Studies on antimicrobial susceptibilities of porcine mycoplasmas in Thailand, rapid detection and genetic diversity of *Mycoplasma hyosynoviae*
Studies on antimicrobial susceptibilities of porcine mycoplasmas in Thailand, rapid detection and genetic diversity of *Mycoplasma hyosynoviae*

A Thesis submitted to the Tokyo University of Agriculture for the Degree of Doctor of Agricultural Science

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2013
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Declaration

I hereby certify that this thesis, submitted to the Tokyo University of Agriculture for the degree of Doctor of Agricultural Science upon Pacharee Thongkamkoon, has not been submitted as an exercise for a degree at any other University. I also certify that the work described in this thesis is entirely my own.

.................................

Pacharee Thongkamkoon

December 5, 2013
Table of contents

Title page……………………………………………………………………………….. i
Declaration…………………………………………………………………………... iii
Table of contents………………………………………………………………………… iv
List of tables………………………………………………………………………… vi
List of figures………………………………………………………………………… vii

General introduction
1 Background on livestock production system in Thailand…………………………………… 2
2 Pig production in Thailand…………………………………………………………………… 3
3 Diseases of pig in Thailand…………………………………………………………………… 5

Chapter 1 Antimicrobial susceptibilities of Mycoplasma hyopneumoniae field isolates and occurrence of enrofloxacin, macrolides and lincomycin resistance
1.1 Introduction……………………………………………………………………………… 9
1.2 Materials and Methods………………………………………………………………… 9
1.3 Results……………………………………………………………………………… 12
1.4 Discussion…………………………………………………………………………… 14
1.5 Abstract…………………………………………………………………………… 18

Chapter 2 Antimicrobial susceptibilities of Mycoplasma hyosynoviae field isolates in Thailand during 2008-2011 and in vitro development of resistance to tylosin and lincomycin in type strain S16 of M. hyosynoviae
3.1 Introduction…………………………………………………………………………… 20
3.2 Materials and Methods………………………………………………………………… 20
3.3 Results……………………………………………………………………………… 23
3.4 Discussion…………………………………………………………………………… 25
3.5 Abstract…………………………………………………………………………… 32

Chapter 3 Development of semi-nested PCR for detection of 16S rRNA gene of Mycoplasma hyosynoviae
3.1 Introduction…………………………………………………………………………… 34
3.2 Materials and Methods………………………………………………………………… 35
3.3 Results……………………………………………………………………………… 39
3.4 Discussion…………………………………………………………………………… 43
Chapter 4 Genetic diversity of *Mycoplasma hyosynoviae* field isolates in Thailand

4.1 Introduction……………………………………… 47
4.2 Materials and Methods…………………………… 48
4.3 Results………………………………………… 50
4.4 Discussion………………………………………… 52
4.5 Abstract…………………………………………… 56

General discussion

1 Antimicrobial susceptibility of porcine mycoplasmas in Thailand……………………………………………… 58
2 Use of semi-nested PCR in detecting *M. hyosynoviae* in Thailand……………………………………………… 60
3 Diversity of *Mycoplasma hyosynoviae* in Thailand……. 61

Abstract………………………………………………… 64
Japanese abstract………………………………………… 71
References………………………………………………… 78
Acknowledgement………………………………………… 90
## List of tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Number of pigs by region in Thailand in 2011</td>
</tr>
<tr>
<td>2</td>
<td>Frequency distribution of minimal inhibitory concentrations (MICs) of 12 antimicrobials for 159 Thai isolates of <em>M. hyopneumoniae</em> isolated during 2006-2011</td>
</tr>
<tr>
<td>3</td>
<td>Comparison on the MIC for <em>M. hyopneumoniae</em> between this study and the previous one</td>
</tr>
<tr>
<td>4</td>
<td>Frequency distribution of minimal inhibitory concentrations (MICs) of 10 antimicrobials for 41 Thai <em>M. hyosynoviae</em> field isolates during 2008-2011</td>
</tr>
<tr>
<td>5</td>
<td>Minimal inhibitory concentrations (MICs) of lincomycin and macrolides for 13 Thai <em>M. hyosynoviae</em> isolates obtained G745A transition in domain II of 23S rRNA</td>
</tr>
<tr>
<td>6</td>
<td>In vitro development of tylosin and lincomycin resistance in <em>M. hyosynoviae</em> strain S16 and MIC level of the mutants</td>
</tr>
<tr>
<td>7</td>
<td>Microorganisms used in the development of semi-nested PCR for <em>Mycoplasma hyosynoviae</em></td>
</tr>
<tr>
<td>8</td>
<td>Comparison between semi-nested PCR and cultivation for the detection of <em>M. hyosynoviae</em> from tonsillar samples</td>
</tr>
</tbody>
</table>
## List of figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>41</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
</tr>
<tr>
<td>6</td>
<td>54</td>
</tr>
<tr>
<td>7</td>
<td>55</td>
</tr>
</tbody>
</table>

1. In vitro development of tylosin and lincomycin resistance in *M. hyosynoviae* strain S16.
2. Oligonucleotide primers specifically amplified 16S rRNA gene of *M. hyosynoviae* and showed the PCR products at the predicted sizes.
3. Detection limit of the semi-nested PCR procedure in detecting extracted genomic DNA of *M. hyosynoviae*.
4. Detection limit of the semi-nested PCR procedure in detecting extracted genomic DNA of *M. hyosynoviae* from simulated lung.
5. PCR products from homogenous tonsils for detection of *M. hyosynoviae* in clinical specimens.
6. Dendrogram of PFGE fragments of 37 *M. hyosynoviae* Thai isolates and the type strain S16.
7. Dendrogram of RAPD profiles of 41 *M. hyosynoviae* Thai isolates and the type strain S16.
General introduction
1. Background on Livestock production system in Thailand

At present, Asian countries comprise more than 4.2 billion people or 60 percent of the world’s population (ESCAP, 2012). Economic development in Asia has generated growing trends of livestock production and increased demand for livestock products. This has led to the implementation of intensive livestock production systems (Cameron, 2000). Although growing rapidly, average meat consumption in ASEAN countries is still low compared to industrialized countries. Nevertheless Thailand has good potential to produce livestock and livestock products. With regards to livestock production, Thailand production system is categorized as:

1. Production for own or domestic consumption: The number of animal raised is minimal but enough for family consumption and some animal can be sold.
2. Commercial production: A cluster of farmers who gain high experience extend their production for sale in the localities. This production system is more developed with systematic and standard animal housing, husbandry and management. However, a higher level of biosecurity and good farming practices are required. These farmer clusters could be strengthened through the formation of farmer cooperatives.
3. Industrialized or intensive production: This category produces large volume of livestock products with sophisticated technology in production practices. Farmers under this category are mostly contract farmers under production scheme of major enterprisers (Limlamthong, 2012).

The scaling up of livestock production became apparent in developing countries after the introduction of modern livestock 30 years ago. In Thailand, it was introduced in the period of 1975 to 1978. As a result, farm size has increased dramatically in all sectors of livestock industry. For example, in a present average broiler farm is now 10,000 birds are reared in one poultry house and a total number
of the birds is between 20,000 and 100,000. An average contract swine farm keeps 100-300 sows since early 2000. The major livestock species produced in Thailand in 2012 were broiler chicken (247.5 million birds), native chicken (82.3 million birds), layer chicken (54.3 million birds), pig (11 million heads), duck (10.4 million birds) and beef cattle (6.4 million heads) (DLD, 2012).

2. Pig production in Thailand

The Department of Livestock Development (DLD) has started the development of pig production in Thailand by importation of exotic pig breeds including Large White, Tamworth, Berkshire, Landrace and Duroc pigs from the United Kingdom and The United States since 1960s. Thus, indigenous native pigs have been increasingly mated with imported breeds to improve their performance in economically important traits. Native pigs have gradually become crossbreeds and are replaced by commercial breeds in the pig and pork industry (Charoensook et al., 2013). Pig breeding has steadily been industrialized in Thailand since 1981. Therefore, pig production increased by 5% annually, the standing stock of sows was 0.8 million in 2011 with production of fattening pigs reaching 12.13 million pigs per year in the last few years.

At present, pigs have been produced by large enterprisers, such as Charoen Pokphand (CP), Betagro, Laem Thong and Mittraparp, which account for more than 20% of the pig industry in Thailand. However, the swine sector is still dominated by the independent producers who access to modern technology and own many large modern farms. In 2011, there were 9.7 million head of pigs throughout the country with 0.71 million native pigs, almost 1 million breeding sows and 6.1 million fattening pigs. The native pigs and breeding pigs were mostly found in the Northern and the lower part of Northern Thailand. The fattening pigs were mostly raised in the Western, Eastern and Central regions of Thailand (Table 1). The Southern part
has the lowest number of pigs due to the fact that a population of Muslim that does not consume pork meat in the region is relatively high.

Thailand ranks the eleventh on pork production (946.25 thousand tons) in the world in 2011. Most of the pork produced in Thailand was consumed domestically (930.25 thousand tons), whereas 16 thousand tons were exported to Hong Kong and Malaysia as fresh product and to Japan, Hong Kong, Singapore and Vietnam as processed pork product.

Table 1. Number of pigs by region in Thailand in 2011

<table>
<thead>
<tr>
<th>Region</th>
<th>Native pig</th>
<th>Breeding boar</th>
<th>Breeding sow</th>
<th>Breeding piglet</th>
<th>Fattening pig</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>29,854</td>
<td>8,280</td>
<td>95,822</td>
<td>189,881</td>
<td>774,866</td>
<td>1,098,703</td>
</tr>
<tr>
<td>Eastern</td>
<td>17,813</td>
<td>9,747</td>
<td>159,108</td>
<td>312,489</td>
<td>1,322,146</td>
<td>1,812,213</td>
</tr>
<tr>
<td>Northeastern (upper)</td>
<td>143,104</td>
<td>29,076</td>
<td>117,559</td>
<td>211,036</td>
<td>583,728</td>
<td>1,084,503</td>
</tr>
<tr>
<td>Northeastern (lower)</td>
<td>91,037</td>
<td>23,034</td>
<td>55,585</td>
<td>143,118</td>
<td>271,180</td>
<td>583,957</td>
</tr>
<tr>
<td>Northern (upper)</td>
<td>204,221</td>
<td>19,354</td>
<td>115,274</td>
<td>207,184</td>
<td>521,474</td>
<td>1,067,507</td>
</tr>
<tr>
<td>Northern (lower)</td>
<td>88,331</td>
<td>33,285</td>
<td>76,284</td>
<td>120,678</td>
<td>348,190</td>
<td>666,768</td>
</tr>
<tr>
<td>Western</td>
<td>51,942</td>
<td>12,801</td>
<td>223,059</td>
<td>385,084</td>
<td>1,580,650</td>
<td>2,253,536</td>
</tr>
<tr>
<td>Southern (upper)</td>
<td>57,201</td>
<td>9,667</td>
<td>46,520</td>
<td>99,691</td>
<td>328,442</td>
<td>541,521</td>
</tr>
<tr>
<td>Southern (lower)</td>
<td>28,440</td>
<td>8,477</td>
<td>53,465</td>
<td>112,121</td>
<td>361,563</td>
<td>564,066</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>711,943</strong></td>
<td><strong>153,724</strong></td>
<td><strong>942,586</strong></td>
<td><strong>1,781,282</strong></td>
<td><strong>6,092,239</strong></td>
<td><strong>9,681,774</strong></td>
</tr>
</tbody>
</table>
3. Diseases of pigs in Thailand

The incidence of pig diseases is high in areas of intensive farming, whereas in the isolated areas the infection rate was low to moderate. The Eastern, Western and Central regions have lots of disease problems since they are the areas of most intensive farming. The exotic diseases entered Thailand through imported breeding pigs from European and North American during the period for genetic improvement of breeding stock in that country. For instance, the first outbreak of Aujeszky’s disease (AD) in Thailand was found in 1977 (Sunyasootcharee et al., 1978) and that of swine fever (SF) in 1980 (Kongsamak, 1980). The Thai AD virus showed a high similarity to the virus in central Europe (Nishimori et al., 1987). SF vaccine (Chinese strain) developed by DLD has been used to prevent the disease since 1976, but severe SF outbreaks occurred during 1986-1988. So lots of commercial vaccines of SF and AD were imported to Thailand and intensive vaccination programs were implemented to control these diseases. At present clinical AD is rarely found due to effective vaccination program but SF still causes problems in Thai pig farms. In recent years emergence of two new viruses, porcine reproductive and respiratory syndrome (PRRS) virus and porcine circovirus type 2 (PCV-2), causes many disease problems all over the world. The US genotype of PRRS virus was introduced to Thailand in 1996 (Damrongwatanapokin et al., 1996) and PCV-2 in 1999 (Tantilertcharoen et al., 1999). Bacterial diarrhea has been a major problem of piglets in almost all of the pig farms in Thailand and *Escherichia coli* is the most common pathogen of the disease. *Clostridium perfringens* is also found in diarrhea affecting neonatal piglets. *Brachyspira hyodysenteriae* and *B. pilosicoli* are rarely found in weaning to grower pigs. Many bacterial pathogens of respiratory organs have been found in weaning and fattening pigs. Mycoplasmal pneumonia has been found in slaughter pigs since late 1970s though the isolation of the causative agent, *Mycoplasma hyopneumoniae*, could not be made until 1988 (Saitanu et al., 1988). The earliest reports on *Streptococcus suis*, *Haemophilus parasuis* and *Actinobacillus*
*pleuropneumoniae* (*App*) were made during 1983–1989 (Suksaithaichana, 1983; Patanasophon et al., 1985; Neramitmansook et al., 1989a; Neramitmansook et al., 1989b) in Thailand.

Before the emergences of PRRS virus and PCV-2 the etiology of respiratory diseases of pigs was not complicated. For example, *M. hyopneumoniae* caused mycoplasmal pneumonia and *App* caused pleuropneumonia though concomitant infections were sometimes observed. But in recent years the word PRDC (porcine respiratory disease complex) has been used, because many organisms including those which were not pathogenic to the respiratory organs of pigs before began to associate with the respiratory diseases. The economic loss due to PRDC is the greatest among disease-related decrease of production in every swine herds. Though the reason why many organisms have to come to associate the recent respiratory diseases has not been fully clarified as yet it has been assumed that PRRS virus enhances the multiplication of these organisms in the lungs by damaging alveolar macrophages (Thanawongnuwech et al., 2004). The PRRS virus infection by itself does not last for a long period, however, if *M. hyopneumoniae* infects the animal simultaneously persistent infection of the virus may occur resulting the severe aggravation of the disease (Thacker et al., 1999). Thus PRRS virus and *M. hyopneumoniae* play an essential role in the etiology of PRDC.

*Mycoplasma hyosynoviae* causes nonpurulent polyarthritis in breeding and fattening pigs. Though the morbidity of the disease is not as high as PRDC, the disease is prevailed worldwide including Thailand leading to decreased production in swine herds. The disease can be a recurring problem in certain farms when new gilts or boars are brought in.

