MOLECULAR CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBLE PROFILES OF THAI *MYCOPLASMA SYNOVIAE* AND IMMUNE RESPONSE CHICKENS RECEIVED LIVE *MYCOPLASMA SYNOVIAE* VACCINE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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คุณลักษณะโมเลกุลและความไวของยาต้านจุลชีพของเชื้อมัยโคพลาสมา ซิโนวิอี สายพันธุ์แยกได้ในประเทศไทย และการตอบสนองภูมิคุ้มกันของไก่ที่ได้รับวัคซีนป้องกันโรคติดเชื้อมัยโคพลาสมา ซิโนวิอี ชนิดเชื้อเป็น

นายเกรียงวิชญ์ ลิมปวิทยากุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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เกรียงวิชญ์ ลิมปวิทยากุล : คุณลักษณะโมเลกุลและความไวของยาต้านจุลชีพของเชื้อมัยโคพลาสมา ซิโนวิอี สายพันธุ์แยกได้ในประเทศไทย และการตอบสนองภูมิคุ้มกันของไก่ที่ได้รับวัคซีนป้องกันโรคติดเชื้อมัยโคพลาสมา ซิโนวิอี ชนิดเชื้อเป็น. (MOLECULAR CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBLE PROFILES OF THAI *MYCOPLASMA SYNOVIAE* AND IMMUNE RESPONSE CHICKENS RECEIVED LIVE *MYCOPLASMA SYNOVIAE* VACCINE) อ.ที่ปรึกษาหลัก : ศ. ดร.สมศักดิ์ ภัคภิญโญ, อ.ที่ปรึกษาร่วม : ผศ. ดร.ณทยา เจริญวิศาล

ปัญหาโรคติดเชื้อมัยโคพลาสมา ซิโนวิอีในไก่ที่พบมากขึ้นทั่วโลกจะถูกควบคุมได้ด้วยการจัดการฝูงไก่แบบปลอดโรค การรักษาด้วยยาต้านจุลชีพในฝูงสัตว์ที่ติดเชื้อ และการทำวัคซีนในฝูงสัตว์ที่มีความเสี่ยงสูง ซึ่งการควบคุมโรคที่มีประสิทธิภาพนั้นจะต้องอาศัยความรู้ความเข้าใจในข้อมูลหลายๆด้านด้วยกัน ทั้งด้านข้อมูลเกี่ยวกับการตรวจวินิจฉัยโรคทางห้องปฏิบัติการที่สามารถตรวจแยกสายพันธุ์ของเชื้อมัยโคพลาสมา ซิโนวิอีได้ ด้านข้อมูลเกี่ยวกับกลไกการตอบสนองทางระบบภูมิคุ้มกันจากการทำวัคซีนชนิดเชื้อเป็น และด้านข้อมูลเกี่ยวกับการใช้ยาต้านจุลชีพที่เหมาะสมกับหลักฐานการตรวจไวรับต่อยาต้านจุลชีพที่ใช้ การศึกษานี้จึงได้ออกแบบมาเพื่อพัฒนาวิธีการทดสอบที่สะดวกในการใช้งานและมีความเหมาะสมสำหรับการนำไปใช้ตรวจติดตามผลภายหลังการทำวัคซีนชนิดเชื้อเป็น เพื่อศึกษากลไกการตอบสนองของระบบภูมิคุ้มกันที่เกิดขึ้นจากการทำวัคซีนชนิดเชื้อเป็น และเพื่อประเมินความไวรับต่อยาต้านจุลชีพของเชื้อมัยโคพลาสมา ซิโนวิอีที่ตรวจพบในปัจจุบัน สำหรับผลการวิเคราะห์ลำดับยีนบางส่วนของยีนวีเอลเอชเอในการศึกษานี้ได้แสดงให้เห็นว่าเชื้อมัยโคพลาสมา ซิโนวิอีที่ตรวจพบจากการเก็บตัวอย่างข้อและตัวอย่างอวัยวะในทางเดินหายใจสามารถจำแนกชนิดตามขนาดความยาวของยีนวีเอลเอชเอได้เป็นเชื้อมัยโคพลาสมา ซิโนวิอีชนิดอีและชนิดแอลที่ประกอบไปด้วยกรดอะมิโน ๑๙ ตัวและ ๓๕ ตัวตามลำดับ ซึ่งต่างจากวัคซีนเชื้อเป็นสายพันธุ์เอชที่ถูกจัดให้เป็นเชื้อมัยโคพลาสมา ซิโนวิอีชนิดซี ที่ประกอบไปด้วยกรดอะมิโนจำนวน ๓๒ ตัว ดังนั้นวิธีการทดสอบพีซีอาร์-อาร์เอฟแอลพีที่พัฒนาขึ้นมาโดยอาศัยเอ็นไซม์ทีเอเอสแอลในการตัดย่อยสายดีเอนเอที่ได้จากการทำพีซีอาร์ที่มีความจำเพาะกับยีนวีเอลเอชเอจึงถูกนำมาทดสอบประสิทธิภาพในการตรวจหาวัคซีนสายพันธุ์เอชในไก่ที่ได้รับวัคซีนและการตรวจแยกความแตกต่างระหว่างสายพันธุ์ที่ไม่ใช่วัคซีน ทั้งสายพันธุ์มาตรฐานดับเบิลยูวียู ๑๘๕๓ (ATCC 25204) และสายพันธุ์อื่นๆที่ตรวจพบในประเทศไทย การศึกษานี้ยังแสดงให้เห็นว่าการทำวัคซีนชนิดเชื้อเป็นสายพันธุ์เอชสามารถกระตุ้นกลไกการตอบสนองของภูมิคุ้มกันได้เหมือนกัน ไม่ว่าจะดำเนินการทำวัคซีนแค่ครั้งเดียวหรือทำสองครั้งก็ตาม ซึ่งการตอบสนองทางซีรัมวิทยาในไก่ที่ได้รับวัคซีนจะสามารถตรวจพบได้ในสัปดาห์ที่ ๒ หลังการทำวัคซีนครั้งแรก และการตอบสนองจะเพิ่มขึ้นอย่างต่อเนื่องจนถึงระดับสูงสุดที่สัปดาห์ที่ ๓ และ ๕ หลังการฉีดวัคซีนครั้งแรกเมื่อตรวจด้วยวิธีการอาร์พีเอและวิธีการอีไลซา ตามลำดับ สำหรับผลการทดสอบด้วยวิธีการเอ็มทีเอส เตตระโซเลียมนั้นก็แสดงให้เห็นว่าการตอบสนองของภูมิคุ้มกันชนิดเซลล์ต่อแอนติเจนชนิดเดียวกันและแอนติเจนต่างชนิดกันนั้นจะมีความคล้ายกันในช่วงแรกหลังจากได้รับวัคซีน แต่หลังจากที่ได้รับวัคซีนไปสักพักแล้ว ภูมิคุ้มกันชนิดเซลล์ต่อแอนติเจนชนิดเดียวกันจะมีการตอบสนองที่ดีกว่าแอนติเจนต่างชนิดกัน นอกจากนี้ ข้อมูลความไวรับต่อยาต้านจุลชีพที่ได้จากการศึกษานี้ได้แสดงให้เห็นว่าตัวเชื้อมัยโคพลาสมา ซิโนวิอีที่ตรวจพบในปัจจุบันนี้มีความไวรับอย่างมากต่อยาไทลวาโลซิน ยาไทโลซิน และยาไทอะมูลิน โดยมีค่าเอ็มไอซี๕๐อยู่ที่ ๐.๐๐๙๘, ๐.๐๓๙๑ และ ๐.๐๗๘๑ ไมโครกรัม/มิลลิลิตร ตามลำดับ; มีความไวรับปานปลางต่อยาด็อกซีไซคลิน ยาอ็อกซีเตตร้าไซคลิน และยาลินโคมัยซิน-สเปคติโนมัยซิน โดยมีค่าเอ็มไอซี๕๐อยู่ที่ ๐.๑๕๖๓, ๐.๑๕๖๓ และ ๐.๖๒๕ ไมโครกรัม/มิลลิลิตร ตามลำดับ; และมีการดื้อต่อยาเอนโรฟลอกซาซิน โดยมีค่าเอ็มไอซี๕๐อยู่ที่ ๑๐ ไมโครกรัม/มิลลิลิตร ซึ่งประเด็นที่น่าสนใจอย่างยิ่งก็คือเชื้อมัยโคพลาสมา ซิโนวิอีสามารถแบ่งกลุ่มได้อย่างน้อย ๓ กลุ่มย่อยที่มีค่าเอ็มไอซี๕๐ที่แตกต่างกันตั้งแต่ ๐.๐๑๙๕-๐.๖๒๕ ไมโครกรัม/มิลลิลิตร หรือตั้งแต่กลุ่มเชื้อที่มีความไวรับสูงไปจนถึงกลุ่มที่ดื้อต่อยาทิลมิโคซิน

สาขาวิชา	อายุรศาสตร์สัตวแพทย์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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Kriengwich Limpavithayakul : MOLECULAR CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBLE PROFILES OF THAI *MYCOPLASMA SYNOVIAE* AND IMMUNE RESPONSE CHICKENS RECEIVED LIVE *MYCOPLASMA SYNOVIAE* VACCINE. Advisor: Prof. SOMSAK PAKPINYO, D.V.M., Ph.D., D.T.B.V.M. Co-advisor: Asst. Prof. NATAYA CHAROENVISAL, D.V.M., Ph.D.

Mycoplasma synoviae infection in chickens which has been increasing worldwide is mostly controlled by pathogen-free flocks' maintenance, medication in infected flocks and vaccination in high-risk flocks. The effective control strategy requires the information of diagnostic assays for detecting and differentiating *M. synoviae* strains, the understanding of immune response mechanisms in vaccinated chickens, and the reliable drug susceptible evidents for making decision in antimicrobial usage. This study aimed to develop a convenient assay for evaluation of quality or uniformity of vaccination with live MS-H vaccine, to characterize the immune response mechanisms in chickens receiving 3 different vaccination programs immunizing single dose at either 9 or 12 weeks of age or two doses at both 9 and 12 weeks of age, and to determine the antimicrobial susceptible profiles of current Thai *M. synoviae* isolates. Based on sequence analysis of partial *vlhA* gene in this study, Thai *M. synoviae* isolates collected from articular joint and respiratory tract of chickens during 2020, consisted of types E and L with 19 and 35 amino acid length, respectively, differing from MS-H vaccine strain classified as type C with 32 amino acid length. Consequently, the developed PCR-RFLP assay using restriction enzyme TasI to digest *vlhA* gene-targeted PCR amplicons, was verified to detect MS-H vaccine strain in vaccinated chickens and differentiate it from non-vaccine strains; WVU1853 reference strain (ATCC 25204) and Thai *M. synoviae* field strains. Besides, this study also demonstrated that vaccination with live MS-H, whether single or two doses, could similarly stimulate immune response mechanisms including both humoral and cellular immune responses. Antibody response in vaccinated chickens were initially detected at 2 weeks post first vaccination, and continuously increased to the highest level at 3 and 5 weeks post first vaccination examined by RPA assay and ELISA, respectively. Cellular immune response against both homologous and heterologous antigens, examined by MTS Tetrazolium assay, were similar at early period post immunization while the responses against homologous antigen was better at late period post immunization. Furthermore, antimicrobial susceptible profiles of current Thai *M. synoviae* isolates was performed strong susceptible to tylvalosin, tylosin, and tiamulin at MIC_{50} value of 0.0098, 0.0391 and 0.0781 µg/ml, respectively; moderate susceptible to doxycycline, oxytetracycline and lincomycin-spectinomycin at MIC_{50} value of 0.1563, 0.1563 and 0.625 µg/ml, respectively; and resistance to enrofloxacin at MIC_{50} value of 10 µg/ml. Interestingly, at least three subpopulations of Thai *M. synoviae* isolates were presented with different MIC values; ranging 0.0195-0.625 µg/ml; from strong susceptibility to resistance to tilmicosin.

Field of Study:	Veterinary Medicine	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....
		Co-advisor's Signature .....

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CHAPTER I

INTRODUCTION

1.1) Importance and Rationale

Mycoplasma synoviae (MS) is an important pathogen in poultry industry worldwide. *M. synoviae* infection frequently appears as subclinical infection of upper respiratory tract in chickens which will be reservoirs or carriers of *M. synoviae* for life. Infected chickens could vertically transmit pathogens to their progeny and could horizontally spread pathogens by direct and indirect contact via contaminated materials, environments, or people (Marois et al., 2000; Fiorentin et al., 2003b; Kleven and Ferguson-Noel, 2008; Buim et al., 2009; Pakpinyo et al., 2009; Feberwee and Landman, 2010; Khiari et al., 2010; Wetzel et al., 2010; Landman, 2014). The co-infection with respiratory pathogens; such as Newcastle disease virus (NDV), infectious bronchitis virus (IBV), and *Escherichia coli* (*E. coli*); can cause airsacculitis, whereas the systemic infection of *M. synoviae* can cause infectious synovitis showing inflammation of tendons or bursa sheath (Kleven and Ferguson-Noel, 2008; Buim et al., 2009; Pakpinyo et al., 2009; Khiari et al., 2010; Wetzel et al., 2010; Landman, 2014; Limpavithayakul et al., 2016).

Consequently, economic consequence and disease severity are variation in pathogenicity among characteristic of *M. synoviae* isolates, from no lesion to severe lesions. Economic impacts of *M. synoviae* infection in poultry industry seem to be noticeable increased worldwide due to the occurrence of synovial membrane infection associating with the infectious synovitis, and the emergence of oviduct infection, affecting the eggshell quality and egg production (Kleven and Ferguson-Noel, 2008; Buim et al., 2009; Pakpinyo et al., 2009; Feberwee and Landman, 2010; Seifi and Shirzad, 2012; Landman, 2014; Limpavithayakul et al., 2016). The clinical lesions of airsacculitis and infectious synovitis in chickens caused by the respiratory tract infection and the synovial membrane infection, respectively, eventually lead to various economic losses including a decrease of poultry flocks' performances; reduction of egg production, hatchability, and growth rate; and an increase of carcasses' condemnation (Landman, 2014). Besides, the oviduct infection could induced the egg

shell apex abnormalities (EAA) without any physical abnormalities (Lockaby et al., 1998; Kleven and Ferguson-Noel, 2008; Pakpinyo et al., 2009; Landman, 2014).

According to disease surveillance worldwide, *M. synoviae* was detected and suspected to be an important pathogen in most commercial layer, breeder and broiler chickens showing respiratory problem or decreased flocks' performances (Fiorentin et al., 2003b; Feberwee et al., 2008; Buim et al., 2009; Pourbakhsh et al., 2010; Seifi and Shirzad, 2012). Most broiler chicken flocks have faced the economic losses due to an occurrence of airsacculitis and infectious synovitis which could respectively increase carcasses' condemnation rate and culling amount of lame chickens (Kleven et al., 1972; Giambrone et al., 1977; Yoder Jr et al., 1977; Landman and Feberwee, 2001), while the infection of *M. synoviae* in oviduct could be associated with egg drops problems and eggshell apex abnormalities (EAA) syndrome in layers flocks showing an altered thinning translucent egg shell surface with easily crack or break (Lockaby et al., 1998; Kleven and Ferguson-Noel, 2008; Feberwee et al., 2009a; Feberwee et al., 2009b; Pakpinyo et al., 2009; Landman, 2014).

In addition to the transmission of *M. synoviae* from old breeders and layers flocks in the multiple-age farms, the infected breeder flocks could predispose various problems associated with *M. synoviae* infection in commercial broiler farms through their congenital infected progeny; day-old-chicks received *M. synoviae* via vertical transmission (Feberwee et al., 2008; Buim et al., 2009; Pourbakhsh et al., 2010; Seifi and Shirzad, 2012). Interestingly, horizontal transmission of *M. synoviae* mostly occurs via respiratory tract by direct contact with infected chickens or indirect contact with contaminated environments, while vertical transmission occurs only in infected chickens during egg production period. So horizontal transmission of *M. synoviae* could be occurred rather than vertical transmission from the breeders (Fahey and Crawley, 1954; Marois et al., 2000; Sentíes-Cué et al., 2005; Tebyanian et al., 2014).

Due to the high capacity in horizontal transmission via contaminated materials, equipment, people, pigeons, ostriches and wild animals (Marois et al., 2000; Tebyanian et al., 2014), *M. synoviae* isolates of the progeny may not be similar to those from the breeder flock, but may be similar to *M. synoviae* isolates, mostly contaminated in environments, from another flock in the same area (Sentíes-Cué et al., 2005). So

diagnostic approaches for detection and differentiation have been required in tracing the spread of pathogens which could persist and contaminate within affected birds, unaffected birds, wild animals, rodents, equipment, dusts, feathers, feeds, or people.

Since culture and isolation are time consuming and laborious diagnostic assays; therefore, molecular assays including conventional polymerase chain reaction (PCR) assay and sequence analysis have been developed and become methods of choice for *M. synoviae* detection and strain differentiation due to their advantages; simple, rapid, highly sensitivity and excellent specificity (Marois et al., 2000; Kleven and Ferguson-Noel, 2008; Buim et al., 2009; Jarquin et al., 2009; Pakpinyo et al., 2009; Feberwee and Landman, 2010; Wetzel et al., 2010; Landman, 2014; Limpavithayakul et al., 2016). *M. synoviae*-specific PCR assay, the 16S rRNA gene-based PCR assay representing a sensitivity of 82% and a specificity of 100%, has been widely used for detecting *M. synoviae* organisms in infected chickens or contaminated environments (Lauerman et al., 1993; Marois et al., 2000; Hong et al., 2004); however, it could not be a reliable diagnostic method for differentiating *M. synoviae* strains. Therefore, the *vlhA* gene-based PCR assay developed by Wetzel in 2010 has been proposed to apply with sequence analysis for differentiating *M. synoviae* strains without culture or isolation methods (Hong et al., 2004; Jeffery et al., 2007; Wetzel et al., 2010; El-Gazzar et al., 2012).

The *vlhA* gene, encoding hemagglutinin and other immunodominant membrane proteins, involves in colonization, antigenic variations, and virulence of *M. synoviae* (Razin et al., 1998; Noormohammadi et al., 2000; Bercic et al., 2008). Besides, the size variation of proline-rich repeat (PRR) region of *vlhA* gene might be interestingly associated with the invasiveness property of each *M. synoviae* strains (Bencina et al., 2001). The 370-400 bp of *vlhA* fragments from the *vlhA* gene-based conventional PCR assay are amplified using the revised Hammond primers; MSRH-F (5'- GGC CAT TGC TCC TRC TGT TAT-3') and MSRH-R (5'- AGT AAC CGA TCC GCT TAA TGC-3') and subsequently analyzed their sequences of partial gene (Wetzel et al., 2010; El-Gazzar et al., 2012). Based on the molecular basis of a size variation in the N-terminal fragment MSPB, the PRR region of *vlhA* gene, is useful for typing of *M. synoviae* strains based on insertion/deletion of nucleotides while the RIII region is useful for subtyping of *M.*

synoviae strains by nucleotide polymorphisms (Bencina et al., 2001; Hong et al., 2004; Jeffery et al., 2007; Hammond et al., 2009; Wetzel et al., 2010; El-Gazzar et al., 2012). Besides, the current 12 types of *M. synoviae* isolates worldwide including types A, B, C, D E, F, G, H, I, J, K and L, have been classified on the basis of the size of PRR region, consisting of 38, 45, 32, 23, 19, 36, 51, 46, 28, 20, 12 and 35 amino acids, respectively (Bencina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Bayatzadeh et al., 2014; Limpavithayakul et al., 2016).

