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Novel Genotypes of Type 2 Porcine Circovirus (PCV2) in PMWS Pigs in China Between 2008 and 2009

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Abstract: The present study analyzed genetic variation and genotypes of 17 strains of type 2 Porcine Circovirus (PCV2) from different epidemic regions in China in 2008 and 2009. All the genomic sequences were 1.767 bp in length. Sequence comparison of complete genomic sequences revealed 95.6-99.9% identity among 17 PCV2 strains and the most variable regions within 1.000-1.700 nt (located in the coding region of ORF2). Comparative analysis of amino acids of the two ORFs revealed that variation extend of ORF2 (93.1-100%) was greater than ORF1 (98.4-100%) and the third codon position showed much more variable than the first and second sites. Mutations in T and B lymphocyte epitopes were also detected by comparative analysis and it was found that T lymphocyte epitopes were more conserved than those of B lymphocytes. Phylogenetic analysis revealed 6 novel genotypes of PCV2 in addition to the 5 known geneotypes (PCV-2a, PCV-2b, PCV-2c, PCV-2d, PCV-2e) reported. Of these genotypes, the PCV-2b, PCV-2d and 3 unidentified genotypes were the most prevailing, within 13, 17 and 16 epidemic provinces, respectively. For the 17 Chinese PCV2 strains examined in this study, 5 strains represented PCV-2b genotype, 6 strains were PCV-2d, 1strain was PCV-2e and other 5 strains were novel genotypes while no strains were PCV-2a and PCV-2c genotype. These findings demonstrated the usefulness and attributes of complete genomic sequences for genetic variation and genotyping of PCV2 and have implications for the studies of population biology, molecular epidemiology and genetic structure of PCV2 and for the effective control of PMWS as well.

Key words: Genetic variation, genotype, PCV2, PMWS, epidemiology, China

INTRODUCTION

Porcine Circovirus (PCV), a non-enveloped virus with single-stranded and circular DNA (Jiang *et al*., 2010) was a member of the Circoviridae family (Lukert *et al*., 1995) and it can be classified into two types (PCV1 and 2) according to its antigenicity, pathogenicity and genomic difference (Allan *et al*., 1998; Morozov *et al*., 1998; Larochelle *et al*., 2002). PCV1 is considered non-pathogenic (Allan *et al*., 1994; Olvera *et al*., 2007) while PCV2 is described as the pivotal and primary causative agent of Postweaning Multisystemic Wasting Syndrome (PMWS) (Ellis *et al*., 1998; Fenaux *et al*., 2002, 2003; Ma *et al*., 2007; Shang *et al*., 2007; Li *et al*., 2010) and it also is associated with Porcine Dermatitis and Nephropathy Syndrome (PDNS), porcine reproductive disorders and other disease syndromes (Ladekjaer-Mikkelsen *et al*., 2001; Meehan *et al*., 2001; Segales *et al*., 2005; Shang *et al*., 2007).

PMWS is a newly emerging and economically important disease in pigs originally reported in Canada (Allan *et al*., 1998; Ellis *et al*., 1998; Allan and Ellis, 2000; Wen *et al*., 2005; Stevenson *et al*., 2007). It has been acknowledged in many European countries, some Asian countries and the US (Wen *et al*., 2005; Liu *et al*., 2007; Stevenson *et al*., 2007). Typically, clinical PMWS associated with PCV2 affects pigs from 5-12 weeks old (Wen *et al*., 2005; Jiang *et al*., 2010) with 5-50% morbidity and nearly 100% mortality (Morozov *et al*., 1998; Shen *et al*., 2009; Jiang *et al*., 2010). Clinical signs of the disease include pallor, fever, jaundice and progressive weight loss, together with respiratory and digestive disorders (Clark, 1997; Liu *et al*., 2007; Olvera *et al*., 2007; Shen *et al*., 2009). It can also be pathologically characterized by lymphocyte depletion and granulomatous inflammation of lymphoid tissues (Rosell *et al*., 1999; Olvera *et al*., 2007). Now a days,

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PMWS has caused great concern throughout the swine industry because of its rapid, Simultaneous and worldwide emergence as well as the uncertain transmission mode and high mortality rates in pigs (Jiang *et al*., 2010).