As mentioned above mycoplasmal diseases are very important in swine production, however, little is known on porcine mycoplasmas in Thailand because the organisms are too fastidious to be handled in ordinary laboratories and their
isolation and cultivation are very laborious. The present investigations were carried out to get information which will improve the diagnostic techniques of porcine mycoplasmosis and contribute to the control of mycoplasmal diseases in that country.
Chapter I

Antimicrobial susceptibilities of *Mycoplasma hyopneumoniae* field isolates and occurrence of enrofloxacin, macrolides and lincomycin resistance
1.1 Introduction

*Mycoplasma hyopneumoniae* is recognized as one of the most important pathogens in pigs. Management practices, medication and vaccination are control measures of the disease (Maes et al., 2008). In Thailand, antimicrobials are generally given to piglets to control diarrhea and respiratory problems during weaning to fattening as well as to gilts and sows during acclimatization and lactation (Prapasaranukul et al., 2010). Excessive medication may cause a decrease of susceptibility of mycoplasmas against antimicrobial agents (Le Carrou et al., 2006; Vicca et al., 2004; Yamamoto et al., 1986). To date, antimicrobial resistance of porcine mycoplasmas has been reported to tetracyclines, macrolides, lincomycin and flumequine, the first generation fluoroquinolone in some countries (Bousquet et al., 1997; Hannan et al., 1997b; Stakenborg et al., 2005a; Vicca et al., 2004; Vicca et al., 2007). In Thailand susceptibility of *M. hyopneumoniae* to antimicrobial agents was investigated for the isolates collected in 1997-1998 and no resistant isolates were found in that period (Narongsak and Thongkamkoon, 2002). In this study, susceptibilities of recent field isolates of *M. hyopneumoniae* collected during 2006-2011 were examined. *M. hyopneumoniae* field isolates showing resistance to macrolides and lincomycin were examined for 23S rRNA transition as an evidence of *in vivo* acquired resistance of *M. hyopneumoniae* to macrolides and lincomycin in Thailand.

1.2 Materials and Methods

*Mycoplasma strains*: One hundred and fifty nine Thai isolates of *M. hyopneumoniae* and the type strain J obtained from National Institute of Animal Health (NIAH), Japan were used. Of the Thai isolates, 7 were isolated from pneumonic lungs of pigs from 5 farms in 2006, and 20, 39, 76, 14 and 3 isolates were isolated from 10, 11, 13, 6 and 1 farms in 2007, 2008, 2009, 2010 and 2011, respectively.
**Culture media:** BHL broth and BHL agar medium described previously (Yamamoto et al., 1986) were used throughout the study. Briefly, the basal medium was prepared by dissolving the following constituents [Brucella broth (BD), 5.8 g; Lactalbumin hydrolysate (Difco), 2.0 g; NaCl, 4.0 g; KCl, 0.2 g; Na₂HPO₄ 12H₂O, 0.06 g; KH₂PO₄, 0.03 g; glucose, 1.0 g; 0.4% phenol red solution, 7.5 ml] in 750 ml of distilled water and autoclaved at 115°C for 15 minutes. For agar medium 10 grams of Difco™ Agar Noble was added before autoclaving. The basal medium was supplemented with 100 ml each of porcine and horse serum, 50 ml of fresh extract of baker’s yeast (Fermipan®) and 10 ml of methicillin solution (10mg/ml). The final pH was adjusted to 7.8 with 5% Na₂CO₃ solution.

**Isolation procedures:** The lung samples collected from pneumonic lesions of pigs, either by the farmers whose pigs were clinically affected with respiratory problems or by the veterinarians at the slaughter house to monitor the respiratory diseases in the farms, were submitted to NIAH, Thailand for identification of the causative agents. Focusing on *M. hyopneumoniae* infection, the primary isolation was carried out in BHL broth and BHL agar medium. Cultures identified as *M. hyopneumoniae* by colony characterization and by specific PCR (Mattsson et al., 1995) were stocked at -80°C until use.

**Antimicrobial agents:** The following 12 antimicrobial agents generally applied in pig industry in Thailand were used; Chlortetracycline (CTC) [Sigma, St. Louis, MO, U.S.A.], Oxytetracycline (OTC) [Sigma], Doxycycline (DOXY) [Sigma], Lincomycin (LCM) [Sigma], Josamycin (JM) [Sigma], Kitasamycin (KT) [Bayer, Leverkusen Germany], Spiramycin (SPM) [Sigma], Tylosin (TS) [Sigma], Tiamulin (TM) [Sandoz, Kundl, Austria], Erythromycin (EM) [Sigma], Florfenicol (FFC) [Zhangjiagang Hengsheng Pharmaceutical Co. Ltd., Jiangsu, China] and Enrofloxacin (ERFX) [Bayer].
**Determination of minimal inhibitory concentration (MIC):** Tests were carried out by broth microdilution method with some modification (Hannan, 2000; Senterfit et al., 1983) using flat bottomed microplates with 96 wells. Serial two-fold dilutions of a drug were made from column 1 to 10. Test organisms were diluted to $10^3$ to contain $10^4$-$10^5$ color changing unit (ccu)/ml and dispensed into two rows of column 1 to 11 per each strain. The column 11 served as organism control and column 12 as medium control. To obtain a constant growth of test organisms, they were passaged in BHL broth until they showed good growth within 5 to 7 days before use. Tests were read after incubation of 7 days at 37°C and the lowest concentration of a drug inhibiting any detectable color change of the medium was regarded as MIC of the drug.

**PCR amplification and sequencing of domain V of 23S rRNA of M. hyopneumoniae:** PCR amplification and sequencing of domain V of 23S rRNA of *M. hyopneumoniae* were performed as follows, One ml of well grown *M. hyopneumoniae* isolates showing high MICs level of macrolides and lincomycin was centrifuged at 12,000 x g for 10 min (model 1920, Kubota, Tokyo, Japan). After washing the pellet with 1 ml PBS, the pellet was mixed with Instagene matrix (Biorad, Hercules, CA, U.S.A.) to prepare the DNA template for PCR according to the manufacturer’s instructions. The specific primer sequences for amplification of domain V of 23S rRNA of *M. hyopneumoniae* were as follows: Mhp Dom5F 5´-AAG CGG AGA TGT TAG CCT A -3´ and Mhp DomR 5´-TCT CCC ACC TAT CCT ACA CA -3´, amplification size of 277 bp (Kobayashi et al., 2008). Amplification was performed in a total volume of 50 µl containing Go taq green (Promega, Medison, WI, U.S.A.), 25 µl; 0.8 µM of each primer; 4 µl of DNA template and DW up to 50 µl. The PCR assay was carried out in a Thermal cycler (Hybaid, Thermo electron, Needham, MA, U.S.A.), comprising pre-incubation at 94°C for 2 min, followed by 30 cycles of 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 68°C. Final extension was performed for 5 min at 68°C. The amplicons, purified
by 2% agarose gel electrophoresis, were sequenced with ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.), according to the manufacturer’s instructions.

1.3 Results

The distribution of the MICs obtained for 159 Thai isolates tested is shown in Table 2. MIC for type strain J as well as the MIC breakpoint values of the 12 tested antimicrobials is also presented in Table 2. Tiamulin (TM) showed the lowest MICs of 0.013 or lower to 0.2 µg/ml against 158 of 159 isolates (MIC\(_{90}\)=0.1 µg/ml). Next to TM, tylosin (TS) and lincomycin (LCM) showed high activity with MIC\(_{90}\) of 0.39 µg/ml, however two isolates were resistant to these drugs showing a MIC value of higher than 12.5 µg/ml. Of the two isolates, one was isolated from an affected pig in a farm in 2007, and the other was isolated from a healthy pig in another farm in 2010. However, another two isolates obtained from different pigs in the latter farm were sensitive to lincomycin and macrolides. Three macrolides, spiramycin (SPM), josamycin (JM) and kitsamycin (KT) also showed high potency with MIC\(_{90}\) of 0.78, 0.78 and 1.56 µg/ml, respectively. Of the five macrolides tested, erythromycin (EM) showed exceptionally low activity with MIC\(_{90}\) of 200 µg/ml. Florfenicol (FFC) showed moderately high activity with MIC range from 0.2 to 6.25 µg/ml and MIC\(_{90}\) of 1.56 µg/ml. Oxytetracycline (OTC) and doxycycline (DOXY) showed similar activity with FFC and their MIC range distributed from 0.39 to 12.5 µg/ml with MIC\(_{90}\) of 6.25 µg/ml. On the other hand activity of chlortetracycline (CTC) to Thai isolates of *M. hyopneumoniae* was low with MIC range from 3.12 to 100 µg/ml and MIC\(_{90}\) of 50 µg/ml. MIC values of enrofloxacin (ERFX) distributed in a broad range from 0.1 to 25 µg/ml and MICs for 76 of 159 (47.7 %) Thai isolates, including 13 from affected pigs, exceeded 2 µg/ml which is the breakpoint of this drug.
Table 2. Frequency distribution of minimal inhibitory concentrations (MICs) of 12 antimicrobials for 159 Thai isolates of *M. hyopneumoniae* isolated during 2006-2011

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Number of strains with MIC (µg/ml) of</th>
<th>MIC (µg/ml)</th>
<th>Strain Breakpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>400 200 100 50 25 12.5 6.25 3.12 1.56 0.78 0.39 0.2 0.1 0.05 0.025 0.013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tiamulin</td>
<td>1 4 54 60 22 18(≤) 0.05 ≥16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lincomycin</td>
<td>2(&gt;) 10 32 80 32 2 1(≤) 0.05 NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tylosin</td>
<td>2(&gt;) 1 10 15 37 60 21 13(≤) 0.05 ≥4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spiramycin</td>
<td>2 1 1 4 25 61 51 14 0.39 NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Josamycin</td>
<td>2(&gt;,1) 3 5 39 53 35 21 0.2 NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kitasamycin</td>
<td>1(&gt;,1) 2 3 35 77 33 6 1 0.39 NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>2(&gt;),11 23 14 40 55 10 4 25 ≥4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Florfenicol</td>
<td>1 2 100 50 3 3 0.39 ≥8 d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxycline</td>
<td>24 79 46 9 1 0.39 NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>8 82 52 13 4 0.78 ≥16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>8 44 b) 60 39 4 4 3.12 NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>1 1 24 50 17 18 27 4 17 0.2 ≥2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) : > : equal or higher than MIC indicated  ≤ : equal or lower than MIC indicated
b) : The underline indicates that the group includes macrolides and lincomycin resistant strain
c) : data from Hannan (2000)
d) : data from CLSI (2010)
Table 3. shows the comparison of susceptibilities of the present 159 isolates with those isolated from 1997 to 1998 in Thailand (Narongsak and Thongkamkoon, 2002). In the previous data CTC, OTC, JM, TS, LCM and TM were tested against 27 Thai isolates of *M. hyopneumoniae*. Decreases of susceptibilities of the present isolates to some of the drugs were noted. The MIC\(_{90}\) values of CTC, OTC, JM and TS for previous isolates were 6.25, 0.78, 0.2 and 0.1 and those for present isolates were 50, 6.25, 0.78 and 0.39 µg/ml, respectively. No change was observed in MIC\(_{90}\) of LCM and TM between the present isolates and the previous ones.

The sequences of domain V of 23 rRNA of the two macrolides and lincomycin resistant isolates were compared with the sequences of *M. hyopneumoniae* J strain and ATCC 27719 strain (Genbank accession number AE017243 and X68421, respectively). The result revealed a point mutation at A2058G (*E.coli* coordinates) in both isolates. The MIC values of the antimicrobials for these isolates were shown (underlined) in Table 2.

1.4 Discussion

It was noteworthy that MICs of ERFX distributed in a broad range from 0.1 to 25 µg/ml and MICs for 76 of 159 (47.7%) Thai isolates exceeded 2 µg/ml which is considered the breakpoint of this drug (CLSI, 2010). The ERFX resistant animal mycoplasmas have rarely been reported. Hannan et al. (1997a) reported that some isolates of animal pathogenic mycoplasmas including *M. hyosynoviae* and *M. hyorhinis* were resistant to flumequine (breakpoint: >16 µg/ml), the first-generation fluoroquinolone which has been removed from clinical use and is no longer being marketed, while all of these flumequine resistant isolates were sensitive to ERFX. Vicca et al. (2004) described that five of 21 field isolates of *M. hyopneumoniae* were resistant to flumequine (MIC: >16 µg/ml) and had lower susceptibility to ERFX (MIC: 4 isolates; 0.5 µg/ml, one isolate; >1 µg/ml) than the other isolates (MIC: = 0.06 µg/ml). In all five isolates with lower susceptibility to ERFX one point
### Table 3. Comparison on the MIC for *M. hyopneumoniae* between this study and the previous one

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>MIC values for Thai <em>M. hyopneumoniae</em> isolates (µg/ml)</th>
<th>1997-1998 (N=27)</th>
<th>2006-2011 (N=159)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC range</td>
<td>MIC 50</td>
<td>MIC 90</td>
</tr>
<tr>
<td>Tiamulin</td>
<td>≤.006-0.1</td>
<td>0.05</td>
<td>0.1</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>0.025-0.39</td>
<td>0.2</td>
<td>0.39</td>
</tr>
<tr>
<td>Tylosin</td>
<td>≤.006-0.1</td>
<td>0.05</td>
<td>0.1</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Josamycin</td>
<td>≤.006-0.2</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Kitasamycin</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Florfenicol</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxycycline</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>0.1-1.56</td>
<td>0.39</td>
<td>0.78</td>
</tr>
<tr>
<td>Chlorotetracycline</td>
<td>0.2-12.5</td>
<td>3.12</td>
<td>6.25</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) data from the previous study (Narongsak and Thongkamkoon, 2002)
ND: not determined;
mutation in quinolone resistant-determining regions of parC of topoisomerase IV was found and in one isolate with MIC value of >1 µg/ml an extra mutation of gyrA was also found. Thai isolates tested here showed higher resistance to ERFX with MIC values of 3.2–25 µg/ml and in addition, the rate of ERFX resistant isolates was also higher than those reported in the previous papers. This may reflect the overuse of ERFX in pig farming in Thailand especially for treatment of diarrhea of lactating piglets and at nursery up to 9 weeks (Prapasaranukul et al., 2010). Since the mechanisms of resistance to ERFX of Thai isolates were not examined in the present study, further study is needed to elucidate whether the resistance to ERFX of Thai isolates comes from alteration of the genes coding for DNA gyrase or reduction of quinolone accumulation by active efflux (Hooper, 1999).

The MICs of chlortetracycline (CTC) for isolates derived from 2006 to 2011 were higher than those for isolates collected during 1997 to 1998 (MIC90: 50 vs 6.25 µg/ml). Similar results were obtained by previous workers (Yamamoto et al., 1986). Most of the Japanese isolates isolated in 1970 were susceptible to CTC at 5 µg/ml or less, while isolates isolated in 1979 to 1981 were clearly separated into two categories (bimodal frequency distribution), one of which was susceptible at 5 µg/ml or less and the other 20 µg/ml or more (Yamamoto et al., 1986). In Thailand tiamulin (TM), tylosin (TS), CTC and amoxicillin have been widely used in fattening pigs, pregnant and lactating sows and replacement gilts during acclimatization. In this respect, increase of CTC resistant isolates of M. hyopneumoniae is considered reasonable, however these CTC resistant isolates were still susceptible to oxytetracycline (OTC) and doxycycline (DOXY) though MICs were higher for isolates isolated from 2006 to 2011 than for those from 1997 to 1998 (Narongsak and Thongkamkoon, 2002).

Lincomycin and all macrolide drugs except erythromycin (EM) showed a bimodal distribution of MICs. MICs of these drugs were clearly higher for two isolates, indicating acquired resistance. In one farm from which one of the two
macrolide resistant strains were derived, additional two strains were isolated and examined for their susceptibility. As a result, these two strains were susceptible to all macrolide drugs tested except erythromycin indicating that *M. hyopneumoniae* strains with different antimicrobial susceptibility coexist among pigs within a same farm. The resistance of two strains to macrolides and lincomycin were shown to be attributable to a transition of A to G at 2058 (*E. coli* coordinates) in the central loop of domain V of the 23S rRNA. It was in agreement to the occurrence in Japan and Belgium (Kobayashi et al., 2008; Stakenborg et al., 2005). Erythromycin is highly active to *M. pneumoniae* (Kenny and Cartwright, 1991) and *M. gallisepticum* (Gautier-Bouchardon et al., 2002), however *M. hyopneumoniae* has natural resistance to this drug as indicated not only by the present data but also by the previous reports (Takahashi et al., 1978; Tanner et al., 1993; Ter Laak et al., 1991; Yamamoto et al., 1986). This natural resistance of *M. hyopneumoniae* to EM may be due to a G to A transition at 2057 (*E. coli* coordinates) in domain V of the 23S rRNA as suggested by Furneri et al (2000).

