Chapter 3

Materials and methods

Part 1 Restriction fragment length polymorphism of ORF5 in selected Thai isolates of PRRSV.

1 Virus isolation

Ten Thai PRRSV isolates were obtained from the Chulalongkorn University, Veterinary Diagnostic Laboratory (CU-VDL). The virus was isolated from swine sera or tissues submitted from swine farms throughout Thailand using MARC-145 cell line (kindly provided by Chris Morrisy, CSIRO, Geelong, Australia) or pulmonary alveolar macrophages (PAMs). PRRSV-free pigs were kindly provided by CP group from Thacham, Chonburi.

1.1 Preparation of MARC-145

MARC-145 seed stock (- 70°C) was thawed in 37°C waterbath. The cell suspension was diluted with 5 ml of MEM and then centrifuged at 2,000 rounds per minute (rpm) for 10 minutes and then the supernatant was aspirated out. The cell pellet was mixed with 10% FCS (Fetal calf serum) MEM (Minimal essential medium) (Hyclone, USA) 20 ml and the cell suspension was placed in a 75 mm^3 culture bottle (Corning Incorporated, USA). The culture bottle was incubated in 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 versience

1 ml at 37°C, 5 min. 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 were diluted in the media until reaching 24 ml and filled in a 24 well plate (Corning Incorporated, USA), 1 ml 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 hour for viral absorption. After that, 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 infected onto the cell line, similarly to the 1st passage. After 2 days, the cells and media was collected in an eppendorf tube at - 70°C until used.

1.3 Indirect immunoperoxidase monolayer assay (IPMA)

IPMA was used to confirm if the PRRSV was present in the cell culture. Breifly, the cell preparing for IPMA were trypsinized MARC-145 and filled 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 2nd passage was inoculated onto the cell line and incubated for 48 hours.

The IPMA, monolayer was fixed with 4% formalin* 100 µl / well at room temperature (RT) for 30 min and washed 3 times with 0.5% PBST*. The N protein of

PRRSV was detected by the monoclonal mouse anti-PRRSV antibody (SDOW17 : South Dakota State University, USA). Fifty μl of SDOW17 (1:300) was filled in each well and incubated in 5% CO_2 at 37°C for 1 hour and washed 3 times with 0.5%PBST. Then, 50 μl of the conjugated antimouse immunoglobulin G (IgG) (Dako, USA) (1:300) of the secondary antibody was added to well and incubated in 5% CO_2 at 37°C for 1 hour and washed 3 times with 0.5% PBST. The substrate was filled 100 μl / well in room temperature for 1 hour and washed with tap water. The conjugated IgG reacted with the substrate and given darked brown granule in the cytoplasm of PRRSV infected cells under light microscope (Bautista et al., 1993). The viral titer (tissue culture infectious dose; $TCID_{50}$ / ml) was calculated using Reed and Muench (1938) method.

2. Restriction Fragment Length Polymorphism based on RT PCR

2.1 RNA extraction

The viral RNA was extracted from the mixture of cell and media by QIAamp® Viral RNA Mini Kit (QIAGEN, germany). Briefly, 40 μl of the mixture was lysed with buffer AVL* 560 μl at room temperature for 10 min. Five hundred fifty μl of absoluted ethanol was added to the solution and centrifuged at 8000 rpm for 1 min. The washing solution (AW1 and AW2*) was added and centrifuged 8000 rpm for removing contamination. The RNA was eluted from membrane by adding 60 μl of buffer AVE* and 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) was 50 μl in volumn containing 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'-CCATTCTGTTGGCAATTTGA-3' (sense) and P620, 5'-GGCATATATCA TCACTGGCG-3' (antisense) (Andreyev et al., 1997) and for the EU isolates were ETS5L, 5'-GGATCCATGAGATGTTCTCACAAATTGG-'3 (sense) and ETR5L, 5'-GGATCCCATCTA 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 with the following thermocycling programs: cDNA synthesis at 50°C for 30 min, inactivation of reverse transcriptase and denaturation at 95°C for 15 min, 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, final extension at 72°C for 10 min and holding at 4°C (Andreyev et al., 1997). The PCR products were detected in a 2% agarose gel (FisherChemical, USA) by electrophoresis at 100 V, 1.5 A for 1 hour. The gel electrophoresis was stained with ethidium bromide (Promega, USA) for 30 min then washed with tap water. The DNA band of ORF5 was analyzed in the UV illuminator.

