ลักษณะทางพันธุกรรมและความไวรับต่อยาต้านจุลชีพของเชื้อมัยโคพลาสมาที่แยกได้จากสุกรในประเทศไทย

นางสาวเมตตา เมฆานนท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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GENETIC CHARACTERISTICS AND ANTIMICROBIAL SUSCEPTIBILITY OF PORCINE MYCOPLASMA ISOLATED IN THAILAND

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เมตตา เมฆานนท์ : ลักษณะทางพันธุกรรมและความไวรับต่อยาต้านจุลชีพของเชื้อมัยโคพลาสมาที่แยกได้จากสุกรในประเทศไทย (GENETIC CHARACTERISTICS AND ANTIMICROBIAL SUSCEPTIBILITY OF PORCINE MYCOPLASMA ISOLATED IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.น.สพ.ดร.ณุวีร์ ประภัสระกูล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.น.สพ.ดร.รุ่งโรจน์ ธนาวงษ์นุเวช, รศ.น.สพ.ดร.เผด็จ ธรรมารักษ์, 131 หน้า

มัยโคพลาสมาในสุกรเป็นโรคที่ก่อปัญหาทั้งทางทางเดินหายใจและข้ออักเสบในสุกรหลายอายุ โรคนี้สามารถลดผลผลิตของสัตว์จึงมีผลกระทบทางเศรษฐกิจในระบบการเลี้ยงสุกร เนื่องจากความซับซ้อนของการวินิจฉัยโรคทำให้การศึกษาเรื่องของมัยโคพลาสมาในประเทศไทยมีความจำกัด การศึกษาในครั้งนี้จึงมีประโยชน์ในการให้ข้อมูลที่จำเป็นและแนวทางในการตรวจวิเคราะห์ ความชุก ระบาดวิทยา การจัดการและการรักษา เพื่อลดปัญหาจากโรคติดเชื้อมัยโคพลาสมาในระบบอุตสาหกรรมการเลี้ยงสุกรไทย

เชื้อมัยโคพลาสมาที่พบในสุกรอนุบาลและสุกรขุนที่โรงฆ่า ได้แก่ *M. hyopneumoniae* (15.6%ในปอด), *M. hyosynoviae* (7.9%ในทอนซิล), และ *M. hyorhnis* (61.1%ในจมูก, 18.2% ในปอดและ 63.5%ในทอนซิล) ลักษณะทางพันธุกรรมของมัยโคพลาสมาพบมีความแตกต่างกัน 11- 20 แบบ โดยพบว่าสายพันธุ์ที่ดื้อต่อยาต้านจุลชีพซึ่งแยกจากคนละฟาร์มและจากสัตว์ป่วยจะมีความแตกต่างกันมากขึ้น เชื้อ *M. hyorhinis* เป็นชนิดที่มีความแตกต่างทางพันธุกรรมสูงที่สุด และมีค่าความไวรับต่อยาต้านจุลชีพในระดับที่สูงเมื่อเทียบกับเชื้อมัยโคพลาสมาอื่น โดยพบมีอัตราการดื้อต่อ tylosin, enrofloxacin และ lincomycin ที่ 48.8%, 41.8% และ 6.5% ตามลำดับ เชื้อ *M. hyopneumoniae* มีอัตราการดื้อต่อ enrofloxacin ที่ 34.6% และ *M. hyosynoviae* มีอัตราการต่อ doxycycline และ tylosin ที่ 46.2% และ 7.7% ไม่พบการดื้อต่อยา tiamulin และ valnemulin การทำวัคซีนมีผลในการควบคุม *M. hyopneumoniae* ดีกว่าการใช้ยาซึ่งจะให้ผลดีต่อ *M. hyosynoviae* และ *M. hyorhinis* ปัจจัยเสี่ยงที่มีผลต่อการลดปริมาณมัยโคพลาสมาได้แก่ การเลี้ยงสุกรแยกฟาร์ม ระบบเข้าหมดออกหมด วิธีการปรับสภาพสุกรทดแทน และการแทรกซ้อนจากไวรัสอื่นๆ จากความแตกต่างทางพันธุกรรม ความไวรับต่อยาและปัจจัยเสี่ยงต่างๆ การวินิจฉัยโรคที่แม่นยำจึงมีความสำคัญต่อการควบคุมโรคติดเชื้อมัยโคพลาสมาในสุกรเช่นกัน

ภาควิชา พยาธิวิทยา ลายมือชื่อ .....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

#5075954531: MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS : MYCOPLASMA / SWINE / ANTIMICROBIAL SUSCEPTIBILITY / GENETIC CHARACTERISTICS

METTA MAKHANON : GENETIC CHARACTERISTICS AND ANTIMICROBIAL SUSCEPTIBILITY OF PORCINE MYCOPLASMAS ISOLATED IN THAILAND. ADVISOR : ASST.PROF. NUVEE PRAPASARAKUL, Ph.D, CO-ADVISOR : PROF. ROONGROJE THANAWONGNUWECH, Ph.D, ASSOC.PROF. PADET TUMMARUK, Ph.D., 131 pp.

Porcine mycoplasmosis is the importance disease caused the respiratory problems, arthritis and synovitis in several ages of pigs. The diseases suppressed the animal performance and caused the economic loss in pig industry. The complicated diagnosis limits the study of *Mycoplasma* spp. in Thailand. The advantage of this study is to provide the necessary information and guideline of diagnosis, occurrence, epidemiology, management and treatment for the reduction of porcine mycoplasmosis from Thai pig industry.

The available species and occurrence of mycoplasmas from nursery and slaughtered pigs were *M. hyopneumoniae* (15.6% from lung), *M. hyosynoviae* (7.9% from tonsil), and *M. hyorhnis* (61.1% from nasal swab, 18.2% from lung, and 63.5% from tonsil). The DNA fragment of the field strains were varied from 11 to 20 patterns while the genetic variation of the antimicrobial resistant strains was increased in the different farms and the sick pigs. *M. hyorhinis* showed the highest variation and conferred high MICs level and resistance to tylosin (48.8%), enrofloxacin (41.8%), and lincomycin (6.5%) comparing to other species. The resistance to enrofloxacin (34.6%) was detected in *M. hyopneumoniae* while *M. hyosynoviae* was resisted to doxycycline (46.2%) and tylosin (7.7%). There was no resistance to tiamulin and valnemulin. *M. hyopneumoniae* vaccination was more effective than medication for *M. hyopneumoniae* control while medication were effective for *M. hyosynoviae* and *M. hyorhinis*. The risk factors to the occurrence of porcine mycoplasmas were included the multiple sites, all-in-all-out in fattening, gilt acclimatization procedure, and other viral diseases. Because there were variation of genetic, antimicrobial susceptibility, and risk factors of the difference species, the accurate diagnosis was also important for porcine mycoplasmosis control.

Department : Pathology Student's Signature .....

Field of Study : Pathobiology Advisor's Signature .....

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LIST OF ABBREVIATIONS

ADG	=	Average daily gain
APP	=	*Actinobacillus pleuropneumoniae*
BHL	=	Brucella broth-Hank's-Lactalbumin
DNA	=	Deoxyrhibonucleic acid
FCR	=	Feed conversion ratio
HAM	=	Hayflick's enriched with arginine and mucin
µg	=	Microgram
MIC	=	Minimum inhibitory concentration
µl	=	Microliter
OR	=	Odd ratio
PCV2	=	Porcine circovirus type2
PFGE	=	Pulsed-field gel electrophoresis
PRDC	=	Porcine respiratory disease complex
PRRSV	=	Porcine reproductive and respiratory virus
RNA	=	Ribonucleic acid

CHAPTER I

1.1 INTRODUCTION

The Genus *Mycoplasma* is the primary pathogen of enzootic pneumonia in pigs. It consists of *Mycoplasma hyopneumoniae, M. hyorhinis, M. hyosynoviae*, *M. flocculare,* and *M. suis* (Hoelzle et al., 2011). These mycoplasmas cause endemic infections, either porcine respiratory disease complex (Calsamiglia et al., 1999) or arthritis. The term PRDC is used to describe the interaction of respiratory pathogens such as bacteria and viruses at the site of pneumonic lesions (Palzer et al., 2008).

M. hyopneumonia and *M. hyorhinis* are closely associated with the appearance of Porcine Reproductive and Respiratory Syndrome Virus (Cho et al., 2006), porcine circovirus type 2 (Dorr et al., 2007a). *P. multocida, B. bronchiseptica,* and alpha-haemolytic streptococci. A combination of *M. hyopneumoniae* with other bacterial infections correlates significantly with respiratory clinical signs and lung scores. *M. hyopneumoniae* can be detected in the upper and lower respiratory tract of pigs as early as 1- 3 weeks of age and results in coughing piglets (Sibila et al., 2007a) . *M. hyopneumoniae* may start relatively early in the production system, as a result of vertical (sow-to-piglet) and horizontal (piglet-to-piglet) transmission in farrowing barns (Sibila et al., 2007a). *M. hyorhinis* infection causes swine pneumonia, arthritis, polyserositis, pleuritis and peritonitis in young pigs. It is also reported as the primary pathogen of porcine otitis media (Friis, 1974; Kobayashi et al., 1996a; Lin et al., 2006; Morita et al., 1995). In a study examining arthritic joints of pigs at slaughter, in some cases, *M. hyorhinis* was the only isolate found (Hariharan et al., 1992). *M. hyorhinis* accelerates a severity of clinical Influenza A virus and PCV2 (Kixmoller et al., 2008;Palzer et al., 2007). *M. hyosynoviae* is the primary cause of acute and severe lameness with macroscopic osteochondrotic lesions in grower-finisher pigs accounting for at least 47% of pigs presenting with lameness.

As a result of their pathogenicity and ability to cause these respiratory and athritic diseases, porcine mycoplasmosis have a significant impact on the economic performance of pig production not least of all the additional budget required for disease prevention and control (Escobar et al., 2002; Hogg and Ross, 1986; Nielsen et al., 2001).

Mycoplasmas appear to be a common cause of porcine pneumonia during late stage of fattening to slaughter pigs. For identification and epidemiological monitoring purposes, mycoplasmal culture and polymerase chain reaction (PCR) are routinely used to identify disease in herds. By contrast serological detection has no significant, direct correlation to current disease and production losses in herds (Abele-Horn et al., 1998; Martinez et al., 2009). Therefore epidemiological and laboratory results, in combination with observed clinical signs, are often used to identify the appropriate disease control measures. Additionally, looking at geographical disease patterns may help when trying to establish a suitable national control program (Stark et al., 2007). In Germany, a survey of *M. hyopneumoniae* in 50 farms showed a high prevalence of infection which was closely associated with single site-production and inappropriate gilt acclimatization (Moorkamp et al., 2009). Mycoplasmas are very capable of antigenic variation especially in response to the alteration of host environment including medication, vaccination, and co-infection. *Mycoplasma* spp., strains with different genetic characteristics have been described by several studies in humans and animals (Kokotovic et al., 2002a; Kokotovic et al., 2002b; Lauritsen et al., 2008; Le Carrou et al., 2006; Razin, 2006; Schaeverbeke et al., 1998; Stakenborg et al., 2005b).

Strategic medication with antimicrobials against *M. hyopneumoniae* and major secondary bacteria is routinely useful for disease prevention. In agreement with the recommendation of International Research Programme on Comparative Mycoplasmology (Hannan, 2000), minimal inhibitory concentration should be used for mycoplasmal susceptibility test in each pig industrial area. To date, an increase of

resistant porcine mycoplasmas has been observed in several countries especially against beta-lactam, tetracycline, fluoroquinolone and macrolide antimicrobials (Bousquet et al., 1997; Hannan et al., 1997; Stakenborg et al., 2005a; Vicca et al., 2007; Vicca et al., 2004).

A study of Thai isolates of porcine mycoplasmas, defining their genetic characteristics, prevalence and antimicrobial susceptibility as well as identifying the key risk factors associated with farm management practices would be highly advantageous for the Thai pig production industry.

1.2 OBJECTIVES

The objective of this study are:

1. To investigate the diagnostic techniques and the prevalence of porcine mycoplasmas isolated in Thailand.

2. To evaluate the relationship between the presence of porcine mycoplasmas and farm risk factors.

3. To determine the antimicrobial susceptibility of porcine mycoplasmas.

4. To characterize the genetic variation of porcine mycoplasmas using pulsed-field gel electrophoresis and to determine any relationship between this and pig age, scores for clinical signs, and/or antimicrobial susceptibility.

The questions the study aims to address are:

1. How does the result of diagnosis by PCR compare with the 'Gold Standard' diagnosis of porcine mycoplasmas by culture?

2. What were the key risk factors related to the presence of porcine mycoplasmas in the nine farms selected for the study?
3. Are there any differences in the degree of antimicrobial susceptibility among porcine mycoplasmas?
4. Are there any diversities of PFGE patterns of porcine mycoplasmas that can be related to pig age, clinical signs, and antimicrobial susceptibility?

The conceptual framework for this study was as follows:

1. Diagnose and isolate *Mycoplasma* spp., from various organs obtained from pigs on nine Thai pig farms using two diagnosistic methods - culture prior to PCR (CPP) and direct PCR (DP).
2. Explore the risk factors associated with the presence of porcine mycoplasmas in nine Thai pig farms using questionnaires to evaluate the relationship between management risks and mycoplasmal isolation (by culture method).
3. Conduct a susceptibility study of *Mycoplasma* spp., isolates and compare the MICs of the different species of these mycoplasmas isolated from pigs of different age groups.
4. Determine the genetic characteristics (by PFGE) of mycoplasma isolates (with known MIC values) and to investigate the relation of their DNA fragment to the antibiogram (the profile of antibiotic resistance), pig age and farm location.

1.3 LITERATURE REVIEW

The pig industry is continuously expanding in the Asia-Pacific region which now accounts for 55% of the global pig production (Cameron, 2000). Since 1990, the percentage of intensive farm systems has been on the rise resulting in fewer, larger production systems accounting for a majority of pigs and pig meat at slaughter. In

Thailand these large systems make up 80% of the pig production market and 56% of these farms contain over 1,000 sows (Cameron, 2000). In the current industrial production system, pigs are grouped according to different production phases. Weanlings are grouped at three to four weeks of age and remain in nursery for five to six weeks after which they enter the fattening period between10 to 25 weeks. Farm management practices vary depending on the herd status, environment, biological vectors and disease distribution patterns. Any and all of these may influence the infectivity of pathogens in the farm (Cameron, 2000).

1. Porcine Mycoplasmosis

Mycoplasmas are classified in class *Mollicutes*, family *Mycoplasmataciae.* Mycoplasmas are the smallest self-replicating organisms and they lack a cell wall (Friis, 1974). *Mycoplasma* spp*.,* possess limited biosynthetic capabilities (Friis, 1974;Rosenbusch, 1994) as they are unable to metabolise amino acids or lipids nor can they carry out the biosynthesis of cofactors. As a result:

1. Because mycoplasmas lack the genes that confer amino acid and cofactor biosynthesis they require all essential amino acid and vitamin supplements to be provided by the host.

2. Because mycoplasmas lack a cell wall they are more sensitive to the osmotic change. This means they need to remain in a stable host cell environment.

3. Because mycoplsmas lack periplasma space essential proteins can leak out from their cells (Razin, 2006). Therefore mycoplasmas stabilize their proteins by binding with long acyl chains forming lipoproteins. These lipoproteins are responsible for the ability of mycoplasmas to adhere to host cells as well as evade the host immune system

by nature of their antigenic diversity (Momynaliev and Govorun, 2001; Razin, 2006; Razin et al., 1998).

The mycoplasmal chromosome is a dynamic component capable of recombination in response to biological and chemical changes in the environment changes such as antimicrobial treatment, co-infection and vaccination. This dynamic chromosome gives mycoplasma an ability for antigenic variation (Lauritsen et al., 2008; Le Carrou et al., 2006). The diversity of mycoplasmal genomic characteristics has been reported by several studies including the DNA fingerprint pattern of *M. hyopneumoniae* and *M. hyosynoviae* isolates from the different herds. The diversity of *Mycoplasma* spp., can be demonstrated by the amplified fragment length polymorphism analysis, pulsed-field gel electrophoresis (Arcangioli et al., 2011) and 16s ribosomal DNA sequencing. There is evidence to indicate the variability of *M. hyopneomoniae* proteins isolated from several countries and farms as well as the genomic diversity of *M. hyosynoviae* isolates from a number of different farms (Kokotovic et al., 2002b). In addition, different strains of *M. hyosynoviae* have also been found in pigs from the same herd (Kokotovic et al., 2002b).

2. Epidemiology of porcine mycoplasmosis

M. hyopneumoniae, M. hyorhinis, M. flocculare, M. suis and *M. hyosynoviae* have been reported in pigs from several countries (Calus et al., 2007; Kawashima et al., 1996; Kokotovic et al., 2002b; Stakenborg et al., 2005b; Ter Laak et al., 1991; Vicca et al., 2004). Porcine mycoplasmosis clearly associates with Porcine Respiratory Disease Complex (Calsamiglia et al., 1999) and is accelerated by PRRSV infection. *M. hyopneumoniae* locates mainly in bronchial and bronchiolar epithelial cells at 7 to 28 days post experimental inoculation. The attachment and disruption of *M. hyopneumoniae* to the airway epithelium increases the secondary infection of both the

virus and the bacteria (Kwon et al., 2002; Thacker et al., 1999). Therefore, concurrent infection of *M. hyopneumoniae*, *M. hyorhinis*, *APP*, *Haemophilus parasuis*, alpha-haemolytic streptococci, *Pasteurella multocida* and PRRSV is a common cause of PRDC (Kawashima et al., 1996; Thacker et al., 1999). Moreover, the high prevalence of *M. hyopneumoniae* in weanling piglets can be an indicator for predicting the disease severity in grower pigs (Fano et al., 2007).

3. Mode of transmission

Healthy or subclinical pigs may be the mycoplasma carriers with the organisms hiding in the tonsils and lymph nodes. These carriers can be introduced to the herd as replacement breeding stock or as outsourced piglets for fattening and are capable of carrying the pathogen for up to 185 days after exposure. Direct contact and airborne transmission are the methods of mycoplasma transmission (Sibila et al., 2009). Direct contact is a common route of transmission in the nursery, fattening, and during acclimatization of replacement gilts in breeder units. Contact transmission is also frequent between persistently infected sows and their offspring during lactation. The contamination of inhaled droplets from sow nostrils is distributed into piglets via the respiratory route. During acclimatization young sows may adopt *Mycoplasma* spp., from high parity sows with a persistent infection, this may also occur later when living in the same breeder house. By contrast, in a different study, it was shown that *M. hyopneumoniae* infected weanling piglets can be a source of enzootic pneumonia in older grower pigs (Fano et al., 2005; Fano et al., 2007; Sibila et al., 2009).

Airborne transmission is considered a common route of transmission over distance from pen to pen, shed to shed and farm to farm. Seroconversion is used as a means of screening new pigs exposed in an endemic area to confirm mycoplasma detection. This should be within 42 days of exposure. In contrast, formites and worker

transmission are uncommon routes when good biosecurity and hygiene, such as changing the clothes and showering before and after visiting farms, are deployed as part of routine farm management practice (Batista et al., 2004; Sibila et al., 2009).

4. Pathogenesis of mycoplasmosis

After *M. hyopneumoniae* reaches the respiratory tract, the pathogen colonizes and adheres to the ciliated epithelium of respiratory tract by its adhesion proteins. The adhesion of *M. hyopneumoniae* is established by means of the host specificity of glycolipid receptors. Binding with *M. hyopneumoniae* adhesins, such as P97, causes the changes and damage to the cilia alteration including tangling, clumping and longitudinal splitting (Djordjevic et al., 2004; Zielinski and Ross, 1993). *M. hyosynoviae* and *M. hyorhinis* also act as pathogens associated with arthritis in wide age range of pigs. Nursery pigs (3-10 weeks old) presenting with pneumonia, pleuritis and peritonitis lesions have been shown to be infected with *M. hyorhinis* (Friis, 1974; Lin et al., 2006; Morita et al., 1995). *M. hyosynoviae* is disseminated systemically and as a result, can be isolated from blood, joint, and internal organs including tonsil, lymph nodes, spleen, lung and kidney. The systemic spread of *M. hyosynoviae* and its ability to result in arthritic disease appears dependent on several factors including age, immunity, virulenance factor, infection pressure, and other 'triggers' such as stress (Hagedorn-Olsen et al., 1999b; Ross and Karmon, 1970). Initially *M. hyosynoviae* was shown to be localized in the tonsils of carriers and this tonsilar infection presented several weeks before clinical arthritis ocurred in the herd. Polyserositis and polyarthritis are commonly associated with *M. hyosynoviae*. *M. hyosynoviae* infection These infections tend to lead to arthritis in older fattening and breeder pigs (Hagedorn-Olsen et al., 1999b; Magnusson et al., 1998; Sokoloff, 1973). In Denmark, *M. hyosynoviae* is a common pathogen causing acute and severe lameness in grower-finisher pigs. In addition, *M.*

hyosynoviae has been isolated from the nasal secretion of piglets and lame piglets (6 weeks old) (Lauritsen et al., 2008).

5. Mycoplasma identification

The culture method is the 'Gold Standard' for *Mycoplasma* spp., identification. It is important to note that to grow mycoplasma on agar enrichment media is required as well as specific environmental conditions e.g. a 5% CO_2, There are some differences in biological traits between *M. hyosynoviae* and other mycoplasmas that may be used to help differentiate these species. For example, based on metabolic cascade, *M. hyosynoviae* utilizes arginine while *M. hyorhinis* utilizes glucose but in the case of *M. hyopneumoniae* such differentiation is not so clear (Assuncao et al., 2005). According to arginine hydrolysis, *M. hyosynoviae* produces ammonia while the glucose fermented mycoplasmas will produce acid. The color change of specific indicator can indicate the growth of organisms. *M. hyosynoviae* colonies are not only the shape of a fried egg but are also characterized by giving a film and spots (Ross and Karmon, 1970) (Figure 1).

6. The control of mycoplasmosis

6.1 Vaccination

There are several bacterin *Mycoplasma hyopneumoniae* vaccines used in pig farms. The benefit of *M. hyopneumoniae* vaccination is that it has been shown to improve production (2-8% ADG and 2-5% FCR) as well as reduce clinical signs and lung lesions. Unfortunately, vaccination does not prevent colonization of *M. hyopneumoniae* (Gutierrez-Martin et al., 2006; Haesebrouck et al., 2004). The attempt to initiate more effective vaccines such as the recombinant or DNA vaccines is still under

way (Razin, 2006). Some studies have investigated the impact of applying one or two shots of *M. hyopneumoniae* vaccine to sows and piglets (including sow vaccination, post-farrowing) on the severity of lung lesions at slaughter (Sibila et al., 2008; Sibila et al., 2007b). The antigenic and genetic variation of field strains is well documented and this is the probable cause for unsatisfactory vaccine protection in different geographical regions (Haesebrouck et al., 2004; Stakenborg et al., 2005b).

6.2 Antimicrobials

Antimicrobial medication is an alternative tool for infection prevention and control in the animal production industry. However there is a trend towards reducing the use of antimicrobials in animal production – this is clearly reflected in the increasing number of European Directives relating to such use. Much of this has been in relation to the use of low doses of antimicrobials for growth promotion but those dose levels are significantly lower than the levels used for feed medication in disease treatment and prevention (Burch, 2005). Nevertheless antimicrobial resistance among bacteria isolated from livestock is on the increase and there are not many new antimicrobial break through on the horizon. The antimycoplasmal drugs of choice are pleuromutilins (tiamulin and valnemulin), tetracyclines (chlortetracycline and oxytetracycline), macrolides (Ayling et al., 2000), lincosamides (Aarestrup and Friis, 1998), and fluoroquinolones (Aarestrup and Friis, 1998; Burch, 2005; Maes et al., 2008; Stipkovits et al., 2001).

6.2.1 Antimicrobial resistance mechanisms

Mycoplasmas develop antimicrobial resistance by gene mutation, acquisition of a resistant gene and active efflux mechanism (Razin, 2006). The resistance of *M. hyorhinis* to tylosin and lincomycin can be detected after three passages and six

passages of antimicrobial exposes, respectively. The location of the mutation for tylosin resistant strains has been identified as domain V of 23s rRNA while for lincomycin resistant strains the mutation is in both the II and V domains of the 23s rRNA (Gautier-Bouchardon et al., 2002; Kobayashi et al., 2005; Wu et al., 2005). It is clearly noted that individual antimycoplasma drugs require their own specific mechanism for mycoplasmas ro acquired resistance to them (Flutt et al., 2001).

