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A Candidate Subunit Porcine Circovirus Type 2 Vaccine Offered Similar Immune Protection Effect as Commercial Licensed Vaccine

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PCV2, Cap protein vaccine, Inactivated vaccine, Vaccine efficacy, Immunization effect evaluation

ABSTRACT

Porcine circovirus type 2 (PCV2) vaccination is used extensively and has been effective in protecting pigs from post-weaning multisystemic wasting syndrome (PMWS). Most PCV2 vaccines used in China were whole virus inactivated vaccine, while no standard has been used to evaluate efficacy of vaccines. The aim of this study was to establish a evaluation method for vaccine immunization. For this purpose, twenty-five Changbai piglets were randomly divided into five groups each of 5 pigs: group A vaccinated with cap protein and challenged with PCV2; group B vaccinated with commercial PCV2 recombinant subunit vaccine and challenged with PCV2; group C vaccinated with commercial PCV2 Vaccine and challenged with PCV2; group D was sham-vaccinated and challenged with PCV2; group E was sham-vaccinated and sham-challenged. Antibody level was detected on day 0 (dpv 0) and day 28(dpv 28). A-C groups were challenged with PCV2 SD (dpv 28) and all pigs were necropsied 28 days post-challenge (dpc 28). Temperature, relative daily weight gain and immunohistochemical test were used as the standard to evaluate the vaccine effect. The cap protein vaccine and commercial PCV2 recombinant subunit vaccine immune results showed that the four pigs comply with the above three standards. The PCV2 inactivated vaccine results showed that five pigs comply with the above three standards. The vaccine protection results were 4/5, 4/5 and 5/5, respectively, identical with the serology results. The results showed that cap protein vaccine has similar immune protection effect as commercial licensed inactivated PCV2 vaccine. Compared with the difficult problem of raising and improving the titer of PCV2 in PK15 cells in the production of inactivated vaccine, prokaryotic expression systems have obviouse advantages in expressing cap protein. This offers another safety and efficacy vaccines for prevention and control of porcine circovirus-associated diseases (PCVD).

INTRODUCTION

Porcine circovirus (PCV) is a small, nonenveloped, icosahedral virus containing a circular single-stranded DNA genome, assigned to the Circoviridae family (Tischer *et al*., 1982). An earlier study showed that PCV comprises of non-pathogenic PCV1 and pathogenic PCV2, which is closely related with postweaning multisystemic wasting syndrome (PMWS) characterized by progressive weight loss, dyspnea, tachypnea, and icterus. Weaned pigs and fattening pigs were mainly infected with PCV2 (Segalés *et al*., 2005). Now PCV is divided into four genotypes(PCV1, PCV2, PCV3, PCV4). PCV3 is a newly emerging virus, which was first reported in USA with unexplained cardiac and multi-organ inflammation in pigs (Phan *et al*., 2017; Wen *et al*., 2017; Ku *et al*., 2017). PCV 4 is a novel porcine circovirus that was just discovered in 2019, and the relevant studies are still in their infancy (Palinski *et al*., 2016; Ku

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et al., 2017; Shen *et al*., 2018; Zhang *et al*., 2020). PCV2 has become one of the most important pathogens affecting the swine industry worldwide. PCV2 was divided into five gentypes: PCV2a, PCV2b, PCV2c, PCV2d and PCV2e. In recent years, PCV2d has widely disseminated throughout the world and has become epidemic strain. There are two major open reading frames (ORFs) within PCV2 genomes: ORF1 econding replication associated proteins (Rep and Rep') and ORF2 encoding structural capsid protein (Cap). Cap was found to be immunogenic and had self-assembly ability into virus-like particles (VLPs) *in vitro*, which becomes suitable candidate for vaccine development against a subsequent challenge with PCV2. Numerous field and experimental trials have shown that PCV2 infection, viremia and lesions could be reduced with PCV2 vaccination compared to non-vaccinated pigs (Blanchard *et al*., 2003; Fu *et al*., 2011; Park *et al*., 2017; Segalés, 2015; Wang *et al*., 2013). Today PCV2 vaccination is the most important prevention strategy. The first vaccine in the market was Circovacr (Merial), an inactivated PCV2, oil-adjuvanted (O/W) vaccine for use in sows and gilts. Nowadays, Boehringer Ingelheim and intervet International B.V. subunit vaccines based on the product of the *ORF2* gene expressed on baculovirus systems were external vaccine licensed in China. Others are mostly whole virus inactivated vaccines with the virus titers $10^{5.5}TCID_{50}$/mL. Increase in the virus titers and their accurate measurements are still the major problems to be solved in the vaccine industry.

