คุณลักษณะในระดับโมเลกุลและการจำแนกสายพันธุ์ของเชื้อ*มัยโคพลาสมา ซินโนวีอี* ที่พบในประเทศไทยด้วยข้อมูลลำดับเบสของยีน*วีแอลเอชเอ*บางส่วน

นายเกรียงวิชญ์ ลิมปวิทยากุล

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MOLECULAR CHARACTERIZATION AND STRAIN DIFFERENTIATION OF THAI *MYCOPLASMA SYNOVIAE* ISOLATES BY SEQUENCING OF PARTIAL *VLHA* GENE

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เกรียงวิชญ์ ลิมปวิทยากุล : คุณลักษณะในระดับโมเลกุลและการจำแนกสายพันธุ์ของเชื้อ*มัยโคพลาสมา ซินโนวีอี* ที่พบในประเทศไทยด้วยข้อมูลลำดับเบสของยีน*วีแอลเอชเอ*บางส่วน (MOLECULAR CHARACTERIZATION AND STRAIN DIFFERENTIATION OF THAI *MYCOPLASMA SYNOVIAE* ISOLATES BY SEQUENCING OF PARTIAL *VLHA* GENE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ดร. สมศักดิ์ ภัคภิญโญ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. น.สพ. ดร. จิโรจ ศศิปรียจันทร์, 48 หน้า.

เชื้อ*มัยโคพลาสมา ซินโนวีอี* (เชื้อเอ็มเอส) เป็นเชื้อจุลชีพก่อโรคที่มีความสำคัญในระบบอุตสาหกรรมการเลี้ยงสัตว์ปีกเนื่องจากสามารถก่อปัญหาในลักษณะของโรคติดเชื้อแบบไม่แสดงอาการในระบบทางเดินหายใจส่วนบนและโรคอักเสบติดเชื้อของข้อขาโดยเฉพาะบริเวณปลอกเอ็นกล้ามเนื้อ ในขณะที่การควบคุมป้องกันโรคโดยการทำวัคซีนฟาร์มเลี้ยงสัตว์ปีกก็ยังมีข้อมูลงานวิจัยสนับสนุนไม่มากพอโดยเฉพาะข้อมูลเกี่ยวกับวิธีการตรวจวินิจฉัยสำหรับแยกความแตกต่างระหว่างเชื้อเอ็มเอสจากวัคซีนและเชื้อเอ็มเอสที่ก่อปัญหาในพื้นที่ แม้ว่าการตรวจวินิจฉัยด้วยการตรวจหายีน 16S rRNA ของเชื้อเอ็มเอสโดยอาศัยวิธีการตรวจในระดับอณูชีววิทยาหรือพีซีอาร์นั้นจะเป็นวิธีการตรวจที่มีความจำเพาะสูงและได้รับความนิยมอย่างแพร่หลายในปัจจุบัน แต่การวิเคราะห์ลำดับเบสหรือนิวคลีโอไทด์ของยีน 16S rRNA นี้ก็ยังไม่สามารถนำมาประยุกต์ใช้สำหรับการจำแนกความแตกต่างของเชื้อเอ็มเอสแต่ละสายพันธุ์ได้ ดังนั้นการตรวจวินิจฉัยด้วยวิธีพีซีอาร์ที่มีความจำเพาะต่อยีน*วีแอลเอชเอ*ของเชื้อเอ็มเอสจึงได้รับการออกแบบและพัฒนาขึ้นมาเพื่อประโยชน์ในการตรวจเพื่อจำแนกความแตกต่างของเชื้อเอ็มเอสแต่ละสายพันธุ์เนื่องจากยีน*วีแอลเอชเอ*นี้ทำหน้าที่ควบคุมการสังเคราะห์โปรตีนฮีแมกกลูตินินและโปรตีนต่างๆที่เยื่อหุ้มเซลล์ของเชื้อเอ็มเอส โดยโปรตีนเหล่านี้ล้วนแล้วแต่มีกลไกการทำงานที่เกี่ยวข้องกับการเจริญเติบโตของเชื้อเอ็มเอสในอวัยวะเป้าหมายซึ่งจะมีความเกี่ยวข้องกับความรุนแรงและความหลากหลายในการก่อโรคของเชื้อด้วย การวิเคราะห์ลำดับเบสของยีน*วีแอลเอชเอ*สามารถแยกเชื้อเอ็มเอสสายพันธุ์ต่างๆออกเป็นกลุ่มหลัก (typing) และกลุ่มย่อย (subtyping) ได้โดยอาศัยข้อมูลขนาดความยาวเบสของส่วน Proline-rich repeat (PRR) ในยีน*วีแอลเอชเอ* และข้อมูลความหลากหลายของรูปแบบลำดับเบสของส่วน RIII ในยีน*วีแอลเอชเอ* การศึกษาครั้งนี้ได้รับการออกแบบขึ้นมาเพื่อศึกษาข้อมูลคุณลักษณะในระดับโมเลกุลของเชื้อเอ็มเอสที่พบในประเทศไทยและศึกษาถึงการนำวิธีการวิเคราะห์ลำดับเบสของยีน*วีแอลเอชเอ*บางส่วนมาใช้สำหรับการแยกสายพันธุ์ของเชื้อเอ็มเอสที่พบในพื้นที่กับเชื้อเอ็มเอสจากวัคซีน สำหรับเชื้อเอ็มเอสจากพื้นที่ทั้ง 20 isolate จากการสำรวจตัวอย่างฟาร์มไก่ในประเทศไทยในช่วงปี พ.ศ. 2558 นั้นได้รับการจัดแบ่งอยู่ในกลุ่ม C1 (1 isolate) C2 (4 isolate) E1 (9 isolate) E2 (1 isolate) และ L (5 isolate) นอกจากนี้ยังพบอีกว่าเชื้อเอ็มเอสทั้ง 9 isolate ที่แยกได้จากไก่ที่มีอาการป่วยของระบบทางเดินหายใจจะได้รับการจัดอยู่ในกลุ่ม E1 ถึง 6 isolate ในขณะที่เชื้อเอ็มเอสทั้ง 4 isolate ที่แยกได้จากไก่ที่แสดงอาการขากะเผลกจะถูกจัดอยู่ในกลุ่ม L ทั้งหมดด้วยขนาดความยาวเบสของส่วน PRR 105 เบสซึ่งถือว่าเป็นข้อมูลหลักฐานใหม่ของเชื้อเอ็มเอสสายพันธุ์ L ที่มีความสามารถในการก่อโรคข้ออักเสบในไก่ด้วย และยังพบอีกว่าฟาร์มที่เข้าร่วมการศึกษาในครั้งนี้เกือบทั้งหมดนั้นจะตรวจพบเชื้อเอ็มเอสเพียงแค่ 1 สายพันธุ์ ยกเว้นเพียงฟาร์มแห่งเดียวเท่านั้นที่ตรวจพบเชื้อเอ็มเอสทั้งสายพันธุ์ E และ L ผลการศึกษาในครั้งนี้ช่วยยืนยันได้ว่าการวิเคราะห์ลำดับเบสของยีน*วีแอลเอชเอนั้น*สามารถนำมาใช้ตรวจแยกสายพันธุ์ของเชื้อเอ็มเอสได้

ภาควิชา อายุรศาสตร์	ลายมือชื่อนิสิต .....
สาขาวิชา อายุรศาสตร์สัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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KRIENGWICH LIMPAVITHAYAKUL: MOLECULAR CHARACTERIZATION AND STRAIN DIFFERENTIATION OF THAI *MYCOPLASMA SYNOVIAE* ISOLATES BY SEQUENCING OF PARTIAL *VLHA* GENE. ADVISOR: ASSOC. PROF. SOMSAK PAKPINYO, D.V.M., Ph.D., D.T.B.V.M., CO-ADVISOR: PROF. JIROJ SASIPREEYAJAN, D.V.M., Ph.D., D.T.B.V.M., 48 pp.

Mycoplasma synoviae (MS), a remarkable pathogen in poultry industry, causes subclinical infection of upper respiratory tract and an infectious synovitis especially in the tendon sheaths and synovial membranes of joints. In addition, vaccination at farm level might have limitation because the information on diagnostic tests to differentiate field and vaccine strains was deficient. Although the specific detection of MS, 16S rRNA gene-based PCR, has been widely used to detect MS infected flocks, the sequencing of these gene is not suitable for strain differentiation. The *vlhA* gene-based PCR was designed to differentiate MS strains because it is encoding for hemagglutinin protein and other immunodominant membrane proteins which can be involving in colonization, antigenic variations, and virulence. The sequence analysis of *vlhA* gene were useful for typing and subtyping of MS strains based on the nucleotide insertion/deletion of proline-rich repeat (PRR) region and the nucleotide polymorphisms of RIII region in *vlhA* gene fragments. This study was designed to characterize Thai MS field isolates and to determine the strain differentiation between Thai field strains and vaccine strain by using sequence analysis of partial *vlhA* gene. In total, 20 MS field isolates submitted from registered chicken farms in Thailand during 2015, were identified as C1 (n=1), C2 (n=4), E1 (n=9), E2 (n=1), and L (n=5). The results revealed that six of nine isolates resulting in respiratory signs were type E1. In addition, four isolates from lame chickens showing joint swelling were type L with 105 nucleotides length. This study provides the first molecular data of Thai MS isolates and the first evidence of type L for being arthropathic strain. Furthermore, co-infection of MS types E and L was observed in one farm while other farms were affected by only one type of MS. The result indicated that sequence analysis of partial *vlhA* gene can be used as a tool for tracing MS characterization.

Department:	Veterinary Medicine	Student's Signature	.....
Field of Study:	Veterinary Medicine	Advisor's Signature	.....
Academic Year:	2015	Co-Advisor's Signature	.....

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LIST OF ABBREVIATIONS

AA = Amino acid

CFU = Colony forming unit

EAA = Eggshell apex abnormalities

ELISA = Enzyme-linked immunosorbent assay

FA = Fluorescent antibody

FMS = Frey's media supplemented with 15% swine serum

MG = *Mycoplasma gallisepticum*

MS = *Mycoplasma synoviae*

MSPB = Major surface protein B

PBS = Phosphate buffered saline

PCR = Polymerase chain reaction

PRR = Proline-rich repeat

RFLP = Restriction fragment length polymorphism

SPA = Serum plate agglutination test

vlhA = Variable lipoprotein hemagglutinin A

CHAPTER I
INTRODUCTION

1.1) Importance and Rationale

Mycoplasma synoviae (MS) is a remarkable pathogen in poultry industry worldwide. MS infection frequently appears in the subclinical infection of the upper respiratory tract in chickens (Kleven and Ferguson-Noel, 2008; Buim et al., 2009; Pakpinyo et al., 2009; Khiari et al., 2010; Wetzel et al., 2010; Landman, 2014). The systemic infection of MS causes infectious synovitis showing an inflammation of tendons or synovial membranes of joints (Kleven and Ferguson-Noel, 2008; Pakpinyo et al., 2009; Wetzel et al., 2010). The co-infection with respiratory viruses (Newcastle disease virus and/or infectious bronchitis virus) or respiratory bacteria (*Escherichia coli*) induces airsaculitis. The airsacculitis and infectious synovitis lesions can cause economic losses including the decreasing of egg production and hatchability, the reducing of growth performance and the increasing of carcass condemnations (Landman, 2014). However, the economic impact and disease severity are variation in pathogenicity among characteristic of MS isolates, from no lesion of disease to severe airsaculitis. In addition to respiratory tract tropism strains and the arthropathic strains, the oviduct tropism strains are able to induce eggshell apex abnormalities (EAA) without any physical abnormalities (Lockaby et al., 1998; Kleven and Ferguson-Noel, 2008; Pakpinyo et al., 2009; Landman, 2014).

Besides, the most horizontal transmission of MS occurs via respiratory tract by direct contact with MS contaminated materials or environments, while vertical transmission occurs only during egg production period of infected chickens. The infected chickens could be reservoirs or carriers for life and can transfer MS organisms to their progeny at anytime (Kleven and Ferguson-Noel, 2008; Pakpinyo et al., 2009; Khiari et al., 2010).

The elimination of infected breeder flocks seems to be the most efficient measure in disease control because it can stop both vertical and horizontal transmission but the biosecurity or hygiene measures could just minimize the

horizontal transmission (Stipkovits and Kempf, 1996; Landman, 2014). Therefore, the sustainability of MS-free flocks should be concerned by obtaining the MS-free birds, rearing in clean environment and strictly compliance in biosecurity program (Markham et al., 1998).

Nonetheless, the control of MS is quite difficult because the MS-free flocks maintenance and the elimination of infected flocks are not economically compliances. So, medical treatments and vaccination programs have been suggested as alternative methods for chicken flocks, having risk for MS field infection (Stipkovits and Kempf, 1996; Markham et al., 1998). Therapeutic and vaccine strategies can be used as preventive measures, which can improve poultry flock performance by reducing clinical signs and economic losses (Stipkovits and Kempf, 1996; Markham et al., 1998). However, therapeutic strategy is unable to completely eliminate the MS infection from affected chickens, whereas the effectiveness of vaccination strategy remains questionable for disease control and eradication programs. The vaccination strategy may be limited at the farm level because the information on disease transmission and control, including diagnostic tests to differentiate between field and vaccine strains, is inadequate (Ley, 2008; Landman, 2014).

