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Concurrent porcine circovirus type 2a (PCV2a) or PCV2b infection increases the rate of amino acid mutations of porcine reproductive and respiratory syndrome virus (PRRSV) during serial passages in pigs

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) has a high degree of genetic and antigenic variability. The purpose of this study was to determine if porcine circovirus type 2 (PCV2) infection increases genetic variability of PRRSV during serial passages in pigs and to determine if there is a difference in the PRRSV mutation rate between pigs concurrently infected with PCV2a or PCV2b. After 8 consecutive passages of PRRSV alone (group 1), PRRSV with PCV2a (group 2), or PCV2b (group 3) in pigs, the sequences of PRRSV structural genes for open reading frame (ORF) 5, ORF6, ORF7 and the partial non-structural protein gene (Nsp) 2 were determined. The total number of identified amino acid mutations in ORF5, ORF6, ORF7 and Nsp2 sequences was 30 for PRRSV infection only, 63 for PRRSV/PCV2a concurrent infection, and 77 for PRRSV/PCV2b concurrent infection when compared with the original VR2385 virus used to infect the passage 1 pigs. Compared to what occurred in pigs infected with PRRSV only, the mutation rates in ORF5 and ORF6 were significantly higher for concurrent PRRSV/PCV2b infected pigs. The PRRSV/PCV2a pigs had a significantly higher mutation rate in ORF7. The results from this study indicated that, besides ORF5 and Nsp2, the PRRSV structural genes ORF6 and ORF7 were shown to mutate at various degrees when the PRRSV was passaged over time *in vivo*. Furthermore, a significantly higher mutation rate of PRRSV was observed when pigs were co-infected with PCV2 highlighting the importance of concurrent infections on PRRSV evolution and control.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is commonly characterized by respiratory disease and increased mortality in growing pigs and reproductive disease in breeding herds (Halbur et al., 1996; Mengeling et al., 1998). PRRS was first recognized in the United States in 1987 and in Europe in 1990 (Paton et al., 1991) and the causative agent, PRRS virus (PRRSV), since then has become an important pathogen globally. It has been estimated that PRRS costs the US swine industry approximately $640 million per year due to production losses, treatment costs and excessive mortality (Holtkamp et al., 2013).

PRRSV is a small, single-stranded positive sense, enveloped, RNA virus that is a member of the family *Arteriviridae* in the order *Nidovirales* (Cavanagh, 1997). The genome of PRRSV is about 15 kb in size and contains 10 open reading frames (ORF), designated as ORF1a, ORF1b, ORF2a, ORF2b, ORF3, ORF4, ORF5, ORF5a, ORF6 and ORF7 (Firth et al., 2011; Johnson et al., 2011). PRRSV can be divided into two main genotypes: type 1 (European type) and type 2 (North American type) (Shi et al., 2010). Similar to many other RNA viruses, PRRSV is characterized by a high mutation rate and the potential for the emergence of new genetically diverse strains (Pirzadeh et al., 1998; Rowland et al., 1999; Forsberg et al., 2001; Hanada et al., 2005). There is evidence that PRRSV isolates with varying degrees of virulence continue to emerge worldwide (Nelsen et al., 1999; Han et al., 2006; Fang et al., 2007) and this is possibly due to a high degree of viral recombination and/or mutation (Yuan et al., 1999, 2000, 2001, 2004). Quasispecies evolution of PRRSV has been demonstrated during sequential passages in pigs (Chang et al., 2002). Specifically, three independent groups of pigs were infected

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with type 2 PRRSV strain VR-2332 and the PRRSV infection was maintained for 367 days by pig-to-pig passage of infectious material at 60-day-intervals. The authors found ORF1b and ORF7 to be highly conserved whereas 48 nucleotide mutations were identified within ORF5 (Chang et al., 2002).

In 2006, a "pig high fever disease" emerged in China and was initially attributed to pigs coinfected with PRRSV, porcine circovirus type 2 (PCV2) and classical swine fever virus (Tian et al., 2007). The disease was characterized by high morbidity and mortality and subsequently spread to more than 10 Chinese provinces affecting over 2 million pigs with morbidity rates of 50–100% and mortality rates of 20–100% (Li et al., 2007; Tian et al., 2007; Li et al., 2011). A PRRSV variant designated as high pathogenic PRRSV or HP-PRRSV which is characterized by a unique discontinuous deletion of 30 amino acids in the Nsp2 gene was identified (Yu et al., 2012).

