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A survey on porcine circovirus type 2 infection and phylogenetic analysis of its ORF2 gene in Hangzhou, Zhejiang Province, China*

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Abstract: Porcine circovirus type 2 (PCV2) is closely related to the postweaning multisystemic wasting syndrome (PMWS). In this study, the pig serum and tissue samples collected from different regions of Hangzhou District in Zhejiang Province of China between 2003 and 2005 were analyzed by enzyme-linked immunosorbent assay (ELISA) for PCV2 antibody and by polymerase chain reaction (PCR) for ORF2 gene. The results show that out of 1250 randomly collected serum samples, 500 sera (40%) were seropositive for PCV2. PCR results demonstrate that Hangzhou PCV2 with more than 50% Chinese PCV2 strains and French PCV2 formed Cluster A. Only one PCV2 from Hangzhou belonged to Cluster B with some other Chinese PCV2 and Netherlands's isolates. Cluster C consisted of PCV2 isolates from China, US, Canada, UK and Germany. The results indicate that the PCV2 infection was widespread in Hangzhou.

Key words: Porcine circovirus type 2 (PCV2), Seroprevalence, ORF2, Phylogenetic analysis
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INTRODUCTION

Porcine circovirus (PCV), a member of the Circoviridae family, is a small, naked DNA virus with a diameter of 17 nm. PCV isolated as a persistent contaminant from a porcine kidney cell line is non-pathogenic and designated as PCV1 (Allan *et al*., 1995; Zhou *et al*., 2005). Postweaning multisystemic wasting syndrome (PMWS) emerged in Canada in 1991 (Fenaux *et al*., 2002; Harding, 1997), and its causative agent is believed to be PCV2, a pathogenic strain of PCV (Allan *et al*., 1999). PMWS is a disease that can affect nursery and fattening pigs, and is reported in pigs throughout the world (Allan *et al*., 1998; Kennedy *et al*., 1998; Segalés *et al*., 2002; Wen *et al*., 2005). The disease is characterized by weight loss, dyspnoea and jaundice, as well as the pathological findings of interstitial pneumonia, generalized and enlarged lymph nodes, hepatitis and nephritis (Ladekjær-Mikkelsen *et al*., 2002). Recently, PCV2 was found to be associated with porcine dermatitis and nephropathy syndrome (PDNS) (Wellenberg *et al*., 2004), respiratory disease complex (Kim *et al*., 2003) and reproduction failure (Sanchez *et al*., 2001).

The complete genomic sequence of PCV2 has been determined (Fenaux *et al*., 2000; Hamel *et al*., 1998; Meehan *et al*., 1998; Morozov *et al*., 1998), containing a single-stranded and closed-circular DNA 1767 or 1768 genome. PCV2 has two major open reading frames (ORFs). ORF1 (Rep gene) is essential for virus DNA replication, while ORF2 (Cap gene) encodes a major capsid protein (Liu *et al*., 2001; Nawagitgul *et al*., 2000). The ORF2 of PCV2 may have a potential causative role in the PCV2 related disease (Larochelle *et al*., 2002). Sequence analysis revealed that PCV2 shares only about 75% nucleotide sequence identity with PCV1. Sequence conservation

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between the capsid and Rep genes of PCV1 and PCV2 differs considerably. The Rep gene region is greater than 85% homologous, while the capsid gene region is only 62% homologous (Mankertz *et al*., 2003).

To better understand the seroprevalence and genetic diversities of PCV2, enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and sequence analysis were carried on serum and tissue samples from pigs suspected of having PMWS from Hangzhou District, Zhejiang Province, China. These sequences were compared with those from other regions in China and several other nations' PCV2 strains published in GenBank.

