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Phylogenetic Analysis of the *ORF2* Genes of Porcine Circovirus 2 Strains Isolated in Yunnan Province, China

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Abstract: The *ORF2* genes of 10 isolated Porcine Circovirus type 2 (PCV2) strains in Yunnan Province of China were sequenced, analyzed and further compared with the sequences of 24 strains retrieved from GenBank. The results showed that the *ORF2* genes of the 10 strains in Yunnan Province shared sequence identity of 98.7-100.0% on nucleotide acid level and of 96.2-100.0% on amino acid level. Moreover, Yunnan strains and the other 24 strains retrieved from GenBank shared 91.0-99.7% nucleotide and 80.3-97.4% amino acid sequence similarity. Based on the phylogenetic tree analysis, these strains could be divided into five genotypes (PCV2a-PCV2e) and all the Yunnan strains fall into PCV2b genotype. Compared with other PCV2b strains, nine point mutations of amino acid residues were identified only in Yunnan strains which might be essential for controlling PCV2 infection in Yunnan Province, China.

Key words: Porcine circovirus type 2, *ORF2* gene, phylogenetic analysis, GenBank, Yunnan Province, China

INTRODUCTION

Porcine Circovirus (PCV) belongs to the family Circoviridae and the genus *Circovirus*. There exist two phenotypically distinct but genetically related strains of PCVs (Meehan *et al*., 1998). One is the Porcine Circovirus type 1 (PCV1) which was originally discovered in 1974 as a persistent contaminant of the porcine kidney PK-15 cell line ATCCCCL-33 (Tischer *et al*., 1982). This nonpathogenic virus is widespread in the swine population but does not cause clinical disease. The other type of strain PCV2 was found in association with Postweaning Multisystemic Wasting Syndrome (PMWS) in Canadian weaning piglets in 1991 (Cheung *et al*., 2007). PMWS is characterized by wasting or poor performance in weaned pigs and by lymphoid depletion and histiocytic replacement of follicles in lymphoid tissues (Krakowka *et al*., 2008). PCV2 is also associated with sporadic reproductive failure, enteritis, Porcine Dermatitis and Nephropathy Syndrome (PDNS), porcine respiratory disease complex and congenital tremor. Consequently, all these syndromes are widely called Porcine Circovirus Associated Disease (PCVAD) (Harding, 2004). PCVAD is a globally emerging disease, resulting in huge economic losses especially in swine-producing countries.

Moreover, PCVAD has become the main cause of many losses in pig herds in China recently (Menghou, 2005).

PCV2 has been divided into two distinct genotypes: Group 1 and 2 (Olvera *et al*., 2007). Originally, only group 2 isolates were found in the United States but in late 2005, several outbreaks with higher mortality were reported to be associated with PCV2 group 1 isolates in Kansas, North Carolina and Iowa (Ellis *et al*., 2004). At the same time, North American laboratories proposed to group PCV2 into North American isolates (PCV2a) and European-like isolates (PCV2b). PCV2b falls into group 1 while PCV2a into group 2 (Gagnon *et al*., 2007a). Within the PCV2 genotype, there are several sub-types (PCV2a-2e). PCV2a, PCV2b and PCV2d have existing in different geographic regions of China (Ge *et al*., 2012). The new genotype PCV2e has been found in pigs from Thailand (Jaganathan *et al*., 2011).

PCV2 is a single-stranded DNA virus containing a circular genome of 1,767-1,768 bp. The virus is predicted to possess 11 overlapping Open Reading Frames (ORFs). ORF1 (nucleotides 51-995) encodes the Rep and Rep' proteins which are absolutely essential for PCV2 replication. ORF2 (nucleotides 1735-1034) and *ORF3* (nucleotides 671-357) genes are encode the capsid protein and apoptotic protein, respectively (Nguyen *et al*., 2012).

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In this study, the *ORF2* genes of 10 PCV2 strains isolated in Yunnan Province of China were sequenced and analyzed to provide some basis for the molecular characterization of PCVs.

MATERIALS AND METHODS

Sample collection: A total of 10 PCV2 samples from lung, lymph node, liver, spleen and kidney were collected from swines with typical clinical syndromes of PCVAD in various districts in Yunnan Province of China from 2007-2008. All samples were stocked at -80°C until use.

