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Viral Challenge Pig Model with Porcine Circovirus Disease

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Abstract

Porcine circoviruse (PCV), causes PCV disease (PCVD), are small non-enveloped DNA viruses containing a single-stranded circular genome. Previously, PCV was even considered a nonpathogenic agent for pigs. However, a novel PCV, designated PCV2, has been associated with various disease syndromes in pigs in the last 5 years. PCV2 can induce primarily postweaning multisystemic wasting syndrome with a variety of clinical signs as debility, dyspnea, palpable lymphadenopathy, diarrhea, and pallor or icterus. PCV2 is now regarded as an important emerging pathogen. Although vertical transmission has been documented, the epidemiology of PCV2 infections is poorly understood. PCV2 have been demonstrated linked to other pathogens to cause abortion and reproductive failure in pigs that seriously affects the economic loss of pig farmers. Therefore, the research and development (R&D) of vaccines against PCVD is very important. Development of a PCV challenge pig model that complies with the development of PCVD vaccines will shorten the R&D time of vaccines and accelerate the PCVD vaccines into the market. It can be seen from our results of the development of PCV challenge pig model. Abnormal clinical symptoms were found in the viral challenge pigs with lower appetite, lower excretion, and slighter to severer breathe. Relative weight gain rate is significant increase in the viral challenge pigs than that in the normal control pigs. All pigs were survival until the end of the experiment with a mortality rate of 0% (0/12). High expressions of PRRSV RNA in porcine serum and nasal specimen were detected 4 weeks post viral-challenge. Viraemia lasts for 4 weeks. After sacrifice of pigs, gross examination was performed and the lung, spleen, and lymph nodes (LNs) etc were collected and the lesions on lung and LNs were evaluated. It can be seen that pneumonia in the viral challenge pigs is significantly severer than that in the normal control group. In the viral challenge group, all pigs presented as the diffuse interstitial pneumonia, Pericardial effusion in the pericardial cavity, the enlarged spleen, the lymph nodes were swollen and tan, and some of them showed redness and congestion, especially in hilar lymph nodes (HLN), mesenteric lymph nodes (MLN), and superficial inguinal lymph nodes (SILN). A slight to severe loss of lymph follicles can be observed in HLN, MLN, and SILN. The positive percentage (%) of PCV2 antigen and the sum of PCV2 antigen index in the viral challenge pigs is higher than that in the normal control pigs. According to the results of this study, the PCV challenge pig model has been successfully established, which can be provided to related units for R&D of PCVD vaccines. The model will be applied in the future and promoted the development of vaccines in pigs.

Keywords: Pig; Porcine Circovirus Disease; Vaccine; Viral Challenge Model

Introduction

Porcine circovirus disease (PCVD) is a viral disease of pigs that has recently emerged as a major problem in the world. This disease causes illness in piglets with the progressive loss of body weight, the enlarged lymph nodes, abnormal in breathing, diarrhea, pale skin, and jaundice. PCVD is very damaging to the pig-producing industry and has been reported worldwide [1-4].

Porcine circovirus (PCV), a non-enveloped, single stranded DNA virus, is a member of the Circoviridae family in the genus Circovirus. Among of porcine circovirus (PCV), PCV1 was first recognized as a non-disease-causing virus. Unfortunately, most swine are infected with PCV2. PCV2 can cause postweaning multisystemic wasting syndrome (PMWS) in pigs. PCV2 has a near universal distribution in most pig herds worldwide. Moreover, only PCV2 itself can't cause PMWS. PMWS is a multifactorial disease that is necessary in combination with other factors. PCV2 co-infects with porcine parvovirus or porcine reproductive and respiratory syndrome virus can lead to increased replication of PCV2 and produce the more severe disease in pigs [5-8].