Although the specific 16S rRNA gene-based PCR for rapid detection of MS has been widely used to detect MS infected flocks (Hong et al., 2004), the conserved nature of the 16S rRNA gene is not suitable for the sequencing differentiation (Hong et al., 2004; Buim et al., 2010). The *vlhA* gene-based PCR was designed to differentiate MS strains without any culture or isolation method (Hong et al., 2004; Jeffery et al., 2007). The *vlhA* gene of MS, encoding for hemagglutinin protein and other immunodominant membrane proteins, involves in colonization, antigenic variations, and virulence of MS (Razin et al., 1998; Bercic et al., 2008). The *vlhA* gene expresses proteins that can cleave post-translationally into MSPA (C-terminal hemagglutinin fragment) and MSPB (N-terminal lipoprotein fragment). The N-terminal of the *vlhA* gene (nucleotides 1-410), encoding for major surface protein B (MSPB), is a single chromosomal copy which each MS strain owns variable DNA sequences. Nevertheless, the downstream region of the N-terminal of the *vlhA* gene must be replaced with the pseudogenes sequence (Noormohammadi et al., 2000; Bencina et al., 2001). Therefore,

the recombination of the expressed *vlhA* gene with the one of pseudogenes causes antigenic variations in different strains of MS (Noormohammadi et al., 2000).

According to *vlhA* gene-based PCR and their sequence analysis, the proline-rich repeat (PRR) region of *vlhA* gene is useful for typing of MS strains based on insertion/deletion of nucleotides; the RIII region is useful for subtyping of MS strains by nucleotide polymorphisms (Bencina et al., 2001; Hammond et al., 2009). Due to the molecular basis of a size variation in the N-terminal fragment MSPB, the longer PRR region is associated with the higher invasiveness of MS strains, causing infectious synovitis in chicken (Bencina et al., 2001). The types of MS isolates, including type A, B, C, D, E, F, G, H, I, J, and K, have been classified on the basis of the size of the PRR region (Bencina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Bayatzadeh et al., 2014).

Based on the description of the *vlhA* fragment, MS isolates of the USA and Canada were typed as A, B, C, D, and E (Bencina et al., 2001), while those of Europe were typed as A, C, and E. In the United Kingdom, they were typed as C, E and F. In Asia, MS isolates of Iran were typed as F, G, H, I, J, and K (Hong et al., 2004; Hammond et al., 2009; Bayatzadeh et al., 2014). However, no available information of MS type in Thailand has been reported even if MS infection determined by serological methods, culture technique and PCR assays has been detected in commercial chicken and native chicken flocks (Pakpinyo et al., 2009).

Recently the revised *vlhA* gene-based PCR assay has shown a single copy of the *vlhA* gene which could be used for the genotyping of MS with higher discriminatory power (El-Gazzar et al., 2012). The revised *vlhA* gene-based PCR primers (Wetzel et al., 2010); producing PCR amplicons 370 bp; were used in this study to determine the molecular characterization of Thai MS field isolates and to determine the strain differentiation between Thai field and vaccine strains in relation to the clinical outcomes.

1.2) Objectives of study

1) To determine the molecular characterization of Thai *Mycoplasma synoviae* field isolates by sequence analysis of partial *vlhA* gene

2) To determine the strain differentiation among the MS-H live vaccine strain and other Thai *Mycoplasma synoviae* field strains by sequence analysis of partial *vlhA* gene

1.3) Questions of study

1) How to use the sequence analysis of partial *vlhA* gene providing the molecular characterization of Thai *Mycoplasma synoviae* field isolates?

2) Can the sequence analysis of partial *vlhA* gene differentiate the MS-H vaccine strain from other Thai *Mycoplasma synoviae* field strains?

1.4) Keywords (Thai)

ไก่ เชื้อ*มัยโคพลาสมา ซินโนวีอี* พีซีอาร์ การแยกสายพันธุ์ของเชื้อ ประเทศไทย ยีน*วีแอลเอชเอ*

1.5) Keywords (English)

Chickens, *Mycoplasma synoviae*, PCR, Strain differentiation, Thailand, *vlhA* gene

1.6) Hypothesis of study

1) The sequence analysis of partial *vlhA* gene, resulting in the basis of a length of PRR-coding region and nucleotides of RIII region, could provide the molecular characterization of Thai *Mycoplasma synoviae* field isolates.

2) The sequence analysis of partial *vlhA* gene can differentiate among the MS-H live vaccine strain and other Thai *Mycoplasma synoviae* field strains.

1.7) Advantages of study

The present study will provide the first molecular characterization of Thai *Mycoplasma synoviae* isolates by the sequencing analysis of partial *vlhA* gene. In addition, the molecular basis of *vlhA* gene will be applied to develop the convenient differentiation procedure in diagnostic methods, which can differentiate Thai field strains from vaccine strain.

CHAPTER II
LITERATURE REVIEW

2.1) General information of *Mycoplasma synoviae* (MS)

Mycoplasma synoviae (MS) is a remarkable pathogen in poultry industry worldwide. MS organisms lack of cell wall and appear as round in shape organisms with diameter around 300 nm. MS infection frequently appears in the subclinical infection of the upper respiratory tract in chickens (Kleven and Ferguson-Noel, 2008; Buim et al., 2009; Pakpinyo et al., 2009; Khiari et al., 2010; Wetzel et al., 2010; Landman, 2014). The co-infection with respiratory viruses (Newcastle disease virus and/or infectious bronchitis virus) or respiratory bacteria including *E.coli* can induce airsaculitis, whereas the systemic infection of MS causes the infectious synovitis showing inflammation of the 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). The airsacculitis and infectious synovitis lesions cause economic losses including the decreasing of egg production and hatchability, the reducing of growth performance and the increasing of carcass condemnation (Landman, 2014).

In addition, the systemic infection of MS may involve other organs such as the keel bursa, liver, spleen and brain including nerves, choroids of the eye and skeletal muscle (Sentíes-Cué et al., 2005). The pathogenesis of MS depends on the routes of infection. The food pad injection and the intravenous inoculation are more frequently to cause synovitis lesions, while the respiratory inoculation and aerosol exposure show mild lesions of synovitis (Lockaby et al., 1998; Landman and Feberwee, 2004). In natural infection, the spread of MS to the joints possibly occurs after the colonization of MS organisms in the respiratory tract via the haematogenous route (Kawakubo et al., 1980) by invading into chicken erythrocytes (Dušanić et al., 2009). Therefore, the epithelial damage of respiratory tract could enhance the prevalence of joint lesions in MS infected birds (Landman and Feberwee, 2004) because MS can easily spread via the bloodstream to the joints (Olson and Kerr, 1967; Kawakubo et al., 1980). Moreover, the induction of EAA caused by MS infection is also dependent on the route of

inoculation. The spread of MS organisms from the airsacs to the oviduct is more efficient than the colonization of the oviduct via the bloodstream (Feberwee et al., 2009).

2.2) Clinical signs and economic impacts of *Mycoplasma synoviae* infection

Clinical signs and economic relevance of MS seem to be noticeable increased, which is caused by the emergence of the reproductive tract tropism strains affecting the eggshell quality and egg production and the arthropathic strains associating with the infectious synovitis (Kleven and Ferguson-Noel, 2008; Landman, 2014).

Although economic impacts of MS infected flocks may not affect to the trade limitation, the MS infection remains as an economic important factor in the poultry industry including the co-infection with other pathogens such as Newcastle disease virus, infectious bronchitis virus and *Escherichia coli* (Kleven and Ferguson-Noel, 2008; Feberwee et al., 2009; Landman, 2014).

In layer flocks, infected with arthropathic strains, may loss due to the reducing of growth rate and the culling of lameness birds. Furthermore, the number of affected birds, which generally ranges from 5 to 15% of flock, may exceed to 75% of flock increasing more economic losses. In rearing pullets of brown layers, infected with arthropathic MS field isolate via intra-articular route, decrease 26% of body weight. In infected breeder flocks, the subclinical signs with the decreasing in egg production 5-10% and in hatchability 5-7%, and the increasing in mortality more than 5% are determined (Landman and Feberwee, 2004; Kleven and Ferguson-Noel, 2008).

In broiler chickens, the economic impact of MS infection has been more extensively losses by the increasing in carcass condemnations due to the occurrence of airsacculitis in the respiratory form disease. The incidence of air sac lesions is greatly influenced by co-infection with respiratory viruses and bacteria, environmental factors and/or immunosuppression (Kleven et al., 1972; Springer et al., 1974; Giambrone et al., 1977; Yoder Jr et al., 1977; Hopkins and Yoder Jr, 1982). Moreover, the performance

reduction of infected broiler flocks can determine by body weight reduction and high level of feed conversion ratio (Kleven et al., 1972; King et al., 1973; Goren, 1978).

In addition to respiratory tract tropism strains, the arthropathic strains of MS can be associated with the infectious synovitis and the oviduct tropism strains are able to induce eggshell apex abnormalities (EAA) with no macroscopic abnormalities (Lockaby et al., 1998; Kleven and Ferguson-Noel, 2008; Pakpinyo et al., 2009; Landman, 2014). The economic losses from eggshell apex abnormalities occurs 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) Transmission of *Mycoplasma synoviae* pathogens

The lateral or horizontal transmission of MS frequently occurs via respiratory tract by direct contact with MS contaminated materials or environments. The vertical transmission of MS occurs only in infected chickens especially during egg production period. The highest rate of egg transmission can be found during the first 4-6 weeks after infection. These infected chickens will be reservoirs or carriers of MS for life and transfer MS organisms to their progeny at anytime (Kleven and Ferguson-Noel, 2008; Pakpinyo et al., 2009). 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 MS has been demonstrated that horizontal could be occurs by indirect contact via people, wild animals or maybe contaminated equipment (Marois et al., 2000).

2.4) Prevention and control of *Mycoplasma synoviae* infection

MS organism is unstable at temperatures higher than 39^{O}C, but it can survive at room temperature up to 3 days on feathers and less than 1 day on other contaminated materials (Christensen et al., 1994; Marois et al., 2000). MS is an egg-transmitted pathogen and can persist within the host for long periods of time,

therefore; the control of MS infection is quite difficult. The successful procedures are the elimination method to break down the cycle of vertical transmission and also stop horizontal transmission (Stipkovits and Kempf, 1996; Landman, 2014). The sustainability of MS-free flocks should be done by obtaining the MS-free birds, rearing in clean environment and strictly compliance in biosecurity program (Markham et al., 1998). Nonetheless, the biosecurity control or hygiene measures can minimize the horizontal transmission (Landman, 2014).

Because the MS free-flocks condition and elimination of MS infected flocks are not economically compliances, the medical treatment and vaccination programs have been suggested as alternative methods for chicken flocks, having risk for MS field infection. Therapeutic and vaccine strategies can be used as alternative measures which can reduce clinical signs and economic losses in poultry flock (Stipkovits and Kempf, 1996; Markham et al., 1998). However, therapeutic strategy is unable to completely eliminate the MS infection from affected chickens, whereas the effectiveness of vaccination strategy remains questionable for disease control and eradication programs. Vaccination strategy, using MS-H live vaccine, may be limited at the farm level because the information on disease transmission and control, including diagnostic tests to differentiate between field and vaccine strains, is deficient (Ley, 2008; Landman, 2014). Moreover, the MS control strategies are based on the disease detection and require a risk assessment of pathogen entering to poultry farm (Landman, 2014).

2.5) Diagnosis approaches for *Mycoplasma synoviae* infection

2.5.1) Antibodies detection

Conventional detection programs for diagnosis the MS infection rely on serological tests including serum plate agglutination (SPA) or rapid plate test (RPT), hemagglutination inhibition (HI) and enzyme linked immunosorbent assay (ELISA). All serological methods should be used as screening tools in monitoring programs of MS infection in poultry flocks (Hong et al., 2004). Positive serological samples should be

confirmed by using the culture or isolation method or the molecular assay (Ewing et al., 1996; Luciano et al., 2011).

2.5.2) Culture and isolation

Swab samples and MS isolates are firstly inoculated into FMS broth, Frey's broth medium supplemented with 15% swine serum, and incubated at 37°C in a humidified chamber for 5-7 days until the broth color changed from pink-red to orange-yellow. The cultured FMS broths are then diluted for culture on FMS agar and incubated at 37°C in humidified condition until mycoplasma colonies are observed. Moreover, mycoplasma colonies on FMS agar need to be isolated and identified as the MS colony by an immunofluorescent assay (Kleven, 1998; Kleven and Ferguson-Noel, 2008).

However, culture and isolation method is the time consuming and laborious method as a result of the fastidious nature of MS. The molecular assays including polymerase chain reaction (PCR) has been developed and become being the method of choice for MS detection due to their advantages, simple, rapid, and highly sensitive (Kleven and Ferguson-Noel, 2008; Pakpinyo et al., 2009).

2.5.3) Direct immunofluorescent assay

To detect and identify the MS colonies, direct fluorescent antibody (FA) test was performed in a biosafety cabinet as described by Talkington and Kleven (1983). This method was accomplished by using fluorescent-conjugated, MS-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).

