สัญญาเลขที่ PDF/27/2544

รายงานวิจัยฉบับสมบูรณ์

โครงการ:

การศึกษาความหลากหลายทางพันธุกรรมของไวรัสพีอาร์อาร์เอส ในสุกร ในประเทศไทย

Study on genetic variation and phylogenetic relationships of porcine reproductive and respiratory syndrome virus (PRRSV): Thai isolates

โดย ผศ.น.สพ.ดร.รุ่งโรจน์ ธนาวงษ์นุเวช และคณะ

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

ตัวอย่างไวรัสพีอาร์อาร์เอส จำนวนทั้งหมด 137 ตัวอย่าง จากงานบริการของหน่วยชันสูตรโรคสัตว์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ได้รับการทดสอบยืนยันการปรากฏตัวของไวรัสโดยใช้ Monoclonal antibody (SDOW-17) และยืนยันชนิดของสายพันธุ์โดยวิธี nested multiplex RT-PCR (nm RT-PCR) ของ ORF-1b สามารถจำแนกสายพันธุ์ได้เป็นสายพันธุ์อเมริกา (US) จำนวน 46 ตัวอย่าง (28.97%) และสายพันธุ์ ยุโรป (EU) จำนวน 91 ตัวอย่าง (71.03%) โดยใช้ไวรัสควบคุม SVI25 (US strain) สำหรับสายพันธุ์ US และ Lelystad virus สำหรับสายพันธุ์ EU รวมทั้งได้ทดสอบความไวของ nm RT-PCR พบว่าสามารถตรวจพบไวรัสที่ปริมาณไตเตอร์น้อยที่สุดน้อยกว่า 10^1 $TCID_{50}$ /ml ทำการคัดเลือกกลุ่มตัวอย่างของทั้งสองสายพันธุ์ เพื่อนำมาตรวจหาการเรียงตัวของลำดับเบสของ ORF-5 พบว่าตัวอย่างไวรัสพีอาร์อาร์เอส สายพันธุ์อเมริกาที่แยกได้ในเมืองไทย (00CS1, 01NP1, 01UD6, 02CB13, 02KK1, 02PB1, 02SP2 และ 02SP3) มีสายพันธุกรรมใกล้เคียงกับ ไวรัสพีอาร์อาร์เอสที่แยกได้จากประเทศแคนาดา (IAF-EXP91) ส่วนตัวอย่างไวรัสพีอาร์อาร์เอส สายพันธุ์ยุโรป (01CB1, 01RB1, 02BR1, 02CB12, 02SB2 และ 03RB1) มีสายพันธุกรรมใกล้เคียงกับ ไวรัสพีอาร์อาร์เอส ต้นแบบ (Lelystad virus) บ่งบอกถึงการระบาดของไวรัสพีอาร์อาร์เอสในประเทศไทยอาจมาจากการนำเข้าสุกรพันธุ์จากประเทศดังกล่าว นอกจากนี้พบว่าการกระจายของไวรัสพีอาร์อาร์เอสในแต่ละท้องที่ไม่ขึ้นอยู่กับภูมิประเทศเนื่องจากมีความเหมือนของไวรัสพีอาร์อาร์เอสที่แยกได้แต่ละท้องที่ที่ห่างไกลกัน ไวรัสพีอาร์อาร์เอสสายพันธุ์ EU 02CB12 มีความเหมือนของการเรียงตัวของลำดับเบสของ ORF5 มากกว่า 99% กับไวรัสวัคซีน (Porcillis) ที่ยังไม่เคยมีรายงานการใช้ในประเทศไทย ไม่พบ genetic recombination ของทั้งสองสายพันธุ์

Abstracts

The Thai isolates of porcine reproductive and respiratory syndrome virus (PRRSV) were obtained from the Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL). Virus isolation was confirmed by immunoperoxidase monolayer assay (IPMA) using SDOW-17. The virus genotype was determined using nested multiplex RT-PCR (nm RT-PCR) of ORF-1b. SVI275 (US genotype) and Lelystad virus (EU genotype) were used as the positive control. The nm RT-PCR was able to detect at least 10^1 TCID 50 /ml of PRRSV. Of 137 Thai isolates, 71.03% belonged to the European genotype and 28.97% was the US genotype. ORF-5 products of the US strain (00CS1, 01NP1, 01UD6, 02CB13, 02KK1, 02PB1, 02SP2 and 02SP3) and the EU strain (01CB1, 01RB1, 02BR1, 02CB12, 02SB2 and 03RB1) were amplified for DNA sequencing. The US strains of the Thai isolates are clustered within the same group and are more closely related to the IAF-EXP91 from Canada (89-90% nucleotide identity), whereas the EU strains were very similar to the EU prototype, Lelystad virus (87-97.5% nucleotide identity). The ORF5 nucleotide identities within the US genotype tested in this study compared with the US prototype, VR-2332 varied from 83.7-85.2%, whereas 83.5-85.5% amino acid identities were found. Based on the phylogenetic tree, each pair of the Thai isolates (01NP1 and 02KK1, 00CS1 and 01UD6, and 01CB1 and 01RB1) were identical, despite they were from different provinces. Therefore, there was no geographic influence on the spreading of PRRSV in Thailand. Interestingly, 02CB12 (EU) shared over 99% similarity of the ORF-5 nucleotide sequence and 98.6% of amino acid identity with the Porcillis vaccine (AF378819). No evidence of genetic recombination between the two genotypes was found in this study.

Executive summary

ทำการแยกชนิดไวรัสพีอาร์อาร์เอส โดยวิธี nested multiplex RT-PCR (nm RT-PCR) ของ ORF-1b จากตัวอย่างน้ำล้างปอด ชิ้นเนื้อ หรือซีรั่มจากฟาร์มสุกรที่มีปัญหาการระบาดของโรคพีอาร์อาร์เอส และจากตัวอย่างที่ได้จากงานบริการของหน่วยชันสูตรโรคสัตว์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย จำนวนทั้งหมด 137 ตัวอย่าง จำแนกสายพันธุ์ได้เป็นสายพันธุ์อเมริกา (US) จำนวน 46 ตัวอย่าง (28.97%) และสายพันธุ์ ยุโรป (EU) จำนวน 91 ตัวอย่าง (71.03%) โดยมีไวรัสควบคุม SVI25 (US strain) และไวรัสที่ได้จากวัคซีนเชื้อเป็น สำหรับสายพันธุ์ US และ Lelystad virus สำหรับสายพันธุ์ EU รวมทั้งได้ทดสอบความไวของ nm RT-PCR พบว่าสามารถตรวจพบไวรัสที่ปริมาณไตเตอร์น้อยที่สุดน้อยกว่า 10^1 $TCID_{50}$/ml ทำการคัดเลือกกลุ่มตัวอย่างของทั้งสองสายพันธุ์ เพื่อนำมาตรวจหาการเรียงตัวของลำดับเบสของ ORF-5 พบว่าตัวอย่างไวรัสพีอาร์อาร์เอส สายพันธุ์อเมริกาที่แยกได้ในเมืองไทยมีสายพันธุกรรมใกล้เคียงกับ ไวรัสพีอาร์อาร์เอส ที่แยกได้จากประเทศแคนาดา ส่วนตัวอย่างไวรัสพีอาร์อาร์เอส สายพันธุ์ ยุโรปมีสายพันธุกรรมใกล้เคียงกับ ไวรัสพีอาร์อาร์เอส ต้นแบบที่ชื่อว่า Lelystad virus บ่งบอกถึงการระบาดของไวรัสพีอาร์อาร์เอสในประเทศไทยอาจมาจากการนำเข้าสุกรพันธุ์จากประเทศดังกล่าว นอกจากนี้พบว่าการกระจายของไวรัสพีอาร์อาร์เอสในแต่ละท้องที่ไม่ขึ้นอยู่กับภูมิประเทศเนื่องจากมีความเหมือนของไวรัสพีอาร์อาร์เอสที่แยกได้แต่ละท้องที่ที่ห่างไกลกัน ไวรัสพีอาร์อาร์เอสสายพันธุ์ EU 02CB12 มีความเหมือนของการเรียงตัวของลำดับเบสของ ORF5 มากกว่า 99% กับไวรัสวัคซีน (Porcillis) ที่ยังไม่เคยมีรายงานการใช้ในประเทศไทย

เนื้อหางานวิจัย

- รวบรวมเชื้อไวรัสพีอาร์อาร์เอสที่แยกได้ในประเทศไทย
- เพาะเลี้ยงไวรัสและทำให้บริสุทธิ์โดยวิธี Plaque purification ในเซลล์ Marc –145 เก็บ seed virus ที่แยกได้ไว้ใน –80 C เพื่อเก็บไว้อ้างอิงและใช้ทดลองในงานวิจัยในอนาคต ต่อไป
- แยก genotype ของไวรัส โดยวิธี RNA Single tube RT-PCR ของ ORF 1b ซึ่งเป็นแม่แบบ polymerase ของไวรัสโดยใช้ universal primers ที่ได้รับการตีพิมพ์แล้ว จากนั้นทำ Multiplex PCR โดยใช้ primers 2 คู่ ที่สามารถแยกชนิดของไวรัสได้ (Gilbert *et. al.*, J. Clin. Microbiol. 1997, 35:264-267) จำแนกสายพันธุ์ได้เป็นสายพันธุ์อเมริกา (US) จำนวน 46 ตัวอย่าง และสายพันธุ์ ยุโรป (EU) จำนวน 91 ตัวอย่าง รวม 137 ตัวอย่าง
- ทำการเพิ่มปริมาณ ORF-5 ของไวรัสพีอาร์อาร์เอส กลุ่มสายพันธุ์ US ด้วย primers ที่ได้รับการตีพิมพ์แล้ว (Andreyev et al., Arch. Virol.1997, 993-1001) และของไวรัสพีอาร์อาร์เอสกลุ่มสายพันธุ์ EU ด้วย primers คู่ใหม่จากงานวิจัยที่ได้ตีพิมพ์แล้ว (Pirzadeh et al., Can. J. Vet. Res. 1998, 62: 170-177) และทำให้บริสุทธิ์
- ORF-5 PCR products ของไวรัสพีอาร์อาร์เอสทั้ง 2 กลุ่มสายพันธุ์ ประกอบด้วย

  US: 00CS1, 01NP1, 01UD6, 02KK1, 02CB13, 02PB1, 02SP2 และ 02SP3

  EU: 01CB1, 01RB1, 02BR1, 02CB12, 02SB2 1และ 03RB1
- clone และเพิ่มปริมาณ ORF5 โดย Ligation และ Transformation ลงใน electrocompetent DH5α *E. coli* cells ก่อนส่งตรวจหาลำดับสายพันธุกรรม (sequencing)
- นำมาศึกษาหาลำดับการเรียงตัวของสายนิวคลีโอไทด์และ amino acid sequence alignment ของ ORF-5 และวิเคราะห์เปรียบเทียบในแต่ละกลุ่มสายพันธุ์ (Phylogenetic relationship) (Fig. A, B, X, and Y)
- ส่งลำดับการเรียงตัวของสายนิวคลีโอไทด์ของ ORF-5 เพื่อใช้สำหรับอ้างอิง ณ GenBank (ตารางที่ 1)

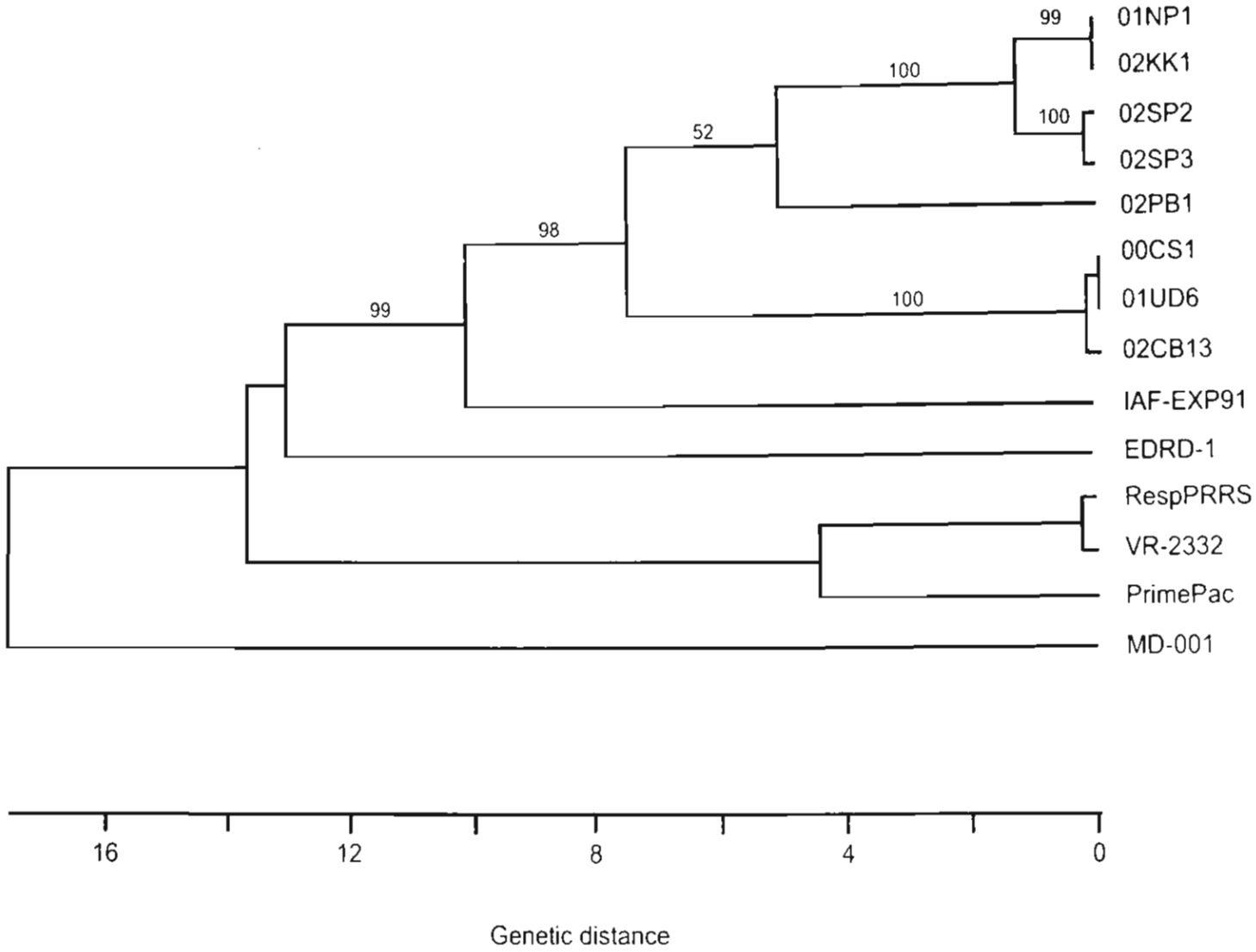

Fig. A. Dendrogram based on the nucleotide sequence of the ORF5 gene of PRRS-US strains. The phylogenetic tree was generated using Clustal method with weighted residue weight table, MegAling program (DNASTAR, Madison, WI). Bootstrap analysis was performed using PAUP version 4.02b with 1,000 bootstrap replications. The value adjacent to each node represent the percentage of 1,000 bootstrap trees that support the clustering.