Tiamulin (TM) had the highest activity among 12 antimicrobials tested with the MIC\(_{90}\) of 0.1 µg/ml and MICs for 158 of 159 strains were lower or equal to 0.2 µg/ml. The MIC for the remaining isolate was 0.78 µg/ml. Although this value was far lower than the breakpoint of this drug, the strain might be the intermediate stage to resistance. Hence, monitoring of antimicrobial susceptibility at regular interval is inevitable.

In conclusion, this study provided the susceptibilities of Thai isolates of *M. hyopneumoniae* to 12 antimicrobials widely used in Thailand. Resistances against CTC, LCM, ERFX and macrolides were detected. In addition, the proportion of ERFX resistant Thai isolates of *M. hyopneumoniae* was remarkably high. Hence, mechanisms of resistance should be elucidated hereafter. Acquired resistance to various antimicrobials might pose a serious problem for the treatment of
Mycoplasma pneumonia of swine. Prudent use of antimicrobials is an urgent necessity in pig industry in Thailand.

1.5 Abstract

A total of 159 Thai isolates of Mycoplasma hyopneumoniae isolated from pneumonic lungs of pigs during 2006-2011 were investigated for their in vitro susceptibility to 12 antimicrobial agents. Low activity of chlortetracycline was indicated by the MIC range from 3.12-100 µg/ml and MIC₉₀ of 50 µg/ml. Seventy six isolates showed resistance to enrofloxacin, whereas 2 isolates showed resistance to macrolides and lincomycin. A point mutation at A2058G which conferred the macrolides and lincomycin resistances was revealed by sequence analysis of 23S ribosomal RNA in both isolates. The present results confirmed the rapid increase of resistant M. hyopneumoniae isolates against chlortetracycline, enrofloxacin, macrolides and lincomycin in Thailand. Selection of drugs to control swine diseases in Thailand must be done more prudently in consideration of reducing the antimicrobial resistance.
Chapter II

Antimicrobial susceptibilities of *Mycoplasma hyosynoviae* field isolates in Thailand during 2008-2011 and in vitro development of resistance to tylosin and lincomycin in type strain S16 of *M. hyosynoviae*
2.1 Introduction

*Mycoplasma hyosynoviae* is one of the porcine pathogenic mycoplasmas and distributes world-wide. Adult pigs served as an important reservoir of infection to piglets by direct contact. Pigs are occasionally infected and developed pneumonic lesion, non-suppurative arthritis and lameness (Hagedorn-Olsen et al., 1999). Nowadays, arthritis caused by *M. hyosynoviae* becomes increasing problem in many countries (Assuncao et al., 2005; Dahlia et al., 2009; Nielsen et al., 2001). Control measures depend on farm management and medication. Primary isolation from tissues of pigs is difficult due to the presence and overgrowth of other mycoplasmas as well as other bacteria (Friis et al., 1991). Hence, reports on the incidence and susceptibility of *M. hyosynoviae* to antimicrobial agents were limited (Aarestrup and Friis, 1998; Friis and Szancer, 1994; Hannan et al., 1997a; Kobayashi et al., 1996c). In addition, the medication programs in each country might vary according to the differences of susceptibilities of the organisms. Therefore, susceptibility testing of the field isolates in each country is essential. Recently, we reported the occurrence of *M. hyosynoviae* infection identified by isolation technique and PCR (Makhanon et al., 2012).

In the present study, the susceptibility of *M. hyosynoviae* field isolates collected from 2008-2011 were determined against 10 antimicrobial agents used in the swine production. All *M. hyosynoviae* isolates were examined for 23S rRNA transition at domain II and V and compared with the MIC levels of macrolides and lincomycin. In addition, macrolides and lincomycin resistant *M. hyosynoviae* S16 mutant was also developed to analyze the mechanisms of the resistance to these antimicrobials.

2.2 Materials and methods

*Mycoplasma isolates and type strain*: Forty one Thai isolates of *M. hyosynoviae* collected during 2008-2011 and kept at -80°C were used. The type strain S16
obtained from National Institute of Animal Health, Japan was also included. Of the Thai isolates, 18 were isolated from tonsils, synovial fluid and lung of pigs from 5 farms in 2008, 13 were isolated from tonsils of pigs from 2 farms in 2009 and 10 were isolated from lungs and tonsil of pigs from 3 farms in 2011.

**Culture medium and cultivation technique:** Mycoplasma cultivation was primarily carried out both from the lung and tonsil samples collected from healthy pigs at slaughterhouse and synovial fluid samples collected from the swollen joint of pigs in the farm using Hayflick’s medium supplemented with arginine and mucin (HAM) as described previously (Friis et al., 1991). After the mycoplasma was cloned three times, *M. hyosynoviae* was identified by colony characterization, film and spot production and a specific PCR (Kobayashi et al., 1996a). Finally, the culture was kept at -80°C as our culture collection. For determination of the susceptibility, all isolates from the collection were sub-cultured for 1 to 2 passages to ensure that each isolate was able to grow well within 2-3 days. Then, the colony forming units per milliliter of the culture were enumerated and aliquots of each culture were kept at -80°C until used.

**Antimicrobial agents:** The following 10 antimicrobial agents generally applied in pig industry in Thailand were used; Oxytetracycline (OTC) [Sigma], Doxycycline (DOXY) [Sigma], Lincomycin (LCM) [Sigma], Josamycin (JM) [Sigma], Spiramycin (SPM) [Sigma], Tylosin (TS) [Sigma], Kitasamycin (KT) [Bayer], Enrofloxacin (ERFX) [Bayer], Tiamulin (TM) [Sandoz] and Florfenicol (FFC) [Zhangjiagang Hengsheng Pharmaceutical Co. Ltd.]. The antimicrobial stock solution was prepared and serially diluted as described in CLSI (2010) at ten times of MIC test concentrations. The test concentrations ranged from 0.008-8 µg/ml for SPM, KT, JM, LCM and TM; 0.016-16 µg/ml for ERFX, FFC and TS and 0.125-128 µg/ml for OTC and DOXY.
**MIC determination:** The determination of MICs was carried out using agar dilution method (Hannan, 2000). HAM agar dilution plates containing antimicrobial agents were prepared by mixing 2 ml of each dilution of antimicrobial agent with 18 ml of molten HAM agar and pouring into a Petri dish. Agar plates without antimicrobial agents for growth control were prepared in the same manner. When the agar was solidified and dried completely, the plates were stored in 2-8°C for a night. MIC assay was performed in the following day. *M. hyosynoviae* was diluted to 10⁸ CFU/ml in 0.5 ml HAM broth and transferred into the well of the replicator seed block. One µl each of *M. hyosynoviae* isolate was inoculated on the agar plate using the replicator so that there would be 10⁵ CFU of *M. hyosynoviae* per spot. The inoculated plates were incubated at 37°C in a humidified incubator with 5% CO₂ for 5 days. The lowest concentration of antimicrobial agent that caused ≥50% inhibition of growth compared with that on the growth control plate was interpreted as the MIC value at the fifth day post incubation.

**PCR amplification and sequencing of domain II and V of 23S rRNA of M. hyosynoviae:** One ml of each *M. hyosynoviae* culture was harvested at 12,000 x g for 10 min (model 1920, Kubota). Then, the pellet was washed once with 1 ml PBS and mix with Instagene matrix (Biorad, U.S.A.) to prepare the DNA template for PCR according to the manufacturer’s instruction. Six specific primers designed based on 23S rRNA partial sequence of *M. hyosynoviae* S16 (GenBank accession number JQ670924) for sequencing of domain II and V of 23S rRNA were as follows: Mhs D2F 5´-ACC CAT GAG CAG GTT GAA GC-3´, Mhs D2R 5´-CCA TCC CAT ATT CAG TGC TC-3´, Mhs D5-1F 5´-CAC GAA AGG AGT AAT TAT TCT-3´, Mhs D5-1R 5´-AAC TAC CCA CCA CAC ACT GTC-3´, Mhs D5-2F 5´-CTC ATC GCA TCC TGG AGC TGG-3´ and Mhs D5-2R 5´-CCC GCT TAG ATG CCT TCA GCG-3´. Amplification was performed for each pair of the primers in a total volume of 50 µl containing Go taq green (Promega, U.S.A.), 25 µl; 0.8 µM of each primer; 4 µl of DNA template and DW up to 50 µl. The PCR assay was carried
out in a Thermal cycler (Hybaid, Thermo electron, U.S.A.), comprising pre-incubation at 94°C for 2 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. Final extension was performed for 5 min at 72°C. The amplicons, separated by 2% agarose gel electrophoresis, were purified by gel extraction kit (Qiagen, Germany) and sequenced with ABI 3130 Genetic Analyzer (Applied Biosystems) according to the manufacturer’s instruction.

**In vitro development of tylosin and/or lincomycin resistance in type strain S16 of *M. hyosynoviae***: In vitro development of tylosin or lincomycin resistance was carried out in the same procedure as previous study (Hannan et al., 1997b) by serial passage in HAM broth containing tylosin or lincomycin at concentrations covering the MIC determination. After four days incubation, the culture showing growth which contained the highest concentration of the drug was inoculated to a fresh series of drug dilutions in HAM broth. The passages were repeated at four days interval for six passages. The S16 mutants from each passage were examined for susceptibility to antimicrobial agents and mutation in 23S rRNA.

2.3 Results

The distribution of the MICs obtained from the 41 isolates tested and MIC for type strain S16 were presented in Table 4. Of 10 antimicrobials tested, tiamulin (TM) showed the lowest MICs of 0.016-0.125 µg/ml against all *M. hyosynoviae* field isolates. Next to TM, lincomycin and josamycin showed high activity with MIC<sub>90</sub> of 1 µg/ml. Kitasamycin also showed high activity with MIC<sub>90</sub> of 2 µg/ml, whereas spiramycin and tylosin (TS) showed the lower activity with MIC<sub>90</sub> of 8 µg/ml and 13 isolates were resistant to TS according to the MIC breakpoint (Hannan, 2000). Florfenicol showed moderately high activity with MIC range from 0.5 to 4 µg/ml and MIC<sub>90</sub> of 4 µg/ml. On the other hand, activity of doxycycline to Thai isolates of *M. hyosynoviae* was low with MIC range from 4 to 16 mcg/ml and MIC<sub>90</sub> of 16 µg/ml. Oxytetracycline (OTC) showed the lowest activity with MIC
Table 4. Frequency distribution of Minimal inhibitory concentrations (MICs) of 10 antimicrobials for 41 Thai *M. hyosynoviae* field isolates during 2008-2011

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Number of strains with MIC (µg/ml) of</th>
<th>MIC 90 µg/ml</th>
<th>MIC for S16 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>128 64 32 16 8 4 2 1 0.5 0.25 0.125 0.063 0.032 0.016 0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tiamulin</td>
<td>1 17 22 1</td>
<td>0.063</td>
<td>0.016</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>11 23 7</td>
<td>1</td>
<td>0.125</td>
</tr>
<tr>
<td>Josamycin</td>
<td>7 7 25 2</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>Kitasamycin</td>
<td>1 17 22 1</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>5 13 21 2</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Tylosin</td>
<td>7 6 9 18 1</td>
<td>8</td>
<td>0.032</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>6 29 5 1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>6 27 8</td>
<td>16</td>
<td>0.5</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>1 23 15 2</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>15 2 20 4</td>
<td>2</td>
<td>0.25</td>
</tr>
</tbody>
</table>
range from 16 to 128 µg/ml, suggesting all Thai isolates of *M. hyosynoviae* were resistant to OTC according to the MIC breakpoint (11). MIC values of enrofloxacin ranged from 0.25 to 2 µg/ml and MICs for 15 of 41 (36.6%) Thai isolates reached 2 µg/ml which is the breakpoint of this drug (Hannan, 2000).

Results from sequencing of the 23S rRNA demonstrated the intrinsic resistance of all *M. hyosynoviae* isolates to 14-membered macrolide since the nucleotide at 2057 (*E. coli* numbering) was adenine (A2057). Neither first nor second part of domain V showed other acquired transition. On the other hand, an acquired G745A transition in domain II was found in thirteen isolates of our Thai *M. hyosynoviae*. This G745A transition conferred the moderate resistance to tylosin at 4-8 µg/ml but not to lincomycin and the other macrolides tested. The MIC levels of macrolides and lincomycin for those isolates were shown in Table 5.

Before exposure to the antimicrobial agent *M. hyosynoviae* S16 was highly susceptible to both tylosin and lincomycin, with the MIC of 0.032 and 0.125 µg/ml, respectively. In the first and second passage, the highest concentration of tylosin and lincomycin that S16 survived were the same as that for a wild type. From the third passage, the S16 mutant rapidly obtained resistance to TS or LCM showing four to thirty two fold higher MICs in each passage. At the sixth passage MICs of the drugs reached 256 µg/ml or higher which was 4,000 fold for tylosin and 1,000 fold increase for lincomycin (Fig 1). In lincomycin selection, the A2058C/G or A2059C transitions were obtained in S16 mutants resulting in lincomycin resistance and macrolides cross resistance. While, the A2062G transition was obtained in tylosin selection which conferred only macrolides resistance. Sequence analysis and MIC results for those S16 mutants were shown in Table 6.

