



RESEARCH ARTICLE

Comparative Observations on the Piglets Infected with Porcine Circovirus Type 2 and Porcine Reproductive and Respiratory Syndrome Virus

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ABSTRACT

Porcine circovirus type 2 (PCV2) is often associated with subclinical infections and swine post weaning multisystemic wasting syndrome (PMWS). In the present study, 28-day-old piglets were inoculated with PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), as well as PCV2+PRRSV. Macroscopic lesions, histologic lesions, virus distribution in tissues were systematically examined. Histologic lesions were found in both single PCV2 and dual PCV2+PRRSV-inoculated piglets, including lymphoid depletion and intracytoplasmic inclusion bodies within histiocytes, as well as bronchointerstitial pneumonia and granulomatous inflammation in the lungs. The lesions were more severe in piglets inoculated with PCV2+PRRSV than in those with PCV2. PRRSV-inoculated piglets showed lymphoid depletion and moderate interstitial pneumonia, but no inclusion body or granulomatous inflammation. PCV2 antigen intensity in many tissues was higher in the coinfection group than in the single PCV2, but PRRSV antigen intensity had no difference between the PRRSV and PCV2+PRRSV groups. Therefore, PCV2, not PRRSV, induced the inclusion bodies and granulomatous inflammation that characterizes PMWS. PRRSV+PCV2 coinfection also promoted the replication of PCV2, but not of PRRSV.

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INTRODUCTION

Porcine circovirus type 2 (PCV2) is often associated with subclinical infections and swine post weaning multisystemic wasting syndrome (PMWS) a new disease affecting pigs between 5 and 12 weeks old (Allan and Ellis, 2000; Harms *et al.*, 2002; Harding and Clark, 1997) by airway (Kristensen *et al.*, 2013). PMWS is characterized by progressive weight loss, anemia, dyspnea, and occasionally, diarrhea, jaundice, and the microscopic lesions of PMWS include lymphocyte depletion and infiltration of lymphoids with some basophilic cytoplasmic inclusion bodies (Chae, 2004; Choi and Chae, 2000; Fan *et al.*, 2013).

The porcine reproductive and respiratory syndrome (PRRS) is a severe disease in sows characterized by reproductive failure (Key *et al.*, 2001). PRRS often has manifestations of stillbirth, piglet mortality, and respiratory disorders in all types of pigs (Johnson *et al.*, 2004). The replication site of PRRS causal agent is recognized as in mononuclear macrophages (Tsai *et al.*, 2012).

Many studies convey the vital function of dual or multiple infections in the pathogenesis of PCV2-associated diseases (Allan and Ellis, 2000; Allan *et al.*, 2000; Cao *et al.*, 2005; Cecere *et al.*, 2012). A retrospective study of natural PMWS infection in Spain has revealed that PCV2 alone is found in only 17.3% of all investigated cases. PRRSV in combination with PCV2 is found in 56.6% of all cases (Sorden *et al.*, 1998). The present experiment investigated the pathogenicity of PCV2 and PRRSV when inoculated either individually or in combination into conventional pigs. The purpose was to systematically document gross, histopathological lesions, virus distributions and relations between PCV2 or PRRSV and lesions.

MATERIALS AND METHODS

Animals: Twenty 28-day-old Large White piglets were obtained from a breeding pig farm in Hubei province, which examined negative for both PCV2 and PRRSV, and were raised in controlled lab conditions.

Virus: The PCV2-WH strain (GenBank accession number: FJ870967) was isolated from a natural PMWS-affected pig in China (Cao *et al.*, 2005). The viral titer for the inoculation of the piglets determined by titration in PK-15 cells was $10^{7.3}$ TCID₅₀/mL. The PRRSV WUHI strain was isolated from a clinical case of PRRSV in China, and confirmation as PRRSV was performed by a complete genome sequence analysis (Li *et al.*, 2009). This virus titer was $10^{5.8}$ TCID₅₀/mL determined by above same method.