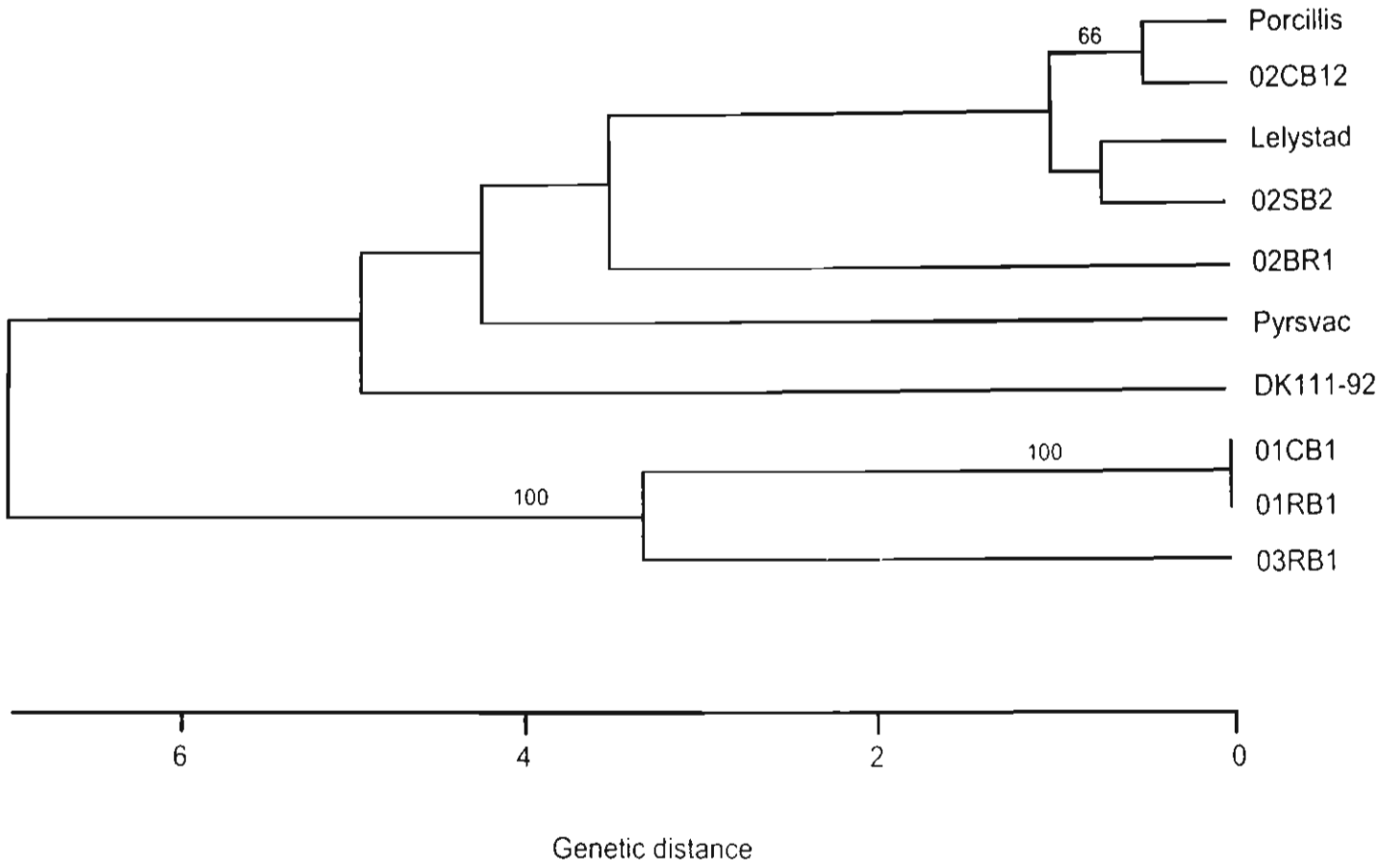

Fig. B. Dendrogram based on the nucleotide sequence of the ORF5 gene of PRRS-EU strains. The phylogenetic tree was generated using Clustal method with weighted residue weight table, MegAling program (DNASTAR, Madison, WI). Bootstrap analysis was performed using PAUP version 4.02b with 1,000 bootstrap replications. The value adjacent to each node represent the percentage of 1,000 bootstrap trees that support the clustering

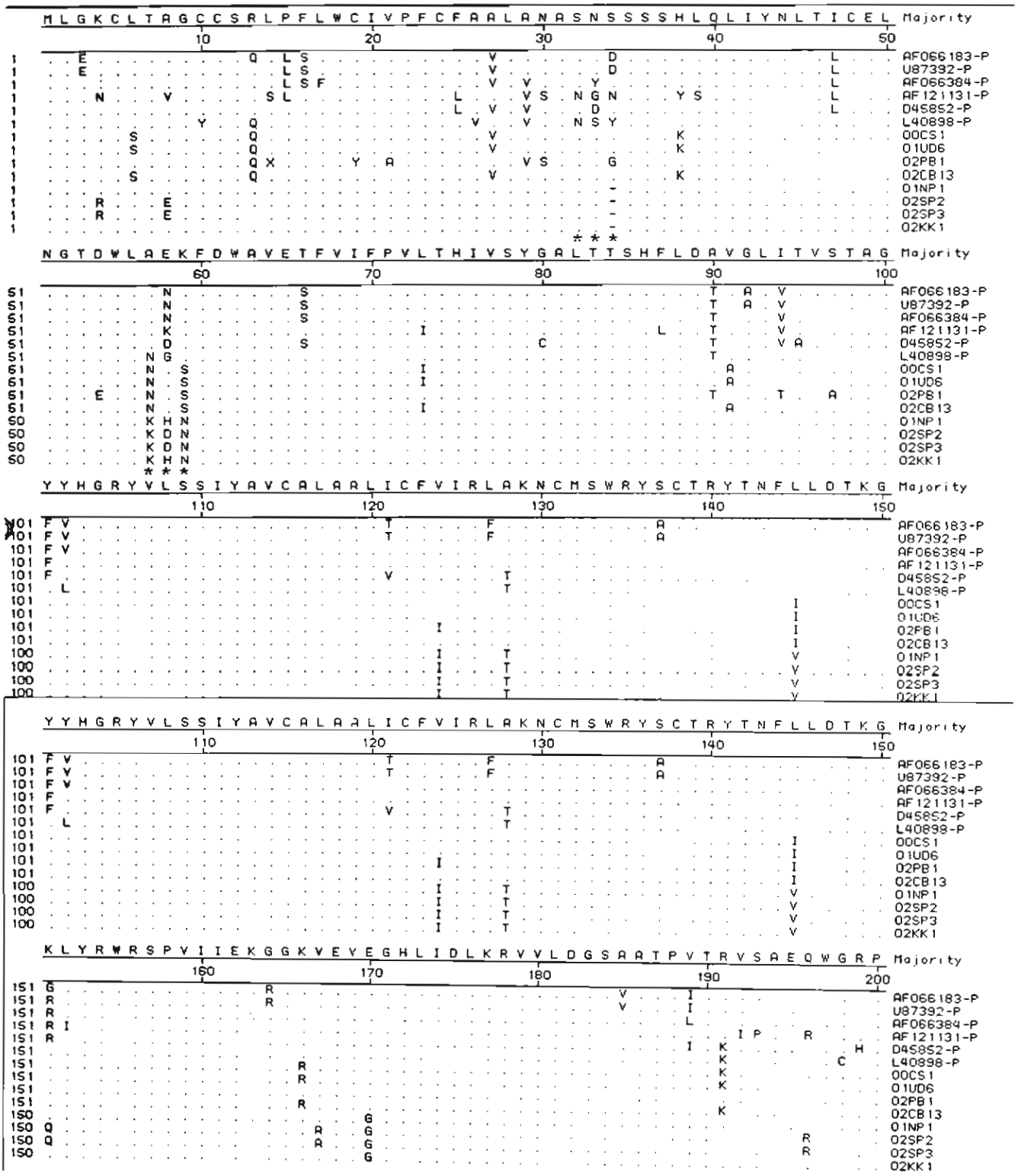

Fig. X. Amino acid sequence alignment of ORF5 of PRRS-US strains. The consensus sequence is shown on the top. The amino acid identities are indicated by period (.). The hypervariable regions are indicated by asterisk (*). The amino acid deletion region (34) is indicated by hyphen (-).

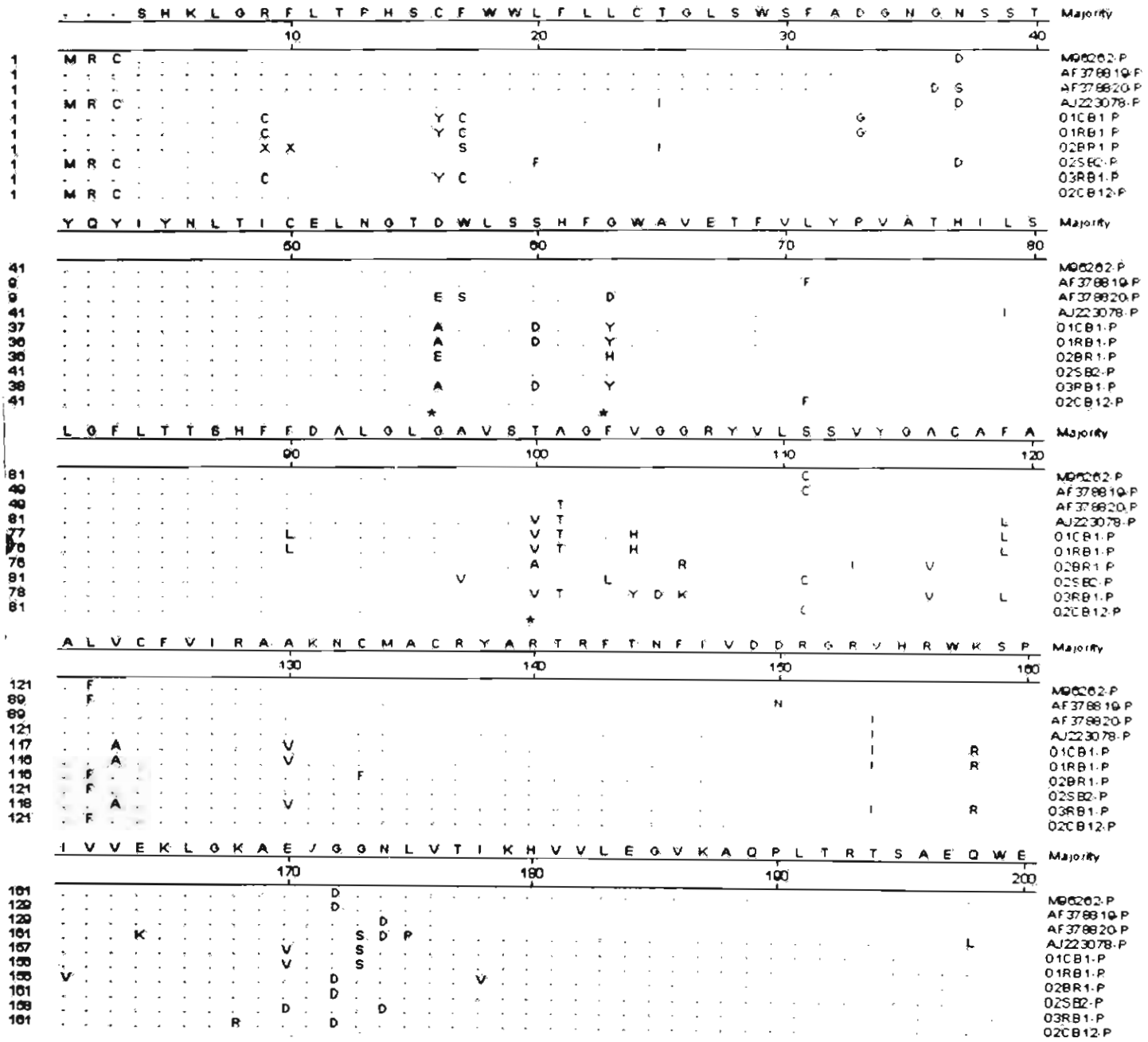

Fig. Y. Amino acid sequence alignment of ORF5 of PRRS-EU strains. The consensus sequence is shown on the top. The amino acid identities are indicated by period (.). The hypervariable regions are indicated by asterisk (*).