Porcine circovirus associated disease (PCVAD) was first described in the early 1990s and since then has become a serious economic problem for the swine industry worldwide (Opriessnig et al., 2007). PCV2 is a small, non-enveloped, single-stranded, circular DNA virus can be divided into PCV2a and PCV2b genotypes which are present worldwide. A third genotype, PCV2c, has only been identified in archived samples from pigs in Denmark (Dupont et al., 2008; Grau-Roma et al., 2008).

The 2005/2006 outbreaks of PCVAD in Canada, North Carolina, and the Midwestern U.S. raised concerns over the introduction of a new and more virulent PCV2 variant into North America. In most cases, PCV2b strains were identified in severe outbreaks (Cheung et al., 2007); however, current experimental evidence does not support major differences in virulence or pathogenicity between PCV2a and PCV2b (Fort et al., 2008; Opriessnig et al., 2008). PCV2 has been shown to impair both the onset of protective immunity (Segalés et al., 2001; Darwich et al., 2002) and induction of proinflammatory cytokines (Darwich et al., 2003; Vincent et al., 2005) which may indirectly enhance the cellular uptake, replication and survivability of concurrent PRRSV infection. Previous studies demonstrated that concurrent infection of PRRSV and PCV2a or PCV2b prolongs PCV2 viremia and shedding when compared to pigs infected with PCV2 alone (Rovira et al., 2002; Opriessnig et al., 2008; Sinha et al., 2011). Similarly, pigs co-infected with PRRSV and PCV2a or PCV2b had more severe macroscopic and microscopic lung lesions and a stronger anti-PRRSV IgG response compared to pigs infected with PRRSV alone (Opriessnig et al., 2012). Taken together, these studies emphasize the importance of co-infection of both pathogens in disease expression and pathogenesis of PRRS and PCVAD.

The objectives of this study were to determine if PCV2 infection increases the ability of PRRSV to mutate over time during serial passages in pigs and to determine if there is a difference in the PRRSV amino acid mutation rate between pigs concurrently infected with PCV2a or PCV2b.

2. Materials and methods

2.1. Experimental design and inoculation

All study procedures were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC# 4-09-6729-S) and the Institutional Biosafety Committee (IBC# 09-I-011-A). Sixty-four conventional crossbred pigs were purchased from a PRRSV negative source farm. Approximately every 2 months a new group of eight pigs was purchased. The eight pigs were randomly assigned to four groups and rooms with two pigs in each room.

For the initial inoculation, pigs from groups 1, 2 and 3 were inoculated intranasally with 4 ml of PRRSV VR2385 at a dose of $10^{5.69}$ $TCID_{50}$ derived from a DNA-launched infectious clone (P0) as described (Ni et al., 2011). In addition, pigs from groups 2 and 3 were inoculated intranasally (3 ml) and intramuscularly (2 ml) with PCV2a (strain 40895) or PCV2b (strain NC-16845) at a dose of $10^{4.5}$ $TCID_{50}$ per ml. Pigs in group 4 served as non-inoculated control pigs. A total of 56 pigs were used in the subsequent serial passages (P2-8). The pigs were re-infected intranasally with 2 ml of a pooled tissue homogenate from the previous passage. Blood samples were collected weekly from all pigs to monitor PCV2 and PRRSV viremia and seroconversion until the termination of each serial passage at 42 days post inoculation (dpi).

2.2. Inocula and inoculation

For the inocula production, tissue homogenate in 20% (w/v) cold phosphate-buffered saline (PBS) was produced using sections of lungs, tonsil, tracheobronchiolar lymph node and spleen collected from all pigs of the same group. After the tissues were finely minced, the pool was homogenized in a Stomacher 80 (Thomas Scientific, Swedesboro, NJ, USA) for 3 min and then centrifuged at 4000 × *g* for 30 min at 4 °C. Homogenates were stored at −80 °C until use.

2.3. Necropsy and sample collection

All pigs were necropsied at 42 dpi. At necropsy, tracheobronchioal lymph node, tonsil, spleen and lungs were collected and stored immediately at −80 °C until further testing.

