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# Genetic Characterization of Porcine Circovirus Type 2 (PCV2) from Pigs in Auhui Province

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**Abstract:** To understand the molecular characteristics in PCV2 strains from Anhui province in China, the whole genomic sequences of the 6 PCV2 Anhui isolates which were associated with PMWS were cloning, sequencing and analyzing. The results show that full genomic sequences of all of 6 PCV2 Anhui isolates were 1767 nt in length and belong to the different clusters in the PCV2b, PCV2-1A, PCV2-1B, PCV2-1C. The genome of 6 PCV2 Anhui isolates have 99.5-99.7% homology in whole nucleotide sequence have 97.2-99.8% homology in ORF1 nucleotide sequence and 93.6-99.9% homology in ORF2 nucleotide sequence and have 97.0-100% homology in ORF2 amino acid sequence. About 6 PCV2, Anhui isolates have the 93-99.5% homology in the whole nucleotide sequence, 96.2-100% in ORF1, 89.8-100% in ORF2 with 42 strains of domestic and foreign. About 30 PCV2b isolates having the identity of 93.2-100% in ORF2 nucleotide sequence and 91.0-100% in ORF2 amino acid sequence. Researchers conclude that PCV2 genome nucleotide sequences were more stable and do not have stronger variations of different characteristics with the change of time and regions. PCV2 strains in Anhui region are close relatives but showed some of the regional characteristics; the variation was mainly concentrated in PCV2 ORF2. PCV2-1C gene cluster have changed in the ORF2 of the two main strains of the immune response zone.

**Key words:** PCV2, whole genomic sequence, ORF2, sequence analysis, variation, regions

## INTRODUCTION

Porcine Circovirus (PCV) is a small, non-enveloped, circular, single-stranded DNA virus of about 1.76 kb that belongs to the genus Circovirus of the family Circoviridae and was first recognized as a contaminant of the continuous porcine kidney cell line PK-15 (ATCC-CCCL33) (Tischer *et al*., 1974, 1982).

The two genetic types of PCV have been recognized: PCV1 is associated with non-pathogenic whereas PCV2 is the virus associated with Postweaning Multisystemic Wasting Syndrome (PMWS), Porcine Dermatitis and Nephropathy Syndrome (PDNS), Porcine Respiratory Disease Complex (PRDC) (Allan *et al*., 1998; Hamel *et al*., 1998; Kim *et al*., 2004; Shibahara *et al*., 2000). Currently, PCV2 has a great impact on the swine industry around the world and PCV2 infection has the increasing tendency in China year by year (Wang *et al*., 2002; Shuai *et al*., 2007).

In 2000, it was reported that PCV2 in Canada could be categorized into five different profles (A-E) by PCR-RFLP based on ORF2 of PCV2 (Hamel *et al*., 2000). In 2005, PCV2 in clinical tissue specimens collected from the pigs of different regions in China was analyzed by PCR-RFLP and the results showed that nine different genotypes (A-I) were identified and CHI-2H is the dominant genotype of PCV2 prevailing in China (Wen *et al*., 2005). Recently, it was analyzed that the phylogenetic tree of 148 PCV2 full genomic sequences and PCV2 genomes were commonly referred to two genetic groups (37-39): group 1 (it was called PCV 2b) that could be divided into three clusters (1A-1C) and group 2 (it was called PCV2a) that could be divided into five clusters (2A-2E) (Olvera *et al*., 2007).

North American laboratories started to group PCV2 field isolates into European-like isolates or PCV2b (falls into PCV2 group 1) and into North American-like isolates or PCV2a (falls into PCV2 group 2) (Lager *et al*., 2007; Gagnon *et al*., 2007; Opriessnig *et al*., 2008; Racine *et al*., 2004).

