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Research Article

Establishment of a Viral Challenge Pig Model with Porcine Reproductive and Respiratory Syndrome

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2. Keywords

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1. Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs mainly causes miscarriage and still birth of sows and respiratory tract lesions of nursery pigs, which seriously affects the economic loss of pig farmers. Therefore, the research and development (R&D) of vaccines against PRRSV infection is very important. Development of a PRRSV challenge pig model that complies with the development of PRRS vaccines will shorten the R&D time of vaccines and accelerate the PRRS vaccines into the market. It can be seen from our results of the development of PRRSV challenge pig model, which fever in pigs was seen after the viral challenge and continued until the end of the experiment. Four pig died after the viral challenge with a mortality rate of 33.33% (4/12). Abnormal clinical symptoms were found in the viral challenge pigs. After sacrifice of pigs, the lung tissue and hilar lymph nodes (HLN) were collected and the lesions on lung were evaluated. It can be seen that pneumonia in the viral challenge pigs is significantly severer than that in the normal control group. Expressions of PRRSV RNA in porcine serum, lung tissue, and HLN were detected post viral challenge. Viraemia lasts for 2 weeks and PRRSV RNA in lung tis- sue and HLN were also detected. Additionally, the indirect fluorescent antibody in serum could be detected post viral challenge. However, the neutralizing antibody in serum was not detected post viral challenge. According to the results of this study, the PRRSV challenge pig model has been successfully established, which can be provided to related units for R&D of PRRS vaccines. The model will be applied in the future and promoted the development of vaccines in pigs.

3. Introduction

Porcine reproductive and respiratory syndrome virus (PRRS) is caused by PRRS virus (PRRSV) that is a positive-stranded enveloped RNA virus which belongs to the genus *Arterivirus*, family *Arteriviridae* and order *Nidovirales*. There are two well recognized PRRSV genotypes: type 1 (European-like; prototype Lelystad) and type 2 (North American-like; prototype VR-2332) [1, 2].

PRRS was first recognized in the United States in 1987, then the causative virus was identified in the Netherlands in 1991. Since the symptoms of PRRS can cause cyanosis of the ear in pigs, PRRS is also named blue ear pig disease. This disease is a widespread disease that affected lots of domestic pigs. The symptoms of this disease include the reproductive failure, pneumonia, and increased susceptibility to secondary bacterial infection [2, 3].

It seems that the transmission of PRRSV in practical terms is most often due to the movement of infected pigs. Pig born to infected dams may not show disease signs but can still be virus shedders. Additionally, PRRSV is also found in faeces, urine, and semen.

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There is evidence that the disease can also spread through artificial insemination when semen is contaminated with the virus. PRRSV can be also spread by vehicles, supplies, windborne, or insects have been found as a potential source of spread [4-6].

According to the information as the outbreaks of PRRSV in vaccinated herds, epidemiological monitoring data, and molecular evolutionary analysis, PRRSV is constantly evolving to cause new outbreaks and is becoming more virulent with ability to evade vaccine-induced immunity [7-10]. Therefore, an effective vaccine to target constantly evolved PRRSV is a top priority for controlling PRRS outbreaks and preventing economic losses. Currently, R&D of PRRS vaccines was performed continuously such as modified live virus (MLV) vaccines, inactivated PRRSV vaccines, DNA PRRSV vaccines, and subunit and virus-vectored PRRSV vaccines etc. Although Australia, New Zealand, several European countries, parts of Africa and India are still currently free of the disease, PRRS is currently found in most areas of the world where pigs are raised. In order to promote the development of PRRS vaccines, the establishment of a viral challenge pig model with PRRS suitable for R&D of vaccines is very important and need.

4. Materials and Methods

4.1. 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 fluorescein isothiocyanate (FITC) conjugated goat anti-pig IgG antiserum (Bio-Rad®, Cat No.: AAI41F).

4.2. Cell Lines and Culture

A monkey kidney cell line used was MARC-145 (ATCC® CRL-12231™). MARC-145 cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO®) supplemented with 10% fetal bovine serum (FBS; HyClone®), 2 mM L-glutamine (Invitrogen®), 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen®) in a humidified 5% CO_2 incubator at 37°C.