ตารางที่ 1: Sequences summary

Isolate ID	Genotype	Source	Year	Accession no.
00CS1	US	Chachoengsao, Thailand	2000	AY297111
01NP1	US	Nakorn Pathom, Thailand	2001	AY297112
01UD6	US	UdornThani, Thailand	2001	AY297113
02CB13	US	Chonburi, Thailand	2002	AY297114
02KK1	US	Khonkhen, Thailand	2002	AY297115
02PB1	US	Prachinburi, Thailand	2002	AY297116
02SP2	US	Suphanburi, Thailand	2002	AY297117
02SP3	US	Suphanburi, Thailand	2002	AY297118
01CB1	EU	Chonburi, Thailand	2001	AY297119
01RB1	EU	Rachaburi, Thailand	2001	AY297120
02BR1	EU	Burirum, Thailand	2002	AY297121
02CB12	EU	Chonburi, Thailand	2002	AY297122
02SB2	EU	Saraburi, Thailand	2002	AY297123
03RB1	EU	Rachaburi, Thailand	2003	AY297124

Output ที่ได้จากโครงการ (ภาคผนวก)

- อมรรัตน์ ทัศนกิจ และ รุ่งโรจน์ ธนาวงษ์นุเวช การทดสอบชนิดของ Monoclonal antibodies เพื่อตรวจไวรัสพีอาร์อาร์เอส ที่แยกได้ในประเทศไทยโดยวิธี Immunoperoxidase monolayer assay ประมวลการนำเสนอผลงานวิจัย พ.ศ. 2544-2545 คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย วันที่ 18-19 เมษายน 2545 (ใช้งบประมาณบางส่วนจาก PDF/27/2544)
- R. Thanawongnuwech, A. Tatsanakit, S. Damrongwatanapokin, and E. Thacker 2002. Typing of PRRSV isolates in Thailand by a nested multiplex RT-PCR. Proceeding of the 17th International Pig Veterinary Society, Ames, Iowa June 2-5, 2002
- R. Thanawongnuwech, S. Disatian, R. Saiyasombat, S. Napakanaporn, and A. Rungsipipat 2002. Detection of IFN-γ positive cell in PRRSV-infected lungs: A comparison between the low and the high virulence strains. Proceeding of the 17th International Pig Veterinary Society, Ames, Iowa June 2-5, 2002 (ใช้งบประมาณบางส่วนจาก PDF/27/2544)
- R. Thanawongnuwech, A. Rungsipipat, S. Disatian, R. Saiyasombat, S. Napakanaporn, and P.G. Halbur 2003. Immunohistochemical staining of IFN-γ positive cells in PRRSV-infected lungs Vet Immuno Immunopathol. 91:73-77. (ใช้งบประมาณบางส่วนจาก PDF/27/2544)
- R. Thanawongnuwech, A. Tatsanakit and K. Tatinij. 2003. Prevalence of PRRSV in Thailand Proceeding of the 11th International Symposium of the World Association of Veterinary Laboratory Diagnosticians, Bangkok, Thailand (ใช้งบประมาณบางส่วนจาก PDF/27/2544)
- R. Thanawongnuwech, A. Amornsin, A. Tatsanakit, and S. Damrongwatanapokin. 2003. Genetic and geographical variation of porcine reproductive and respiratory syndrome virus in Thailand Vet. Microbiol. (อยู่ระหว่างเขียนต้นฉบับ)

การทดสอบชนิดของ Monoclonal antibodies (Mabs) เพื่อตรวจไวรัสพีอาร์อาร์เอส ที่แยกได้ในประเทศไทยโดยวิธี Immunoperoxidase monolayer assay

อมรรัตน์ ทัศนกิจ [1] และ รุ่งโรจน์ ธนาวงษ์นุเวช [1]

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บทนำ

โรคพีอาร์อาร์เอส (Porcine Reproductive and respiratory syndrome) เป็นโรคสำคัญที่ก่อให้เกิดปัญหาทางเศรษฐกิจการเลี้ยงสุกรในประเทศไทย จากการตรวจพบแอนติบอดีต่อไวรัสพีอาร์อาร์เอสในตัวอย่างซีรั่มสุกรที่เก็บไว้ตั้งแต่ปี 2532 จากฟาร์มสุกรที่มีประวัติเกี่ยวกับการแท้งและการเกิดมัมมี่ (Damrongwatanapokin et al., 1996) จนปัจจุบันพบว่าเชื้อไวรัสพีอาร์อาร์เอสได้แพร่กระจายทุกพื้นที่ของประเทศไทย โดยการตรวจพบระดับแอนติบอดีที่ตอบสนองต่อการติดเชื้อ (คณิศักดิ์ และคณะ 2538) เชื้อไวรัสพีอาร์อาร์เอส เป็นอาร์เอ็นเอไวรัส จัดอยู่ในกลุ่ม Arterivirus แฟมิลี Arteriviridae (De Vries et al., 1997) แบ่งได้ 2 กลุ่มหลักตามลักษณะแตกต่างของแอนติเจนคือ กลุ่มทางประเทศยุโรป และกลุ่มทางประเทศ สหรัฐอเมริกา ซึ่งในกลุ่มนี้พบความแตกต่างทางแอนติเจนโดยสามารถแยกเป็นกลุ่มย่อยๆ ได้ตามความรุนแรงของโรค(Nelson et al., 1993) ชนิดของไวรัสพีอาร์อาร์เอส ที่แยกได้ในประเทศไทย โดยสุดารัตน์ และคณะ (1996) พบว่ามีความคล้ายคลึงทางพันธุกรรมกับชนิดของไวรัสดังกล่าวในประเทศสหรัฐอเมริกา ดังนั้นในการศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อคัดเลือก monoclonal antibodies ที่ให้ผลตอบสนองไวรัสพีอาร์อาร์เอส สายพันธุ์ในประเทศไทย ที่ดีที่สุดมาใช้ในการตรวจวินิจฉัยโดยวิธี IPMA

อุปกรณ์และวิธีการ

Monoclonal antibodies :12 ชนิด โดยแบ่งเป็น 2 กลุ่ม

กลุ่มที่มีความจำเพาะต่อ 15 kDnucleocapsid protein ได้แก่ A, B, C, D, E, BP7, SDOW17 และ SR30

กลุ่มที่มีความจำเพาะต่อ 25 kD enveloped protein ได้แก่ P-1S-1, P6-28, P6-246 และ 4bB,

เซลล์เพาะเลี้ยง : MARC 145 ใน minimum essential media (MEM)

ไวรัสทดสอบ : ไวรัสที่แยกได้ในประเทศไทย 5 Isolates จากภาคกลาง ภาคตะวันออกและภาคตะวันออกเฉียงเหนือตั้งแต่ เดือนธันวาคม 2543 - ตุลาคม 2544

ไวรัสมาตรฐาน : สายพันธุ์ US (SVI 275, MLV Vaccine),สายพันธุ์ EU(Lelystad virus)

การทดสอบ : เพาะเลี้ยงเซลล์ MARC 145 ใน MEM, 5% FBS, ใส่ 96 well plateหลุมละ200µLที่อุณหภูมิ 37°C, 5%CO_2, 48 ชั่วโมง นำมาใส่ไวรัสพีอาร์อาร์เอส หลุมละ 50µL. ใน MEM, 2% FBS, 37°C, 5%CO_2, 48 ชั่วโมง ตรวจหาการตอบสนองของไวรัสพีอาร์อาร์เอสต่อ monoclonal antibodies ทั้ง 12 ชนิดโดยวิธี immunoperoxidase monolayer assay(IPMA)

ผลการศึกษา

ดังตารางที่ 1

วิจารณ์และสรุป

การทดสอบไวรัสพีอาร์อาร์เอสที่แยกได้ในประเทศไทยจำนวน 5 isolates แบ่งเป็นสายพันธุ์ทางประเทศสหรัฐ (US) 2 isolates (01CS2 และ 01NP2) สายพันธุ์ทางประเทศยุโรป (EU) 3 isolates (01RB1, 01CB1, 01UD2) โดยมีกลุ่มไวรัสควบคุมผลบวกสายพันธุ์ US (SVI-275 และ MLV Vaccine) และสายพันธุ์ EU (Lelystad virus) พบว่า Mabs ทั้งสองกลุ่มให้ผลการตอบสนองที่ระดับมากกว่าหรือเท่ากับ2 (0-3) กับกลุ่มไวรัสพีอาร์อาร์เอสควบคุมผลบวกทั้งหมดและไวรัสพีอาร์อาร์เอสที่แยกได้ในประเทศไทยสายพันธุ์ US ยกเว้น Mabs E ที่ให้ผลให้ผลตอบสนองระดับ 1 ต่อ 01CS2 (สายพันธุ์ US) และ 01RB1(สายพันธุ์ EU) จากการศึกษาพบว่า ไวรัสพีอาร์อาร์เอส สายพันธุ์ EU ให้ผลบวกที่ไม่สม่ำเสมอต่อ Mabs ทั้งหมดที่ทดสอบโดยเฉพาะ 01RB1 และ 01CB1 ไม่ให้ผลตอบสนองต่อ C และ D เลยตามลำดับ โดยสรุปจากการศึกษาครั้งนี้ทำให้สามารถเลือก Mabs ชนิดที่เหมาะสมและให้ผลตอบสนองที่ดีที่สุดต่อเชื้อไวรัสพีอาร์อาร์เอสที่แยกได้ในประเทศไทยทุกสายพันธุ์ที่ทดสอบคือ SDOW 17, SR 30, P-1S-1, P6-28 และ 4bB, มาใช้ในการตรวจวินิจฉัยโรคพีอาร์อาร์เอสในประเทศไทยต่อไป

กิตติกรรมประกาศ

ขอบคุณ Dr. Kenneth Platt ที่อนุเคราะห์ Mabs (A, B, C, D, E, P-1S-1, P6-28, and P6-246) Dr. Eileen Thacker ที่อนุเคราะห์ Mabs (SDOW17 และ SR30) และ Dr. Yan Jin Zhang ที่อนุเคราะห์ Mabs (BP7 และ 4bB,) ในการศึกษาครั้งนี้ และคณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย และงานประมาณบางส่วนจาก สำนักงานกองทุนสนับสนุนการวิจัย # PDF/27/2544 ที่สนับสนุนเงินวิจัย

เอกสารอ้างอิง

คณิศักดิ์ อรวิวะกุล ดวงใจ พนธ์อารีวัฒนา สุพล เลื่องยศถือชากุล วิชัย ทันตศุภารักษ์ และอรรถพ คุณาวงษ์กฤต. 2538. ความชุกของโรคพีอาร์อาร์เอส ของฟาร์มสุกรพ่อแม่พันธุ์ในภาคกลางและภาคตะวันออกเฉียงเหนือของประเทศไทย เวชชสารสัตว์แพทย์ 25(3) : 233-240

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ตารางที่ 1. ผลการตอบสนองของไวรัสพีอาร์อาร์เอสต่อMonoclonal antibodies ชนิดต่างๆ

ไวรัส พี อาร์ อาร์ เอส	ชนิดของ Monoclonal antibodies											
	A	B	C	D	E	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
SVI 275(US)	3	3	3	3	3	3	3	3	3	2	3	3
MLV(US)	3	3	3	3	3	3	3	3	3	3	3	3
LV (EU)	3	3	3	2	2	2	3	3	2	2	2	3
01CS2(US)	3	3	3	3	1	3	3	3	3	3	3	3
01NP2(US)	3	2	3	3	3	3	3	3	2	2	2	3
01RB1(EU)	3	3	0	1	1	1	3	3	3	2	1	2
01CB1(EU)	1	1	1	0	2	1	2	1	2	3	1	2
01UD1(EU)	1	1	1	1	2	1	3	2	1	3	1	2

TYPING OF PRRSV ISOLATES IN THAILAND BY A NESTED MULTIPLEX RT-PCR

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Keywords: RT-PCR, PRRSV, serotype

Introduction and Objective

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the arterivirus first observed in the United States (US) in 1987 and in Europe (EU) in 1990 (1). A serological survey of swine sera during 1988-1999 in Thailand demonstrated the earliest detection of seropositive animals in 1989 and the percentage of seropositive animals increased annually from 8.6 in 1991 to over 79 in 1999 (4). However, PRRSV was first isolated in Thailand from suckling and nursery pigs with severe chronic respiratory distress and the virus was identified as the US serotype (2). Since Thailand has continuously imported swine breeders from both European and North American countries for genetic improvement of breeding stocks, both EU and US serotypes may be presence in the swine population in Thailand. The objective of this study was to isolate and determine the serotypes of the PRRSV recently isolated from clinical samples in Thailand using a multiplex PCR (1).

Materials and Methods

Virus isolation: Virus isolation (VI) was performed from pooled sera or bronchoalveolar lavage fluid of pigs from central, Eastern, and Northeastern parts of Thailand during December 2000-October 2001 using either porcine alveolar macrophages (PAMs) or MARC-145. VI was confirmed by immunoperoxidase monolayer assay (IPMA) using SDOW-17.

Multiplex RT-PCR: Cultured supernates from 13 Thai isolates were tested for nested multiplex PCR using a modified protocol previously described (Gilbert et al., 1997). The sizes of the expected PCR products (ORF 1b) are 107 and 186 bp for the US and EU serotypes, respectively. Positive control of both EU (Lelystad virus or LV) and US (SVI275) serotypes were included in each test.

Results and Discussion

The nested multiplex PCR demonstrated that out of 13 Thai isolates, 8 isolates belong to the EU serotype and 5 isolates belong to the US serotypes (Fig. 1). Undoubtedly, both EU and US serotypes have been present in Thailand since Thailand has continuously imported swine breeders from both European and North American countries. PRRSV might spread into the country via imported pigs as evidence from the detection of anti-PRRSV antibodies in the imported pigs (2). This is the first report of the presence of the EU serotype in Thailand.

Collectively, our results provided additional information on the mixed population of PRRSV serotypes even within the same herd. Extensive diversity among PRRSV isolates has been documented (3). Phylogenetic and monoclonal antibody analyses are undergoing in our lab.

