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Short Communication

Genetic Characterization of Porcine Parvovirus 7 (PPV7) Isolates in Fujian, China

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Abstract

In this study, we sequenced and analyzed the complete genomes of the porcine parvovirus 7 (PPV7) isolated in Fujian, Southeast China. Genomic comparison revealed that PPV7 isolates in Fujian shared higher (more than 98.5%) nucleotide homology and closer relationship with other PPV7 strains. Phylogenetic analysis based on NS indicated that PPV7 formed a distinct cluster (proposed *Chapparvovirus)* closer to subfamily *Parvovirinae*. However, phylogenetic analysis based on genome and VP showed that PPV7 isolates formed a distinct cluster closer to subfamily *Densovirinae*. No recombination event observed suggesting recombination did not contribution to the genetic diversity of PPV7. These findings demonstrated that PPV7 continuous circulating in China, which may help us to understand the evolution diversity of porcine parvoviruses.

Keywords: *Porcine parvovirus 7, Genomic comparison, Phylogenetic analysis*

Çin'in Fujian Eyaletinde Domuz Parvovirus 7 (PPV7) İzolatlarının Genetik Karakterizasyonu

Öz

Bu çalışmada, Güneydoğu Çin'in Fujian Eyaletinde elde edilen domuz parvovirus 7 (PPV7) izolatlarının tüm genomu sekanslandı ve analiz edildi. Genomik karşılaştırmada Fujian'dan elde edilen PPV7 izolatlarının diğer PPV7 suşları ile yüksek nükleotid benzerlik (%98.5'den daha fazla) göstererek yakın ilişkili olduğu gözlemlendi. NS temelli filogenetik analiz PPV7'nin Parvovirinae alt familyasına yakın farklı topluluk oluşturduğunu (*Chapparvovirus* olabileceği önerilen) gösterdi. Ancak, genom ve VP temelli filogenetik analiz PPV7 izolatlarının *Densovirinae* alt familyasına daha yakın topluluk oluşturduğunu gösterdi. Rekombinasyonun gözlemlenmemesi nedeniyle PPV7'nin genetik farlılıklarının oluşmasında rekombinasyonun katkısının olmadığı belirlendi. Bu bulgular, Çin'de PPV7'nin halen mevcut olduğunu göstermiş ve domuz parvovirusunun kalıtsal farklılaşmasını anlamamıza yardımcı olabilir.

Anahtar sözcükler: *Domuz parvovirus 7, Genomik karşılaştırma, Filogenetik analiz*

INTRODUCTION

The family *Parvoviridae* consists of many small, non-enveloped, single-stranded DNA viruses which can infect a wide variety of animal species. Parvoviruses genomes are approximately 5.0 kb in size, with two major open reading frames (ORFs), ORF1 and ORF2. The ORF1 encodes the non-structural protein (NSP) which has replicase (REP) activity, while the ORF2 encodes the structural or capsid protein (VP) [1]. Many different parvoviruses species had been identified in swine herds, including the PPV1 (also namely classic PPV), PPV2, PPV3 (also namely PARV4, hokovirus or partetravirus), PPV4, PPV5, PPV6, and porcine Bocaviruses (PBoV). Although these recently identified emerging PPV genotypes have been found in many pig breeding countries, but the clinical significance of PPV2 through porcine Bocaviruses infections remains unclear [2-4]. Moreover, no commercial vaccines are available at present.

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Currently, the family *Parvoviridae* includes two subfamilies, *Densovirinae* and *Parvovirinae*. The subfamily *Densovirinae* contains six genera: *Ambidensovirus, Brevidensovirus, Hepandensovirus, Iteradensovirus, Penstyldensovirus* and an unassigned genus. Meanwhile, the subfamily *Parvovirinae* contains eight genera: *Amdoparvovirus*, *Aveparvovirus, Bocaparvovirus* (including PBoV), *Copiparvovirus* (including PPV4, PPV5 and PPV6), *Dependoparvovirus, Erythroparvovirus, Protoparvovirus* (including PPV1) and *Tetraparvovirus* (including PPV2 and PPV3) [1]. Additionally, there are a number of unassigned members in the subfamily *Parvovirinae*.

