Comparison of porcine circovirus type 2 (PCV2)-associated lesions produced by co-infection between two genotypes of PCV2 and two genotypes of porcine reproductive and respiratory syndrome virus

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The objective of this study was to compare the virulence and pathogenicity of a combination of concurrent infections of two genotypes of porcine circovirus type 2 (PCV2) and two genotypes of porcine reproductive and respiratory syndrome virus (PRRSV) in terms of PCV2 viraemia, and PCV2-associated lesions and antigens in co-infected pigs. Pigs with PCV2a (or 2b)/type 1 (or type 2) PRRSV had significantly ($P<0.05$) higher mean clinical respiratory scores and lower average daily weight gain compared with pigs with PCV2a (or 2b). Co-infection induced significantly lower levels of anti-PCV2 and anti-PRRSV IgG antibodies than infection with one genotype alone, regardless of the genotype of the two viruses. Pigs with PCV2a (or 2b)/type 2 PRRSV had significantly ($P<0.05$) higher levels of PCV2 viraemia, more severe PCV2-associated lesions, and more PCV2 DNA within the lesions compared with pigs with PCV2a (or 2b)/type 1 PRRSV. However, there was no significant difference in these parameters in pigs with PCV2a/type 2 PRRSV or PCV2b/type 2 PRRSV. The results of this study demonstrate significant differences in the virulence and pathogenicity of type 1 and type 2 PRRSV but no significant differences in the virulence and pathogenicity of PCV2a and PCV2b with respect to the production of PCV2-associated lesions.

INTRODUCTION

Porcine circovirus type 2 (PCV2) is a non-enveloped, single-stranded, circular DNA virus belonging to the family Circoviridae (Mankertz et al., 1997) that can be divided into at least two major genotypes, PCV2a and PCV2b, which are present worldwide (Grau-Roma et al., 2008; Segales et al., 2008). PCV2 has been implicated as the aetiological agent for postweaning multisystemic wasting syndrome (PMWS) and other associated diseases, which are collectively called porcine circovirus-associated disease (PCVAD) (Chae, 2005). Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, single-stranded RNA virus belonging to the family Arteriviridae (Cavanagh, 1997) that causes reproductive failure in sows and respiratory disease in grower-finisher pigs (Zimmerman et al., 2012). The type 1 (European) and type 2 (North American) genotypes of PRRSV are two distinct genotypes (Allende et al., 1999; Murtaugh et al., 2010).

A global shift in the prevalence of the main PCV2 genotype from the previously dominant PCV2 genotype PCV2a to PCV2b suggests a link between PCV2b and the occurrence of PMWS (Cheung et al., 2007; Gagnon et al., 2007; Dupont et al., 2008; Wiederkehr et al., 2009). These data suggest that PCV2b may potentially be more pathogenic than PCV2a; however, neither genotype was found to be directly correlated with a more severe disease in controlled experimental infections (Fort et al., 2008; Opriessnig et al., 2008a).

PCV2 and PRRSV are considered to be important viral pathogens and cause tremendous economic losses. Mixed infection of PRRSV and PCV2 is one of the most common co-infections associated with swine disease under current field conditions. Currently, two genotypes of PCV2 and PRRSV are found in most countries, including Asian countries (Chen et al., 2011; Thanawongnuwech et al., 2004). In a co-infection study, the virulence and pathogenicity of PCV2a/type 2 PRRSV and PCV2b/type 2 PRRSV
were not different in conventional specific-pathogen-free pigs (Opriessnig et al., 2012). However, an in-depth comparison of the virulence and pathogenicity of concurrent infections with the two genotypes of the two viruses has yet to be undertaken. Hence, the objective of this study was to evaluate the virulence and pathogenicity of concurrent infection with the two genotypes of the two viruses (PCV2a/type 1 PRRSV, PCV2a/type 2 PRRSV, PCV2b/type 1 PRRSV, and PCV2b/type 2 PRRSV) in terms of PCV2 viraemia, and PCV2-associated lesions and antigens in co-infected pigs.

RESULTS

Clinical observations

Pigs with PCV2a (or 2b)/type 2 PRRSV had significantly ($P<0.05$) higher mean clinical respiratory scores than pigs with PCV2a (or 2b) from 4 to 23 days post-inoculation (p.i.). Pigs with PCV2a (or 2b)/type 1 PRRSV had significantly ($P<0.05$) higher mean clinical respiratory scores than pigs with PCV2a (or 2b) from 7 to 9 days p.i. Pigs with type 2 PRRSV had significantly ($P<0.05$) higher mean clinical respiratory scores than pigs with PCV2a (or 2b) from 4 to 16 days p.i. No respiratory distress was observed in negative control pigs throughout the experiment.

