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Research Article

Turk. J. Vet. Anim. Sci.
2010; 34(2): 173-179
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doi:10.3906/vet-0807-13

Immune response to type-2 porcine circovirus in weaned pigs*

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Received: 10.07.2008

Abstract: Sixteen crossbred barrows (Duroc × Landrace × Yorkshire) weaned at 28 days with an initial body weight of 5.12-8.64 kg and no antibodies to PCV2 were used in a 5-week trial to evaluate the immune response after intranasal inoculation with type-2 porcine circovirus. The percentage of peripheral blood ANAE$^+$ T cells, PCV2 antibody titer, immunoglobulins, and serum IFN-γ, IL-2, and sIL-2R levels were determined. The results show that the percentage of peripheral blood ANAE$^+$ T cells was notably higher in inoculated pigs 5 days post-inoculation (dpi) and significantly lower 21 dpi than in the control group. The anti-PCV2 titer in the inoculated group was significantly higher than that in the control group 14 dpi. The immunoglobulin profile of the 2 groups also differed. The IFN-γ level in the inoculated group decreased significantly from 7 to 28 dpi, as compared with the control group. The results suggest that PCV2 challenge results in an immune system response.

Key words: Immune response, type-2 porcine circovirus, weaning piglets

Introduction

Porcine circovirus 2 (PCV2) is a member of the family Circoviridae. It is a very small (~17 nm), non-enveloped, single-stranded DNA virus with a circular genome, and is ubiquitous in the global swine population (1,2). PCV2 infection is associated with post-weaning multisystemic wasting syndrome (PMWS) (3,4). In addition, it is associated with a number of other porcine diseases, including porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex, reproductive failure, granulomatous enteritis, exudative epidermitis, necrotizing lymphadenitis, and congenital tremor (4,5). In China, approximately 20%-60% of PCV2-infected piglets reportedly have PMWS (6,7), resulting in considerable economic losses to the pig industry.

Researchers have attempted to establish an experimental PCV2 inoculation protocol, but the immune response to the virus has not been fully characterized. The objective of the present study was to characterize the immune response of weaned pigs after intranasal inoculation with PCV2.

* This work was supported by grants from the Innovative Research Team in University (No. IRTO555-5), and the Key Laboratory for Animal Disease-Resistance Nutrition, Ministry of Education, China.

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Materials and methods

Experimental animals and diets

This experiment was conducted according to a protocol approved by the Sichuan Agricultural University Animal Care and Use Committee. The animal feeding trial was performed using 16 crossbred barrows (Duroc × Landrace × Yorkshire) weaned at 28 days, with an initial body weight of 5.12-8.64 kg and no antibodies to PCV2. The experimental diet was formulated according to NRC***.

Experimental design

Testing for maternal antibodies were conducted using a PCV2-specific enzyme-linked immunosorbent assay (ELISA). An S/P ratio (sample mean/positive mean – negative mean) below 0.15 was considered negative for maternal antibody levels (8). Sixteen piglets without PCV2 antibodies were included. They were divided into a control and treatment group according to body weight, litter, and gender. The 2 groups were housed in separate nursery rooms and raised individually. All the piglets were fed the same diet, with free access to feed and water, and were handled according to the practices of animal care established by the Institutional Animal Care Committee.

All piglets were bled weekly by puncturing the vena cava. Blood samples were collected into 5-mL tubes and centrifuged (3500 ×g for 5 min) to collect serum, which was stored at –20 °C until analysis for IgG, IgM, and IgA, and interferon-γ, IL-2, and sIL-2R. One pig from each group with the lowest growth rate was sacrificed and necropsied 7, 14, 21, 28, and 35 dpi.

Infection protocol

On day 1 of the experiment pigs in the treatment group were inoculated with 1 mL of PCV2-infected PK-15 cell lysate [1 mL of a PCV2 pool containing $1.0 \times 10^{5.5}$ tissue culture infective dose of 50 ($TCID_{50}$) mL^{-1}] and the control group was inoculated intranasally with 1 mL of saline solution. Biosecurity measures were implemented to prevent contamination of the uninfected room with PCV2. PCV2-infected PK-15 cell lysates were a gift from Dr. Xu-Zhiwen.

Cellular immune response

By using acid α-naphthyl acetate esterase (ANAE) staining (9), the percentage of peripheral blood large granule $ANAE^+$ T cells (l g of ANAE T cells) in all pigs was assayed 3, 5, 7, 14, 21, 28, and 35 dpi.

Humoral immune response

The level of PCV2 serum antibodies was assayed via enzyme-linked immunosorbent assay (ELISA) using a specific immunoassay kit (Beijing Anheal Laboratories Co. Ltd.). OD values were read and the S/P ratio [sample mean/positive – negative means] was calculated. Serum samples were tested for IgA, IgM, and IgG isotype-specific antibodies by immunoturbidimetry, using an assay kit (Sichuan Maker Science Technology Co., LTD., China).

