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Short communication

Understanding the local molecular epidemiology of *Mycoplasma hyopneumoniae* from pig herds in northern-Thailand

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Abstract

Mycoplasma hyopneumoniae, a causative agent of Enzootic pneumonia (EP), is one of the most important issues contributing to economic losses in swine industry. A study of their genetics can further an understanding of its epidemiology assisted in developing the control strategy. Multilocus sequence typing (MLST) is widely accepted as a tool for this type of determination. In this study, 15 local strains of *M. hyopneumoniae* were isolated from consolidated lungs of slaughtered pigs in Chiang Mai and Lamphun provinces between 2018 and 2019 and genotyped by MLST. The variations of three housekeeping genes, adk, rpoB and tpiA were explored to query the specific sequence types (STs). Eleven STs were determined. Nine (ST135–ST143) were assigned as newly detected STs. The remaining two were ST117 and ST106. Of those strains recently detected, only one identified as ST106 was similar to others previously found by locals; ten STs recently investigated had not previously been found. New strains were carried by newly acquired pigs, and a high rate of overall mutation was emerged in a short period of time. The hygienic quarantine and disease detection, especially in pigs introduced to the region, should be taught and implemented in field practices.

Keywords: Enzootic pneumonia, Multilocus sequence typing, *Mycoplasma hyopneumoniae*, Pig, Thailand

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INTRODUCTION

There have been widespread reports of *Mycoplasma* pneumonia in intensive pig farm production, and this problem has led to serious economic losses (Sibila et al., 2009; Stakenborg et al., 2005; Thacker et al., 1999). Mild to severe intermittent non-productive cough persisting through the pig's lifespan is the primary clinical manifestation (Fano et al., 2005). A rubbery, red-purple to gray "*Mycoplasma*-like lung lesion or lung consolidation" is usually found (Straw et al.,1989). A reduction in growth performance and feed efficacy due to loss of lung function (Kuhnert and Overesch, 2014; Vicca et al., 2003) combined with secondary bacterial infections such as *Actinobacillus pleuropneumoniae*, *Pasteurella multocida* and *Streptococcus suis* are the commonly-detected consequences (Paisley et al.,1993).

In Thailand, Makhanon et al. (2012) reported a 32% prevalence of *M. hyopneumoniae* from the central, north and north-eastern region. In the northern region, a later report showed that 57.7% of the lungs collected contained consolidations, and nearly half of them involved *M. hyopneumoniae* (Tadee et al., 2019). These results signify that, without an effective control against the disease, there is no possibility of eradication.

Current biogenetic information can help us develop an understanding of the condition's epidemiology. Tracing possible routes of disease spreading is important for disease surveillance and investigation (Brown et al., 2015; Thongkamkoon et al., 2013). Multilocus sequence typing (MLST) is one of the most popular molecular tools accepted globally (Kuhnert and Overesch, 2014; Patchanee et al., 2015). In a previous study of MLST profiles of *M. hyopneumoniae* in northern Thailand, variations of the three targeted housekeeping genes, Adenylate kinase (*adk*), RNA polymerase beta-subunit (*rpo*B), and Triosephosphat isomerase (*tpi*A) showed evidence of contamination across pig farms in nearby areas through the sharing of a supplied root. Point mutation during the period of the pathogen persisted in a herd was also noticed (Tadee et al., 2018). However, since the *M. hyopneumoniae* global database was established (Mayor et al., 2008), only approximately 150 Sequence Types (STs) have been documented. That is far fewer than the numbers in other bacterial databases, e.g., *Escherichia coli*, *Salmonella enterica*, for which more than 1000 STs have been detailed (Wirth et al.,2006; Alikhan et al.,2018). Therefore, it is essential to encourage more study of the pathogen's genetic diversity.

MATERIALS and METHODS

M. hyopneumoniae strains

M. hyopneumoniae strains were isolated from consolidated lungs of slaughtered pigs in Chiang Mai and Lamphun provinces, Thailand during 2018 and 2019. In the culturing and identification methods, lung samples were suspended and inoculated into the Hank's–lactalbumin (BHL) broth medium [1%phenol red (Ajax Finechem, Auckland, Australia), 0.82% (w/v) Brucella broth (HIMEDIA, Nashik, India), 0.28% (w/v) lactalbumin hydrolysate (Biobasic, Marham Otario, Canada), 8.2% (v/v) NaCl, 2% yeast extract (HIMEDIA, Nashik, India), 15% (v/v) inactivated swine serum (Gibco®, Auckland, New Zealand), and 15% (v/v) horse serum (Gibco®, Auckland, New Zealand)