In this study, a subunit vaccine based on the product of an optimized expression Cap protein expressed on prokaryotic system was produced. The aim of this study was to establish a standard for evaluation of vaccine immunization effect and to compare the protection effect of the subunit Cap protein vaccine with commercial inactivated PCV2 vaccines, which may be another vaccine for prevention and control of porcine circovirus-associated diseases (PCVD).

MATERIALS AND METHODS

Prokaryotic expression system

The *ORF2* gene used in this study was the PCV2d subtype. To improve the expression of *ORF2* gene in prokaryotic system, we generated a synthetic *ORF2* gene sequence in which wild-type codons were replaced with codons from the highly expressed in *E.coli*, the first 40 amino acids of the ORF2, coding for the eukaryotic nuclear localization signal. The synthetic genes were cloned in a prokaryotic expression system plasmid pET-30a. *E. coli* BL21 were transformed into new recombinant plasmids and induced by IPTG. SDS-PAGE and western-blot were used to detect the recombinant protein expression. Transmission electron microscopy was also used to observe the recombinant capsid protein virus-like particles.

Animals,vaccine and experimental design

Twenty-five two weeks old commercial Landrace piglets tested negative for PCV2 antigen-antibody from Shandong Huahong Biological Engineering Co., Ltd. were selected for vaccine and randomly divided into five groups (A-E), each of five piglets and housed separately.

The piglets were vaccinated intramuscularly (i.m.) in the neck. Recombinant proteins were purified and emulsified with Seppic MONTANIDETM ISA 15A VG adjuvant to make an O/W vaccine with Cap protein concentration 25 μg/mL.Group A was vaccinated with 2 mL of O/W vaccine with Cap protein per pig. Group B was vaccinated with the commercial PCV2 Recombinant Subunit Vaccine (Lot No. (2014)150131096) 2 mL per head. Group C was vaccinated with the commercial PCV2 vaccine, inactivated strain LG (Lot No. (2010)080011071) as 2 mL per head. Group D and E were vaccinated with 2 mL saline per head. The experimental plan is shown in Figure 1.

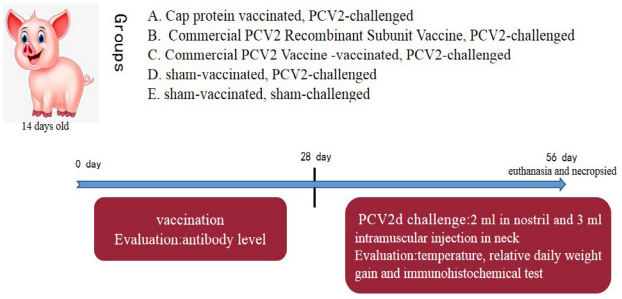

Fig. 1. Animal experimental design.

Assay of pigs blood antibody levels

Blood samples were collected upon the piglets' arrival at the research facility and four weeks after vaccination, centrifuged at 3000g for 10 min at 4°C, and the serum was separated and stored at -80°C until testing. PCV2 antibody levels were detected with PCV2-dCap-ELISA kit (Tianjin Ringpu Biotechnology Limited by Share Ltd) according to the manufacturer's directions. The 200 times dilution serum samples were double diluted and the S/P was checked.

The ELISA antibody titer of the serum was determined by the maximum dilution when the S/P was no less than 0.25. At least 4 pigs in the immunization groups should not be less than 1:1600, and the control pigs should not be more than 1:400. S/P = (OD_{450} of sample-OD_{450} mean of negative control)/(OD_{450} mean of positive control -OD_{450} mean of negative control).

