A peer-reviewed version of this preprint was published in PeerJ on 8 May 2018.

Tuanthap S, Phupolphan C, Luengyosluechakul S, Duang-in A, Theamboonlers A, Wattanaphansak S, Vongpunsawad S, Amonsin A, Poovorawan Y. 2018. Porcine rotavirus C in pigs with gastroenteritis on Thai swine farms, 2011–2016. PeerJ 6:e4724
https://doi.org/10.7717/peerj.4724

Molecular characterization of porcine rotavirus C in pigs with gastroenteritis in Thailand, 2011 – 2016

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Swine are economically important food animals, but highly contagious enteric viruses can affect entire swine herds and contribute significantly to piglet morbidity and mortality. The most frequent viruses associated with pig gastroenteritis have been reported as porcine epidemic diarrhea virus (PEDV) and rotavirus. Rotavirus is an important cause of diarrhea in piglets and pigs worldwide, and group A and C types are those that pig herds are mostly affected by. In Thailand, studies on rotavirus group A (RVA) have been reported continuously, whereas information on group C is still limited. In this study, we aimed to identify rotavirus group C (RVC) from the feces and intestinal contents of pigs affected with diarrhea. Seven hundred and sixty-nine samples were collected from swine herds located in difference provinces throughout Thailand. The specimens were tested using virus-specific RT-PCR to detect the gene encoding RVC capsid protein VP7 and VP4. Sequencing analyses showed that 6.6% (51/769) of samples were positive for RVC, one third of which tested as single positive for RVC (34/51). Co-infections with the most frequent enteric viruses, RVA and PEDV were also analyzed. Co-infections of RVA/RVC accounted for 21.6% (11/51) of samples and of PEDV/RVC for 7.8% (4/51) of samples, while three samples (5.9%) tested positive for all three viruses. Infections were not associated with seasonality, since the virus was detected throughout the year. RVC was detected in pigs up to 8 weeks old. Analysis of the partial VP7 gene sequences was suggestive that the predominant genotype was G1, which was closely related to the prototype Cowden strain. Due to P[5] was the most prevalent of VP4 genotype. This study demonstrated the low prevalence of RVC in Thailand, a virus not previously documented in this country.

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Running Head: Molecular characterization of Thai PRVC

Keywords: Molecular characterization, pigs, porcine rotavirus C, Thailand, VP7, VP4

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ABSTRACT

Swine are economically important food animals, but highly contagious enteric viruses can affect entire swine herds and contribute significantly to piglet morbidity and mortality. The most frequent viruses associated with pig gastroenteritis have been reported as porcine epidemic diarrhea virus (PEDV) and rotavirus. Rotavirus is an important cause of diarrhea in piglets and pigs worldwide, and group A and C types are those that pig herds are mostly affected by. In Thailand, studies on rotavirus group A (RVA) have been reported continuously, whereas information on group C is still limited. In this study, we aimed to identify rotavirus group C (RVC) from the feces and intestinal contents of pigs affected with diarrhea. Seven hundred and sixty-nine samples were collected from swine herds located in difference provinces throughout Thailand. The specimens were tested using virus-specific RT-PCR to detect the gene encoding RVC capsid protein VP7 and VP4. Sequencing analyses showed that 6.6% (51/769) of samples were positive for RVC, one third of which tested as single positive for RVC (34/51). Co-infections with the most frequent enteric viruses, RVA and PEDV were also analyzed. Co-infections of RVA/RVC accounted for 21.6% (11/51) of samples and of PEDV/RVC for 7.8% (4/51) of samples, while three samples (5.9%) tested positive for all three viruses. Infections were not associated with seasonality, since the virus was detected throughout the year. RVC was detected in pigs up to 8 weeks old. Analysis of the partial VP7 gene sequences was suggestive that the predominant genotype was G1, which was closely related to the prototype Cowden strain. Due to P[5] was the most prevalent of VP4 genotype. This study demonstrated the low prevalence of RVC in Thailand, a virus not previously documented in this country.

INTRODUCTION

The viral gastroenteritis associated with high morbidity and mortality rates in suckling and post-weaning piglets is caused by porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus and rotavirus. For these viruses, there could be a single infection or mixed infections, for which the naturally infected pigs display similar symptoms and fecal appearances. Moreover, the pathogenesis of rotavirus and PEDV infection are similar, because the target cells of the viral replication are the villous enterocytes in the animal's intestine. Blunting of the villi of infected enterocytes and atrophy results in electrolyte imbalance, dehydration due to intestinal malabsorption, osmotic irregularities, watery diarrhea and eventually death (*Jung et al., 2015; Chang et al., 2012)*.

PEDV infection results in acute diarrhea in very young piglets and can occur throughout the year. Rotaviruses are also important causes of diarrhea in animals and can manifest in different disease severity depending on the age of the animals (*Ciarlet et al., 2002; Neog et al., 2011; Pott et al., 2012; Riepenhoff et al., 1982*). Although pigs of all ages are susceptible to rotavirus infection, neonatal and even post-weaned piglets are frequently infected (*Bohl et al., 1982; Lecce 1978)*. It is known that PEDV is endemic in some countries in Asia; in addition, several previous studies have shown that rotavirus was also responsible for diarrhea within swine herds.

Rotavirus is a member of the order *Piconavirales*, family *Reoviridae*, genus *Rotavirus*. The rotavirus particle is 75 nm in diameter, icosahedral in shape and a non-enveloped RNA virus with a triple layer capsid structure. The total genome size is approximately 18,522 bp, with 11 segments of double-stranded RNA, each segment encodes only one protein except segment 11, which can encode two nonstructural proteins in some species. Based on the serological

differences and diverse virus types, rotavirus has been classified into eight serogroups (groups A, B, C, D, E, F, G and H) using the VP6 sequence (*Matthijnssens et al., 2008*).