In addition, despite the report of *M. synoviae* infection in commercial chicken and native chicken flocks in Thailand determined by serological assays, culture method and PCR assays, (Pakpinyo et al., 2009), sequence analysis of partial *vlhA* gene recently provides an available information of Thai *M. synoviae* field isolates; identified as types C, E, and L with 19, 32, and 35 amino acid lengths, respectively (Limpavithayakul et al., 2016). Thai *M. synoviae* types C and E, isolated from chickens showing respiratory signs, were possibly associated with respiratory tract infection and could not induce the egg shell apex abnormality syndrome like Dutch *M. synoviae* isolate types C and E, which could infect the reproductive tract of chickens (Feberwee et al., 2009a). In addition, Thai *M. synoviae* isolates type L causing infectious synovitis and lameness in both commercial and native chickens could be the highly invasive strain like the *M. synoviae* K1968 strain classified as *M. synoviae* type B with long PRR region which could induce infectious synovitis in chicken (Bencina et al., 2001; Limpavithayakul et al., 2016).

A pathogenesis of *M. synoviae* infection depends on modes of infection but a presence of antibodies against *M. synoviae* in chickens (Sun et al., 2017), a virulence of difference strain (Lockaby et al., 1998; Narat et al., 1998; Khiari et al., 2010; Limpavithayakul et al., 2016) and an immune response mechanism to infection (Washburn et al., 1985) could also be the crucial factors of the *M. synoviae* reproduction in chickens consequently affecting the pathogenesis (Sun et al., 2017). In case of natural infection, the spread of *M. synoviae* to joints possibly occurs via a hematogenous route after colonization in respiratory tract (Kawakubo et al., 1980) by invading into chicken erythrocytes (Dušanić et al., 2009). Therefore, *M. synoviae* could easily spread to the joints via bloodstream and could enhance the prevalence of joint

lesions in infected chickens which show highly respiratory tissue damage caused by environmental distress or co-infection with other respiratory pathogens (Olson and Kerr, 1967; Yoder Jr et al., 1977; Kawakubo et al., 1980; Landman and Feberwee, 2004). Furthermore, both environmental temperature and relative humidity in the poultry house could also affect the incidence and severity of lesions in *M. synoviae* infected chickens so the difference ventilation management affecting temperature and relative humidity in the poultry house could be an important predisposing factor of *M. synoviae* prevalence in the poultry flocks (Yoder Jr et al., 1977).

M. synoviae colonization in the chicken respiratory mucosa could induce both local and systemic immune response (Razin et al., 1998). Because the *vlhA* haemagglutinin and other embedded lipoproteins classified as pathogen associated molecular patterns (PAMPs) of *M. synoviae* could play a vital role in the interactions to Toll-like receptors (TLR) of chicken heterophils and macrophages which are an essential cellular component of the innate immune system connecting the acquired immunity (Noormohammadi et al., 1998; Razin et al., 1998; Bencina et al., 2001; Fukui et al., 2001; Iqbal et al., 2005). Besides, the systemic infection of *M. synoviae* could activate lymphoid tissues in several organs showing cellular infiltration (Sentíes-Cué et al., 2005). The activation of the TLR2 signal transduction in the chicken tracheal mucosa reveals the up-regulation of cytokine genes expression associated with innate immune response, inflammation process and macrophage activation such as interleukin-18 (IL-18), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), inducible nitric oxide synthase (iNOS) and macrophage inflammatory proteins (MIPβ, CCL-4) (Beutler et al., 2006; Lavrič et al., 2007; Bolha et al., 2013; Majumder and Silbart, 2016).

During acute phase of *M. synoviae* infection, the secretion of pro-inflammatory cytokines; IL-6, IL-1β and iNOS; could activate macrophages which subsequently stimulate and recruit B-lymphocytes and T-lymphocytes (Wigley and Kaiser, 2003; Lavrič et al., 2007). The numbers of macrophages in tracheal mucosa perform an important function in development of protective immunity associated with cell mediated immune response (Gaunson et al., 2006). Besides, to control the

dissemination and clearance of *M. synoviae* in infected tracheal epithelial cells and other infected organs, other cytokines including IL-2, IL-6, IL-12, IL-18, IL-21, TGF-β and IFN-γ also present in infected tissues for recruitment and activation of leukocytes; macrophages, NK-cells, Th1 cells, Th17 cells and B-lymphocytes (Swain et al., 2012; Bolha et al., 2013; Majumder and Silbart, 2016).

In addition, the immune response during acute phase of *M. synoviae* infection could represent the upregulated cytokines associated with Th1 cells; including IL-2, IL-12 and IFN-γ; associated with Th17 cells; including IL-6, IL-21 and TGF-β and associated with cell-mediated immune functional of macrophages and NK-cells; including IL-12, IL-18 and IFN-γ (Gaunson et al., 2006; Swain et al., 2012; Majumder and Silbart, 2016). While the combination effect of IL-12 and IL-18 could consequently express IFN-γ stimulating the IgG production in B-lymphocytes which usually present in the follicular tracheal lesions after 3 weeks post infection (Gaunson et al., 2006). According to the co-infection with other respiratory pathogens like NDV could be associated with the decreased immune function of macrophages or NK-cells and the reduced antibody response in B-cells follicles and could predispose the severe airsacculitis or severe respiratory disease in *M. synoviae* infected chicken (Silva et al., 2008); therefore, the humoral immunity related to the development of B-cells follicles of the infected organs and the immune function of both macrophages and NK-cells could be more important factor associated with the pathogenesis, recovery process and protective immunity (Gaunson et al., 2006).

Disease control strategy of *M. synoviae* infection in poultry flocks mostly consist of three approaches including pathogen-free flocks maintenance, medication, and vaccination (Umar et al., 2017). However, the successful of disease control strategy requires an early detection of disease and the corrected risks assessments in poultry farms (Stipkovits and Kempf, 1996). Besides, poultry farms; breeder, layer and broiler flocks; have been concerned to initially maintain *M. synoviae*-free flocks by obtaining only the *M. synoviae*-free chickens originated from *M. synoviae*-free parent flocks and subsequently fulfill disease control by rearing *M. synoviae*-free chickens in clean environment with strict biosecurity system or maintaining the channeling measure

separating uninfected flocks and their eggs from *M. synoviae*-infected flocks (Markham et al., 1998b; Landman, 2014).

Although *M. synoviae*-free flocks maintenance and disease control strategies in breeder flocks are important measures to reduce economic losses and treatment cost in broiler flocks (Buim et al., 2009; Landman, 2014; Hong et al., 2015) or the single-age management and elimination of infected flocks are notified as the strongest measure in breeder and layer chickens due to the efficacy in reducing the incidence and prevalence of mycoplasma disease in poultry flocks, in breaking down the cycle of vertical transmission from hens to their progeny, and in reducing the horizontal transmission transferring pathogens to new rearing flocks when introduced into the *M. synoviae* infected farms (Stipkovits and Kempf, 1996; Feberwee and Landman, 2012; Landman, 2014). However, prevalence of *M. synoviae* infection has been increasing in many countries because *M. synoviae*-free flocks' maintenance, single-age management and elimination are difficult practices and are not the economic compliances in poultry industry business. Therefore, medication in infected flocks and vaccination in high risk flocks have been suggested as the alternative measures representing an obvious efficacy in reducing clinical signs or improving production performance in poultry industrial level (Stipkovits and Kempf, 1996; Markham et al., 1998b; Fiorentin et al., 2003b; Kleven and Ferguson-Noel, 2008; Ley, 2008; Feberwee et al., 2009b; Landman, 2014; Hong et al., 2015; Kreizinger et al., 2017).

The alternative strategies; medication and vaccination; have been improved to show attractive optimal cost-benefit relation and to present capacity in reducing clinical signs and economic losses in *M. synoviae* infected flocks (Stipkovits and Kempf, 1996; Markham et al., 1998b; Feberwee et al., 2009b). Although medication is unable to completely eliminate the *M. synoviae* infection from affected breeder or layer chickens, medication has been more common practice in poultry industry than vaccination because of the limited countries having available live vaccine registration (Fiorentin et al., 2003b; Kleven and Ferguson-Noel, 2008; Ley, 2008; Catania et al., 2010; Landman, 2014; Hong et al., 2015; Kreizinger et al., 2017). In addition, although vaccination with commercial live attenuated MS-H vaccine; temperature-sensitive strain; has been dramatically increasing in several countries challenged with

antimicrobial resistance argument, vaccination is still questionable in efficacy and strain differentiation (Fiorentin et al., 2003b; Kleven and Ferguson-Noel, 2008; Ley, 2008; Catania et al., 2010; Landman, 2014; Hong et al., 2015; Kreizinger et al., 2017).

Due to the concurrent or the re-entry of *M. synoviae* pathogen in poultry industry, the successful control strategy, showing *M. synoviae* growth reduction and finally *M. synoviae* eradicating from infected chicken flocks, requires the continuous intensive medication with mycoplasma susceptible antimicrobials inhibiting protein synthesis including tetracyclines (oxytracycline, chlortetracycline, and doxycycline), macrolides (tylosin, tilmicosin, tylvalosin, erythromycin, spiramycin), lincosamides (lincomycin), quinolones (enrofloxacin), and pleuromutilins (tiamulin) (Landman et al., 2008; Hong et al., 2015; Kreizinger et al., 2017). Although antimicrobials have been widely used in poultry production for decades; some are used as therapeutic drug to treat bacterial infectious diseases and improve the well-being of animals but most antimicrobials are used as prophylactic substances in term of antimicrobial growth performance promoters (AGPs) to improve growth rate and feed conversion efficiency (Edens, 2003; Huyghebaert et al., 2011).

Although medication in complete eradication program could decrease the usage of antimicrobials in next flocks or progeny flocks, the long term of intensive treatment is not generally acceptable in poultry industry (Fiorentin et al., 2003b). In addition, the beneficial effects of AGPs in improving the production performance or reducing the clinical signs have been challenged by the emergence of antimicrobial resistance issue. European Union (EU) banned the use of sub-therapeutic dosing of some antimicrobials in poultry since June 1999 (EC Regulation No. 1831/20031) because some potential human pathogens found on processed poultry carcasses were revealed the resistant property to some antimicrobials, not due to single but to multiple antimicrobials. Consequently, the current argument of antimicrobial resistance issues has influenced the practical use of antimicrobials in poultry production including the medication strategy for *M. synoviae* control requiring the prescription from poultry veterinarian and the reliable susceptible profiles like minimum inhibitory concentration (MIC) of *M. synoviae* isolated from each farms (Edens, 2003; Behbahan et al., 2008).

Therefore, in addition to the obvious efficacy in reducing clinical signs or improving production performance of *M. synoviae* infected flocks, the live *M. synoviae* MS-H vaccine has been proposed to use in risk farms and dramatically increasing in several countries challenged with antimicrobial resistance argument (Markham et al., 1998a; Fiorentin et al., 2003b; Kleven, 2008; Kleven and Ferguson-Noel, 2008; Ley, 2008; Feberwee et al., 2009b; Nicholas et al., 2009; Catania et al., 2010; Landman, 2014; Hong et al., 2015; Kreizinger et al., 2017). However, the vaccination with live MS-H strain is uncommon practice in the field situation because the efficacy in protecting clinical signs and disease shedding remains questionable at commercial farm level and the information of diagnostic tests distinguishing between field and vaccine strains is also deficient (Ley, 2008; Feberwee et al., 2009b; Landman, 2014).

Consequently, sequence analysis of partial *vlhA* gene of *M. synoviae* isolates worldwide recently provides an available information of Thai *M. synoviae* field isolates; identified as types C, E, and L; which could differentiate from MS-H vaccine strain (Bencina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Limpavithayakul et al., 2016). However, sequence analysis is considered as time-consuming assay (Jeffery et al., 2007; Hammond et al., 2009; Landman, 2014; Limpavithayakul et al., 2016); therefore, the PCR-restriction fragment length polymorphism (PCR-RFLP) assay, using the restriction enzyme TasI to digest *vlhA* gene-targeted PCR product, has been developed for being the convenient assay with good sensitivity and specificity for detecting MS-H vaccine strain and differentiating it from non-vaccine strains circulating in Thailand including *M. synoviae* WVU 1853 reference strain and Thai *M. synoviae* types C, E and L.

Accordingly, this study aimed to characterize the partial *vlhA* gene of *M. synoviae* isolates recently circulated in Thailand by sequence analysis and to verify the developed PCR-RFLP assay as a convenient approach for strain differentiation in epidemiological study. In addition, to fulfill the missing information in vaccination and medication, the present study was also conducted to characterize the immune response mechanisms, humoral and cellular immune responses, in layer type chickens receiving a single dose or two doses of live MS-H vaccine in experimental study and to determine the antimicrobial susceptibility profiles by using microbroth dilution method

with common antimicrobials used in veterinary practice against avian mycoplasmosis including enrofloxacin, oxytetracycline, doxycycline, tiamulin, tylosin, tilmicosin, tylvalosin and lincomycin-spectinomycin.

1.2) Objectives of this study

1) To characterize the partial *vlhA* gene of *M. synoviae* isolates recently circulated in Thailand by sequence analysis.

2) To verify the PCR-RFLP assay as a convenient approach for strain differentiation in epidemiological study.

3) To characterize the humoral and cell-mediated immune response mechanisms due to vaccinated with live MS-H vaccine.

4) To determine the antimicrobial susceptibility profiles by using microbroth dilution method with common antimicrobials including enrofloxacin, oxytetracycline, doxycycline, tiamulin, tylosin, tilmicosin, tylvalosin and lincomycin-spectinomycin used in veterinary practice against avian mycoplasmosis

1.3) Questions of this study

1) How is the difference of molecular characterization of *M. synoviae* MS-H vaccine strain and Thai *M. synoviae* field strains?

2) How could the developed PCR-RFLP assay be a convenient approach for strain differentiation in epidemiological study?

3) How could the live *M. synoviae* MS-H vaccine stimulate the humoral and cell-mediated immune response mechanisms against homologous and heterologous strains?

4) What are the antimicrobial susceptibility profiles of current Thai *M. synoviae* field isolates?

1.4) Keywords (Thai):

ข้อมูลความไวรับต่อยาต้านจุลชีพ ไก่ การตอบสนองทางภูมิคุ้มกัน เชื้อมัยโคพลาสมา ซิโนวีอี การจำแนกสายพันธุ์ ไทย วัคซีน

1.5) Keywords (English):

Antimicrobial susceptible profiles, Chickens, Immune response, *Mycoplasma synoviae*, Strain differentiation, Thai, Vaccine

1.6) Hypothesis

1) Molecular characterization *M. synoviae* MS-H vaccine strain is different from other Thai *M. synoviae* field strains.

2) The developed PCR-RFLP assay is the suitable diagnostic assay for *M. synoviae* surveillance in poultry industry.

3) Vaccination with live MS-H vaccine could stimulate the specific immune mechanisms including humoral and cell-mediated immune response against homologous and heterologous strains.

4) Antimicrobial susceptibility profiles of current Thai *M. synoviae* field isolates could notify the susceptible or resistance antimicrobials.

1.7) Advantages of study

The present study established the missing knowledges; an information of diagnostic assays for detecting and differentiating *M. synoviae* strains, an understanding of immune response mechanisms in vaccinated chickens, and a reliable drug susceptible evident for making decision in antimicrobial usage; which are necessary to design the *M. synoviae* control procedures suitable for poultry flocks in Thailand.

CHAPTER II

Literature review

2.1) General information of *M. synoviae*

M. synoviae, appearing as round organisms of the class Mollicutes with diameter around 300 nm and lacking cell wall, is an important pathogen in poultry industry worldwide. The infection of *M. synoviae* mostly appears in term of subclinical infection in upper respiratory tract of chickens but clinical signs of respiratory system could be more severity when *M. synoviae* infection occurs with other predisposing factors (Kleven and Ferguson-Noel, 2008; Buim et al., 2009; Pakpinyo et al., 2009; Khiari et al., 2010; Wetzel et al., 2010; Landman, 2014). Clinical signs in *M. synoviae* infected chickens are different due to variable in disease induction among *M. synoviae* strains. In addition to subclinical infection in the upper respiratory tract caused by most *M. synoviae* strains, other severe clinical signs like airsacculitis, eggshell apex abnormalities and infectious synovitis could be induced by specific *M. synoviae* strains with appropriate predisposing factors (Lockaby et al., 1998; Landman and Feberwee, 2012; Landman, 2014; Catania et al., 2016).

Although a systemic infection of *M. synoviae* can cause the infectious synovitis showing inflammation of tendons or bursa sheath, predisposing factors; environmental distress, immunosuppressive condition, and co-infection with other respiratory pathogens such as Newcastle disease virus (NDV), infectious bronchitis virus (IBV), and *Escherichia coli* (*E. coli*); could strongly trigger severe airsacculitis or respiratory disease in infected chickens (Kleven et al., 1972; Springer et al., 1974; Kleven et al., 1975; Giambrone et al., 1977; Yoder Jr et al., 1977; Hopkins and Yoder Jr, 1982; Lockaby et al., 1998; Landman and Feberwee, 2004; Kleven and Ferguson-Noel, 2008; Buim et al., 2009; Feberwee et al., 2009a; Pakpinyo et al., 2009; Feberwee and Landman, 2010; Khiari et al., 2010; Wetzel et al., 2010; Landman, 2014; Limpavithayakul et al., 2016). In addition to the systemic infection of *M. synoviae* may involve other organs such as keel bursa, liver, spleen, skeletal muscle and brain including nerves, choroids of eyes (Sentíes-Cué et al., 2005), the infection of *M. synoviae* in synovial membrane and

oviduct could be associated with the infectious synovitis and the eggshell apex abnormalities (EAA), respectively.

Besides, some Dutch and Australian *M. synoviae* isolates causing respiratory disease could not induce the infectious synovitis (Landman and Feberwee, 2004) but some *M. synoviae* isolates obtained from synovitis lesion of chickens could infect chickens' joint, causing the infectious synovitis lesion, via respiratory infection or intra-articular inoculation (Lockaby et al., 1998; Bencina et al., 2001; Limpavithayakul et al., 2016). Moreover, some *M. synoviae* isolates obtained from reproductive tract of chickens could perform ability to infect the reproductive tract of chickens via respiratory infection and could cause the egg shell apex abnormalities (Feberwee et al., 2009a). The clinical lesions of airsacculitis, infectious synovitis and eggshell abnormalities eventually lead to various economic losses including a decrease of poultry flocks' performances; reduction of egg production, hatchability, and growth rate; and an increase of carcasses' condemnation (Landman, 2014).

2.2) Economic impacts of *M. synoviae* infection in chickens

Although *M. synoviae* infected flocks may not affect to the trade limitation, economic impacts of *M. synoviae* infection seem to be noticeable increased because of the emergence of reproductive tract infection, affecting the eggshell quality and egg production, and the occurrence of arthropathic infection associating with the infectious synovitis (Kleven and Ferguson-Noel, 2008; Landman, 2014). Therefore, *M. synoviae* is well-known as an important pathogen inducing large economic losses in poultry industry worldwide including Brazil, Netherlands and Thailand (Buim et al., 2009; Pakpinyo et al., 2009; Seifi and Shirzad, 2012; Landman, 2014; Limpavithayakul et al., 2016). According to surveillance of *M. synoviae* disease worldwide especially in Brazil and Netherlands, *M. synoviae* was detected and suspected to be an important pathogen in commercial chickens showing problems of respiratory signs or egg-production reduction (Fiorentin et al., 2003b; Buim et al., 2009; Pourbakhsh et al., 2010; Seifi and Shirzad, 2012). Although the impacts of *M. gallisepticum* worldwide has been concerned much more than *M. synoviae* (Goren, 1979; Mohammed et al., 1987);

however, the prevalence of *M. synoviae* in poultry flocks in some countries like Netherlands was higher than that of *M. gallisepticum* (Feberwee et al., 2008).

