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New Multi-Locus Sequence Typing of *Mycoplasma hyorhinis* Isolated from Pig Farms in Central Thailand

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ABSTRACT

Mycoplasma hyorhinis (*M. hyorhinis*) is an important pathogen in the pig industry, especially during the nursery period. Multi-locus sequence typing (MLST) is a specific method used to identify many bacterial species. At present, 108 MLST schemes of *M. hyorhinis* have been reported around the world. This study aimed to investigate the variable multi-locus sequence typing (MLST) schemes of *M. hyorhinis* in pig herds from bacterial stock at the large animal hospital, Faculty of Veterinary Science, Chulalongkorn University, since 2010. Bacteria were collected from 98 deceased pigs sent for autopsy at this veterinary hospital. Samples were collected from at least one lesion per pig located in the joint capsule, lung, thoracic cavity, abdominal cavity, and pericardium. Bacteria were cultured and confirmed the species by PCR. MLST was detected by PCR and DNA sequencing. Sequence data were reported to GenBank and PubMLST databases. In this study, the positive results of *M. hyorhinis* were found on 75 samples, while 23 samples gave negative results. The highest population of this pathogen was shown on the joint organ but no significant difference with the other organs. Only nine positive samples could be cultured, purified, and sent for sequencing. Sequencing results revealed 6 MLST schemes, while 5 of them were defined as new ST types (ST71-75) defined for the first time in Thailand. A diverse array of MLST in this location, some of which are novel, implied that bacteria might adapt to their environment. MLST information might play a role in vaccine development and preventative strategies.

Keywords: Multilocus sequence typing; Mycoplasma hyorhinis; pigs

INTRODUCTION

Mycoplasmas are a common cause of respiratory disease in humans and animals, especially in pigs (Lee *et al*., 2016). *Mycoplasma hyorhinis* (*M. hyorhinis*) has been identified in pig herds since 1955 (Switzer, 1955). This pathogen can be found in any age of pigs and impact the pig welfare and economy of the pig industry. In nursery pigs, polyserositis and arthritis are commonly observed (Clavijo *et al*., 2017), while in adult pigs, many symptoms are found, such as arthritis, polyserositis, conjunctivitis, otitis, pneumonia, eutachitis, and abortions (Neto *et al*., 2012; Clavijo *et al*., 2017). Generally, the infection of *M. hyorhinis* is found as co-infected with other pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), and the other porcine mycoplasma. It causes severe sickness and death in piglets (Lee *et al*., 2016). Lung lesion is a significant clinical sign of *M. hyorhinis*. Acute lung lesions are displayed on co-infection of *M. hyorhinis* and PRRSV (Lee *et al*., 2016). The worsening of lung lesions also occurred in pigs co-infected with *M. hyorhinis* and PCV2 (Chen *et al*., 2016). However, the lung lesions of porcine mycoplasma infection are similar, especially in *M. hyorhinis* and *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) infections (Luehrs *et al*., 2017).

The distribution of *M. hyorhinis* has been reported in some countries such as Switzerland (Luehrs *et al*., 2017), the USA (Clavijo *et al*., 2017), and Brazil (Cibulski *et al*., 2016). A high percentage of *M. hyorhinis* was discovered with polyserositis lesion (Clavijo *et al*., 2017). There are limited reports about *M. hyorhinis* infection (Makhanon *et al*., 2012; Clavijo *et al*., 2019; Clavijo *et al*., 2017; Xu *et al*., 2013). Specifically, no genotyping report of *M. hyorhinis* is available in Thailand, resulting in difficulty managing and preventing disease. The distribution of clonal bacteria aids in the development of vaccinations. At present, there are only a few genotyping reports for *M. hyorhinis* that have been conducted (Trueb *et al*., 2016). At present, 70 MLST schemes of *M. hyorhinis* have been reported worldwide, especially in France. In Asia, a clonal report of *M. hyorhinis* has been presented in China, but no study has been conducted in South East Asia (www.pubmlst.com).

This retrospective study aimed to study target organs and the variable multi-locus sequence typing (MLST) schemes of *M. hyorhinis* in pig herds from bacterial stock at the large animal hospital, Faculty of Veterinary Science, Chulalongkorn University, since 2010. The clonal identification of this pathogen will help us characterize local clones, find clonal relationships, and plan disease-control strategies.

MATERIALS AND METHODS

Samples

This experiment used the stored bacteria kept between 2010-2011. Bacteria were retrieved from 98 deceased pigs from 18 farms in Nakhon Pathom Province, a major pork-producing province (ICT, 2020). Carcasses were sent for autopsy at the large animal hospital of Faculty of Veterinary Science, Chulalongkorn University between 2010-2011. During sample collection in 2010-2011, there was no university legislation to regulate or permit the use of pathogens isolated from necropsy cases for research. Therefore, no application for IACUC permission was shown in this study. All pigs were suspected of infection with *M. hyorhinis*. The samples were identified species from 2010 to 2011 at the Veterinary Diagnostic Laboratory at the Faculty of Veterinary Science, Chulalongkorn University, which is the largest animal hospital in the country. With the sterile technique, samples (1 g of an organ or 1 cc of fluid) were collected from at least one lesion per pig located in the joint capsule, lung, thoracic cavity, and abdominal cavity pericardium. Samples were divided into 4 types; Joint, Lung, Thoracic and pericardial fluid, and Pooled organ samples. Pooled organ samples were used in pigs using two criteria: 1) the target organ size or weight was less than 1 g, and 2) Two target organs were collected within the same pig. In addition, pooled samples were collected via organ separation consisting of thoracic and abdominal fluid, pericardial fluid and abdominal fluid, joint and pericardial fluid, as well as lung and joint capsule. Likewise, samples were checked for *M. hyorhinis* by directed PCR (Kobayashi *et al*., 1996).

