Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Veterinary Microbiology 158 (2012) 69–81

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Effect of porcine circovirus type 2a or 2b on infection kinetics and pathogenicity of two genetically divergent strains of porcine reproductive and respiratory syndrome virus in the conventional pig model

Tanja Opriessnig [a,*], Phillip C. Gauger [a], Kay S. Faaberg [b], Huigang Shen [a], Nathan M. Beach [c], Xiang-Jin Meng [c], Chong Wang [a,d], Patrick G. Halbur [a]

[a] *Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, USA*
[b] *Virus and Prion Research Unit, National Animal Disease Center, USDA-Agricultural Research Service, Ames, IA, USA*
[c] *Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Diseases, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA*
[d] *Department of Statistics, College of Liberal Arts & Sciences, Iowa State University, Ames, IA, USA*

ARTICLE INFO

Article history:
Received 12 December 2011
Received in revised form 3 February 2012
Accepted 9 February 2012

Keywords:
Coinfection
Interaction
Porcine circovirus type 2 (PCV2)
Porcine reproductive and respiratory syndrome virus (PRRSV)

ABSTRACT

To determine differences in infection kinetics of two temporally and genetically different type 2 porcine reproductive and respiratory syndrome virus (PRRSV) isolates *in vivo* with and without concurrent porcine circovirus (PCV) type 2a or 2b infection, 62 pigs were randomly assigned to one of seven groups: negative controls ($n = 8$); pigs coinfected with a 1992 PRRSV strain (VR-2385) and PCV2a (CoI-92-2a; $n = 9$), pigs coinfected with VR-2385 and PCV2b (CoI-92-2b; $n = 9$), pigs coinfected with a 2006 PRRSV strain (NC16845b) and PCV2a (CoI-06-2a; $n = 9$), pigs coinfected with NC16845b and PCV2b (CoI-06-2b; $n = 9$), pigs infected with VR-2385 ($n = 9$), and pigs infected with NC16845b ($n = 9$). Blood samples were collected before inoculation and at day post-inoculation (dpi) 3, 6, 9 and 12 and tested for the presence of PRRSV antibody and RNA, PCV2 antibody and DNA, complete blood counts, and interferon gamma (IFN-γ) levels. Regardless of concurrent PCV2 infection, VR-2385 initially replicated at higher levels and reached peak replication levels at dpi 6. Pigs infected with VR-2385 had significantly higher amounts of viral RNA in serum on both dpi 3 and dpi 6, compared to pigs infected with NC16845b. The peak of NC16845b virus replication occurred between dpi 9 and dpi 12 and was associated with a delayed anti-PRRSV antibody response in these pigs. PCV2 coinfection resulted in significantly more severe macroscopic and microscopic lung lesions and a stronger anti-PRRSV IgG response compared to pigs infected with PRRSV alone. This work further emphasizes *in vivo* replication differences among PRRSV strains and the importance of coinfecting pathogens.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), a single-stranded, positive-sense RNA virus, is characterized by a high mutation rate with the potential of genetically diverse strains evolving over time (Forsberg et al., 2001; Hanada et al., 2005; Pirzadeh et al., 1998; Rowland et al., 1999). In the past, 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 mutation and recombination (Yuan et al., 1999, 2000, 2001, 2004). More recently, attention has focused on the occurrence of high mortality in Chinese swine herds which

* Corresponding author at: Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA. Tel.: +1 515 294 1137; fax: +1 515 294 3564.
E-mail address: tanjaopr@iastate.edu (T. Opriessnig).

0378-1135/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2012.02.010

was associated with novel PRRSV isolates and described as porcine high fever disease in 2006 (Tian et al., 2007; Tong et al., 2007; Wu et al., 2009). The PRRSV isolates involved in porcine high fever disease contained unique nucleotide differences compared to other isolates. Specifically, a discontinuous, 30 amino acid deletion was identified within the nsp2 region which was initially suggested to be correlated with the pathogenicity of the virus (Tian et al., 2007; Wu et al., 2009). However, more recent reports have concluded that this deletion is unrelated to virulence (Zhou et al., 2009) in spite of the high mortality that was initially associated with this PRRSV variant (Tian et al., 2007; Tong et al., 2007). Interestingly, analysis of 582 samples from affected pigs resulted in the identification of PRRSV, porcine circovirus type 2 (PCV2) and classical swine fever virus as the most common co-infection pathogens, suggesting that a potential synergistic interaction among these viruses may account for the unusually high mortality (Lv et al., 2008; Wu et al., 2009).

PCV2 is a small, circular, non-enveloped DNA virus belonging to the *Circoviridae* family in the genus *Circovirus*. PCV2 can be further divided into several subtypes of which PCV2a and PCV2b are prevalent worldwide (Patterson and Opriessnig, 2010). To date, experimental infections comparing PCV2a and PCV2b in gnotobiotic and conventional pigs have not demonstrated major differences in virulence (Beach et al., 2010; Fort et al., 2008; Lager et al., 2007; Opriessnig et al., 2008b). PCV2 is the cause of porcine circovirus associated disease (PCVAD) with multiple clinical manifestations including respiratory disease (Harms et al., 2002). PRRSV has become an important component of the porcine respiratory disease complex (PRDC) with major economic impact on the swine industry (Chae, 2005). Retrospective studies identified PRRSV as the most common cofactor in cases of PCVAD (Pallarés et al., 2002). Experimental coinfection with PRRSV and PCV2 has yielded mixed results. One study completed in 2002 reported minimal clinical disease or death loss in conventional pigs coinfected with PCV2 and PRRSV (Rovira et al., 2002). In contrast, in another study, severe clinical disease and death in 10 of 11 pigs between 10 and 21 days post-infection (dpi) was reported in dually infected, caesarian-derived and colostrum-deprived (CDCD) pigs (Harms et al., 2001). Despite differences in severity of clinical presentation, experimental coinfection of pigs with PCV2 and PRRSV has consistently resulted in up-regulation of PCV2 replication (enhanced viremia and PCV2 tissue load) and increased severity of PRRSV-induced lesions in lung tissues (Allan et al., 2000; Harms et al., 2001; Rovira et al., 2002).

In North America, both PRRSV and PCV2b have been identified in PCVAD outbreaks characterized by excessive mortality suggesting a synergistic relationship between these two viruses (Cheung et al., 2007; Gagnon et al., 2007; Horlen et al., 2007). The objective of this study was to characterize the infection dynamics and pathogenicity of two different type 2 PRRSV isolates in a conventional pig model under the influence of concurrent PCV2a or PCV2b infection. The severity of clinical disease, macroscopic and microscopic lesions, amount of PRRSV and PCV2 antibodies and nucleic acids in sera, amount of PRRSV and PCV2 antigen associated with lesions, and interferon gamma (IFN-γ) concentrations in serum were measured and compared between groups.

2. Materials and methods

2.1. Animals, housing, and experimental design

Fifty-three colostrum-fed, crossbred pigs were derived from sows known to be free of PCV2, PRRSV and *Mycoplasma hyopneumoniae* in two separate batches, 44 pigs in batch 1 (B1) and 9 pigs in batch 2 (B2). In addition, batch 3 (B3) consisted of 9 colostrum-fed crossbred pigs derived from sows free of PRRSV and *M. hyopneumoniae* but seropositive for PCV2. B2 and B3 pigs were challenged at the same age as B1 pigs but the experiment was conducted approximately 24 months after B1 pigs. Insufficient numbers of pigs were available from the source herd for singularly PRRSV-infected groups to be included with the original experiment. The experimental design and group designations are summarized in Table 1. All pigs were housed under the same conditions and treated in a similar way. All pigs were weaned at three weeks of age and transported to the Livestock Infectious Disease Isolation Facility at Iowa State University, Ames, Iowa. On the day of arrival, all B1 pigs were comingled and randomly assigned to one of five rooms each containing 8 or 9 pigs: negative controls ($n = 8$); pigs coinfected with a 1992 isolate of PRRSV (VR-2385) and PCV2a (CoI-92-2a; $n = 9$), pigs coinfected with PRRSV VR-2385 and PCV2b (CoI-92-2b; $n = 9$), pigs coinfected with a 2006 isolate of PRRSV (NC16845b) and PCV2a (CoI-06-2a; $n = 9$) and pigs

Table 1
Experimental design and group designations.

Group designation	Batch[a]	Number of pigs	PCV2 inoculum	PRRSV inoculum
Negative controls	1	8	None	None
CoI-92-2a	1	9	PCV2a	VR-2385
CoI-92-2b	1	9	PCV2b	VR-2385
CoI-06-2a	1	9	PCV2a	NC16845b
CoI-06-2b	1	9	PCV2b	NC16845b
PRRSV-I-92	2	4	None	VR-2385
PRRSV-I-06	2	5	None	NC16845b
B3-PRRSV-I-92	3	5	None	VR-2385
B3-PRRSV-I-06	3	4	None	NC16845b

[a] Batch 1 and 2 pigs were derived from the same source herd free of PRRSV and PCV2 whereas batch 3 pigs were derived from a different source herd seropositive for PCV2.

coinfected with PRRSV NC16845b and PCV2b (CoI-06-2b; $n = 9$). B2 pigs were randomly assigned to one of two rooms each containing 4 or 5 pigs which were infected with PRRSV VR-2385 (PRRSV-I-92) and PRRSV NC16845b (PRRSV-I-06), respectively. Similarly, B3 pigs were comingled and randomly assigned to one of two rooms each containing 4 or 5 pigs that were infected with PRRSV VR-2385 (B3-PRRSV-I-92) and PRRSV NC16845b (B3-PRRSV-I-06), respectively.

The pigs from the different batches were kept in different but identical rooms. Each room had 18 m^2 of solid concrete floor space, separate ventilation systems and one nipple drinker. Inoculation was conducted at approximately 23 days of age. Blood samples were collected from all pigs prior to inoculation and at dpi 3, 6, 9 and 12 in serum separator tubes (8.5 ml BD Vacutainer, Benton Dixon, Franklin Lakes, NJ, USA). The blood was centrifuged at $2000 \times g$ for 10 min at 4 °C and serum was stored at −80 °C until testing. Serum samples were analyzed for levels of anti-PCV2 IgG antibody, anti-PRRSV-IgG antibody, IFN-γ, PCV2 DNA, and PRRSV RNA. In addition, EDTA tubes (8.5 mL MONOJECT™ 15% EDTA liquid, Tyco Healthcare Group LP, Mansfield, MA, USA) were collected at dpi 3, 6, 9, and 12, stored at room temperature and used within 12 h after collection to determine blood cell counts. All pigs were necropsied on dpi 12 and tissues collected during necropsy were analyzed by immunohistochemistry (IHC) for the presence of PCV2 and PRRSV antigens. The experimental protocol was approved by Iowa State University Institutional Animal Care and Use Committee (IACUC approval # 7-08-6595-S).