Pigs with PCV2a (or 2b)/type 2 PRRSV and pigs with type 2 PRRSV had significantly ($P<0.05$) higher mean rectal temperatures than pigs in the other eight groups from 2 to 7 days p.i. Pigs with PCV2a (or 2b)/type 1 PRRSV and pigs with type 1 PRRSV had significantly ($P<0.05$) higher mean rectal temperatures than pigs with PCV2a (or 2b) and negative control pigs at 3 and 4 days p.i. (Fig. 1).

Growth performance

From 6–14 days p.i. (6–8 weeks of age), pigs with PCV2a (or 2b)/type 2 PRRSV and pigs with type 2 PRRSV had significantly ($P<0.05$) lower average daily weight gain (ADWG) than pigs with PCV2a (or 2b), pigs with type 1 PRRSV and negative control pigs. Pigs with PCV2a (or 2b)/type 1 PRRSV had significantly ($P<0.05$) lower ADWG than pigs with PCV2a (or 2b) and negative control pigs. From 14–28 days p.i. (8–10 weeks of age), pigs with PCV2a (or 2b)/type 1 (or type 2) PRRSV had significantly ($P<0.05$) lower ADWG than pigs with PCV2a (or 2b), pigs

Fig. 1. Mean rectal temperature in pigs from nine groups (PCV2a/type 1 PRRSV, ◆; PCV2a/type 2 PRRSV, ●; PCV2b/type 1 PRRSV, □; PCV2b/type 2 PRRSV, ■; PCV2a, ○; PCV2b, ●; type 1 PRRSV, △; and type 2 PRRSV, ▲; Negative Control, X). Symbols (*, †, ‡) indicate statistically significant differences ($P<0.05$) among groups. Error bars represent SD.
Anti-PCV2 IgG antibodies

Regardless of the genotype of PCV2, PCV2-infected pigs were seropositive by ELISA at 10 days p.i. Pigs with PCV2a (or 2b) had significantly (P<0.05) higher anti-PCV2 IgG antibody levels than pigs with PCV2a (or 2b)/type 1 (or type 2) PRRSV (Fig. 3a). No anti-PCV2 IgG antibodies were detected in negative control pigs.

Anti-PRRSV IgG antibodies

Regardless of the genotype of PRRSV, PRRSV-infected pigs were seropositive by ELISA at 10 days p.i. Pigs with type 1 (or type 2) PRRSV had significantly (P<0.05) higher anti-PRRSV IgG antibody levels than pigs with a PCV2a (or 2b)/type 1 (or type 2) PRRSV (Fig. 3b). No anti-PRRSV IgG antibodies were detected in negative control pigs.

Quantification of PCV2 DNA in sera

Regarding PCV2a viraemia, pigs with PCV2a/type 2 PRRSV had a significantly (P<0.05) higher number of genomic copies of PCV2a in their sera than pigs with PCV2a/type 1 PRRSV or pigs with PCV2a at 10 and 14 days p.i. Pigs with PCV2a/type 1 PRRSV had a significantly (P<0.05) higher number of genomic copies of PCV2a in their sera than pigs with PCV2a at 14, 21, and 28 days p.i. (Fig. 4a). Regarding PCV2b viraemia, pigs with PCV2b/type 2 PRRSV had a significantly (P<0.05) higher number of genomic copies of PCV2b in their sera than pigs with PCV2b at 14, 21, and 28 days p.i. (Fig. 4a). The number of genomic copies of PCV2a and PCV2b in PCV2a-infected pigs and PCV2b-infected pigs was not significantly different. No PCV2a or PCV2b was detected in the blood of negative control pigs.
Quantification of PRRSV RNA in sera

The number of genomic copies of type 2 PRRSV in pigs with PCV2a (or 2b)/type 2 PRRSV and pigs with type 2 PRRSV was significantly $(P<0.05)$ higher than that of type 1 PRRSV in pigs with PCV2a (or 2b)/type 1 PRRSV and pigs with type 1 PRRSV. Pigs with PCV2a (or 2b)/type 1 PRRSV had significantly $(P<0.05)$ higher lung lesion scores than pigs with PCV2a (or 2b) and pigs with type 1 PRRSV. No macroscopic lung lesions were noted in negative control pigs.