Acknowledgements
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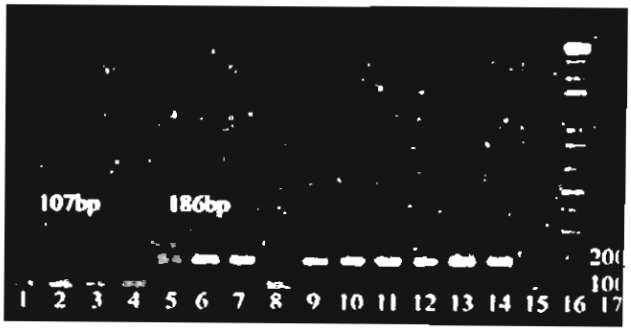

Figure 1. Nested multiplex PCR for PRRSV typing. Expected sizes of PCR products are 107bp for the US serotype and 186bp for the EU serotype. Lanes: 1=00CS1, 2=01CS2, 3=01NP1, 4=01NP2, 5=01CB2, 6=01RB1, 7=01CB1, 8=01UD6, 9=01UD5, 10=01UD4, 11=01UD3, 12=01UD2, 13=01UD1, 14=LV, 15=SVI275, 16=100bp DNA ladder, 17= negative control.

DETECTION OF IFN-γ POSITIVE CELLS IN PRRSV-INFECTED LUNGS: A COMPARISON BETWEEN A LOW AND A HIGH VIRULENCE STRAINS

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Keywords: IFN-γ, lung, lymphocytes, macrophages, PRRSV

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) infection induces T-cell mediated immune responses (1). Gorcyca et al. founded that PRRSV-infected pigs were protected to the second PRRSV challenge (3). In addition, PRRSV specific lymphocytes secrete IFN-γ upon stimulation in vitro (2). These suggested that the mechanism to control PRRSV in vivo might be antiviral activity of IFN-γ. However, there are variations in sensitivity to IFN-γ among different strains of virus (4). Our objective was to compare the IFN-γproduction in the lungs infected with different strains of PRRSV.

Materials and Methods

Seventy five 3-weeks-old PRRSV-free pigs were divided into three groups un-infected control group, low virulence-infected (LV, modified live virus-vaccine) group and high virulence-infected (HV, VR-2385) group Necropsy was performed on 5 pigs from each group at 3, 7, 10, 14 or 28 days post-inoculation (DPI) as previous described (6). Lungs were collected at necropsy and staining for IFN-γ positive cells using polyclonal antibodies (ENDOGEN, Woburn, USA)

Results and Discussion

The microscopic lung lesions induced by the HV group were more severe than those in the LV group. In the LV group, the number of infiltrating cells tended to increase at 10 to 28 DPI. The most severe changes of lung lesions in the HV group were demonstrated at 10 DPI as previously described (6). Significant increase in number of lymphocytes in the HV group was observed at 10 DPI (24.9%), 14 DPI (22 %) and 28 DPI (28.95 %) ($P< 0.05$). Relative decrease in number of macrophages was observed and correlated well with the increase of lymphocytic number when the disease progressed. IFN-γpositive cells were demonstrated in both lymphocytes and macrophages (Figure 1). Significant increase of IFN-γ positive cells was found at 7 DPI (15.9 %), 10 DPI (46.95 %), 14 DPI (10.9 %) and 28 DPI (13.4 %) in the HV group ($P<0.05$) (Table 1).

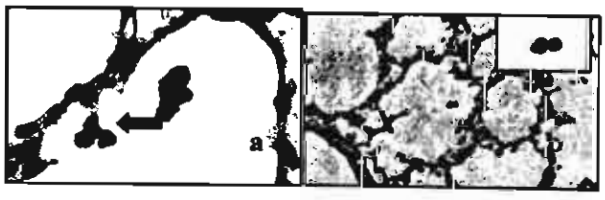

Figure 1: Lung: positive staining of IFN-γ in lymphocytes (arrow, a) and in macrophage (inset, b)

Rossow et al. showed that PRRSV titers in the lung were higher at 7 DPI and 14 DPI than those at 28 DPI and the virus could be cleared out of the lung as early as 28 DPI (5). In addition, a previous study demonstrated that IFN-γ interfered with PRRSV replication (1). Our results suggested that the increased number of IFN-γ positive cells in the HV group correlated well with the severity of lung lesions, which may be because of the presence of PRRSV in the lung. IFN-γ inhibits the replication of PRRSV in the lung. The increased IFN-γ production and the severity of the lung lesions may relate to the presence of PRRSV in the lung. The control strategies of PRRSV may involve in proper stimulation of IFN-γ production in pigs.

Acknowledgments

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Table 1: Percent of IFN-γ positive cells in PRRSV–infected lungs

Cells	Control	LV group	HV group
Lymphocytes			
3 DPI	0.20±0.44	0.70±0.97	1.30±2.91
7 DPI	0.00	0.50±0.86	1.50±1.97
10 DPI	0.85±0.80[a]	1.80±2.13[a]	23.9±8.70[b]
14 DPI	0.00[a]	0.40±0.42[a]	2.10±0.96[b]
28 DPI	1.50±1.83[a]	4.25±2.24[a]	6.95±4.18[b]
Macrophages			
3 DPI	3.70±2.68	3.50±5.72	5.00±6.60
7 DPI	0.40±0.65[a]	3.90±6.82[a]	14.40±12.07[b]
10 DPI	1.55±1.01[a]	4.25±1.08[a]	23.10±10.02[b]
14 DPI	2.90±2.43[a]	4.20±3.21[a]	8.80±4.72[b]
28 DPI	2.80±3.25[a]	7.35±4.26[b]	6.45±1.81[a]
Total			
3 DPI	3.90±2.74	4.20±6.58	6.30±9.46
7 DPI	0.40±0.65[a]	4.40±7.68[a]	15.90±13.65[b]
10 DPI	2.25±1.79[a]	6.05±2.90[a]	46.95±13.79[b]
14 DPI	2.90±2.43[a]	4.60±3.59[a]	10.90±5.13[b]
28 DPI	4.30±5.03[a]	11.60±6.36[a]	13.40±4.89[b]

*Mean (%)±SD, n=5, DPI: days post inoculation
a, b means within the same row with different superscripts in each DPI differ significantly at $P<0.05$

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Short communication

Immunohistochemical staining of IFN-γ positive cells in porcine reproductive and respiratory syndrome virus-infected lungs

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Abstract

Paraffin-embedded lungs were obtained from a previous porcine reproductive and respiratory syndrome virus (PRRSV)-challenged experiment involving three groups: an uninfected control group, a low virulence (LV, Resp PRRSV/Repro™)-infected group, and a high virulence (HV, VR-2385)-infected group. Tissues were collected at 3, 7, 10, 14 or 28 days post-inoculation (DPI) ($n = 5$). Lungs were examined to detect IFN-γ positive cells by immunohistochemical staining using polyclonal antibodies to IFN-γ. The microscopic lung lesions induced by the HV group were more severe than those in the LV group. A significant increase in number of lymphocytes in the HV group was observed at 10 DPI (24.90 ± 9.79%), 14 DPI (22.00 ± 11.47%) and 28 DPI (28.95 ± 15.11%) ($P < 0.05$). A relative decrease in macrophage numbers was observed and correlated well with the increase in lymphocyte numbers when the disease progressed. IFN-γ positive cells were demonstrated in both lymphocytes and macrophages, particularly pulmonary alveolar macrophages. A significant increase in IFN-γ positive cells was found at 7 DPI (15.90 ± 13.65%), 10 DPI (46.95 ± 13.79%), 14 DPI (10.90 ± 5.13%) and 28 DPI (13.40 ± 4.89%) in the HV group ($P < 0.05$). The results suggested that the increase in IFN-γ positive cells in the HV group correlated well with the severity of the lung lesions, which may be because of the presence of PRRSV in the lung.

Keywords: IFN-γ; Immunohistochemistry; Lymphocytes; Macrophages; Porcine reproductive and respiratory syndrome virus

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is caused by the porcine reproductive and respiratory syndrome virus (PRRSV). PRRSV is a positive-stranded enveloped, RNA virus classified in the family Arteriviridae order Nidovirales (Cavanagh, 1997). PRRSV infection is characterized by abortion in gilts and sows and respiratory diseases of variable severity in growing pigs. PRRSV strains vary in the severity of clinical disease and gross and microscopic lesions induced experimentally (Halbur et al., 1996; Thanawongnuwech et al., 2000). Although they differ in severity and duration of pneumonia induced, all strains induce interstitial pneumonia characterized by

Abbreviations: DPI, days post-infection; HV, high virulence; LV, low virulence; PRRSV, porcine reproductive and respiratory syndrome virus

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type 2 pneumocyte hypertrophy and hyperplasia, septal infiltration by mixed mononuclear cells, and increase amounts of mixed inflammatory and necrotic exudates in airways.

Alveolar macrophages are the predominant susceptible cells for PRRSV replication as evidenced by detection of PRRSV antigens or viral RNA (Halbur et al., 1996). Macrophages have important physiological functions and play a major role in cell-mediated immune response either as phagocytic cells or as antigen presenting cells (Hamilton, 1993). The most potent activating factor for the macrophage is IFN-γ or type II IFN, released from virus-infected cells. IFN-γ stimulates the production of many new proteins, which have antiviral activities. IFN-γ is produced by T-lymphocytes, large granular lymphocytes or NK cells, but not B-lymphocytes and monocytes during immune responses (Domeika et al., 2002; Rodriguez-Carreno et al., 2002).

PRRSV infection induces T-cell-mediated immune responses as demonstrated by lymphocyte proliferation and delayed-type hypersensitivity in infected pigs (Bautista and Molitor, 1997). It has been demonstrated that PRRSV specific lymphocytes secrete IFN-γ upon stimulation in vitro (Meier et al., 1997), and the PRRSV-infected pigs become protected from subsequent virus challenge (Gorcyca et al., 1995). It is likely to suggest that one mechanism of the protective immune response controlling PRRSV infection in vivo might be mediated through the antiviral mechanism induced by IFN-γ on macrophages (Bautista and Molitor, 1999).

In this study, we determined IFN-γ positive cells in the lungs at 3, 7, 10, 14 or 28 days post-inoculation (DPI) by immunohistochemistry compared between the low virulent (LV, Resp PRRSV/Repro™) and the high virulent (HV, VR-2385) strains of PRRSV. We hypothesized that different strains of PRRSV may have different effects on IFN-γ production.

2. Materials and methods

2.1. Experimental design

This experiment used archived lung tissue sections, embedded in paraffin blocks from a previous experiment (Thanawongnuwech et al., 1998). Briefly, the samples were collected from 75, 3-week-old, crossbred pig from a PRRSV-free herd. Pigs in the PRRSV-infected groups were inoculated by intranasal instillation with the HV (VR 2385) or the LV (Resp PRRS/Repro™) PRRSV. The control group was inoculated with media without virus. Five pigs from each group were euthanized and necropsied at 3, 7, 10, 14 or 28 DPI. Lung tissue sections were collected from cranial and middle lobes, fixed in 10% neutral buffer formalin. Each tissue section was embedded in paraffin and cut at 5 μm. The sections were stained with hematoxyline and eosin (H and E) staining and examined microscopically.

2.2. Immunohistochemistry

Avidin-Biotin peroxidase complex staining procedure was used and modified from a previous method (Numata et al., 1991). Paraffin-embedded lungs were cut at 5 μm, mounted on 3-aminopropyl triethoxysilane coated slides and dried for 24 h (overnight) at 45 °C. Sections were deparaffinized in xyline twice and followed by rehydration in graded ethanols 100, 95, 80 and 70%, respectively. Antigen retrieval treatment was done using 0.1% trypsin at 37 °C for 30 min. Sections were washed three times (5 min each) in PBS. Sections were then incubated with 0.3% H_2O_2 in methanol at room temperature for 30 min and with 10% BSA (Fluka, Switzerland) at 37 °C for 30 min. Sections were incubated with primary antibody using rabbit polyclonal anti swine IFN-γ antiboby (Endogen, Woburn, USA; 10 μg/ml) overnight at 4 °C, and were then incubated with biotinylated secondary antibody using goat anti rabbit IgG antibody (Dako, Glostrup, Denmark) at 37 °C for 1 h. Sections were incubated with avidin-biotinylated horseradish peroxidase complex (Dako, Glostrup, Denmark) at 37 °C for 1 h then, stained with 0.05% 3.3-diaminobenzidine tetrahydrochloride (0.01 M in Tris–HCl, pH 7.6; Sigma, USA), and followed by counter staining with Mayer's hematoxyline. A negative control slide was included at each batch of staining by substitution of Tris/PBS in place of the primary antibody. Two hundred mononuclear cells were identified and counted morphologically as macrophages or lymphocytes and the percent of IFN-γ positive staining cells in each cell type was recorded.

2.3. Statistic analysis

Data were expressed as a mean ± S.D. and were evaluated using one-way analysis of variance. The results were compared with the differentiation between groups by least significant difference. Statistical package for the social science was used to determine statistical significance among the groups in each DPI at $P < 0.05$.

3. Results and discussion

The microscopic lung lesions induced by the HV group persisted for 28 days and were more severe than those in the LV group. The lung lesions at 3 DPI looked normal in both LV and HV groups. The lung lesions at 7 DPI in the LV group were characterized by mild increase in the number of mononuclear cell infiltration, whereas, in the HV group, there were a few foci of type II pneumocyte hypertrophy and hyperplasia. Moderate interstitial pneumonia with severe mononuclear cell infiltration was found at 10 and 14 DPI in the HV group. Mild multifocal interstitial pneumonia with multifocal thickening of alveoli was characterized by mild pneumocyte type II hypertrophy and mild infiltration of mononuclear cells in the LV group at 10, 14 and 28 DPI. Lungs of the control group were microscopically normal.

Our study suggests that the LV strain may need more time to replicate in pigs because the number of infiltrating cells tended to increase at 10–28 DPI in the LV group. In contrast to the HV group, the most severe changes of lung lesions were demonstrated at 10 DPI after that the microscopic lung lesions were milder, but still more severe than those in the LV group. The population of lymphocytes and macrophages in alveolar spaces was counted and summarized in Table 1. A significant increase in lymphocyte numbers was found in the HV group at 10 DPI (24.90 ± 9.79%), 14 DPI (22.00 ± 11.47%) and 28 DPI (28.95 ± 15.11%) compared with the control group. However, in the LV group, lymphocyte numbers was slightly increased at 28 DPI (14.70 ± 9.81%). A relative decrease in macrophage numbers was correlated well with the increase in lymphocyte numbers.