Porcine rotaviruses are divided into five serogroups (A, B, C, E and H). Group A is the major cause of diarrhea affecting piglets between 1 and 3 weeks of age. Also, it is the most common causative agent associated with diarrhea in both young humans and animals. Groups B is detected sporadically, while group C commonly causes diarrhea in pre-post weaning piglets (*Gouvea et al., 1991; Kim et al., 1999; Martella et al., 2007; Marthaler et al., 2013; Médici et al., 2011*). Group E has only been detected in pigs in the United Kingdom (*Chasey et al., 1986*). Interestingly, a new group H has been recently discovered (*Molinari et al., 2012; Wakuda et al., 2011*).

Rotavirus is easily transmitted via the fecal-oral route and the incubation period is 18-96 hours. The target site of rotavirus replication is the villous enterocytes in the small intestine, especially the jejunum and ileum; rotavirus also replicates in the duodenum, cecum and colon. Infection leads to cell lysis, villi blunting and atrophy. The disease severity is likely depends on whether piglets are co-infected with other viral enteric pathogens (*Amimo et al., 2013; Martella et al., 2007; Saif, 1999*).

Porcine rotavirus group C (RVC) was first identified in 1980 and considered as an enteric pathogen with a moderate prevalence rate of between 4 and 31% (*Saif et al., 1980*). There have been reports from many countries with an incidence rate of 4.4-46% (*Collins et al., 2008; Jeong et al., 2009; Martella et al., 2007; Moutelikova et al., 2015; Stipp et al., 2015; Suzuki et al., 2015; Theuns et al., 2016; Will et al., 1994*). However, RVC infections are often reported in piglets coinfected with other viruses, rather than a single infection. The infection was also found in asymptomatic pigs (*Collins et al., 2008; Marthaler et al., 2013; Saif et al., 1980; Theuns et*

al., 2016; Zhou et al., 2016). Currently, RVC genotypes based on the VP7 gene have been established as 10 G genotypes. Porcine RVCs are shown in G1, G3 and G5-G10, bovine RVCs are exhibited in G2, and human RVCs are exhibited in G4 (*Collins et al., 2008*; *Moutelikova et al., 2015; Rahman et al., 2005*).

For pig herds in Thailand, reports on infection and molecular characterization of porcine RVC are still limited. Thus, the objective of this study was to investigate the occurrence and molecular characterization of RVC in pigs with acute gastroenteritis from swine herds in Thailand between 2011 and 2016.

MATERIALS AND METHODS

The research followed the guidelines of Ethical Principles and the Use of Animals for Scientific Purposes from The National Research Council of Thailand. The protocol was approved by the Animal Care and Use Committee (IACUC) (animal use protocol number 1731020) and the Institutional Biosafety Committee (CU-VET-IBC) (protocol number IBC 1731008) of Chulalongkorn University, Bangkok, Thailand.

Specimen collection

The specimens in this study were stools and small intestine contents from pigs of various ages with clinical signs of watery diarrhea, and were submitted to the Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, located in Nakorn Pathom province, Thailand. Seven hundred and sixty-nine samples were collected in 2011 (n=40), 2012 (n= 95), 2013 (n= 87), 2014 (n= 158), 2015 (n= 164) and 2016 (n= 225) from 123 commercial swine farms in different provinces throughout Thailand. One hundred and seventy three swine

samples were collected from the central provinces (Lop Buri, Samut Songkhram, Suphan Buri, Saraburi, Phra Nakhon Si Ayutthaya and Nakhon Pathom); 316 samples were from the western provinces (Kanchanaburi**,** Prachuap Khiri Khan**,** Phetchaburi and Ratchaburi); 109 samples were from the eastern provinces (Chon Buri and Chachoengsao); 80 samples were from the northeastern provinces (Ubon Ratchathani, Udon Thani and Nakhon Ratchasima); 26 samples were from the southern provinces (Trang and Nakhon Si Thammarat); and 65 samples were from unspecified locations. The locations of the sample collection areas are shown in Fig. 1. Moreover, samples were categorized into age groups: 0-6 days, 1-4 weeks (pre-weaning), ≥4-8 weeks (early nursery), ≥8-12 weeks (late nursery), >12 weeks (starter-finisher), and sow (both pregnant and lactating).

Specimen preparation

The intestinal mucosa samples were collected by tissue scraping technique from the duodenum and upper part of the jejunum, especially the thin walled area with gas accumulation inside the lumen. An approximately 10% (v/v) sample suspension in sterile phosphate-buffered saline solution (0.1 M, pH 7.2) was centrifuged at 3,000 *g* for 20 minutes and only the supernatant collected. The supernatant was kept at −80 °C until testing. Fecal samples were prepared with the same protocol.

Viral nucleic acid extraction

Viral genome extractions were performed in the biosafety level 2 laboratory at the Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, located in Nakorn Pathom province, Thailand. Viral RNA was extracted using a Ribospin vRD

II viral RNA purification kit (GeneAll, Seoul, Korea) according to the manufacturer's instructions and kept at −80°C. Afterwards, the purified RNAs were delivered to the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, for the RNA amplification step.

Viral detection

PEDV and rotavirus group A (RVA) detection

Samples were screened using one-step RT-PCR (SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA polymerase; Invitrogen, Carlsbad, CA, USA) to amplify the partial S gene of PEDV and VP7 gene primers were used to screen for RVA. Cycling parameters were: reverse transcription at 48°C for 45 minutes; initial denaturation at 95°C for 2 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute and extension at 72°C for 90 seconds; and a final extension at 72°C for 5 minutes. The PCR amplicon was separated by 1% agarose gel electrophoresis, stained with 0.002% ethidium bromide solution (200 ul of ethidium bromide (concentration 10mg/ml)/ 1 ml of distilled water) and visualized under UV with a transilluminator. The primer sequences, annealing temperatures and amplicons with product sizes are shown in Table 1.

RVC detection

All specimens were also screened for RVC. Amplification of the VP7 gene region was performed by using specifically designed primers from our center. Partial VP4 gene amplification was subsequently performed for all VP7-positive samples. The RT-PCR and sequencing was carried out using a set of VP4 primers from Amimo, reported in 2013, for which

the amplicons were located at the VP8 segment (*Amimo et al., 2013*). The primer sequences, annealing temperatures and amplicons with product sizes are shown in Table 1.