2.2. Inocula and inoculation

2.2.1. PRRSV

PRRSV isolate VR-2385 with a RFLP pattern 1-3-4 was recovered in 1992 from pig tissues obtained from a 160 sow herd in southwestern Iowa affected by severe respiratory disease in 3–16-week-old pigs and high numbers of late term abortions (Halbur et al., 1995b; Meng et al., 1994). The passage 5 virus of the original VR-2385 isolate was used to inoculate pigs in 2001 as described previously (Opriessnig et al., 2002). Serum from the pigs infected with VR-2385 in 2001 was used to re-isolate the virus followed by two subsequent passages in MARC-145 cells to produce the virus stock of VR-2385 for this study. PRRSV isolate NC16845b with a RFLP pattern 1-18-2 was isolated in 2006 from a clinically affected 9-week-old pig with systemic PCVAD from a group of pigs from North Carolina with a history of severe respiratory disease in 50% of the pigs and approximatley 20% mortality in the group (Gauger et al., 2012). The passage 2 virus of the original isolate was used to experimentally infect a set of PRRSV-free conventional pigs (data not shown) and the lung tissues from these pigs collected two weeks after infection were used for re-isolation of the NC16845b virus followed by two subsequent passages in MARC-145 cells to produce the NC16845b virus stock for this study. The two inocula were separated in different aliquots, stored at −80 °C, and virus of the same lot was used for all batches of pigs. On dpi 0, CoI-92-2a, CoI-92-2b, PRRSV-I-92, and B3-PRRSV-I-92 groups received 2 ml of PRRSV isolate VR-2385 at a dose of $10^{5.0}$ median tissue culture infective dose ($TCID_{50}$) per ml. All pigs in groups CoI-06-2a, CoI-06-2b, PRRSV-I-06, and B3-PRRSV-I-06 received 2 ml of PRRSV isolate NC16845b at a dose of $10^{5.0}$ $TCID_{50}$ per ml. Inoculation was intranasal by holding the pig in the upright position and slowly dripping 1 ml of the inoculum into each nostril using a 3 ml syringe (Fisher Scientific, Inc.).

2.2.2. PCV2

Two different PCV2 subtypes were used for the inoculation of pigs. Pigs in groups CoI-92-2a and CoI-06-2a were inoculated with the PCV2a isolate 40895, which was recovered from an Iowa farm in 1998 (Fenaux et al., 2000) and has been well characterized genetically (Fenaux et al., 2000) and in the conventional specific pathogen free (SPF) pig model (Opriessnig et al., 2003, 2004a). Both PCV2a and PCV2b viruses were produced as described previously (Opriessnig et al., 2008b) and used for inoculation in this study at a titer of $10^{4.0}$ $TCID_{50}$ per ml. Pigs in groups CoI-92-2b and CoI-06-2b were inoculated with PCV2b isolate NC16845 which was isolated in 2006 from a pig farm in North Carolina (Opriessnig et al., 2008b). Both, PCV2b NC16845 and PRRSV NC16845b originated from the same tissues. The PCV2 groups were inoculated intranasally (3 ml) and intramuscularly (2 ml) with their respective PCV2 subtype by injecting 2 ml of the inoculum intramuscularly into the right neck area and 3 ml (1.5 ml per nostril) intranasally by holding the pig in the upright position and slowly dripping 1.5 ml of the inoculum into each nostril using a 3 ml syringe (Fisher Scientific, Inc.).

2.3. Leukogram

EDTA-treated blood samples were analyzed for white blood cells using a multispecies hematology instrument (Hemavet HV950FS, Drew Scientific, Inc.). The white blood cell (WBC) count was reported as actual numbers of neutrophils, lymphocytes and total WBC per μl of whole blood. In addition to WBC, a ratio was determined between the total neutrophil count and the total lymphocyte count reported as the N/L ratio. Values from negative control pigs were considered as baseline for the infected pigs on each dpi.

2.4. Serology

2.4.1. PRRSV

Serum samples from all pigs were also tested for the presence of anti-PRRSV antibodies by a commercial PRRSV ELISA (HerdChek PRRS virus antibody test kit 2XR, IDEXX Laboratories Inc. Westbrook, MA, USA), according to the instructions of the manufacturer. Samples were considered positive if the calculated S/P ratio was equal to 0.4 or greater.

2.4.2. PCV2

All serum samples were tested for the presence of anti-PCV2 IgG antibodies based on an open reading frame 2 (ORF2) ELISA (Nawagitgul et al., 2002). Samples were considered positive if the calculated sample-to-positive (S/P) ratio was equal to 0.2 or greater.

2.4.3. Other viruses

On dpi 12, three samples were randomly chosen from each group and room and tested for the presence of swine influenza virus (SIV) antibodies by an *in house* nucleoprotein NS1 ELISA (Richt et al., 2006) and for the presence of antibodies to porcine parvovirus (PPV) by hemagglutination inhibition (HI) assay (Mengeling et al., 1988).

2.5. Interferon gamma (IFN-γ)

A commercial ELISA kit (Swine IFN-γ; Invitrogen, Camarillo, CA, USA) was used to detect and quantify IFN-γ concentrations in serum according to the instructions of the manufacturer.

2.6. Clinical evaluation

Following PRRSV/PCV2 coinfection, the pigs were monitored daily for respiratory disease (dyspnea, sneezing, coughing, nasal discharge). Rectal temperatures and behavioral changes such as lethargy and inappetence/anorexia were also recorded daily. The observers were aware (not blinded) to the treatment status.

2.7. Quantitative real-time PCR

2.7.1. PRRSV RT-PCR

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 AgPATH-ID PRRSV multiplex reagent kit (Applied Biosystems, Foster City, CA, USA) was used for the real-time, reverse transcriptase PCR (RT-PCR) on each extracted RNA sample. All samples were run in duplicate. Each PCR consisted of 8 μl template RNA and 17 μl of PCR master mix. The PCR master mix contained 12.5 μl of 2× RT-PCR buffer, 2.5 μl 10× PRRSV primer probe mix, 1.25 μl 20× multiplex RT-PCR enzyme mix, 0.25 μl of ZenoRNA-01 internal control RNA and 0.5 μl nuclease-free water. Each reaction included eight progressive 1:10 dilutions of a known copy number of PRRSV to generate a standard curve for quantification. Each plate was run in the sequence detection system (GeneAmp 5700 Sequence Detection System, Applied Biosystems) using the AgPATH-ID company specific conditions (10 min at 45 °C, 10 min at 95 °C, followed by 40 cycles of 2 s at 97 °C and 40 s at 60 °C). Samples were considered negative when no signal was observed within the 40 amplification cycles.

2.7.2. PCV2 ORF1-based PCR

DNA extraction on serum collected on dpi 0, 3, 6, 9, and 12 days was performed using the QIAamp DNA blood mini kit (Qiagen, Valencia, CA, USA) and subsequently used for detection of PCV2 DNA by quantitative real-time PCR utilizing primers and a probe designed for PCV2 ORF1 as described (Opriessnig et al., 2003). The real-time PCR reaction consisted of a 25 μl PCR mixture containing 12.5 μl commercially available master mix (TaqMan® Universal PCR master mix, Applied Biosystems by Life Technologies), 2.5 μl DNA, 1 μl (0.4 μM) of each primer, and 0.5 μl (0.2 μM) probe. The reaction was run in a 7500 Fast Real-Time PCR system (ABI, Foster City, CA, USA) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All samples were run in duplicate. Serial dilutions of a recombinant PCV2 DNA clone were included on each plate to generate a standard curve. Viral concentrations were expressed as the DNA copy numbers per ml of sample. Samples were considered negative when no signal was observed within the 40 amplification cycles.

2.7.3. PCV2a/b ORF2-based differential PCR

All DNA extracts were also tested for presence of PCV2a and PCV2b DNA by utilizing a forward primer (5′-GCAGGGCCAGAATTCAACC-3′), a reverse primer (5′-GGCGGTGGACATGATGAGA-3′), a probe specific for PCV2a (5′-Cal Fluor Orange 560-GGGGACCAACAAAATCTCTA-TACCCTTT-BHQ-3′), and a probe specific for PCV2b (5′-Quasar 670-CTCAAACCCCCGCTCTGTGCCC-BHQ-3′), which were designed in the PCV2 ORF2 as described (Opriessnig et al., 2010). The multiplex real-time PCR reaction consisted of a total volume of 25 μl containing 12.5 μl of the commercially available master mix (Applied Biosystems), 5 μl DNA, 0.4 μM of each primer, and 0.2 μM of each probe. All samples were run in duplicate. The reactions were carried out under the following conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The sensitivity and specificity of the real-time PCR reaction was evaluated using known PCV2a and PCV2b isolates as well as PPV, PRRSV, and PCV type 1 (PCV1) isolates. Samples were considered negative when no signal was observed within the 40 amplification cycles.

2.8. Sequencing

Open reading frame (ORF) 5 of one PRRSV RT-PCR positive pig in each group was sequenced on dpi 12 as previously described (Gauger et al., 2012).

2.9. Necropsy

On dpi 12, all pigs were humanely euthanized by intravenous pentobarbital overdose (Fatal-Plus® Vortech Pharmaceutical, Ltd., Dearborn, MI, USA). Macroscopic lung lesions were estimated based on the percentage of the lung surface affected by pneumonia ranging from 0 to 100% (Halbur et al., 1995b). The scoring system was based on the approximate volume that each lung lobe contributes to the entire lung: the right cranial lobe, cranial part of the left cranial lobe, and the caudal part of the left cranial lobe contribute 10% each to the total lung volume, the accessory lobe contributes 5%, and the right and left caudal lobes contribute 27.5% each (Halbur et al., 1995b). Additionally, lymph node size was scored ranging from 0 (normal) to 3 (four times the normal size) (Opriessnig et al., 2006). Lungs were insufflated with fixative as previously described (Halbur et al., 1995b). Sections of lymph nodes (tracheobronchial, mesenteric, mediastinal, superficial inguinal, and external iliac), tonsil, thymus, ileum, kidney, colon, spleen, heart, liver, and brain were collected at necropsy and fixed in 10% neutral-buffered formalin and routinely processed for histological examination.

2.10. Histopathology

Microscopic lesions were evaluated independently by two veterinary pathologists (TO, PCG) blinded to the treatment status. Sections of lung were scored for the presence and severity of interstitial pneumonia ranging from 0 (normal) to 6 (severe, diffuse) (Halbur et al., 1995b). Sections of heart, liver, kidney, ileum, colon and brain were evaluated for the presence of lymphohistiocytic inflammation and scored from 0 (none) to 3 (severe). Lymphoid tissues including lymph nodes (trachea-bronchial, mediastinal, mesenteric, external iliac and superficial inguinal), tonsil, spleen and thymus were evaluated for the presence of lymphoid depletion ranging from 0 (normal) to 3 (severe) and histiocytic inflammation and replacement of follicles ranging from 0 (normal) to 3 (severe) (Opriessnig et al., 2006).