Interestingly, most poultry farms including layer farms, breeder farms and broiler farms could present the *M. synoviae* infection and some *M. synoviae* infected flocks could show the co-infection with *M. gallisepticum* (Feberwee et al., 2008; Buim et al., 2009; Pourbakhsh et al., 2010; Seifi and Shirzad, 2012). Therefore, day-old-chicks originated from infected breeder flocks with high *M. synoviae* prevalence could be an important predisposing cause of *M. synoviae* problem in commercial broiler farms (Feberwee et al., 2008; Buim et al., 2009). Moreover, *M. synoviae* circulation among breeder and layer flocks could be influenced by high concentration of pathogens in environments, by inadequate sanitary barriers, and by weak biosecurity system but the long period of remaining at the farm is the most important predisposing factor of *M. synoviae* in breeder and layer flocks. Because *M. synoviae* could remain feasible in farm location for long periods, so economic impacts of *M. synoviae* infection in commercial broiler farms seem to be increased likewise the increased age of their parent stocks farms. Besides, the old breeder and layer flocks could also be the major source of *M. synoviae* possibly transferred to the new flocks when introduced into the *M. synoviae* infected farms (Buim et al., 2009; Pourbakhsh et al., 2010; Seifi and Shirzad, 2012).

Most broiler chicken flocks have faced extensive economic losses due to the occurrence of airsacculitis which could increase carcasses' condemnation rate and could decrease production performance determining by body weight reduction and high level of feed conversion ratio (Kleven et al., 1972; King et al., 1973; Goren, 1978). The severe airsacculitis or the huge economic losses in infected broiler chickens could be greatly influenced by co-infection with various respiratory pathogens, environmental factors and/or immunosuppression (Kleven et al., 1972; Giambrone et al., 1977; Yoder Jr et al., 1977). Moreover, the synovial membrane infection causing infectious synovitis could impact economic losses in the poultry flocks due to culling of lame chickens and decreasing in growth rate of affected flocks (Landman and Feberwee, 2001).

Besides, layer flocks, the arthropathic infection, may observable loss due to the decreasing of growth rate and the culling of lameness birds. In addition to the decrease of 26% body weight in the rearing pullets of brown layers infected with arthropathic field isolate via intra-articular route, the huge economic losses in infected layer flocks could be influenced by the number of affected birds which generally ranges from 5 to 15% and may exceed to 75% of flock. Besides, in infected breeder flocks, the subclinical respiratory signs with the decreasing in egg production 5-10% and in hatchability 5-7%, and the increasing in mortality more than 5% are also determined (Landman and Feberwee, 2004; Kleven and Ferguson-Noel, 2008). In addition to the respiratory tract and arthropathic infection, the oviduct infection is able to induce eggshell apex abnormalities presenting an altered thinning translucent eggshell surface with easily crack or break (Lockaby et al., 1998; Kleven and Ferguson-Noel, 2008; Feberwee et al., 2009a; Feberwee et al., 2009b; Pakpinyo et al., 2009; Landman, 2014). The economic losses from eggshell apex abnormalities could occur both at the farm level and at the egg packing station due to breakage of eggs, downgrading and labor costs for eggs selection (Landman, 2014; Moreiraa et al., 2014).

2.3) Incubation period and transmission of *M. synoviae*

Incubation period of *M. synoviae* disease depends on modes of transmission. Horizontal and vertical transmissions could induce disease within 11–21 days and up to only 6 days, respectively (Sentíes-Cué et al., 2005). As a result of maternal derived antibodies (MDA) against *M. synoviae*, the commercial broiler chickens should not be detected clinical signs earlier than 35 days of age (Sun et al., 2017). In addition to the presence of antibodies against *M. synoviae* in young chickens especially in first 50 days of age (Sun et al., 2017), the virulence of difference *M. synoviae* strain (Lockaby et al., 1998; Narat et al., 1998; Khiari et al., 2010; Limpavithayakul et al., 2016) and the immune responses to *M. synoviae* infection (Washburn et al., 1985) could also predispose a reproduction of *M. synoviae* organisms consequently affecting the incubation period (Sun et al., 2017).

Interestingly, the infected chickens could be reservoirs or carriers of *M. synoviae* for life. They can horizontally spread pathogens to environments and can vertically transmit organisms to their progeny (Kleven and Ferguson-Noel, 2008; Buim et al., 2009; Pakpinyo et al., 2009; Khiari et al., 2010; Wetzel et al., 2010; Landman, 2014). Besides, the horizontal transmission of *M. synoviae* frequently occurs via respiratory tract by direct contact with *M. synoviae* contaminated environments or materials, while the vertical transmission occurs only in the infected chickens especially during egg production period. In naturally and artificially infected chickens, vertical transmission or egg transmission usually occurs during 1-4 weeks post infection and the highest rate of egg transmission is always found during 4-6 weeks post infection which *M. synoviae* organisms could be isolated at day-old chickens approximately 6.09% in egg yolk and 10.5% in trachea (Roberts and McDaniel, 1967; Vardaman, 1976; Kleven and Ferguson-Noel, 2008; Buim et al., 2009; Pakpinyo et al., 2009; Khiari et al., 2010; Wetzel et al., 2010). Although the percentage of vertically-infected progeny is low during chronic phase; however, the horizontal transmission, spreading to non-infected chickens, could occur anytime (Fahey and Crawley, 1954). Even if transmission via indirect contact is rather unexpected for wall-less bacteria because their sensitivity to osmotic shock, heating or chemical treatments, the high dissemination capacity of *M. synoviae* has been demonstrated that horizontal transmission could be occurs by indirect contact via contaminated materials, equipment, people, pigeons, ostriches and wild animals (Marois et al., 2000; Tebyanian et al., 2014). Therefore, *M. synoviae* isolates of the progeny flock might not be similar to those from the breeder flock, but may be similar to *M. synoviae* isolates contaminated in environments or obtained from another flocks in the same areas (Sentíes-Cué et al., 2005).

2.4) Pathogenesis of *M. synoviae*

M. synoviae is lack of cell wall. The surface proteins embedded in the cell membrane could play an important role in the interactions with host cells; both adherence and invasion (Razin et al., 1998; Buim et al., 2011). Hemagglutinin protein encoded by *vlhA* gene is an important surface membrane protein apparently

associated with the antigenic variability involving in adherence, colonization, virulence and antigenic variations among different strains (Noormohammadi et al., 1998; Razin et al., 1998; Noormohammadi et al., 2000; Bercic et al., 2008). Besides, the pathogenesis of *M. synoviae* infection was affected by immune responses, transmissible routes, and predisposing factors involving the incubation period of disease and the proliferation of *M. synoviae* in chickens (Washburn et al., 1985; Lockaby et al., 1998; Narat et al., 1998; Khiari et al., 2010; Limpavithayakul et al., 2016; Sun et al., 2017).

The pathogenesis of *M. synoviae* infection depends on the transmissible routes. The intravenous inoculation and the food pad injection are more frequently causing progressive synovitis lesions, while the respiratory inoculation and the aerosol exposure usually present mild synovitis lesions (Lockaby et al., 1998; Landman and Feberwee, 2004). In addition, the induction of EAA caused by *M. synoviae* infection is also depended on the route of inoculation. Translocation of *M. synoviae* organisms from the airsacs to the oviduct is more efficient than the oviduct colonization via the bloodstream (Feberwee et al., 2009a). Besides, *M. synoviae* has been proved the *in vitro* capacity to invade epithelial cell line, HEp-2 cells, and non-phagocytic chicken cells, including chicken erythrocytes, chicken embryonic cell line and chicken chondrocytes, within 3 hours and 24 hours of incubation, respectively (Dušanić et al., 2009; Buim et al., 2011). Interestingly, the internalization property of *M. synoviae* could be a crucial factor in transposition strategy from the mucous barrier to the more favorable niches which *M. synoviae* could resist to the antibiotics treatment and could evade the host immune response (Buim et al., 2011).

In natural infection, after colonization in the upper respiratory tract, *M. synoviae* possibly spread to joints via a hematogenous route by invading in chicken erythrocytes like *M. gallisepticum (Dušanić et al., 2009)*. Severe respiratory tissue damage in infected chickens predisposed by environmental distress or co-infection with other respiratory pathogens could enhance the spread of *M. synoviae* via bloodstream and the occurrence of joint lesions (Olson and Kerr, 1967; Yoder Jr et al., 1977; Kawakubo et al., 1980; Landman and Feberwee, 2004; Dušanić et al., 2009). Besides, an environmental temperature and a relative humidity in poultry house could also predispose the prevalence, the incidence, and the severity of *M. synoviae* infection in

the poultry flocks, so the difference ventilation management affecting temperature and relative humidity in the poultry house could be an important predisposing factor of various impacts of *M. synoviae* infection in poultry industry (Yoder Jr et al., 1977)

2.5) Immune responses associated with *M. synoviae* infection

Cytokines are soluble polypeptides and glycopeptides produced by a broad range of cell types to affect behaviors or cellular responses of other cells such as proliferation, differentiation, activation, and apoptosis (Arai et al., 1990; Kogut, 2000; Giansanti et al., 2006). Besides, cytokines mediating cellular responses via autocrine, paracrine, and endocrine activities like hormones (Arai et al., 1990) could work together as complex network for control both inflammatory and immune responses to invasive pathogens (Grossberg, 1987; Kogut, 2000). Based on the variation in type of immune response mechanisms for protecting the host, cytokines are mostly classified based on their activities and functional roles in regulating inflammation and immunity such as pro-inflammatory cytokines, T1 cytokines, T2 cytokines, T3 cytokines, interferons, chemokines, and colony stimulating factors (Giansanti et al., 2006). Therefore, the successful defense mechanism to any pathogens depends on the stimulation of proper cytokines which subsequently induce proper effector cells and immune responses (Kogut, 2000; Giansanti et al., 2006).

The colonization of *M. synoviae* in upper respiratory mucosa could induce both local and systemic immune response (Razin et al., 1998) whereas the systemic infection of *M. synoviae* could activate the lymphoid tissue in several organs showing cellular infiltration (Sentíes-Cué et al., 2005). The embedded haemagglutinin in cell membrane of *M. synoviae* has been classified as the major membrane immunogen or pathogen associated molecular patterns (PAMPs) playing a vital role in the interactions to Toll-like receptors (TLR) of chicken heterophils and macrophages which are essential cellular component of the innate immune system connecting the acquired immunity (Noormohammadi et al., 1998; Razin et al., 1998; Bencina et al., 2001; Fukui et al., 2001; Iqbal et al., 2005).

Interestingly, the numbers of macrophages in the tracheal mucosa could perform an important function in the protective immunity development associated with the cell mediated immune response (Gaunson et al., 2006). *M. synoviae* infection could activate the TLR2 signal transduction in the chicken tracheal mucosa which subsequently induce the up regulation of cytokine genes expression associated with innate immune response, inflammation process and macrophage activation such as interleukin-18 (IL-18), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), inducible nitric oxide synthase (iNOS) and macrophage Inflammatory proteins (MIPβ, CCL-4) (Beutler et al., 2006; Lavrič et al., 2007; Bolha et al., 2013; Majumder and Silbart, 2016). Besides, the secretion of pro-inflammatory cytokines; IL-6, IL-1β and iNOS during acute phase of infection could activate macrophages which subsequently stimulate and recruit B-lymphocytes and T lymphocytes (Wigley and Kaiser, 2003; Lavrič et al., 2007).

The cytokines IL-2, IL-6, IL-12, IL-18, IL-21, TGF-β and IFN-γ associated with recruitment and activation of leukocytes, including macrophages, NK-cells, Th1 cells, Th17 cells and B-lymphocytes could be presented in infected tracheal epithelial cells and other infected organs to manipulate a dissemination and a clearance of *M. synoviae* (Swain et al., 2012; Bolha et al., 2013; Majumder and Silbart, 2016). The immune responses during acute phase of *M. synoviae* infection could represent the upregulated cytokines associated with Th1 cells; including IL-2, IL-12 and IFN-γ; associated with Th17 cells; including IL-6, IL-21 and TGF-β and associated with cell-mediated immune functional of macrophages and NK-cells; including IL-12, IL-18 and IFN-γ (Gaunson et al., 2006; Swain et al., 2012; Majumder and Silbart, 2016). The combination effect of IL-12 and IL-18 could consequently express IFN-γ stimulating the IgG production in B-lymphocytes which usually present in the follicular tracheal lesions after 3 weeks post infection (Gaunson et al., 2006). Interestingly, the co-infection with other respiratory pathogens like NDV predisposes severe airsacculitis or severe respiratory disease in *M. synoviae* infected chickens because NDV infection could induce the downregulation of cytokines gene especially IL-1β, IL-2, IL-6, IL-18, iNOS and IFN-γ which were mostly upregulated by *M. synoviae* infection. Besides, the

reduced immune responses to *M. synoviae* due to co-infected with NDV may be associated with the reduced antibody response in B-cells follicles and the reduced function of macrophages or NK-cells (Silva et al., 2008). Therefore, the humoral immunity related to the development of B-cells follicles in infected organs and the immune function of both macrophages and NK-cells could also be the remarkable factors associated with the pathogenesis, recovery process and protective immunity (Gaunson et al., 2006).

2.6) Diagnosis approaches for *M. synoviae* infection

Diagnosis assays for *M. synoviae* infection mostly consist of antibody detection using serological assays, *M. synoviae* isolation using culture method, *M. synoviae* identification using molecular assays, and strain differentiation using sequence analysis (Hong et al., 2004; Jeffery et al., 2007; Kleven and Ferguson-Noel, 2008).

2.6.1) Antibody detection

Conventional diagnosis assay for *M. synoviae* infection has rely on the serological tests including serum plate agglutination (SPA) or rapid plate agglutination (RPA) assay, hemagglutination inhibition (HI) test, and enzyme linked immunosorbent assay (ELISA). All serological methods are improper tools for *M. synoviae* diagnosis in case of early infection as a result of the seroconversion gap period after infection; a minimum 1 week and up to 3 weeks requirement before antibodies will be detected by RPA assay and HI test, respectively (Kleven, 1975). Therefore, serological assays are suitably applied as screening tools in surveillance or monitoring programs of *M. synoviae* infection in poultry flocks (Kleven, 1975; Hong et al., 2004). Besides, the positive serological samples should be further confirmed by using culture method and molecular assays (Ewing et al., 1996; Luciano et al., 2011).

2.6.2) *M. synoviae* isolation using culture method

Swab samples and *M. synoviae* isolates are firstly cultured in the FMS broth; Frey's medium supplemented with 15% swine serum. The FMS broths are incubated

at 37°C in a humidified chamber for 5–7 days until their color changed from pink-red to orange-yellow. Then, the orange-yellow FMS broths, showing mycoplasma growth, are diluted for culture on FMS agar and incubated at 37°C in humidified condition until mycoplasma colonies could be observed. Moreover, mycoplasma colonies on FMS agar need to be isolated and identified as the *M. synoviae* colony by an immunofluorescent assay (IFA) or molecular assays (Kleven, 1998; Kleven and Ferguson-Noel, 2008).

To detect and identify the *M. synoviae* colonies, a direct fluorescent antibody test or an immunofluorescent assay (IFA) could be performed in a biosafety cabinet as described by Talkington and Kleven (1983). The IFA is accomplished by using fluorescent-conjugated *M. synoviae*-specific antibody, provided by S.H. Kleven (Poultry Disease Research Center, Department of Avian Medicine, College of Veterinary Medicine, University of Georgia, Athens, GA 30605). Briefly, *M. synoviae* colonies on FMS agar medium are enclosed in a stainless-steel cylinder, washed with phosphate-buffered saline (PBS), stained with fluorescent-conjugated, *M. synoviae*-specific antibody, and then incubated at 37°C in a humidified chamber. After incubation, *M. synoviae* colonies are washed and soaked with PBS at 4°C. Thereafter, stainless steel cylinders are carefully removed, while the stained mycoplasma colonies are identified on fluorescent microscope. Besides, all IFA steps should be done at room temperature unless indicated (Talkington and Kleven, 1983).

2.6.3) DNA analysis by molecular assays

Because *M. synoviae* isolation using culture method with immunofluorescent assay is the time consuming and laborious method as a result of the fastidious nature of *M. synoviae* organisms, so molecular assays including conventional polymerase chain reaction (PCR) assay and sequence analysis have been developed and become the method of choice for *M. synoviae* detection and strain differentiation due to their advantages, simple, rapid, highly sensitivity and excellent specificity (Kleven and Ferguson-Noel, 2008; Pakpinyo et al., 2009).

DNA templates are extracted from FMS broth culture using the modified rapid boiling DNA extraction (Ley et al., 1997). Briefly, the broth cultures are centrifuged at

16,000× g for 6 min, washed twice with sterile phosphate-buffered saline (PBS), and resuspended in 50 µl of sterile PBS. Packed cells are boiled at 100°C for 10 min, placed on ice for 10 min, and centrifuged at 16,000× g for 6 min. The supernatant containing DNA templates is collected and stored at -20°C until used (Hong et al., 2004). In addition, The DNA template concentration is determined using a NanoDrop™ spectrophotometer. The DNA templates are prepared for further molecular assays including the 16S rRNA gene-based PCR assays and the *vlhA*-based PCR assays.

A) The 16S rRNA gene-based PCR assay

The 16S rRNA gene-based PCR assay with highly specific to *M. synoviae* has been widely used to monitor and detect *M. synoviae* infection in poultry flocks (Hong et al., 2004). Analysis of *M. synoviae*, using the 16S rRNA gene-based PCR assay, indicated a sensitivity of 82% and a specificity of 100% (Lauerman et al., 1993). Briefly, the primers, MSLF (5' GAG AAG CAA AAT AGT GAT ATC A 3') and MSLR (5' CAG TCG TCT CCG AAG TTA ACA A 3'), can produce PCR amplicons size 214 bp (Lauerman et al., 1993; Lauerman, 1998). Interestingly, because *M. synoviae* could survive in the environments such as feed, dust, fly, or soil; so the 16S rRNA PCR assay could be applied to detect the contaminated *M. synoviae* organisms (Christensen et al., 1994; Marois et al., 2000). Even if, the sequence analysis of partial 16S rRNA gene may show genetic variability of different *M. synoviae* strains; however, the conserved nature of 16S rRNA gene is not suitable for the strain differentiation because the polymorphic patterns of 16S rRNA gene could not be related to the origin area of *M. synoviae* strains and/or the occurrence of genotypic diversity (Hong et al., 2004; Buim et al., 2010). Therefore, the hemagglutinin gene-based PCR assay was developed and designed to differentiate *M. synoviae* strains depending on their virulence (Hong et al., 2004; Jeffery et al., 2007).

B) The *vlhA* gene-based PCR assay

The hemagglutinin protein encoded by *vlhA* gene is apparently associated with antigenic variability by involving in adherence, colonization, virulence, and antigenic variations among different strains of *M. synoviae* (Noormohammadi et al., 1998; Razin

et al., 1998; Noormohammadi et al., 2000; Bercic et al., 2008). The *vlhA* gene expresses proteins could be cleave post-translationally into the C-terminal hemagglutinin fragment MSPA (50-55 kDa of major surface protein A) and the N-terminal lipoprotein fragment MSPB (40-50 kDa of major surface protein B) (Noormohammadi et al., 2000; Bencina et al., 2001). The size of MSPB differs among *M. synoviae* isolates due to insertions or deletions in the *vlhA* gene region encoding proline-rich repeats (PRR) (Bencina et al., 2001). In addition, the truncated forms of MSPB or tMSPB (20-30 kDa of MSPB) could occur in some *M. synoviae* organisms which have transforming in their hemagglutinating phenotype. Besides, both MSPB and tMSPB are highly immunogenic proteins inducing local and systemic antibody responses in the early period of infectious synovitis (Narat et al., 1998; Noormohammadi et al., 1998).

