Differentiation of Strains and Phylogenetic Analysis of Thai PRRSV Strains based on ORF7 sequence.

ABSTRACT

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is a major problem faced by pork producers in Thailand. In order to properly solve PRRS problems in the field, a rapid diagnostic method is required for the differentiation of North American and European isolates of the PRRSV. This paper reports on a Reverse Transcription-nested PCR (RT-nPCR) method for the detection and differentiation of PRRSV strains based on the ORF7 sequence.
viral open-reading frame 7 (ORF7) gene sequences in serum from 300 PRRS-infected pigs. The results of the PRRSV RT-nPCR assay show that this method is highly sensitive and specific for the detection of North American and European PRRSV strains. The RT-nPCR of North American and European strains showed the PCR products of 287 and 257 bps, respectively. In addition, phylogenetic analysis on the Thai isolates of PRRSV in the present study shows the existence of both North American (95%) and European (98%) genotype. Moreover, the phylogenetic study also confirmed the specificity of RT-nPCR for the detection and differentiation of PRRSV strain in Thailand.

**Key Words:** PRRSV, North American and European strains, Nested PCR assay, Phylogenetic trees

**INTRODUCTION**

Porcine Reproductive and Respiratory Syndrome (PRRS) is characterized by reproductive failure of sows and respiratory problems of piglets and growing pigs. PRRS occurs in most major pig-producing areas throughout the world. The reproductive failure is characterized by infertility, late fetal mummification, abortions, stillbirths, and the birth of weak piglets that often die soon after birth from respiratory disease and secondary infections. Older pigs may demonstrate mild signs of respiratory disease, sometimes complicated by secondary infections. Animals other than pigs do not seem to be affected by PRRS (OIE, 2004). PRRS virus (PRRSV) has a genome of approximately 15 kb containing 7 open reading frames (ORFs) (Meulenberg, 2000). PRRSV isolated from North America and Europe were difference genetically. It has been demonstrated that a high genetic diversity exists among isolates belonging to the North American type of PRRSV (Goldberg et al., 2000; Forsberg et al., 2002). However, the average genetic diversity of European type of PRRSV has
been found to be much lower (Le Gall et al., 1998). Several PCR assays have been developed for the detection of PRRSV (Suarez et al., 1994; Van Woensel et al., 1994; Christopher-Hennings et al., 1995). Most of these methods used primers designed to amplify nucleocapsid protein (ORF7). The fact that their assays were less efficiently amplification from a very small amount of virus. In this study, we designed the specific primers based on strains of PRRSV circulating in other countries for the detection and differentiation of the strain of PRRSV in Thailand. We also studied the genetic variation of the nucleocapsid gene and compared the nucleotide and amino acid sequences with those strains of PRRSV originated in other areas.

**MATERIALS AND METHODS**

**PRRSV isolates**

Three hundred swine serums collected from the different areas of Thailand were used for RNA extraction. The RNA templates were aliquoted in microcentrifuge tubes and stored at −80°C until further studies. As a positive PCR control for the North American or European PRRSV isolates, the PRRS vaccines, Ingelvac PRRS MLV (Boehringer Ingelheim Vetmedica, USA) and European Amervac (Hipra, Spain) were used, respectively.

**Primers**

The oligonucleotide sequences and positions of the primers specific to PRRSV genome for the RT-PCR and nested-PCR steps were shown in the Table 1. These primers were designed according to the VR-2332 (accession no.AY150564), IAF- EX91 (accession no.L40898), HN1 (accession no.AY457635), IAF-Klop (accession no. U64928), NVSL-14 (accession no.AF396841), NADC-9 (accession no.AF396838), 01NP1.2 (accession no.DQ056373), HB-2(sh)/2002 (accession no.AY262352), LV4.2.1 (accession no. AY588319), AGS-96 (accession no.AF512378), DV (accession no. AF511526) and NL2.2 (accession no.Z92533) isolates of PRRSV.

**RNA Extraction and One-step Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Viral RNA was extracted using acid phenol-guanidinium thiocyanate-chloroform extraction. The RNA templates were subjected to reverse transcription polymerase chain reaction protocol (RT-PCR) technique using C-F and C-R primers and SuperScript™ III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, USA) according to the manufacturer instruction. The RT-PCR step was 1 cycle of cDNA synthesis at 45°C for 1 hour and 1 cycle of pre-denaturation at 94°C for 7 minutes and 35 cycles of PCR amplification at 94°C for 45 seconds, 50°C for 1 minute, 72°C for 1 minute and 1 cycle of final extension at 72°C for 10 minutes.
Differentiation Nested Polymerase Chain Reaction (Nested-PCR)