The number of lymphocytes in the HV group was greater than that in the LV group. This suggests that the HV isolate of PRRSV induced much more infiltration of mononuclear cells and more inflammation than the LV strain. The number of lymphocytes in the HV group obviously increased when the disease progressed due to the immune responses. Similar to the previous study (Zhou et al., 1992), there was a significant increase in the number of T-helper cells in the peripheral circulation after PRRSV infection. The increase in the proportion of lymphocytes in the HV group could be from the enhanced immune response of pigs against PRRSV or from lymphocyte proliferation due to PRRSV-induced IL-1 stimulation (Van Reeth and Nauwynck, 2000).

Similar to our study, Nauwynck et al. (2001) found that PRRSV replication in the lung was associated with a decrease in the proportion of macrophage in lung lavage fluids. The proportion of macrophage was reduced because it could be related with the concurrent increase in lymphocyte numbers or because the macrophages were destroyed by PRRSV (Done and Paton, 1995).

IFN-γ positive cells were examined and the results are summarized in Table 2. A significant increase of IFN-γ positive cells was found at 7 DPI (15.90 ± 13.65%), 10 DPI (46.95 ± 13.79%), 14 DPI (10.90 ±

Table 1
Means (%) of lymphocytes and macrophages in alveoli of PRRSV-infected lungs[a]

Cells	Control pigs	LV-infected pigs[b]	HV-infected pigs[c]
Lymphocytes			
3 DPI[d]	3.30 ± 1.02[e]	2.95 ± 2.18	3.95 ± 3.46
7 DPI	7.90 ± 5.42	7.05 ± 3.44	15.65 ± 13.12
10 DPI	2.30 ± 0.99 a	2.95 ± 1.67 a	24.90 ± 9.79 b
14 DPI	6.20 ± 2.53 a	4.25 ± 2.05 a	22.00 ± 11.47 b
28 DPI	3.35 ± 1.43 a	14.70 ± 9.81 a	28.95 ± 15.11 b
Macrophages			
3 DPI	96.70 ± 1.02	97.05 ± 2.18	96.10 ± 3.45
7 DPI	92.10 ± 5.42	92.95 ± 3.44	84.35 ± 13.12
10 DPI	96.20 ± 3.73 a	97.05 ± 1.67 a	75.15 ± 9.77 b
14 DPI	94.30 ± 2.39 a	95.75 ± 2.05 a	77.40 ± 11.47 b
28 DPI	97.10 ± 1.09 a	85.3 ± 9.81 a	70.96 ± 15.15 b

[a] a, b means within the same row with different letters in each DPI differ significantly at $P < 0.05$.
[b] Low virulence.
[c] High virulence.
[d] Days post-inoculation.
[e] Mean (%) ± S.D., $n = 5$.

Table 2
Means (%) of IFN-γ positive cells in PRRSV-infected lungs*

Cells	Control pigs	LV-infected pigs	HV-infected pigs
Lymphocytes			
3 DPI	0.20 ± 0.44	0.70 ± 0.97	1.30 ± 2.91
7 DPI	0.00	0.50 ± 0.86	1.50 ± 1.97
10 DPI	0.85 ± 0.80 a	1.80 ± 2.13 a	23.9 ± 8.70 b
14 DPI	0.00 a	0.40 ± 0.42 a	2.10 ± 0.96 b
28 DPI	1.50 ± 1.83 a	4.25 ± 2.24 a	6.95 ± 4.18 b
Macrophages			
3 DPI	3.70 ± 2.68	3.50 ± 5.72	5.00 ± 6.60
7 DPI	0.40 ± 0.65 a	3.90 ± 6.82 a	14.40 ± 12.07 b
10 DPI	1.55 ± 1.01 a	4.25 ± 1.08 a	23.10 ± 10.02 b
14 DPI	2.90 ± 2.43 a	4.20 ± 3.21 a	8.80 ± 4.72 b
28 DPI	2.80 ± 3.25 a	7.35 ± 4.26 b	6.45 ± 1.81 a
Total			
3 DPI	3.90 ± 2.74	4.20 ± 6.58	6.30 ± 9.46
7 DPI	0.40 ± 0.65 a	4.40 ± 7.68 a	15.90 ± 13.65 b
10 DPI	2.25 ± 1.79 a	6.05 ± 2.90 a	46.95 ± 13.79 b
14 DPI	2.90 ± 2.43 a	4.60 ± 3.59 a	10.90 ± 5.13 b
28 DPI	4.30 ± 5.03 a	11.60 ± 6.36 a	13.40 ± 4.89 b

* See key in Table 1.

5.13%) and 28 DPI (13.40 ± 4.89%) in the HV group compared to the control group ($P < 0.05$). Both lymphocytes and macrophages had similar percentages of positive cells. The highest total number of IFN-γ positive cells in the LV group was at 28 DPI, but in the HV group was at 10 DPI.

In our study, IFN-γ positive cells in PRRSV-infected pigs were found in both lymphocytes and macrophages in different proportions. The possible reasons are that IFN-γ commonly acts on macrophages to activate them or macrophages may produce a significant amount of IFN-γ when stimulated. A recent report demonstrated that human alveolar macrophages were able to produce IFN-γ in disease states (Frucht et al., 2001).

The number of IFN-γ positive lymphocytes was increased in the HV group. IFN-γ was produced shortly after the infection process. IFN-γ production is stimulated by macrophage-derived cytokines, especially TNF-α and IL-12 (Trinchieri, 1995). This is the reason why IFN-γ positive lymphocytes tend to increase at 3–10 DPI. The number of IFN-γ positive cells was greatly increased at 10 DPI because PRRSV-infected lung was the most severe.

At 28 DPI in the HV group, the number of infiltrating cells tends to decrease when compared to that number at 10 DPI. Rossow et al. (1995) showed that PRRSV titers in the lung were higher at 7 and 14 DPI than those at 28 DPI. This suggests that PRRSV could be cleared out of the lung as early as 28 DPI resulting in the reduction of the lung lesion. Pretreatment with IFN-γ could affect PRRSV replication in porcine macrophages evaluated by reduction in titer and percentage of PRRSV-infected cells (Bautista and Molitor, 1999). These suggested that IFN-γ produced more at 10 DPI may induce an inhibitory effect on PRRSV replication. The IFN-γ might play an important role in PRRSV protection. However, the increased IFN-γ production in the lung may have some negative inflammatory effects on the lung lesions.

In conclusion, IFN-γ might inhibit the replication of PRRSV in the lung. The increase in IFN-γ production in the lung may relate to the presence of PRRSV. The strategies of PRRSV control may involve controlled stimulation of IFN-γ production in pigs. Further studies should investigate the involvement of other cytokines in the PRRSV-infected pig.

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สัญญาเลขที่ PDF/27/2544

PREVALENCE OF PRRSV IN THAILAND

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Keywords: genotype, prevalence, PRRSV

Introduction and Objective

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the arterivirus first observed in the United States (US) in 1987 and in Europe (EU) in 1990 (2). A serological survey of swine sera during 1988-1999 in Thailand demonstrated the earliest detection of seropositive animals in 1989 and the percentage of seropositive animals increased annually from 8.6 in 1991 to over 67 in 2002 based on the data from the Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL). However, PRRSV was first isolated in Thailand from suckling and nursery pigs with severe chronic respiratory distress and the virus was identified as the US genotype (1). Since Thailand has continuously imported swine breeders from both European and North American countries for genetic improvement of breeding stocks, both EU and US genotypes are present in Thailand. The objective of this study is to isolate and determine the genotypes of the PRRSV recently isolated from clinical samples submitted from each region throughout Thailand using a nested multiplex RT-PCR (Fig. 1-3).

Materials and Methods

Virus isolation: Virus isolation (VI) was performed from pooled sera, bronchoalveolar lavage fluid or minced tissues of pigs from the central, southern, northern and northeastern parts of Thailand during December 2000-December 2002 using either porcine alveolar macrophages (PAMs) or MARC-145. VI was confirmed by immunoperoxidase monolayer assay (IPMA) using SDOW-17.

Nested Multiplex RT-PCR: Samples were tested for nested multiplex RT-PCR using a modified protocol previously described (2). The sizes of the expected PCR products (ORF 1b) are 107 and 186 bp for the US and EU serotypes, respectively (Fig. 1). Positive control of both EU (Lelystad virus or LV) and US (SVI275) genotypes were included in each test (4).

Results and Discussion

The nested multiplex RT-PCR demonstrated that out of 137 samples throughout Thailand, 91 samples (66.42%) were the EU genotype and 46 samples (33.58%) were the US genotypes (Table 1). Undoubtedly, both EU and US genotypes have been present in Thailand since Thailand has continuously imported swine breeders from both European and North American countries. PRRSV might spread into the country via imported pigs or semen as evidence from the detection of anti-PRRSV antibodies in the imported pigs (1). This is the update report of the prevalence of the PRRSV in Thailand. Porcine respiratory disease complex (PRDC) induced by PRRSV and other pathogens, currently, is the major problem of the Thai swine industry causing severe losses in the weanling and fattening pigs. Additional information from the Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL) demonstrates the presence of approximately 7% of the mixed population of PRRSV genotypes within the same herd. Prevention and control strategies in the mixed infection herds are much more complicated than the single strain infection.

Extensive diversity among PRRSV isolates has been documented (3). The preliminary data suggested that the US genotypes in Thailand were similar to the Canadian isolates, whereas the EU genotypes were similar to the Denmark isolates (Thanawongnuwech et al., unpublished data). Phylogenetic and monoclonal antibody analyses are undergoing in our lab.

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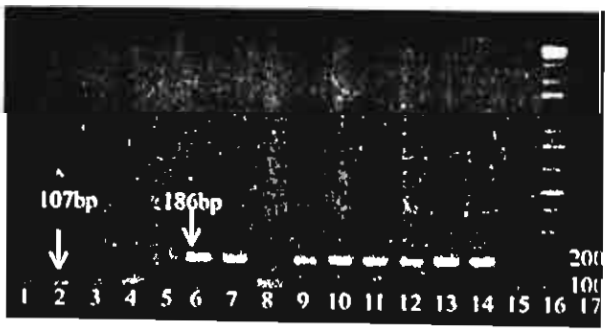

Fig. 1. Nested multiplex RT-PCR for PRRSV. Expected sizes of PCR products are 107bp for the US genotype and 186bp for the EU genotype. Lanes: 1=00CS1, 2=01CS2, 3=01NP1, 4=01NP2, 5=01CB2, 6=01RB1, 7=01CB1, 8=01UD6, 9=01UD5, 10=01UD4, 11=01UD3, 12=01UD2, 13=01UD1, 14=LV, 15=SVI275, 16=100bp DNA ladder, 17= negative control.

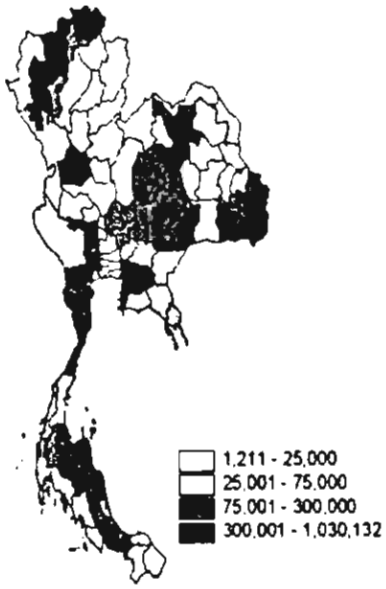

Figure 1: Pig population in each province of Thailand (Kindly provided by Dr. Wantanee Kalpravidh, Department of Livestock Development, Thailand)

Fig. 3 Map of Thailand

Table 1: Prevalence of PRRSV genotypes in each region

Regions	EU	US	Total
Central	61	31	92
North	15	4	19
Northeast	10	4	14
South	3	4	7
Unidentified	2	3	5
Total	91	46	137

Title:

Genetic and geographical variation of porcine reproductive and respiratory syndrome virus (PRRSV) in Thailand

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Abstract

The Thai isolates of porcine reproductive and respiratory syndrome virus (PRRSV) were obtained from the Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL). Virus isolation was confirmed by immunoperoxidase monolayer assay (IPMA) using SDOW-17. The virus genotype was determined using nested multiplex RT-PCR (nm RT-PCR) of ORF-1b. The nm RT-PCR was able to detect at least 10^1 $TCID_{50}$/ml of PRRSV. Of 137 Thai isolates, 71.03% belonged to the European genotype and 28.97% to the US genotype. ORF-5 products of the 8 US strains (00CS1, 01NP1, 01UD6, 02CB13, 02KK1, 02PB1, 02SP2 and 02SP3) and the 6 EU strains (01CB1, 01RB1, 02BR1, 02CB12, 02SB2 and 03RB1) were sequenced for genetic variation analysis. The US strains of the Thai isolates are clustered within the same group and are more closely related to the IAF-EXP91 from Canada (89-90% nucleotide identity), whereas the EU strains were very similar to the EU prototype, Lelystad virus (87-97.5% nucleotide identity). The ORF5 nucleotide identities within the US genotype tested in this study compared to the US prototype, VR-2332 varied from 83.7-85.2%, whereas 83.5-85.5% amino acid identities were found. Based on the phylogenetic tree, each pair of the Thai isolates (01NP1 and 02KK1, 00CS1 and 01UD6, and 01CB1 and 01RB1) was identical despite they were collected from different provinces. Therefore, there was no geographic influence on the spreading of PRRSV in Thailand. Interestingly, 02CB12 (EU genotype) shared over 99% similarity of the ORF5 nucleotide sequence and 98.6% of amino acid identity with the European vaccine, Porcillis (AF378819). No evidence of genetic recombination based on ORF5 sequences between the two genotypes was found in this study.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is classified within the genus *Arterivirus* in the family *Arteriviridae* in the order *Nidovirales* (Cavanagh, 1997). The disease caused by PRRSV is characterized by reproductive failure in gilts and sows and by a respiratory disease in young pigs. PRRSV was first observed in the North American countries in 1987 and in the European countries in 1990 (Gilbert et al., 1997). Sequence comparison has shown that two genotypes of PRRSV recognized as the American (US) and the European (EU) strains currently exist (Meng, 2000). A serological survey of swine sera during 1988-1999 in Thailand demonstrated the earliest detection of seropositive animals in 1989 and the percentage of the seropositive animals increased annually from 8.6 in 1991 to over 67 in 2002 based on the data from the Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL). PRRSV was first isolated in Thailand in 1996 from the suckling and nursery pigs with severe chronic respiratory distress and the virus was later identified as the US genotype (Damrongwatanapokin et al., 1996). Both EU and US genotypes have been reported in Thailand with the high prevalence of the EU genotype (Thanawongnuwech, 2002). Since its first appearance in Thailand, PRRSV has been the major cause of economic losses in the swine industry. Until now, the Thai authority does not allow to use any modified live virus vaccine of PRRSV, but the killed virus vaccine is commercially available since 1996.