The resistance mechanism of MLS (Macrolides, Lincosamides, and Streptogramin) is mainly the alteration of the common binding site of MLS antimicrobials in 23s rRNA. The alteration of the binding site may cause the cross resistance to MLS. Other mechanisms are the efflux mechanism to pump the antimicrobials out of the bacterial cells and the enzymatic inactivation to modify MLS to be inactivated (Flutt et al., 2001). Similar to MLS, pleuromutilins (tiamulin and valnemulin) inhibit the protein synthesis of bacteria at 23s rRNA in 50s ribosome. The binding site of pleuromutilins is located on the peptidyl transferase center (Long et al., 2009). Although the resistance to pleuromutilins is rare, the experimental resistant occurs by alteration of ribosomal protein L3 and L4 of *Escherichia coli.* This may imply a lower affinity of bacterial ribosome to bind tiamulin (Bosling et al., 2003; Hunt, 2000; Schlunzen et al., 2004).

Fluoroquinolones inhibit the bacterial topoisomerase enzymes including DNA gyrase and topoisomerase IV. DNA gyrase is encoded by *gyr*A and *gyr*B. DNA topoisomerase IV is encoded by *par*C and *par*E. The resistant mechanism is commonly the alteration of chromosomal mutation conferred enzymatic function. Another mechanism is the efflux pump (Flutt et al., 2001). Tetracyclines penetrate into bacterial cells and bind to 30s ribosomal subunit RNA. This result in protein synthesis inhibition. The resistant mechanism is the efflux pump and the protection of ribosome by bacterial synthesis of a protein to inactivate tetracyclines efficacy (Flutt et al., 2001).

6.2.2 Susceptibility study

The Guidelines of Minimum Inhibitory Concentration (MIC) determination of Mycoplasmal susceptibility is proposed (Hannan, 2000;Vicca et al., 2004). The MIC breakpoints for veterinary pathogens are corresponded to the achievable level in target organs (Jones et al., 2002). According to MIC study, *M. hyopneumoniae* indicates acquired resistance to macrolides, fluoroquinolines and lincosamides whereas there is no incidence of resistance to spectinomycin, oxytetracycline, doxycycline, gentamicin, florfenicol and tiamulin (Aarestrup and Friis, 1998; Kobayashi et al., 1996a; Le Carrou et al., 2006; Vicca et al., 2004).

M. hyorhinis is evidently resistant to macrolides and lincosamides (Kobayashi et al., 1996a; Kobayashi et al., 1996b). Fluoroquinolones are less effective to *M. hyorhinis* than *M. hyopneumoniae* (Ter Laak et al., 1991). Tiamulin shows the highest efficacy to *M. hyosynoviae* when compared with those of enrofloxacin, lincomycin and tetracycline. Tylosin shows highly resistant to *M. hyosynoviae* (Aarestrup and Friis, 1998). Tylosin resistant *M. hyosynoviae* has no co-resistance to lincomycin while there is a cross resistance of *M. hyorhinis* to josamycin, spiramycin and kitasamycin to lincomycin in Japanese herds (Aarestrup and Friis, 1998; Kobayashi et al., 2005; Vicca et al., 2004).

6.3 Herd management

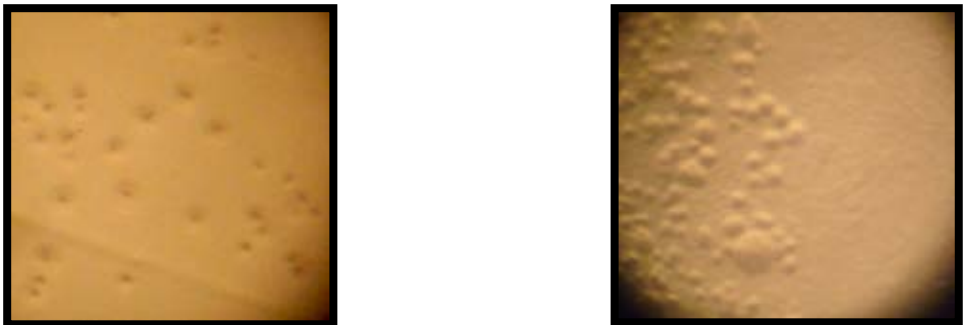
Risk factors of porcine mycoplasmas associated with overall management factors have been reported in several area comprising of medication and vaccination program, housing, age grouping, herd size, stock density, biosecurity, source of replacement gilts and piglets, and other endemic pathogens such as PRRSV or PCV2. These factors directly associated with the incidence of *Mycoplasma* spp. among differences area and management system. In addition, susceptibility of detection

methods including microbiological assay, molecular assay, clinical signs and lung scores will verify the incident rate (Bargen, 2004; Fraile et al., 2010; Maes et al., 2008; Sibila et al., 2004).

7. Genetic characteristics

Mycoplasmas are the perfect parasite which developed themselves to stay with host, successfully. They use the antigenic variation to be the key mechanism for their parasitic living. Therefore, the antimicrobial treatment is also challenged by mycoplasmal adaptation, depended on the types of antimicrobials and strains of *Mycoplasma* spp., especially, in the intensive use of antibiotic and intensive farming area (Elbers et al., 2006; Hege et al., 2002; Oakeshott et al., 2004). The diversity of mycoplasmal genetic characteristics can occur between different herds with different management and disease control program (Calus et al., 2007; Kokotovic et al., 2002a; Kokotovic et al., 2002b; Stakenborg et al., 2005b). However, the geographic difference causes the lower diversity of clones than the local outbreaks of *M. hyopneumoniae* in herds (Mayor et al., 2007).

Pulsed-field gel electrophoresis (PFGE) is an effective tool for revealing of genome fingerprinting. Diversity of mycoplasmal strains can be monitored by using identical genomic pattern in any area of study. The method clearly reflects the intraspecies genomic variations among *Mycoplasma* spp. (Kokotovic et al., 2002a). The application of PFGE provides a high discrimination and a good reproductivity for typing of *Mycoplasma* spp. that is a tool for epidemiological surveillance of *Mycoplasma* spp. (Razin, 2006; Stakenborg et al., 2005b).



A. *M. hyorhinis* colonies B. *M.hyosynoviae* colonies

Figure 1.1 Mycoplasmal colonies observed by spectroscope. A. shows the fried-egg appearance on Modified Hayflick's agar; B shows film and spot character on Hayflick's+Arginine+Mucin (HAM) agar.

CHAPTER II

Comparison of detection procedures of *Mycoplasma hyopneumoniae*, *Mycoplasma hyosynoviae*, and *Mycoplasma hyorhinis* in lungs, tonsils, and synovial fluid of slaughtered pigs and their distribution in Thailand

2.1 Introduction

Porcine mycoplasmas are the causative agent of enzootic pneumonia and polyserositis (Kobisch and Friis, 1996). Several studies have found a correlation between conventional culture and PCR methods for mycoplasmal detection from sputum, nasal flushing, biopsy samples, and ear and throat swabs in animals including poultry, goats, cats and humans (Amores et al., 2010; Dorigo-Zetsma et al., 1999; El Sayed Zaki et al., 2009; Kahya et al., 2010). Although PCR is more sensitive, several studies have relied on combining both methods for diagnosis. Since mycoplasmal culture, the gold standard, is quite time-consuming, PCR has become a preferred rapid method for mycoplasmal detection. Previously, single PCRs (sPCR), nested PCRs, and real-time PCRs (rPCR) have been recommended for mycoplasmal diagnosis, together with the traditional culture method (Caron et al., 2000; Fano et al., 2007; Lin et al., 2006). The inconsistency of the results derived from each method implies variation in suitability of sampling methods and tissues of choice - such as lungs, tonsils or synovial fluids (Fablet et al., 2010). In order to prevent false positive/negative results of field cases when using PCR, there is a need to evaluate the PCR method in comparison with culture-based method using different tissues (Mattsson et al., 1995; Stakenborg et al., 2006).

This study aimed to determine the samples of choice among lungs, tonsils and synovial fluids for directed polymerase chain reaction (DP) and culture prior to PCR

(CPP) for detection of mycoplasmas from slaughtered pigs including *Mycoplasma hyopneumoniae*, *Mycoplasma hyosynoviae* and *Mycoplasma hyorhinis*. The relationship between the presence of lesions and mycoplasmal occurrence in each tested organ also was evaluated.

2.2 Materials and Methods

2.2.1 Sample collection

A total of 724 samples comprising 270 lungs, 266 tonsils and 188 synovial fluid samples were collected from 270 slaughtered pigs at nine abattoirs in areas of intensive pig farming in Thailand, including Suphanburi, Cholburi, Nakornrachasrima, Burirum, Udonthani, Chiangmai, and Lumpoon provinces. Lung scoring was graded according to a previous study (Ostanello et al., 2007). The cranioventral parts of lung were randomly collected during lung scoring. Prior to synovial fluid collection, the swelling of the joint was recorded. All samples were kept on ice and submitted to the laboratory within 24 hours of collection.

2.2.2 Sample preparation

At least 20 gram of lung and whole tonsil samples were ground and suspended in Brucella Hank's –Lactalbumin (BHL) broth medium [1% phenol red (Wako Pure Chemical industries), 0.82% (w/v) Brucella broth (Becton Dickenson), 0.28% (w/v) lactalbumin hydrolysate (Difco), 8.2% (v/v) NaCl, 2% yeast extract (Gibco), 15% (v/v) inactivated swine serum (Gibco), and 15% (v/v) horse serum (Gibco) at 7.6 pH] (modified from (Kawamura et al., 1982b). After centrifugation, the supernatant was prepared for the DP and mycoplasma culture.

Synovial fluid samples were prepared in Hayflick's+Arginine+Mucin (HAM) broth (modified from (Friis, 1972)), comprising 0.5% phenol red (Wako, Japan), 2% (w/v) PPLO broth (Becton), 0.085% (w/v) yeast extract (Becton), 0.084 % (w/v) L-arginine (Sigma), 2.8% (w/v) mucin (Sigma), 15% (v/v) inactivated swine serum (Gibco), 2% yeast extract (NIAH), 15% (v/v) horse serum (Gibco), 200 unit/ml penicillin G (M&H Manufacturing), and 0.01% thallium acetate (Merck) at 7.6 pH. The samples were also placed in 10 ml modified Hayflict's broth for MHR culture [2% PPLO broth (Becton), 0.0002% phenol red (Wako Pure Chemical industries) and 20% horse serum (Gibco); 3.3% Yeast extract (NIAH), 0.2% DNA (Sigma), 1.2 ml; penicillin G (200.000 unit) (M&H Manufacturing), 0.5 ml; 0.026% thallium acetate (Merck), 1.3% glucose (Merck), at 7.6 pH (Friis, 1972;Masover et al., 1975). Sample suspension in BHL, HAM and modified Hayflick's were used in the DP and culture procedures.

2.2.3 Culture Methods

Briefly, the sample suspensions were filtered through a 0.45 μm membrane filter (Minisart, Sartorius Stedim Biotech), and were inoculated into the appropriate media broth: Friis's or BHL, HAM, and modified Hayflick's for isolation of *M. hyopneumoniae*, *M. hyosynoviae* and *M. hyorhinis*, respectively (Friis, 1972; Friis et al., 1991; Kawamura et al., 1982; Kobayashi et al., 1996a). The suspensions were incubated at 37°C for at least 4 days with changing of indicator. Then the sample suspension were inoculated into the appropriate agar media and incubated at 37°C under 5% CO_2 humid condition in a CO_2 gas incubator (WJ-121, Hirasawa work Model) for 4 days. Mycoplasmal colonies were collected and re-inoculated into the appropriate broth media at 37°C for 4 days under 5% CO_2 humid condition in CO_2 gas incubator. The culturing process was repeated three times. At the third cultivation, pure isolates were identified as *Mycoplasma* spp. by polymerase chain reaction (PCR) (Kobayashi et al., 1996a;

Mattsson et al., 1995). Positive samples were defined by the appearance of the mycoplasmal colony with a positive PCR result. Negative samples were defined by either the lack of mycoplasmal colony for culture or lack of specific PCR bands for DP and CPP.

2.2.4 DNA extraction and polymerase chain reaction

DNA was directly extracted from the ground tissues suspension and the cultured isolates using the phenol/choroform extraction method (Bashiruddin, 1998; Caron et al., 2000).

The PCR procedure used with the purified DNA was modified from the previous methods (Kobayashi et al., 1996a; Mattsson et al., 1995). *M. hyopneumoniae* J-Strain, *M. hyosynoviae* S16, and *M. hyorhinis* BTS-7 obtained from The National Institute of Animal Health, Tsukuba, Japan, were used as the control strains. The heterogeneous species of mycoplasma were used as an internal negative control of the PCR reactions. The PCRs were run in a Px2 Thermal Cycler (Thermo Electron Coperation) and the products were analysed by 1.5% agarose gel by electrophoresis (GIBCO BRL Horizontal Gel Electrophoresis Apparatus) and staining with ethidium bromide. The oligonucleotide primers for amplifying the fragment of 16S rDNA of the three porcine mycoplasmas were listed in table 2.1.

2.2.5 Statistical analyses

Agreement analysis was performed by Cohen's Kappa method (Blackman and Koval, 2000). The relation between CPP and DP result of the same organs was analyzed by Chi-Square. Values of $P<0.05$ were defined as statistically significant. The sensitivity

and specificity of the directed PCR and the culture with PCR methods were calculated (Amores et al., 2010; Gunnarsson and Lanke, 2002). Validity variable determination between CPP and DP included positive predictive value (PPV) and negative predictive value (NPV). The true status was the result of the positive result either from the CPP or DP test, or all positive cases. The relation between lesions (lung lesions and swelling joint) and *Mycoplasma* spp. detection were analyzed by Chi-Square. Values of $P<0.05$ were defined as statistically significant.

2.3 Results

M. hyopneumoniae, *M. hyosynoviae* and *M. hyorhinis* detection from the samples are summarized in table 2.2. *M. hyopneumoniae* was detected in 15.6 - 31.5% of lungs by CPP and DP, respectively, but no significant difference was determined between both procedures. All synovial fluid and tonsils were negative to *M. hyopneumoniae* detection. Only 6.8 - 7.9% of tonsils were positive for *M. hyosynoviae* by the two methods, respectively. *M. hyorhinis* were detected in 70/188 (37.2%) of synovial fluid samples by DP whereas all were negative by CPP. In contrast, using the tonsils and lung, *M. hyorhinis* was detected by CPP apparently yielded higher positive than that of the DP. The results obtained from tonsil for *M. hyosynoviae* detection and lung for *M. hyorhinis* detection showed a significant agreement between both procedures at *kappa=0.309* and *kappa=0.189*, respectively, and significance at $P<0.05$ for the relation of the CPP as well as DP result.

Validity variable determination between CPP and DP is described in table 2.3 and 2.4. For *M. hyopneumoniae* detected in lungs, the positive samples derived from the CPP were much lower than that of DP (PPV, 42 versus 85). Comparing to the true positive result, the sensitivity (95%) CI of CPP and DP were 38.5% and 77.9%, respectively. For MHS detected from tonsils, the sensitivity of CPP is closed to that of DP

at 65.63% and 56.25%, respectively. For *M. hyorhinis* detected, positive result obtained from CPP was much higher than those of DP in lungs (PPV, 49 versus 14) and tonsils (169 versus 14) with sensitivity at 89% and 98.2%, respectively. In table 2.3, the samples positive to either CPP or DP were included as true positive and no false positive was detected in this study. Therefore the specificity and PPV was 100% for all tested organs and procedures. The negative predictive value (NPV) of *M. hyorhinis* detected from tonsils by directed PCR is much lower than that of CPP whereas the value obtained from lung was resembled.

The relation of lung and joint lesion was also statistically analyzed, described in table 2.5. Appearance of pneumonic lung was related to *M. hyopneumoniae* positive obtained from only DP ($P<0.05$). *M. hyorhinis* positive from lungs was also related with the lung lesion ($P<0.05$) by using CPP. Additionally, There was a significant relationship between joint swelling and *M. hyorhinis* detection by DP ($P<0.05$), but not for CPP of *M. hyosynoviae* and *M. hyorhinis*.

2.4 Discussion

In several previous studies, many techniques have been proposed for improvement of detection sensitivity, but the correlation of sample types was not determined. Generally, use of PCR technique could not determine dead and alive of the organism, whereas culturing can detect only living bacteria (Dussurget and Roulland-Dussoix, 1994). In this study, we used both positive and negative controls to ensure the validity of PCR technique in all reactions and the results obtained from culture could be used for confirmation of alive isolates. The detection of the 3 mycoplasmas is the first reported incidence in the country. Previously, seropositive of *M. hyopneumoniae* among slaughtered pigs were epidemiologically reported at a level of only 8-13% in Germany (Holmgren et al., 1999; Maes et al., 1999). The *M. hyopneumoniae* detected by CPP is

recommended as a gold standard diagnostic technique (Thacker, 2004). However, use of gold standard yielded lower detection rate in this study. The relation between DP and pneumonic lung could be deductible the accuracy. Hence, using lung sample for DP is more reliable for carrier and diseased pigs in slaughter. Moreover, (Moorkamp et al., 2008) revealed that the use of lung sample was more appropriate than bronchoalveolar lavage fluid BALF, whereas it became a non-reliable indicator for a nested PCR in experimental pigs (Kurth et al., 2002).

The incidence of MHS in tonsils detected from CPP and DP was proximate with statistical agreement between both procedures. However, the number of *M. hyosynoviae* persistent in tonsil did not reflect the infective level in herds that was regardless of the occurrence of joint lesions or arthritis (Nielsen et al., 2005). This might be explained by the fact that the pathogen might disappear about three weeks post-infection or during chronic stage of arthritis at slaughter (Hagedorn-Olsen et al., 1999a). Additionally, other prevalent pathogens (*Erysipelothrix rhusiopathiae, Streptococcus suis*, *Actinomyces pyogenes*, *Mycoplasma hyorhinis*, staphylococci, and *Streptococcus* spp.) are also concurrently etiologic of arthritis (Hariharan et al., 1992; Thacker, 2004) which are not included in this study.

Detection of *M. hyorhinis* from synovial joint by DP had a statistical relation of with joint swelling whereas the high incidence of cultivable *M. hyorhinis* in tonsil might represent persistent infection. Even though, use of DP determination for *M. hyorhinis* in lungs was in moderate agreement with the culture with PCR technique but CPP was still strongly recommended since the higher sensitivity value. Nevertheless, a disagreement between DP and CPP was previously reported *M. hyorhinis* in tissue of polyserositis, spleen and pericardium (Kim et al., 2010; Palzer et al., 2006). The higher yield from tonsil by CPP and the higher yield from synovial fluid by DP were determined with high sensitivity and NPV in this study. This implies that the fluid samples from the chronic stage at slaughter may be inappropriate for cultivable mycoplasmas. Therefore, the

detection of *M. hyorhinis* from synovial fluid should be detected by DP that had also clinical relevance. However, collecting synovial fluid from living animals is not practical unlike that for abattoir surveillance. Recently, *M. hyorhinis* isolation was recommended for during acute and sub-acute phase infection (Kobisch and Friis, 1996). For *M. hyorhinis* detection, the anatomical sites and stage of infection should be taken into consideration in each diagnostic tool.

In this study, the number of mycoplasmal infected pigs might be overestimated since the true positive was included from all procedures. Therefore, co-consideration of clinical lesion should be raised the valid of interpretation rather than only existence of mycoplasma. Use of DP showed the higher sensitivity and NPV to *M. hyopneumoniae* from lung and *M. hyosynoviae* from tonsils, whereas CPP showed the higher sensitivity and NPV to *M. hyorhinis*. Given the need of improved understanding of porcine mycoplasma occurrence, these results are providing chooser of the proper sample types and detection procedure for approved mycoplasmal surveillance (Assuncao et al., 2005; Lin et al., 2006; Nielsen et al., 2001; Thacker, 2004).

Table 2.1 The oligonucleotide primers for amplifying the fragment of 16SrDNA of the three porcine mycoplasmas

Species	Primers	Product size (bp)
M. hyopneumoniae	Forward 5´-GAG CCT TCA AGC TTC ACC AAG A-3´, Reverse 5´-TGT GTT AGT GAC TTT TGC CAC C-3´	649
M. hyosynoviae	Forward 5´-GCA GTT GAG GAA ATG CAA CTG-3´, Reverse 5´-TTA GCT GCG TCA GTG ATT TGG-3´	400
M. hyorhinis	Forward 5´-TAT CGC ATG ATG AGT AAT AG-3´, Reverse 5´-GCT GCG TTA GTG AAA TTA T-3´	676

Table 2.2 The incident rate of mycoplasmal positive rates derived from culture prior to PCR (CPP) and directed PCR in each tissue samples.

Samples	No.	Positive samples (%)					
		M. hyopneumoniae		*M. hyosynoviae*		*M. hyorhinis*	
		CPP*	DP	CPP	DP	CPP	DP
Lungs	270	42[a] (15.6%)	85[a] (31.5%)	0	0	49[c] (18.1%)	14[c] (5.2%)
Tonsils	266	0	0	21[b] (7.9%)	18[b] (6.8%)	169[d] (63.5%)	14[d] (5.3%)
Synovial fluid	188	0	0	0	3 (1.6%)	0	70 (37.2%)
Total	724	42 (5.8%)	85 (11.7%)	21 (2.9%)	21 (2.9%)	218 (30.1%)	98 (13.5%)

[a,b,c,d] Cohen's Kappa Value (95%CI), *P* value of chi-square significance at $P<0.05$

[a] 0.095 (-0.021-0.211) = disagreement, $P=0.084$

[b] 0.309 (0.103-0.515) = agreement, $P<0.001$

[c] 0.189 (0.047-0.331) = agreement, $P<0.001$

[d] 0.025 (-0.013-0.063) = disagreement, $P=0.269$

Table 2.3 Comparison of positive and negative number (percentages) for porcine mycoplasmas in each tissue sample between culture prior to PCR (C) and directed PCR (DP)

Mycoplasmas	Organs	No	Number of sample (%)				
			Positive[a]	C+[c],DP+[d]	C+,DP-	C- ,DP+	Negative[b]
Mycoplasma hyopneumoniae	All	724	109 (15.06)	18 (2.49)	24 (3.31)	67 (9.25)	615 (84.95)
	Lungs	270	109 (40.37)	18 (6.67)	24 (8.89)	67 (24.81)	161 (59.63)
	Tonsils	266	0	0	0	0	266 (100)
	Synovial	188	0	0	0	0	188 (100)
Mycoplasma hyosynoviae	All	724	35 (4.83)	7 (0.97)	14 (1.93)	14 (1.93)	689 (95.17)
	Lungs	270	0	0	0	0	270 (100)
	Tonsils	266	32 (12.03)	7 (2.63)	14 (5.26)	11 (4.14)	234 (87.97)
	Synovial	188	3 (1.60)	0	0	3 (1.60)	185 (98.40)
Mycoplasma hyorhinis	All	724	297 (41.02)	19 (2.62)	199 (67.00)	79 (10.91)	427 (58.98)
	Lungs	270	55 (20.37)	8 (2.96)	41 (15.19)	6 (2.22)	215 (79.63)
	Tonsils	266	172 (64.66)	11 (4.14)	158 (59.40)	3 (1.13)	94 (35.34)
	Synovial	188	70 (37.23)	0	0	70 (37.23)	118 (62.77)

[a]All positive results from either culture prior to PCR (C) or directed PCR

[b]Negative samples from C and DP

[c]Positive to C

[d]Positive to DP

Table 2.4 Validity variable values of culture prior to PCR (CPP) and directed PCR for porcine mycoplasmal detection in lungs, tonsils, and synovial fluid.

Mycoplasmas	Organs	Sensitivity (95%CI)		Specificity (95%CI)*		PPV (95%CI)**		NPV (95%CI)	
		CCP	DP	CCP	DP	CCP	DP	CCP	DP
Mycoplasma hyopneumoniae	Lungs	38.53	77.98	100	100	100	100	70.61	87.03
Mycoplasma hyosynoviae	Tonsils	65.63	56.25	100	100	100	100	95.51	94.35
Mycoplasma hyorhinis	Lungs	89.01	25.45	100	100	100	100	97.29	83.98
	Tonsils	98.26	8.14	100	100	100	100	96.91	37.3

Table 2.5 Correlation of mycoplasmal detection from culture prior to PCR (CPP) and directed PCR comparing with pneumonic lung and swelling joint pigs by chi-square .