The N-terminal of *vlhA* gene (nucleotides 1-410), encoding for major surface protein B (MSPB), is a single chromosomal copy with high variation among *M. synoviae* strains. The downstream region of the N-terminal of *vlhA* gene is replaced with the pseudogenes sequence (Noormohammadi et al., 2000; Bencina et al., 2001). Therefore, the antigenic variations among different *M. synoviae* strains could be influenced by a recombination of the expressed *vlhA* gene with one of pseudogenes (Noormohammadi et al., 2000). Consequently, the *vlhA* gene-based PCR assay was developed to differentiate *M. synoviae* strains based on sequence analysis of a single copy of the *vlhA* gene showing high discriminatory. Briefly, the 370-400 bp of *vlhA* gene fragments are amplified using the Hammond *vlhA* gene-targeted PCR assay with the primer MSRH-F (5'- GGC CAT TGC TCC TRC TGT TAT-3') and the primer MSRH-R (5'- AGT AAC CGA TCC GCT TAA TGC-3') (Wetzel et al., 2010; El-Gazzar et al., 2012).

2.6.4) Strain differentiation by sequence analysis of partial *vlhA* gene

Since surface proteins embedded in a cell membrane of *M. synoviae* could play an important role in the interactions with host cells (Razin et al., 1998; Buim et al., 2011). Besides, hemagglutinin protein encoded by *vlhA* gene is apparently associated with the antigenic variability of *M. synoviae*. Therefore, the *vlhA* gene-based PCR assay was developed to differentiate *M. synoviae* strains, using sequence analysis, without culture or isolation methods (Noormohammadi et al., 1998; Razin et al., 1998;

Noormohammadi et al., 2000; Hong et al., 2004; Jeffery et al., 2007; Bercic et al., 2008). Briefly, the proline-rich repeat (PRR) region of *vlhA* gene is useful for typing of *M. synoviae* strains based on insertion/deletion of nucleotides, while the RIII region is useful for subtyping of *M. synoviae* strains by nucleotide polymorphisms (Bencina et al., 2001; Hammond et al., 2009).

According to the molecular basis of N-terminal fragment MSPB, the size variation in PRR region could be associated with the invasiveness of *M. synoviae* strains, causing systemic infection and infectious synovitis in infected chickens (Bencina et al., 2001). The current 12 types of *M. synoviae* isolates, including types A, B, C, D E, F, G, H, I, J, K and L, have been classified on the basis of the size of PRR region, consisting of 38, 45, 32, 23, 19, 36, 51, 46, 28, 20, 12 and 35 amino acids, respectively (Bencina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Bayatzadeh et al., 2014; Limpavithayakul et al., 2016). Based on the description of the *vlhA* fragment, *M. synoviae* isolates found in the USA and Canada were types A, B, C, D, and E (Bencina et al., 2001), while those from Europe were types A, C, and E. In the United Kingdom, they were types C, E, and F. In Asia, *M. synoviae* isolates of Iran were types F, G, H, I, J, and K (Bencina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Bayatzadeh et al., 2014).

However, despite the report of Thai *M. synoviae* infection in commercial chicken and native chicken flocks determined by serological methods, culture technique and PCR assays, (Pakpinyo et al., 2009), there is an available information of Thai *M. synoviae* field isolates which could be identified as types E, C, and L with 19, 32, and 35 amino acid lengths, respectively, indicating that there were at least 3 genogroups of *M. synoviae* circulated in Thailand (Limpavithayakul et al., 2016). In addition, Thai *M. synoviae* types C and E, isolated from chickens showing respiratory signs, were possibly associated with respiratory tract infection, but they could not induce the egg shell apex abnormality syndrome like Dutch *M. synoviae* isolate types C and E, which could infect the reproductive tract of chickens (Feberwee et al., 2009a). Interestingly, in addition to the highly invasive *M. synoviae* strain K1968 classified as *M. synoviae* type B with long PRR region which could also cause infectious synovitis in chickens, Thai *M. synoviae* isolates type L causing infectious synovitis and lameness in

chickens was identified and confirmed as the arthropathic strain (Bencina et al., 2001; Limpavithayakul et al., 2016).

2.6.5) Complete genome sequence of *M. synoviae*

The next-generation sequencing technology representing ability to sequence large number of genomes cheaply could generate the extended or complete genome sequence information which possibly fulfill the genetic background and promote the understandings of how genetic differences can affect disease virulence and pathogenesis (Voelkerding et al., 2009; Metzker, 2010). So the comparative analyses of the complete mycoplasma genomes could provide a chance to identify the strain-specific regions associated with pathogenicity, to characterize the function of gene composition and to design the proper strategies for disease control (Yogev et al., 1991; Yogev et al., 1995; Voelkerding et al., 2009; Metzker, 2010).

Complete genome sequence of *M. synoviae* WVU 1853 strain (ATCC 25204) was analyzed by using the PacBio RS Modification and Motif Analysis module version 2.2.0 to provide the epigenetic modifications data of non-pathogenic strain which was deposited in GenBank under the accession number CP011096. The closed circular genome of *M. synoviae* WVU 1853 strain is 846,495 bp in nucleotide length with predicted 28.3% of G+C content (May et al., 2015). In addition, the complete genome alignments of whole 799,000 bp of Brazilian low virulent *M. synoviae* strain composing of 694 coding DNA sequences was compared with the complete genome sequence of other porcine mycoplasma pathogens such as *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Mycoplasma suis* to determine strain-specific region which could be probably involved in the pathogenesis (Fiorentin et al., 2003a; Vasconcelos et al., 2005; Liu et al., 2010; Guimaraes et al., 2011). Besides, the complete genome sequence of Brazilian low virulence *M. synoviae* strain presented 464 known protein coding DNA sequences (CDSs) in total 694 CDSs. The organization of hemagglutinin genes in *M. synoviae* comprised 70 CDSs with a single locus differentiating from *Mycoplasma gallisepticum* comprising 43 CDSs with five loci (Papazisi et al., 2003; Allen et al., 2005; Vasconcelos et al., 2005). Unfortunately, virulence factors and pathogenesis of *M. synoviae* are not well established, and the complete genome

sequence of Thai *M. synoviae* isolates has not been determined because of the requirement of high quality of purified DNA using with high performance sequencing machine. However, strain-specific regions, genome rearrangements and alterations in adhesin sequences could be potentially associated with pathogenicity as previous observation in *Mycoplasma hyopneumoniae* strains (Vasconcelos et al., 2005).

2.7) Prevention and control strategies of *M. synoviae* infection

The disease control strategy of *M. synoviae* infection in breeder, layer and broiler flocks normally consists of three general approaches; pathogen-free flocks' maintenance, medication, and vaccination (Umar et al., 2017). Besides, the successful strategy requires the early detection of disease and the corrected assessments of risks in poultry farms (Stipkovits and Kempf, 1996). The poultry farms have been concerned to initially maintain *M. synoviae*-free flocks by obtaining only the *M. synoviae*-free chickens originated from *M. synoviae*-free parent flocks and subsequently fulfill disease control by rearing *M. synoviae*-free chickens in the clean environment with strict biosecurity system or maintaining the channeling measure separating uninfected flocks and their eggs from *M. synoviae*-infected flocks (Markham et al., 1998b; Landman, 2014).

In broiler flocks, *M. synoviae* organisms in hatching eggs obtaining from *M. synoviae*-infected breeder flocks were reported to inactivated by a pre-incubation heat treatment procedure at hatchery (Yoder Jr, 1970). Besides, medication in *M. synoviae* infected broiler chickens usually done for controlling the co-infection with other respiratory pathogens like *Escherichia coli* (*E. coli*) or *Ornithobacterium rhinotracheale* (ORT). However, medications are uncommon economically practice in commercial broiler production industry concentrating to produce high quality and low-cost products (Fiorentin et al., 2003b). Therefore, *M. synoviae*-free flocks' maintenance and disease control strategies in breeder flocks are the key measures to reduce economic losses and treatment cost in broiler flocks (Buim et al., 2009; Landman, 2014; Hong et al., 2015).

In breeder and layer flocks, single-age management and elimination of infected flocks are notified as the strongest measure due to the efficacy in decreasing an incidence and a prevalence of *M. synoviae* disease in poultry flocks. Although the most effective strategy could stop horizontal transmission transferring *M. synoviae* to new rearing flocks when introduced into the *M. synoviae* infected farms and could break down the cycle of vertical transmission from hens to their progeny; however, single-age management and elimination of infected flocks are not friendly practices and are not the economic compliances in poultry industry business (Stipkovits and Kempf, 1996; Markham et al., 1998b; Feberwee and Landman, 2012; Landman, 2014). Due to an obvious efficacy in reducing clinical signs or improving production performances in the poultry industrial level, medication and vaccination have been suggested as alternative practices representing an attractive optimal cost-benefit relation of each methods (Stipkovits and Kempf, 1996; Markham et al., 1998b; Fiorentin et al., 2003b; Kleven and Ferguson-Noel, 2008; Ley, 2008; Feberwee et al., 2009b; Landman, 2014; Hong et al., 2015; Kreizinger et al., 2017).

Medication strategy which mostly performs with mycoplasma susceptible antimicrobials inhibiting protein synthesis of *M. synoviae* organism is the common practice in poultry industry due to the limited countries having available live vaccine registration and the obvious efficacy of medication in reducing clinical signs or improving production performance of *M. synoviae* infected flocks (Fiorentin et al., 2003b; Kleven and Ferguson-Noel, 2008; Landman et al., 2008; Ley, 2008; Catania et al., 2010; Landman, 2014; Hong et al., 2015; Kreizinger et al., 2017). Besides, the mycoplasma susceptible antimicrobials commonly use in poultry industry consist of tetracyclines (oxytracycline, chlortetracycline, and doxycycline), macrolides (tylosin, tilmicosin, tylvalosin, erythromycin, spiramycin), lincosamides (lincomycin), quinolones (enrofloxacin), and pleuromutilins (tiamulin) (Landman et al., 2008; Hong et al., 2015; Kreizinger et al., 2017). Moreover, medication is effective in preventing economic losses associated with *M. synoviae* infections because of the efficacy in reducing the populations of *M. synoviae* in the respiratory tract like *M. gallisepticum*, in reducing the risks of horizontal transmission in poultry farms, in reducing the clinical signs in

infected chickens, and in reducing the vertical transmission via eggs (Yoder Jr et al., 1977; Stanley et al., 2001).

M. synoviae could be accidental eradicated from the infected flocks after the intensive medication for treating *E. coli* infection; however, only medication could not completely eliminate *M. synoviae* from the infected chicken flocks (Stanley et al., 2001; Fiorentin et al., 2003b; Kleven, 2008; Kleven and Ferguson-Noel, 2008; Ley, 2008; Catania et al., 2010; Landman, 2014; Hong et al., 2015; Kreizinger et al., 2017). Interestingly, in the high-risk farm facing a concurrent and a re-entry of *M. synoviae* pathogen, the successful control strategy for reducing *M. synoviae* growth and eradicating *M. synoviae* from infected chicken flocks could be presented by using the continuous intensive medication with excessive mycoplasma susceptible antibiotics (Landman et al., 2008; Hong et al., 2015; Kreizinger et al., 2017). Although complete disease control program associated with intensive medication method in the infected flock could decrease amount and cost of medication in the next flocks, the long term of intensive medication predisposing the antimicrobial resistance problems is not widely economically acceptable in the infected poultry flocks. Consequently, the current argument of antimicrobial resistance issues has influenced the practical medication in poultry production including a requirement of the prescription from poultry veterinarian and the reliable susceptible evident like minimum inhibitory concentration (MIC) of *M. synoviae* field strains (Edens, 2003; Behbahan et al., 2008).

Accordingly, the live *M. synoviae* vaccine has been dramatically increasing in several countries which are challenged with antimicrobial resistance argument and especially proposed to use in risk farms due to the obvious efficacy in reducing clinical signs or improving production performance in *M. synoviae* infected flocks (Fiorentin et al., 2003b; Kleven, 2008; Kleven and Ferguson-Noel, 2008; Ley, 2008; Nicholas et al., 2009; Catania et al., 2010; Landman, 2014; Hong et al., 2015; Kreizinger et al., 2017). Besides, the available live commercial vaccine, MS-H strain originated from Australian field strain, is the temperature-sensitive mutant strain growing better at 33 ^{o}C and has been shown to be safe and efficacious for use in chickens (Markham et al., 1998b; Kleven, 2008). Vaccination with live MS-H strain could experimentally present an ability in reducing clinical signs and improving production performance in infected flocks such

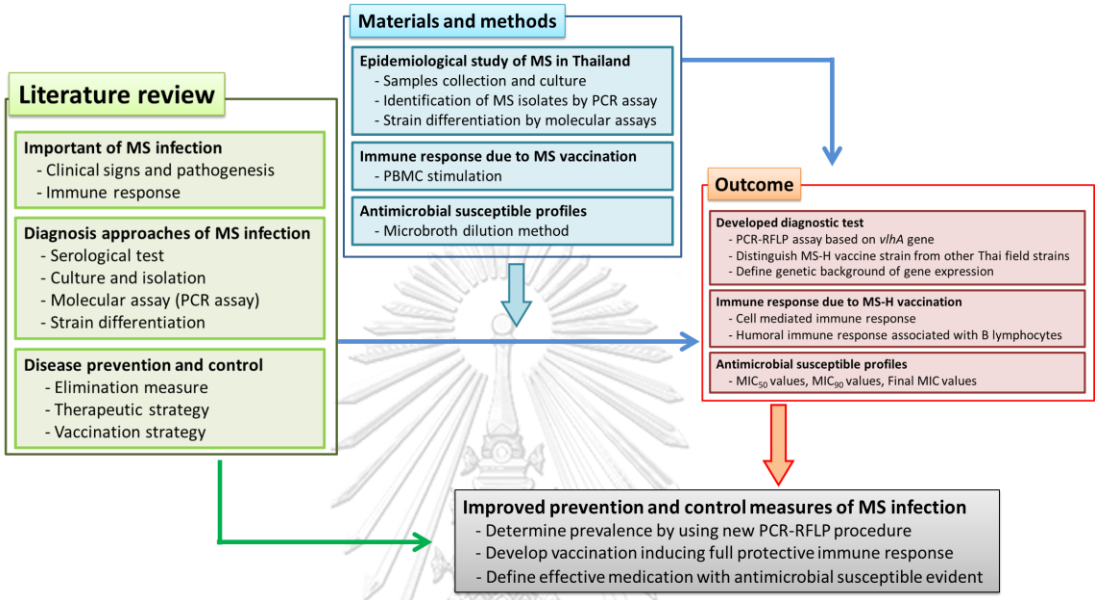
as 10% decreasing in occurrence of egg shell apex abnormalities (Feberwee et al., 2009b) and 80% reducing in airsacculitis caused by co-infection of *M. synoviae* and IBV (Markham et al., 1998a).

As a results of live *M. gallisepticum* vaccines, the live *M. synoviae* vaccine could also present the inducing fluctuated antibody response over time and from flock to flock (Hein, 2004), the replacement of field strains by the vaccine strain (Barbour et al., 2000; Kleven, 2008) and the reducing horizontal shedding (Feberwee et al., 2006a). However, vaccination with live MS-H strain is uncommon practice in a field situation because the efficacy in protecting clinical signs and in preventing pathogen shedding remains questionable at commercial farm level and the information of diagnostic assays distinguishing between field and vaccine strains is also deficient (Ley, 2008; Feberwee et al., 2009b; Landman, 2014).