2.4. Laboratory testing

2.4.1. Serology

Successful passaging of PCV2 and PRRSV in pigs was evaluated and monitored by PRRSV and PCV2 serology. Selected serum samples were tested with an in-house PCV2 ELISA based on a recombinant capsid protein as previously described (Nawagitgul et al., 2002) and with the ELISA HerdChek PRRS X3 (IDEXX Laboratories, Inc., Westbrook, ME, USA) according to the manufacturer's label instructions.

2.4.2. Detection of PCV2 DNA

DNA from all serum samples was extracted by using a commercially available kit (QIAamp® DNA Blood Kit; Qiagen, Valencia, CA, USA) according to the manufacturer's specifications. PCV2 DNA was detected using previously described primers and probes targeting a signature motif located in the ORF2 capsid gene of PCV2 capable of differentiation between PCV2a and PCV2b (Opriessnig et al., 2010) with a total reaction volume of 25 μl consisting of 12.5 μl of commercially available master mix (TaqMan® Universal PCR master mix), 2.5 μl of DNA, 0.4 μM of each primer, and 0.2 μM of each probe. The cycling conditions were as follows: one cycle of 2 min at 50 °C, one cycle of 10 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. A sample with a C_T value greater than 40 was considered negative.

2.4.3. Detection of PRRSV RNA

RNA extraction on serum samples collected at dpi 0, 7, 14, 21, 28, 35 and 42 was performed using a QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA). The PRRSV RNA was detected by real-time PCR with TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems®, Foster City, CA, USA) with a pair of primers and a probe targeting the conserved region of ORF7 of the PRRSV genomes. The RT-PCR reaction mixture consisted of 12.5 μl of 2× RT-PCR Master Buffer, 0.625 μl of 40× MultiScribe Mix, 1 μl of each primer (0.4 μM), 0.5 μl of probe (0.2 μM), 5 μl of RNA, and 4.375 μl Rnase free water. The RT-PCR cycling conditions were as follows: one cycle of 30 min at 50 °C, one cycle of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Table 1
Primers used for the RT-PCR assays.

Primers	Sequences	Length of product (bp)
ORF5F	GGCAATGTGTCAGGCATCGTG	1091
ORF5R	AGCAAGCACAAACGGCATCTG	
PSVF	CTATTACCTACACGCCAGTGATGAT	960
PSVR	GTGTGGTTCTCGCCAATTAAATCT	
Nsp2F	CACCCGAGTCAGTGACACCT	460
Nsp2R	GGGTCTTTGGTAGGTTGGTC	

2.4.4. PRRSV sequencing

Reverse transcription was performed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo d(T). For the amplification of PRRSV genes including ORF5, ORF6, ORF7, and partial Nsp2 (sequencing attempts of the entire Nsp2 were not successful for all samples while the selected Nsp2 fragment was obtained consistently), the second step of the RT-PCR assay was performed in a total reaction volume of 25 μl containing 5 pmol/primer (Table 1), 2 μl of cDNA template and 22 μl of PCR SuperMix High Fidelity (Invitrogen, Grand Island, NY, USA). The PCR was carried out for 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 90 s (Nsp2 extension at 72 °C for 50 s). After the last cycle, the extension time was maintained at 72 °C for 6 min. The amplified products were visualized by ethidium bromide staining after 2% agarose gel. If the initial PCR products were barely visible on the gel (approximately 50% of products), 2 μl reaction mixture from the amplified sample was re-amplified to get enough DNA for subsequently sequencing. The amplified PCR products with anticipated size were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced at the DNA Sequencing Facility at Iowa State University by standard Sanger sequencing using an Applied Biosystems 3730xl DNA Analyzer. The PCR products were sequenced with both sense and antisense primers, and those with ambiguous reads were re-sequenced. The obtained sequences were compared with that of the original PRRSV VR2385 (GenBank: JX044140) by DNASTAR software.

2.5. Statistics

The association between PRRSV and PCV2a or PCV2b co-infections with amino acid changes in ORF5, ORF6, ORF7 and partial Nsp2 gens was tested using a two-tailed Fisher's exact test. For these calculations, only one amino acid substitution per position was included, regardless of the possibility of mutation back to the original amino acid after serial passage. Differences with p values <0.05 were considered significant. GraphPad Prism (version 5.00; GraphPad Software, San Diego, CA, USA) was used for statistical analysis.