Mycoplasmas	Procedures	No. of pneumonic presence (%) (n=270)		No. of swelling joint presence (%) (n=188)	
		-	+	-	+
Mycoplasma hyopneumoniae	CCP	10 (3.7%) [a]	32 (11.9%) [a]	0	0
	DP	16 (5.9%) [a]	69 (25.6%) [b]	0	0
Mycoplasma hyosynoviae	CCP	0	0	0	0
	DP	0	0	2 (1.1%) [a]	1(0.5%) [a]
Mycoplasma hyorhinis	CCP	9 (3.3%) [a]	40 (14.8%) [b]	0	0
	DP	2 (0.7%) [a]	12 (4.4%) [a]	58 (30.9%) [a]	12 (6.4%) [b]

[a,b] Different superscripts between column differ significantly $P< 0.05$

CHAPTER III

Risk factors analysis of porcine mycoplasma isolated from nursery and slaughtered pigs in Thailand

3.1 Introduction

Mycoplasma spp. is a bacteria without cell wall classified in genus *Mollicutes* and family *Mycoplasmataciae*. In pigs, *Mycoplasma* spp. that has been reported included *Mycoplasma hyopneumoniae*, *M. hyorhinis*, *M. flocculare*, *M. suis* and *M.hyosynoviae*. *Mycoplasma* spp. is a common bacteria causing endemic infections in the pig farming which are clinically implicated, either respiratory diseases or arthritis or both. Furthermore, porcine mycoplasmosis causes major economic losses due to an incretion of the cost concerning health managements, such as laboratory diagnosis, medical treatment, culling and man power (Escobar et al., 2002; Friis, 1974; Hogg and Ross, 1986; Nielsen et al., 2001; Rosenbusch, 1994)

Porcine mycoplasmosis is associated with porcine respiratory disease complex (PRDC) and porcine reproductive and respiratory syndrome virus (PRRSV) infection. In general, *M. hyopneumonia* is located in the respiratory tract of pigs and causes the enzootic pneumonia and often co-infected with PRRSV and resulted in PRDC. The common clinical signs of enzootic pneumonia includes coughing, laboured breathing and retarded growth. In practice, mycoplasmal pneumonia is widely and easily spread in the pig farms (Maes et al., 2008; Thacker et al., 1999). An earlier study has demonstrated that even though *Mycoplasma* spp. vaccination in pigs could reduce the clinical symptoms of the pig, no data on infective protection of vaccinated pigs compared to non-vaccinated pigs has been published (Gutierrez-Martin et al., 2006).

M. hyosynoviae and *M. hyorhinis* are classified as the invasive blood-borne infection of mycoplasmal diseases. These mycoplasmas can penetrate epithelial barriers and enter the bloodstream under the stress situation. Invasive mycoplasmas are localized in an inflamed joints leading to polyserositis and arthritis in pigs. The nursery pigs (age 3-10 weeks) are commonly infected with *M. hyorhinis* and cause polyserositis, polyarthritis, peritonitis and pleuritis in the infected animals. *M. hyosynoviae* also implicates arthritis in fattening pigs and gilts. Joint lesions are similar to *M. hyorhinis* infection. So far, no vaccine is available for these two *Mycoplasma* spp. (Browning et al., 2010; Chae, 2011; Hagedorn-Olsen et al., 1999a; Hagedorn-Olsen et al., 1999b; Magnusson et al., 1998; Ross and Duncan, 1970; Sokoloff, 1973). *M. hyosynoviae* can turn a systemic dissemination and subsequently being isolated from blood, joint, and internal organs including tonsil, lymph nodes, spleen, lung and kidney. In Denmark, *M. hyosynoviae* is a common pathogen that causes acute and severe lameness in grower-finisher pigs (Lauritsen et al., 2008). Moreover, *M. hyosynoviae* has been isolated from nasal secretion in lameness piglets at six weeks old (Sibila et al., 2009).

The carrier of *Mycoplasma* spp. in pig farm, either healthy or subclinical pigs, had the pathogen located in their tonsil. The carriers are usually replacement gilts and the nursery pig that are imported from other swine herds. In general, the pig can carry the pathogen for 185 days after exposure. Major sources of the disease transmission within the swine herds are direct contact and airborne transmission (Fano et al., 2005; Fano et al., 2007; Sibila et al., 2009). Direct contact transmission is usually occurred in nursery and fattening pigs and during the acclimatization of replacement gilts. In perspective study, *M. hyopneumoniae* infected since weaned pigs may be a cause of colonization severity of pneumonia in the fattening pigs (Bargen, 2004; Maes et al., 2008; Sibila et al., 2009).

Many risk factors of porcine mycoplasmas are associated general managements of the herds e.g. medication and vaccination, housing conditions, pig flow (all-in-all-out

versus continuous flow), multiple-sites systems, herd size, stock density, biosecurity, management of replacement gilts (gilt acclimatization), and co-infection with other pathogens e.g. porcine reproductive and respiratory syndrome virus (PRRSV), circovirus type 2 (PCV-2) and swine influenza virus (SIV). Earlier studies have demonstrated that the transmission of *Mycoplasma* spp. from sows to piglets can be diminished via the control of potential risk factors (Dorr et al., 2007b; Martinez et al., 2009; Straw et al., 1986). In Thailand, the potential risk factors for *Mycoplasma* spp. has not been comprehensively evaluated.

The present study aimed to evaluate the relation of risk factors and clinical signs to the occurrence of *M. hyopneumoniae*, *M. hyosynoviae* and *M. hyorhinis* isolates in nursery and fattening pigs from different types of tissue samples.

3.2 Materials and method

3.2.1 Selected herds

In the first part, a cross sectional study was carried out in nine commercial pig herds in the northern (A, B, C), northeastern (D, E, F), eastern (G), and western (H, I) parts of Thailand during a period from 2008 to 2010. The number of sow on production varied from 1,000 to 5,000 sows. The replacement gilts were produced within the herds using their own grandparent stock. The housing for fattening pigs were either located within the same area as the sows and piglets (so call “one-site” production system, i.e., herds A, B, D and F) or separated from the sow herd (so call “two-sites” production system, i.e., herds C, E, G and I). The sows and piglets in herds A, B, C, D, E and G were kept in a closed housing system equipped evaporative cooling system to reduce the effect of hot climate, while the sows in herds F, H and I were kept in a conventional open house system equipped with fan and water sprinkler to reduce the effect of

environmental temperature. In the second part, two herds (D and H), with the nearest and convenient location and almost similar size, were selected with their owners' permission. A longitudinal study was conducted by collecting samples from nursery and slaughtered pigs twice from February, 2009 to March, 2011. The samples were obtained from nursery pigs at 6-8 weeks old having respiratory and arthritis. The number of sows on production in herd D was 1,000 sows and in herd H was 1,300 sows. Herd D was located in the northeast part and herd H was located in west part of Thailand. *M. hyopneumoniae* vaccination was performed in both herds (table 3.1).

3.2.2 Herd health management

In general, the gilts were vaccinated against foot-and-mouth disease virus (FMD), classical swine fever virus (CSFV), ADV, and PPV between 22 and 30 weeks of age. Apart from these, vaccination in herd C, E, G, H, and I also included PRRSV modified lived vaccine. *M. hyopneumoniae* vaccination was applied in piglets at 2-3 weeks of age in herd A, C, D, E, F, G, H, and I, while in herd B, *M. hyopneumoniae* vaccination was not applied. All herds were PRRSV sero-positive herds.

3.2.3 Collection of tissue samples

In the first part, all samples were randomly collected from nursery and slaughtered pigs. Nasal swabs were collected at 30 samples per farm from 6-8 weeks old pigs and kept in transported media (Brucella broth -Hank's –Lactalbumin: BHL) at 4 $^{\circ}$C. Samples were transported to the laboratory within 24 hours. Samples from slaughtered pigs included lungs, tonsils and synovial fluids. A total of 30 samples were obtained from each organ. In the second part, tissue samples of lungs, tonsils, lymph nodes, and synovial tissue and fluid were collected from six nursery pigs with severe

respiratory and arthritis signs. Ten lung samples from the consolidation of cranio-ventral part were collected. All samples were collected twice from each farm at six-month interval (Aarestrup and Friis, 1998; Friis, 1974; Friis et al., 1991; Kobayashi et al., 1996a; Kobayashi et al., 2005; Yamamoto et al., 1986).

3.2.4 Scoring of lung and swelling joint

Lung scoring was observed by palpation and visual method. The scoring unit was the percentage of lung consolidated lobes, 10% each for the right and left cranial lobes, 10% each of the right and left middle lobes and 10% for accessory lobes, 25% each for the right and left caudal lobes In the second part, the longitudinal study, tissue samples of lungs, tonsils, lymph nodes, and synovial tissue and fluid were collected from six nursery pigs with severe respiratory and arthritis signs. Ten lung samples from the consolidation of cranio-ventral part were collected. All observation and sampling were collected twice from each farm at six-month interval.

3.2.5 Mycoplasmal culture and identification

All samples were cultured in appropriated media including BHL for *M. hyopneumoniae*, Modified Hayflick's broth for *M. hyorhinis* and Hayflick's enriched with alginine and mucin (HAM) for *M. hyosynoviae* isolation (Caron et al., 2000; Kobayashi et al., 1996a; Mattsson et al., 1995). After pure colonies were multiplied, single polymerase chain reaction (sPCR) was used for the identification of *Mycoplasma* spp. (Sibila et al., 2008; Thusfield, 2005). Briefly, the sample suspensions were filtered by 0.45 μm membrane filter (Minisart, Sartorius Stedim Biotech) and were inoculated into the appropriate media broth. The sample suspensions were incubated at 37°C for at least 4 days with changing of indicator. Then the sample suspension were inoculated into the

appropriate agar media and incubated at 37°C under 5% CO_2 humid condition in a CO_2 gas incubator (WJ-121, Hirasawa work Model) for 4 days. The mycoplasma-liked colonies were collected and re-inoculated into the appropriate broth media at 37°C for 4 days. The culturing process was repeated for 3 times (C1-C3). At the 3rd cultivation, the pure isolate was identified *Mycoplasma* spp. by sPCR.

3.2.6 Statistical analyses

Data from swabs and organs cultivation, clinical signs, lung score, swelling joint (yes, no) were analyzed by SAS version 9.0 (SAS Inst. Inc., Cary, NC., USA.). Lung score was analyzed by Wilcoxon rank sum test. Categorical data were analyzed by descriptive analysis, and Chi-square test. The odd ratio and 95% confident interval of the risk factors (i.e., sites, pig flow, gilt acclimatization, *M. hyopneumoniae* vaccination, etc.) and clinical signs (lung lesions and the presence of swelling joints) were calculated. $P<0.05$ was regarded to be statistically significance.

3.3 Results

3.3.1 *Mycoplasma* spp. occurrence

In the first part, *M. hyopneumoniae* was isolated from lungs of slaughtered pigs in 15.6% (42/270) of the total lung samples. *M. hyopneumoniae* was found from only one sample out of 270 nasal swab samples (0.4%). *M. hyosynoviae* was found in 7.9% (21/266) of the tonsils. *M. hyorhinis* was found in nasal swabs of nursery and organs (lungs and tonsils) of fattening pigs in 61.1% (165/270), 18.2% (49/270), 63.5% (169/266) from nasal swabs, lungs and tonsils, respectively. No *Mycoplasma* spp. was isolated from synovial fluid of fattening pigs (table 3.2). The total occurrence in the

North, Northeast, East, and West areas were 17.6% (31/176), 3.3% (6/182), 0, and 4.9% (6/122) for *M. hyopneumoniae*, 0%, 3.3% (9/272), 40% (40/100) and 0% for *M. hyosynoviae*, and 41.2% (108/262), 40.2% (110/272), 46% (46/100), and 64.7% (119/184) for *M. hyorhinis*, respectively (figure 3.1).

In the second part, *M. hyopneumoniae* was detected from lung samples only 7.7% (10/130) of total lung samples. *M. hyosynoviae* was detected from lungs and tonsil samples at 1.5% (2/130) and 1.0% (1/99), respectively. *M. hyorhinis* was detected from nasal swabs, lungs, tonsils, lymph nodes, and synovial tissue and fluid at 48.3% (29/60), 48.5% (63/130), 82.8% (82/99), 22.2% (4/18), and 7.8% (9/115), respectively (table 3.2).

3.3.2 Risk factors and clinical signs

Mycoplasma spp. obtained from lungs sample of the longitudinal study in different seasons, summer, rainy, and winter, were illustrated in table 3.3. There was no significant different among seasons at $P<0.05$. Lung score and present lung lesion in slaughtered pigs were illustrated in table 3.4. Present of *M. hyopneumoniae* had no significant correlation to lung score and lesion while there was significant correlation for *M. hyorhinis* and lung lesion at $P<0.05$.

Risk factors of porcine mycoplasma were illustrated in table 3.5-3.7. Factors causing the higher risk of the *M. hyopneumoniae* and *M. hyorhinis* detection in lungs were including one site management (odd ratio =1.3 (1.2-1.4), $P<0.001$ and odd ratio =4.6 (2.1-10), $P<0.001$, respectively), no all-in-all-out management in fattening period (odd ratio =1.9 (1.0-3.8), $P<0.05$ and odd ratio =4.9 (2.6-9.5), $P<0.001$, respectively). In addition, *M. hyopneomoniae* was higher detected in the gilt acclimatization with only sow donor (odd ratio = 4.6 (95% CI = 2.2-9.5), $P<0.001$), co-infection with PRRSV in herds (odd ratio = 3.6 (95% CI = 1.4-9.0), $P=0.003$) and no *M. hyopneumoniae*

vaccination for weaning piglets (odd ratio = 13.3 (95% CI = 5.5-32.3), *P*<0.001). Medication program had no significant correlation with *M. hyopneumoniae* occurrence in the lung tissue whereas there was significant correlation for *M. hyorhinis* occurrence in the herd with no medication for sow and nursery (odd ratio = 2.8 (95% CI = 1.3-6.2), *P*=0.011) (table 3.5).

Risk factors for the detection of *M. hyosynoviae* and *M. hyorhinis* from tonsils were the absent of finisher medication (*P*=0.005). *M. hyosynoviae* was significant higher detection from tonsils in the gilt acclimatization by PRRSV vaccine (*P*<0.001), and no medication for piglet (odd ratio = 2.9 (95%CI = 1.2-7.0), *P*=0.019), starter (odd ratio = 2.8 (95%CI = 1.1-7.1), *P*=0.029) and grower (*P*<0.001) pigs. *M. hyorhinis* was detected from tonsils in the management of no all-in-all-out in fattening period (odd ratio = 2.2 (95%CI = 1.2-3.8), *P*=0.006) (table 3.6).

There are the correlation of *M. hyorhinis* occurrence from nasal swab and the risk factors including the gilt acclimatization by PRRSV vaccine (odd ratio = 8.4 (95%CI = 3.8-18.4), *P*<0.001) and co-infection with PRRSV+PCV2 (odd ratio = 13.1 (95%CI = 5.2-32.9), *P*<0.001) in the nursery with swelling joint while *M. hyorhinis* occurrence in nasal swab has no correlation to the present of swelling joint in nursery at *P*<0.05 (table 3.7).

3.4 Discussion

Different farm management and medication caused the different occurrence of *Mycoplasma* spp. derived from various organs. From this study, the occurrence of porcine mycoplasmas detected from lung was similar among different seasons in Thailand. If there was no seasonal effect to porcine mycoplasmas from pig farms, the management, medication, and vaccination might be the key factors.

The clinical signs and lesions, including lung lesion and swelling joint, observed during the sample collection showed the correlation to the occurrence of porcine mycoplasmas at the presence of risk factors. The evidence of *M. hyorhinis* obtained from lung lesions in the present study supported the significant correlation of the cranio-ventral consolidation of lungs in slaughtered pigs to *M. hyorhinis* (Fablet et al., 2010; Maes et al., 2008). Furthermore, the presence of *M. hyorhinis* co-infection with *M. hyopneumoniae* caused the worse lung lesion had been reported by the previous study (Browning et al., 2010). *M. hyorhinis* was an invasive blood-born infection and had the capacity to penetrate epithelial barrier to bloodstream during stress situation and, in the chronic case, *M. hyorhinis* penetrated epithelial barrier to serosal and synovial cavities and caused polyserositis, arthritis and synovitis syndromes. The affected joints contained the nonsuppurative serosanguineous synovial fluid and showed the swelling of joints (Browning et al., 2010; Friis and Feenstra, 1994). From the present study, the management of gilt acclimatization by PRRSV vaccine and co-infection with PRRSV and PCV2 might promote more *M. hyorhinis* occurrence from nasal swabs of the nursery pigs with present swelling joint. The stress from PRRSV and other viral diseases from lived vaccine or natural infection could damage the respiratory tract and promote mycoplasmal infection in sow and gilt. The *M. hyorhinis* loaded in nostril of nursery was the consequence from the lactating direct contact transmission (Browning et al., 2010; Dorr et al., 2007b; Sibila et al., 2009; Thacker et al., 1999; Thacker et al., 2000b).

In the cross-sectional study, no mycoplasmas could be derived from synovial joints of slaughtered pigs because the organism might be absent in the chronic infection in the fattening. Nevertheless, the synovial fluid and tissue from slaughtered pigs might not be the appropriated sample for culture method while the acute lesion of lung consolidation found in the slaughtered pigs were appropriated for both *M. hyopneumoniae* and *M. hyorhinis* isolation (Kobisch and Frey, 2003; Sibila et al., 2007b).

The management to protect the after weanling direct contact such as two-sites system and all-in-all-out management in fattening period, diminished the lung infection of *Mycoplasma* spp. In addition, the breeder management to promote the direct contact and immune-suppression including gilt acclimatization by sow donor and co-infection with PRRSV in herd, resulted in the incretion of *M. hyopneumoniae* present in lung of slaughtered pigs from this study. The result was complied to the previous studies that the infected sow maintained the carrier stage for a long time. The sow donor should be selected carefully to be free from the diseases. Furthermore, the management to control of mycoplasmas in breeder herd including vaccination and medication should be concerned (Alves et al., 2007; Browning et al., 2010; Hagedorn-Olsen et al., 1999a; Magnusson et al., 1998; Ross and Karmon, 1970; Sokoloff, 1973). All-in all-out management in breeder was one of the important factor and being reduced the seroprevalence of slaughtered pigs to other respiratory pathogens including *Actinobacillus pleuropneumoniae* (APP) and Porcine Reproductive and Respiratory Syndrome virus (PRRSV) (Sibila et al., 2009).

Medication was not correlated the occurrence of *M. hyopneumoniae* in lung whereas the sow and nursery medication diminished the occurrence of *M. hyorhinis* in lung of slaughtered pigs. *M. hyopneumoniae* was classified as the localized extension infection which involved in bronchitis, bronchiolitis and pneumonia in animals. Besides the susceptibility, the pharmacokinetic and pharmacodynamic of antimicrobials in respiratory tract was the important quality to diminish *M. hyopneumoniae* due to rarely chance to inhibit this pathogen in serum (Bousquet et al., 1997).

There were several studies reported that *M. hyopneumoniae* vaccination in piglets, before or after weanling, did not stop the transmission and colonization of *M. hyopneumoniae*, whereas the lung lesion was reduced in vaccinated pigs (Okada et al., 2000;Okada et al., 1999;Sheldrake, 1990;Sibila et al., 2007b). After the vaccination, *M. hyopneumoniae* vaccinated pigs were decreased in the tumor necrosis factor (TNF)-α

level, inflammatory cell infiltration, and T-cells accumulation around bronchi, therefore, the lung lesion was reduced as the consequence (Maes et al., 2008). From the present study, vaccination by *M. hyopneumoniae* bacterin in piglets, one or two doses, showed highly significant to limit *M. hyopneumoniae* occurrence from lung of slaughtered pigs. The result supported that the immunity from vaccine diminished the growth of *M. hyopneumoniae* in lung lesions at slaughter. The result was not complied to the previous studies that reported the failure of the vaccination to control the transmission and colonization of *M. hyopneumoniae* (Chae, 2011; Meyns et al., 2006). Artificial immunity from *M. hyopneumoiae* vaccination might stimulate T-lymphocytes to promote opsonization and phagocytosis in the lungs as well as the memory for adaptive immunity response to the infection of field strains. Since the protection had no correlation to antibody response, the vaccine might activate phagocytic cells such as neutrophils, macrophages and NK cells to kill *M. hyopneumoniae* localized on cilia of respiratory epithelium. From the nowadays technology of antigen and adjuvant, the immune stimulation could be as fast as the colonization of pathogens, then, vaccination could diminish the presence of *M. hyopneumoniae* in lung lesion from the present study. For *M. hyopneumoniae* control in herd, vaccination in breeder should be considered for reduction of *M. hyopneumoniae* loaded in lung of breeder (Browning et al., 2010; Djordjevic et al., 1997; Haesebrouck et al., 2004; Rodriguez et al., 2007; Sibila et al., 2008; Thacker et al., 2000a).

Tonsil carriers of *M. hyosynoviae* and *M. hyorhinis* derived from slaughtered pigs were correlated to all-in-all-out management in fattening period and gilt acclimatization by PRRSV vaccine. No all-in-all-out management promoted the tonsil carriers of *M. hyorhinis* due to such management could increase the direct contact transmission of the infected pigs from lactating period to nursery and fattening units. The previous study reported the incidence in weaning period might lead to the higher incidence in fattening period for localized mycoplasmal infection (Fano et al., 2007). Gilt

acclimatization by PRRSV vaccine was correlated to higher *M. hyosynoviae* occurrence in tonsil of slaughtered pigs. *M. hyosynoviae* was as well as *M. hyorhinis*, being classified as the invasive blood-borne infection. The stress from movement or vaccination stimulated the arthritis caused by *M. hyosynoviae*. The stress or immune-suppression might have an effect to tonsil carriers as the present result (Browning et al., 2010). The medication in piglet and fattening period diminished the *M. hyosynoviae* tonsil carriers in the present study. Medication was still the important tool for control *M. hyosynoviae* in herds. The successful of medication gave the advantages to the breeder producers during the time that the vaccine for the protection of this mycoplasma was still developing (Aarestrup and Friis, 1998; Fraile et al., 2010; Friis et al., 1991; Hagedorn-Olsen et al., 1999b; Lauritsen et al., 2008; Nielsen et al., 2001). Medication for breeder and piglet from sucking to nursery, reduced the present of swelling joint in nursery. This medication might be useful for the breeder producers to protect the breeder swelling joint and reduced the culling rate of grower breeder during the selection. Furthermore, the occurrence of *Mycoplasma* spp. in nursery pigs with severe arthritis were *M. hyorhinis* detected from synovial tissue and fluid. From the present study, the suitable sample for diagnosis of Mycoplasmal arthritis was synovial tissue from infected nursery pigs in spite of slaughtered pigs (Chae, 2011; Djordjevic et al., 1997; Haesebrouck et al., 2004; Maes et al., 2008; Sibila et al., 2008; Sibila et al., 2009; Thacker et al., 2000a; Villarreal et al., 2011).

3.5 Conclusion

In conclusions, the risk factors for lung infection, tonsil carrier and nostril load of porcine mycoplasmas were including the promotion of direct contact (one-sites, no all-in-all-out, and gilt acclimatization by sow donor), immune-suppression (gilt acclimatization by PRRS vaccine and co-infection with PRRSV and PCV2) in breeder

and fattening herds. *M. hyopneumoniae* vaccination in piglet diminished *M. hyopneumoniae* reduction from lung of slaughtered pigs. Medication was not effective for *M. hyopneumoniae* present in lung, however, it could control tonsil carrier of *M. hyosynoviae*, lung infection and tonsil carrier of *M. hyorhinis*.