Ninety-eight samples were collected from pigs with polyserositis, lameness, and dyspnea. Likewise, 70 samples were collected from joint fluid and capsules in cases of polyserositis and lameness. Twelve lung samples were collected from pigs with dyspnea. Of these, 5 of the animals had acute lung lesions leading to a lack of thoracic and pericardial fluid. As a result, only seven thoracic and pericardial fluids could be collected in this study. Types of pooled samples included those composed of thoracic and abdominal fluid (2 samples), pericardial fluid and abdominal fluid (3 samples), joint and pericardial fluid (1 sample), and lung and joint capsule (3 samples).

Bacterial Culture

All *M. hyorhinis* positive samples were cultured immediately (Makhanon *et al*., 2012). Briefly, samples (smashed organs or fluid) were put into 10 mL of Brucella broth Hank's-Lactalbumin (BHL broth) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and mixed well. Then, the suspension was centrifuged at 6682 rpm for 10 min. The supernatant (2.5 mL) was duplicated to 2 mL of BHL broth for culturing and gene detection. Samples were incubated in a 5% CO_2 incubator at 37 °C for 7-14 days until the media changed from red to yellow. Finally, a one-loop-full of *M. hyorhinis* was placed onto Noble agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in a 5% CO_2 incubator at 37 °C for 7 days. Purified colonies were kept in skim milk with glycerol at -80 °C until used.

DNA Extraction and Species Identification

For DNA extraction, all suspensions of samples in BHL broth were centrifuged at 6682 rpm for 10 min. Then, the supernatant was removed and placed in 70 µL ddH_2O. Suspensions were boiled for 10 min at 100 °C. All medium was centrifuged for 10 min at 6682 rpm. The supernatant was moved to a 1.5 mL new microcentrifuge tube. All purified samples were identified by species using 16S rRNA gene composed of *Mhr1* (5′-TATCGCATGATGAGTAATAG-3′) and *Mhr2* (5′-GCTGCGTTAGTGAAATTAT-3′) (Kobayashi *et al*., 1996). A total of 50 µL of PCR reaction mixture containing 25 µL Master Mix (Promega, Madison, WI, USA) and 1.25 units of Taq-DNA polymerase were used. PCR conditions were 30 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s. After finishing, 5 µL of PCR product was put into a 1.2% agarose gel containing 2 µL of nucleic acid gel stain (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea) and run at 80 V for 1-1.5 h.

MLST Identification

All samples were re-cultured in November 2016. The only sample that could grow was used for MLST identification. MLST identification included chromosomal replication of initiation protein (*dnaA*), the RNA polymerase β subunit (*rpoB*), the DNA gyrase subunit B (*gyrB*), the glutamyl-tRNA synthetase (*gltX*), the adenylate kinase (*adk*), and the guanylate kinase (*gmk*) (Cibulski *et al*., 2016; Clavijo *et al*., 2017). A total of 50 µL of PCR reaction mixture contained 25 µL Master Mix (Promega, Madison, WI, USA) and 1.25 units of Taq-DNA polymerase. The unpurified amplification products of the six genes from one strain were sent for sequencing using the Sanger sequencing method (1st BASE, Singapore Science Park II, Singapore). Cycling conditions were 95 °C for 30 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s. The final extension step was 72 °C for 7 min. PCR products were loaded onto 1.5% agarose, which contained 2 µL of nucleic acid gel stain (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea), and ran at 100 V for 40 min.

All sequences were analyzed using PHYLOViZ Online (https://online.phyloviz.net/index). The data of new alleles and allele combinations were sent to the PubMLST database (www.pubmlst.org) (Bayatzadeh *et al*., 2014).

Statistical Analysis

The positive samples of *M. hyorhinis* were recorded as percentages. The Chi-square test with 99% confidence interval (CI) was used for analyzing positive samples among organs. Significant difference cut-off was 95% ($p<0.05$). The samples were calculated with Statistic 22 for Microsoft Windows (SPSS Inc.; Chicago, IL, USA). Gene sequences were analyzed by Applied Biosystems Sequence Scanner 2.0. Sequence data were reported to GenBank and PubMLST databases.

RESULTS

Prevalence of *M. hyorhinis*

Of 98 samples, *M. hyorhinis* were detected in positive samples from 75 samples, while 23 samples gave negative results. The highest positive sample population was discovered at the joint organ with no significant differences among organs (Table 1). The lowest percent of *M. hyorhinis* positive organs was seen in Thoracic and pericardial fluid samples (6.7%). Most *M. hyorhinis* was not discovered in pooled organ samples on joint and pericardial fluid, while positive results were shown in 2 samples of lung and joint capsule (2/3; 66.67%).

MLST Results

With sequencing results, 6 MLST schemes were discovered in this study while 5 new alleles were defined as new ST types composed of ST71 (*dna*_13/ *rpoB*_1/ *gyrB*_1/ *gltX*_4/ *adk*_1/ *gmk*_3), ST72 (*dna*_7/ *rpoB*_1/ *gyrB*_6/ *gltX*_1/ *adk*_1/ *gmk*_3), ST73 (*dna*_13/ *rpoB*_1/ *gyrB*_1/ *gltX*_9/ *adk*_1/ *gmk*_3), ST74 (*dna*_7/ *rpoB*_1/ *gyrB*_1/ *gltX*_4/ *adk*_1/ *gmk*_3), and ST75 (*dna*_7/ *rpoB*_1/ *gyrB*_4/ *gltX*_4/ *adk*_1/ *gmk*_3) (Figure 1). The most prevalent *M. hyorhinis* scheme was ST71. ST56 was discovered from 2 joint fluid samples (Table 2-3).

