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PCR-RFLP ANALYSIS IN AN OPEN READING FRAME 5 OF VARIANTS OF PRRSV ISOLATED IN THAILAND

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Abstract

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PCR-RFLP ANALYSIS IN AN OPEN READING FRAME 5 OF VARIANTS OF PRRSV ISOLATED IN THAILAND

Ten selected Thai isolates of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) were amplified in an open reading frame 5 by a reverse, transcriptase, polymerase chain reaction (RT-PCR) and cut with restriction enzymes: *Mlu*I, *Hinc*II, *Sac*II and *Hae*III for the US genotype and *Pst*I, *Hae*II and *Cla*I for the EU genotype. None of the field isolates had a similar cutting pattern when compared to modified live virus vaccines, however, several cutting patterns were obtained from this study. The results suggested that genetic variation was present among the Thai isolates, even within the same genotype. The variations may be the result of the introduction of a new variant into the endemic area or by local evolution.

Keywords : Porcine Reproductive and Respiratory Syndrome Virus, restriction fragment length polymorphism, open reading frame 5

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การตัดด้วยเอนไซม์ตัดจำเพาะที่ยีนสำหรับสังเคราะห์โปรตีนที่ 5 ของไวรัสพี อาร์ อาร์ เอส ที่แยกได้ในประเทศไทย

ไวรัสพี อาร์ อาร์ เอส ที่แยกได้ในประเทศไทยจำนวน 10 ตัวอย่าง ถูกนำมาเพิ่มจำนวนที่ยีนสำหรับสังเคราะห์โปรตีนที่ 5 โดยวิธีปฏิกิริยารีเวอร์สทรานสคริปเตส ลูกโซ่โพลิเมอเรส แล้วตัดด้วยเอนไซม์ตัดจำเพาะสำหรับสายพันธุ์อเมริกา คือ *Mlu*I *Hinc*II *Sac*II *Hae*III และสายพันธุ์ยุโรป คือ *Pst*I *Hae*II *Cla*I ไม่พบรูปแบบการตัดที่เหมือนกับวัคซีนเชื้อเป็นทั้งสองสายพันธุ์ แต่พบการตัดได้หลายรูปแบบในแต่ละสายพันธุ์ สรุปได้ว่ามีการแปรผันทางพันธุกรรมของไวรัสนี้ในประเทศไทย ซึ่งอาจเกิดจากการนำสายพันธุ์ใหม่เข้ามาในพื้นที่หรือจากการวิวัฒนาการของไวรัสภายในพื้นที่

คำสำคัญ : ไวรัสพี อาร์ อาร์ เอส การตัดด้วยเอนไซม์ตัดจำเพาะ ตำแหน่งสังเคราะห์โปรตีนที่ 5

Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) is one of several major swine diseases causing economic loss in the swine industry worldwide, including in Thailand. Clinically, PRRS is characterized by reproductive failure in gilts and sows, as well as respiratory diseases in young pigs. Morever, PRRS virus (PRRSV) increased the susceptibility of the pigs to other respiratory bacterial infections (Thanawongnuwech et al., 2000; Wills et al 2000). The syndrome was first recognized in 1987 in swine herds in North Carolina, Iowa and Minnesota (Keffaber, 1989). However, the etiological agent responsible for the disease was only first isolated in the Netherlands in 1991 and was named the Lelystad virus and identified as the European genotype. Later in the United States, the prototype of the American strain (US), VR-2332 was isolated in 1992 (Collins et al., 1992). In Thailand, serological studies suggest widespread PRRSV infection among herds and the earliest detection of seropositive animals was in 1989 (Damrongwatanapokin et al., 1996). The virus was first successfully isolated from suckling and nursery piglets with a severe chronic respiratory distress in June 1995. Indirect immunofluorescent staining and reverse-transcription polymerase chain reaction (RT-PCR) tests indicated that this particular Thai isolate was more closely related to the American strain than the Lelystad virus (Damrongwatanapokin et al., 1996). Recently, the nested multiplex PCR demonstrated that both the EU and US genotypes are present in Thailand, since Thailand has continuously imported swine from both Europe and North America (Thanawongnuwech et al., 2002).