The cDNA synthesis was performed by one-step RT-PCR by using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). The conditions were: reverse transcription at 48°C for 45 minutes; initial denaturation at 95°C for 2 minutes; followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 1 minute and extension at 72°C for 90 seconds; and final extension at 72°C for 5 minutes. The PCR amplicon was separated by 1.5% agarose gel electrophoresis, stained with 0.002% ethidium bromide solution and visualized under UV with a transilluminator. The 1046 bp amplicon of VP7 and the 1222 bp amplicon of VP4 were cut from the agarose gel and purified by using a Purification Kit (GeneAll, Seoul, Korea). Consequently, the purified products were sent for sequencing (First BASE Laboratories, Selangor, Malaysia).

Molecular characterization and phylogenetic analysis of RVC

The nucleotide sequences were assembled using SeqMan sequence analysis software Version 6 (DNASTAR Inc., Madison, WI). The nucleotide sequence alignment was performed by using Clustral X multiple alignment, version 2.0.11 (*Larkin et al., 2007*). The nucleotide sequence was compared with reference sequences from the GenBank database. Phylogenetic trees were reconstructed by MEGA software (version 6) using the maximum-likelihood method and 1,000 replicates of bootstrap pseudo-replicates to determine the genetic variation and the relationships with reference sequences (*Tamura et al., 2013*). Bootstrap values >85% were considered significant for the VP7 gene and >80% for the VP4 gene. The VP7 nucleotide sequences from this study are available in the GenBank database under accession numbers KX911667-KX911708, MF139507-MF139509 and MF139516-MF139517. For the VP4 nucleotide sequences, the accession numbers are MG575522-MG575532.

RESULTS

Viral detection, and seasonal and age distribution

Among 769 samples from pigs of all ages submitted between May 2011 and Aug 2016, RVC were detected in 6.6% (51/769) of the samples, while 19.9% (153/769) of the samples were positive for PEDV and 9.5% (73/769) were positive for RVA. The total number of samples from the 5 year summary and the number of positive cases each month are shown in Fig. 2. Co-infection with two or more viruses in the samples was not very common. Mixed rotavirus species infection such as RVC and RVA were identified at the 21.6% (11/51) level, while RVC/PEDV was found at the 7.8% (4/51) level. Meanwhile, three samples (5.9%) tested positive for all three viruses. Overall, the most frequent co-infection was PEDV/RVA.

The RVC and RVA infections were found at the highest occurrence rate in >4- to 8-week-old piglets, whereas PEDV was most prevalent in piglets less than a week old (32.9%) (Fig. 3). All of the RVC-positive samples were collected from piglets with clinical signs of diarrhea. A list of the 47 RVC strains, the co-infections with RVA or PEDV, or single RVC infection status, the age of pigs, sample types, and G and P genotypes of the RVC strains, are shown in Table 2.

Molecular and phylogenetic analysis of VP7 and VP4 genes

To better understand the genetic relationship between RVC and genotype, 47 RVC-positive samples were selected for VP7 and VP4 gene sequencing. The VP7 gene was used for RVC screening, and VP4 gene amplification was performed subsequently.

The nearly full length of the VP7 sequences and the partial VP8 segment of VP4 were

determined (expected product lengths 1046 bp and 1222 bp, respectively). A phylogenetic tree was reconstructed to compare 47 RVC strains and known RVC sequences that were available from the GenBank database. The 85% and 80% nucleotide percent identity cut-off values were proposed to divide the phylogenetic tree for VP7 and VP4 genotypes, respectively.

VP7 gene sequence

The G genotype identification (VP7 gene) was obtained for 47 from a total of 51 RVC-positive samples (4 samples were not successfully sequenced). We identified four G genotypes as G1, G3, G6 and G9. G1 was the most frequently detected in 54.9% (28/51) samples, G6 was detected in 19.6% (10/51), G9 was detected in 15.7% (8/51) and G3 was the least frequently found in 2% (1/51) samples. The phylogenetic tree is shown in Fig. 4.

The nearly full-length VP7 sequence (nt 112-952) encoding a protein of 279 aa was analyzed. We analyzed residues 38 to 316, which were located between the VR2 and VR8 regions, while the VR-1 region was non-applicable. Several amino acid changes within the variable region were found. The open reading frame within variable region-2 (VR-2) of RVC G1 and G9 represented three variable sites at residues 39, 53 and 57. Almost all G6 strains (9/10 strains) were found to have a 4 aa insertion between residues 245 and 248 (SSSV/SSTL/SSTM/SSSM), located at the carboxy-terminus of the VR8 variable region, except for strain RVC/Pig/THA/CU146C/16/G6 (Supplementary data; Table S1). The VR-4 region, the potential *N*-linked glycosylation sites (located at residues 67-69 and 225-227 (Asn-X-Ser/Thr)) and the putative signal cleavage site (residues 49-50 (A/G-Q)) were conserved in all strains in this study.

An analysis of genetic relationship between strains in this study and previous RVC strains was included. The results showed G1 strains were closely related to the prototype strain Cowden (86.1-91.7% nucleotide identity). G6 strains shared high nucleotide identities to a porcine rotavirus strain from Italy (strain ITA/43/06, which was isolated in 2005) (88.6-90.9%). G9 strains were closely related to a Vietnamese porcine rotavirus strain (strain RVC/Pig-wt/VNM/14175_22) (86.3-89.5% nucleotide identity). G4 strains were diverse from human RVC, due to a G3 strain RVC/Pig/THA/CU-PY/12/G3 that shared low relatedness to the porcine G3 prototype strain HF (78%).

VP4 gene sequence

The nearly full length of the VP8 segment (nt 43-1155), encoding a protein of 371 aa, was selected to determine the genotype. We were able to identify only 11 VP4 sequences from the total of 47 VP7 sequences. Four P genotypes, P[1], P[4], P[5] and P[7] were detected. P[5] was the most frequently detected (54.5 %, 6/11), while P[4] was detected in 18.2% (2/11) VP4 sequences, P[7] in 18.2% (2/11) and P[7] in 9.1% (1/11).