at 7.6 pH] at 37 °C for at least seven days until the indicator changed. Materials were then incubated at 37 °C under 5% CO_2 humid conditions in a CO_2 gas incubator for four days. Presumptive colonies were collected and identified as *M. hyopneumoniae* by polymerase chain reaction (PCR), following Makhanon et al. (2012). A total of 15 strains were included. The procedures were organized and conducted in coordination with the Bacteriology Section of the Veterinary Research and Development Center (Upper Northern Region), Lampang, Thailand.

Multilocus Sequence Typing (MLST)

Genomic DNA was extracted by NucleoSpin® DNA extraction kits (Macherey-Nagel, Düren, Germany). All DNA samples were amplified with the primers of the three targeted housekeeping genes, Adenylate kinase (*adk*), RNA polymerase beta-subunit (*rpo*B) and Triosephosphate isomerase (*tpi*A), according the protocol set forth by Mayor et al. (2008). Primers used were detailed in Table 1. Gene sequences were processed by the Macrogen Service Center (Seoul, Republic of Korea). All's acquired were explored to query the allelic numbers and specific STs from the *Mycoplasma hyopneumoniae* MLST Database (https://pubmlst.org/mhyopneumoniae/). Any strain that could not be identified in the existing STs were then registered to the database for curation with the new nomenclature.

Data Analyses

Phylogenetic tree of *M. hyopneumoniae* at the current 15 and the 16 northern-Thai strains previously detected in the geographical matching were analyzed by Bionumerics® software version 7.6 (Applied Maths, Ghent, Belgium). The unweighted pair group with arithmetic mean algorithms (UPGMA) through individual similarity matrices of all three housekeeping genes was used as the performing method.

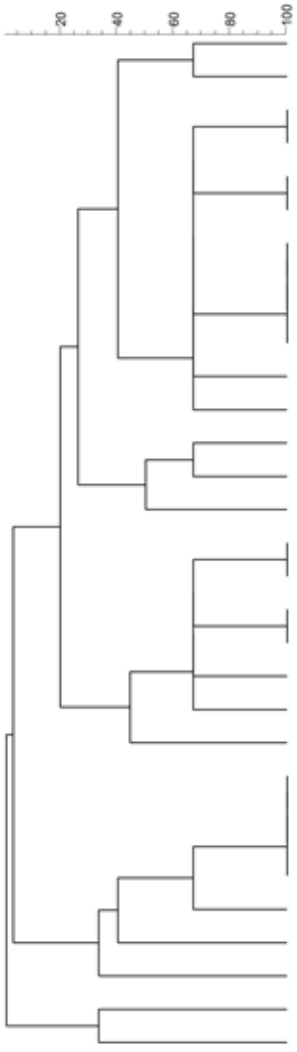
Table 1 Targeted housekeeping genes used in *M. hyopneuniae* MLST analysis

Housekeeping genes	Primer	(5'-3')	Size (bp)
Adenylate kinase	Forward	GGAGCTCCTGGCTCAGGTAAAG	581
	Reverse	GTTTCTTCAAGGGTTTGCTCG	
RNA polymerase b-subunit	Forward	AAACGGATAGTTAGTGTTGGCG	602
	Reverse	TGTTCGGCATCAAGGACAAG	
Triosephosphat isomerase	Forward	GAAATTGAAAAATGAATAAAACCGTAAG	641
	Reverse	GATGCTTTTCTGGGATACTAACTCG	

RESULTS

Fifteen strains of *M. hyopneumoniae*, recovered from consolidated lungs of slaughtered pigs in Chiang-Mai and Lamphun provinces during 2018 and 2019 were genotyped by MLST. The three targeted housekeeping genes sequences from each were submitted to *M. hyopneumoniae* database to find the allelic number and corresponding STs. In the current study, all 15 strains were collected from 3 farms (SN, CO and YP farms) in 5 slaughtered batches. Eleven genotypic characters were identified from the allelic construction. Initially, only two characters could be assigned in STs, ST106 (*adk*_6 / *rpo*B_37 / *tpi*A_20) and ST117 (*adk*_23 / *rpo*B_18 / *tpi*A_20). The gene sequences of the other nine characters were then registered to the MLST database curator to determine the corresponding STs. The nine newly reported STs were named ST135–ST143. ST143 (*adk*_26 / *rpo*B_54 / *tpi*A_24) was the most frequently found (n=4), followed by the two strains grouped in ST138 (*adk*_23 / *rpo*B_37 / *tpi*A_20).