Table 3.1 Summary of herd management

Farm	Sow on production	Production system	Housing	*M. hyopneumoniae* vaccination	PRRSV vaccination
A	1,200	1-site	close	yes	no
B	3,000	1-site	close	no	no
C	3,000	2-sites	close	yes	yes
D	1,000	1-site	close	yes	no
E	5,400	2-sites	close	yes	yes
F	4,300	1-site	open	yes	no
G	4,100	2-sites	close	yes	yes
H	1,300	1-site	open	yes	yes
I	1,300	2-sites	open	yes	yes

Table 3.2 Porcine mycoplasmas isolation from cross-sectional (Study I) and longitudinal (Study II) studies

Type of samples	Number. of samples	Mycoplasmal isolation (%)		
		M. hyopneumoniae	*M. hyosynoviae*	*M. hyorhinis*
Study I (n=1,024)				
Nasal swab	270	1 (0.4)	0 (0)	165 (61.1)
Lung	270	42 (15.6)	0 (0)	49 (18.2)
Tonsil	266	0 (0)	21 (7.9)	169 (63.5)
Synovial fluid	218	0 (0)	0 (0)	0 (0)
Study II (n=422)				
Nasal swab	60	0 (0)	0 (0)	29 (48.3)
Lung	130	10 (7.7)	2 (1.5)	63 (48.5)
Tonsil	99	0 (0)	1 (1%)	82 (83.8)
Lymph node	16	0 (0)	0 (0)	4 (22.2)
Synovial tissue and fluid	115	0 (0)	0 (0)	9 (7.8)

Table 3.3 *Mycoplasma spp.* detected from lung by seasons

Mycoplasma spp.	No. (%) of lung detection			*P*-value
	Summer	Rainy	Winter	
M. hyopneumoniae	0/34 (0)	6/47 (12.8)	4/49 (8.2)	0.103
M. hyosynoviae	2/34 (5.9)	0/47 (0)	0/49 (0)	0.057
M. hyorhinis	20/34 (58.8)	24/47 (51.1)	19/49 (38.8)	0.180

Table 3.4 Lung score and present lung lesion correlated to *Mycoplasma* spp. occurrence from lung in cross-sectional study

Mycoplasma spp.	Mycoplasmal occurrence	Lung score		Present lung lesion		
		Mean ±SD	*P*-value	No. (%)	OR (95%CI)	*P*-value
M. hyopneumoniae	no	6.5±10.3	0.776	10/86 (11.6)	1.6 (0.7-3.4)	0.223
	yes	6.0±7.4		32/184 (17.4)		
M. hyorhinis	no	5.3±8.3	<0.001	9/86 (10.5)	2.4 (1.1-5.2)	0.025
	yes	11.6±14.2		40/184 (21.7)		

Table 3.5 Risk factors and medication correlated to *Mycoplasma* spp. detected from lung in cross-sectional study indicated by Odd Ratio (OR: 95% Confidence Interval (95% CI)) and bivariable analysis by Chi-square ($P<0.05$)

Risk factor	*M. hyopneumoniae occurrence*			*M. hyorhinis occurrence*		
	%	OR (95% CI)	*P*-value	%	OR (95% CI)	*P*-value
One-site	24.8	1.3 (1.2-1.4)	<0.001	26.8	4.6 (2.1-10)	<0.001
No all-in-all-out in fattening	21.5	1.9 (1-3.8)	0.05	34.4	4.9 (2.6-9.5)	<0.001
Gilt acclimatization by sow donor only	26.3	4.6 (2.2-9.5)	<0.001	22	1.6 (0.9-3.0)	0.144
Co-infection with PRRSV	20.2	3.6 (1.4-9.0)	0.003	19.1	1.1 (0.6-2.4)	0.572
No *M. hyopneumoniae* vaccination	61.5	13.3(5.5-32.3)	<0.001	11.5	0.6 (0.2-2.0)	0.435
No sow medication	12.5	0.8 (0.3-2.5)	0.797	34.4	2.8 (1.3-6.2)	0.011
No nursery medication	12.5	0.8 (0.3-2.5)	0.797	34.4	2.8 (1.3-6.2)	0.011

Table 3.6 The relation of risk factors and medication to *Mycoplasma* spp. occurrence in tonsils in cross-sectional study indicated by Odd Ratio (OR: 95% Confidence Interval (95% CI)) and bivariable analysis by Chi-square (P<0.05)

Risk factor	*M. hyosynoviae occurrence*			*M. hyorhinis occurrence*		
	%	OR (95% CI)	*P*-value	%	OR (95% CI)	*P*-value
No all-in-all-out in fattening	0	-	-	74.7	2.2 (1.2-3.8)	0.006
Gilt acclimatization by PRRSV Vaccine	14	-	<0.001	68	1.6 (0.9-2.6)	0.085
No piglet medication	13.3	2.9 (1.2-7.0)	0.019	68.9	1.4 (0.8-2.5)	0.194
No starter medication	15	2.8 (1.1-7.1)	0.029	43.3	0.3 (0.2-0.6)	<0.001
No grower medication	18.1	-	<0.001	63.8	1.0 (0.6-1.7)	0.938
No finisher medication	10.2	-	0.005	68	2.3 (1.3-4.1)	0.005

Table 3.7 Relation of two risk factors and the presence of swelling joints in nursery to *M. hyorhinis* occurrence in nasal swab of nursery in cross-sectional study indicated by Odd Ratio (OR: 95% Confidence Interval (95% CI)) and bivariable analysis by Chi-square (P<0.05)

Clinical sign	Risk Factor	*M. hyorhinis* occurrence		
		%	OR (95% CI)	*P*-value
Swelling joint present		64.9	1.5 (0.9-2.5)	0.104
	+Gilt acclimatization with PRRSV vaccine	77	8.4 (3.8-18.4)	<0.001
	+Co-infection of PRRSV+PCV2	91.5	13.1 (5.2-32.9)	<0.001

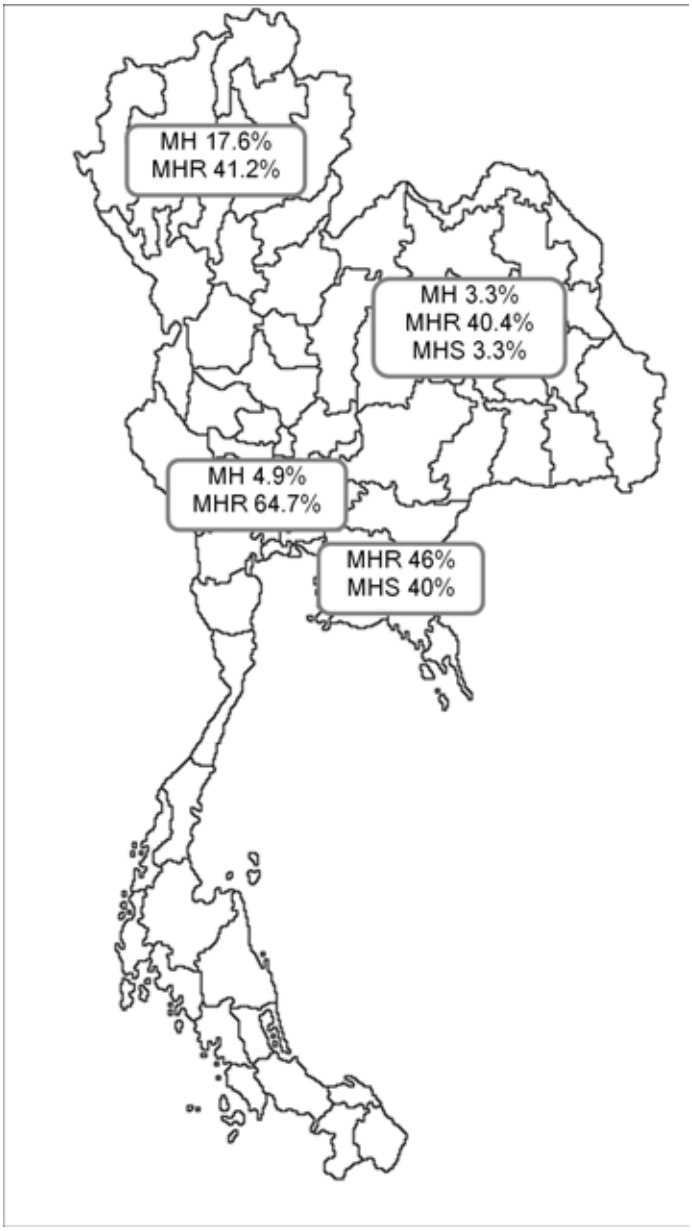


Figure 3.1 Illustration of *Mycoplasma* spp. occurence (%) obtained from all samples in the different areas by the cross-sectional study, MH= *M. hyopneumoniae*; MHS= *M. hyosynoviae*; MHR= *M. hyorhinis*

CHAPTER IV

Antimicrobial susceptibility of porcine mycoplasmas isolated from nine commercial pig farms in 2009-2011

4.1 Introduction

Porcine mycoplasmas are the important organisms cause respiratory diseases and arthritis in pigs from nursery to fattening pigs as well as the young breeders (Fraile et al., 2010; Friis et al., 1991; Hagedorn-Olsen et al., 1999a; Hagedorn-Olsen et al., 1999b; Lin et al., 2006). The protection by vaccination can be produced for mycoplasmosis causing by *Mycoplasma hyopneumoniae* only. Furthermore, the bacterin vaccine seems to reduce the consequence of *M. hyopneumoniae* infection such as lung lesion and growth performance in spite of the transmission and colonization from the recent study (Chae, 2011; Villarreal et al., 2011). Therefore, the antimicrobials are generally the important tools for both therapeutic and prophylactic purpose. The frequency of medication is depended on the clinical level in each farm. Farms in the endemic area seem to be the most frequent users. Medicated program for mycoplasmal control is generally applied from weaning to fattening as well as some specific period in breeders such as during gilt acclimatization and lactation. The evidence of antimicrobial resistance from *Mycopalsma spp*. have been reported recently. Nevertheless, the susceptibility study of porcine mycoplasmas including *M. hyopneumoniae, M. hyosynoviae*, and *M. hyorhinis*, are complicated and no international standard for the procedure such as The Minimum Inhibitory Concentration (MIC). Therefore, the data of susceptibility for these species is limited (Hannan, 2000; Kobayashi et al., 2005; Vicca et al., 2007).

Since mycoplasmas are the small organisms without cell wall, they are intrinsically resistant to antimicrobials inhibiting cell wall synthesis such as the β-lactam group. The drug of choice for porcine mycoplasmas are commonly included macrolides, lincosamides, pleuromutilins, tetracyclines, and fluroquinolones. The available reports of mycoplasmal susceptibility indicate an increasing in the resistant strains against widely used antimicrobial agents. The 23S ribosomal RNA mutation of the mycoplasmas causes resistance against macrolide, lincosamide and streptogramine (MLS) antibiotics and confers cross-resistance among these antibiotics. Point mutation in the *Par*C quinolone resistance-determining region (QRDR) or the mutations in a gene encoding for DNA topoisomerase IV have been described for *M. hyopneumoniae*. Other activities conferred the resistance are including the efflux pump of the antimicrobials out of the cell membrane of the organisms to free the targeted ribosome from antimicrobials, the production of protein for ribosomal protection, e.g. to tetracyclines, and the enzymatic inactivation to lincomycin, erythromycin and chloramphenicol (Flutt et al., 2001; Le Carrou et al., 2006; Stakenborg et al., 2005b).

The Minimum Inhibitory Concentration (MIC) is an *in vitro* susceptibility study to define the lowest concentration of antimicrobials inhibiting the visible growth or metabolism of the organisms. The MIC technique against *Mycoplasma* spp. can be carried on both liquid (broth) and solid (agar) media specified to each mycoplasma. Due to the diversity of nutritional requirements, the isolation and cultivation of mycoplasma needs the specific enriched media for differentiating mycoplasmal growth. *M. hyopneumoniae* and *M. hyorhinis* utilize glucose and result in acidic environment from their specific growth condition. Phenol red indicator can detect by media color that change from red to yellow after the growth of such mycoplasmas. This color change is useful for the visual detection of mycoplasmal growth during the MIC test. The color change of amino acid utilized mycoplasma, such as *M. hyosynoviae*, confers the alkaline condition in the growth media by phenol red indicator that convert from red to

red-bluish color. The MIC has been reported as MIC_{50}, MIC_{90} and MIC range that described the minimum concentration of antimicrobial inhibited mycoplasmas at 50% and 90% of the population tested and the minimum to maximum range of the inhibitory concentration, respectively. The value over breakpoint is used for resistant level (Aarestrup and Friis, 1998; Hannan, 2000; Ter Laak et al., 1991).

The present study aims to determine the antimicrobial susceptibility of three porcine mycoplasmas including *M. hyopneumoniae*, *M. hyosynoviae*, and *M. hyorhinis*, for six antimicrobials including tetracyclines (doxycycline), fluoroquinolones (enrofloxacin), lincosamides (lincomycin), macrolides (tylosin), pleuromutilins (tiamulin and valnemulin) by the broth micro-dilution method.

4.2 Materials and Methods

4.2.1 Data collection

Nine pig farms from different areas of Thailand (North, Northeast, East and West) were selected for sample collection from their nursery and slaughtered pigs from 2009 to 2011. The questionnaires were used for collection of farm disease, medication, and management information.

4.2.2 Mycoplasma strain selection

Type strains J, S16 and BTS-7 were obtained from National Institute of Animal Health, Japan, to be the reference strains for *M. hyopneumoniae*, *M. hyosynoviae*, and *M. hyorhinis*, respectively. Twenty-six field strains of *M. hyopneumoniae* were isolated from the lungs at slaughterhouse of five pig farms in 2009. Thirteen filed strains of *M. hyosynoviae* were isolated from tonsils and lungs at slaughterhouse of two pig farms in

2009 and 2011. All isolates were selected for the MIC test of these two species. One hundred and seventy field strains of *M. hyorhinis* isolated from nasal swab samples, lungs, tonsils, lymph nodes, synovial fluid and tissue samples of nursery pigs at 4-8 weeks old and from lungs and tonsils of slaughtered pigs of nine pig farms from 2009 to 2011 were selected by choosing the isolates from every farms, all organs and period at least three isolates per category. The two hundreds and nine isolates of *Mycoplasma* spp. were multiplied and counted by plate count method at the concentration 10^7 cfu/ml for *M. hyopneumoniae* and 10^8 cfu/ml for *M. hyosynoviae* and *M. hyorhinis* (Hannan, 2000; Ter Laak et al., 1991; Vicca et al., 2004) to confirm the concentration before the test.

Plate count of *Mycoplasma* spp. were conducted in the appropriate agar for *Mycoplasma* spp., BHL for *M. hyopneumoniae*, HAM for *M. h*yo*synoviae*, and Modified Hayflick's for *M. hyorhinis*. Ten fold dilution of *Mycoplasma* spp. were diluted from 10^{-4} to 10^{-7} cfu/ml. 10 µl of mycoplasmal dilution were dropped to agar from higher to lower concentration. Five concentration were dropped in one plate. The dilution in agar was incubated in 37°C under 5% CO_2 for at last four days. The colony present on agar was counted.

4.2.3 Mycoplasma culture dilution

Stock culture of field strains and type strains were diluted in the appropriate media and adjusted into concentration of $5x10^5$ cfu/ml by dilution of the stock solution, and incubated at 37°C for 3 hours. *M. hyopneumonia*, *M. hyosynoviae* and *M. hyorhinis* were diluted in Bruclla Hank's Lactalbumin (BHL), Hayflick's with arginine and mucin (HAM) and Modified Hayflick's broth, respectively (Friis, 1972; Kawamura et al., 1982; Masover et al., 1975).

4.2.4 Antimicrobial dilution

The six antimicrobial agents were used comprising doxycycline (Fluka Chemie GmbH, China), enrofloxacin (Sigma, China), lincomycin (Sigma, China), tylosin (Sigma, China), tiamulin (Sandoz, Austria), and valnemulin (Sandoz, Austria). Antimicrobials were diluted to prepare the stock solution at the concentration 800 µg/ml in distilled water except enrofloxacin which was diluted in 10% methanol for seconds and added 0.1 M PBS. All stock solution were sterilized by filtration through a 0.45 µm membrane filter (Minisart, Sartorius Stedim Biotech).

4.2.5 Minimum inhibitory concentration (MIC) determination

The MICs were carried out using broth micro-dilution method (Hannan, 2000). Each antimicrobial stock solution was serially diluted two fold at 25 µl/well in 96-wells microtiter plate. The test concentration ranged from 0.006-12.5 µg/ml for lincomycin, tiamulin, tylosin, and valnemulin, and from 0.048-100 µg/ml for doxycycline and enrofloxacin. Stock cultures of mycoplasmas were added to each well at 175 µl, duplicating by two rows used per one strain. Two rows were filled with 175 µl media broth instead of stock culture were used as negative control. Plates were sealed and incubated at 37°C for seven days. Mycoplasmal growth was observed by color change indicator and microscopic observation. The lowest concentrations of each antimicrobial showing inhibition of visible color change were interpreted as the MIC values at the seventh days post incubation. Breakpoint values were used for interpretation of the resistance and susceptibility in percentage of the isolation number (Hannan, 2000).

4.2.6 Statistical analyses

MICs were described by species, ages, and farms. The relation between susceptibility level and certain associated factors such as age of pigs, *Mycoplasma* species, were analyzed by chi-square at $P<0.05$.

4.3 Results

The antimicrobial programs of nine pig farms before and during the period of sample collection were illustrated in Table 4.1. The medication programs of each farm had a verity of antimicrobials and durations. Tiamulin, tylosin, chlortetracycline (CTC), and amoxicillin had been used in breeder pigs including gilt replacement and sow (pregnant and lactating), generally. Enrofloxacin was used for the treatment of piglet diarrhoea during lactating to nursery period. Some farms used colistin, phosphomycin and halguinol premix for the enteric problem. Tiamulin and tylosin or tylosin plus sulfonamindes had been generally used for the treatment of respiratory diseases during 0-9 weeks old. Tiamulin, tylosin, CTC, and amoxicillin had been used during fattening period. Because of the withdrawing period before slaughter, most of farms had no medication for finisher pigs or 4-6 weeks before slaughter. Tiamulin, tylosin, and CTC was the major antimicrobial used in all farms in this study applied by oral route and enrofloxacin by injection, whereas doxycycline and lincomycin were not used before and during the period of sample collection. Macrolides, tylosin and valosin, were used in two farms in 2009 to 2010. Florfenicol, the derivative of chloramphenical, was used in one farm from breeder to fattening pigs in 2009 to 2011.

A total of 209 *Mycoplasma* strains, they showed the highest MIC_{90} to enrofloxacin at 25 µg/ml, the resistant rate (%) was 38.3% (80/209). Tylosin was the highest resistant antimicrobial to *Mycoplasma* spp. at 40.2% (84/209) with the MIC_{90} at 12.5 µg/ml. Its maximum range of MIC was exceed at >12.5 µg/ml. Doxycycline and lincomycin were the susceptible antimicrobials to the tested isolates with lower resistant

rate at only 2.9% (6/209) and 5.3% (11/209), and MIC_{90} at 6.25 and 3.12 µg/ml, respectively. The highest MIC value to doxycycline was 25 µg/ml while the highest value to lincomycin was exceed the highest concentration in the test (>12.5 µg/ml). All tested mycoplasmas were susceptible to tiamulin and valnemulin, the members of pleuromutilin group, Furthermore, the lowest MIC_{90} to valnemulin was 0.024 µg/ml, while that of tiamulin MIC_{90} was 0.78 µg/ml. The MIC_{90} to pleuromutilin antimicrobials was less than 1 µg/ml to all mycoplasmal strains from this study (Table 4.2).

As the susceptibility results, the levels of MIC_{90} were analyzed together with the difference of mycoplasmal species at $P<0.05$. The MIC_{90} of *M. hyorhinis* for all antimicrobials were higher than those of *M. hyopneumoniae* and *M. hyosynoviae* had the highest MIC_{90}., except *M. hyosynoviae* that showed more resistance to doxycycline. *M. hyopneumoniae* had the lowest MICs among the three species for all antimicrobials except enrofloxacin (3.12 µg/ml) and tiamulin (0.097 µg/ml). *M. hyopneumoniae* showed the highest MIC_{90} to doxycycline and enrofloxacin at 3.12 µg/ml. *M. hyosynoviae* showed the highest MIC_{90} to doxycycline at 25 µg/ml. For *M. hyorhinis*, the highest MIC_{90} was 25 µg/ml to enrofloxacin, whereas MIC range of lincomycin and tylosin exceeded the maximum range of the tested concentration (12.5 µg/ml) (Table 4.3).

The susceptibility to doxycycline, enrofloxacin and tylosin among three porcine *Mycoplasma* spp. was statistically different at $P<0.05$. *M. hyosynoviae* was resistant to doxycycline and tylosin at 46.2% (6/13) and 7.1% (1/13) of the tested strains, respectively. *M. hyopneumoniae* was resistant to only enrofloxacin at 34.6% (9/26) of the tested strains. The most *M. hyorhinis* was resistant to tylosin, enrofloxacin and lincomycin at 48.8% (83/170), 41.8% (71/170), and 6.5% (11/170) of the tested strains, respectively. There was no resistance to tiamulin and valnemulin from all tested mycoplasmas (table 4.4).

M. hyorhinis was the sole porcine *Mycoplasma* spp. that had been isolated from different ages of pigs in nursery and finisher period at 91 and 79 strains, respectively. The susceptibility level between nursery and finisher pigs were not statistically different for all tested antimicrobials. Actually, the MIC ranges of *M. hyorhinis* to all antimicrobials, except valnemulin, were similar between nursery and finisher pigs (table 4.5).

The MIC_{90} (µg/ml) and the resistance (%) of *Mycoplasma* spp. obtained from individual farms were illustrated in table 4.6. 80% (4/5) of farms had *M. hyopneumoniae* resistant strains to enrofloxacin. All two farms had *M. hyosynoviae* resistant strains to doxycycline but the strains derived from PN farm were more resistant than AK. The MIC_{90} of *M. hyorhinis* was significant different among farms at $P<0.05$. There were *M. hyorhinis* resistant strains to enrofloxacin, tylosin, and lincomycin in at 66.7% (6/9), 100% (9/9), and 11.1% (1/9) of farms, respectively.

4.4 Discussion

Porcine mycoplasmal field strains had been occasionally determined for susceptibility to the antimicrobial agents in different farms, areas and periods. The genetic variation of mycoplasmas such as the mutation in 23S ribosomal RNA was a major mechanism for macrolides and lincosamides resistances. Moreover, the point mutation of *Par*C gene coding for topoisomerase IV and the ribosomal protection by the production of a protein to bind ribosome coding by the mutant gene of mycoplasmas also conferred fluoroquinolone resistance, and tetracyclines resistance (Flutt et al., 2001; Kobayashi et al., 2005; Le Carrou et al., 2006; Stakenborg et al., 2005a). The resistance of porcine mycoplasmas to tylosin, enrofloxacin, lincomycine and doxycycline was confirmed in this study. Although the use of lincosamides had not been recorded before 2009 and during the sample collection from 2009 to 2011 but the resistance of lincomycin was still detected in *M. hyorhinis* derived from both young and

slaughtered pigs in a farm which the resistance to tylosin and enrofloxacin occured. An *in vitro* study showed the cross-resistance between lincomycin and tylosin, the lincomycin resistant strains of *M. hyorhinis* BTS-7 strains in tylosin passages was reported to be high resistant to tylosin whereas the lincomycin resistant strains conferred only moderate resistance to tylosin (Kobayashi et al., 2005). If this phenomenon occurred also in field condition, the frequency of either tylosin or macrolides used in farms could induce the resistant strain to lincomycin. According to this study *M. hyosynoviae* was highly resistant to doxycycline while there was no doxycycline in the program during the sample collection. The continuous use of tetracyclines in farms for a long time might cause the cross-resistant for *M. hyosynoviae* obtained from slaughtered pigs in these two observed farms (Flutt et al., 2001).

In the present study, the high resistant strains to enrofloxacin were detected in *M. hyopneumoniae* and *M. hyorhinis*, espectially in the farms currently used enrofloxacin in young pigs. The result was not consistent to that of previous study in Japan in 2005 which enrofloxacin and tiamulin were the satisfied antimicrobials (Kobayashi et al., 2005). Whereas the result was complied to some European studies. *M. hyopneumoniae* resistant to tylosin, lincomycin, and enrofloxacin was detected in Belgian strains, corrected from 2000 to 2002. There were the resistant strains of *M. hyosynoviae* to tylosin in Netherlands since 1995-1996 which complied with the present study (Aarestrup and Friis, 1998; Stakenborg et al., 2005a; Ter Laak et al., 1991; Vicca et al., 2004). From the present study, there was no resistant strains of *M. hyosynoviae* to enrofloxacin. This result related to the occurrence of *M. hyosynoviae* during the grower to finisher pigs and the young breeders which was not the period of enrofloxacin application in most farms. The different environment of pig production, medication, and diseases, might be the factor caused the different resistance of porcine mycoplasmas in different regions.