2.4 Discussion

The occurrence of tylosin resistant *M. hyosynoviae* among our Thai isolates was found in this study. In Denmark, *M. hyosynoviae* strains showed less
Table 5. Minimal inhibitory concentrations (MICs) of lincomycin and macrolides for 13 Thai *M. hyosynoviae* isolates obtained G745A transition in domain II of 23S rRNA

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>MIC (µg/ml)</th>
<th>Lincomycin</th>
<th>Josamycin</th>
<th>Kitasamycin</th>
<th>Spiramycin</th>
<th>Tylosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>982/51 T2</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>982/51 T5</td>
<td>0.25</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>982/51 T6</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>982/51 T8</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>982/51 T11</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>982/51 Lg1</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>904/54 Lg13</td>
<td>0.5</td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>904/54 Lg56</td>
<td>0.5</td>
<td>0.25</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>904/54 Lg57</td>
<td>0.5</td>
<td>0.25</td>
<td>2</td>
<td>8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>4087/54 T2</td>
<td>0.5</td>
<td>0.25</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>4087/54 Lg26</td>
<td>0.5</td>
<td>0.5</td>
<td>2</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>4087/54 Lg35</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>6654/54 Lg37</td>
<td>0.5</td>
<td>0.25</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. In vitro development of tylosin and lincomycin resistance in *M. hyosynoviae* strain S16
Table 6. In vitro development of tylosin and lincomycin resistance in *M. hyosynoviae* strain S16 and MIC level of the mutants

<table>
<thead>
<tr>
<th>Passage ID</th>
<th>Mutation pattern</th>
<th>MIC range (µg/ml)</th>
<th>Lincomycin</th>
<th>Tylosin</th>
<th>Spiramycin</th>
<th>Josamycin</th>
<th>Kitasamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P1</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>normal</td>
<td>0.5-2</td>
<td>0.25-2</td>
<td>2-8</td>
<td>0.25-4</td>
<td>0.5-8</td>
<td></td>
</tr>
<tr>
<td><strong>P2</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>normal</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td><strong>P3T1, P4T4</strong>, P5T4, P5T16, <strong>P5T32</strong>, P6T64, <strong>P6T128</strong></td>
<td>A2062G</td>
<td>64-256</td>
<td>32-256</td>
<td>256-256</td>
<td>&gt;256</td>
<td>256-256</td>
<td></td>
</tr>
<tr>
<td><strong>P1L0.063, P2L0.125</strong>, P3L0.5, P4L0.125, P5L2</td>
<td>normal</td>
<td>0.5-2</td>
<td>0.25-2</td>
<td>1-4</td>
<td>0.25-8</td>
<td>0.5-8</td>
<td></td>
</tr>
<tr>
<td><strong>P3L1, P3L2, P4L2</strong></td>
<td>A2058C</td>
<td>64-&gt;256</td>
<td>32-&gt;256</td>
<td>256-&gt;256</td>
<td>&gt;256</td>
<td>256-&gt;256</td>
<td></td>
</tr>
<tr>
<td><strong>P4L0.5, P4L1</strong></td>
<td>A2059C</td>
<td>64-&gt;256</td>
<td>32-&gt;256</td>
<td>256</td>
<td>&gt;256</td>
<td>256-&gt;256</td>
<td></td>
</tr>
<tr>
<td><strong>P5L16, P5L32, P6L128</strong></td>
<td>A2058G</td>
<td>&gt;256</td>
<td>64-&gt;256</td>
<td>64&gt;256</td>
<td>&gt;256</td>
<td>256-&gt;256</td>
<td></td>
</tr>
</tbody>
</table>

**a)** Number of passage

**b)** Abbreviation of antimicrobial selected (T: tylosin; L: lincomycin)

**c)** Concentration of antimicrobial in the *M. hyosynoviae* S16 culture broth

**d)** The bold indicates the passage and the highest concentration of antimicrobial that *M. hyosynoviae* S16 could survived
sensitivity to tylosin but the MIC levels did not reach the breakpoint (Aarestrup and Friis, 1998; Friis and Szancer, 1994), whereas the macrolides and lincomycin resistant *M. hyosynoviae* strains were found in Japan (Kobayashi et al., 1996c). In the United States, tylosin tended to have less activity to *M. hyosynoviae* field strains since 1970, however the recent US *M. hyosynoviae* strains did not show the resistance against tylosin (Schultz et al., 2012). In addition, macrolide resistant *M. hyorhinis* could revert to sensitive by subculturing in macrolide free broth (Kobayashi et al., 1996b). The above results suggested the occurrence of macrolide resistant strains was induced by the presence of antimicrobials in its environment. Hence, the MIC data may reflect the use of antimicrobial agents in the certain period of time.

As it is known, ribosomal peptidyl transferase center (PTC) is located at the interface side of the 50S subunit of bacterial ribosome and is responsible for protein synthesis. The peptidyl transferase cavity forms by the nucleotides of central loop of domain V in 23S rRNA, and A2058, A2059 implicate as an entrance of the tunnel. Loop of hairpin 35 of domain II in 23S rRNA also forms the wall of the exit tunnel opposite to A2058 (Polacek and Mankin, 2005). Actions of macrolides and lincosamides antimicrobials are binding to the PTC and inhibiting protein synthesis. Therefore, one of the defense mechanisms of bacteria is an alteration of specific nucleotides at the target site in 23S rRNA. As the evidences, various mutations at domain V of 23S rRNA conferred macrolides or lincomycin resistance and cross resistance in the number of bacteria were reported (Vester and Douthwaite, 2001). The mutation of 23S rRNA conferred macrolides and lincomycin cross resistance in *M. hyopnemoniae* and *M. hyorhinis* were also reported (Kobayashi et al., 2005; Stekenborg et al., 2005). In this study, we confirmed the occurrence of tylosin resistance in *M. hyosynoviae* Thai strains which had a point mutation at G745A. Tylosin contains a mycinose sugar at position 14 of the lactone ring. Therefore, tylosin not only binds at the upper position of the entrance of the tunnel and reaches
to the PTC cavity, but also reaches across a tunnel to interact with loop of hairpin 35 (Pelacek and Mankin, 2005). This might be the reason why the G745A in our M. hyosynoviae strains conferred tylosin resistance. However, the level of tylosin resistance of this mutant was not high. This result was in agreement to the previous study (Kobayashi et al., 2005).

To our knowledge, in vitro resistance mechanism of M. hyosynoviae strain against tylosin or lincomycin has never been examined. In this study, M. hyosynoviae S16 mutants developed quickly within 3 passages for both tylosin and lincomycin. The result was similar to the studies for M. hyopneumoniae by Hannan and for M. hyorhinis by Kobayashi (Hannan et al., 1997b; Kobayashi et al., 2005). The interaction of lincomycin with E.coli 23S rRNA revealed that lincomycin strongly interact with A2058 but did not interact with A2059 and A2062 (Douthwaite, 1992). However, the mutation at A2059 of 23S rRNA conferred macrolides and lincomycin resistance in M. hyorhinis and Helicobacter pylori (Kobayashi et al., 2005; Wang and Taylor, 1998). In our study, the S16 mutated at A2058C/G or A2059C in lincomycin selection also conferred the lincomycin resistance and macrolides cross resistance. Whereas the S16 mutated at A2062G in tylosin selection conferred only macrolides resistance. This result was similar to the study on josamycin selected M. hominis which mutated at A2062 and showed resistance to macrolides but was susceptible to lincosamides (Funeri et al., 2001). According to the results, we believed that lincomycin might have some part reach to nucleotide A2059 but not to A2062 of central loop of domain V in 23S rRNA of M. hyosynoviae since A2059G S16 mutants could be obtained and predominated in lincomycin selection resulting in lincomycin resistance and macrolides cross resistance, but A2062G mutants obtained from the tylosin selection showed the resistance only to tylosin but not to lincomycin.
It was noted that 15 of 41 (36.6%) Thai isolates of *M. hyosynoviae* were resistant to enrofloxacin. The result was different from the previous reports (Aarestrup and Friis, 1998; Hannan et al., 1997a; Kobayashi et al., 1996c; Schultz et al., 2012). On the other hand, enrofloxacin resistant *M. hyopneumoniae* was found in Belgium and the resistance mechanism has been studied (Vicca et al., 2007). It should be carried out whether the same mechanism would be detected in our *M. hyosynoviae* strains. Tiamulin, among antimicrobials tested, showed the highest activity against *M. hyosynoviae* strains which is in agreement with the previous reports (Aarestrup and Friis, 1998; Friis and Szancer, 1994; Hannan et al., 1997a; Kobayashi et al 1996c).

Interestingly, all *M. hyosynoviae* strains were highly resistant to oxytetracycline. In Japan, also the activity of oxytetracycline to *M. hyosynoviae* has decreased, but MIC level did not reach the break point (Kobayashi et al 1996c). The resistance to oxytetracycline of *M. hominis* and *Ureaplasma parvum* was associated with acquisition of *tet*(M) determinant (Mardassi et al., 2012). The 16S rRNA mutation also conferred tetracycline resistance in *Helicobacter pylori, M. hominis* and *M. pneumonia* (Degrange et al., 2008; Wu et al., 2005). The resistance mechanisms of *M. hyosynoviae* against tetracyclines including oxytetracycline and doxycycline should be elucidated.

In conclusion, this study provided the susceptibilities of Thai isolates of *M. hyosynoviae* to 10 antimicrobials used in Thailand. Resistances against oxytetracycline, enrofloxacin and tylosin were detected. The resistance mechanism of *M. hyosynoviae* field isolates against tylosin was associated with G745A transition in domain II of 23S rRNA. The veterinarians should carefully choose appropriate drug for the treatment and control of *M. hyosynoviae* infection to minimize the increase of resistant strains in Thailand.
2.5 Abstract

*In vitro* susceptibilities of 41 Thai isolates of *Mycoplasma hyosynoviae* isolated from lung and tonsil of pigs to 10 antimicrobial agents were investigated. All isolates showed resistance to oxytetracycline, while 15 isolates (36.6%) showed resistance to enrofloxacin. The examination for the mutation in domain II and domain V of 23S rRNA revealed 13 Thai isolates of *M. hyosynoviae* obtained G745A transition (*E. coli* numbering). The result was correlated to the resistance to tylosin with MIC ranged 4-8 µg/ml. The development of *M. hyosynoviae* S16 resistance to tylosin and lincomycin showed the A2058C, A2059C or A2058G transitions in lincomycin selection which conferred marked resistance to lincomycin and all macrolides tested. While, A2062G transition was obtained from tylosin selection and this point mutation conferred resistance to macrolides but not to lincomycin.
Chapter III

Development of semi-nested PCR for detection of 16S rRNA gene of *Mycoplasma hyosynoviae*


3.1 Introduction

*Mycoplasma hyosynoviae* (*M. hyosynoviae*), a non-cell wall prokaryote, belongs to the class Mollicutes, Family Mycoplasmataceae is one of the porcine mycoplasma pathogens and appears world-wide (Kobisch and Friis, 1996). At present, arthritis caused by *M. hyosynoviae* becomes increasing problem in many countries (Neilsen et al., 2001; Assuncao et al., 2005; Schultz et al., 2012). *M. hyosynoviae* is the host specific bacterium in pigs and commonly inhabits upper respiratory tract such as nasal cavity, pharynx and tonsil of convalescent and adult animals. Adult pigs are an important reservoir of infection to piglets by direct contact. Pigs may occasionally develop pneumonic lesion and arthritis (Hagedorn-Olsen et al., 1999). *M. hyosynoviae* is an arginine metabolizing mycoplasma, which can be cultivated in Hayflick’s medium supplemented with mucin and arginine. Primary isolation from tissues of pigs is often complicated because of overgrowth of *M. hyorhinis* as well as other bacteria (Friis et al, 1991) making it difficult to detect *M. hyosynoviae* by cultivation.

Although certain breeders have experienced osteoarthritis lesions similar to *M. hyosynoviae* arthritis in breeding pigs in Thailand, there has been no report confirming the detection of this bacterium. However, *M. hyosynoviae* has been a common pathogen causing acute and severe lameness in grower-finisher pigs in Denmark. Time consumed for the surveillance and treatment of the disease was 30-90 minutes per 1000 pigs daily (Nielsen et al., 2001). Among pathogenic porcine mycoplasmas, *M. hyopneumoniae* is known as the most important mycoplasma that causes enzootic pneumonia and economic losses in pig industry. Therefore, there have been several techniques developed for specific and rapid detection and identification including PCR based method with high sensitivity such as nested PCR and real time PCR (Stark, et al., 1998; Calsamiglia et al., 1999; Kurth et al., 2002; Dubosson et al., 2004). Although the 16S-23S intergenic spacer PCR was established to differentiate the porcine mycoplasmas in the culture medium, it was
not evaluated for use in clinical samples (Nathues et al., 2011). A few PCR protocols for identification of *M. hyosynoviae* in clinical samples have been developed, however, the assays had limit of detection about 10⁴ CFU of the organism per gram of lung tissue (Ahrens et al., 1996; Kobayashi et al., 1996a).

In this study we developed a semi-nested PCR assay with improving limit of detection targeted to 16S rRNA gene for detection of *M. hyosynoviae* from tissues of pigs.

### 3.2 Materials and Methods

**Bacterial strains and growth conditions:** The mycoplasmas and other bacteria used in this study are listed in Table 7. Mycoplasma type strains including *M. hyopneumoniae* strain J, *M. hyorhinis* BTS7 and *M. hyosynoviae* S16 were obtained from National Institute of Animal Health (NIAH), Japan. *M. flocculare*, *Arcanobacterium pyogenes* and *Escherichia coli* were purchased from American Type Culture Collection. The other bacteria were the local isolates derived from naturally infected pigs and collected in our culture collection.

*M. hyopneumoniae* was cultured in BHL broth, *M. hyorhinis*, *M. arginini* and *M. bovigenitalium* in Hayflick’s broth, and *M. hyosynoviae* in Hayflick’s broth supplemented with mucin and arginine. *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis* were incubated on chocolate blood agar and the other bacteria on 5% sheep blood agar. The incubation times were 3-5 days for mycoplasmas and 18-24 hours for bacteria. Mycoplasma cells were collected in 1.5 ml microtube by centrifugation at 13,000 rpm for 10 minutes. The pellets were washed one time with phosphate buffered saline and followed by DNA preparation step.
Table 7. Microorganisms used in the development of semi-nested PCR for *Mycoplasma hyosynoviae*

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycoplasma hyosynoviae</em> S16</td>
<td>NIAH, Japan</td>
</tr>
<tr>
<td><em>Mycoplasma hyopneumoniae</em> J</td>
<td>NIAH, Japan</td>
</tr>
<tr>
<td><em>Mycoplasma hyopneumoniae</em> 1-10</td>
<td>Local isolate</td>
</tr>
<tr>
<td><em>Mycoplasma hyorhinis</em> BTS 7</td>
<td>NIAH, Japan</td>
</tr>
<tr>
<td><em>Mycoplasma hyorhinis</em> 1-10</td>
<td>Local isolate</td>
</tr>
<tr>
<td><em>Mycoplasma flocculare</em> ATCC 27399</td>
<td>ATCC, U.S.A.</td>
</tr>
<tr>
<td><em>Mycoplasma arginini</em> PG 230</td>
<td>NIAH, Japan</td>
</tr>
<tr>
<td><em>Mycoplasma bovigenitalium</em> PG 11</td>
<td>NIAH, Japan</td>
</tr>
<tr>
<td><em>Arcanobacterium pyogenes</em> ATCC 49698</td>
<td>ATCC, U.S.A.</td>
</tr>
<tr>
<td><em>Actinobacillus pleuropneumoniae</em></td>
<td>Local isolate</td>
</tr>
<tr>
<td><em>Bordetella bronchiseptica</em></td>
<td>Local isolate</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC 35150</td>
<td>ATCC, U.S.A.</td>
</tr>
<tr>
<td><em>Hemophilus parasuis</em></td>
<td>Local isolate</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>Local isolate</td>
</tr>
<tr>
<td><em>Pasteurella multocida</em></td>
<td>Local isolate</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Local isolate</td>
</tr>
<tr>
<td><em>Streptococcus suis</em></td>
<td>Local isolate</td>
</tr>
</tbody>
</table>
**DNA preparation:** All mycoplasmal and bacterial DNA were prepared using Instagene (Bio-Rad, U.S.A.) following the manufacturer’s protocol. After the cell lysate was centrifuged at the end of the process, the supernatant was collected and kept as DNA template at -20°C until use. For *M. hyosynoviae* S16, the concentrations of DNA were determined by spectrophotometer (Nanodrop® ND-1000, Nanodrop Technology, U.S.A.). The DNA was then diluted to 1 ng/µl and serial ten-fold dilution were made to $10^{-6}$ and used to test the detection limit of this semi-nested PCR assay.

**Selection of primers and PCR reactions:** The specific forward primer was selected based on the alignments of 16S rRNA gene of *M. hyosynoviae* (Genbank accession number U26730) with other porcine mycoplasmas including *M. hyopneumoniae* (E02783), *M. flocculare* (X63377), *M. hyorhinis* (M24658), *M. arginini* (NR041743) and *M. hyopharyngis* (U58997). The outer specific reverse primer was designed by Kobayashi et al. (1996a). The inner specific reverse primer was also selected from the multi-alignments of 16S rRNA genes as well. Nucleotide sequences of the primers were MHS_F (A): 5' GAA GCG TTT GCT TCA CTA AGA GAT 3' (nt 196-219), MHS_R (B1): 5' TTA GCT GCG TCA GTG ATT GG 3' (nt 825-844) and MHS_R (B2): 5' GCT TTC TAA CAA GGT ACC GTC AGT 3' (nt 468-491).