The genetic relatedness of 31 northern-Thai *M. hyopneumoniae* strains with complete three housekeeping gene sequences was generated using UPGMA (Figure 1). The overview of the phylogenetic trees showed that only the CO and YP farms were resampled during different sampling periods (demonstrated in different font colors). Almost all the strains obtained under the temporal variation were not clones, except the two indistinguishable strains grouped in ST106 (TH-A3-16 and TH-Yout). These two strains were derived from different provinces. The close relationship at the next below (66.67% similarity) was demonstrated in strains obtained from YP farm (TH-A3-16 and TH-A-2). Based on the study's period of 2018–2019, SN and CO farms yielded the diverse of *M. hyopneumoniae* genotypes. Five STs were identified from each farm. Nevertheless, groups of the clonal strains (2 strains of ST138, and 4 of ST143) obtained from the same slaughtered batches samplings were also detected.



adk	*rpoB*	*tpiA*	Strain	ST-type	Collection Date	Farm	ZIP-Code
23	18	20	TH-Thai	117	2018-08-27	CO farm	51000
20	18	20	TH-Jan	139	2019-09-17	SN farm	51000
6	18	30	TH-H-17	101	2017	PD Farm	50000
6	18	30	TH-NONG-17	101	2017	ST Farm	51000
6	18	20	TH-DM4-16	48	2016	CO Farm	51000
6	18	20	TH-SUPORN-16	48	2016	CO Farm	51000
6	18	3	TH-CR5-16	102	2016	CO Farm	51000
6	18	3	TH-DEAN-16	102	2016	JR Farm	51000
6	18	3	TH-CR2-16	102	2016	CO Farm	51000
6	18	3	TH-Y1-16	102	2016	CD Farm	51000
6	18	27	TH-CR6-17	103	2017	PD Farm	50000
6	18	49	TH-CR-17	104	2017	AN Farm	51000
6	38	3	TH-CHI-17	105	2017	AP Farm	51000
6	38	4	TH-D3-17	108	2016	CO Farm	51000
26	38	3	TH-Sukit	137	2018-08-27	SN farm	51000
23	37	20	TH-Wittaya	138	2019-09-17	CO farm	51000
23	37	20	TH-Wirachai	138	2019-09-17	CO farm	51000
6	37	20	TH-A3-16	106	2016	YP Farm	50220
6	37	20	TH-Yout	106	2019-09-17	CO farm	51000
39	37	20	TH-Nung	141	2019-09-17	CO farm	51000
38	37	20	TH-A-2	142	2019-09-17	YP farm	51000
6	37	19	TH-KAI-17	107	2017	YP Farm	50220
26	54	24	TH-Kanjana-1	143	2018-08-27	SN farm	51000
26	54	24	TH-S-5	143	2018-08-27	SN farm	51000
26	54	24	TH-S-6	143	2018-08-27	SN farm	51000
26	54	24	TH-Nong-2	143	2018-08-27	SN farm	51000
26	54	39	TH-Dang	135	2018-08-27	CO farm	51000
27	54	39	TH-Nong-1	136	2018-08-27	SN farm	51000
23	54	3	TH-Kanjana-2	140	2019-09-17	SN farm	51000
32	21	45	TH-CR62-17	99	2017	YP Farm	50220
32	49	27	TH-JAN-17	100	2017	CO Farm	51000

Figure 1 Phylogenetic trees analysis based on three targeted housekeeping genes of 31 northern-Thai *M. hyopneumoniae* recovered from consolidated lungs of slaughtered pigs during 2016-2019, with their origins. Font labeled in blue and red represent for the samples collected in 2016-2017 and 2018-2019, respectively.

DISCUSSION

Generally, five to eight targeted housekeeping genes have been used in *Mycoplasma* MLST schemes. However, only for *M. hyopneumoniae*, a reduction to the 3 targets (*adk*, *rpo*B, *tpi*A) is demonstrated highest variation results in matching STs as set of five genes, or more (Mayor et al., 2008). Therefore, number of three genes have been evaluated, suitably.

