Original Article

Development of Semi-nested PCR for Detection of 16S rRNA Gene of *Mycoplasma hyosynoviae*

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Abstract

In Thailand, some breeders in pig farms have had osteoarthritis lesions for which *M. hyosynoviae* might be one of the causative agents. Therefore, the semi-nested polymerase chain reaction assay was developed for detection of *Mycoplasma hyosynoviae* in organs of pigs in Thailand using three oligonucleotide primers specific to 16S rRNA gene of *M. hyosynoviae*. The detection limit of purified DNA was 10 femtogram per reaction and of the simulated lung sample was 10^3 CFU per gram of sample. Preliminary study for tonsil-carrier state of *M. hyosynoviae* in slaughtered pigs revealed the presence of *M. hyosynoviae* in 5 out of 10 farms. Thus, the semi-nested PCR is a useful tool for presumptive screening of *M. hyosynoviae* presenting in pig herds.

Keywords: *Mycoplasma hyosynoviae*, pig, semi-nested PCR

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บทคัดย่อ

การพัฒนาปฏิกิริยาลูกโซ่โพลิเมอเรสแบบเซมิเนสเต็ดสำหรับตรวจหา 16S rRNA จีนของเชื้อมัยโคพลาสมา ไฮโอซินโนวิเอ้ จากสุกร

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ในประเทศไทยพบว่าในฟาร์มสุกรพันธุ์มีสุกรที่แสดงอาการข้ออักเสบโดยที่เชื้อมัยโคพลาสมา ไฮโอซินโนวิเอ้ อาจเป็นสาเหตุหนึ่งของการเกิดโรค ดังนั้นปฏิกิริยาลูกโซ่โพลิเมอเรสแบบเซมิเนสเต็ด จึงได้รับการพัฒนาขึ้นเพื่อใช้สำหรับตรวจหาเชื้อมัยโคพลาสมา ไฮโอซินโนวิเอ้ ในอวัยวะของสุกรในประเทศไทย โดยการใช้ไพรเมอร์ 3 เส้นที่จำเพาะต่อจีน 16S rRNA ของเชื้อชนิดนี้ เทคนิคนี้สามารถตรวจพบดีเอ็นเอของเชื้อในปฏิกิริยาได้ตั้งแต่ 10 เฟมโตแกรม และสามารถตรวจพบผลบวกในตัวอย่างอวัยวะปอดของสุกรที่มีเชื้ออยู่ได้ตั้งแต่ 10^3 CFU ต่อตัวอย่าง 1 กรัม การศึกษาเบื้องต้นโดยการตรวจหาดีเอ็นเอของเชื้อมัยโคพลาสมา ไฮโอซินโนวิเอ้ จากตัวอย่างทอนซิลสุกรที่เก็บจากโรงฆ่าสัตว์ พบว่ามีสุกรติดเชื้อจากฟาร์มต่างๆ จำนวน 5 ฟาร์มจากทั้งหมด 10 ฟาร์ม แสดงให้เห็นว่าสามารถนำเทคนิคนี้ไปใช้ในการคัดกรองเบื้องต้นการติดเชื้อมัยโคพลาสมา ไฮโอซินโนวิเอ้ ในฟาร์มสุกร

คำสำคัญ: มัยโคพลาสมา ไฮโอซินโนวิเอ้ สุกร ปฏิกิริยาลูกโซ่โพลีเมอเรสแบบเซมิเนสเต็ด

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Introduction

Mycoplasma hyosynoviae (*M. hyosynoviae*), a non-cell wall prokaryote, belongs to the class Mollicutes. Family Mycoplasmataceae is one of the porcine mycoplasma pathogens and appears world-wide (Kobisch and Friis, 1996). At present, arthritis caused by *M. hyosynoviae* becomes an increasing problem in many countries (Neilsen et al., 2001; Assuncao et al., 2005; Schultz et al., 2012). *M. hyosynoviae* is the host specific bacterium in pigs and commonly inhabits upper respiratory tract such as nasal cavity, pharynx and tonsil of convalescent and adult animals. Adult pigs are important reservoirs of infection to piglets by direct contact. Therefore, pigs may occasionally develop pneumonic lesion and arthritis (Hagedorn-Olsen et al., 1999). *M. hyosynoviae* is an arginine metabolizing mycoplasma, which can be cultivated in Hayflick's medium supplemented with mucin and arginine. Primary isolation from tissues of pig is often complicated because of overgrowth of *M. hyorhinis* as well as other bacteria (Friis et al, 1991). Therefore, it is difficult to detect *M. hyosynoviae* by cultivation.