Based on nucleotide or protein sequences of the complete genome or single (or combined) gene, phylogenetic and evolutionary studies are of increasing importance in molecular epidemiological studies on viral and bacterial pathogens (Jiang *et al*., 2010). The genome of PCV2 contains 1.767-1.768 Nucleotides (nt) which comprise 6 Open Reading Frames (ORFs) encoding putative proteins (Meehan *et al*., 1998; Stevenson *et al*., 2007). However, only 3 proteins encoded by ORF1, ORF2 and ORF3 have been detected mainly in PCV2 infected cells (Cheung, 2003). Among them, ORF1 encodes 2 replication associated proteins (Rep and Rep) (Mankertz *et al*., 1998; Cheung, 2003; Shang *et al*., 2007), ORF2 encodes a viral Capsid Protein (Cap) which is involved in the host immune responses (Meehan *et al*., 1998; Mahe *et al*., 2000; Nawagitgul *et al*., 2000; Shang *et al*., 2007).

Previous studies indicated that the PCV2 was a kind of multi-genotype virus based on the ORF2 and the whole genomic sequences as well (Larochelle *et al*., 2002; Olvera *et al*., 2007) which would increase viral complexity in the PMWS pigs in different epidemic regions or eras (Shuai *et al*., 2007; Segales *et al*., 2008; Wang *et al*., 2009).

To monitor the latest genotypic variation of PCV2 in PMWS pigs in China, especially in the high-production provinces, 17 PCV2 strains were collected from 11 epidemic provinces in China in 2008 and 2009. The present study described the complete genomic sequence analysis of these 17 PCV2 strains including their genetic variations and genotypes.

MATERIALS AND METHODS

Field samples and DNA preparation: From January 2008 to December 2009, clinical samples (lungs, lymph nodes, tonsil, livers and spleens) of pigs presenting clinical signs and lesions associated with PMWS were collected from different farms of 11 provinces in China. PCR and histopathologic analysis were used to confirm the samples and the PCV2 positive samples were used for genome amplification.

Representative PCV2 stains with detailed information including the designation, date of isolation, geographic origin, genome size, GenBank™ accession number are shown in Table 1.

For tissue samples, homogenates comprising 1:5 tissue/Phosphate Buffer Saline (PBS) were obtained after grinding in a glass homogenizer and were disrupted by freeze-thawing three times. The supernatants containing viruses were recovered by centrifugation and then were treated using penicillin and streptomycin and filter (0.22 μm). The final solutions were added to the cell monolayer of PK-15 cells for 1.5-2 h infection at 37°C and then discarded the infection solution and added maintenance media to culture the PCV2 virus. After 3-5 blind passages, the supernatants of cultural cells were used to extract total viral DNA with DNAZol extraction kit (Invitrogen) according to the manufacturer's recommendations. RNA was removed with the RNAase treatment step of the kit. DNA was recovered with centrifugation and then it was dried and dissolved in sterile water.

Enzymatic amplification and sequencing: The complete genome of PCV2 was amplified with primers P1 (-CCGggatccCCGCGGGCTGGCTGAACTTTTAAAAGT-3)