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* detail in appendix

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 volumn and filled into the provided column, centrifuged at 8000 rpm. DNA (100 bp -10 kp) was absorbed in the high-salt silica membrane. Buffer PE* was added about 0.75 ml and centrifuged at 8000 rpm for 1 min. PCR product was eluted with 50 µl of RNAse free water, centrifuged at 13000 rpm for 1 min and kept in - 20°C until needed.

2.4 Restriction Fragment Length Polymorphism

The purified PCR products were measured for the concentration by diluting 100 times (product 7 µl, DW 700 µl) using the optical density (OD) at wavelength 260 and 280 nanometer (nm) (Spectronic 20 genesys, Germany). The concentration was using the formula : [PCR product] (µg/ml) = OD_{260} x 50**x 100 (dilution factor). The purity of nucleic acid was measured by a ratio of OD_{260}/OD_{280} (Sambrook et al., 1989).

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 (in table 1) at 37°C for 2 hours (MBI Fermentas, USA). The cutting patterns were electrophoresed on 2% agarose gel, 100 V, 1.5 A for 1 hour, stained with ethidium bromide (Promega, USA) and analyzed with the UV illuminator.

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* detail in appendix

** The average absorbance 1 = DNA 50 µg/ml (Sambrook et al., 1989)

Table 1 Restriction sites, sources and appropriate buffer for retriction enzymes used for US genotype (*Mlu*II , *Hinc*II , *Sac*II and *Hae*III) and EU genotype (*Pst*I , *Hae*II and *Cla*I).

Restriction Fragment Length Polymorphism				
Geno type	Restriction enzymes	Source	Cutting site	Buffer*
US	*Mlu*II[a]	*Micrococcus luteus*	5' A^{v}CGCGT TGCGC^{v}A	Buffer R^{+} or 2xγ^{+} TANGO™
	*Hinc*II[b]	An *E. coli* strain that carries the cloned *hincII* gene from *Haemophilus influenzae* Rc (ATCC 49699)	5' GTY^{v}RAC CAR^{v}YTG	1x NEBuffer3
	*Sac*II (*Cfr*42I)[a]	An *E. coli* strain that carries the cloned *cfr42I* gene from *Citrobacter freundii* RFL42	5' CCGC^{v}GG GG^{v}CGCC	Buffer B^{+} or 1xγ^{+} TANGO™
	*Hae*III (*Bsu*RI)[a]	*Bacillus subtilis* R	5' GG^{v}CC CC^{v}GG	Buffer R^{+} or 1xγ^{+} TANGO™
EU	*Pst*I[a]	*Providencia stauarti*	5' CTGCA^{v}G G^{v}ACGTC	Buffer R^{+} or 1xγ^{+} TANGO™
	*Hae*II (*Bsp*143II)[a]	An *E. coli* strain that carries the cloned *bsp143IRI* gene from *Bacillus species* RFL143	5' RGCGC^{v}Y Y^{v}CGCGR	1xγ^{+} TANGO™
	*Cla*I (*Bsu*15I)[a]	*Bacillus subtilis* 15	5' AT^{v}CGAT TAGC^{v}TA	1xγ^{+} TANGO™

[a] MBI Fermentas, USA, [b] Biolabs Inc., England

Code Y, pyrimidine (C/T); R, purine (A/G).

Part 2 Pathological study of the selected Thai isolates of PRRSV.