Polymerase Chain Reaction (PCR): DNA was extracted from individual homogenous samples using the AxyPrepTM viral DNA miniprep kit (Axygen Scientific, USA) according to the manufacturer's instructions. PCR amplification was performed using ExTaq polymerase (Takara Biotechnology Co., Dalian, China). A pair of primers (ORF2-forward: 5'-AGT GAG CGG GAA AAT GCA GA-3', ORF2-reverse: 5'-TCC TCC GTG GAT TGT TCT GT-3') was used to amplify the *ORF2* gene from PCV2 viral genome and a fragment in length of 702 bp was expected to be amplified.

The PCR consisted of an initial enzyme-activation step at 94°C for 5 min followed by 29 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 45 sec with a final extension at 72°C for 10 min.

The amplified PCR product of the PCV2 *ORF2* gene was excised from the gel, purified and subsequently cloned into the pMD18-T vector (Takara Biotechnology Co.) with the sequence determined by an external company (Sangon Biological Engineering Technology and Service Co., Shanghai, China). The 10 sequences were named as YN01, YN02, YN03, YN04, YN05, YN07, YN08, YN09, YN12, YN13.

Phylogenetic analysis: Together with 24 references strains (Table 1), the 10 sequences from the study (Table 2) were analyzed. Nucleotide sequences and deduced amino acid sequences were aligned using the program CLUSTAL V.

Phylogenetic analysis was performed using the MEGA program, version 5.0.5. The minimum-evolution method was chosen to generate the phylogenetic tree. The reliability of the phylogenetic tree obtained for the ORF2 region was evaluated by running 1,000 replicates in the bootstrap test.

Table 1: 24 PCV2 strains with their geographic origins and GenBank accession numbers

Strains	Geographic origin	GenBank accession No.	Genotypes
Porcine circovirus Type II	Canada	AF055392	2a/reference
Japan	Japan	AB426905	2a
SPA	Spain	AF201308	2a
US	United states	AF264042	2a
TAINAN	China	AF166528	2a
CAN412	Canada	AF085695	2a
CAN34464	Canada	AF264043	2a
HLJ2	China	EF592575	2b
Porcine circovirus Type II	France	AF055394	2b/reference
QD	China	AY291316	2b
Yuanyang	China	EU521709	2b
BJ0804	China	EU921257	2b
BJ05	China	FJ644557	2b
GX0853	China	GQ359007	2b
GX08142	China	GQ359008	2b
Canada-FMV-05-6302	Canada	DQ220739	2b
DK1980PMWSfree	Denmark	EU148503	2c/reference
DK1987PMWSfree	Denmark	EU148504	2c
TJ06	China	EF524539	2d
TJ	China	AY181946	2d
HZ0301	China	AY510375	2d
THKUL16	Thailand	HQ701665	2e
THKUB98	Thailand	HQ735203	2e
THKUF49	Thailand	HQ701666	2e

Table 2: PCV2 strains isolated in Yunnan

Isolates	Nomination	GeneBank accession No.
YN01	YN01-02	FJ384966
YN02	YN02-03	FJ384967
YN03	YN03-04	FJ384968
YN04	YN04-06	FJ384969
YN05	YN05-08	FJ384965
YN07	YN07	JN989553
YN08	YN08	JN989554
YN09	YN09	JN989555
YN12	YN12	JN989556
YN13	YN13	JN989557

RESULTS AND DISCUSSION

To investigate the phylogenetic relationships of strains from Yunnan Province of China with other PCV2 strains, a phylogenetic tree based on the nucleotide acid sequence from 1-702 nt of the *ORF2* gene was constructed. As shown in Fig. 1, all PCV2 strains can be classified into fine genotypes (PCV2a-PCV2e) with the 10 PCV2 strains from Yunnan Province were grouped into PCV2b genotype (Fig. 1).

Sequence comparisons showed that the *ORF2* gene researchers obtained from Yunnan Province of China shared approximately 98.7-100.0% nucleotide and 96.2-100.0% amino acid sequence identities. Yunnan strains and other strains obtained from the GenBank shared 91.0~99.7 % nucleotide and 80.3~97.4% amino acid sequence identities.