Mycoplasma colonies in FMS agar medium were enclosed in a stainless steel cylinder, washed with phosphate-buffered saline (PBS), stained with fluorescent-conjugated, MS-specific antibody, and then incubated at 37°C in a humidified chamber. After incubation, mycoplasma colonies were washed and soaked with PBS at 4°C. Thereafter, stainless steel cylinders were carefully removed; stained mycoplasma

colonies were identified on fluorescent microscope. All steps were done at room temperature unless indicated (Talkington and Kleven, 1983).

2.5.4) The 16S rRNA gene-based PCR assay

A 16S rRNA gene-based PCR with highly specific to MS has been widely used to monitor and detect MS infection in poultry flocks (Hong et al., 2004). Analysis of MS, using the 16S rRNA gene-based PCR, indicated a sensitivity of 82% and a specificity of 100% (Lauerman et al., 1993). The primers, MSL-F (5'-GAA GCA AAA TAG TGA TAT CA-3') and MSL-R (5'-GTC GTC TCC GAA GTT AAC AA-3'), can produce PCR amplicons size 207 bp (Lauerman et al., 1993).

In addition, MS could survive in the environments such as feed, dust, fly, or soil (Christensen et al., 1994) and the 16S rRNA PCR assay could be applying to detect the contaminated MS organisms (Marois et al., 2000). Even if, the sequence analysis of partial 16S rRNA gene may show genetic variability of different MS strains; however, the conserved nature of the 16S rRNA gene is not suitable for the strain differentiation because the polymorphic patterns 16S rRNA gene cannot relate to the origin area of MS 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 MS strains depending on their virulence (Hong et al., 2004; Jeffery et al., 2007).

2.5.5) The *vlhA* gene-based PCR assay

Due to the lack of cell wall, surface proteins anchored by acyl moieties or embedded in the cell membrane play the important role in the interactions of mycoplasmas and host cells (Razin et al., 1998). The *vlhA* gene, encoding for hemagglutinin protein and other immunodominant membrane proteins, is associated with the antigenic variability by involving in colonization, antigenic variations, and virulence (Razin et al., 1998; Bercic et al., 2008). The *vlhA* gene expresses proteins that can 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, major surface protein B, differs among MS 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) can occur in some MS organisms, which have transforming in their hemaglutinating phenotype. Both MSPB and tMSPB are highly immunogenic proteins which can induce local and systemic antibody responses in the early period of infectious synovitis (Narat et al., 1998; Noormohammadi et al., 1998).

The N-terminal of the *vlhA* gene (nucleotides 1-410), encoding for major surface protein B (MSPB), is a single chromosomal copy with high variation among the MS strains. Nevertheless, the downstream region of the N-terminal of the *vlhA* gene can be replaced with the pseudogenes sequence (Noormohammadi et al., 2000; Bencina et al., 2001). Therefore, the recombination of the expressed *vlhA* gene with the one of pseudogenes causes antigenic variations among different strains of MS (Noormohammadi et al., 2000). Recently, *vlhA* gene-based PCR assay has shown a single copy of the *vlhA* gene which could be used for the genotyping of MS with higher discriminatory power (El-Gazzar et al., 2012). The 370 bp of *vlhA* fragments 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') (Wetzel et al., 2010).

2.6) Strain differentiation by using the sequence analysis of partial *vlhA* gene

The *vlhA* gene-based PCR has been designed to differentiate MS strains, using sequence analysis, without any culture or isolation method (Hong et al., 2004; Jeffery et al., 2007). The proline-rich repeat (PRR) region of *vlhA* gene is useful for typing of MS strains based on insertion/deletion of nucleotides and the RIII region is useful for subtyping of MS strains by nucleotide polymorphisms (Bencina et al., 2001; Hammond et al., 2009). Due to the molecular basis of a size variation in the N-terminal fragment MSPB, the longer PRR region is associated with the higher invasiveness of MS strains, causing infectious synovitis in chicken (Bencina et al., 2001). The current 11 types of

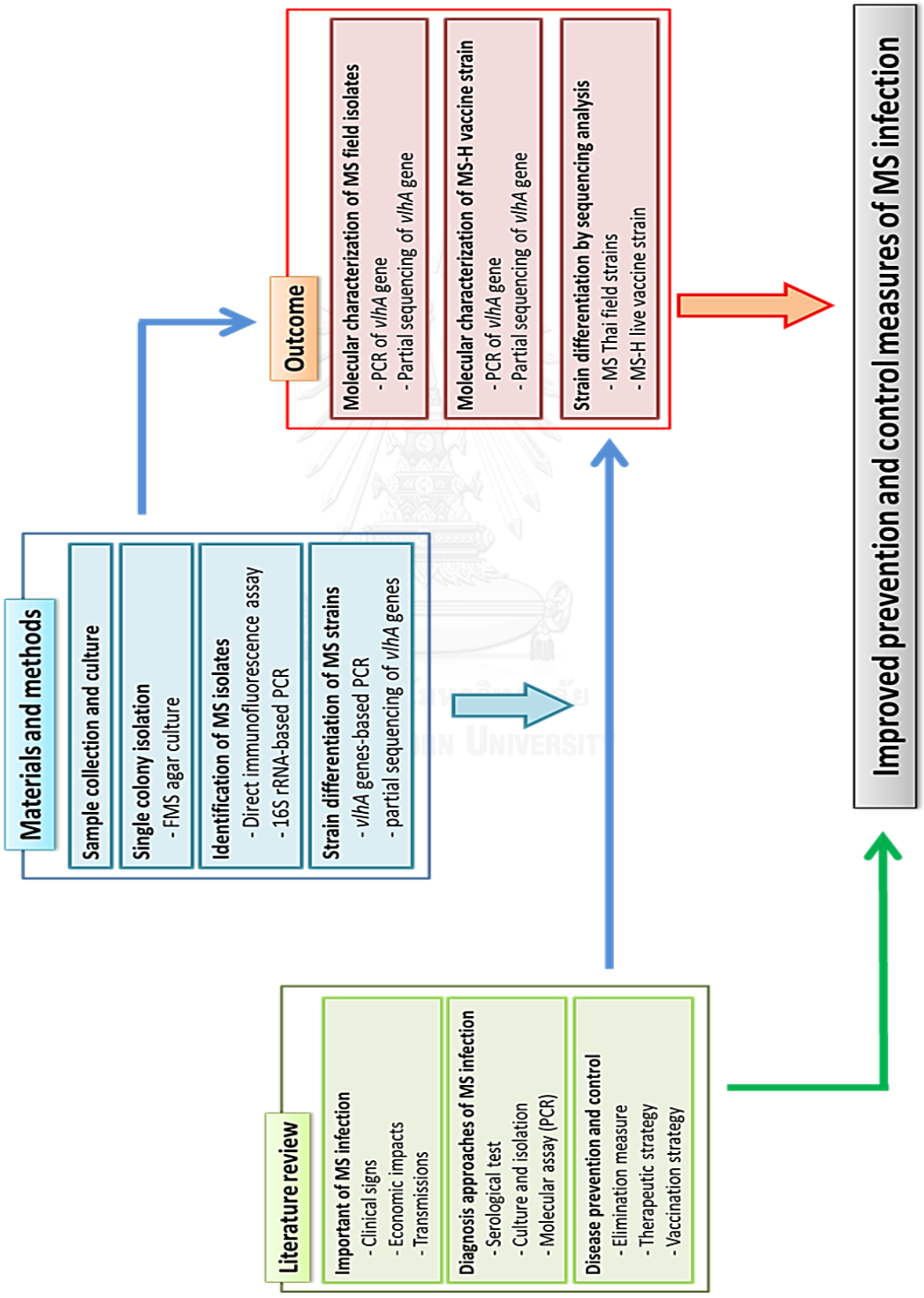
MS isolates, including type A, B, C, D E, F, G, H, I, J, and K, have been classified on the basis of the size of PRR region, consisting with 38, 45, 32, 23, 19, 36, 51, 46, 28, 20, and 12 amino acids, respectively (Bencina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Bayatzadeh et al., 2014).

Based on the description of the *vlhA* fragment, MS isolates found in the USA and Canada were type A, B, C, D, and E (Bencina et al., 2001), while those from Europe were type A, C, and E. In the United Kingdom, they were types C, E, and F. In Asia, MS isolates of Iran were type F, G, H, I, J, and K (Bencina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Bayatzadeh et al., 2014). However, there is no available information of MS type in Thailand, despite the report of MS infection, determined by serological methods, culture technique and PCR assays, in commercial chicken and native chicken flocks (Pakpinyo et al., 2009).

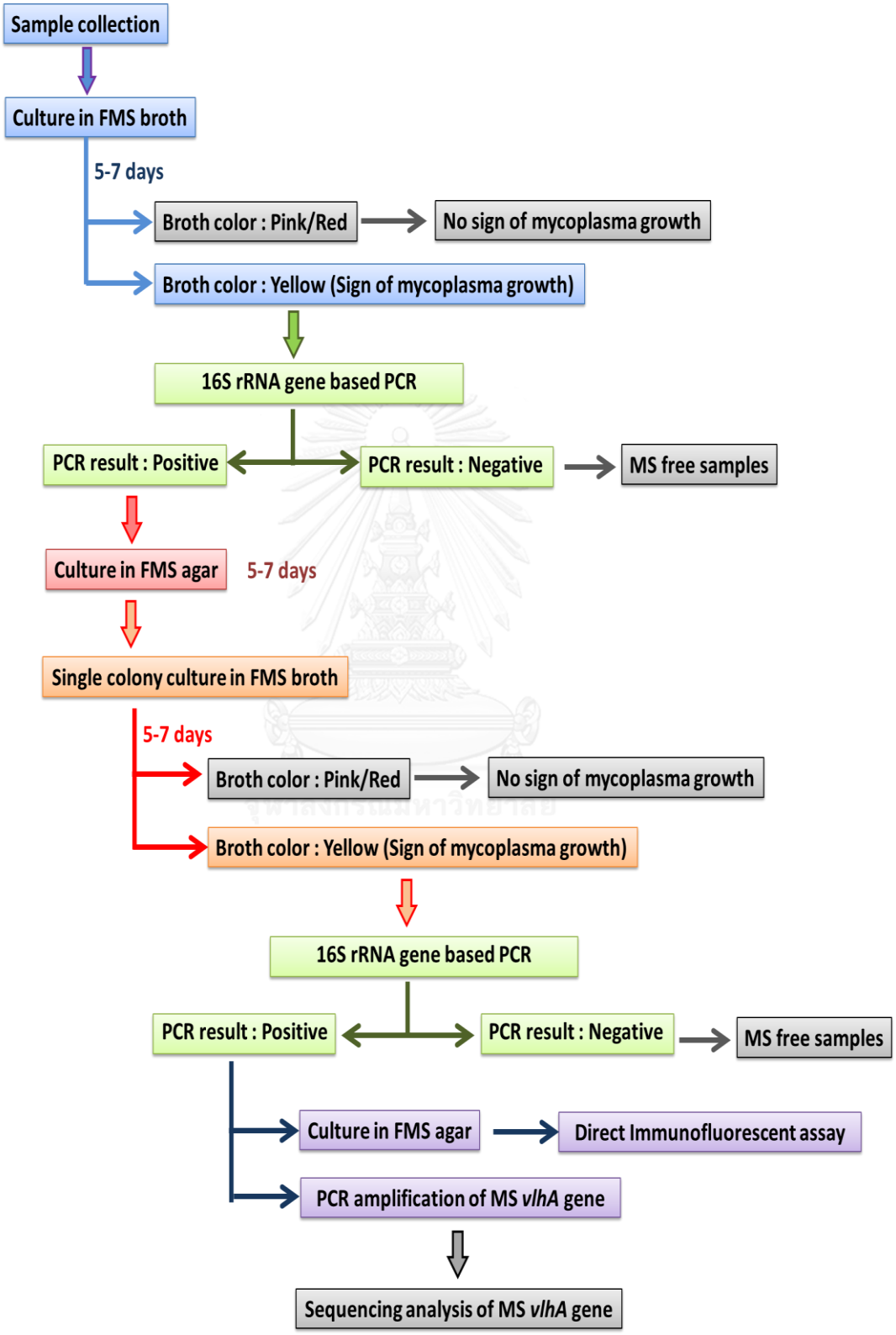
CHAPTER III

Materials and Methods

3.1) Conceptual framework of study



3.2) Research plan of study



3.3) Sample collection

In total, twenty samples of MS field isolates submitted from registered chicken farms in Thailand during 2015 were included. All samples were obtained from non-vaccinated commercial chicken flocks, including breeder, broiler, and layer flocks. Individual chickens were swabbed at choanal cleft, trachea, or joint by using a sterilized cotton swab. Swab samples were identified by inoculating into 2 ml of FMS broth, Frey's broth medium supplemented with 15% swine serum (Kleven, 1998). All FMS broth samples were then submitted for DNA analysis by the 16S rRNA gene-based PCR assay and the *vlhA* gene-targeted PCR assay.

3.4) *Mycoplasma synoviae* culture method

All FMS broth samples were first incubated at 37°C in a humidified chamber for 5-7 days until the broth color changed from pink-red to orange-yellow. Each broth samples, showing mycoplasma growth, were divided into 2 portions. The first portion was approached in the DNA extraction for *Mycoplasma synoviae* specific PCR, 16S rRNA gene-based PCR. The remaining portion was then diluted for culture on FMS agar and incubated at 37°C in humidified condition before sampling the single colony of MS.

