Original Article

Comparison of GTS and RAPD assays to characterize Thai *Mycoplasma gallisepticum* strains

Arithat Limsatanun[1] Jiroj Sasipreeyajan[1] Somsak Pakpinyo[1*]

Abstract

Mycoplasma gallisepticum (MG) is one of the important pathogens which have economic impact on the poultry industry worldwide. Molecular characterization is the effective method to study the relation among international strains and the epidemiology of MG transmission. In this study, 17 Thai MG strains were characterized by using 2 molecular assays including random amplified polymorphic DNA (RAPD) and gene-targeted sequencing (GTS) assays. Results showed that the RAPD assay could classify the Thai MG strains into 3 patterns. Most MG strains obtained from the same area were in the same pattern. Partial *mgc2* gene was used to distinguish between the Thai MG strains and MG strains from various countries. The phylogenetic tree of nucleotide sequence of *mgc2* gene showed that 11 Thai MG strains had 100% similarity sequence to Indian MG strains and one strain was 100% similar to Israel MG strain. The targeted partial *mgc2* gene could characterize the Thai MG strains into 4 groups while the RAPD pattern classified the Thai MG strains into 3 groups. These results provided interesting data about the relation of Thai MG strains and MG strains from other countries. GTS assay might be the new optional assay to monitor MG outbreak in Thailand.

Keywords: gene-targeted sequencing (GTS) assay, *mgc2* gene, *Mycoplasma gallisepticum*, random amplified polymorphic DNA (RAPD) assay, Thailand

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Introduction

Mycoplasma gallisepticum (MG) has been one of the significant pathogens in the poultry industry in Thailand. MG causes chronic respiratory disease in chicken, decrease in egg production and carcass degradation (Raviv and Ley, 2013). MG is an important respiratory pathogen worldwide. It can be transmitted to egg from infected hen and contaminate the environment (Raviv and Ley, 2013). Eradication of MG from infected farm is the best prevention strategy. However, it is impractical to do in Thailand. Therefore, biosecurity, surveillance and vaccination become the important management of MG prevention. There are 3 available commercial types of MG vaccines, including MG bacterin, attenuated live MG vaccines (F, 6/85 and ts-11 strains) and recombinant fowlpox-MG vaccine (rFP-MG) (Carpenter et al., 1981; Whithear et al., 1990; Evans and Hafez, 1992; Zhang et al., 2010). Because of the increasing use of attenuated live MG vaccine, effective assays to differentiate between MG vaccine strain and field strain are required. A common method of differentiation is the random amplification of polymorphic DNA (RAPD) assay (Geary et al., 1994; Fan et al., 1995). However, RAPD assay has several disadvantages including low reproducibility and difficulty in comparing results among laboratories (Tyler et al., 1997; Mettifogo et al., 2006). Interestingly, Ferguson et al. (2005) introduced the new molecular assay known as gene-targeted sequencing (GTS) assay for MG strain differentiation. The GTS assay has high reproducibility and can compare results among laboratories; however, this assay is time-consuming and more expensive compared to the RAPD assay. *Mgc2* gene is one of the targeted genes which have been used in several epidemiological studies of MG (Gharaibeh et al., 2011; Sprygin et al., 2011; Moretti et al., 2013; Khalifa et al., 2014). This gene encodes Mgc2 protein, which works with GapA protein related to cell attachment of MG (Boguslavsky et al., 2000). Considering the molecular result, *mgc2* gene is one of the MG genes which have been used in MG molecular characterization (Gharaibeh et al., 2011; Sprygin et al., 2011; Moretti et al., 2013; Khalifa et al., 2014), and showed high discriminatory power in differentiating MG strains. Armour et al. (2013) identified MG sequences with IGSR, *mgc2*, MGAL0319 and *gapA* genes. The result showed that *mgc2* gene was the best sequence to discriminate between MG strains.

The purposes of this study were to determine and compare the capability of RAPD assay and GTS assay to discriminate *mgc2* gene of Thai MG strains. The hypothesis of this study was that GTS assay showed higher discriminatory power than RAPD assay. In addition, the *mgc2* gene of Thai MG strains was analyzed and compared to that of other strains from various countries.