Enzootic pneumonia and porcine respiratory disease complex remain the major problems in swine production in Thailand. *M. hyopneumoniae* has been isolated from infected pigs since 1986 (Saitanu et al., 1989). In addition, *M. hyorhinis* causing pneumonia, polyserositis and arthritis in fattening pigs was also reported (Thongkamkoon et al., 2008). Although certain breeders have had osteoarthritis lesions similar to *M. hyosynoviae* arthritis, there has been no report confirming the detection of this bacterium. However, *M. hyosynoviae* has been a common pathogen causing acute and severe lameness in grower-finisher pigs in Denmark. Time consumed for the surveillance and treatment of the disease was 30-90 min per 1000 pigs daily (Nielsen et al., 2001). Among pathogenic porcine mycoplasmas, *M. hyopneumoniae* is known as the most important mycoplasma that causes enzootic pneumonia and economic losses in pig industry. Therefore, there have been several techniques developed for specific and rapid detection and identification including PCR based method with high sensitivity such as nested PCR and real-time PCR (Stark et al., 1998; Calsamiglia et al., 1999; Kurth et al., 2002; Dubosson et al., 2004). Although the 16S-23S intergenic spacer PCR was established for differentiation of the porcine mycoplasmas in the culture medium, it was not evaluated to be used in the clinical samples (Nathues et al., 2011). A few PCR protocols for identification of *M. hyosynoviae* in clinical samples have been developed. The assays had limit of detection about at least 10^4 CFU of the organism per gram of lung tissue

(Ahrens et al., 1996; Kobayashi et al., 1996).

In this study we developed a semi-nested PCR assay with improving limit of detection targeted to 16S rRNA gene for detection of *M. hyosynoviae* from tissues of pigs. This assay is a useful tool for demonstrating the presence of *M. hyosynoviae* in pig farms in Thailand.

Materials and Methods

Bacterial strains and growth conditions: The mycoplasmas and other bacteria used in this study are listed in Table 1. Mycoplasma type strains including *M. hyopneumoniae* strain J, *M. hyorhinis* BTS7 and *M. hyosynoviae* S16 were obtained from National Institute of Animal Health (NIAH), Japan. *M. flocculare*, *Arcanobacterium pyogenes* and *Escherichia coli* were purchased from American Type Culture Collection. The other bacteria were the local isolates derived from naturally infected pigs and collected in our culture collection.

M. hyopneumoniae was inoculated in BHL broth. *M. hyorhinis*, *M. arginini* and *M. bovigenitalium* were inoculated in Hayflick's broth, whereas *M. hyosynoviae* was inoculated in mucin and arginine supplemented Hayflick's broth. *Actinobacillus pleuropneumoniae* and *Hemophilus parasuis* were inoculated on chocolate blood agar and the other bacteria were inoculated on 5% sheep blood agar. The incubation times were 3-5 days for mycoplasma strains and 18-24 hours for bacteria strains. Mycoplasma cells were collected in 1.5 ml microtube by centrifugation at 13,000 rpm for 10 min. The pellets were washed one time with phosphate buffered saline and followed by DNA preparation step.

DNA preparation: All mycoplasmas and bacterial DNA were prepared using Instagene (Bio-Rad) following the manufacturer's protocol. After the cell lysate was centrifuged at the end of the process, the supernatant was collected and kept as DNA template at -20^{0}C until used. For *M. hyosynoviae* S16, the concentrations of DNA were determined by spectrophotometer (Nanodrop® ND-1000, Nanodrop Technology, USA). The DNA was then diluted to 1 ng/µl and serially ten-fold diluted to 10^{-6} fold and used to test for the limit of detection of this semi-nested PCR assay.

Selection of primers and PCR reactions: The specific forward primer was selected based on the alignments of 16S rRNA gene of *M. hyosynoviae* (Genbank accession No U26730) with other porcine mycoplasmas including *M. hyopneumoniae* (E02783), *M. flocculare* (X63377), *M. hyorhinis* (M24658), *M. arginini* (NR041743) and *M. hyopharyngis* (U58997). The outer specific reverse primer was designed by Kobayashi et al. (1996). The inner specific reverse primer was also selected from the multi-alignments of 16S rRNA genes as well. Nucleotide sequences of the primers were MHS_ F (A): 5' GAA GCG TTT GCT TCA CTA AGA GAT 3' (nt 196-219), MHS_R (B1): 5' TTA GCT GCG TCA GTG ATT GG 3' (nt 825-844) and MHS_R (B2): 5' GCT TTC TAA CAA GGT ACC GTC AGT 3' (nt 468-491).