The PCR was carried out in 0.2 ml tube in a reaction volume of 20 µl. All PCR mixture contained 1x PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% TritonX-100), 1.5 mM MgCl$_2$, 200 µM each of dATP, dTTP, dCTP and dGTP; 0.5 U Taq polymerase (HotstarTaq, Qiagen, Germany), and 0.5 µM of each forward and reverse primers. Then DNase/RNase-free water was added up to 18 µl. For the first round of the semi-nested PCR, primer A and B1 were used and 2 µl of DNA sample was added in each reaction. The amplification performed in thermal cycler (Hybaid, Thermo electron, U.S.A.) with an initial denaturation at 95°C 15 min, followed by 35 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 1 min,
then a final extension at 72°C for 5 min. For the second round of the semi-nested PCR, primer A and B2 were used. The 0.5 µl of PCR product from the first round of the semi-nested PCR used as a DNA template and 1.5 µl DNase/RNase-free water were added in each reaction. The amplification performed in the same condition as the first PCR, but the amplification ended after cycle 25. The PCR products from both amplifications were analyzed by electrophoresis through 1.5% agarose gels containing 0.1 µg/ml ethidium bromide. The gels were run at 100 volts for 30 min and visualized by ultraviolet light in gel documentation (GelDoc. It. UVP, U.S.A.).

**Specificity:** The specificity of the primers was examined by amplification of these primers with the other porcine mycoplasmal and bacterial species (Table 7) which associate with pneumonia and arthritis in pigs.

**Limit of detection:**

**Purified M. hyosynoviae DNA**

The serial ten-fold dilutions of *M. hyosynoviae* S16 DNA started from 1 ng/µl were tested by semi-nested PCR. The minimum concentrations showing positive result in the semi-nested PCR were noted.

**Simulated lung samples**

A piece of lung collected from a mycoplasma free pig was homogenized and put into a bag approximately 1 gram per bag. Five ml of phosphate buffered saline was added to make a lung suspension and serial 10-fold dilutions of *M. hyosynoviae* S16 from $10^7$ CFU to 10 CFU were added into each bag. Afterwards, the DNA of simulated lung samples was extracted as described by Kobayashi et al. (1996a). Then semi-nested PCR was performed to determine the minimum number of mycoplasma cells in simulated lung sample that showed positive result.

**Detection of M. hyosynoviae from slaughtered pigs:** Thirty tonsil samples per farm were collected from slaughtered pigs in 10 farms and tested for *M. hyosynoviae* by semi-nested PCR as described above as well as by cultivation following to the
modified method from Friis et al. (1991). The rate of detection of *M. hyosynoviae* by each method was compared.

### 3.3 Results

The expected sizes of the PCR products from the first and the second round of the semi-nested PCR were 649 bp and 295 bp, respectively, because the primers were designed based on 16S rRNA gene of *M. hyosynoviae* accession number U26730 from the following positions. The position of a forward primer was at 196nt to 219nt whereas the positions of an outer reverse primer and an inner reverse primer were at 825nt to 844nt and 468nt to 491nt, respectively. Using the adequate concentration of the *M. hyosynoviae* DNA, the first round of semi-nested PCR could generate an amplified fragment about 649 bp followed by an amplified fragment about 295 bp for the second round of semi-nested PCR as shown in Fig 2.

![Oligonucleotide primers specifically amplified 16S rRNA gene of *M. hyosynoviae* and showed the PCR products at the predicted sizes of 649 bp (A) and 295 bp (B) with the primers A&B1 and A&B2, respectively. Lane M, 100 bp DNA marker; Lane 1-4, DNA of *M. hyosynoviae* strain S16](image)
The first and the second round of semi-nested PCR were examined for their specificity with porcine mycoplasmas and other bacterial species commonly associated with pneumonia and/or arthritis in pig as listed in Table 7. None of the primer pairs yielded PCR products or non-specific bands with DNA from the other mycoplasmas and bacterial species. The semi-nested PCR detected as little as 10 fg of purified *M. hyosynoviae* DNA in a reaction (Fig 3) and showed positive result with lung samples containing $10^7$-$10^3$ CFU/g of *M. hyosynoviae* (Fig 4).

![Figure 3. Detection limit of the semi-nested PCR procedure in detecting 10 fold dilution of 1 ng of extracted genomic DNA of *M. hyosynoviae* (M. hs) from 1 ng to 0.1 fg, Lane M, 100 bp DNA marker; Lane 1 through 8, 1 ng of M. hs DNA to 0.1 fg of M. hs DNA, respectively; Lane 9, DW (negative control); Lane 10, M. hs DNA (positive control)
Figure 4. Detection limit of the semi-nested PCR procedure in detecting extracted genomic DNA of *M. hyosynoviae* (M. hs) from simulated lung with $10^7$-$10^9$ CFU/g. Lane M, 100 bp DNA marker; Lane 1 through 7, DNA from simulated lung with M. hs $10^7$ CFU to 10 CFU, respectively; Lane 8, DW (negative control); Lane 9, M. hs DNA (positive control)

To evaluate the applicability of this method a total of 300 tonsillar samples were collected at a slaughterhouse and compared the detection rate with that by cultivation. As shown in Table 8, 45 of 300 (15%) samples were positive by semi-nested PCR while 17 of 300 (5.7%) were positive by cultivation. As for farm, 5 of ten farms were positive by semi-nested PCR (Fig 5) and 2 of 10 by culture. In cultivation positive two farms, positive rates by cultivation were 7/30 (23.3%) and 10/30 (33.3%) while those by semi-nested PCR were 14/30 (47%) and 21/30 (70%) respectively. Thus the semi-nested PCR was shown to be a useful tool for rapid and sensitive detection of *M. hyosynoviae* in clinical materials in pig herds.
Table 8. Comparison between semi-nested PCR and cultivation for the detection of *M. hyosynoviae* from tonsillar sample

<table>
<thead>
<tr>
<th>Semi-nested PCR</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of sample</td>
<td>No. of farm</td>
<td>No. of sample</td>
</tr>
<tr>
<td>Cultivation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>17</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>28</td>
<td>3</td>
<td>255</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td>5</td>
<td>255</td>
</tr>
</tbody>
</table>

Figure 5. PCR products from homogenate tonsils for detection of *M. hyosynoviae* (*M. hs*) in clinical specimens. Lane M, 100 bp DNA marker; Lane 1 through 28, DNA extract of tonsil samples from pigs; Lane 29, *M. hs* S16 DNA (positive control); Lane 30, DW (negative control)
3.4 Discussion

To our knowledge, there have been limited simplex PCR assays for detection of *M. hyosynoviae* in clinical samples with a limit of detection at $10^4$ CFU/g of sample (Ahrens et al., 1996; Kobayashi et al., 1996a). In this study, we developed the semi-nested PCR with a lower limit of detection at $10^3$ CFU/g. This might be of benefit to laboratory workers in detecting *M. hyosynoviae* infection in various clinical samples using the higher sensitivity PCR assay.

The oligonucleotide primers used in this study were designed based on the multi-alignment of 16S rRNA genes of both pathogenic porcine mycoplasmas and rarely isolated non-pathogenic porcine mycoplasmas to select species specific primers for *M. hyosynoviae*. *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* are genetically related and belong to *M. neurolyticum* cluster (Johansson and Petersson, 2002) so that the nucleotide sequences of selected primers completely differed from those mycoplasmas. *M. hyopharyngis* is a non-pathogenic and occasionally isolated from pigs. Like *M. hyosynoviae*, *M. hyopharyngis* hydrolyzes arginine and produces film and spot on the agar medium. It is likely to get confused between these two mycoplasmas by some workers. *M. hyopharyngis* belongs to the *M. lipophilum* cluster (Pettersson et al., 2001), whereas *M. hyosynoviae* belongs to the *M. hominis* cluster (Johansson and Petersson, 2002). However, we found that the nucleotide sequences of the primers partially matched the 16S rRNA gene of *M. hyopharyngis*. Moreover, the observation by BLAST indicated that the forward primer completely (100%) matched the 16S rRNA sequences of *M. buccale*, a close genetic relative of *M. hyosynoviae*, while the other two primers were partially matched. Although *M. buccale* is rarely isolated from pigs, the semi-nested PCR shall not produce a non-specific amplified product with this mycoplasma when using the appropriate annealing temperature.
Comparative study of the detection of *M. hyosynoviae* in tonsil samples of pigs from 10 farms by semi-nested PCR and cultivation revealed the presence of *M. hyosynoviae* in pig farms in Thailand. The selected farms were located in areas of intensive pig farming in the north, northeastern, eastern and the central part of Thailand. The use of semi-nested PCR yielded a higher infection rate than culture method. Hence, PCR assay seemed to be an effective method for diagnosis of *M. hyosynoviae* as described by Strait, et al. (2006). Isolation of *M. hyosynoviae* was successful in only 2 farms that showed a high rate of infection by semi-nested PCR. This high infection rate might reflect the recent outbreak of arthritis (Friis et al., 1991). On the other hand, although *M. hyosynoviae* colonizes in tonsils, the invasion of the organisms to the lung or joint which results in pneumonia or arthritis might depend on several factors such as age, immunity, infection pressure and stress (Hagedorn-Olsen et al., 1999; Nielsen et al., 2005). Therefore, the occurrence of pneumonia, arthritis and lameness must be further investigated in the semi-nested PCR positive farms and the samples from target organs should be taken for diagnosis to prove the infection level and clinical impact in the farm.

In conclusion, the semi-nested PCR developed here is useful for rapid and sensitive screening of *M. hyosynoviae* in pig herds in Thailand. Further study for the prevalence and incidence of the disease should be carried out.

### 3.5 Abstract

The semi-nested polymerase chain reaction assay was developed for detection of *Mycoplasma hyosynoviae* in organs of pigs in Thailand using three oligonucleotide primers specific to 16S rRNA gene of *M. hyosynoviae*. The detection limit of purified DNA was 10 femtogram per reaction and of the simulated lung sample was $10^3$ CFU per gram of sample. Comparative study for the detection of *M. hyosynoviae* in tonsils revealed that the detection rate by semi-nested PCR was
higher than that by cultivation both in samples and farms. Thus, the semi-nested PCR was suggested to be a useful tool for screening *M. hyosynoviae* in pig herds.
Chapter IV

Genetic diversity of

*Mycoplasma hyosynoviae* field isolates

in Thailand
4.1 Introduction

Arthritis caused by *M. hyosynoviae* becomes one of the most important increasing problems in many countries (Neilsen et al., 2001; Assuncao et al., 2005; Dahlia et al., 2009). Recently, the occurrence of *M. hyosynoviae* infections in Thailand identified by isolation technique, PCR and semi-nested PCR were reported (Makhanon et al., 2012; Thongkamkoon et al., 2012). The organisms commonly inhabit the upper respiratory airways and/or tonsils and occasionally develop non-suppurative arthritis and lameness. The development of arthritis might depend on many factors including variation of virulent factors and the antigenicity of different strains (Hagedorn-Olsen et al., 1999). It is essential to know the epidemiology of the disease to establish control measures for *M. hyosynoviae* arthritis. Recently genetic heterogeneity of casual organisms has been widely used to get the epidemiological information in various infectious diseases. It was reported that *M. hyosynoviae* showed highly genetic differences among isolates by amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE) analysis techniques (Kokotovic et al., 1999; 2002a). These two techniques yielded the comparable results for differentiation of *M. hyosynoviae* strains obtained from different geographical locations. The identical patterns were detected only for the strains obtained from the same country suggesting the usefulness of the methods in monitoring the epidemiology of the disease. Random amplified polymorphic DNA (RAPD) analysis was developed and found to be useful to investigate the epidemiological relatedness of avian mycoplasmas. For instance, Fan et al., (1995) used the technique to differentiate the subspecies level of *M. synoviae*. Marois et al. (2001) found that typability and discriminatory power of RAPD were greater than PFGE. For a genetically homogeneous species, *M. pneumoniae*, PFGE and RAPD had a little advantage over RFLP analysis of the P1 gene (Cousin-Allery et al., 2000). A high diversity was observed for the strains of *M. bovis* by RAPD technique (McAuliffe et al., 2004). It seemed to have lower discriminatory power for
differentiation of *M. hyopneumoniae* strains (Stakenborg et al., 2006). The technique has yet not been applied for *M. hyosynoviae*.

The aim of the present study was to compare the applicability of PFGE and RAPD techniques for molecular typing of *M. hyosynoviae* and to investigate the genetic diversity as an epidemiological data of Thai *M. hyosynoviae* isolates.

**4.2 Materials and Methods**

*Mycoplasma strains*: Forty one Thai isolates of *M. hyosynoviae* isolated from slaughtered pigs during 2008-2011 at National Institute of Animal Health, Thailand and kept at -80°C were used. The type strain S16 obtained from National Institute of Animal Health, Japan was also included. Of the Thai isolates, 18 were isolated from tonsils, synovial fluid and lung of pigs from 5 farms located in Chiang Mai (1), Buri Ram (2), Nakhon Sawan (1) and Nakhon Ratchasima (1) in 2008, 13 were isolated from tonsils of pigs from one farm located in Chon Buri in 2009 and 10 were isolated from lungs and tonsil of pigs from 3 farms located in Suphan Buri, Nakhon Ratchasima and Saraburi in 2011. Each of *M. hyosynoviae* isolate was propagated in 30 ml Hayflick’s broth supplemented with arginine and mucin (Friis et al., 1991) and incubated in 37°C for 4 days.

**Pulsed-field gel electrophoresis (PFGE):** *M. hyosynoviae* cells were harvested from the broth culture by centrifugation at 10,000 xg for 30 minutes at 4°C (Himac CR22GII, Hitachi, Japan). The packed cells were washed three times in 2 ml washing buffer (50mM Tris-HCl, 10mM EDTA, 100mM NaCl, pH 7.2) and centrifuged at 13000 xg for 5 minutes at 4°C (Model 1920, Kubota, Japan). The cells were resuspended in 200 µl of normal saline. The suspension was mixed with an equal volume of 1.6% low melting agarose (Bio-Rad, U.S.A.) and loaded into the plug mould (Bio-Rad, U.S.A.) to set the plug at 4°C for 10 minutes. Then, the agarose plugs were left in 2 ml lysis buffer (10 mM Tris-HCl pH 8.0, 50mM EDTA pH8.0, 1% N-lauroyl sarcosine, 0.1 mg/ml proteinase K) at 50°C for overnight.
After incubation, the plugs were washed three times in DW for 15 minutes at 50°C, followed by washing in TE for 15 minutes at 50°C for two times. Prior to the DNA digestion, the plugs were equilibrated in 1x restriction enzyme buffer at 37°C for 10 minutes. The thirty units per plug of BssHII restriction enzyme (New England Biolabs, U.S.A.) was used for the digestion of genomic DNA of *M. hyosynoviae* at 37°C for 5 hours.

PFGE was performed with a CHEF-DR III Pulsed Field Electrophoresis System (Bio-Rad, U.S.A.). After digestion by restriction enzyme, plugs were rinsed one time by 0.5x TBE buffer and loaded into 1.0% pulsed field certified agarose (Bio-Rad, U.S.A.). Electrophoresis was run at 14°C and 6V/cm from 0.5-8.5 seconds switching times for 18 hours. After electrophoresis, agarose gels were stained with 0.1µg/ml ethidium bromide in DW, destained in DW and DNA fragments were visualized and photographed under ultraviolet light in a gel documentation system (GelDoc-It, UVP, U.S.A.).

**Random amplified polymorphic DNA (RAPD):** One ml of each *M. hyosynoviae* culture was harvested at 10,000 xg for 10 min (Model 1920, Kubota, Japan). Then, the pellet was washed once with 1 ml PBS and mix with Instagene matrix (Bio-Rad, U.S.A.) for DNA preparation following the manufacturer’s instruction. RAPD was performed for all samples in one single PCR using Ready-To-Go RAPD Analysis kit (GE healthcare, Lifesciences, U.S.A.) following the manufacturer’s instructions. Briefly, five µl of DNA template, 25 pmol of RAPD analysis primer 6 - [5'- d (CCCGTCAGCA) -3'] and 15 µl of distilled water were added into a tube containing RAPD analysis bead. The contents were mixed well by gently vortex. A sample was placed in a thermal cycler (Hybaid, Thermo electron, U.S.A.) and cycle using the following program: 1 cycle of 95°C for 5 minutes; 45 cycles of 95°C for 1 minute, 36°C for 1 minute and 72°C for 2 minutes. Amplification products were electrophoresed through 2% agarose gel containing 0.1 µg/ml ethidium bromide at
100 volts for 50 minutes. RAPD patterns were also visualized and photographed under ultraviolet light.