Materials and Methods

Sample collection: Seventeen MG samples were collected from different regions in Thailand (provided by Somsak Pakpinyo, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University). These samples were obtained from broiler breeder, broiler and layer farms, which were not vaccinated with attenuated live MG vaccines, located in the central, eastern and western parts of Thailand during 2003-2009 (Table 1). The samples were taken from choanal cleft and confirmed as pure MG isolates by MG PCR and direct immunofluorescent assays. All MG cultures were stored in Frey's broth medium at -80°C. All MG samples were thawed and re-propagated in 2 ml Frey's medium supplemented with 15% swine serum (FMS) until broth color changed to orange and were re-confirmed MG positive and MS negative results by PCR (Lauerman, 1998). All samples were prepared for the *mgc2* gene-targeted PCR and RAPD assays (Table 1).

Table 1 Description of Thai MG strains used in this study and RAPD type

Isolate	Year	Part of Thailand	GenBank accession	RAPD type	GTS type
AHRU/2002/CU0111.3	2002	Central part	KX268616	1	4
AHRU/2003/CU0103.3	2003	Central part	KX268617	2	4
AHRU/2003/CU0701.2	2003	Eastern part	KX268618	3	1
AHRU/2003/CU0802.2	2003	Eastern part	KX268619	3	1
AHRU/2003/CU3101.2	2003	Eastern part	KX268620	3	1
AHRU/2003/CU3215.1	2003	Eastern part	KX268621	3	1
AHRU/2003/CU3302.3	2003	Eastern part	KX268622	3	1
AHRU/2003/CU3401.1	2003	Eastern part	KX268623	2	1
AHRU/2003/CU5004.2	2003	Central part	KX268624	2	2
AHRU/2003/CU5113.2	2003	Central part	KX268625	1	2
AHRU/2003/CU5311.2	2003	Eastern part	KX268626	1	1
AHRU/2003/CU5415.2	2003	Eastern part	KX268627	3	1
AHRU/2003/CU5505.3	2003	Eastern part	KX268628	3	1
AHRU/2003/CU5507.3	2003	Eastern part	KX268629	3	1
AHRU/2003/CU5713.2	2003	Eastern part	KX268630	3	1
AHRU/2003/CU5808.2	2003	Central part	KX268631	3	3
AHRU/2009/CU2006.1	2009	Western part	KX268632	3	2

Four reference strains were used in this study. F strain was a vaccine strain which was provided by a local distributor (MSD, Thailand) and S6 strain was obtained from the ATCC (15302). The ts-11 and R strain sequences were retrieved from GenBank data (Table 2).

For MG differentiation analysis, eighteen *mgc2* gene sequences retrieved from 7 countries were used to compare alignments of *mgc2* gene sequences (Table 2). All *mgc2* gene sequences were obtained from GenBank data.

Table 2 Description of MG reference strains used in this study

Isolate	Country	GenBank accession
K5152ACK01	USA	AY556289
K4669ATK98	USA	AY556303
K4781ATK99	USA	AY556272
K5033ATK00	USA	AY556278
K5109BCK01	USA	AY556286
K4705CK99	USA	AY556271
K5120CK01	USA	AY556288
K4902TK00	USA	AY556284
Au94043CK94	Australia	AY556300
Au96022 CK96	Australia	AY556301
Au99169CK99	Australia	AY556304
KS2	Israel	AY556293
SA1Y12	South Africa	KC130903
ZM1Y12	Zimbabwe	KC130907
Eis10-17	Egypt	KY421065
MGS849	India	KP300756
MGS1345	India	KP300762
MGS19B	India	KP279743
F	Vaccine strain	KX268633
ts 11	Vaccine strain	JQ770175
S6	Laboratory strain	KX268634
Strain R	Laboratory strain	AY556228

Polymerase chain reaction (PCR) assay: To confirm MG cultured samples, all FMS broth samples were subjected to DNA extraction. Target DNA was amplified by polymerase chain reaction (PCR) following the protocol described by Lauerman (1998). Briefly, the cultured broth was centrifuged at 15,000xg for 6 min. MG pellets were washed with distilled water and centrifuged twice. The pellets were re-suspended with approximately 30 µl of distilled water, depending on the size of the pellets. The samples were boiled for 10 min, then immediately placed on ice for 5 min and centrifuged at 15,000xg for 2 min. The supernatants containing the DNA template were collected and kept at -20°C until use. The PCR mixtures were prepared at 25 µl volume containing 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 1.25 mM $MgCl_2$, 1 mM dNTP (Thermo Scientific, Vilnius, Lithuania), 10 pmole each of primer F (5'-GAGCTAATCTGTAAAGTTGGTC-3) and primer R (5'-GCTTCCTTGCGGTTAGCAAC-3') (Qiagen®, Valencia, CA, USA), 1.25 U Taq polymerase (Promega, Madison, WI, USA) and 2.5 µl (250 ng) of the DNA template. MG S6 strain (ATCC 15302) was used as a positive control. The PCR mixtures were amplified in a DNA thermal cycler, PCR Sprint® (Thermo Electron Corporation, USA) at 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec for 40 cycles, followed by at 72°C for 5 min. The PCR product was analyzed by 2% agarose gel electrophoresis (Pharmacia Biotech AB, Uppsala, Sweden), stained with ethidium bromide 0.20 µg/ml, and visualized by E-BOX VXII UV transilluminator (Vilber Lourmat, Eberhardzell, Germany).