4.3. 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.: 104058)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: 104-T20). The 24 pigs were housed 12pigs 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.

4.4. Experimental Animals and Grouping

Twenty-four eight-week-old SPF pigs (negative for PRRS Ab and Ag) were obtained from ATRI, Taiwan. All SPF pigs were randomly divided into two groups (12 pigs/group), normal control group and viral challenge group.

4.5. Viral Challenge Test

The Taiwan local strong virulence of PRRSV (strain MD-005, viral titer is 10^5 $TCID_{50}$/mL) was challenged to the viral challenge group by respective 1 mL nasal cavity- and intra-muscle-administration. At the each designed experimental points, the detection of clinical behavior, 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.

4.6. Monitor of Clinical Behavior and Survival, and Detection of Body Weight and Body Temperature in Pigs

In this study, the monitor 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).

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

Score	Spirit	Appetite	Excretion	Breathe	Gait	Body appearance
1	Normal	Normal	Normal	Normal	Normal	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

4.7. Gross Pathologic Examination

At the end of the experiment, all pigs were sacrificed and dissected. Then, the collection and gross appearance examination of pig's lung and hilar lymph nodes (HLN) were performed by a senior pathologic veterinarian. Percentage (%) of pneumonia lesions was compared between the normal control group and the viral challenge group. Percentage (%) of pneumonia lesions is calculated as 100 ×[(0.10 × left anterior lobe) + (0.10 × left cardiac lobe) + (0.25 × left diaphragmatic lobe) + (0.10 × right anterior lobe) + (0.10 × right cardiac lobe) + (0.25 × diaphragmatic lobe) + (0.10 × intermediate lobe)]. Additionally, the HLN were collected for the further the evaluation of viral loading.

4.8. Collection of Peripheral Blood

Collection of peripheral blood was performed before viral challenge and 3 days-, 5 days-, 7 day-s, and 10 days-post challenge with PRRSV (strain MD-005). These sera were applied to the detection of viraemia and the titers of naturalizing antibody (NA) and indirect fluorescent antibody (IFA).

4.9. Quantification of PRRSV RNA

PRRSV RNA was extracted from sera, lung tissues and HLN for the quantitative detection of the viral genomic DNA copy numbers. Viral RNA extraction was done using Lab Turbo Viral RNA Mini kit (Taigen). Copy number of viral RNA was then quantified using previously published TaqMan® probe-based real-time RT-PCR with minor modification. Primers and probe were as follow: reverse primer, 5'-ACA CGG TCG CCC TAA TTG-3'; forward primer 5'-ATG ATG RGC TGG CAT TCT-3'; probe 5'-Vic-TGT GGT GAA TGG CAC TGA-MGB-3'. The qRT-PCR method was performed in a 25 μL volume containing 6.25 μL of 4 × TaqMan® Fast Virus One-Step Master Mix (Thermo Fisher Scientific), 0.5 μL of each primer (5 μM), 0.625 μL of probe (10 μM), 12.125 μL of DEPC treated water, and 5 μL of viral RNA. The qRT-PCR was carried out using 7500 Fast Real-time PCR System (Applied Bio systems).

4.10. Detection of the Titers of Naturalizing Antibody

For the neutralization test, serum and virus are reacted together in equal volumes and inoculated into a susceptible animal host or cell culture. Firstly, the sera were performed inactivation at 56°C for 30 minutes. Then, the serial twice dilution of the inactivated sera was performed in a 96-well culture dish. Later, an equal amount of 200 $TCID_{50}$/ well of PRRSV solution was added and then inoculated at 37°C for 1 hour. Then, the mixed solution of serum and virus was taken out and added into MARC-145 cell-rich culture plate. After 7 days of culture, the cytopathy of MARC-145 cells was observed and determined the antibody titer. If the cytopathy cannot be observed under the neutralization test, the IFA staining was following used.

