title **Instructor Dr. Anusak Kijtawornrat** **Chairman**	**Name and Title** **Assoc. Prof. Dr. Alongkorn Amonsin** **Associate Dean (Academic, Research, International and Student Affairs)**
The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals. *This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.*	

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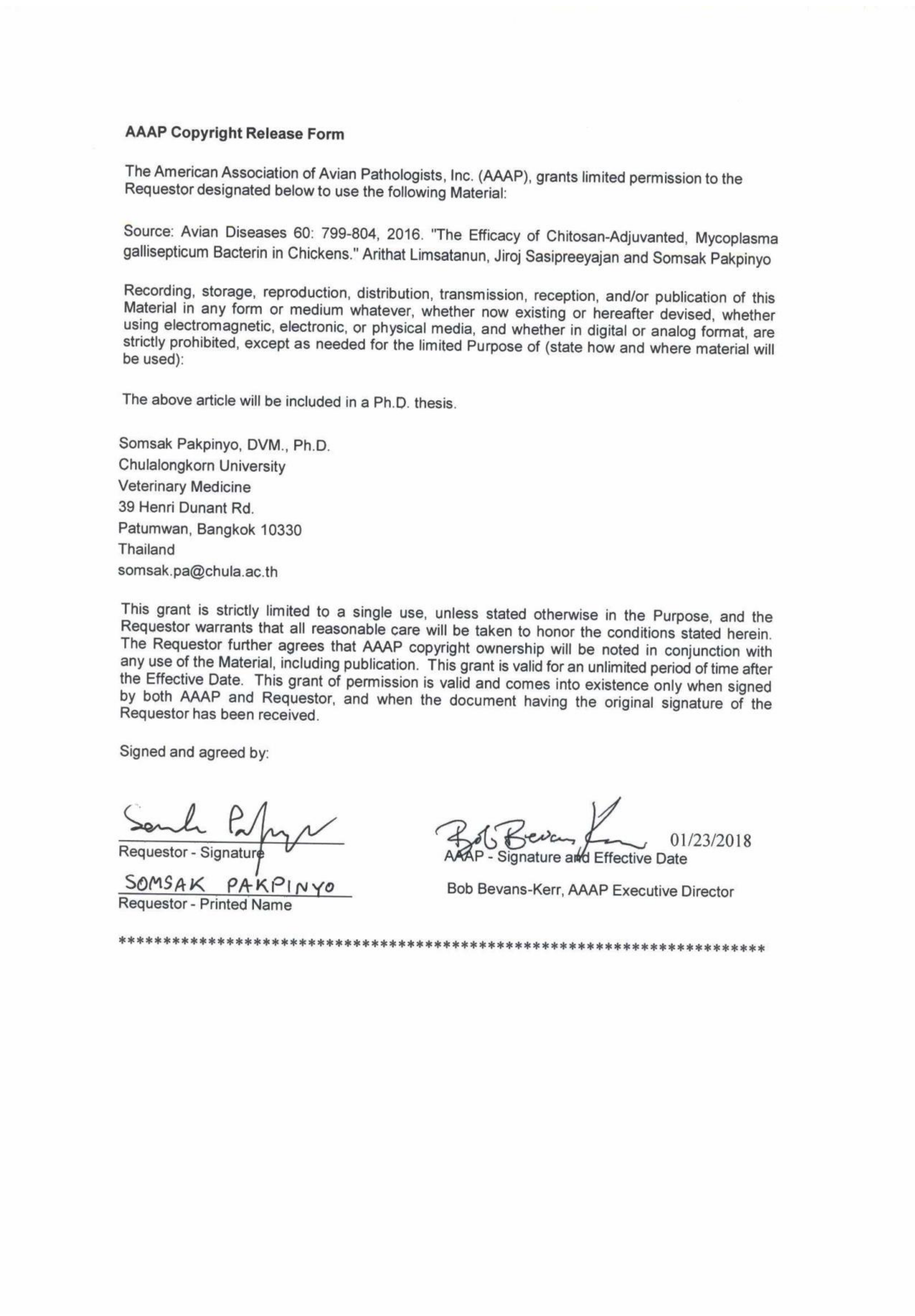

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Chulalongkorn University
Veterinary Medicine
39 Henri Dunant Rd.
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somsak.pa@chula.ac.th

