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SHORT COMMUNICATION

Molecular epidemiological survey of canine parvovirus in domestic dogs in four provinces, China

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Abstract Canine parvovirus (CPV) can cause severe disease in animals, especially in dogs, and continuously generates new variants. In this study, the complete VP2 genes of 59 CPV isolates from clinical diseased dogs between 2015 and 2016 in 4 Chinese provinces were sequenced and analyzed. The results showed that new CPV-2a was still the prominent CPV genotype, followed by CPV-2c and new CPV-2b. CPV-2c with Ala5Gly and Gln370Arg mutations was first detected in Henan, Guangxi and Jiangsu provinces in China. A phylogenetic tree based on VP2 sequences showed that all isolates in this study formed a close lineage which separated from foreign isolates. The above results indicated that point mutations in VP2 were constantly occurring along with the widespread of CPV in China.

Keywords Canine parvovirus · Epidemiological survey · VP2 gene · Point mutation

Canine parvovirus 2 (CPV-2) is a member of the genus *Protoparvovirus* within the family *Parvoviridae*. It is a small, highly contagious, non-enveloped, single stranded DNA virus that causes acute gastroenteritis in domestic dogs, cat and several wild carnivore species [5, 13]. CPV-2 genome contains 2 large open reading frames (ORFs). One ORF (2007 bp) encodes 2 non-structural proteins (NS1 and NS2). Another ORF (2256 bp) encodes 2 structural proteins (VP1 and VP2), by alternative splicing of the same mRNAs. VP2 is highly antigenic and plays an important role in determining viral host range and tissue tropism [6]. Amino acid substitutions in VP2 protein can cause the changes of virus antigenic properties.

CPV-2 was first identified and described in 1978 in young puppies with fatal myocarditis and haemorrhagic gastroenteritis in the United States and Australia which can infect dogs and other members of the family Canidae but not cats [11]. Although CPV is a DNA virus, its genomic substitution rate is similar to RNA viruses [4]. A few years later, the original CPV-2 was replaced worldwide by series of new antigenic variants (i.e., CPV-2a and CPV-2b) based on key amino acid substitution (CPV-2a: Val555Ile, Asp305Tyr, Ala300Gly, Ile101Thr and Met87Leu; CPV-2b: Ile555Val and Asp426Asn) in VP2 protein [2, 10–12]. In the past decades, CPV-2a/2b with the Ser297Ala mutation was designated as new CPV-2a/2b [8]. Currently, new CPV-2a and new CPV-2b are circulating in many countries and appear to replace the prototype CPV-2a and CPV-2b [9, 17, 19]. In 2000, another antigenic variant having an amino acid substitution (Asp426Glu), named as CPV-2c, was first reported in Italy, and then rapidly spread to other countries. Compare to original CPV-2, the above antigenic variants showed high pathogenicity to dogs, and the susceptible hosts were also extended from dogs to cats [8]. Recently, CPV infections of vaccinated dogs were reported [2]. Therefore, it is imperative to perform epidemiological survey and molecular characterization of

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CPV strains that are currently circulating in the field. In this study, 59 VP2 genes of Chinese CPV isolates between 2015 and 2016 were analyzed to determine whether epidemic CPV genotype has changed.

A total of 89 feces or anal swabs from domestic dogs with diarrhea were collected in animal hospitals during 2015–2016. The samples were obtained from Henan, Jiangsu, Zhejiang and Guangxi provinces in China. Viral DNA was extracted using a viral nucleic acid extraction kit II (Geneaid Biotech Ltd, Taiwan, China) according to manufacturer's instructions. PCR primers were CPV-F: 5′-GCACATCAAGATACAGGAAG-3′ and CPV-R: 5′-CCTTAACATATTCTAAGGGCAA-3′, which amplifies an 800 bp fragment of VP2 gene. The PCR reaction volume was in 15 μl which included 7.5 μl of 2 × prime STAR Max premix (Takara Biotechnology Co., LTD, Dalian, China), 0.75 μl of each primer (10 μM), 1.5 μl of viral DNA, and 4.5 μl of ultrapure water. The PCR reaction included an initial denaturation step at 98 °C for 5 min followed by 33 cycles of 95 °C for 30 s, 52 °C for 40 s, 72 °C for 60 s, and 72 °C for 10 min. A commercial vaccine (Nobivac® Puppy DP, MSD Animal Health (Shanghai) Trading Co., Ltd.) was used as a positive control and sterile water was used as a negative control.