3. Results

3.1. PCV2 and PRRSV infection kinetics in experimentally infected pigs

Serology and PCR results indicated that PRRSV, PCV2a and PCV2b passages in pigs were successful through all eight serial passages conducted in this study. Throughout the passages, animals tested positive for PRRSV RNA by RT-PCR from dpi 7 to dpi 42 and started being viremic for PCV2a or PCV2b between dpi 14 and 28 and remained PCV2 DNA positive until the end of each passage. Cross-contamination between groups was not observed based on the PCR monitoring of serum samples for PCV2a and PCV2b during each passage and in the pooled tissue homogenate produced after each passage. Negative control pigs remained negative for PCV2a, PCV2b and PRRSV.

3.2. Genetic changes and mutations of PRRSV VR2385 genome through eight consecutive passages in pigs

3.2.1. Sequence analysis of ORF5 (major envelope protein) and comparison to VR2385-P0

For the purpose of this study it was assumed that VR2385-P0 has the same sequence as the original virus used for infection (JX044140), but it needs to be considered that the cellular passages carried out for virus preparation could have resulted in mutations or partial mutations not detected in a consensus sequence. Relative to VR2385-P0, the PRRSV-only group 1 had one ORF5 amino acid

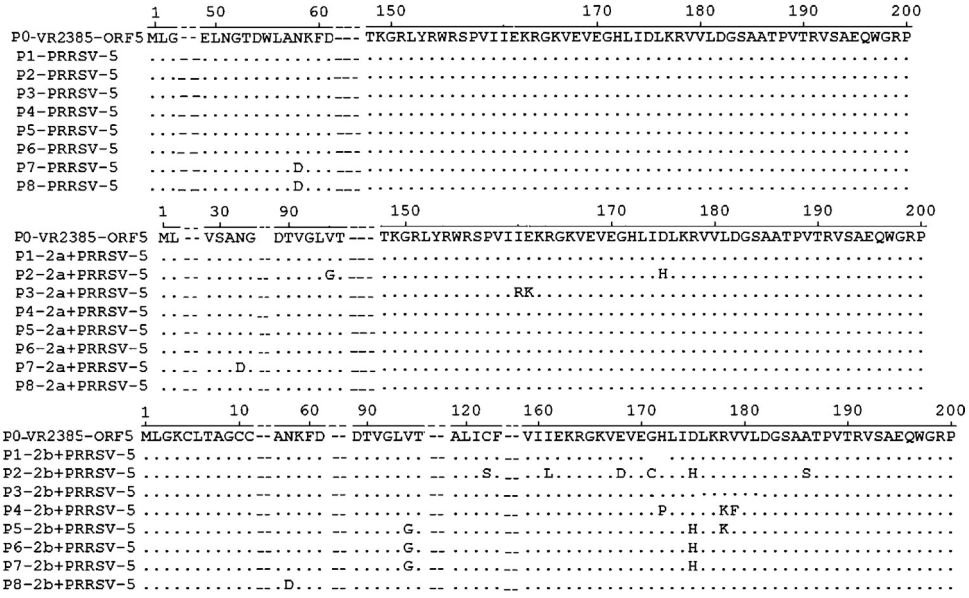

Fig. 1. A multiple sequence alignment of the deduced amino acid sequences of ORF5 from viruses recovered at different serial passages in pigs. All amino acid sequences were compared to the original VR2385 sequence from GenBank. Dots indicate residues identical to those in VR2385, and letters indicate amino mutations. Dashes (–) represent the conserved amino acid sequences throughout the passages that were not displayed in the alignment.