Design of experiment: The piglets were randomly divided into four groups of five piglets each, and were placed in four different rooms. Namely, PCV2 treatment, PRRSV treatment, PCV2+PRRSV treatment, and control group. Each pig was inoculated at day 0 in the following manner as the conference (Song, 2010; Li *et al.*, 2013). The PCV2 group was infected both intranasally and intramuscularly with PCV2 inoculum (2.5mL each route). The PRRSV group was infected intranasally with PRRSV inoculum (3mL). The PCV2+PRRSV group was infected both intranasally and intramuscularly with PCV2 inoculum (2.5mL each route), as well as intramuscularly with PRRSV inoculum (3mL). The control group was infected both intranasally and intramuscularly with sham inoculum (2.5mL each route). All piglets were humanely sacrificed at 28 days post-inoculation (DPI) for necropsy.

Clinical assessment: The piglets were observed daily for clinical symptoms, also recorded rectal temperatures and body weight changes from the time of inoculation until the test end.

Histopathology: Samples of lymph nodes (superficial inguinal, submaxillary, and mesenteric), Peyer's patches, tonsils, spleen, heart, liver, kidneys, and lungs at autopsy were collected and fixed by immersion in 4% neutral-buffered paraformaldehyde for histological examination.

Microscopic assessment was carried out in accordance with the references (Opriessning *et al.*, 2004). In brief, lung lesions were scored for the presence and severity of interstitial pneumonia from 0 (normal) to 6 (severe). Kidney, heart, and liver were scored for the presence and severity of interstitial nephritis, myocarditis and hepatitis from 0 (normal) to 3 (severe). Lymphoid tissues were evaluated for the presence of lymphoid depletion from 0 (normal) to 3 (severe), histiocytic inflammation was scored from 0 (normal) to 3 (severe). The presence of inclusion bodies was also determined.

Immunohistochemistry of PCV2 and PRRSV antigens:

The sections of the lung, lymph tissues, kidney, heart and liver of all piglets were sampled for immunohistochemical analysis. A monoclonal antibody anti-PCV2 or anti-PRRSV (given by State Key Lab of Agricultural Microbiology, Wuhan, China. 1:100 in PBS) as the primary antibodies and the biotinylated goat anti-mouse linking antibody (Boster Biological Technology Ltd, Wuhan, China) were used. Tissue distribution of PCV2 and PRRSV antigens were carried out as previously described (Chianini *et al.*, 2003), and the antigen integrated optical density (IOD) for each slide was

obtained using the IPP 6.0 software. The mean values were also calculated.

Statistical analysis: Data were represented as mean±SD. Statistical analysis of the data was performed using ANOVA for all groups. Results with $P \leq 0.05$ were considered statistically significant.

RESULTS

Clinical presentation and gross lesions: No clinical sign was observed in the control group. However, mild respiratory signs characterized of drowsiness and shortness of breath were observed in both PRRSV and PCV2+PRRSV-inoculated piglets, but were more severe in the latter. Most of the PCV2+PRRSV-infected pigs had rough hair coats. The temporary drowsiness and respiratory symptoms were found in the PCV2 group.

No mean rectal temperature (MRT) above 40°C was observed in both control and PCV2 groups. In the PRRSV group, the MRT rose above 40°C at 11DPI, and maintained the high level until 22DPI. In the PCV2+PRRSV group, the changes of MRT were similar to those of the PRRSV group through 8DPI but continued the high level until 15DPI, and then decreased.

The average daily weight gain had the following trend: control (0.71 kg/day) > PCV2 (0.53 kg/day) > PRRSV (0.45 kg/day) > PCV2+PRRSV (0.39 kg/day). The significant differences ($P < 0.05$) were found between the control and any of the other three treatment groups. However, no significant difference was found between the pigs with individual inoculations and those with the combined inoculation. All three treatment groups exhibited clinical lesions, severe edema and hemorrhage showed in lung and lymph nodes, especially in coinfection group. The gross lesions of spleen, heart, liver and kidney were relative moderate. The results are summarized in Table 1.

Microscopic lesions: Histopathological lesions were observed in many pig lymphoid and non-lymphoid tissues in the PCV2, PRRSV, and PCV2+PRRSV groups. No gross lesion was observed in the control group.