Five single colonies of MS selected were passaged in fresh FMS broth and incubated at 37°C in humidified condition until the broth color changed from pink to orange-yellow. FMS broth samples, showing mycoplasma growth, were then equally divided into 3 portions. The first portion was cultured on FMS agar and further identified for MS colonies by direct immunofluorescent assay. The second portion was extracted for PCR assays, the 16S rRNA gene-based PCR assay and the *vlhA* gene-targeted PCR assay. The last portion was stored at -80°C as stock of each pure MS isolate (Hong et al., 2004).

3.5) Direct immunofluorescent assay

To detect MS colony, the direct fluorescent antibody (FA) test was performed in biological hazard cabinet as described by Talkington and Kleven (1983). This method was accomplished by using fluorescent MS-specific antibody conjugates, provided by S.H. Kleven (Poultry Disease Research Center, Department of Avian Medicine, College of Veterinary Medicine, University of Georgia, Athens, GA 30605).

Mycoplasma colonies on FMS agar medium were enclosed by stainless steel cylinders, washed with 200 µl of phosphate-buffered saline (PBS) for 15 min, stained with fluorescent MS-specific antibody conjugates, and then incubated at 37°C in a humidified chamber for 30 min. After incubation, mycoplasma colonies were washed for two times with PBS for 15 min and then soaked with PBS at 4°C for overnight. Thereafter, stainless steel cylinders were carefully removed; stained mycoplasma colonies were identified on microscope equipped with fluorescence illuminator. The colony showing fluorescent reaction was regarded as the MS organism. All steps were done at room temperature unless indicated (Talkington and Kleven, 1983).

3.6) DNA extraction and DNA template preparation

FMS broth samples were individually approached in the modified rapid boiling DNA extraction designed by Ley et al. (1997). The DNA extraction procedure was performed in biological hazard cabinet. Briefly, the FMS broth sample was centrifuged at 16,000 x g for 6 min, washed twice with sterile PBS, and the pellet was resuspended with sterile PBS (Ley et al., 1997). The suspension, afterwards, was boiled for 10 min, placed on ice for 10 min, and centrifuged at 16,000 x g for 2 min. The supernatant, finally, was collected and stored at -20°C until used.

3.7) 16s rRNA gene-based PCR for MS detection

DNA templates were tested for the 16s rRNA gene of *Mycoplasma synoviae* with specific primers (Lauerman et al., 1993). The 50 µl of PCR mixture contained 35 µl of nuclease-free water, 5 µl of 5x PCR Buffer, 2.5 µl of 1.25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 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'), 0.5 µl of 5 U/µl DNA Polymerase, and 5 µl (250 ng) of DNA template. MG S6 strain (ATCC 15302) and MS WVU 1853 strain (ATCC 25204) were used as negative and positive controls, respectively.

PCR mixtures were amplified in the DNA thermal cycler (Life express, BIOER®). It started with 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and ended with 72°C for 5 min. The PCR products were analyzed in 2% agarose gel, stained with ethidium bromide, visualized by UV transilluminator (E-BOX VX2, Vilber-Lourmat®), and photographed.

3.8) PCR amplification of *vlhA* gene

The *vlhA* fragments of *Mycoplasma synoviae* were amplified and sequenced with revised Hammond primers, which were specific to *vlhA* gene of *Mycoplasma synoviae* (Wetzel et al., 2010). The 50 µl of PCR mixture contained 35 µl of nuclease-free water, 5 µl of 5x PCR Buffer, 2.5 µl of 1.25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 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'), 0.5 µl of 5 U/µl DNA Polymerase and DNA template 5 µl (250 ng). MG S6 strain (ATCC 15302) and MS WVU 1853 strain (ATCC 25204) were used as negative and positive controls, respectively.

PCR mixtures were amplified in the DNA thermal cycler (Life express, BIOER®). It started with 95°C for 3 min, followed by 40 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and ended with 72°C for 5 min. The PCR products were analyzed in 2% agarose gel, stained with ethidium bromide, visualized by UV transilluminator (E-BOX VX2, Vilber-Lourmat®), and photographed.

3.9) Sequence analysis of MS *vlhA* gene

PCR products containing *vlhA* DNA fragment were amplified with the 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'), visualized by an electrophoresis on 2 % agarose gel and then subjected to sequencing (First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor, Malaysia). After the sequencing of PCR products, each of the nucleotide sequences was examined for the similarity using the BLAST program of NCBI (www.ncbi.nlm.nih.gov/BLAST). The type of MS isolates was classified on the basis of the size of the PRR region; the subtype of MS isolates was identified by using the point mutation of the RIII region (Bencina et al., 2001).

Based on the description of the *vlhA* fragment, types of MS isolates, including type A, B, C, D, E, F, G, H, I, J, and K, were classified on the PRR fragment of 38, 45, 32, 23, 19, 36, 51, 46, 28, 20, and 12 amino acids, respectively (Bencina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Bayatzadeh et al., 2014). Phylogenetic and sequencing alignment analyses were performed by using the Molecular Evolutionary Genetic Analysis (MEGA 6) software (http://www.megasoftware.net). The evolutionary distances were computed with the same software by using the Maximum Composite Likelihood method (Tamura et al., 2011).

3.10) Experimental study of arthropathic strain

Twenty-five, day-old broiler chicks were obtained from a local hatchery and housed in isolation rooms at the Avian Health Research Unit, Faculty of Veterinary Science, Chulalongkorn University. Feed and water were supplied ad libitum. At 5 weeks old, all birds were divided into 3 groups; 5 were negative control, 10 served as challenged treatment via intra hock joint, and 10 served as challenged treatment via intra foot pad. The challenged groups were inoculated with 0.2 ml of 2.5 X 10^7 CFU/ml Thai MS, isolated from the lame chickens (Isolate ID AHRU2015CU2807.1).

The clinical signs of lameness and joint swelling were observed daily. At 42 days of age, all birds were euthanized and necropsied. Gross pathologic lesions of air sacs and joints were determined. Swab samples from synovial fluid were individually taken from birds showing clinical lameness and swollen hock-joint to determine the presence of MS by culture and PCR assays. The use of experimental animals in this study was approved by IACUC, protocol No. 1631009.

CHAPTER IV
RESULTS

4.1) Detection of *Mycoplasma synoviae*

Choanal cleft swab samples were collected from approximately forty flocks of registered chicken farms in Thailand during 2015 to monitor MS status. From this monitoring program, three different MS flock statuses, including MS-free, MS-positive, MS- and MG-positive flocks were observed. Among the identified 20 field isolates, 10, 4, 4, and 2 isolates were from Lopburi (central part of Thailand), Chonburi (eastern part of Thailand), Satun (southern part), and Saraburi provinces, respectively (Table 1). All MS isolates used in this study were cultured and confirmed by direct fluorescent antibody test and 16S rRNA gene-based PCR assay amplified 207 bp (Figures 1-3). The cultured MS isolates were then characterized by sequence analysis of *vlhA* gene.

Based on historical details of the Thai *Mycoplasma synoviae* field isolates in Table 1, swab samples were collected and submitted to laboratory due to 2 major aims; to monitor status of mycoplasma infection in flocks, and to diagnose suspected cases showing clinical signs of mycoplasma infection or having seropositive of ELISA test in serology monitoring program. In addition to both detection of MS field isolates in normal birds and suspected birds, MS isolates could be found in wide range of age from few weeks to a year.

Table 1: Historical details of the Thai *Mycoplasma synoviae* field isolates used in this study.

Thai field Isolates ID	Chicken type [A]	Age	Clinical signs	Province	Proposal of samples collection
AHRU2015CG0202.1	BB female / CC	62 weeks	No clinical sign	Lopburi	ELISA positive (monitor)
AHRU2015CG0208.1	BB female / CC	62 weeks	No clinical sign	Lopburi	ELISA positive (monitor)
AHRU2015CG0212.1	BB male / CC	62 weeks	No clinical sign	Lopburi	ELISA positive (monitor)
AHRU2015CG0301.1	BB male / CC	88 weeks	No clinical sign	Saraburi	ELISA positive (monitor)
AHRU2015CG0306.1	BB female / CC	88 weeks	No clinical sign	Saraburi	ELISA positive (monitor)
AHRU2015CU3502.1	BB / CC	40 weeks	No clinical sign	Chonburi	Monitoring program
AHRU2015CU3505.1	BB / CC	50 weeks	No clinical sign	Chonburi	Monitoring program
AHRU2015CU3001.1	B / CC	37 days	Respiratory signs	Lopburi	Laboratory diagnosis
AHRU2015CU3010.1	B / CC	37 days	Respiratory signs	Lopburi	Laboratory diagnosis
AHRU2015CU3019.1	B / CC	39 days	Respiratory signs	Lopburi	Laboratory diagnosis
AHRU2015CU3021.1	B / CC	42 days	Respiratory signs	Lopburi	Laboratory diagnosis
AHRU2015CU3022.1	B / CC	42 days	Respiratory signs	Lopburi	Laboratory diagnosis
AHRU2015HU1427.1	BB / CC	Laying periods	Respiratory signs	Chonburi	Laboratory diagnosis
AHRU2015CU1303.1	BB / CC	Laying periods	Respiratory signs	Chonburi	Laboratory diagnosis
AHRU2015CU2006.1	BB / CC	57 weeks	Respiratory signs	Lopburi	Laboratory diagnosis
AHRU2014CU5801.2	BB / CC	Laying periods	Respiratory signs	Lopburi	Laboratory diagnosis
AHRU2015CU2802.1	TNC / J	50 days	Joint swollen	Satun	Laboratory diagnosis
AHRU2015CU2803.1	TNC / J	50 days	Joint swollen	Satun	Laboratory diagnosis
AHRU2015CU2806.1	TNC / J	50 days	Joint swollen	Satun	Laboratory diagnosis
AHRU2015CU2807.1	TNC / J	50 days	Joint swollen	Satun	Laboratory diagnosis

[A]**Chickens type :** BB is broiler breeder type; B is broiler type; TNC is Thai native chicken
CC is choanal cleft swab samples; J is synovial fluid swab samples

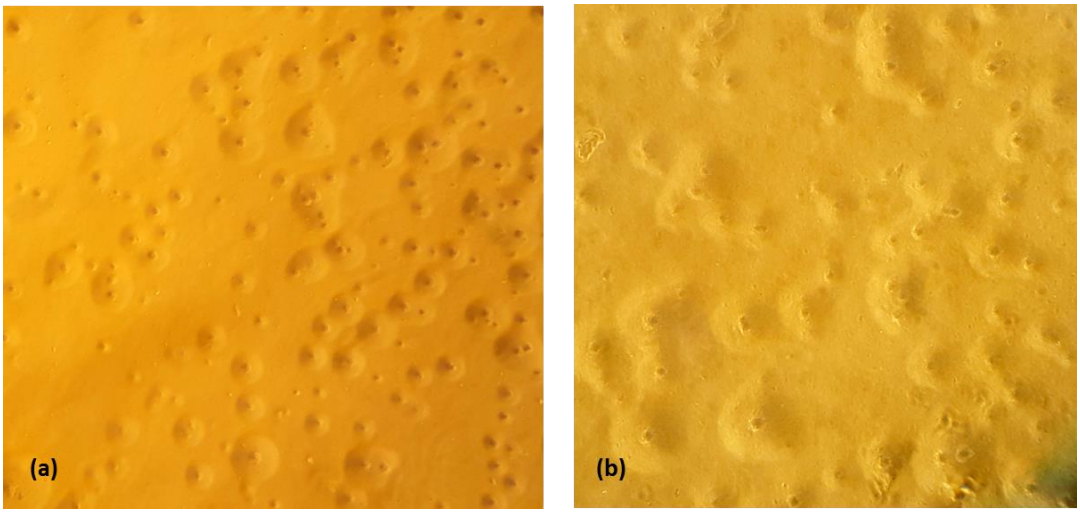


Figure 1: *Mycoplasma synoviae* colonies on agar, under light microscopes with magnification 100x (a) and 400x (b). The colonies show typical morphology of the mycoplasma, fried-egg appearance.

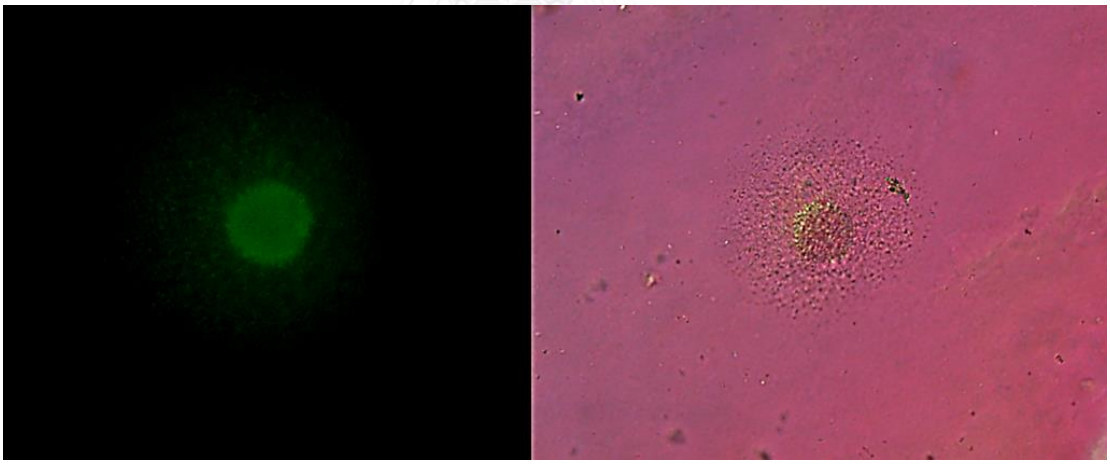


Figure 2: *Mycoplasma synoviae* colony on agar, stained with fluorescent MS-specific antibody conjugates, under microscope equipped with fluorescence illuminator with magnification 400x. The colony show solid apple green color with typical morphology of the mycoplasma, fried-egg appearance.

