CHAPTER V

DISCUSSION AND CONCLUSSION

The current Thai PEDV isolates collected during 2011-2012 were investigated their genetic relationship with reference isolates based on partial spike (S) and nucleocapsid (N) gene sequences. The 1000 replicates of bootstrap values were used for accurate distance estimation of each clade.

- **The partial S gene sequences**

The 428 bp of partial S gene sequence was amplified from nucleotide positions 1516-1965, encoding for 141 amino acids, which situated on positions 514-655 of S1 C-terminal domain (Lee et al., 2010). The amplicon was compared with reference S gene sequence of strain CV777, accession number AF353511.1 with a total length of 4152 nt or 1383 amino acids of PEDV reference strain (PEDV strain CV777). The phylogenetic tree was generated based on partial S gene sequences, resulting in a classification of current Thai isolates into 3 groups along with 4 subgroups of group 3 (subgroups 3.1, 3.2, 3.3 and 3.4). Most of current Thai isolates were located in subgroup 3.1 as same as previous Thai, Chinese, Vietnamese and Korean isolates, except for TH/NP-68/12 that was separated into subgroup 3.4 (Table5).

From a previous report, the Thai isolates showed a highly similarity to Chinese isolates such as JS-2004-2 and LJB/03 (Puranaveja et al., 2009). Other previous reports by Lee et al. (2010) and Duy et al. (2011) revealed that Chinese isolates were genetically related to Korean and Vietnamese isolates, respectively. From this circumstance, it indicates that the current Thai PEDV had a close genetic relationship to previous isolates, which were report in 2009 and also exhibited more closed related to Chinese isolates as GD-1 and GD-A.

There were some evidences of genetic diversity between current Thai isolates: TH/NP-68/12 and TH/RB-1421/11 were separated out of main Thai clusters, Noteworthy, the farm represented TG/RB-1421/11 isolate had subsequently two re-outbreaks as TH/RB-KHF/11 and TH/RB-123/12 which occurred in 1 and 5 month interval. The phylogenetic tree showed the genetic relationship between TH/RB-KHF/11 and TH/RB-123/12 had higher

nucleotide identity to each other than TH/RB-1421/11. It possibly signifies that both isolates might be mutations of TH/RB-1421/11.

The other re-outbreak samples indicated genetic similarity with few point mutations, especially samples from a couple re-outbreaks as TH/AY-2.2/12 and TH/AY-2.7/12, which had 100% nucleotide identity without any mutations. The isolate of AY-2.2/12 was found in February and TH/AY-2.7/12 occurred later in July 2012. The circulating PEDV isolates within the same farms still occurred with minor genetic mutation except for TH/RB-1421/12 and its re-outbreak samples (TH/RB-KHF/11 and TH/RB-123/12). In the case that farm contained with various hosts species, the viral genetic might be diverted from the original at high variable sites and became a new variant strain (Bataille et al., 2011).

For comparison of TH/NP-68/12 which was divided into subgroup 3.3, TH/NP-68/12 shared a low level of similarity to other current Thai isolates. The results showed that only 90.50-93.55% shared similarity, including with 24 nucleotide positions (or 12 amino acid positions).

The partial S gene sequence in S1 C-terminal domain of current Thai PEDV isolates showed genetic diversity. The S1 C-terminal domain seems to be a good candidate for PEDV genetic differentiation among field Thai isolates. However, Lee et al. (2010) reported that the S1 C-terminal domain was not appropriated for use as a full S glycoprotein sequence analysis.

- **The partial N gene sequences**

Nucleocapsid gene of PEDV is a highly conservative sequence in the coronaviruses (Li et al., 2009). The partial N gene sequence of current Thai isolates contained 1260 bp which located between nucleotide positions 12-1271, encoding for 419 amino acids (positions 5-423). The full length of N gene sequence composed of 1326 nucleotide (Lee and Yeo, 2003), encoding for 419 amino acids of PEDV reference strain (PEDV strain CV777, accession number AF353511.1).