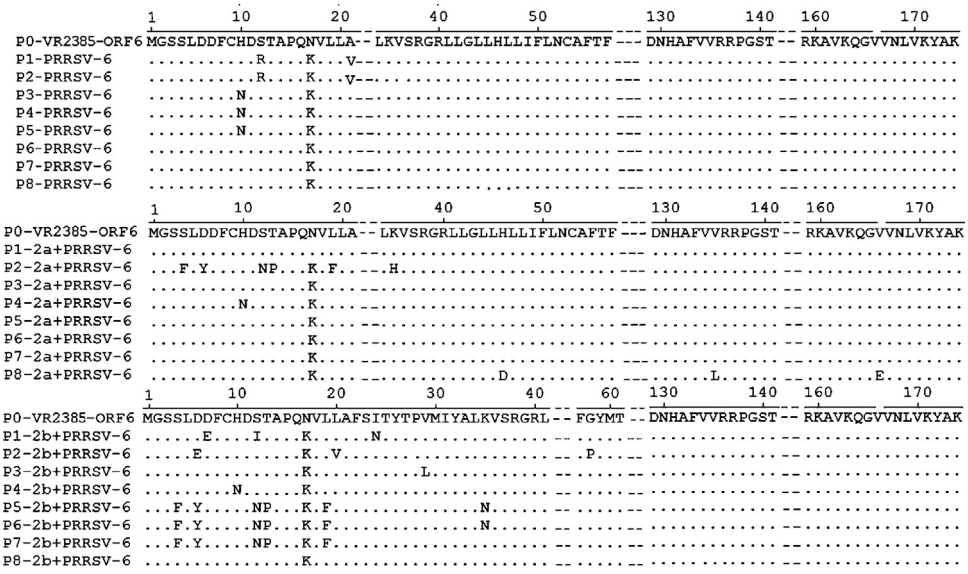

Fig. 2. A multiple sequence alignment of the deduced amino acid sequences of ORF6. Dots indicate amino acid residues identical to those in VR2385, and letters indicate amino substitutions. Dashes (–) represent the conserved amino acid sequences throughout the passages that were not displayed in the alignment.

mutation at position 58, where an asparagine changed to aspartic acid (Fig. 1). This mutation occurred during P7 and remained in P8. PRRSV and PCV2a coinfection resulted in five amino acid mutations in ORF5: N32D in P7, V94G in P2, I161R and E162 K in P3, and D175H in P2. In PRRSV/PCV2b coinfected group 3, a total of 11 amino acid mutations were identified in ORF5 when compared to the original P0 VR2385 sequence (Fig. 1). PRRSV and PCV2b coinfection had higher numbers of ORF5 amino acid mutations compared to the PRRSV infection alone ($p < 0.01$). There was no difference between PCV2a or PCV2b coinfection groups ($p = 0.20$) (Fig. 1).

3.2.2. Sequence analysis of ORF6 (matrix protein, M) and comparison to VR2385-P0

For PRRSV-only group 1 pigs, four amino acid mutations occurred in ORF6 throughout the study: S12R and A21V in P1 and P2, and H10N in P3 through P5. The amino acid mutations at these three positions disappeared in the subsequent passages. In PRRSV/PCV2a coinfected group 2, a total of 12 amino acid mutations were identified, three amino acid mutations occurred in P8: H46D, V135L and V166E, the remaining amino acid mutations were randomly distributed. In the PRRSV/PCV2b coinfected group 3, 16 amino acid mutations were identified at 14 different positions in ORF6 compared to P0 VR-2385 (Fig. 2). Some of the changes appeared in certain passages but disappeared in subsequent passages, such as the S4F mutation. One amino acid mutation, N17K, was common to all groups and remained stable from P1 (groups 1 and 3) or P2 (group 2) through P8. PRRSV and PCV2b coinfection had higher numbers of amino acid mutations in ORF6 compared to PRRSV infection alone ($p = 0.02$). There was no difference between PCV2a or PCV2b coinfection groups ($p = 0.83$) (Fig. 2).

3.2.3. Sequence analysis of ORF7 (nucleocapsid protein, N)

In group 1, the ORF7 sequence had three unique amino acid mutations appearing at three different positions over the serial

