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Genetic diversity and global relationships of *Mycoplasma hyopneumoniae* from slaughter age pigs in Chiang Mai and Lamphun Provinces, Thailand

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Abstract

***Mycoplasma hyopneumoniae* is a causative agent of enzootic pneumonia concerning for economic burdens in pig herds. Detection in genetic variable of the agent is an essential tool, which can be traced the route of disease spreading. Multilocus Sequence Typing (MLST) is accepted as one of the methods widely used. This study investigated genotypic characteristics of 16 *M. hyopneumoniae* strains recovered from consolidated lungs of slaughter age pigs in Chiang Mai and Lamphun provinces during 2016–2017 and 229 pig-originated strains previously submitted to global MLST database during 2001–2017, focusing on the locus numbers of three housekeeping genes, *adk*, *rpoB* and *tpiA* in MLST analysis to expand an understanding of the epidemiological knowledge for enzootic pneumonia. Of the 16 local strains, ten novel-first reported sequence types (ST99–ST108) from 14 strains were discovered, while the remaining 2 were identified in ST48. ST102 denoted for the majority, which accounted for four strains. Strains delivered at different collection times were not clones. However, some groups of strains collected at identical period belonging long distances were determined to be clones. At the global level, one hundred and eight STs were distributed in 15 countries. Almost all strains grouped in any STs were allied to the single country. The one exception is the strains grouped in ST48, which were derived from Thailand and Greece. In summary, MLST has the advantage of providing precise typing outcomes for epidemiology. The findings from this study provide advice regarding effective prevention and control of enzootic pneumonia in the study area.**

Key Words: enzootic pneumonia, MLST, *Mycoplasma hyopneumoniae*, pig, Thailand

Introduction

Mycoplasma hyopneumoniae, a causative agent of enzootic pneumonia, is one of the most important economic concerns in the pig industry worldwide[3,7,13)]. An infection of a pig respiratory tract by this organism results in a dry and non-productive cough and is associated with high

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morbidity rates[8,11]. Red-purple to gray rubbery changing mainly located at cranio-vantral parts of the affected lung, "lung consolidation lesion or *M. hyopneumoniae*-like gross lung lesion" is the lesion mostly correlated with the disease[14,24]. Reduction in growth performance and feed efficacy due to loss of lung function are resulted[1]. The infection also makes the host more susceptible to secondary pathogenic agents which has been documented to be the main complication of enzootic pneumonia[6,32].

In Thailand, *M. hyopneumoniae* is frequently detected in lung samples of slaughter age pigs. In 2012, detection rates of 32% were reported from intensive pig farming areas, including Suphanburi, Chonburi, Nakornrachasrima, Buriram, Udonthani, Chiang Mai, and Lamphun provinces[15]. Four years later, a detection rate of 43% was reported in the Chiang Mai-Lamphun area[26], indicating that *M. hyopneumoniae* or enzootic pneumonia has been a significant and growing problem in Chiang Mai and Lanphun. Vaccination, which is widely used, can reduce clinical signs and lung lesions, but cannot protect against infection[14,16]. On top, the organism can persist in some organs even after antimicrobial treatment, so reinfection can be occured[12,23]. Control measures over and above a vaccination program, e.g., farm biosecurity and optimal housing conditions as well as appropriated farm management, are needed as well[7,19].

Molecular techniques are the essential tools for tracing possible routes of disease spread. The bacterial geno-informatics approach can provide a better understanding of the epidemiology of many diseases and outbreaks[23,27,32]. Multilocus Sequence Typing (MLST) is a global-based bacterial genotyping technique that utilizes allelic variations in nucleotide sequences of several housekeeping gene loci[10]. It overcomes the limitation of the gel-based technique, Pulsed-field Gel Electrophoresis (PFGE), which does not allow for direct comparison of results from different laboratories[17,29]. In mycoplasma MLST schemes, there are normally five to eight targeted housekeeping genes[2,5,18,22,28]. Nevertheless, for *M. hyopneumoniae*, only Adenylate kinase (*adk*), RNA polymerase beta-subunit (*rpoB*), and Triosephosphat isomerase (*tpiA*) are normally sufficient[11,17].

