RESEARCH REPORT
SWINE HEALTH

**Title:** Understanding the effect of concurrent PCV2a or PCV2b infection on the evolution of the PRRSV during serial passage in pigs – **NPB #09-200**

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**Industry Summary**

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) infections are among the most important swine viruses facing the industry today. Although vaccines have been highly effective overall, cases of porcine circovirus associated disease (PCVAD) have re-emerged in the United States in recent years. The outbreaks have raised concerns over introduction of a new more virulent PCV2-variant (PCV2b) into North America. Simultaneous with the PCVAD increase, we have observed a marked increase in submissions of PRRSV-associated pneumonia to the Veterinary Diagnostic Laboratory at Iowa State University. PRRSV, an RNA virus, is capable of continuous genomic changes through innate error of RNA polymerase or PRRSV recombination. The goal of the study was to investigate the effect of PCV2 infection on genetic and pathogenic evolution of PRRSV during serial passages in pigs. We hypothesized that concurrent PCV2b infection during passage of PRRSV in pigs would result in a substantial increase in mutations of the PRRSV genome compared to concurrent PCV2a infection or singular PRRSV infection. Eight, 2-week-old conventional PRRSV and PCV2 free pigs were randomly divided into 4 groups and rooms of 2 pigs each. Group 4 served as the negative control group. Pigs in groups 1, 2 and 3 were challenged intranasally with PRRSV VR2385. In addition, pigs in groups 2 and 3 were inoculated intranasally and intramuscularly with PCV2a or PCV2b. Forty-two days after inoculation, the pigs were euthanized and lungs and lymphoid tissues were collected, tissue homogenate was produced which was utilized to inoculated the next set of pigs. This process was repeated seven times to account for a total of eight *in vivo* passages. PRRSV open reading frames (ORF) 5, 6, 7 as well as a portion of ORF4 were sequenced and compared at the end of each passage. In this study, overall 13 nucleotide mutations were detected in the PRRSV only group 1, 4 nucleotide mutations were detected in the PRRSV/PCV2a coinfected group and 2 nucleotide mutations were detected in the PRRSV/PCV2b coinfected group 3 after eight consecutive passages in pigs. Seven of these mutations resulted into amino acid alterations. One of the amino acid substitutions in the PRRSV only group 1 resulted in a change of polarity, that is, the hydrophilic 16Ser in ORF4 mutated into hydrophobic 16Tyr. An influence of PCV2 on the PRRSV mutation rate was not identified based on sequencing of ORF5, ORF6, ORF7 and partial ORF4.

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**Keywords:** PRRSV, PCV2, *in vivo* passage, pigs.

**Scientific Abstract**

PRRSV is recognized as possessing a high degree of genetic and antigenic variability. Viral diversity has led to questions regarding the association of virus mutation and persistent infection in the host and has raised concerns on protective immunity, the ability of diagnostic assays to detect novel variants, and the possible emergence of virulent strains. The purpose of this study was to determine if PCV2 increases the ability of PRRSV to change over time during serial passage 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) or PRRSV with PCV2a (group 2) or PCV2b (group 3) in pigs, the sequences of the complete genes encoding the structural proteins of envelope (ORF 5), matrix (ORF6), nucleocapsid (ORF7) and partial ORF4 were obtained and analyzed. Thirteen, four, and two different nucleotide mutations were detected in group 1 (PRRSV only), group 2 (PRRSV-PCV2a), and group 3 (PRRSV-PCV2b) during the consecutive passages, respectively. 5 and 2 of the mutations resulted into amino acid alterations in group 1 and group 2, respectively. One of the amino acid substitutions present in group 1 had changed their polarity, that is, the hydrophilic 16Ser in ORF4 mutated into hydrophobic 16Tyr. The results from this study indicated that, besides ORF5, structural genes of ORF4 and ORF6 were shown to mutate and these results further confirm that PRRSV evolves continuously in *in vivo*.

**Introduction**

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small, positive sense, enveloped, single-stranded RNA virus of major economic importance in swine herds worldwide. PRRSV costs the US swine industry approximately $560 million per year due to production losses, treatment costs and excessive mortality. The virus was first recognized in the United States (US) in 1987 (Keffaber, 1989) and Europe in 1990 (Paton et al., 1991) and has become ubiquitous in the swine population. PRRSV is a member of the family *Arteriviridae* and the order *Nidovirales* (Cavanagh, 1997) which includes lactate dehydrogenase-elevating virus of mice, equine arteritis virus and simian hemorrhagic fever virus (Plagemann and Moennig, 1992). Clinical manifestation of PRRSV includes systemic and respiratory disease in grow finish pigs and reproductive failure in naïve females (Halbur et al., 1996; Mengeling et al., 1998).