Challenge

After 4 weeks (dpv 28 or dpc 0), A-D groups were challenged with 2 ml in nostril and 3 ml intramuscular injection in neck PCV2 cells virulent strain SD (PCV2d, No.DQ478947, $10^{6.0}$ $TCID_{50}$/ml). Pigs in group E were sham-inoculated with 5 ml saline with the same way.

Temperature measurement

The rectal temperature of piglets in each group was measured daily on the first 7 days after challenge at about 10:00 am. The temperature (≥40°C) was judged fervescence. Fervescence maintain more than 3 days was judged with PMWS symptoms after PCV2 challenge. Note that the pigs should be quiet when measuring the temperature.

Relative daily weight gain

The change of all pigs body weight was recorded at the time before challenge (dpc 0) and 4 weeks later (dpc 28). Group average relative daily gain (the sum of IRDG/5) was calculated to evaluate the vaccine effection.

Individual relative daily gain (IRDG)= (dpc28 weight- dpc0 weight)/dpc0 weight/28

Group average relative daily gain (GARDG)= The sum of IRDG/5

Immunohistochemical test

Four weeks after challenge, the pigs were euthanized by intravenous pentobarbital sodium overdose (Fatal Plus, Vortech Pharmaceuticals, LTD, Dearborn, MI, USA) and necropsied. Samples from inguinal lymphoid tissues were fixed in 10% neutral-buffered formalin solution, sectioned. Immunohistochemical test was used to detect PCV2 antigen.

Immune protection standard

I. Temperature standard: The temperature was not ≥40°C or fervescence (≥ 40°C), not appear for more than 3 days.
II. Relative daily weight gain standard: The group average relative daily gain was calculated within 28 days after challenge. If there was no significant difference between the experimental group and the blank control group (P > 0.05), the immune protection of this group was judged. P value was calculated by SPSS20.0.
III. Immunohistochemistry standard: Immunohistochemical test was used to detect the inguinal lymph nodes of piglets. The PCV2 antigen should not be detected.

Compliance with any two of the above three standard shall be identified as immune protection.

Statistical analysis

All data was statistically analyzed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to do the statistical analyses followed by Tukey's post hoctest, respectively. Differences were considered significance at $p < 0.05$.

RESULTS

Prokaryotic expression system

153 of 235 codons in the *ORF2* gene have been optimized with no change in the amino acid sequence. A recombinant plasmid with the synthetic *ORF2* gene was constructed and the cap protein were induced with 0.8 mmol/LIPTG for 5 h. SDS-PAGE and western-blot indicated that cap protein were expressed as 37KDa which was recognized by anti-PCV2 monoclonal antibody.

Serology

Four weeks after vaccination, the blood samples were collected, double diluted and antibody levels were determined with PCV2-dCap-ELISA kit. The maximum dilution when the S/P was no less than 0.25 is shown in Table III. The results showed that most piglets in the immunization groups were not less than 1:1600, except for two pigs (No.A2, B4). Four piglets in group D were immunoprotected with not more than 1:400. The vaccine protection results were 4/5, 4/5 and 5/5 protection of cap protein vaccine , commercial PCV2 recombinant subunit vaccine, and PCV2 inactivated vaccine, respectively.

Temperature measurement

The rectal temperature of all piglets on the first 7 days after challenge is shown in Table I. The results showed that most piglets in immunization groups and group E had temperature under 40°C, except for two pigs (No. A2, B4), in which fervescence was maintained for over 3 days.

There were four pigs in group D judged with PMWS symptoms after PCV2 challenge with fervescence maintained for more than 3 days (Fig. 2).

Relative daily weight gain

The GARDG is summarized in Table II. The P-value based on the GARDG was calcuted. There was no significant difference between group A and E (P =0.055), B and E (P =0.077) and C and E (P =0.093). So the experimental groups (A,B,C) were judged immune protected, with no PMWS symptoms after PCV2 challenge. There was significant difference between groups D and E (P <0.05), so the experimental group D was judged with PMWS symptoms after PCV2 challenge.