The nucleotide sequence analysis indicated that among the P[4] genotype, Thai RVC strains shared 79.5% nucleotide identity with each other, while P[7] strains shared 80.7% nucleotide identity and identity was more than 99.8% for P[5] strains. A phylogenetic tree of the VP4 gene sequences was also reconstructed (Fig. 5).

For all the Thai strain amino acid sequences, no insertions or deletions were found in their VP8 segments, including the cleavage site. Hypervariable amino acid positions were found for many sites, such as positions 228, 236 and 241. Conserved regions were found behind position 260 (Fig. 6).

The G/P combination of 11 Thai RVC strains was classified into six combinations, as G6P[5], G1P[1], G1P[4], G1P[5], G9P[4] and G9P[7]. G6P[5] was the predominant G/P genotype in this study (45.5%, 5/11).

DISCUSSION

In the past, most reports of porcine rotavirus prevalence in Thailand involved only RVA (approximately 10-23%), while epidemiological study of porcine RVC was limited (*Chan-it et al., 2008; Khamrin et al., 2007; Maneekarn et al., 2014; Yodmeeklin et al., 2016*). RVC infections were found with a lower prevalence rate than RVA in symptomatic piglets with diarrhea and had been detected as a single infection or in combination with other enteric viruses (*Collins et al., 2008; Marthaler et al., 2014; Nagesha et al., 1988; Theuns et al., 2016; Zhou et al., 2016*). Our study demonstrated a RVC from symptomatic pigs and piglets in low occurrence rate around 6.6%. We found that RVC appeared most frequently in pigs of >4-8 weeks old, while samples from pigs older than 12 weeks were decreased for RVC positivity. The results correlated with previous reports that indicated RVC is the cause of acute gastroenteritis in various age of piglets (pre-post weaned piglets) (*Amimo et al., 2013; Jeong et al., 2009; Kim et al., 1999; Martella et al., 2007; Marthaler et al., 2013;Suzuki et al., 2015*).

Rotavirus could be detected as a single infection or a mixed infection with enteric viruses, which RVC are often reported in mixed infections (*Martell et al., 2007; Médici et al., 2011*). In the case of mixed infection, the intestinal epithelium damage and/or viral replication were increased, resulting in more severe diarrhea being found often (*Amimo et al., 2013; Jeong et al., 2009; Ishimaru et al., 1991; Martella et al., 2007*). In this study, we found cases of dual infections between PEDV and rotavirus in younger piglets (<4 weeks old) often showed a higher

morbidity rate. Likewise, several previous studies reported that younger piglets showed higher morbidity and mortality than older pigs (*Annamalai et al., 2015; Shibata et al., 2000; Steyer et al., 2008*). It was probably that PEDV infection could contribute to the rapid turnover rate of enterocytes and have an influence in rapid disease recovery (*Jeong et al., 2015*). Apart from piglets, we also found dual infection between PEDV and RVA in sows, even though sows are usually asymptomatic in either disease. The possibility of multiple enteric viruses circulating and persisting within swine herds might be increased from vertical transmission at the beginning.

For seasonal factor, most of the previous studies indicated the rotavirus infection was frequently found in the winter season. Thailand is located in the tropics, with warm weather year round but the rotavirus could be detected throughout the year in this study. Nevertheless, there are several studies suggested rotavirus infection is less seasonally influenced in the tropical zone, because relatively high humidity may facilitate increased rotavirus infection (*Cook et al., 2004; Levy et al., 2009*).

For the study of genetic relationships, the VP7 nucleotide sequences of the Thai RVC strains were compared. Most of field Thai RV strains had close genetic relationship, such as the G1 strains shared nucleotide sequence identities of between 83.7 and 100%, the G6 and G9 strains shared 82.2-100% and 83.2-100% nucleotide identities in their genotypes.

Even though, there was some evidence of genetic diversity among G6 Thai strains. Strain RVC/Pig/THA/CU146C/16/G6 was separated out of the genotype clusters and also shared lower nucleotide identity among Thai strains (82.2-84.4%). The amino acid sequence of RVC/THA/CU146C/16/G6 lacked four amino acid residues between positions 245 and 246; these deletions may give the minor genetic diversity of this strain. This finding is in

concordance with an Irish RVC strain (strain 1GA/05/Cork) in a 2008 study (*Collins et al., 2008*).

Apart from strain RVC/Pig/THA/CU146C/16/G6, we found the G6 genotype has four amino acid additions at the carboxy-terminus of the variable region VR8 (between residues 245 and 248). These findings were related to several strains from Italy (strain 344/04-7, 43/06-16, 43/06-22 and 134/04-2), the Czech Republic (strain CZE/P8/2011) and Japan (strain CJ3-6) (*Martella et al., 2007; Moutelikova et al., 2015; Suzuki et al., 2015*). However, there are no documented reports linking this substitution with any disease severity.

G1 was the predominant genotype and G3 a rare genotype detected in this study, unlike in a Korean report, which suggested G3 and G7 were the most frequently detected genotypes (*Jeong et al., 2015*). However, this finding of a predominant G1 genotype was correlated with previous reports from Ireland, the USA, Canada and the Czech Republic (*Collins et al., 2008; Marthaler et al., 2013; Moutelikova et al., 2015*).

The mixed G genotypes within the same farms were also detected in this study. The specific genotype distributions among each year were not clear, even though the G1 genotype has been the most commonly detected since 2014. Likewise, the report from Martella 2007 suggested inappropriate management and overcrowded conditions may lead to multiple enteric pathogens or mixed viral infections (*Martella et al., 2007*).

Due to the fact that we were not able to sequence all the VP4 gene (for a total of 47 RVC strains), there were probably issues such as high variability in the region that we used for amplification (VP8 segment, aa positions 1-231) or RNA degradation from long-term sample storage (*Diaz-Salinas et al., 2013*). P[5] genotype was the most often found because the P[5]

strains were collected from the same herd and the same period, it was probably those strains that had high genetic similarity rather than other genotypes such as P[4] or P[7].