The PCR was carried out in 0.2 ml tube in a reaction volume of 20 µl. All PCR mixture contained 1x PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% TritonX-100), 1.5 mM $MgCl_2$, 200 µM each of dATP, dTTP, dCTP and dGTP; 0.5 U Taq polymerase (HotstarTaq, Qiagen, Germany), and 0.5 µM of each forward and reverse primers. Then, DNase/RNase-free water was added up to 18 µl. For the first round of the semi-nested PCR. Primer A and B1 were used and 2 µl of DNA sample was added in each reaction. The amplification performed in Thermal cycler (Hybaid, Thermo electron, Germany) with an initial denaturation at 95^{0}C for 15 min, followed by 35 cycles of 94^{0}C for 1 min, 59^{0}C for 1 min and 72^{0}C for 1 min, then a final extension at 72^{0}C for 5 min. For the second round of the semi-nested PCR, primer A and B2 were used. 0.5 µl of PCR product from the first round of the semi-nested PCR was used as a DNA template and 1.5 µl DNase/RNase-free water was added in each reaction. The amplification performed in the same condition as the first PCR, but the amplification ended after cycle 25. The PCR products from both amplifications were analyzed by electrophoresis through 1.5% agarose gels containing 0.1 µg/ml ethidium bromide. The gels were run at 100 volts for 30 min and visualized by ultraviolet light in gel documentation (GelDoc It UVP, USA).

Specificity: The specificity of the primers was examined by amplification of these primers with the other porcine mycoplasmas and bacterial strains (Table 1) that are associated with pneumonic lesion and arthritis in pigs to prove the absence of cross reaction of the primers to those samples.

Limit of detection: Purified *M. hyosynoviae* DNA: The serial dilution of *M. hyosynoviae* S16 DNA starting from 1 ng/µl were tested by semi-nested PCR. The minimum concentrations showing positive result in the semi-nested PCR were noted.

Simulated lung samples: Lung collected from mycoplasma free pig was homogenized and put into a bag approximately 1 gram per bag. Five ml of phosphate buffered saline, was added to make a lung suspension and 10-fold serial dilution of *M. hyosynoviae* S16 from 10^7 CFU to 10 CFU was added into each bag. Afterwards, the DNA of simulated lung samples was extracted as described by Kobayashi et al. (1996). Then, semi-nested PCR was performed to determine the minimum number of mycoplasma cells in simulated lung sample that showed positive result.

Detection of M. hyosynoviae from the slaughtered pigs: Thirty tonsil samples per farm were collected from the slaughtered pigs from 10 farms and tested for *M. hyosynoviae* by semi-nested PCR as described above as well as by cultivation following to the modified method from Friis et al. (1991). The presence of *M. hyosynoviae* by each method was recorded.

Results

The expected sizes of the PCR products from the first and the second round of the semi-nested PCR were 649 bp and 295 bp, respectively, because the primers were designed based on 16S rRNA gene of *M.*

hyosynoviae accession number U26730 from the following positions. The position of a forward primer was at 196nt to 219nt whereas the positions of an outer reverse primer and an inner reverse primer were at 825nt to 844nt and 468nt to 491nt, respectively. Using the adequate concentration of the *M. hyosynoviae* DNA, the first round of semi-nested PCR could generate an amplified fragment about 649 bp followed by an amplified fragment about 295 bp for the second round of semi-nested PCR as shown in Fig 1.

The first and the second round of semi-nested PCR were examined for their specificity with porcine mycoplasmas and other bacterial species commonly causing pneumonia and/or arthritis in pig as listed in Table 1. None of the primer pairs yielded PCR products or non specific bands with DNA from the other mycoplasmas and bacteria species. The semi-nested PCR could detect as little as 10 fg of purified *M. hyosynoviae* DNA in a reaction (Fig 2). However, the semi-nested PCR showed positive result with the lung containing *M. hyosynoviae* DNA sample from 10^7-10^3 CFU/g (Fig 3). Therefore, the limit of detection of the semi-nested PCR assay is at least 10^3 CFU/g of sample.