Table I. Pigs temperature after challenge.

Temperature of different days after challenge (℃)							
No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Group A							
A1	39.2	39.6	39.1	39.1	38.9	39.2	39.4
A2	39.4	40.4	40.1	40.3	40.1	40.4	40.2
A3	39.1	39.4	39.1	39.5	39.0	39.4	39.0
A4	39.5	40.3	38.9	39.2	39.8	39.7	39.3
A5	39.9	39.5	39.1	39.3	39.5	39.8	39.5
Group B							
B1	39.8	39.0	39.1	39.9	39.2	39.2	38.6
B2	39.1	39.9	38.8	39.2	39.8	39.5	39.1
B3	40.3	38.9	39.6	39.1	39.0	39.3	40.4
B4	40.4	40.1	40.5	39.8	40.4	40.2	39.9
B5	39.4	39.3	39.1	39.9	39.4	39.4	39.1
Group C							
C1	39.1	39.0	39.3	39.1	39.9	38.8	38.9
C2	38.8	39.4	39.2	40.3	38.9	39.6	39.1
C3	39.9	39.4	39.4	39.1	39.5	38.8	39.1
C4	39.9	40.1	39.5	38.9	39.2	39.7	38.7
C5	39.1	38.9	37.9	38.2	39.1	39.4	38.9
Group D							
D1	39.8	41.3	40.6	40.1	41.0	41.2	40.8
D2	39.8	41.3	40.4	39.8	39.9	39.7	39.5
D3	40.9	41.2	40.0	40.9	41.3	41.3	40.2
D4	40.1	40.4	40.1	41.2	40.0	41.3	40.1
D5	41.2	41.3	39.8	40.2	40.7	41.3	40.5
Group E							
E1	39.7	39.0	38.9	39.2	39.7	39.8	39.3
E2	39.8	39.5	39.1	39.0	39.5	39.8	39.5
E3	40.1	39.0	38.8	39.4	39.9	39.1	39.9
E4	39.3	39.2	39.3	39.8	39.9	39.3	39.9
E5	38.9	39.3	38.9	39.1	39.3	38.9	39.3

The rectal temperature of piglets in each group was measured daily on the first 7 days after challenge at about 10:00 am. The temperature (≥ 40℃) was judged fervescence. Fervescence maintain more than 3 days was judged with PMWS symptoms after PCV2 challenge.

This results indected that infection with PCV2 does not affect weight gain after vaccine and PCV2 cap protein vaccination was effective against PCV2 challenge. Also, the results showed that the subunit vaccine candidate had no adverse effect on the weight gain (Fig. 3).

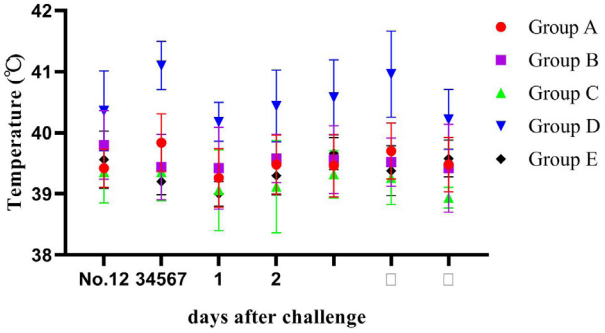

Fig. 2. Pigs temperature after challenge.
The rectal temperature of piglets in each group was measured daily on the first 7 days after challenge at about 10:00 am. The temperature(≥40℃) was judged fervescence. Fervescence maintain more than 3 days was judged with PMWS symptoms after PCV2 challenge.