2.11. Immunohistochemistry

2.11.1. PRRSV

Detection of PRRSV-specific antigen was performed by IHC staining on lung sections as previously described (Halbur et al., 1995a). Sections were scored for presence of PRRSV antigen independently by two veterinary pathologists (TO, PCG) blinded to the treatment groups.

2.11.2. PCV2

IHC for detection of PCV2-specific antigen was performed on sections of lung, lymph nodes (tracheobronchial, mediastinal, mesenteric, superficial inguinal and external iliac), tonsil, spleen, thymus and small intestine using a rabbit polyclonal antiserum (Sorden et al., 1999). Sections were scored for presence and amount of PCV2 antigen independently by two veterinary pathologists (TO, PCG) blinded to the treatment groups. If the results obtained by the two pathologists on a certain tissue differed, the mean of the two scores was used. PCV2 scores ranged from 0 (no antigen) to 3 (more than 50% of the lymphoid follicles contain cells with PCV2-antigen staining) (Opriessnig et al., 2006). Any tissue or tissue pool with detectable staining was given at least a score of 1. For the purpose of determining prevalence rates, a score of 0 was considered negative and scores of 1, 2 and 3 were considered positive.

2.12. Statistical analysis

For data analysis, JMP® software version 8.0.1and SAS® software version 9.2.0 (both SAS Institute, Cary, NC, USA) were used. Summary statistics were calculated for groups to assess the distributional property and data that were not distributed normally (PCR data) were log transformed prior to analysis. As log transformation can only be applied to numbers above 0, a constant number (1) was added to each number in the data set prior to log transformation. A linear mixed model with the random effects "Source" (Source A: B1 and B2 and Source B: B3) and "Batch" (B1, B2, B3, nested within "Source") and the fixed effects "PRRSV strain" (none, VR-2385, NC16845b) and "PCV2 subtype" (none, PCV2a, PCV2b) was used first on all outcomes. From this, it was determined that the random effect "Source" contributed to the overall variation whereas "Batch" did not. To decrease the heterogeneity of the animals in the analysis, all data obtained from the second source, B3, were removed from the analysis but were provided as supplemental information throughout the "Results" and Tables. The final model to analyze continuous data collected over time (rectal temperatures, blood cell counts, log transformed PCV2 and PRRSV genomic copies, and ELISA S/P ratios) was a repeated measures analysis of variance (ANOVA), where PRRSV strain, PCV2 subtype, DPI and their interactions were the fixed effects and pig was the subject of repeated measures. Compound symmetry variance-covariance structure was used to model the within pig correlation. A one-way ANOVA was used to analyze cross-sectional data (macroscopic and microscopic lung lesions) where PRRSV strain, PCV2 subtype, and their interaction were the fixed effects. Differences among the interacting groups (PRRSV strain $\times$ PCV2 subtype) in the repeated measures ANOVA or the one-way ANOVA were assessed using Tukey's *t*-test. A *p*-value of less than 0.05 was considered significant. Differences in prevalence of PRRSV and PCV2 antigen between groups (IHC staining) were determined by Fisher's exact test.

3. Results

3.1. Clinical presentation

Mild, transient lethargy and inappetence were observed in all inoculated groups, although coughing or sneezing was not a feature. Pigs in all inoculated groups regardless of coinfection status developed a transient to persistent fever ranging from 40.0 °C to 41.8 °C between dpi 3 and dpi 12. The mean rectal temperature time by group interaction after inoculation was significant ($P < 0.05$). All six inoculated groups had rectal temperatures significantly higher than the negative controls at dpi 6 and dpi 9. By dpi 12, the mean group rectal temperatures in the PRRSV-I-92, PRRSV-I-06, CoI-92-2a, CoI-92-2b and CoI-06-2a groups were significantly ($P < 0.05$) higher compared to the negative controls. When the effect of "PRRSV strain" was evaluated across groups, no differences were found. Compared to pigs infected with PRRSV alone, coinfected pigs had higher mean rectal temperatures at dpi 3, 6 and 9. When the effect "PCV2 subtype" was evaluated among coinfected groups, PCV2a pigs had significantly ($P < 0.01$) higher rectal temperatures on dpi 9 compared to PCV2b pigs (data not shown). B3 pigs (B3-PRRSV-I-92 and B3-PRRSV-I-06) had similar rectal temperatures as B2 (PRRSV-I-92 and PRRSV-I-06) pigs.

3.2. Whole blood counts

Hematology results are summarized in Table 2. There was an effect of "PRRSV strain" on white blood cell counts at dpi 3 with pigs infected with NC16845b having significantly ($P = 0.03$) higher levels of white blood cells compared to pigs infected with VR-2385 (10.5 ± 0.9 *versus* 8.1 ± 0.5). Also, there was a significant effect of "PCV2" ($P < 0.05$): PCV2-infected pigs had higher levels of white blood cells at dpi 9 and 12 compared to non-PCV2-infected pigs (17.1 ± 0.9 *versus* 9.4 ± 0.6 and 20.3 ± 1.0 *versus* 14.5 ± 1.2). There was no effect of "PRRSV strain" on numbers

Table 2
Mean group leukocyte values ($\times 1000/\mu l$ of whole blood except for ratios) in the different treatment groups on days post-inoculation (dpi) 0, 3, 6, 9 and 12. Data obtained from B3-PRRSV-I-92 and B3-PRRSV-I-06 pigs (gray shaded area) were not included in the analysis.

Group	Hematology[a]	0	3	6	9	12
Negative controls ($n = 8$)	WBC	8.6 ± 1.0	6.0 ± 0.7^{A}	12.0 ± 0.8	19.8 ± 2.8^{A}	$17.8 \pm 0.9^{A,B}$
	Neutrophils	2.7 ± 0.3	2.7 ± 0.3	4.9 ± 0.3	$9.1 \pm 1.5^{A,B}$	$7.7 \pm 0.7^{A,B}$
	Lymphocytes	4.1 ± 0.3	2.4 ± 0.3	4.9 ± 0.6	$6.4 \pm 0.4^{A,B}$	7.2 ± 0.3
	N/L ratio	0.6 ± 0.3	1.1 ± 0.1	1.1 ± 0.1^{A}	$1.5 \pm 0.2^{A,B}$	1.1 ± 0.2^{A}
CoI-92-2a ($n = 9$)	WBC	13.6 ± 2.1	$8.3 \pm 0.8^{A,B}$	8.4 ± 1.2	$17.5 \pm 1.7^{A,B}$	$19.5 \pm 1.3^{A,C}$
	Neutrophils	5.5 ± 1.3	4.6 ± 0.8	4.5 ± 0.7	10.2 ± 1.0^{A}	$12.0 \pm 0.8^{B,C}$
	Lymphocytes	6.0 ± 0.9	2.5 ± 0.3	2.7 ± 0.6	$4.7 \pm 0.6^{A,B,C}$	5.0 ± 0.6
	N/L ratio	1.0 ± 0.2	2.1 ± 0.5	$1.9 \pm 0.3^{A,B}$	2.3 ± 0.2^{A}	2.6 ± 0.3^{B}
CoI-92-2b ($n = 9$)	WBC	12.1 ± 1.6	$8.6 \pm 0.8^{A,B}$	10.9 ± 1.1	20.2 ± 1.4^{A}	23.5 ± 2.0^{A}
	Neutrophils	4.6 ± 1.1	4.7 ± 0.7	6.2 ± 0.7	11.3 ± 0.8^{A}	14.1 ± 1.1^{C}
	Lymphocytes	5.7 ± 0.7	2.7 ± 0.2	3.4 ± 0.4	6.6 ± 0.9^{A}	6.9 ± 1.2
	N/L ratio	0.9 ± 0.2	1.8 ± 0.3	$1.9 \pm 0.2^{A,B}$	$1.9 \pm 0.2^{A,B}$	$2.4 \pm 0.3^{B,C}$
CoI-06-2a ($n = 9$)	WBC	10.8 ± 0.9	$8.7 \pm 1.0^{A,B}$	9.8 ± 0.7	$14.8 \pm 2.1^{A,B}$	$18.7 \pm 1.7^{A,B}$
	Neutrophils	3.4 ± 0.3	3.9 ± 0.5	4.0 ± 0.3	$4.7 \pm 0.6^{A,B,C}$	$9.9 \pm 0.8^{A,B,C}$
	Lymphocytes	5.3 ± 0.6	3.4 ± 0.5	3.4 ± 0.4	$4.0 \pm 0.5^{B,C}$	6.4 ± 0.8
	N/L ratio	0.7 ± 0.4	1.2 ± 0.2	$1.3 \pm 0.1^{A,B}$	2.3 ± 0.3^{A}	$1.7 \pm 0.2^{A,B,C}$
CoI-06-2b ($n = 9$)	WBC	12.8 ± 2.4	$10.4 \pm 1.5^{A,B}$	11.7 ± 1.7	$16.0 \pm 1.6^{A,B}$	$19.5 \pm 2.0^{A,B}$
	Neutrophils	6.0 ± 1.8	5.3 ± 0.8	6.6 ± 1.1	$9.8 \pm 1.2^{A,B}$	$11.7 \pm 1.6^{C,B}$
	Lymphocytes	5.1 ± 0.7	2.8 ± 0.3	3.4 ± 0.5	$4.1 \pm 0.5^{A,B,C}$	6.1 ± 0.8
	N/L ratio	0.9 ± 0.5	2.0 ± 0.2	2.1 ± 0.3^{B}	2.5 ± 0.1^{A}	$2.0 \pm 0.2^{A,B,C}$
PRRSV-I-92 ($n = 4$)	WBC	7.8 ± 0.9	6.6 ± 0.6^{A}	6.9 ± 1.0	9.1 ± 0.8^{B}	$16.4 \pm 1.3^{A,B}$
	Neutrophils	3.0 ± 0.6	1.8 ± 0.2	2.6 ± 0.4	$3.8 \pm 0.6^{B,C}$	$7.4 \pm 1.3^{A,B}$
	Lymphocytes	4.1 ± 0.6	2.7 ± 0.3	2.5 ± 0.6	2.6 ± 0.2^{C}	6.0 ± 1.1
	N/L ratio	0.7 ± 0.1	0.7 ± 0.0	$1.3 \pm 0.5^{A,B}$	$1.4 \pm 0.1^{A,B}$	$1.3 \pm 0.2^{A,C}$
PRRSV-I-06 ($n = 5$)	WBC	10.6 ± 0.4	13.9 ± 2.3^{B}	10.4 ± 1.5	9.5 ± 0.9^{B}	13.3 ± 1.7^{B}
	Neutrophils	3.4 ± 0.3	5.6 ± 1.7	3.4 ± 0.5	3.1 ± 0.2^{C}	5.1 ± 1.1^{A}
	Lymphocytes	4.8 ± 0.6	2.6 ± 0.4	4.2 ± 1.0	3.3 ± 0.4^{C}	4.5 ± 0.4
	N/L ratio	0.7 ± 0.1	2.1 ± 0.5	$1.0 \pm 0.3^{A,B}$	1.0 ± 0.1^{B}	1.1 ± 0.2^{A}
B3-PRRSV-I-92 ($n = 5$)	WBC	12.1 ± 2.1	11.0 ± 1.1	11.1 ± 1.0	14.6 ± 2.0	21.6 ± 1.8
	Neutrophils	5.9 ± 1.4	4.8 ± 2.6	4.1 ± 0.6	8.3 ± 1.8	13.8 ± 1.6
	Lymphocytes	3.7 ± 0.5	2.6 ± 0.8	2.1 ± 0.4	2.3 ± 0.4	4.0 ± 0.8
	N/L ratio	1.6 ± 0.3	1.9 ± 0.3	2.1 ± 0.2	3.6 ± 0.6	3.6 ± 0.4
B3-PRRSV-I-06 ($n = 4$)	WBC	13.3 ± 1.2	13.4 ± 0.6	10.9 ± 0.8	14.0 ± 1.2	17.9 ± 3.2
	Neutrophils	5.6 ± 1.1	4.3 ± 0.5	4.2 ± 0.7	6.0 ± 1.0	9.1 ± 2.1
	Lymphocytes	5.3 ± 1.0	2.7 ± 0.7	2.8 ± 0.3	2.5 ± 0.2	3.7 ± 0.3
	N/L ratio	1.2 ± 0.2	2.6 ± 1.0	1.5 ± 0.2	2.5 ± 0.5	2.5 ± 0.6