The phylogenetic tree based on partial N gene sequence was divided into 5 groups. The current Thai isolates belonged to group 4 which Chinese isolates and Korean isolate as Virulent DR13 are situated (Table6). The current Thai isolates indicated a close genetic relationship to Chinese isolates, which showed the nucleotide identity between

94.00-99.52% to each other and also exhibited more closed related to SWK1, SWK2, SWK3, SWK4, SWK5 and SWK6. Moreover, the current Thai isclates were clearly separated from reference isolates of group 2 such as European isolates (CV777 and BR1/87) and some Asian isolates (Chinese isolates: CV777 Chinese, LZC and Korean isolate: SM98). The European isolates shared genetic relatedness to current Thai isolates as ranging 94.28-95.31% nucleotide identity. In addition, the deduced amino acid sequence comparisons of Asian references (excluded Asian isolates from group 2) and current Thai isolates indicated 4 residue differences from the European isolates at position 381 ($^{L}381^{P}$), 395 ($^{L}395^{Q}$), 398 ($^{H}398^{N}$), and 408 ($^{A}408^{V}$) (Appendix B: Figure 22).

All current Thai isolates were organized into the same group and those isolates shared high similarity ranging from 97.67-100% to each other. This result was unlike partial S gene sequence comparisons. This suggested that partial N gene sequence of current Thai isolates were less genetic diversity than their partial S gene sequences and our results were consistent with previous reports (Li et al., 2009; Ren et al., 2011; Li et al., 2012).

Samples from re-outbreaks represented that nucleotide sequences were almost identical to previous outbreak samples by sharing high similarity between 98.93-100%. Although, TH/RB-1421/11 was still differed from its re-outbreak samples (TH/RB-KHF/11 and TH/RB-123/12), but their partial N gene sequences showed a closer relationship than partial S gene sequences. In addition, TH/RB-1421/11 also showed a close relationship to other current Thai isolates such as TH/NP-795/11, TH/AY-2.2/12 and TH/AY-2.7/12 with nucleotide identity as 99.11%.

The nucleotide substitutions were found on partial N gene sequence of some current Thai isolates such as TH/NP-657/12, TH/NP-68/12 and TH/RB-79/12. The TH/NP-657/12 and TH/NP-68/12 isolates had some nucleotide and amino acid deletions, but TH/RB-79/12 had some nucleotide and amino acid insertion. Nevertheless, the minor nucleotide substitution on three isolates could not give the genetic diversity among other current Thai isolates. In addition, piglets infected with TH/NP-657/12 and TH/RB-79/12 showed low morbidity (< 30%) and low mortality rate (<10%). In contrast, piglets infected with TH/NP-68/12 were all died. So, the relatedness between N gene sequence substitutions and viral pathogenicity still remain unclear. However, Li et al. (2012)

suggested that the full S gene sequence of some PEDV strains with amino acid insertion could be effective factor to increase their virulent.

Furthermore, the herd with TH/NP-68/12 infection had been reoccurred continually since 2007 and became more impact in the year of 2011. For control of PEDV infection in this herd, the pregnant sows were immunized by feeding with minced intestinal content from infected piglets, diluted sow feces, and vaccinated sows with PEDV vaccine (K1 vaccine). However, piglets still suffered from PEDV infection and mortality rate in this herd still showed 100%. It is possible that continuously stocking new susceptible sows and keeping PEDV infected piglets circulate in the herd seem to be predisposing factors for PEDV persists in herd (Pospischil et al., 2002; Pensaert, 2005; Puranaveja et al., 2009) or making PEDV virulent variation (Yang et al., 2013), especially in TH/NP-68/12 infected herd.