DISCUSSION

From 75 of 98 samples collected from 18 farms were positive (76.53%) to *M. hyorhinis* from PCR detection, but only nine samples could be cultured and used for MLST detection. The most frequent *M. hyorhinis* was

Table 1. The percentile of *Mycoplasma hyorhinis* on joint fluid and capsule, lung, thoracic and pericardial fluid, and pooled samples from pigs

Position*	Describe	Negative	Positive	Total
Joint	Count	14	56	70
	% within Position	20.00%	80.00%	100.00%
	% within Positive	60.90%	74.70%	71.40%
Lung	Count	2	10	12
	% within Position	16.70%	83.30%	100.00%
	% within Positive	8.70%	13.30%	12.20%
Pool	Count	5	4	9
	% within Position	55.60%	44.40%	100.00%
	% within Positive	21.70%	5.30%	9.20%
Thorax	Count	2	5	7
	% within Position	28.60%	71.40%	100.00%
	% within Positive	8.70%	6.70%	7.10%
Total	Count	23	75	98
	% within Position	23.50%	76.50%	100.00%
	% within Positive	100.00%	100.00%	100.00%

Note: *= No significant difference among organs (Chi-square test, $p<0.05$; CI= 0.095-0.110).

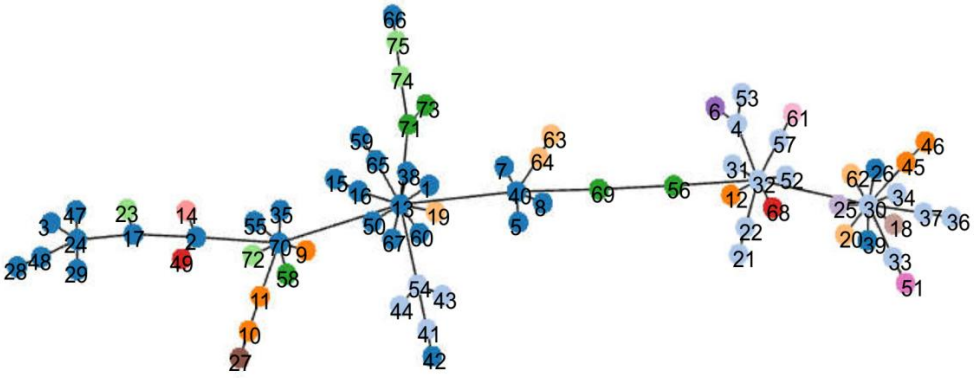

Figure 1. Minimal spanning tree of *Mycoplasma hyorhinis* sequence types (ST) based on allele numbers using PHYLOViZ Online (https://online.phyloviz.net/index). The color of the circle correlated with the type of *dnaA* gene. The number in each color is mentioned as a sequencing type.

Table 2. Background information of *Mycoplasma hyorhinis* samples (n= 9) from pigs and their multilocus sequencing type (MLST) typing results

Aliases	Disease	Source	*dnaA*	*rpoB*	*gyrB*	*gltX*	*adk*	*gmk*	ST
CU_TH1	arthritis	joint fluid	13	1	4	1	1	3	56
CU_TH2	arthritis	joint fluid	13	1	1	4	1	3	71
CU_TH3	arthritis	joint fluid	13	1	1	4	1	3	56
CU_TH4	arthritis	joint fluid	7	1	6	1	1	3	72
CU_TH5	arthritis	joint fluid	13	1	1	4	1	3	71
CU_TH6	arthritis	joint fluid	13	1	1	4	1	3	71
CU_TH7	arthritis	joint fluid	13	1	1	9	1	3	73
CU_TH8	pneumonia	thoracic and pericardial fluid	7	1	1	4	1	3	74
CU_TH9	Acute cough	thoracic and pericardial fluid	7	1	4	4	1	3	75

Note: All samples were collected from pig farms, Nakhon Pathom Province, Thailand since 2010 and deposited at Chulalongkorn University.

Table 3. The sequencing results and GenBank accession numbers of *aA*, *rpoB*, *gyrB*, *gltX*, *adk*, and *gmk* of 5 new alleles from *Mycoplasma hyorhinis* in pigs