RAPD analysis: The RAPD assay was modified from Ley et al. (1997). Briefly, PCR reaction was performed using 25 µl of PCR mixtures. Each RAPD mixture consisted of 2 mM $MgCl_2$, 1 mM dNTP (Fermentas, USA), 500 ng Geary primer set (Geary et al., 1994) (5'-CCGCAGCCAA-3') (Qiagen, Germany), 2.5 U of Taq polymerase (Promega, USA), and 0.5 µl of MG DNA containing 50 ng DNA. Four cycles of amplification reactions were performed, of 94°C for 5 min, 36°C for 5 min, and 72°C for 5 min, ending with 30 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 1 min, and final elongation of 72°C for 10 min. The PCR banding pattern or genotypic profile was analyzed by agarose gel electrophoresis. The samples were interpreted as indistinguishable when no major band differences were found (Fig. 1).

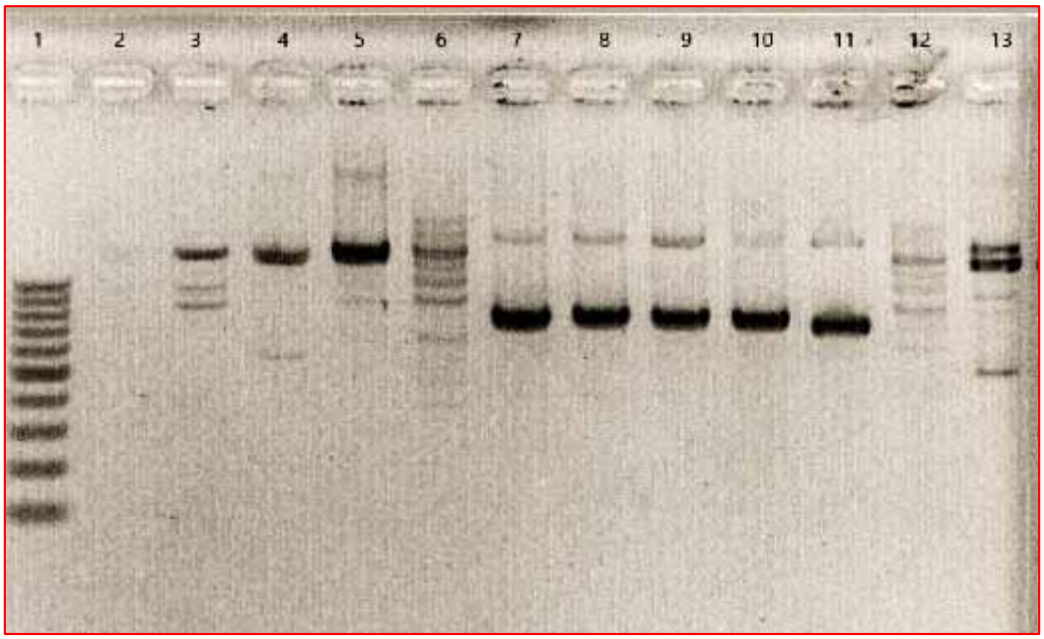

Figure 1 RAPD analysis of Thai MG strains with primer set described by Geary et al. (1994). Lane 1, molecular mass ladder; Lane 2, negative sample; Lane 3, F strain; Lane 4, S6 strain; Lane 5, AHRU/2003/CU5004.2; Lane 6, AHRU/2003/CU5113.2; Lane 7, AHRU/2003/CU5311.2; Lane 8, AHRU/2003/CU5415.2; Lane 9, AHRU/2003/CU5505.3; Lane 10, AHRU/2003/CU5507.3; Lane 11, AHRU/2003/CU5713.2; Lane 12, AHRU/2003/CU5808.2; Lane 13, AHRU/2009/CU2006.1.