In this study, MLST was used to characterize and investigate the genetic diversity, distribution, and microevolution of *M. hyopneumoniae* circulating in northern Thai pig herds. For the population structure of the pathogen during the two different sampling periods, numerous STs were registered as newly reported. From 2016–2017, 16 local strains were assigned to 11 STs; 10 of these were newly detected STs. Additionally, from 2018–2019, 15 local strains detected with geographical matching were identified to 11 STs, with 9 of them being newly reported. Since the *M. hyoneumoniae* database were established (Mayor et al., 2008), only 150 genetic characters the Sequence types (STs) have been reported. Therefore, findings of the novel STs are not unusual situation could be detected. However, the novels can be explained by alteration in only some points of gene sequences. For example, ST137 is the novel identified due to the shift of 390C to T from ST105 in adk gene (*adk*_6 to *adk*_26). Microevolution in the housekeeping genes of a clone might be occurred during the short period (Tadee et al.,2018). Actually, in a normal pig production cycle, gilts are replaced and transferred to breeding herd, routinely (Tamiozzo, 2013). For another reason, new *M. hyopneumoniae* strains carried by newly acquired pigs might be able to be occurred. It is not surprising that pigs in the same slaughtered batches are infected with the same STs. Consequently, good hygienic quarantine and disease detection in the new purchasing breeders should be taught and fulfilled in field practices.

The SN and CO farms yielded the most diverse *M. hyopneumoniae* genotypes, providing evidence that more than one strain can exist within a herd (Thongkamkoon et al., 2013). The study by Michiels et al. (2017) mentioned that batches of slaughtered pigs with different *M. hyopneumoniae* strains had a higher prevalence and severity of *Mycoplasma*-like lung lesions. However, their explanation was not clarified. Vicca et al. (2003) and Villarreal et al. (2009) stated that infection with a low virulent strain did not protect against a subsequent infection with high virulence, and new strains introduced to the herds often are more virulent. Implying that, reducing the number of different strains may lead to better respiratory health of the pigs.

Strains with the identical typing (ST106) originating from different provinces in different sampling period were identified. In facts, aerosol transportation is not possible (Otake et al., 2010). Due to the wide geographical parting of those, the cause of the relationship is appeared to be uncertain. A common pig supply chain or derivation from a mutual ancestor could be possible epidemiologic explanations (Tadee et al., 2018).

CONCLUSION

Understanding of *Mycoplasma hyopneumoniae* genetics can expand knowledge of their epidemiology. MLST is accepted as a tool for routine works. From the findings in this study, 11 STs were determined. Nine of them were newly detected. Proper hygienic quarantine and disease detection for new breeders, and reduce number of different strains at herd level are recommended. However, since the study was performed in small group samples, consequent monitoring *M. hyopneumoniae* genotypes in pig population should be functioned continuously. In addition, the updates of antimicrobial susceptibility tests as well as vaccine efficacy assessments are needed to create a disease control strategy for further study.

CONFLICT of INTEREST

There is no conflict of interest

REFERENCES

Alikhan, N.F., Zhou, Z., Sergeant, M.J., Achtman, M., 2018. A genomic overview of the population structure of *Salmonella*. PLoS Genet. 14, e1007261. https://doi: 10.1371/journal.pgen.1007261.

Brown, R.J., Holden, M.T.G., Spiller, O.B., Chalker, V.J., 2015. Development of a Multilocus sequence typing scheme for molecular typing of *Mycoplasma* pneumoniae. J. Clin. Microbiol. 53, 3195-3203.

Fano, E., Pijoan, C., Dee, S., 2005. Dynamics and persistence of *Mycoplasma hyopneumoniae* infection in pigs. Can. J. Vet. Res. 69, 223-228.

Kuhnert, P., Overesch, G., 2014. Molecular epidemiology of *Mycoplasma hyopneumoniae* from outbreaks of enzootic pneumonia in domestic pig and the role of wild boar. Vet. Microbiol. 174, 261-266.

Makhanon, M., Tummaruk, P., Thongkamkoon, P., Thanawongnuwech, R., Prapasarakul, N., 2012. Comparison of detection procedures of *Mycoplasma hyopneumoniae*, *Mycoplasma hyosynoviae*, and *Mycoplasma hyorhinis* in lungs, tonsils, and synovial fluid of slaughtered pigs and their distributions in Thailand. Trop. Anim. Health Pro. 44, 313-318.

Mayor, D., Jores, J., Korczak, B.M., Kuhnert, P., 2008. Multilocus sequence typing (MLST) of *Mycoplasma hyopneumoniae*: a diverse pathogen with limited clonality. Vet. Microbiol. 127, 63-72.

