CHAPTER IV

RESULTS

1. Viral RNA detection

Viral RNA extracted from samples was examined for partial S and N genes of PEDV by RT-PCR. The PCR product size was predicted to be 651 bp of S gene (Figure6), 738 bp of N1 gene (Figure7), 845 bp of N2 gene (Figure8), 760 bp of N3 gene (Figure9) and 848 of N4 gene (Figure10).

All nucleotide sequences of both genes were aligned and adjusted the length fragment with program package, ClustralX multiple alignment versions 2.0.11 (multiple alignments of nucleic acid and protein sequences; GNU Lesser General Public License) and BioEdit sequence alignment editor version 7.1.3.0, respectively. Then, the full length nucleotide sequences were presented in 428 bp of partial S gene and 1260 bp of partial N gene.

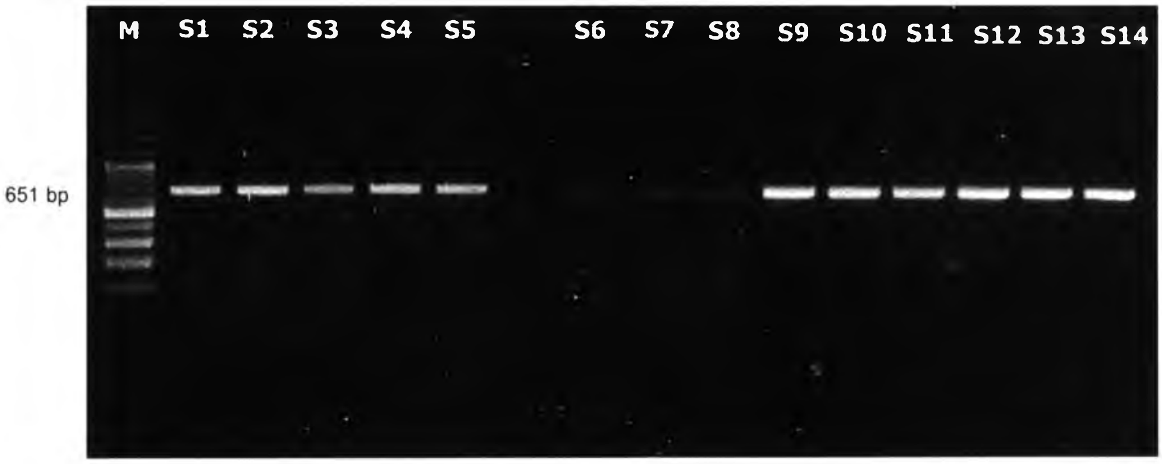


Figure6: RT-PCR product of S gene fragments; M: 100 bp DNA ladder, S1-S14: samples.

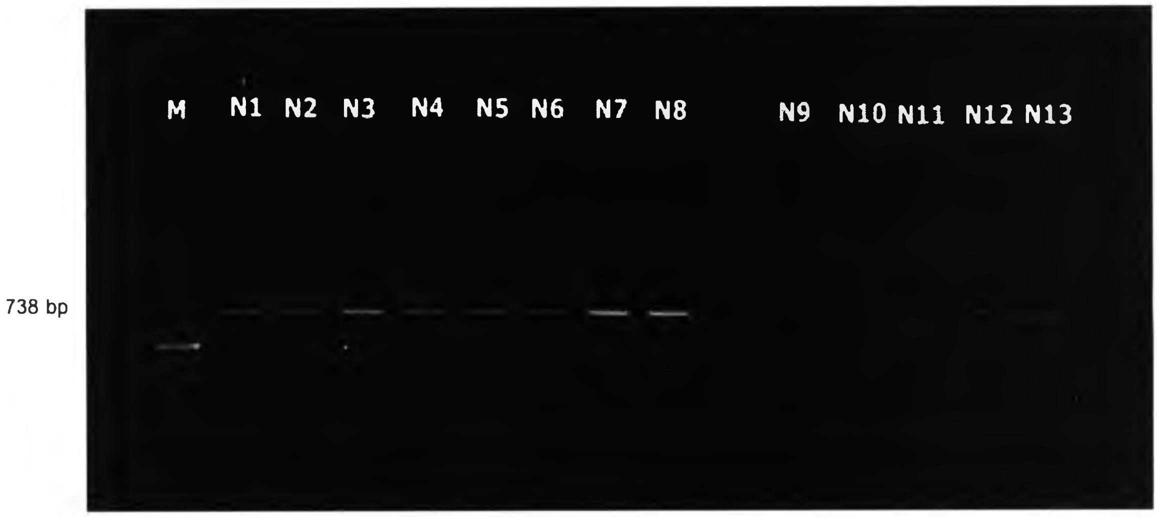


Figure7: RT-PCR product of N1 gene fragments; M: 100 bp DNA ladder, N1-N13: samples.

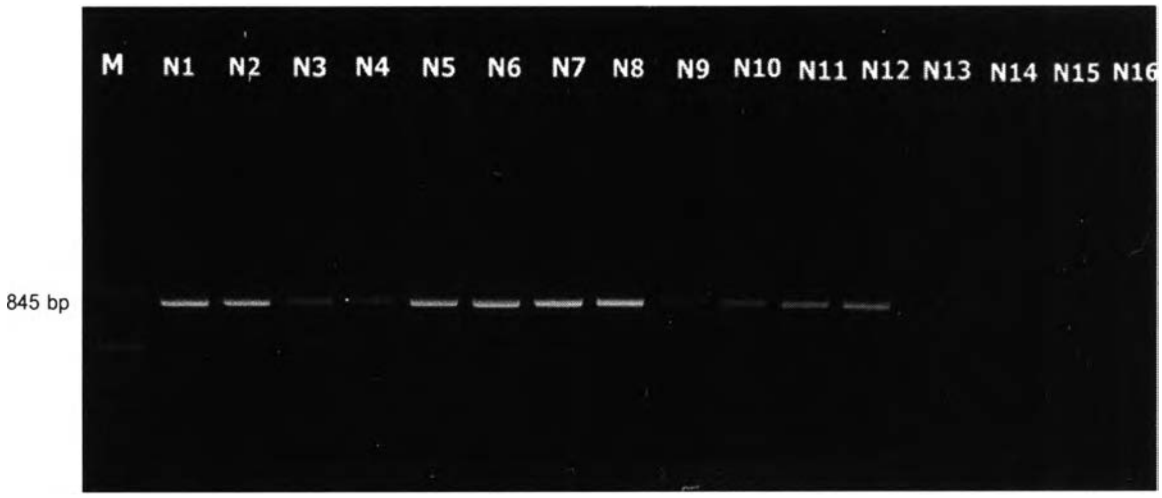


Figure8: RT-PCR product of N2 gene fragments; M: 100 bp DNA ladder, N1-N16: samples.

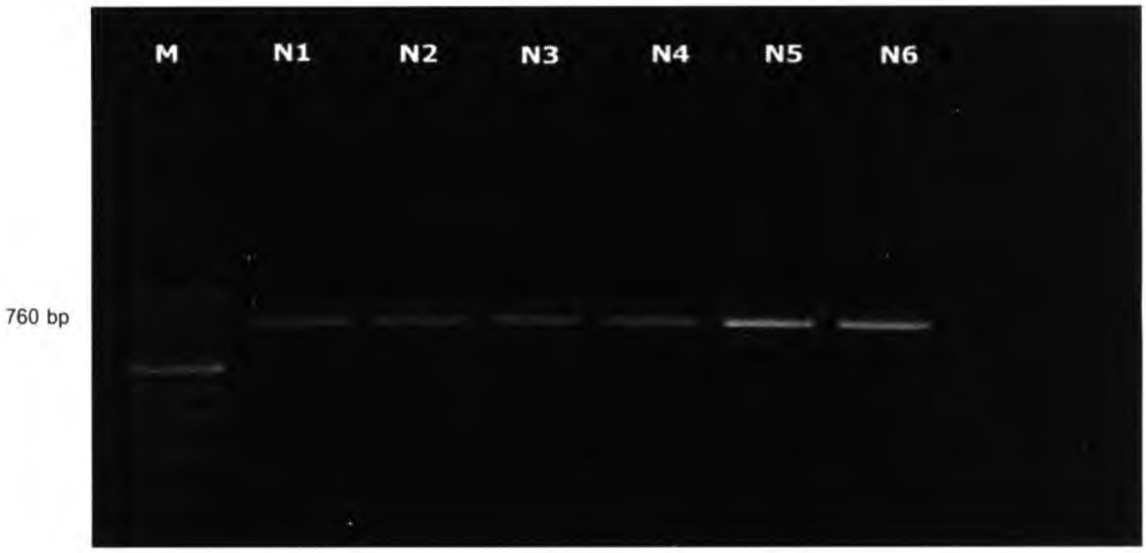


Figure9: RT-PCR product of N3 gene fragments; M: 100 bp DNA ladder, N1-N6: samples.