PRRSV, similar to most RNA viruses, is characterized by a high mutation rate and the potential emergence of new, genetically diverse, strains (Forsberg et al., 2001; Hanada et al., 2005; Pirzadeh et al., 1998; Rowland et al., 1999). More recently, PRRSV isolates have emerged within the swine population with varying degrees of virulence (Fang et al., 2007; Han et al., 2006; Nelsen et al., 1999) possibly due to a high degree of viral recombination (Yuan et al., 1999; Yuan et al., 2000; Yuan et al., 2001; Yuan et al., 2004). Recently, evolution of PRRSV has been shown during sequential passages in pigs (Chang et al., 2002). In this experiment, 3 independent lines of *in vivo* replication were maintained for 367 days by pig-to-pig passage of virus at 60-day-intervals. The authors found ORF 1b and 7 to be highly conserved whereas there were 48 nucleotide variants within ORF5 (Chang et al., 2002).

Interestingly, in 2006, a "high fever disease" emerged in China and was initially attributed to pigs coinfected with PRRSV, PCV2 and hog cholera virus (Tian et al., 2007). The disease is characterized by high morbidity and mortality with apparent spread to more than 10 Chinese provinces affecting over 2,000,000 pigs with mortality rates 40% or higher on individual farms. Recent investigative teams from the U.S. who worked closely with Chinese scientists reported PRRSV and PCV2b as the most common cofactors identified in samples from diseased pigs. Interestingly, PCV2a was not identified in any of these samples. The 2005/06 outbreaks of PCVAD in Canada, North Carolina, and the Midwest U.S. raised concerns over the introduction of a new more virulent PCV2-variant into North America. In most cases PCV2b strains were identified in severe outbreaks (Cheung et al., 2007). However, it appears that current evidence does not support major differences in virulence among PCV2a and PCV2b isolates when based on information from experimental models where pigs were singularly inoculated with PCV2 (Opriessnig et al., 2008). PCV2 has been shown to impair both the onset of protective immunity (Darwich et al., 2002; Segalés et al., 2001) 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.

**Objectives**

- To determine if PCV2 increases the ability of PRRSV to change over time during serial passage in pigs.
- To determine if there is a difference in the PRRSV mutation rate between pigs concurrently infected with PCV2a or PCV2b.

**Materials and Methods**

*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). Eight pigs were randomly assigned to four groups and rooms with two pigs in each room. Pigs in group 4 served as non-inoculated control pigs.

**Table 1:** Experimental design.

| **Passage** | **Group 1** PRRSV only | **Group 2** PRRSV PCV2a | **Group 3** PRRSV PCV2b | **Group 4** Negative controls |
|---|---|---|---|---|
| 1 | A1 | B1 | C1 | D1 |
| | ↓ | ↓ | ↓ | ↓ |
| 2 | A2 | B2 | C2 | D2 |
| | ↓ | ↓ | ↓ | ↓ |
| 3 | A3 | B3 | C3 | D3 |
| | ↓ | ↓ | ↓ | ↓ |
| 4 | A4 | B4 | C4 | D4 |
| | ↓ | ↓ | ↓ | ↓ |
| 5 | A5 | B5 | C5 | D5 |
| | ↓ | ↓ | ↓ | ↓ |
| 6 | A6 | B6 | C6 | D6 |
| | ↓ | ↓ | ↓ | ↓ |
| 7 | A7 | B7 | C7 | D7 |
| | ↓ | ↓ | ↓ | ↓ |
| 8 | A8 | B8 | C8 | D8 |

For the initial inoculation and first *in vivo* passage, Groups 1, 2, 3 were inoculated intranasally with 4 ml of PRRSV VR2385 at a dose of $10^{5.69}$ $TCID_{50}$ rescued from an infectious DNA-launched clone as described (Ni et al., 2011). In addition, groups 2 and 3 were inoculated intranasally (3 ml) and intramuscularly (2 ml) with PCV2a (40895) or PCV2b (NC-16845) at a dose of $10^{4.5}$ $TCID_{50}$ per ml. Blood samples were collected from all pigs weekly until termination of each passage at 42 days post inoculation (dpi).