Nucleotide sequences were translated into amino acid sequences to identify amino acid mutations. Critical

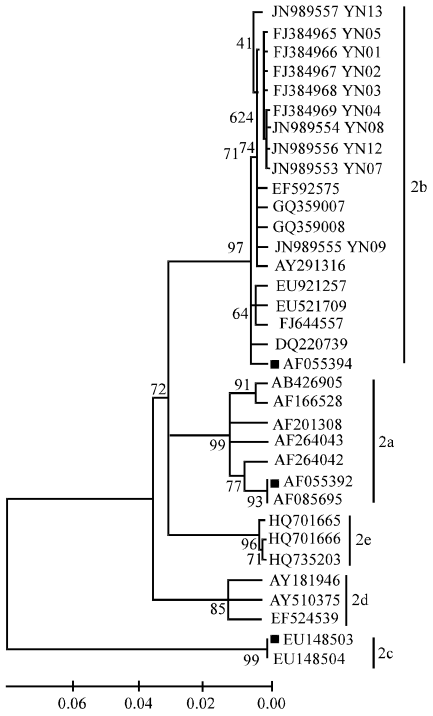

Fig. 1: Phylogenetic analysis of the *ORF2* gene of porcine circovirus 2 strains; unrooted minimum-evolution tree constructed from aligned nucleotide acid sequences of 10 strains from Yunnan Province of China and 24 sequences from GenBank; Original names and GenBank accession numbers are given in Table 1; The ORF2 sequences from prototype viruses of PCV2 genotypes are marked with a black rectangle

positions of the PCV2 *ORF2* gene products of isolates sequenced in this study were shown in Fig. 2. The amino acid differences were found at position 8 (F-S and YN09), 15 (L-S and YN07), 26 (L-P, YN07 and YN08), 34 (R-S, YN04, YN07, YN08, YN11 and YN12), 59 (W-R and YN12), 140 (V-A and YN07), 162 (S-P and YN09), 171 (R-C, YN04, YN07, YN08, YN11 and YN12) and 205 (E-D and YN09) for Yunnan Province PCV2 strains vs. other PCV2b strains in China (Fig. 2).

Genetically, PCV2 is a relatively stable virus. However, genomic variation of the virus has been noticed all over the world. Earlier studies have indicated that the ORF2 encoding for the major structural capsid protein exhibited a higher rate of variation in comparison with *ORF1* and *ORF3* genes among different strains of PCV2. PCV2 sequences isolated during 2001-2010 in China can be divided into three genotypes; genotype PCV2a, PCV2b and PCV2d (Ge *et al*., 2012).

PCVAD has been a globally emerging disease with huge impacts on swine-producing countries and is arguably the most economically important disease affecting the global swine industry. PCVAD is mainly caused by PCV2 in pigs but the prevalence is different in many countries and regions (Walker *et al*., 2000). Recently, Chinese scholars conducted a serological survey on PCV2 infection in swine and found that the positive rate of PCV2 antibodies were 75.6% indicating that PCV2 is also widespread in swine herds in China (Yanhong *et al*., 2007).

As shown in the phylogenetic tree (Fig. 1), all of the 10 strains isolated in Yunnan Province formed a cluster together with QD strain and Porcine circovirus Type II (France strain) which were in a PCV2b genotype cluster indicated by the previous surveys (Gagnon *et al*., 2007b). Similarly, the 10 isolates in Yunnan Province were also clustered into the PCV2b genotype. It is obvious that the PCV2b genotype is more prevalent in Yunnan Province than the PCV2a genotype (Li *et al*., 2009). Demonstrating that PCV2b was the predominant genotype in most regions of China. Similar results were also reported in Hong Kong in 2005 that the prevalence of PCV2b strains accorded with the simultaneous increases in clinical PMWS cases (Ma and Leung, 2005).

The two distinct PCV2 genotypes may imply marked differences in clinical pathogenicity, i.e., PCV2b is more virulent (Opriessnig *et al*., 2008). However, some other evidences did not support distinct genomic differences among PCV2 isolates recovered from healthy pigs and diseased pigs (Larochelle *et al*., 2002; Grierson *et al*., 2004) and molecular studies on the genetic variations of PCV2 have revealed that minor branches of PCV2 were associated with geographical origin rather than with differences in virulence (Meehan *et al*., 2001). These studies led to the assumption that there is no difference in pathogenicity among PCV2 isolates. In the study, all the 10 isolates were recovered from diseased pigs therefore, researchers still could not conclude whether the pathogencity of PCV2a and PCV2b was the same or different.