Twenty - one 3 weeks old pigs, PRRSV – free pigs kindly provided by C.P. Group, Thacham, Chonburi. The pigs were devided into 3 groups : a control group (3), US genotype-infected (9) pigs and EU genotype-infected (9) group. Each group was separated in each room using the strick biosecurity system.

All pigs were injected with antibiotic, Ceftiofur (Excenel®, pharmacia USA) 3 days for acclimatization. Pigs were inoculated intranasally (IN) 1 ml and intramuscularly (IM) 1 ml with 01NP1* (US genotype, $10^{2.5}$ $TCID_{50}$/ml), 02SB3** (EU genotype, $10^{2.5}$ $TCID_{50}$/ml) or uninfected media*** in an appropriated group. Three pigs from each PRRSV – infected group and 1 pig from the control were subjected for necropsy at 5, 9 or 15 days post inoculation (dpi.).

1. Clinical signs

The pigs were observed and scored for respiratory signs everyday as the following criteria : 0, normal, 1, dyspnea and/or mild tachypnea when stress, 2, dyspnea and/or mild tachypnea when rest, 3, dyspnea and/or moderate tachypnea when stress, 4, dyspnea and/or moderate tachypnea when rest, 5, dyspnea and/or severe tachypnea when stress, 6, dyspnea and/or severe tachypnea when rest (Halbur et al., 1995b). The stress was induced daily by handling the animal for rectal temperature. Other relevant

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* Nakorn pathom, 2001

** Saraburi, 2002

*** The media of non infected MARC-145 cell.

clinical observation (coughing, diarrhea, inappetence or lethargy) was noted separately and was not reflected in the respiratory disease score. The relative humidity in the rooms was also recorded.

2. Macroscopic findings

The pigs were sacrified with pentobarbital sodium (Nembutal®, USA) injection for a complete necropsy at the assigned dates. The total point for all the lung lobes as an estimation of the percentage of the entire lung with grossly visible pneumonia. The pig lung composes of 7 lobes : 2 for cranial lobes, 2 for cardiac lobes, 2 for diaphramatic lobes, and 1 intermediate lobe. The scoring system is summarized in Fig 4. Other organs consisting of brain, tonsil, lung, heart, liver, kidney, spleen, ileum and mesenteric lymph node were preserved in 10% buffer formalin and in - 70°C until needed.

3. Microscopic findings

The formalin fixed tissues were trimmed and processed in the tissue processor. Briefly, tissues were dehydrated by 70, 80, 95 and 100% ethanol and xylene, respectively. The tissues were embeded in a paraffin block and cut to 4 - 6 μm by a rotary microtome, then placed on slide (for routine Hematoxylin & Eosin; H & E staining) or poly-L-lysine coat slide (for Immunohistochemistry; IHC).

The 4 - 6 μm tissues were deparafinized and rehydrated by xylene, 100, 95, 85, 70% ethanol and DW, respectively. The slides were stained with Harris's hematoxylin and then stained with eosin. The slides were dehydrated by 70, 80, 95, 100 % ethanol

and xylene, respectively. The microscopic lesions were noted and estimated score of the severeity of the interstitial pneumonia in the lung as the following : 0, no microscopic lesions, 1, mild interstitial pneumonia, 2, moderate multifocal interstitial pneumonia, 3, moderate diffuse interstitial pneumonia, 4, severe interstitial pneumonia (Halbur et al ., 1995b).

4. Viral detection

PRRSV infection was confirmed by immunohistochemistry on lung tissue and virus isolation from sera. The virus genotypes were confirmed using nested multiplex PCR (Thanawongnuwech et al., 2002).