The genetic relationship between VP4 sequences in this study and previous RVC isolates was also determined. The sequence identity among Thai RVC strains and porcine prototype strains was quite low, such as the Cowden strain was between 59.6 and 66%, for the human strain Bristol was 52.2-62.7% and for bovine strain Shintoku was 59.5-66.1%. Most of the Thai strains had nucleotide identities close to Asian RVC strains (Korean and Japanese strains) (*Jeong et al., 2015; Suzuki et al., 2015*). This finding was suggestive that the same RVC genotype might circulate and be maintained within Asian countries.

Conclusion

The reports of porcine RVC prevalence, genomic information and G/P genotypes in Thailand are very limited. Therefore, these finding provide information about RVC surveillance and molecular characteristics based on VP7 and VP4 that might be useful for a better understanding of the re-occurring, genetic variation among Thai RVC strains, or of the possibility of interspecies transmission. However, further studies on porcine RVC molecular characterization are required to reduce the serious economic loss in the swine industry from single RVC infection or mixed infection with the other enteric viruses.

ACKNOWLEDGEMENTS

We thank the staff of the Center of Excellence in Clinical Virology and the Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University for their helpful support and assistance.

Funding

This work was supported by the 100th Anniversary Chulalongkorn University Fund for doctoral scholarship to Supansa Tuanthap in Inter-Department Program of Biomedical Sciences, Faculty of Graduate School, Chulalongkorn University, Bangkok, Thailand, the National Research Council of Thailand, the Research Chair Grant from NSTDA (P-15-50004) and the Center of Excellence in Clinical Virology (GCE 59-009-30-005).

Grant Disclosures

The 100th Anniversary Chulalongkorn University Fund for doctoral scholarship
The National Research Council of Thailand, the Research Chair Grant from NSTDA (P-15-50004)
The Center of Excellence in Clinical Virology (GCE 59-009-30-005)

Conflict of Interest

The authors have no conflicts of interest to declare.

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LEGENDS

Fig. 1 Thailand map and provinces where samples were collected.

Fig. 2 Bar graph indicating the total number of samples from the 5 year summary and the number of positive cases each month.

Fig. 3 Age distribution of PEDV, RVA and RVC infection cases.

Fig. 4 Phylogenetic tree of the RVC VP7 gene. The black dot symbols in front of the names represent porcine RVC strains in this study, bold with underline indicates porcine RVC prototypes. For more detail, the phylogenetic tree branches of genotypes G1, G9 and G6 are represented in separate columns as follows, 4a, 4b and 4c, respectively

Fig. 5 Phylogenetic tree of the RVC VP4 gene. The black triangle symbols in front of the names represent porcine RVC strains in this study.

Fig. 6 Amino acid alignment sequence of RVC VP4. The colored, bold letters represent conserved positions, dashes indicate gaps, bold with underline indicates *N*-glycosylation sites, and bold represents hypervariable positions.

Table 1. Oligonucleotide primers used in this study.

Table 2. Porcine RVC strain information.

Table S1. Multiple sequence alignments of the eight variable regions (VR1–VR8) in each RVC genotypes.

Table 1(on next page)

Oligonucleotide primers used in this study.

1 **Table 1**. Oligonucleotide primers used in this study.

Primers	Nucleotide sequence (5′ to 3′)	Position	T_m	Product size
PEDV S gene	TTCTGAGTCACGAACAGCCA	1466-1485	55°C	651 bp
(*Kim et al., 2001*)	CATATGCAGCCTGCTCTGAA	2097-2116		
RVA VP7 gene	VP7-CU-RVAF: CGGTTAGCTCCTTTTAATGT	33-52	55°C	891 bp
(accession number AB176677.1)	VP7-CU-RVAR: CATTTCTTCCAATTTACTCGC	903-924		
RVC VP7	VP7-CU-RVCF: GAAGCTGTCTGACAAACTGG	17-36	52°C	1046 bp
(accession number M61101.1)	VP7-CU-RVCR: GCCACATGATCTTGTTTACGC	1042-1061		
RVC VP4	VP4-17Fdeg: GATCRATGGCGTCYTCAC	17-34	55°C	1222 bp
(*Amimo et al., 2013*)	VP4-1238R: CCTGATGAATGTAATCCWGGAT	1216-1238		

2

Table 2(on next page)

Porcine RVC strain information.

Table 2. Porcine RVC strain information.

Collection year	Strain name	Age of host (week)	Sample	RVC genotype		RVA	PEDV
				VP7	VP4		
2012	RVC/Pig/THA/CU-PY/12/G3	1-4	Small intestine	G3			
2013	RVC/Pig/THA/CU571/13/G6	n/a	Feces	G6			
	RVC/Pig/THA/CU264-U12/13/G9	n/a	Feces	G9	P[7]		
2014	RVC/Pig/THA/CU875-1C/14/G1	5-8	Small intestine	G1		+	
	RVC/Pig/THA/CU1035/14/G1	1-4	Feces	G1			+
	RVC/Pig/THA/CU781-2/14/G1	1-4	Small intestine	G1			
2015	RVC/Pig/THA/CU-SUN/15/G9	5-8	Feces	G9		+	
	RVC/Pig/THA/CU-BDN-C/15/G1	5-8	Feces	G1		+	
	RVC/Pig/THA/CUSB-N/15/G1	5-8	Feces	G1		+	
	RVC/Pig/THA/CU-CHN/15/G1	5-8	Feces	G1			
	RVC/Pig/THA/CU4-6C/15/G1	5-8	Small intestine	G1			
	RVC/Pig/THA/CU5-1C/15/G1	5-8	Small intestine	G1			
	RVC/Pig/THA/CU5-3/15/G1	5-8	Small intestine	G1			
	RVC/Pig/THA/CU12/15/G6	5-8	Feces	G6			
	RVC/Pig/THA/CU13/15/G9	1-4	Feces	G9			
	RVC/Pig/THA/CU14/15/G1	5-8	Feces	G1			
	RVC/Pig/THA/CU40/15/G9	5-8	Feces	G9	P[4]	+	
	RVC/Pig/THA/CU48/15/G1	5-8	Feces	G1	P[4]		
	RVC/Pig/THA/CU49/15/G9	1-4	Feces	G9			
	RVC/Pig/THA/CU54/15/G6	5-8	Small intestine	G6			
	RVC/Pig/THA/CU60/15/G1	5-8	Small intestine	G1	P[5]		+
	RVC/Pig/THA/CU62C/15/G1	5-8	Small intestine	G1			
	RVC/Pig/THA/CU68C/15/G1	5-8	Small intestine	G1			
	RVC/Pig/THA/CU69C/15/G1	5-8	Small intestine	G1			
	RVC/Pig/THA/CU74C/15/G1	1-4	Small intestine	G1			+
	RVC/Pig/THA/CU79C/15/G1	0-6 d	Small intestine	G1			+
	RVC/Pig/THA/CU84/15/G9	5-8	Feces	G9	P[7]	+	+