PCV2 genome contains at least two major Open Reading Frames (ORFs); ORF1 encodes the Rep proteins involved in viral replication, ORF2 encodes the immunogenic capsid protein that is the major structural protein and the ORF2 nucleotide sequence has more obvious changes than ORF1 (Nawagitgul *et al*., 2000;

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Mahe *et al*., 2000; Lekcharoensuk *et al*., 2004). Although, there are many reports about the whole genome sequence analysis of PCV2 at home and abroad, there are no solid data about PCV2 whole genome for analysis in Anhui region. Anhui province is located in Yangtze Delta, across Huaihe river, Yangtze river, Xin'an river which were three major river systems and is topography. At sometimes, Anhui province is also China's Anhui province pig. Therefore, investigating and researching the epidemiological genetic characteristics of PCV2 in Anhui province have an important significance for the prevention and control of diseases caused by PCV2. In this study, the 5 PCV2 were isolated from the pigs presenting clinical signs and lesions associated with PMWS in Huangshan (HS), Xuancheng (XC), Dingyuan (DY), Feixi (FX), Feidong (FD) and Beihu (BH) districts, and 1 PCV2 isolate from domesticated wild boar (YZ) in Anhui province. Their whole genome and ORF1, ORF2 sequence analysis was carried out and ORF2 amino acid sequences were compared. It aims to better service for research of PCV2 isolates from Anhui in future.

## MATERIALS AND METHODS

**Samples and strains:** Treated disease which was associated with PMWS and was identified as positive by PCR was inoculated in PK-15 cells. Then the PCV2 strains were identified by PCR, indirect immunofluorescence assay, electron microscopy and the ORF2 gene sequence analysis (Yu *et al*., 2007; Jin-Cun *et al*., 2010; Rong *et al*., 2010). About 6 PCV2 strains were designated BH0801 (GenBank accession No. GQ 915289), DY 0801 (GenBank accession No. GU 017735), FD0801 (GenBank accession No. GQ 996404), XC 0801 (GenBank accession No. GQ 915288), FX 0901 (GenBank accession No. GU 252369), YZ 0901 (GenBank accession No. GU 252370).

**Complete sequencing of the PCV2 genome:** Two pairs of primers were designed based on the genomic sequence of the PCV2 strain (GenBank accession No. AY 188355.1). PCV2 was divided into A and B fragments for PCR. The length of A fragment has 827 bp. The length of B fragment was 1197 bp. Two fragments overlapping district 275 bp. The forward primer of the A fragment is 5'-TAGAAA CAAGTGGTGGGATGGT-3' and the reverse primer is 5'-CCGCTCTGTGCCCTTTGAAT-3'.

The forward primer of the B fragment is 5'-GGAG TAGTTTACATAGGGGTCAT-3' and the reverse primer is 5'-GTAGATCATCCCAGGGCAG-3'. The amplification of PCR reaction in A and B fragment were all 25 μL including 2× TaqMasterMix 12.5 μL, the forward primer 0.5 μL, the reverse primer 0.5 μL, DNA sample 0.5 μL, $ddH_2O$ 6.5 μL. The A or B fragment of PCR amplification conditions: the PCR consisted of an initial enzyme activation step at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min for A fragment (60°C for 1 min for B fragment), extension at 72°C for 1.5 min and a final extension at 72°C for 10 min. The two PCR products were ligated into a pMD18-T vector (Takara, Japan) and transformed in DH5α cells.