PCR amplification of mgc2 gene: Genomic DNA was extracted from individual sample in FMS broth by using the QIAamp DNA Mini Kit (Qiagen®; Valencia, CA) according to the manufacturer's recommendations. To amplify *mgc2* gene, the primers and reaction were conducted following previously described protocols (Ferguson et al., 2005). Briefly, the 25 µl of PCR mixture contained 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 1.25 mM $MgCl_2$, 1 mM dNTP (Thermo Scientific, Vilnius, Lithuania), 10 pmole each of primer F (5-GCTTTGTGTTCTCGGGTGCTA-3) and primer R (5'-CGGTGGAAAACCAGCTCTTG-3') (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, PCR Sprint® (Thermo Electron Corporation, USA), with condition at 94°C for 3 min, and 40 cycles of 94°C for 20 sec, 58°C for 40 sec, 72°C for 60 sec, and 72°C for 5 min. The PCR product was analyzed by 2% agarose gel electrophoresis (Pharmacia Biotech AB, Uppsala, Sweden), stained with ethidium bromide 0.20 µg/ml, and visualized by E-BOX VXII UV transilluminator (Vilber Lourmat, Eberhardzell, Germany). The amplified *mgc2* gene product was 824 base pairs.

Sequencing and sequence analysis of mgc2 gene: Products of amplified *mgc2* gene were subjected to sequence (Malaysia Genomics Resource Centre, Malaysia). All sequence data were analyzed with Editseq program (Lasergene, DNASTAR Inc., USA) and constructed the consensus with Seqman program (Lasergene, DNASTAR Inc., USA). Thai and reference gene sequence data were aligned to construct phylogenetic tree by clustal-W method with Molecular Evolutionary Genetic Analysis (MEGA 7) software (http://www.megasoftware.net).

Results

From the RAPD analysis, the samples were divided into 3 different RAPD patterns based on major band presence (Table 1). The Thai MG strains, AHRU/2002/CU0111.3, AHRU/2003/CU5113.2 and AHRU/2003/CU5311.2 MG, showed similar pattern of major band to F and S6 strains. Eleven Thai MG strains were categorized as the same group. Nine out of 11 strains were collected from the same geographical area.

The sequences of *mgc2* gene of the Thai MG strains were compared with MG strains from various countries based on the Maximum likelihood method of MEGA7 program. The data showed that the similarity sequence ranged from 93.85-100% (Fig. 2). The similarity of *mgc2* gene sequences of the Thai strains and American strains ranged from 93.85-99.61%, while the Australian strains showed a range of 96.77-99.01%. There were 11 Thai MG strains which showed 100% similarity to MGS1345 and MGS19B strains from India, and KS2 strain from Israel. Comparing the sequence of the Thai MG strains with vaccine strains, the Thai strains showed 93.85-95.8% and 96.77-98.40% similarity to F and ts-11 strains, respectively (Table 1).

Discussion

To control MG infection, attenuated live MG vaccines have been used worldwide. Therefore, an assay which can differentiate between wild type and vaccine strains becomes more important. An effective assay will be useful to rapidly recognize and track the source of MG outbreak. In this study, the RAPD assay

and molecular assays were used to differentiate the Thai MG strains. The RAPD procedure, which was introduced by Geary et al. (1994), was used in this study. All of the Thai MG strains were categorized into 3 groups based on the visual band pattern. Three of the Thai MG strains showed the same band pattern as F and S6 strains. Two strains were isolated from the central part and one was isolated from the eastern part of Thailand. However, all Thai MG strain samples in the present study were obtained from unvaccinated poultry farms; therefore, these three strains could be exposed or contaminated with the F strain-vaccinated flock. Most MG strains from the same area were grouped together, suggesting that MG outbreak might take place in the same or nearby area and spread to other farms in other areas.