Different superscripts (A, B, C) within columns indicate significant ($P < 0.05$) differences among groups.
[a] WBC: white blood cells; N/L ratio: neutrophil to lymphocyte ratio.

of neutrophils; however, there was a significant effect of "PCV2" on mean group neutrophil counts at dpi 6, 9, and 12 with PCV2-infected pigs having elevated levels compared to pigs not infected with PCV2 (5.3 ± 0.4 *versus* 3.1 ± 0.3, 10.0 ± 0.6 *versus* 3.4 ± 0.3, and 11.9 ± 6.1 *versus* 6.1 ± 0.9, respectively). Differences in mean group lymphocyte counts were only observed on dpi 9 (Table 2) and there was an effect of PRRSV strain ($P = 0.048$): pigs infected with NC16845b had lower levels of lymphocytes compared to pigs infected with VR-2385 (3.9 ± 0.3 *versus* 5.1 ± 0.5). Additionally, pigs coinfected with PCV2 had higher levels of lymphocytes ($P = 0.016$) compared to pigs infected with PRRSV alone (4.8 ± 0.3 *versus* 3.0 ± 0.3) suggesting an effect of "PCV2".

3.3. Antibody levels

3.3.1. Anti-PRRSV-IgG antibody levels

All pigs in all groups were negative for PRRSV-specific antibodies at 0 dpi and negative control pigs remained negative for anti-PRRSV antibody throughout the study. Prevalence and group mean S/P ratios are summarized in Table 3. Overall, there was a significant effect of "PRRSV strain" and "PCV2" on the anti-PRRSV antibody response at dpi 9. Specifically, pigs infected with VR-2385 had a significantly ($P = 0.017$) higher anti-PRRSV antibody response compared to those infected with NC16845b. Similarly, coinfected pigs had significantly ($P = 0.028$) higher anti-PRRSV S/P ratios compared to pigs singularly infected with PRRSV. There was no effect of "PCV2 subtype" on the magnitude of the anti-PRRSV-antibody response among coinfected groups.

3.3.2. Anti-PCV2-IgG antibody levels

All pigs in B1 and B2 were negative for PCV2-specific anti-IgG antibodies at 0 dpi and the negative controls and B2 pigs remained PCV2 seronegative throughout the trial. In the PCV2 coinfected groups, seroconversion was observed at dpi 9. The prevalence and mean group anti-PCV2-IgG S/P ratios

Table 3
Prevalence of anti-PRRSV antibodies and mean group sample-to-positive (S/P) ratios in the different treatment groups on days post-inoculation (dpi) 0, 3, 6, 9 and 12. Data presented as prevalence (mean S/P ratio ± SE). Data obtained from B3-PRRSV-I-92 and B3-PRRSV-I-06 pigs (gray shaded area) were not included in the analysis.

Group	0	3	6	9	12
Negative controls	0/8 (0.01 ± 0.00)	0/8 (0.02 ± 0.00)	0/8 (0.02 ± 0.00)	0/8 (0.02 ± 0.01)^{A}	0/9 (0.02 ± 0.00)^{A}
CoI-92-2a	0/9 (0.01 ± 0.00)	0/9 (0.01 ± 0.00)	0/9 (0.03 ± 0.01)	7/9 (0.74 ± 0.19)^{B}	9/9 (0.84 ± 0.09)^{B}
CoI-92-2b	0/9 (0.02 ± 0.01)	0/9 (0.01 ± 0.00)	0/9 (0.02 ± 0.01)	6/9 (0.82 ± 0.26)^{C}	9/9 (0.98 ± 0.24)^{B}
CoI-06-2a	0/9 (0.08 ± 0.07)	0/9 (0.08 ± 0.07)	0/9 (0.08 ± 0.06)	4/9 (0.40 ± 0.08)^{A,B}	7/9 (0.70 ± 0.11)^{B}
CoI-06-2b	0/9 (0.01 ± 0.00)	0/9 (0.01 ± 0.01)	0/9 (0.01 ± 0.00)	6/9 (0.52 ± 0.08)^{A,B}	6/9 (0.68 ± 0.12)^{B}
PRRSV-I-92	0/4 (0.01 ± 0.00)	0/4 (0.01 ± 0.00)	0/4 (0.00 ± 0.00)	2/4 (0.35 ± 0.06)^{A,B}	4/4 (0.83 ± 0.13)^{B}
PRRSV-I-06	0/5 (0.02 ± 0.00)	0/5 (0.01 ± 0.00)	0/5 (0.00 ± 0.00)	0/5 (0.08 ± 0.03)^{A,B}	2/5 (0.36 ± 0.19)^{B}
B3-PRRSV-I-92	0/5 (0.01 ± 0.00)	0/5 (0.01 ± 0.00)	0/5 (0.00 ± 0.00)	3/5 (0.56 ± 0.02)	5/5 (0.77 ± 0.21)
B3-PRRSV-I-06	0/4 (0.02 ± 0.00)	0/4 (0.01 ± 0.00)	0/4 (0.01 ± 0.00)	2/4 (0.20 ± 0.12)	1/4 (0.30 ± 0.14)

Different superscripts (A, B, C) within columns indicate significant ($P < 0.05$) differences in mean group S/P ratios among groups.

Table 4
Prevalence of anti-PCV2 IgG antibodies and mean group sample-to-positive (S/P) ratios in the different treatment groups except PRRSV-I-92 and PRRSV-I-06 on day post-inoculation (dpi) 0, 3, 6, 9 and 12. Data presented as prevalence (mean S/P ratio ± SE). Grey shaded areas indicate the presence of PCV2 seropositive pigs (S/P ratio > 0.2) within a treatment group.

Group	0	3	6	9	12
Negative controls	0/8 (−0.01 ± 0.01)	0/8 (−0.01 ± 0.01)	0/8 (−0.03 ± 0.05)	0/8 (−0.15 ± 0.16)^{A}	0/8 (0.00 ± 0.0)^{A}
CoI-92-2a	0/9 (0.00 ± 0.01)	0/9 (0.02 ± 0.03)	0/9 (0.02 ± 0.01)	4/9 (0.17 ± 0.04)^{B}	5/9 (0.22 ± 0.05)^{B}
CoI-92-2b	0/9 (0.00 ± 0.01)	0/9 (−0.02 ± 0.03)	0/9 (0.02 ± 0.01)	0/9 (0.05 ± 0.01)^{A,B}	3/9 (0.16 ± 0.03)^{A,B}
CoI-06-2a	0/9 (0.02 ± 0.02)	0/9 (−0.06 ± 0.08)	0/9 (0.03 ± 0.01)	3/9 (0.13 ± 0.03)^{A,B}	7/9 (0.29 ± 0.04)^{B}
CoI-06-2b	0/9 (0.00 ± 0.00)	0/9 (−0.00 ± 0.02)	0/9 (0.03 ± 0.01)	2/9 (0.11 ± 0.04)^{A,B}	4/9 (0.26 ± 0.06)^{B}

Different superscripts (A, B) within columns indicate significant ($P < 0.05$) differences in mean group S/P ratios among groups.

are summarized in Table 4. There was no effect of "PRRSV strain" or "PCV2 subtype" on the magnitude of the anti-PCV2-antibody response. B3 pigs were seropositive for PCV2 at the time of arrival (mean PCV2 ELISA S/P ratio: 1.58 ± 0.09) and the S/P ratios remained at a similar level for the duration of the study (data not shown).

3.3.3. Other viruses

At termination of the study, pigs randomly selected from each batch tested negative for antibodies against PPV, SIV H1N1 and SIV H3N2 (data not shown).

3.4. IFN-γ levels

Prevalence of IFN-γ positive samples and mean group IFN-γ concentrations are summarized in Table 5. There was no effect of "PRRSV strain" or "PCV2" on the IFN-γ levels and no differences were found among groups; however, analysis of an effect of "PCV2 subtype" among coinfected groups revealed that on dpi 6, PCV2b-inoculated pigs had significantly ($P = 0.028$) higher levels of IFN-γ compared to PCV2a-inoculated pigs (0.46 ± 0.17 pg/ml *versus* 0.06 ± 0.05 pg/ml).

3.5. PRRSV and PCV2 viremia

3.5.1. Prevalence and amount of PRRSV RNA

All pigs were negative for PRRSV-RNA in serum at 0 dpi and the negative controls remained negative for PRRSV RNA throughout the study. The prevalence of PRRSV RNA positive pigs and group mean genomic copy numbers/ml are summarized in Table 6. Sequencing of the ORF5 gene of PRRSV and comparison with the original inocula confirmed that the correct PRRSV isolates were present in the

Table 5
Prevalence of IFN-γ and mean group concentration (pg/ml) in the different treatment groups on day post-inoculation (dpi) 0, 3, 6, 9 and 12. Data presented as prevalence (mean $\log_{10}$ group concentration ± SE). Data obtained from B3-PRRSV-I-92 and B3-PRRSV-I-06 pigs (gray shaded area) were not included in the analysis.