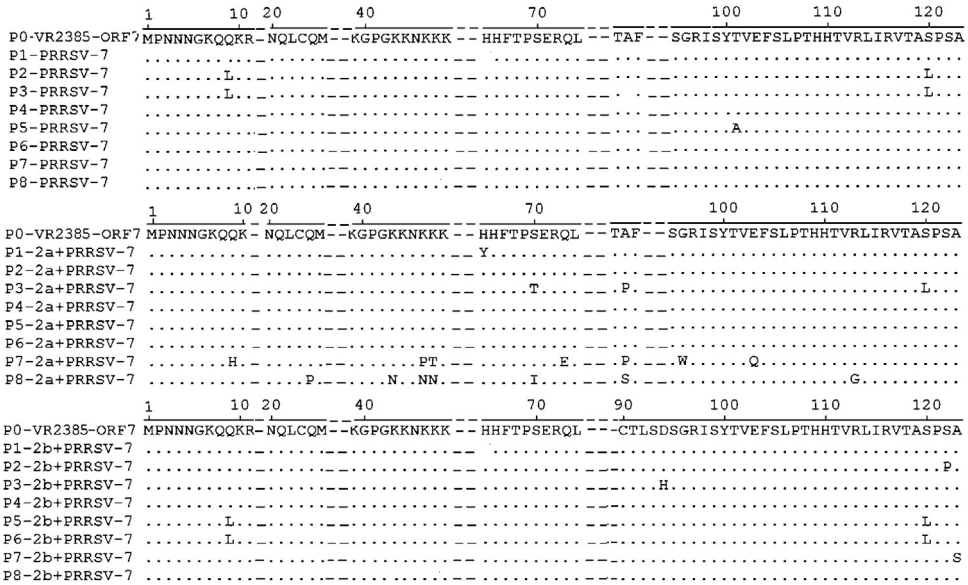

Fig. 3. A multiple sequence alignment of deduced amino acid sequences of ORF7. Dots indicate residues identical to those in VR2385, and letters indicate amino substitutions. Dashes (–) represent the conserved amino acid sequences throughout the passages that were not displayed in the alignment.

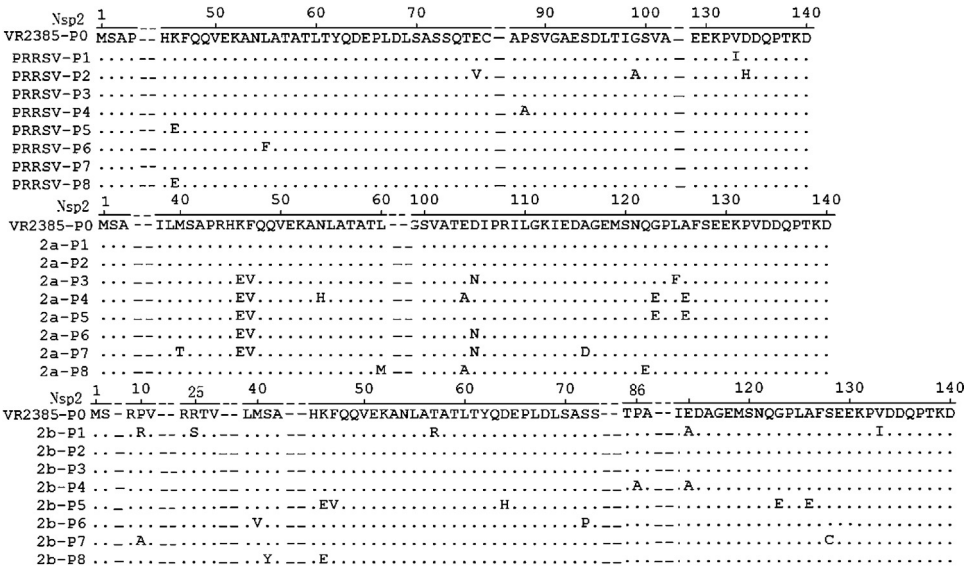

Fig. 4. A multiple sequence alignment of deduced amino acid sequences of partial Nsp2 gene. Dots indicate residues identical to those in VR-2385, and letters indicate amino substitutions. Dashes (–) represent the conserved amino acid sequences throughout the passages that were not displayed in the alignment.

passages: Q9L and S120L in P2 and P3. The other amino acid mutation was T101A in P5. These mutations disappeared in later passages. In PRRSV/PCV2 coinfected group 2, seven unique amino acid mutations were present in P8 (Fig. 3). The remaining mutations appeared and disappeared randomly in different passages. There were seven amino acid mutations at five different positions in the ORF7 in PRRSV/PCV2b coinfected group 3. These changes were not consistent substitution(s) but appeared occasionally and disappeared in subsequent passages. PRRSV and PCV2a coinfection had higher numbers of ORF7 amino acid mutations compared to PRRSV infection alone ($p = 0.01$). There was no difference between PCV2a or PCV2b coinfection groups ($p = 0.08$) (Fig. 3).