Table 1: Information on all PCV2 full genomes used in this study

| Accession no. | Clade | Place | Years | Back ground | Accession no. | Clade | Place | Years | Back ground |
|---|---|---|---|---|---|---|---|---|---|
| DQ 141322 | 1A | Shandong, China | 2005 | Unknown | AB 072302 | 2A | Japan | 2001 | PMWS |
| AY 188355 | 1A | Zhejiang, China | 2005 | Unknown | AF 109398 | 2A | Canada | 2000 | Unknown |
| AY 969004 | 1A | Zhejiang, China | 2005 | Unknown | AF 117753 | 2A | Canada | 2000 | Unknown |
| AY 849938 | 1A | Changsha, China | 2005 | Unknown | AY 180397 | 2B | Taiwan | 2003 | Unknown |
| AY 916791 | 1A | Guangzhou, China | 2005 | Unknown | AY 146993 | 2B | Taiwan | 2002 | Unknown |
| AY 484416 | 1A | Netherlands | 2004 | PMWS | AY 180396 | 2B | Taiwan | 2003 | Unknown |
| AY 484412 | 1A | Netherlands | 2004 | Healthy | AY 256459 | 2C | Hungary | 2003 | PMWS |
| AY 613854 | 1A | Guangdong, China | 2004 | PMWS | AY 256455 | 2C | Hungary | 2003 | PDNS |
| AY 484407 | 1B | Netherlands | 2004 | Healthy | AF 381176 | 2D | China | 2001 | Unknown |
| AY 556475 | 1B | Guangxi, China | 2004 | PMWS | AF 109399 | 2D | Canada | 2000 | Unknown |
| AY 682992 | 1B | Conghua, China | 2004 | Unknown | AY 322004 | 2D | France | 2004 | Healthy |
| AY 682995 | 1B | Qingyuan, China | 2004 | Unknown | AY 256458 | 2D | Hungary | 2003 | PMWS |
| AY 691169 | 1B | Zhejiang, China | 2004 | PMWS | DQ 104423 | 2E | China | 2005 | Unknown |
| AY 847748 | 1B | Beijing, China | 2005 | PMWS | AY 325495 | 2E | South Africa | 2003 | PMWS |
| AY 678532 | 1B | Zhejiang, China | 2004 | Unknown | DQ 104419 | 2E | China | 2005 | Unknown |
| AY 556477 | 1C | Hunan, China | 2004 | PMWS | AF 465211 | 2E | Taiwan | 2002 | Unknown |
| AY 556476 | 1C | Hunan, China | 2004 | PMWS | AF 465211 | 2E | Taiwan | 2002 | Unknown |
| AY 181946 | 1C | Tianjin, China | 2003 | PMWS | GQ 996404.1 | 1A | Anhui, China | 2008 | PMWS |
| AY 510375 | 1C | Zhejiang, China | 2004 | PMWS | GU 017735.1 | 1A | Anhui, China | 2008 | PMWS |
| AY 713470 | 1C | Germany | 2005 | Healthy | GU 252369.1 | 1B | Anhui, China | 2009 | PMWS |
| AY 035820 | 1C | China | 2001 | PMWS | GQ 915288.1 | 1C | Anhui, China | 2008 | PMWS |
| AY 484410 | 1C | Netherlands | 2004 | Healthy | GU 252370.1 | 1C | Anhui, China | 2009 | PMWS |
| AY 291317 | 1C | HuBei, China | 2003 | PMWS | GQ 915289.1 | 1B | Anhui, China | 2008 | PMWS |
| AY 943819 | 1C | Hunan, China | 2005 | Unknown | AF 264042 (USA40895) | | USA | 2000 | Unknown |

The recombinant plasmids were confirmed by PCR and sequencing (Shanghai Sangon Bioloical Engineering Technology and Services Co. Ltd). For sequencing, the universal primers consisting of M13F (-20), 5' GTA AAA CGA CGG CCA GT 3' and M13R (-20), 5' GCG GAT AAC AAT TTC ACA CAG G 3' were used. These primer sequences flanking the recombination site between the PCR product and pMD18-T vector are commercially available in Shanghai Sangon Bioloical Engineering Technology and Services Co. Ltd.

**Sequence analysis:** There are 42 PCV2 isolates at home and abroad and 6 PCV2 Anhui isolates have been used to make the whole genome sequence phylogenetic tree and compare the sequences by MEGA 4.1 software. DNASTAR software application derived 24 PCV2b strains at home and abroad and 6 strains of PCV2 isolates from Anhui ORF2 amino acid sequence and comparative analysis are shown in Table 1.

## RESULTS

**The results from cloning and sequencing of PCV2 Anhui isolates:** Sequence analysis showed that full genomic sequences of all of 6 PCV2 isolates were 1767 nt in length and these sequences have all been incorporated into GenBank database. Accession numbers were GQ 915289 (BH 0801), GU 017735 (DY 0801), GQ 996404 (FD 0801), GQ 915288 (XC 0801), GU 252369 (FX 0901) and GU 252370 (YZ 0901).

**PCV2 isolate genome sequence homology:** The homology of the genome nucleotide between 6 PCV2 Anhui isolates is 95.5-99.7%. They were more divergent from those of other countries or other regions of China with lower level of similarities ranging from 95.5-99.7%. About 6 PCV2 Anhui isolates have the 95.5-99.5% homology with 26 strains of domestic and 93-98% homology with 16 foreign reference strains are shown in Table 2.