Group	3	6	9	12
Negative controls	0/8	1/8 (0.09 ± 0.09)	0/8 (0.00 ± 0.00)	1/8 (0.05 ± 0.05)
CoI-92-2a	0/9	1/9 (0.03 ± 0.03)	7/9 (1.16 ± 0.30)	1/9 (0.27 ± 0.27)
CoI-92-2b	0/9	2/9 (0.39 ± 0.26)	6/9 (0.77 ± 0.33)	2/9 (0.39 ± 0.28)
CoI-06-2a	0/9	1/9 (0.09 ± 0.09)	3/9 (0.46 ± 0.24)	1/9 (0.17 ± 0.17)
CoI-06-2b	0/9	4/9 (0.53 ± 0.22)	6/9 (1.01 ± 0.28)	2/9 (0.31 ± 0.21)
PRRSV-I-92	0/4	0/4	0/4	1/4 (0.35 ± 0.35)
PRRSV-I-06	1/5 (0.25 ± 0.25)	1/5 (0.24 ± 0.24)	1/5 (0.32 ± 0.32)	1/5 (0.40 ± 0.40)
B3-PRRSV-I-92	0/5	0/5	0/5 (0.03 ± 0.01)	2/5 (0.70 ± 0.49)
B3-PRRSV-I-06	0/4	1/4 (0.32 ± 0.32)	0/4	0/4

Table 6
Prevalence of PRRSV and group mean $\log_{10}$ PRRSV genomic copies per ml in the different treatment groups on days post-inoculation (dpi) 3, 6, 9 and 12. Data presented as prevalence ($\log_{10}$ PRRSV RNA ± SE). Data obtained from B3-PRRSV-I-92 and B3-PRRSV-I-06 pigs (gray shaded area) were not included into the analysis.

Group	3	6	9	12
Negative controls	0/8 (0.00 ± 0.00)^{A}	0/8 (0.00 ± 0.00)^{A}	0/8 (0.00 ± 0.00)^{A}	0/8 (0.00 ± 0.00)^{A}
CoI-92-2a	9/9 (5.44 ± 0.23)^{B,D}	9/9 (6.74 ± 0.14)^{B}	9/9 (6.63 ± 0.05)^{B}	9/9 (6.17 ± 0.08)^{B}
CoI-92-2b	9/9 (5.42 ± 0.22)^{B,D}	9/9 (6.93 ± 0.16)^{B}	9/9 (6.23 ± 0.18)^{B}	9/9 (5.76 ± 0.22)^{B}
CoI-06-2a	9/9 (4.66 ± 0.16)^{B,C}	9/9 (6.35 ± 0.17)^{B,C}	9/9 (6.93 ± 0.12)^{B}	9/9 (6.56 ± 0.08)^{B}
CoI-06-2b	9/9 (5.35 ± 0.36)^{B,D}	9/9 (6.53 ± 0.28)^{B}	9/9 (6.90 ± 0.16)^{B}	9/9 (6.76 ± 0.14)^{B}
PRRSV-I-92	4/4 (6.36 ± 0.25)^{D}	4/4 (7.45 ± 0.18)^{B}	4/4 (7.11 ± 0.09)^{B}	4/4 (7.11 ± 0.16)^{B}
PRRSV-I-06	3/5 (2.99 ± 1.26)^{C}	4/5 (4.82 ± 1.47)^{C}	5/5 (6.00 ± 0.92)^{B}	5/5 (6.25 ± 0.02)^{B}
B3-PRRSV-I-92	4/5 (4.52 ± 1.14)	5/5 (7.47 ± 0.12)	5/5 (7.43 ± 0.14)	5/5 (7.28 ± 0.09)
B3-PRRSV-I-06	4/4 (3.84 ± 0.63)	3/4 (4.57 ± 1.53)	4/4 (6.12 ± 1.33)	4/4 (7.13 ± 0.44)

Different superscripts (A,B,C,D) within columns indicate significant ($P < 0.05$) differences in mean group S/P ratios among groups.

respective groups and rooms. When results were analyzed for a possible effect of "PRRSV strain", a significantly ($P < 0.05$) higher amount of PRRSV RNA was detected in pigs infected with VR-2385 at dpi 3 and 6 compared to pigs infected with NC16845b (Fig. 1). When the pigs infected with PRRSV alone were removed from the analysis, coinfected pigs with NC16845b had significantly higher amounts of PRRSV RNA in serum compared to pigs infected with VR-2385 on dpi 9 (6.49 ± 0.10 *versus* 5.99 ± 0.11) and dpi 12 (6.24 ± 0.08 *versus* 5.55 ± 0.12), respectively. An effect of "PCV2" or "PCV2 subtype" on PRRSV replication was not evident.

3.5.2. Prevalence and amount of PCV2 DNA

All pigs were negative for PCV2-DNA in serum at 0 dpi and the negative controls and B2 and B3 pigs remained PCV2 DNA negative throughout the study (data not shown). At dpi 3, 28/36 PCV2-inoculated pigs were positive for PCV2-DNA, and all PCV2-inoculated pigs were positive for PCV2-DNA by 6 dpi and remained positive until dpi 12. The $\log_{10}$ group mean PCV2 DNA amounts are summarized in Fig. 2. When results were analyzed for a possible effect of "PRRSV strain" it was found that there was a significantly higher amount of PCV2 DNA in pigs infected with VR-2385 (7.99 ± 0.19) compared to pigs infected with NC16845b (7.01 ± 0.21) on dpi 12. There was a significant effect of "PCV2 subtype" on dpi 3; groups infected with PCV2b had significantly higher amounts of PCV2 DNA in serum compared to groups infected with PCV2a (4.63 ± 0.40 *versus* 2.96 ± 0.51, respectively). An effect of "PCV2 subtype" was not evident in the later stages of infection.

3.5.3. PCV2 subtypes

All pigs in the PCV2a or PCV2b groups were correctly infected with their respective subtype as determined by multiplex real-time PCR (data not shown) and cross-contamination between groups and rooms was not detected.

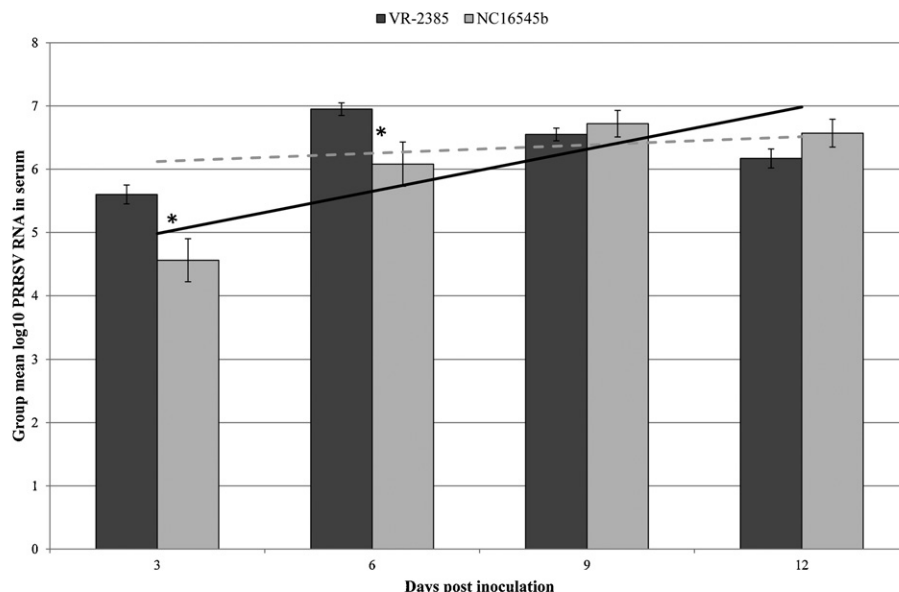

Fig. 1. Log_{10} transformed mean PRRSV RNA genomic copies (±SE) in VR-2385 and NC16845b infected pigs regardless of coinfection status on day post-inoculation (dpi) 0, 3, 6, 9 and 12. Significant ($P < 0.05$) differences between groups within a dpi are indicated by asterisks. The lines indicate the linear trend for pigs infected with VR-2385 (gray, dashed) or NC16845b (black, solid).

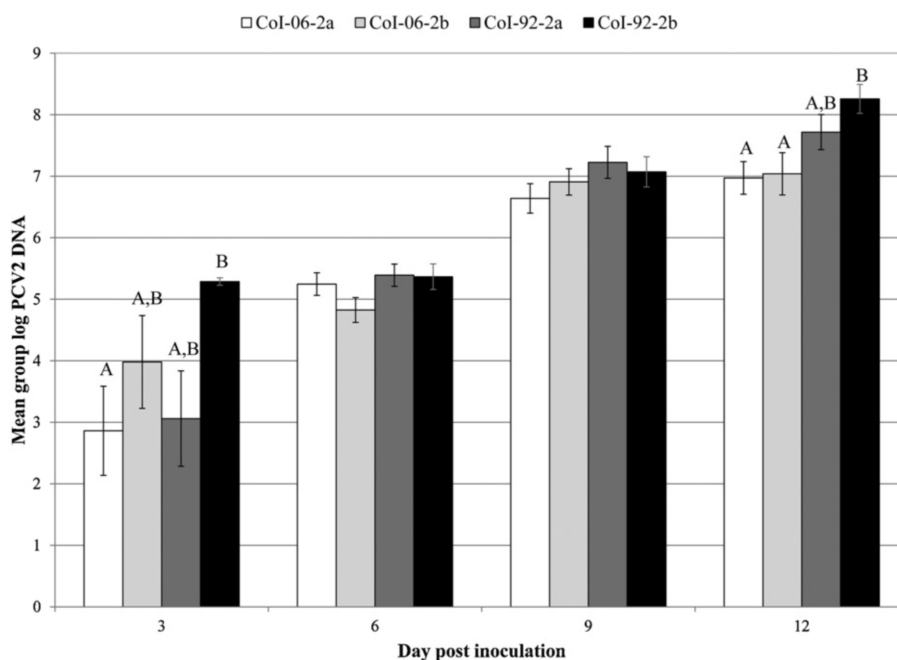

Fig. 2. Log_{10} transformed group mean PCV2 DNA amounts ($\pm$SE) in the PCV2-PRRSV coinfected groups on day post-inoculation (dpi) 0, 3, 6, 9 and 12. Significant ($P < 0.05$) differences between groups within a dpi are indicated by different superscripts (A, B).