MATERIALS AND METHODS

Sample collection

A total of 1250 pig sera were randomly collected from 6 counties in Hangzhou District, Zhejiang Province, China from December, 2003 to August, 2005 to test for PCV2 seroconversion by ELISA. All serum samples were stored at −20 °C until further use. Tissue samples of suspected PMWS pigs (n=200), including spleens, livers and lymph nodes, were also collected to test for the presence of PCV2 DNA by PCR.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA kit (KEQIAN Co., Ltd., Hubei, China) was used for detection of PCV2 antibodies. It was performed according to the manufacturer's instruction. A sample was considered positive if the value of OD_{650} (optical density at 650 nm) was 0.5 or greater. The known positive and negative sera were included as controls.

Polymerase chain reaction (PCR)

The procedure used for DNA purification has been previously described (Yang *et al*., 2007). Genomic DNA was dissolved in 50 μl of distilled water as template. The primers for detection of PCV2 were designed by using PrimerSelect program in DNAStar package (DNASTAR Inc., Madison, WI, USA) according to the published PCV2 sequences (GenBank accession No. AF027217). The primer sequences were as follows: 939 forward (nt 939 to nt 958), 5′-GCC GAG GTG CTG CCG CT-3′; 33 reverse (nt 33 to nt 17), 5′-CAG TTC GTC ACC CTT TCC CC-3′. PCR was performed on a Perkin-Elmer GeneAmp PCR System. Two microlitres of DNA sample were added to a 50 μl total volume reaction mixture, with each primer at a concentration of 0.4 μmol/L, 0.2 mmol/L (each) dNTP, 10 mmol/L Tris-Cl (pH 9.0), 10 mmol/L KCl, 2 mmol/L $MgCl_2$, 8 mmol/L $(NH_4)_2SO_4$, 0.05% NP-40, and 2.5 units Taq DNA polymerase (SNBC, China).

The amplification involved incubation for 5 min at 94 °C as first denaturation, followed by 30 cycles consisting of 45 s at 94 °C (denaturation); 45 s at 59 °C (annealing), 1 min at 72 °C (extension), and a final incubation for 8 min at 72 °C. The amplicons of 863 bp were detected by electrophoresing through 1% agarose gels containing 0.5 μg/ml ethidium bromide (EB). ORF2 products of 11 PCV2 strains isolated in Hangzhou were sent for DNA sequencing. The origins of the Hangzhou isolates are shown in Table 1.

Table 1 Geographic origin, year of isolation of PCV2 examined in this study

Isolate ID	Origin	GenBank accession No.	Year
AA	Fuyang	DQ231511	2004
BB	Tonglu	DQ231512	2004
CC	Chun'an	DQ231513	2005
DD	Lin'an	DQ231514	2005
EE	Lin'an	DQ231515	2005
FF	Yuhang	DQ231516	2003
GG	Xiaoshan	DQ231517	2003
HH	Xiaoshan	DQ231518	2005
II	Jiande	DQ231519	2003
JJ	Fuyang	−	2004
HZ1	Xiaoshan	AY539828	2004

Nucleotide sequencing and analysis

Before sequencing, PCR products were purified using a commercial kit according to the manufacture's instructions (BioDev PCR purification kit, Bio. Tec. Inc., China). Purified PCR products were sent to the AuGCT Co., Ltd. (Beijing, China) for sequencing using standard automated method.

Sequence alignments were done using the Editseq and MegAlign computer programs (DNASTAR Inc., Madison, WI, USA). The sequences of PCV2 isolates from other regions used in this study were retrieved from GenBank (Table 2).