**Phylogenetic analysis of 6 PCV2 full genomic sequences:** The phylogenetic tree showed that 6 PCV2 viruses isolated from Anhui in this study were predominantly included in the PCV2b group (Fig. 1).

Interestingly, 6 PCV2b viruses were not concentrated on one cluster. However, two strains, FD 0801 and DY 0801 belong to PCV2b-1A. They all have a higher affinity with AY 849938 (Changsha, China) and DQ 141322 (Shandong, China). The other two strains, BH 0801 and FX 0901 belong to PCV2b-1B. BH 0801 has a higher affinity with AY 691169 (Zhejiang, China). FX 0901 has a higher affinity with AY 556475 (Guangxi, China ). XC 0801 and YZ 0901 were included in the PCV2b-1C cluster and all have a higher affinity with AY 943819 (Hunan, China) and AY 291317 (Hubei, China).

**Homology analysis of 6 PCV2 ORF1 and ORF2 sequences:** The homology in ORF1 comparison, the 6 PCV2 isolates from Anhui have fewer nucleotide changes and were closely related to each other with high nucleotide sequence identity from 97.2-99.8%. Sometimes they keep a higer identity with the others from domestic and international isolates (96.2-100%). The homology in ORF2 comparison, the 6 PCV2 isolates from Anhui have fewer nucleotide changes and were closely related to each other with high nucleotide sequence identity from 93.6-99.9%. However, they keep the identity of 93.6-99.9% with others from domestic and international isolates. Moreover, it is closely related to 30 PCV2b isolates with identity of 93.2-100% (Table 2).

**Comparison of deduced amino acids of ORF2 in PCV2:** The alignment of the deduced 6 ORF2 Anhui isolates of ORF2 amino acid sequences indicated that the divergence at amino acid level was showing high identity of 97.0-100% with each other and was greater than that of the nucleotide sequence (93.6-99.9%).

As well, ORF2 amino acid sequences among three clusters (1A-1C) in PCV2b groups have been kept the high identity (91.0-100%). However, it has the lower similarities compared with isolates from the PCV2a and PCV2b (86.8-100%) (Table 2). In order to know the changes in PCV2b, deduced three clusters (1A-1C) amino acids sequences from ORF2 were compared without considering the splicing mechanism. Some single amino acid differences were found in three clusters in PCV2b. The amino acid sequences of PCV2-1A and PCV2-1B

Table 2: The results of the homology comparison between 6 Anhui PCV2 isolates

| Source strain | Comparison project | Comparison between PCV2 isolates Anhui (%) | PCV2 Anhui isolates compared with 42 domestic and foreign strains (%) | PCV2 Anhui isolates compared with 26 domestic strains (%) | PCV2 Anhui isolates compared with 16 foreign strains (%) | Comparison of 30 PCV2b strains (%) |
|---|---|---|---|---|---|---|
| 6 Anhui | Whole | 99.5-99.7 | 93-99.5 | 95.5-99.5 | 93-98 | - |
| PCV2 isolates | Genome (nt) | - | - | - | - | - |
| 26 strains of China | ORF1 (nt) | 97.2-99.8 | 96.2-100 | - | - | - |
| | ORF2 (nt) | 93.6-99.9 | 89.8-100 | - | - | 93.2-100 |
| 16 foreign strains | CAP (aa) | 97.0-100 | 86.8-100 | - | - | 91.0-100 |