According to the previous study, the resistance of tylosin to *M. hyorhinis* was high incidence in Japan and there was a different detail of the MIC_{90} to the age of pigs. The previous study reported that nursery pigs conferred the higher MIC_{90} than finisher pigs at slaughter for tylosin, lincomycin, and tiamulin. The investigator explained that this was the result from the more extensive use of the macrolides in piglet in Japan than the finisher pigs (Kobayashi et al., 2005). From the present study there was no difference of MIC and susceptibility to all antimicrobials tested between the young and finisher pigs. The factors could be the treatment frequency and major antimicrobials used in nursery were not different to the older pigs. Moreover, the resistant strains of *M. hyorhinis* in lived young animals developed no more or less resistant when those pigs grew up to slaughter. The rotation of some medication from young pigs while the resistance occurred might alter the resistant situation of *M. hyorhinis* in farm.

In Thailand, tetracyclines such as chlortetracycline and oxytetracycline were commonly used in pig industry. The previous study in Netherlands in 1991 reported the low MIC_{90} of tetracycline members including doxycycline to *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* whereas higher MIC and resistance (%) for doxycycline was detected in *M. hyosynoviae* from the present study (Ter Laak et al., 1991). Doxycycline was the better pharmacokinetic tetracyclines than the simple tetracyclines such as CTC or OTC. Due to the limited cost of pig production, doxycycline was seldom used in fattening pig production in Thailand. However, because of the excess use of other tetracyclines, the cross-resistance showed its evidence according to the present study.

Pleuromutilins, tiamulin and valnemuin, were the most satisfactory sole group resulting no resistant mycoplasmal strains for all ages although tiamulin had been used in all tested farm at least in one period of production. Pleuromutilins activity is protein synthesis inhibition by interacting at the peptidyl transferase center (PTC) in 23S of the 50S subunit of bacterial ribosome. There was the experimentally resistance to tiamuin from inducing the L3 mutation in plasmid of an *Escherichia coli* strain. Nevertheless, the

plasmid borne mutation was not frequent enough to be a manifest resistant phenotype. Furthermore, tiamulin binding pocket in bacterial ribosome was tight lined with the essential nucleotides for cell growth. These were considered as the reasons why there was no tiamulin-resistant ribosomal RNA in the field condition (Bosling et al., 2003;Hunt, 2000). Valnemulin had never used in Thailand before the present study while tiamulin was widely applied in Thai pig farms. Although there was no report of cross-resistant, valnemulin was selected for the detection of the cross-resistant among the pleuromutilins in the present study. According to this study, porcine mycoplasmas had not resisted to this group. Tiamulin could be the effective drug of choice for porcine mycoplasmas in term of no resistance from the target species while valnemulin could be the next generation of pleuromutilins in the countries being widely used tiamulin due to its lower MICs.

The MIC study is an important phenotypic testing tool for monitoring the susceptibility level of *Mycoplasma* spp. to antimicrobials used in the specific period. The following of the MIC study would provide the predictable information for the veterinary practitioners to choose the drug of choice for disease controls and to avoid the resistant antimicrobials.

4.5 Conclusion

The resistance of porcine mycoplasmas was different among *Mycoplasma* spp. whereas there was no different between young and older pigs. The highest resistant mycoplamas strains conferred the high MICs level were found in *M. hyorhinis*. Tiamulin and valnemulin were the most effective agents for all species while enrofloxacin, tylosin, and lincomycin were partially usable for porcine mycoplasmas. Doxycycline should be avoided for the indication of *M. hyosynoviae* control because of the high occurrence of resistance. The identification of *Mycoplasma* spp. being available in farms was

important for the selection of appropriated antimicrobials for porcine mycoplasma control.

Table 4.1 The antimicrobial program of nine pig farms during 2007-2011

Farms[a]	Antimicrobials[c] on different ages[b]			Period
	Breeder	Piglet	Fattening	
PN	N	N	CTC	Before 2009
	tia/CTC	tia/colis/enro	tia/amox	2009
AK	N	N	N	Before 2009
	flor	Tia	tia/amox/CTC/flor	2009-2011
CC	N	N	N	Before 2009
	tylo/tia	tylo	tylo/tia	2009
FT	N	N	N	Before 2009
	tylo/sulfa/tia	tia	tia	2009
PD	N	N	N	Before 2009
	tia	tia	no	2009
KP	tia/CTC	enro/tia/phos	tylo/sulfa/ CTC	Before 2009
	tia/amox	enro/tia/amox/valo	tia/amox	2009-2011
MT	tia/CTC	enro/tia/phos	tylo/sulfa/ CTC	Before 2009
	tia/amox	enro/tia/amox/valo	tia/amox	2009
RM	tylo/CTC	enro/tylo/phos	tylo/CTC	Before 2009
	tylo/tia/amox	enro/tia/amox/hal	no	2009
MI	N	N	N	Before 2009
	tylo/CTC/amox	enro/tia/amox	tia/CTC/OTC/amox	2009

[a] AK and KP farms were longitudinally collected samples for three time from 2009-2011

[b] Breeder (gilt and sow); Piglet (0-9 wk old); Fattening (10 wk old to slaughter)

[c] tia=tiamulin; amox=amoxicillin; enro=enrofloxacin; colis= colistin; tylo= tylosin; sulfa= sulfonamides; phos= phosphomycin; hal= halguinol; CTC=chlortetracycline; tylo=tylosin; enro=enrofloxacin; OTC=oxytetracycline; flor=florfenicol (chemical); valo=valocin (the second generation tylosin); N=no record

Table 4.2 MIC (μg/ml) and resistance (%) of all *Mycoplasma* spp.[a] isolated from nursery and slaughtered pigs (N=209) to six antimicrobials

Antimicrobials	MIC (μg/ml)			Resistance (%)	Breakpoint[b] (μg/ml)
	Range	MIC_{50}	MIC_{90}		
Doxycycline	<0.048-25	1.56	6.25	6/209 (2.9)	≥ 16
Enrofloxacin	<0.048-50	1.56	25	80/209 (38.3)	≥ 2
Lincomycin	<0.006- >12.5	0.78	3.12	11/209 (5.3)	> 8
Tiamulin	<0.006-1.56	0.19	0.78	0/209 (0)	≥ 16
Tylosin	<0.006- >12.5	3.12	12.5	84/209 (40.2)	≥ 4
Valnemulin	<0.006-0.048	<0.006	0.024	0/209 (0)	≥ 16

[a] *Mycoplasma* spp. tested are 26, 13 and 170 strains of *M. hyopneumoniae. M. hyosynoviae*, and *M. hyorhinis*, respectively.

[b] Hannan, 2000.

Table 4.3 The MICs (µg/ml) and the correlation of the different *Mycoplasma* spp. to six antimicrobials.

Antimicrobials	Species	MIC (µg/ml)				*P*-Value
		Range	MIC_{50}	MIC_{90}	Ref[b]	
Doxycycline	MH	0.78-6.25	1.56	3.12	0.78	<0.001
	MHS	<0.048-25	25	25	0.78	
	MHR	<0.048-12.5	1.56	6.25	0.78	
Enrofloxacin	MH	0.37-3.12	0.78	3.12	0.19	<0.001
	MHS	0.048-1.56	0.78	1.56	1.56	
	MHR	<0.048-50	1.56	25	1.56	
Lincomycin	MH	0.097-0.78	0.19	0.39	0.39	<0.001
	MHS	0.097-3.12	0.78	3.12	0.39	
	MHR	<0.006- >12.5	1.56	6.25	0.78	
Tiamulin	MH	0.024-0.19	0.097	0.097	0.097	<0.001
	MHS	<0.006-0.024	0.012	0.024	0.012	
	MHR	<0.006-1.56	0.39	0.78	0.19	
Tylosin	MH	0.024-0.39	0.097	0.19	0.19	<0.001
	MHS	0.78-6.25	1.56	3.12	0.048	
	MHR	<0.006- >12.5	3.12	12.5	0.19	
Valnemulin	MH	<0.006	<0.006	<0.006	<0.006	<0.001
	MHS	<0.006	<0.006	<0.006	<0.006	
	MHR	<0.006-0.048	<0.006	0.024	<0.006	

[a] MH=*M. hyopneumoniae*; MHS=*M. hyosynoviae*; MHR=*M. hyorhinis*, at N= 26, 13, and 170 strains, respectively.

[b] Reference type strains: J, S-16, and BTS-7 for *M. hyopneumoniae, M. hyosynoviae*, and *M. hyorhinis*, respectively.

Table 4.4 The susceptibility (%) and the correlation of the different *Mycoplasma* spp. to six antimicrobials.

Antimicrobials	Species	Susceptibility (%)		*P*-Value
		Susceptible	Resistant	
Doxycycline	MH	25/26 (96.2)	0/26 (0)	<0.001
	MHS	2/13 (15.4)	6/13 (46.2)	
	MHR	148/170 (87.1)	0/170 90)	
Enrofloxacin	MH	10/26 (38.5)	9/26 (34.6)	<0.001
	MHS	4/13 (30.8)	0/13 (0)	
	MHR	7/170 (4.1)	71/170 (41.8)	
Lincomycin	MH	26/26 (100)	0/26 (0)	0.436
	MHS	13/13 (100)	0/13 (0)	
	MHR	159/170 (93.5)	11/170 (6.5)	
Tiamulin	MH	26/26 (100)	0/26 (0)	-
	MHS	13/13 (100)	0/13 (0)	
	MHR	170/170 (100)	0/170 (0)	
Tylosin	MH	26/26 (100)	0/26 (0)	<0.001
	MHS	3/13 (23.1)	1/13 (7.1)	
	MHR	15/170 (8.8)	83/170 (48.8)	
Valnemulin	MH	26/26 (100)	0/26 (0)	-
	MHS	13/13 (100)	0/13 (0)	
	MHR	170/170 (100)	0/170 (0)	

[a] MH=*M. hyopneumoniae*; MHS=*M. hyosynoviae*; MHR=*M. hyorhinis*, at N= 26, 13, and 170 strains, respectively.

Table 4.5 The correlation between MIC (µg/ml) and age of pigs being isolated *M. hyorhinis* isolated from their organs.

Antimicrobials	Age[a]	MIC (µg/ml)			Resistance[b]	P-value
		Range	MIC_{50}	MIC_{90}	(%)	
Doxycycline	Nursery	<0.048-12.5	1.56	6.25	0/91 (0)	0.639
	Finisher	<0.048-12.5	1.56	3.12	0/91 (0)	
Enrofloxacin	Nursery	<0.048-50	1.56	25	37/91 (40.7)	0.749
	Finisher	<0.048-50	1.56	25	34/79 (43.0)	
Lincomycin	Nursery	<0.006->12.5	0.78	3.12	4/91 (4.4)	0.107
	Finisher	<0.006->12.5	1.56	6.25	7/79 (8.9)	
Tiamulin	Nursery	<0.006-1.56	0.39	0.78	0/91 (0)	0.065
	Finisher	<0.006-1.56	0.39	0.78	0/79 (0)	
Tylosin	Nursery	<0.006->12.5	3.12	12.5	39/91 (42.9)	0.306
	Finisher	<0.006->12.5	6.25	>12.5	44/79 (55.7)	
Valnemulin	Nursery	<0.006-0.024	<0.006	0.024	0/91 (0)	0.226
	Finisher	<0.006-0.048	<0.006	0.024	0/79 (0)	

[a] nursery 6-8 wk old (N=91); finisher at slaughter (N=79)

[b] Hannan, 2000

Table 4.6 MIC_{90} (μg/ml) and the resistance (%R) of *Mycoplasma* spp. obtained from different farms.

Species	Doxycycline		Enrofloxacin		Lincomycin		Tiamulin		Tylosin		Valnemulin	
/Farms	MIC_{90}	%R	MIC_{90}	%R	MIC_{90}	%R	MIC_{90}	%R	MIC_{90}	%R	MIC_{90}	%R
M. hyopneumoniae												
AK	1.56	0	3.12	75	0.19	0	0.097	0	0.097	0	<0.006	0
CC	3.12	0	3.12	12.5	0.39	0	0.097	0	0.097	0	<0.006	0
FT	3.12	0	3.12	40	0.78	0	0.19	0	0.19	0	<0.006	0
PD	6.25	0	1.56	0	0.19	0	0.097	0	0.19	0	<0.006	0
KP	3.12	0	3.12	50	0.39	0	0.19	0	0.39	0	<0.006	0
M. hyosynoviae												
PN	25	62.5	1.56	0	3.12	0	0.024	0	3.12	0	<0.006	0
AK	25	20	1.56	0	3.12	0	0.024	0	6.25	20	<0.006	0
*M. hyorhinis**												
PN	6.25	0	6.25	87.5	3.12	0	0.78	0	12.5	62.5	0.012	0
AK	12.5	0	3.12	11.5	1.56	0	0.39	0	6.25	26.2	0.012	0
CC	3.12	0	1.56	0	3.12	0	0.78	0	12.5	100	0.048	0
FT	1.56	0	3.12	20	3.12	0	0.39	0	>12.5	100	0.024	0
PD	3.12	0	1.56	0	6.25	0	0.78	0	12.5	87.5	0.024	0
KP	3.12	0	50	80.3	>12.5	18	0.78	0	>12.5	42.6	0.024	0
MT	1.56	0	1.56	0	3.12	0	0.39	0	12.5	66.7	0.012	0
RM	3.12	0	12.5	100	3.12	0	0.39	0	12.5	80	0.024	0
MI	6.25	0	12.5	25	6.25	0	0.78	0	>12.5	100	0.024	0

*$P<0.05$ for MIC_{90}

CHAPTER V

Genetic characteristic of Thai porcine mycoplasmas using Pulsed-field gel electrophoresis (PFGE)

5.1 Introduction

Porcine mycoplasmas including *M. hyopneumoniae*, *M. hyosyno*viae, and *M. hyorhinis* are the major etiology of enzootic pneumonia, non-suppurative arthritis, and synovitis in piglets and fattening pigs as well as young breeders. During chronic infection period, this may be an importance cause of the economic loss in pig industry (Escobar et al., 2002; Hogg and Ross, 1986). To improve the control strategy of porcine mycoplasmas, the information of genetic variation together with distributing direction by molecular epidemiology have still need to be prepared (Kokotovic et al., 2002b).

However, the data based on genetic diversity among mycoplasmal strains have been very limited because of the difficulty in isolation method of these species. Moreover, the routine method in conventional laboratory techniques cannot differentiate the phenotypic and genetic characteristics. Certain molecular typing methods such as Amplified fragment length polymorphism analysis (AFLP), the polymerase chain reaction (PCR)-based method for genomic fringerfrinting of short DNA fragment produced by two different restriction enzyme, random amplified pholymorphic DNA (RAPD), the PCR-based method, and pulsed-field gel electrophoresis (PFGE). Recently, the studies are indicated the variable number of tandem repeats (VNTR) and multiple-locus VNTR, the genetic amplifying method to characterize bacteria without cultivation, as the alternative tools for mycoplasmal differentiation. The genomic diversity information is the useful data interpreting epidemiological distribution by determination of heterogeneity and homogeneity strains. The pattern data could reflect source of origins and the path of

transmission especially during chronic stage (Kokotovic et al., 2002a; Kokotovic et al., 2002b; Nathues, 2011; Stakenborg et al., 2005b; Vranckx et al., 2011). Moreover, the relation between the variation and antimicrobial resistant phenotypes as described in the Chapter 4 has never reported in porcine mycoplasmas.

PFGE is one of the most practical technique for revealing the genetic and can concretely illustrate of phylogenic location in the level of intra-species differentiation. This tool is used in general laboratory for genetic differentiation of several bacteria. The method has been recognized as being highly efficacy for revealing intraspecies genomic variations in mycoplasma (Kokotovic et al., 2002a; Prapasarakul et al., 2012; Tenover et al., 1995). The restriction enzymes used for PFGE of mycoplasmas were proposed by several studies. Use of *Sal*I and *Apa*I enzyme could reveal the similar efficacy for *M. hyopneumoniae* differentiation (Stakenborg et al., 2005b; Stakenborg et al., 2006) whereas *Bss*HII was used for *M. hyosynoviae* study (Kokotovic et al., 2002a). There were some restriction enzyme including *Bst*EII, *Xho*I, and *Sac*I recommended as the enzyme of choice for *M. hyorhinis* study (Darai et al., 1982). However, certain restriction enzymes for study of *M. hyorhinis* by PFGE did not give a consensual group by DNA bands in the later studies (Stakenborg et al., 2005b). Thus, various types of enzymes and condition had been tested to find out until finally, *Bst*EII has been chosen because of its most discriminated bands of *M. hyorhinis* differentiation.

This study aimed to reveal the genetic characteristics of porcine mycoplasmas by PFGE and to evaluate their relation to the distribution in the farms, area, age of pigs, and the antibiogram patterns of resistance. The genetic characteristics of the strains isolated from the sick pigs during the longitudinal sample collection were also evaluated.

5.2 Materials and methods

5.2.1 Mycoplasmal strains selection

The total of sixty-seven mycoplasmal field strains were selected from nursery and fattening pigs after MICs of isolates were tested as the following details:

- Fifteen field strains of *M. hyopneumoniae* were obtained from lung of slaughtered pigs in five pig farms (AK1, CC, FT, PD, and KP). Three isolates per farm were selected. Lung lesion observed during sample collection were represented the clinical symptom of pigs.

- Twelve field strains of *M. hyosynoviae* were obtained from tonsils (N=8) and lungs (N=4) of slaughtered pigs in two farms (PN and AK3). Seven and five strains were from PN an AK3 farms, respectively.

- Forty field strains of *M. hyorhinis* were obtained from various organs of nursery and slaughtered pigs from nine pig farms (PN, Ak1, CC, FT, PD, KP, MT, RM, and MI). When divided by the collected type, twenty-seven strains were obtained by one time collection from nine farms and thirteen strains were obtained by longitudinal collection every six months for one year from the sick pigs in two farms (AK and KP) named AK2 and AK3 and KP2 and KP3 for each samples collection. From one time collection, *M. hyorhinis* were obtained from nasal swab of nursery pigs (N=8), tonsil (N=14) and lung (N=5) of slaughtered pigs while six and seven strains were obtained from lung (N=7), tonsil (N=3), synovial fluid (N=1) and synovial tissue (N=2) of nursery and slaughtered pigs, respectively, by longitudinal collection.

The mycoplasmal type strains comprising J-strain, S16, and BTS-7 for *M. hyopneumoniae, M. hyosynoviae*, and *M. hyo*rhinis, respectively were derived from National Institute of Animal Health, Tsukuba, Japan.

5.5.2 Culture and identification

Mycoplasma strains were grown on their appropriated broth medium including BHL, HAM, and Modified Hayflick's for *M. hyopneumoiae*, *M. hyosynoviae*, and *M. hyorhinis*, respectively as described in Chapter 2 and 3 (Friis, 1972; Friis et al., 1991; Kawamura et al., 1982; Kobayashi et al., 1996a; Kokotovic et al., 2002a)

5.2.3 PGFE

5.2.3.1 Cell and DNA preparation

Mycoplasmal packed cells were harvested from 50 ml broth culture for *M. hyopneumoniae* and 30 ml broth for *M. hyosynoviae* and *M. hyorhinis* by centrifugation at 10,000 rpm X g for 50 minutes at 4°C. Cell pellets were washed for three times in 2 ml mycoplasma washing buffer (50mMTris-HCl, 10mM EDTA, 100mM NaCl, pH 7.2) and centrifuged at 13,000 rpm X g for 5 minutes. The washed pellets were resuspended in 200 ml of 0.85% NaCl. The suspension was mixed with an equal volume of 1.6% Low-melting-point agarose (Bio-Rad, USA) before loading into the plug molds (Bio-Rad, USA) at 80-100 µl/plug to set into block at 4°C for 10 minutes. After separating the plug from molds, the agarose plugs were soaked in 2 ml lysis buffer (50 mM EDTA, 1% N-lauroyl sarcosine, 0.1 mg/ml Proteinase K, 10 mM Tris-HCl, pH 8) at 50°C, for overnight. After incubation, the lysis process was repeated at 50°C, for overnight. The plugs were washed three times by distilled water at 50°C for a time and TE buffer (10mM Tris, 1mM EDTA, pH 7.6) at 50°C for two times and stored at 4°C until digested (Kokotovic et al., 2002a; Stakenborg et al., 2005b).

5.2.3.2 DNA digestion

Prior to the DNA digestion, the plugs were equilibrated in 1 X restriction enzyme buffer (delivered with the enzyme) at 37°C for 10 minutes. The restriction enzymes; *Sal*I (BioLabs, USA), *Bss*HII (BioLabs, USA), and *Bst*EII (BioLabs, USA) were used for the digestion of genomic DNA of *M. hyopneumoniae*, *M. hyosynoviae*, and *M. hyorhinis*, respectively. The genomic DNA were digested by the restriction enzyme at 30 units/plug and incubated at 37°C for 5 hours.

5.2.3.3 Gel electrophoresis

1.0 % Agarose gel prepared by the dilution of 1 gram pulsed field certified agarose (Bio-Rad, USA) in 100 ml 0.5XTBE buffer (44.5mM Tris, 44.5mM boric acid, 1mM EDTA, pH8.0) warmed by microwave, then, poured into PFGE tray (Bio-Rad, USA). After insertion of 15-wheels comb, gel was left in room temperature for 30 minutes. Removing the comb and loading 0.5 X TBE buffer into the wheels for lubrication. In case of 30-wheel comb and large tray, 150 ml of 1.0% agarose gel dilution were prepared. Lambda Ladder PFG Marker size range 50-1,000 kb (Biolabs, USA) was loaded as a marker for *M. hyopheumoniae* and *M. hyosynovoae* and CHEF DNA Size Standard size range 8-48 kb (Bio-Rad, USA) was loaded as a marker for *M. hyorhinis*.

Pulsed-field gel electrophoresis was performed with a CHEF-DR® III Pulsed Field Electrophoresis System (Bio-Rad, USA). After digestion by restriction enzyme, plugs were rinsed one time by 0.5XTBE buffer and loaded in1.0% pulsed field certified agarose (Bio-Rad, USA). 0.5 X TBE buffer (44.5mM Tris, 44.5mM boric acid, 1mM EDTA, pH8.0) plus 100mM thiourea (Sigma-Aldrich, USA) was added and electrophoresis was run at 14°C and 6V/cm, run parameters was set to *M. hyopneumoniae* and *M. hyosynoviae* at pulse parameters switching times from 0.5-8.5 seconds and run time 18 hours, and *M. hyorhinis* at 2.0-10.0 seconds, 10 hours.

5.2.3.4 Gel illustration

After electrophoresis, agarose gels were stained with dilution of ethidium bromine (Bio-Rad, USA) in distilled water 300 ml at the concentration of 1µg/ml, for 30 minutes and destrained with distilled water for 30 minutes and photographed under ultraviolet light (Kokotovic et al., 2002a; Stakenborg et al., 2005b).

5.2.4 Data analysis

DNA fragments were visualized and analyzed in a gel documentation system Bio-1 D++ software (Vilber-Lourmat, Germany). The dendrogram with homology coefficient 1.0% by the unweighted pair group method with arithmetic mean (UPGMA) was used for clustering the PFGE patterns. The same pattern was 100% similarity. The patterns were grouped at 80% similarity according to Dice similarity cut off (Valsesia et al., 2010). The profiles of individual strain including the antibiogram (the antimicrobial resistance profile of individual strains), farm, area, age, and organ were analyzed for the correlation with the DNA fragment patterns by descriptive analysis.

5.3 Result

The profiles of mycoplasmal strains chosen for PFGE study were illustrated in table 5.1-5.4.

5.3.1 *M. hyopneumoniae*

The DNA pattern consisted of DNA fragments at the band size ranged from approximately 700-970 kb. The cluster patterns of *M. hyopneumoniae* were varied to 16 patterns in four groups of 80% similarity and no 100% similarity of DNA fragment was detected. The highest homologous of DNA fragment similarity among *M. hyopneumoniae* were 95% similarity between Pattern 6 and 7 and between Pattern 11 and 12 of Group A which was the largest group consisted of 13 strains (Pattern 1-13). The type strain, J-strain, was the only strain in Pattern 14 in Group B (figure 5.1).

The distribution of groups in the different farms and the antibiogram patterns were illustrated in table 5.5. Group A was found in all five farms. The groups solely found in AK and KP farms were Group C and D, respectively. The antibiograms of *M. hyopneumoniae* strains consisted of two patterns, no resistance and enrofloxacin resistance. *M. hyopneumoniae* obtained from PD farm showed only no resistant pattern.