A total of 56 pigs were used in subsequent passages 2-8. After confirmation of their negative PCV2 and PRRSV status, the pigs were challenged intranasally with 2 ml of tissue homogenate (tracheobronchiolar lymph node, lungs, tonsil) from the previous passage. For the tissue homogenate, sections of lungs, tonsil, tracheobronchiolar lymph node and spleen were suspended in cold PPS at approximately 20% (wt/vol). The pool was homogenized in a Stomacher 80 for 3 min and then centrifuged at 4,000 x g for 30 minutes at 4°C. The supernatant was used to inoculate the next passage of pigs.

*2. Necropsy and sample collection*

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

*3. Laboratory testing*

*Serology.* Successful passaging of PCV2 and PRRSV was evaluated and monitored by PRRSV and PCV2 serology. Selected samples were tested with the ISU-VDL ORF2-PCV2 ELISA as previously described (Nawagitgul et al., 2002) and with the IDEXX PRRSV ELISA.

*Detection of PRRSV RNA.* RNA extraction on serum collected at dpi 0, 3, 6, 9, and 12 was performed using a QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA).The PRRSV RNA were detected by real-time PCR with TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems®) with a pair of primers and a probe targeting the conserved region of ORF7 of the PRRSV genomes. The PCR reagents were as follows: 12.5 µl of 2×RT-PCR Master Buffer, 0.625µl of 40×MultiScribe Mix, 1µl of each primer (0.4 µM), and 0.5 µl of probe (0.2 µM), 5 µl of RNA, and 4.375 µl Rnase free water. The cycling conditions were as follows: one cycle of 30 min at 50°C, one cycle of 10 min at 95°C, 40 cycles of 15 sec at 95°C, and 40 cycles of 1 min at 60°C.

*PRRSV sequencing.* After each passage, the PRRSV ORF5, ORF6, ORF7, and partial ORF4 present in the tissue homogenate were sequenced (Chang et al., 2002). All sequences were compared to each other and to the prototype.

*Detection of PCV2 DNA.* DNA from all serum and colostrum samples was obtained by using a commercially available extraction kit (QIAamp® DNA Blood Kit; Qiagen, Valencia, CA, USA) according to the manufacturers' specifications. PCV2 DNA was detected using previously described primers and probes targeting a signature motif located in ORF2 of PCV2 capable of differentiation between PCV2a and PCV2b was achieved (Opriessnig et al., 2010) with a total reaction volume of 25µl consisting of 12.5 µl of commercially available master mix (TaqMan® Universal PCR master mix), 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, 40 cycles of 15 sec at 95°C, and 40 cycles of 1 min at 60°C. A sample with a $C_T$ value greater than 40 was considered negative.

**Results**

*1. PCV2 and PRRSV infection kinetics in experimentally infected pigs.*

Serology and PCR results indicated that PRRSV, PCV2a and PCV2b passages were successful through all eight passages conducted. Cross-contamination between groups was not observed. Negative control pigs remained negative for PCV2a, PCV2b and PRRSV.

*2. Genetic evolution of PRRSV VR2385 thought eight consecutive passages in pigs*

Complete ORF 5 nucleotide sequences of passage 1 and 8 were obtained from pigs of groups 1, 2 and 3, no PRRSV RNA were detected in pigs of control group 4. Compared with the original sequence of passage 1, in passage 8 seven variable positions were identified, and four nucleotide changes resulted into amino acid changes (Table 2).

**Table 2:** Nucleotide and amino acid variants of ORF5, 6, 7 and partial ORF4 during eight passages in pigs.

| Group | Gene | Nucleotide change | | Amino acid change | |
|---|---|---|---|---|---|
| | | **Passage 1** | **Passage 8** | **Passage 1** | **Passage 8** |
| **Group 1* PRRSV only** | ORF4 | 47C | 47A | 16Ser | 16Tyr |
| | | 59T | 59C | 20Val | 20Ala |
| | | 93C | 93T | - | - |
| | ORF5 | 16C | 16T | - | - |
| | | 58A | 58G | 20Ile | 20Val |
| | | 169A | 169G | - | - |