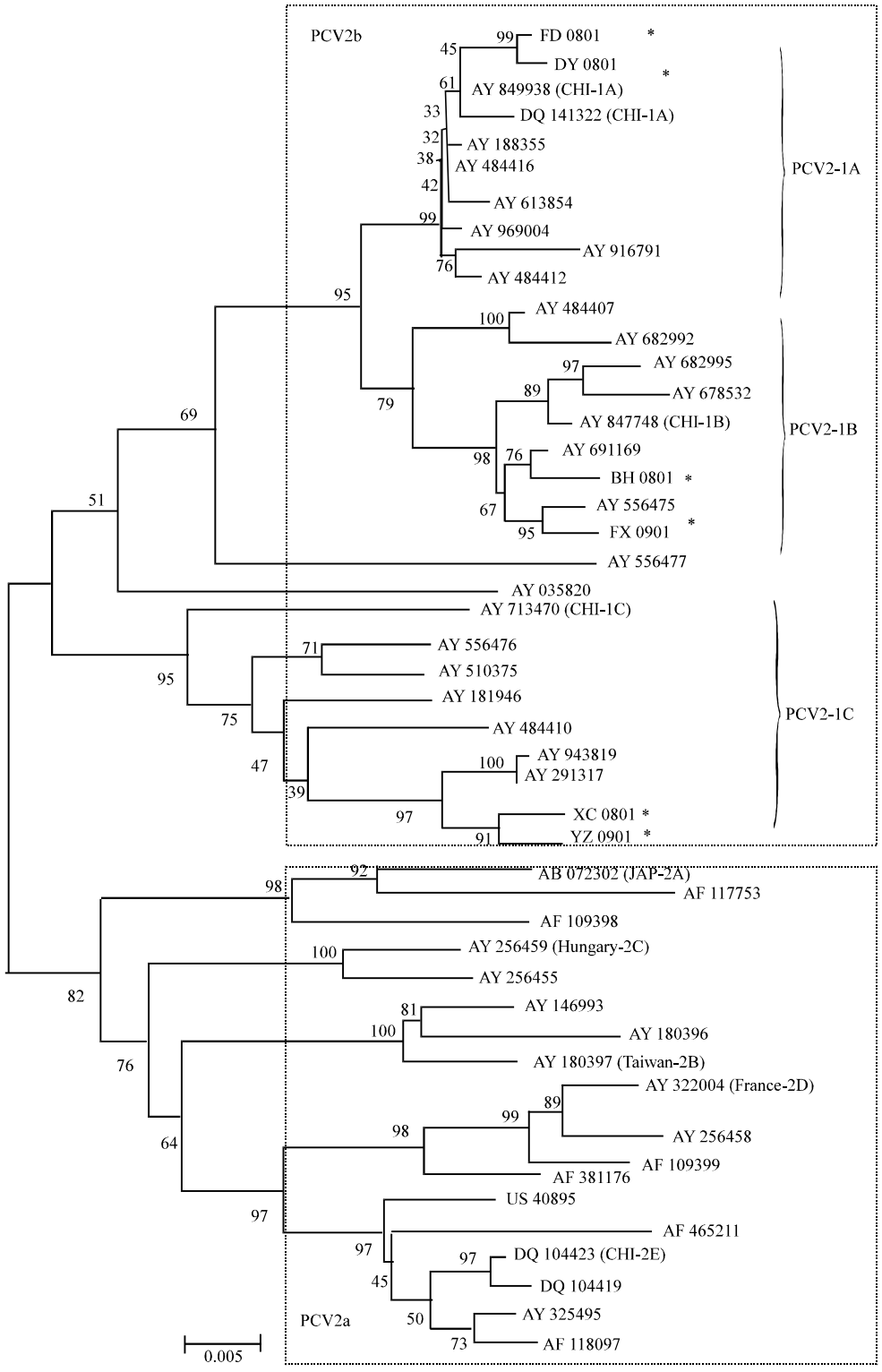


Fig. 1: Phylogenetic tree based on the NJ method for the PCV2 full genomic sequences used. About 48 PCV2 is divided into two subtypes (PCV2b and PCV2a) which is delimitation of the black box. The asterisk appeared on behalf of the location of the strain in this Phylogenetic tree. FD 0801 and DY 0801 belong to the PCV2b-1A; BH 0801 and FX 0901 belong to the PCV2b-1B; XC 0801 and YZ 0901 were included in the PCV2b-1C cluster