All three treatment groups exhibited lymphocytic depletion, histiocytic infiltration, and lymphocytic necrosis in lymphoid nodes, macrophage infiltration in the spleen, and intracytoplasmic inclusion bodies in Peyer's patches and tonsils. Lymphocytic depletion of MSN was observed to resemble a star shape in the PCV2 (Fig.1a) and PCV2+PRRSV (Fig.1b) groups. Only the PCV2+PRRSV group exhibited amphophilic intracytoplasmic inclusion bodies in the MSN (Fig.1e). Inflammation and lymphocyte depletion in the PCV2+PRRSV group were more severe than the PCV2 or PRRSV group, especially in the submaxillary lymph node ($P < 0.05$). The lymphoid tissue lesions are summarized in Table 2.

All three inoculated groups had interstitial pneumonia. In the PCV2 and coinfection groups also had moderate to severe peribronchiolar and perivascular lymphocyte infiltration and nodule proliferation and alveolar exudates. Focal to multifocal granulomatous lesions were observed in the dual infection groups

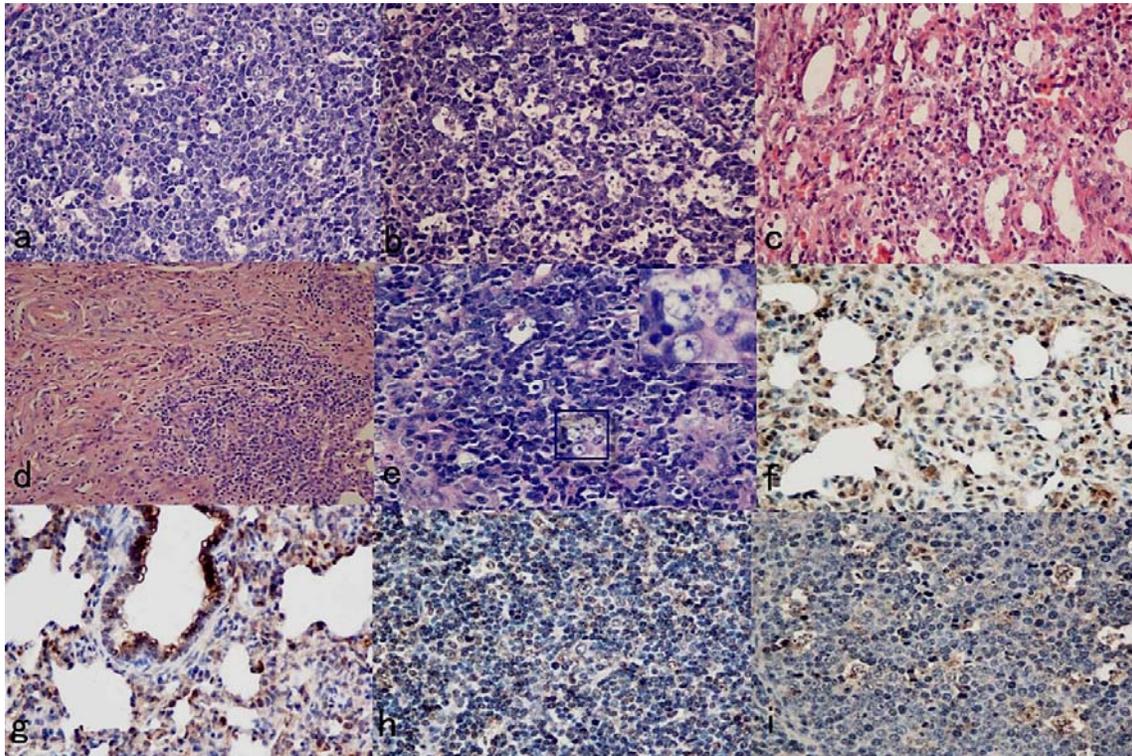


Fig. 1: Microscopic lesions and virus distribution in piglets upon different viral inoculations. Mild lymphocytic depletion in submaxillary lymph node (SLN) from a PCV2-infected pig (Fig. 1a), HE; 400 \times . And severe lymphocytic depletion in macrophages in SLN from a coinfecting pig (Fig. 1b), HE; 400 \times . And renal interstitia and hemorrhage in filtrated with inflammatory cells from a PRRSV-infected pig (Fig. 1c), HE; 400 \times . And granulomatous formation in the lung (Fig. 1d), HE; 200 \times ; and amphophilic intracytoplasmic inclusion bodies in the SLN (Fig. 1e, HE; 400 \times) from a coinfecting pig. The Lungs from a PCV2-infected pig (Fig. 1f) and a coinfecting pig (Fig. 1g), the macrophage cells of PCV2 antigens show cytoplasmic labeling in septa; the macrophage cells of PRRSV antigens show cytoplasmic labeling in lymph nodules of mesenteric lymph node (MLN) from a PRRSV-infected pig (Fig. 1h), and the macrophage cells of PCV2 antigens show cytoplasmic labeling in lymph nodules of the MLN from a coinfecting pig (Fig. 1i). IHC; 400 \times .