CHAPTER III

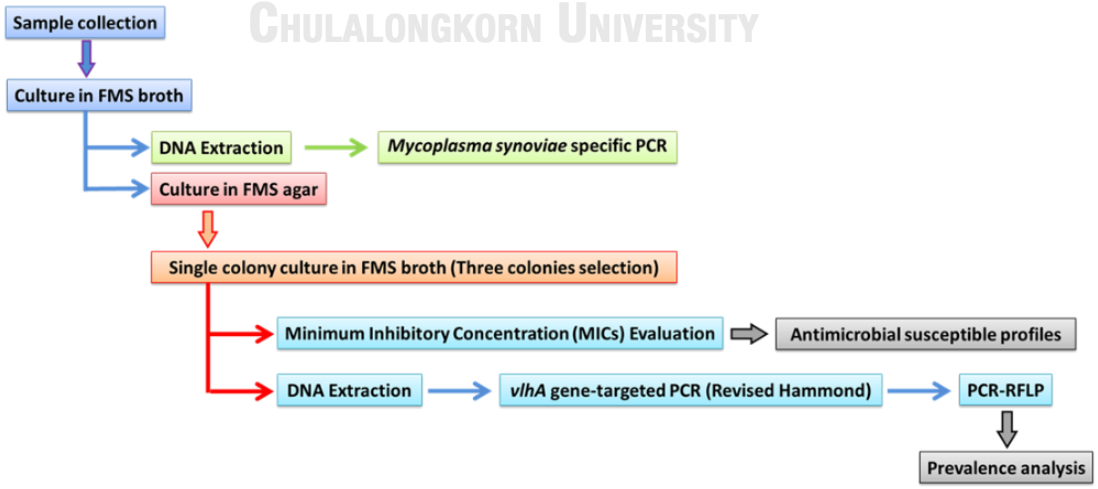
Materials and Methods

3.1) Conceptual framework of this study



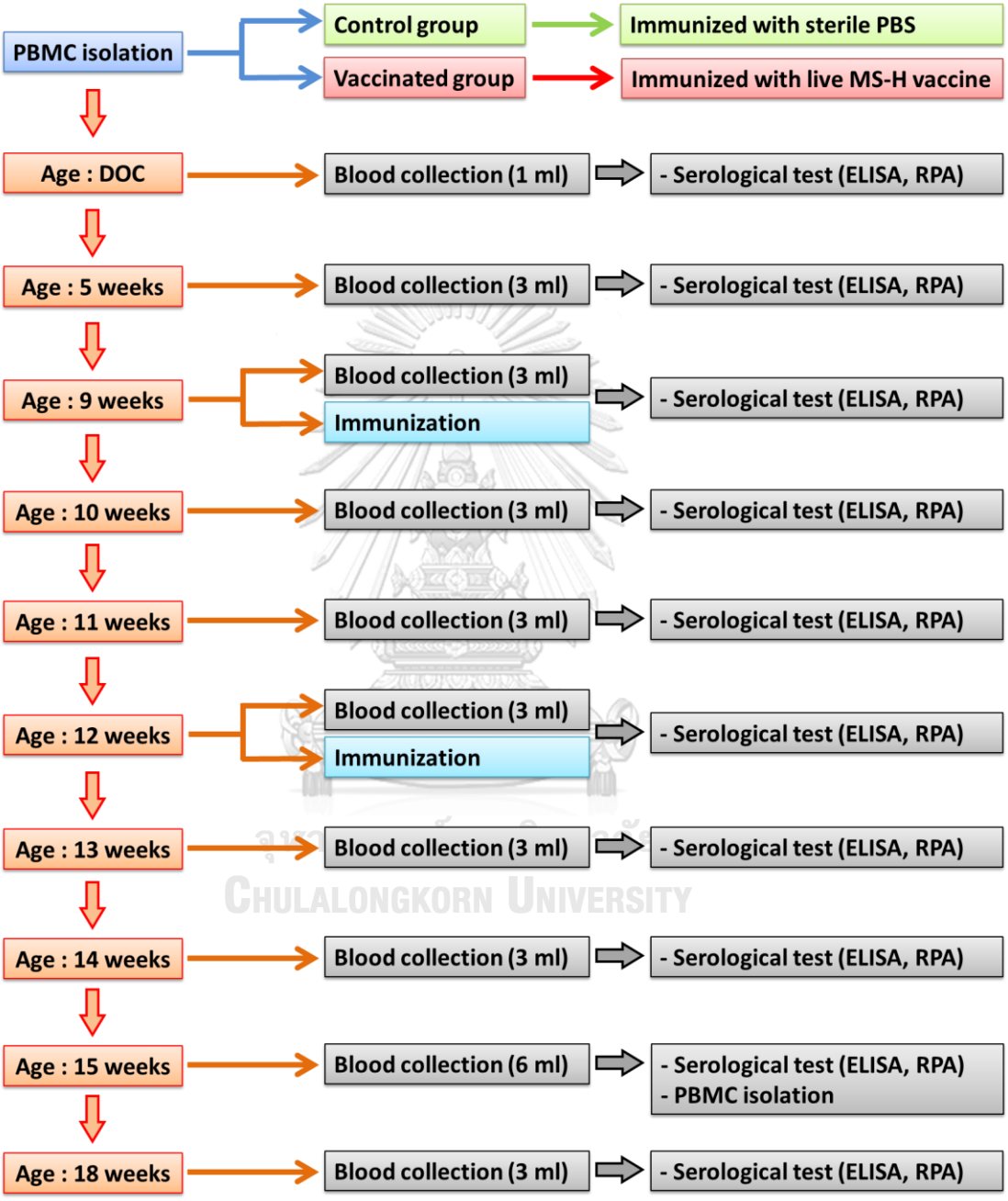
3.2) Research plan of study

3.2.1) Surveillance and sequence analysis of Thai *M. synoviae* strains

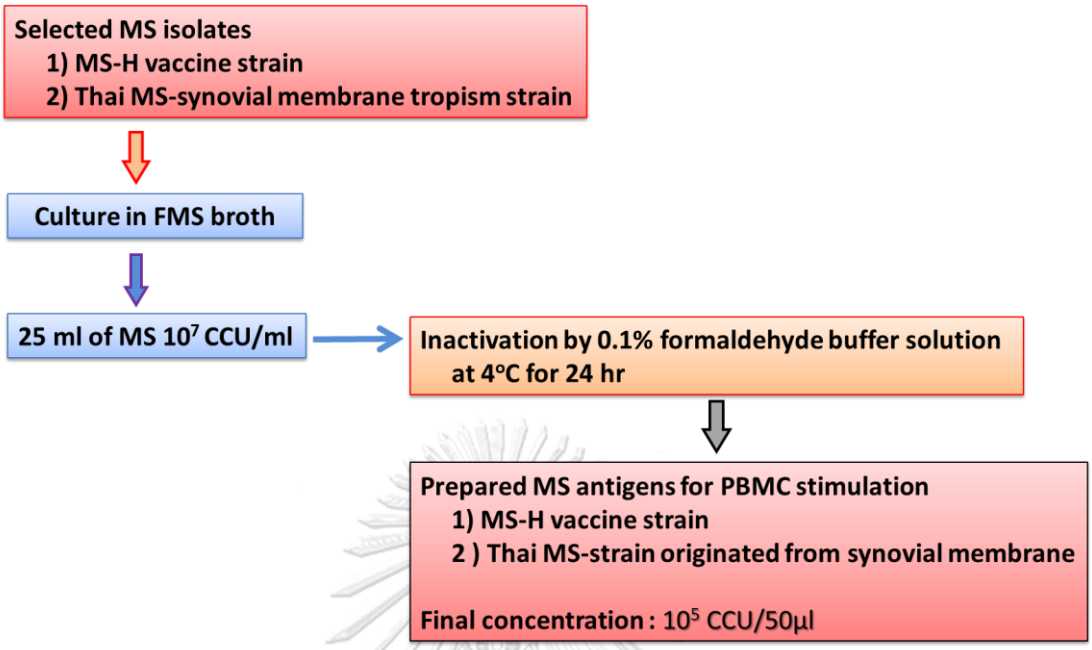


3.2.2) Immune responses due to *M. synoviae* infection in chickens

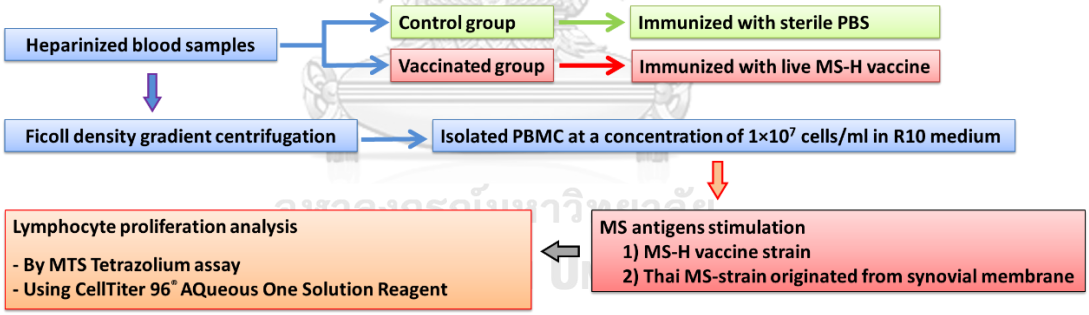
A. Experimental design and sample collection protocol



B. *M. synoviae* antigens preparation



C. PBMC isolation and stimulation



3.3) *M. synoviae* vaccine, reference and field strains

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

3.4) Sample collection in *M. synoviae* surveillance

M. synoviae field isolates were obtained from registered commercial chicken farms consisting of breeder flocks, broiler flocks, and layer flocks in Thailand during the period of 2020. Chickens were individually swabbed at articular joint or respiratory tract; choanal cleft, trachea, and airsac; using a sterilized cotton swab. The swab samples were identified and inoculated into 2 ml of FMS broth immediately (Kleven, 1998). Then, broth samples were submitted using triple pack system to Avian Health Research Laboratory Unit, Faculty of Veterinary Science, Chulalongkorn University for DNA analysis based on the 16S rRNA gene and the *vlhA* gene. All steps of DNA extraction were done in biosafety cabinet (BSL) class II. Besides, waste products were inactivated in Clorox® dilution 1:10 or 0.6% concentration for overnight before discard. The use of experimental animals in this study was approved by Chulalongkorn University Animal Care and Use Committee (IACUC), protocol No.1931051. All methods were performed in accordance with the relevant guidelines and regulations. Good practice principles were respected to minimize the discomfort and provide the well-being to chickens.

3.5) Culture method

Swab samples, *M. gallisepticum* S6 strain, *M. synoviae* WVU 1853 strain, *M. synoviae* MS-H vaccine isolate and Thai *M. synoviae* field isolates identified as types C, E, and L were individually cultured in 2 ml of FMS broth and consequently analyzed by PCR assays. Briefly, FMS broth samples were firstly incubated at 37°C in a humidified chamber for 5-7 days until the broth color changed from pink-red to orange-yellow. Then, the orange-yellow FMS broths, showing mycoplasma growth, were divided into 2 portions. First portion was subjected to extract the DNA for *M. synoviae* specific PCR assay, 16S rRNA gene-based PCR assay. The remaining portion was immediately diluted for culture on FMS agar and incubated at 37°C in humidified condition before sampling a single colony of *M. synoviae* isolate. Furthermore, the incubation time of suspected negative broth samples were extended up to 3 weeks.

Five selected single *M. synoviae* colonies were individually cultures into the fresh FMS broths and incubated at 37°C in humidified condition until the broth color changed from pink to orange-yellow. The FMS cultured broths, showing mycoplasma growth, were then equally divided into 3 portions. The first portion was extracted the DNA for the 16S rRNA gene and the *vlhA* -based PCR assays. The second and third portion were stored at -80°C as frozen stock of each pure *M. synoviae* isolate for further study (Hong et al., 2004).

3.6) DNA templates preparation

DNA templates were extracted from FMS broth culture using the modified rapid boiling DNA extraction (Ley et al., 1997). Briefly, the broth cultures were centrifuged at 16,000× g for 6 min, washed twice with sterile phosphate-buffered saline (PBS), and resuspended in 50 µl of sterile PBS. Packed cells were boiled at 100°C for 10 min, placed on ice for 10 min, and centrifuged at 16,000× g for 6 min. The supernatant containing DNA templates is collected and stored at -20°C until used (Hong et al., 2004). In addition, The DNA template concentration is determined using a NanoDrop™ spectrophotometer. The DNA templates are prepared for further molecular assays including the 16S rRNA gene-based PCR assays and the *vlhA*-based PCR assays.

In addition to DNA templates of *M. gallisepticum* S6 strain, *M. synoviae* WVU 1853 strain, *M. synoviae* MS-H vaccine isolate and Thai *M. synoviae* field isolates identified as types C, E, and L, the DNA template mixtures of Thai M. synoviae field strains and M. synoviae MS-H vaccine strains were also prepared by mixing the same DNA amount of two strains for further molecular approaches.

3.7) *M. synoviae*-specific PCR assay (16S rRNA gene-based PCR assay)

DNA templates were used in the Lauerman 16S rRNA gene-based PCR assay (Lauerman et al., 1993). PCR mixture of 50 µl contained 5 µl of 5× Green GoTaq® Flexi Buffer (Promega, Madison, WI, USA), 2.5 µl of 1.25 mM $MgCl_2$, 1 µl of 10 mM dNTP (Fermentas, Leon-Rot, Germany), 0.5 µl of each 10 µM primer MSL-1 (5'-GAA GCA AAA TAG TGA TAT CA-3') and primer MSL-2 (5'-GTC GTC TCC GAA GTT AAC AA-3') (Qiagen), 0.5 µl of 5 U/µl GoTaq® Flexi DNA Polymerase (Promega, Medison, WI, USA), and DNA template of 100–200 ng. *M. gallisepticum* S6 strain and *M. synoviae* WVU 1853 strain were used as negative and positive controls, respectively.

PCR mixtures were amplified in a DNA thermal cycler (Life express, BIOER®, ROC) starting with 94°C for 5 min and 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and then followed by 72°C for 5 min at the final extension. The PCR products were analyzed in 2% agarose gel (Vivantis Technologies, Malaysia), in a 1× TBE buffer at 100 volts for 35 min, pre-stained with MaestroSafeTM dye (Maestrogen, Las Vegas, NV, USA), visualized by UV transilluminator, and photographed. Each *M. synoviae*-specific PCR amplicon size was compared to the standard 100 bp DNA ladder (New England Biolab, UK).

3.8) PCR amplification of partial *vlhA* gene

The *vlhA* gene fragment of *M. synoviae* positive samples was amplified using the revised Hammond *vlhA* gene-targeted PCR assay (Wetzel et al., 2010). PCR mixture of 50 µl contained 5 µl of 5× Colorless GoTaq® Flexi Buffer (Promega, Madison, WI, USA), 2.5 µl of 1.25 mM $MgCl_2$, 1 µl of 10 mM dNTP (Fermentas, Leon-Rot, Germany),

0.5 µl of each 10 µM primer MSRH-1 (5'- GGC CAT TGC TCC TRC TGT TAT -3') and primer MSRH-2 (5'- AGT AAC CGA TCC GCT TAA TGC -3') (Qiagen®, Valencia, CA, USA), 0.5 µl of 5 U/µl GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA), and DNA template of 100–200 ng. *M. gallisepticum* S6 strain and *M. synoviae* WVU 1853 strain were used as negative and positive controls, respectively.

PCR mixtures were amplified in a DNA thermal cycler (Life express, BIOER®, ROC) starting with 95°C for 3 min and 40 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min and then followed by 72°C for 5 min at the final extension. The PCR products were analyzed in 2% agarose gel (Vivantis Technologies, Malaysia), in a 1x TBE buffer at 100 volts for 35 min, pre-stained with MaestroSafe™ dye (Maestrogen, Las Vegas, NV, USA), visualized by UV transilluminator, and photographed. Each *vlhA* gene-targeted PCR amplicon size was compared to standard 100bp DNA ladder (New England Biolab, UK).

3.9) Sequence analysis of partial *vlhA* gene

The *vlhA* gene PCR products from the revised Hammond *vlhA* gene-targeted PCR assay containing *vlhA* DNA fragment were purified and subjected to sequencing at A T G C Co. Ltd. (Thailand Science Park, Pathum Thani, Thailand). A similarity of nucleotide sequence was analyzed by using the BLAST program (www.ncbi.nlm.nih.gov/BLAST). Sequencing alignment analyses, corresponding to the N-terminal *vlhA* gene of *M. synoviae* K1968 strain classified as type B, were performed by using the molecular evolutionary genetic analysis (MEGA 6) software (http://www.megasoftware.net). *M. synoviae* isolates were typed based on the description of *vlhA* fragment. *M. synoviae* types A, B, C, D, E, F, G, H, I, J, K, and L were classified based on the length of PRR fragment of 38, 45, 32, 23, 19, 36, 51, 46, 28, 20, 12 and 35 amino acids, respectively (Bencina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Bayatzadeh et al., 2014; Limpavithayakul et al., 2016).

3.10) The PCR-RFLP assay

PCR-RFLP assay was developed by using the restriction enzyme map analysis tool on the Genescript webpage (https://www.genscript.com/tools/restriction-enzyme-map-analysis) to reveal the restriction enzyme TasI (ThermoFisher Scientific, San Jose, CA, USA), cutting best at 65°C in B buffer, as the suitable restriction enzyme which could digest *vlhA* gene-targeted PCR products of *M. synoviae* positive samples. Briefly, 20 µl of *vlhA* gene-based PCR product containing DNA at least 0.05 µg/ µl were added to the 23 µl of TasI mixture containing 4 µl of 10× Buffer B, 1 µl of TasI (10 U/ µl; ThermoFisher Scientific, San Jose, CA, USA), and 18 µl of distilled water. *M. gallisepticum* S6 strain and *M. synoviae* WVU 1853 strain were used as negative and positive controls, respectively. After incubation at 65°C for 2 h, all digested PCR products were separated and analyzed in 2% agarose gel (Vivantis Technologies, Malaysia), in a 1× TBE buffer at 100 volts for 45 min, pre-stained with MaestroSafe^{TM} dye (Maestrogen, Las Vegas, NV, USA), visualized by UV transilluminator, and finally photographed. Each restriction fragment size was compared to the standard 100 bp DNA ladder (New England Biolab, UK).

3.11) Inactivated *M. synoviae* antigens preparation

Homologous and heterologous *M. synoviae* antigens, MS-H and MS-L, were respectively prepared from 10^7 CFU/ml of MS-H vaccine strain and Thai *M. synoviae* arthropathic strain classified as *M. synoviae* type L (Limpavithayakul et al., 2016). About 25 ml FMS of both *M. synoviae* isolates were finally inactivated with 0.1% formaldehyde in a sterile PBS at pH 7.2. Briefly, after *M. synoviae* cultured broths were centrifuged at 12,000× g at 4°C for 30 min, the pellets were washed three times with PBS, followed by centrifugation of another 12,000× g at 4°C for 30 min, and finally resuspended in 0.1% formaldehyde-buffered solution at 4°C for 24 h. Then, treated *M. synoviae* solutions were centrifuged at 12,000× g at 4°C for 20 min, washed three times with PBS, and subsequently confirmed the complete inactivation using the culture method (Kleven, 1998; David et al., 2010). Finally, inactivated *M. synoviae* antigens were resuspended in 10 ml of R10 medium, the RPMI 1640 medium (Gibco,

USA) supplemented with 10% fetal calf serum and 500 IU penicillin-streptomycin. Antigen solutions for *in vitro* study were prepared at 2 × 10^6 cells/ml concentration for peripheral blood mononuclear cell (PBMC) stimulation, aliquoted, and stored at -20°C until use. An appropriate risk assessment of this study was approved by the Institution Biosafety Committee (CU-VET-BC), protocol No. IBC1931023.

3.12) Chickens and experimental design

A total of sixty 1-day-old healthy chicks of brown egg layer (ISA Babcock B-380 breed) obtained from a commercial hatchery were equally divided into four treatment groups comprising 15 chickens each and placed in wired cages in separated rooms at chicken experimental units of Veterinary Student Training Center, Faculty of Veterinary Science, Chulalongkorn University, Nakorn Prathom, Thailand. The control group was immunized with 30 µl sterile PBS at 9 and 12 weeks of age via the intra-ocular route, whereas three vaccinated groups consisting of groups 9V, 12V, and 9–12V were vaccinated with a dose (30 µl) of a commercial live MS-H vaccine via the same route at 9, 12, and combined 9 and 12 weeks of age, respectively. At least 10 birds from each treatment group were randomly bled via the jugular vein before immunization; at 1 day, 5 and 9 weeks of age; and after immunization; at 10, 11, 12, 13, 14, 15, and 18 weeks of age.

Serum samples were separated and immediately examined using the rapid plate agglutination (RPA) assay. The remaining serum samples were stored at -20°C before determining the *M. synoviae* antibody level using the enzyme-linked immunosorbent assay (ELISA). At 15 weeks of age, additional 3 ml of blood samples were collected from four birds randomly and suddenly mixed with heparin for further isolating PBMCs. In addition, palatine cleft swab samples from 10 chickens in each treatment group were randomly obtained before immunization; at 1 day and 9 weeks of age; and after immunization; at 10 and 13 weeks of age. The presence of *M. synoviae*-specific DNA in all swab samples was determined by inoculating into 2 ml FMS broths incubated at 37°C in a humidified chamber for 5–7 days before running the *M. synoviae*-specific PCR and PCR-RFLP assays.

Experimental chickens were reared in full-day lighting management with feed and water *ad libitum*. The live MS-H vaccine (Vaxsafe® MS, Bioproperties Pty Ltd, Australia), provided by Zoetis Animal Health Thailand Company, contained a living *M. synoviae* of ≥ $10^{5.7}$ CCU/dose. The use of experimental animals in the present study was approved by Chulalongkorn University Animal Care and Use Committee (IACUC; protocol No.1931051). All methods were performed following the relevant guidelines and regulations. Good practice principles were respected to minimize discomfort and improve the well-being of chickens.

3.13) Serology

Serum samples obtained before and after immunization were immediately tested by RPA assay using a commercial *M. synoviae* antigen (NISSEIKEN Co. Ltd, Japan). Briefly, the serum was serially diluted twofold to 1:8 in PBS pH 7.2. Then, the 30 µl undiluted and 1:8 diluted serums were tested with the same volume of RPA *M. synoviae* antigen to determine the presence of *M. synoviae*-specific antibodies presenting agglutination at dilution of 1:8 or higher (Feberwee et al., 2005).

The remaining serum samples were later examined for *M. synoviae* antibodies level using the commercial ELISA test kit (ProFlock, Synbiotics Corporation, USA). ELISA plates were read and recorded an optical density (OD) value at 450 nm using the absorbance reader (BioTek Model EL800, USA). The sample-to-positive (S/P) ratio was calculated by subtracting the average negative control OD from each sample OD and dividing the difference by the corrected positive control. Both RPA assay and ELISA were performed according to the manufacturer's instructions.

3.14) PBMC isolation and stimulation

PBMCs were purified from each heparinized peripheral blood sample at 15 weeks of age by Ficoll density gradient centrifugation (Dalgaard et al., 2010b). Then, 3 ml of heparinized blood sample was diluted with 3 ml of PBS and layered on an equal volume of Ficoll-Paque™ PLUS (density 1.077 g/ml; Amersham Biosciences, Uppsala,

Sweden) before centrifugation at 400× g 20°C for 30 min. Subsequently, 2 ml of the interface was transferred to a new tube and washed twice with 10 ml of PBS by centrifugation at 300× g 4°C for 5 min. Besides, during the final PBMC washing, any contaminating erythrocytes were lysed by resuspending with 5 ml of sterile distilled water before immediately adding 5 ml of 2× PBS.