The present study used *M. hyopneumoniae* MLST results to achieve two objectives. The first was to characterize and investigate the geno-diversity and evolution of *M. hyopneumoniae* strains recovered from consolidated lungs of slaughter age pigs in the Chiang Mai-Lamphun area during 2016–2017 for local epidemiological study. The second was to compare spatial associations among both the strains identified locally as well as all global strains previously submitted to the MLST database to expand understanding of global epidemiology and to provide advice regarding enzootic pneumonia control in the study area.

Materials and Methods

M. hyopneumoniae strains: Sixteen strains of *M. hyopneumoanie* were isolated from consolidated lungs of slaughter age pigs obtained in Chaing Mai-Lamphun provinces, Thailand during 2016–2017 (Table 1). Polymerase chain reaction techniques (PCR) were used to confirm the identification of materials obtained by bacterial cultivation methods. The procedures were completed according to previously described[15]. All strains were organized in coordination with the Bacteriology Section, Veterinary Research and Development Center (Upper Northern Region), Lampang, Thailand and included samples received from local pig farmers.

Multilocus sequence Typing (MLST): All strains were focused in 3 loci of housekeeping genes, Adenylate kinase (*adk*), RNA polymerase beta-subunit (*rpoB*), and Triosephosphat isomerase (*tpiA*). The 3 targeted housekeeping genes were amplified using the primers listed in Table 2 following the protocol of Mayor *et al.* (2008)[20].

Table 1. Origins of *M. hyopneuniae* isolated from slaughter age pigs in Chiang Mai-Lamphun during 2016–2017

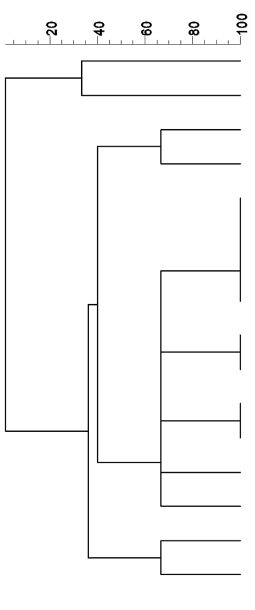
Strain ID	Sampling date	Farm origin	Zipcode
TH-DEAN-16	2/7/2016	JR farm	51000
TH-A3-16	2/7/2016	YP farm	50220
TH-Y1-16	2/7/2016	CD farm	51000
TH-CR5-16	5/8/2016	CO farm	51000
TH-D3-16	5/8/2016	CO farm	51000
TH-CH2-16	5/8/2016	CO farm	51000
TH-DM4-16	5/8/2016	CO farm	51000
TH-SUPORN-16	5/8/2016	CO farm	51000
TH-CR6-17	25/6/2017	PD farm	50000
TH-CHI-17	25/6/2017	AP farm	51000
TH-CR-17	25/6/2017	AN farm	51000
TH-NONG-17	25/6/2017	ST farm	51000
TH-KAI-17	17/7/2017	YP farm	50220
TH-CR6/2-17	17/7/2017	YP farm	50220
TH-H-17	17/9/2017	PD farm	50000
TH-JAN-17	6/8/2017	CO farm	51000

Table 2. Primers of 3 targeted housekeeping genes used in *M. hyopneuniae* MLST analysis

Target	Primer	(5'-3')	Size (bp)
Adenylate kinase	MH_*adk*-L	GGAGCTCCTGGCTCAGGTAAAG	581
	MH_*adk*-R	GTTTCTTCAAGGGTTTGCTCG	
RNA polymerase b-subunit	MH_*rpo*B-L	AAACGGATAGTTAGTGTTGGCG	602
	MH_*rpo*B-R	TGTTCGGCATCAAGGACAAG	
Triosephosphat isomerase	MH_*tpi*A-L	GAAATTGAAAAATGAATAAAACCGTAAG	641
	MH_*tpi*A-R	GATGCTTTTCTGGGATACTAACTCG	

Sequencing of the targeted genes was accomplished by the Macrogen Service Center, (Seoul, Republic of Korea). All gene sequences were submitted to the *Mycoplasma hyopneumoniae* MLST Database (https://pubmlst.org/mhyopneumoniae/) to query allelic numbers and Sequence Types (STs)[10]. For the new findings of typing results, all sequences were registered to the *M. hyopneumoniae* MLST database for the curator to evaluate for the strains ST[11].