Cytokine response

Serum levels of interferon-γ, IL-2, and soluble IL-2 receptor (sIL-2R) were determined by ELISA, using an immunoassay kit (RapidBio Lab, USA). The coating antibody was goat anti-pig and the sensitivity of these assays was 1.0 pg mL^{-1}.

Statistical analysis

One-way analysis of variance (ANOVA) was performed using SPSS v.13.0 to compare differences between the groups. Data are presented as mean ± SEM. P values < 0.05 were considered statistically significant.

Results

Adaptive immune response

Cellular immune response

The percentage of $ANAE^+$ T cells in the control group increased with time, while in the inoculated group there was a sudden increase 5 dpi, followed by a rapid decrease 7 dpi. The percentage of $ANAE^+$ T cells in the inoculated group was 176% higher than in the control group 5 dpi ($P < 0.01$). After 7 dpi there was not a significant difference between the 2 groups, except 21 dpi when the percentage of $ANAE^+$ T cells in the control group was significantly higher than in the control group ($P < 0.05$, Table 1).

*** NRC (National Research Council): Nutrient Requirements of Swine. 10th edn., Washington DC, National Academy Press, 1998)

Table 1. Effect of PCV2 infection on the percentage of peripheral blood ANAE$^+$ T cells and the PCV2 antibody titer in piglets.

ANAE+%	control		PCV2-inoculated		
	n	Mean ± SEM	n	Mean ± SEM	P
3 dpi	8	3.59 ± 0.62	8	3.53 ± 0.56	0.95
5 dpi	8	3.00 ± 0.67^B	8	12.51 ± 3.14^A	0.01**
7 dpi	8	5.10 ± 0.85	7	7.62 ± 1.67	0.21
14 dpi	7	10.72 ± 1.61	6	8.78 ± 0.75	0.31
21 dpi	6	16.71 ± 0.53^a	5	15.25 ± 0.41^b	0.05*
28 dpi	5	15.78 ± 0.93	4	16.62 ± 1.18	0.60
35 dpi	4	11.94 ± 1.15	3	15.45 ± 1.36	0.12

PCV2				(*S/P*)			
antibody titre	n	(S/P < 0.15)	Mean ± SEM	n	(S/P > 0.15)	Mean ± SEM	P
0 dpi	8	0	0.047 ± 0.01	8	0	0.071 ± 0.02	0.33
7 dpi	8	0	0.051 ± 0.01	7	1	0.107 ± 0.05	0.25
14 dpi	7	0	0.084 ± 0.01^b	6	3	0.149 ± 0.03^a	0.02*
21 dpi	6	0	0.102 ± 0.01^B	5	4	0.169 ± 0.02^A	0.01**
28 dpi	5	0	0.111 ± 0.01^b	4	4	0.243 ± 0.06^a	0.04
35 dpi	4	0	0.104 ± 0.01^B	3	4	0.361 ± 0.05^A	0.01**

1) Within a row, values of the same item with different capital letter superscripts are highly significantly different (P < 0.01) and different small letter superscripts show significant difference (P < 0.05), nothing means no significant difference (P > 0.05)
2) * means P < 0.05, ** means P < 0.01.

Humoral immune response

PCV2 serum antibody titers at different time points are shown in Table 2. Dramatic changes in PCV2 antibody levels were not observed, and PCV2 antibodies remained undetectable in the control group during the entire study period. In the inoculated group PCV2 antibody titers were significantly higher than in the control group after 14 dpi. This result indicates that pigs in the inoculated group were infected with PCV2 after inoculation.

The effect of PCV2 infection on serum immunoglobulin levels is shown in Table 3. The maximum serum IgM concentration in the inoculated group was observed 7 dpi, which dramatically decreased to the minimum concentration 21 dpi. There was a significant difference between the 2 groups 21 dpi. IgG levels in the control group decreased to the lowest point 14 dpi, increased until 28 dpi, and then dropped again. IgG levels in the inoculated group 21 dpi and 28 dpi were significantly different (P < 0.01, P < 0.02) from those in the control group. The peak concentration in the inoculated group was observed 28 dpi, which was 1 week later than in the control group. Two peaks in the serum IgA concentration in the inoculated group appeared 7 and 28 dpi. The second peak was observed 2 weeks later in the control group.

Cytokine response

IL-2, sIL-2R, and IFN-γ response

Serum IFN-γ levels in the control group remained stable until 21 dpi (Table 3). Serum IFN-γ levels differed significantly between the control and experimental groups (P < 0.01).