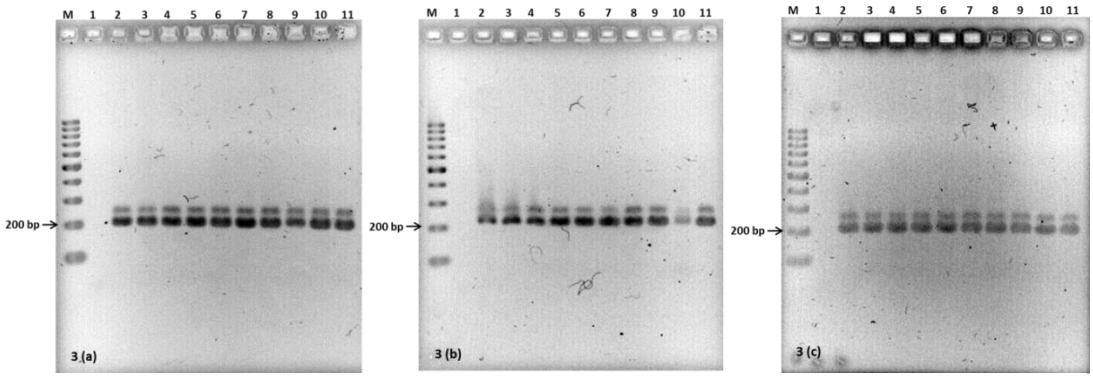


Figure 3: PCR electrophoresis gel demonstrating 16S rRNA gene amplicons size of 207 bp from *Mycoplasma synoviae* isolates.

(a) Lane M, 1000 bp DNA ladder;
Lane 1, MG strain S6 negative control; Lane 2, MS strain WVU 1853 positive control;
Lane 3, AHRU2015CG0202.1; Lane 4, AHRU2015CG0208.1;
Lane 5, AHRU2015CG0212.1; Lane 6, AHRU2015CG0301.1;
Lane 7, AHRU2015CG0306.1; Lane 8, AHRU2015CU3502.1;
Lane 9, AHRU2015CU3505.1; Lane 10, AHRU2015CU3502.2;
Lane 11, AHRU2015CU3505.2.

(b) Lane M, 1000 bp DNA ladder;
Lane 1, MG strain S6 negative control; Lane 2, MS strain WVU 1853 positive control;
Lane 3, AHRU2015CU3001.1; Lane 4, AHRU2015CU3010.1;
Lane 5, AHRU2015CU3019.1; Lane 6, AHRU2015CU3021.1;
Lane 7, AHRU2015CU3022.1; Lane 8, AHRU2015HU1427.1;
Lane 9, AHRU2015CU1303.1; Lane 10, AHRU2015CU2006.1;
Lane 11, AHRU2015CU2006.1.

(c) Lane M, 1000 bp DNA ladder;
Lane 1, MG strain S6 negative control; Lane 2, MS strain WVU 1853 positive control;
Lane 3, AHRU2014CU5801.2; Lane 4, AHRU2015CU2802.1;
Lane 5, AHRU2015CU2803.1; Lane 6, AHRU2015CU2806.1;
Lane 7, AHRU2015CU2807.1; Lane 8, AHRU2015CU2807.2;
Lane 9, AHRU2015CU2807.1Ch1Jt; Lane 10, AHRU2015CU2807.1Ch2Jt;
Lane 11, AHRU2015CU2807.1Ch3Jt.

4.2) DNA sequence analysis of MS *vlhA* gene

The *vlhA* gene fragments from all MS isolates were amplified (Figure 4). Sequences of *vlhA* gene were submitted to the GenBank Database. The details of each MS isolate including sizes of amplicons, PRR nucleotide and amino acid sequences, and GenBank accession number were shown in Table 2.

Based on the *vlhA* sequence analysis from alignment of nucleotide or amino acid sequences corresponding to MS strain K1968 (Figure 5), the PRR type of MS WVU 1853 and MS-H vaccine strains were identified as groups A and C, respectively. The twenty Thai MS field isolates were identified as groups E, C and L with 19, 32 and 35 amino acid length, respectively (Figure 5). The twenty Thai MS field isolates were further classified as subgroups C1 (1 isolate), C2 (4 isolates), E1 (9 isolates), E2 (1 isolate), and L (5 isolates) (Figures 6 and 7).

Most of the farms were identified as only one type. In addition, several isolates collected from different flocks within the same farm in Chonburi: AHRU2015CU3502.1 and AHRU2015CU3505.1 were types E2 and L, respectively. The MS isolates of Chonburi belonged to types C2, E1, E2, and L; those of Lopburi belonged to types C1, C2, and E1; those of Saraburi belonged to types C2, and those of Satun belonged to type L. MS isolates collected from male and female chickens within the same flock belonged to the same type: AHRU2015CG0202.1, AHRU2015CG0208.1, and AHRU2015CG0212.1 were type E1, while AHRU2015CG0301.1 and AHRU2015CG0306.1 were type C2.

Nine Thai MS isolates of chickens showing respiratory signs in different farms and flocks were types E1, C2, and C1 as follows. Isolates AHRU2015CU3001.1, AHRU2015CU3010.1, AHRU2015CU3019.1, AHRU2015CU3021.1, AHRU2015CU3022.1, and AHRU2015CU1303.1 were type E1, AHRU2015HU1427.1 and AHRU2015CU2006.1 were type C2, and AHRU2014CU5801.2 was type C1. Four Thai MS isolates of lame chickens showing joint swelling were type L: AHRU2015CU2802.1, AHRU2015CU2803.1, AHRU2015CU2806.1, and AHRU2015CU2807.1.

In addition to PRR typing, the nucleotide polymorphism of RIII region could be also used for subtyping the PRR types C and E (Figures 6 and 7). The 346th-351st

nucleotide sequences of subtype C1 (ACAGAC) were different from subtype C2 (ACAGAA). The subtype E1 and E2 were different in 2 portions, the 319th-321st and 346th-351st nucleotide sequences of subtype E1 were TCT and GCAGAC, respectively; while the 319th-321st and 346th-351st nucleotide sequences of subtype E2 were GCT and ACAGAA, respectively.

4.3) Experimentally infected study of arthropathic strain

Five birds in non-challenged group were not shown any abnormalities associated with infectious synovitis or airsaculitis. Otherwise, nine birds in both challenged groups; 6 and 3 birds from intra hock joint inoculated group and intra foot pad inoculated group, respectively; were shown clinical lameness and hock-joint swelling within 7 days after the inoculation with Thai MS isolate ID AHRU2015CU2807.1. Grossly, the infectious synovitis with viscous yellowish synovial fluids was observed in lame birds, while the airsaculitis lesion was not found in both non-challenged and challenged birds. Swab samples of synovial fluid were positive results to MS organisms detected by culture method and the 16S rRNA PCR assay (Figure 3).

In addition, the presence of MS in the experimental study was confirmed by direct fluorescent antibody test and the 16S rRNA PCR assay. Some isolates were randomly selected for analysis of the *vlhA* gene fragments (Figure 4). MS isolates ID. AHRU2015CU2807.1Ch1Jt, AHRU2015CU2807.1Ch2Jt, and AHRU2015CU2807.1Ch3Jt were classified as type L with 35-amino acid fragment of PRR which were similar to the inoculated MS strain (Table 2).

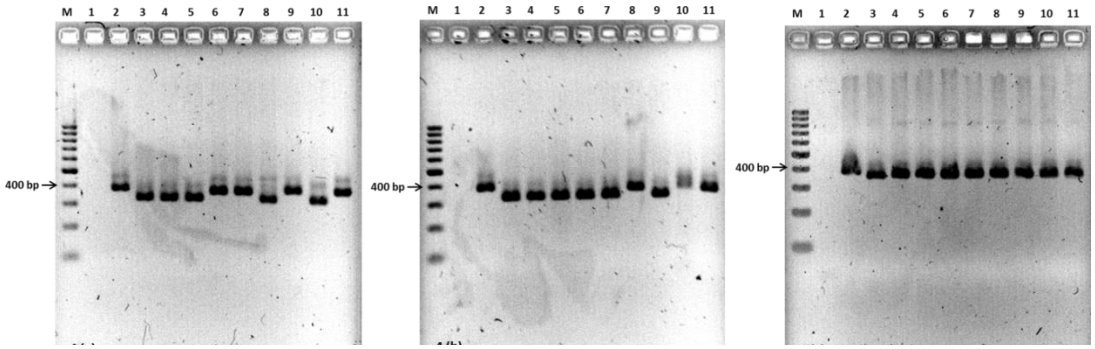


Figure 4: PCR electrophoresis gel demonstrating MS *vlhA* gene amplicons size of 350-370 bp from *Mycoplasma synoviae* isolates.

(a) Lane M, 1000 bp DNA ladder;
Lane 1, MG strain S6 negative control; Lane 2, MS strain WVU 1853 positive control;
Lane 3, AHRU2015CG0202.1; Lane 4, AHRU2015CG0208.1;
Lane 5, AHRU2015CG0212.1; Lane 6, AHRU2015CG0301.1;
Lane 7, AHRU2015CG0306.1; Lane 8, AHRU2015CU3502.1;
Lane 9, AHRU2015CU3505.1; Lane 10, AHRU2015CU3502.2;
Lane 11, AHRU2015CU3505.2.

(b) Lane M, 1000 bp DNA ladder;
Lane 1, MG strain S6 negative control; Lane 2, MS strain WVU 1853 positive control;
Lane 3, AHRU2015CU3001.1; Lane 4, AHRU2015CU3010.1;
Lane 5, AHRU2015CU3019.1; Lane 6, AHRU2015CU3021.1;
Lane 7, AHRU2015CU3022.1; Lane 8, AHRU2015HU1427.1;
Lane 9, AHRU2015CU1303.1; Lane 10, AHRU2015CU2006.1;
Lane 11, AHRU2015CU2006.1.

(c) Lane M, 1000 bp DNA ladder;
Lane 1, MG strain S6 negative control; Lane 2, MS strain WVU 1853 positive control;
Lane 3, AHRU2014CU5801.2; Lane 4, AHRU2015CU2802.1;
Lane 5, AHRU2015CU2803.1; Lane 6, AHRU2015CU2806.1;
Lane 7, AHRU2015CU2807.1; Lane 8, AHRU2015CU2807.2;
Lane 9, AHRU2015CU2807.1Ch1Jt; Lane 10, AHRU2015CU2807.1Ch2Jt;
Lane 11, AHRU2015CU2807.1Ch3Jt.

Table 2*:* Information and molecular characteristic details of the MS isolates and MS strains used in this study.

Isolates ID	Additional information [A]	PCR amplicons size (bp)	*vlhA* gene PRR (nt)	Size of PRR (aa)	Type and subtype	GenBank Accession No.
WVU1853	ATCC 25204	376	114	38	A	KX168667, AM998371
MS-H	Vaccine strain (Australia origin)	358	96	32	C1	KX168666, JX960401
AHRU2015CG0202.1	Farm No. 1 Flock 1A	319	57	19	E1	KX168668
AHRU2015CG0208.1	Farm No. 1 Flock 1A	319	57	19	E1	KX168669
AHRU2015CG0212.1	Farm No. 1 Flock 1A	319	57	19	E1	KX168670
AHRU2015CG0301.1	Farm No. 2 Flock 2A	358	96	32	C2	KX168671
AHRU2015CG0306.1	Farm No. 2 Flock 2A	358	96	32	C2	KX168672
AHRU2015CU3502.1	Farm No. 3 Flock 3A	319	57	19	E2	KX168673
AHRU2015CU3505.1	Farm No. 3 Flock 3B	367	105	35	L	KX168674
AHRU2015CU3502.2	Cultured from AHRU MS35/58 2.1P	319	57	19	E2	KX168675
AHRU2015CU3505.2	Cultured from AHRU MS35/58 5.1P	367	105	35	L	KX168676
AHRU2015CU3001.1	Farm No. 4 Flock 4A	319	57	19	E1	KX168677
AHRU2015CU3010.1	Farm No. 4 Flock 4A	319	57	19	E1	KX168678
AHRU2015CU3019.1	Farm No. 4 Flock 4B	319	57	19	E1	KX168679
AHRU2015CU3021.1	Farm No. 4 Flock 4C	319	57	19	E1	KX168680
AHRU2015CU3022.1	Farm No. 4 Flock 4C	319	57	19	E1	KX168681
AHRU2015HU1427.1	Farm No. 5 Flock 5A	358	96	32	C2	KX168682
AHRU2015CU1303.1	Farm No. 6 Flock 6A	319	57	19	E1	KX168683
AHRU2015CU2006.1	Farm No. 7 Flock 7A	358	96	32	C2	KX168684
AHRU2014CU5801.2	Farm No. 8 Flock 8A	358	96	32	C1	KX168685
AHRU2015CU2802.1	Farm No. 9 Flock 9A	367	105	35	L	KX168690
AHRU2015CU2803.1	Farm No. 9 Flock 9A	367	105	35	L	KX168686
AHRU2015CU2806.1	Farm No. 9 Flock 9A	367	105	35	L	KX168687
AHRU2015CU2807.1	Farm No. 9 Flock 9A	367	105	35	L	KX168688
AHRU2015CU2807.2	Cultured from AHRU MS28/58 7.1P	367	105	35	L	KX168689
AHRU2015CU2807.1Ch1Jt	Challenge study	367	105	35	L	KX168691
AHRU2015CU2807.1Ch2Jt	Challenge study	367	105	35	L	KX168692
AHRU2015CU2807.1Ch3Jt	Challenge study	367	105	35	L	KX168693