PRRSV is an enveloped virus with a positive-sense, single-stranded RNA genome containing nine open reading frames (ORFs) which code for the viral replicase (ORF1a and 1b), four membrane-associated glycoproteins (ORF2a to 5), two unglycosylated proteins (ORF2b and 6) and the nucleocapsid protein (ORF7) (Meng et al., 1994). It has been shown that the ORF5 gene sequence of PRRSV is very polymorphic (Andreyev et al., 1997). Unlike the EU isolates, the

US isolates of PRRSV display a high degree of variability in the ORFs 2, 3, 5 and 7 (Meng et al., 1995; Murtaugh et al., 1998). Monoclonal antibodies against GP4 and GP5 proteins were demonstrated to have neutralizing ability to the virus (Pirzadeh and Dea, 1997). The objective of this study was to isolate and determine the genotypes of the PRRSV recently isolated during 2000-2003 from clinical samples submitted from each region throughout Thailand using a nested multiplex RT-PCR. DNA sequencing was also done on the selected isolates to determine the genetic variation of the Thai isolates from different regions. Genetic comparison of the ORF5 gene sequences of the Thai isolates to those of PRRSV prototypes and some available sequences from other countries may provide insight into the genetic evolution and origin of the Thai isolates.

2. Materials and Methods

2.1 Virus isolation (VI)

Virus isolation (VI) was performed from sera, bronchoalveolar lavage fluid or minced tissues of pigs from all regions of Thailand during December 2000-January 2003 using either porcine alveolar macrophages (PAMs) or MARC-145 (Kindly provided by Dr. E. Thacker, Iowa State University, USA). The presence of the virus was confirmed by immunoperoxidase monolayer assay (IPMA) using SDOW-17 (kindly provided by Dr. E. Thacker, Iowa State University, USA) modified from the immunofluorescent assay (IFA) as previously described (Thanawongnuwech et al., 1998). The stock virus was kept at –80 °C until needed.

2.2 Nested Multiplex RT-PCR (nm RT-PCR)

The stock viruses were tested for nested multiplex RT-PCR (nm RT-PCR) using a modified protocol previously described (Gilbert et al., 1997). SVI275 (US genotype) and Lelystad virus (EU genotype) were used as the positive control. Sensitivity of the assay was performed using ten-fold dilution of the control viruses. The final dilution contained approximately 10^1

TCID 50/ml. Viral RNA templates were isolated from the culture media using the commercial kit (QIAGEN, USA). In this study, we used one step RT-PCR (QIAGEN, USA) to amplify the common genome of ORF1b at the position 8628-8882 yielding the PCR product of 255 bp using the thermoregulator PTC-200 (MJ Research, USA). Two microliters of the PCR product was then utilized as the template in the nested multiplex PCR with the same condition. The sizes of the expected multiplex PCR products (ORF 1b) were 107 and 186 bp for the US and EU genotypes, respectively. Positive control of both EU (Lelystad virus) and US (SV1275) genotypes were included in each test.

2.3 Amplification and purification of ORF5 products

ORF-5 products of the US strain (00CS1, 01NP1, 01UD6, 02CB13, 02KK1, 02PB1, 02SP2 and 02SP3) and the EU strain (01CB1, 01RB1, 02BR1, 02CB12, 02SB2 and 03RB1) isolated in Thailand were selected and amplified for DNA sequencing. The origins of the Thai isolates are shown in Fig. 1 and Table 1. One step RT-PCR (QIAGEN, USA) using the same RNA template from the nm RT-PCR was used for ORF-5 DNA amplification. The condition and primers used for the US strain were similar to the previous report (Andreyev et al., 1997) with slight modification by performing 40 cycles of the amplification generating a 716 bp DNA fragment. The primers used for the EU strain were obtained from the previous report (Pirzdah et al., 1998), but using the same condition as the DNA amplification of the ORF5 of the US strain. The amplified PCR products were examined by gel electrophoresis and were then purified using QIAquick® spin (QIAGEN, USA) according to the manufacturer's procedure and submitted for sequencing.

2.4 Nucleotide sequencing and analysis

Purified PCR products were sequenced using an ABI Prism sequencer, ABI 3700 (Applied Biosystem). The ORF5 sequences of the Thai isolates reported in this study have been deposited with the GenBank database under the accession numbers AY297111-AY297124 (Table 1). Sequence alignments were done using the EditSeq and MegAlign computer programs (DNASTAR, Madison, WI). The ORF5 gene sequences of other known PRRSV isolates used in this study were retrieved from the GenBank (Table 1). The percentages of sequence identity among different PRRSV isolates were determined and the phylogenetic analysis was performed using Clustal method with weighted residue weight table, MegAling program (DNASTAR). Imported alignments were then analyzed further for Bootstrap analysis with PAUP program version 4.02b with 1,000 bootstrap replications (D. L. Swofford, University of Illinois, Urbana-Champaign). Predicted amino acids were analyzed in a similar manner to nucleotide sequences.

Antigenic determinants in the ORF5 protein of selected sequences were predicted using a computer program, PROTEAN (DNASTAR, Madison, WI), which takes into account hydrophilicity, surface probability, chain flexibility, hydropathy and secondary structure.

3. Results

3.1 Genotyping of PRRSV in Thailand

The nm RT-PCR was able to detect at least 10^1 $TCID_{50}$/ml of PRRSV (data not shown). The nm RT-PCR demonstrated that out of 137 Thai PRRSV isolates, 91 samples (66.42%) were the EU genotype and 46 samples (33.58%) were the US genotype (Table 2). The central region, the highest density of pig production of Thailand was the major source of the submitted samples (92/137). Approximately, two-third of the Thai isolates were the EU genotype, except for the southern region having similar numbers between the genotypes. However, the number of the submitted samples from the southern region was somewhat low.

3.2 Genomic variations of the ORF5 genes between the Thai isolates

A total of 14 partial ORF5 sequences representing the Thai PRRSV isolates were determined. Eight Thai isolates (00CS1, 01NP1, 01UD6, 02CB13, 02KK1, 02PB1, 02SP2 and 02SP3) were the US genotype, while the other six isolates (01CB1, 01RB1, 02BR1, 02CB12, 02SB2 and 03RB1) were belonged to the EU genotype. Together with the selected ORF5 sequences available in GenBank including some available PRRSV vaccines (Table 1), two phylogenetic trees were constructed based on the virus genotypes (Fig. 2). The alignment of the deduced amino acid sequences for those isolates is also shown (Fig. 3A and Fig. 3B). It should be noted that the envelope glycoprotein amino acid sequence of the Thai isolates (US genotype) consisted of 200 residues. Only 4 isolates of the US genotype including 01NP1, 02KK1, 02SP2 and 02SP3 had one amino acid deletion at position 34. There was no deletion of those amino acid in the EU genotype tested.

Based on the phylogenetic tree, each pair of the Thai isolates (01NP1 and 02KK1, 00CS1 and 01UD6, and 01CB1 and 01RB1) was identical. No distinct epidemiological feature based on geography or date of isolation was apparent except for the two isolates, 02SP2 and 02SP3 submitted from the farms in the same province. Those isolates shared 99.8% nucleotide identity. Interestingly, 02CB12 (EU) shared over 99% similarity of the ORF5 nucleotide sequence and 98.6% of amino acid identity with the Porcillis vaccine (AF378819). The Porcillis vaccine has an asparagine residue at amino acid position 150, whereas the other EU strains have an aspartic acid residue. The other substitution occurs at amino acid position 168 where a lysine residue in the EU isolates tested in this study as well as the Porcillis vaccine is replaced by an arginine residue in 02CB12.

The US strains of the Thai isolates are clustered within the same group and are more closely related to the IAF-EXP91 from Canada (89-90% nucleotide identity), whereas the EU strains were very similar to the EU prototype, Lelystad virus (87-97.5% nucleotide identity). The ORF-5 nucleotide identities within the US genotype tested in this study compared with the US prototype, VR-2332 varied from 83.7-85.2%, whereas 83.5-85.5% amino acid identities were observed. Most of the amino acid substitutions of the US strain locate in two short hypervariable regions at the amino terminal region (amino acid positions 32-34 and 57-59), whereas the amino acid positions 56, 63 and 100 were hypervariable in the EU strain (Fig. 3).

Six moderately conserved regions with greater than 10 residues each were identified in the envelope glycoprotein (aa 39-53, 60-72, 74-89, 98-123, 129-165 and 171-190) of the US strain tested. In conserved region 1 (a.a. 39-53), only the 02PB1 differed from the consensus sequence with a conservative an aspartic acid residue to a glutamic acid residue substitution at position 54. Region 5 (a.a. 129-165) has a leucine residue at position 145 for the US prototype whereas an isoleucine residue substitution (00CS1, 01UD1, 02PB1 and 02CB13) and a valine residue substitution (01NP1, 02KK1, 02SP2, and 02SP3) were found.

In the EU strain tested, four conserved regions were also identified (a.a. 38-55, 64-89, 134-153, and 179-200). In conserved region 2 (a.a. 64-89), only the 02CB12 differed from the consensus sequence with a conservative a leucine residue to a phenylalanine residue substitution at position 71 similar to the Porcillis vaccine.

3.3 Predicted antigenic differences

Our finding of the diversity in ORF5 sequences in the Thai isolates of both genotypes led us to compare the antigenicity profiles of the selected Thai isolates to the known ORF5 sequences including the live PRRSV vaccines (Fig. 4). The proximal end of the N-terminal part of the ORF5

proteins of 00CS1 and 02SP2 (US genotype) had higher predicted antigenicity than those selected US genotype sequences including both US live vaccines. The residue 89-109 region of only 03RB1 in the EU genotype had higher predicted antigenicity than those selected EU genotype sequences including the EU live vaccines.

4. Discussion

Porcine respiratory disease complex (PRDC) induced by PRRSV and other pathogens, currently, is the major problem of the Thai swine industry causing severe losses in the weanling and fattening pigs. The first isolate in Thailand was identified as the US genotype in 1996 (Damrongwatanapokin et al., 1996). Our study demonstrated the official report of the presence of the EU genotype in Thailand and two-third of the Thai PRRSV isolates was the EU genotype. Undoubtedly, both EU and US genotypes have been present in Thailand since Thailand has continuously imported swine breeders from both the European and North American countries. PRRSV might spread into the country via imported pigs or semen as evidence from the detection of anti-PRRSV antibodies in the imported pigs from the retrospective study (Damrongwatanapokin et al., 1996). Additional information from the CU-VDL using the nm RTPCR demonstrated the presence of approximately 7% of the mixed genotypes of PRRSV within the same herd (Unpublished data). The mixed infection may occur from bringing in PRRSV-positive replacement gilts or boars from the herds carrying different PRRSV strains. Prevention and control strategies in the mixed infection of both genotypes may be more complicated than the single strain infection. RNA recombination has been reported in PRRSV as a mechanism of generating heterogeneity (Murtaugh et al., 2001). However, no evidence of genetic recombination between the two genotypes was found in this study.

Ideally, studies on the genetic diversity of the Thai isolates should include a large number of isolates from all provinces and preferably cover the period from the first detection until now. We were unable to retrieve the samples before 2000 due to the technical problems. Our collection in the past three years is considered to be sufficient for this study. However, the origins of the PRRSV in Thailand were not clearly identified in this study. Within the genotypes, several minor branches were also observed (Fig. 2). Similar to the previous investigation, the clustering of the PRRSV isolates was not associated with geographic origins (Key et al., 2001). Those minor branches did not appear to be associated with the geographic origins of the Thai isolates except for 02SP2 and 02SP3, which shared 99.8% nucleotide identity of ORF5 and 99.50% amino acid identity. The two isolates were from the farms in closed geographical proximity. In addition, some Thai isolates are identical despite from different provinces. Introducing the PRRSV-positive animals into the herd may be the cause of PRRSV spreading throughout Thailand. This may explain why the presence of identical virus was found in different provinces.

Extensive diversity among PRRSV isolates has been documented (Andreyev et al., 1997; Key et al., 2001; Pirzdah et al., 1998). The preliminary data suggested that the US strain of the Thai isolates were closely related to the Canadian isolates (IAF-EXP91), whereas the EU isolates in Thailand were similar to the EU prototype, Lelystad virus. Most of the amino acid substitutions of the US strain locate in two short hypervariable regions at the terminal region (amino acid positions32-34 and 57-59) similar to the previous report having the hypervariable regions at amino acid positions 32-39 and 57-61 of the ORF5 protein (Key et al., 2001).

Based on theoretical antigenicity prediction, some Thai ORF5 protein sequences are different from those of the prototypes of both genotypes including the currently available live vaccines outside Thailand (Fig. 4). The differences at the N-terminal part of the ORF5 protein of

the US genotypes, 00CS1 and 02SP2, may make these particular Thai isolates unresponding to the neutralizing antibodies-induced by the currently available US vaccines since this protein was believed to be involved in neutralization (Dea et al., 2000). Similarly, 03RB1 (EU genotype) had higher predicted antigenicity than others including the EU vaccines. These evidences may imply of antigenic variation occurring in the Thai PRRSV.