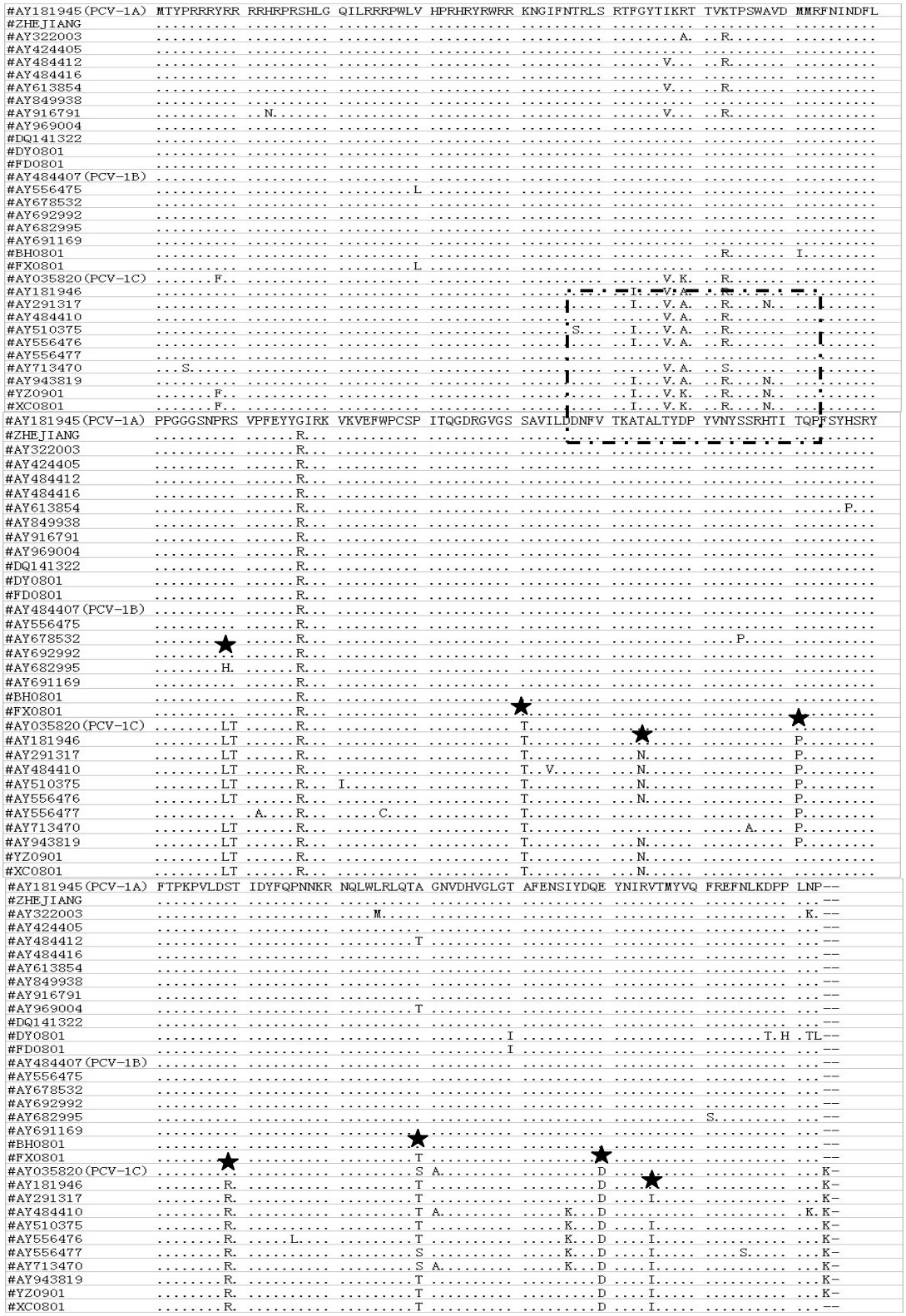


Fig. 2: Deduced amino acid sequences of ORF2 in 30 PCV2 full genomes. The PCV2-1C with the other two clusters in PCV2b group have more difference. We presented them with the black box and the asterisks. There were one major region (located at positions 53-68) and some positions (at 89, 90, 121, 134, 151, 169, 190, 210 and 215)

clusters were similar although, it has some sporadic changes in individual virus. The amino acid sequences of PCV2-1C cluster has an obviously difference so can clearly distinguish from the other two clusters. There were one major region (located at positions 53-68) and other positions (at 89, 90, 121, 134, 151, 169, 190, 210 and 215) in Fig. 2.