Table 1: PCV2 strains sequenced in the present study

Designation	Geographic origin	Date of the isolation (Month/Year)	GenBank™ accession number
FJ-3	Fujian (Qiuhui)	4/2009	HM776437
FJ-4	Fujian (Qiuhui)	4/2009	HM776438
HLJ-7	Heilongjian	11/2008	HM776439
HLJ-10	Heilongjian	11/2008	HM776440
HUB-5	Hubei (Wuhan)	3/2009	HM776441
HUN-2	Hunan (Loudi)	6/2009	HM776442
HUN-11	Hunan (Changsha)	5/2009	HM776443
HUN	Hunan (Changde)	5/2009	HM776444
JX-1	Jiangxi (Xinjian)	4/2008	HM776445
JX-2	Jiangxi (Xinjian)	4/2008	HM776446
LN-3	Liaoning (Anshan)	5/2009	HM776447
LN-19	Liaoning (Anshan)	5/2009	HM776448
SC-10	Sichuan (Leshan)	3/2009	HM776449
SD	Shandong	4/2009	HM776450
SX-1	Shaanxi	3/2009	HM776451
YN-8	Yunnan (Kunming)	5/2009	HM776452
ZJ-38	Zhejiang	11/2008	HM776453

Table 2: Genome sequences of PCV2 strains deposited in the GenBank

Designation	Geographic origin	Year of the isolation	Genome size (nt)	GenBank™ accession number	Reference
J0955b	Zhejiang	2009	1767	GU450329	Unpublished
GXWM	Guangxi	Unknown	1767	EF675241	Unpublished
HBsy-21	Hubei	2008	1767	FJ870972	Unpublished
HZ08	Zhejiang	2008	1767	FJ644927	Li *et al.* (2010)
SD-1	Shandong	Unknown	1767	AY556473	Unpublished
TJ06	Tianjin	2006	1767	EF524539	Wang *et al.* (2009)
CG08	Shanghai	2008	1767	FJ644931	Li *et al.* (2010)
GXHZ-1	Guangxi	Unknown	1767	EF675230	Unpublished
CHL	Guangdong	Unknown	1767	AY682991	Unpublished
GL08	Jiangsu	2008	1767	FJ644929	Li *et al.* (2010)
ZJjh-s	Zhejiang	2008	1767	FJ870970	Unpublished
HBwh-24	Hubei	2008	1767	FJ870971	Unpublished
JZ	Shandong	Unknown	1767	DQ206444	Unpublished
TW	Taiwan	Unknown	1768	AF166528	Unpublished
MLTW98	Taiwan	Unknown	1768	AF154679	Unpublished
Zhejiang2006	Zhejiang	Unknown	1767	EF210106	Unpublished
CHST	Guangdong	Unknown	1767	AY682992	Unpublished
BJ0602	Beijing	2006	1768	EF524540	Wang *et al.* (2009)
HB0603	Hebei	2006	1768	EF524538	Wang *et al.* (2009)
FJ	Fujian	Unknown	1768	AY556474	Unpublished
GXGG-2	Guangxi	Unknown	1768	EF675229	Unpublished
HN0601	Henan	2006	1768	EF524531	Unpublished
LN05	Liaoning	2005	1767	EF524526	Wang *et al.* (2009)
HB0602	Hebei	2006	1768	EF524537	Wang *et al.* (2009)
WX06	Shanghai	2006	1767	FJ644926	Li *et al.* (2010)
GXHK	Guangxi	Unknown	1767	EF675238	Unpublished
HNyz-3b	Henan	2008	1767	FJ870976	Unpublished
QY	Guangdong	Unknown	1767	AY682995	Unpublished
SH0822	Shanghai	2008	1767	GU450330	Unpublished
BJW	Beijing	2005	1767	AY847748	Liu *et al.* (2007)
GSLZ	Gansu	Unknown	1767	FJ447482	Unpublished
DQ08	Zhejiang	2008	1767	FJ644928	Li *et al.* (2010)
CQ08	Chongqing	2008	1767	FJ608544	Wang *et al.* (2009)
GX	Guangxi	Unknown	1767	AY556475	Unpublished
SC07	Sichuan	2007	1767	FJ608538	Wang *et al.* (2009)
JX0601	Jiangxi	2006	1767	EF524535	Wang *et al.* (2009)
SD-2	Shandong	2002	1767	AY181947	Wang *et al.* (2009)
HaiNan	Hainan	Unknown	1767	AY556476	Unpublished
SDrc-1b	Shandong	2008	1767	FJ870975	Unpublished
ShenZhen	Shenzhen	2008	1767	FJ870969	Unpublished
HKS03-04	Hongkong	2004	1767	DQ997816	Ma *et al.* (2007)
HKS091-04	Hongkong	2004	1767	DQ997817	Ma *et al.* (2007)
HEN0901	Henan	2009	1767	GU450328	Unpublished
AH0901	Anhui	2009	1767	GU450327	Unpublished
HuB08	Hubei	2008	1767	FJ608542	Wang *et al.* (2009)
GXLC	Guangxi	Unknown	1767	EF675240	Unpublished
HuNan	Hunan	Unknown	1767	AY556477	Unpublished
GY06	Jiangsu	2006	1767	FJ644921	Li *et al.* (2010)
JXI	Jiangxi	Unknown	1767	AY686762	Unpublished
ZJ0401	Zhejiang	2004	1767	EF524521	Wang *et al.* (2009)
TJ04	Tianjin	2004	1767	EF524520	Wang *et al.* (2009)
GS04	Gansu	2004	1767	EF524517	Wang *et al.* (2009)
SX04	Shanxi	2004	1768	EF524523	Wang *et al.* (2009)
ZC	Guangdong	Unknown	1767	AY682997	Unpublished
SX04New	Shanxi	Unknown	1767	AY604430	Unpublished
HB0702	Hebei	2007	1767	FJ608541	Wang *et al.* (2009)
SH1	Shanghai	2002	1767	FJ644919	Li *et al.* (2010)
BJ0401	Beijing	2004	1767	EF524515	Wang *et al.* (2009)
HT06	Shanghai	2006	1767	FJ644925	Li *et al.* (2010)
SD05	Shandong	2005	1767	FJ644563	Zhao *et al.* (2009a)
QZ05	Fujian	2005	1767	FJ644562	Zhao *et al.* (2009b)
Stoon-1010	Canada	Unknown	1768	AF055392	Meehan *et al.* (1998)
48285	France	Unknown	1767	AF055394	Meehan *et al.* (1998)
DK1980 PMWSfree	Denmark	Unknown	1767	EU148503	Dupont *et al.* (2008)
TJ	Tianjin	2002	1767	AY181946	Wang *et al.* (2009)
GX0601	Guangxi	2006	1768	EF524532	Wang *et al.* (2009)