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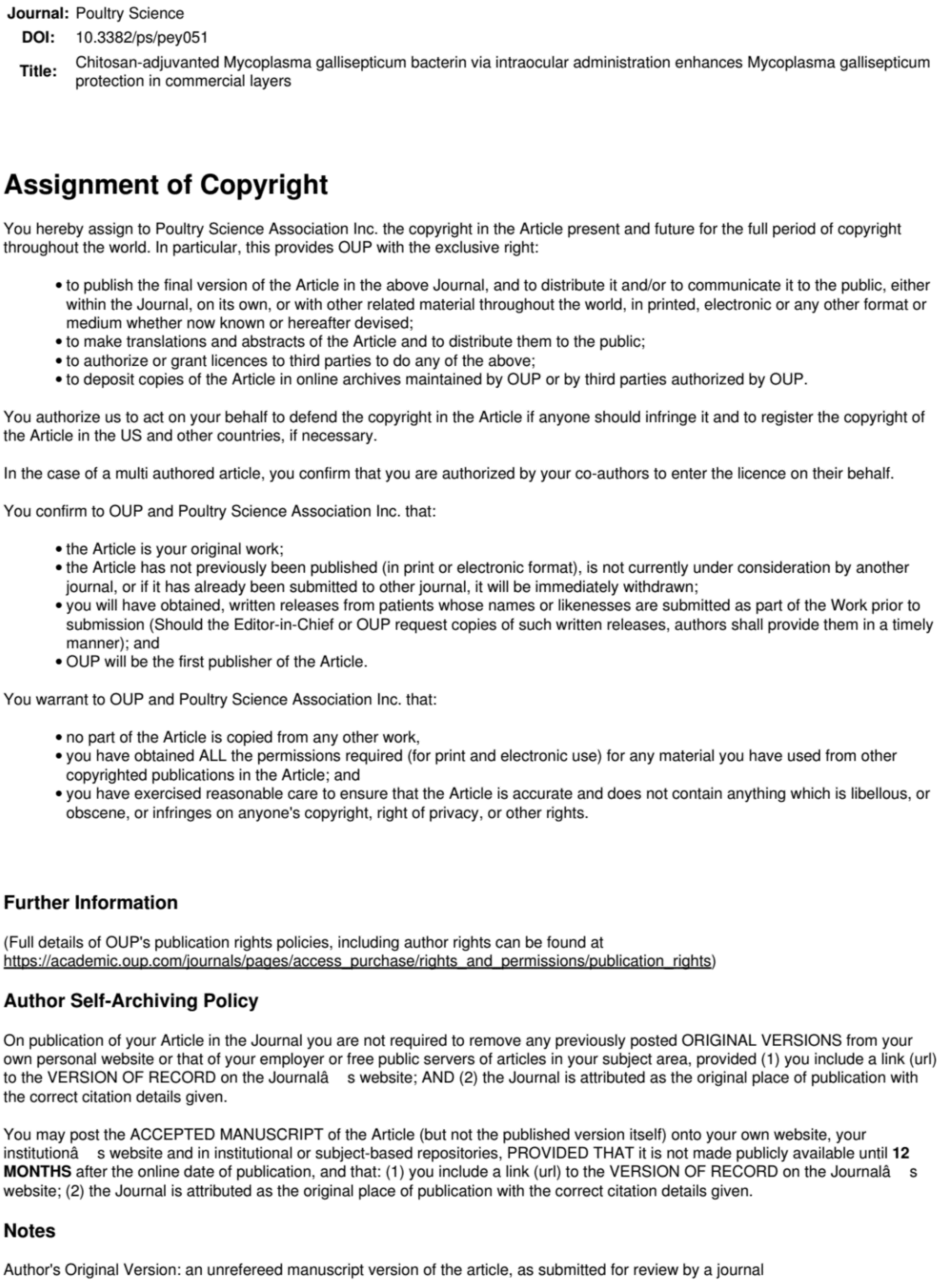

Journal: Poultry Science
DOI: 10.3382/ps/pey051
Title: Chitosan-adjuvanted Mycoplasma gallisepticum bacterin via intraocular administration enhances Mycoplasma gallisepticum protection in commercial layers

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Notes

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Appendix D: Chitosan-adjuvanted vaccine preparation

1. Thai MG strain 20/52 AHRL was propagated in 20 ml Frey's broth and then incubated at 37 °C until the color of broth changed from pink to orange-yellow color
2. After propagation, the cultured broth was aliquoted in 1 ml and made 10-fold dilution on Frey's agar plate and incubated at 37 ^{o}C for 7 days. MG colony will be counted and calculated as colony forming unit (CFU)/ml. In this study, MG colony was prepared approximately 1X10^{8} CFU/ml for intramuscular route and 1X10^{10} CFU/ml for intraocular route.
3. MG cells was washed with phosphate-buffered saline (PBS) and centrifuged at 12,000xg 30 minutes at 4°C.
4. Thimerosal is added in PBS containing MG cells to inactivated MG organism as a final concentration 0.01% thimerosal (Sigma-Aldrich Corp., USA).
5. The treated MG cells was incubated at 4°C for 7 days (Chukiatsiri et al., 2009)
6. Confirmed cell death by MG culture method
7. Killed MG cells were washed with phosphate-buffered saline (PBS) and diluted in PBS with 0.01% thimerosal.

8. 1% (w/v) chitosan (Sigma-Aldrich Corp., USA) was prepared in 1% acetic acid solution (pH 5.0)
9. Chitosan was added as a final concentration of 1%, 0.5% and 0.25% (w/v) chitosan (Ghendon et al., 2009) and stored at 4°C.

Appendix E: Viscosity test (test is determined by measuring stop time (second)) (Stone et al., 1978; Stone and Xie, 1990).

1. Chitosan-adjuvanted vaccine and commercial MG inactivated vaccine were drawn with 1-ml plastic pipette
2. Pipette containing vaccine was held vertically
3. The time was measured when vaccine was flowing from 0.0-ml. to 0.4-ml

Appendix F: Gross lesion of thoracic air sac evaluation

Thoracic air sac is determined by visual scoring system from 0-4 as the following table

Table. Gross thoracic air sac lesion score (Kleven et al., 1972).

Score	Lesion
0	No air sac lesion score.
1	Slight cloudiness of air sac membrane is found.
2	Air sac membrane is slightly thick and usually presents small accumulations of cheesy exudates.
3	Air sac membrane is obviously thick and meaty in consistency, with large accumulations of cheesy exudates in one air sac.
4	Lesions are observed the same as 3, but 2 or more air sacs are found. and histopathologic trachea.

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APPENDIX

VITA

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