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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

Shuang-Hui Yin[a], Chao-Ting Xiao[a], Priscilla F. Gerber[a,b], Nathan M. Beach[c], Xiang-Jin Meng[c], Patrick G. Halbur[a], Tanja Opriessnig[a,d,*]

[a] Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, USA
[b] Laboratório de Pesquisa em Virologia Animal, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Minas Gerais, Minas Gerais, Brazil
[c] Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA
[d] The Roslin Institute and The Royal (Dick) School of Veterinary Studies, University of Edinburgh, Midlothian EH25 9RG, United Kingdom

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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

* Corresponding author at: Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, USA. Tel.: +1 515 294 1137; fax: +1 515 294 3564.
E-mail address: tanjaopr@iastate.edu (T. Opriessnig).

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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.