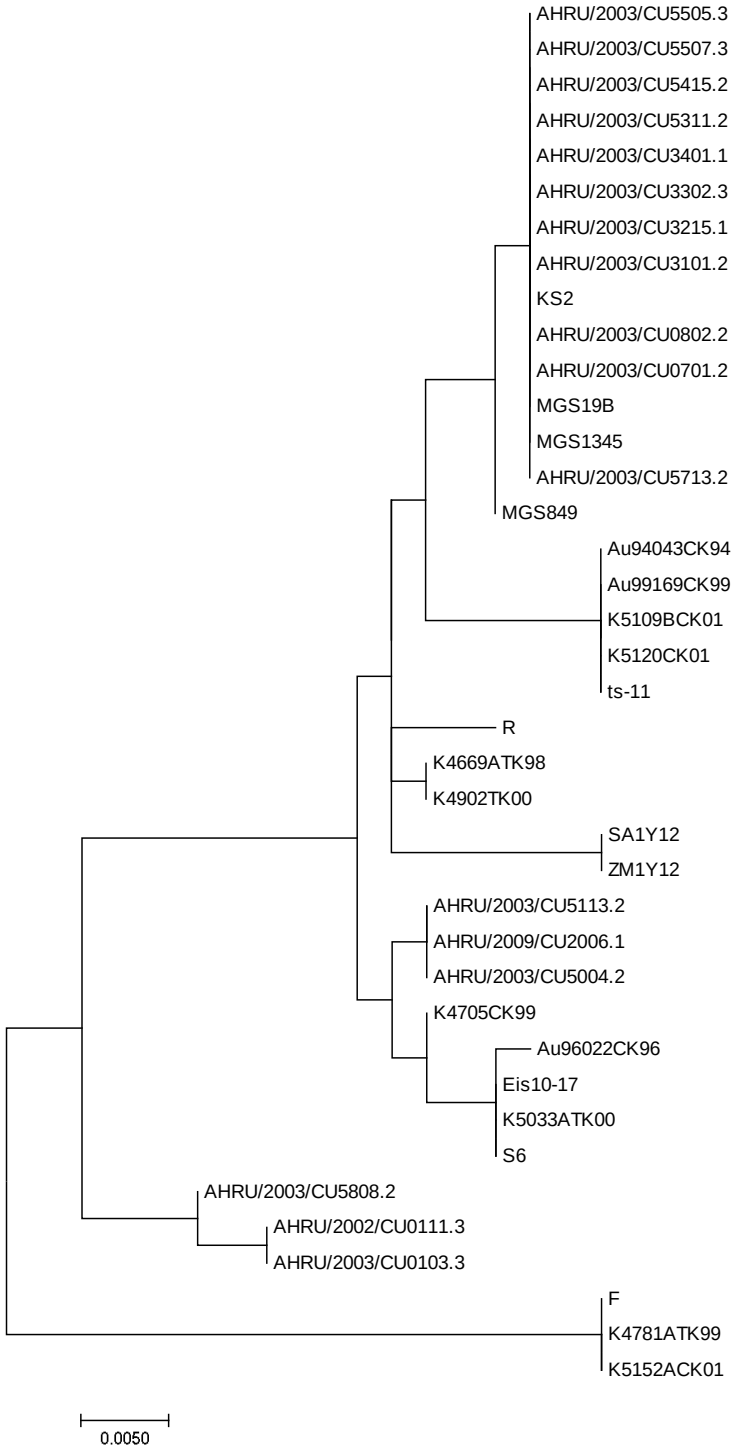

Figure 2 Phylogenetic tree of *mgc2* gene constructed by Maximum Likelihood method using MEGA7 software. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 39 nucleotide sequences. There were a total of 509 positions in the final data set.

From the GTS analysis, 11 out of the 17 Thai MG strains had 100% partial *mgc2* gene similarity to MG strains of India and Israel. AHRU/2003/CU5004.2, AHRU/2003/CU5113.2 and AHRU/2009/CU2006.1 were the Thai MG strains which showed the highest similarity of partial *mgc2* sequence to MG strains of USA (99.61%), Australia (99.01%), Egypt (99.21%) and S6 strain (99.21%). Compared to vaccine strain, none of the Thai MG strains shared 100% similarity with F and ts-11 strains. In addition, the GTS assay could classify the Thai MG strains from the eastern part of Thailand into GTS type 1, while the Thai MG strains from the central part were classified into GTS types 2, 3 and 4. From the RAPD results, the Thai MG strains from the eastern and central parts were RAPD types 1, 2 and 3. The results of the GTS assay were inconsistent with those of the RAPD assay; 6 Thai MG strains were categorized into different groups from both assays, suggesting that the GTS assay gives more accurate results of the characterization of Thai MG strains. However, these inconsistent results might be explained by the error of visual band observation and the undetectable bands which might have different patterns of RAPD assay. In this study, partial *mgc2* gene was used to characterize all samples. There might be an error during the process due to shortness of sequencing reads. The advantage of RAPD assay is that it saves time and cost (Maurer et al., 1998), in contrast to the GTS assay. However, the sequencing assay can present global comparison of MG strain typing with high discriminatory power (Ferguson-Noel et al., 2005).