3.6. Macroscopic lesions

Macroscopic lesions were characterized by mild-to-moderate enlargement of lymph nodes (especially tracheobrochiolar lymph nodes and mediastinal lymph nodes) and mottled-tan lungs with varying degrees of the lung surface affected by visible pneumonia lesions. The group mean lung lesion severity scores are summarized in Table 7 and were significantly ($P < 0.05$) lower for negative controls compared to all coinfected groups. There were no significant differences in lung lesions severity between the negative controls and the pigs infected with PRRSV alone. There was an effect of "PCV2" on the mean group macroscopic lung lesion scores as evidenced by the coinfected pigs having more severe macroscopic lung lesions compared to pigs infected with PRRSV alone. However, there was no effect of "PRRSV strain" or "PCV2 subtype" on the severity of the observed macroscopic lung lesions.

Table 7
Mean group macroscopic (percentage of lung surface affected by lesions) and microscopic (interstitial pneumonia ranging from 0 = normal to 6 = severe, diffuse) lung lesions (mean group amount ± SE). Data obtained from B3-PRRSV-I-92 and B3-PRRSV-I-06 pigs (gray shaded area) were not included in the analysis. Significant ($P < 0.05$) differences between groups are indicated by different superscripts (A, B, C).

Group	Macroscopic lung lesions (0–100%)	Microscopic lung lesions (0–6)
Negative controls	0.1 ± 0.1^{A}	0.75 ± 0.25^{A}
CoI-92-2a	54.8 ± 4.3^{B}	4.44 ± 0.24^{B}
CoI-92-2b	56.3 ± 4.5^{B}	4.67 ± 0.17^{B}
CoI-06-2a	52.8 ± 6.4^{B}	4.78 ± 0.32^{B}
CoI-06-2b	$48.7 \pm 4.7^{B,C}$	4.44 ± 0.24^{B}
PRRSV-I-92	$31.8 \pm 8.3^{B,C}$	2.50 ± 0.87^{A}
PRRSV-I-06	$4.3 \pm 2.0^{A,C}$	1.80 ± 0.80^{A}
B3-PRRSV-I-92	43.8 ± 5.7	4.60 ± 0.24
B3-PRRSV-I-06	25.8 ± 10.2	4.00 ± 0.71

3.7. Microscopic lesions

Lung tissues had multifocal-to-diffuse, mild-to-severe, lymphohistiocytic interstitial pneumonia. The mean microscopic lung lesion scores, which are summarized in Table 7, were significantly ($P < 0.0001$) lower in the negative controls compared to the four coinfected groups; however, the scores in the negative controls were not significantly lower than observed in the groups singularly infected with PRRSV. There was a significant effect of "PCV2" ($P < 0.001$) on microscopic lung lesions but there was no effect of "PRRSV strain" or "PCV2 subtype" on the severity of the observed microscopic lung lesions.

Lymphoid lesions were either not observed or were characterized by mild depletion of follicles and minimal granulomatous lymphadenitis in all coinfected groups. Significant differences in lymphoid lesion scores were not observed among the groups (data not shown).

3.8. Prevalence of PRRSV and PCV2 antigens in tissues

3.8.1. PRRSV

All control pigs were negative for PRRSV antigen by IHC on sections of lung. The prevalence of PRRSV antigen in lung sections was 16/23 pigs in the NC16845b-inoculated group (B3: 4/4 pigs) compared to 21/22 pigs in the VR-2385-inoculated group (B3: 5/5 pigs). There were no significant differences in the prevalence rates of PRRSV

antigen in lungs between the virus-inoculated groups. The prevalence of PRRSV antigen in lungs was independent of "PRRSV strain" or "PCV2 subtype".

3.8.2. PCV2

All control pigs and all B2 and B3 pigs were negative for PCV2 antigen by IHC. Low-to-high amounts of PCV2-antigen in lung sections were detected in 8/9 CoI-92-2a pigs, 7/9 CoI-92-2b pigs, 8/9 CoI-06-2a pigs and in 4/9 CoI-06-2b pigs which corresponds to16/18 PCV2a-inoculated pigs and 11/18 PCV2b-inoculated pigs. Moreover, PCV2 antigen was detected in 15/18 VR-2385-inoculated pigs and in 12/18 NC16845b-inoculated pigs. The prevalence of PCV2 antigen in lung tissues was independent of "PRRSV strain" or "PCV2 subtype". In lymphoid tissues, low-to-high amounts of PCV2 antigen were detected in 8/9 CoI-92-2a pigs, 7/9 CoI-92-2b pigs, 8/9 CoI-06-2a pigs and in 5/9 CoI-06-2b pigs which corresponds to 16/18 pigs inoculated with PCV2a and 12/18 pigs inoculated with PCV2b, as well as 13/18 pigs inoculated with NC16845b and 15/18 pigs inoculated with VR-2385. The prevalence of PCV2 antigen in lymphoid tissues was independent of "PRRSV strain" or "PCV2 subtype".

4. Discussion

The objective of this study was to characterize the infection dynamics and pathogenicity of two different type 2 PRRSV isolates recovered from pigs in 1992 and 2006 in a conventional pig model. To mimic field conditions where coinfections frequently occur, the pigs were concurrently infected with either PCV2a or PCV2b. The effect of each PRRSV isolate was also evaluated in singularly inoculated pigs. However, due to limitations in numbers of available pigs from the source herd, the experiments with singularly PRRSV-inoculated groups were conducted separately but under the same study conditions, using the same inocula and assays to analyze the samples.

The PRRSV isolate VR-2385 used in this experiment has been well-characterized in the CDCD and the conventional pig models and is considered a relatively highly pathogenic PRRSV isolate from the 1990s (Halbur et al., 1995b, 1996; Meng et al., 1996). In contrast, NC16845b represents a more recent PRRSV isolated from an outbreak of respiratory disease on a farm characterized by high morbidity and mortality in 2006 (Gauger et al., 2012). The ORF2-7 sequence homology between VR-2385 (GenBank accession PRU20788 and PRU03040) and NC16845b (GenBank accession HQ699067) was approximately 90.4%. The ORF5 region demonstrated the least nucleotide and amino acid homology at 87.4% and 87.1%, respectively (Gauger et al., 2012).

In the past, dual infections with PRRSV and porcine respiratory coronavirus (PRCV) or PRRSV and SIV were studied using conventional pigs (Van Reeth et al., 1996) and gnotobiotic pigs (Van Reeth and Nauwynck, 2000) and in general disease was found to be more pronounced in dually inoculated pigs. Interestingly, in gnotobiotic pigs the effect of the coinfection appeared additive rather than synergistic (Van Reeth and Nauwynck, 2000). More recent studies have shown that PRRSV modifies the innate immune response, induces immunosuppression and enhances the inflammatory response to PRCV in pigs (Jung et al., 2009; Renukaradhya et al., 2010). In another study, dual infection of specific pathogen-free pigs with PRRSV and pseudorabies virus (PRV) resulted in more severe clinical signs and increased pneumonia in pigs inoculated with both viruses compared to pigs infected with PRRSV or PRV alone (Shibata et al., 2003). It is also well recognized that PCV2 replication is enhanced by concurrent PRRSV infection in both CD and conventional pigs compared to singularly inoculated pigs (Allan et al., 2000; Rovira et al., 2002). To the authors' knowledge, the pathogenicity of genetically different PRRSV isolates in the presence of concurrent viral infection has not been evaluated *in vivo*.

The combination of PRRSV and PCV2 is one of the most common coinfections associated with swine respiratory disease under field conditions (Dorr et al., 2007; Pallarés et al., 2002). Both PRRSV isolates used in the current study were isolated from field cases of high mortality and experimental infection of pigs with PRRSV VR-2385 has resulted in severe lesions and high levels of viremia (Halbur et al., 1995b, 1996). The two PCV2 isolates were initially recovered from typical field cases of PCVAD in Iowa and North Carolina and have been characterized in the conventional pig model side by side without identifiable differences between PCV2 subtypes (Opriessnig et al., 2008b; Sinha et al., 2011). In the current study, clinical disease in the treatment groups was characterized by variable numbers of infected pigs experiencing transient, mild lethargy, mild respiratory disease and inappetence. Coinfected groups had significantly higher mean rectal temperatures compared to pigs infected with PRRSV alone and the negative controls. Interestingly, when organized by coinfection status and analyzed by PCV2 subtype, pigs inoculated with PCV2a had significantly higher mean group rectal temperatures compared to pigs inoculated with PCV2b on dpi 9 which was associated with an anti-PCV2-antibody response in 38.9% (7/18) of the PCV2a-inoculated pigs on dpi 9 whereas a delayed antibody response was seen in PCV2b- inoculated pigs (11.1%; 2/18).

It is well documented that pathogenic differences between type 2 PRRSV isolates exist (Halbur et al., 1995b, 1996). The uniqueness of the current study is the utilization of two temporally and genetically different PRRSV isolates both from cases of high morbidity and mortality in the field but isolated 15 years apart. In a separate *in vitro* study comparing phenotypic traits of the two PRRS viruses, NC16845b demonstrated reduced growth characteristics compared to VR-2385 (Gauger et al., 2012). NC16845b plaque sizes were slightly smaller than VR-2385 and the peak viral titer demonstrated by NC16845b was approximately 13-fold lower than the VR-2385 peak titer. This is in contrast to the *in vivo* growth characteristics demonstrated in this report. There were clear differences in initial replication between the two PRRSV isolates used in this study. The VR-2385-inoculated pigs had significantly higher levels of PRRSV RNA in serum on dpi 3 and 6. Moreover, NC16845b replicated at higher levels at dpi 9 and dpi 12 compared to VR-2385 which was associated with significantly lower levels of lymphocytes at dpi 9 and a significantly lower N/L ratio at dpi 12. These

results suggest that highly pathogenic PRRSVs may replicate more efficiently *in vivo* in contrast to their decreased growth properties *in vitro* as previously suggested (Johnson et al., 2004; Wang et al., 2008). This is further supported by the data obtained from the pigs infected with PRRSV alone (B2 and B3) which clearly show an increase in replication in pigs infected with NC16845b in the later stages of the *in vivo* study.