PRRSV is classified within the genus *Arterivirus*, in the family *Arteriviridae* and placed in the order Nidovirales (Cavanagh, 1997). PRRSV has a positive, single-stranded, polyadenylated, RNA molecule, approximately 15 kb in length and containing eight open reading frames (ORFs). The genome contains two large open reading frames (ORF 1a and 1b), encoding the non-structural polyproteins (viral RNA polymerase and associated protease) and other six smaller ORFs (ORF2-ORF7), encoding the structural proteins. Envelope glycoprotein E, encoded by ORF5, is a major viral glycoprotein consisting of a 25 kDa polypeptide. Both glycoprotein E and the glycoprotein encoded by ORF4 are able to induce neutralizing antibodies (Andreyev

et al., 1997). These principal envelope glycoproteins, containing a hypervariable region, are responsible for generating the diversity of PRRSV. The variability in the ectodomain of ORF5 associated with antigenic variability of the GP5 of PRRSV is the result of positive or negative neutral selection by antibodies or other host defences (Pirzadeh et al., 1998; Rowland et al., 1999). Mutations within the antigenic domains of GP5 may contribute to the establishment of a chronic form of the disease and eventually a persistent infection (Pirzadeh et al., 1998). The antigenic drifts of the hydrophilic domains of GP5 could be the result of the host's selective humoral immune response directed against the exposed domains of this envelope glycoprotein. These genome variants may be an effective mechanism for evading the host's immune surveillance.

In order to control PRRSV, a commercial modified live virus (MLV) vaccine has been used in the United States since late 1994 and has had some limited use in other countries, including Thailand. Although the vaccine virus is attenuated, it occasionally persist for at least several weeks in a vaccinated pig (Wesley et al., 1998). Furthermore, the live attenuated virus has been reported to be capable of spreading to non-vaccinated sows. The vaccine virus may change genetically or revert to virulence under field conditions (Nielsen et al., 2001). Several incidences of this occurred in Denmark (Madsen et al., 1998), in Korea (Cheon and Chae, 2000), in Japan (Itou et al., 2001) and in Canada (Cai et al., 2002). Coexistence between the vaccine virus and the field strain is possible and may induce clinical disease (Rossow et al., 1999). It is hard to distinguish a particular vaccine viruse from PRRSV field strains by any routine laboratory procedure. Recently, a Polymerase, Chain Reaction-based, Restriction Fragment Length, Polymorphism (PCR-based RFLP) analytic method has been developed in order to group the PRRSV (Wesley et al., 1998). The selected restriction enzymes are cut at the precise 4-6 position in the DNA sequence. Any mutation of the sequence would change the cutting site of the DNA resulting in different RFLP patterns. Various patterns are used for grouping the viruses. The grouping using RFLP patterns will be a valuable tool in farm management and epidemiologic studies. The RFLP may indicate a mutation of the virus or may differentiate the infected pig from the vaccinated pig (Wesley et al., 1998). However, the MLV vaccine is not yet officially allowed for use in Thailand.

The objectives of this study was to study the RFLP patterns of Thai isolates from a previous study using selected enzymes and from computer program based on available ORF5 sequences from GenBank. The PCR- based RFLP of the open reading frame 5 (ORF5) analysis may be applied for grouping the Thai isolates of PRRSV.

Materials and Methods

1. Virus isolation and viruses used

Ten Thai PRRSV isolates were obtained from Chulalongkorn University, Veterinary Diagnostic Laboratory (CU-VDL). The viruses used for this study were 01NP2, 02SP2, 02SP3, 02SP4 and 02PB1 (US genotypes) and 01CB1, 02NP2, 02RB1, 02BR1 and 03RB1 (EU genotypes), using multiplex RT-PCR for genotyping. The viruses had been isolated from swine sera or tissues submitted by farm owners and veterinarians throughout Thailand, by using a MARC-145 cell line (provided by Chris Morrisy, CSIRO, Geelong, Australia) or pulmonary alveolar macrophages (PAMs). PRRSV-free pigs were provided by the CP group, Thacham, Chonburi. Resp PRRS/Repro™ (BI, USA) was used as a control for the US genotypes while Porcillis® (Intervet, The Netherlands) was used as a control for the EU genotypes.