The isolated PBMC pellet was resuspended in the R10 medium at 2×10^6 cells/ml concentration and subsequently transferred to 96-well plates at a volume of 50 µl/well (1×10^5 cells/well). While each stimulated well was added with 50 µl of inactivated MS-H and MS-L antigen solutions, respectively, non-stimulated control wells were added with a similar volume of the R10 medium. The PBMC culture plates containing duplicated wells of non-stimulation, homologous stimulation, and heterologous stimulation were placed in a humidified 5% CO_2 incubator at 37°C for 60 h before identifying lymphocyte proliferation.

3.15) Lymphocyte proliferation

Lymphocyte proliferation was determined by the MTS Tetrazolium assay using inner salt MTS reagent (3-4,5-dimethylthiazol-2-yl-5-3-carboxymethoxyphenyl-2-4-sulfophenyl-2H-tetrazolium) following the manufacturer's instructions. Briefly, at the end of PBMC culture incubation, 20 µl of CellTiter 96® AQueous One Solution Reagent (Promega, Madison WI, USA) was simultaneously added to each PBMC well and incubated in a humidified 5% CO_2 at 37°C conditions for 5 h. The incubated MTS plates were read and recorded an OD at 490 nm using the absorbance reader (BioTek Model 800™ TS, USA). Lymphocyte proliferation index was calculated as OD values of antigen-stimulated cells were divided by that of non-stimulated cells.

3.16) Determination of antimicrobial susceptible profiles

A viable count of *M. synoviae* isolates; 10 field strains and WVU 1853 strain; in color changing unit (CCU) was obtained using the most probable number (MPN) which is determined using an MPN table (Meynell and Meynell, 1970; Rowe et al., 1977).

Briefly, 20 µl of each *M. synoviae* isolate culture broth from frozen stocks was filled into each well of 1st column of the 96-well plate containing 180 µl of FMS broth and then serially 10-fold diluted from 1st until 11th column; with the 12th column containing only 200 µl of FMS broth. Each cultured plate was incubated at 37°C in humidified condition for 14 days before the number of wells in the last 3 columns showing color change from red to yellow were counted and estimated the CCU.

The frozen *M. synoviae* stocks were also used as inocula for evaluating the MIC (minimum inhibitory concentration) of antimicrobials including enrofloxacin, oxytetracycline, doxycycline, tiamulin, tylosin, tilmicosin, tylvalosin and lincomycin-spectinomycin. The eight tested antimicrobials; registered and approved by the Food and Drug Administration (FDA), Ministry of Public Health, Thailand; were formulated and diluted in FMS broth. Antimicrobial susceptible profiles were determined by final MIC values using serial broth dilution method (Wang et al., 2001).

Briefly, duplicate wells of antimicrobials were two-folded, serially diluted in a 100 µl of FMS broth in the sterile 96-well, flat-bottomed microtitration plates. The 100 µl of FMS broth containing *M. synoviae* organisms approximately 10^5 CCU/ml was added to each well at 1st until 11th column containing same amount of antimicrobial at final concentrations 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.1563, 0.0781, 0.0391, 0.0195 and 0.0098 µg/ml. Positive control consisting only *M. synoviae* cultured broth was also included at the 12th column in each plate. The MIC values were recorded daily after the positive control broth color changing and final MIC values were assessed at 14 days after incubation. The lowest concentration of each tested antimicrobial that completely prevented the broth change of color from pink to orange-yellow was considered as MIC.

3.17) Statistical analyses

RPA assay and ELISA results were analyzed by using independent samples t-test, analysis of variance (ANOVA), and Duncan test. Serological information of all treatment groups was compared among groups and time of sample collection. Lymphocyte proliferation index arising from both homologous and heterologous

antigens stimulation were compared within treatment groups by using paired samples t-test and compared between treatment groups by using independent samples t-test.

ELISA S/P ratio and lymphocyte proliferation index of identical blood samples at 15 weeks of age were analyzed the correlations by using Pearson's correlation coefficient with two-tailed statistical significance and were categorized into 3 level for the Cohen's kappa analysis determining the agreement between antibody titer and cellular immunocompetence. Comparison of MIC values between Thai *M. synoviae* field isolates, which were classified as types E and L or originated from choanal cleft and joint, was analyzed by using independent samples t-test. Statistical analyses were performed using IBM SPSS Statistics 22 for windows and differences were considered as significant at $P < 0.05$.

CHAPTER IV
RESULTS

4.1) Sequence analysis of partial *vlhA* gene

Swab samples, from articular joint or respiratory tract, were collected from approximately ninety flocks of registered chicken farms in Thailand during 2020. All identified *M. synoviae* field isolates, confirmed by the 16S rRNA gene-based PCR assay, were selected for characterization of partial *vlhA* gene which nucleotide sequences were submitted to the GenBank Database. Details including sizes of amplicons, PRR nucleotides and amino acid sequences, and GenBank accession numbers were shown in Table 1. The datasets including sequence data generated and analyzed during this study are available at the NCBI Nucleotide (https://www.ncbi.nlm.nih.gov/nuccore).

According to sequence analyses of partial *vlhA* gene, the PRR type of *M. synoviae* WVU 1853 and MS-H vaccine strains were respectively identified as groups A and C. Besides, most Thai *M. synoviae* isolates during 2020 were identified as types E and L with 19 and 35 amino acid length, respectively. Thai *M. synoviae* isolates collected from articular joint were classified as type E while Thai field isolates from respiratory tract were classified as types E (3 isolates) and L (4 isolates).

Table 1 Information and molecular characteristic details of *M. synoviae* isolates and *M. synoviae* strains used in this study.

Isolates ID	Source of MS [A]	Age (weeks)	Province	Sequence analysis of partial *vlhA* gene-			Genbank Accession No.
				PCR amplicons (bp)	PRR length (nt / aa)	MS Type	
WVU1853 strain (ATCC 25204)	Reference strain			376	114/38	A	KX168667, AM998371, ON191513
MS-H strain (Australia origin)	Vaccine strain			358	96/32	C	KX168666, JX960401, ON191514
AHRU2020CK0615	Broiler / C	4	Prachin Buri	319	57/19	E	ON191515
AHRU2020CK0301	Layer / J	19	Chon Buri	319	57/19	E	ON191516
AHRU2020CK0305	Layer / J	19	Chon Buri	319	57/19	E	ON191517
AHRU2020CK0404	Layer / J	22	Chon Buri	319	57/19	E	ON191518
AHRU2020CK0709	Layer / J	16	Chon Buri	319	57/19	E	ON191519
AHRU2020CU1401	Layer / C	60	Chachoengsao	367	105/35	L	ON191520
AHRU2020CU1409	Layer / C	60	Chachoengsao	367	105/35	L	ON191521
AHRU2020CU1505	Broiler / Y	1	Prachin Buri	367	105/35	L	ON191522
AHRU2015CU2802	Native broiler / J	9	Satun	367	105/35	L	KX168690, ON191523
AHRU2018CK0301	Layer / J	15	Chon Buri	319	57/19	E	ON191524
AHRU2020CU1104	Layer / C	55	Chon Buri	319	57/19	E	ON191525
AHRU2020CU1101	Layer / C	55	Chon Buri	319	57/19	E	ON191526
AHRU2020CU1323	Layer breeder / C	7	Nakhon Nayok	358	96/32	C	ON191527
AHRU2020CK1206	Layer breeder / C	22	Chaiyaphum	367	105/35	L	ON191528
MS-1 strain (NAD-independent)	Vaccine strain			376	114/38	A	ON191529

[A] Abbreviations C, J and Y were represented the swab samples from choanal cleft, synovial fluid and yolk sac.

4.2) PCR-RFLP assay

PCR amplicons of partial *vlhA* gene from *M. synoviae* isolates were presented in Figure 1 consisting of the full-length amplicons size 350-400 bp (Figure 1A and 1B) and the two digested fragments size 100-250 bp (Figure 1A and 1C). The MS-H isolates whether from vaccine, 1st passage, 2nd passage, or vaccinated chicken were presented two smaller digested fragments size 100 and 134 bp while other non-vaccine isolates; *M. synoviae* WVU 1853 strain and Thai *M. synoviae* field isolates identified as types C, E, and L; were presented the specific two digested fragments size 130 and 160-210 bp. In any palatine cleft swab samples before immunization; at 1 day and 9 weeks of age, *M. synoviae* MS-H vaccine strain and non-vaccine isolates were not detected (Table 2). Although palatine cleft swab samples post immunization; at 10 and 13 weeks of age; were positive using the developed MS-H detection assay; PCR-RFLP assay; only palatine cleft swab samples obtained from 10-week-old vaccinated chickens in group 9V are shown in Lane 6 of Figure 1B and 1C.

The developed PCR-RFLP assay was validated with *M. synoviae* field isolates recently circulating in Thailand. Consequently, PCR amplicons of partial *vlhA* gene in Figure 2; full-length amplicons size 350-400 bp (Figure 2A) and two digested fragments size 100-250 bp (Figure 2B); were presented specific two digested fragments size 100 and 134 bp of *M. synoviae* MS-H strain (Figure 2B: Lane 21) in Thai *M. synoviae* isolate AHRU2020CU1323 (Figure 2B: Lane 17) which were different from other non-vaccine isolates; *M. synoviae* WVU 1853 strain (Figure 2B: Lanes 3 and 20) and Thai *M. synoviae* field strains (Figure 2B: Lanes 5-16 and 22); which concisely presented specific two digested fragments size 130 and 160-210 bp.

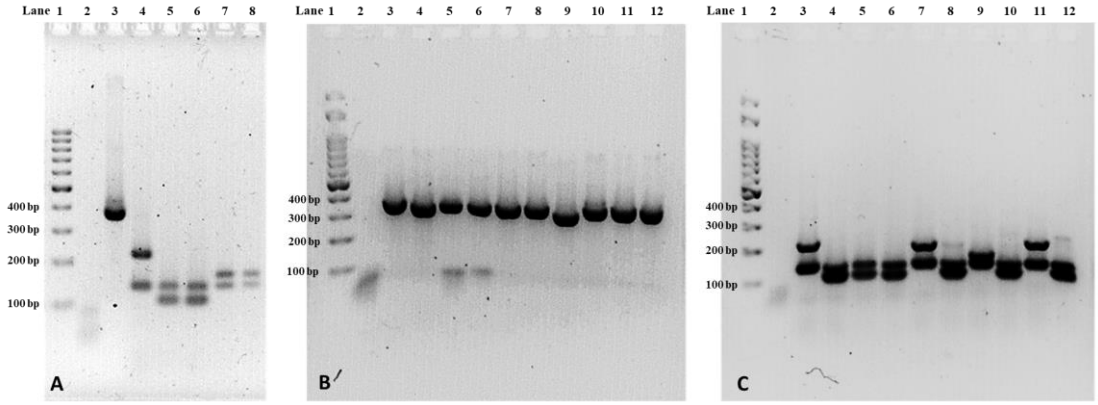


Figure 1 Electrophoresis gels demonstrating PCR products from *M. synoviae* isolates consisting of the full-length of partial *vlhA* gene amplicons size of 350-400 bp (A, B) and two digested fragments partial *vlhA* gene amplicons size of 100-250 bp (A, C).

(A) Lane 1, 1000 bp DNA ladder;

Lane 2, *M. gallisepticum* S6 strain as negative control;

Lane 3, *M. synoviae* WVU 1853 strain as undigested positive control;

Lane 4, *M. synoviae* WVU 1853 strain as digested positive control;

Lane 5, digested amplicons of 1st passage *M. synoviae* MS-H vaccine strain;

Lane 6, digested amplicons of 2nd passage *M. synoviae* MS-H vaccine strain;

Lane 7, digested amplicons of 1st passage Thai *M. synoviae* type E;

Lane 8, digested amplicons of 2nd passage Thai *M. synoviae* type E.

(B. Undigested PCR amplicons, C. TasI digested PCR amplicons)

Lane 1, 1000 bp DNA ladder; Lane 2, *M. gallisepticum* S6 strain as negative control;

Lane 3, *M. synoviae* WVU 1853 strain as positive control;

Lane 4, 1st passage *M. synoviae* MS-H vaccine strain;

Lane 5, 2nd passage *M. synoviae* MS-H vaccine strain;

Lane 6, *M. synoviae* MS-H vaccine strain from vaccinated chickens;

Lane 7, Thai *M. synoviae* type C;

Lane 8, Thai *M. synoviae* type C with *M. synoviae* MS-H vaccine strain;

Lane 9, Thai *M. synoviae* type E;

Lane 10, Thai *M. synoviae* type E with *M. synoviae* MS-H vaccine strain;

Lane 11, Thai *M. synoviae* type L;

Lane 12, Thai *M. synoviae* type L with *M. synoviae* MS-H vaccine strain.

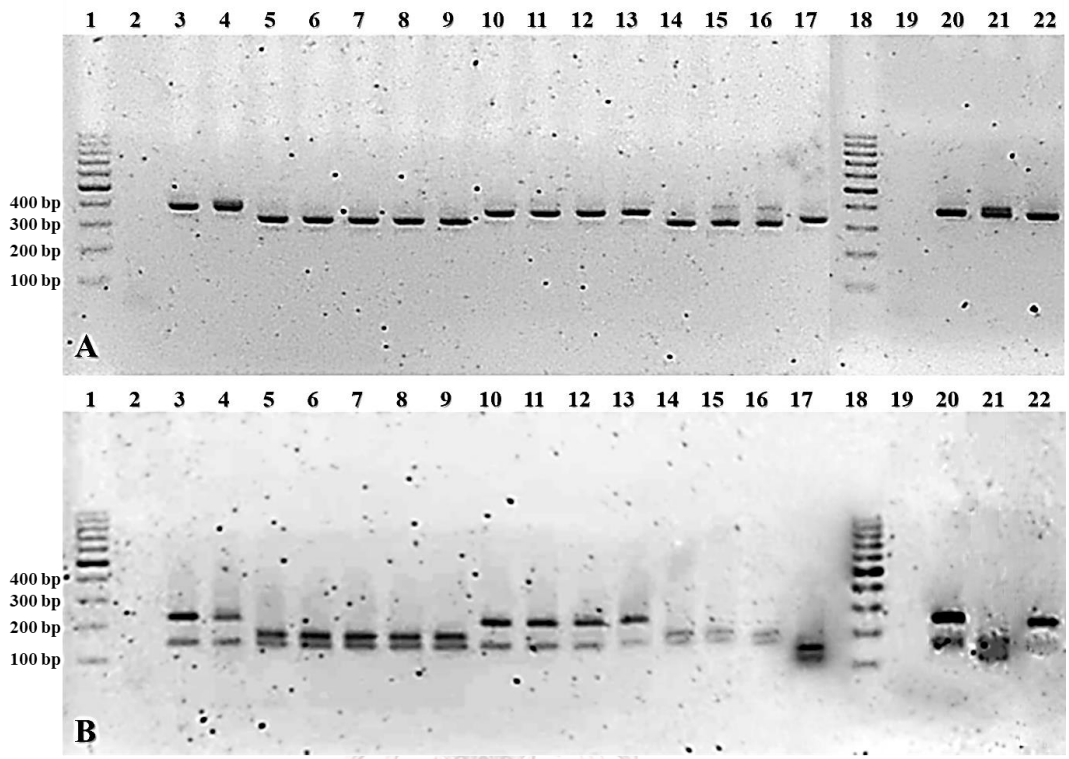


Figure 2 PCR electrophoresis gel demonstrating PCR products from *M. synoviae* isolates consisting of the full length of partial *vlhA* gene amplicons size of 350-400 bp (A) and the two digested fragments of partial *vlhA* gene amplicons size of 100-200 bp (B).

Lane 1 and 18, 1000 bp DNA ladder;

Lane 2 and 19, *M. gallisepticum* S6 strain as negative control;

Lane 3 and 20, *M. synoviae* WVU 1853 strain as positive control;

Lane 4, *M. synoviae* MS-1 strain; Lane 5, AHRU2020CK0615;

Lane 6, AHRU2020CK0301; Lane 7, AHRU2020CK0305;

Lane 8, AHRU2020CK0404; Lane 9, AHRU2020CK0709;

Lane 10, AHRU2020CU1401; Lane 11, AHRU2020CU1409;

Lane 12, AHRU2020CU1505; Lane 13, AHRU2015CU2802;

Lane 14, AHRU2018CK0301; Lane 15, AHRU2020CU1104;

Lane 16, AHRU2020CU1101; Lane 17, AHRU2020CU1323;

Lane 21, MS MS-H vaccine strain; Lane 22, AHRU2020CK1206

Table 2 Percentage of palatine cleft swab samples positive by *M. synoviae*-specific PCR assay and PCR-RFLP assay in non-vaccinated chickens (Control) and vaccinated chickens (12V, 9-12V, and 9V).

In addition, palatine cleft swab samples from 10 chickens in each treatment groups were randomly obtained prior to immunization; at 1 day and 9 weeks of age; and post immunization; at 10 and 13 weeks of age.

Age	No. samples [1]	Treatment groups							
		Control		12V		9-12V		9V	
		MS-PCR	PCR-RFLP	MS-PCR	PCR-RFLP	MS-PCR	PCR-RFLP	MS-PCR	PCR-RFLP
1 day *	10	0	0	0	0	0	0	0	0
9 weeks	10	0	0	0	0	0	0	0	0
10 weeks	10	0	0	0	0	100	100	100	100
13 weeks	10	0	0	100	100	100	100	-	-

* Palatine cleft swab samples obtained from chickens at arrival.

[1] Number of palatine cleft swab samples in each group.

4.3) Dynamic of serological responses

At 9 weeks of age, all serum samples were negative by RPA assay (data not shown). At 2 weeks after the first vaccination (at 14 weeks of age in group 12V and 11 weeks of age in groups 9V and 9–12V), 17–20% of serum samples collected from vaccinated chickens were positive by RPA assay, and 20–25% were positive by ELISA (Tables 3 and 4). At 3 weeks after the first vaccination (at 15 weeks of age in group 12V and 12 weeks of age in groups 9V and 9–12V), 70–100% of serum samples were positive when tested by RPA assay and ELISA. The percentage of positive serum samples from 3 weeks after the first vaccination to the end of the study remained steady.

Dynamic of serological responses characterized by ELISA was shown that the mean S/P ratio of three vaccinated groups continuously increased, reached the highest level, and then maintained at baseline level during 6-9 weeks after the last vaccination of each group. The baseline mean S/P ratio at 18 weeks of age in groups 12V, 9-12V, and 9V was insignificantly different at 2.169, 2.253, and 2.341, respectively (Tables 4).

Table 3 Percentage of serum samples positive to *M. synoviae* by RPA assay in non-vaccinated chickens (Control) and chickens receiving 3 different vaccination schedules (12V, 9-12V, and 9V).

Age	No. samples [1]	Treatment groups							
		Control		12V		9-12V		9V	
		UD [2]	D	UD	D	UD	D	UD	D
1 day *	15	100	98	100	98	100	98	100	98
5 weeks	10	0	0	0	0	0	0	0	0
10 weeks	12	0	0	0	0	0	0	0	0
11 weeks	12	0	0	0	0	16.67	16.67	16.67	16.67
12 weeks	10	0	0	0	0	70	70	70	70
13 weeks	10	0	0	0	0	100	70	100	70
14 weeks	10	0	0	20	20	100	80	100	80
15 weeks	10	0	0	100	70	100	80	100	90

* Serum samples collected from chickens at arrival.

[1] Number of serum samples obtained from each group.

[2] Serum samples, undiluted (UD) and 1:8 diluted (D) serum, were tested with *M. synoviae* antigen.