4 **continued*

Collection year	Strain name	Age of host (week)	Sample	RVC genotype		RVA	PEDV
				VP7	VP4		
2016	RVC/Pig/THA/CU108C/16/G1	5-8	Feces	G1			
	RVC/Pig/THA/CU109C/16/G1	1-4	Feces	G1			
	RVC/Pig/THA/CU111C/16/G1	1-4	Feces	G1			
	RVC/Pig/THA/CU150C/16/G1	5-8	Small intestine	G1			
	RVC/Pig/THA/CU115C/15/G1	5-8	Feces	G1			
	RVC/Pig/THA/CU99C/16/G1	5-8	Feces	G1		+	+
	RVC/Pig/THA/CU100C/16/G1	5-8	Feces	G1		+	+
	RVC/Pig/THA/CU122/16/G6	0-6 d	Feces	G6	P[5]		
	RVC/Pig/THA/CU123/16/G6	0-6 d	Feces	G6	P[5]		
	RVC/Pig/THA/CU124/16/G6	0-6 d	Feces	G6	P[5]		
	RVC/Pig/THA/CU125/16/G6	0-6 d	Feces	G6	P[5]		
	RVC/Pig/THA/CU135/16/G6	1-4	Feces	G6	P[5]		
	RVC/Pig/THA/CU136/16/G6	1-4	Feces	G6		+	
	RVC/Pig/THA/CU146C/16/G6	5-8	Feces	G6			
	RVC/Pig/THA/CU200/16/G1	5-8	Feces	G1	P[1]	+	
	RVC/Pig/THA/CU201C/16/G1	1-4	Feces	G1			
	RVC/Pig/THA/CU202/16/G1	5-8	Feces	G1			
	RVC/Pig/THA/CU275C/16/G9	1-4	Feces	G9			
	RVC/Pig/THA/CU276C/16/G9	1-4	Feces	G9			
	RVC/Pig/THA/CU330C/16/G1	5-8	Feces	G1			

5

Figure 1

Thailand map and provinces where samples were collected.

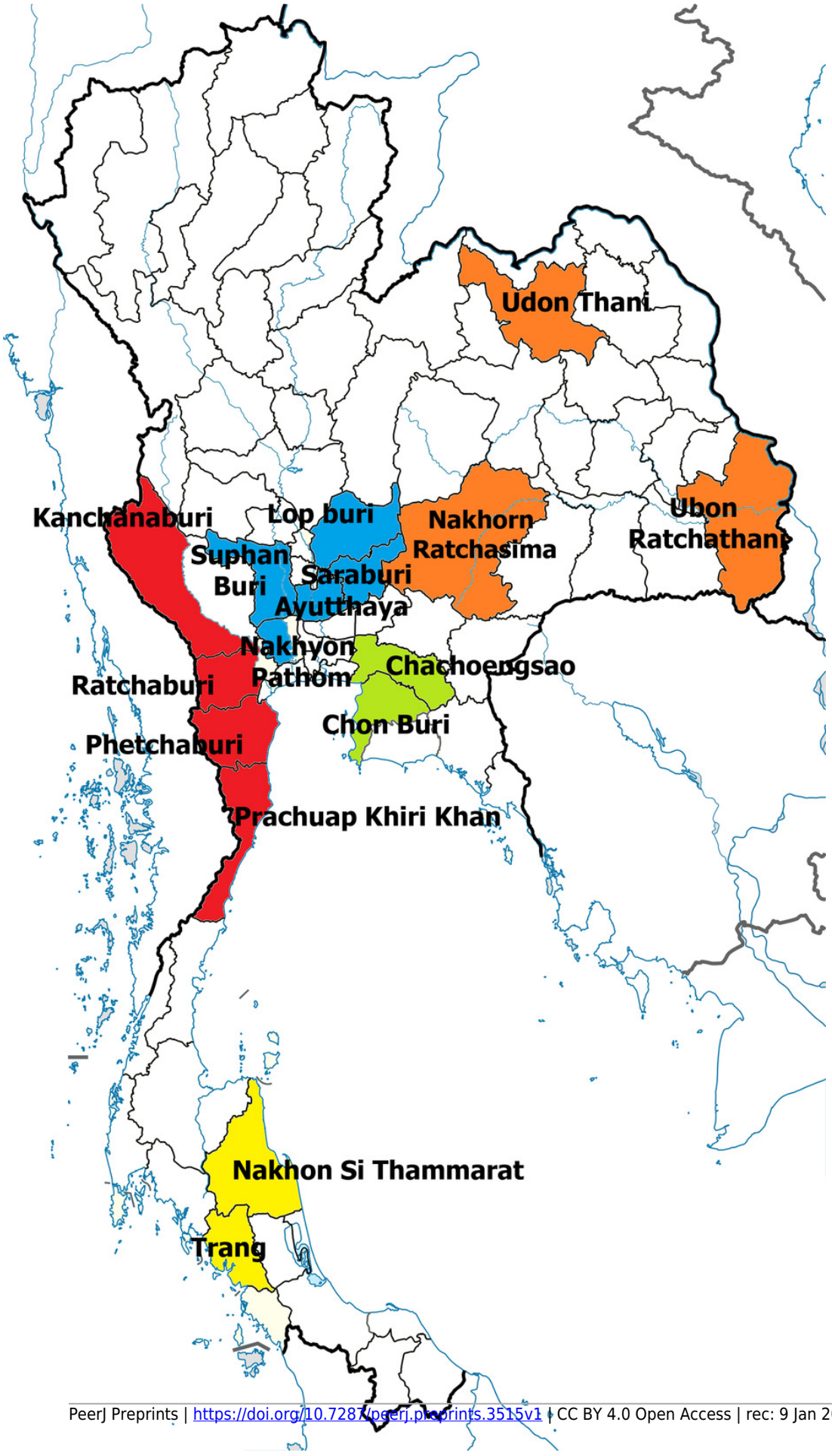

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Figure 2

Bar graph indicating the total number of samples from the 5 year summary and the number of positive cases each month.