**Data analysis:** The dendrogram, based on the PFGE patterns, was constructed using BIO-PROFIL Bio-1D++ v11.11 software (Vil-Lourmat, Germany). Similar matrix between PFGE profiles was calculated using Dice similarity coefficient. Cluster analysis was performed with unweighted pair group method using average linkages (UPGMA). Isolates that had 100% similarity were considered to be the same strain and isolates that had more than 90% similarity were considered to be genetically related isolates and to be derived from a common parent. The dendrogram, based on the RAPD patterns, was also constructed with the same software.

### 4.3 Results

**PFGE:** Of 41 *M. hyosynoviae* Thai isolates, 37 isolates were typable by PFGE technique whereas 4 isolates which consisted of 2 isolates from farm B and one isolate each from farm D and F did not yielded the well-separated bands. The banding patterns of typable strains consisted of 6-12 fragments in the size range of 2-48 kb. Twenty two different patterns were detected among 37 *M. hyosynoviae* Thai isolates and the type strain S16 (Figure 6). Six isolates obtained from farm A were differentiated into 4 PFGE patterns including pattern 1, 2, 12 and 13. The similarity between pattern 1 and 2 was more than 90%. The same result was also shown between pattern 12 and 13. Of the 5 isolates obtained from farm B, 4 isolates had 100% similarity of PFGE pattern 9 whereas the other isolate showed PFGE pattern 17 which had 100% similarity to the isolate obtained from farm C. Three isolates obtained from farm E showed PFGE pattern 4 and 11. Eight isolates obtained from the first fattening pig herd in farm F showed PFGE pattern 3, 7, 8, 10 and 14, whereas 5 isolates obtained from another fattening pig herd that was raised two months later in the same farm showed the same PFGE pattern 10. Three isolates obtained from farm G showed the PFGE pattern 5 and 15. The other isolate obtained
from different fattening pig herd that was raised four months later in the same farm showed the PFGE pattern 16 which had 75% similarity to the pattern 15. The isolate obtained from farm I showed the PFGE pattern 20. Five isolates obtained from farm H showed the PFGE pattern 6, 18, 19 and 21. The *M. hyosynoviae* type strain S16 showed the PFGE 22 which was distinguished from the local isolates.

In this study, the typability of PFGE to 41 *M. hyosynoviae* Thai isolates was 91%. PFGE technique had a high reproducibility with the identical banding pattern obtained for replicate samples, even when the DNA extraction and electrophoresis were performed at different time.

**RAPD:** Five *M. hyosynoviae* randomly selected Thai isolates and the type strain S16 were typable by RAPD technique using 6 RAPD analysis primers and RAPD beads, however different discriminatory powers were found among the primers (result not shown). RAPD analysis primer 6 was selected due to the highest discriminatory power. The reproducibility was stable only when RAPD was performed using the RAPD beads since the results obtained from different pre-prepared commercial mastermix showed different fingerprint (result not shown). The banding patterns consisted of 1-12 fragments in the size range of 200-1500 bp. Thirty nine RAPD patterns were detected among 41 *M. hyosynoviae* Thai isolates and the type strain S16 (Figure 6). All isolates obtained from the different farms showed different RAPD patterns whereas almost all isolates obtained from the same farms also showed different RAPD patterns. The exception was 4 isolates obtained from farm F showing 100% similarity of pattern 33 which was in agreement with the result obtained from PFGE analysis. The three isolates from farm C of which 2 isolates could not be typed by PFGE showed their RAPD patterns (pattern 6, 7, 8) within a group with 67% similarity (Fig 5.2). The RAPD pattern 7 and 8 which had about 83% similarity belonged to the isolates obtained from the same farm whereas RAPD 3 and 4 having about 77% similarity belonged to the isolates with the same PFGE profile.
**Relationship between genetic profiles and sequences of domain II and V of 23S rRNA:** A acquired G745A transition in domain II was found in *M. hyosynoviae* Thai isolates including all 6 isolates from farm A, 4 isolates from farm G and 3 isolates from farm H which showed an identical or similar PFGE patterns. In addition, 2 isolates from farm H showing an identical PFGE pattern obtained an acquired C739T transition in domain II.

**4.4 Discussion**

As revealed by PFGE, the clonal appearance was found in farm A, B, E, F, G and H. The same clone found in different organs of pigs including pattern 9 in farm B and pattern 12 in farm A might indicate the invasion ability of *M. hyosynoviae* strains from tonsil to either joint or lung of the pig. The 92% similarity between pattern 1 and 2, and the 95% similarity between pattern 12 and 13 in farm A demonstrated the alteration in genetic composition of one clone during its colonization in the herd. Danish strains of *M. hyosynoviae* obtained from the same farms consisted of identical patterns and 1 to 4 different clonal lines by AFLP (Kokotovic et al., 2002b). Similar result was found in this study since 2 to 5 clonal lines were also found in farm A, B, E, F, G and H supporting the evidence of highly genetic heterogeneity of *M. hyosynoviae*. Sharing of identical pattern between two strains obtained from the different geographic location in Denmark was observed once in the previous study (Kokotovic et al., 2002b). We also found the same clonal pattern obtained in the different two farms (farm B and farm C) within the same province suggesting that they might be derived from the same source.

Although RAPD seemed to have typability and discriminatory potential greater than PFGE in this study, we preferred using PFGE to reveal genetic diversity of *M. hyosynoviae* in Thailand. The most important drawback of RAPD was the limited reproducibility of the technique although all standard reagents and material were used. Number and intensity of the bands might vary due to more or less DNA
template and resulted in analysis problems. In addition, RAPD result was not related to the farms or origins of the isolates, whereas PFGE results showed a good correlation of its patterns and the origins of the isolates. In addition, the relationship between alteration of 23S rRNA gene and PFGE pattern of *M. hyosynoviae* isolates was demonstrated which also suggested that PFGE would be a suitable and useful tool for epidemiological study of *M. hyosynoviae*.

In conclusion, the typability of RAPD and PFGE for *M. hyosynoviae* Thai isolates in this study was 100% and 91%, respectively. RAPD profiles showed very high genetic heterogeneity of *M. hyosynoviae* isolates inside the herd and among the herds, whereas PFGE profiles could be grouped and revealed a single or multiple clonal lines of *M. hyosynoviae* inside a herd and between the herds within the same province. As *M. hyosynoviae* has shown to have a highly genetic heterogeneity, a longitudinal observation of their PFGE patterns among the pig population throughout the country would be useful to elucidate the epidemiology of *M. hyosynoviae* infections in Thailand.
Figure 5. Dendrogram of PFGE fragments of 37 *M. hyosynoviae* Thai isolates and the type strain S16. Cluster analysis was performed with UPGMA using the Dice similarity coefficient and 2% interval of confidence for band matching.
Figure 6. Dendrogram of RAPD profiles of 41 M. hyosynoviae Thai isolates and the type strain S16. Cluster analysis was performed with UPGMA using the Dice similarity coefficient and 2% interval of confidence for band matching.
4.5 Abstract

Pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) analyses were performed to compare their usefulness to investigate the genetic diversity as an epidemiological data of *M. hyosynoviae* Thai isolates. A total of 41 isolates and a type strain S16 were typable and consisted 39 different patterns by RAPD technique, whereas 37 isolates (97%) were typable and consisted 22 different patterns by PFGE technique. Based on PFGE patterns, multiple clones of *M. hyosynoviae* existed within a farm and high genetic heterogeneity of *M. hyosynoviae* was observed among the farms. No identical PFGE pattern between the pig farms was found except two farms that located in the same province. This finding might indicate the distribution of the organism from the same source. Monitoring the genetic diversity of *M. hyosynoviae* strains using PFGE analysis would be useful to elucidate the epidemiology of *M. hyosynoviae* infections in Thailand.
General discussion
1. Antimicrobial susceptibility of porcine mycoplasmas in Thailand

*M. hyopneumoniae* has been recognized as a cause of enzootic pneumonia in Thailand for more than forty years. Antimicrobials have long been given to piglets, gilts and sows to control mycoplasmas as well as other enteric and respiratory bacterial pathogens. Antimicrobial susceptibility tests of *M. hyopneumoniae* were performed for the isolates collected in 1988 and during 1997-1998. No resistant isolates were found in those periods (Saitanu et al., 1989; Narongsak and Thongkamkoon, 2002). To date, antimicrobial resistance of porcine mycoplasmas has been reported to tetracyclines, macrolides, lincomycin and flumequine, the first generation fluoroquinolone in some countries. However, the medication programs used for swine production in each country might vary according to the differences of susceptibility information. Therefore, susceptibilities of recent field isolates of *M. hyopneumoniae* collected during 2006-2011 and *M. hyosynoviae* field isolates collected from 2008-2011 were performed against antimicrobial agents widely used in the swine production in Thailand in this study.

The results revealed the MICs of enrofloxacin, which is the second choice of drug for *M. hyopneumoniae* distributed in a broad range and MICs for 47.7% of *M. hyopneumoniae* and 36.6% of *M. hyosynoviae* Thai isolates exceeded the breakpoint of this drug. This may reflect the overuse of enrofloxacin in pig farming in Thailand especially for treatment of diarrhea of lactating piglets and at nursery up to 9 weeks (Prapasaranukul et al., 2010).

In Thailand, tiamulin, tylosin, chlortetracycline and amoxicillin are also widely used in fattening pigs, pregnant and lactating sows and replacement gilts during acclimatization to maintain health status of the pigs. This might be the main factor that MICs of chlortetracycline and oxytetracycline for recent *M. hyopneumoniae* isolates were gradually increasing as compared to those for isolates in the previous study. The 70% of *M. hyopneumoniae* isolates was considered
chlotetracycline resistant. It was in agreement with the study of MICs of tetracyclines for *M. hyorhinis* Thai isolates collected during 1999-2004 that MICs for more than 50% of *M. hyorhinis* exceeded the breakpoint (Maknanon et al., 2006).

In addition, MICs of lincomycin and macrolides including tylosin, spiramycin, josamycin and kitasamycin were obviously higher for two *M. hyopneumoniae* isolates, indicating acquired resistance that attributed to a transition of A2058G in the central loop of domain V of the 23S rRNA of the isolates. One *M. hyorhinis* isolate in our previous study was also resistant to lincomycin and macrolides although the resistance mechanism of the isolate was not examined (Maknanon et al., 2006). Moreover, *M. hyosynoviae* Thai isolates which obtained a point mutation at G745A and showed a low level of resistance (MIC: 4-8 µg/ml) to tylosin were also found in our study.

Furthermore, in vitro resistance mechanism of *M. hyosynoviae* S16 strain against lincomycin and tylosin was examined. Lincomycin selected *M. hyosynoviae* S16 mutant strains obtained A2058C/G or A2059C transition, which conferred not only lincomycin resistance but also macrolides cross resistance. On the other hand tylosin selected S16 mutant strains obtained A2062G which conferred macrolides resistance only.

The pig producers usually use the same pattern of medication along the production line. Sows are medicated to reduce the transmission of an infectious agent to their piglets. Therefore, suckling piglets might get antimicrobials via milk. Weaning pigs also receive some form of antimicrobials primarily for *E. coli* control. Thereafter different antimicrobials might be given to grower and finishing pigs to control respiratory or systemic infections. Thus, antimicrobial resistant commensals and pathogens probably generate and dominate under this selection pressure and antimicrobial resistance determinants would be able to transfer horizontally to other bacteria as well. At present, acquired resistance to various antimicrobials might pose
a serious problem for the treatment of mycoplasma pneumonia of swine. Hence, prudent use of antimicrobials by monitoring the infections presenting in the farm and selecting suitable drugs for control programs should be an urgent necessity in pig industry in Thailand.

2. Use of semi-nested PCR in detecting *M. hyosynoviae* in Thailand

In Thailand, importation of exotic pig breeds from the United Kingdom and the United States has been started since 1960s. Then, pig breeding was industrialized steadily twenty years later (Charoensook et al., 2013). At present, Thailand ranks the eleventh on pork production in the world. It is known that *M. hyopneumoniae, M. hyosynoviae* and *M. hyorhinis* infections in pigs are very common in countries where swine production is one of their livestock industries (Maes et al., 2011). As a result, porcine mycoplasmas might enter Thailand via the imported breeders. Although the isolation of *M. hyopneumoniae* in Thailand was first successful in 1988, lung lesions resembling mycoplasmal pneumonia of swine have been shown in slaughter pigs since late 1970s (Saitanu et al., 1988), moreover, the prevalence of *M. hyopneumoniae* infection as well as the concurrent infection of *M. hyorhinis* was reported in 2000 (Thongkamkoon et al., 2000). In contrast, *M. hyosynoviae* infection has never been reported officially in Thailand although some breeders have experienced arthritis lesions similar to *M. hyosynoviae* infection.

The gold standard for diagnosis of various mycoplasmas is based on isolation technique. Although the isolation technique has been developed more than 30 years ago, these fastidious organisms always take at least a week to several weeks to grow on solid agar. Therefore, PCR becomes an alternative method for diagnosis of mycoplasmosis by its advantages including sensitivity and rapidity. A number of PCR protocols have been developed for the detection of *M. hyopneumoniae*, an etiological agent of enzootic pneumonia, which is a major pathogen and generally the most fastidious organism among porcine mycoplasmas. However, isolation
technique is recommended for detection of *M. hyorhinis* infection in pigs since *M. hyorhinis* can grow easily and overgrow *M. hyopneumoniae* and *M. hyosynoviae* (Scheiber and Thacker, 2012). So far, a few PCR protocols for detection of *M. hyosynoviae* in clinical samples have been developed. The assays had limit of detection about at least $10^4$ CFU of the organism per gram of lung tissue (Ahrens et al., 1996; Kobayashi et al., 1996a).

In this study, the semi-nested PCR with a lower limit of detection at $10^3$ CFU/g was developed. This is of benefit to Thai swine practitioners and farmers in confirming the existence of *M. hyosynoviae* in pigs in Thailand. As shown in the comparative study of the detection methods for *M. hyosynoviae* infection in tonsils of pigs semi-nested PCR revealed that *M. hyosynoviae* was presented in 5 of 10 farms and 45 of 300 (15%) samples, while the detection rate by cultivation was 20% (2/10) for farms and 5.7% (17/300) for samples. The PCR assay is considered to be an effective method for diagnosis of *M. hyosynoviae* by Strait, et al. (2006) also. Thus, the semi-nested PCR is a useful tool for presumptive screening of *M. hyosynoviae* presenting in pig herds in Thailand.

3. **Diversity of Mycoplasma hyosynoviae in Thailand.**

The semi-nested PCR was successfully developed in Chapter 3 of the study which enabled the screening of *M. hyosynoviae* infection in the farms. In chapter 2 *M. hyosynoviae* Thai isolates were examined for the antimicrobial susceptibility and some of them showed a point mutation by partial sequencing of 23S rRNA gene of the isolates. This resistance genotype might be a useful marker to search for genetic diversity and epidemiological relatedness of Thai *M. hyosynoviae* isolates.