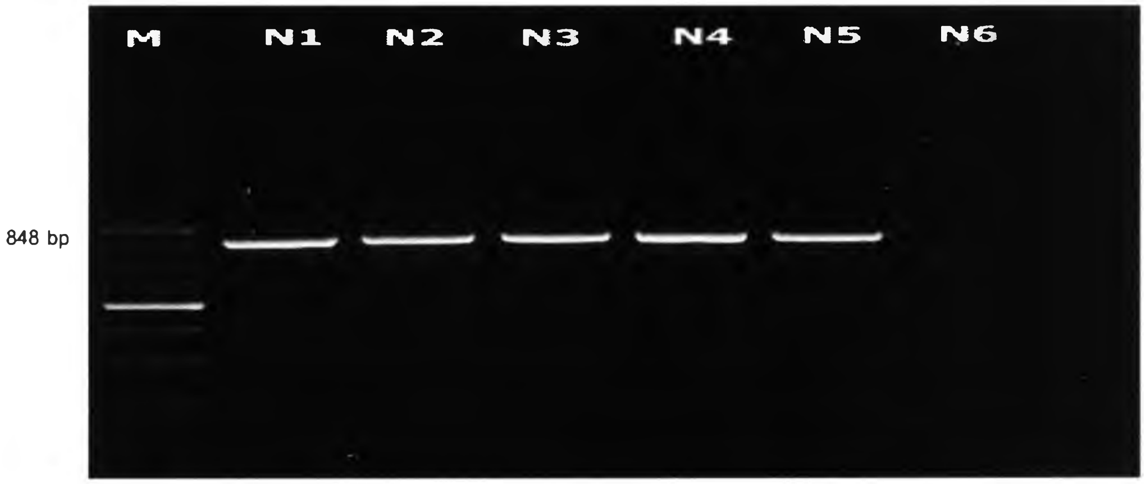


Figure10: RT-PCR product of N4 gene fragments; M: 100 bp DNA ladder, N1-N6: samples.

2. Sequence and phylogenetic analyses

The relationship between current Thai isolates and PED vaccine strains were analyzed based on partial S and N gene sequences. Phylogenetic trees were constructed by using the maximum likelihood method in Mega version 5.

- Partial S gene sequences

The partial S gene sequences including epitope region of current Thai isolates and PED lived vaccine strains (K1 and VCP-3 vaccines) were used to construct the phylogenetic tree. The 428 bp partial S gene sequences located between nucleotide positions 1538-1965 of a total of 4152 nucleotide in length were investigated.

The phylogenetic tree based on current Thai isolates, PED vaccine strains and reference isolates from NCBI database was divided into 3 groups along with 4 subgroups of group 3 (including subgroups 3.1, 3.2, 3.3 and 3.4) (Figure11). The current Thai isolates and PED vaccine strains were grouped into group 3. The results are shown in Table 5.

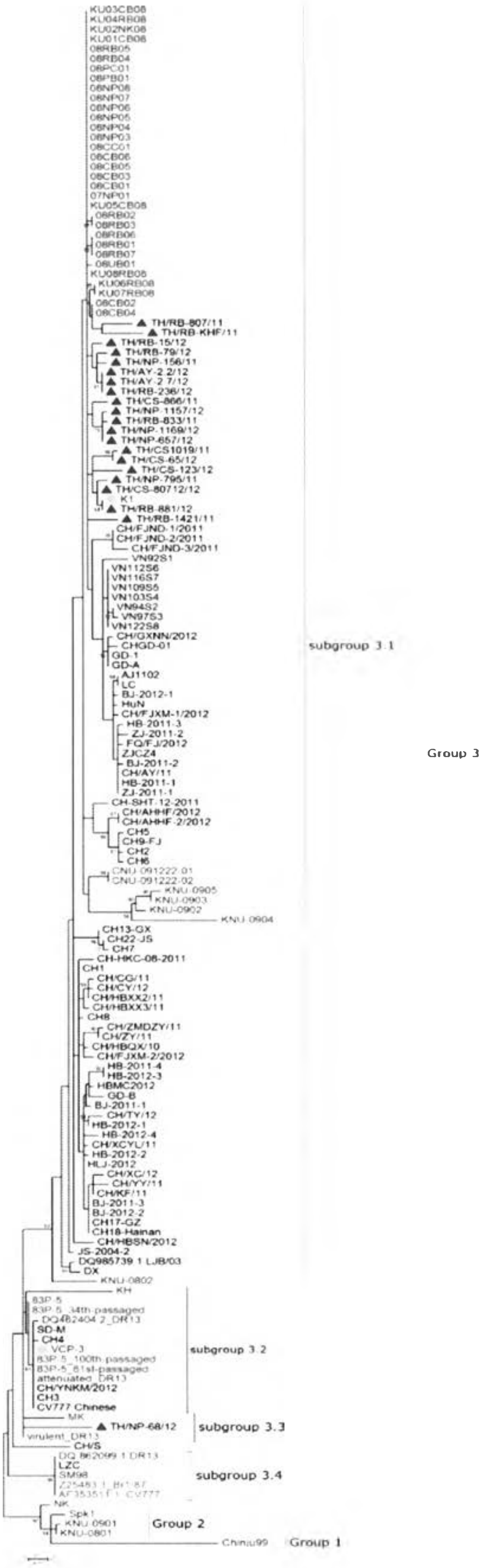
Table5. PEDV isolate groups were categorized base on partial S gene sequence*.

Group	Isolates
Group 1	**Korean isolate** : Chinju99
Group 2	**Korean isolates** : Spk1, KNU-0901 and KNU-0801
Group 3	
Subgroup 3.1	**Previous isolates** : KU07RB08, KU05CB08, KU03CB08, KU01CB08, 08RB07, 08RB05, 08RB01, 08RB03, 08PB01, 08NP07, 08NP05, 08NP03, 08CC01, 08CB05, 08CB03, 08CB01, KU08RB08, KU06RB08, KU04RB08, KU02NK08, 08UB01, 08RB06, 08RB04, 08RB02, 08PC01, 08NP08, 08NP06, 08NP04, 08CB06, 08CB04, 08CB02, 07NP01 **Current Thai isolates** : TH/NP-156/11, TH/NP-795/11, TH/RB-1421/1, TH/RB-807/11, TH/RB-833/11, TH/RB-KHF/11, TH/CS-866/11, TH/CS-1019/11, TH/RB-15/12, TH/RB-123/12, TH/CS-65/12, TH/RB-236/12, TH/NP-79/12, TH/NP-657/12, TH/CS-80712/12, TH/RB-881/12, TH/AY-2.2/12, TH/AY-2.7/12, TH/NP-1157/12, TH/NP-1169/12 **PED vaccine strain** : K1 **Vietnamese isolates** : VN92S1, VN103S4, VN109S5, VN112S6, VN122S8, VN116S7, VN94S2 and VN97S3 **Korean isolates** : CNU-0901222-01, CNU-0901222-02, KNU0902, KNU0903, KNU0904, KNU0905, KNU-0802