According to the farm areas, Group C and D were the specific groups in the Northeast and West areas, respectively. Group A was found in the North, Northeast, and the West areas. J strain was the sole strain in Group B (table 5.9). Among the *M. hyopneumoniae* resistant strains to enrofloxacin, the strains obtained from the different farms showed more different than the strains obtained from the same farm (table 5.10).

5.3.2 M. hyosynoviae

For *M. hyosynoviae*, twelve isolates collected from two positive farms (PN and AK) were genetically analyzed. The DNA pattern was in the size range of approximately 770-960 kb. The cluster patterns of *M. hyosynoviae* strains were varied to 11 patterns in five groups. There were two clusters with100% similarity of DNA fragment, Pattern 1 and 3, obtained from the same farm, PN. The high homologous strains, more than 80%

similarity, were obtained from the same farm, both PN and AK3. The type strain, S16, was found in Group C (figure 5.2).

The distribution of group in two farms and the antibiogram patterns were illustrated in table 5.6. Group B were obtained from both farms. Group A and C were solely obtained from PN farm and Group D and E were solely obtained from AK farm. Antibiogram patterns of no resistance and doxycycline resistance were found in both farms while the pattern of tylosin resistannce and enrofloxacin resisitance were solely found in PN and AK farms, respectively. The distribution of *M. hyosynoviae* in various areas was similar to the farm distribution because *M. hyosynoviae* strains were obtained from only PN and AK farms from two different areas, East and Northeast, respectively (table 5.9). Among the *M. hyosynoviase* doxycycline resistant strains, the strains obtained from the same farm showed less different DNA fragment than the strains obtained from the different farms (table 5.10).

5.3.3 *M. hyorhinis*

5.3.3.1 Cross-sectional collection

The relation of 27 isolates collected from the nine farms in a period of collection was generally analyzes. The DNA patterns consisted of 1-9 fragments from approximately 20-130 kb. The cluster patterns of *M. hyorhinis* strains were varied to 20 patterns in 15 groups. There were four clusters with100% similarity of DNA fragment, Pattern 3, 5, 7, and 18, obtained from FT, RM, MT, AK1 and CC farms. Pattern 5, 7, and 18 were obtained from the same farm within the patterns whereas Pattern 3 was obtained from several farms, AK1, CC, and FT. The Pattern 20 of Group O was totally different from other strains with no homologous score. Furthermore, the type strain, BTS-7, was very low homologous to other *M. hyorhinis* strains, at 13% homology.

The distribution of groups in these nine farms and the antibiogram patterns were illustrated in table 5.7. More than one group were distributed in each farm except RM farm. There was one antibiogram pattern in CC, PD, PN, and MT farms including the pattern of tylosin resistance and enrofloxacin and tylosin resistance. There was the distribution of varied groups in nursery and fattening pigs. Group B, C, E, G, and H were found in both ages. Group D and F were found in only nursery pig and Group J, K, L, M, and O were solely found in fattening pigs.

BTS-7 type strain was the sole strain in Group N. There were specific groups of the different areas including Group C and D for the North, Group E and L for the Northeast, Group A and O for the East, and Group F, K, and M for the West areas. Group B was distributed in the most areas except the West. Group H and I were distributed in the North and the West areas while Group G and J were distributed in the Northeast and the West areas (table 5.9).

5.3.3.2 Longitudinal collection

The DNA fragment obtained from the sick pigs were consisted of 4-11 fragments and size range from approximately 15-105 kb. The cluster patterns of *M. hyorhinis* strains were varied to 11 patterns in six groups. There were two clusters with100% similarity of DNA fragment, Pattern 8, and 9, obtained from the same farm at the different collecting period, KP2 and KP3 in Pattern 8, and the same period, AK3 in Pattern 9 (figure 5.4).

The group obtained from sick nursery of AK farm were Group A and E and Group C for the second and the third collection, respectively. The groups obtained from lung consolidation lesions and tonsils at slaughter were Group B and D for the second and the third collection of AK farm. Group C was the sole group obtained from sick pigs

during the two collection of KP farm. There were two antibiogram patterns for both collection of AK farm, no resistance and tylosin resistance. The antibiogram of the seconded collection in KP farm were enrofloxacin resistance and tylosin resistance whereas, in the third collection, the antibiogram patterns were changed to enrofloxacin resistance, enrofloxacin and tylosin resistance, and enrofloxacin, lincomycin, and tylosin resistance (table 5.8).

The PFGE different bands from sick pigs in longitudinal collection were more than the cross-sectional collection for the DNA fragment of tylosin resistant and enrofloxacin and tylosin resistant strains (table 5.10).

5.4 Discussion

The intraspecies diversity of porcine mycoplasmas were reported globally. The present study was one of the systematized report from the field strains of three species, *M. hyopneumoniae*, *M. hyosynoviae*, and *M. hyorhinis*, obtained from 2009 to 2011. The external factors including farms and areas, age of pigs, clinical signs (longitudinal collection) and antimicrobial susceptibility were counted for the possible relation with the genetic characteristics of porcine mycoplasmas by PFGE.

From the previous studies, the studies by PFGE of porcine mycoplasma were mostly detected the diversity of strains for molecular epidemiological study than the antimicrobial susceptibility relation while there were the alternation to utilize PFGE for investigation of antimicrobial susceptibility in other bacteria such as Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Prapasarakul et al., 2012; Stakenborg et al., 2005b; Stakenborg et al., 2006; Tenover et al., 1995). According to the present study, the different cluster of DNA fragment at 80% similarity did not well detect the genetic diversity to antimicrobial susceptibility whereas the different antibiogram patterns were

showed in the same pattern and group of 80% similarity of porcine mycoplasmas. The number of different DNA fragment (bands) among the isolates can be better detection of this slightly change in antimicrobial resistance from the present study. The present study showed the advantage of PFGE to detect the antimicrobial resistance of porcine mycoplasma by the diversity of DNA fragment since this is the first study to compare the genetic diversity of porcine mycoplasma to antimicrobial resistance. According to this method, the higher diversity from the strains obtained from the different farms was revealed as well as the increased diversity between the strains obtained from the sick pigs during the longitudinal sample collection to the antimicrobial resistance.

From the present study, the field strains of porcine mycoplasmas illustrated the intraspecies heterogeneity, especially, between the field strains. Furthermore, there was the high diversity between field strains and their type strains, J-strain, S16 and BTS-7. This was one of the evidence to cause high resistance to antimicrobials and the difficulty of mycoplasmal control. There were both the unique and similar groups of porcine mycoplasmas distributed in the different areas from the present study. The largest intraspecies diversity trended to be within *M. hyorhinis* field strains which were the wide range of infection and antimicrobial used from piglets to finisher pigs (Browning et al., 2010; Lin et al., 2006; Magnusson et al., 1998). From the present study, the genetic of *M. hyopneumoniae* were not highly diversity among five pig farms as well as the type strain, J-strain, and their antimicrobial susceptibility whereas the high DNA fragment diversity to the resistance for enrofloxacin was detected between different farms. Conversely, the high deviation of genetic characteristics of systemic mycoplasmas including *M. hyosynoviae* and *M. hyorhinis* might cause the difficulty of vaccine development and disease control by medication as well. The high genetic diversity of *M. hyorhinis* was related to the high resistance and MICs level analyzed in Chapter 4.

There were previous evidence shown the mutation of *M. hyorhinis* after several passages in some antimicrobials such as macrolides and lincosamides. The resistant

strains were detected the mutation of domains II and V in 23S rRNA of *M. hyorhinis* (Kobayashi et al., 2005; Ter Laak et al., 1991). *M. hyorhinis* isolated from slaughtered pigs were obtained from the young carriers to their house mates after weaning. The effective prophylactic strategies had to consider the management to decrease or eliminate the young carriers with biosecurity, compartment, and medication. The management during lactating period might be necessary because the young carriers obtained the organisms from upper respiratory tract of sows (Kobisch and Friis, 1996; Maes et al., 2008; Palzer et al., 2008; Stark et al., 2007). Furthermore, *M. hyorhinis* strains obtained from nursery pigs from the present study were slightly less variation than the strains from fattening pigs. The elimination of *M. hyorhinis* during nursery period might be more effective than in fattening pigs.

According to the diversity of porcine mycoplasmas from the present study, an isolate might not be the representative for whole strains in a farm. For the further study including the susceptibility and epidemiological study, the several isolates from different ages and time were strongly recommended.

5.5 Conclusion

In conclusions, the genetic characteristics of porcine mycoplasmas obtained from pig farms were varies among species and their diversity was occurred intraspecies at the different characters. The *M. hyopneumoniae* field strains were less diversity than *M. hyorhinis* and *M. hyosynoviae*. The high diversity caused the difficulty of vaccine development of these two species. The diversity of *M. hyorhinis* strains in nursery pigs was slightly less than the strains obtained from slaughtered pigs. There was no relation between the variation of genetic characteristics to the strains distributed in the different areas and the antimicrobial resistant patterns. Moreover, there was the different diversity between the antimicrobial resistant strains obtained from the same and different farms.

The antimicrobial resistant strains of *M. hyorhinis* obtained from the sick pigs showed more diversity than other field strains from this study.

Table 5.1 The profile of *M. hyopneumoniae* 15 strains chosen for PFGE and the lungscoring observed during sample collection

Isolates	Farm	Year of collection	Area	Age of pigs	Lung scoring (%)
1AK1lg109	AK1	2009	Northeast	Finisher	0
2AK1lg209	AK1	2009	Northeast	Finisher	10
3AK1lg409	AK1	2009	Northeast	Finisher	3
5CClg309	CC	2009	North	Finisher	1
6CClg409	CC	2009	North	Finisher	34
11CClg1609	CC	2009	North	Finisher	16
13FTlg209	FT	2009	North	Finisher	16
14FTlg509	FT	2009	North	Finisher	1
16FTlg1809	FT	2009	North	Finisher	17
18PDlg1709	PD	2009	North	Finisher	4
19PDlg2609	PD	2009	North	Finisher	7
20PDlg2809	PD	2009	North	Finisher	6
22KP1lg409	KP1	2009	West	Finisher	8
24KP1lg709	KP1	2009	West	Finisher	0
25KP1lg809	KP1	2009	West	Finisher	6

Table 5.2 The profile of *M. hyosynoviae* 12 strains chosen for PFGE

Isolates	Farm	Year of collection	Area	Age of pigs	Organ
1PNton309	PN	2009	East	Finisher	tonsil
2PNton909	PN	2009	East	Finisher	tonsil
3PNton1009	PN	2009	East	Finisher	tonsil
4PNton1109	PN	2009	East	Finisher	tonsil
5PNton1909	PN	2009	East	Finisher	tonsil
6PNton1309	PN	2009	East	Finisher	tonsil
8PNton2309	PN	2009	East	Finisher	tonsil
10AK3lg111	AK3	2011	West	Finisher	lung
11AK3lg2611	AK3	2011	West	Finisher	lung
12AK3lg2711	AK3	2011	West	Finisher	lung
13AK3lg3511	AK3	2011	West	Finisher	lung
15AK3ton211	AK3	2011	West	Finisher	tonsil

Table 5.3 The profile of *M. hyorhinis* 27 strains chosen for PFGE by cross-sectional collection

Isolates	Farm	Year of collection	Area	Age of pigs	Organ
1PNns2N09	PN	2009	East	Nursery	nasal swab
4PNton1F09	PN	2009	East	Finisher	tonsil
8PNlg14F09	PN	2009	East	Finisher	lung
11AK1ns28N09	AK1	2009	Northeast	Nursery	nasal swab
13AK1ton9F09	AK1	2009	Northeast	Finisher	tonsil
17AK1lg23F09	AK1	2009	Northeast	Finisher	lung
19CCns12N09	CC	2009	North	Nursery	nasal swab
21CCton3F09	CC	2009	North	Finisher	tonsil
23CClg6F09	CC	2009	North	Finisher	lung
26FTton1F09	FT	2009	North	Finisher	tonsil
27FTton2F09	FT	2009	North	Finisher	tonsil
29FTlg11F09	FT	2009	North	Finisher	lung
33PDns7N09	PD	2009	North	Nursery	nasal swab
34PDton1F09	PD	2009	North	Finisher	tonsil
38PDton14F09	PD	2009	North	Finisher	tonsil
39KP1ns3N09	KP1	2009	West	Nursery	nasal swab
43KP1ton2F09	KP1	2009	West	Finisher	tonsil
47KP1ton14F09	KP1	2009	West	Finisher	tonsil
49MTns4N09	MT	2009	West	Nursery	nasal swab
51MTton1F09	MT	2009	West	Finisher	tonsil
53MTton3F09	MT	2009	West	Finisher	tonsil
56RMns3N09	RM	2009	Northeast	Nursery	nasal swab
57RMton11F09	RM	2009	Northeast	Finisher	tonsil
58RMton15F09	RM	2009	Northeast	Finisher	tonsil
60MIns27N09	MI	2009	Northeast	Nursery	nasal swab
62MIton2F09	MI	2009	Northeast	Finisher	tonsil
66MIlg20F09	MI	2009	Northeast	Finisher	lung

Table 5.4 The profile of *M. hyorhinis* 13 strains chosen for PFGE by longitudinal collection

Isolates	Farm	Year of collection	Area	Age of pigs	Organ
21AK2lg4N10	AK2	2010	Northeast	Nursery	lung
23AK2ton1N10	AK2	2010	Northeast	Nursery	tonsil
28AK2lg4F10	AK2	2010	Northeast	Finisher	lung
34KP2lg1N10	KP2	2010	West	Nursery	lung
48KP2lg3F10	KP2	2010	West	Finisher	lung
53KP2ton4F10	KP2	2010	West	Finisher	tonsil
80AK3sf5N11	AK3	2011	Northeast	Nursery	synovial fluid
84AK3st5N11	AK3	2011	Northeast	Nursery	synovial tissue
86AK3lg9F11	AK3	2011	Northeast	Finisher	lung
89AK3ton2F11	AK3	2011	Northeast	Finisher	tonsil
91KP3lg2N10	KP3	2010	West	Nursery	lung
101KP3st1N10	KP3	2010	West	Nursery	synovial tissue
104KP3lg17F10	KP3	2010	West	Finisher	lung

Table 5.5 The relation between PFGE groups of *M. hyopneumoniae* to farms and antibiogram patterns

Farm	Distribution of groups*	Antibiogram patterns**
CC	A	1, 2
FT	A	1, 2
PD	A, D	1
AK	A, C	1, 2
KP	A	1, 2

*Group of 80% similarity threshold (figure 5.1)

**Pattern 1=no resistance; 2=enrofloxacin resistance

Table 5.6 The relation between PFGE groups of *M. hyosynoviae* to farms and antibiogram patterns

Farm	Distribution of groups*	Antibiogram patterns**
PN	A, B, C	1, 2, 3
AK	B, D, E	1, 2, 4

*Group of 80% similarity threshold (figure 5.2)

**Pattern 1=no resistance; 2=doxycycline resistance; 3=tylosin resistance; 4=doxycycline and enrofloxacin resistance

Table 5.7 The relation between PFGE groups of *M. hyorhinis* to farms, age of pigs, and antibiogram patterns in cross-sectional collection.

Farm	Distribution of groups*			Antibiogram patterns**
	by Farm	by Age		
		Nursery	Fattening	
CC	B, C	C	B	3
FT	E, C	-	B, C	3, 4
PD	D, H, I	D	H, I	3
AK	B, L	B	B, L	1, 3
RM	E	E	E	2, 4
MI	G, J	G	G, J	3, 4
PN	A, B, O	A	B, O	4
KP	F, I, K	F	K, I	2, 5
MT	H, M	H	M	3

*Group of 80% similarity threshold (figure 5.3)

**Pattern 1=no resistance; 2=enrofloxacin resistance; 3=tylosin resistance; 4=enrofloxacin and tylosin resistance; 5=enrofloxacin, lincomycin, and tylosin resistance

Table 5.8 The relation between PFGE groups of *M. hyorhinis* to farms, age of pigs, and antibiogram patterns obtained from sick pigs in longitudinal collection.

Farm	Year of collection	Distribution of groups*			Antibiogram patterns**
		by Farm	by Age		
			Nursery	Fattening	
AK2	2010	A, B, E	A, E	B	1, 3
AK3	2011	C, D	C	D	1, 3
KP2	2010	C	C	C	2, 3
KP3	2010	C	C	C	2, 4, 5

*Group of 80% similarity threshold (figure 5.4)

**Pattern 1=no resistance; 2=enrofloxacin resistance; 3=tylosin resistance; 4= enrofloxacin and tylosin resistance; 5=enrofloxacin, lincomycin, and tylosin resistance

Table 5.9 The PFGE group of porcine mycoplasmas distributed in areas

Species	Specificity group to area	Group* in area and type strains				
		North	Northeast	East	West	Type strain**
M.	yes	-	C	-	D	B
hyopneumoniae	no	A	A	-	A	-
M. hyosynoviae	yes	-	*D, E*	*A*	-	-
	no	-	*B*	*B, C*	-	*C*
M. hyorhinis	yes	**C, D**	**E, L**	**A, O**	**F, K, M**	**N**
	no	**B, H, I**	**B, G, J**	**B**	**G, H, I, J**	-

*Groups of each *Mycoplasma* spp. were different and indicated by normal, italic and bold typing of group names

**J, S16, and BTS-7 for *M. hyopneumoniae, M. hyosynoviae, and M. hyorhinis*, respectively

Table 5.10 Comparison of pulsed-field gel electrophoresis (PFGE) band difference of porcine mycoplasma and the antimicrobial resistance

Mycoplasma spp.*	No. of PFGE different bands***				
	Antimicrobial resistance **				
	Doxy	Enro	Linco +Tylo +Enro	Tylo	Tylo +Enro
MH from same farm		1[a]			
MH from different farms		4-9[d]			
MHS from same farm	0-1[a]				
MHS from different farms	4[c]				
MHR from one collection		4[c]	3[b]	0-2[b]	0-2[b]
MHR from longitudinal collection		0-4[c]		1-4[c]	0-4[c]

* MH= *M. hyopneumoniae*; MHS= *M. hyosynoviae*; MHR= *M. hyorhinis*

**Doxy= doxycycline; Enro= enrofloxacin; Linco= lincomycin; Tylo= tylosin

***Interpreting DNA fragment patterns is indicated by superscripts: [a]indistinguishable, [b]closely related, [c]possibly related, and [d]different.

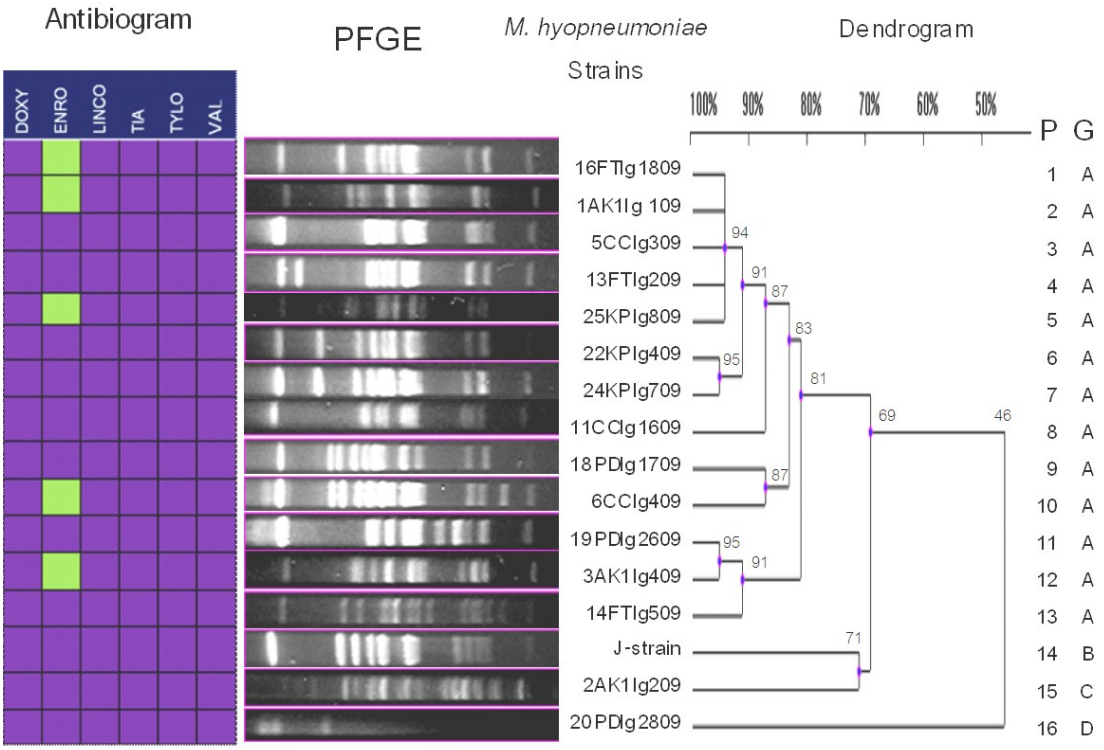


Figure 5.1 Dendrogram of *M. hyopneumoniae* 15 field strains from five farms (AK1, CC, FT, PD, KP1) and 1 type strain, J. Antibiogram, showed the resistant patterns, are depicted in green, and violet for resistant and no resistant effect (DOXY, doxycycline; ENRO, enrofloxacin; LINCO, lincomycin; TIA, tiamulin; TYLO, tylosin; VAL, valnemulin). P is PFGE pattern. G is Group of the 80% similarity threshold. Percent homology is calculated at homology coefficient 1.0% and is represented by UPGMA.

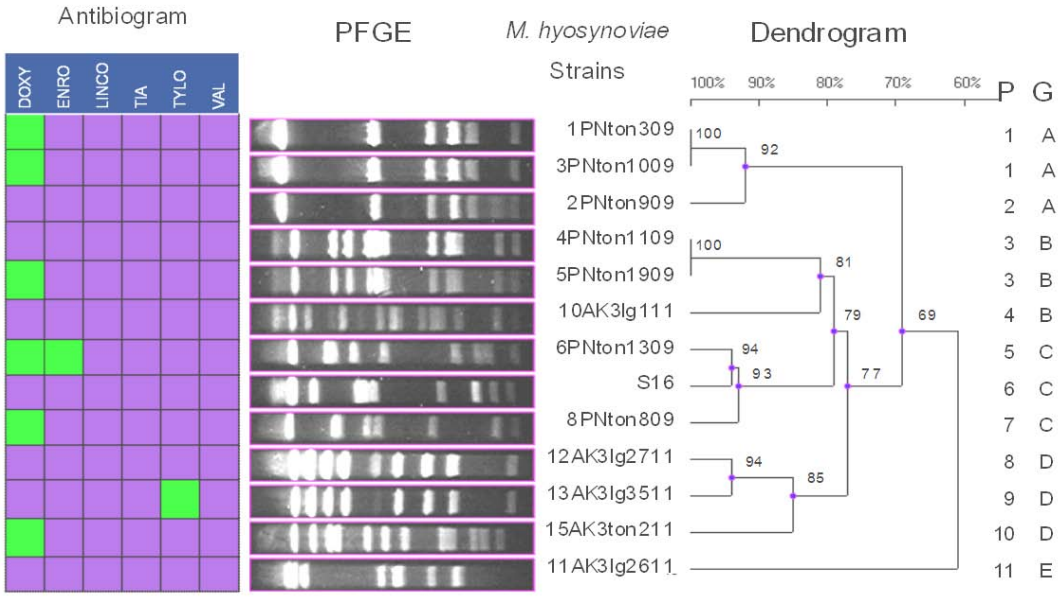


Figure 5.2 Dendrogram of *M. hyosynoviae* 12 field strains from two farms (PN, AK3) and 1 type strain, S16. Antibiogram, showed the resistant patterns, are depicted in green and violet for resistant and no resistant effect (DOXY, doxycycline; ENRO, enrofloxacin; LINCO, lincomycin; TIA, tiamulin; TYLO, tylosin; VAL, valnemulin). P is PFGE pattern. G is Group of the 80% similarity threshold. Percent homology is calculated at homology coefficient 1.0% and is represented by UPGMA.