1.1 Preparation of MARC-145: MARC-145 seed stock, kept at -70°C, was thawed in a 37°C waterbath. The cell suspension was diluted with 5 ml of MEM (Minimal essential medium) (Hyclone, USA) and then centrifuged at 2,000 rounds per minute (rpm) for 10 mins before the supernatant was aspirated out. The cell pellet was mixed with 10% FCS (Fetal calf serum) MEM and the cell suspension was placed in a 75 mm^3 culture

bottle (Corning Incorporated, USA). The culture bottle was incubated at 37°C under 5% CO_2 and was observed until the monolayer was seen. For splitling the cells, the supernatant was aspirated from the culture bottle and the monolayer was trypsinized by trypsin versene, 1 ml at 37°C for 5 mins. The detached cells were pipetted, up and down for cell separation, with 5 ml of media.

1.2 Primary and secondary passage

Primary passage: one ml of trypsinized MARC-145 was diluted in the media until it reached 24 ml and use to fill a 24 well plate (Corning Incorporated, USA), 1 ml in each well and incubated in 5% CO_2 at 37°C until the monolayer was seen. The supernatant was aspirated and the sample was inoculated onto the monolayer. The cell line was incubated in 5% CO_2 at 37°C for 1 hr, for viral adsorption. The supernatant was aspirated and 2 ml of 2% FCS media was filled and observed everyday for the CPE and kept at -70°C until needed.

Secondary passage: the supernatant from the primary passage was inoculated onto the cell line, similarly to the primary passage. After 2 days, the cells and media were collected in an Eppendorf tube and kept at -70°C until used.

1.3 Indirect Immunoperoxidase Monolayer Assay (IPMA): IPMA was used to confirm if the PRRSV antigen was present in the cell culture. The cells in preparation for IPMA were trypsinized MARC-145 and placed in a 96-well-plate (Corning Incorporated, USA), 200 µl/well, incubated in 5% CO_2 at 37°C until the monolayer was seen. The supernatant from the secondary passage was inoculated onto the cell line and incubated for 48 hrs. The monolayer was fixed in a 4% formalin 100 µl/well, at room temperature, for 30 mins. The N protein of PRRSV was detected by mouse, monoclonal, anti-PRRSV antibody (SDOW17: South Dakota State University, USA). Fifty µl of SDOW17 (1:300) was placed in each well and incubated in 5% CO_2 at 37°C for 1 hr. Fifty 50 µl of the conjugated, anti-mouse immunoglobulin G (Dako, USA; 1:300) of the secondary antibody was added and incubated in 5% CO_2 at 37°C for 1 hr. The substrate was added (100 µl/well) at room temperature for 1 hr. The conjugated IgG reacted with the substrate and showed a dark brown granule in the cytoplasm of PRRSV infected cells, when examine under a light microscope. The viral titer (tissue culture, infective dose; $TCID_{50}$/ml) was calculated using the Reed and Muench (1938) method.

2. Restriction Fragment Length Polymorphism based on RT PCR

2.1 RNA extraction: Viral RNA was extracted from the mixture of cells and media using a QIAamp® Viral RNA Mini Kit (QIAGEN, Germany). Forty µl of the mixture was lysed with buffer AVL 560 µl, at room temperature for 10 mins. Five hundred and fifty µl of absolute ethanol was added to the solution and centrifuged at 8000 rpm for 1 min. The washing solutions (AW1 and AW2) were added and centrifuged at 8000 rpm to remove any contamination. The RNA was eluted from the membrane by adding 60 µl of buffer AVE, then centrifuged at 13000 rpm and kept at -20°C until needed.