	Chinese isolates : CH/FJND-1/2011, CH/FJND-2/2011, CH/FJND-3/2011, GD-1, GD-A, BJ-2012-1, CHGD-01, CH/FJXM-1/2012, CH/GXNN/2012, HuN, FQ/FJ/2012, HB-2011-3, BJ-2011-2, ZJCZ4, LC, AJ1102, BJ-2012-1, CHGD-01, CH/FJXM-1/2012, CH/GXNN/2012, CH/AY/11, ZJ-2011-2, HB-2011-1, ZJ-2011-1, CH-SHT-12-2011, CH/AHHF/2012, CH/AHHF-2/2012, CH2, CH5, CH6,CH9-FJ, CH13-GX, CH22-JS, CH7, CH-HKC-08-2011, CH/ZMDZY/11, CH1, CH/ZY/11, CH/FJXM-2/2012, CH/HBQX/10, CH/HBXX2/11, CH8, CH/HBXX3/11, CH/CG/11, CH/XCYL/11, CH/TY/12, CH/CY/12, HB-2012-1, HB-2012-4, HB-2012-2, HB-2012-3, HB-2011-4, HLJ-2012, HBMC2012, GD-B, CH/XC/12, CH18-Hainan, CH17-GZ, BJ-2012-2, BJ-2011-3, CH/YY/11, CH/KF/11, BJ-2011-1, CH/HBSN/2012, JS- 2004-2, DQ985739.1 : Cloning LJB/03
Subgroup 3.2	**PED vaccine strain** : VCP-3 **Japanese isolates** : KH, 83P-5, 83P-5 34th passaged, 83P-5 : 61st passaged and 83P-5 : 100th passaged **Korean isolates** : DR13 (accession number : DQ462404.2) and attenuated DR13 **Chinese isolates** : CH3, CH4, CV777, CH/YNKM/2012 and SD-M
Subgroup 3.3	**Current Thai isolate** : TH/NP-68/12 **Japanese isolate** : MK **Korean isolate** : Virulent DR13
Subgroup 3.4	**Chinese isolate** : LZC **Korean isolates** : SM98, DR13 (accession number : DQ 862099.1) **European isolates** : BR1/87 and CV777

* PEDV isolates group were organized as reported by Park et al. (2007)

Figure11. Phylogenetic tree based on partial S gene sequences. The markers were shown in difference colors depending on country of origin such as red color for previous Thailand isolates in 2007-2008, blue color for Vietnamese isolates, light blue color for Korean isolates, pink color for Japanese isolates and black color for Chinese isolates. Twenty-one current Thailand isolates during 2011-2012 were shown included red triangle symbols in front of their name and two PED vaccine strains were shown in yellow square symbols in front of their name.



- Partial N gene sequences

The partial N gene sequences of the current Thai isolates and PED vaccine strains were used to construct the phylogenetic tree. Unfortunately, the genomic sequences of TH/RB-807/12 and TH/RB-236/12 were failed to be amplified.

The partial N gene sequences contained with 1260 bp that were located between nucleotide positions 12-1271 of a total of 1326 nucleotide in length. The phylogenetic tree was categorized into 5 groups, which PED vaccine strains were grouped into group 3 and the current Thai isolates were grouped into group 4 (Table6 and Figure12).

Table6: PEDV isolate groups were categorized base on partial N gene sequence*.

Group	Isolates
Group 1	**Korean isolate** : Chinju99
Group 2	**Korean isolate** : SM98 **Chinese isolate** : LZC, CV777 Chinese (accession number : JN599150.1) **European isolate** : BR1/87 and CV777
Group 3	**PED vaccine strains** : K1, VCP-3 **Japanese isolate** : 83P-5, 83P-5 34th passaged, 83P-5 61st passaged and 83P-5 100th passaged **Korean isolate** : attenuated DR13 **Chinese isolates** : strain S, CH/S, JN.173270 Chinese, SD-M, HLJBY, CH/HLJHG/2010, CH/HLJQ/2010, CH/HNZZ/2011, CH/HLJHH/2011, CH/XIUrumqi/2011, CH/HLJHG/2011, CH/GDQY-1/2011, CH/SDRZ-2/2011, CH/BJYQ-1/2011, CH/BJYQ-2/2011
Group 4	**Current Thai isolates** : TH/NP-156/11, TH/NP-795/11, TH/RB-1421/1, TH/RB-807/11, TH/RB-833/11, TH/RB-KHF/11, TH/CS-866/11, TH/CS-1019/11, TH/RB-15/12, TH/NP-68/12, TH/RB-123/12, TH/CS-65/12, TH/RB-236/12, TH/NP-79/12, TH/NP-657/12, TH/CS-80712/12, TH/RB-881/12, TH/AY-2.2/12, TH/AY-2.7/12, TH/NP-1157/12, TH/NP-1169/12 **Chinese isolates** : CH/IMT/06, JN12-3, CH/HNH/07, JY12-3, CH/HLJM/07, JN12-2, CH/JL/09, CQ12-2, CH/BJSY/2011, GD-B, CH/HNCH/06, DZ12-1, CH/HLJHRB/2011, CH/GXQZ/2011, CH/FJND-3/1011, CH/FJND/2011, CH/GXWP/2011, GDYE10, GDST1, GDST2, GDST3, GDST4, GDST5, GDST6, GDST7, GDST8, GDYG, GDYD, GDYE, GDSTY, GDXS1, GDXS2, GDXS4, GDXS5, GDXS6, GDXS7, GDXS8, LY12-3, CH/SHH/06, DZ12-3, FJ473395.1 Chinese, YT12-4, CH/IMB/06, TA12-3, CH/GXNN/2011, BJ2010, SWK1, SWK2, SWK3, SWK4, SWK5, SWK 6, BJ-2011-1, WS12-2, CH/JSX/06, HB/HS, HB/GY, CH/GSJ/07, CH/ZMDZY/11, JS-2004-2, GDYB,

	GDYE70, GDYE67, DX, CH/HLJH/06, LJB/03, GDYE4, GDYE6, GDYE7, GDYE8, GDYE9, QYA1, HuN, GDDWC, DWA1, DBA1 **Korean isolate** : Virulent DR13
Group 5	**Chinese isolates** : GD-A, LC, AJ1102, GD-1, GDLC, GDYA, CH/GDQY-2/1011, CH/GDQY-3/1011, QY1, QY2, XG1, XG2, JKA1, GHGD-01, GDHSY, CH/GDS/09, CH/GXWN/2011, ZJCZ4, GDCB6, GDCB9

* PEDV isolate groups were organized as reported by Pan et al. (2012)