and P2 (5-CAT ctcg ag ACCCGCGG AAA TTTCT GA CAAACGTTACA-3). The primer pairs were designed using software Oligo (Version 6.0) according to the published sequences of PCV2 in the GenBank™ (Table 2). PCR mix (25 μL) included 2.5 μM of each primer, 2.5 μL of 10×rTaq buffer, 0.2 mM of each dNTP, 1.25U of rTaq DNA

polymerase (TAKARA) and 1 μL of DNA sample in a thermocycler (Biometra). The PCR was performed with the following conditions: an initial denaturation at 94°C for 35 min, followed by 94°C for 1 min (denaturation); 54°C for 1 min (annealing); 72°C for 1.5 min (extension) for 35 cycles then with a final extension at 72°C for 10 min. These optimized cycling conditions for the specific and efficient amplification of individual DNA fragments were obtained after varying annealing temperatures). Samples without genomic DNA (no-DNA controls) or with host genomic DNA (host-DNA controls) were included in each amplification run and in no case were amplicons detected in the no-DNA and host-DNA controls. Each amplicon (5 μL) was examined by agarose gel electrophoresis to validate amplification efficiency (Zhao *et al*., 2009a, b).

The PCR products were purified using spin columns (Wizard™ PCR-Preps DNA Purification System, Promega) and ligated with pGEM-T Easy plasmid vector (Promega) according to the manufacturer's recommendations. The recombinant plasmid was then transformed into *Escherichia coli* DH5α competent cells (Promega) and positive transformants were selected and checked by PCR amplification. Cell cultures with confirmed recombinant plasmid were cultured and sent to Songon Co. Ltd. (Shanghai) for sequencing. For each sample, 3 colonies were sequenced from both ends.

