Targeted Sequencing Analysis Of *Mycoplasma Gallisepticum* Isolates In Chicken Layer And Breeder Flocks In Thailand.

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Research Article

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Abstract

Mycoplasma gallisepticum (MG) is one of the most economically significant pathogens worldwide. MG affects the respiratory system and cause a poor growth performance in poultry. In some developing countries, the conventional PCR assay are still the widely used technique. In this study, 24 collected Thai MG isolates from the unvaccinated farms during 2002-2020 were characterized by gene-targeted sequencing (GTS), followed by phylogenetic using UPMGA analysis. From the results, 24 Thai MG isolates could be differentiated from vaccine strains including F, ts-11 and 6/85. One of Thai MG isolates showed 99.5-100% genetic similarity to F strain with 4 partial genes analysis. The contamination of F strain from the vaccinated farms could be a possible reason because F strain is the most used vaccine strain in Thailand. However, The GTS analysis using the partial MG genes in this study showed that Thai MG isolates were grouped in different patterns based on individual gene sequences. The phylogenetic of partial *mgc2*, *gapA*, *pvpA* and *lp* gene sequences could classified Thai MG isolates into 7, 11, 7 and 2 groups, respectively. In conclusion, at least 2 partial MG genes especially partial *gap*A and *mgc*2 genes should be determined to differentiate the MG isolates.

Introduction

Mycoplasma gallisepticum (MG) is still one of the most important bacterial pathogens worldwide, causing respiratory disease called as chronic respiratory disease (CRD) in infected flocks and monetary losses for treatment and control[1]. MG can transmit through horizontal and vertical transmissions, which economically affect the poultry industry. MG infection can cause high feed conversion ratio, egg production loss, poor hatchability, and carcass degradation[1]. Stipkovits and Kempf [2] investigated the economic loss from MG and found that there were 10-20% of egg production drop in infected layers and the body weight loss up to 10-20% in infected broilers. In Thailand, there were approximately 25% of all laying hen in poultry industry having MG infection causing around 15 million U.S. dollar loss due to the decrease of egg production[3].

Due to the widespread MG infection, vaccination has been the important preventive strategy generally using in poultry industry in Thailand. There are live vaccine strains including F, ts-11, and 6/85 and MG inactivated vaccine have been used for years[1]. Especially, F strain is one of the most effective strain and it is widely used in Thailand. Therefore, the differentiation technique to identify between vaccine and field MG strains in suspected MG infection flock is needed. Several studies have investigated the techniques for MG classification[4-7]. The most used technique in Thailand is random amplification of polymorphic DNA (RAPD). However, RAPD showed low reproducibility and results from different laboratories could not be compared[8,9]. Sequencing has become a potential technique for MG classification. It could differentiate MG strains with some partial DNA sequences and can be used to compare MG strains among laboratories in different area or countries[4,10,11]. In addition, Gene targeted sequencing (GTS) is cost efficient and affordable for developing countries including Thailand, where is the advanced techniques which are not applicable in general.

The important genes of MG including *gap*A, *mgc*2, *pvp*A and MGA_0319 (*lp*) have been investigated in several MG epidemiological studies[4,12,13]. In Thailand, Limsatanun et al.[14] determined Thai MG strains classification with partial *mgc*2 gene sequences. The results showed that partial *mgc*2 gene could classify Thai MG strains from vaccine strains and various strains from different countries. However, only partial *mgc*2 gene classification could not be used as a reliable method for MG characterization[4].

The aim of this study was to determine the GTS technique which could classify Thai MG field strains isolated from vaccine strains in commercial chicken flocks from different regions. This is the first study using 4 partial MG

gene sequences for MG classification in commercial in Thailand.

Results

PCR amplification. All twenty-four Thai MG isolates were detected by MG specific PCR amplification following Lauerman method[15]. For the amplification of partial *mgc*2 genes, 22 Thai MG isolates were successfully amplified and sequenced which had 615 bp in size. Following with the result of specific partial *gap*A PCR, 21 Thai MG isolates were positive and proceeded for phylogenetic analysis with 306 bp, while 20 samples of Thai MG isolates were successfully amplified using *pvp*A and MGA_0319 (lp) primer with respectively 456 and 495 bp in length. All nucleotide sequences from Thai MG under this study were submitted to Genbank for accession numbers (Table. 2).