Similar to other PCV2-PRRSV coinfection studies (Allan et al., 2000; Harms et al., 2001), macroscopic and microscopic lesions in coinfected groups were enhanced compared to pigs singularly infected with PRRSV. Recently, it has been shown that pigs infected with VR-2385 had significantly prolonged (until 70 DPI) PCV2 viremia and shedding in PRRSV-PCV2 coinfected pigs (Sinha et al., 2011). A similar approach using PRRSV NC16845b, which replicated differently from VR-2385 in the early stages of infection, could potentially offer new insights on viral interactions in pigs. In the current study, PCV2b replication was significantly up-regulated shortly after initiation of the study at dpi 3 compared to PCV2a. Furthermore, the CoI-92-2b group had significantly higher quantities of PCV2b in the serum compared to CoI-06-2a (dpi 3 and 12) and CoI-06-2b (dpi 12) which was associated with a higher prevalence of PCV2 antigen in tissues (93.8% *versus* 75.0%) indicating a synergistic relationship between PRRSV-1992 (VR-2385) and PCV2. Unlike previous studies where the average trial length ranged from 21 to 32 days (Allan et al., 2000; Rovira et al., 2002), this trial was terminated at dpi 12 to evaluate PRRSV-induced lung lesions which tend to be most severe between dpi 10 and dpi 14. It remains unknown if the observed trend would have resulted in a difference in clinical disease in the later stages of infection. As expected, and similar to a previous study (Yu et al., 2007), the pathological lesions associated with PCV2 were either not present or they were mild; however, PCV2 antigen was detected in most tissues in coinfected pigs. In this study, PCV2 naïve pigs were utilized, thus the relevance of the model to actual field situations is limited considering the majority of young pigs have high levels of passively acquired anti-PCV2 antibodies (Opriessnig et al., 2004b). Therefore, the impact of anti-PCV2 immunity on the PCV2 infection could not be ascertained in the experiment; however, this was not a major concern as we know from several experiments that pigs with passively derived antibodies, although protected from clinical PCV2 associated disease, can still be infected with PCV2 (McKeown et al., 2005; Opriessnig et al., 2008a). Therefore, we believe that a PCV2 naïve pig model increases the ability to identify trends and associations between PRRSV and PCV2.

In the current study, PRRSV-PCV2 coinfection was administered intranasally on the same day. This model of simultaneous dual inoculation does not fully mimic the population dynamics due to the variability in timing of exposure to these two pathogens within and between herds in field situations. On many conventional farms, endemic exposure and seroconversion to PRRSV often occurs earlier than exposure to PCV2. Infection of pigs with PRRSV prior to PCV2 may contribute to the manifestation of more severe PCV2-induced clinical disease and lesions. PRRSV is immunosuppressive, primarily infecting porcine alveolar macrophages (Drew, 2000), which decreases the pig's ability to clear subsequent infections. In contrast, prior PRRSV infection may induce an immunostimulatory effect on the host immune response that serves to enhance PCV2 replication and lesions (Krakowka et al., 2001).

It is possible that amino acid mutations acquired during serial passaging of PRRSV on MARC-145 cells could result in attenuation as reported previously (Allende et al., 2000; An et al., 2011). While this is also applicable to the current study, we attempted to minimize this risk, by using a relatively low passage of both viruses with a pig passage followed by only two *in vitro* passages in MARC-145 cells. Inoculation was completed two days after weaning and transport of the pigs to the research facility. It is also possible that the stress from weaning, transport, new socialization, and adjusting to a new environment may have affected the ability of the pigs to respond to concurrent PRRSV-PCV2 infection and influenced the level of PRRSV replication in the pigs. However, the data obtained from pigs infected with PRRSV alone indicate that this was not the case and that the ability of the pigs to develop a humoral immune response was normal.

5. Conclusions

Overall, the data indicate no significant differences between the two PRRSV isolates based on clinical signs, gross pathology, histology or hematology even though the PRRSV isolates we utilized in this study were isolated from geographically separated herds (VR-2385 from Iowa and NC16845b from North Carolina) over a period of 15 years. Differences in *in vivo* replication kinetics were identified. VR-2385 initially replicated more quickly and to higher levels and peaked at dpi 6 and the amount of VR-2385 RNA steadily declined thereafter. In contrast, pigs infected with NC16845b had lower levels of PRRSV RNA in serum initially and this steadily increased through termination of the study at dpi 12. Concurrent PCV2 viremia was enhanced by PRRSV VR-2385 infection but not by concurrent PRRSV NC16845b infection. A higher prevalence of PCV2 antigen was demonstrated in the lungs of pigs coinfected with VR-2385 (83.3%) compared to pigs coinfected with PRRSV NC16845b (66.7%). This work further emphasizes *in vivo* replication differences among PRRSV strains and the importance of coinfecting pathogens on PRRSV kinetics. Additional investigations are necessary to further elucidate the specific mechanisms of the PCV2-PRRSV interaction in pigs.

Acknowledgment

The authors thank the Iowa Livestock Health Advisory Council for funding of this study.

References

An, T.Q., Tian, Z.J., Zhou, Y.J., Xiao, Y., Peng, J.M., Chen, J., Jiang, Y.F., Hao, X.F., Tong, G.Z., 2011. Comparative genomic analysis of five pairs of virulent parental/attenuated vaccine strains of PRRSV. Vet. Microbiol. 149, 104–112.

Allan, G.M., McNeilly, F., Ellis, J., Krakowka, S., Meehan, B., McNair, I., Walker, I., Kennedy, S., 2000. Experimental infection of colostrum deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. Arch. Virol. 145, 2421–2429.

Allende, R., Kutish, G.F., Laegreid, W., Lu, Z., Lewis, T.L., Rock, D.L., Friesen, J., Galeota, J.A., Doster, A.R., Osorio, F.A., 2000. Mutations in the genome of porcine reproductive and respiratory syndrome virus responsible for the attenuation phenotype. Arch. Virol 145, 1149–1161.

Beach, N.M., Ramamoorthy, S., Opriessnig, T., Wu, S.Q., Meng, X.J., 2010. Novel chimeric porcine circovirus (PCV) with the capsid gene of the emerging PCV2b subtype cloned in the genomic backbone of the non-pathogenic PCV1 is attenuated in vivo and induces protective and cross-protective immunity against PCV2b and PCV2a subtypes in pigs. Vaccine 29, 221–232.

Chae, C., 2005. A review of porcine circovirus 2-associated syndromes and diseases. Vet. J. 169, 326–336.

Cheung, A.K., Lager, K.M., Kohutyuk, O.I., Vincent, A.L., Henry, S.C., Baker, R.B., Rowland, R.R., Dunham, A.G., 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. Arch. Virol. 152, 1035–1044.

Dorr, P.M., Baker, R.B., Almond, G.W., Wayne, S.R., Gebreyes, W.A., 2007. Epidemiologic assessment of porcine circovirus type 2 coinfection with other pathogens in swine. J. Am. Vet. Med. Assoc. 230, 244–250.

Drew, T.W., 2000. A review of evidence for immunosuppression due to porcine reproductive and respiratory syndrome virus. Vet. Res. 31, 27–39.

Fang, Y., Schneider, P., Zhang, W.P., Faaberg, K.S., Nelson, E.A., Rowland, R.R., 2007. Diversity and evolution of a newly emerged North American Type 1 porcine arterivirus: analysis of isolates collected between 1999 and 2004. Arch. Virol. 152, 1009–1017.

Fenaux, M., Halbur, P.G., Gill, M., Toth, T.E., Meng, X.J., 2000. Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. J. Clin. Microbiol. 38, 2494–2503.

Forsberg, R., Oleksiewicz, M.B., Petersen, A.M., Hein, J., Botner, A., Storgaard, T., 2001. A molecular clock dates the common ancestor of European-type porcine reproductive and respiratory syndrome virus at more than 10 years before the emergence of disease. Virology 289, 174–179.

Fort, M., Sibila, M., Allepuz, A., Mateu, E., Roerink, F., Segales, J., 2008. Porcine circovirus type 2 (PCV2) vaccination of conventional pigs prevents viremia against PCV2 isolates of different genotypes and geographic origins. Vaccine 26, 1063–1071.

Gagnon, C.A., Tremblay, D., Tijssen, P., Venne, M.H., Houde, A., Elahi, S.M., 2007. The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada. Can. Vet. J. 48, 811–819.

Gauger, P., Faaberg, K.S., Guo, B., Kappes, M.A., Opriessnig, T., 2012. Genetic and phenotypic characterization of a 2006 United States porcine reproductive and respiratory virus isolate associated with high morbidity and mortality in the field. Virus Res. 163, 98–107.

Halbur, P.G., Miller, L.D., Paul, P.S., Meng, X.J., Huffman, E.L., Andrews, J.J., 1995a. Immunohistochemical identification of porcine reproductive and respiratory syndrome virus (PRRSV) antigen in the heart and lymphoid system of three-week-old colostrum-deprived pigs. Vet. Pathol. 32, 200–204.

Halbur, P.G., Paul, P.S., Frey, M.L., Landgraf, J., Eernisse, K., Meng, X.J., Lum, M.A., Andrews, J.J., Rathje, J.A., 1995b. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet. Pathol. 32, 648–660.

Halbur, P.G., Paul, P.S., Meng, X.J., Lum, M.A., Andrews, J.J., Rathje, J.A., 1996. Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. J. Vet. Diagn. Invest. 8, 11–20.

Han, J., Burkhart, K.M., Vaughn, E.M., Roof, M.B., Faaberg, K.S., 2006. Replication and expression analysis of PRRSV defective RNA. Adv. Exp. Med. Biol. 581, 445–448.

Hanada, K., Suzuki, Y., Nakane, T., Hirose, O., Gojobori, T., 2005. The origin and evolution of porcine reproductive and respiratory syndrome viruses. Mol. Biol. Evol. 22, 1024–1031.

Harms, P.A., Halbur, P.G., Sorden, S.D., 2002. Three cases of porcine respiratory disease complex associated with porcine circovirus type 2 infection. J. Swine Health Prod. 10, 27–30.

Harms, P.A., Sorden, S.D., Halbur, P.G., Bolin, S.R., Lager, K.M., Morozov, I., Paul, P.S., 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Vet. Pathol. 38, 528–539.

Horlen, K.P., Schneider, P., Anderson, J., Nietfeld, J.C., Henry, S.C., Tokach, L.M., Rowland, R.R., 2007. A cluster of farms experiencing severe porcine circovirus associated disease: clinical features and association with the PCV2b genotype. J. Swine Health Prod. 15, 270–278.

Johnson, W., Roof, M., Vaughn, E., Christopher-Hennings, J., Johnson, C.R., Murtaugh, M.P., 2004. Pathogenic and humoral immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) are related to viral load in acute infection. Vet. Immunol. Immunopathol. 102, 233–247.

Jung, K., Renukaradhya, G.J., Alekseev, K.P., Fang, Y., Tang, Y., Saif, L.J., 2009. Porcine reproductive and respiratory syndrome virus modifies innate immunity and alters disease outcome in pigs subsequently infected with porcine respiratory coronavirus: implications for respiratory viral co-infections. J. Gen. Virol. 90, 2713–2723.

Krakowka, S., Ellis, J.A., McNeilly, F., Ringler, S., Rings, D.M., Allan, G., 2001. Activation of the immune system is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2). Vet. Pathol. 38, 31–42.