Using the semi-nested PCR, *M. hyosynoviae* DNA was detected in 5 out of 10 farms with an infection rate at 15% or 45 out of 300 tonsil samples (Fig 4). However, the infection rate by cultivation was 20% farms (2/10) and 5.7% individuals (17/300). Farms where *M. hyosynoviae* was isolated showed high rate of infection at 47% (14/30) and 70% (21/30) by semi-nested PCR.

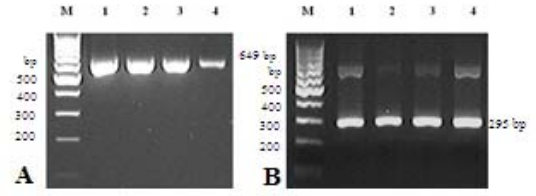

Figure 1 Oligonucleotide primers specifically amplified 16S rRNA gene of *M. hyosynoviae* and showed the PCR products at the predicted sizes of 649 bp (A) and 295 bp (B) with the primers A&B1 and A&B2, respectively. Lane M, 100 bp DNA marker; Lane 1-4, DNA of *M. hyosynoviae* strain S16

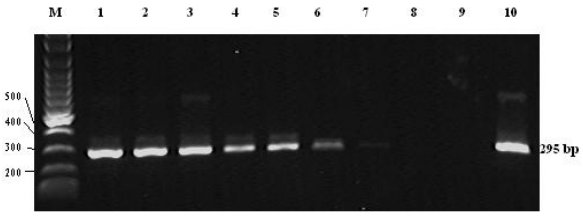

Figure 2 Detection limit of the semi-nested PCR procedure in detecting 10 fold dilution of 1 ng of extracted genomic DNA of *M. hyosynoviae* (M. hs) from 1 ng to 0.1 fg, Lane M, 100 bp DNA marker; Lane 1 through 8, 1 ng of M. hs DNA to 0.1 fg of M. hs DNA, respectively; Lane 9, DW (negative control); Lane 10, M. hs DNA (positive control)

Table 1 Microorganisms used in this study

Microorganism	source
Mycoplasma hyosynoviae S16	NIAH, Japan
Mycoplasma hyopneumoniae J	NIAH, Japan
Mycoplasma hyopneumoniae 1-10	Local isolate
Mycoplasma hyorhinis BTS 7	NIAH, Japan
Mycoplasma hyorhinis 1-10	Local isolate
Mycoplasma flocculare ATCC 27399	ATCC, USA
Mycoplasma arginini PG 230	NIAH, Japan
Mycoplasma bovigenitalium PG 11	NIAH, Japan
Arcanobacterium pyogenes ATCC 49698	ATCC, USA
Actinobacillus pleuropneumoniae	Local isolate
Bordetella bronchiseptica	Local isolate
Escherichia coli ATCC 35150	ATCC, USA
Hemophilus parasuis	Local isolate
Klebsiella pneumoniae	Local isolate
Pasteurella multocida	Local isolate
Staphylococcus aureus	Local isolate
Streptococcus suis	Local isolate

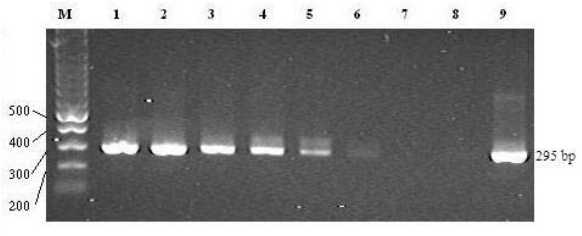

Figure 3 Detection limit of the semi-nested PCR procedure in detecting extracted genomic DNA of *M. hyosynoviae* (M. hs) from simulated lung with 10^{7-10} CFU/g, Lane M, 100 bp DNA marker; Lane 1 through 7, DNA from simulated lung with M. hs 10^7 CFU to 10 CFU, respectively; Lane 8, DW (negative control); Lane 9, M. hs DNA (positive control)