[A] **Additional information of each MS isolate:** "ATCC 25204" is the Reference strain; "Vaccine strain" is live vaccine strain; "Cultured from" means MS isolates which were re-cultured from other isolates; "Challenge study" means MS isolates cultured from experimental inoculation; The same farm number and flock name represent same origin of MS field isolates

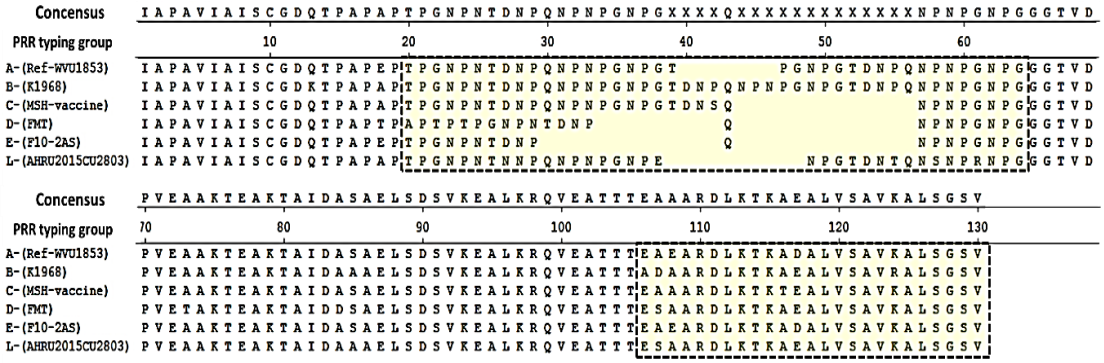
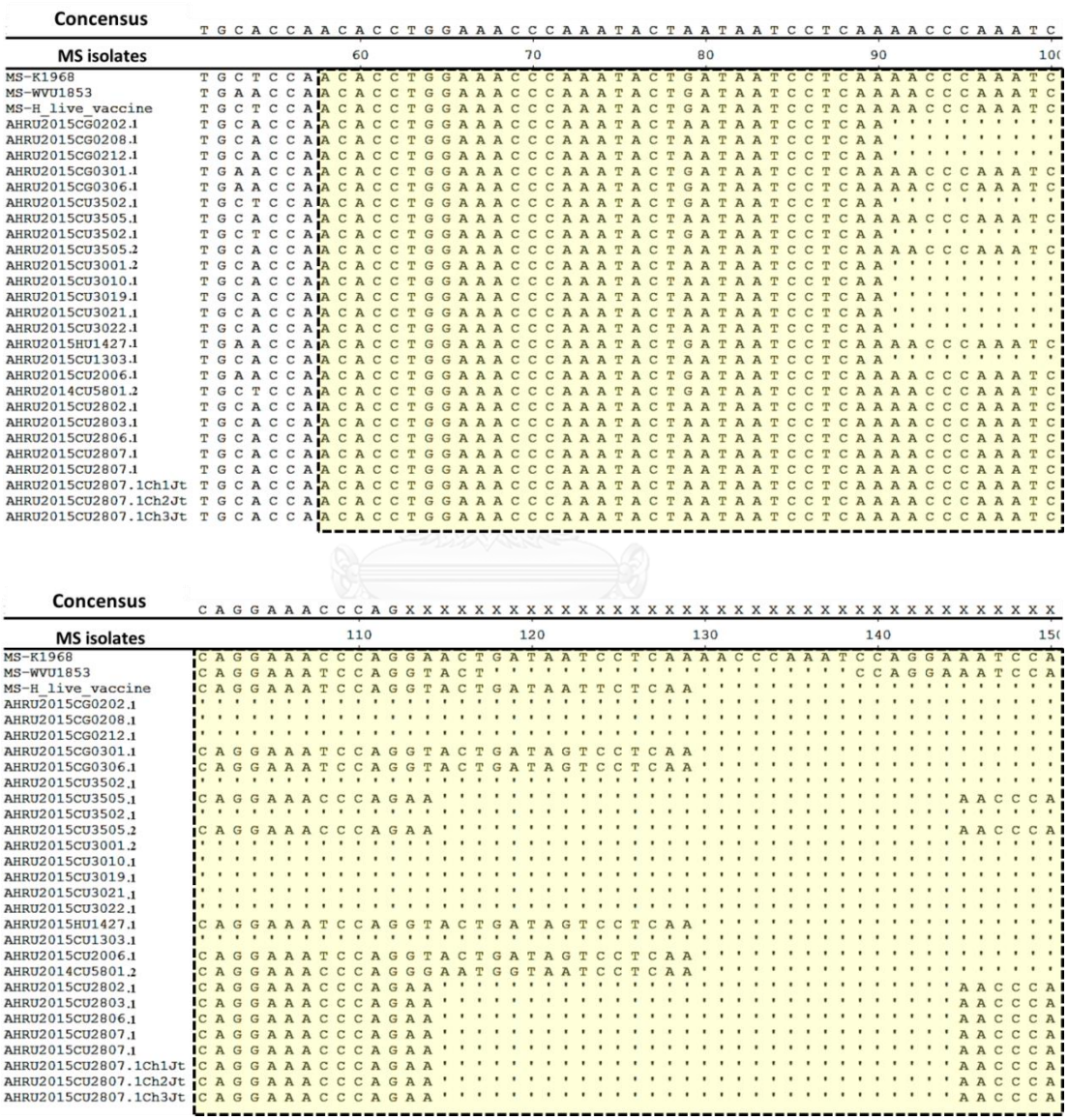


Figure 5: Amino acid alignment representing size polymorphism of the proline-rich-repeat (PRR) region. The sequences corresponded to amino acids (AA) 20-64 in strain K1968 N-terminal *vlhA* gene. PRR typing group A has 38 AA, PRR typing group B has 45 AA, PRR typing group C has 32 AA, PRR typing group D has 23 AA, PRR typing group E has 19 AA, and PRR typing group L has 35 AA. In addition, the RIII region corresponded to the nucleotide sequences 106-130 in strain K1968 N-terminal *vlhA* gene.

Figure 6: Nucleotide sequence alignment of the partial *vlhA* gene from MS isolates and MS strains used in this study.

(a) The proline-rich-repeat (PRR) region of N-terminal *vlhA* gene corresponds to the nucleotide sequences 58th-192nd in strain K1968.

(b) The RIII region of N-terminal *vlhA* gene corresponds to the nucleotide sequences 316th-393rd in strain K1968.



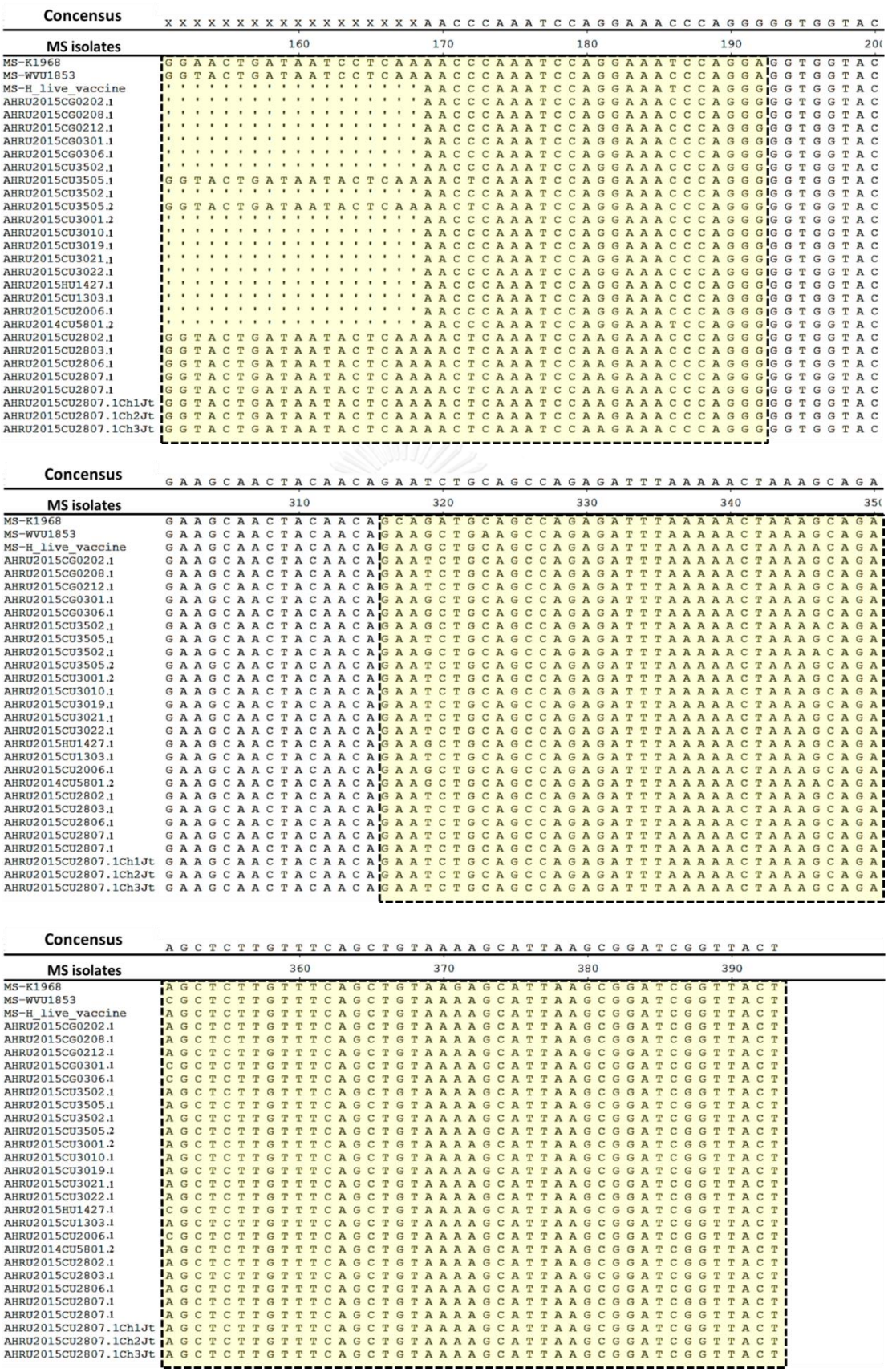
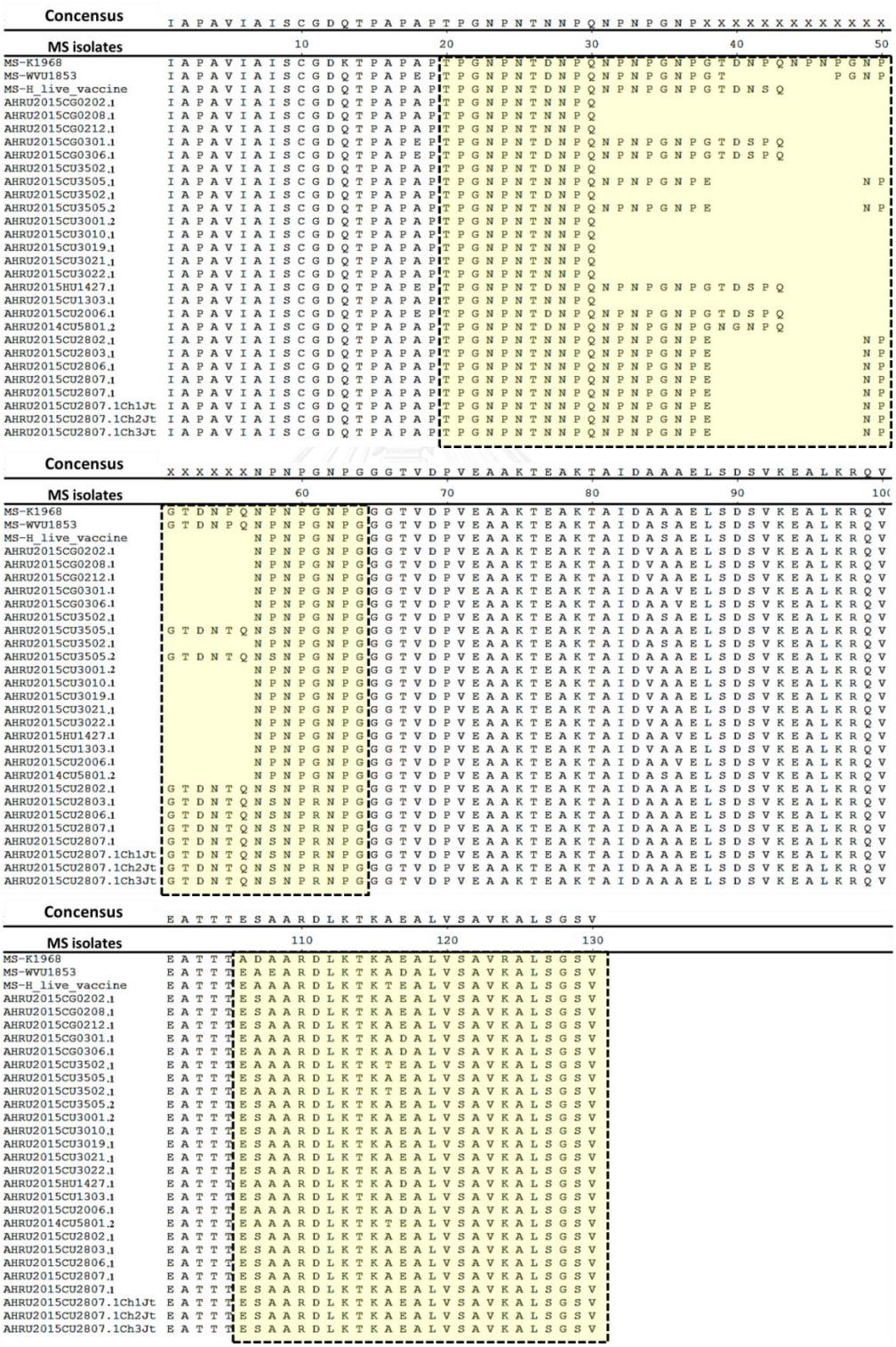