Lager, K.M., Gauger, P.C., Vincent, A.L., Opriessnig, T., Kehrli Jr., M.E., Cheung, A.K., 2007. Mortality in pigs given porcine circovirus type 2 subgroup 1 and 2 viruses derived from DNA clones. Vet. Rec. 161, 428–429.

Lv, J., Zhang, J., Sun, Z., Liu, W., Yuan, S., 2008. An infectious cDNA clone of a highly pathogenic porcine reproductive and respiratory syndrome virus variant associated with porcine high fever syndrome. J. Gen. Virol. 89, 2075–2079.

McKeown, N.E., Opriessnig, T., Thomas, P., Guenette, D.K., Elvinger, F., Fenaux, M., Halbur, P.G., Meng, X.J., 2005. Effects of porcine circovirus type 2 (PCV2) maternal antibodies on experimental infection of piglets with PCV2. Clin. Diagn. Lab. Immunol. 12, 1347–1351.

Meng, X.J., Paul, P.S., Halbur, P.G., 1994. Molecular cloning and nucleotide sequencing of the 3′-terminal genomic RNA of the porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 75, 1795–1801.

Meng, X.J., Paul, P.S., Halbur, P.G., Lum, M.A., 1996. Characterization of a high-virulence US isolate of porcine reproductive and respiratory syndrome virus in a continuous cell line, ATCC CRL11171. J. Vet. Diagn. Invest. 8, 374–381.

Mengeling, W.L., Ridpath, J.F., Vorwald, A.C., 1988. Size and antigenic comparisons among the structural proteins of selected autonomous parvoviruses. J. Gen. Virol. 69, 825–837.

Nawagitgul, P., Harms, P.A., Morozov, I., Thacker, B.J., Sorden, S.D., Lekcharoensuk, C., Paul, P.S., 2002. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. Clin. Diagn. Lab. Immunol. 9, 33–40.

Nelsen, C.J., Murtaugh, M.P., Faaberg, K.S., 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J. Virol. 73, 270–280.

Opriessnig, T., Halbur, P.G., Yoon, K.J., Pogranichniy, R.M., Harmon, K.M., Evans, R., Key, K.F., Pallares, F.J., Thomas, P., Meng, X.J., 2002. Comparison of molecular and biological characteristics of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (Ingelvac PRRS MLV), the parent strain of the vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. J. Virol. 76, 11837–11844.

Opriessnig, T., Halbur, P.G., Yu, S., Thacker, E.L., Fenaux, M., Meng, X.J., 2006. Effects of the timing of the administration of *Mycoplasma hyopneumoniae* bacterin on the development of lesions associated with porcine circovirus type 2. Vet. Rec. 158, 149–154.

Opriessnig, T., Patterson, A.R., Elsener, J., Meng, X.J., Halbur, P.G., 2008a. Influence of maternal antibodies on efficacy of porcine circovirus type 2 (PCV2) vaccination to protect pigs from experimental infection with PCV2. Clin. Vaccine Immunol. 15, 397–401.

Opriessnig, T., Prickett, J.R., Madson, D.M., Shen, H.-G., Juhan, N.M., Pogranichniy, R.R., Meng, X.-J., Halbur, P.G., 2010. Porcine circovirus type 2 (PCV2)-infection and re-inoculation with homologous or heterologous strains: virological, serological, pathological and clinical effects in growing pigs. Vet. Res. 41, 31.

Opriessnig, T., Ramamoorthy, S., Madson, D.M., Patterson, A.R., Pal, N., Carman, S., Meng, X.J., Halbur, P.G., 2008b. Differences in virulence among porcine circovirus type 2 isolates are unrelated to cluster type 2a or 2b and prior infection provides heterologous protection. J. Gen. Virol. 89, 2482–2491.

Opriessnig, T., Thacker, E.L., Yu, S., Fenaux, M., Meng, X.J., Halbur, P.G., 2004a. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma hyopneumoniae* and porcine circovirus type 2. Vet. Pathol. 41, 624–640.

Opriessnig, T., Yu, S., Gallup, J.M., Evans, R.B., Fenaux, M., Pallarés, F.J., Thacker, E.L., Brockus, C.W., Ackermann, M.R., Thomas, P., Meng, X.J., Halbur, P.G., 2003. Effect of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. Vet. Pathol. 40, 521–529.

Opriessnig, T., Yu, S., Thacker, E., Halbur, P.G., 2004b. Derivation of porcine circovirus type 2-negative pigs from positive breeding herds. J. Swine Health Prod. 12, 186–191.

Pallarés, F.J., Halbur, P.G., Opriessnig, T., Sorden, S.D., Villar, D., Janke, B.H., Yaeger, M.J., Larson, D.J., Schwartz, K.J., Yoon, K.J., Hoffman, L.J., 2002. Porcine circovirus type 2 (PCV-2) coinfections in US field cases of postweaning multisystemic wasting syndrome (PMWS). J. Vet. Diagn. Invest. 14, 515–519.

Patterson, A.R., Opriessnig, T., 2010. Epidemiology and horizontal transmission of porcine circovirus type 2 (PCV2). Anim. Health Res. Rev. 11, 217–234.

Pirzadeh, B., Gagnon, C.A., Dea, S., 1998. Genomic and antigenic variations of porcine reproductive and respiratory syndrome virus major envelope GP5 glycoprotein. Can. J. Vet. Res. 62, 170–177.

Renukaradhya, G.J., Alekseev, K., Jung, K., Fang, Y., Saif, L.J., 2010. Porcine reproductive and respiratory syndrome virus-induced immunosuppression exacerbates the inflammatory response to porcine respiratory coronavirus in pigs. Viral Immunol. 23, 457–466.

Richt, J.A., Lekcharoensuk, P., Lager, K.M., Vincent, A.L., Loiacono, C.M., Janke, B.H., Wu, W.H., Yoon, K.J., Webby, R.J., Solorzano, A., Garcia-Sastre, A., 2006. Vaccination of pigs against swine influenza viruses by using an NS1-truncated modified live-virus vaccine. J. Virol. 80, 11009–11018.

Rovira, A., Balasch, M., Segalés, J., Garcia, L., Plana-Dúran, J., Rosell, C., Ellerbrok, H., Mankertz, A., Domingo, M., 2002. Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. J. Virol. 76, 3232–3239.

Rowland, R.R., Steffen, M., Ackerman, T., Benfield, D.A., 1999. The evolution of porcine reproductive and respiratory syndrome virus: quasispecies and emergence of a virus subpopulation during infection of pigs with VR-2332. Virology 259, 262–266.

Shibata, I., Yazawa, S., Ono, M., Okuda, Y., 2003. Experimental dual infection of specific pathogen-free pigs with porcine reproductive and respiratory syndrome virus and pseudorabies virus. J. Vet. Med. B: Infect. Dis. Vet. Public Health 50, 14–19.

Sinha, A., Shen, H.G., Schalk, S., Beach, N.M., Huang, Y.W., Meng, X.J., Halbur, P.G., Opriessnig, T., 2011. Porcine reproductive and respiratory syndrome virus (PRRSV) influences infection dynamics of porcine circovirus type 2 (PCV2) subtypes PCV2a and PCV2b by prolonging PCV2 viremia and shedding. Vet. Microbiol. 152, 235–246.

Sorden, S.D., Harms, P.A., Nawagitgul, P., Cavanaugh, D., Paul, P.S., 1999. Development of a polyclonal-antibody-based immunohistochemical method for the detection of type 2 porcine circovirus in formalin-fixed, paraffin-embedded tissue. J. Vet. Diagn. Invest. 11, 528–530.

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., Hou, L., Wang, X., Yang, X., Kang, L., Sun, M., Jin, P., Wang, S., Kitamura, Y., Yan, J., Gao, G.F., 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical prrs in china and molecular dissection of the unique hallmark. PLoS ONE 2, e526.

Tong, G.Z., Zhou, Y.J., Hao, X.F., Tian, Z.J., An, T.Q., Qiu, H.J., 2007. Highly pathogenic porcine reproductive and respiratory syndrome. China. Emerg. Infect Dis 13, 1434–1436.

Van Reeth, K., Nauwynck, H., 2000. Proinflammatory cytokines and viral respiratory disease in pigs. Vet. Res. 31, 187–213.

Van Reeth, K., Nauwynck, H., Pensaert, M., 1996. Dual infections of feeder pigs with porcine reproductive and respiratory syndrome virus followed by porcine respiratory coronavirus or swine influenza virus: a clinical and virological study. Vet. Microbiol. 48, 325–335.

Wang, Y., Liang, Y., Han, J., Burkhart, K.M., Vaughn, E.M., Roof, M.B., Faaberg, K.S., 2008. Attenuation of porcine reproductive and respiratory syndrome virus strain MN184 using chimeric construction with vaccine sequence. Virology 371, 418–429.

Wu, J., Li, J., Tian, F., Ren, S., Yu, M., Chen, J., Lan, Z., Zhang, X., Yoo, D., Wang, J., 2009. Genetic variation and pathogenicity of highly virulent porcine reproductive and respiratory syndrome virus emerging in China. Arch. Virol. 154, 1589–1597.

Yu, S., Opriessnig, T., Kitikoon, P., Nilubol, D., Halbur, P.G., Thacker, E., 2007. Porcine circovirus type 2 (PCV2) distribution and replication in tissues and immune cells in early infected pigs. Vet. Immunol. Immunopathol. 115, 261–272.

Yuan, S., Mickelson, D., Murtaugh, M.P., Faaberg, K.S., 2001. Complete genome comparison of porcine reproductive and respiratory syndrome virus parental and attenuated strains. Virus Res. 79, 189–200.

Yuan, S., Murtaugh, M.P., Faaberg, K.S., 2000. Heteroclite subgenomic RNAs are produced in porcine reproductive and respiratory syndrome virus infection. Virology 275, 158–169.

Yuan, S., Murtaugh, M.P., Schumann, F.A., Mickelson, D., Faaberg, K.S., 2004. Characterization of heteroclite subgenomic RNAs associated with PRRSV infection. Virus Res. 105, 75–87.

Yuan, S., Nelsen, C.J., Murtaugh, M.P., Schmitt, B.J., Faaberg, K.S., 1999. Recombination between North American strains of porcine reproductive and respiratory syndrome virus. Virus Res. 61, 87–98.

Zhou, L., Zhang, J., Zeng, J., Yin, S., Li, Y., Zheng, L., Guo, X., Ge, X., Yang, H., 2009. The 30-amino-acid deletion in the Nsp2 of highly pathogenic porcine reproductive and respiratory syndrome virus emerging in China is not related to its virulence. J. Virol. 83, 5156–5167.