Chapter 5

Discussion

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

Among the 5 selected Thai isolates from each genotype, the US genotype had 3 different cutting patterns and the EU genotype also had 3 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 Suphan Buri (02SP2, 02SP3) and 1 isolate from Nakorn pathom (01NP2) had the same *Hinc*II cutting pattern as the MLV vaccine. Despite having the same *Hinc*II RFLP pattern, the Suphan Buri isolates could be distinguished from the MLV vaccine virus by different *Hae*III cutting patterns. Taken together, the 3 enzymes could be used for differentiation. Similar to the previous study, the MLV and its parent virus, VR2332 could be distinguished from other

PRRSV field isolates by *Mlu*I, *Hinc*II and *Sac*II. The other restrictive enzymes, *Hae*III (Madsen et al., 1998) and *Msp*I (Itou et al., 2001) were added, for more precise cutting patterns. In the EU genotypes, *Pst*I and *Cla*I were able to differentiate the EU isolates from the EU MLV vaccine except for the Burirum isolates (02BR1) which had the same pattern as the MLV vaccine. However, the Burirum isolate could be differentiated from the vaccine using *Hae*II. In conclusion, using *Pst*I, *Cla*I combining with *Hae*II with the EU ORF5 product, the EU MLV vaccine could be distinguish from the EU isolates.

In this study, we did not see any field isolate having the same cutting pattern as the MLV vaccines either in the US or the EU isolates. In contrast to the previous reports, the field isolates had the 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 the US MLV vaccines a few years before the investigation. The MLV vaccine using in those countries might have reverted to a more virulent strain (Nielsen et al., 2001). Fortunately, Thailand does not allow the use of MLV vaccine. Only the killed PRRSV vaccine is available. In these case, to distinguish the PRRSV vaccine strain from other PRRSV field isolates, it is necessary to have series of restriction enzymes to obtain more specific RFLP patterns. Alternatively, nucleotide sequence would be better to differentiate wild type PRRS viruses from the vaccine virus (Cheon and Chae, 2000). However, our number of PRRSV in this study was limited. More recent field isolates needed to be studied for a better result, if the MLV vaccine virus was present in

Thailand. If so, the vaccine virus may come with the imported breeder stocks or illegal smuggling of the vaccine.

Our results were able to demonstrate the different cutting patterns of the selected Thai PRRSV isolates. Those cutting patterns of the open reading frame 5 (ORF5) were able to divide the PRRSV isolates suggesting of the presence of genetic variation in each genotype isolated in Thailand. ORF5 is suitable for the RFLP study for genetic variation since the antibodies neutralize the virus in these 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). In case of evading the host immune system, the particular virus may have greater potential to be shed to other pigs and to establish persistent infections. The differences of the cutting pattern probably caused by 1) the introduction of a new variant into the area or 2) local evolution. In case of finding the different variants at the same time or in the vicinity areas, the genetic variability was possibly caused by the introduction of animals or using contaminated semen rather by the local evolution (Martelli et al., 2003). However, the virus probably maintained in the sows and underwent evolution gradually driven by several factors including recombination, random mutation or natural selection. Regarding to 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 determine the presence of the recombination. However, based on our ORF5 sequencing results, no evidence of recombination 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 the experimental infection in the cell culture system (Yuan et al., 1999; Joke et al., 2001) or in the pigs (Mengeling et al., 2000). In addition, the genetic drift has occurred as suggested by Murtaugh et al (2003) that many strains isolated in the early 1990's had no close relation to the current isolates. Interestingly, most ORF5 variants underwent negative selection and disappeared from continuing passages in pigs in one study (Yoon et al., 2003). In conclusion, the genetic variation certainly is present among the Thai isolates, even within the same genotype.

In order to study the pathogenesis of the Thai isolates of PRRSV in both genotypes, 01NP1 (US genotype) and 02SB3 (EU genotype), the selected PRRSV isolates, were used as the infection model using pigs from a PRRSV - free herd. After inoculation, the pigs had continuous fever in both inoculated groups and some transient fever in the control group due to the high humidity. The duration of fever in the 01NP1 - infected pigs (2 - 10 days) was longer than those in the 02SB3 - infected pigs (2 - 3 days). Other relevant clinical signs including moderate lethargy, anorexia and rough hair coat were observed in both inoculated groups but more severe symptoms were seen in the 01NP1 - infected pigs. One pig (US/4) from the 01NP1 - infected group had severe weakness and died at 14 dpi due to mismanagement on blood collection. Necropsy revealed severe bleeding at the anterior vena cava. The results suggested that severe clinical signs were induced by the US genotype of the Thai isolates similar to the previous report (Halbur et al., 1995a). It should be noted that, the pigs in the EU

group had greater respiratory scores than those in the US group. However, the increasing of relative humidity in the room of the EU group was higher.