Among genetic comparison between TH/NP-68/12 and other current Thai isolates, the partial S gene sequence revealed that TH/NP-68/12 shared less genetic relationship to current Thai isolates between 90.50-93.55% nucleotide identities. Sato et al. (2011) suggested that S gene sequence was less conservation than M and N genes. With the respect to partial N gene sequence, TH/NP-68/12 shared high genetic relatedness between 97.85-98.86% nucleotide identity to current Thai isolates although TH/NP-68/12 revealed minor deletions at both of nucleotide and amino acid of partial N gene sequences.

For genetic comparisons between TH/NP-68/12 and K1 vaccine based on partial S and N gene sequences, the results indicated that TH/NP-68/12 had low nucleotide similarity as of 93.55% and 94.73%, respectively. It is possible that the genetic variations of TH/NP-68/12 were associated with viral virulence or antigenicity. Therefore, any immunized techniques did not give a complete PEDV protection to their herd. This investigation is consistent with the previous report that suggested PEDV mutations may be associated to viral pathogenicity or antigenicity (Li et al., 2012; Pan et al., 2012).

PEDV isolates from Chachoengsao Province: TH/CS-866/11, TH/CS-1019/11, TH/CS-65/12 and TH/CS-80712/12 had unique amino acid residues at amino acid position 239 (Figure18). This point of mutation might be a specific characterization of PEDV based on geographic areas.

The phylogenetic tree based on partial S gene sequence was useful for studying on genetic diversity among PEDV strains, genetic mutations and PEDV epidemiology because

this gene sequence was less conservation than other genes (M and N gene sequences) (Sato et al., 2011). Although, N gene sequence was high conserved sequence but could differentiate between PEDV strains from different origins (Li et al., 2012) or among field strain and vaccine strain (Lee et al., 2008) by genotypic comparisons.

In conclusion, both partial S and N gene sequence analysis indicates that current Thai isolates had closely genetically related to Chinese isolates. Therefore, the PEDV Chinese isolates might be circulating within the Southeast Asian neighboring countries (Duy et al., 2011; Song and Park, 2012; Weiss et al., 2012) and entering into Thailand with unknown route of transmission since 2007 (Parunaveja et al., 2009).

It was possible that current PEDV isolates in Thailand might have originated from Chinese lineage. As known, PEDV had spread across from Europe (1970s) into Asia in early 1980s (Kubota et al., 1999; Chen et al., 2011). For more than 30 years ago since PEDV had been introduced in Asia and the recombination between coronavirus genome is high frequency to occur (Pan et al., 2012) so the genetic diversity of PEDV from originated strain to new endemic variants based on distributed areas could be evolved naturally likewise the several previous reports in Asia (Chen et al., 2008; Li et al., 2009; Parunaveja et al., 2009; Lee et al., 2010; Duy et al., 2011; Park et al., 2011; Fan et al., 2012; Li et al., 2012; Pan et al., 2012; Chen et al., 2013). Therefore, genotypic changes of PEDV isolates may prefer to viral characteristic that could be genetic information for further study of molecular epidemiology based on different geographic areas (Li et al., 2012), viral antigenic identity related to clinical presentation (Li et al., 2012; Pan et al., 2012) or antigenic size variation associates to neutralizing antibody production (Park et al., 2007).

The relationship between farm locations and disease widespread

The farm locations of each current Thai isolate were analyzed together with their genetic diversity. The results showed that TH/NP-795/11 from Nakornpathom province was highly homologous to other two current Thai isolates from 2 different farm locations which were TH/CS-80712/12 (99.53%) from Chachoengsao province and TH/RB-881/12 (99.29%) from Ratchaburi province. Moreover, TH/CS-80712/12 had also presented highly similarity to TH/RB-881/12, TH/RB-236/12 from Ratchaburi province (98.81%) as same as TH/AY-2.2/12 and TH/AY-2.7/12 from Ayuthaya province. Isolate TH/AY-2.2/12 and TH/AY-2.7/12

had 100% nucleotide identity to TH/RB-236/12 from Ratchaburi province. Phylogenetic tree analysis of partial N gene sequences indicated that TH/NP-795/11 had a close relationship to various Thai isolates that had originated from different provinces such as TH/AY-2.2/12 (99.84%), TH/AY-2.7/12 (99.84%), TH/RB-881/12 (99.68%), TH/CS-1019/12 (99.02%) and TH/CS-65/12 (99.02%). Interestingly, the farm locations between those isolates are located in different part of Thailand.