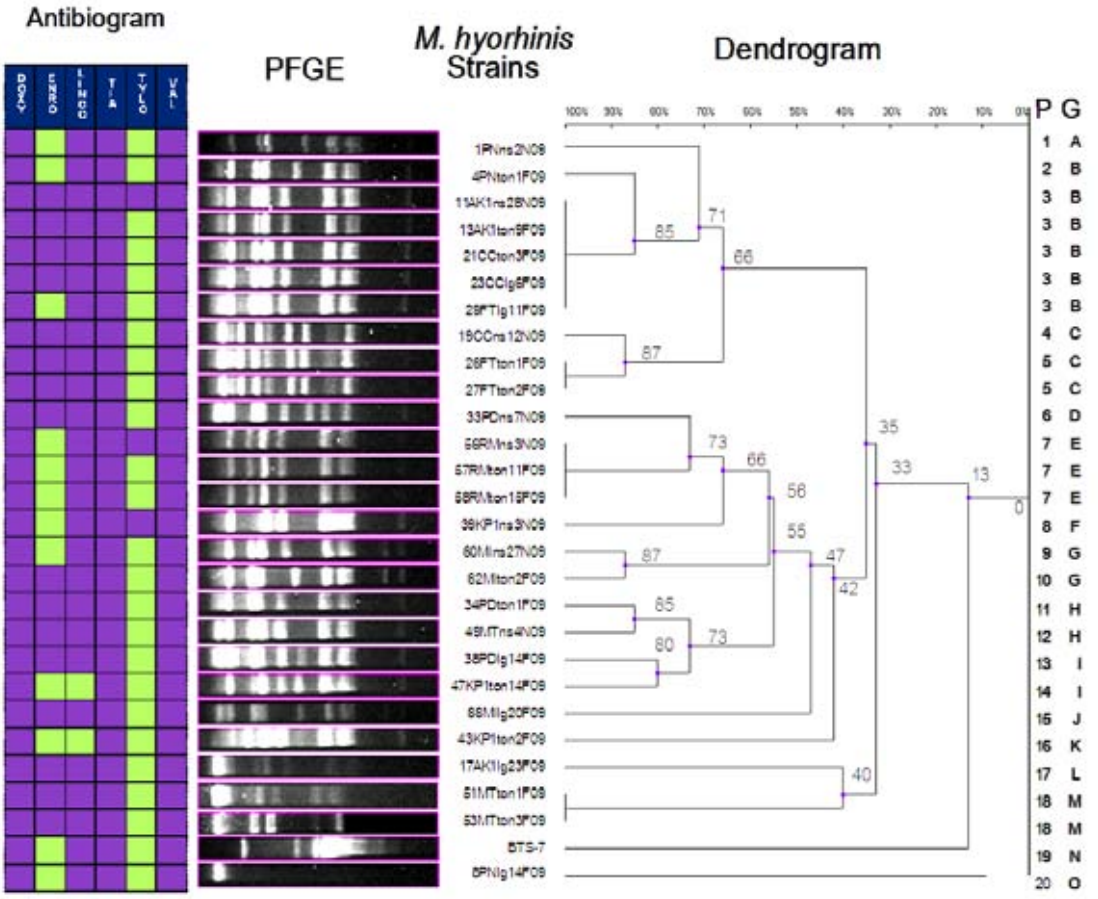


Figure 5.3 Dendrogram of *M. hyorhinis* 27 field strains from nine farms (AK1, PN, CC, FT, PD, KP1, MT, RM, MI), cross-sectional collection, and 1 type strain, BTS-7. Antibiogram, showed the resistant patterns, are depicted in green and violet for resistant and no resistant effect (DOXY: doxycycline; ENRO: enrofloxacin; LINCO: lincomycin; TIA: tiamulin; TYLO: tylosin; VAL: valnemulin). P is PFGE pattern. G is Group of the 80% similarity threshold. Percent homology is calculated at homology coefficient 1.0% and is represented by UPGMA.

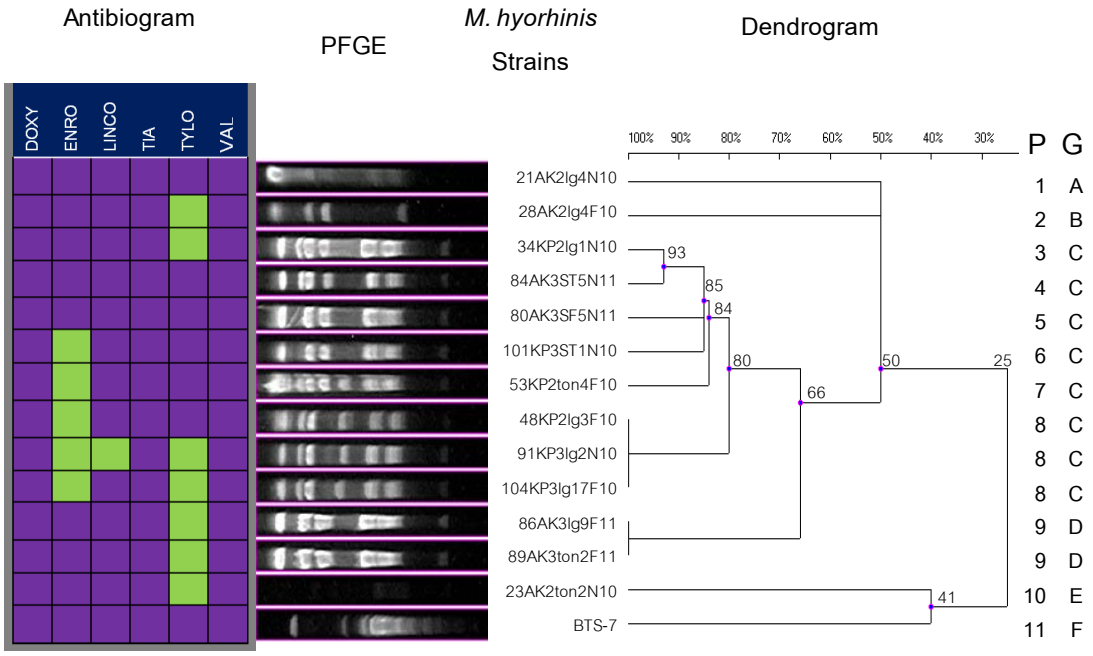


Figure 5.4 Dendrogram of *M. hyorhinis* 13 field strains, longitudinal collection from the sick pigs of two farms (AK and KP), and 1 type strain, BTS-7. Antibiogram, showed the resistant patterns, are depicted in green and violet for resistant and no resistant effect (DOXY, doxycycline; ENRO, enrofloxacin; LINCO, lincomycin; TIA, tiamulin; TYLO, tylosin; VAL, valnemulin). P is PFGE pattern. G is Group of the 80% similarity threshold. Percent homology is calculated at homology coefficient 1.0% and is represented by UPGMA.

CHAPTER VI

CONCLUSION

This dissertation aimed to investigate the four objectives included in the topic "genetic characteristics and antimicrobial susceptibility of porcine mycoplasma isolated in Thailand". The study covered into four objectives of investigations and determinations. Firstly, the procedures of mycoplasmal diagnosis by using various organs were determined in term of validation and the results could correctly lead to the occurrence of three porcine *Mycoplasma* spp. distributing in Thai commercial farms. Thereafter, factors associated managements and strategic controls obtained from questionnaires were statistically analyzed for the risk factors of mycoplasmal occurrence in pig farms. Thirdly, the susceptibility levels of the selected isolates derived from the surveillance were determined in term of minimum inhibitory concentration (MIC) at MIC_{50} and MIC_{90} against the routine antimicrobials used in Thai pig farms. Finally, the genetic characteristics of the different MICs isolates obtained from various farms and areas were analyzed by the pulsed-field gel electrophoresis (PFGE) technique and their dendrogram was illustrated with the antibiogram of the antimicrobial resistant patterns. The result showed the intraspecies variation of *M. hyopneumoniae, M. hyosynoviae*, and *M. hyorhinis* strains and their relation to antimicrobial susceptibility as well as other factors including the difference of farms, areas, pig ages, and clinical diseases of the sources of mycoplasmal isolates.

To investigate whether direct PCR (DP) gives similar results to culture prior to PCR (CPP) for the detection of mycoplasmas in different types of pig tissues a total of 724 samples obtained from lungs, tonsils, and synovial fluids from 270 slaughtered pigs were assessed. The history of clinical signs, lung score and the presence of joint lesions were recorded during sample collection. The rates of detection of *M. hyopneumoniae*, *M. hyosynoviae* and *M. hyorhinis* isolates using both procedures were evaluated. DP

gave a higher detection rate for *M. hyopneumoniae* (31.5%) than CCP (15.6%) from lung tissues. The tonsil detection was much different between *M. hyorhinis* detected by CPP (63.5%) than DP (5.3%).The positive rate of *M. hyosynoviae* derived from tonsil by CPP (7.9%) was closed to DP (6.8%). The synovial fluid samples did not yield any positive *M. hyorhinis* from CPP whereas 37.2% were positive using DP. In contrast, using tonsil sample for detection by CPP showed a much higher positive number than that of DP, 63.5% and 5.3%, respectively. Both procedures yielded similar results for *M. hyosynoviae* from tonsils and *M. hyorhinis* from lungs while the sensitivity of the DP test was higher than CPP for *M. hyopneumoniae* detection in lung tissue. Synovial fluid of slaughtered pigs was unable to culture any mycoplasma organisms but this may be because it is unsuited to the procedure. It appears the accuracy of mycoplasmal detection test may depend upon the type of sample used and the detection procedure deployed.

Using the culture method, porcine mycoplasmas were identified in 270, 270, 266, and 213 samples of nasal swabs lung tissue, tonsil tissue, and synovial fluid of slaughtered nursery pigs, respectively (2009). *M. hyopneumoniae* was found in 0.4% of the lung samples and 15.6% of nasal swabs taken from slaughtered nursery pigs. *M. hyosynoviae* was found in 7.9%. of the tonsil amples while at *M. hyorhinis* was found to present in 61.1%, 18.2%, and 63.5% of nostril, lungs, and tonsil samples, respectively. No mycoplasma could be isolated from the synovial fluid of the slaughtered pigs.

The presence of mycoplasma detected varied regionally as follows, in the North, Northeast, East, and West 17.6%, 3.3%, 0, and 4.9% for *M. hyopneumoniae*, 0%, 3.3%, 40% and 0% for *M. hyosynoviae*, and 41.2%, 40.2%, 46%, and 64.7% for *M. hyorhinis*, respectively. The longitudinal sample collection from various organs of the sick nursery pigs and the lung lesions and tonsils of slaughtered pigs was collected from 2009 to 2011. The occurrence of *M. hyopneumoniae* in lung samples was 7.7%. for *M. hyosynoviae* in the lungs and tonsils it was 1.5% and 1%, respectively. *M. hyorhinis* was

found to be present in 48.3% of lung samples and 48.5%, of tonsil samples 22.3% of lymph nodes and 7.8%, of the synovial tissue samples.

The risk factors including management, vaccination and medication on the occurrence of porcine mycoplasmas were assessed. Two selected farms were used for longitudinal sampling to find the seasonal effect on mycoplasmal occurrence in lung tissue. Data on management and prophylactic programs were recorded by means of a questionnaire. Logistic regression analyses were conducted to evaluate the risk factors at $P < 0.05$. The result of longitudinal study illustrated that seasonality, in Thailand, had no effect to the number of positive lung samples at $P<0.05$. The factors influencing the increase in *M. hyopneumoniae* positive lung samples were the gilt acclimatization (direct contact), single site management, without all-in all-out in the fattening unit, and lack of *M. hyopneumoniae* vaccination. The increase of *M. hyorhinis* obtained from lung samples was due to single site production system, without all in all out in fattening, and no sow and nursery medication. The factors that promoted the increase in systemic *Mycoplasma* spp., in tonsils including the no all-in-all-out in fattening for *M. hyorhinis*, no medication in piglet, starter, and grower medication for *M. hyosynoviae* incretion and no finisher medication for both *M. hyorhinis* and *M. hyosynoviase*. The occurrence of *M. hyorhinis* from lungs was associated with lung lesion of slaughtered pigs and mainly recovered from nursery pigs in all sample organs. *M. hyopneumoniae* vaccination was the key factor to reduce the occurrence of *M. hyopneumoniae* in lung in stead of medication. The herd with gilt acclimatization vaccinated with PRRSV influenced to high number of *M. hyosynoviae* in tonsils and *M. hyorhinis* in nasal swab. In conclusions, the promotion of direct contact and the immune-suppression in breeder and fattening units influenced the occurrence of porcine mycoplasmas. To improve of farm management, the vaccination and medication were the important factors to be managed by specific *Mycoplasma* spp.

To determine the antimicrobial susceptibility levels of porcine mycoplasmas, a total of 209 filed isolates were determined by the values of MIC by the broth microdilution method against six antimicrobials, including doxycycline, enrofloxacin, lincomycin, tiamulin, tylosin, and valnemulin. The type strains; *M. hyopneumoniae* J-strain, *M. hyosynoviae* S-16, and *M. hyorhinis* BTS-7 were used for control of the method. MICs were report by MIC_{50}, and MIC_{90} and the susceptibility breakpoint interpretations according to the previous reports (Hannan, 2000). The antimicrobial susceptibility levels were statistically analyzed for the relation between antimicrobial susceptibility and mycoplasmal species and age of pig. The results showed that enrofloxacin and tylosin were not the satisfied antimicrobials, *in vitro*, with high rate of resistance (38-40%) and MIC_{90} ranging from 12.5-25 µg/ml. There were a few resistant isolates to doxycycline and lincomycin at 2.9% and 3.3%, with MIC_{90} ranging from 6.25 and 3.12 µg/ml, respectively. *In vitro* study, tiamulin and valnemulin were the most effective antimicrobials for inhibiting the tested mycoplasma isolates and gave MIC_{90} ranging from 0.024-0.78 µg/ml. Regarding to age comprising nursery and finisher groups, there was no statistic relation to the resistant rate at $P< 0.05$. The susceptibility among the different *Mycoplasma* spp. was significant for doxycycline, enrofloxacin and tylosin. *M. hyosynoviae* was highly resistant to doxycycline and tylosin at 46.2% and 7.1%. respectively. *M. hyopneumoniae* was highly resistant to enrofloxacin at 34.6%. *M. hyorhinis* was the most resistant mycoplasmal species that was highly resistant to tylosin, enrofloxacin and lincomycin at 48.8%, 41.8%, and 6.5%, respectively. On the basic of the susceptibility levels, the results could be strongly predictable the satisfactory use of pleuromutilin group and the use of enrofloxacin and doxycycline might not be suitable for controlling of porcine respiratory disease complex.

The genetic variation of the selected porcine mycoplasmas were demonstrated using the pulsed-field gel electrophoresis (PFGE) and aligned into dendrogram and their antibiogram. The tested isolates were selected by mean of different antimicrobial

susceptibility and their relations to ages, clinical signs, and susceptibility level was analyzed. The restriction enzymes comprising *Sal*I, *Bss*HII and *Bst*EII were used for digestion the DNA derived from *M. hyopnemoniae*, *M. hyosynoviae*, and *M. hyorhinis*, respectively. The dendrogram with homology coefficient 1.0% by the unweighted pair group method with arithmetic mean (UPGMA) was used for clustering the DNA finger print patterns at 80% similarity for the same group. The PFGE patterns of *M. hyopneumoniae* showed a variety into 16 cluster patterns in four groups and no 100% homologous DNA fragment was detected among isolates. The PFGE patterns of *M. hyosynoviae* clustered into 11 cluster patterns in five groups of 80% similarity. The PFGE patterns of *M. hyorhinis* showed a variation of 20 cluster patterns in 15 groups. Although the antibiogram profiles had no relation to the DNA cluster profiles but the different DNA fragment band provided the evidence of mycoplasmal diversity obtained from different farms and the sick pigs. The diversity of porcine mycoplasmas had no relation to the different areas as well as the age of pigs. However, there is the slightly less diversity of *M. hyorhinis* strains obtained from nursery than the strains from slaughtered pigs. The high variation of *M. hyorhinis* strains were complied to their high antimicrobial resistance whereas *M. hyopneumoniae* strains diversity were low.

The advantages of this investigation were provided the evidence of three porcine mycoplasmas occurrence in Thai pig farms. The risk factors, antimicrobial susceptibility, and the genetic characteristics provided the practical points to eliminate porcine mycoplasmas from pig farms. According to the different risk factors and the antimicrobial susceptibility of the different *Mycoplasma* spp., the surveillance and identification was needed to find out the target *Mycoplasma* spp. in the farm. MICs were necessary for the selection of effective antimicrobial agents to be a tool for treatment during the process of eradication. Because of the slightly less diversity in nursery, the medication should be applied for the young animal treatment with the strict management to inhibit the direct contract among pigs including the two-sites management and all in

all out management in fattening period or young breeders. The sow donor need to be tested for free mycoplasma load in nostrils before the acclimatization procedure. The PRRSV vaccination should be avoid in the *M. hyorhinis* and *M. hyosynoviae* positive farms. The reduction of viral load in farm environment and in the nostril of pigs should be focused as well. *M. hyopneumoniae* vaccine was necessary to control *M. hyopneumoniae* occurrence in lungs of fattening and the vaccination should be applied for the reduction of the occurrence in breeder pigs. Since the sick animals caused the higher diversity strains of antimicrobial resistance, the depopulation or culling of sick pigs was better than repeated treatment. Furthermore, the scientific investigation was the key success for educating the farm owners, managers and workers to understand the reasons supported the important to eliminate porcine mycoplasmas in pig farms.

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APPENDICES

APPENDIX A

Sample collection

1. Organ collection for cross-sectional collection

Nursery	Nasal swab 30 samples/farm
Finisher	Lung 30 samples/farm, tonsil 30 samples/farm, synovial fluid 30 samples/farm

2. Organ collection for longitudinal collection from AK and KP farms

Nursery	Lung, tonsil, lymph node, synovial fluid and tissue
Finisher	Lung and tonsil

3. Questionnaires

แบบสอบถามงานวิจัย

ลักษณะทางพันธุศาสตร์และความไวรับต่อยาต้านจุลชีพของเชื้อมัยโคพลาสมาที่แยกได้จากสุกรในประเทศไทย

แบบสอบถามแบ่งออกเป็น 2 ส่วน ดังนี้

1. ข้อมูลทั่วไปของผู้ตอบแบบสอบถาม (General Background)

2. มาตรการสำหรับการป้องกันโรค (Prevention)

ส่วนที่ 1 ข้อมูลทั่วไปของผู้ตอบแบบสอบถาม (General Background)

1. สถานที่ตั้งฟาร์ม อำเภอ_____จังหวัด_____

2. ชนิดของสุกรในฟาร์ม

☐ 2.1 แม่สุกร จำนวน_____ตัว

☐ 2.2 พ่อสุกร_____ตัว

☐ 2.3 สุกรสาวทดแทน_____ตัว

แหล่งที่มาของสุกรสาวทดแทน

☐ เป็นสุกรที่ผลิตได้ในฟาร์ม ☐ นำเข้ามาจากแหล่งอื่น

☐2.4 สุกรอนุบาล_____ตัว

☐ 2.5 สุกรขุน จำนวน_____ ตัว

แหล่งสุกรขุน

☐ เป็นสุกรที่ผลิตได้ในฟาร์ม ☐ นำเข้ามาจากแหล่งอื่น

3. สถานที่เลี้ยง (Sites) การจัดการสุกรอนุบาล-ขุน

☐ สุกรขุนเลี้ยงรวมอยู่กับฟาร์มสุกรพ่อ-แม่พันธุ์ (one-site)

☐ เลี้ยงแยกสุกรขุนและสุกรพ่อ-แม่พันธุ์ (2-sites)

☐ สุกรขุนแยกกับฟาร์มแม่พันธุ์ตั้งแต่หย่านมและเลี้ยงในโรงเรือนเดียวถึงขาย (Wean to Finish)

☐ โรงเรือนอนุบาลอยู่กับฟาร์มแม่พันธุ์แต่โรงเรือนขุนแยกออกไป

☐ โรงเรือนอนุบาลอยู่ที่ฟาร์มสุกรขุน

4. การไหลของสุกร (Pig Flow)

☐ เข้าหมดออกหมดในโรงเรือนเดียวกัน (All-in-all-out) อายุต่างกันไม่เกิน 1 สัปดาห์

☐ มีสุกรอายุต่างกัน 1-2 สัปดาห์อยู่ในโรงเรือนเดียวกัน

☐มีสุกรต่างอายุเกินกว่า 2 สัปดาห์อยู่ในโรงเรือนเดียวกัน

5. สภาพโรงเรือนสุกร

กลุ่มสุกร	**โรงเรือนเปิด**	**โรงเรือน** EVAP	**พัดลม**
พ่อพันธุ์			
อุ้มท้อง			
คลอด			
ทดแทน			
อนุบาล			
ขุน			

6. โรคทางเดินหายใจจากไวรัสที่พบในฟาร์ม (ตอบได้มากกว่า 1 ข้อ)

☐ พีอาร์อาร์เอส

☐ เซอร์โคไวรัส

☐ หวัดสุกร

☐ อื่นๆ (ระบุ)_____

ส่วนที่ 2 มาตรการสำหรับการป้องกันโรค (Prevention)

นิยาม มาตรการสำหรับการป้องกันโรค (Prevention) หมายถึง การปฏิบัติที่ใช้เพื่อการป้องกันก่อนสุกรเกิดอาการป่วย

มาตรการทั่วไป (Biosecurity)

7. การตรวจสอบคุณภาพอาหาร หรือ วัตถุดิบอาหารสัตว์ที่ซื้อเข้าฟาร์ม

☐ มีการตรวจทุกชุดที่นำเข้า อย่างน้อยทางกายภาพ เช่น กลิ่น สี ความสะอาด การปนปลอม

☐ไม่มีการตรวจเป็นประจำ

☐ไม่เคยตรวจเลย

8. การตรวจสอบคัดเลือกสุกรพันธุ์ที่นำเข้าฟาร์ม หรือสุกรที่ส่งมาจากหน่วยผลิตในฟาร์ม

☐ มีการตรวจทุกชุดที่นำเข้าไม่นำสุกรที่มีอาการป่วยเข้าฝูง

☐ ตรวจเฉพาะสุกรที่นำเข้าจากภายนอกฟาร์ม

☐ ไม่เคยตรวจเลย

9. การตรวจสอบคัดเลือกสุกรขุนที่นำเข้าฟาร์ม หรือสุกรที่ส่งมาจากหน่วยผลิตในฟาร์ม

☐ มีการตรวจทุกชุดที่นำเข้า ไม่นำสุกรที่มีอาการป่วยเข้าฝูง

☐ ตรวจเฉพาะสุกรที่นำเข้าจากภายนอกฟาร์ม

☐ไม่เคยตรวจเลย

10. ระยะห่างระหว่างโรงเรือนขุน

☐ น้อยกว่า 10 เมตร

☐ ตั้งแต่ 10 เมตรขึ้นไป

11. พื้นที่ต่อสุกรขุน 1 ตัว

☐ น้อยกว่า 1 ตารางเมตร

☐ ตั้งแต่ 1 ตารางเมตรขึ้นไป

12.การควบคุมคนภายนอกเข้าออกฟาร์ม

☐ ไม่มีมาตรการใดๆ

☐ มีมาตรการชัดเจน เช่น จำกัดจำนวนคน อาบน้ำเปลี่ยนเสื้อผ้าที่ฟาร์มจัดไว้ให้

☐ ไม่อนุญาตคนภายนอกเข้าฟาร์มเลย

13.การควบคุมคนงานเข้าออกฟาร์ม

☐ ไม่มีมาตรการใดๆ

☐ มีมาตรการชัดเจน เช่น อาบน้ำเปลี่ยนเสื้อผ้าที่ฟาร์มจัดไว้ให้

14. การพ่นยาฆ่าเชื้อในโรงเรือนระหว่างการเลี้ยง

☐ ไม่มี

☐ มีเป็นประจำแต่น้อยกว่าวันละครั้ง (ระบุ)_____วัน/ครั้ง

☐ มีเป็นประจำวันละครั้ง หรือมากกว่า

☐ มีบ้างแต่ไม่เป็นประจำ

การป้องกันเ โรคมัยโคพลาสมา

15. การใช้ยาในอาหารเพื่อควบคุมมัยโคพลาสมาในปัจจุบัน

15.1 การใช้ยาผสมอาหาร

□ไม่ใช้

□ใช้ โปรดระบุรายละเอียดการใช้ยา

อายุสุกร	ชื่อยา	ขนาดการใช้	ระยะเวลา(วัน)
□พ่อแม่พันธุ์			
□เลี้ยราง			
□อนุบาลอายุ___สัปดาห์			
□สุกรเล็กอายุ___สัปดาห์			
□สุกรรุ่นอายุ___สัปดาห์			
□สุกรขุนอายุ___สัปดาห์			
□สุกรทดแทนอายุ___ สัปดาห์			

15.2 การใช้ยาละลายน้ำ

□ไม่ใช้

□ใช้ โปรดระบุรายละเอียดการใช้ยา

อายุสุกร	ชื่อยา	ขนาดการใช้	ระยะเวลา(วัน)
□พ่อแม่พันธุ์			
□อนุบาลอายุ___ สัปดาห์			
□ขุนอายุ___สัปดาห์			
□ทดแทนอายุ___ สัปดาห์			

15.3 การใช้ยาฉีด

□ไม่ใช้

□ใช้ โปรดระบุรายละเอียดการใช้ยา

อายุสุกร	ชื่อยา	ขนาดการใช้	ระยะเวลา(วัน)
☐พ่อแม่พันธุ์			
☐อนุบาลอายุ___ สัปดาห์			
☐ขุนอายุ___สัปดาห์			
☐ทดแทนอายุ___ สัปดาห์			

การสร้างภูมิคุ้มกันต่อมัยโคพลาสมา

16. การใช้วัคซีนป้องกันมัยโคพลาสมา ไฮโอนิวมอเนีย

☐ไม่ใช้

☐ใช้ โปรดระบุรายละเอียดการใช้วัคซีน

อายุสุกร	ชื่อวัคซีน	ขนาดการใช้ (ซีซี/ตัว)	อายุที่ฉีดครั้งที่ 1	อายุที่ฉีดครั้งที่ 2 (ถ้ามี)
☐พ่อแม่พันธุ์				
☐ลูกดูดนม				
☐อนุบาล				
☐ขุน				
☐ทดแทน				

ขอขอบพระคุณทุกท่านที่ได้ให้ความร่วมมือในการตอบแบบสอบถามครั้งนี้

สพ.ญ.เมตตา เมฆานนท์

นิสิตผู้ทำการวิจัย

สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX B

Media for mycoplasmal culture and PCR-component

BHL Broth

Brucella broth	2.9	g
Lactalbumin hydrolysate	1.0	g
Stock salt	25	ml
Distiled water (DW)	350	ml
1% Phenol red	0.5	ml
Noble agar	5.1	g
Autoclave 115°C, 15 minutes, cool down in ice		
Swine serum, inactivated at 56°C, 30 minutes	50	ml
Horse serum	50	ml
25% Fresh yeast extract	25	ml
Adjust pH 7.5-7.6 with 5% Na_2CO_3		

BHL Agar

Replaced Brucella broth 2.9 g by Brucella agar 4.31 g and no 1% Phenol red added.