Molecular genetic analysis: Sequences of the complete genome were aligned using the Clustal X 1.81 (Thompson *et al*., 1997) and mVISTA (http://genome.lbl.gov/vista/mvista/submit.shtml). Alignments were checked by eye and modifications were made as required. Megalign procedure within the DNAStar 5.0 (Burland, 2000) was used to analyze sequence identity and to calculate base composition and the divergence. The Neighbor-Joining (NJ) method in Mega version 4.0 (Tamura *et al*., 2007) was used to examine the genetic relationship under default settings. The consensus tree was obtained after bootstrap analysis, with 1,000 replications and with values above 50% reported. We divided PCV2 genotypes with the following criteria as described by previous studies (Segales *et al*., 2008; Wang *et al*., 2009): when the ORF2 genetic distance between them is >0.035 and agrees with the distance between viral sequence groups in the phylogenetic trees revealed by complete genomic sequences, the two PCV2 genotypes are regarded as different. Phylograms were drawn using the Tree View program version 1.65 (Page, 1996).

RESULTS AND DISCUSSION

Sequence analysis of nucleotide and amino acid: Genomic DNA was prepared from 17 stains in 11 epidemic provinces of China. The complete genomes were amplified successfully from all of the samples. The amplicons were subjected to agarose gel electrophoresis, sequenced and then deposited into the GenBank™ with accession numbers HM776437-HM776453 (Table 1). No size variation was detected in all of the genomic sequences which were 1.767 bp in length. The A+T contents of complete genomic sequences were 51.10-51.78%, slight higher than G+C contents. All the complete genomic sequences of PCV2 in the present study contained at least six Open Reading Frames (ORFs), encoding 2 major proteins (Rep and Cap) as previous studies reported (Shang *et al*., 2007; Wang *et al*., 2009). The comparison of complete genomic sequences revealed 95.6-99.9% identity in the 17 PCV strains and the most variable regions were within 1.000-1.700 nt (located in the coding region of ORF2) (Fig. 1) which was consistent with previous study in China (Wang *et al*., 2009). Among these viruses, ZJ-38 and HLJ-10 showed the highest identity with a percentage nearly to 100%. Comparative analysis of amino acids of the two ORFs revealed that the variation extend of ORF2 (93.1-100%) was greater than ORF1 (98.4-100%) and the third codon position showed more variable than the first and second sites.

To date, at least 6 linear immunodominant regions and 5 overlapping conformational B lymphocyte epitopes within 47-85, 156-202 and 230-233 residues in the PCV2 Cap were revealed by PEPSCAN analysis and mAbs scanning (Mahe *et al*., 2000; Shang *et al*., 2007; Lekcharoensuk *et al*., 2004). Previous study also showed that 2 immunodominant regions encoded by PCV2 ORF1 (amino acid residues 81-100 and 201-220) and 1 region encoded by PCV2 ORF3 (amino acid residues 31-50) appeared to be able to induce T lymphocyte proliferation in porcine Peripheral Blood Mononuclear Cells (PBMCs) from infected pigs (Stevenson *et al*., 2007). It suggested that the T lymphocytes responses to PCV2 are primarily directed toward epitopes of the nonstructural proteins of ORF1 and ORF3. Comparative analysis of these regions detected 13 mutations (Table 3) in ORF2, 2 in ORF1 and 1 in ORF3 and T lymphocyte epitopes were more conserved than those of B lymphocytes (Table 3).