Data analysis: Cluster analyses were performed using Bionumerics® software version 7.6 (Applied Maths, Ghent, Belgium) by the unweighted pair group method with arithmetic mean algorithms (UPGMA) through individual similarity matrices of the 3 genes. Local epidemiology results of strains obtained from the Chiang Mai-Lamphun area during the 2 year period are displayed in phylogenetic networks. In addition, Epi Info™ version 7 (CDC, Atlanta, USA) and Pixel Map Generator (amCharts, Vilnius, Lithuania) were used to identify the precise geographic locations of each of the farms using GPS coordinates on a street map to determine the relationship of *M. hyopneumoniae* strains grouped in 100% similarity threshold by MLST. Global epidemiology results were demonstrated using minimum spanning tree (MST) analysis. All of 229 *M. hyopneumoniae* strains previously submitted during 2001–2017



adk	*rpoB*	*tpiA*	Strain	Year	ST	Zip code	Farm
32	21	45	TH-CR62-17	2017	99	50220	YP farm
32	49	27	TH-JAN-17	2017	100	51000	CO farm
6	38	3	TH-CHI-17	2017	105	51000	AP farm
6	38	4	TH-D3-16	2016	108	51000	CO farm
6	18	3	TH-CR5-16	2016	102	51000	CO farm
6	18	3	TH-DEAN-16	2016	102	51000	JR farm
6	18	3	TH-CR2-16	2016	102	51000	CO farm
6	18	3	TH-Y1-16	2016	102	51000	CD farm
6	18	20	TH-DM4-16	2016	48	51000	CO farm
6	18	20	TH-SUPORN-16	2016	48	51000	CO farm
6	18	30	TH-H-17	2017	101	50000	PD farm
6	18	30	TH-NONG-17	2017	101	51000	ST farm
6	18	27	TH-CR6-17	2017	103	50000	PD farm
6	18	49	TH-CR-17	2017	104	51000	AN farm
6	37	20	TH-A3-16	2016	106	50220	YP farm
6	37	19	TH-KAI-17	2017	107	50220	YP farm

Fig. 1. Phylogenetic networks and origins of *M. hyopneumoniae* strains recovered from consolidated pig lungs in Chiang Mai-Lamphun during 2016–2017. Cluster analysis was completed by Bionumerics® software version 7.6 based on *adk*, *rpoB* and *tpiA* sequences.

in MLST database (https://pubmlst.org/bigsdb?db=pubmlst_mhyopneumoniae_isolates&page=query), including strains derived from Switzerland ($n = 150$), Hungary ($n = 42$), Cuba ($n = 11$), Greece ($n = 11$), Slovakia ($n = 3$), Brazil ($n = 2$), France ($n = 2$), United Kingdom ($n = 2$), Australia ($n = 1$), Belgium ($n = 1$), Canada ($n = 1$), China ($n = 1$), Czech Republic ($n = 1$) and the United States ($n = 1$), as well as the 16 local stains in the current study, were analyzed together. Stratification of relationships between STs by geographic area was achieved using "advanced cluster analysis for categorical data" in Bionumerics® software version 7.6 (Applied Maths, Ghent, Belgium).

Results

All sequencing data of *adk*, *rpoB* and *tpiA* were submitted to the *M. hyopneumoniae* definition's database to query an allelic numbers and STs. Of the 16 strains recovered from consolidated lung lesions originating from slaughter age pigs in Chiang Mai-Lamphun during 2016–2017, two of them were identified in ST48 (TH-DM4-16 and TH-SUPORN-16), which were typed in "*adk*_6 / *rpoB*_18 / *tpiA*_20". Even though, the other of 14 were not initially identified in any ST; all "target-gene" sequences were registered and submitted to MLST database curator for the determination of corresponding ST. The 10 novel-first reported ST were then assigned in ST99–ST108. ST102 was the most frequency found, which were typed in "*adk*_6 / *rpoB*_18 / *tpiA*_3" (TH-DEAN-16, TH-Y1-16, TH-CR5-16 and TH-CH2-16).