Table 2 Sequences of PCV2 isolates from other countries retrieved from GenBank were used for comparison

Isolate ID/origin name	Geographic origin	GenBank accession No.	References
2A	Canada	AF027217	Hamel *et al*., 2000
M226	Canada	AF086836	–[1]
2D	Canada	AF117753	Hamel *et al*., 2000
Imp.999	US	AF055391	Meehan *et al*., 1998
ISU-31	US/Iowa	AJ223185	Morozov *et al*., 1998
FRA3	France	AF201311	Mankertz *et al*., 2000
Imp.1011-48121	France	AF055393	Meehan *et al*., 1998
SPA1	Spain	AF201308	Mankertz *et al*., 2000
Jap	Japan	AB072303	–[2]
NL_Control_4	Netherlands	AY484410	Grierson *et al*., 2004
GER1	Germany	AF201305	Mankertz *et al*., 2000
GER3	Germany	AF201307	Mankertz *et al*., 2000

[1]No reference and the year in GenBank is 1998; [2]No reference and the year in GenBank is 2001

The percentages of sequence identity among different PCV2 strains were determined and the phylogenetic tree was constructed using Clustal V method with weighted residue weight table (MegAlign program, DNAStar). Predicted amino acids were analyzed in a similar manner. Antigenic determinants in the ORF2 protein of selected sequences were predicted using a computer program, PROTEAN (DNASTAR Inc., Madison, WI, USA), which takes into account hydrophilicity, surface probability, chain flexibility and secondary structure.

Table 3 Prevalence of PCV2 in each county of Hangzhou by ELISA or PCR

County	ELISA		PCR	
	n_1	n_2 (percentage)	n_1	n_2 (percentage)
Xiaoshan	350	230 (66%)	90	81 (90%)
Lin'an	150	52 (35%)	25	15 (60%)
Yuhang	200	75 (38%)	20	16 (80%)
Fuyang	150	45 (30%)	20	17 (85%)
Tonglu	150	38 (25%)	15	13 (87%)
Jiande	150	45 (30%)	15	10 (67%)
Chun'an	100	15 (15%)	15	13 (87%)
Total	1250	500 (40%)	200	165 (83%)

n_1: Number of tested pigs; n_2: Number of samples positive for PCV2

RESULTS

Overall PCV2 seroprevalence

Out of 1250 randomly chosen serum samples tested, 500 (40%) were seropositive for PCV2 (Table 3). The positive rate in 1- to 50-day-old pigs was 40%, 63% for those at 50- to 160-day-old, and 31.5% for boars and sows.

PCV2 DNA detection by PCR

PCR amplification demonstrated that 165 were positive for PCV2 from 200 tissue samples. Prevalence of PCV2 in pigs from each county ranged from 60% to 90% (average 83%) by PCR. Xiaoshan County, the highest density area of pig production of Hangzhou, was the major source of submitted samples, and among 90 pigs from Xiaoshan County, 81 (90%) were PCV2 positive (Table 3).

The complete ORF2 nucleotide sequence of the 11 PCV2 strains were determined and found to be 702 (majority) or 705 nt (FF strain) in length. In pairwise comparisons, these 11 strains shared 93.3%~100% overall nucleotide homology and 91.5%~99.6% amino acid sequence identity. Except for FF, the nucleotide and amino acid similarity among them was 99.3% and 97.9%, respectively.

Besides the 11 PCV2 sequences reported in the present paper, 66 ORF2 gene sequences from China deposited in GenBank database were included for alignment. The nucleotide homology between the 77 PCV2 strains varied from 90% to 100%, whereas the variation at the amino acid level was 89.3%~99.6%. Sequence alignment of the capsid protein revealed areas of greater heterogeneity at residues 59~91, 121~134, 185~191, 206~215 and 232~234.

Phylogenetic analysis of ORF2 gene of PCV2 strains

The phylogenetic tree was generated by comparing Hangzhou PCV2 strains of this study with 68 Chinese PCV2 sequences and those of foreign countries (Fig.1). The sequences were segregated into