According to the information as the outbreaks of PCV in vaccinated herds, epidemiological monitoring data, and molecular evolutionary analysis, PCV is constantly evolving to cause new outbreaks. This disease is becoming more difficult prevention with ability to evade vaccine-induced immunity. Therefore, an effective

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vaccine to target constantly evolved PCV is a top priority for controlling PCVD outbreaks and preventing economic losses [9,10]. Currently, R&D of PCVD vaccines was performed continuously such as modified live virus (MLV) vaccines, inactivated PCV vaccines, and subunit PCV vaccines etc. The commercially available PCV2 vaccines are major inactivated or subunit vaccines. Currently, a live-attenuated PCV2 vaccine based on a chimeric PCV1-2 were being also found. Since PCVD is a major problem in pig industry in the world and is currently found in most areas of the world where pigs are raised, vaccination is a method to prevent PCV infection [1,3,8]. In order to promote the development of PCVD vaccines, the establishment of a viral challenge pig model with PCVD suitable for R&D of vaccines is very important and need.

Materials and Methods

Experimental reagents

Experimental reagents included as phosphate buffered saline (PBS; No. P3813, Sigma-Aldrich®), Zoletil 50 (Vibac Laboratories, Carros, France), azaperone (Stresnil®; Elanco Animal Health, USA), and Porcine Circovirus 2 (PCV2) ELISA Kit (BioChek®, Cat No.: SK105).

Cell lines and culture

A porcine kidney cell line used was PK-15 (ATCC® CCL-33™). PK-15 cells were grown in Eagle's Minimum Essential Medium (MEM; Corning®) supplemented with 10% fetal bovine serum (FBS; HyClone®), 2mM L-glutamine (Invitrogen®), 100U/mL penicillin and 100mg/mL streptomycin (Invitrogen®) in a humidified 5% CO_2 incubator at 37°C.

Animal care

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Animal Technology Laboratories, Agricultural Technology Research Institute (ATRI), Miaoli, Taiwan. Twenty-four eight-week-old specific pathogen free (SPF) pigs were ordered from ATRI, Miaoli, Taiwan (the ATRI approval No.: 105111C2) and experimented in the GMO veterinary building, Animal Drugs Inspection Branch (ADIB), Animal Health Research Institute, Council of Agriculture, Executive Yuan, Miaoli, Taiwan (the ADIB approval No.: 106-T09). The 12 pigs were housed 6 pigs per animal room under a 12-h light/dark cycle at 22-24°C and 70-75% humidity. Normal laboratory diet (FWUSOW industry, Taichung, Taiwan) and fresh water were supplied to pigs continuously ad libitum.

Experimental animals and grouping

Twelve nine-week-old SPF pigs (negative for PCVD Ab and Ag) were obtained from ATRI, Taiwan. All SPF pigs were randomly divided into two groups (6 pigs/group), normal control group and viral challenge group.

Viral challenge test

The Taiwan local strong virulence of PCV (strain CYC08, viral titer is 105 TCID50/mL) was challenged to the viral challenge group by 2mL nasal cavity administration. At the each designed experimental points, the detection of clinical symptoms, survival, and detection of body weight (BW) and body temperature (BT) in each group was performed to compare the difference of these above indexes between two groups.

Monitor of clinical symptoms and survival, and detection of body weight and body temperature in pigs

In this study, the monitoring of clinical behavior and survival, and the detection of BW and BT in each group were performed once per day. Six indexes of clinical behavior as spirit, appetite, excretion, breathe, gait, and body appearance are used for the score (Table 1). Incidence (%) of the poor growth and body weight loss in pigs was calculated as the percentage of the individual body weight was below 75% average body weight of all pigs.

Gross and immuno-histiopathologic examination

At the end of the experiment, all pigs were sacrificed and dissected. Then, the collection and gross appearance examination of pig's lung, kidney, hilar lymph nodes (HLN), mesenteric lymph nodes (MLN), and superficial inguinal lymph nodes (SILN) were performed by a senior pathologic veterinarian. The pathologic score followed as the lesion score in the interstitial pneumonia: 0 = no microscopic lesions; 1 = mild interstitial pneumonia; 2 = moderate multifocal interstitial pneumonia; 3 = moderate diffuse interstitial pneumonia; 4 = severe interstitial pneumonia; the lesion score in the interstitial nephritis: 0 = no microscopic lesions; 1 = mild interstitial nephritis; 2 = moderate multifocal interstitial nephritis; 3 = moderate diffuse interstitial nephritis; 4 = severe interstitial nephritis; the score in the lymphoid depletion: 0 = normal; 1 = mild lymphoid depletion; 2 = moderate lymphoid depletion; 3 = severe lymphoid depletion; 4 = complete lymphoid depletion; the score in the immunohistochemistry (IHC) examination: 0 = absent; 1 = focal; 2 = sporadic; 3 = multifocal; 4 = abundant [11].