Recently, a novel genus parvoviruses (proposed *Chapparvovirus*) has been discovered from turkeys [5], Eidolon helvums [6], wild rats [7] and pigs (namely porcine parvovirus 7, PPV7) [8,9]. Previously study also demonstrated that *Chapparvovirus* still have unclear species. PPV7 was firstly discovered by metagenomic sequencing from porcine rectal swabs of healthy adult pigs in the USA, in 2016 [8], then was identified in Guangdong Province in southern China [9] with no significant clinical signs observed. In this study, we sequenced and analysis the PPV7 isolates from lung tissues in Fujian, which help us to understand the genetic diversity of porcine parvoviruses in China.

MATERIAL and METHODS

Ethics Statement

The animal protocol used in this study was approved by the Research Ethics Committee of the Institute of Animal Husbandry and Veterinary Medicine, Fujian Academy of Agriculture Sciences (Permit Number AHVMFAAS20170012). All samples were handled in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of China.

Collection of Samples

The samples (lung tissues) were collected from healthy pigs (with the age about 180 d) after slaughtered from three commercial slaughterhouses in October, 2017. These three slaughterhouses located in Fuzhou city, Zhangzhou city and Longyan city in Fujian province (Southeast China), respectively. The total 30 lung tissues (10 samples for each slaughterhouse) were collected and kept at -80°C until processing.

DNA Extraction and PCR

These samples were homogenized in sterile phosphate-buffered saline (PBS, pH 7.2) and centrifuged at 4 000 rpm for 15 min at room temperature. The supernatants of each sample were used for DNA extraction with the MagicPure Viral DNA/RNA Kit (Transgen Biotek, Beijing, China) according to the manufacturer's instructions. The eluted DNAs were stored at -20°C until PCR detection. The presence of PPV7 DNA was initially detected by PCR with primers (PPV7-3434-F and PPV7-3654-R, see *Table 1*) targeting a 241 bp fragment of the VP gene as described previously [9]. The amplifications were performed using 2×TransTaq-T PCR SuperMix (+dye) (Transgen Biotek, Beijing, China) in a 50 μL PCR reaction containing 25 μL of 2X TransTaq-T PCR SuperMix, 1 μL PPV7-3434-F and PPV7-3654-R primer (10 μM each), 2 μL of the extracted DNA and 21 μL of PCR-grade water under the following conditions: denaturation for 5 min at 94°C followed by 35 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec, then with a final extension step at 72°C for 10 min. The PCR products were visualised by 1.0% agarose gel electrophoresis.

DNA Sequencing

Primers for genome sequencing *(Table 1)* were designed according to the similar strategy described previously [9] after optimized, with overlapped fragments encompassed the PPV7 genome. All the PCR positive samples' genomes were amplified by PCR technology. Amplicons were purified by QIAquick PCR Purification Kit (QIAGEN, Germany) and inserted into the pEASY-Blunt Zero Cloning Kit (Transgen Biotek, Beijing, China). In each case, five positive clones were randomly selected and sent to Sangon (Sangon Biotech, Shanghai, China) for sequencing.

Genomic Sequence Alignment and Phylogenetic Analysis

The sequences were assembled by the SeqMan program of Lasergene 7.0 software (DNASTAR Inc., Madison, Wisconsin, USA) [10] in order to obtain the PPV7 genome, and submitted to GenBank. Sequence comparison and genomic homology was determined using the ClustalW method [8]. Phylogenetic trees based on the genome (nucleotide sequences), NS (amino acid sequences), and VP (amino acid sequences) were constructed by using the neighbor-joining method implemented in MEGA v6.0 [11] with other reference sequences downloaded from GenBank. Support for individual nodes was determined by 1000 bootstrap replicates (Bootstrap=1000). *Chapparvovirus*, which were discovered from turkeys (turkey parvovirus, TuPV, GenBank No. KF925531), Eidolon helvums (Eidolon helvums parvovirus 2, GenBank No.JX885610, only had NS gene which had not used for analysis in this study) and wild rats (rat parvovirus 2, RPV2, GenBank No. KX272741) were used for analysis. Additionally, *Ambidensovirus* (Galleria mellonella densovirus, GmDNV, GenBank No. NC004286), *Brevidensovirus* (Anopheles gambiae densovirus, AgDNV, GenBank No. NC011317), *Hepandensovirus* (Decapod hepandensovirus 1, DhDNV, GenBank No. KT316242)**,** *Iteradensovirus* (Bombyx mori densovirus 5, BmDNV, GenBank No. NC004287) and *Penstyldensovirus* (Decapod penstyldensovirus 1, DpDNV, GenBank No. KT316259) in the subfamily *Densovirinae* also used as out-group.