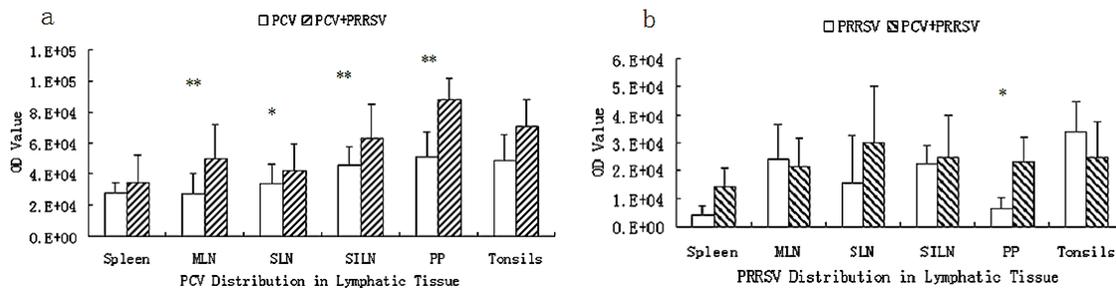


Fig. 2: Integrated optical density of antigens in immune organs; Note: Alone infection group compared with the co-infection group (** $P < 0.01$; * $P < 0.05$).

(Fig. 1d). Lesion score of the PCV2+PRRSV group was significantly higher than that of the PCV2 group ($P < 0.05$), whereas no significant difference was demonstrated between the PCV2 and PRRSV groups. The lung tissue lesions are summarized in Table 3.

The heart, liver, and kidney in the three treatment piglets showed degeneration, necrosis and infiltration. The heart and kidney lesions in the PCV2 group were milder than the PRRSV groups or the co-infection group, and there was no difference between in the PRRSV and co-infection group ($P < 0.05$). In the kidney of PRRSV group, severe lymphohistiocytic inflammation (Fig. 1c) often can be found. The liver lesions in the PRRSV group were milder than the PCV2 groups or the co-infection group, and there was no difference between in the PCV2 and co-

infection group ($P < 0.05$). The lesions are summarized in Table 4.

PCV2 distribution: The PCV2 antigens were mainly concentrated in the cytoplasm, and occasionally, in the nuclei of the macrophages and monocytes of lymph nodes and follicular karyorrhectic debris (Fig. 1i). There were abundant antigens in the follicular and inter follicular macrophages in ileal Peyer's patches, spleen and tonsil. The antigens were observed in pneumocyte, macrophages, and bronchial epithelial cells of lung (Fig. 1f; 1g) and renal tubular epithelial cells of kidney. No antigen was observed in heart and liver. The antigen IODs in mesenteric lymph nodes, superficial inguinal lymph nodes, and Peyer's patches were significantly higher in

the coinfection group than in the PCV2 group ($P < 0.01$). The IODs in submaxillary lymph nodes showed significant difference between in the coinfection group and the PCV2 group ($P < 0.05$). The IODs in the spleen and tonsil tissues of the two groups did not significantly differ ($P > 0.05$) (Fig. 2a).

PRRSV distribution: The antigens were mainly observed in the cytoplasm. In lymph nodes, PRRSV antigens were labeled in the macrophages of the cortex and medulla (Fig. 1h) and follicular karyorrhectic debris. Abundant PRRSV antigens were located in follicular and inter follicular macrophages of ileal Peyer's patches, and mononuclear cells and macrophages in lymphoid follicles of spleen, and in the follicular macrophages of tonsil tissue. In lung tissue, the antigens were observed in pneumocytes and macrophages. The PRRSV antigen was also mildly observed in renal tubular epithelial cells and mononuclear cell infiltrates. No antigen was observed in heart and liver. The PRRSV antigen IODs in Peyer's patches had significant difference between in the coinfection group and the PCV2 group ($P < 0.05$). The antigen IODs in the lymph nodes, as well as in spleen and tonsil tissues of the two groups did not significantly differ ($P > 0.05$) (Fig. 2b).