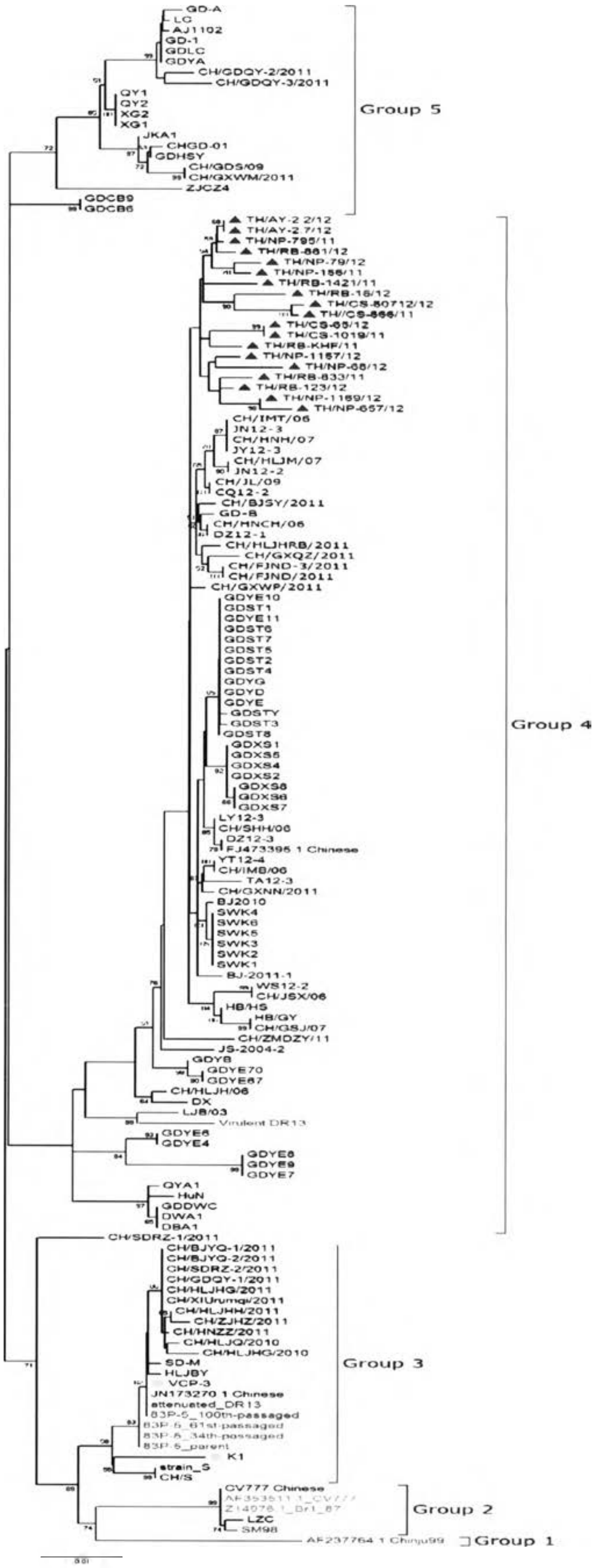


Figure12. Phylogenetic tree based on partial N gene sequences. The markers were shown in difference colors depending on country of origin such as light blue color for Korean isolates, pink color for Japanese isolates and black color for Chinese isolates. For nineteen current isolates in 2011-2012 were shown included green triangle symbols in front of their name and two PED vaccine strains were shown with yellow circle symbols in front of their name.

3. Sequence homology analysis

- **Partial S gene sequence**

- Nucleotide sequence homology

Most of the current Thai isolates belonged to group 3 as same as previous Thai, Chinese, Vietnamese and Korean isolates (Table5). The current Thai isolates had closely related to previous Thai and Chinese isolates by sharing nucleotide identity between 93.22-99.53% and 91.20-98.82%, respectively. The percentage identity of partial S gene sequence as compared between current Thai isolates and reference isolated are shown in Table7 and nucleotide sequence alignment comparisons between PEDV isolates are shown in Appendix A: Figure19.

The current Thai isolates represented 96.24-100% nucleotide identity as compared to each other, that some isolates shared nucleotide comparison as 100% nucleotide identity such as TH/NP-657/12, TH/NP-1169/12, TH/RB-236/12, TH/AY-2.2/12 and TH/AY-2.7/12. Interestingly, TH/RB-1421/11 and TH/NP-68/12 were separated out of majority of current Thai isolate cluster, with a range of 96.24-98.06% and 90.50-93.55% nucleotide identity, respectively. TH/RB-1421/11 remained belonging to subgroup 3.1 as same as other current Thai isolates, whereas TH/NP-68/12 was categorized into subgroup 3.3 which shared high nucleotide homology with Korean isolate (virulent DR13) for 96.77% and Japanese isolate (MK) for 94.94%.

The genetic comparisons between re-outbreak samples such as TH/CS-866/11, TH/CS-80712/12, TH/NP-795/11, TH/NP-1169/12, TH/CS-1019/11, TH/CS-65/12, TH/AY-2.2/12, TH/AY-2.7/12, TH/RB-1421/11, TH/RB-KHF/11 and TH/RB-123/12 represented high similarity as ranging from 98.81-100%, except TH/RB-1421/11 that showed lower similarity between 96.24-96.78% to the isolates found in subsequent outbreaks (TH/RB-123/12 and TH/RB-KHF/11).

The genetic relationship between PED vaccine strains which are K1 vaccine and VCP-3 vaccine were also compared. The phylogenetic tree divided both of vaccine strains into group 3. However, those vaccine strains were subdivided into different subgroups. K1 vaccine was subdivided into subgroup 3.1 as same as the current and previous Thai isolates, which shared similarity ranging from 93.55-100% and 99.05-99.53%, respectively. Moreover, the current Thai isolate as TH/RB-881/12 had 100% nucleotide identity to K1 vaccine. In contrast to VCP-3 vaccine, that showed lower genetic similarity to current Thai isolates than K1 vaccine in ranging from 94.09-96.28%. VCP-3 vaccine was subdivided into subgroup 3.2 and sharing 100% nucleotide identity to reference isolates such as Japanese isolates (83P-5: 61st and 83P-5: 100th passaged cell culture isolates), Chinese isolates (CV777 Chinese, SD-M, CH3 and CH/YNKM/2012) and Korean vaccine strain (attenuated DR13). Nevertheless, K1 and VCP-3 vaccines shared nucleotide similarity of 95.48% to each other.

The current Thai isolates were categorized in a different group from reference vaccine strains (such as CV777 strains, CV777 derived Chinese strain, attenuated DR13 strain and 83P-5 100th passage (Figure11) and also shared low sequence similarities to CV777 strain (European strain) between 91.43-94.36%. The CV777 Chinese strain (Chinese vaccine strain) shared the same nucleotide identity value with attenuated DR13 strain (Korean isolate) and 83P-5 100th passaged strain (Japanese vaccine strain) compared to current Thai isolates with a range of 94.09-96.28%. In this study, the J-Vac strain (Japanese vaccine strain) was not included for partial S gene comparisons because its sequence data was not yet available in the database.

- **Deduced amino acid sequence**

The amino acid comparisons of 141 amino acid residues, which are located between positions 514-655 represented by current Thai isolates had closely related to previous Thai isolates and Chinese isolates by sharing amino acid identity between 93.27-99.53% and 91.65-98.82%, respectively.

The current Thai isolates represented amino acid identity ranging from 92.46-100% as compared to each other, that some isolates such as TH/NP657, TH/NP1169, TH/RB-236/12, TH/AY-2.2/12 and TH/AY-2.7/12 showed 100% amino acid identity. In contrast, TH/NP-68/12 had less amino acid identity between 92.46-93.80% to other current Thai isolates with 11 amino acid position differences (Figure13).

The amino acid similarity between re-outbreak samples indicated low diversity between 96.37-100% by showing minor changes at some positions (Figure14). However, TH/RB-1421/12 revealed variable sequence as compared to TH/RB-KHF/12 and TH/RB-123/12 (14 amino acid position differences) by sharing similarity 96.37- 96.87% amino acid identities to each other.

The amino acid comparisons between K1 and VCP-3 vaccines showed 96.61% amino acid similarity by representing 6 amino acid residue differences at positions 517 (${}^{S}517^{A}$), 521 (${}^{R}521^{H}$), 549 (${}^{S}549^{T}$), 594 (${}^{S}594^{G}$), 605 (${}^{D}605^{E}$) and 633 (${}^{E}633^{Q}$) (Appendix A: Figure20). The K1 vaccine shared high identity to current Thai and previous isolates as ranging from 93.80-100% and 99.06-99.29%, respectively. In contrast to VCP-3, it showed low amino acid similarity to current Thai isolates between 94.32-96.37%. Interestingly, VCP-3 vaccine showed identical amino acid sequence as 100% identity to reference isolates from Japan (83P-5 61th and 100th-passaged), China (CV777 Chinese, SD-M, CH3 and CH/YNKM/2012) and Korean vaccine strains (attenuated DR13).

Table7: The partial S gene sequence identity between current Thai isolates and reference isolated divided by the groups.

20 Current Thai isolates (except TH/NP-68/12)	Group		Percentage identity	
			Nucleotide identity (%)	Amino acid identity (%)
TH/NP-156/11, TH/NP-795/11, TH/RB-1421/1, TH/RB-807/11, TH/RB-833/11, TH/RB-KHF/11, TH/CS-866/11, TH/CS-1019/11, TH/RB-15/12, TH/RB-123/12, TH/CS-65/12, TH/RB-236/12, TH/NP-79/12, TH/NP-657/12, TH/CS-80712/12, TH/RB-881/12, TH/AY-2.2/12, TH/AY-2.7/12, TH/NP-1157/12, TH/NP-1169/12	Group 1		81.66-85.25	84.32-87.07
	Group 2		89.87-92.96	90/54-96.62
	Group 3	Subgroup 3.1	91.20-99.53	91.65-99.53
		Subgroup 3.2	89.92-96.55	90.54-96.62
		Subgroup 3.3 (TH/NP-68/12 was included to compare with 20 current Thai isolates)	92.18-96.77	92.46-96.87
		Subgroup 3.4	91.43-94.36	91.92-94.58

- Partial N gene sequence

- Nucleotide sequence homology

All current Thai isolates were divided into group 4 as same as Chinese and Korean isolates (Table6). The current Thai isolates showed high genetic similarity to Chinese isolates such as SWK1, SWK2, SWK3, SWK4, SWK5 and SWK6 by sharing 97.99-99.52% nucleotide identity. The percentage identity of partial N gene sequence as compared between current Thai isolates and reference isolated are shown in Table8 and nucleotide sequence alignment comparisons between PEDV isolates are shown in Appendix B: Figure21.