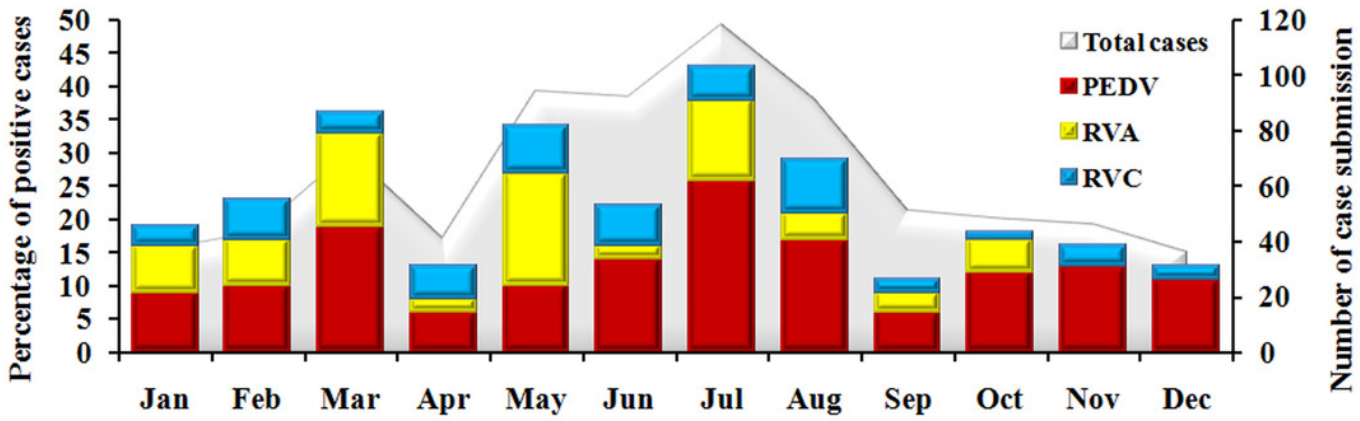

Figure 3

Age distribution of PEDV, RVA and RVC infection cases.

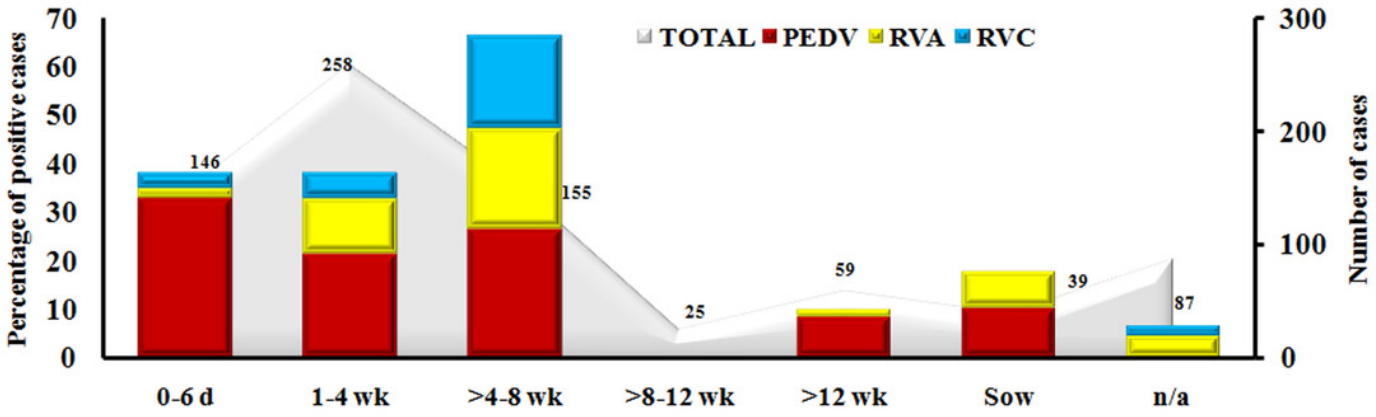

Figure 4

Phylogenetic tree of the RVC VP7 gene.

The black dot symbols in front of the names represent porcine RVC strains in this study, bold with underline indicates porcine RVC prototypes. For more detail, the phylogenetic tree branches of genotypes G1, G9 and G6 are represented in separate columns as follows, 4a, 4b and 4c, respectively.