Microscopic lung lesions

Regardless of the genotype of the viruses, typical granulomatous inflammatory reaction and lymphoid depletion consistent with histopathological lesions of PMWS were observed in the lymph nodes of pigs with PCV2/PRRSV. At 14 days p.i., pigs with PCV2a (or 2b)/type 2 PRRSV had significantly $(P<0.05)$ higher lung lesion scores than pigs with PCV2a (or 2b)/type 1 PRRSV (Fig. 5a, b), pigs with PCV2a (or 2b), and pigs with type 1 (or type 2) PRRSV. Pigs with PCV2a (or 2b)/type 1 PRRSV had significantly $(P<0.05)$ higher lymphoid lesion scores than pigs with PCV2a (or 2b) and pigs with type 1 (or type 2) PRRSV. Pigs with PCV2a (or 2b)/type 1 PRRSV had significantly $(P<0.05)$ higher lymphoid lesion scores than pigs with PCV2a (or 2b) and pigs with type 1 (or type 2) PRRSV. Pigs with PCV2a (or 2b) had significantly $(P<0.05)$ higher lymphoid lesion scores than pigs with type 1 (or type 2) PRRSV (Fig. 6a).

Microscopic lung lesions in pigs with PCV2a (or 2b)/type 2 PRRSV and pigs with type 2 PRRSV were characterized by...
thickened alveolar septae, and increased numbers of interstitial macrophages and lymphocytes. At 14 days p.i., pigs with PCV2a (or 2b)/type 2 PRRSV had significantly ($P<0.05$) higher scores for interstitial pneumonia lesions than pigs with PCV2a (or 2b). At 28 days p.i., pigs with PCV2a (or 2b)/type 1 (or type 2) PRRSV had a significantly ($P<0.05$) higher number of PCV2-positive cells per unit in their lymph nodes and lungs than pigs with PCV2a (or 2b). No PCV2a or PCV2b DNA was detected in the lymph nodes or lungs of negative control pigs (Fig. 7).

**In situ hybridization of PRRSV**

PRRSV was detected in macrophages of the lymph nodes and lungs. At 14 days p.i., pigs with PCV2a (or 2b)/type 2 PRRSV had a significantly ($P<0.05$) higher number of PRRSV-positive cells per unit in their lungs than pigs with PCV2a (or 2b)/type 1 PRRSV or pigs with type 1 PRRSV. No type 1 or type 2 PRRSV RNA was detected in the lymph nodes or lungs of negative control pigs (Fig. 8).

**In situ hybridization of PCV2**

PCV2 was detected in mononuclear cells of the lymph nodes and lungs. At 14 days p.i., pigs with PCV2a (or 2b)/type 2 PRRSV had a significantly ($P<0.05$) higher number of PCV2-positive cells per unit in their lymph nodes and lungs than pigs with PCV2a (or 2b)/type 1 PRRSV or pigs with PCV2a (or 2b). Pigs with PCV2a (or 2b)/type 1 PRRSV had significantly ($P<0.05$) higher scores for interstitial pneumonia lesions than pigs with type 1 PRRSV. Pigs with PCV2a (or 2b)/type 1 PRRSV had significantly ($P<0.05$) higher scores for interstitial pneumonia lesions than pigs with type 1 PRRSV or pigs with PCV2a (or 2b). The lymph nodes and lungs of negative control pigs were normal (Fig. 6b).

**Fig. 6.** Microscopic lymph node (a) and lung (b) lesion scores in pigs from eight groups (PCV2a/type 1 PRRSV, PCV2a/type 2 PRRSV, PCV2b/type 1 PRRSV, PCV2b/type 2 PRRSV, PCV2a, PCV2b, type 1 PRRSV, and type 2 PRRSV). Symbols (*, †, ††, and ‡) indicate statistically significant differences ($P<0.05$) among groups. Error bars represent SD.

**Fig. 7.** Number of PCV2-positive cells per unit in lymph nodes (a) and lungs (b) from pigs in six groups (PCV2a/type 1 PRRSV, PCV2a/type 2 PRRSV, PCV2b/type 1 PRRSV, PCV2b/type 2 PRRSV, PCV2a, PCV2b). Symbols (*, †, ††, and ‡) indicate statistically significant differences ($P<0.05$) among groups. Error bars represent SD.
Comparison of PCV2-associated lesions