Collection of peripheral blood and nasal specimen

Collection of peripheral blood and nasal specimen (by swab) was performed before viral challenge and per week after PCV (strain CYC08) challenge. These sera and nasal specimen were applied to the quantification of PCV DNA. The titer of PCV antibody in serum was also detected.

Quantification of PCV DNA

PCV DNA was extracted from specimen of sera and nasal and, quantitative the viral genomic DNA copy numbers by real-time PCR. The viral DNA was extracted by LabTurbo Viral DNA Mini kit (Taigen). The following primers and probe were used: forward primer 5'-GTA ACG GGA GTG GTA GGA GAA-3'; reverse primer, 5'-CCA CAG CCC TAA CCT ATG AC-3' and probe 5'- Fam-ATG TAA ACT ACT CCT CCC GCC ATA CAA TC-Tamra -3'. The volume of real-time PCR reaction was 25μL included 12.5μL of Taqman Fast Universal PCR Master Mix (Thermo Fisher Scientific), 0.5μL of each primer (5μM), 0.625μL of the probe (10μM), 5.875μL of water, and 5μL of viral DNA. The real-time PCR mixture solution reacted in 7500 Real-time PCR system (Applied Biosystems) and analyzed viral copy numbers.

Detection of antibody titer serum

The PCV2 antibody test was used to measure the amount of antibodies to all types of PCV2 present in the serum of pigs according to the manufacture's protocol.

Statistical analysis

Statistical analysis was performed using one-way analysis of

Table 1: Six indexes of clinical symptoms as spirit, appetite, excretion, breathe, gait, and body appearance for the score.

Score	Spirit	Appetite	Excretion	Breathe	Gait	Body appearance
1	Normal					
2	Inactive / weak	Suboptimal	Atherosclerosis	Slight	Slight limp	Petechial bleeding / Scabs
3	Lying down	Unable to eat	Watery diarrhea	Severe	Severe limp	Anemia / Jaundice

Table 2: The average weight gain and average daily weight gain of the viral challenge group and the normal control group. Data were presented as mean ± SD; $^{**}p < 0.01$.

Group	No.	Before viral challenge	Sacrifice	Average weight gain (kg)	Average daily weight gain (kg)
Viral challenge group	6	15.85 ± 2.08	32.39 ± 3.85	16.54 ± 2.56	0.59 ± 0.09
Normal control group	6	21.07 ± 3.05	35.89 ± 5.07	14.83 ± 3.14	0.53 ± 0.11

variance (one-way ANOVA), Student's *t*-test, Fisher's exact test, and Kruskal-Wallis one-way ANOVA. Survival in the group comparisons was performed using Fisher's exact test. Clinical examination and IHC examination in the group comparisons was performed using Kruskal-Wallis test and/or Dunn's multiple comparison method [12]. Others in the group comparisons was performed using ANOVA. Differences between groups were considered statistically significant at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

Results

Average weight gain and average daily weight gain in pigs

From the beginning to the end of the experiment, the average daily weight gain (ADWG) of the normal control group was 0.53 ± 0.11 kg and ADWG of the viral challenge group was 0.59 ± 0.09 kg; the average weight gain (AWG) of the normal control group was 14.83 ± 3.14 kg and AWG of the viral challenge group was 16.54 ± 2.56 kg. There were not significantly different between two groups on the AWG and ADWG (Table 2).

Incidence (%) of the poor growth and body weight loss in pigs

At the end of the experiment, the individual body weight (BT) was below 75% average body weight of all pigs was calculated. The average BT (ABT) is 34.14 ± 4.67 kg and 75% ABT is 25.60 ± 3.50 kg. The individual BT of all pigs (n = 12) was higher than 75% ABT. Therefore, the incidence (%) of the poor growth and BT loss in pigs is 0% (0/12).