Thailand currently does not have any modified live virus (MLV) vaccine of PRRSV available commercially. Interestingly, the EU strain isolated from the eastern part of Thailand, 02CB12 shared over 99% similarity of the ORF5 nucleotide sequence and 98.6% of amino acid identity with the Porcillis vaccine (AF378819). There are only two amino acid differences in the ORF5 between 02CB12 and the Porcillis vaccine. The Porcillis vaccine has an asparagine residue at amino acid position 150, whereas 02CB12 has an aspartic acid residue. The other substitution occurs at amino acid position 168 where a lysine residue in the Porcillis vaccine is replaced by an arginine residue in 02CB12. However, the ORF5 data from this study do not fully support the origin of 02CB12. Clearly, further studies are needed to determine the similarity of those viruses.

It is likely that the presence of genetic heterogeneity among PRRSV isolates will continue to be the major obstacle to effective prevention and control of PRRSV infection. In the near future, the MLV vaccines will be available in Thailand. The use of homologous MLV vaccine may lessen the clinical signs of the PRRSV-induced diseases, but there are still ambiguous on heterologous infections (Mengeling et al., 1999; Mengeling et al., 2003). The multi-strain attenuated PRRSV vaccines may be needed. The control strategies in the mixed infection of the two genotypes may be more complicated than the single genotype infection. Until now, the Thai swine practitioners have implemented several strategies including supportive treatment, culling

sick piglets and sows, minimized fostering, prolonged gilt acclimatization for at least 60-90 days and other husbandry management and biosecurity systems to control the outbreak.

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Table 1: Genotype, geographic origin, year of isolation and accession no. of PRRSV isolates examined in this study

Isolate ID	Genotype	Origin	Year	Accession no.
RespPRRS	US	USA vaccine	NA	AF066183
PrimePac PRRS	US	USA Vaccine	NA	AF066384
MD-011	US	Taiwan	NA	AF121131
EDRD-1	US	Japan	1992	D45852
IAF-EXP91	US	Quebec, Canada	1995	L40898
Lelystad	EU	The Netherlands	1991	M96262
DK111-92	EU	Denmark	1992	AJ223078
Porcillis	EU	The Netherlands, vaccine	NA	AF378819
Pyrsvac	EU	Spain, vaccine	NA	AF378820
00CS1	US	Chachoengsao (13)*, Thailand	2000	AY297111
01NP1	US	Nakorn Pathom (6), Thailand	2001	AY297112
01UD6	US	UdornThani (35), Thailand	2001	AY297113
02CB13	US	Chonburi (14), Thailand	2002	AY297114
02KK1	US	Khonkhen (28), Thailand	2002	AY297115
02PB1	US	Prachinburi (18), Thailand	2002	AY297116
02SP2	US	Suphanburi (56), Thailand	2002	AY297117
02SP3	US	Suphanburi (56), Thailand	2002	AY297118
01CB1	EU	Chonburi (14), Thailand	2001	AY297119
01RB1	EU	Rachaburi (57), Thailand	2001	AY297120
02BR1	EU	Burirum (21), Thailand	2002	AY297121
02CB12	EU	Chonburi (14), Thailand	2002	AY297122
02SB2	EU	Saraburi (11), Thailand	2002	AY297123
03RB1	EU	Rachaburi (57), Thailand	2003	AY297124

* See Fig 1 for the province location

Table 2: Prevalence of PRRSV genotypes in each region

Regions	EU	US	Total
Central	61	31	92 (67.15%)
North	15	4	19 (13.87%)
Northeast	10	4	14 (10.22%)
South	3	4	7 (5.11%)
Unidentified	2	3	5 (3.65%)
Total	91	46	137

Table 3A: Pair-wise comparison of nucleotides and amino acid sequences of the ORF5 gene of the PRRSV (US genotype).

PRRSV Isolates	Resp PRRS	VR-2332	PrimePac PRRS	MD-001	EDRD-1	IAF-Exp91	00CS1	01UD6	02PB1	02CB13	01NP1	02SP2	02SP3	02KK1
RespPRRS		99.70[b]	90.90	84.70	89.90	87.60	85.20	85.20	83.70	85.20	84.30	84.20	84.30	84.30
VR-2332	99.00		91.20	85.10	90.20	87.60	85.20	85.20	83.70	85.20	84.70	84.30	84.50	84.70
PrimePac PRRS	93.00	94.00		88.60	91.70	88.10	86.10	86.10	84.90	86.10	86.00	84.80	85.00	86.00
MD-001	84.00	85.00	88.50		88.40	85.40	83.30	83.30	82.80	83.30	82.80	83.00	83.20	82.80
EDRD-1	89.00	90.00	93.00	87.00		90.70	87.90	87.90	86.20	87.90	87.20	86.80	87.00	87.20
IAF-EXP91	86.00	85.50	89.00	85.50	89.00		90.00	90.00	89.10	90.00	89.80	89.00	89.20	89.80
00CS1[a]	85.50	85.00	87.50	83.50	87.00	90.00		100.00	89.40	100.00	90.20	89.00	89.20	90.20
01UD6	85.50	85.00	87.50	83.50	87.00	90.00	99.50		89.40	100.00	90.20	89.00	89.20	90.20
02PB1	85.00	84.50	87.50	85.00	86.00	90.00	90.50	90.50		89.40	89.30	88.70	88.80	89.30
02CB13	85.50	85.00	87.50	83.50	87.00	90.00	99.50	99.50	90.50		90.20	89.00	89.20	90.20
01NP1	85.00	85.50	87.50	84.00	87.00	90.50	90.00	90.00	90.00	90.00		97.50	97.30	100.00
02SP2	83.00	83.50	85.50	84.00	85.50	88.00	87.50	87.50	87.50	87.50	96.50		99.80	97.50
02SP3	83.00	83.50	85.50	84.00	85.50	88.00	87.50	87.50	87.50	87.50	96.50	99.50		97.30
02KK1	85.00	85.50	87.50	84.00	87.00	90.50	90.00	90.00	90.00	90.00	99.50	96.50	96.50	

[a] The Thai isolates are shown in bold.

[b] The values in the table represent percentage of nucleotide (upper right half) or amino acid (lower left half) sequence identities.

Table 3B: Pair-wise comparison of nucleotides and amino acid sequences of the ORF5 gene of the PRRSV (EU genotype).

PRRSV Isolates	Lelystad	Porcilis	Pyrsvac	DK111-92	01CB1	01RB1	02BR1	02SB2	03RB1	02CB12
Lelystad		98.60[b]	93.30	92.00	87.50	87.50	93.30	97.80	87.00	98.50
Porcilis	97.9		93.1	89.4	84	84	93.1	96.3	83.6	99.1
Pyrsvac	92.4	91		90.3	84.5	84.5	89.8	91.2	85.6	92.6
DK111-92	93	89.6	92.4		88.7	88.7	87.2	90.3	87.5	91.3
01CB1[a]	88.8	86.1	88.2	89.8		100	84	86.2	93.5	86.5
01RB1	88.7	86.1	88.2	89.7	100		84	86.2	93.5	86.5
02BR1	92.3	92.4	89.6	87.7	85.6	85.6		91.5	85.5	93.3
02SB2	98.5	96.5	91	91.5	87.2	87.2	90.8		85.6	96.6
03RB1	88.3	85.4	88.9	89.3	95.4	95.4	86.7	86.8		86.2
02CB12	98.5	98.6	91	91.5	88.3	88.2	91.8	96.5	87.8	

[a,b] See Table 3A for key

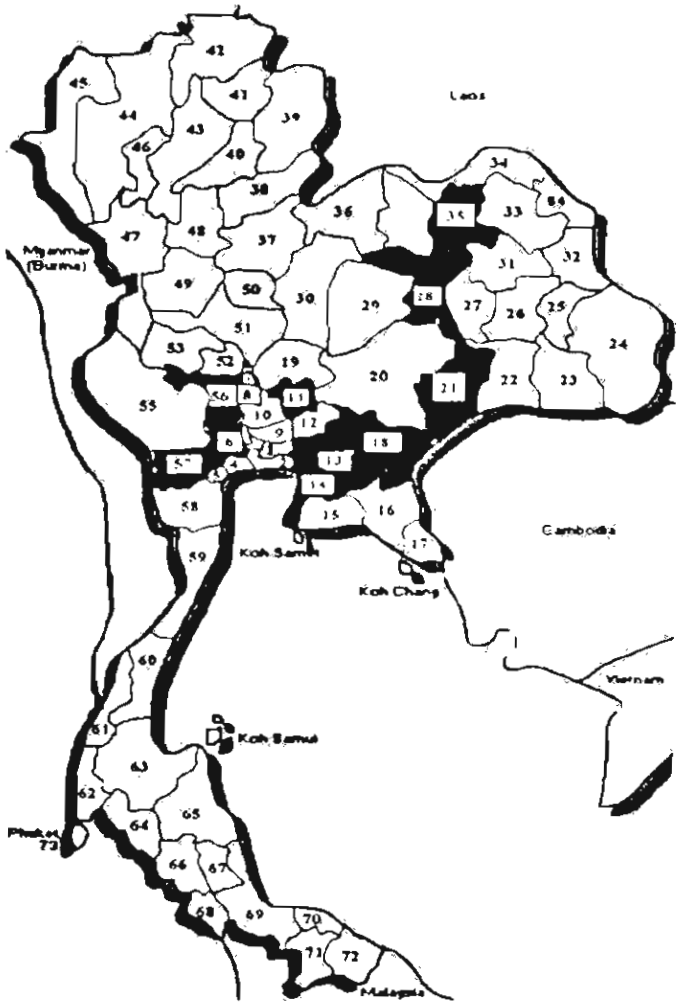

Fig. 1 Map of Thailand. Dark areas represent the provinces where the selected viruses were tested in this study. See Table 1 for the virus origin.

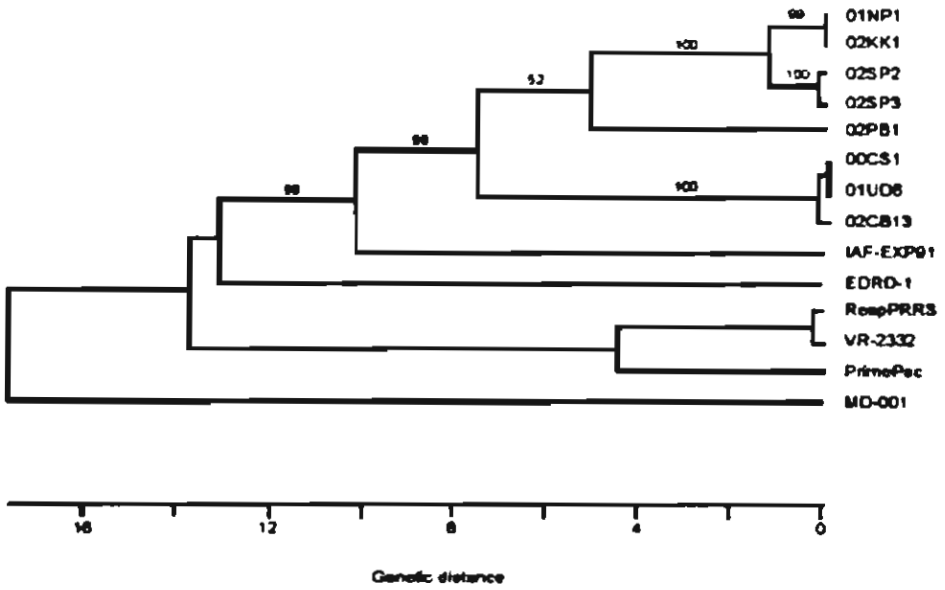

Fig. 2A

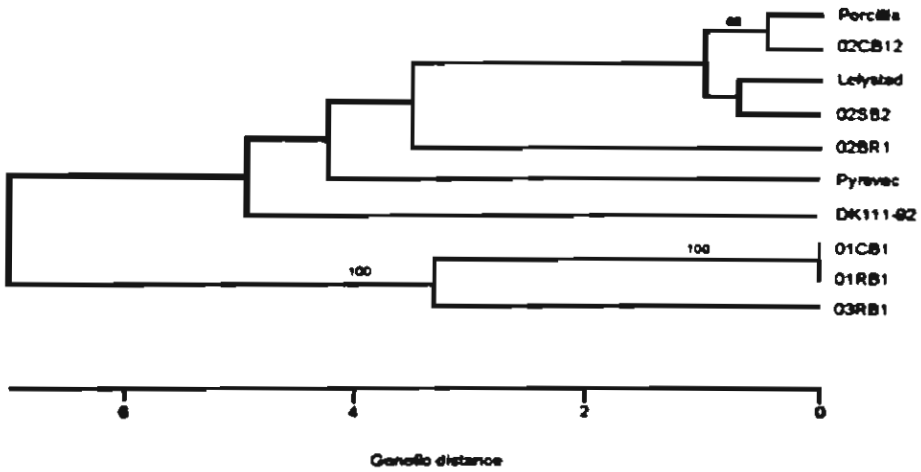

Fig. 2B

Fig. 2 Dendrogram based on the nucleotide sequence of the ORF5 gene of PRRSV (Fig. 2A = US genotype, Fig. 2B = EU genotype). The phylogenetic tree was generated using Clustal method with weighted residue weight table, MegAling program (DNASTAR, Madison, WI). Bootstrap analysis was performed using PAUP version 4.02b with 1,000 bootstrap replications. The values adjacent to each node represent the percentage of 1,000 bootstrap trees that support the clustering.