***Table 1**. Primers used in this study*

Primers	Sequcences(5′→3′)	Position *	Length (bp)
PPV7-3434-F	CCTCCATCAGCAGCGACCAGT	3434-3454	241
PPV7-3654-R	ACCAGGGTTCCGTTTTCGTCT	3654-3674	
PPV7-F1	ATATGCACACCCTAGGCCAC	448-467	1375
PPV7-R1	GTCCCAGATAAATATGAACA	1803-1822	
PPV7-F2	TGCCGAGAACACCCATCATCA	1719-1739	1205
PPV7-R2	TGTGGTACCCGGTCTCGTA	2905-2923	
PPV7-F3	GTTCAATCCCATTCCCATCACA	2802-2823	851
PPV7-R3	TGAGTGGTATTCCTTTGATGAAGT	3629-3652	
PPV7-F4	GAACACCAACAAACCGTATCC	3102-3122	943
PPV7-R4	TAACAGGAAGGGCCAAAGATAGTT	4021-4044	

** Postion reference strain PPV 7 isolate 42 (GenBank number KU563733)*

Recombination Analysis

Recombination analysis is a major evolutionary mechanism which has been observed in several porcine parvovirus species. We performed a recombination analysis using the Simplot 3.5.1 and the Recombination Detection Program (RDP 4.0 software). Porcine parvovirus into the subfamily *Parvovirinae*, such as PPV1, PPV2, PPV3, PPV4, PPV5, PPV6, PBoV and PPV7 were used for recombination analysis.

Characteristic Amino Acids Motifs of PPV7 VP Gene

Phospholipase A_2 (PLA_2) motif resembling the catalytic site of secretary phospholipase A_2 ($sPLA_2$) founded in parvoviruses, which located on the N-terminal of the capsid protein [12]. The capsid protein of PPV7s were used to analysis based on the conserved motif in Ca^{2+} binding loop (YXGXG) and catalytic site of $sPLA_2$ (HDXXY), compared with PPV1 (GenBank No. NC001718).

RESULTS

Genomic Comparison

Three samples in Fuzhou, none in Zhangzhou and two samples in Longyan were detected positive by PCR, with the positive rate of 30%, 0 and 20%, respectively. The five positive samples were sequenced and assembled; the genomic comparison revealed that the three samples collected in Fuzhou city shared 100% genomic nucleotide identity (designated as PPV7 FJFZ2017). Meanwhile, two samples collected in Longyan city also shared 100% genomic nucleotide identity (designated as PPV7 FJLY2017). The sequenced PPV7 genome has a NS coding region of 2019 nucleotides (672 amino acids) and a VP coding region of 1401 nucleotides (466 amino acids). The complete genome of PPV7 strains FJFZ2017 and FJLY2017 were entered in to the NCBI GenBank under the accession number MG696111 and MG696112.

Based on sequence analysis of complete coding regions, the sequenced sequences (PPV7 FJFZ2017 and PPV7 FJLY2017) shared 99.7% nucleotide similarity with each other, shared 98.8% and 99.0% with PPV7 strain 42 [6] which was first identified in the US swine, shared 98.5% to 99.0% with PPV7 strains (GD-2014-1, GD-2014-2 and GD-2014-3) [9] which were first identified in Southern China. Phylogenetic analysis based on genome sequences under the family *Parvoviridae* (subfamily: *Densovirinae* and *Parvovirinae*) indicated that all the PPV7 isolates, together with TuPV and RPV2, were formed a distinct cluster *(Chapparvovirus),* different with other porcine parvoviruses. Moreover, the *Chapparvovirus* cluster is closer to subfamily *Densovirinae* rather than subfamily *Parvovirinae (Fig. 1A)*.

NS Comparison and Phylogenetic Analysis

The PPV7 NS shared 98.7%-99.9% and 96.6%-99.7% similarity with each other at the nucleotide and amino acid (aa) levels, respectively. Phylogenetic analysis based on NS aa sequences under the family *Parvoviridae*, the results demonstrated that all the PPV7 isolates shared a distinct cluster with TuPV and RPV2, formed a distinct *Chapparvovirus* cluster. Meanwhile, based on the NS evolution, the *Chapparvovirus* cluster shared the similar relationship with other porcine parvoviruses, under the subfamily *Parvovirinae (Fig. 1B)*.