DISCUSSION

In this study, piglets were inoculated with PCV2 and subjected to an experimental model involving concurrent infection with PRRSV that can increase the pathological injury of single PCV2 infection. However, none of the piglet duplicated a PMWS model with typical symptoms, although a clinically silent PCV2 infection apparently associated with decreased weight gain developed in some of the piglets. In the PCV2 group, histopathological lesions typical of PMWS (Allan *et al.*, 2000; Cao *et al.*, 2005) were observed, including viral inclusion bodies formation in cytoplasm of macrophages and lymphocyte depletion in immune organs, and chronic granuloma formation in the lungs. PCV2 infection caused lymphocyte depletion of CD4⁺ and CD8⁺T lymphocytes (Opprissing *et al.*, 2004; Seo *et al.*, 2012; He *et al.*, 2013).

However, myocarditis, hepatitis, and interstitial nephritis were not observed. There were many differences between our experimentally induced lesions and those

found in PMWS field cases may be attributed to host susceptibility or environmental factors (Magar *et al.*, 2000). In generally, heat, cold, wet, poor ventilation and crowd caused by excessive breeding density have all been suggested as factors that can accelerate the development of disease. Coinfection with other pathogens may also aggravate PMWS. Previous study showed that the disease mainly occurs in pigs without main respiratory or enteric diseases, but reportedly affects PPV-infected pigs (Allan and Ellis, 2000) and Torque tenosus virus co-infection (Ellis *et al.*, 2008). Moreover, granuloma appeared in several tissues of neonatal piglets inoculated with PCV2 and PPV (Kennedy *et al.*, 2000).

In the present study, weight gain of the pigs in the PRRSV group showed more severely reduced than that of the PCV2 group. Histopathological lesions such as lymphocytic depletion and macrophage infiltration, interstitial pneumonia, myocarditis, hepatitis, and interstitial nephritis were observed in the PRRSV group. The lesions were different from those found by Harms (2002). They have observed less severe interstitial pneumonia and myocarditis in PRRSV-inoculated pigs. The discrepancies may be attributed to the different PRRSV strains and infection doses (the strains used in the present study were highly pathogenic PRRSV mutants). Li *et al.* (2007) have observed the same lesions as those caused by highly virulent strains.

The lesions were more severe in the PCV2+PRRSV group than in the PCV2 or PRRSV group. Chae *et al.* (2004) have reported that PCV2 infection in pigs can cause the characteristic lesions of PMWS. They posit that these lesions are induced by both PCV2 and PCV2+PRRSV infections, but are more serious when induced by the latter because the main lesions are replicated in a PCV2+PRRSV infection. The level of injury in the dual inoculation of PCV2 and PRRSV were significantly increased compared with infection of PCV2 or PRRSV only (Allan *et al.* 2000; Park *et al.*, 2013; Tsai *et al.*, 2012). In our study, the mixed infection can induce more severe myocarditis, hepatitis, and interstitial nephritis than alone infection, more showing that coinfection can aggravate pathological damages.

PRRSV antigens were detected in the lung, spleen, kidney, lymph nodes, and tonsils in the PRRSV and PCV2+PRRSV groups. By antigen strength analysis, the PRRSV antigens induced in the PRRSV group had no

Table 1: Pathological changes in pig tissues upon different viral inoculations^a

Organ or tissue	Lesions	PCV2	PCV2+PRRSV	PRRSV	Control
Lung	Edema	2/5	5/5	4/5	0/5
	Flea-bitten	4/5	5/5	4/5	0/5
Superficial inguinal lymph	Edema	2/5	5/5	5/5	0/5
	Flea-bitten	3/5	3/5	3/5	0/5
Mesenteric lymph node	Edema	2/5	3/5	2/5	0/5
	Flea-bitten	1/5	2/5	0/5	0/5
Submaxillary lymph node	Edema	2/5	2/5	3/5	0/5
	Flea-bitten	1/5	2/5	1/5	0/5
Spleen	Membrane thickening	1/5	1/5	0/5	0/5
	Swelling	0/5	0/5	1/5	0/5
	Marginal infarction	0/5	1/5	1/5	0/5
Heart	Pericardial effusion	2/5	4/5	2/5	0/5
	Endocardial bleeding	0/5	0/5	1/5	0/5
Liver	White mottling	1/5	0/5	1/5	0/5
Kidney	Color taupe	1/5	0/5	3/5	0/5
	Flea-bitten	1/5	3/5	1/5	0/5