The nucleotide comparisons between current Thai isolates represented 97.67-100% nucleotide identity. Some isolates such as TH/CS-1019/11, TH/CS-65/12, TH/AY-2.2/12, and TH/AY-2.7/12 shared 100% nucleotide identity. For TH/NP-68/12, it was organized into group 4 as same as the current Thai isolates by sharing 97.85-98.78% nucleotide identity to each other. Moreover, the nucleotide sequence of current Thai isolates exhibited T at nucleotide position 372 that was changed only current Thai isolates (Figure15). The nucleotide substitutions were also observed, some of current Thai isolates represented minor deletions and insertions. Some deletions were found with TH/NP-657/12 at positions 23, 24, 31 and 36, likewise as TH/NP-68/12 that showed between positions 750-752. For some insertion, it was found in TH/NP-79/12 at position 1267 (as T) (Figure16).

The samples that came from the re-outbreak herd (TH/CS-866/11, TH/CS-80712/12, TH/NP-795/11, TH/NP-1169/12, TH/RB-1421/11, TH/RB-KHF/1, TH/RB-123/12, TH/CS-1019/11, TH/CS-65/12, TH/AY-2.2/12 and TH/AY-2.7/12) represented high genetic similarity in ranging from 98.86-100% as compared to each other. For TH/RB-1421/11 and its re-outbreak samples (TH/RB-123/12 and TH/RB-KHF/11), the nucleotide sequence from those samples showed less nucleotide variations by sharing 98.86-98.93% identity to each other.

The genetic relationships among PED vaccine strains were also compared. The phylogenetic tree divided both of vaccine strains into group 3, which those strains shared diverse similarity as 98.51% identity. Interestingly, Korean vaccine strain (attenuated DR13) represented close relatedness to VCP-3 vaccine (100%) and K1 vaccine (98.51%), respectively.

The PED vaccines and current Thai field isolates were compared, the results found that K1 and VCP-3 vaccine shared nucleotide identity to current Thai isolates in ranging from 94.51-95.21% and 96.06-95.98%, respectively. Moreover, TH/AY-2.2/12 and TH/AY-2.7/12 had high relatedness to both vaccines.

The other reference vaccine strains (such as CV777 strains, CV777 Chinese strain, attenuated DR13 strain and 83P-5 100th passaged were also organized out of current Thai isolate cluster (Figure12) like partial S gene sequence comparisons. Most of reference vaccine strains shared low sequence similarities to current Thai isolates (CV777 strain and

CV777 Chinese strain shared nucleotide identities between 94.28-95.31%, attenuated DR13 strain and 83P-5 100th passaged strain shared 95.06-95.98%). While J-Vac strain (Japanese vaccine strain) was not included for partial N gene comparisons because full length N gene sequence is too short (approximate 612 bp).

- Deduced amino acid sequence

For amino acid comparisons at location between positions of 4-423, current isolates had closely related to Chinese isolates by sharing amino acid identity between 94.16-99.52%. With in current Thai isolates, amino acid identities were compared ranging from 97.79-100%. Some isolates such as TH/CS-1019/12, TH/CS-65/12, TH/AY-2.2/12 and TH/AY-2.7/12 showed identical sequences as 100% amino acid identity. For TH/NP-68/12, the sequence similarity was showed between 97.79%-98.79% amino acid identity as compared to other current isolates and representing 12 variable amino acid residues (Appendix B: Figure22).

In addition, some current Thai isolates represented minor insertions and deletions such as TH/NP-657/12, TH/NP-68/12 and TH/RB-79/12. TH/NP-657/12 was found 3-residue deletion at positions 8, 11 and 12 as well as TH/NP-68/12 was found 2-residue deletion at positions 250 and 251. Moreover, TH/RB-79/12 represented 1 amino acid insertion at position 423 (as C) (Figure17).

The amino acid comparisons between re-outbreak samples shared a ranging from 98.79-100% identity. Some isolates such as TH/CS-1019/12, TH/CS-65/12, TH/AY-2.2/12 and TH/AY-2.7/12 showed the identical sequence as 100% identity. The most variable sequences were found in TH/RB-1421/12, TH/RB-KHF/12 and TH/RB-123/12 isolates. Those isolates represented amino acid identity between 98.79-98.95% to each other. Interestingly, the amino acid sequences of current Thai isolates from Chachoengsao Province (TH/CS-866/12, TH/CS-80712/12, TH/CS1019/12 and TH/CS-65/12) represented amino acid residue differences from other current Thai isolates at position 239. The TH/CS-866/12 and TH/CS-80712/12 were changed from Proline (P) to Leucine (L) whereas TH/CS1019/12 and TH/CS-65/12 were changed to Threonine (T) (Figure18).

The K1 and VCP-3 vaccines shared 98.46% amino acid identity to each other by representing 4 amino acid residue differences at positions 218 ($^{A}218^{S}$), 350 ($^{I}350^{T}$), 376 ($^{N}376^{K}$) and 380 ($^{K}380^{I}$) (appendix B: Figure22). In addition, TH/NP-795/11 and TH/RB-

123/12 showed the highest similarity to K1 vaccine (95.31%) as same as VCP-3 vaccine (96.01%). The comparisons between PED vaccines and reference isolates revealed 100% amino acid identity to Korean vaccine strain (attenuated DR13).

Table8: The partial N gene sequence identity between current Thai isolates and reference isolates divided by the groups.

Current Thai isolates	Group	Percentage identity	
		Nucleotide identity (%)	Amino acid identity (%)
TH/NP-156/11, TH/NP-795/11, TH/RB-1421/1, TH/RB-807/11, TH/RB-833/11, TH/RB-KHF/11, TH/CS-866/11, TH/CS-1019/11, TH/RB-15/12, TH/NP-68/12, TH/RB-123/12, TH/CS-65/12, TH/RB-236/12, TH/NP-79/12, TH/NP-657/12, TH/CS-80712/12, TH/RB-881/12, TH/AY-2.2/12, TH/AY-2.7/12, TH/NP-1157/12, TH/NP-1169/12	Group 1	93.32-94.39	93.62-94.51
	Group 2	94.00-95.31	94.16-95.40
	Group 3	94.40-96.07	94.51-96.09
	Group 4	95.35-99.52	95.40-99.52
	Group 5	94.31-97.06	94.43-97.12

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                         500       510       520       530       540       550       560
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CV777            SHEQPISFVTLPSFNDHSFVNITVSAAFGGLSSANLVASDTTINGFSSFCVDTRQFTITLFYNVTNSYGY

TH/NP-156/11                            ...S...H.G...I.....................S...........
TH/RB-1421/11                           ...S...Q.G...I.....................S...........
TH/NP-795/11                            ...S...H.G...I.....................S...........
TH/RB-807/11                            ...S...R.G.D.I.....................S...........
TH/RB-833/11                            ...S...H.G...I.....................S...........
TH/RB-KHF/11                            ...S...H.G...I..........F..........S...........
TH/CS-866/11                            ...S...H.G...I.....................S...........
TH/CS-1019/11                           ...S...H.G...I.....................S...........
TH/RB-15/12                             ...S...H.G...I.....................S...........
TH/CS-123/12                            ...S...H.G...I..........F..........S...........
TH/RB-236/12                            ...S...H.G...I.....................S...........
TH/CS-65/12                             ...S...H.G...I.....................S...........
TH/RB-79/12                             ...S...H.G...I.....................S...........
TH/NP-657/12                            ...S...H.G...I.....................S...........
TH/RB-881/12                            ...S...R.G...I.....................S...........
TH/CS-80712/12                          ...S...H.G...I.....................S...........
TH/NP-1157/12                           ...S...H.G...I.....................S...........
TH/NP-1169/12                           ...S...H.G...I.....................S...........
TH/AY-2.2/12                            ...S...H.G...I.....................S...........
TH/AY-2.7/12                            ...S...H.G...I.....................S...........
TH/NP-68/12                             .....D.R.G.D.I..............E..................