Table 4 Percentage of serum samples positive and mean S/P ratio by ELISA in vaccinated chickens (12V, 9-12V, and 12V) and non-vaccinated chickens (Control)

Age	No. samples [3]	Treatment groups			
		Control	12V	9-12V	9V
1 day*	**15**	1.589 ± 0.523[1]	1.589 ± 0.523	1.589 ± 0.523	1.589 ± 0.523
		100%[2]	100%	100%	100%
5 weeks	**10**	0.030 ± 0.022	0.030 ± 0.022	0.030 ± 0.022	0.030 ± 0.022
		0%	0%	0%	0%
10 weeks	**12**	0.004 ± 0.004 ^{B}	0.004 ± 0.004 $^{B, b}$	0.003 ± 0.004 $^{B, c}$	0.003 ± 0.004 $^{B, c}$
		0%	0%	0%	0%
11 weeks	**12**	0.003 ± 0.006 ^{B}	0.003 ± 0.006 $^{B, b}$	0.186 ± 0.343 $^{A, c}$	0.186 ± 0.343 $^{A, c}$
		0%	0%	25%	25%
12 weeks	**10**	0.005 ± 0.008 ^{B}	0.005 ± 0.008 $^{B, b}$	0.810 ± 1.037 $^{A, bc}$	0.810 ± 1.037 $^{A, bc}$
		0%	0%	50%	50%
13 weeks	**10**	0.027 ± 0.030 ^{B}	0.030 ± 0.054 $^{B, b}$	1.470 ± 0.899 $^{A, ab}$	1.855 ± 1.416 $^{A, ab}$
		0%	0%	100%	100%
14 weeks	**10**	0.004 ± 0.013 ^{B}	0.385 ± 0.764 $^{B, b}$	2.042 ± 1.073 $^{A, a}$	2.661 ± 1.410 $^{A, a}$
		0%	20%	100%	100%
15 weeks	**10**	0.017 ± 0.014 ^{B}	1.764 ± 1.035 $^{A, a}$	1.278 ± 0.717 $^{A, ab}$	2.037 ± 0.970 $^{A, ab}$
		0%	100%	100%	100%
18 weeks	**10**	0.014 ± 0.022 ^{B}	2.169 ± 0.925 $^{A, a}$	2.253 ± 0.691 $^{A, b}$	2.341 ± 1.017 $^{A, a}$
		0%	100%	100%	100%

[1] Data represents mean S/P ratio ± standard error.

[2] Data represents percentage of birds positive to *M. synoviae* by MS-ELISA test kit.

[3] Number of serum samples obtained from each group.

* Serum samples collected from chickens at arrival.

^{A}, ^{B} In each row, values with the same uppercase superscript letters are not significantly different (P ≥ 0.05).

^{a}, ^{b}, ^{c} In each column, values with the same lowercase superscript letters are not significantly different (P ≥ 0.05).

4.4) Evidence of lymphocyte proliferative response

According to *in vitro* stimulation of PBMC obtained from chickens at 15 weeks of age, the lymphocyte proliferation index of vaccinated chickens in groups 12V, 9–12V, and 9V represented an ability to enhance the bacterin-specific lymphocyte proliferative response against both MS-H vaccine and MS-L field antigens (Figure 3). Besides, the index of PBMC stimulated with the MS-H antigen in the control, 12V, 9–12V, and 9V groups was 1.135, 1.400, 1.423, and 1.533, respectively, whereas the index of PBMC stimulated with MS-L antigen in the control, 12V, 9–12V, and 9V groups was 1.113, 1.425, 1.363, and 1.393, respectively. Within the homologous antigen (MS-H) stimulation, the indices in the 12V and 9–12V groups were significantly lower than that in group 9V. However, the index within the heterologous (MS-L) antigen stimulation was not significantly different among treatment groups. Pearson's correlation coefficient and Cohen's kappa values within homologous or heterologous antigens stimulation were 0.455 or 0.410 and 0.069 or 0.341, respectively. A significant different index between homologous and heterologous antigens stimulation in the same treatment group was presented in groups 9–12V and 9V. In addition to the mean S/P ratio of 0.012 in four non-vaccinated chickens, the four randomly selected vaccinated chickens in groups 12V, 9–12V, and 9V were presented the mean S/P ratios of 2.031, 1.876, and 2.017, respectively.

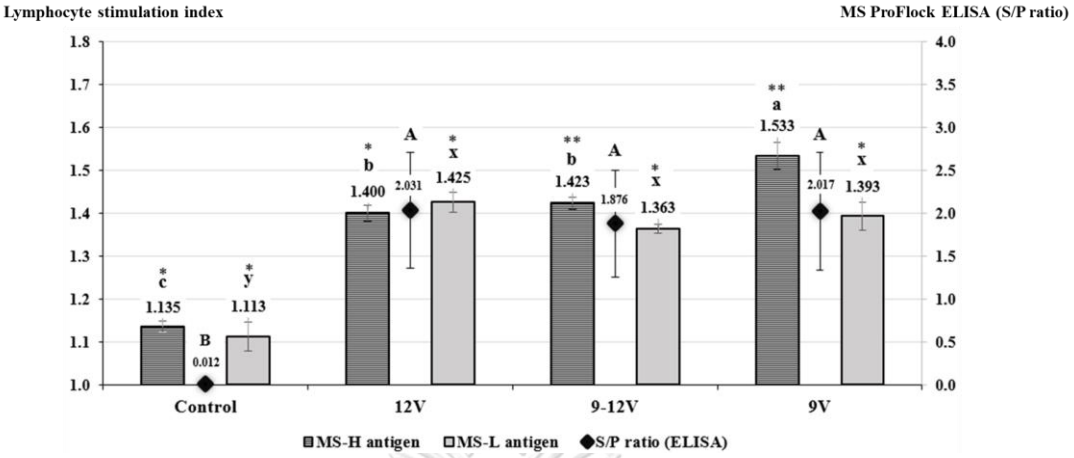


Figure 3 Lymphocyte stimulation index (Primary axis) and mean ELISA titer (Secondary axis) of the four randomly selected chickens from four treatment groups; non-vaccinated (Control) and vaccinated groups (12V, 9–12V, and 9V) at 15 weeks of age.

Data labels on each pillar represent the lymphocyte proliferation index as a result of stimulating PBMCs with inactivated antigen solution of MS-H or MS-L.

The mean S/P ratio from four serum samples in each group is shown as diamond markers.

*, ** In each treatment groups, values marked with the same number of asterisk are not significantly different (P ≥ 0.05).

a, b, c, x, y Between treatment groups, values for MS-H antigen or MS-L antigen with the same lowercase superscript letters are not significantly different (P ≥ 0.05).

A, B Between treatment groups, mean S/P ratio with the same uppercase superscript letters are not significantly different (P ≥ 0.05).

4.5) Antimicrobial susceptible profiles

The MIC values of tested antimicrobials; enrofloxacin, doxycycline, oxytetracycline, tylosin, tilmicosin, tylvalosin, lincomycin in combination with spectinomycin and tiamulin; against Thai *M. synoviae* field and reference strains were shown in Tables 5 and 6. *M. synoviae* WVU 1853 strain and Thai *M. synoviae* isolate AHRU2020CU1323 (or MS-H like strain) were presented low final MIC values of tylosin, tilmicosin and tylvalosin at 0.0098 µg/ml and high final MIC values of enrofloxacin and lincomycin-spectinomycin at 0.3125 and 0.625 µg/ml, respectively. Thai *M. synoviae* isolates AHRU2020CU1323 and AHRU2020CU1409 were respectively showed the lowest final MIC value of enrofloxacin (at 0.3125 and 2.5 µg/ml) and oxytetracycline (at 0.0195 and 0.0391 µg/ml). Thai *M. synoviae* field isolates AHRU2020CK0301, AHRU2020CK0305 and AHRU2020CK0404 were performed the high final MIC value of tilmicosin (at 0.625 µg/ml) and tiamulin (at 0.1563 and 0.3125 µg/ml).

Although Thai *M. synoviae* types E and L were insignificantly presented the difference of mean MIC values among each antimicrobial, mean MIC values of tylosin, tilmicosin, tylvalosin, and tiamulin against Thai *M. synoviae* types E were seem to be higher level than Thai *M. synoviae* types L. In addition, mean MIC values of tylosin and tilmicosin against Thai *M. synoviae* isolates originated from respiratory tract were significantly lower than isolates originated from joint; however, mean MIC values of other antimicrobials were also insignificant lower in respiratory tract origin isolates than joint origin isolates.

Besides, most Thai *M. synoviae* field isolates showed the resistance to enrofloxacin and lincomycin-spectinomycin at MIC_{50} value of 10 and 0.625 µg/ml, respectively, and presented the susceptibility to tylosin, tilmicosin, tylvalosin, and tiamulin at MIC_{50} value of 0.0391, 0.0781, 0.0098 and 0.0781 µg/ml, respectively. Doxycycline and oxytetracycline were also performed a good activity against Thai *M. synoviae* field isolates at MIC_{50} value of 0.1563 µg/ml.

In addition, except enrofloxacin and lincomycin-spectinomycin, MIC_{90} values of doxycycline, oxytetracycline, tylosin, tilmicosin, tylvalosin, and tiamulin were slightly higher than MIC_{50} values. Tylvalosin, tylosin and tiamulin could performed quick

activity against Thai *M. synoviae* isolates at MIC_{90} values of 0.0195, 0.1563 and 0.1563 µg/ml, respectively.

Table 5 Details of strains and final MIC values of *M. synoviae* isolates.

Isolates ID	Type	MIC values (µg/ml)							
		EFX	DX	OTC	TYL	TIL	TVN	LC-SP	TIA
AHRU2020CK0615	E	10	0.1563	0.1563	0.0391	0.0781	0.0098	0.6250	0.0391
AHRU2020CK0301	E	10	0.1563	0.3125	0.1563	0.6250	0.0195	0.6250	0.1563
AHRU2020CK0305	E	10	0.1563	0.1563	0.0781	0.6250	0.0195	0.6250	0.1563
AHRU2020CK0404	E	10	0.3125	0.3125	0.1563	0.6250	0.0391	0.6250	0.3125
AHRU2020CK0709	E	10	0.3125	0.1563	0.0391	0.0781	0.0098	0.3125	0.0781
AHRU2020CU1409	L	2.5	0.1563	0.0391	0.0098	0.0781	0.0098	0.6250	0.0391
AHRU2020CU1401	L	10	0.1563	0.1563	0.0098	0.0195	0.0098	0.6250	0.0781
AHRU2020CU1505	L	10	0.3125	0.3125	0.0098	0.0195	0.0098	0.6250	0.0781
AHRU2015CU2802	L	10	0.3125	0.3125	0.0781	0.1563	0.0098	0.1563	0.0391
AHRU2020CU1323	C	0.3125	0.0391	0.0195	0.0098	0.0098	0.0098	0.6250	0.0195
WVU1853	A	0.6250	0.1563	0.1563	0.0098	0.0098	0.0098	0.6250	0.0781

Abbreviations of antibiotics: EFX enrofloxacin, DX doxycycline, OTC oxytetracycline, TYL tylosin, TIL tilmicosin, TVN tylvalosin, LC-SP lincomycin-spectinomycin, TIA tiamulin

Table 6 Summary of MIC range, MIC_{50} and MIC_{90} of *M. synoviae* isolates.

Antibiotics	MIC values (µg/ml)					
	Reference	MS-H like	MS type E	MS type L	Cleft origin	Joint origin
	WVU 1853	AHRU2020CU1323	Mean ± SE (n=5)	Mean ± SE (n=4)	Mean ± SE (n=4)	Mean ± SE (n=5)
EFX	0.625	0.3125	10.00 ± 0.00	8.125 ± 1.875	8.125 ± 1.875	10.00 ± 0.00
DX	0.1563	0.0391	0.219 ± 0.038	0.234 ± 0.045	0.195 ± 0.039	0.250 ± 0.038
OTC	0.1563	0.0195	0.219 ± 0.038	0.205 ± 0.066	0.166 ± 0.056	0.250 ± 0.038
TYL	0.0098	0.0098	0.094 ± 0.026	0.027 ± 0.017	0.017 ± 0.007	0.102 ± 0.023
TIL	0.0098	0.0098	0.406 ± 0.134	0.068 ± 0.032	0.049 ± 0.017	0.422 ± 0.125
TVN	0.0098	0.0098	0.020 ± 0.005	0.010 ± 0.000	0.010 ± 0.000	0.020 ± 0.005
LC-SP	0.625	0.625	0.563 ± 0.063	0.508 ± 0.117	0.625 ± 0.000	0.469 ± 0.099
TIA	0.0781	0.0195	0.148 ± 0.047	0.059 ± 0.011	0.059 ± 0.011	0.148 ± 0.047

Antibiotics	MIC values (µg/ml)			
	MS field isolate			
	Mean ± SE (n=9)	Range	MIC_{50}	MIC_{90}
EFX	9.167 ± 1.157	2.5 - 10	10	10
DX	0.226 ± 0.031	0.1563 - 0.3125	0.1563	0.3125
OTC	0.213 ± 0.034	0.0391 - 0.3125	0.1563	0.3125
TYL	0.064 ± 0.018	0.0098 - 0.1563	0.0391	0.1563
TIL	0.256 ± 0.087	0.0195 - 0.6250	0.0781	0.625
TVN	0.015 ± 0.003	0.0098 - 0.0391	0.0098	0.0195
LC-SP	0.538 ± 0.053	0.1563 - 0.6250	0.625	0.625
TIA	0.109 ±0.028	0.0391 - 0.3125	0.0781	0.1563

Abbreviations of antibiotics:

EFX enrofloxacin, DX doxycycline, OTC oxytetracycline, TYL tylosin, TIL tilmicosin, TVN tylvalosin, LC-SP lincomycin-spectinomycin, TIA tiamulin

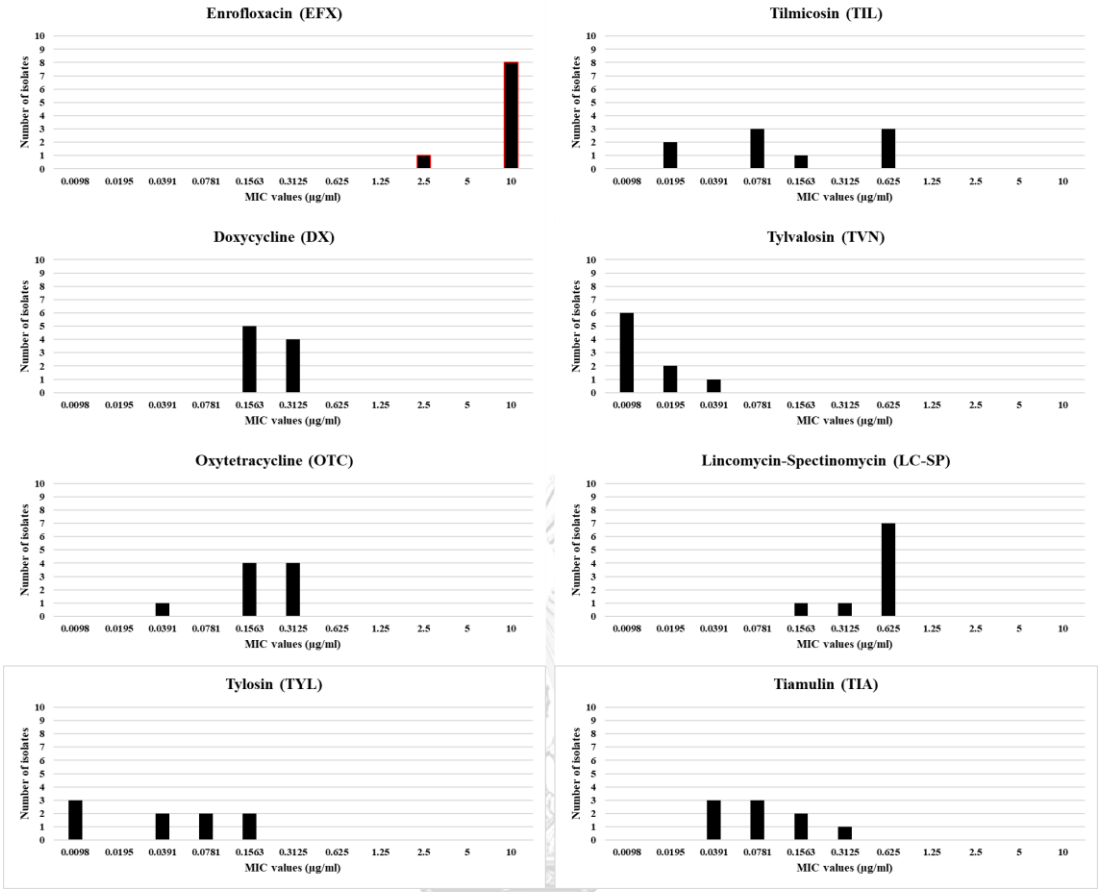


Figure 4 Bar charts illustrate the distribution of *M. synoviae* isolates among the final MIC values of tested antibiotics including enrofloxacin, doxycycline, oxytetracycline, tylosin, tilmicosin, tylvalosin, lincomycin-spectinomycin and tiamulin.

CHAPTER V
DISSCUSSION

5.1) Sequence analysis of partial *vlhA* gene

The present study was conducted to investigate the current *M. synoviae* surveillance information of poultry industry in Thailand and to define the genetic characterization of Thai *M. synoviae* isolates by sequence analysis of partial *vlhA* gene. Based on the basis of PRR region length, Thai *M. synoviae* isolates during 2020 classified as types E and L with 19 and 35 amino acid length, respectively; were presented at least 2 genotypes of *M. synoviae* circulated in Thailand which differed from the previous study in 2015 showing at least 3 genotypes and showing Thai *M. synoviae* type L as arthropathic strain (Limpavithayakul et al., 2016). Besides, both Thai *M. synoviae* types E and L were currently isolated from articular joint and Thai *M. synoviae* type L was isolated from respiratory tract; therefore, both Thai *M. synoviae* types E and L were possibly characterized as the cause of airsaculitis and synovitis but they might not be associated with oviduct infection, causing egg shell apex abnormality syndrome like Dutch *M. synoviae* isolates types C and E (Feberwee et al., 2009a).

Because pathogenesis of *M. synoviae* infection depends on the transmissible routes; an intravenous inoculation and a food pad injection are more frequently causing progressive synovitis lesions, while a respiratory tract inoculation or aerosol exposure usually presents mild synovitis lesions (Lockaby et al., 1998; Landman and Feberwee, 2004). Therefore, the experimental inoculation of each Thai *M. synoviae* types E and L via intranasal and intravenous routes in chickens should be further studied to clarify the tissue tropism and the internalization properties which could be the important factors in transposition strategy of *M. synoviae* from the mucous barrier to reach more favorable niches which *M. synoviae* could resist to antibiotics treatment and could evade the host immune response (Buim et al., 2011).

In addition, in several countries which are challenged with antimicrobial resistance argument, immunization with live MS-H commercial vaccine has been dramatically increasing and especially proposed to use in risk farms due to possibly

protection capability against respiratory signs, airsacculitis, egg production losses and egg transmission (Kleven, 2008; Kleven and Ferguson-Noel, 2008; Ley, 2008; Nicholas et al., 2009; Landman, 2014). Because the available live commercial vaccine, MS-H strain which is the temperature-sensitive mutant strain growing well at 33°C (Markham et al., 1998b), was classified as type C with 32 amino acid length of PRR region, so vaccination with live MS-H vaccine might present the better protection against *M. synoviae* field isolates type C than types E and L. Besides, live MS-H vaccine might effectively control and reduce the horizontal shedding of *M. synoviae* field isolates type C in outbreak areas as the results of immunization with live MG vaccines (Barbour et al., 2000; Feberwee et al., 2006a; Kleven, 2008).