3.2.4. Comparison of the partial amino acid sequence of the Nsp2 gene (2813–3273 nt)

Seven amino acid mutations were identified in the PRRSV only group 1 compared to P0 VR2385, although no stable mutation was identified through the eight consecutive passages. In PRRSV/PCV2a group 2, two amino acid mutations were identified in P8: L60M and Q122E. In PRRSV/PCV2b group 3, only one mutation, S41Y, was identified in P8 only. Another amino acid mutation, K46E, occurred in P5, disappeared in P6 and P7, and reappeared in P8 (Fig. 4). There was no difference in the amino acid mutations rates between groups for the partial Nsp2 sequence ($p > 0.05$) (Fig. 4).

3.3. Analysis of rates and positions of mutations

All the 8 sequences from each group were compared to the original VR2385-P0 sequence and the mutation rates for ORF5, 6, 7 and Nsp2 were calculated (Table 2). In addition, the numbers of synonymous and non-synonymous mutations were also calculated (Table 3). At the termination of the study, the total number of amino acid mutations was 30 (group 1), 63 (group 2) and 77 (group 3) compared to the sequences of the original P0 VR2385 PRRSV. Genetic variation and evolution of PRRSV VR2385 during pig passages were further analyzed by combining the amino acid sequence data for all passages at different positions. Most of the amino acid mutations were conserved during the serial pig passages in ORF5, ORF6, ORF7 and Nsp2 of PRRSV. In comparison to data for PRRSV infection only group, the total rates of positions with amino acid mutations in pigs co-infected with PRRSV and PCV2a or PCV2b (regardless of genotype) were higher (15, 42 and 45 respectively, $p < 0.01$).

Table 2
Occurrence of amino acid mutations in ORF5, ORF6, ORF7 and partial Nsp2 of PRRSV (strain VR2385) through eight consecutive passages *in vivo* (*i.e.* P1 through P8). A "–" indicates that no mutation was identified.

Passage	Group 1 (PRRSV)				Group2 (PRRSV + PCV2a)				Group3 (PRRSV + PCV2b)			
	ORF5	ORF6	ORF7	Nsp2	ORF5	ORF6	ORF7	Nsp2	ORF5	ORF6	ORF7	Nsp2
P1	–	3	–	1	–	–	1	–	–	4	–	5
P2	–	3	2	3	2	8	–	–	6	4	1	–
P3	–	2	2	–	2	1	3	4	–	2	1	–
P4	–	2	–	1	–	2	–	6	3	2	–	2
P5	–	2	1	1	–	–	–	4	3	8	2	5
P6	–	1	–	1	–	–	–	3	2	8	2	2
P7	1	1	–	–	1	–	7	5	2	6	1	2
P8	1	1	–	1	–	4	7	3	1	1	–	2
Number of positions with mutations	1	4	3	7	5	12	13	12	11	14	5	15
Total numbers of mutations	2	15	5	8	5	15	18	25	17	35	7	18

Table 3
Numbers of non-synonymous (N) and synonymous (S) mutations of the nucleotide sequences of ORF5, ORF6, ORF7 and partial Nsp2 in PRRSV infected pigs with and without concurrent PCV2 infection.

Group	ORF5		ORF6		ORF7		Nsp2	
	N	S	N	S	N	S	N	S
1 (PRRSV)	2	15	7	14	5	9	8	0
2 (PRRSV + PCV2a)	5	7	9	8	18	3	28	10
3 (PRRSV + PCV2b)	17	2	25	12	7	3	17	2

4. Discussion

The objective of this study was to evaluate the genetic mutation rates of PRRSV in pigs when co-infected with PCV2 (PCV2a or PCV2b) during the course of multiple serial passages in pigs under experimental conditions. It is thought that PRRSV in a pig or population of pigs exists as quasispecies variants which help the virus persist in the host and survive the host immune response (Rowland et al., 1999). This makes the interpretation of observed mutations somewhat difficult since one cannot be sure whether a specific mutation was already present in the inoculum or if it occurred during the passage in pigs. To account for this concern, in the current study, a uniform PRRSV population was used for the first passage by using PRRSV derived from an infectious clone as described previously (Ni et al., 2011). However, two pigs were used in each passage and tissues from the pigs were pooled at termination and used for the infecting the next set of pigs. This strategy was done to increases the changes of successful PRRSV transmission from one passage to the next. This could have impacted the results as PRRSV could have evolved differently in each pig which would have been masked by using a tissue pool for reinfection.