Isolates	*Genes*	GenBank accession numbers	Sequence (5'→3')
CU_TH2	*dnaA*	BankIt2091406 Seq1 MH018247	TTTTCTAAACCTACAAAAGAAGATTTTTTAAAGGTTTTAAAATATAAACTTGA ATTTCAAAATATAAATCCTGAAAAATTTGAAAAAGATGCTTTAGATTTTTTAG TTTATAACAAAAAATCGGTTAGAGAAATCGAAGGTGTAGTTAATAGAATTAA ATTTTTTTCAGAAGGAGAAGCCATTCAAATTTATTCATTAGAACTTATAGAG AGGATCTTCAAAGGTATTGTTAAAAATAAAGAAAACTTAACACATAACAAA ATAATAGAAGAAGTTGCTAATTATTTCCAAATAAACAAAGAAGAAATCATTG GGACTTCAAGAAAAACAGAAATTGTTATGGCTAGAGATATATCAATTTGGTT
	rpoB	BankIt2090774 Seq1 MH010352	CTCCTTATGTTGCTACAGGAATTGAAGCAGATGTAGCTAAATTCTCATCAACT AACATCAGAGCTAGCGAAGATGGAGAAGTAGTTTTTGTTGATTCTAAATCAA TTCAAATCAAAAACGAACAAGGAAATATTAAAACTTATCACCTACGTTATTT TGAAAGATCAAACCAAGAAACTTTGATTTTACAACGTCCAATAGTTGAAGTT GGTCAATTTGTTAAGCAAGGTGATTTAATTTGTGATGGTCCTTCTACAGATCA AGGTGAATTGGCTTTGGGTAAAAACGTTTTAGTAGCTTTTTCCACTTGATATG GATACAATTACGAAGATGCGATTATTATTAGTGAAAAACTAGTTAAAGATGA TGTTTTTACTTCAATTCATATTCAAGAACAAACTATTCAATTTAGATCTTCAA AAGCAGGTAATGATGATCTTACTAGAGAAATTCCAAATGCTTCACAAAGAAG TAAAAGACACTTAGATGAAAACGG
	gyrB	BankIt2092793 Seq1 MH029377	TTTACATAGCACAACCTCCACTTTACAAAATTTCAGCAAATAAAAAAGTTGT TTATGCATATTCAGATAAGCAAAAAGAACAAATAATTGAACAAGAATTTTCA AATCAAAAAGTTAGCATTCAAAGATACAAAGGTCTTGGAGAGATGAATCCA GATCAACTCTGAGAAACCACTATGGACCCAAAAACAAGAAAAATGATTCAA GTACAAATTCAGGATGCAATTATTGCTTCACAAGTTTTTGAAGAATTAATGG GAACAGATGTAGCTCCTAGAAAATTATTTATTG
	gltX	BankIt2092386 Seq1 MH025800	AAGAAAATATAGATATTAAACTTGAAAATATTTCAGCAGATTGATTGGATTT TGCTATTGATACTTACAAAGCAAGTTGTACAACTTATAAAGATTTAGAAAAA GAAATTAACTCATTTGCTCAAGTATTGCAAACTTGTCACAATGAAAAAGACC AACAACTTTACGAACAAATTGATGATAGTTTACTCAAAACATTTACTTCATTT ATCGATTGGGATAATTTTACTGTTGCAATGTTAGAACAAGCAATTGAATCAA CAAAACAAGCTAGCAATTTAAGTGGTAAAAAGCTTCTTTTACCAATTAGAAT AGCTACAACTCTTCGTTCTTC
	adk	BankIt2089996 Seq1 MH000222	TTATAATTTTCATAAAGTAGTAATAACTTCTTCTGTTAGCTTGTTTAAATCTTG ATTTGCATCAAATTCAATTAAGAGATTTTGTGTTGCGTAATAGTTAATTAAAG GTGCTGTTTGTTCTTGATAAATTGCAATTCTTTTTTTAATTGCTTCTGGTTCAT CATCTTTTCTTCTTTGCACTACAGTATTGTCTTTAAAACAATATTTTCCTTCTT TTGCTGGTTTGAATTTATCATGATAAGTGCTTTGACAAGTTGGACAATATTTT CTAAGTGAT
	gmk	BankIt2088753 Seq1 MG975779	TTTTTATCTTCTTTTAAAATTATTTTTTCAACTTCTTTTGCTGCTACTTTTAAAT CTTTATTAACAACAATATATTGGAATTTATGTTGTTGACTTAATTCTTCTAAA GATTTATCAATTCTTAATTGAATTTGTTCATGTGAATCAGTTTTTCTACTAATT ATTCTTTTTTTAAGATCATCAAATGTTGGAGGAGCTATGAAAATGGTAACAA GTTGATCACTTTTGTTTGCTTGCTCATACATTTTCATAATGTTTAGTGCTCCAA CAGTTTCAATTTCTAAAATAGGACTATAACCTTCTTTAATAATGTTATCAACT TCTGATTTTAGTGTTCCGTAATAATTATTAAAGTGTTTGTTTCATTCAAGAAA TTCATTATTTTTGATTTTATTTTTAAAATCT