Phylogenetic analysis. The phylogenetic tree based on partial *mgc*2 gene of Thai MG isolates demonstrated that 3 Thai MG isolates were closely related to F strain. AHRU/2014/CU4508.1 was grouped together with F strain while AHRU/2020/CU0143.1 and AHRU/2020/0147.1 showed 97.6% genetic similarity to F strain (Figure. 1). According to phylogenetic tree of partial *gapA* gene, AHRU/2014/CU4508.1 still showed the genetic similarity ranged 99.5% to F strain. AHRU/2020/CU3704.1 was also grouped with F strain (Figure. 2). The phylogenetic analysis of partial *pvp*A gene demonstrated that All Thai MG isolates were characterized into the same cluster except reference strain, S6 (Figure. 3). There were four Thai MG isolates showed 94.3 % genetic similarity to 6/85 strain. While AHRU/2014/CU450 8.1 was grouped with F strain with 100% similarity. The partial *lp* gene sequences of Thai MG were compared with reference strains. 6/85 strain was the one which being grouped in different cluster. AHRU/2014/CU4508.1 and AHRU/2020/CU0143.1 had 100% genetic similarity to F strain and 99.2 % comparing with ts-11 strain (Figure. 4). The phylogenetic with DNA sequences data is available in the Supplementary information

Discussion

Avian mycoplasmosis have been recognized as an important pathogen causing economic effected disease in poultry industry. Live, inactivated, and recombinant MG vaccines have been used in Thailand for a long time. Due to the increased use of MG vaccines, differentiation between field and vaccine strains is warranted. Molecular MG characterization have been investigated in many countries[4,5,7,16,17]. This study is the first study which GTS technique have been done on Thai MG strains with 4 partial MG gene sequences. Partial *mgc* gene was used for MG characterization in many epidemiological studies[6,14,18]. *mgc2* gene encodes MGC2 protein which works with *gapA* gene encoded protein for cell attachment[19], involving with MG immunogenicity[20,21].

In previous study, Armour et al.[16] investigated MG isolates from South Africa using intergenic spacer region (IGSR), *mgc2* and *gapA* genes. Thirty-six MG isolates were classified by *mgc2* gene into 8 types and by *gapA* into 2 types. The results showed that *mgc2* gene had the higher discriminatory power than *gapA* gene. MG epidemiological investigation also was reported by the other study in Russia[7]. The results showed the mgc2 had the good discriminatory power while gapA was unable to show the good discriminatory index for MG classification. However, using only single gene for MG classification could not indicate the similarity between MG isolates. There were MG isolates were negative by PCR assay with *mgc2* gene detection resulting in a failure to obtain *mgc2* sequences[5,6,13,16], indicating that only one partial gene sequence cannot be used to characterize MG gene.

In the present study, *lp* gene of Thai MG isolates were more conserved than *gapA, mgc2* and *pvpA* genes, eighteen out of 20 Thai MG isolates had 100% genetic similarity. While partial *gapA* showed the highest genetic variation among Thai isolates. The results were contradicted comparing with the previous studies[4,7,16] indicating that MG isolates from the same area could have lower genetic diversity than MG isolates from different regions[16]. Thai MG isolates were

identified with 4 genes using phylogenetic tree (UPMGA) method. AHRU/2014/CU4508.1 showed the closest genetic relationship to F strain. The results from UPMGA showed that AHRU/2014/CU4508.1 was grouped with F strain with all 4 partial genes analysis. Interestingly, all Thai MG isolates in this study were collected from unvaccinated farm. Possibly, AHRU/2014/CU4508.1 isolate in this farm was contaminated from vaccinated chicken farm via horizontal transmission. Other Thai MG isolates in this study showed the variation of genetic classification in each gene analysis. The results of *gapA* and *mgc2* genes analysis showed that AHRU/2003/CU5113.2 and AHRU/2003/CU5808.2 were grouped with S6 strain with 97% and 99.4% genetic similarity. While using partial *pvpA* gene sequence comparison, S6 strain was separated from all Thai MG isolates including AHRU/2003/CU5113.2 and AHRU/2003/CU5808.2. It could be indicated that AHRU/2003/CU5113.2 and AHRU/2003/CU5808.2 might have genetic relation with S6 strain. There were some Thai MG isolates could not be obtained DNA sequences with all 4 virulence genes. For example, Thai MG isolate AHRU/2009/CU3704 could only be classified by phylogenetic of *gapA* and *pvpA* genes because it was negative with PCR method using *mgc*2 and *pvpA* primers specific. Plausibly, it might be the quality of DNA and/or the genetic mutation in inter- and intra-strain MG[6,22,23].