The PCR products from the RT-PCR step were used as the template for the nested-PCR step. The nested PCR was performed by using 2 pairs of the specific primers which were specific for the European strain (EU-F and EU-R) and the North American strain (US-F and US-R) (Table 1). The PCR mixture was made up of 100 µl of RT-PCR products, 1.25 mM of MgCl₂, 1xbuffer, 0.2 mM of the dNTPs, 1 U of Taq DNA polymerase, 100 pmol of the primer of each segment. The nested PCR cycle was 1 cycle of pre-denaturation at 94°C for 5 minutes and 35 cycles of PCR amplification at 94°C for 45 seconds, 52°C (European strain) or 56°C (North American strain) for 1 minute, 72°C for 1 minute and 1 cycle of final extension at 72°C for 15 minutes. The amplified nested PCR products were analyzed by electrophoresing 10 µl aliquots through 1.5% agarose gels in TAE buffer (0.04M Tris-acetate [pH8.5], 0.002 M EDTA) for approximately 30 minutes at 100 Volts and gels were stained with ethidium bromide. The electrophoresis results were visualized by gel documentation system.

**Nested-PCR amplification for cloning**

Ten PCR products of each the European and North American strains from the RT-PCR step were used as the templates for another nested-PCR amplification. Two pairs of the primers were designed specifically for the ORF7 gene. The forward primer of the European strain was 5’ ATGGCCGGTGAAAAACCGAG 3’ and the reverse primer was 5’ TTAACCTGACCCCTGACTGG 3’. The forward primer for the North American strain was 5’ ATGCACAAATAAACCAGGCA 3’ and the reverse primer was 5’ TCATGCTGAGGGTGATGC 3’.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Position in genome</th>
<th>Size of PCR product (bps)</th>
<th>Type detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>External for the One-Step RT-PCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-F: TGTAAACGGGGAGTT</td>
<td>EU;14566 – 15022</td>
<td>EU; 456</td>
<td>common</td>
</tr>
<tr>
<td>C-R: TTGAATAGGTGACTTAGAGGC</td>
<td>US;14856 – 15356</td>
<td>US; 500</td>
<td></td>
</tr>
</tbody>
</table>

| Internal for the nested PCR | | | |
| EU-F: AAAGAAAAGTACAGCTCCA | 14628 – 14884 | 257 | European |
| EU-R: CTGGATGAAACGCGACGCA | | | |

| US-R: CATCATGCTGAGGGTGATGC | | | |

**Table 1** Oligonucleotide primers for the RT-PCR and the nested-PCR steps for the detection and typing of the strain of PRRS virus
PCR mixture was made up of 10 μl of RT-PCR products, 1.25 mM of MgCl₂, 1x buffer, 0.2 mM of the dNTPs, 1 U of Taq DNA polymerase, 100 pmol of the primer of each segment. The PCR cycle was 1 cycle of pre-denaturation at 94°C for 5 minutes and 35 cycles of PCR amplification at 94°C for 45 seconds, 55°C (European strain) or 51°C (North American strain) for 1 minute, 72°C for 1 minute and 1 cycle of final extension at 72°C for 15 minutes. The purified PCR products were cloned into pGEM-T Easy vector (Promega, USA) and the recombinant plasmids were use to transform DH5α competent cells. The positive clones were identified by PCR and restriction endonuclease digestion. The correct recombinant plasmids were subjected to DNA sequencing.

Sequence alignments and phylogenetic analysis

The nucleotide and deduced amino acid sequences were aligned using the multiple alignment program CLUSTALW and compared with the reported sequences in Genbank [LV4.2.1. (European strain prototype) (accession no. AY588319), NL2.2 (accession no.Z92533), DV (accession no. AF511526), AGS-96 (accession no. AF512378), VR-2332 (North American strain prototype) (accession no.AY150564), HB-(sh)/2002 (accession no.AY262352), O1NP1.2 (accession no.DQ056373), NVSL-14 (accession no.AF396841), IAF-EX91 (accession no.L40898), IAF-Klop (accession no. U64928), LDV (accession no. NC001639), EAV (accession no.NC002532) and SHFV (accession no.AF180391)]. The boot strap option was carried out on 1000 pseudoreplicate data sets to assess the robustness of interior branches of the tree. Phylogenetic analysis of the same alignment was also performed by the maximum parsimony method using the PHYLIP package (Felsenstein, 1989).

RESULTS

Nested RT-PCR for the detection of North American and European PRRSV strains

The optimum PCR products were achieved by using 1.25 mM MgCl₂, annealing temperature of 50°C for the RT-PCR step and 53°C or 58°C for the Nested PCR step of the European and North American strains, respectively. The optimum time for annealing and extension was 1 minute. The PCR products of the European and the North American strains were 257 and 287 bps, respectively (Figure 1A and 1B). The North American strain gave the negative results by primers EU-F and EU-R. On the other hand, the European strain also gave the negative results by primers US-F and US-R.