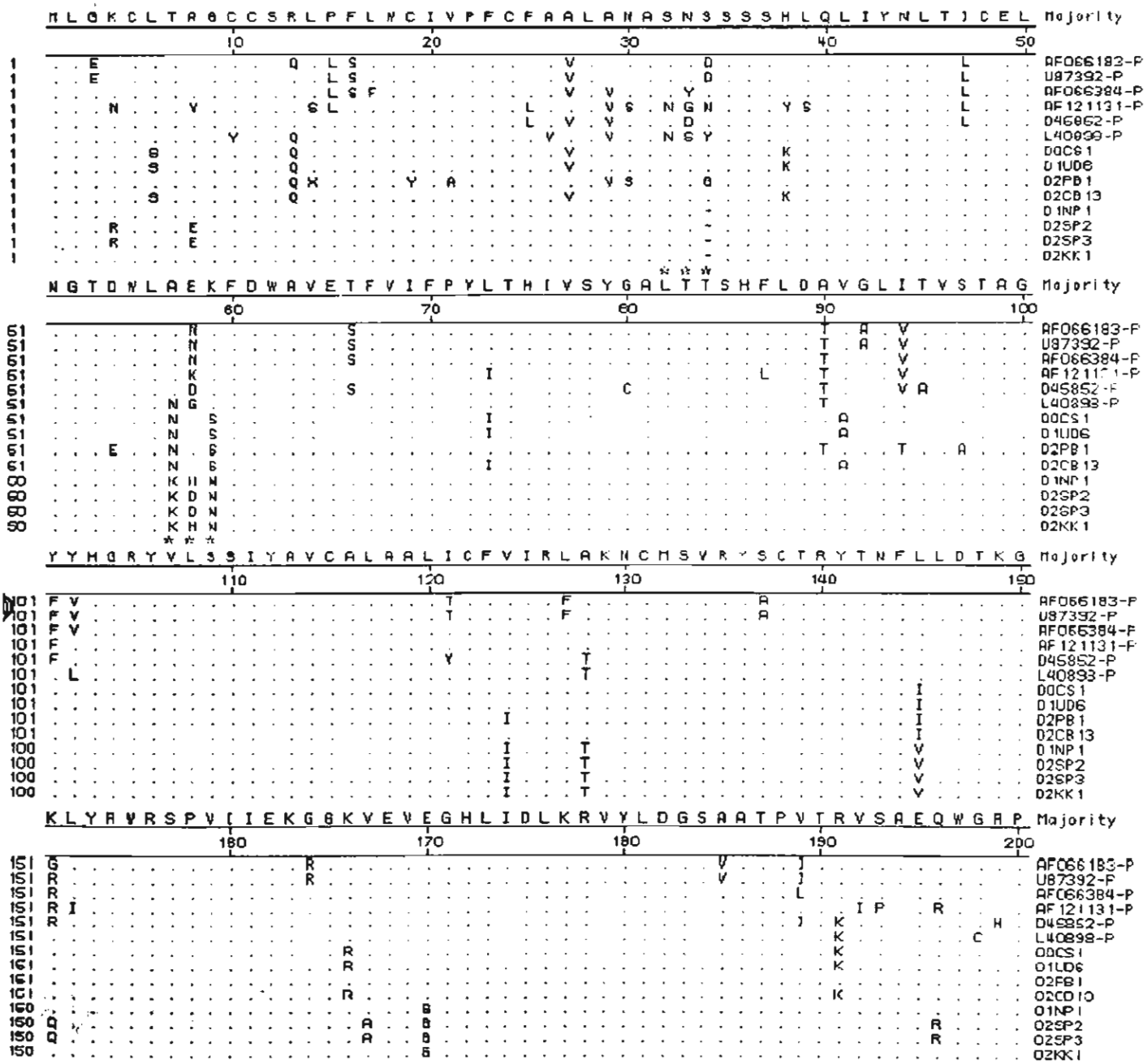
Majority
AF066183-P
U87392-P
AF066384-P
AF121131-P
D46862-P
L40899-P
D0CS1
D1UD6
D2PB1
D2CB13
D1NP1
D2SP2
D2SP3
D2KK1

Fig. 3A

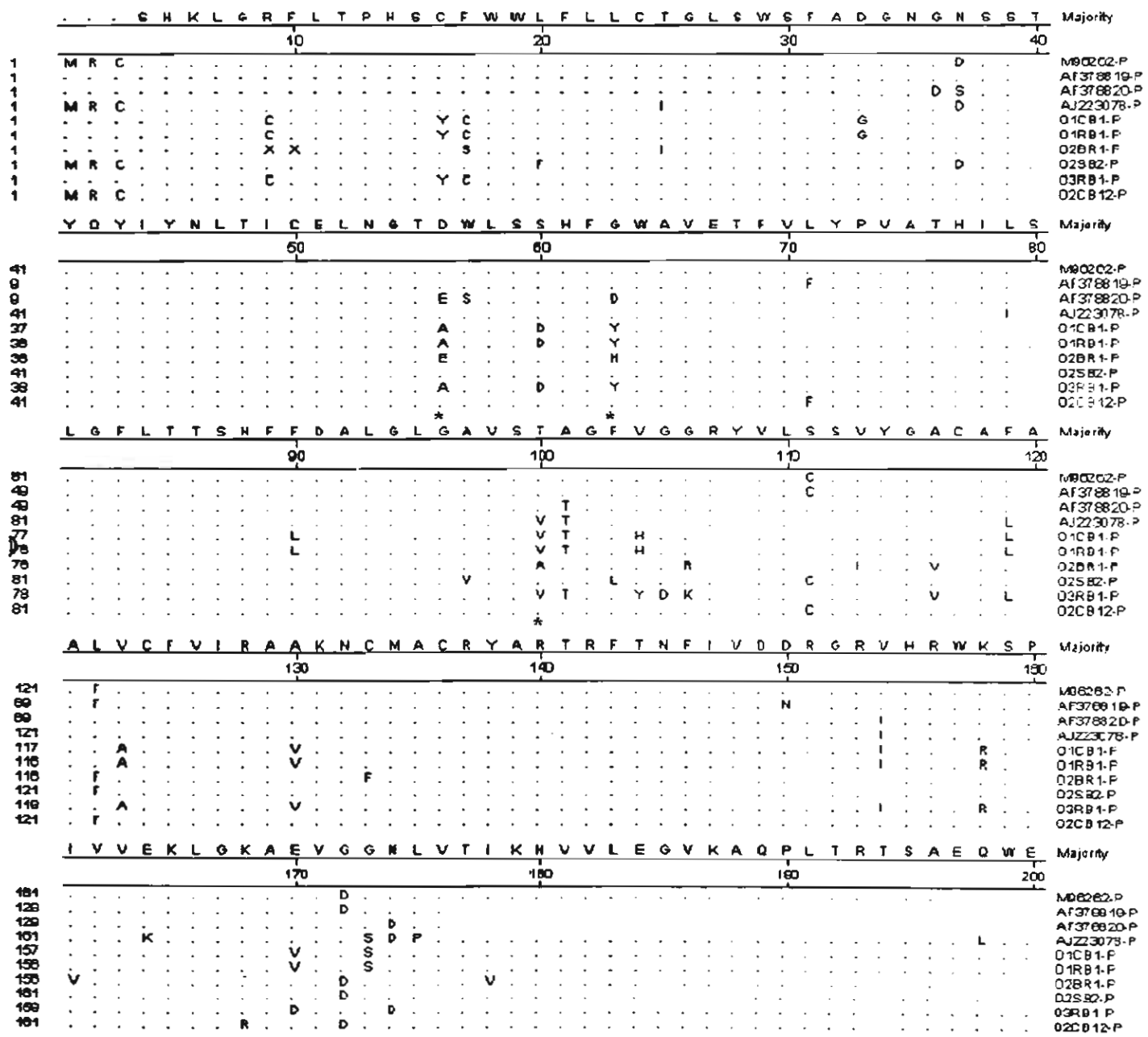

Fig. 3B

Fig. 3 Amino acid sequence alignment of ORF5 of PRRSV (Fig. 3A = US genotype, Fig. 3B = EU genotype). The consensus sequence is shown on the top. The amino acid identities are indicated by period (.). The hypervariable regions are indicated by asterisk (*).

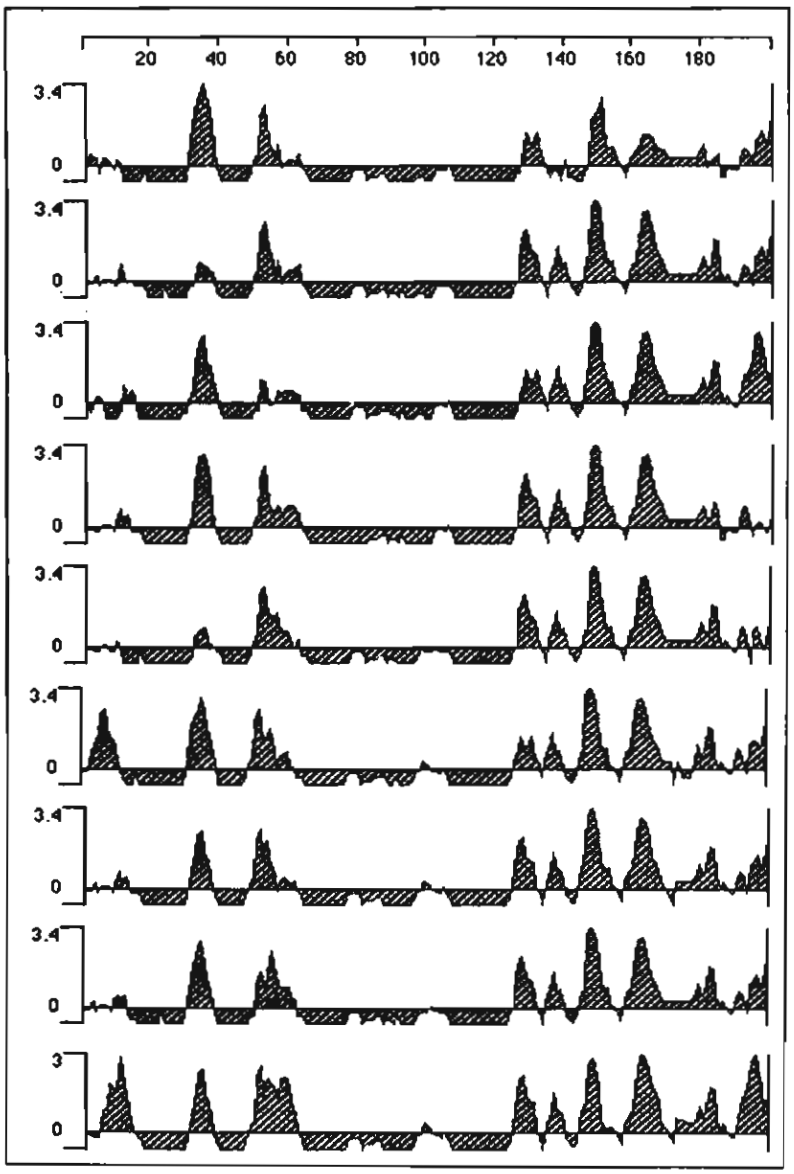
20
40
60
80
100
120
140
160
180
3.4
0
3
RespPRRS
PrimePac PRRS
MD-011
EDRD-1
IAF-EXP91
00CS1
01NP1
02PB1
02SP2

Fig. 4A

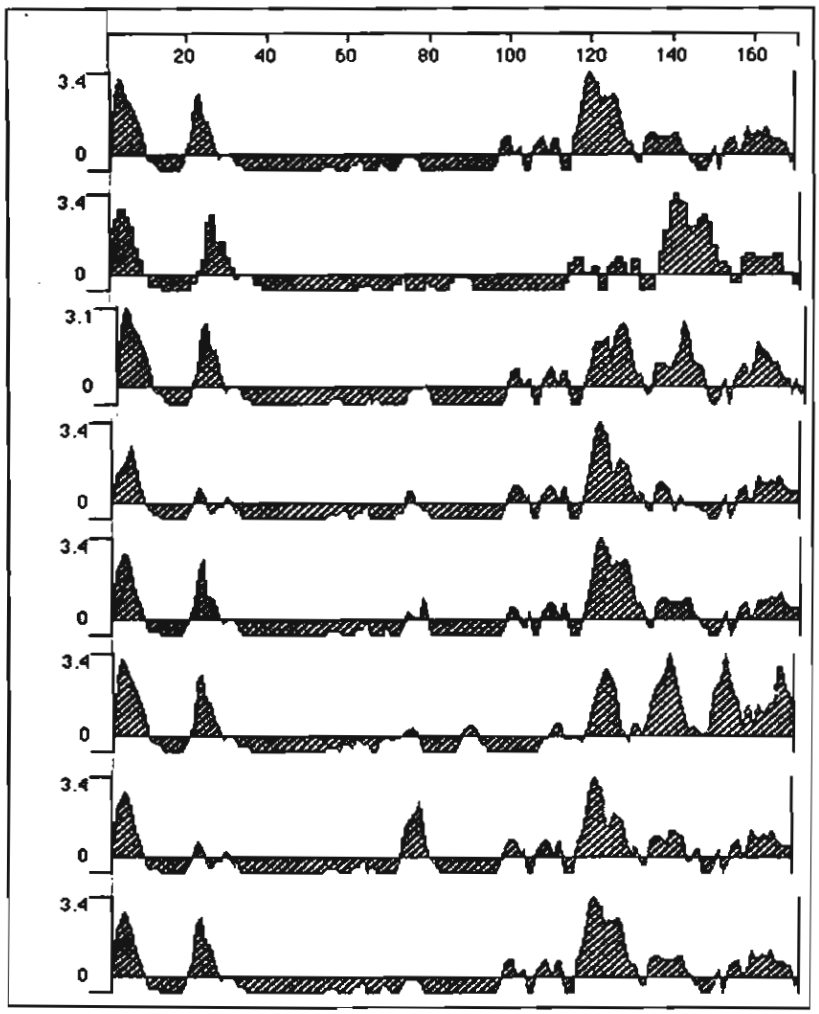

Fig. 4B

Fig. 4 Antigenicity plot (Fig. 4A =US strain and Fig. 4B = EU strain) of selected PRRSV based on the ORF5 trees in Fig. 2. The Jameson-Wolf antigenicity plot was generated using a computer program, PROTEAN (DNASTAR, Madison, WI). A high score indicates high antigenicity. A detailed description of the selected sequences is given in Table 1. The residue at the amino acid position 33 was used at the beginning of the plot in Fig 4B.