                         570       580       590       600       610       620       630
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CV777            VSKSQDSNCPFTLQSVNDYLSFSKFCVSTSLLAGACTIDLFGYPAFGSGVKLTSLYFQFTKGELITGTPK

TH/NP-156/11     ..S..............................S..........E......F..............S...
TH/RB-1421/11    ..........................I......S..........E......F..................
TH/NP-795/11     .......K.....................N...S..........D......F..................
TH/RB-807/11     ..N....................R.........S..........E......F.................G
TH/RB-833/11     .................................S..........E......F..................
TH/RB-KHF/11     ..N............I......G..........S..........E..G...F..................
TH/CS-866/11     ..N..............................S..........E......F..................
TH/CS-1019/11    .................................S..........D......F..................
TH/RB-15/12      ..N..............................S.................F........E.........
TH/CS-123/12     .......................R........DS..........D.........................
TH/RB-236/12     ..N..............................S..........E......F..................
TH/CS-65/12      .................................S..........D......F..................
TH/RB-79/12      ..N..N...........................S..........E......F..................
TH/NP-657/12     .................................S..........E......F..................
TH/RB-881/12     .................................S..........D......F..................
TH/CS-80712/12   .................................S..........D......F..................
TH/NP-1157/12    .................................S..........E......F..................
TH/NP-1169/12    .................................S..........E......F..................
TH/AY-2.2/12     ..N..............................S..........E......F..................
TH/AY-2.7/12     ..N..............................S..........E......F..................
TH/NP-68/12      ......G................R....................D......F..........D.......

                         640       650       660       670       680       690       700
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CV777            PLEGITDVSFMTLDVCTKYTIYGFKGEGIITLTNSSILAGVYYTSDSGQLLAFKNVTSGAVYSVTPCSFS

TH/NP-156/11     ....V....................
TH/RB-1421/11    ....V.....I..G...........
TH/NP-795/11     ....V....................
TH/RB-807/11     ....V....................
TH/RB-833/11     ....V............T.......
TH/RB-KHF/11     ..V.V....................
TH/CS-866/11     ....VK...................
TH/CS-1019/11    ..G.V....................
TH/RB-15/12      ....V....................
TH/CS-123/12     ..Q.V....................
TH/RB-236/12     ....V....................
TH/CS-65/12      ....V....................
TH/RB-79/12      ....V....................
TH/NP-657/12     ....V....................
TH/RB-881/12     ....V....................
TH/CS-80712/12   ....V....................
TH/NP-1157/12    ....V....................
TH/NP-1169/12    ....V....................
TH/AY-2.2/12     ....V....................
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```
TH/AY-2.7/12      ....V....................
TH/NP-68/12       ....V.....I..............
```

Figure13: The amino acid sequences alignment of current Thai isolates based on partial S gene.

```
                         500       510       520       530       540       550       560
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CV777             SHEQPISFVTLPSFNDHSFVNITVSAAFGGLSSANLVASDTTINGFSSFCVDTRQFTITLFYNVTNSYGY

TH/RB-1421/11                            ...S...Q.G...I.....................S...........
TH/RB-KHF/11                             ...S...H.G...I..........F..........S...........
TH/CS-123/12                             ...S...H.G...I..........F..........S...........

TH/NP-795/11                             ...S...H.G...I.....................S...........
TH/NP-1169/12                            ...S...H.G...I.....................S...........

TH/CS-866/11                             ...S...H.G...I.....................S...........
TH/CS-80712/12                           ...S...H.G...I.....................S...........

TH/CS-1019/11                            ...S...H.G...I.....................S...........
TH/CS-65/12                              ...S...H.G...I.....................S...........

TH/AY-2.2/12                             ...S...H.G...I.....................S...........
TH/AY-2.7/12                             ...S...H.G...I.....................S...........

                         570       580       590       600       610       620       630
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CV777             VSKSQDSNCPFTLQSVNDYLSFSKFCVSTSLLAGACTIDLFGYPAFGSGVKLTSLYFQFTKGELITGTPK

TH/RB-1421/11     ..........................I......S..........E......F..................
TH/RB-KHF/11      ..N............I......G..........S..........E..G...F..................
TH/CS-123/12      .......................R........DS..........D.........................

TH/NP-795/11      .......K.....................N...S..........D......F..................
TH/NP-1169/12     ......  .........................S..........E......F..................

TH/CS-866/11      ..N..............................S..........E......F..................
TH/CS-80712/12    .................................S..........D......F..................

TH/CS-1019/11     .................................S..........D......F..................
TH/CS-65/12       .................................S..........D......F..................

TH/AY-2.2/12      ..N..............................S..........E......F..................
TH/AY-2.7/12      ..N..............................S..........E......F..................

                         640       650       660       670       680       690       700
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CV777             PLEGITDVSFMTLDVCTKYTIYGFKGEGIITLTNSSILAGVYYTSDSGQLLAFKNVTSGAVYSVTPCSFS

TH/RB-1421/11     ....V.....I..G...........-
TH/RB-KHF/11      ..V.V....................-
TH/CS-123/12      ..Q.V....................-

TH/NP-795/11      ....V....................
TH/NP-1169/12     ....V....................

TH/CS-866/11      ....VK...................
TH/CS-80712/12    ....V....................

TH/CS-1019/11     ..G.V....................
TH/CS-65/12       ....V....................

TH/AY-2.2/12      ....V....................
TH/AY-2.7/12      ....V....................
```

Figure14: The amino acid sequences alignment of re-outbreak samples based on partial S gene.

```
                            360       370       380       390       400       410       420
                     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CV777                AAAGCCAATCATTCCAAAATTCTCTCAACAGCTCCCCAGTGTAGTTGAGATTGTTGAACCTAACACACCT

TH/NP-156/11         ..................T..T...........T.....C..............................
TH/RB-1421/11        .........T........T..T...........T.....C..............................
TH/NP-795/11         ..................T..T...........T.....C..............................
TH/RB-833/11         ..................T..T...........T.....C..............................
TH/RB-KHF/11         ..................T..T...........T....TC..............................
TH/CS-866/11         ............C.....T..T...........T.....C..............................
TH/CS-1019/11        ..................T..T...........T.....CA.............................
TH/RB-15/12          ............C.....T..T...........T.....C..............................
TH/RB-123/12         ..................T..T...........T.....C..............................
TH/CS-65/12          ..................T..T...........T.....CA.............................
TH/NP-79/12          ..................C..T...........T.....C..............................
TH/NP-657/12         ..................T..T...........T.....C..............................
TH/CS-80712/12       ............C.....T..T...........T.....C..............................
TH/RB-881/12         ..................T..T...........T.....C..............................
TH/NP-1157/12        ..................T..T...........T.....C..............................
TH/NP-1169/12        ..................T..T...........T.....C..............................
TH/AY-2.2/12         ..................T..T...........T.....C..............................
TH/AY-2.7/12         ..................T..T...........T.....C..............................
TH/NP-68/12          ..................T..T...........T.....C...............C..............

VCP-3                .........T............................................................
K1                   .........T.....T......................................................