The results of partial S and N gene sequence analysis indicated that the disease could spread from farm to farm, although those farms were situated in different locations. The infected animal movement or vehicle transportation between infected areas might be an important mode of disease transmission (Parunaveja et al , 2009). Furthermore, PEDV can exist in the environment that outside host by physicochemical properties of PEDV particle showed the resistance of acidity between pH 4-7 at 4°C and pH 6.5-7.5 at 37 °C. The heat at 50°C for 180 minutes, PEDV is still able to infect the cell culture but PEDV infectivity had loss at more than 60°C for 30 minutes (Lee and Yeo, 2003).

Although fecal-oral route is the major route of PEDV transmission among pigs (Pensaert and Yeo, 2006) but the PEDV particle had also dispersed via aerosol route similar to the SARS-CoV and TGEV mechanism (Goh et al., 2012). Thus, the airborne transmission could occur, especially the short distance between infected farms (Bataille et al., 2011).

The time periods of PEDV outbreaks

The current Thai isolates had taken from several months during 2011-2012. PEDV cases were submitted sporadically in January, February, May, July, August, September, October, November, and December. The maximum numbers of cases were submitted in October and subsequently in May. Interestingly, Thailand located in tropical zone area with warm weather throughout the year (approximately 27 °C). The average temperature during 2011-2012 in May and October is 29.0-29.9 °C and 27.8-28.5 °C, respectively. This prevalence was differed from previous reports that the PEDV outbreaks are often found in the month with lower temperature (Shibata et al., 2000) or during the winter (Pensaert, 1999).

The age of susceptible pigs and clinical presentation

All samples in this study were derived from various ages of pigs with watery diarrhea. The ranges of affected ages were from 2 days to 20 weeks old, included lactating sows. The most common affected ages were less than 7 days, except some isolates such as TH/CS-65/12, TH/NP-1157/12 and TH/NP-1169/12 that were obtained from 15 days old piglet, 14 days old piglet and 20 weeks finishing pig, respectively. In addition, TH/RB-236/12 was isolated from lactating sow feces.

The morbidity and mortality rates had also recorded, the morbidity rates indicated between 20-100% and the mortality rates between 10-100%. The high morbidity and mortality rates were found with young piglets (< 7 days old), except TH/CS-65/12 that was 15 days old piglet. Interestingly, TH/CS-65/12 derived from a PEDV re-occurred herd in which mortality rate reached to 100%.

The PEDV re-outbreaks and herd immunity

The phylogenetic analysis based on partial S and N genes indicates most of re-outbreak samples fall in the same group. Although some point mutations on nucleotide or amino acid sequences were discovered, but the results represented those isolates had closely genetic relationship to each other.

According to disease history of farms in this study, it indicated that some sow herds suffered from mastitis and agalactia. This data is consistent with the previous report that PEDV affected sows herds resulting in symptom of mastitis. Therefore their piglets may lose the complete protective immunity due to insufficient colostrums intake (Park et al., 2009) along with fasting onset of diarrhea (1.7 days after exposure), and several days of viral shedding (within 7 days after infection) (de Arriba et al., 2002) could maintain the chain of infection within the infected herds even though PEDV antibody could be detected in serum at least 1 year after exposure (Song and Park, 2012).