Phylogenetic networks using UPGMA algorithms generated the genetic relatedness of the *M. hyopneumoniae* 16 strains for which complete in all genes defining (Fig. 1). Of the samples obtained during different sampling periods in 2016 and 2017 for which genotypes were identified, none were found to be clones. The closest relationship was a 66.67% similarity between the two strains which originated from YP farm, TH-A3-16 and TH-KAI-17. The genotypic characters of strains derived from CO farm in

2016 and YP farm in 2017 were diverse. In terms of spatial analysis, four strains (TH-DEAN-16, TH-Y1-16, TH-CR5-16 and TH-CH2-16) originated from three different farms in nearby areas (CO farm, JR farm, and CD farm). Additionally, two clonal strains were identified (TH-H-17 and TH-NONG-17) which were obtained from areas approximately 30 km apart. The 100% similarity threshold relationship of *M. hyopneumoniae* strains in geographic locations marked with GPS coordinates on a street map are displayed in Fig. 2.

To expand epidemiological knowledge of *M. hyopneumoniae*, minimum spanning tree (MST) analysis of 16 local strains and 229 strains previously submitted to MLST database was conducted (Fig. 3). Total of 245 strains derived from 15 countries were analyzed together. Of all strains analyzed, three-fifths were referenced as the Swiss strains. More than 100 typing results for 3 housekeeping genes schemes were demonstrated. ST26 was the most frequently noted (26 strains; 10.61%), followed by ST36 (12 strains; 4.90%) and ST82 (11 strains; 4.48%), respectively. Almost all the *M. hyopneumoniae* strains circulating all regions of the world were unique to a single country with the exception of the strains grouped in ST48 which were derived from both Thailand and Greece. Moreover, two neighboring countries, Switzerland and France, a 66.67% similarity was found among strains belonged.

Discussion

In this study, Multilocus Sequence Typing (MLST) was used as the main tool for determining the genotypic characteristics of *M. hyopneumoniae*, a causative agent of enzootic pneumonia. That process involved the sequencing of 3 housekeeping genes, *adk*, *rpoB* and *tpiA*. Initially, only two of 16 strains recovered from slaughter age pigs in Chiang Mai-Lamphun during 2016–2017 could be noted into the Sequence Type (ST), ST48. The 14 other strains using were only successful in locus numbers determination. This study is the first report of MLST analysis of *M. hyopneumoniae* in Thailand as well as the other ASEAN countries. Therefore, the 14 *M. hyopneumoniae* strains grouping in ST99–ST108 represents a novel discovery. Although limitations of some laboratories in determining the genetic identification of *M. hyopneumoniae*, only 108 different STs have been recorded since the global database were established in 2001[10,17]. That is far fewer than other bacterial database, e.g., *Streptococcus suis*, *Escherichia coli, Salmonella enterica*, etc., for which more than 1000 STs have been detailed[9,21,30].

Phylogenetic networks generated the genetic relatedness of 16 *M. hyopneumoniae* in this local epidemiologic study. The strains collected in different sampling periods during 2016 and 2017 are displayed independently. New strains carried by newly acquired pigs might be able to be occurred in some herds[4]. Remarkably, the two strains obtained from YP farm with 33% un-matched genotypes, *tpiA*_19 and *tpiA*_20, were documented with an identity of 99.15%. A one locus difference was happened through five nucleotide variations, including 196T>C, 199C>T, 224A>G, 225C>T, and 229G>A. (Details were obtained from the allele sequence comparison analysis of *M. hyopneumoniae* MLST database). Point mutation can be explained to the finding[11]. Genetic composition of a clone might be altered during the period it persists in a pig herd[27]. Diverse MLST genotypes were detected in two farms at the identical time. The existence of more than one strain within a single herd is evidenced[25]. Four strains sharing identical typing belonging to 3 different farms located nearby areas were identified. Those farms share the same zip code, indicating that they are part of the same local sub-district area. In fact, nose to nose transmission among littermates or pen mates is the main route of disease spread[3,32]. Nevertheless, aerosol transportation of *M. hyopneumoniae* up