The Mortality rate post viral challenge

After viral challenge, all pig survived in two groups with a mortality rate of 0% (0/12).

Clinical symptoms of pigs post viral challenge

The clinical symptoms of the pigs in each group can be found that the pigs in the viral challenge group began to appear soft stool on DPC 3 and watery diarrhea occurred at the later stage of the viral challenge, and the clinical score of excretion was at 2 score (5/6). On DPC 16, the pigs in the viral challenge group showed a decrease in appetite, which lasted for 13 consecutive days (DPC 16-DPC 28), and the clinical score of appetite was at 2 score (5/6). Partly, one pig were found cough symptom after the viral challenge and the clinical score of breathe was at 2 score (1/6). The spirit, gait, and body appearance of the pigs in two groups were normal, and the clinical score of spirit, gait, and body appearance was respectively at 1 score (12/12).

Macroscopic lesions of pig post viral challenge

After viral challenge, all pigs survived (n = 12) and their body appearance were normal. After sacrifice and dissection, the gross examination was performed and the lung, spleen, and lymph nodes (LNs) etc were collected and the lesions on lung and LNs were evaluated. It can be seen that pneumonia in the viral challenge pigs is significantly severer than that in the normal control group. In the viral challenge group, all pigs presented as the diffuse interstitial pneumonia, Pericardial effusion in the pericardial cavity, the enlarged spleen, the lymph nodes were swollen and tan, and some of them showed redness and congestion, especially in hilar lymph nodes (HLN), mesenteric lymph nodes (MLN), and superficial inguinal lymph nodes (SILN). Some pigs have a paler liver, and the rest of the organs including the tonsils, stomach, kidney, ileocecal valve, bladder, and larynx were normal (data not shown).

Microscopic lesions of pig post viral challenge

A slight to severe loss of lymph follicles can be observed in HLN, MLN, and SILN of the viral challenge pigs. The severity of lymph follicle loss in HLN, MLN, and SILN in the viral challenge pigs was higher than that in the normal control group ($p < 0.05$). On the other hand, some pigs presented the mild to moderate multiple interstitial pneumonia and interstitial nephritis according to the histio-pathologic examination in the viral challenge group (data not shown).

Immunohistochemistry staining of pig's lymph nodes post viral challenge

After sacrifice and dissection, the immunohistochemistry (IHC) examination was performed on LNs of all pigs, HLN, MLN, and SILN (n = 12). In the viral challenge group, all pigs were scored above 4 score in LNs, HLN, MLN, and SILN. The positive percentage (%) of PCV2 antigen in the viral challenge pigs was higher than that in the normal control pigs ($p < 0.001$). The sum of PCV2 antigen index in the viral challenge group and the normal control group was 5.00 ± 1.09 and 0.00 ± 0.00. The sum of PCV2 antigen index in the viral challenge was also higher than that in the normal control pigs ($p < 0.001$) (Table 3).

Quantification of PCV2 DNA in the nasal specimen in pigs

PCV2 DNA load in the nasal specimen (collection by swab) was

Table 3: The sum of PCV2 antigen index in two groups. Data were presented as mean ± SD; $^{***}p < 0.001$.

	Viral challenge group	Normal control group
Sum of PCV2 antigen index	5.00 ± 1.09	0.00 ± 0.00***

Table 4: PCV2 DNA load in the nasal specimen was detected at DPC 0, 7, 14, 21, and 28 in the experiment. Data were presented as mean ± SD; $^{***}p < 0.001$.

Mean log10 of PCV2 (copies/mL)	DPC 0	DPC 7	DPC 14	DPC 21	DPC 28
Viral challenge group	0.00 ± 0.00	6.98 ± 0.78***	9.19 ± 0.46***	8.67 ± 0.51***	9.20 ± 0.31***
Normal control group	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 5: PCV2 DNA load in the serum was detected at DPC 0, 7, 14, 21, and 28 in the experiment. Data were presented as mean ± SD; $^{***}p < 0.001$.