Michiels, A., Vranckx, K., Piepers, S., Del Pozo Sacristán, R., Arsenakis, I., Boyen, F., Haesebrouck, F., Maes, D., 2017. Impact of diversity of *Mycoplasma hyopneumoniae* strains on lung lesions in slaughter pigs. Vet. Res. 48, 2. https://doi: 10.1186/s13567-016-0408-z.

Otake, S., Dee, S., Corzo, C., Oliveira, S., Deen, J., 2010. Long-distance airborne transport of infectious PRRSV and *Mycoplasma hyopneumoniae* from a swine population infected with multiple viral variants. Vet. Microbiol. 145, 198-208.

Paisley, L.G., Vraa-Andersen, L., Dybkjaer, L., Møller, K., Christensen, G., Mousing, J., Agger, J.F., 1993. An epidemiologic and economic study of respiratory diseases in two conventional Danish swine herds. II: Associations between lesions present at slaughter and mean daily gains during specific intervals of the growth period. Acta. Vet. Scand. 34, 331-344.

Patchanee, P., Eiamsam-Ang, T., Vanaseang, J., Boonkhot, P., Tadee, P., 2017. Determination of regional relationships among *Salmonella* spp. isolated from retail pork circulating in the Chiang Mai municipality area using a WGS data approach. Int. J. Food Microbiol. 254, 18-24.

Sibila, M., Pieters, M., Molitor, T., Maes, D., Haesebrouck, F., Segalés, J., 2009. Current perspectives on the diagnosis and epidemiology of *Mycoplasma hyopneumoniae* infection. Vet. J. 181, 221-231.

Stakenborg, T., Vicca, J., Butaye, P., Maes, D., Minion, F.C., Peeters, J., De Kruif, A., Haesebrouck, F., 2005. Characterization of In Vivo acquired resistance of *Mycoplasma hyopneumoniae* to macrolides and lincosamides. Micro. Drug Resist. 11, 290-294.

Straw, B.E., Tuovinen, V.K., Bigras-Poulin, M., 1989. Estimation of the cost of pneumonia in swine herds. J. Am. Vet. Med. Assoc. 195, 1702-1706.

Tadee, P., To-in, P., Thongjamroon, J., Patchanee, P., Thongkamkoon, P., Tadee, P., 2019. Lung consolidations assessment and associated pathogens detection in slaughter-aged pigs in Chiang Mai - Lamphun, Thailand. Vet. Int. Sci. 17, 1-10.

Tadee, P., Chanrittisen, K., Thainoi, N., Thongkamkoon, P., Patchanee, P., 2018. Genetic diversity and global relationships of *Mycoplasma hyopneumoniae* from slaughter age pigs in Chiang Mai and Lamphun Provinces, Thailand. Jpn. J. Vet. Res. 66, 251-259.

Tamiozzo, P.J., 2013. Monitoring for *Mycoplasma hyopneumoniae* before and after a partial depopulation program using a typing scheme based on the polyserine repeat motif of p146. J. Swine Health Prod. 21, 309-312.

Thacker, E.L., Halbur, P.G., Ross, R.F., Thanawongnuwech, R., Thacker, B.J., 1999. *Mycoplasma hyopneumoniae* potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. J. Clin. Microbiol. 37, 620-627.

Thongkamkoon, P., Yuwapanichsampan, S., Kobayashi, H., Pathanasophon, P., Kishima, M., Yamamoto, K., 2013. Genetic diversity of *Mycoplasma hyosynoviae* field isolates in Thailand. Thai J. Vet. Med. 43, 359-365.

Vicca, J., Stakenborg, T., Maes, D., Butaye, P., Peeters, J., de Kruif, A., Haesebrouck, F., 2003. Evaluation of virulence of *Mycoplasma hyopneumoniae* field isolates. Vet. Microbiol. 97, 177-190.

Villarreal, I., Maes, D., Meyns, T., Gebruers, F., Calus, D., Pasmans, F., Haesebrouck, F., 2009. Infection with a low virulent *Mycoplasma hyopneumoniae* isolate does not protect piglets against subsequent infection with a highly virulent *Mycoplasma hyopneumoniae* isolate. Vaccine. 27, 1875-1879.

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L.H., Karch, H., Reeves, P.R., Maiden, M.C.J., Ochman, H., Achtman, M., 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol. Microbiol. 60, 1136-1151.

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