Phylogenetic relationships among PCV2 sequences: The NJ phylogenetic tree of the 17 Chinese PCV2 strains sequenced in the present study, together with 66 PCV2

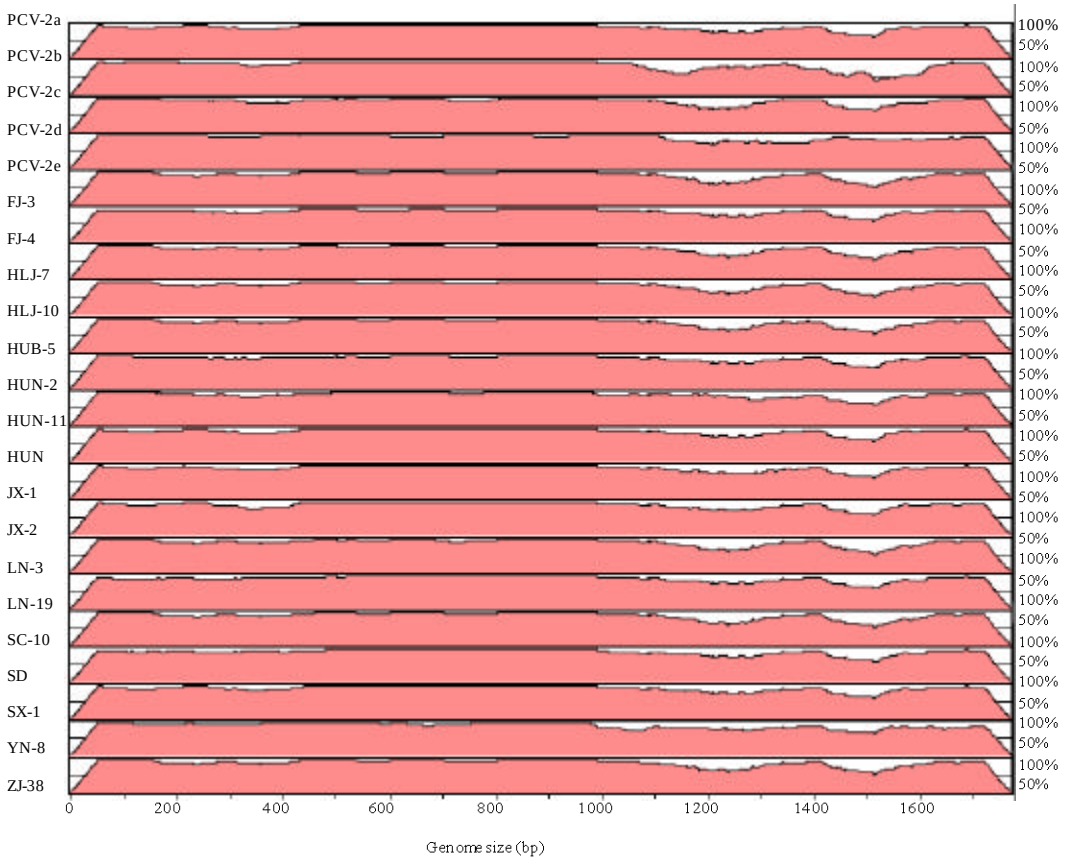

Fig. 1: Comparative analysis of complete genomic sequences of PCV2 in China using mVISTA

Table 3: Mutations in the sequences of T and B lymphocyte epitopes of PCV2

Epitope region (aa)	Mutation sites
47-85	47 (T-A), 53 (F-I), 57 (V-I), 59 (R-K-A), 63 (R-K-T), 68 (A-N)
165-200	168 (S-R), 175 (P-R), 185 (L-M), 190 (A-T-S), 191 (G-A)
231-233	232 (N-H), 233 (P-T)

strains available in the GenBank™ (Table 2) with complete genomic nucleotide sequences, are shown in Fig. 2. All of these Chinese PCV2 strains were isolated from 25 epidemic provinces, respectively. Phylogenetic analysis showed 6 novel genotypes of PCV2 in addition to the 5 known geneotypes (PCV-2a, PCV-2b, PCV-2c, PCV-2d, PCV-2e) reported (Segales *et al*., 2008; Wang *et al*., 2009). The sequences were segregated into two large groups A and B. The upper part of the tree contained 2 genotypes (PCV-2d and PCV-2b) in previous studies and 2 novel genotypes (unidentified type 2 and 3).