4.11. Detection of the Titers of Indirect Fluorescent Antibody

For the IFA staining, MARC-145 cells were cultured in 96-well culture plates and inoculated with 100 $TCID_{50}$/well of PRRSV solution. The tested serum was continuously diluted with PBS and then added to the cell plate. After 1 hour inoculation of serum and PRRSV, the total of 3 times wash with PBS for cell culture disc was performed. Then, FITC conjugated goat anti-pig IgG antiserum was added and inoculated for 1 hour. Later, the total of 3 times wash with PBS for cell culture disc was performed and observed the fluorescence to interpret the IFA potency.

4.12. Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (one-way ANOVA), Student's 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 in the group comparisons was performed using Kruskal-Wallis test. Others in the group comparisons was performed using ANOVA. Differences between groups were considered statistically significant at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

5. Results

5.1. Changes of Body Temperature in Pigs

The BT of pigs were measured before the viral challenge. BT of all pigs is between 39.01-39.18 °C. After viral challenge with PRRSV, BT of two groups is measured once a day until the end of the experiment. From the next day of viral challenge (days post-challenge 1; DPC1), the average BT of the pigs in the viral challenge group continuously rise with the average BT of pigs between 40.09-41.10 °C. In the normal control group, the average BT of the pigs maintained between 38.56-39.85 °C. On the DPC 3 and DPC 8-14, the average BT value of the pigs in the viral challenge group was significantly lower than that in the normal control group ($p < 0.05$-$p < 0.001$) (Table 2)

Table 2: The trend of average body temperature in the each group after viral challenge.

Average of body temperature (°C)	DPC														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Viral challenge group	39.01	40.33	40.69	40.80**	40.44	40.6	40.62	40.54	40.93***	41.10***	40.83**	40.79***	40.15*	40.28*	40.09***
Normal control group	39.18	39.25	39.4	38.56	39.22	39.25	39.85	38.78	39.2	39.66	39.33	39.78	38.98	39.65	39.83

5.2. 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 0.14 ±0.02 kg and ADWG of the viral challenge group was 0.04± 0.02 kg; the average weight gain (AWG) of the normal control group was 3.9 ± 0.5 kg and AWG of the viral challenge group was 1.1 ± 0.5 kg. AWG and ADWG of the normal control group was significantly higher than that of the viral challenge group (p <0.01-p < 0.001) (Table 3).

Table 3: The average weight gainand average daily weight gain of the viralchallenge group and the normal control group.Data were presented as mean ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$.

Group	No.	Before viral challenge	Sacrifice	Average weight gain (kg)	Average daily weight gain (kg)
Viral challenge group	8	10.3 ± 0.8	11.4 ± 1.0	1.1 ± 0.5	0.04 ± 0.02
Normal control group	12	14.1 ± 0.6	18.0 ± 0.6	3.9 ± 0.5***	0.14 ± 0.02**

5.3. The Mortality Rate Post Viral Challenge

After viral challenge, 4 pig deaths occurred in the viral challenge group with a mortality rate of 33.33% (4/12). Moreover, all pigs were survived in the normal control group with a mortality rate of 0% (0/12).Survival rate of pig after the viral challenge in the viral challenge group was significantly lower than that in the normal control group (p <0.001).

5.4. 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 inactive and weak, and the clinical score of the spirit was between 1-2.1 score on the DPC 10. On DPC 3, the pigs in the viral challenge group showed a decrease in appetite, which lasted for 14 consecutive days (DPC 1-DPC 14), and the clinical score of appetite was at 2 score. On DPC 4, pigs in the viral challenge group began to see diarrhea, the clinical score of excretion was between 1-1.25 score, while the normal control group were all normal and the clinical score was 1 score. On DPC 4, the pigs in the viral challenge group began to cough and the clinical score for breathe ranged from 1-1.38 score, while the pigs in the normal control group were all normal with a score of 1. On DPC 9, the pigs in the viral challenge group can see that the lameness, the clinical score of gait ranged from 1-1.3 score, while all the pigs in the normal control group were normal with a score of 1.The body appearance of the pigs in two groups was almost normal under viral challenge. The clinical score of the body appearance ranges from 1-1.5 score (Figure 1).