VP Comparison and Phylogenetic Analysis

The PPV7 VP shared 98.0%-99.9% and 94.6%-99.1% similarity with each other at the nucleotide and aa levels, respectively. Phylogenetic analysis based on VP aa sequences under the family *Parvoviridae* suggested that the PPV7 isolates together with TuPV and RPV2, shared a distinct cluster *(Chapparvovirus)*. Similar like the genome evolution relationship, the *Chapparvovirus* cluster is closer to subfamily *Densovirinae* rather than subfamily *Parvovirinae (Fig. 1C)*.

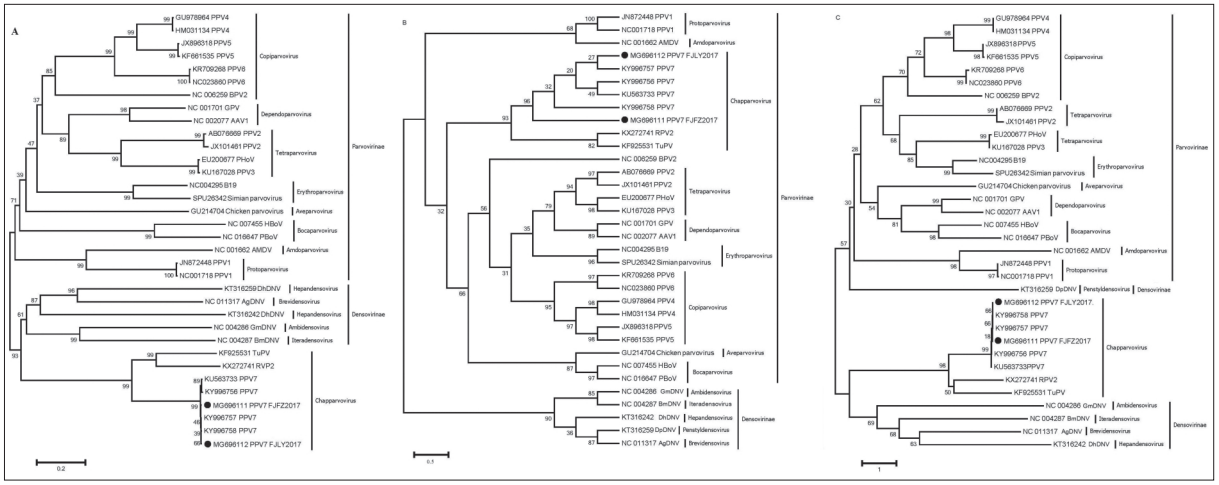

Fig 1. Phylogenetic relationship of PPV7s within family *Parvoviridae*. A- Phylogenetic tree constructed based on the genome nucleotide sequences, B- Phylogenetic tree constructed based on the NS protein seqences, C- Phylogenetic tree constructed based on the VP protein seqences. Phylogenetic trees were constructed by using the neighbor-joining method implemented in MEGA v6.0. Support for individual nodes was determined by 1000 bootstrap replicates (Bootstrap =1000). The evolutionary distance scale is shown at the bottom. The PPV7 sequenced in this study were indicated with filled circle (●). Reference sequences obtained from GenBank are indicated by accession number

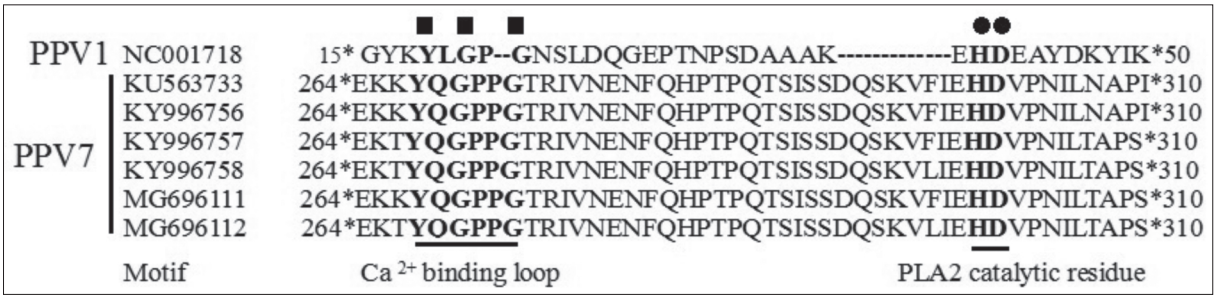

Fig 2. Sequence alignment of the putative phospholipase A_2 motif of PPV7s. The Ca^{2+} binding loop is indicated by filled squares (■) and the catalytic residues by filled circle (●)