Isolates	*Genes*	GenBank accession numbers	Sequence (5'→3')
CU_TH4	*dnaA*	BankIt2091406 Seq2 MH018248	TTTTCTAAACCTACAAAAGAAGATTTTTTAAAGGTTTTAAAATATAAACTTGA ATTTCAAAATATAAATCCTGAAAAATTTGAAAAAGATGCTTTAGATTTTTTAG TTTATAACAAAAAATCAGTTAGAGAAATCGAAGGTGTAGTTAATAGAATTAA ATTTTTTTCAGAAGGAGAAGCCATTCAAATTTATTCATTAGAACTTATAGAG AGGATCTTCAAAGGTATTGTTAAAAATAAAGAAAACTTAACACATAACAAA ATAATAGAAGAAGTTGCTAATTATTTCCAAATAAACAAAGAAGAAATCATTG GGACTTCAAGAAAAACAGAAATTGTTATGGCTAGAGATATATCAATTTGGTT TGTTAAAAACTTGTTAGATTTAACTTTGAAAAGCATCGGCCAGA
	rpoB	BankIt2090774 Seq2 MH010353	CTCCTTATGTTGCTACAGGAATTGAAGCAGATGTAGCTAAATTCTCATCAACT AACATCAGAGCTAGCGAAGATGGAGAAGTAGTTTTTGTTGATTCTAAATCAA TTCAAATCAAAAACGAACAAGGAAATATTAAAACTTATCACCTACGTTATTT TGAAAGATCAAACCAAGAAACTTTGATTTTACAACGTCCAATAGTTGAAGTT GGTCAATTTGTTAAGCAAGGTGATTTAATTTGTGATGGTCCTTCTACAGATCA AGGTGAATTGGCTTTGGGTAAAAACGTTTTAGTAGCTTTTTCCACTTGATATG GATACAATTACGAAGATGCGATTATTATTAGTGAAAAACTAGTTAAAGATGA TGTTTTTACTTCAATTCATATTCAAGAACAAACTATTCAATTTAGATCTTCAA AAGCAGGTAATGATGATCTTACTAGAGAAATTCCAAATGCTTCACAAAGAAG TAAAAGACACTTAGATGAAAACGG
	gyrB	BankIt2092793 Seq2 MH029378	TTACATAGCACAACCTCCACTTTACAAAATTTCAGCAAATAAAAAAGTTGTT TATGCATATTCAGATAAGCAAAAAGAACAAATAATTGAACAAGAATTTTCAA ATCAAAAAGTTAGCATTCAAAGATATAAAGGTCTTGGAGAGATGAATCCAG ATCAACTCTGAGAAACCACTATGGACCCAAAAACAAGAAAAATGATTCAAG TACAAATTCAGGATGCAATTATTGCTTCACAAGTTTTTGAAGAATTAATGGG AACAGATGTAGCTCCTAGAAAATTATTTATTG
	gltX	BankIt2092386 Seq2 MH025801	AAGAAAATATAGATATTAAACTTGAAAATATTTCAGCAGATTGATTGGATTT TGCTATTGATACTTACAAAGCAAGTTGTACAACTTATAAAGATTTAGAAAAA GAAATTAACTCATTTGCTCAAATATTGCAAACTTGTCACAATGAAAAAGACC AACAACTTTACGAACAAATTGATGATAGTTTACTCAAAACATTTACTTCATTT ATCGATTGGGATAATTTTACTGTTGCAATGTTAGAACAAGCAATTGAATCAA CAAAACAAGCTAGCAATTTAAGTGGTAAAAAGCTTCTTTTACCAATTAGAAT AGCTACAACTCTTCGTTCTTC
	adk	BankIt2089996 Seq2 MH000223	TTATAATTTTCATAAAGTAGTAATAACTTCTTCTGTTAGCTTGTTTAAATCTTG ATTTGCATCAAATTCAATTAAGAGATTTTGTGTTGCGTAATAGTTAATTAAAG GTGCTGTTTGTTCTTGATAAATTGCAATTCTTTTTTTAATTGCTTCTGGTTCAT CATCTTTTCTTCTTTGCACTACAGTATTGTCTTTAAAACAATATTTTCCTTCTT TTGCTGGTTTGAATTTATCATGATAAGTGCTTTGACAAGTTGGACAATATTTT CTAAGTGAT
	gmk	BankIt2088753 Seq2 MG975780	TTTTTATCTTCTTTTAAAATTATTTTTTCAACTTCTTTTGCTGCTACTTTTAAAT CTTTATTAACAACAATATATTGGAATTTATGTTGTTGACTTAATTCTTCTAAA GATTTATCAATTCTTAATTGAATTTGTTCATGTGAATCAGTTTTTCTACTAATT ATTCTTTTTTTAAGATCATCAAATGTTGGAGGAGCTATGAAAATGGTAACAA GTTGATCACTTTTGTTTGCTTGCTCATACATTTTCATAATGTTTAGTGCTCCAA CAGTTTCAATTTCTAAAATAGGACTATAACCTTCTTTAATAATGTTATCAACT TCTGATTTTAGTGTTCCGTAATAATTATTAAAGTGTTTGTTTCATTCAAGAAA TTCATTATTTTTGATTTTATTTTTAAAATCT
CU_TH7	*dnaA*	BankIt2091406 Seq3 MH018249	TTTTCTAAACCTACAAAAGAAGATTTTTTAAAGGTTTTAAAATATAAACTTGA ATTTCAAAATATAAATCCTGAAAAATTTGAAAAAGATGCTTTAGATTTTTTAG TTTATAACAAAAAATCGGTTAGAGAAATCGAAGGTGTAGTTAATAGAATTAA ATTTTTTTCAGAAGGAGAAGCCATTCAAATTTATTCATTAGAACTTATAGAG AGGATCTTCAAAGGTATTGTTAAAAATAAAGAAAACTTAACACATAACAAA ATAATAGAAGAAGTTGCTAATTATTTCCAAATAAACAAAGAAGAAATCATTG GGACTTCAAGAAAAACAGAAATTGTTATGGCTAGAGATATATCAATTTGGTT
	rpoB	BankIt2090774 Seq3 MH010354	CTCCTTATGTTGCTACAGGAATTGAAGCAGATGTAGCTAAATTCTCATCAACT AACATCAGAGCTAGCGAAGATGGAGAAGTAGTTTTTGTTGATTCTAAATCAA TTCAAATCAAAAACGAACAAGGAAATATTAAAACTTATCACCTACGTTATTT TGAAAGATCAAACCAAGAAACTTTGATTTTACAACGTCCAATAGTTGAAGTT GGTCAATTTGTTAAGCAAGGTGATTTAATTTGTGATGGTCCTTCTACAGATCA AGGTGAATTGGCTTTGGGTAAAAACGTTTTAGTAGCTTTTTCCACTTGATATG GATACAATTACGAAGATGCGATTATTATTAGTGAAAAACTAGTTAAAGATGA TGTTTTTACTTCAATTCATATTCAAGAACAAACTATTCAATTTAGATCTTCAA AAGCAGGTAATGATGATCTTACTAGAGAAATTCCAAATGCTTCACAAAGAAG TAAAAGACACTTAGATGAAAACGG