In conclusion, the identification of Thai MG isolates in this study could be differentiated with partial MG genes including *gap*A, *mgc2*, *pvp*A and MGA_0319 (*lp*) genes. All Thai MG isolates could be classified with at least 2 out of 4 partial genes sequences especially partial *gap*A and *mgc*2 genes which had satisfactory discriminatory power for Thai MG characterization. Using the partial DNA sequencing for MG characterization is effective, reproducible to establish the genetic relationship between MG strains and differentiate between vaccine and field strains. In addition, this study was the first epidemiological data of Thai MG strains using 4 partial MG genes sequencing, presenting the genetic diversity of circulating MG strains in Thailand. In further study, the 23S rRNA of Thai MG strains will be investigated on the association between the mutation and antibiotic resistance, providing more epidemiological and evolutionary data to improve monitoring technique of vaccine and field MG strains in Thailand.

Materials And Methods

MG isolates. Twenty-four Thai MG isolates were used in this study. All isolates were collected during 2003-2020 by Prof. Somsak Pakpinyo, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University. All MG isolates were collected from choanal cleft and were propagated in FMS medium supplemented with 15% swine serum as reported[24] and incubated at 37° C until broth's color changed from pink to orange. All isolates were confirmed as MG by polymerase chain reaction (PCR) assay[15].

Molecular typing. The DNA from each Thai MG isolate was extracted with an equal volume of phosphate buffered saline (PBS) then amplified by the PCR assay. The primers in this study were designed by Ferguson[4] (Table. 1). PCR assay was performed to detect *gap*A, *pvp*A, MGA_0319 and *mgc*2 partial genes. The PCR mixture consisted of 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 1.25 mM MgCl2, 1 mM dNTP (Thermo Scientific, Vilnius, Lithuania) 10 pmole each of primer (Qiagen®, Valencia, CA, USA), 1.25 µl of Taq polymerase (Promega, Madison, WI, USA) and 2.5 µl (125 ng) of the DNA template. The amplification reaction was performed in a DNA thermal cycler with condition at 94° C for 3 min, followed by 40 cycles of 94° C for 20 s, 55-60° C for 40 s, 72° C for 60 s, and 72° C for 5 min for *gap*A, *pvp*A, MGA_0319 (*lp*) and *mgc*2 genes. The PCR products are 332, 702, 590 and 824 bp, respectively[4].

Reference sequences. Four reference strains were used in this study. F strain which is the vaccine strain provided by local distributor (MSD, Thailand). S6 strain was obtained from the ATCC (15302). The ts-11 and 6/85 strain sequences were obtained from Prof. Somsak Pakpinyo, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University.

DNA Sequence analysis. Amplified PCR products of MG targeted gene-positive extracts were submitted to determine the sequence. All sequences were analyzed with Editseq program (Lasergene, DNASTAR Inc., USA) and constructed the consensus with Seqman program (Lasergene, DNASTAR Inc., USA). Thai MG isolates and reference gene sequence data were aligned to construct phylogenetic tree by Bionumeric version 7.6 software (Applied Maths, Sint-Martens-Latem, Belgium). The cluster analysis was done with UPGMA method. The similarity coefficients of Thai MG isolates and reference strains were determined on multiple sequence alignments.

Declarations

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Disclosure statement

No potential conflict of interest was reported by the authors.

Author contributions

A.L. conceptualized and designed the study, performed the experiment and carried all analyses, interpreted the results and drafted the manuscript; T.P. constructed the phylogenetic trees; K.L. collected swab samples data; S.P. supervised the study, reviewed and edited the manuscript.

Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available

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Tables

Table 1. Thai MG strains and GenBank accession

Strain	Source	Accession number			
		*mgc*2	*gap*A	*pvp*A	lp gene
F	Vaccine	MW617973	MW617946	MW617933	MW617913
S6	Laboratory	MW617972	MW617947	MW617934	MW617934
TS-11	Vaccine	MW617971	MW617954	MW617945	MW617945
6/85	Vaccine	MW617974	MW617966	MW617930	MW617930
AHRU/2002/CU0001.3	Central part	MW617980	MW617957	MW617943	MW617919
AHRU/2002/CU0111.3	Central part	KX268616	MW617959	MW617944	MW617920
AHRU/2003/CU0103.3	Central part	KX268617	MW617958	MW617940	MW617918
AHRU/2003/CU0701.2	Eastern part	KX268618	MW617948	MW617931	MW617908
AHRU/2003/CU0802.2	Eastern part	KX268619	MW617949	MW617935	MW617909
AHRU/2003/CU3101.2	Eastern part	KX268620	MW617969	MW617929	MW617917
AHRU/2003/CU3201.1	Eastern part	MW617977	MW617960	MW617926	-
AHRU/2003/CU3215.1	Eastern part	KX268621	MW617970	MW617925	MW617904
AHRU/2003/CU3302.3	Eastern part	KX268622	MW617965	MW617924	MW617916
AHRU/2003/CU5004.2	Central part	KX268624	MW617955	MW617927	MW617905
AHRU/2003/CU5113.2	Central part	KX268625	MW617956	MW617923	MW617902
AHRU/2003/CU5311.2	Eastern part	KX268626	MW617964	MW617922	MW617903
AHRU/2003/CU5415.2	Eastern part	KX268627	MW617950	MW617938	MW617907
AHRU/2003/CU5505.3	Eastern part	KX268628	MW617963	MW617936	MW617901
AHRU/2003/CU5507.3	Eastern part	KX268629	MW617962	MW617937	MW617900
AHRU/2003/CU5613.3	Western part	MW617975	MW617961	-	MW617899
AHRU/2003/CU5713.2	Eastern part	KX268630	MW617951	MW617939	MW617910
AHRU/2003/CU5808.2	Central part	KX268631	MW617952	MW617941	MW617911
AHRU/2009/CU2006.1	Western part	KX268632	MW617953	MW617942	MW617912
AHRU/2009/CU3704.1	Western part	-	MW617968	MW617932	-
AHRU/2014/CU4508.1	Western part	MW617976	MW617967	MW617928	MW617906
AHRU/2020/CU0143.1	Central part	MW617979	-	-	MW617915
AHRU/2020/CU0147.1	Central part	MW617978	-	-	MW617912