## DISCUSSION

Earlier, some scholars analyzed the whole genome of PCV2 isolates (Wang *et al*., 2005; Zhou *et al*., 2006) but there are the lack of the analysis of the whole genome sequence about the PCV2 isolates from China's Anhui province. In this study, sequencing the 6 PCV2 Anhui isolates of the whole genome shows that the complete genomes of 6 PCV2 viruses from Anhui province in China are 1767 nt.

About 6 PCV2 isolates of the nucleotide sequence have high homology not only with each other (95.5-99.7%) but also with the national and international reference strains (93-99.5%).

That evidence is that PCV2 does not have the greater variation of different characteristics along with the change of time and regions. The phylogenetic analysis of 6 PCV2 full genomic sequences indicate, 6 PCV2 viruses isolated from Anhui all belong to PCV2b but were not concentrated on one cluster. They belong to three gene clusters, respectively FD 0801 and DY 0801 belong to the PCV2b-1A; BH 0801 and FX 0901 belong to the PCV2b-1B; XC 0801 and YZ 0901 were included in the PCV2b-1C cluster. They have the higher affinity with the isolates from China, respectively such as Shandong, Changsha, Zhejiang, Guangxi, Hunan, Hubei, etc.

This illustrates that the source of the PCV2 isolates from Anhui is widespread and complex. Anhui province in East China region is the intensive pig farming area and is geographically diverse both North and South the color, the introduction of pigs and diverse ways resulting in molecular PCV2 strains in the region show different regional affinity. The ORF1 nucleotide of all PCV2 strains in this study demonstrates a high degree of conservative, exactly as Claire reported (Boisseson *et al*., 2004).

The replicated genes of PCV2 are a strong selection pressure exerted by its functions and are not prone to variations. In this study, 6 PCV2 Anhui isolates of ORF1 nucleotide sequence have higher homology with each other (97.2-99.8%) and with domestic and international reference strains (96.2-100%). Boisseson found that the variations among the PCV2 genomic sequences were mainly due to variability within ORF2 (Marit *et al*., 2004). In this study, 6 PCV2 Anhui isolates of ORF2 nucleotide sequence have 93.6-99.9% homology with each other and 89.8-100% homology with domestic and international reference strains. This shows that the mutation rate in ORF2 gene-encoding the major structural protein of PCV2Cap is high.

Therefore, the PCV2 could be categorized to different profiles by PCR-RFLP based on ORF2 of PCV2 in Canada and China (Hamel *et al*., 2000; Wen *et al*., 2005). Recently, several studies have suggested that the 28-kDa PCV2 Cap protein is the major immunogenic protein and the principal bearer of type-specific epitopes (Wen *et al*., 2005; Racine *et al*., 2004). Therefore, the Cap proteins were recognized differentially by polyclonal anti-PCV2 antibodies (Olvera *et al*., 2007; Liu *et al*., 2001) and may be a candidate gene of PCV2 for recombinant vaccine (Magar *et al*., 2000; Blanchard *et al*., 2003).

Fenaux demonstrated that the 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). A total of nine amino acid changes in ORF2 and two amino acid changes in ORF1 were identified between the PCV2-4838 and the PCV2-40895 isolates, animal experiments with these two viruses showed that the occuring time of the viremia and the micro-level of lymphoid tissue lesions were different (Wang *et al*., 2005).

In this study, the result of comparing ORF2 amino acids sequences from 30 PCV2b indicated that PCV2b-1A and PCV2b-1B have no significant difference but they both have a significance difference with ORF2 amino acids sequences the in PCV2b-1C. Especially, there are changes in one major region (located at positions 53-68) and other positions (at 89, 90, 121, 134, 151, 169, 190, 210 and 215) in the 1C. These changes were all in the two main immunogenic areas (Mahe *et al*., 2000). Whether, changes in availability of these sites lead to changes in virulence still needs to confirmed by further animal experiments.

## CONCLUSION

The results of this study, clearly present the molecular characteristics of the genome in PCV2 strains from Anhui under different circumstances and in different regions in China between the nucleotide sequences of PCV2 strains in close relatives have a little variability. The PCV2b-1A-B of the ORF2 amino acid sequence have a significant difference with PCV-1C. So for further, molecular epidemiology of PCV2 and the prevention and control of diseases it has an important guiding significance.

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