Isolates	*Genes*	GenBank accession numbers	Sequence (5'→3')
	gyrB	BankIt2092793 Seq3 MH029379	TTTACATAGCACAACCTCCACTTTACAAAATTTCAGCAAATAAAAAAGTTGT TTATGCATATTCAGATAAGCAAAAAGAACAAATAATTGAACAAGAATTTTCA AATCAAAAAGTTAGCATTCAAAGATACAAAGGTCTTGGAGAGATGAATCCA GATCAACTCTGAGAAACCACTATGGACCCAAAAACAAGAAAAATGATTCAA GTACAAATTCAGGATGCAATTATTGCTTCACAAGTTTTTGAAGAATTAATGG GAACAGATGTAGCTCCTAGAAAATTATTTATTG
	gltX	BankIt2092386 Seq3 MH025802	AAGAAAATATAGATATTAAACTTGAAAATATTTCAGCAGATTGATTGGATTT TGCTATTGATACTTACAAAGCAAGTTGTACAACTTATAAAGATTTAGAAAAA GAAATTAACTCATTTGCTCAAATATTGCAAACTTGTTACAATGAAAAAGACC AACAACTTTACGAACAAATTGATGATAGTTTACTCAAAACATTTACTTCATTT ATCGATTGGGATAATTTTACTGTTGCAATGTTAGAACAAGCAATTGAATCAA CAAAACAAGCTAGCAATTTAAGTGGTAAAAAGCTTCTTTTACCAATTAGAAT AGCTACAACTCTTCGTTCTTC
	adk	BankIt2089996 Seq3 MH000224	TTATAATTTTCATAAAGTAGTAATAACTTCTTCTGTTAGCTTGTTTAAATCTTG ATTTGCATCAAATTCAATTAAGAGATTTTGTGTTGCGTAATAGTTAATTAAAG GTGCTGTTTGTTCTTGATAAATTGCAATTCTTTTTTTAATTGCTTCTGGTTCAT CATCTTTTCTTCTTTGCACTACAGTATTGTCTTTAAAACAATATTTTCCTTCTT TTGCTGGTTTGAATTTATCATGATAAGTGCTTTGACAAGTTGGACAATATTTT CTAAGTGAT
	gmk	BankIt2088753 Seq3 MG975781	TTTTATCTTCTTTTAAAATTATTTTTTCAACTTCTTTTGCTGCTACTTTTAAATC TTTATTAACAACAATATATTGGAATTTATGTTGTTGACTTAATTCTTCTAAAG ATTTATCAATTCTTAATTGAATTTGTTCATGTGAATCAGTTTTTCTACTAATTA TTCTTTTTTTAAGATCATCAAATGTTGGAGGAGCTATGAAAATGGTAACAAG TTGATCACTTTTGTTTGCTTGCTCATACATTTTCATAATGTTTAGTGCTCCAAC AGTTTCAATTTCTAAAATAGGACTATAACCTTCTTTAATAATGTTATCAACTT CTGATTTTAGTGTTCCGTAATAATTATTAAAGTGTTTGTTTCATTCAAGAAAT TCATTATTTTTGATTTTATTTTTAAAATCT
CU_TH8	*dnaA*	BankIt2091406 Seq4 MH018250	TTTTCTAAACCTACAAAAGAAGATTTTTTAAAGGTTTTAAAATATAAACTTGA ATTTCAAAATATAAATCCTGAAAAATTTGAAAAAGATGCTTTAGATTTTTTAG TTTATAACAAAAAATCAGTTAGAGAAATCGAAGGTGTAGTTAATAGAATTAA ATTTTTTTCAGAAGGAGAAGCCATTCAAATTTATTCATTAGAACTTATAGAG AGGATCTTCAAAGGTATTGTTAAAAATAAAGAAAACTTAACACATAACAAA ATAATAGAAGAAGTTGCTAATTATTTCCAAATAAACAAAGAAGAAATCATTG GGACTTCAAGAAAAACAGAAATTGTTATGGCTAGAGATATATCAATTTGGTT TGTTAAAAACTTGTTAGATTTAACTTTGAAAAGCATCGGCCAGA
	rpoB	BankIt2090774 Seq4 MH010355	CTCCTTATGTTGCTACAGGAATTGAAGCAGATGTAGCTAAATTCTCATCAACT AACATCAGAGCTAGCGAAGATGGAGAAGTAGTTTTTGTTGATTCTAAATCAA TTCAAATCAAAAACGAACAAGGAAATATTAAAACTTATCACCTACGTTATTT TGAAAGATCAAACCAAGAAACTTTGATTTTACAACGTCCAATAGTTGAAGTT GGTCAATTTGTTAAGCAAGGTGATTTAATTTGTGATGGTCCTTCTACAGATCA AGGTGAATTGGCTTTGGGTAAAAACGTTTTAGTAGCTTTTTCCACTTGATATG GATACAATTACGAAGATGCGATTATTATTAGTGAAAAACTAGTTAAAGATGA TGTTTTTACTTCAATTCATATTCAAGAACAAACTATTCAATTTAGATCTTCAA AAGCAGGTAATGATGATCTTACTAGAGAAATTCCAAATGCTTCACAAAGAAG TAAAAGACACTTAGATGAAAACGG
	gyrB	BankIt2092793 Seq4 MH029380	TTTACATAGCACAACCTCCACTTTACAAAATTTCAGCAAATAAAAAAGTTGT TTATGCATATTCAGATAAGCAAAAAGAACAAATAATTGAACAAGAATTTTCA AATCAAAAAGTTAGCATTCAAAGATACAAAGGTCTTGGAGAGATGAATCCA GATCAACTCTGAGAAACCACTATGGACCCAAAAACAAGAAAAATGATTCAA GTACAAATTCAGGATGCAATTATTGCTTCACAAGTTTTTGAAGAATTAATGG GAACAGATGTAGCTCCTAGAAAATTATTTATTG
	gltX	BankIt2092386 Seq4 MH025803	AAGAAAATATAGATATTAAACTTGAAAATATTTCAGCAGATTGATTGGATTT TGCTATTGATACTTACAAAGCAAGTTGTACAACTTATAAAGATTTAGAAAAA GAAATTAACTCATTTGCTCAAGTATTGCAAACTTGTCACAATGAAAAAGACC AACAACTTTACGAACAAATTGATGATAGTTTACTCAAAACATTTACTTCATTT ATCGATTGGGATAATTTTACTGTTGCAATGTTAGAACAAGCAATTGAATCAA CAAAACAAGCTAGCAATTTAAGTGGTAAAAAGCTTCTTTTACCAATTAGAAT AGCTACAACTCTTCGTTCTTC
	adk	BankIt2089996 Seq4 MH000225	TTATAATTTTCATAAAGTAGTAATAACTTCTTCTGTTAGCTTGTTTAAATCTTG ATTTGCATCAAATTCAATTAAGAGATTTTGTGTTGCGTAATAGTTAATTAAAG GTGCTGTTTGTTCTTGATAAATTGCAATTCTTTTTTTAATTGCTTCTGGTTCAT CATCTTTTCTTCTTTGCACTACAGTATTGTCTTTAAAACAATATTTTCCTTCTT TTGCTGGTTTGAATTTATCATGATAAGTGCTTTGACAAGTTGGACAATATTTT CTAAGTGAT