Concensus
MS isolates
MS-K1968
MS-WVU1853
MS-H_live_vaccine
AHRU2015CG0202.1
AHRU2015CG0208.1
AHRU2015CG0212.1
AHRU2015CG0301.1
AHRU2015CG0306.1
AHRU2015CU3502.1
AHRU2015CU3505.1
AHRU2015CU3502.1
AHRU2015CU3505.2
AHRU2015CU3001.2
AHRU2015CU3010.1
AHRU2015CU3019.1
AHRU2015CU3021.1
AHRU2015CU3022.1
AHRU2015HU1427.1
AHRU2015CU1303.1
AHRU2015CU2006.1
AHRU2014CU5801.2
AHRU2015CU2802.1
AHRU2015CU2803.1
AHRU2015CU2806.1
AHRU2015CU2807.1
AHRU2015CU2807.1
AHRU2015CU2807.1Ch1Jt
AHRU2015CU2807.1Ch2Jt
AHRU2015CU2807.1Ch3Jt

Figure 7: Amino acid alignment of partial *vlhA* protein of MS isolates and MS strains used in the present study. Proline-rich-repeat (PRR) region and RIII region corresponds to the amino acid sequences 20th-64th and 106th-130th in MS strain K1968, respectively.



CHAPTER V
DISSCUSSION

5.1) Detection of *Mycoplasma synoviae*

Due to historical details of the Thai *Mycoplasma synoviae* field isolates in Table 1, MS field isolates could be found in wide range of age from few weeks to a year and could be detected in normal and suspected birds within both monitoring program and laboratory diagnosis. In addition to vertical transmission during egg production period in infected flocks, the horizontal transmission also frequently occurs via respiratory tract by both direct and indirect contact via MS contaminated materials, people, pigeons, ostriches, wild animals, equipment (Marois et al., 2000; Tebyanian et al., 2014). MS infection frequently appear in the subclinical infection of the upper respiratory tract in chickens which will be reservoirs or carriers of MS, will spread pathogens to environments, and will transferred organisms to progeny for life (Kleven and Ferguson-Noel, 2008; Buim et al., 2009; Pakpinyo et al., 2009; Khiari et al., 2010; Wetzel et al., 2010; Landman, 2014). Interestingly, horizontally transmission of MS could be occurred rather than vertically from the breeders. The MS isolates of the progeny may not be similar to those from the breeder flock, but may be similar to MS isolates from another flock or MS isolates contaminated in environments (Sentíes-Cué et al., 2005).

5.2) Experimental study and the sequence analysis of partial *vlhA* gene

This study was conducted to determine the characterization of Thai MS isolates by the partial sequence analysis of the *vlhA* gene, which could differentiate the MS strains. PCR products containing *vlhA* DNA fragment were amplified and sequenced with the primers used by Wetzel et al. (2010). In addition, field isolates of MS in Thailand were classified on the basis of the size of the PRR region and the point mutation of the RIII region described by Bencina et al. (2001). Twenty Thai MS field isolates could be identified as groups E, C, and L with 19, 32, and 35 amino acid lengths, respectively, indicating that there were at least 3 genogroups of MS circulated in

Thailand. Furthermore, the present study is the first report identifying the MS type L among the Thai MS isolates. The MS groups E and C were genetically similar to the MS strains reported from other countries, including UK, USA, Slovenia, France, and Hungary (Bencina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Bayatzadeh et al., 2014).

This study provided the first molecular data of the Thai MS types C and E isolated from chickens showing respiratory signs. It was possible that Thai MS isolate types C and E were associated with respiratory tract infection, but they could not induce the eggshell apex abnormality syndrome like Dutch MS isolate types C and E, which could infect the reproductive tract of chickens (Feberwee et al., 2009). Moreover, this study provided the first molecular evidence of Thai MS isolates type L causing infectious synovitis and lameness in both commercial and native chickens. In experimental study, broiler chickens showed infectious synovitis within 7 days after inoculation with Thai MS isolate type L via intra hock joint and intra foot pad routes. All inoculated broilers showing clinical lameness and hock-joint swelling with viscous yellowish synovial fluids, were not shown any gross lesion of airsac. Therefore, the MS type L could be the new arthropathic strain, in addition to the highly invasive MS strain K1968 classified as MS type B with long PRR region which could also cause infectious synovitis in chicken (Bencina et al., 2001).

Based on the description of the *vlhA* fragment, types of MS isolates classified on the PRR fragment, and amino acid alignment analyses were performed by using the Molecular Evolutionary Genetic Analysis (MEGA 6) software. The 5'-end of the *vlhA* gene sequence of 20 Thai MS isolates encoded almost two identical amino acids at the N-terminal region of the MSPB protein, which consisted of the conserve region (19 amino acids) and PRR region (Noormohammadi et al., 1998). Due to amino acid alignment analyses in this study and previous studies, the *vlhA* sequences corresponded to amino acids (AA) 20^{th}-64^{th} in N-terminal region of MS strain K1968; at least 12 types of MS strain with variation in length of PRR fragments were noticed. The PRR typing groups A, B, C, D, E, F, G, H, I, J, K, and L have 38, 45, 32, 23, 19, 36, 51, 46, 28, 20, 12, and 35 amino acids in N-terminal fragment of MSPB, respectively (Bencina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Bayatzadeh et al., 2014).

The *vlhA* sequences of all 12 types of MS encoded a conserved end of the signal peptide (IAPAVIAIS). The first eight amino acids of their predicted MSPB had an identical sequence (CGDQTPAP), except those of strain K1968 (Group B), in which the fourth amino acid was changed from CGDQ to CGDK. Furthermore, MS strain K1968 (Group B) have been described as the arthropathic strain (Lockaby et al., 1998; Lockaby et al., 1999) and had the longest PRR by an addition of repeated motif DNPQNPN within its PRR, AA 40^{th}-46^{th} (Bencina et al., 2001), while the Thai MS type L in this study, described as the arthropathic strain, had the different PRR from MS strain K1968. Considering AA, Thai MS type L had 35 AA in N-terminal fragment of MSPB but MS type B had 45 AA. Besides, the 38^{th} AA of MS type L was acidic polar side-chain AA, Glutamic acid (E), while the 38^{th} AA of MS type B was nonpolar side-chain AA, Glycine (G). In addition to the higher invasiveness of the longer PRR region, the folding of N-terminal fragment of MSPB; resulting from alignment of polar and non-polar side chain amino acids; may also be the virulence factor affecting the adhesive property of MS.

Moreover, re-culture MS isolates; AHRU2015CU3502.2, AHRU2015CU3505.2, and AHRU2015CU2807.2; presented the molecular characteristic details as same as their original isolates especially in nucleotide and amino acid sequences, PRR typing groups, and nucleotide polymorphism of RIII region. Although the Thai MS field isolate AHRU2014CU5801.2 and the MS-H vaccine strain were the same subtype C1; however, their 39^{th}-42^{nd} amino acid sequences were different; AA of AHRU2014CU5801.2 and vaccine strain were NGNP and TDNS, respectively. Thereby, the consistence molecular details of *vlhA* gene and the different of amino acid sequences in the same size of PRR region; which has been found in MS of many countries including UK, USA, Slovenia, France, and Hungary (Bencina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Bayatzadeh et al., 2014).; could ensure the genotyping potential of MS *vlhA* gene in epidemiological studies.

Interestingly, almost farms in this study were found a single type of MS isolate except one farm, which showed co-infection of types E and L. Based on the questionable disease transmission model, the co-infection of MS strains in this study may be represented in the multiple-strains infection in the same farm. Because several chicken farms in Thailand have been vaccinated with live MS vaccine, the sequence

analysis of partial *vlhA* gene; therefore, was able to differentiate the Thai MS field isolates from the vaccine strain, which has been more useful in MS control strategies (Hammond et al., 2009). Moreover, further studies on the efficacy of MS live vaccine in a transmission model should be performed consecutively as same as the effect of vaccination on the transmission and displacement ability of *Mycoplasma gallisepticum* in the experimental study and/or field situations.

The prevalence of MS infection has been increasing in many countries, including Thailand. Diagnostic methods for strain 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. The sequencing of partial *vlhA* gene could be used as a tool for tracing MS characterization and strain differentiation between Thai field and vaccine strains. Although the usefulness of the *vlhA* gene was demonstrated in this study, the sequencing of *vlhA* gene was considered the time consuming method for strain typing and epidemiological studies (Jeffery et al., 2007; Hammond et al., 2009). However, the use of live MS vaccine has been dramatically increasing in several countries. Therefore, the development of the convenient procedure, including PCR-RFLP procedure to differentiate vaccine strain from field strain will be necessary.

In conclusion, the present study provided the first molecular characterization of Thai MS isolates by the sequence analysis of partial *vlhA* gene. At least 3 types of MS could be identified in Thailand. Thai MS isolates type C and E were associated with respiratory tract infection without evidence of reproductive tract infection. Thai MS isolates type L, causing infectious synovitis and lameness, was first reported, indicating that the MS type L could be the new arthropathic strain apart from MS type B. In addition, further studies of the efficacy of MS live vaccine in transmission model should be performed together with the development of the convenient differentiation procedure.

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APPENDICES

Appendix 1: Frey's medium supplemented with 15% swine serum (FMS)

The FMS media were modified from: a Laboratory Manual for the Isolation, Identification, and Characterization of Avian Pathogens. 5th Edition (Dufour-Zavala, 2008). This media were prepared each time to use in sample collection procedure, MS field isolates culture, and MS propagation in laboratory. Fungizone is added to broth medium for potentially contaminated field samples.

Preparation

1) Set water bath at 56°C
2) Thaw 75 ml of heat inactivated swine serum
   (Swine serum has been heated previously at 56°C for 30 minutes)
3) Clean and sterile the laminar flow hood with 70% EtOH and UV light

Procedure to prepare 500 ml of broth medium

1) Add 11.25 g Frey's Broth Base to 385 ml distilled water in a sterile flask
2) Adjust pH to 8.00 with 1N NaOH
3) Autoclave at 121°C for 15 minutes
4) Allow to cool to 56°C in water bath
5) Add aseptically all followed compositions to flask:
   75 ml swine serum
   25 ml 20% Dextrose
   10 ml 1% NAD/Cysteine
   2.5 ml 5% Thallium acetate
   2.5 ml Penicillin G (200,000 IU/ml) for final conc. of 1000 IU/ml
   1.25 ml 1% phenol red
6) Aseptically add Fungizone for final concentration of 1% Fungizone
   For potentially contaminated field samples
7) Store at 4°C
8) Dispense 2 ml into sterile polystyrene snap-cap culture tubes as needed
9) Label date of preparation
10) Quality control (QC) broth medium using cultures of MG and MS