Three genotypes (PCV-2a, PCV-2c, PCV-2e) with 3 novel genotypes were in the other clade. Of these genotypes, PCV-2b, PCV-2d and the 3 unidentified genotypes were the most prevailing, within 13, 17 and 16 epidemic provinces, respectively (Table 4). For the Chinese PCV2 strains in this study, 5 strains were in PCV-2b, 6 strains were in PCV-2d, 1 strains in PCV-2e and other 5 strains were in novel genotypes but no strains were in genotypes of PCV-2a and PCV-2c (Fig. 2).

The study of the evolution of the genetic diversity of virus strains and the existence of variants are very important. It provides a better understanding of the pathogenesis of these potentially emerging diseases and has implications for the development of prophylactic measures (Larochelle *et al*., 2002). On the other hand, point mutation and recombination are major mechanism of viral evolution (Ma *et al*., 2007).

In the present study, complete genomes of 17 PCV2 strains in 11 epidemic provinces were sequenced and analyzed. The sequences contained at least 6 ORFs and it was found that the Cap protein encoded by ORF2 was the most variable regions and it exhibited a higher rate of variation (the lowest homology reported being 90%) compared with ORF1 which was consistent with previous studies (Brunborg *et al*. 2004; Wen *et al*., 2005; Wang *et al*., 2009). The result of the present study further confirmed that capsid protein was the major structural protein responsible for viral pathogenecity (Hamel *et al*., 2000; Mankertz *et al*., 2000; Larochelle *et al*., 2002).