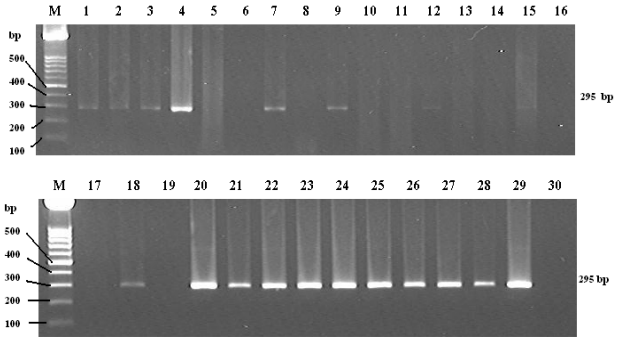

Figure 4 PCR products from homogenate tonsils for detection of *M. hyosynoviae* (M. hs) in clinical specimens. Lane M, 100 bp DNA marker; Lane 1 through 28, DNA extracted of tonsil samples from pigs; Lane 29, M. hs S16 DNA (positive control); Lane 30, DW (negative control)

Discussion

To our knowledge, there has been limited simplex PCR assays for detection of *M. hyosynoviae* in clinical samples with a limit of detection at 10^4 CFU/g of sample (Ahrens et al., 1996; Kobayashi et al., 1996). In this study, we developed the semi-nested PCR with a lower limit of detection at 10^3 CFU/g. This might be of benefit to laboratory workers in detecting *M. hyosynoviae* infection in various clinical samples using the higher sensitivity PCR assay.

The oligonucleotide primers used in this study were designed based on the multi-alignment of 16S rRNA genes of both pathogenic porcine mycoplasmas and rarely isolated non-pathogenic porcine mycoplasmas to select species specific primers for *M. hyosynoviae*. *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* are genetically related and belonged to *M. neurolyticum* cluster (Johansson and Petersson, 2002), so that the nucleotide sequences of selected primers completely differed from those mycoplasmas. *M. hyopharyngis* is non-pathogenic and occasionally isolated from pigs. Like *M. hyosynoviae*, *M. hyopharyngis* hydrolyzes arginine and produces film and spot on the agar medium. It is likely to get confused between these two mycoplasmas by some workers. *M. hyopharyngis* belongs to the *M. lipophilum* cluster (Pettersson et al., 2001), whereas *M. hyosynoviae* belongs to the *M. hominis* cluster (Johansson and Petersson, 2002). However, we found that the nucleotide sequences of the primers partially matched the 16S rRNA gene of *M. hyopharyngis*. Moreover, the observation by BLAST indicated that the forward primer completely (100%) matched the 16S rRNA sequences of *M. baccule*, a close genetic relative of *M. hyosynoviae*, while the other two primers were partially matched. Although *M. buccule* is rarely isolated from pig, the semi-nested PCR shall not produce a non-specific amplified product with this mycoplasma when using the appropriate annealing temperature.

Preliminary study of the detection of *M. hyosynoviae* infection in tonsil samples of pigs from 10 farms by semi-nested PCR and cultivation revealed the presence of *M. hyosynoviae* in pig farms in Thailand. The selected farms were located in areas of intensive pig farming in the North, Northeastern, Eastern and the central part of Thailand. Some farms had a history of *M. hyopneumoniae* and *M. hyorhinis* infection in the fattening pigs, however, the clinical impact was not determined. The use of semi-nested PCR yielded a higher infection rate than culture method. Hence, PCR assay seemed to be an effective method for diagnosis of *M. hyosynoviae* (Strait et al., 2006). Isolation of *M. hyosynoviae* succeeded in only 2 farms that showed a high rate of infection by semi-nested PCR might reflect high persistent of *M. hyosynoviae* and might be due to recent outbreak of arthritis (Friis et al., 1991). On the other hand, although *M. hyosynoviae* detected in tonsil demonstrated the presence of *M. hyosynoviae* in a pig herd, the spread of bacteria to the lung or joint that results in pneumonic lesion or arthritis might vary due to several factors such as age, immunity, infection pressure and stress (Hagedorn-Olsen et al., 1999; Nielsen et al., 2005). Therefore, the occurrence of pneumonia, arthritis and lameness must be further investigated in the semi- nested PCR positive farm and the samples from target organs should be taken for diagnosis to prove the infection level and clinical impact in the farm.

In conclusion, the semi-nested PCR was developed and is a useful tool for presumptive screening the presence of *M. hyosynoviae* in pig herds in Thailand. Further study for the prevalence and incidence of the disease as well as a genetic diversity of *M. hyosynoviae* field isolates should be carried out.

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