4.1 Immunohistochemistry

PRRSV antigen was detected by monoclonal mouse anti - PRRSV antibody (SDOW17, USA, kindly provied by Dr. E. Thacker, Ames, Iowa). Briefly, sections on poly-L- lysine coated slides were deparafinized and rehydrated by xylene and graded ethanol, DW and PBS, respectively. The tissues were digested by trypsin at 37°C for 30 min and washed with PBS 3 times. The endogenous peroxidase enzyme was removed using 3% hydrogen peroxide (H_2O_2) in methanol 30 min in room temperature and then with DW 5 min and wash with PBS 3 times. The nonspecific background staining was blocked with 10%BSA (Fluka, Switzerland) in DW at 37°C for 30 min and washed with PBS 3 times. The PRRSV antigen was attached with SDOW17 : DW (1 : 1000) at 4°C for 15 hours and washed with PBS 3 times. The IgG was attached with peroxidase

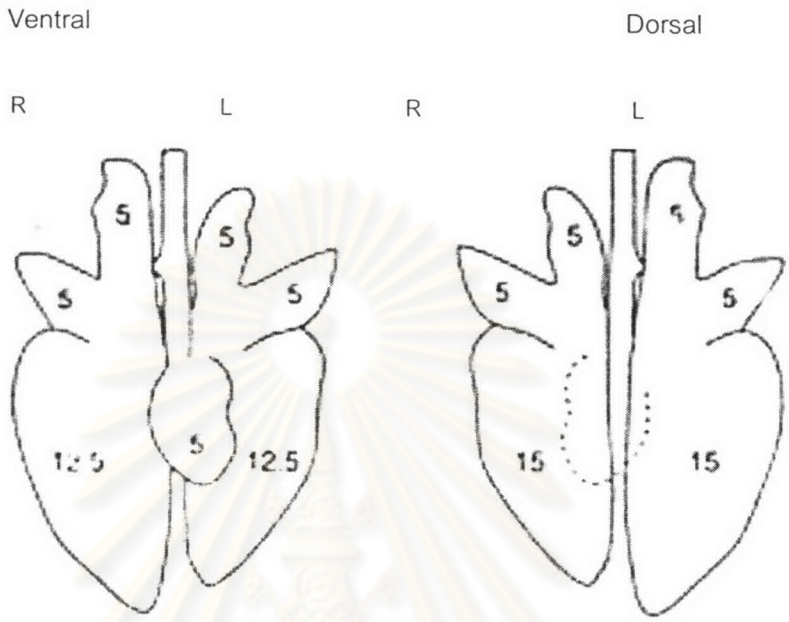

Fig 4 Gross lung lesion scoring system (100 points) (Halbur et al., 1995b)

antiperoxidase (Nichirei, Japan) at 37°C for 1 hour and then washed with PBS 3 times. The Diaminobenzidinetetra chloride (DAB)* solution was prepared before use. The DAB was reacted with the Ag - Ab complex and then the brown granular staining was appeared in the cytoplasm of the PRRSV - infected cells within 5 min. The reaction was stoped by rinsing with DW. The slides were counter stained with hematoxylin for 1 min and then dehydrated with graded ethanol and xylene (Halbur et al., 1994).

4.2 Virus isolation (VI)

VI was performed on serum, bronchial larvage and tissue homogenize from all pigs as described previously in chapter 3, part 1, page 19.

4.3 Polymerase chain reaction

Swine fever and porcine circo virus were ruled out from the animals used before inoculation (pooled sera at 0 dpi) using routinely PCR performed at the CU - VDL. The strains of virus in pigs post inoculation (pooled sera in each group at 0, 5, 9, 15 dpi) was confirmed by Nested Multiplex RT - PCR* as previously described by Gilbert et al. (1997).

5. Serology

Serological tests were performed at the CU - VDL. The antibody titers to other swine pathogens such as, Aujeszky's disease virus and swine fever virus from pigs before inoculation were measured at 0 dpi. by serum neutralization*. The antibody response to PRRSV from all pigs was tested using Enzyme - Linked Immunosorbent

Assay (ELISA)* (Herdchek®, Idexx, USA) at 0, 5, 9, 15 dpi as described by the manufacture's instruction.

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