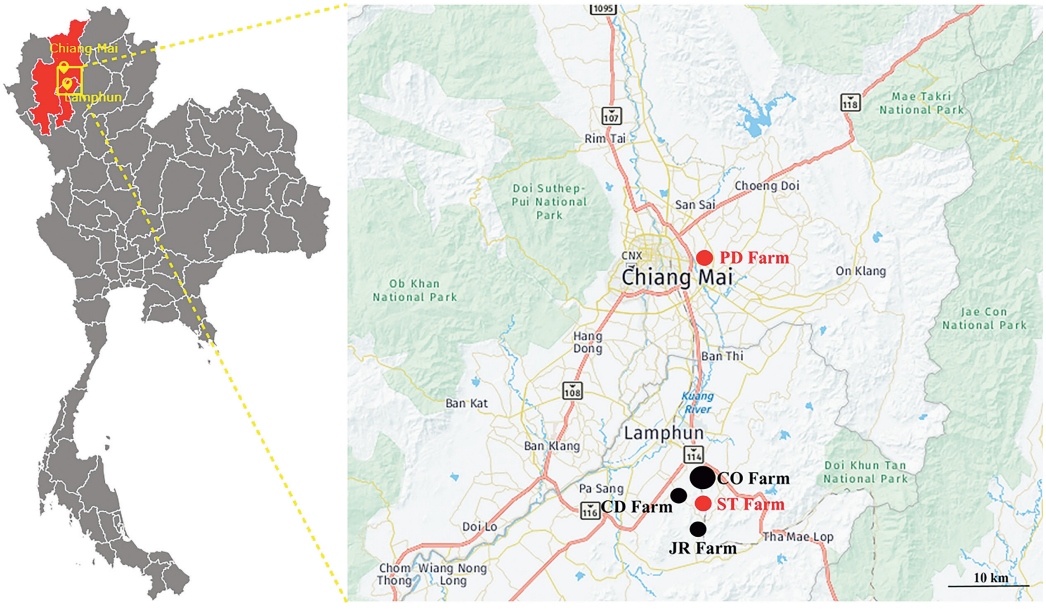


Fig. 2. Geographic location of farms with the groups of clonal *M. hyopneumoniae* strains. Red dots: strains grouped in ST101; Black dots: strains grouped in ST102