Various molecular typing methods were used to reveal the intraspecies genomic variations and facilitating epidemiological studies. By pulsed-field gel electrophoresis (PFGE), *M. hyosynoviae* showed highly genetic differences among isolates (Kokotovic et al., 1999). The identical patterns were detected only for the
strains obtained from the same country suggesting the ability of the methods in monitoring the epidemiological relatedness of the strains. Random amplified polymorphic DNA (RAPD) was useful to investigate the epidemiology of avian mycoplasmas and had a discriminatory power greater than PFGE (Fan et al., 1995; Marois et al., 2001). However, it seemed to have lower discriminatory power for differentiation of *M. hyopneumoniae* strains (Stakenborg et al., 2006).

In this study PFGE and RAPD techniques for molecular typing of *M. hyosynoviae* was studied and compared since RAPD have never been used for intraspecies study of *M. hyosynoviae*. The result showed that RAPD seemed to have typability and discriminatory potential greater than PFGE, but RAPD result was not related to the farms or origins of the isolates. In addition, the same banding patterns were not necessarily obtained by repeated analyses of the same strains. In contrast, PFGE results showed a good correlation of its patterns and the origins of the isolates and the test showed a high reproducibility with identical banding patterns for replicate samples.

As revealed by PFGE, the clonal appearance was found in 5 farms since more than 2 clonal lines existed in the same farm supporting the evidence of highly genetic heterogeneity of *M. hyosynoviae*. One common profile was detected in each isolate derived from different two farms in one occasion.

Interestingly, all isolates from farm A confirmed to have G745A transition showed an identical PFGE pattern. Two of G745A *M. hyosynoviae* strains and another G745A mutant from farm G also showed an identical PFGE pattern and related PFGE pattern, respectively. The same result was also demonstrated in farm H. Additionally, another two isolates from farm H confirmed as C739T mutant also showed an identical PFGE pattern. The relationship between alteration of 23S rRNA gene and PFGE pattern of *M. hyosynoviae* isolates within farm A, G and H
supported the reliability of PFGE results and obviously confirmed that PFGE would be a suitable and useful tool for epidemiological study of *M. hyosynoviae*. 
Abstract

Mycoplasmosis is one of the most important porcine diseases. It has a great impact on the productivity and the economic performance in swine industry worldwide including Thailand where the pig production system has been becoming more and more industrialized. ‘Industrialized’ means ‘intensive management’ in which numerous pigs are reared in confined air space. In these environments chronic diseases including respiratory diseases, diarrhea and arthritis are most common in every country. Recently catastrophic disease called PRDC (porcine respiratory disease complex) prevails all over the world. Among various etiological agents which are claimed to be associated with the disease Mycoplasma hyopneumoniae is considered to play an essential role together with PRRS (porcine reproductive and respiratory syndrome) virus. It not only causes pneumonia (mycoplasmal pneumonia of swine: MPS) by itself but also aggravates infections caused by other agents especially PRRS virus, which escalates the economic losses induced by PRDC. Mycoplasma hyosynoviae causes nonpurulent polyarthritis in breeding and fattening pigs. The disease is also prevailed all over the world. Little is known on porcine mycoplasmas in Thailand because of their difficulties of isolation and cultivation of the organisms. The present investigations were carried out to get information which will improve the diagnostic techniques of porcine mycoplasmosis and contribute to the control of the diseases in that country. In the first chapter antimicrobial susceptibilities of M. hyopneumoniae were investigated since it is an important etiological agent of not only MPS but also PRDC. For isolates with resistance to macrolides, the first choice drugs in chemotherapy for mycoplasmosis, mechanisms of the resistance were examined by sequencing 23S rRNA of resistant isolates. In chapter 2 antimicrobial susceptibilities of M. hyosynoviae were also investigated and in vitro development of macrolide and lincomycin resistance was examined to elucidate the mechanisms of the resistance using type strain S16 of this organism. Since early medication is required for the effective chemotherapy of arthritis due to
M. hyosynoviae, rapid detection of this organism was developed in chapter 3. Finally methods to see the genetic diversity of field isolates of M. hyosynoviae in Thailand were evaluated from the perspective of getting epidemiological information of the disease.

1. Antimicrobial susceptibilities of Mycoplasma hyopneumoniae field isolates and occurrence of enroflaxacin, macrolides and lincomycin resistance

To assess the in vitro susceptibilities of M. hyopneumoniae to antimicrobial agents is inevitable for the chemotherapy of MPS. In this chapter a total of 159 Thai isolates of M. hyopneumoniae derived from pneumonic lungs of pigs in 41 farms during 2006-2011 were subjected for susceptibility testing against 12 antimicrobial agents widely used in Thailand; chlortetracycline (CTC), oxytetracycline (OTC), doxycycline (DOXY), lincomycin (LCM), josamycin (JM), kitasamycin (KT), spiramycin (SPM), tylosin (TS), erythromycin (EM), tiamulin (TM), florfenicol (FFC) and enrofloxacin (ERFX). The type strain J of M. hyopneumoniae was also included in the test as the control strain. Obtained data were compared with those for 27 isolates collected during 1997-1998.

Tiamulin showed the lowest minimal inhibitory concentrations (MICs) among drugs tested ranged between 0.013 and 0.2 µg/ml and its MIC₉₀ (MIC at which 90% of the isolates tested were inhibited) was 0.1 µg/ml. Next to TM, TS and LCM showed high activities with MIC₉₀ of 0.39 µg/ml, however, two isolates were resistant to these drugs showing a MIC value of higher than 12.5 µg/ml. Three macrolides, SPM, JM and KT also showed high potency with MIC₉₀ of 0.78, 0.78 and 1.56 µg/ml, respectively. Erythromycin showed exceptionally low activity among five macrolides tested with MIC₅₀ of 50 µg/ml. Florfenicol showed moderately high activity with MIC₉₀ of 1.56 µg/ml. Both OTC and DOXY showed similar activities with FFC and their MIC range distributed from 0.39 to 12.5 µg/ml with MIC₉₀ of 6.25 µg/ml. On the other hand, activity of CTC to Thai isolates of M.
**Mycoplasma hyopneumoniae** was low with MIC range from 3.12 to 100 µg/ml, MIC\(_{90}\) of 50 µg/ml and MIC\(_{50}\) of 25 µg/ml. The MICs of ERFX distributed in a broad range from 0.1 to 25 µg/ml and those for 76 of 159 (47.7 %) Thai isolates exceeded 2µg/ml which is the breakpoint of this drug.

The comparison of susceptibilities of the present 159 isolates with those of isolates obtained from 1997 to 1998 in Thailand revealed that susceptibilities of the present isolates to CTC, OTC, JM and TS were decreased considerably. The MIC\(_{90}\) for previous isolates were 6.25, 0.78, 0.2 and 0.1, whereas those for present isolates were 50, 6.25, 0.78 and 0.39 µg/ml, respectively, showing 4- to 8-fold higher MIC\(_{90}\). No change was observed in MIC\(_{90}\) of LCM and TM between the present isolates and the previous ones. The sequences of domain V of 23S rRNA of the two macrolides and LCM resistant isolates were compared with those of *M. hyopneumoniae* strain J and strain ATCC 27719 (Genbank accession number AE017243 and X68421, respectively). The result revealed a point mutation at A2058G (*E.coli* coordinates) in both macrolides and LCM resistant isolates.

2. Antimicrobial susceptibilities of *Mycoplasma hyosynoviae* field isolates in Thailand during 2008-2011 and in vitro development of resistance to tylosin and lincomycin in type strain S16 of *M. hyosynoviae*

No information is available on antimicrobial susceptibilities of field isolates of *M. hyosynoviae* in Thailand. In this chapter 41 Thai isolates of *M. hyosynoviae* derived from tonsils (29), lungs (10) and joint fluids (2) of pigs from 9 farms during 2008-2011 were investigated for their in vitro susceptibilities against 10 antimicrobial agents widely used in Thailand, namely OTC, DOXY, LCM, JM, KT, SPM, TS, TM, FFC and ERFX. The type strain S16 of *M. hyosynoviae* was also included in the test. All isolates and type strain S16 were examined for 23S rRNA transitions at domain II and V which relate to macrolide resistance and compared with MIC levels of macrolides and lincomycin for each strain. In vitro development
of macrolides and lincomycin resistance in type strain S16 of \textit{M. hyosynoviae} were also investigated to analyze the mechanisms of macrolides and lincomycin resistance.

Of 10 antimicrobials tested, TM showed the lowest MICs of 0.016-0.125 µg/ml against all \textit{M. hyosynoviae} field isolates. Next to TM, LCM and JM showed high activities with MIC$_{90}$ of 1 µg/ml. Both KT and FFC showed moderately high activities with MIC$_{90}$ of 2-4 µg/ml, whereas SPM and TS showed lower activities with MIC$_{90}$ of 8 µg/ml. Activities of tetracyclines to Thai isolates of \textit{M. hyosynoviae} were low and MICs for OTC ranged from 16 to 128 µg/ml and those for DOXY from 4 to 16µg/ml. The MIC range of ERFX was between 0.25 and 2 µg/ml. Of the Thai isolates of \textit{M. hyosynoviae} considerable portion was resistant to some of the drugs; 41 of 41 for OTC (break point; ≥16 µg/ml), 15 of 41 (36.6%) for ERFX (≥2µg/ml) and 13 of 41 (31.7%) for TS (≥4µg/ml).

An acquired G745A transition in domain II was found in 13 isolates by sequence analysis of domain II and V of 23S rRNA of all Thai isolates and type strain S16 of \textit{M. hyosynoviae}. The MICs of TS were of 4-8 µg/ml for these 13 isolates and 0.5-2.0µg/ml for remaining 28 isolates, suggesting strongly that this G745A transition in \textit{M. hyosynoviae} may confer a mild resistance to TS. MIC values of other macrolides and LCM were not correlated to this transition.

Type strain S16 of \textit{M. hyosynoviae} was highly susceptible to both TS and LCM with the MICs of 0.032 and 0.125 µg/ml respectively. In vitro development of resistance to TS and LCM were investigated by passaging this strain in broth medium containing various concentrations of TS or LCM. At the first and second passage, the highest concentration of TS and LCM that allowed the growth of S16 was the same as that for parent strain. From the third passage, the strain began to get resistance to TS or LCM rapidly and could grow at about 4 to 32 times higher concentrations in each passage. At the sixth passage the highest concentration of the
drug that S16 mutant strain could grow was 4,000 or 1,000 times higher for TS or LCM respectively. In LCM selection, the A2058C/G or A2059C transitions were obtained in S16 mutants. This mutant was resistant not only to LCM but also to all macrolide drugs tested. In TS selection the A2062G transition was obtained which conferred the resistance to macrolides but not to LCM.

3. Development of semi-nested PCR for detection of 16S rRNA gene of *Mycoplasma hyosynoviae*

Successful treatment of arthritis due to *M. hyosynoviae* in pigs is best achieved by injecting effective antimicrobials as early as possible. It needs several days, however, to diagnose the disease since the isolation of mycoplasmas is time-consuming. In this chapter semi-nested PCR to detect *M. hyosynoviae* using three oligonucleotide primers was developed for the rapid diagnosis of the disease. The primers were designed based on 16S rRNA gene of *M. hyosynoviae* accession number U26730 from the three positions. The position of a forward primer was at 196nt to 219nt whereas the positions of an outer reverse primer and an inner reverse primer were at 825nt to 844nt and 468nt to 491nt, respectively. The expected sizes of the PCR products from the first and the second round of the semi-nested PCR were 649 bp and 295 bp, respectively. Using the adequate concentration of the *M. hyosynoviae* DNA, the first round of semi-nested PCR could generate an amplified fragment about 649 bp followed by an amplified fragment about 295 bp for the second round of semi-nested PCR. The specificity of the test was examined with porcine mycoplasmal and bacterial species commonly encountered in pneumonic and/or arthritic lesions in pigs. The organisms tested were as follows: *Mycoplasma hyopneumoniae, M. hyorhinis, M. flocculare, M. arginini, M. bovigenitalium, Arcanobacterium pyogenes, Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Escherichia coli, Haemophilus parasuis, Klebsiella pneumoniae Pasteurella multocida Staphylococcus aureus* and *Streptococcus suis*. None of the primer pairs yielded PCR products or non-specific bands with DNA from those
mycoplasmal and bacterial species. The semi-nested PCR developed here detected as little as $10^{-14}$ g of purified *M. hyosynoviae* DNA in a reaction and the limit of detection for clinical materials was at least $10^3$ CFU (colony forming unit) per gram of the lung samples. To evaluate the applicability of this method a total of 300 tonsillar samples were collected at a slaughterhouse and compared the detection rate with that by cultivation. As a result 45 of 300 (15%) samples were positive by semi-nested PCR while 17 of 300 (5.7%) were positive by cultivation. Thus the semi-nested PCR was shown to be a useful tool for rapid detection of *M. hyosynoviae* in clinical materials in pig herds.

4. Genetic diversity of *Mycoplasma hyosynoviae* field isolates in Thailand

It is essential to know the epidemiology of the disease such as source of infection or mode of transmission for the establishment of control measures for arthritis due to *M. hyosynoviae*. Recently genetic heterogeneity of causal organisms has been widely utilized to get epidemiological information in various infectious diseases. In this chapter genetic characterization of field isolates of *M. hyosynoviae* was investigated by pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) analyses. *Mycoplasma hyosynoviae* strains used were the same as those in chapter 2. Well-separated DNA fragments were obtained from 37 of 41 Thai isolates and type strain S16 with PFGE. Four Thai isolates did not yield clear bands. The banding patterns of the typable strains consisted of 6-12 fragments in the size range of 2-48 kbp. Twenty-two different patterns were detected among these strains. Among isolates derived from the same farm, two to five different profiles were also detected in addition to the same profiles. Though different profiles were usually detected among isolates derived from different farms, one common profile was detected in two isolates from two different farms on an occasion. These two farms were located in the same province, suggesting ‘farm to farm’ transmission of these isolates with the same profile. The PFGE technique had a high reproducibility with identical banding patterns for replicate samples. Thus it
revealed that *M. hyosynoviae* had high genetic heterogeneity both within a pig farm and among the farms by PFGE. On the other hand it was suggested that PFGE might be a useful tool for the epidemiological studies of *M. hyosynoviae* infection from the fact that common profile was detected in each isolate derived from different farm in one occasion.

The RAPD analyses were carried out after various preliminary experiments. The clear patterns were obtained by using RAPD primer 6 and RAPD beads (Ready-To-Go RAPD Analysis kit, GE healthcare, Life Science, USA). The 41 Thai isolates and type strain S16 were all typed with banding patterns consisting of 1-12 fragments in size range of 200-1,500 bp. Thirty-nine different patterns were detected among 42 strains and two isolates with the same profile by PFGE derived from different farms showed different pattern by RAPD. On the other hand, the same banding patterns were not necessarily obtained by repeated analyses of the same strains. Due to this low reproducibility RAPD analyses were not considered appropriate to obtain epidemiological information of the disease despite its high discriminatory power.