5.2) PCR-RFLP assay

The PCR-RFLP assay in this study was developed for detecting *M. synoviae* MS-H vaccine strain and differentiating it from *M. synoviae* non-vaccine strains circulating in Thailand including *M. synoviae* WVU 1853 strain, Thai *M. synoviae* types C, E, and L. Based on the analytical result from the Genescript webpage, the full-length amplicons and the two digested fragments of partial *vlhA* gene would be 350-400 bp and 100-250 bp, respectively. Besides, *M. synoviae* MS-H vaccine strain whether from vaccine product, a few passages, or vaccinated chickens was expectedly presented the specific two digested fragments size 100 and 134 bp while other *M. synoviae* non-vaccine isolates; *M. synoviae* WVU 1853 strain, Thai *M. synoviae* field strains identified as types C, E, and L; were probably presented the specific two digested fragments size 130 and 160-210 bp. In addition, the two digested fragments of *M. synoviae* WVU 1853 strain (GenBank accession no. KX168667) classified as *M. synoviae* type A were 133 and 218 bp; MS K1968 strain (GenBank accession no. KJ606929) classified as *M. synoviae* type B were 133 and 239 bp; *M. synoviae* type C (GenBank accession no. KX168685) were 133 and 200 bp; *M. synoviae* type E (GenBank accession no. KX168683) were 133 and 161 bp; and *M. synoviae* type L (GenBank accession no. KX168688), Thai arthropathic strain, were 133 and 209 bp. Consequently, the specific two digested fragments of MS-H vaccine strain, which were greatly expressed in this study whether pure isolates, 1st or 2nd passage (Figure 1A and 1C), were different from digested amplicons of 1st and 2nd Thai *M. synoviae* field strains (Figure 1A). Interestingly, DNA template mixtures of Thai *M. synoviae* field strains and *M. synoviae* MS-H vaccine strain were presented the two digested fragments of MS-H vaccine strain (Figure 1C); hence, the PCR-RFLP assay might be further applied with other methods, i.e., bacterial culture, molecular and serological assays; in *M. synoviae* surveillance.

However, due to the detection ability of the MS-H vaccine strain whether pure or mixed isolates, the palatine cleft swab samples of vaccinated chickens at 1-week post-immunization were tested using the PCR-RFLP assay to determine the live MS-H vaccine administration. Based on the size of the proline-rich repeat region of *vlhA* gene, Thai *M. synoviae* field isolates were identified as types C, E, and L, whereas *M. synoviae* types B, C, and E were reported in other countries, including the UK, USA, Slovenia,

France, Netherland, Hungary, and Australia (Bencina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Landman, 2014; Limpavithayakul et al., 2016). Therefore, the PCR-RFLP assay could be a convenient assay for detecting and differentiating MS-H vaccine from non-vaccine strains circulating worldwide and could be further verified with other *M. synoviae* field strains recently circulating in other country to affirm the differentiating efficacy and to fulfill the information of using vaccination to control *M. synoviae* disease (Fiorentin et al., 2003b; Kleven, 2008; Kleven and Ferguson-Noel, 2008; Ley, 2008; Feberwee et al., 2009b; Nicholas et al., 2009; Catania et al., 2010; Landman, 2014; Hong et al., 2015; Kreizinger et al., 2017).

However, in chickens infected with multiple different strains simultaneously, the PCR-RFLP assay might not be a suitable assay similar to that of the previous study developing the pulsed-field gel electrophoresis using three restriction enzymes, *Sma*I, *Bln*I, and *Bam*HI, because chickens infected with multiple strains should be carefully isolated and subjected to molecular investigations (Harada et al., 2009). Apart from *vlhA* gene, the *obg* PCR-HRM system, i.e., high-resolution melting-curve analysis based on *obg* gene, was also developed and verified for being a rapid and reliable genotyping method, which could be performed in laboratories containing limited facilities using basic real-time PCR machine or conventional PCR thermocycler with agarose gel electrophoresis (Shahid et al., 2014; Kreizinger et al., 2015).

In addition, the present study provided the first validated information of using the PCR-RFLP assay for detecting *M. synoviae* MS-H vaccine strain and differentiating from non-vaccine strains circulating in Thailand; *M. synoviae* WVU 1853 strain, Thai *M. synoviae* field isolares types E and L. Interestingly, Thai *M. synoviae* isolate AHRU2020CU1323 showing the specific two digested fragments size 100 and 134 bp of *M. synoviae* MS-H strain might be spread horizontally from other vaccinated chicken in the same areas of high commercial farm density and heavy poultry traffic (Sentíes-Cué et al., 2005; Bergeron et al., 2021). Furthermore, the PCR-RFLP assay should be verified with normal MS-H vaccine strain (ts MS-H) and non-temperature-sensitive MS-H strain, which could grow at 39.5°C and could be re-isolated from vaccinated field chickens (Markham et al., 1998b).

5.3) Immune response mechanisms due to live vaccine

Vaccination with booster dose of live MS-H has been remarkably applied in some chicken farms in Thailand to substitute *M. synoviae* bacterin, an unavailable commercial vaccine, due to a previous concern in its protective efficacy of bacterin, which could stimulate long-lasting protective immunity, showing high serum antibody levels for protecting chickens and their progeny against *Mycoplasma* infection (Glisson and Kleven, 1984; Yoder Jr et al., 1984; Feberwee et al., 2006b; Jones et al., 2006b; Gole et al., 2012; Gong et al., 2020). Therefore, the present study was conducted to characterize the immune response mechanisms, including humoral and cellular immune responses, in layer-type chickens receiving a single or two doses of live MS-H vaccine.

This study revealed the immune response mechanisms in immunized layer chickens received live MS-H vaccine, whether a single dose or two doses, which was similar to that of the previous study in commercial pathogen-free chickens, hybrid white leghorn received live MS-H vaccines (Jones et al., 2006a). The serological response was initially detected at 2 weeks after vaccination, and the protective immunity in vaccinated chickens could be developed within 7–14 days thereafter (Jones et al., 2006c). Interestingly, after 3 weeks after vaccination which MS-H-vaccinated chickens were 100% seropositive, serological response and protective immunity were continuously maintained at least 40 weeks after vaccination (Jones et al., 2006b). Based on the baseline mean S/P ratio (range 2.169–2.341) in three vaccinated groups at 6–9 weeks after the last vaccination, vaccinated chickens received a single dose or two doses could similarly perform serological response. Although triggering B-cell activation was upregulated and humoral immunity was induced after the first immunization; however, this opposite phenomenon after the second immunization may involve with vaccination in seropositive animals which the presence of antibodies could interfere the humoral immune response against mycoplasma disease (Martelli et al., 2006).

In addition, in the lymphocyte proliferative response study at 15 weeks of age, vaccinated chickens received a single dose or two doses could insignificantly present the humoral immune response as shown in the mean S/P ratio of only four randomly

selected chickens (Figure 3). However, the correlation and agreement between the S/P ratio and lymphocyte proliferation index were low regardless of the stimulation of PBMC with homologous or heterologous antigens because the protection against *M. synoviae* infection in the upper respiratory tract is associated with macrophage activation, an important part of the cellular immune response, despite the upregulation of several immune response genes, a pro-inflammatory cytokine, and TH1 cytokines which significantly correlated with the humoral immune response (Guo et al., 2008; Lavrič et al., 2008; Bolha et al., 2013; Oven et al., 2013).

Cellular immune mechanisms against homologous and heterologous strains were not different in the early period after the first immunization (Figure 3, 12V). However, when stimulating with *M. synoviae* MS-H antigen, the better lymphocyte proliferation index of chickens in groups 9–12V and 9V received the first vaccination at 9 weeks of age could indicate better cellular immune response against homologous than heterologous strains at the late period after the first vaccination. Interestingly, the index from homologous antigen stimulation in group 9–12V received the second vaccine at 12 weeks of age was significantly lower than that in group 9V, whereas the index within heterologous antigen stimulation was insignificantly different. According to the dynamic of lymphocyte populations in *M. gallisepticum* infection, macrophages and NK-cells ($CD^{8+}TCR^{0}$), associated with initial innate immune response, pathogenesis, and recovery process are upregulated and recruited to the infected site at the early period post-infection, whereas at the late stage of infection, $CD^{4+}TCR^{\alpha\beta 1+}$ cell counts are decreased and $CD^{4+}TCR^{\alpha\beta 2+}$ cell proportion are increased (Gaunson et al., 2006). Although the specific immune mechanism against *Mycoplasma* infection of $CD^{4+}TCR^{\alpha\beta 1+}$ and $CD^{4+}TCR^{\alpha\beta 2+}$ cells are still questionable, better cellular immune responses against homologous strain at the late period after the first vaccination in this study (group 9–12V and 9V) might be involved with a more specific activity of $CD^{4+}TCR^{\alpha\beta 2+}$ cells against a homologous antigen. Besides, during the early period after the second infection, the upregulation of macrophages and NK-cells ($CD^{8+}TCR^{0}$) may predispose the reducing proportion of $CD^{4+}TCR^{\alpha\beta 2+}$ cells affecting the decreased cellular immune response against homologous strains (group 9–12V). However, the

dynamics of lymphocyte populations in *M. synoviae* infection including macrophages, NK-cells ($CD^{8+}TCR^{0}$), $CD^{4+}TCR^{\alpha\beta 1+}$ cells, and $CD^{4+}TCR^{\alpha\beta 2+}$ cells should be further investigated to identify the protective cellular immune response in *M. synoviae* field strain-infected chickens.

Consequently, clarifying the immune response mechanisms in this study showed that immunized chickens could present the resembling characteristics of immune responses. However, the successful immunization under field conditions requires good vaccination practices; including careful handle or administer, storage or transfer in constant freezing condition, and thawing as the procedure described in the manufacturer's instructions (Jones et al., 2006a); to perform the obvious efficacy of reducing clinical signs or improving the production performance of *M. synoviae* infected flocks (Kleven and Ferguson-Noel, 2008; Ley, 2008; Landman, 2014).

In naturally and artificially infected chickens, vertical or egg transmission usually occurs during 1–4 weeks post-infection (Roberts and McDaniel, 1967). *M. synoviae* organisms could be isolated from 1-day-old chickens approximately 6.09% in egg yolks and 10.5% in trachea (Vardaman, 1976). Although the percentage of vertically infected progeny is low during the chronic phase; however, horizontal transmission, spreading to non-infected chickens, could occur anytime (Fahey and Crawley, 1954). Experimental chickens in this study were uninfected with any pathogens, including *M. synoviae*, during the study by rearing in separated rooms at the chicken experimental units with strict biosecurity and biosafety procedures. The seropositive at 1-day-old represented the normal passive immunity from their commercial parent layer flock, which were immunized with the available live MS-H vaccine and were monthly monitored for the occurrence of *M. synoviae* organisms in the flock; thus, experimental chickens have confirmed an *M. synoviae*-free condition in experimental units before immunization using molecular assays at 1-day-old and 9-weeks-old (Table 2) and serological assays at 5-weeks-old and 9-weeks-old (Tables 3 and 4).

In addition, the lymphocyte proliferation assays for determining the cell-mediated immunity from vaccines using in this study was the MTS Tetrazolium assay which is a convenient assay for measuring the metabolic activity of cells including cell viability and proliferation (Mattson et al., 1947; Sitz and Birx, 1999; Long et al., 2017;

Nikbakht et al., 2019). Besides, most previous studies were presented a parameter of cellular immune response as the PBMC proliferation using the Tetrazolium assay (Alvarez et al., 2020). Due to the critical limitations, the colorimetric MTT or MTS assay have become the alternative assays because the present techniques including staining with BrdU, CFSE, and antibodies of surface markers have been designed to develop more sensitive and harmless methods that evaluate cell proliferation and identify the cell phenotype (Motobu et al., 2002; Dalgaard et al., 2010a; Alvarez et al., 2020). The cellular immune mechanism using the flow cytometric phenotyping of peripheral PBMC will be further studied with the antibodies set of surface markers consisting of CD3-FITC, CD4-PE/CY7, CD8α-APC, Bu1-PE, RPE-conjugated rabbit polyclonal anti-chIFN-γ IgG antibody and anti-BrdU-APC.

5.4) Antimicrobial susceptible profiles

Medication; mostly performing with mycoplasma susceptible antimicrobials inhibiting protein synthesis of mycoplasma organism (Landman et al., 2008; Hong et al., 2015; Kreizinger et al., 2017); is the common practice in poultry industry due to an obvious efficacy in improving production performance of *M. synoviae* infected flocks by reduce clinical signs or economic losses associated with *M. synoviae* infections (Stanley et al., 2001; Fiorentin et al., 2003b; Kleven and Ferguson-Noel, 2008; Ley, 2008; Catania et al., 2010; Landman, 2014; Hong et al., 2015; Kreizinger et al., 2017). Although the susceptible antimicrobials could reduce the populations of *M. synoviae* in respiratory tract like *M. gallisepticum*, could decrease the risks of horizontal transmission (Cummings et al., 1986), and could reduce the vertical transmission via eggs (Stanley et al., 2001); however, the long term intensive medication, predisposing the antimicrobial resistance problems, is not economically acceptable in the infected poultry flocks (Fiorentin et al., 2003b; Kleven, 2008; Kleven and Ferguson-Noel, 2008). The current argument of antimicrobial resistance issues has influenced the practical medication in poultry production requiring the prescription from poultry veterinarian and the reliable drug susceptible evidents (Edens, 2003; Behbahan et al., 2008).

In this study, mycoplasma susceptible antimicrobials commonly use in poultry industry (Kleven and Ferguson-Noel, 2008; Landman et al., 2008; Hong et al., 2015; Kreizinger et al., 2017) consisting of tetracyclines (oxytracycline and doxycycline), macrolides (tylosin, tilmicosin, tylvalosin), lincosamides (lincomycin), quinolones (enrofloxacin) and pleuromutilins (tiamulin); were determined the MIC values using the liquid method because of its simplicity and convenience compared with the agar or solid method (Hannan, 2000). This study firstly provided the *in vitro* antimicrobial sensitivity information of each type of Thai *M. synoviae* isolates. Although antibiotic resistance levels are difficult to compare with the past antibiotic resistance levels; however, the present antimicrobial susceptible profiles of *M. synoviae* field isolates could be the reliable evidents for poultry veterinarian to make the prescription and design the suitable medication strategy for controlling *M. synoviae* problem in each farms (Edens, 2003; Behbahan et al., 2008).

Interestingly, the excellent susceptibility to tylosin, tilmicosin, tylvalosin and tiamulin in this study and other study in Europe, represented the effective antimicrobials against *M. synoviae* and the attractive drug of choice in the poultry farms (Kreizinger et al., 2017). Besides, tetracyclines; doxycycline and oxytetracycline; could also performed a good activity against Thai *M. synoviae* isolates like a previous study in *M. gallisepticum* isolates showing good sussceptibility to oxytetracycline (Behbahan et al., 2008) and doxycycline (Pakpinyo and Sasipreeyajan, 2007). In addition, the resistance to enrofloxacin in this study could notify that Thai *M. synoviae* isolates might be able to develop antimicrobial resistance against quinolones similar to the previous studies in *M. synoviae* (Kreizinger et al., 2017) and *M. gallisepticum* (Pakpinyo and Sasipreeyajan, 2007; Behbahan et al., 2008) performing the high MIC values for fluoroquinolones.

In this study, the tested *M. synoviae* isolates were only the second or third passage, so the numbers of microbial passages did not affect on the resistant test. It is unlikely that the resistant test was induced during the microbial passages, which could lead to the selection of resistant mutants (Gautier-Bouchardon et al., 2002). Therefore, the antibiotic resistance development needs further study about the history of antibiotics usage in the poultry farms including the effect of frequent or long period usage. Interestingly, an evident of the presence of many subpopulations that differ distinctly in their MIC values was reveal in this study (Figure 4). Although most *M. synoviae* field isolates in this study represented one population performing strong susceptible to tylosin, tylvalosin and tiamulin; moderate susceptible to doxycycline, oxytetracycline and lincomycin-spectinomycin; and resistance to enrofloxacin, distribution of *M. synoviae* field isolates in tilmicosin could reveal at least three subpopulations with different MIC values; ranging 0.0195-0.625 µg/ml; from strong susceptiblity to resistance to tilmicosin.

Some Thai *M. synoviae* isolates; AHRU2020CK0301, AHRU2020CK0305 and AHRU2020CK0404; were presented the final MIC value (at 0.625 µg/ml) of tilmicosin higher than other isolates; and some field isolates; AHRU2020CU1323 and AHRU2020CU1409; could perform the lower final MIC value than other isolates. In addition, the emergence of few isolates with high MIC values could have an

asymmetrically high influence on MIC_{50} and MIC_{90} values (Schwarz et al., 2010) as the difference between MIC_{50} and MIC_{90} values in doxycycline, oxytetracycline, tylosin, tilmicosin, tylvalosin, and tiamulin. Besides, the presence of a mixed population with different MIC level in the field may contribute to resist against antimicrobials when a minor component with high MIC level is growing with longer time of incubation as evident in fluoroquinolones (Gerchman et al., 2008). However, *in vitro* resistance might also be the result of multi-step mutation affecting the permeability of cells, the uptake of the drug and the binding to the ribosomes. Accordingly, the different MIC level among respiratory tract isolates and joint origin isolates in this study may be predisposed by the competence of tissues origin isolates including ability to survive in the environment, ability to subsequent re-infection of chickens, ability to live in respiratory organ or synovial tissue and ability to invade host cells for long periods or to reach subcellular fractions which antimicrobials would not be active (Le Carrou et al., 2006).

Furthermore, due to Thai *M. synoviae* field isolates were currently presented strong resistance to enrofloxacin, in addition to studying the effect of many microbial passages, enrofloxacin-resistance mutants associated with alteration of the genes coding for DNA gyrase and topoisomerase IV will be determined the amino acid substitution. The quinolone resistance-determining regions (QRDR) of DNA gyrase gene could presented in *gyrA* and *gyrB* genes while QRDR of topoisomerase IV could showed in *parC* and *parE* genes (Le Carrou et al., 2006).

CHAPTER VI

CONCLUSION

In addition to recently defining the genetic characterization of Thai *M. synoviae* isolates types E and L by sequence analysis of partial *vlhA* gene, this study provided the first validation of the PCR-RFLP assay as the convenient diagnostic tool for strain differentiation in epidemiological study of *M. synoviae* disease in Thailand, for evaluating the uniformity of vaccine administration in poultry farm, and for further determining the antimicrobial susceptible profiles of *M. synoviae* isolates especilally in the vaccinated chicken flocks which firstly presented in this study. Besides, the immunized chickens receiving live MS-H vaccine; whether single dose or two doses; produced the similar pattern of immune response mechanisms including the serological response and the cellular immune response. Furthermore, the developed PCR-RFLP assay using in this study presented ability to detect and differentiate MS-H vaccine strain from non-vaccine strains circulating in Thailand. Interestingly, in addition to an information of diagnostic assays for detecting and differentiating *M. synoviae* strains and an understanding of immune response mechanisms in vaccinated chickens, this study was also provided the antimicrobial susceptible profiles of current Thai *M. synoviae* performed strong susceptible to tylosin, tylvalosin, and tiamulin; moderate susceptible to doxycycline, oxytetracycline and lincomycin-spectinomycin; and resistance to enrofloxacin which are necessary to design the *M. synoviae* control procedures suitable for poultry flocks in Thailand.

Further studies are necessary to determine the protective efficacy of vaccination in challenge trial or at farm level, and to monitor the antimicrobial susceptible profiles and other factors predisposing drug resistance effect.

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