In general, PEDV was found in small intestine (Debouck et al., 1981), so mucosal immunity such as IgA antibody response was effective immunological defense against PEDV infection. Unfortunately, the local protection by secretory IgA might be not prolonged (Saif et al., 2012). Piglets could be obtained the specific IgA antibody from passive maternal colostrum of sows immunized with feeding back techniques (either diluted

infected sow feces or minced infected intestine) (Saif, 1999; Song et al., 2007; Song and Park, 2012). After PEDV was given to late-term pregnant sows by oral administration, the lymphocytes and monocytes were stimulated and circulated within blood stream. The mature plasma cells (IgA producing monocytes) migrate into internal organ including gut and mammary gland, then secretory IgA is produced and secreted via colostrum and milk. Milk-bound secretoty IgA is rapidly absorbed within newborn piglet gut, it can bind pathogen in intestinal lumen, lamina propia or during antigen transcytosis into epithelial cell. IgA antibody plays important role in blocking viral attachment, penetration into intestinal mucosa, and viral neutralizing function (Lamm, 1998; Saif, 2012). In addition, Song et al. (2007) reported that IgA antibody had more neutralizing PEDV ability in experimental suckling piglets than IgG. The proteolytic resistant property of IgA is important for neutralizing orally infected pathogens (Offit and Clask, 1985). IgA antibody could resist the acid conditions within intestinal tract rather than IgG and IgM antibodies. Therefore, IgA antibody in colostrum of immunized sow may only stimulate immunological defense of suckling piglets, but could not protect their intestine from PEDV infection (Song and Park, 2012).

However, Song et al. (2003) suggested the other factors that had relationship to piglet immunity were quantity of colostrum intake by suckling piglet, litter size, quality of colostrum and antibody concentration. Lactogenic immunity is one of effective strategies in control and prevention of PEDV infection. Therefore, colostrum and milk from immunized sow should be containing high antibody titer (Song et al., 2003).

In Thailand during the last decade, oral immunizations of pregnant sows and replacement gilts with either diluted sow feces, or minced intestinal content of infected piglets had been practiced to battle PEDV infection. These immunized techniques were expected to generate lactogenic immunity and reduce piglet losses, but these were not always the best way for PEDV protection (Olanratmanee et al., 2010). Many limitations of feeding back techniques were found such as unknown viral titers of feeding content, lacking of precise PEDV identification and perhaps contamination with other enteric pathogens such as bacteria (*E. coli*, *Salmonella* spp.), or viruses (PRRS, porcine circovirus type 2 or porcine hepatitis E viruses) (Yang et al., 2003; Kanai et al., 2010; Olanratmanee et al., 2010). Nevertheless, the PEDV re-occurring within infected herds remains continuously

observed. Other field conditions as individual farm management that could contribute to PEDV reemergence within the same herds, for example restocking with new susceptible pigs, improper gilt acclimatization, keeping PEDV infected piglets, and poor biosecurity (de Arriba et al., 2002; Pensaert, 2005; Puranaveja et al., 2009).

The genetic relationship between current Thai isolates and PED vaccine strains

The PED modified live vaccine strains such as K1 vaccine and VCP-3 vaccine are used in several farms in Thailand. Generally, some sow herds used both of vaccines for preventive and control programs. Four current Thai isolates in three vaccinated sow herds were observed in this study. The phylogenetic tree based on partial S gene sequences indicated that K1and VCP-3 vaccines were divided into the same group, but both isolates were subdivided to different subgroups. K1 vaccine was organized to subgroup 3.1 as same as most current Thai isolates, including previous Thai isolates and reference isolates (Chinese, Vietnamese and Korean isolates). In contrast, VCP-3 vaccine was fallen to subgroup 3.2 together with reference isolates from Korea and Japan.