SWK1                 ..................T..............T.....C..............................
SWK2                 ..................T..............T.....C..............................
SWK3                 ..................T..............T.....C..............................
SWK4                 ..................T..............T.....C..............................
SWK5                 ..................T..............T.....C..............................
SWK6                 ..................T..............T.....C..............................
GD-1                 ....................................G..................C....C.........
GD-A                 ....................................G..................C....C.........
JS-2004-2            ..................T..............T.....C..............................
attenuated_DR13      .........T............................................................
SM98                 ......................................T...............................
Chinju99             ................................................A.....................
```

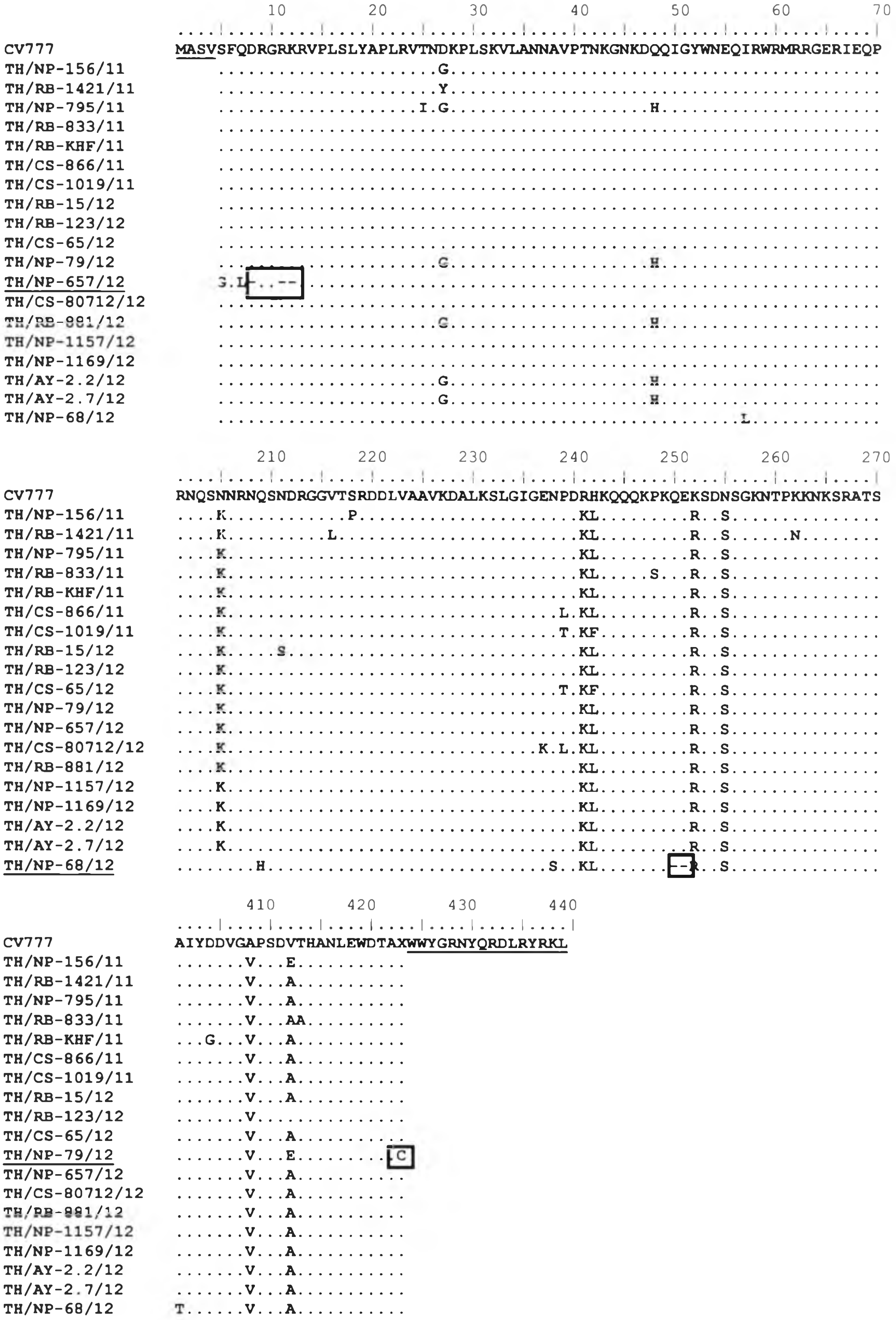
Figure15: The partial N gene nucleotide sequences alignment of current Thai isolates.

```
                          10        20        30        40        50        60        70
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CV777             ATGGCTTCTGTCAGCTTTCAGGATCGTGGCCGCAAACGGGTGCCATTATCTCTCTATGCCCCTCTTAGGG
TH/NP-156/11                  ..T...................................C...................
TH/RB-1421/11                 ..T...................................C...................
TH/NP-795/11                  ..T...................................C...................
TH/RB-833/11                  ..T............................... ...C...................
TH/RB-KHF/11                  ..T...................................C...................
TH/CS-866/11                  ..T..............A....................C...................
TH/CS-1019/11                 ..T...................................C...................
TH/RB-15/12                   ..T..............A...............C....C...................
TH/RB-123/12                  ..T...................................C...................
TH/CS-65/12                   ..T...................................C...................
TH/NP-79/12                   ..T...................................C...................
TH/NP-657/12                  G.T...TT.A--......-....-..............C...................
TH/CS-80712/12                ..T..............A....................C...................
TH/RB-881/12                  ..T...................................C...................
TH/NP-1157/12                 ..T...................................C...................
TH/NP-1169/12                 ..T...................................C...................
TH/AY-2.2/12                  ..T...................................C..A................
TH/AY-2.7/12                  ..T...................................C..A................
TH/NP-68/12                   ..T...................................C...................

                         710       720       730       740       750       760       770
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CV777             GTATTGGAGAAAATCCTGACAGGCATAAGCAACAGCAGAAGCCTAAGCAGGAAAAGTCTGACAACAGCGG
TH/NP-156/11      .......C.....C.......A..T..................C..A.......G........G......
TH/RB-1421/11     .......C.....C.......A..T..................C..A.......G........G......
TH/NP-795/11      .......C.....C.......A..T..................C..A.......G........G......
TH/RB-833/11      .......C..G..C.......A..T................T.C..A.......G........G......
TH/RB-KHF/11      .......C.....C.......A..T..................C..A.......G........G......
TH/CS-866/11      .......C.....C.T.....A..T..................C..A.......G........G......
TH/CS-1019/11     .......C.....CA......A.TT..................C..A.......G........G......
TH/RB-15/12       .......C.....C.......A..T..................C..A.......G........G......
TH/RB-123/12      .......C.....C.....T.A..T..................C..A.......G........G......
TH/CS-65/12       .......C.....CA......A.TT..................C..A.......G........G......
TH/NP-79/12       .......C.....C.......A..T..................C..A.......G........G......
TH/NP-657/12      .......C..G..C.......A..T..................C..A.......G........G......
TH/CS-80712/12    .......CA....C.T.....A..T..................C..A.......G........G......
TH/RB-881/12      .......C.....C.....T.A..T..................C..A.......G........G......
TH/NP-1157/12     .......C..G..C.......A..T..................C..A.......G........G......
TH/NP-1169/12     .......C..G..C.......A..T..................C..A.......G........G......
TH/AY-2.2/12      .......C.....C.......A..T..................C..A.......G........G......
TH/AY-2.7/12      .......C.....C.......A..T..................C..A.......G........G......
TH/NP-68/12       .......C....GC.......A..T..................C..A.---...G........G......