**DISCUSSION**

This study demonstrates that co-infection with PCV2a (or 2b)/type 2 PRRSV can result in a more serious disease than co-infection with PCV2a (or 2b)/type 1 PRRSV or infection with PCV2a (or 2b) alone. There were no differences in the virulence and pathogenicity of PCV2a/type 2 PRRSV and PCV2b/type 2 PRRSV or PCV2a/type 1 PRRSV and PCV2b/type 1 PRRSV. These results agree with previous findings that demonstrated no significant differences in the pathogenicity of PCV2a/type 2 PRRSV and PCV2b/type 2 PRRSV (Opriessnig et al., 2012). Our well-controlled experimental study also does not support major differences in the virulence or pathogenicity of PCV2a and PCV2b. These observations are in contrast with those of field studies reporting differences in the ORF 2 sequences of PCV2 obtained from animals from PMWS-affected farms (majority PCV2b) and animals from non-PMWS-affected farms (all PCV2a) (Grau-Roma et al., 2008). In addition, epidemiological studies have suggested a link between PCV2b and the occurrence of PMWS and a genotype shift from PCV2a to PCV2b (Cheung et al., 2007; Dupont et al., 2008; Wiederkehr et al., 2009). We have no clear explanation for this discrepancy. Both genotypes of the PCV2 isolates used in this study originated from post-weaned pigs with severe PMWS, and thus both genotypes were expected to be of similar virulence. Another explanation to consider based on this study is that a global shift of the PCV2 genotype, from PCV2a to PCV2b, coincidentally occurred at the same time that the PCVAD outbreak occurred worldwide.

Significant differences in the virulence and pathogenicity of PCV2a (or 2b)/type 2 PRRSV and PCV2a (or 2b)/type 1 PRRSV are reasonable because type 2 PRRSV induces a more severe respiratory disease than type 1 PRRSV (Halbur et al., 1996a; Han et al., 2013a; Martínez-Lobo et al., 2011). Moreover, pigs with PCV2a (or 2b)/type 2 PRRSV had a higher PCV2 load in their blood than pigs with PCV2a (or 2b)/type 1 PRRSV. PCV2 viraemia plays a central role in the development of PMWS. High levels of PCV2 viraemia are associated with development of PCVAD (Meerts et al., 2005, 2006). The more virulent type 2 PRRSV replicates faster than the less virulent type 1 PRRSV (Han et al., 2013a). Notably, type 2 PRRSV appears to reach a higher level of viraemia than type 1 PRRSV, as previously described (Han et al., 2013a). These results suggest that the enhancement of PCV2 replication may be explained by a quantitative difference in the viraemia of type 1 and type 2 PRRSV.

In the present study, there were no significant differences in the levels of PRRSV viraemia in pigs with PCV2a/type 2 (or type 1) PRRSV and pigs with PCV2b/type 2 (or type 1) PRRSV. The results of this study suggest that PCV2 does not enhance PRRSV viraemia. In contrast, another experimental study indicated that a highly pathogenic PRRSV infection followed by a PCV2 infection enhanced the replication of both viruses in pigs (Fan et al., 2013). These synergistic effects may have resulted from the high virulence of highly pathogenic PRRSV or the use of different (concurrent vs. sequential) co-infection models. Further studies are needed to compare different co-infection models and the virulence of the strains of the same PRRSV genotype.

The diagnosis of PMWS must include characteristic lesions in the lymphoid tissues and the detection of a PCV2 antigen or PCV2 DNA within these lesions (Chae, 2004). Hence, pathological evaluation and detection of a PCV2 antigen within the lesion are the most important criteria for assessing virulence and pathogenicity in co-infected pigs. A hallmark of the pathological lesions in lymphoid tissues is granulomatous inflammation with lymphoid depletion (Chae, 2004). In the present study, typical histopathological changes were observed in pigs with PCV2a (or 2b)/type 2 PRRSV and pigs with PCV2a (or 2b)/type 1 PRRSV. However, pigs with PCV2a (or 2b)/type 2 PRRSV had more severe PCV2-associated lesions and more PCV2 DNA.