^aPCV2, porcine circovirus type 2; PRRSV, porcine reproductive and respiratory syndrome virus.

Table 2: Microscopic lesions and in pig immune tissues upon different viral inoculations.

Organ or tissue	Lesions	PCV2	PRRSV	PCV2+PRRSV	Control
Submaxillary lymph node	Depletion (0 to 3)	4/5(1.4) ⁱ	4/5(1.0) ⁱ	5/5(2.2) ^{i,ii,iii}	0/5(0.0)
	Inflammation (0 to 3)	4/5(1.0) ⁱ	4/5(0.8) ⁱ	5/5(2.2) ^{i,ii,iii}	0/5(0.0)
	Inclusion body	3/5	0/5	4/5	0/5
Superficial inguinal lymph	Depletion (0 to 3)	5/5(1.8) ⁱ	4/5(1.2) ⁱ	5/5(2.2) ^{i,ii,iii}	0/5(0.0)
	Inflammation (0 to 3)	5/5(1.0) ⁱ	2/5(0.6)	5/5(2.0) ^{i,ii,iii}	0/5(0.0)
	Inclusion body	2/5	0/5	3/5	0/5
Mesenteric lymph node	Depletion (0 to 3)	5/5(1.8) ⁱ	4/5(1.2) ⁱ	5/5(2.2) ^{i,ii,iii}	0/5(0.0)
	Inflammation (0 to 3)	5/5(1.4) ⁱ	4/5(0.8) ⁱ	5/5(1.8) ^{i,ii,iii}	0/5(0.0)
	Inclusion body	2/5	0/5	3/5	0/5
Spleen	Depletion (0 to 3)	5/5(1.0) ⁱ	4/5(0.8) ⁱ	5/5(1.8) ⁱ	0/5(0.0)
	Inflammation (0 to 3)	4/5(0.8) ⁱ	2/5(0.4)	5/5(1.2) ^{i,ii,iii}	0/5(0.0)
	Inclusion body	0/5	0/5	1/5	0/5
Tonsils	Depletion (0 to 3)	5/5(1.2) ⁱ	5/5(1.0) ⁱ	5/5(2.0) ^{i,ii,iii}	0/5(0.0)
	Inflammation (0 to 3)	4/5(0.8)	2/5(0.4)	5/5(1.2) ^{i,ii,iii}	0/5(0.0)
	Inclusion body	2/5	0/5	3/5	0/5
Peyer's patches	Depletion (0 to 3)	5/5(1.2) ⁱ	4/5(1.0) ⁱ	5/5(1.8) ⁱ	0/5(0.0)
	Inflammation (0 to 3)	5/5(1.0) ⁱ	2/5(0.4)	5/5(1.2) ^{i,ii,iii}	0/5(0.0)
	Inclusion body	3/5	0/5	3/5	0/5

Score range for severe lymphoid depletion: 0 (normal) to 3; score range for severe histiocytic inflammation with replacement of follicles: 0 (normal) to 3. ⁱSignificantly different from the control group (P<0.05). ⁱⁱSignificantly different from the PCV2 group (P<0.05). ⁱⁱⁱSignificantly different from the PRRSV group (P<0.05). Data are presented as incidences.