The ORF2 nucleotide sequence identities among the 10 PCV2b isolates were highly conserved, sharing similarities of 98.7-100.0%. It was reported that the immune responses of PCV2 monoclonal antibody against different chimeric virus were different and the rabbit anti-PCV2 hyperimmune serum recognized the combinated chimeric virus motif of 63-85 and 165-185 aa region in the PCV2 ORF2 (Lekcharoensuk *et al*., 2004). In present study, researchers found the R171C mutations in 5 of the 10 strains which may affect PCV2 virus-specific antibody binding. Larochelle *et al*. (2002) identified three

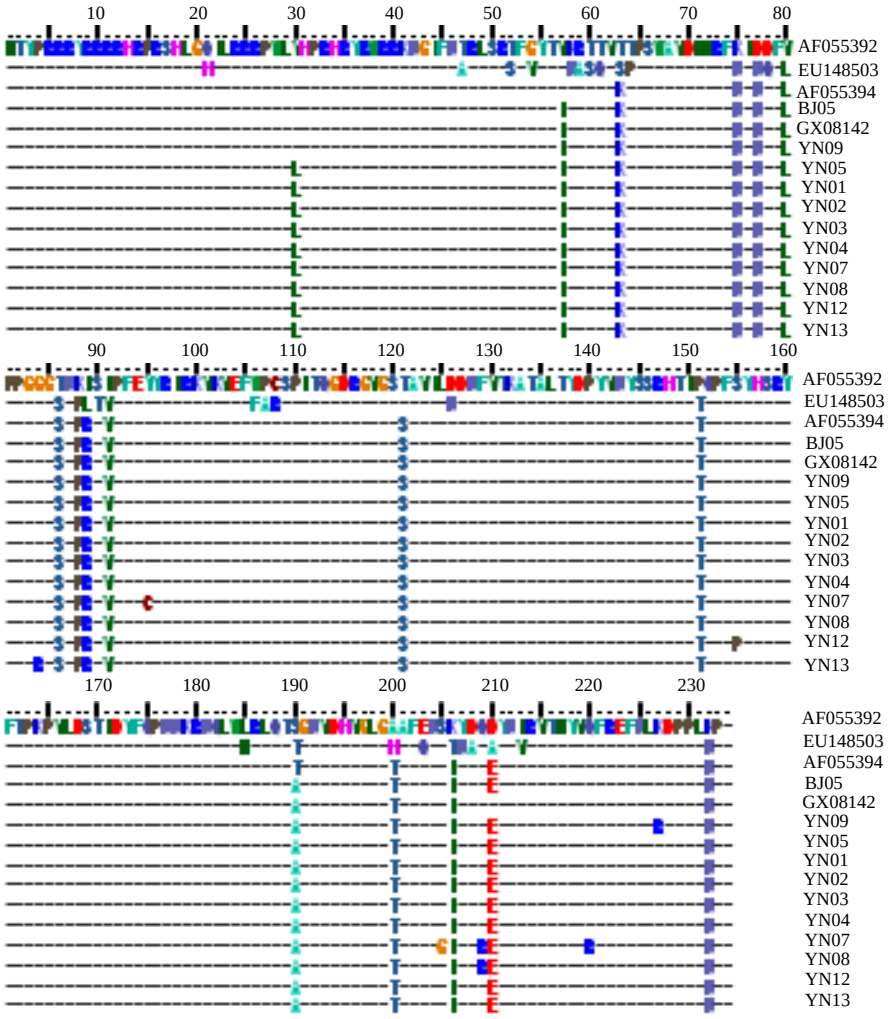

Fig. 2: Comparison of the ORF2 amino acid sequences among Yunnan Province and references strains; Residues that match the consensus are shown in hide as "–"

immunogenic regions in ORF2 at residues 59-80, 121-136 and 180-191 aa. None mutation was found in these three regions in the 10 PCV2 isolates in Southwest China except that the YN12 strain had the mutation at site 59 of the ORF2 encoded protein. These suggested that most PCV2 strains in Southwest China had no variation in the three epitopes. It was reported by Fenaux *et al*. (2004) that the two amino acid differences in ORF2, P110A and R191S could enhance the ability of PCV2 to grow *in vitro* and attenuated the virus *in vivo*. Neither of 10 PCV2 strains in this study had mutations in the two sites, indicating that the PCV2 isolates in Yunnan possessed the same genetic toxicity as the original isolate.

Compared with several other PCV2b strains in China, 21 amino acid mutations were found in 10 strains in Yunnan. In addition to a D-E mutation at 204 aa in YN09 strain, the other mutants were found only in the 10 strains. Furthermore, it was indicated by protein function prediction that there were two amino acid mutations, i.e., L15→S15 in YN07 and W59→R59 in YN12 in the hydrophilic region and potential antigenic sites, respectively. However, whether these mutations will affect the PCV2 caused epidemic in Yunnan is unknown.

CONCLUSION

In summary, the study indicated that PCV2b is the prominent genotype of PCV2 in Yunnan Province of China. Moreover, to control PCV2 infection in Yunnan Province of China, monitoring of PCV2 isolates and detection of genetic mutation and antigenic changes will be necessary in the future.

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