2.2 Reverse Transcriptase-Polymerase Chain Reaction: RT-PCR was used for PRRSV RNA amplification of ORF5. The PCR mix (QIAGEN® One Step RT-PCR Kit, Germany), fifty µl in volume, contained 2 µl of dNTP Mix (each dNTP 10mM), 2 µl of QIAGEN One step RT-PCR Enzyme Mix, 5 µl of Template RNA, 19 µl of RNAse-fee water, 10 µl of 5xQ-solution, 10 µl of 5xQIAGEN One step RT-PCR Buffer, 1 µl of primer sense and 1 µl of antisense. In this study, the primers for the US isolates were P420, 5'-CCATTCTGTTGGCAA TTTGA-3' (sense) and P620, 5'-GGCATATATCATCAC TGGCG-3' (antisense) (Andreyev et al., 1997) and for the EU isolates ETS5L, 5'-GGATCCATGAGATGTTCTCA CAAATTGG-'3 (sense) and ETR5L, 5'-GGATCCCATTA GGCCTCCCATTG'-3 (antisense) (Pirzadeh et al., 1998). The PCR mix was placed in the thermoregulator PTC-200 (MJ Research, USA) and the PCR condition was modified using the following thermocycling programs: cDNA synthesis at 50°C for 30 min, inactivation of reverse transcriptase and denaturation at 95°C for 15 mins,

denaturation at 94°C for 30 sec, primer annealing at 53°C for 30 sec and primer extension at 72°C for 30 sec for 40 cycles with a final extension at 72°C for 10 mins and holding at 4°C (Andreyev et al., 1997). The PCR products were detected in a 2% agarose gel (Fisher Chemical, USA) by electrophoresis at 100 V, 1.5 A for 1 hr. The gel was stained with ethidium bromide (Promega, USA) for 30 mins then washed in tap water. The DNA band of ORF5 was visualized using a UV illuminator.

2.3 PCR product purification: The PCR products were purified by QIAquick® spin (QIAGEN, Germany). The PCR product was mixed with buffer PB at the ratio of 1:5, placed into the column provided and centrifuged at 8000 rpm. DNA (100 bp-10 kp) was adsorbed into high-salt silica membrane. Buffer PE was added (0.75 ml) and centrifuged at 8000 rpm for 1 min. The PCR product was eluted with 50 µl of RNAse free water, centrifuged at 13000 rpm for 1 min and kept at -20°C until needed.

2.4 Restriction Fragment Length Polymorphism: The purified PCR products were measured for their concentration by diluting 100 times (product 7 µl, DW 700 µl) using optical density (OD) at wavelengths of 260 and 280 nm (Spectronic 20 genesys, Germany). The purified PCR products were digested using 1 µg of the product and 1 µl of restrictive enzymes in 50 µl of the appropriate buffer at 37°C for 2 hrs (MBI Fermentas, USA). The cutting patterns were electrophoresed on 2% agarose gel, 100 V, 1.5 A for 1 hr, stained with ethidium bromide (Promega, USA) and visualized using the UV illuminator.

Results

Five Thai isolates (01NP2, 02SP2, 02SP3, 02SP4 and 02PB1) of the US genotype were amplified by RT-PCR and cut with *Mlu*I, *Hinc*II, *Sac*II and *Hae*III as described by Cheon and Chae (2000) (Fig.1). Each isolate had a numeric code for its ORF5 RFLP pattern after being treated with the selected enzymes: *Mlu*I, *Hinc*II, *Sac*II and *Hae*III. *Mlu*I cut (code II) only the vaccine virus. *Hinc*II cut all the samples tested, with 2 different cutting patterns. Code I yielded the products at approximately 320 and 400 bp, while code II yielded the products at approximately 250 and 500 bp. *Sac*II had no cut pattern (code I). *Hae*III had 2 cut patterns. The RFLP cutting patterns of the Thai isolates of the US genotype are shown in table 1.