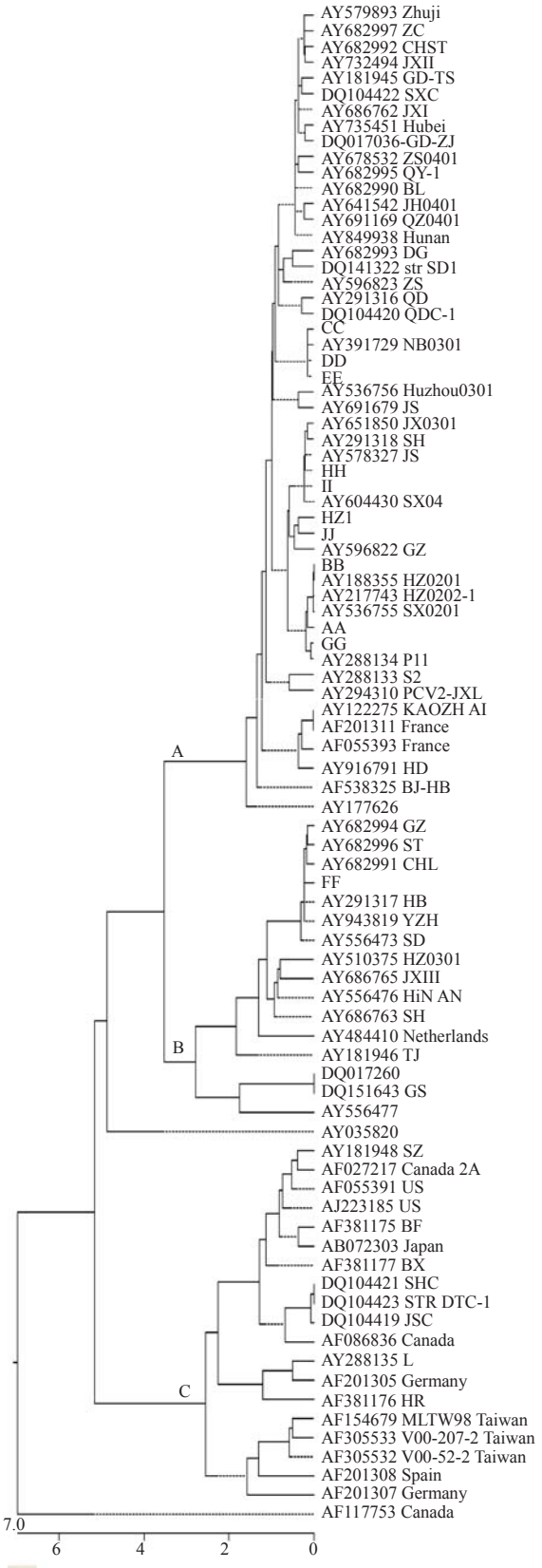

Fig.1 Phylogenetic analysis based on the nucleotide sequence of the ORF2 gene of PCV2

three major groups A, B and C. There was no distinct epidemiological feature based on geography in China, except for the Taiwanese PCV2 strains. In the upper part of the tree, one large cluster contained more than 50% of Chinese PCV2 strains. Within this cluster, some isolates were grouped according to farm origin (e.g., DD and EE from the same farm) or geographic origin (e.g., AA, BB, GG, AY536755, AY188355 and AY217743). But some PCV2 strains from different provinces also belonged to this cluster. For example, AY181945 was closely related to AY849938 originated from Guangdong and Hunan Provinces, respectively.

Isolate FF was closely related to PCV2 isolates from Guangdong Province (AY682994, AY682996 and AY682991). The strain closest to FF in GenBank was the NL_Control_4 (Netherlands) by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Most foreign PCV2 strains fall into Cluster C with a few Chinese PCV2 isolates, including strains BF, BX and HR submitted to GenBank early in 2001. The Taiwanese PCV2 strains also existed in this cluster.

Predicted antigenic differences

Sequences from isolates AA, FF and 2A standing for Clusters A, B and C, as well as U49186 standing for PCV1 were used to predict antigenicity. The residues 206~215 regions of AA, FF and 2A had higher predicted antigenicity than PCV1 (Fig.2).