Stock salt

NaCl	30	g
KCl	4	g
Na_2HPO_4.12H2O	1.2	g
glucose	20	g
Mixing		
KH_2PO_4	0.6	g
DW q.s. to	1,000	g
Stored at 4°C		

HAM Broth

PPLO broth	10	g
Bacto yeast extract	0.4	g
L-arginine	1.3	g
Mucin bacteriology	0.13	g
0.5% Phenol red	1.8	ml
Deionized DW	475	ml
Autoclave 121°C, 5 minutes, cool down in ice		
Horse serum, inactivated at 56°C, 30 minutes	70	ml
25% Fresh yeast extract	25	ml
Swine serum, inactivated at 56°C, 30 minutes	70	ml
Cycloserum	100	ml
Adjust pH 7.5-7.6 with 5% Na_2CO_3		

HAM agar

Replaced PPLO broth by 1%noble agar and no 0.5% Phenol red added.

Modified Hayflick's Broth

PPLO broth	1.58	g
Deionized DW	75	ml
1% Phenol red	0.05	ml
Autoclave 121°C, 15 minutes, cool down in ice		
Horse serum	15	ml
25% Fresh yeast extract	10	ml
0.2% Calve Thymas DNA	1.2	ml
Penicillin G (200,000 units)	0.5	ml
10% Thallium acetate	0.2	ml
10% Glucose	1.0	ml
Adjust pH 7.6-7.8 with 1N NaOH		

Modified Hayflick's Agar

Replace PPLO broth with 1% noble agar and no 1% Phenol red added.

PCR-Component (for 10 samples)

10x PCR Buffer	10.0	µl
Q-solution	20.0	µl
dNTP mix (10mM of each)	2.0	µl
Hot star taq DNA polymerase	0.5	µl
DW	42.5	µl
Primer F (10pmol/ µl)	5.0	µl
Primer R (10pmol/ µl)	5.0	µl
DNA template	15.0	µl
Total	100	µl

APPENDIX C

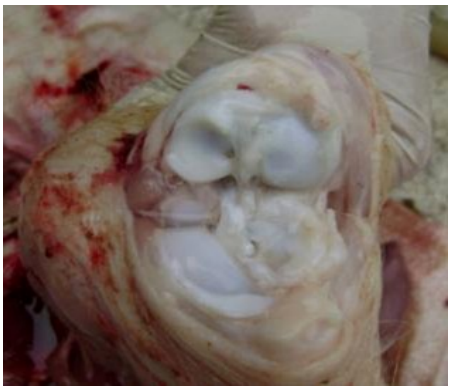
Proceeding

1. Mycoplasmal culture

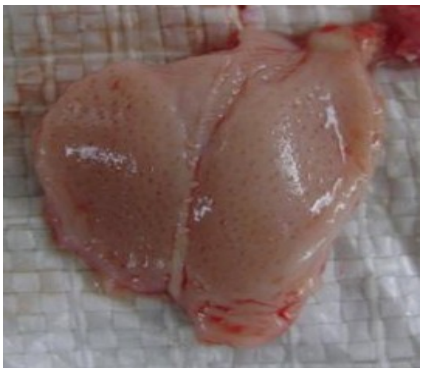
Samples

Nasal swab

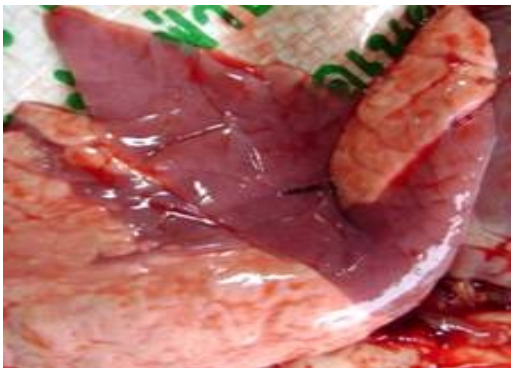
Synovial fluid and tissue



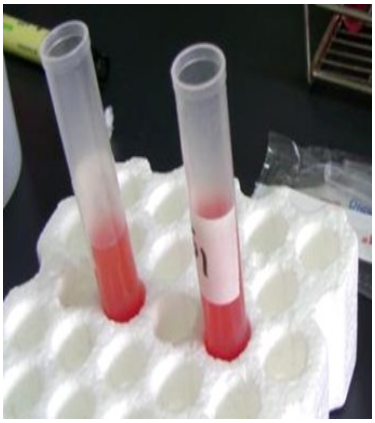
Tonsil



Lung



Centrifuge

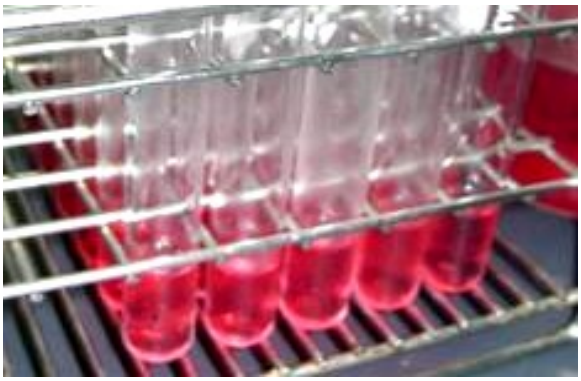


Lungs and tonsils were grinded by scissors and add 10 ml of BHL broth, mixing and centrifuging at 2,000 rpm for 5 minutes. The supernatant was processed in the next step

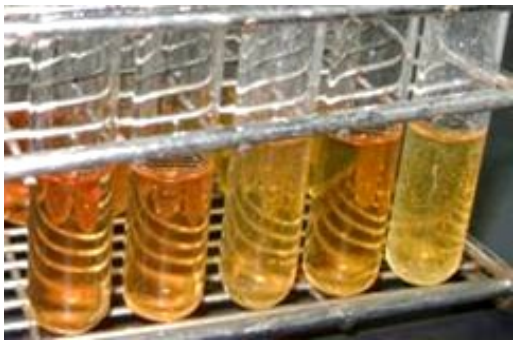
Broth will be filtered by 0.45 µm membrane filter, to get rid of the contaminated bacteria and will be inoculated into media broth and will serially be diluted 10-fold dilution ($10\text{-}10^{-3}$)

The dilution will be incubated in 37°C for 4 days or until there will be observed visible color change of media.

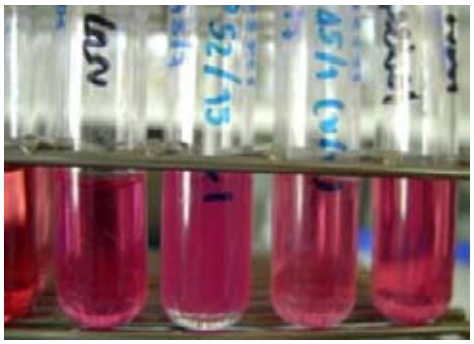
BHL >> red



pH<7 >> yellow

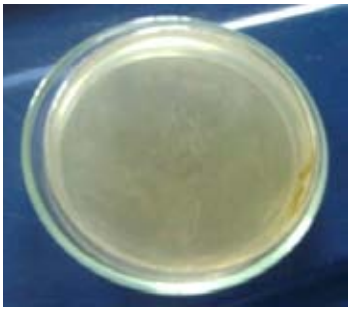


pH>7 >> pink-red

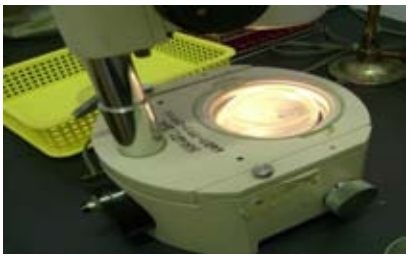


Swabs will be removed from the broth in collecting tubes to continue for the next step.

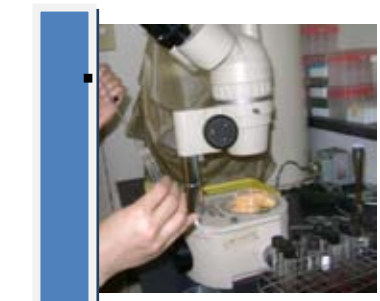
Two to three drops of synovial fluid will be inoculated on agar media at 37°C under 5% CO2 condition for 4 days and the rest synovial fluid will be inoculated in the media broth 2 ml to process in the next step.



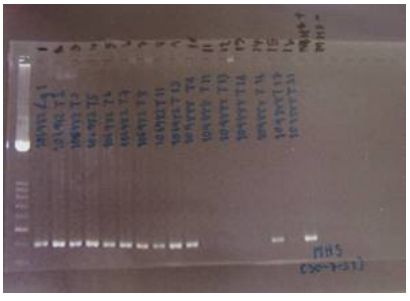
Color change dilution will be inoculated into the appropriate agar media and incubated at 37°C under 5% CO2 condition for 4 days and will be observed the mycoplasmal colonies by Stereoscope



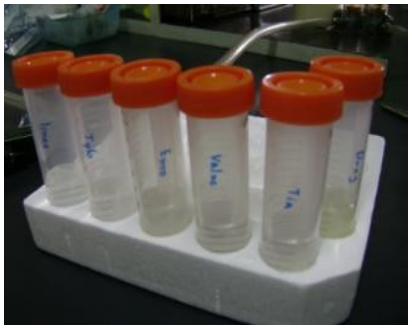
Colony morphologies will be observed and selected in the broth media for three times



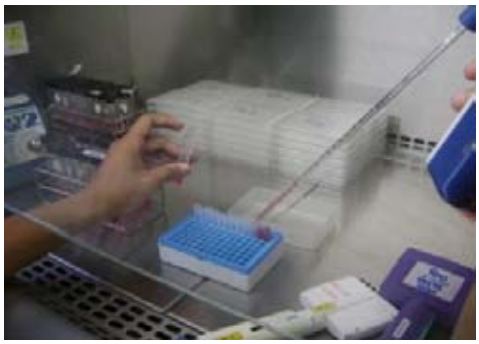
Identification of *Mycoplasma* spp by PCR

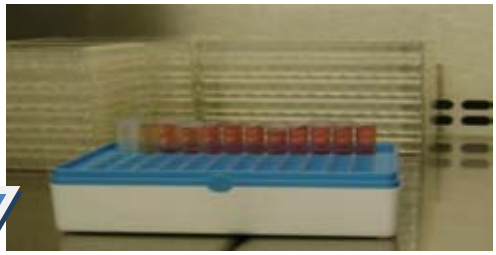


2. MIC



Stock dilution of six antimicrobials 800 μg/ml (20 ml) Diluting in the appropriated broth

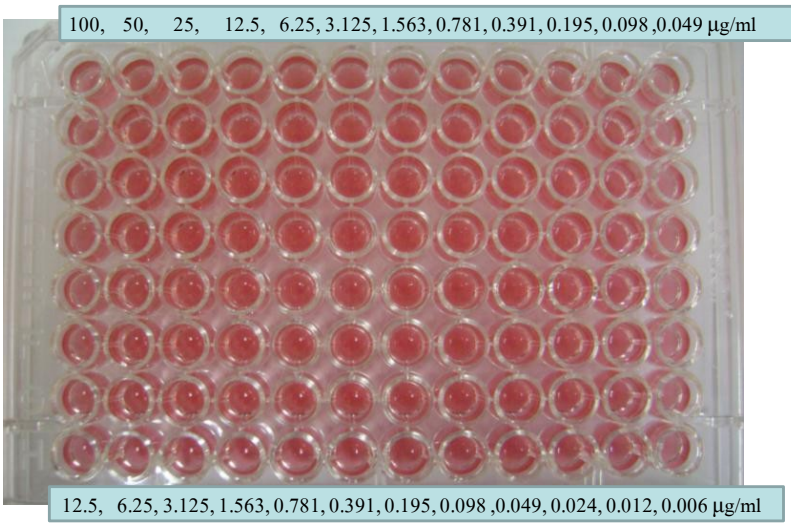




Fill the reference dilution in collection tube

Fill reference dilution in plate with 96-wheel 25 μl/wheel

Fill mycoplasmal dilution (10^5 cfu/ml) 175 µl/wheel



Doxycycline

Enrofloxacin

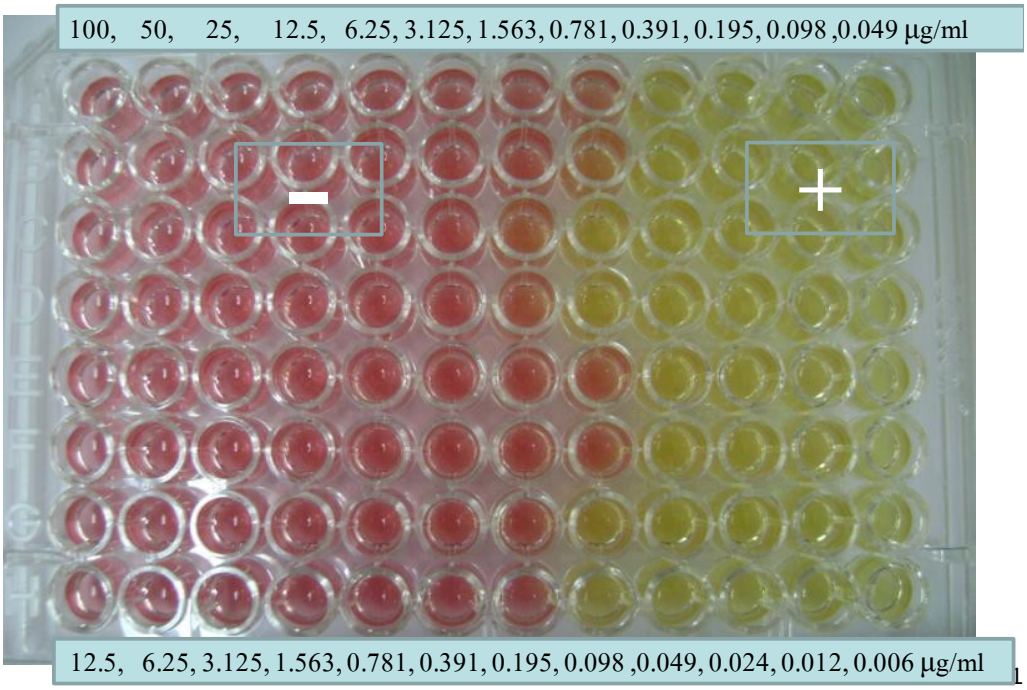
Lincomycin

Tiamulin

Tylosin

Valnemulin

Wet cotton wool to supply humidity inside the plastic pack during incubation at 37°C for 7 days



The result was read by the color change detection

3. PFGE

Culture *Mycoplasma* spp. and multiplying the volume in their appropriate broth media

Incubation at 37°C for 4 days and detection of color change

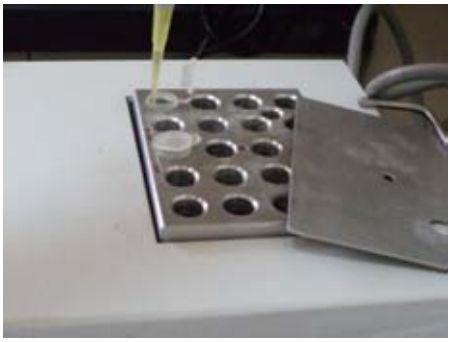
Centrifuge at 10,000 rpm X g, 50 min, 4°C

for harvesting and pelleting the mycoplasmas

Washing the pellet for three time by mycoplasma washing buffer

(50mMTris-HCl, 10mM EDTA, 100mM NaCl, pH 7.2)

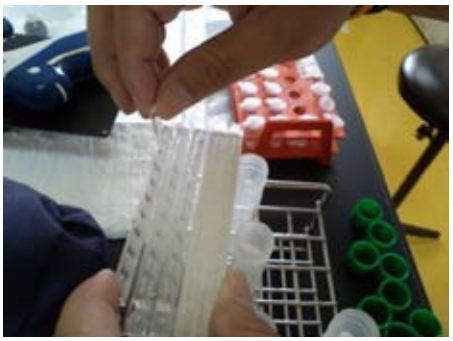
The washed pellets were resuspended in 200 ml of 0.85% NaCl



The suspension was mixed with an equal volume of 1.6% Low-melting-point agarose

loading into the plug molds (Bio-Rad, USA) at

80-100 µl/plug to set into block at 4°C for 10 minutes.



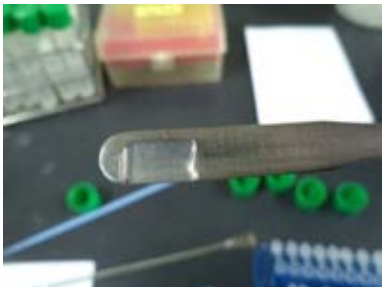
After separating the plug from molds,

the agarose plugs were soaked in 2 ml lysis buffer

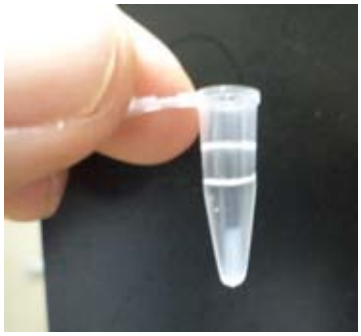
(50 mM EDTA, 1% N-lauroyl sarcosine, 0.1 mg/ml Proteinase K, 10 mM Tris-HCl, pH 8)

at 50°C, for overnight.

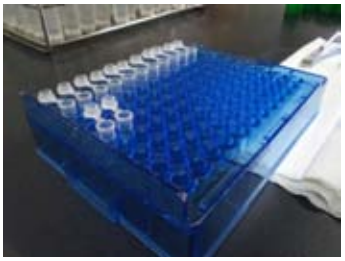
After incubation, the lysis process was repeated at 50°C, for overnight.



The plugs were washed three times by distilled water at 50°C for a time and TE buffer (10mM Tris, 1mM EDTA, pH 7.6) at 50°C for two times and stored at 4°C until digested

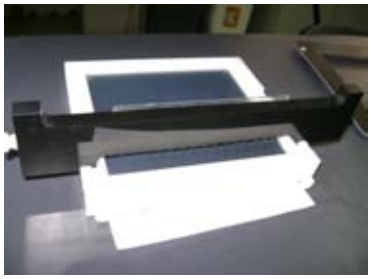


Prior to the DNA digestion, the plugs were equilibrated in 1 X restriction enzyme buffer (delivered with the enzyme) at 37°C for 10 minutes.

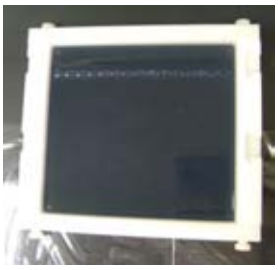


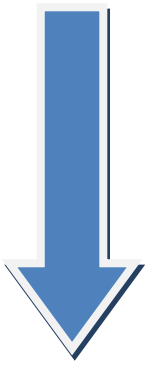
The genomic DNA were digested by the restriction enzyme at 30 units/plug and incubated at 37°C for 5 hours.

1.0 % Agarose gel prepared by the dilution of 1 gram pulsed field certified agarose (Bio-Rad, USA) in 100 ml 0.5XTBE buffer (44.5mM Tris, 44.5mM boric acid, 1mM EDTA, pH8.0) warmed by microwave



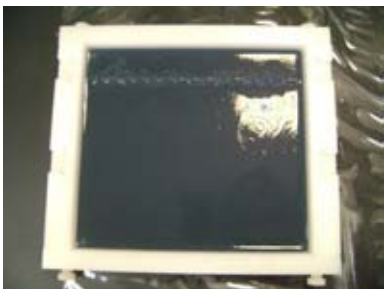
Poured gel into PFGE tray (Bio-Rad, USA) and insertion of 15-wheels comb, gel was left in room temperature for 30 minutes.

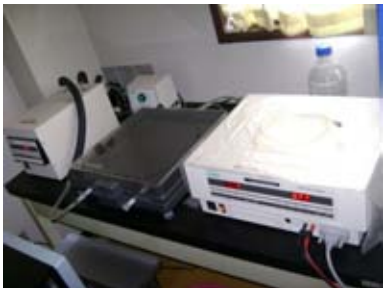
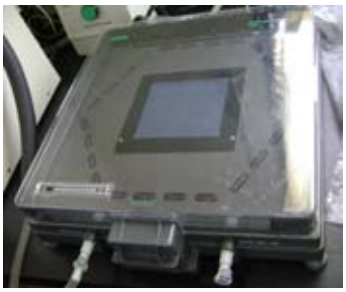




Removing the comb and loading 0.5 X TBE buffer into the wheels for lubrication.

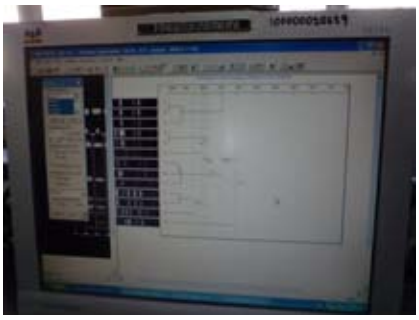
Lambda Ladder PFG Marker size range 50-1,000 kb (Biolabs, USA) was loaded as a marker for *M. hyopheumoniae* and *M. hyosynovoae* and CHEF DNA Size Standard size range 8-48 kb (Bio-Rad, USA) was loaded as a marker for *M. hyorhinis*.



The electrophoresis was run at 14°C and 6V/cm, run parameters was set to *M. hyopneumoniae* and *M. hyosynoviae* at pulse parameters switching times from 0.5-8.5 seconds and run time 18 hours, and *M. hyorhinis* at 2.0-10.0 seconds, 10 hours.

Agarose gels were stained with dilution of ethidium bromine and destrained with distilled water for 30 minutes and photographed under ultraviolet light.



DNA fragments were visualized and analyzed in a gel documentation system

Bio-1 D++ software (Vilber-Lourmat, Germany).

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