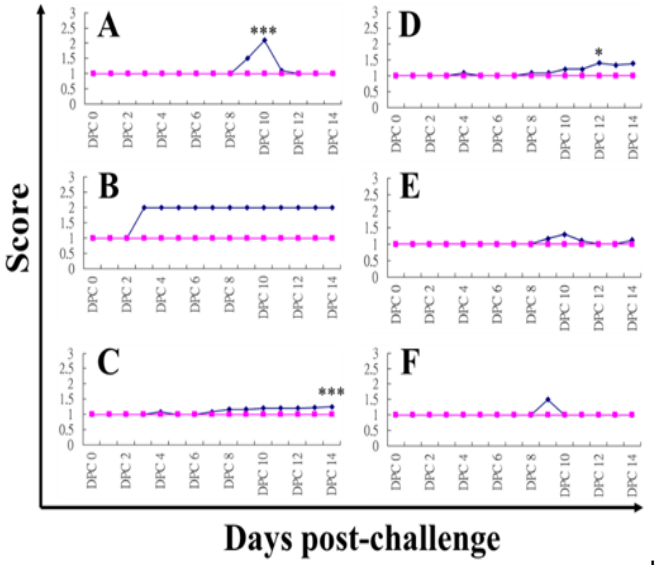

Figure 1: The clinical symptom score of the pigs post viral challenge. (A) spirit; (B) appetite; (C) excretion; (D) breathing; (E) gait; (F) body appearance. Diamond symbol indicated the viralchallenge group; Square symbol indicated the normal control group. $^{*}p < 0.05$; $^{***}p < 0.001$.

5.5. Macroscopic Lesions of Pig's Lung Post Viral Challenge

After viral challenge, the pigs continually died (n = 4) in the viral challenge group. According to the body appearance of these died pigs, only one pig presented the blue ear. After dissection, all pigs in the viral challenge group showed ascites, *Mycoplasmapneumoniae* infection-like lung lesions, hilar lymphatic hemorrhage and swelling, severe gastric ulcers and bleeding. In the normal control group, all pigs were survival (n = 12) and the all pigs showed the normal body appearance. After viral challenge, the results showed that an average percentage (%) of pneumonia lesions was 55.22 ± 23.43% in the viral challenged group (n = 12), which was significantly higher than that of the normal control group (n = 12) of 0.00 ± 0.00% (p < 0.001) (Table 4).

Table 4: The average percentage (%) of pneumonia lesions in two groups. Data were presented as mean ± SEM. $^{***}p < 0.001$.

	Viral challenge group	Normal control group
Average percentage (%) of pneumonia lesions	55.22 ± 23.43	0.00 ± 0.00***

5.6. Quantification of PRRSV RNA in Lung and Hilar Lymph Nodes in Pigs

PRRS RNA load in lung tissues and HLN was detected by quantitative PCR at DPC 14 in the experiment. The results showed that 100% expression in the lung tissues and HLN of all pigs in the viral challenge group was detected. PRRSV RNA content of the lung tissues and HLN of the viral challenge group was significantly higher than that of the normal control group ($p < 0.001$) (Table 5).

Table 5: PRRS RNA load (PRRS copy number/100mg tissue) in lung tissues and hilar lymph nodeswas detected at DPC 14 in the experiment. Data were presented as mean ± SD. $^{***}p < 0.001$.

	Lung	Hilar lymph nodes
Viral challenge group	$7.31 \pm 1.12^{***}$	$6.77 \pm 0.84^{***}$
Normal control group	0.00 ± 0.00	0.00 ± 0.00

5.7. Quantification of PRRSV RNA in Serum

Collection of pig blood before (DPC 0) and after viral challenge (DPC 3, 5, 7, 10 and 14) was performed and detected by quantitative RT-PCR for the evaluation of viral load in the sera. Before the viral challenge, no PRRSV RNA was detected in the sera of the pigs. On DPC 3, PRRSV RNA in the serum of viral challenge group began to be measured and continued to DPC 14. The viraemia continued for 2 week sin the viral challenge pigs (Table 6).

Table 6: PRRS RNA load (PRRS copy number/mL serum) in serumwas detected on DPC 0, 3, 5, 7, 10, and 14in the experiment. Data were presented as mean ± SD. $^{***}p < 0.001$.