The results on antimicrobial susceptibilities of *M. hyopneumoniae* described in this work have great importance for controlling not only MPS but also PRDC in which *M. hyopneumoniae* plays an important role. Rapid detection of *M. hyosynoviae* enables the rapid diagnosis and earlier medication of arthritis due to *M. hyosynoviae* and improves the treatment together with the results of antimicrobial susceptibilities. The elucidation of the mechanisms of macrolide and LCM resistances of *M. hyosynoviae* will contribute to establish the prudent use of antimicrobials in animal husbandry. Lastly, PFGE analyses of *M. hyosynoviae* isolates in Thailand will facilitate the molecular epidemiological study of animal mycoplasmosis in that country.
Japanese abstract

マイコプラズマ病は最も重要な豚疾病の一つであり、養豚システムがますます近代化して来ているタイ国を含め世界中で養豚の生産性、経済性に大きな影響を与えている。「近代化」とは「集約化」と同義であり、密飼が常態化している。このような環境では国を問わず呼吸器病、下痢症、関節炎のような慢性疾患が多発している。近年、豚呼吸器症候群（porcine respiratory disease complex: PRDC）と呼ばれる壊滅的な被害を与える疾病が世界中で流行している。PRDCに関与すると考えられている様々な病原体の中で *Mycoplasma hyopneumoniae* は、豚繁殖呼吸障害症候群（porcine reproductive and respiratory syndrome: PRRS）ウイルスとともに PRDC において最も重要な役割を演じている。すなわち、単独でマイコプラズマ肺炎（mycoplasmal pneumonia of swine: MPS）を惹起するだけでなく、他の病原体、特に PRRS ウイルス感染を増悪することにより PRDC による経済的被害を拡大している。

*Mycoplasma hyopneumoniae* は、繁殖豚や肥種豚に非化膿性多発性関節炎を惹起するが、本病も世界中で流行し、タイ国においても増加して来ている。マイコプラズマは分離培養が難しく、また時日を要することから、タイ国においては豚のマイコプラズマに関する知見は少ない。そこで、同国における豚マイコプラズマ病の診断技術の向上と防遏に資する知見を得ることを目的として本研究を実施した。本論文は先ず、MPS のみならず PRDC の病原体としても重要な *M. hyopneumoniae* の薬剤感受性を明らかにするとともに、マイコプラズマによる疾病によく用いられているマクロライドに耐性を示した菌株について、その耐性機構の解明を試みた。次いで、*M. hyosynoviae* についても同様に薬剤感受性を明らかにするとともに、本菌の耐性機構の解明には、基準株 S16 を用いた in vitro における耐性値上昇試験も合わせて実施した。さらに *M. hyosynoviae* による関節炎は、早期の治療が必須であることから、培養によらない迅速検出法を考案し、また MPS や PRDC のような常在型疾病ではなく、豚の導入により病原体が侵入することが多いと考えられていることから、侵入経路の特定に有用な遺伝学的多様性について検討を加えた。
1. Mycoplasma hyopneumoniae 野外分離株の抗菌剤感受性とエンロフロキサシン、マクロライドおよびリンコマイシン耐性株の出現

MPS の化学療法には M. hyopneumoniae の各種抗菌剤に対する試験管内感受性を評価することが必須である。本章では、2006~2011 年にタイ国の 41 養豚場の豚の肺炎病巣部から分離された 159 株の分離株について、同国で広く用いられている 12 種類の抗菌剤、すなわち、テトラサイクリン系抗生物質のクロールテトラサイクリン (CTC)、オキシテトラサイクリン (OTC)、ドキシサイクリン (DOXY)、リンコマイシン系抗生物質のリンコマイシン (LCM)、マクロライド系抗生物質のジョサマイシン (JM)、キタサマイシン (KT)、スピラマイシン (SPM)、タイロシン (TS)、エリスロマイシン (EM)、プルロムチリン系抗生物質のチアムリン (TM)、合成抗菌剤のフロールフェニコール (FFC) およびニューキノロン系合成抗菌剤で第 2 次選択薬とされているエンロフロキサシン (ERFX) に対する感受性を調べた。試験には対照株として M. hyopneumoniae の基準株 J も用いた。また、得られたデータを 1997~1998 年の分離株 27 株のそれと比較した。

TM が供試薬剤中最も低い最小有効阻止濃度 (minimal inhibitory concentration: MIC) を示し、MIC 値は 0.013 µg/ml から 0.2 µg/ml に分布し、MIC₉₀ (90% の株の発育が阻止される MIC) は 0.1 µg/ml であった。TM に次いで LCM の MIC₉₀ が 0.39 µg/ml と高い活性を示したが、2 株は 12.5 µg/ml よりも高い MIC 値を示し耐性であった。SPM、JM および KT の 3 種のマクロライドも MIC₉₀ がそれぞれ 0.78、0.78 および 1.56 µg/ml と高い活性を示した。5 種のマクロライドのうち EM は例外的に活性が低く MIC₉₀ は 50 µg/ml であった。FFC は比較的活性が高く、MIC₉₀ は 1.56 µg/ml であった。OTC と DOXY は FFC と類似の活性を示し、MIC 域は 0.39 µg/ml から 12.5 µg/ml に分布し MIC₉₀ は 6.25 µg/ml であった。一方、CTC のタイ分離株に対する活性は低く、MIC 域は 3.12 µg/ml から 100 µg/ml に分布し MIC₉₀ は 50 µg/ml、MIC₉₀ は 25 µg/ml であった。ERFX の MIC 値は 0.1 µg/ml から 25 µg/ml まで広く分布し、159 株中 76 株 (47.7 %) が本抗菌剤の耐性限界値である 2 µg/ml を超えていた。
今回の成績を 1997-1998 年に分離された菌株の感受性と比較すると、今回の株は CTC、OTC、JM および TS に対する感受性がかなり低下していることが明らかとなった。すなわち、これらの薬剤に対する MIC₉₀ は 1997-1998 年分離株がそれぞれ 6.25、0.78、0.2 および 0.1 µg/ml であったのに対して 2006-2011 年分離株ではそれぞれ 50、6.25、0.78 および 0.39 µg/ml と 4~8 倍高くなっていた。

タイ国で繁用されているマクロライドおよびマクロライド類似の抗菌剤と抗菌力を示す LCM に耐性を示した 2 株の分離株について、23S rRNA のドメイン V 領域の塩基配列を基準株 J および ATCC 27719 のそれ（Genbank accession number AE017243 および X68421）と比較したところ、これら 2 株の耐性株には、A2058G（E.coli coordinates）の点変異が認められた。

2. 2008~2011 年にタイ国で分離された Mycoplasma hyosynoviae 野外株の抗菌剤感受性と基準株 S16 の試験管内における耐性獲得

タイ国では M. hyosynoviae 野外分離株の抗菌剤感受性に関するデータはない。本章では、2008~2011 年にタイ国の 9 養豚場の豚の扁桃（29 株）、肺（10 株）および関節（2 株）から分離された 41 株の分離株について、同国で広く用いられている 10 種類の抗菌剤、すなわち、オキシテトラサイクリン（OTC）、ドキシサイクリン（DOXY）、リンコマイシン（LCM）、ジョサマイシン（JM）、キタサマイシン（KT）、スピラマイシン（SPM）、タイロシン（TS）、チアムリン（TM）、フロールフェニコール（FFC）およびエンロフロキサシン（ERFX）に対する感受性を調べた。試験には対照株として M. hyosynoviae の基準株 S16 も用いた。また、全ての分離株と基準株 S16 についてマクロライド耐性に関与する 23S rRNA のドメイン II および V の塩基配列を調べ、マクロライドおよび LCM の各菌株に対する MIC 濃度を比較した。さらにマクロライドおよび LCM に対する耐性機構を解析するため、基準株 S16 の試験管内における耐性獲得試験を実施した。

試験した 10 種の抗菌剤のうち、TM が全ての株に対して最も低い MIC 値（0.016~0.125 µg/ml）を示した。TM に次いで LCM と JM が 1 µg/ml の MIC₉₀ で高
い活性を示した。KT と FFC は、MIC_{90} が 2-4 µg/ml と比較的高い活性を示したが、SPM と TS の抗菌力はこれらより低く MIC_{90} は 8 µg/ml であった。テトラサイクリン系薬剤のタイ分離株に対する抗菌力は低く、MIC の分布域は OTC が 16-128 µg/ml、DOXY が 4-16 µg/ml であった。ERFX の MIC 値は 0.25-2 µg/ml に分布していた。数種の薬剤に対しては分離株の相当数が耐性を示した。すなわち、OTC (耐性限界値; ≥16 µg/ml) に対しては 41 株すべて、ERFX (同; ≥2 µg/ml) に対しては 15 株(36.6%)、TS (同; ≥4 µg/ml) に対しては 13 株(31.7%) が耐性であった。

全菌株について 23S rRNA のドメイン II および V の塩基配列を調べたところ、13 株にドメイン II の領域に G745A の置換が認められた。この 13 株は TS の MIC 値が 4-8 µg/ml であったのに対して、他の 28 株では 0.5-2.0 µg/ml であり、この点変異が TS の軽度耐性を付与していることが強く示唆された。他のマクロライドや LCM の MIC はこの変異とは相関していないかった。

基準株 S16 は TS と LCM に対してきわめて感受性で、これらの薬剤の基準株に対する MIC はそれぞれ 0.032 and 0.125 µg/ml であった。本菌株を TS あるいは LCM を様々な濃度に含む液体培地で継代することにより耐性値上昇試験を行った。初代および 2 代目では、S16 株が発育できる薬剤の最高濃度は、親株と同じであったが、3 代目より TS あるいは LCM に対する耐性を急速に獲得し始め、継代ごとに 4-32 倍のより高い濃度で発育した。そして 6 代目には、S16 の変異株が発育した最大薬剤濃度は、TS では 4,000 倍、LCM では 1,000 倍に達した。LCM で選択した S16 変異株では、A2058C/G あるいは A2059C の置換が認められ、この変異株は LCM のみならず全てのマクロライドに対しても耐性であった。一方、TS で選択した S16 変異株では、A2062G の置換が認められ、この変異株はマクロライド耐性だけであり LCM には耐性となっていなかった。

3. *Mycoplasma hyosynoviae* の 16S リボソーム RNA 遺伝子を標的とした semi-nested PCR の考案
豚の *M. hyosynoviae* による関節炎の治療は、有効な抗菌剤を可能な限り早期に投与することで達成される。しかしマイコプラズマの分離には時日を要することから、本病の診断には数日を要している。そこで本章では、本病の早期診断のため、*M. hyosynoviae* を検出できる 3 本のオリゴヌクレオチドプライマーを用いた semi-nested PCR を開発した。プライマーとして用いた 3 領域は GenBank データ（accession number U26730）を利用して選定した。共通の forward プライマー領域は *M. hyosynoviae* 16S リポソーム RNA 遺伝子の 196～219 位から、残りの 2 本の reverse プライマーはそれぞれ、844～825 位、491～468 位とした。したがって、本 semi-nested PCR で予想される増幅バンドの本数は 2 本、サイズは 649bp と 295bp となる。外側プライマーによる PCR では 649bp の産物が増幅され、この増幅産物は後に内側プライマーによる 295bp の増幅のためのテンペレートにもなっている。本試験の特異性を、豚の肺炎や関節炎に関与するマイコプラズマおよび細菌種を用いて検討した。供試した微生物は次のとおりである：*M. hyopneumoniae, M. hyorhinis, M. flocculare, M. arginini, M. bovigenitalium, Arcanobacterium pyogenes, Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Escherichia coli, Haemophilus parasuis, Klebsiella pneumoniae Pasteurella multocida Staphylococcus aureus* および *Streptococcus suis*。これら細菌の DNA から、いずれのプライマー間でも当該サイズの増幅物を認めることなく、また、非特異サイズの増幅物も検出されなかった。本 semi-nested PCR は少なくとも 10^-14 g の *M. hyosynoviae* 精製 DNA を検出可能であった。また、肺材料での検出限界は肺組織 1 g あたり 10^3 CFU であった。

本法の有用性を評価するため、と畜場で採取した扁桃材料 300 検体について検出率を培養と比較した。その結果、semi-nested PCR では 45/300（15%）が陽性であったのに対して培養では 17/300 (5.7%) が陽性であり、本法は *M. hyosynoviae* を迅速かつ鋭敏に検出するのに有用な方法であることが示された。

4. タイ国における *Mycoplasma hyosynoviae* 分離株の遺伝的多様性

感染源や伝播様式のような疫学を知ることは、*M. hyosynoviae* による関節炎の防遏対策を講じる上で欠かすことのできない。近年このような疫学情報を得るため、
さまざまな感染症で原因微生物の遺伝的多様性が利用されている。本章では、パルスフィールトゲル電気泳動 (pulsed-field gel electrophoresis: PFGE) 法および増幅断片多型 DNA (Random amplified polymorphic DNA: RAPD) 法により M. hyosynoviae の遺伝的性状について検討を加えた。供試した M. hyosynoviae 菌株は第 2 章で使用したものと同じである。

PFGE 法では、タイ分離株 41 株中 37 株および基準株 S16 でよく分離した DNA 断片が認められた。タイ分離株のうち 4 株では明確なバンドが得られなかった。型別可能であったバンドパターンは、2〜48kbp の DNA 断片 6〜12 本から成っており、22 の異なるプロフィールが認められた。同一の農場由来株には、同一プロファイルの他に、2 から 5 つの異なるパターンが認められた。異なった農場由来株間では、プロファイルは通常異なっていたが、異なる農場由来の 2 株が同一のプロフィールを示す例が 1 例あった。これら 2 株の農場は同一の地域にあったことから、これらの株の農場間伝播が示唆された。PFGE 法は、繰り返し実験により常に同一のバンドパターンが得られ、再現性に富んでいた。このように M. hyosynoviae は、PFGE 法により農場内および農場間できわめて多様性に富んでいることが明らかとなった。

一方 PFGE 法は、2 農場間で共通のプロファイルを検出した例があったことから、M. hyosynoviae の疫学的研究に有用であると考えられた。

RAPD 法では、種々の予備実験により、Ready-To-Go RAPD Analysis kit (GE healthcare, Life Science, USA) のプライマー 6 および RAPD ビーズを用いることにより、明瞭なパターンが得られた。タイ分離株 41 株および基準株 S16 はすべて型別され、バンドパターンは、200〜1,500 bp の DNA 断片 1〜12 本から成っており、供試した 42 株には 39 のパターンが認められた。また、PFGE 法で同一のパターンを示した異なる農場由来株 2 株は RAPD 法では異なるパターンを示した。一方、本法は同一サンプルを用いた繰り返し実験で同一のパターンが得られないことがしばしばあった。このように RAPD 法は、菌株の識別には優れていたが、再現性が十分ではなかったことから、本病の疫学情報を得るには適当ではないと考えられた。
本論文に記載された *M. hyopneumoniae* の抗菌剤感受性成績は、MPS のみならず、本菌が重要な役割を演じている PRDC を防遏する上でもきわめて重要である。*M. hyosynoviae* の迅速検出は、本マイコプラズマによる関節炎の早期診断と早期の薬剤投与を可能とし、薬剤感受性成績とあいまって治療成績を向上させることができる。また *M. hyosynoviae* のマクロライドおよび LCM に対する耐性機構を明らかにしたことは、畜産領域における抗菌剤の慎重使用の確立に貢献すると考えられる。さらに *M. hyosynoviae* 分離株の PFGE 法による解析は、タイ国におけるマイコプラズマ病の分子疫学研究促進に資するであろう。
References


*Hemophilus parasuis* in swine. Proceedings of the 8th Annual Livestock 

Pleuropneumoniae. Proceedings of the 8th Annual Livestock Conference. 7-9 

Nishimori, T. Imada, T. Sakurai, M. Kitabayashi, T., Kawamura, H. and Nakajima, 
H. 1987. Restriction endonuclease analysis of Aujeszky’s disease virus isolated 

Nielsen, E.O., Lauritsen, K.T., Friis, N.F., Enoe, C., Hagedorn-Olsen, T. and 
state for evaluation of *Mycoplasma hyosynoviae* distributions in pig herds with 


Patanasophon, P., Pipitkul, S., Tanticharoenyoys, T. and Praikanahok, N. 1985. A 
study on serotypes of *Pasteurella multocida*. J. Thai Vet. Med. Assoc. 36: 385- 
393.

the classical *Mycoplasma lipophilum* cluster (Weisburg et al., 1989) and 
description of two new clusters in the hominis group based on 16S rDNA 

Polacek, N. and Mankin, A.S. 2005. The ribose peptidyl transferase center: structure, 

Prapasarakul, N., Tummaruk, P., Niyomtum, W., Tripipat, T. and Serichantalergs, O. 
2010. Virulence genes and antimicrobial susceptibilities of hemolytic and


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