**Fig. 8.** Number of PRRSV-positive cells per unit in lymph node (a) and lung (b) of pigs from 6 groups (PCV2a/type 1 PRRSV, ■; PCV2a/type 2 PRRSV, □; PCV2b/type 1 PRRSV, □; PCV2b/type 2 PRRSV, □; type 1 PRRSV, □; and type 2 PRRSV, □). Symbols (*) and † indicate statistically significant differences (P<0.05) among groups. Error bars represent SD.
within the lesions than pigs with PCV2a (or 2b)/type 1 PRRSV. These results suggest that type 2 PRRSV enhances the replication of PCV2 and can induce more severe lymphoid lesions than type 1 PRRSV. Interestingly, the co-infected pigs produced significantly lower levels of anti-PCV2 and anti-PRRSV IgG antibodies than singularly infected pigs, regardless of the genotype of the two viruses. Pigs that were co-infected with PCV2 and PRRSV were found to have more severe lymphoid depletion and enhanced PCV2 replication in previous studies (Allan et al., 2000; Harms et al., 2001; Rovira et al., 2002) and in the present study. Selective loss of CD3+ and CD4+ cells is observed in pigs with PMWS (Nielsen et al., 2003; Segales et al., 2001). Therefore, the lymphoid depletion induced in pigs by co-infection with PCV2 and PRRSV may render them unable to mount an effective immune response.

Our results should be interpreted carefully because this study has been conducted with only one strain for each genotype of both viruses. Different results could have been obtained from an experiment conducted with a different strain of the same genotype. Variation in the virulence of different PCV2 and PRRSV isolates with the same genotypes in pigs has been documented (Halbur et al., 1996b; Han et al., 2013b; Opriessnig et al., 2008b). Future studies are needed to compare the virulence and pathogenicity of a combination of concurrent infections of each genotype of both viruses using different strains of the same genotype.

Genetically modified PCV2 demonstrated that both the rep gene and the origin of replication (Ori) of PCV1 enhance replication and pathogenicity of the PCV2-based chimeric viruses in vitro (Beach et al., 2010). Although the molecular mechanisms of PRRSV-enhanced PCV2 replication and pathogenesis remain poorly understood, interaction between PRRSV and two genes (rep and Ori genes) of PCV2 may activate PCV2 replication and exacerbate the clinical outcome of infection. Mixed infection with PRRSV and PCV2 is currently one of the most common co-infections associated with swine disease under field conditions. Hence, the four different combinations of concurrent infections involving the two genotypes of the two viruses in this study will provide swine practitioners and producers with clinical information. Regular surveillance of the two genotypes of the two viruses in sick and dead pigs should be undertaken in herds in which both genotypes coexist.

**METHODS**

**Animals and housing.** Ninety conventional crossbred pigs derived from 10 sows known to be free of PCV2, PRRSV and *Mycoplasma hyopneumoniae* were purchased from a commercial PRRSV-free farm. On the day of arrival, all pigs were tested and found negative for PCV2, PRRSV and *M. hyopneumoniae* according to routine serological testing. All piglets were obese for the presence of PCV2 and PRRSV in the blood by real-time PCR (Gagnon et al., 2008; Wasilk et al., 2004). All pigs were housed in an environmentally controlled building as previously described (Kim et al., 2011).

**Viral inocula.** Two different genotypes of PCV2 were used for the inoculation of pigs. PCV2a (SNUVR100032 strain) and PCV2b (SNUVR000463 strain) were isolated in superficial inguinal lymph node from different postweaned pigs with severe PMWS in different herds (Kim et al., 2010; Seo et al., 2012). Inoculum of PCV2a and PCV2b was prepared from tissue culture fluid containing 10^6 tissue culture infective doses 50% (TCID_{50}) ml^{-1} of each genotype (third passage in PCV-free PK-15 cells).

Two different genotypes of PRRSV were used for the inoculation of pigs. Type 2 PRRSV (SNUVR090851 strain) and type 1 PRRSV (SNUVR090485 strain) were isolated in lung samples from newly weaned pigs and neonatal piglets with severe dyspnoea, respectively, from different herds (Han et al., 2012, 2013a). Inoculum of type 2 PRRSV (third passage in MARC-145 cells) and type 1 PRRSV (third passage in alveolar macrophages) was prepared from tissue culture fluid containing 10^5 TCID_{50} ml^{-1} of each genotype.

**Experimental design.** Ninety pigs were placed into one of nine treatment groups (10 pigs in each group) at 2 weeks of age: PCV2a/1 PRRSV, PCV2a/type 2 PRRSV, PCV2b/type 1 PRRSV, PCV2b/ type 2 PRRSV, PCV2a, PCV2b, type 1 PRRSV, type 2 PRRSV, or negative control. At 6 weeks of age, the pigs were inoculated intranasally as follows: PCV2a and 2b, 3 ml of each PCV2 genotype inoculum; type 1 and type 2 PRRSV, 3 ml of each PRRSV genotype inoculum; PCV2/PRRSV, 3 ml of each PCV2 inoculum and PRRSV inoculum. Blood samples were collected from each pig by jugular venipuncture at 0, 5, 7, 10, 14, 21, and 28 days p.i. Five pigs from each group were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution at 14 and 28 days p.i., as previously described (Beaver et al., 2001). Tissues were collected from each pig at necropsy. All of the methods were approved by the Seoul National University Institutional Animal Care and Use Committee.