	DPC 0	DPC 3	DPC 5	DPC 7	DPC 10	DPC 14
Viral challenge group	0.00 ± 0.00	$8.99 \pm 0.59^{***}$	$8.92 \pm 0.54^{***}$	$8.45 \pm 0.46^{***}$	$7.82 \pm 0.54^{***}$	$7.17 \pm 0.66^{***}$
Normal control group	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

5.8. Titers of Neutralizing Antibody

Collection of blood before and after the viral challenge (DPC 0, 3, 5, 7, 10, and 14) was performed and determined the NA titers in serum. The results showed that the normal control group and the viral challenge group did not detect NA titers. NA titers in thetwo groups are 0.00 ± 0.00 (Table 7).

Table 7: Titers of the neutralizing antibody (Log2) in serum was detected on DPC 0, 3, 5, 7, 10, and 14 in the experiment. Data were presented as mean ± SD.

	DPC 0	DPC 3	DPC 5	DPC 7	DPC 10	DPC 14
Viral challenge group	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Normal control group	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

5.9. Titers of the Indirect Fluorescent Antibody

Collection of pig blood before and after the viral challenge (DPC 0, 3, 5, 7, 10, and 14) was performed and detected by IFA staining for the evaluation of IFA titers. The results showed that IFA titers in serum in the viral challenge group gradually increased on DPC 3 until the end of the experiment (Table 8).

Table 8: Titers of the indirect fluorescent antibody (Log2) in serum was detected on DPC 0, 3, 5, 7, 10, and 14 in the experiment. Data were presented as mean ± SD. $^{***}p < 0.001$.

	DPC 0	DPC 3	DPC 5	DPC 7	DPC 10	DPC 14
Viral challenge group	0.00 ± 0.00	$6.33 \pm 0.49^{***}$	$8.50 \pm 0.90^{***}$	$10.08 \pm 0.67^{***}$	$11.27 \pm 0.47^{***}$	$10.88 \pm 0.64^{***}$
Normal control group	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

6. Discussion

PRRS is responsible for substantial animal and economic losses to the pig industry [11]. PRRSV can causes viraemia, pyrexia, pneumonia with abnormal respiratory behavior, and reduced ADWG [1]. PRRSV is highly infectious and spreads via intra-nasal, intra-muscular, haematogenous, and aerosol routes. As pigs are often raised in the areas of high density, the spread of infection is difficult to control [12]. Vaccination is a method to prevent PRRSV infection and spread via reducing clinical signs, viraemia, and lung lesions for improving health and performance in pigs [13, 14].

PRRSV are classified in two different genotypes based on genomic heterogeneity. Martinez-Lobo et al. [15] even compared the pathogenic properties of type 1 PRRSV and type 2 PRRSV in a young pig (3-week-old) infection model. After viral challenge, the results indicated that type 2 PRRSV are more pneumovirulent than type 1 PRRSV. However, no clear differences could be established between genotypes in systemic clinical signs or viral load, and viral distribution after challenge. In our study, clinical signs and viral load in serum, lung tissues, and HLN were established after PRRSV challenge.

ADWG of pig is a primary economic driver of health and performance in swine production [16, 17, 18]. Reduced ADWG results from loss of appetite and reduced feed in take that usually caused by high temperatures and pneumonia. In our viral challenge pig model, the reduced ADWG was presented in the viral challenge pig as a consequence of PRRSV infection.

The objective of this study was to establish a viral challenge pig model with PRRS suitable for the need of R&D of PRRS vaccines. According to our all results, viraemia was found in the viral challenge group compared to that in the normal control group. Additionally, other results about the increase BT, reduced ADWG, increase mortality rate, the expression of PRRSV RNA and lesions in lung tissues and HLN, and increase titers of IFA in serum were presented in the viral challenge pigs. Herein, a PRRSV 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.

7. Conclusion

PRRSV is constantly evolving to cause new outbreaks. This disease

is becoming more virulent with ability to evade vaccine-induced immunity. Therefore, R&D of an effective PRRS vaccine is very important for controlling PRRS outbreaks and preventing economic losses. In order to promote the development of PRRS vaccines, the establishment of a viral challenge pig model with PRRS 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 PRRS. This model will be suitable for the R&D need of PRRS vaccines.

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