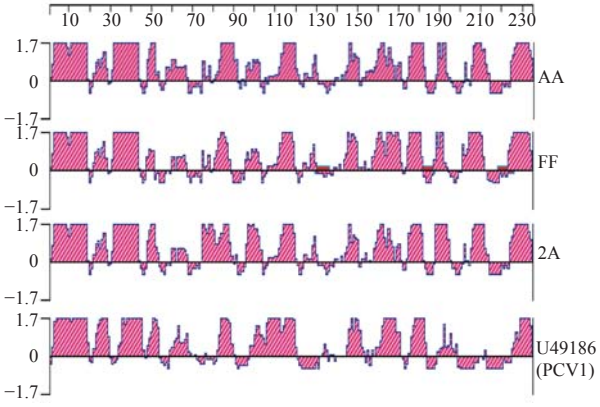

Fig.2 Antigenicity plot of selected PCV2 based on the ORF2 tree in Fig.1

The Jameson-Wolf antigenicity plot was generated using a computer program, PROTEAN (DNASTAR Inc., Madison, WI, USA). A high score indicates high antigenicity. A detailed description of the AA, FF, 2A sequences is given in Tables 1 and 2

DISCUSSION

This study showed that the PCV2 infection was widely spread in swine herds throughout Hangzhou as shown by high seroprevalence from 15% to 66% as well as 78% of the pigs suspected of having PMWS being PCV2 positive by PCR.

The ORF2 gene has been used for molecular epidemiological study by PCR-RFLP (restriction fragment length polymorphism) or sequence alignment (Larochelle *et al*., 2002; Wen *et al*., 2005). In the present study, we sequenced 11 ORF2 genes of PCV2 strains. Sequence analysis indicated that they were closely related to each other (lowest homology of 93.3%) and to other PCV2 ORF2 strains from other regions of China (lowest homology of 90%). Alignment of the PCV2 capsid protein identified five major regions of amino acid heterogeneity between PCV2 strains: amino acid regions 59~91, 121~134, 185~191, 206~215 and 232~234. It is interesting note that three of these regions (59~91, 185~191 and 232~234) correspond with three dominant immunoreative areas identified by Lekcharoensuk *et al*.(2004). Truong *et al*.(2001) verified aa 69~83 and aa 117~131 as immunorelevant epitopes by ELISA using PCV2 polyclonal antibodies. Mahe *et al*.(2000) reported similar findings by Pepscan analysis performed on genes encoding ORF2. P110A and R191S mutations in the capsid of PCV2 enhanced the growth ability of PCV2 in vitro and attenuated the virus in vivo (Fenaux *et al*., 2004). In all of the ORF2 deduced amino acid (including foreign sequences) used in this study, aa 110 is P, and aa 191 is G, R or A. For Hangzhou PCV2 ORF2, all had G at aa 191. It remains unclear if this particular G is related to pathogenicity.

Phylogenetic analysis of Chinese PCV2 isolates based on the ORF2 gene has been reported (Wen *et al*., 2005). We reported here the phylogenetic analysis of 11 Hangzhou PCV2 isolates along with those of 66 Chinese PCV2 ORF2 sequences from GenBank, representing most of strains from China. We found one cluster composed of about more than 50% Chinese PCV2 isolates, including 10 strains from Hangzhou. The majority of Chinese ORF2 genes were closely related to French strains, whereas Canadian and American ORF2 genes distributed apart from this cluster, which suggests that Chinese PCV2 strains could be more closely related to those of Europe. The FF was a distinct one with the unique feature of its His-207, which was different from the Tyr-207 found in all other strains.

It is a noteworthy feature in epidemiology that Clusters A and B were submitted from 2002 to 2005 and 2003 to 2005, respectively, while Cluster C from 1999~2005 (data not shown). In addition, the percentage of the Cluster A has increased to about more than 50% to date. This leads us to think that although PCV2 strains are relatively stable and closely related to each other, their pathogenecity might change with time due to point mutation.

In conclusion, the present experiment demonstrates that PCV2 infection was prevailing in Hangzhou, and PCV2 isolates in this area were closely related to majority isolates from other regions of China.

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