Five Thai isolates (01CB1, 02NP2, 02RB1, 02BR1 and 03RB1) of the EU genotypes were amplified by RT-PCR as described by Pirzadeh et al (1998). The ORF5 products were digested by *Pst*I, *Hae*II and *Cla*I. The enzymes used were obtained from a firstmarket webcutter program. The RFLP patterns are showed in Fig. 2. Similar to the US isolates, the results were given a numeric code as follows: *Pst*I and *Cla*I had 2 cut patterns, no cut (code 1) and cut (code 2). *Hae*II had 3 different cutting patterns, no cut code 1, code 2 yielded the products at approximately 300 bp, while code 3 yielded the products at approximately 300-400 bp. The RFLP patterns are shown in table 1.

Discussion

The nested RT-PCR for PRRSV amplification was 100-1000 folds more sensitive than the regular RT-PCR (Umthun and Mengeling, 1999). However, the results from RT-PCR are not capable to determining the strain differences. A PCR-based RFLP analysis has been developed for the differentiation of PRRSV isolates (Wesley et al., 1998). RFLP analysis is useful in molecular biology for the rapid differentiation of DNA variation in many diseases. The PCR-based RFLP analysis for typing PRRSV isolates, directly from lung specimens, has been proved to be sensitive, accurate and rapid (Cheon and Chae, 2001).

Among the 5 selected Thai isolates, from both genotypes, the US genotype had 3 different cutting patterns and the EU genotype also had 3 but different cutting patterns. In the US genotype, the *Mlu*I cutting site of ORF5 in the vaccine virus was able to distinguish the MLV virus from other PRRSV field isolates. *Hinc*II could differentiate the MLV vaccine from 2 of the 5 field isolates. Only 2 isolates from Suphanburi (02SP2 and

02SP3) and 1 isolate from Nakornpathom (01NP2) had the same *Hinc*II cutting pattern as the MLV vaccine. Despite having the same *Hinc*II RFLP pattern, the Suphanburi isolates could be distinguished from the MLV vaccine virus by having different *Hae*III cutting patterns. Used together, the 3 enzymes could be used for differentiation of the US MLV vaccine from the field isolates. As in the previous study, the US MLV vaccine virus and its parent virus, VR2332 could be distinguished from other PRRSV field isolates by *Mlu*I, *Hinc*II and *Sac*II (Wesley et al., 1998). The other restriction enzymes, *Hae*III (Madsen et al., 1998) and *Msp*I (Itou et al., 2001) were added, to produce more precise cutting patterns. The MLV vaccine virus could be cut by this enzyme because only this vaccine virus has an alanine (A; GCG) at the residue 137, in a moderately conserved region of ORF5. The consensus sequence has a serine (S; TCA, TCT) at the residue 137 for all the PRRSV strains. A, G to T transversion made the 6 nucleotide recognition sequence resulting in a non cut of all the PRRSV isolates by *Mlu*I (Wesley et al., 1998). In the EU genotypes, *Pst*I and *Cla*I were able to differentiate the EU isolates from the EU MLV vaccine virus apart from the Burirum isolate (02BR1) which had the same pattern as the MLV vaccine. However, the Burirum isolate could be differentiated from the EU vaccine using *Hae*II. In conclusion, using *Pst*I, *Cla*I in combination with *Hae*II for the EU ORF5 product, the EU MLV vaccine could be distinguished from the EU isolates.

In this study, we did not see any field isolates with the same cutting pattern as the MLV vaccines either in the US or the EU isolates. In contrast to previous reports from other countries, the field isolates had RFLP cutting patterns similar to the US vaccine in Korea, Japan and Canada (Cheon and Chae, 2000; Itou et al., 2001; Cai et al., 2002). Those countries had been using US MLV vaccines for a few years before the investigation. The MLV vaccine used in those countries might have reverted to a more virulent strain (Nielsen et al., 2001). Fortunately, Thailand does not currently allow the use of MLV vaccines. Only a killed PRRSV vaccine is available. In this situation, to distinguish the PRRSV vaccine strain from other PRRSV field isolates, it is necessary to have series of restriction enzymes in order to obtain more specific RFLP patterns. Alternatively, nucleotide sequencing would be a better way to differentiate wild type PRRSV from the vaccine virus (Cheon and Chae, 2000). However, the number of PRRSV isolates used in this study was limited. More recent field isolates need to be studied for better results, if the MLV vaccine virus was found to be present in Thailand.