Both current and previous Thai isolates represented a closer genetic relationship to K1 vaccine than VCP-3 vaccine. TH/RB-881/12 showed the genetic homology to K1 vaccine by 100% nucleotide identity. Interestingly, this isolate recently derived from vaccinated sow herds which have previously used K1 vaccine since 2009. However, recently this herd does not use any PEDV vaccine. Therefore, it is possible that partial S gene sequence of TH/RB-881/12 might be changed from K1 vaccine. For partial N gene sequence analysis, TH/RB-881/12 and K1 vaccines were classified in different groups and also showed low similarity by 95.01% identity. The partial N gene sequence of TH/NP-881/12 had nucleotide identity differed from K1 vaccine.

For VCP-3 vaccine, present data did not show any current Thai isolates were identical to VCP-3 vaccine except two current Thai isolates from Ayuttaya province (TH/AY-2.2/12 and TH/AY-2.7/12) showed close relatedness as 95.98% identity to VCP-3 vaccine. Moreover, isolates TH/NP-795/11, TH/NP-657/12 and TH/NP-1169/12 were found from herds that used VCP-3 vaccine but those herds still suffered from PEDV re-occurring. Although PEDV remained circulating in their sow herds, but affected piglets showed a low mortality rate (Table1). While the genetic comparisons based on partial S and N gene

sequences among reference vaccine strains (such as CV777 strain, CV777 Chinese strain, attenuated DR13 strain and 83P-5 100th passaged) and current Thai isolates showed low sequence similarities to each other.

The Injection of PEDV vaccine to pregnant sows was optional tool for preventive and control program. Moreover, route of vaccination to pregnant sows are substantial. Song and Park (2012) recommended that oral vaccination with attenuated DR13 is prone to be more effective in prevention from PEDV infection than injection route. Several reports also suggested that immunized sows with orally attenuated PEDV could lower clinical signs of susceptible piglets and reduction of piglet death (Kweon et al., 1999; Song et al., 2003; Song et al., 2007; Park et al., 2009; Lumyai et al., 2011; Li et al., 2012; Song and Park, 2012).

Nevertheless, PEDV recombination between heterogeneous genome including vaccine strain should be considered because the recombination between Coronavirus genome is high frequency to occur (Pan et al., 2012). The errors during mRNA transcription were highly occurrence in Coronavirus genome because of their genome is very large size (Lai, 1996). For better understanding, the full-length S gene sequence between TH/NR-881/12 and K1 vaccine should be further investigated because this study was focused only partial S gene sequence at S1 C-terminal domain which this region does not provided to delicate full S glycoprotein sequence (Lee et al., 2010). Moreover, the modified live vaccine could turn on virulence by itself in the susceptible host if that vaccine was obtained from inadequate attenuated virus (Bestetti et al., 1978; Pensaert an de Bouck, 1978; Lee et al., 2008). In addition, serial passaged of virus might be promote viral growth adaptation within host cell and then become a virulent infection (Shen et al., 2003).

Conclusion

The phylogenetic analyses based on partial S gene sequence including neutralizing epitope region showed that current Thai isolates are homologous to previous Thai isolates during 2007-2008. Both of the PEDV share a close relationship to Chinese isolates which is similar to previous report in 2009, although Chinese isolates had been changed into the other isolates as GD-1 and GD-A. Similar to S gene sequence analysis, the partial N gene sequence represented a close relationship of all current Thai isolates to Chinese isolates.

From this circumstance, the current endemic PEDV isolates in Thailand might have originated from Chinese lineage.

The genetic relationship among current Thai isolates indicated a continuing PEDV circulation in several swine farms possibly caused by live PEDV immunization by feeding back technique or PEDV vaccination could not give the complete protection to those susceptible piglets. Further study on genetic diversity between PEDV isolates in Thailand should be performed for better understanding PEDV molecular characteristics in Thailand.

For prevention and control of PEDV infection, oral immunization with live attenuated vaccine or minced intestinal content from infected pigs should be performed carefully and correctly for preventing continuous outbreak. Moreover, improvement of farm management such as colostrum intake, minimizing animal movement and transportation should be emphasized as primary priority.

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