Mean log10 of PCV2 (copies/mL)	DPC 0	DPC 7	DPC 14	DPC 21	DPC 28
Viral challenge group	0.00 ± 0.00	6.98 ± 0.78***	9.19 ± 0.46***	8.67 ± 0.51***	9.20 ± 0.31***
Normal control group	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 6: Titers of the antibody in serum was detected on DPC 0, 7, 14, 21, and 28 in the experiment. Data were presented as mean ± SD; $^{***}p < 0.001$.

Mean of PCV2 antibody titer (log2)	DPC 0	DPC 7	DPC 14	DPC 21	DPC 28
Viral challenge group	0.00 ± 0.00	1.25 ± 0.10	4.72 ± 2.59***	7.95 ± 2.44***	9.28 ± 1.83***
Normal control group	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

detected by quantitative PCR at DPC 0 and DPC 28 in the experiment. The results showed that 100% expression in the nasal specimen of all pigs in the viral challenge group was detected. Additionally, PCV2 DNA load was gradually increased with the viral challenge time. PCV2 DNA content of the nasal specimen of the viral challenge group was significantly higher than that of the normal control group ($p < 0.001$) (Table 4).

Quantification of PCV DNA in serum

Collection of pig blood before (DPC 0) and after viral challenge (DPC 7, 14, 21, and 28) was performed and detected by quantitative PCR for the evaluation of viral load in the sera. Before the viral challenge, no PCV2 DNA was detected in the sera of the pigs.

Additionally, PCV2 DNA load was gradually increased with the viral challenge time. PCV2 DNA content in the sera of the viral challenge group was significantly higher than that of the normal control group ($p < 0.001$) (Table 5).

The Titer of antibody in serum

Collection of blood before and after the viral challenge (DPC 0, 7, 14, 21, and 28) was performed and determined the antibody titers in serum. The results showed that antibody titers in serum in the viral challenge group gradually increased on DPC 7 until the end of the experiment (Table 6).

Discussion

PCVD is responsible for substantial animal and economic losses to the pig industry. PCV can cause viraemia, pneumonia with abnormal respiratory symptoms, and reduced body weight gain. At present, two main types of PCV, PCV1 and PCV2, have been identified. PCV is a highly infectious virus in the pig farms worldwide as pigs are often raised in the areas of high density, the spread of PCVD is difficult to control. Vaccination is a method to prevent PCVD and spread via reducing clinical symptoms, viraemia, and tissue lesions for improving health and performance in pigs [13-15].

Since pigs infected with PCV2 with clinical symptoms as gradual wasting, dyspnea, and diarrhea, we have successfully established PCV challenge pig model according to clinical symptoms in the viral challenge pigs in this study. Moreover, PCVD caused the histologic lesions as lymphoid depletion and/or lymphohistiocytic to granulomatous inflammation in affected organs. According to the histologic lesions, we also found these microscopic lesions in the three LNs (HLN, MLN, and SILN) in the viral challenge pigs in this study. On the other hand, PCVD-affected pigs were usually died and these PCVD pigs' clinical survivors are severely stunted [16-18]. Based on these clinical symptoms and macroscopic and microscopic lesions, our established PCV challenge pig model with these clinical symptoms and macroscopic and microscopic lesions were almost suitable except the mortality rate of PCV-infected pigs. In our study, all viral challenge pigs were survival with a mortality rate of 0%.

The objective of this study was to establish a viral challenge pig model with PCVD suitable for the need of R&D of PCVD vaccines. According to our all results, a PCV challenge pig model was successfully established. In the future, we hope this viral challenge animal model will be applied in the R&D of swine vaccines.

Conclusion

PCV is constantly evolving to cause new outbreaks. This disease is becoming more difficult prevention with ability to evade vaccine-induced immunity. Therefore, R&D of an effective PCVD vaccine is very important for controlling PCVD outbreaks and preventing economic losses. In order to promote the development of PCVD vaccines, the establishment of a viral challenge pig model with PCVD suitable for R&D of vaccines is very important and need. According to our results, we have successfully established a viral challenge pig model with PCVD. This model will be suitable for the R&D need of PCVD vaccines.

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