Comparison of PRRSV ORF7 sequences

The sequence comparison of ORF7 of both strains showed homology 98% between the European strains, 95% between the North American strains The nucleotide and deduced amino acid sequences of the nucleoprotein gene of PRRSV were aligned (Figure 2A and 2B). The pairwise
The distances between all Thailand isolates of PRRS viruses was more related to LDV than either SHFV or EAV. Moreover, Thailand isolates of the North American strain was more related to LDV than European strain (Figure 2). For Thailand isolates of European strain, the nucleotide sequences were more related to European strain of vaccine virus than wild types (LV4.2.1, DV, NL2.2 and AGS-96 strains) (Figure 2A). The amino acid sequences of the European strains isolated form the Northern

Figure 1 The PCR products of the European using primers EU-F and EU-R (A) and the North American strains using primers US-F and US-R (B) by 1.5% agarose gel electrophoresis. (A) The Nested PCR for the European strain gave 257 bps products for lanes 2, 3, 4, 6 and 11. The North American strain in Lane 13 gave the negative result by using primers EU-F and EU-R. (B) The Nested PCR for the North American strain lanes gave 287 bps products for lanes 3, 4, 5, 8 and 9. The European strain in Lane 12 gave the negative result by using primers US-F and US-R. Negative samples were not shown the products of the both strains. Lane1 = 100 bp marker; Lane 2-11 = samples (samples in panel A were not the same sample in panel B), Lane12 = positive control for the European strain, Lane13, = positive control for the North American strain and Lane14 = negative control.
and Western parts of Thailand were related to LV4.2.1, DV and NL2.2. However, the European strains isolated from other parts of Thailand were more related to European strain of vaccine virus (Figure 2B). For North American strain, the most sample nucleotide sequences were more related to VR-2332 strain including North American strain vaccine virus but amino acid sequences of most

Figure 2. Phylogenetic trees based on nucleotide sequence (A) and amino acid sequence (B) of the European and North American strains of PRRSV inferred from the neighbour-joining method. Numbers on the trees indicate the bootstrap values (percentage) of branches by neighbour-joining (1000 replicates) methods.
isolates including North American strain vaccine virus were related to VR-2332, O1NP1.2, HN1, NADC-9, NVSL-14, IAF-EX91 or IAF-Klop strains except the samples collected from the Eastern part of Thailand (Figure 2A and 2B).

**DISCUSSION**

In this study, the differentiation nested RT-PCR technique for the detection and differentiation of the European and the North American strains of PRRSV were successfully developed. The size of PCR products of the European and North American strains in this study was 257 and 287 bps, respectively, compared with 186 and 107 bps reported previously (Gilbert et al., 1997). The main advantage of this assay described herein is the ability to type PRRS virus to one of two genotypes from the samples. The nested assay was sufficiently sensitive that PCR products could be visualized by ethidium bromide staining without the need for additional detection methodology.

By analyzing the phylogenetic relationships of nucleoprotein gene (ORF7), two strains of PRRSV (European and North American strains) were identified in Thailand. The Northern and Western Thailand isolates of European strains were more related to wild type virus than those viruses isolated from other parts of Thailand which more related to European strain of vaccine. However, all Thailand isolates of North American strains were related to VR-2332 and US strain of vaccine. According to these results, most PRRSVs circulated in Thailand might be originated from the European and Northern American vaccine strains. These results were in agreement with the previous studies in Thailand and Denmark which also showed that the most field isolates of the European strain were more related to European vaccine strain (Thanawongnuwech et al., 2004, Stadejek et al., 2005). These studies also stated that the virus vaccine might mutate become to be the pathogenic virus or the samples were collected at the viraemic period after vaccination (Thanawongnuwech et al., 2004, Stadejek et al., 2005). For the North American strain, the field sample sequences including North American vaccine were closely related to VR-2332. This result was similar to the previous studies which showed that the North American strain in Denmark were significantly closer to the American-type of PRRSV than the previously seen because of North American strain vaccination of PRRSV in Europe after the introduction of the North American strain vaccine into Denmark (Stadejek et al., 2002, Stadejek et al., 2005).

To summarize, we have demonstrated the sensitivity and specificity of the RT-nPCR for the identification of PRRS-positive pigs. This technique is not only useful for diagnosis and discrimination between North American and European isolates of PRRSV, but also for studies of persistent infection by the virus. In addition, genetic variation and phylogenetic relationships of local PRRSV have been revealed. These data should be taken into consideration for control and preventive measures.
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reverse Polymerase Chain Reaction (RT-PCR).  