                        1210      1220      1230      1240      1250      1260      1270
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
CV777             GCCATCTACGATGATGTGGGTGCGCCATCTGATGTGACCCATGCCAATCTGGAATGGGACACAGCT-GTTG
TH/NP-156/11      ......................T...........A...T.........T.................-....
TH/RB-1421/11     ......................T...........C...T.........T.................-....
TH/NP-795/11      ......................T...........C...T.........T.................-....
TH/RB-833/11      ......................T...........C.G.T.........T.................-....
TH/RB-KHF/11      ..........G...........T...........C...T.........T.................-....
TH/CS-866/11      ......................T...........C...T.........T..........T......-..A.
TH/CS-1019/11     ......................T...........C...T.........T..........T......-....
TH/RB-15/12       ......................T...........C...T.........T.................-....
TH/RB-123/12      ......................T...............T.........T.................-....
TH/CS-65/12       ......................T...........C...T.........T..........T......-....
TH/NP-79/12       ......................T...........A...T.........T.................T....
TH/NP-657/12      ......................T...........C...T.........T.................-....
TH/CS-80712/12    ......................T...........C...T.........T..........T......-....
TH/RB-881/12      ......................T...........C...T.........T.................-....
TH/NP-1157/12     ......................T...........C...T.........T.................-....
TH/NP-1169/12     ......................T...........C...T.........T.................-....
TH/AY-2.2/12      ......................T...........C...T.........T.................-....
TH/AY-2.7/12      ......................T...........C...T.........T.................-....
```

Figure16: The nucleotide substitutions of some current Thai isolates that represented minor deletions and insertion based on partial N gene sequence.

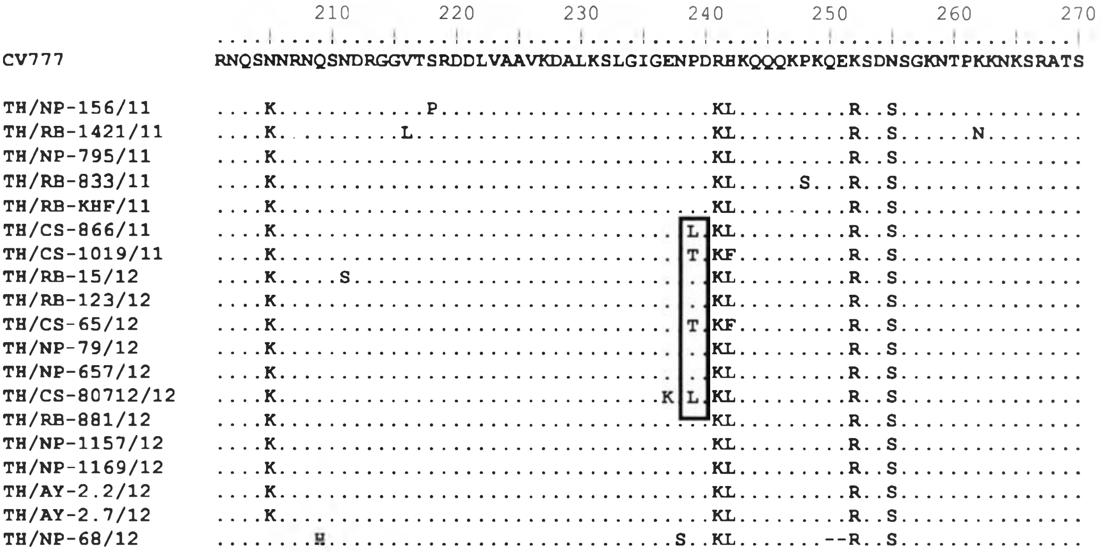


```
                          10        20        30        40        50        60        70
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CV777             MASVSFQDRGRKRVPLSLYAPLRVTNDKPLSKVLANNAVPTNKGNKDQQIGYWNEQIRWRMRRGERIEQP
TH/NP-156/11          ......................G...........................................
TH/RB-1421/11         ......................Y...........................................
TH/NP-795/11          ....................I.G....................H......................
TH/RB-833/11          ..................................................................
TH/RB-KHF/11          ..................................................................
TH/CS-866/11          ..................................................................
TH/CS-1019/11         ..................................................................
TH/RB-15/12           ..................................................................
TH/RB-123/12          ..................................................................
TH/CS-65/12           ..................................................................
TH/NP-79/12           ......................G....................H......................
TH/NP-657/12          G.L...--..........................................................
TH/CS-80712/12        ..................................................................
TH/RB-881/12          ......................G....................H......................
TH/NP-1157/12         ..................................................................
TH/NP-1169/12         ..................................................................
TH/AY-2.2/12          ......................G....................H......................
TH/AY-2.7/12          ......................G....................H......................
TH/NP-68/12           ....................................................L.............

                         210       220       230       240       250       260       270
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CV777             RNQSNNRNQSNDRGGVTSRDDLVAAVKDALKSLGIGENPDRHKQQQKPKQEKSDNSGKNTPKKNKSRATS
TH/NP-156/11      ....K............P......................KL.........R..S...............
TH/RB-1421/11     ....K..........L........................KL.........R..S......N........
TH/NP-795/11      ....K...................................KL.........R..S...............
TH/RB-833/11      ....K...................................KL.....S...R..S...............
TH/RB-KHF/11      ....K...................................KL.........R..S...............
TH/CS-866/11      ....K.................................L.KL.........R..S...............
TH/CS-1019/11     ....K.................................T.KF.........R..S...............
TH/RB-15/12       ....K.....S.............................KL.........R..S...............
TH/RB-123/12      ....K...................................KL.........R..S...............
TH/CS-65/12       ....K.................................T.KF.........R..S...............
TH/NP-79/12       ....K...................................KL.........R..S...............
TH/NP-657/12      ....K...................................KL.........R..S...............
TH/CS-80712/12    ....K...............................K.L.KL.........R..S...............
TH/RB-881/12      ....K...................................KL.........R..S...............
TH/NP-1157/12     ....K...................................KL.........R..S...............
TH/NP-1169/12     ....K...................................KL.........R..S...............
TH/AY-2.2/12      ....K...................................KL.........R..S...............
TH/AY-2.7/12      ....K...................................KL.........R..S...............
TH/NP-68/12       ........H............................S..KL.......--R..S...............

                         410       420       430       440
                  ....|....|....|....|....|....|....|....|
CV777             AIYDDVGAPSDVTHANLEWDTAXWWYGRNYQRDLRYRKL
TH/NP-156/11      .......V...E...........
TH/RB-1421/11     .......V...A...........
TH/NP-795/11      .......V...A...........
TH/RB-833/11      .......V...AA..........
TH/RB-KHF/11      ...G...V...A...........
TH/CS-866/11      .......V...A...........
TH/CS-1019/11     .......V...A...........
TH/RB-15/12       .......V...A...........
TH/RB-123/12      .......V...............
TH/CS-65/12       .......V...A...........
TH/NP-79/12       .......V...E..........C
TH/NP-657/12      .......V...A...........
TH/CS-80712/12    .......V...A...........
TH/RB-881/12      .......V...A...........
TH/NP-1157/12     .......V...A...........
TH/NP-1169/12     .......V...A...........
TH/AY-2.2/12      .......V...A...........
TH/AY-2.7/12      .......V...A...........
TH/NP-68/12       T......V...A...........
```

Figure17: The amino acid substitutions of some current Thai isolates that represented minor deletions and insertion based on partial N gene sequence.



```
                       210       220       230       240       250       260       270
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CV777           RNQSNNRNQSNDRGGVTSRDDLVAAVKDALKSLGIGENPDRHKQQQKPKQEKSDNSGKNTPKKNKSRATS

TH/NP-156/11    ....K............P......................KL.........R..S...............
TH/RB-1421/11   ....K..........L........................KL.........R..S......N........
TH/NP-795/11    ....K...................................KL.........R..S...............
TH/RB-833/11    ....K...................................KL.....S...R..S...............
TH/RB-KHF/11    ....K...................................KL.........R..S...............
TH/CS-866/11    ....K.................................L.KL.........R..S...............
TH/CS-1019/11   ....K.................................T.KF.........R..S...............
TH/RB-15/12     ....K.....S.............................KL.........R..S...............
TH/RB-123/12    ....K...................................KL.........R..S...............
TH/CS-65/12     ....K.................................T.KF.........R..S...............
TH/NP-79/12     ....K...................................KL.........R..S...............
TH/NP-657/12    ....K...................................KL.........R..S...............
TH/CS-80712/12  ....K................................KL.KL.........R..S...............
TH/RB-881/12    ....K...................................KL.........R..S...............
TH/NP-1157/12   ....K...................................KL.........R..S...............
TH/NP-1169/12   ....K...................................KL.........R..S...............
TH/AY-2.2/12    ....K...................................KL.........R..S...............
TH/AY-2.7/12    ....K...................................KL.........R..S...............
TH/NP-68/12     ........H............................S..KL.......--R..S...............
```

Figure18: The amino acid sequences of current Thai isolates from Chachoengsao Province (TH/CS-866/12, TH/CS-80712/12, TH/CS1019/12 and TH/CS-65/12) were compared to the other current Thai isolates.