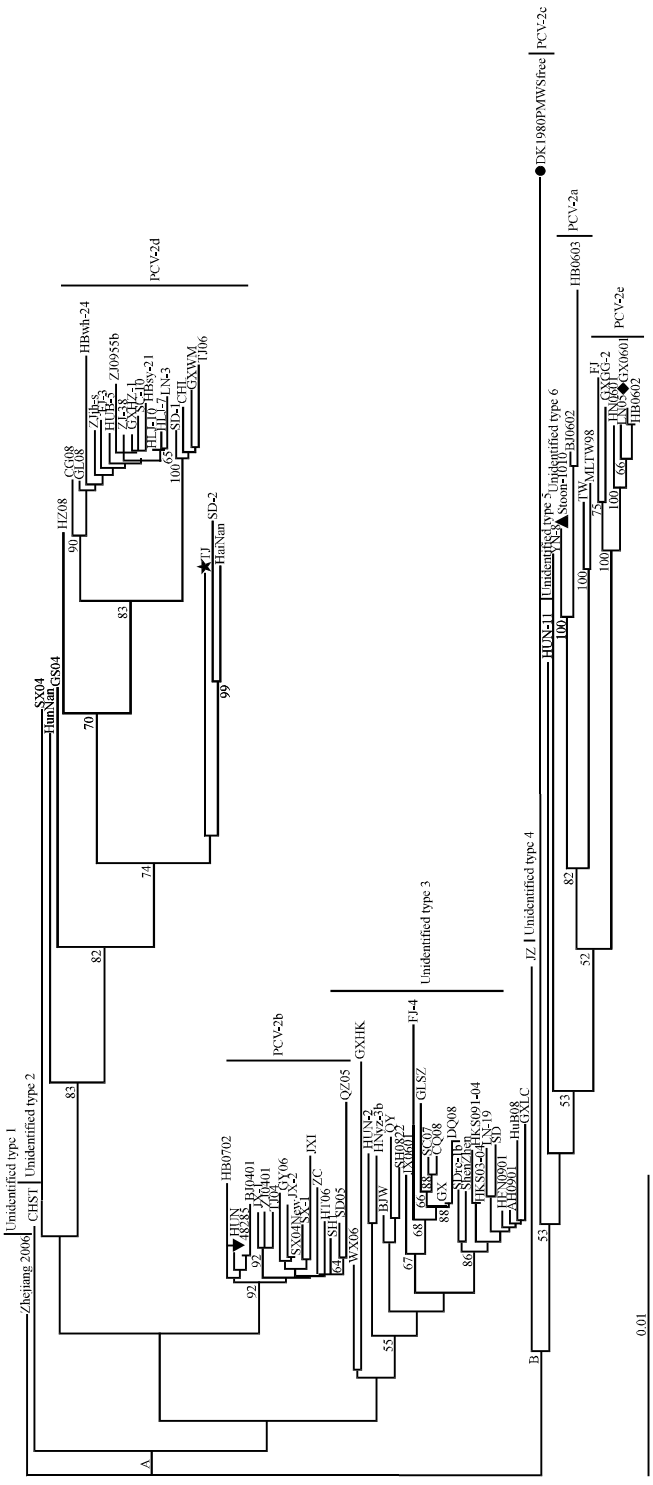

Fig. 2: Phylogenetic analysis of 17 PCV2 strains in this study and 66 strains isolates from other regions of China or other countries based on the complete genomic nucleotide sequences. The tree was constructed using Neighbor-Joining (NJ) algorithm by MEGA 4.0. (▲)Reference sequences for genotype PCV-2a; (▼) reference sequences for genotype PCV-2b; (●) reference sequences for genotype PCV-2c; (✶) reference sequences for genotype PCV-2d; (◆) reference sequences for genotype PCV-2e

Table 4: Genotypes of PCV2 in different provinces in China

Epidemic province	Genotype
Zhejiang	PCV-2b, PCV-2d, unidentified type 1 and 3
Guangxi	PCV-2d, PCV-2e, unidentified type 3
Hebei	PCV-2a, PCV-2b, PCV-2d, PCV-2e, unidentified type 3
Shandong	PCV-2b, PCV-2d, unidentified type 3 and 4
Tianjin	PCV-2b, PCV-2d
Hubei	PCV-2d, unidentified type 3
Shanghai	PCV-2b, PCV-2d, unidentified type 3
Sichuan	PCV-2d, unidentified type 3
Heilongjiang	PCV-2d
Liaoning	PCV-2d, PCV-2e, unidentified type 3
Fujian	PCV-2b, PCV-2d, PCV-2e, unidentified type 3
Jiangsu	PCV-2b, PCV-2d
Taiwan	PCV-2a
Hunan	PCV-2b, PCV-2d, unidentified type 3 and 5
Yunnan	unidentified type 6
Guangdong	PCV-2b, PCV-2d, unidentified type 2 and 3
Beijing	PCV-2a, PCV-2b, unidentified type 3
Henan	PCV-2e, unidentified type 3
Gansu	PCV-2d, unidentified type 3
Chongqing	unidentified type 3
Jiangxi	PCV-2b, unidentified type 3
Shanxi	PCV-2b, PCV-2d
Shaanxi	PCV-2b
Hainan	PCV-2d
Hongkong	Unidentified type 3

Simultaneously, deduced amino acid sequences alignment obtained mutations in epitopes of T and B lymphocytes as previous reports (Larochelle *et al*., 2002; Wen *et al*., 2005). Previous studies indicated that the genetic variations of PCV2 might be associated with geographic origin rather than with differences in tropism (Fenaux *et al*., 2000; Meehan *et al*., 2001). However, the study showed that the genotypes of PCV2 were not relation to their locations. PCV2 strains from different provinces contained some of the same mutation sites, while strains from the same province have different variations. Phylogenetic tree based on complete genomic sequences also showed that strains from the same provinces were categorized into different genotypes, while same genotypes were in different orgins. The genotypes in the same geographical orgins would be changed with years. These results could be one of the reasons for immunity complexity of PCV2 infection.

CONCLUSION

The present study examined genetic variations and genotypes of PCV2 strains from 11 epidemic provinces in China in 2008 and 2009 based on complete genomic sequence. Analyses of these sequences revealed low level genetic differences of PCV2 strains in China. Phylogenetic relationships analyses showed that there are novel genotypes of PCV2 existed in China. These results would have important implications for the effective prevention and control of PCV2 infection.

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