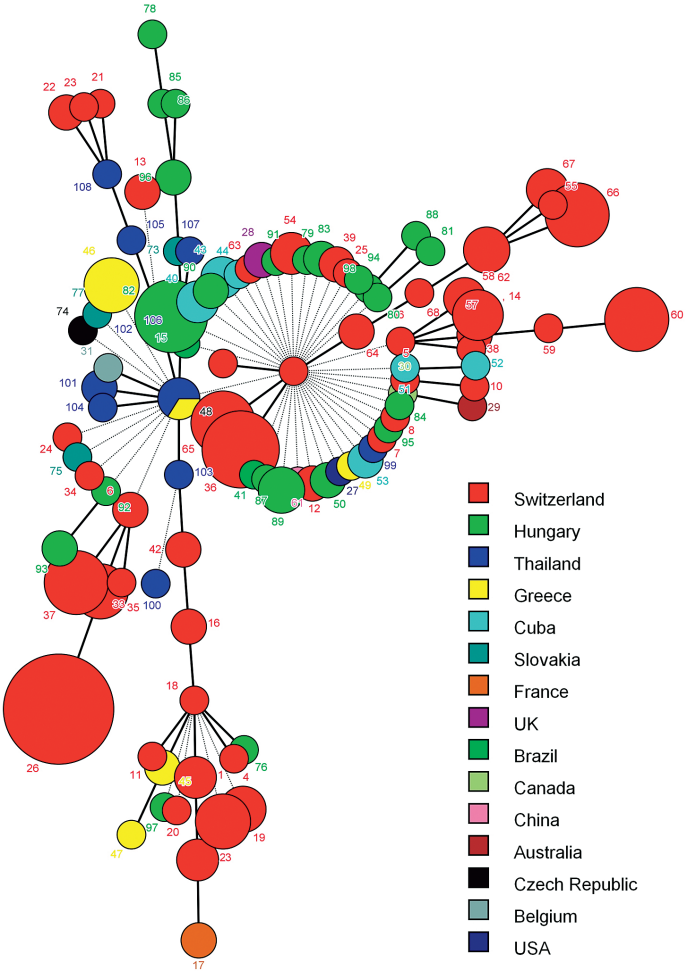


Fig. 3. Minimum spanning tree (MST) analysis of global *M. hyopneumoniae* completed using Bionumerics® software version 7.6 based on *adk*, *rpoB* and *tpiA* sequences. Size of circles indicates the number of strains with identical ST(s). Color indicates the country of origin. Solid thick black lines connect ST-types with one locus difference; dotted thin black lines connect ST-types with double loci differences.

to 9.2 km has described[20]. Furthermore, a group of undistinguishable strains originated at farms located far apart. Pigs passing through the same supply chain or sharing common transportation has been inferred to be an important root for the situation, based on the reported distribution of environmental contamination of the organism[11].

As part of expanding knowledge of global epidemiology, results of ST typing of *M. hyopneumonia* strains derived from several regions of the world have been displayed in MST to demonstrate geographical relatedness. The database was initiated and developed by the University of Bern, Switzerland[11,17], so it is not surprising that the majority of the STs are Swiss. Most of the strains tested were distributed in several STs which were unique to the country of origin. These suggests a low risk of international spread of enzootic pneumonia. On the other hand, the strains calling up in ST48 were obtained from different continents, Asia and Europe. Due to the wide geographic separation of those two areas, the cause of the relationship is seemed to be unclear. A shared pig supply chain or derivation from a common ancestor could be possible epidemiologic explanations. Evolutionary dynamics of target house-keeping genes variation in *M. hyopneumonia* genomes were in the context of strains domestication. It is the same reason explained the finding of 67% genotypic similar in the Swiss and French *M. hyopneumoniae*.

Due to the fastidious nature of bacteria such as *Mycoplasma* spp., recovering the organism using conventional culturing methods is both tedious and time consuming[20]. The PCR-less gel-based typing technique, Pulsed-field Gel Electrophoresis (PFGE), the gold standard for bacterial typing, cannot be performed in non-viable bacteria. To overcome that limitation, MLST was developed as an alternative method for bacterial population structure analysis. PCR-based techniques like as MLST have been used successfully whether the organism is dead or alive[15]. Moreover, comparison of results from various laboratories can be done easily and directly[11,17].

A set of 3 housekeeping genes (*adk*, *rpoB*, *tpiA*) were used to establish the *M. hyopneumoniae* MLST scheme. In conducting comparisons with other mycoplasmas, five to eight targeted housekeeping genes are chosen to define allelic differences[2,5,18,22,28]. However, Mayor *et al.*[17] reported that a number of genes suitable for *M. hyopneumoniae* typing have been evaluated. A reduction to the 3 targets is demonstrated highest variation results in matching STs as set of 7 genes. Varying of one loci number is related to at least one point of nucleotide change. For example, in *rpoB*, changing 86C>G results in a loci shift of *rpoB*_6 to *rpoB*_18.

This study provides information related to both local and global epidemiology of *M. hyopneumoniae*. Multilocus Sequence Typing (MLST) schemes using three housekeeping genes have the advantage of providing precisely typed outcomes which are easy to compare with the strain tracing results of other laboratories. It should be accepted as a tool for routine genotyping, that enough in national or international of enzootic pneumoniae surveillance and investigation. This study highlights associations among strains originating from far distant regions as well as point mutations and aerosol transmission among nearby areas. The information obtained further supports the importance of biosecurity measures and appropriate pig supply chain management as well as the need for knowledge of global genotypes as a means of minimizing the risk of the spread of disease.

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Conflicts of interest

The authors have no conflicts of interest to report.

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