Differences in IL-2 and sIL-2 receptor concentrations were not statistically significant between the 2 groups throughout the entire study (data not shown).

Table 2. Effect of PCV2 infection on IgG, IgA, and IgM (mg/mL).

	group	n	IgG	P	IgM	P	IgA	P
0 dpi	control	8	1.088 ± 0.024	0.16	0.169 ± 0.027	0.50	0.144 ± 0.031	0.80
	inoculated	8	1.179 ± 0.052		0.138 ± 0.035		0.127 ± 0.058	
7 dpi	control	8	0.962 ± 0.024	0.43	0.132 ± 0.012	0.22	0.209 ± 0.039	0.72
	inoculated	8	0.917 ± 0.050		0.164 ± 0.022		0.183 ± 0.059	
14 dpi	control	7	0.743 ± 0.032	0.28	0.097 ± 0.011	0.56	0.021 ± 0.001	0.60
	inoculated	6	0.836 ± 0.016		0.106 ± 0.009		0.022 ± 0.002	
21 dpi	control	6	1.072 ± 0.055^{A}	0.01**	0.173 ± 0.026^{a}	0.02*	0.308 ± 0.106^{a}	0.04*
	inoculated	5	0.839 ± 0.044^{B}		0.085 ± 0.011^{b}		0.053 ± 0.008^{b}	
28 dpi	control	5	0.887 ± 0.036^{b}	0.02*	0.110 ± 0.007	0.14	0.101 ± 0.014	0.08
	inoculated	4	1.067 ± 0.045^{a}		0.128 ± 0.008		0.159 ± 0.028	
35 dpi	control	4	0.906 ± 0.039	0.77	0.159 ± 0.033	0.50	0.201 ± 0.052	0.53
	inoculated	3	0.889 ± 0.039		0.129 ± 0.016		0.156 ± 0.033	

1) Within an array, values of the same item with different capital letter superscripts are highly significantly different ($P < 0.01$) and different small letter superscripts show significant difference ($P < 0.05$), nothing means no significant difference ($P > 0.05$)
2) * means $P < 0.05$, ** means $P < 0.01$.

Table 3. Effect of PCV2 infection on IFN-γ levels.

	Control group (pg/mL)	Inoculated group (pg/mL)	P
0 dpi	62.81 ± 7.19	72.20 ± 12.52	0.54
7 dpi	79.65 ± 9.02^{A}	17.17 ± 4.51^{B}	0.00**
14 dpi	80.12 ± 3.22^{A}	41.23 ± 10.54^{B}	0.00**
21 dpi	96.06 ± 11.13^{A}	41.04 ± 9.46^{B}	0.01**
28 dpi	121.60 ± 30.6	169.37 ± 62.55	0.49
35 dpi	299.87 ± 86.26	246.71 ± 102.58	0.71

1) Within a row, values of the same item with different capital letter superscripts are highly significantly different ($P < 0.01$) and different small letter superscripts show significant difference ($P < 0.05$), nothing means no significant difference ($P > 0.05$)
2) * means $P < 0.05$, ** means $P < 0.01$.

Discussion

In recent years several studies have reported the activity of α-naphthyl acetate esterase (ANAE), a nonspecific esterase, in T-lymphocytes, but not in B-lymphocytes (10,11). It has been postulated that ANAE participates as a lysosomal enzyme in the activation and death of T cell target cells (10). The percentage of $ANAE^{+}$ T cells in the inoculated group increased significantly 5 dpi, which is indicative of a cellular immune response to PCV2 infection. The number of $ANAE^{+}$ T cells decreased quickly 7 dpi, indicating a decrease in the percentage of $ANAE^{+}$ T cells in peripheral blood due to a weak immune response. The percentage of $ANAE^{+}$ T cells was significantly lower ($P < 0.04$) in the treatment group than in the control group 21 dpi, which may have been due to a large number of T-lymphocytes that underwent apoptosis after clearing PCV2 infection. Peripheral blood $ANAE^{+}$ T cell levels increased over time in the control group, which may also indicate that the piglets were more dependent upon their own immune systems to eliminate the antigenic challenge rather than passive immunity from the sows (12).

All the control pigs were negative and seroconversion to PCV2 emerged in our study 14 dpi; the PCV2 titer in all infected animals became positive 21 dpi. This result is consistent with previous reports on the time frame for PCV2 seroconversion in weanling or colostrum-deprived piglets (5,13-16). These previous studies show that numerous factors influence PCV2 antibody production, including the pig model used (i.e. conventional, gnotobiotic, caesarean-delivered, or colostrum-deprived), the PCV2 strain or inoculum used (i.e. infected tissue homogenate or cell culture fluid), the inoculation route (i.e. nasal aspiration, subcutaneous, or abdominal cavity injection), viral dosage, pig rearing management, environmental conditions, animal age, and so forth; however, all the available research indicates that serum PCV2 antibodies emerge at 14-21 dpi and increase stably for about 3 weeks thereafter.