Similar to the previous reports (Halbur et al., 1995b), the respiratory system had the most remarkable lesions. Grossly pneumonia was characterized by multifocal, mottled - tan areas, non collapsing, firm, rubbering with irregular and indistinct borders. Microscopically, multifocal interstitial pneumonia was characterized by septal thickening with mononuclear cells, type 2 pneumocyte hypertrophy and hyperplasia and accumulation cellular debries in the alveolar spaces with peribronchiolar and perivascular cuffing. The gross and microscopic lesions were seen from 5 dpi, peaked at 9 dpi and remained severe until 15 dpi for both inoculated groups. Similarly, Stevenson et al. (2003) reported that the gross and microscopic lesions were observed from 3 to 28 dpi and were most severe at approximately 10 dpi. Normally, the lesions are seen within 48 hours of infection, most severe by 10 days and begin to resolve by 21 - 28 days post infection. Immunohistochemistry was able to demonstrate PRRSV antigen in lungs of all infected pigs at all necropsied dates. No PRRSV antigen was detected in the control pigs. Grossly, affected lymph nodes were enlarged from 2 - 4 times of normal size approximately 5 - 15 dpi and microscopically, mild to severe lymphoid necrosis similar to the previous study (Dea et al., 1992; Halbur et al., 1995a; Rossow et al., 1995). Both of inoculated groups had moderate petechial hemorrhage on the cortex of kidneys as early as 5 dpi and milder at 9 and 15 dpi. The renal lesions in the previous reports were seen from 14 - 42 dpi and were seen in the pelvis and medulla

characterized by mild periglomerular and peritubular lymphohistiocytic aggregates and vasculitis (Cooper et al., 1997; Rossow et al., 1995). Interestingly, petechial hemorrhage on the renal cortex compatible with the lesion of classical swine fever (CSF) was present in this study. However, no evidence of swine fever virus antigen was detected in this study using virus isolation or PCR for CSFV. Neutralization peroxidase linked assay (NPLA) was gradually decreased from 0 until 15 dpi. indicating that the antibody titers were actually maternal immunity. We believed that the petechial hemorrhage found in this study was induced by PRRSV by unknown mechanisms. The nasal mucosa lesions were detected early at 5 dpi similar to the previous reports that rhinitis was seen as early as 12 hours post infection (Collins et al., 1992; Rossow et al., 1995; Halbur et al., 1996). The presence of PRRSV antigen in the nasal tissue (unpublished datas) may imply that nasal discharge would be another route of virus shedding to another pig. The non suppurative encephalitis and non suppurative myocarditis were seen at 15 dpi similar to other experiments that the brain and heart lesions developed after 7 dpi (Collins et al., 1992; Rossow et al., 1995; Halbur et al., 1995a). In conclusion, the severity of the PRRSV lesions varied with the virulence of the virus strains (Halbur et al., 1996). The selected PRRSV - isolates in Thailand especially 01NP1 was able to induce severe clinical signs and lesions than the EU genotype (02SB3). The most consistent gross and microscopic lesions in the PRRSV - inoculated pigs were seen in the lung and the lymphoid tissues. However, microscopic lesions were also seen in the heart and brain.

Less frequently, microscopic lesions had been described in kidney, nasal mucosa (Rossow et al., 1995).

Finally, the RFLP study was able to determine that all selected Thai isolates did not have similar cutting patterns to the MLV vaccine in either genotype. Genetic variation definitely occurred in those isolates in both genotypes. The US genotype (01NP1) was of high virulence than the EU genotype (02SB3). Most lesions found in this study were similar to the previous reports excepted for the kidney lesions. In order to confirm those lesions, more pigs need to be used for the next experiments. However, differential diagnosis of PRRSV infection should be considered when petechial hemorrhage was present at necropsy.