The comparison of Thai MG strains to reference, vaccine and MG strains from various countries in the present study showed the relation among them. However, the relationship among Thai MG strains and MG strains of India and Israel was not concluded in the present study. A possible explanation might be the migration of wild birds and trading route, which is similar to the avian influenza virus (AIV) spreading. Sequencing technique could track down the AIV-infected migratory waterfowl at breeding site in China. H5N1 AIV genomes collected from migratory birds in China showed close relation to the original virus in Hong Kong 2004 and caused the emerging disease in south-east Asia (Liu et al., 2005). In further studies, *pvpA*, *IGSR*, *MGAL0319* or *gapA* genes should be determined to provide more information compared to one target gene analysis.

In conclusion, the *mgc2* gene sequence analysis showed that most Thai MG strains had 100% similarity sequence to MG strains of India and Israel. This assay allows us better understanding of MG epidemiological control. Moreover, it will aid in finding new affordable assay for MG surveillance in Thailand.

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บทคัดย่อ

การเปรียบเทียบวิธีจีทีเอสและอาร์เอพีดีเพื่อจำแนกลักษณะสายพันธุ์ของเชื้อ มัยโคพลาสมา กัลลิเซพติกุม ของประเทศไทย

อริย์ธัช ลิ้มศตนันท์[1] จิโรจ ศศิปรียจันทร์[1] สมศักดิ์ ภัคภิญโญ[1*]

มัยโคพลาสมา กัลลิเซพติกุม (เอ็มจี) เป็นหนึ่งในเชื้อที่ก่อความสูญเสียทางเศรษฐกิจของอุตสาหกรรมการเลี้ยงสัตว์ปีกทั่วโลก การจำแนกลักษณะทางโมเลกุลเป็นวิธีที่มีประสิทธิภาพในการศึกษาความสัมพันธ์ระหว่างสายพันธุ์ของเชื้อประเทศต่างๆและระบาดวิทยาของการแพร่เชื้อเอ็มจี การศึกษาครั้งนี้ใช้เชื้อเอ็มจีของประเทศไทยจำนวน 17 สายพันธุ์นำมาจำแนกลักษณะทางโมเลกุลด้วย 2 วิธีคือ แรนดอมแอมพิฟายด์โพลีมอร์ฟิกดีเอ็นเอ (อาร์เอพีดี) และจีนทาร์เกตซีเควนซิง (จีทีเอส) ผลพบว่าวิธีอาร์เอพีดีสามารถจำแนกเชื้อได้เป็น 3 รูปแบบ เชื้อเอ็มจีที่พบส่วนใหญ่ที่มาจากพื้นที่เดียวกันจะมีรูปแบบเดียวกัน บางส่วนของจีน*เอ็มจีซี2* ถูกนำมาใช้แยกความแตกต่างระหว่างสายพันธุ์ของเชื้อเอ็มจีของไทย และสายพันธุ์ของเชื้อเอ็มจีของประเทศต่างๆ จากการศึกษาแผนภูมิวิวัฒนาการจากลำดับนิวคลิโอไทด์บางส่วนของจีน*เอ็มจีซี2* แสดงให้เห็นว่าสายพันธุ์ของเชื้อเอ็มจีของไทยจำนวน 11 สายพันธุ์ มีความเหมือน 100% กับสายพันธุ์ของเชื้อเอ็มจีของประเทศอินเดีย และมี 1 สายพันธุ์ที่เหมือนกับสายพันธุ์ของประเทศอิสราเอล วิธีการวิเคราะห์บางส่วนของจีน*เอ็มจีซี2* สามารถจำแนกสายพันธุ์เอ็มจีของไทยเป็น 4 กลุ่มขณะที่วิธีอาร์เอพีดีสามารถจำแนกสายพันธุ์เอ็มจีของไทยเป็น 3 รูปแบบ ผลการศึกษาแสดงข้อมูลที่น่าสนใจเกี่ยวกับความสัมพันธ์ของสายพันธุ์เอ็มจีของไทยและของประเทศอื่นๆ วิธีจีทีเอสอาจเป็นทางเลือกหนึ่งในการเฝ้าระวังการระบาดของเชื้อเอ็มจีในประเทศไทย

คำสำคัญ: วิธีจีนทาร์เกตซีเควนซิง (จีทีเอส) จีน*เอ็มจีซี2* มัยโคพลาสมา กัลลิเซพติกุม วิธีแรนดอมแอมพิฟายด์โพลีมอร์ฟิกดีเอ็นเอ (อาร์เอพีดี) ประเทศไทย

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