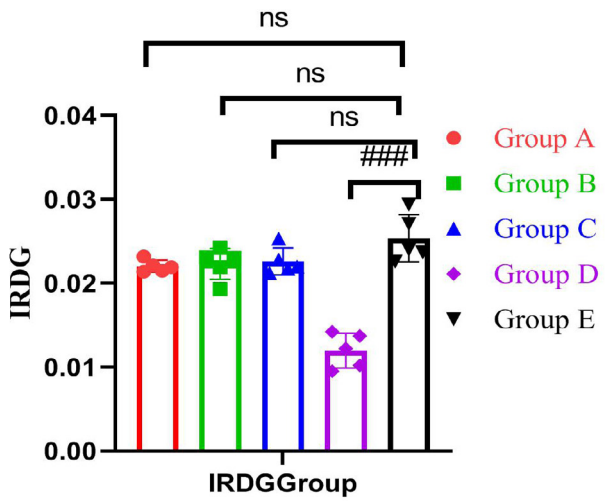

Fig. 3. Pigs body weight changes after challenge.
There was no significant difference between A and E (P =0.055), B and E (P =0.077), C and E (P =0.093), so the experimental groups(A,B,C) were judged immune protection, with no PMWS symptoms after PCV2 challenge. There was significant difference between D and E (P <0.05),so the experimental group D was judged with PMWS symptoms after PCV2 challenge.

Immunohistochemical test

The results of immunohistochemical test used to detect PCV2 antigen are shown in Table III. The results showed that most piglets in the immunization groups were negative for immunohistochemical test, except for two pigs No. A2 and B4. Four pigs in group D were positive except D2. The results showed that the PCV2 cap protein vaccine as well as virus vaccine could prevent virus replication in the pigs.

Table II. Pigs body weight changes after challenge.

No.	Body weight (Kg)				P value
	dpc 0	dpc 28	IRDG	GARDG	
Group A					
A1	11.8	18.9	0.0215	0.0220	0.055
A2	11.9	19.2	0.0219		
A3	12.1	19.6	0.0221		
A4	11.1	18.3	0.0232		
A5	12.2	19.5	0.0214		
Group B					
B1	10.7	17.6	0.0230	0.0223	0.077
B2	11.6	18.7	0.0219		
B3	11.3	17.4	0.0193		
B4	10.9	18.3	0.0242		
B5	11.3	18.6	0.0231		
Group C					
C1	11.5	18.5	0.0217	0.0226	0.093
C2	10.6	18.1	0.0253		
C3	11.3	18.5	0.0228		
C4	11.5	18.6	0.0220		
C5	11.6	18.5	0.0212		
Group D					
D1	12.4	15.7	0.0095	0.0120	0.000
D2	11.2	14.4	0.0102		
D3	11.7	15.7	0.0122		
D4	11.5	15.9	0.0137		
D5	11.1	15.5	0.0142		
Group E					
E1	10.2	18.6	0.0294	0.0254	\
E2	10.4	18.3	0.0271		
E3	11.3	18.9	0.0240		
E4	10.7	17.8	0.0237		
E5	11.4	18.6	0.0226		

There was no significant difference between A and E (P =0.055), B and E (P =0.077), C and E (P =0.093), so the experimental groups(A,B,C) were judged immune protection, with no PMWS symptoms after PCV2 challenge. There was significant difference between D and E (P <0.05),so the experimental group D was judged with PMWS symptoms after PCV2 challenge.

Immuneprotection results

Temperature, relative daily weight gain and immunohistochemical test were used as the standard to evaluate the vaccine effect. Compliance with any two of the above three standard shall be identified as immune protection. The cap protein vaccine and commercial PCV2 recombinant subunit vaccine immune results showed that 4 pigs complied with the three above standard. The PCV2 inactivated vaccine results showed that 5 pigs complianed with three above standard. The vaccine protection results were 4/5, 4/5 and 5/5 protection, identical with the serology results (Table III).

Table III. The results of vaccine immune protection after PCV2 challenge.