| | | | | | |
|---|---|---|---|---|---|
| | ORF6 | 28C | 28A | 10His | 10Asn |
| | | 147C | 147T | - | - |
| | | 193T | 193C | 65Phe | 65Leu |
| | | 453A | 453G | - | - |
| | | 486T | 486C | - | - |
| | ORF7 | 140C | 140T | - | - |
| | | 338T | 338C | - | - |
| **Group 2** PRRSV PCV2a | ORF4 | 53G | 53A | 18Arg | 18Gln |
| | | 125A | 125G | - | - |
| | ORF6 | 62T | 62C | 21Val | 21Ala |
| | | 165C | 165T | - | - |
| **Group 3** PRRSV PCV2b | ORF4 | | | | |
| | ORF5 | 171T | 171C | - | - |
| | ORF6 | 42T | 42C | - | - |
| | ORF7 | | | | |

"-"Indicates that the nucleotide changes didn't result in an amino acid change.

From the comparison of the partial ORF4 (183bp), five sites were revealed to have nucleotide substitutions, which were identified in the PRRSV-only group 1 and in the PRRSV/PCV2b-coinfected group 2 (Table 2). Among them, the amino acid substitutions of 16Ser→16Tyr in group 1 change the polarity.

Specifically for ORF5, in group 1 nucleotide transitions of 16C to16T, 58A to 58G, and 169A to169G were observed between the first and the last passage, and the nucleotide change 58A→58G resulted into an amino acid change from 20Ile to 20Val. One nucleotide (171T→171C) was revealed to be mutated during sequential passages of PRRSV and PCV2b (group 3) (Table 2). Interestingly, no nucleotide mutations were observed during the consecutive passage of PRRSV and PCV2a (group 2) (data not shown).

For ORF6, 8 mutated positions were identified, with three of the mutations producing amino acid alterations (Table 2). In PRRSV-only group 1 pigs, the nucleotides of 28C, 147C, 193T, 453A, 486T present in passage 1 had mutated to 28A, 147T, 193C, 453G, and 486C by passage 8. Two of the changes resulted in amino acid alterations: 10His→10Asn and 65Phe→ 65Leu. In PRRSV-PCV2a coinfected group 2 pigs, two nucleotide substitutions (62T→62C, 165C→165T) occurred during passage 1 and 8, resulting in one amino acid substitution from 21Val to 21Ala. In the PRRSV/PCV2b coinfected group 3, only one mutation (42T→42C) was identified after eight consecutive passages.

For ORF7, only two sites were revealed to be mutated in PRRSV only group 1; however, none of the mutations results in an amino acid change (Table 2).

**Discussion**

PRRSV is a RNA virus and therefore replication is error prone. It is thought that RNA virus isolates are composed of multiple variants (quasipecies) which helps the virus to persistence in organisms and to survive the host immune response. This makes interpretation of observed mutations somewhat difficult since you cannot be sure whether a specific mutation was already present in the inoculum or if it occurred during the pig passage. To account for this in the current study, a uniform PRRSV population was selected for the first passage by utilization of an infectious PRRSV rescued from a DNA clone as described previously (Ni et al., 2011).

In this study, overall 13 nucleotide mutations were detected in the PRRSV only group 1, 4 nucleotide mutations were detected in the PRRSV/PCV2a coinfected group and 2 nucleotide mutations were detected in the PRRSV/PCV2b coinfected group 3 after seven consecutive passages in pigs. Seven of these mutations resulted into amino acid alterations. One of the amino acid substitution in the PRRSV only group 1 resulted in a change of polarity, that is, the hydrophilic 16Ser in ORF4 mutated into hydrophobic 16Tyr (Table 2).

It has been reported that GP5 (encoded by ORF5) which is present in the viral envelope, participates in viral attachment to cells, entry into target cells, and contains a neutralization epitope while ORF6 and ORF7 encode the matrix and the non-glycosylated nucleocapsid protein. ORF5 and ORF6 and ORF7 all play key roles in the formation of virions and infectivity of the virus (Snijder et al., 2003; Hanada et al., 2005). The present study further confirms that PRRSV evolves continuously in pigs infected with PRRSV or concurrently with

PCV2a and PCV2b, with different genes of the viral genome undergoing various degrees of change. Evidence that PCV2a or PCV2b may have a potential effect on genetic and antigenic evolution and subsequent variability of PRRSV was not identified under the study conditions.

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