Our results were able to demonstrate the different cutting patterns of the selected Thai PRRSV isolates. The cutting patterns of the ORF5 were able to divide the PRRSV isolates, suggesting the presence of genetic variation in each genotype of PRRSV isolated in Thailand. ORF5 is suitable for RFLP study for genetic variation since the antibodies neutralize PRRSV in this position (Pirzadeh et al., 1998; Rowland et al., 1999). This variability frequently affects genes encoding for the more exposed parts of the virion (Martelli et al., 2003). If it can invade the host immune system, any particular virus may have a greater potential to be shed and infect other pigs, so establishing persistent infections. The differences in the cutting patterns are probably caused by the introduction of a new variant into the area or by local evolution. When finding different variants, at the same time or in the same areas, the genetic variability is possibly caused by the introduction of PRRSV-infected animals or from using contaminated semen, rather than by the local evolution (Martelli et al., 2003). However, the virus is probably maintained in the sows and can undergo gradual evolution, driven by several factors, including recombination, random mutation or natural selection. Regarding interstrain recombination, a cell has to be infected simultaneously, or almost so, with at least 2 strains of PRRSV (Mengeling, 2002). It should be noted that the RFLP study was not able to confirm the presence of the recombination. However, based on our ORF5 sequencing results, no evidence of recombination

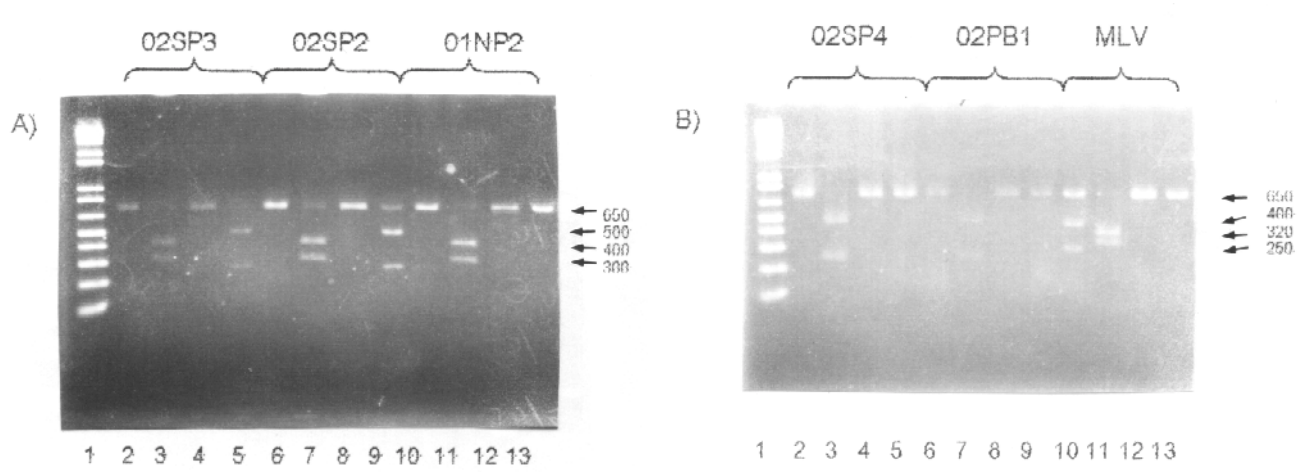

Figure 1: The RFLP patterns of the ORF5 of the Thai isolates (US genotype) and the US-MLV vaccine. ORF5-PCR products were treated with 4 restriction endonuclease enzymes. A) Lane 1, 100 bp ladder; Lane 2, 6, 10 (*Mlu*I); Lane 3, 7, 11 (*Hinc*II); Lane 4, 8, 12 (*Sac*II); Lane 5, 9, 13 (*Hae*III). B) Lane 1 100 bp ladder; Lane 2, 6, 10 (*Mlu*I); Lane 3, 7, 11 (*Hinc*II); Lane 4, 8, 12 (*Sac*II); Lane 5, 9, 13 (*Hae*III).