Isolates	*Genes*	GenBank accession numbers	Sequence (5'→3')
	gmk	BankIt2088753 Seq4 MG975782	TTTTATCTTCTTTTAAAATTATTTTTTCAACTTCTTTTGCTGCTACTTTTAAATC TTTATTAACAACAATATATTGGAATTTATGTTGTTGACTTAATTCTTCTAAAG ATTTATCAATTCTTAATTGAATTTGTTCATGTGAATCAGTTTTTCTACTAATTA TTCTTTTTTTAAGATCATCAAATGTTGGAGGAGCTATGAAAATGGTAACAAG TTGATCACTTTTGTTTGCTTGCTCATACATTTTCATAATGTTTAGTGCTCCAAC AGTTTCAATTTCTAAAATAGGACTATAACCTTCTTTAATAATGTTATCAACTT CTGATTTTAGTGTTCCGTAATAATTATTAAAGTGTTTGTTTCATTCAAGAAAT TCATTATTTTTGATTTTATTTTTAAAATCT
CU_TH9	*dnaA*	BankIt2091406 Seq5 MH018251	TTTTCTAAACCTACAAAAGAAGATTTTTTAAAGGTTTTAAAATATAAACTTGA ATTTCAAAATATAAATCCTGAAAAATTTGAAAAAGATGCTTTAGATTTTTTAG TTTATAACAAAAAATCAGTTAGAGAAATCGAAGGTGTAGTTAATAGAATTAA ATTTTTTTCAGAAGGAGAAGCCATTCAAATTTATTCATTAGAACTTATAGAG AGGATCTTCAAAGGTATTGTTAAAAATAAAGAAAACTTAACACATAACAAA ATAATAGAAGAAGTTGCTAATTATTTCCAAATAAACAAAGAAGAAATCATTG GGACTTCAAGAAAAACAGAAATTGTTATGGCTAGAGATATATCAATTTGGTT TGTTAAAAACTTGTTAGATTTAACTTTGAAAAGCATCGGCCAGA
	rpoB	BankIt2090774 Seq5 MH010356	CTCCTTATGTTGCTACAGGAATTGAAGCAGATGTAGCTAAATTCTCATCAACT AACATCAGAGCTAGCGAAGATGGAGAAGTAGTTTTTGTTGATTCTAAATCAA TTCAAATCAAAAACGAACAAGGAAATATTAAAACTTATCACCTACGTTATTT TGAAAGATCAAACCAAGAAACTTTGATTTTACAACGTCCAATAGTTGAAGTT GGTCAATTTGTTAAGCAAGGTGATTTAATTTGTGATGGTCCTTCTACAGATCA AGGTGAATTGGCTTTGGGTAAAAACGTTTTAGTAGCTTTTTCCACTTGATATG GATACAATTACGAAGATGCGATTATTATTAGTGAAAAACTAGTTAAAGATGA TGTTTTTACTTCAATTCATATTCAAGAACAAACTATTCAATTTAGATCTTCAA AAGCAGGTAATGATGATCTTACTAGAGAAATTCCAAATGCTTCACAAAGAAG TAAAAGACACTTAGATGAAAACGG
	gyrB	BankIt2092793 Seq5 MH029381	TTTACATAGCACAACCTCCACTTTACAAAATTTCAGCAAATAAAAAAGTTGT TTATGCATATTCAGATAAGCAAAAAGAACAAATAATTGAACAAGAATTTTCA AATCAAAAAGTTAGCATTCAAAGATACAAAGGTCTTGGAGAGATGAATCCA GATCAACTCTGAGAAACCACTATGGACCCAAAAACAAGAAAAATGATTCAA GTACAAATTCAGGATGCAATTATTGCTTCACAAGTTTTTGAAGAATTAATGG GAACAGATGTAGCTCCTAGAAAATTATTTATCG
	gltX	BankIt2092386 Seq5 MH025804	AAGAAAATATAGATATTAAACTTGAAAATATTTCAGCAGATTGATTGGATTT TGCTATTGATACTTACAAAGCAAGTTGTACAACTTATAAAGATTTAGAAAAA GAAATTAACTCATTTGCTCAAGTATTGCAAACTTGTCACAATGAAAAAGACC AACAACTTTACGAACAAATTGATGATAGTTTACTCAAAACATTTACTTCATTT ATCGATTGGGATAATTTTACTGTTGCAATGTTAGAACAAGCAATTGAATCAA CAAAACAAGCTAGCAATTTAAGTGGTAAAAAGCTTCTTTTACCAATTAGAAT AGCTACAACTCTTCGTTCTTC
	adk	BankIt2089996 Seq5 MH000226	TTATAATTTTCATAAAGTAGTAATAACTTCTTCTGTTAGCTTGTTTAAATCTTG ATTTGCATCAAATTCAATTAAGAGATTTTGTGTTGCGTAATAGTTAATTAAAG GTGCTGTTTGTTCTTGATAAATTGCAATTCTTTTTTTAATTGCTTCTGGTTCAT CATCTTTTCTTCTTTGCACTACAGTATTGTCTTTAAAACAATATTTTCCTTCTT TTGCTGGTTTGAATTTATCATGATAAGTGCTTTGACAAGTTGGACAATATTTT CTAAGTGAT
	gmk	BankIt2088753 Seq5 MG975783	TTTTATCTTCTTTTAAAATTATTTTTTCAACTTCTTTTGCTGCTACTTTTAAATC TTTATTAACAACAATATATTGGAATTTATGTTGTTGACTTAATTCTTCTAAAG ATTTATCAATTCTTAATTGAATTTGTTCATGTGAATCAGTTTTTCTACTAATTA TTCTTTTTTTAAGATCATCAAATGTTGGAGGAGCTATGAAAATGGTAACAAG TTGATCACTTTTGTTTGCTTGCTCATACATTTTCATAATGTTTAGTGCTCCAAC AGTTTCAATTTCTAAAATAGGACTATAACCTTCTTTAATAATGTTATCAACTT CTGATTTTAGTGTTCCGTAATAATTATTAAAGTGTTTGTTTCATTCAAGAAAT TCATTATTTTTGATTTTATTTTTAAAATCT