Table 3: Microscopic lung lesions in pigs upon different viral inoculations

Group	Type2 pneumocyte hypertrophy (0 to 6)	Septal infiltration (0 to 6)	Peribronchiolar lymphoid hyperplasia (0 to 6)	Peribronchiolar exudate (0 to 6)	Alveolar exudate (0 to 6)	Granulomatous
PCV2	5/5(1.2)	5/5(2.8) ⁱ	5/5(2.6) ⁱ	0/5(0.0)	0/5(0.0)	1/5
PRRSV	5/5(2.4) ⁱ	5/5(3.4) ⁱ	0/5(0.0)	3/5(1.6) ⁱ	1/5(0.4) ^{i,ii}	0/5
PCV2+PRRSV	5/5(3.4) ^{i,ii}	5/5(5.0) ^{i,ii}	5/5(3.0) ^{i,iii}	4/5(2.2) ^{i,ii}	2/5(0.6) ^{i,ii}	1/5
Control	1/5(0.4)	1/5(0.4)	0/5(0.0)	0/5(0.0)	0/5(0.0)	0/5

Score range: from 0 (normal) to 6 (severe diffuse). ⁱSignificantly different from the control group (P<0.05). ⁱⁱSignificantly different from the PCV2 group (P<0.05). ⁱⁱⁱSignificantly different from the PRRSV group (P<0.05). Data are presented as incidences.

Table 4: Microscopic heart, liver, and kidney lesions in pigs upon different viral inoculations

Organ or tissue	Lesions	PCV2	PRRSV	PCV2+PRRSV	Control
Heart	Epicardial edema (0 to 3)	4/5(1.0)	2/5(1.0)	5/5(2.0) ⁱ	0/5(0.0)
	Degeneration and necrosis (0 to 3)	5/5(1.2) ⁱ	5/5(2.2) ⁱ	5/5(2.6) ^{i,ii}	0/5(0.0)
	Infiltration(0 to 3)	1/5(0.2)	5/5(2.4) ⁱⁱ	5/5(2.4) ^{i,ii}	0/5(0.0)
Liver	Degeneration and necrosis (0 to 3)	5/5(1.2) ⁱ	5/5(2.2) ⁱ	5/5(2.6) ^{i,iii}	0/5(0.0)
	Fibroplastic proliferation (0 to 3)	5/5(1.0) ⁱ	5/5(1.8) ⁱ	5/5(1.8) ⁱ	0/5(0.0)
	Infiltration (0 to 3)	5/5(1.0) ⁱ	5/5(1.6) ⁱ	5/5(2.6) ^{i,ii,iii}	0/5(0.0)
Kidney	Degeneration and necrosis (0 to 3)	5/5(1.4) ⁱ	5/5(1.6) ⁱ	5/5(1.8) ⁱⁱ	0/5(0.0)
	Infiltration (0 to 3)	2/5(0.4)	5/5(2.4) ⁱ	5/5(2.2) ^{i,ii}	0/5(0.0)
	Hemorrhage (0 to 3)	0/5(0.0)	5/5(1.8) ⁱ	2/5(1.0) ^{i,ii}	0/5(0.0)

Score range for severe lymphohistiocytic inflammation: 0 (normal) to 3. ⁱSignificantly different from the control group (P<0.05). ⁱⁱSignificantly different from the PCV2 group (P<0.05). ⁱⁱⁱSignificantly different from the PRRSV group (P<0.05). Data are presented as incidences.

significant difference from that in the PCV2+PRRSV group in immune tissues, indicating that PCV2 infection did not potentiate PRRSV replication. PCV2 antigens were detected in the lung and immune organs in the PCV2 and PCV2+PRRSV groups. By antigen strength analysis, the PCV2 antigens induced in the PCV2 group had a significant difference from the PCV2+PRRSV group in immune tissues. PRRSV and PCV2 sequential infection in the susceptible pigs can increase the replication of both viruses and induce more severe injury (Fan *et al.*, 2013). PCV2 vaccine can reduce PCV2 viremia and but PRRSV vaccine increase PCV2 viremia in the dually infected piglets (Park *et al.*, 2013). Our previous study found the serum PCV2 antigen contents increased in the co-infection group compared with the PCV2 group (Song, 2010; the data were not shown), moreover, PCV2 infection of porcine alveolar macrophages may induce systematic infection (Li *et al.*, 2013), which indicated that PRRSV infection potentiated PCV2 replication and distribution.

Author's contribution: CG and QH conceived and designed the experiments. LS GC and XH executed the

experiment and analyzed the tissue samples. LS and WZ analyzed the data. All authors involved in collecting data and discussing the contents of the manuscript and agreed to publication.

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