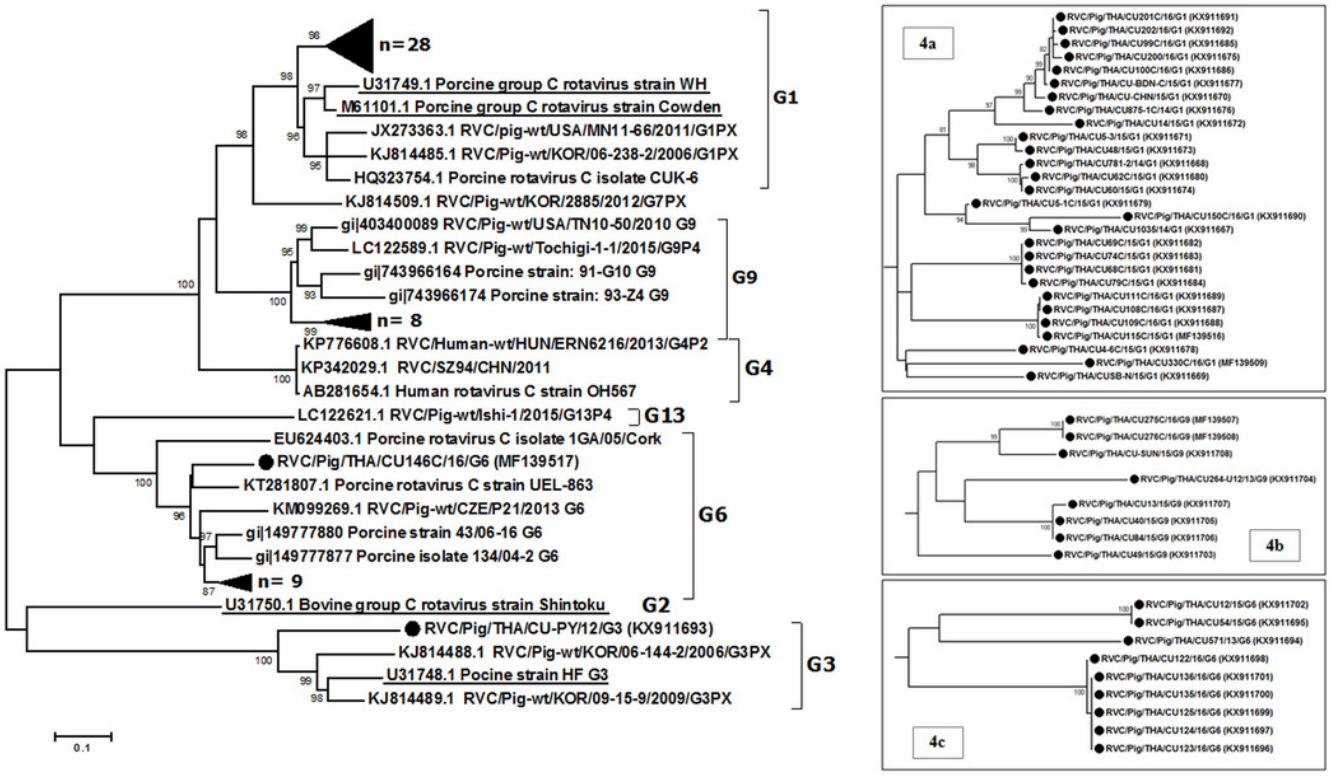

Figure 5

Phylogenetic tree of the RVC VP4 gene.

The black triangle symbols in front of the names represent porcine RVC strains in this study.

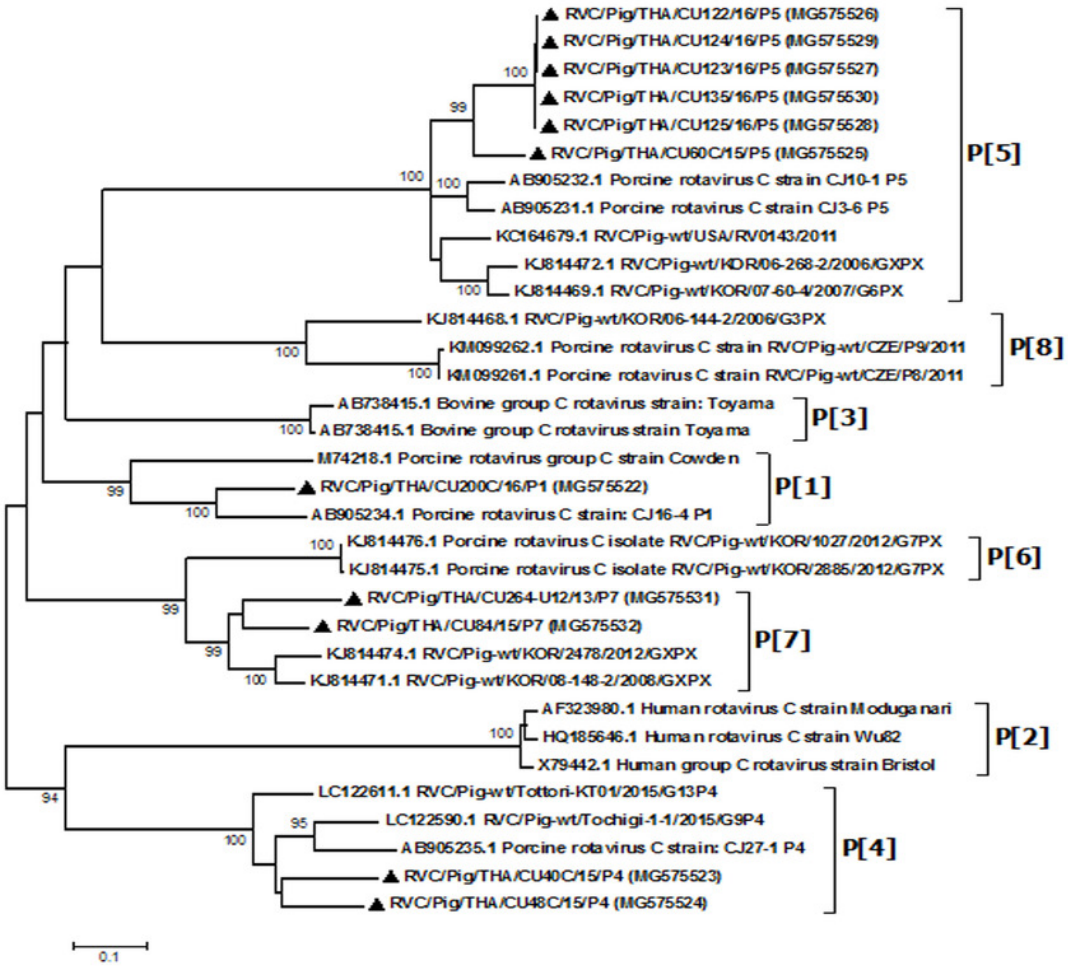

Figure 6

Amino acid alignment sequence of RVC VP4.

The colored, bold letters represent conserved positions, dashes indicate gaps, bold with underline indicates *N*-glycosylation sites, and bold represents hypervariable positions.