To mimic field conditions where co-infections frequently occur, the pigs were concurrently infected with either PCV2a or PCV2b and PRRSV. Evidence from field surveys suggests that co-infections of pigs with PCV2 and PRRSV in the field are common (Sirinarumitr et al., 2001). According to the data from this study, no specific genetic marker in the ORF5, ORF6, ORF7 and partial Nsp2 genes of PRRSV was identified during the serial passages in pigs; however, it is interesting to note the high frequency of amino acid mutations of PRRSV genes in PCV2 co-infection groups relative to PRRSV infection alone. Whether these mutations affect the virulence or *in vivo* replication and shedding of PRRSV remain to be determined.

The present study further confirms that PRRSV evolves continuously in pigs infected with PRRSV alone or concurrently with PCV2a and PCV2b and that different genes of the viral genome undergo various degrees of mutations. Ultimately, this could mean that by controlling PCV2 infection in a herd, a producer can decrease the risks of PRRSV mutations, and thus subsequent PRRS outbreaks associated with novel PRRSV variants. In the past, dual infections with PRRSV and porcine respiratory coronavirus, or PRRSV and swine influenza virus were studied using conventional pigs (Van et al., 1996) or gnotobiotic pigs (Van et al., 1999) and in general, disease was found to be more pronounced in dually inoculated pigs. Interestingly, in gnotobiotic pigs the result of the co-infection was additive rather than synergistic (Van et al., 1999). In another study, co-infection of specific-pathogen-free pigs with PRRSV and Pseudorabies virus resulted in more severe clinical signs and increased pneumonia in pigs inoculated with both viruses compared to pigs infected with either virus alone (Shibata et al., 2000). The mixed infection of PRRSV and PCV2 is one of the most common co-infections associated with swine disease under field conditions today. It is also well recognized that PCV2 replication is enhanced by concurrent PRRSV infection in both colostrum-deprived (Allan et al., 2000a; Harms et al., 2001) and conventional pigs (Opriessnig et al., 2012) compared to singularly infected pig. There is also evidence suggesting that the commonality of porcine immune function target cells may play a key role in this synergism for these viruses due to stimulation of the monocyte/macrophage lineage and other cells of the immune system (Allan et al., 2000b; Rovira et al., 2002).

To our knowledge, a study investigating the mutation rate of PRRSV with concurrent viral infection has not been conducted to date. PCV2 viral load and tissue distribution was enhanced in pigs co-infected with PCV2 and PRRSV compared to pigs infected with PCV2 alone (Allan et al., 2000b; Rovira et al., 2002; Sinha et al., 2011). In the current study, co-infected groups had significantly higher amino acid mutations of PRRSV compared to pigs infected with PRRSV alone. The total number of positions with amino acid mutations of ORF5, 6, 7 and Nsp2 were 15 (group 1), 42 (group 2) and 45 (group 3). When single gene mutations were compared among different groups for ORF5, the group infected singularly with PRRSV had one mutation, the PRRSV/PCV2a co-infection group had 5 mutations at five positions, and the PRRSV/PCV2b co-infection group had 17 mutations at 11 positions.

It has been documented that singular PRRSV infection adversely affects the host's immune system (Lunney and Chen, 2010) and it is reasonable to assume that the effect of coinfection of pigs with virulent strains of PRRSV and PCV2 further enhances the immunosuppression. The genomic variability may be attributed to the lack of proof reading function of viral RNA-dependent RNA polymerase. Concurrence of variable regions with the hydrophilic domains of GP5 can be the result of host's selective humoral immune response directed against the exposed ectodomains of this envelope glycoprotein which in turn favors antigenic drifts. The genomic variations contribute to the emergence of antigenic variants, which is an effective mechanism for evading the host's immune surveillance. Clinical manifestations of PRRS are diverse and complex and they depend in part on the immune status of the host. Whether mutations within the antigenic domains of the GP5 may contribute to the establishment of a chronic form of the disease and eventually a persistent infection remains to be demonstrated (Pirzadeh et al., 1998).

In conclusion, our data demonstrated high genetic variability in the ORF5, ORF6 and ORF7 genes of PRRSV in pigs during co-infection of PRRSV and PCV2a or PCV2b compared with PRRSV infection alone. This work provides evidence that PCV2a or PCV2b may potentially have an important effect on the genetic and antigenic evolution and subsequent genetic variability of PRRSV during the course of multiple passages under experimental conditions.

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