IgM is an early antibody and its concentration in pigs was higher 7 dpi than at 0 dpi, with an upward trend in concentration, indicating that PCV2 inoculation induced a first-time humoral immunoresponse in the infected pigs. Nonetheless, this immune response was weak and significant differences were not observed between the inoculated and control groups 7 dpi. The presence of specific IgG is the hallmark of a vigorous humoral immune response. Previous reports (17) showed that plasma IgG levels differed between breeds ($P < 0.001$) and IgG increased with age in all breeds (age: $P < 0.01$; breed × age: $P < 0.005$).

In the present study the evolution of serum IgG levels in the PCV2-inoculated group differed from that in the control group. The serum IgG concentration in the inoculated group decreased after PCV2 inoculation, and remained depressed until 28 dpi (as shown in Table 3). The second peak in the serum IgG concentration occurred 1 week later in the inoculated group than in the control group. These results suggest that the immune response in the inoculated group returned to normal by 28 dpi, as in the control group. The changes in serum IgG level in the inoculated group suggest that the serum IgG antibody was depleted due to PCV2 infection.

Secretion-type IgA (sIgA) is produced by plasma cells in the respiratory tract, gastrointestinal tract, urinary tract, and in mammary tissue. IgA is produced and secreted into blood by plasma cells in bone marrow, lymph nodes, and the spleen (18). Although 2 types of IgA are synthesized in different regions, antigens that contact mucous membranes or are present systemically induce both types of IgA. In the present study IgA levels were similar to the levels of the other 2 immunoglobulins described above.

The minimal concentration of each antibody isotype occurred at different times because of variation in their half-lives. Frenyó et al. (19) suggested that the minimal concentration occurred at approximately 4 weeks for IgG, while van de Ligt et al. (20) suggested that the minimum concentration occurred at approximately 4.5 weeks for IgG and IgM. In the present study the lowest levels of total IgG, IgM, and IgA all occurred at approximately 6 weeks of age (14 dpi), which is considerably later than published values. Our results show that serum IgM, IgG, and IgA concentrations in the control group rebounded about 1 week after reaching minimal levels, whereas the levels rebounded in the inoculated animals later due to infection. In other words, serum immunoglobulin concentrations were depleted by PCV2-infection and virus infection suppressed the humoral immune response. The results are in concordance with those of previous studies (21,22).

IFN-γ acts as a regulator of antigen presentation, and of proliferation and differentiation in lymphocyte populations. These actions result in immunosuppressive as well as immunostimulatory effects (18). Darwich et al. (23) reported that infection of cell cultures with PCV2 had significant effects on the release of cytokines after stimulation with PHA or SEB. Thus, PCV2-infected cultures stimulated with PHA or SEB produced lower levels of IFN-γ than uninfected cultures, regardless of whether the cells came from animals with PMWS or healthy pigs ($P < 0.05$). Meerts (24) reported that IFN-γ influenced PCV2 infection in porcine kidney cells (PK-15) and monocytic cells (3D4/31). When IFN-γ was added to the culture medium before, during, and after inoculation, the number of PCV2-antigen positive cells increased by 418%, 171%, and 691% in PK-15 cells, respectively, and by 706%, 114%, and 423%, respectively, in 3D4/31 cells. The effect of IFN-γ on PCV2 infection was dose-dependent and could be blocked with IFN-γ neutralizing antibodies (24).

We observed that the serum IFN-γ concentration in the inoculated group decreased after PCV2 inoculation and was dramatically lower than in the control group from 7 to 21 dpi ($P < 0.01$). Previous in vitro research showed that IFN-γ was inhibited after PCV2 infection (24). These previous findings suggest that a great quantity of IFN-γ was consumed, and our results show that serum IFN-γ levels decreased after PCV2 inoculation, but recovered over time.

Although previous cell culture studies (25) have shown that the IL-2 level is depressed by PCV2 replication and that the immune response can differ significantly due to individual differences in immune status and nutritional status, as well as other factors. Overall, these studies show that PCV2 inoculation changed the balance of immunoglobulins and IFN-γ in weaned piglets. Immunoglobulin profiles changed markedly at 21 dpi and all of the secondary peaks after inoculation in the inoculated group occurred about 1 week later than in the control group. The data from the present study may contribute to a feasible model of PCV2 infection and lead to additional research.

Acknowledgements

This study was supported by grants from the Innovative Research Team in University (No. IRTO555-5) and the Key Laboratory for Animal Disease-Resistance Nutrition, Ministry of Education, China (No. 2006D005).

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