Table 2. MG PCR Primers for MG characterization

Primer	Oligonucleotides Sequence (5’-3’)	Reference
*gap*A-3F	TTCTAGCGCTTTAGCCCTAAACCC	Ferguson et al, 2005
*gap*A-3R	CTTGTGGAACAGCAACGTATTCGC	
*pvp*A-3F	GCCAMTCCAACTCAACAAGCTGA	
*pvp*A-3R	GGACGTSGTCCTGGCTGGTTAGC	
lp -F	CCAGGCATTTAAAAATCCCAAAGACC	
lp-R	GGATCCCATCTCGACCACGAGAAAA	
*mgc*2-1F	GCTTTGTGTTCTCGGGTGCTA	
*mgc*2-1R	CGGTGGAAAACCAGCTCTTG	

Figures

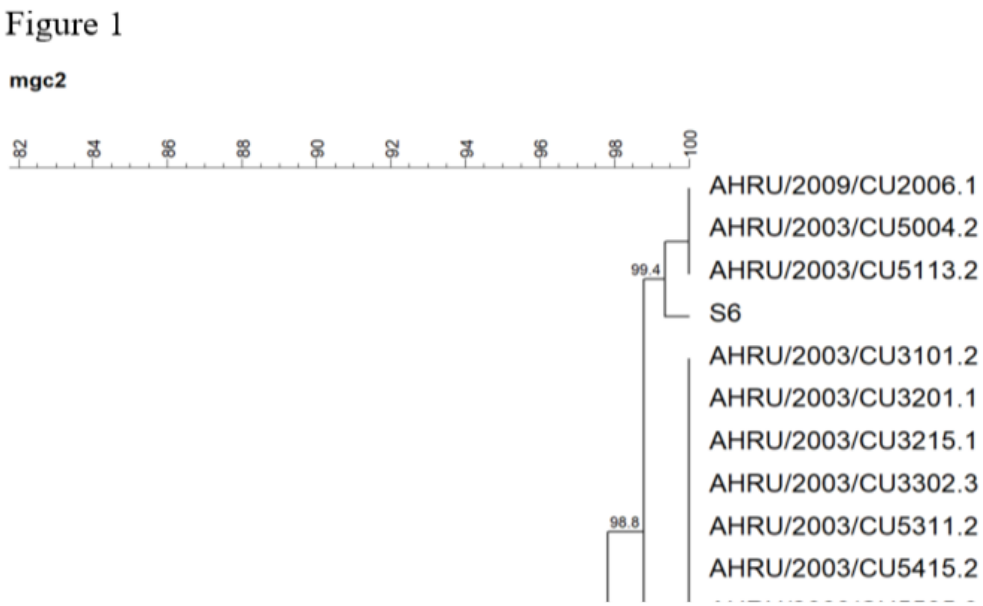

Figure 1

Phylogenetic tree based on the alignment of partial *mgc2* gene of Thai MG and reference strains was performed with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using Bionumeric version 7.6 software.

Figure 2

Phylogenetic tree based on the alignment of partial *gapA* gene of Thai MG and reference strains was performed with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using Bionumeric version 7.6 software.

Figure 3

Phylogenetic tree based on the alignment of partial *pvpA* gene of Thai MG and reference strains was performed with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using Bionumeric version 7.6 software.

Figure 4

Phylogenetic tree based on the alignment of partial *pvpA* gene of Thai MG and reference strains was performed with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using Bionumeric version 7.6 software.

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