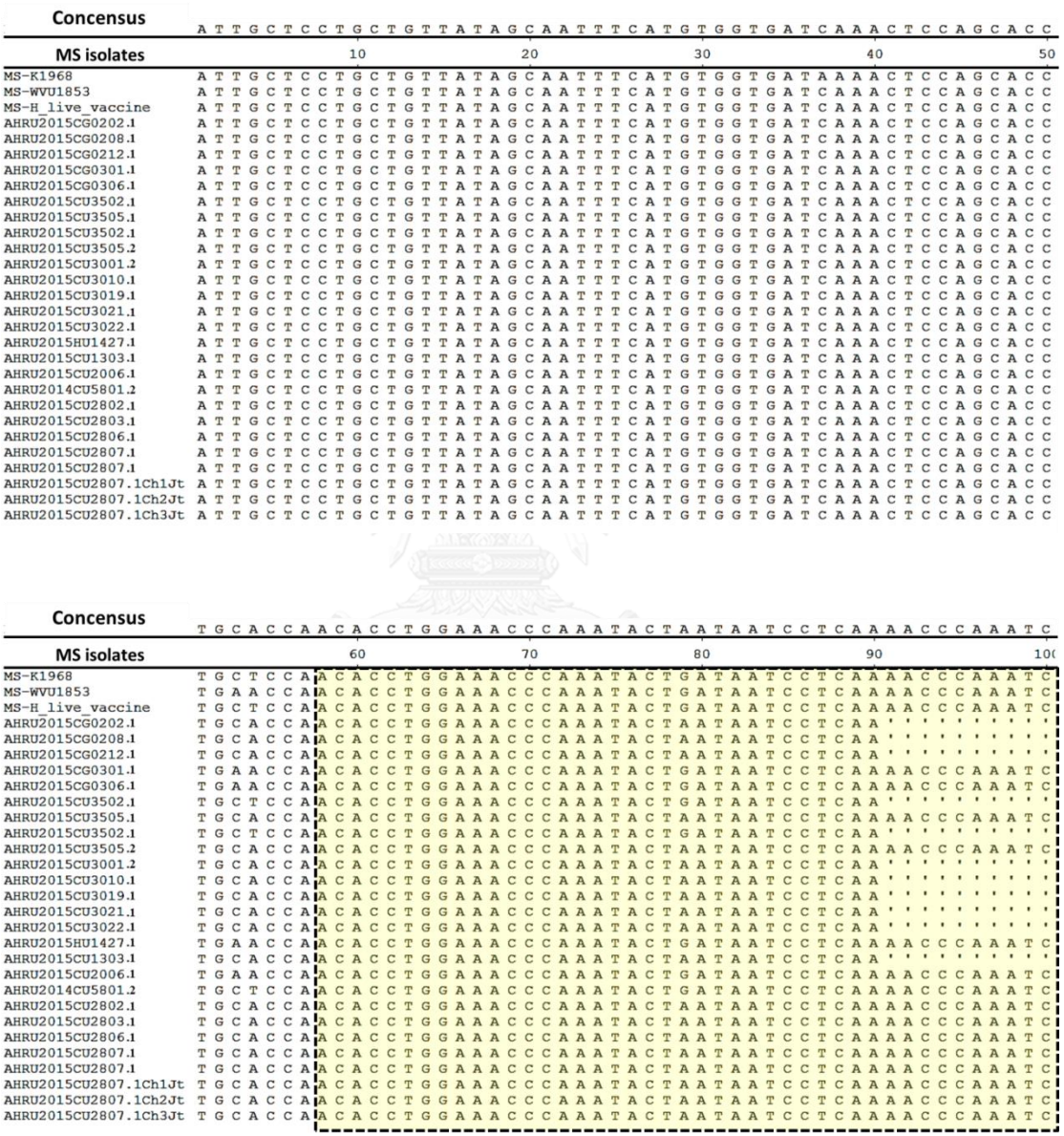
- Individual broths with each of above cultures and incubate at 37°C
- Include an uninoculated broth as a negative control

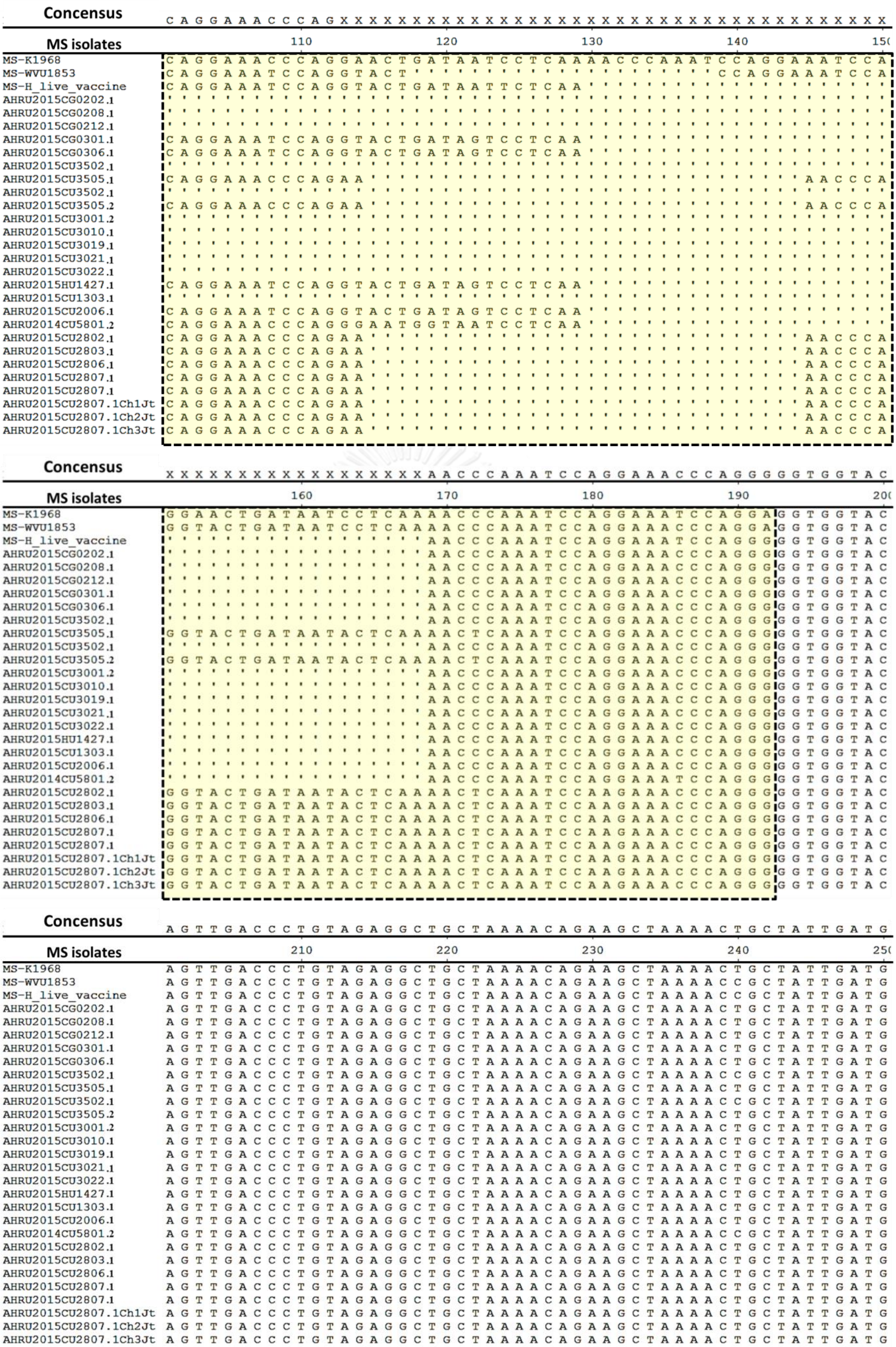
Procedure to prepare 500 ml of agar medium

1) Add 11.25 g Frey's Broth Base to 385 ml distilled water in a sterile flask
2) Adjust pH to 8.00 with 1N NaOH
3) Add 5 g Noble Agar to the above solution
   - Microwave for 5 minutes on high power, stirring occasionally
4) Autoclave at 121°C for 15 minutes
5) Allow to cool to 56°C in water bath
6) Add aseptically all followed compositions to flask:
   75 ml swine serum
   25 ml 20% Dextrose
   10 ml 1% NAD/Cysteine
   2.5 ml 5% Thallium acetate
   2.5 ml Penicillin G (200,000 IU/ml) for final conc. of 1000 IU/ml
   1.25 ml 1% phenol red
7) Aseptically add Fungizone for final concentration of 1% Fungizone
   - For potentially contaminated field samples
8) Add heated mixture to the cooled Frey's agar base in laminar flow hood
   - Mix well, avoiding bubbles or foam, and pour plates
   - Using 15 ml molten medium per 100x15 mm sterilized Petri Dish
   - If bubbles persist, flame agar with torch to remove bubbles
9) Stack solidified-agar plates in plastic bags with agar side down
10) Seal bag with tape and label tape with date of preparation
11) Store at 4°C
11) Quality control (QC) agar medium using cultures of MG and MS
   - Individual sections of agar plate and incubate at 37°C
   - Include an uninoculated agar as a negative control

Appendix 2: Complete nucleotide sequence alignment of the partial *vlhA* gene from *Mycoplasma synoviae* isolates and MS strains used in the present study.

Proline-rich-repeat (PRR) region and RIII region of N-terminal *vlhA* gene corresponds to the nucleotide sequences 58th-192nd and 316th-393rd in MS strain K1968, respectively.

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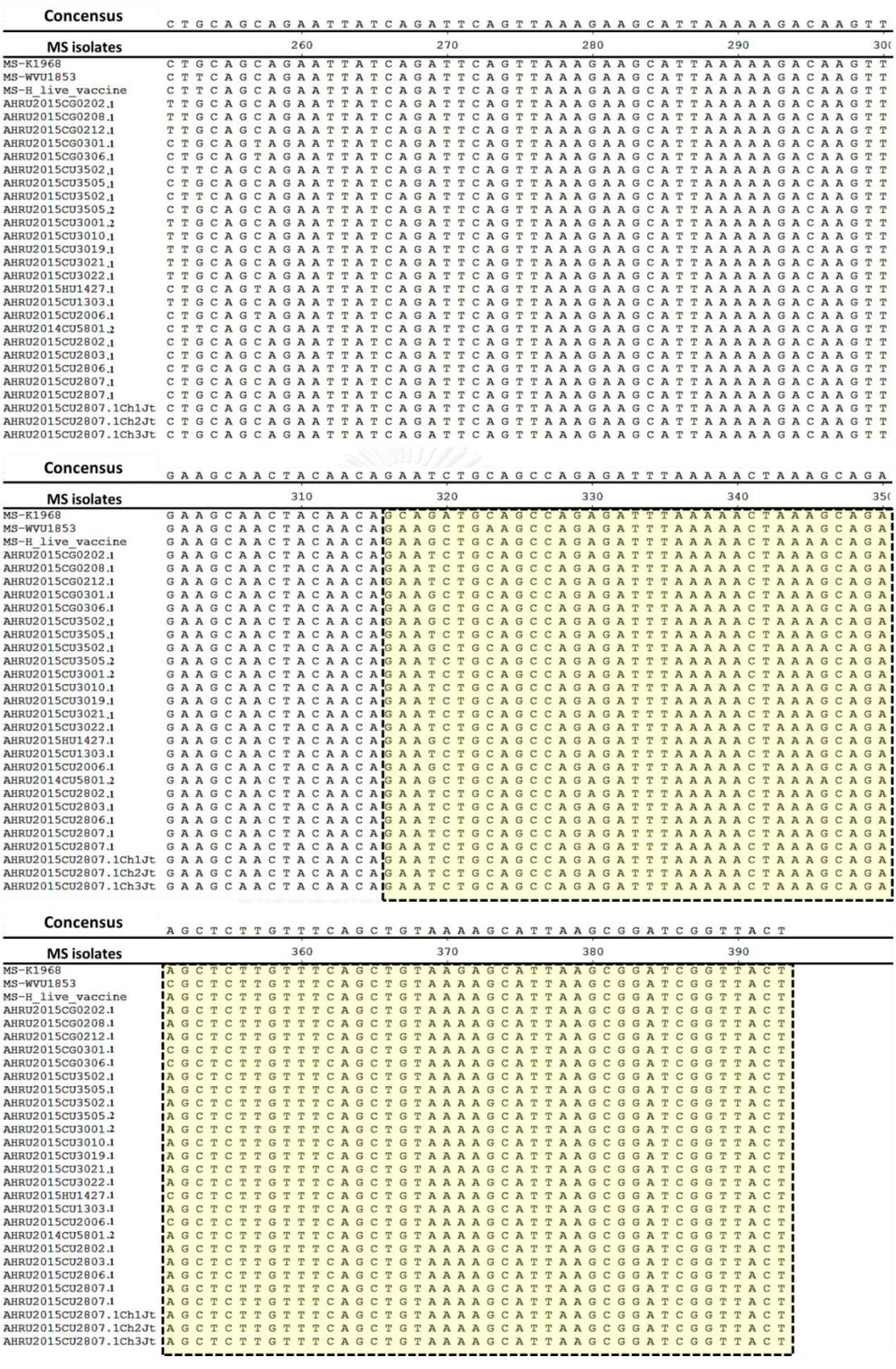
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VITA

Mr. Kriengwich Limpavithayakul was born on April 23, 1987 in Bangkok, Thailand. After finished high school from Debsirin School in 2004, he started being the veterinary student at Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. He graduated the Doctor of Veterinary Medicine (DVM) degree with First class Honor in 2010 and immediately takes his first step of veterinary job in the Poultry Business, Charoen Pokphand Foods, Thailand. He spend first year for working in Thailand before moved to the poultry veterinarian in Charoen Pokphand Foods (India) PVT. LTD. for 2 years. Then, he came back to Thailand in 2013 for studying in graduated program of Master's degree in Veterinary Medicine department, Faculty of Veterinary Science, Chulalongkorn University. In addition, he also is the veterinarian in the Poultry Veterinary Service department of Poultry Business, CPF (Thailand).