No.	Antibody levels	Immune protection standard			No. of PMWS	Results
		I	I I	I I I		
Group A						
A1	1:1600	N	N	-	1	4/5 protection
A2	1:800	P		+		
A3	1:1600	N		-		
A4	1:3200	N		-		
A5	1:6400	N		-		
Group B						
B1	1:1600	N	N	-	1	4/5 protection
B2	1:1600	N		-		
B3	1:3200	N		-		
B4	1:800	P		+		
B5	1:6400	N		-		
Group C						
C1	1:3200	N	N	-	0	5/5 protection
C2	1:1600	N		-		
C3	1:1600	N		-		
C4	1:1600	N		-		
C5	1:1600	N		-		
Group D						
D1	1:400	P	P	+	4	4/5 PMWS
D2	1:1600	N		-		
D3	1:400	P		+		
D4	1:200	P		+		
D5	1:200	P		+		
Group E						
E1	1:200	N	\	-	\	\
E2	1:400	N		-		
E3	1:400	N		-		
E4	1:400	N		-		
E5	1:400	N		-		

N, no symptoms like fervescence and affect weight gain after PCV2 challenge. P, PMWS like fervescence(maintain more than 3 days) and affect weight gain after PCV2 challenge. +, There was PCV2 antigen used Immunohistochemical test. -, There was not PCV2 antigen used Immunohistochemical test. \, There was no such item.

DISCUSSION

Emergence of PCV2 in the swine producing resulted in substantial economic losses to the pig industry across the globe. PCV2 was the primary causative agent of PMWS, mainly infecting the weaned piglets, which can affect the respiratory system and be transmitted oronasally (Allan *et al*., 1998). At present, vaccine immunization has become a fundamental means and measures to prevent diseases caused by PCV2.

There are many factors that affect the expression of foreign genes in prokaryotic expression system, such as the target encoding gene, the expression vector, the competent cell, the cultivation condition of the *E. coli*, and the induction methods (Adam, 2001). Previous studies have indicated that the genome of PCV2 ORF2 contains rare codons, which is difficult to express. To improve the expression of *ORF2* gene in prokaryotic system, 153 triplet codons of ORF2 were replaced with codons for highly expressed in *E. coli*, no change in amino acid sequence. The synthetic *ORF2* genes were cloned into pET-30a to construct prokaryotic expression systems. The expression condition with different IPTG concentrations (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2mmol/L) and different induction time (1, 3, 5, 7, 9, and 11h) were compared. The optimized induction condition was for 5 h with 0.8mmol/L IPTG to the recombinant BL21 at OD_{600} 0.8. The results of optimized expression showed that the quantity of cap protein had been improved and recombinant protein was recognited by anti-PCV2 monoclonal antibody. Other researcher had reported subunit cap vaccine displayed by *Lactococcus lactis* particles, co-expressing with PRRSV GP3/GP5 and recombinant porcine IL-2 or GM-CSF (Li *et al*., 2016; Han *et al*., 2018; Wang *et al*., 2015). Meanwhile, the prokaryotic expression system we used was more simple with higher expression cap vaccine concentration and cost effective. The encoded cap proteins could be self-assembl into VLPs with immune protection against PCV2 challenge.

In the present study, at the time of challenge all of the vaccinated pigs (groups A-C) had detectable anti-PCV2 IgG antibody titers and were significantly higher compared to non-vaccined pigs (groups D-E). Obviously a detectable antibody response to PCV2 vaccination is a good predictor for successful vaccine administration. The results of serology detection, immune protection results showed that cap protein vaccine offered similar immune protection effect as commercial licensed inactivated PCV2 vaccine. Compared with the difficult problem of raising and improving the titer of PCV2 in PK15 cells in produce inactivated vaccine, prokaryotic expression systems have obvious advantages in raising *E. coli* and the expression of cap protein, making another candidate available for prevention and control of PCVD.

CONCLUSION

In conclusion, an evaluation method was established as the standard to evaluate the vaccine immunization effect. Using this methods, we found that the cap protein vaccines offered similar immune protection effect as commercial licensed inactivated PCV2 vaccine, thus giving another safety and efficacy vaccine for prevention and control of PCVD.

DECLARATIONS

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Ethical statement and IRB approval

The whole procedure for experimental animals was performed and carried out in strict accordance with guideline (IACC20060101,1 Jan, 2006) of the Institutional Animal Care and Use Committee of Institute of Animal Science and Veterinary Medicine, Shandong Academy of Agricultural Sciences. All animals used in this study were made to minimize suffering and euthanasia were applied for viscera.

Statement of conflict of interest

The authors have declared no conflict of interest.

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