displayed on the joint organ but not significantly different from the other organs. Five new ST types were the first reports in Thailand, and ST71 acted as the highest population.

M. hyorhinis is the cause of enzootic pneumonia (EP) and porcine respiratory disease complex (PRDC) in pigs. This pathogen can be found in various lesions, especially the upper respiratory tract and the tonsils of pigs (Jang *et al*., 2016; Trueb *et al*., 2016). This study showed that *M. hyorhinis* were discovered in many organs but predominantly at joints, consistent with another study in Thailand (Makhanon *et al*., 2012). The number of the pathogen is dynamic and can spread to many organs, as it has been shown in the previous study (Ennis *et al*., 1971) that monitored pigs for 224 days after injecting the *M. hyorhinis* and found that the pathogen was highly detectable from the joint area for the first 90 days and later decreased substantially. They

suggested that samples be collected from more than one organ (Ennis *et al*., 1971).

In this study, only 9 of 75 PCR-positive samples could be re-cultured from the stock samples, and most of these were derived from synovial fluids from the joint organ. It was believed that this pathogen is susceptible to the environment and difficult to culture. The low success rate of culture *M. hyorhinis* is confirmed by other reports from other countries (Clavijo *et al*., 2019) and from another group in Thailand where *M. hyorhinis* is unsuccessfully cultured (Makhanon *et al*., 2012). It was believed that the success of culture this bacterium may relate to the sampling time and incubation period of bacteria (Ennis *et al*., 1971).

MLST is an essential method for detecting clonal complexes in many species (Urwin & Maiden, 2003). This study presented a unique allelic profile in Thailand. Concerning the results, 3 of 6 housekeeping genes showed similarity among allele types; *rpoB* (*rpoB_1*), *adk* (*adk_1*), and *gmk* (*gmk_3*), while the others displayed high diversity (*dnaA*, *gyrB,* and *gltX*). The *dnaA* is known as a trans-acting control element. This gene helps bacteria replicate DNA under various nutritional and environmental conditions (Magnan & Bates, 2015). Thus, the mutation rate of this conserved gene is slower than other genes. This study suggested that the mutation of *dnaA* might be caused by nutritional and environmental conditions from the European type (O'Dea *et al*., 1996). The *gyrB* gene has a role in ATP hydrolysis in bacteria. In Thailand, about 75% of pigs farms use antibiotics for the purpose of growth stimulators, disease prevention, and treatment. The most common antibiotics used in pig farms are amoxicillin, enrofloxacin, and tetracycline (Lekagul *et al*., 2020). The mutation of *gyrB* causes the loss of ATP-dependent functions and is related to resisting fluoroquinolone antibiotics. In this case, it might be related to nutritional and environmental factors, especially fluoroquinolone antibiotics in food supplements (Maes *et al*., 2020; Trouchon *et al*., 2016). Finally, *gltX* is a gene influencing temperature sensitivity (Bubnov *et al*., 2020), and thus the mutation we found in this gene may be related to the thermal conditions in this region.

Interestingly, ST56 that has been reported in France was also discovered in Thailand (www.pubmlst.org), although Thailand has never imported pigs from France. It could be speculated that bacteria contamination may come from other countries from the EU, but the incidence of *M. hyorhinis* is minimal. Due to low reports of *M. hyorhinis*, the clonal relationship among each region is still unclear. However, variable MLST schemes of *M. hyorhinis* have been increasingly reported (www.pubmlst.org). We suggested that *M. hyorhinis* might have a high ability to adapt to different environments, leading to various gene mutations.

CONCLUSION

This study unveiled five novel genomic variations of *Mycoplasma hyorhinis* by multi-locus sequence typing (MLST) and a previously recognized type from Thailand's diseased pig. The know fledge of the MLST scheme provides promising information for future vaccine development and disease prevention strategy.

CONFLICT OF INTEREST

The authors declare no conflict of interest in this study.

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