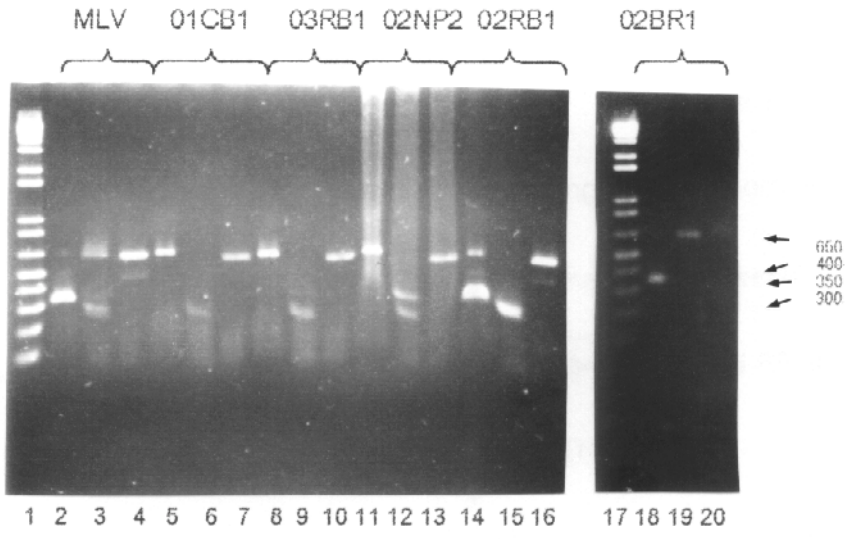

Figure 2: The RFLP patterns of the ORF5 of the Thai isolates (EU genotype) and the EU-MLV vaccine. ORF5-PCR products were treated with 3 restriction endonuclease enzymes. Lane 1, 17 100 bp ladder; Lane 2, 5, 8, 11, 14 and 18 were treated with *Pst*I; Lane 3, 6, 9, 12, 15 and 19 were treated with *Hae*II and Lane 4, 7, 10, 13, 16 and 20 were treated with *Cla*I.

Table 1. Sources and ORF 5 RFLP patterns of PRRSV isolates in Thailand.

Genotypes	Isolates	Samples	Sources	Year	Cutting frequency[a]
US	02SP2	Serum	Suphanburi	2002	1-1-1-2
	02SP3	Serum	Suphanburi	2002	1-1-1-2
	02SP4	Serum	Suphanburi	2002	1-2-1-1
	01NP2	Serum	Nakornpathom	2001	1-1-1-1
	02PB1	Serum	Pracheenburi	2001	1-2-1-1
	MLV-vacc	Resp PRRS™	-	-	2-1-1-1
EU	02RB1	Serum	Ratchaburi	2002	2-2-1
	01CB1	Serum	Chonburi	2001	1-2-1
	03RB1	Serum	Ratchaburi	2003	1-2-1
	02NP2	Serum	Nakornpathom	2002	1-3-1
	02BR1	Serum	Burirum	2001	2-1-2
	MLV-vacc	Porcilis®	-	-	2-2-2

[a]The cutting patterns were derived from ORF5 products after being treated with *Mlu*I, *Hinc*II, *Sac*II and *Hae*III in the US genotype or with *Pst*I, *Hae*II and *Cla*I in the EU genotype, respectively.

occurred in this study (unpublished data). The evidence of PRRSV recombination, in the field, has been reported (Kapur et al., 1996; Forsberg et al., 2002) as well as in the experimental infection in a cell culture system (Yuan et al., 1999; Joke et al., 2001) and in pigs (Mengeling et al., 2000). In addition, genetic drift has occurred, as is suggested by Murtaugh et al. (2003) so that many strains isolated in the early 1990's have no close relationship to current isolates. Interestingly, most ORF5 variants underwent negative selection and disappeared after repeat passage in pigs, in one study (Yoon et al., 2003). In conclusion, genetic variation certainly exists among the Thai isolates, even within the same genotype.

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