**Clinical observations.** The pigs were scored daily for clinical respiratory disease severity using scores ranging from 0 (normal) to 6 (severe dyspnoea and abdominal breathing) (Halbur et al., 1995). Observers were blinded to the vaccination status. Rectal temperatures were recorded daily at the same time by the same personnel.

**Growth performance.** The live weight of each pig was measured at 0 days p.i. (6 weeks of age), 14 days p.i. (8 weeks of age), and 28 days p.i. (10 weeks of age). The ADWG (g per pig per day) was analysed over two time periods: (i) between 0 and 14 days p.i. and (ii) between 14 and 28 days p.i. The ADWG during the different production stages was calculated as the difference between the starting and final weights divided by the duration of the stage.

**Serology.** The serum samples were tested using the commercial PCV2 ELISA (Symbiotics) and PRRS ELISA (HerdCheck PRRS 3XR, IDEXX Laboratories).

**Quantification of PCV DNA in blood.** DNA extraction from serum samples was performed using the QIamp DNA mini kit. DNA extracts were used to quantify PCV2a and PCV2b genomic DNA copy numbers by real-time PCR as previously described (Gagnon et al., 2008).

**Quantification of PRRSV RNA in blood.** RNA extraction from serum samples was performed as previously described (Wasilk et al., 2004). Real-time PCR for the type 1 and type 2 PRRSVs was used to quantify PRRSV genomic cDNA copy numbers using RNA extractions from serum samples as previously described (Wasilk et al., 2004).

**In situ hybridization.** Genotype-specific *in situ* hybridization (ISH) was used to detect and differentiate PCV2a and PCV2b, respectively,
in formalin-fixed, paraffin-embedded tissues (Kim et al., 2010). Genotype-specific ISH was used to detect and differentiate type 1 and type 2 PRRSV, respectively, in formalin-fixed, paraffin-embedded tissues (Han et al., 2013a).

**Morphometric analysis.** For morphometric analysis of the macroscopic pulmonary lesion score, lung lesions were scored to indicate the percentage of total lung that was consolidated (Halbur et al., 1995). For morphometric analysis of the microscopic pulmonary and lymphoid lesion score, tissue sections were examined blindly. Lung sections were given an estimated score of the severity of the interstitial pneumonia with the scores ranging from 0 (normal) to 4 (severe diffuse) based on five areas of the lung. These were one from the dorsomedial part of right anterior lobe, one from the dorsomedial part of right middle lobe, one from the dorsomedial part of right caudal lobe, one from the ventromedial part of right caudal lobe and one from the dorsomedial part of the accessory lobe (Halbur et al., 1995). Three superficial inguinal lymph node sections were given an estimated score ranging from 0 (normal, i.e. no lymphoid depletion or granulomatous replacement) to 5 (severe lymphoid depletion and granulomatous replacement) (Kim & Chae, 2004).

For morphometric analysis of in situ hybridization, three sections were cut from each of three blocks of tissue from one entire right pulmonary lobe and superficial inguinal lymph node of each pig. To obtain quantitative data, slides were analysed with the NIH Image J 1.43 m Program (http://rsb.info.nih.gov/ij). In each slide, 10 fields were randomly selected, and the number of positive cells per unit area (0.95 mm² for lung and 0.25 mm² for lymph node) was determined as previously described (Kim et al., 2011; Halbur et al., 1996a). The mean values were also calculated.

**Statistical analyses.** The summarized statistics for all of the groups were calculated to assess the overall quality of the data, including the normality. The values for the genomic copies of PCV2 and the PRRSV viraemia were transformed to log₁₀ values prior to analysis. Continuous data (genomic copies of PCV2 DNA and of PRRSV RNA) were evaluated using a repeated measures analysis of variance (ANOVA). If the ANOVA revealed a significant effect, a one-way ANOVA with pairwise testing using Tukey’s adjustment was performed for each time point. If the distribution of the variables was not normal, non-parametric Kruskal–Wallis and Mann–Whitney tests were used to analyse the data. Discrete data (pulmonary and lymphoid lesion scores) were analysed using the chi-squared and/or Fisher’s exact test. P<0.05 was considered significant.

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