Recombination Analysis

Using Simplot 3.5.1 and RDP 4.0 software, PPV7s shared no potential recombination signal with other parvoviruses discovered in pigs, including PPV1, PPV2, PPV3, PPV4, PPV5, PPV6 and PBoV. These data indicated that PPV7 is not a recombination of other PPVs and also suggesting recombination did not contribution to the genetic diversity for PPV7.

Identification of A Phospholipase A_2 Motif

In the aligments of the predicted VP protein sequence of PPV7 with PPV1, the conserved motif of Ca^{2+} binding loop (YXGXG) was different due to the single aa insertion at 271 aa (P) changed as (YXGXXG). Moreover, the catalytic residues (HDXXY) of Phospholipase A_2 (PLA_2) were different due to the single aa mutation at 304 aa (Y to N) *(Fig. 2)*.

DISCUSSION

Recent investigations demonstrated that the age of the family *Parvoviridae* may exceed 40 to 50 million years [13]. Besides the long history for parvoviruses, the genomes were exhibit similar high mutation and recombination rates as RNA viruses [14-16]. These data may explain for the high diversity and vast genetic divergence under the family *Parvoviridae* [17].

Genomic comparison revealed that PPV7 isolates analysed in this study shared higher (more than 98.5%) nucleotide homology with other PPV7 strains reported previously. Further genomic comparison analysis revealed that PPV7 FJFZ2017 and PPV7 FJLY2017, similar like the GD-2014-2 and GD-2014-3 which were identified in a co-infection with porcine circovurs 2 (PCV2), possess a consecutive 9-nt deletion (ATGTCTGTA) near the 5' end of the VP gene which leads to a three aa deletion. These PPV7 positive samples also tested positive with PCV2 by PCR [9].

The NS protein encode by the diversity PPV genome is commonly used to classify PPV genotype [1]. Phylogenetic analysis based on NS protein showed PPV7 isolates sharing closer relationship, which formed a closer genetic cluster, different with other eight genera under the subfamily

Parvovirinae. But phylogenetic analyses express the differences between genome (also the VP protein) and NS protein based phylogenetic analysis. Though based on genome and VP protein also demonstrated that PPV7 isolates together with TuPV and RPV2, shared a distinct cluster *(Chapparvovirus)*. But the relationship shared closer to subfamily *Densovirinae* rather than subfamily *Parvovirinae*. Until now, the PPV7 had not been classified by International Committee on Taxonomy of Viruses (ICTV), genomic comparison and phylogenetic analysis revealed that the PPV7s (together with TuPV and RPV2) may be classified in to a novel genus (proposed *Chapparvovirus*), these data suggesting that *Chapparvovirus* may be classified under subfamily *Densovirinae* rather than subfamily *Parvovirinae*.

Previously research indicated that potential recombination events can be detected both in domestic and wild boar origin recombinant PPVs [18]. But in this study, no potential recombination event can be observed with other parvoviruses discovered in swine herds. These data suggested that recombination did not contribution to the genetic diversity for PPV7. Although mutation of critical amino acids strongly reduce both PLA_2 activity and proportionally, viral infectivity, but cell surface attachment, entry and endocytosis by PLA_2-deficient virions were not affected [12]. Compared with PPV1, the PPV7 Ca^{2+} binding loop and catalytic residues were different due to the single aa mutation. Similar as turkey parvovirus, which also lack the PLA_2 domain [1,8]. Whether these changes can affect the PLA_2 activity needs further study.

In summary, this report confirms the PPV7 continuous circulating in China. Regardless of how the PPV7 generated, the PPV genomic characterization provide new sights for different PPVs in swine herds, which may help us to understand the evolution diversity of porcine parvoviruses.

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