The complete VP2 nucleotide sequence of positive samples was amplified using the primers: VP2F: 5′-AGAGACAATCTTGCACCAAT-3′ and VP2R: 5′-ATGTTAATATAATTTTCTAGGTGCT-3′. The full-length of VP2 gene is 1776 bp. The PCR reaction volume was in 50 μl which included 25 μl of 2 × prime STAR Max premix (Takara Biotechnology Co., LTD, Dalian, China), 2 μl of each primer, 4 μl of viral DNA, and 17 μl of ultrapure water. The PCR reaction included an initial denaturation step at 95 °C for 3 min followed by 33 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 100 s, and 72 °C for 10 min. After purification, PCR products were cloned into pEASY blunt vector (TransGen Biotech Co., Ltd, Beijing, China) and sequenced at least 3 times (GENEWIZ, Inc., Beijing, China). The complete VP2 nucleotide sequences and deduced amino acid sequences were aligned with 26 reference CPV sequences from GenBank (Supplemental Table 1) using DNAStar Software. The genotypes of CPV isolates in this study were classified based on key amino acid sequences in VP2. The phylogenetic analyses based on the VP2 nucleotide sequences were conducted by the neighbor-joining method and a Maximum Composite Likelihood model in Mega 5.0 software (bootstrap replicates = 1000).

The conventional PCR results showed that 79 samples of 89 (89.0%) clinical samples from domestic dogs with diarrhea were positive for CPV-2. The full-length VP2 genes were successfully amplified from 59 out of 79 CPV-2 positive samples. Since some samples have complete same nucleotide sequences with each other, 19 haplotypes with different VP2 genes were selected for further analysis (Supplemental Table 2). The above 19 haplotypes shared 99.0–99.9% nucleotide and 98.6–99.8% amino acid similarities with each other, 99.0–100% nucleotide and 98.6–99.8% amino acid similarities with other reported Chinese CPV isolates, and 98.4–99.7% nucleotide and 97.3–99.5% amino acid similarities with CPV isolates from other countries (data not shown).

The genotypes of CPV isolates in this study were classified based on residues 297Ala and 426 (i.e., 426Asn in new CPV-2a, 426Asp in new CPV-2b, and 426Glu in CPV-2c) of VP2 gene. Among 59 CPV-positive samples, 38 CPVs were classified as new CPV-2a genotype, 8 as new CPV-2b genotype and 13 as CPV-2c genotype. Detailed information on genotypes and percentage rates in CPV-2 positive samples was summarized in Table 1. Twelve nonsynonymous nucleotide mutations of VP2 genes (14, 259, 302, 800, 889, 899, 913, 970, 1109, 1256, 1318 and 1665) were found which resulted in the changes of amino acid residues at 5, 87, 101, 267, 297, 300, 305, 324, 370, 419, 440 and 555, respectively (Supplemental Table 2).

Phylogenetic tree was constructed based on 59 full-length VP2 sequences isolated in this study and 26 sequences retrieved from GenBank. The results showed that Chinese CPVs formed a distinct cluster which separated from vaccine strains as well as isolates from other countries including France, South Korea, India, New Zealand, Japan, Italy, the United States, Ecuador, Germany and Uruguay (Fig. 1). Compared to local CPV isolates, new CPV-2a isolates in this study, resulting in 13 haplotypes, were most related to previously reported strains from Henan (KJ438805, Henan 42, 2013), Jiangsu (KF676668, CPV-JS2, 2013) and Heilongjiang provinces (KM386822, HLJ-02, 2014). New CPV-2b isolates in this study were most related to previously reported strains from Hubei (KC881278, wuhan2, 2010), Gansu (JQ268284, LZ2, 2011) and Heilongjiang provinces (JQ743894, BM-11, 2011). CPV-2c isolates in this study were most related to previously reported strains from Jilin (GU380303, 06–09, 2009) and Heilongjiang provinces (KT156832, HRB-A6, 2014).

In China, CPV-2 was first reported in 1983. Subsequently, several antigenic variants (include CPV-2a, CPV-2b, new CPV-2a, new CPV-2b and CPV-2c) appeared in recent years. At present, CPV-2c is broadly distributed in

Table 1 Summary of CPV genotypes in this study

Genotype	Cases	Rates (%)
New CPV-2a	38	64.4
New CPV-2b	8	13.6
CPV-2c	13	22.0

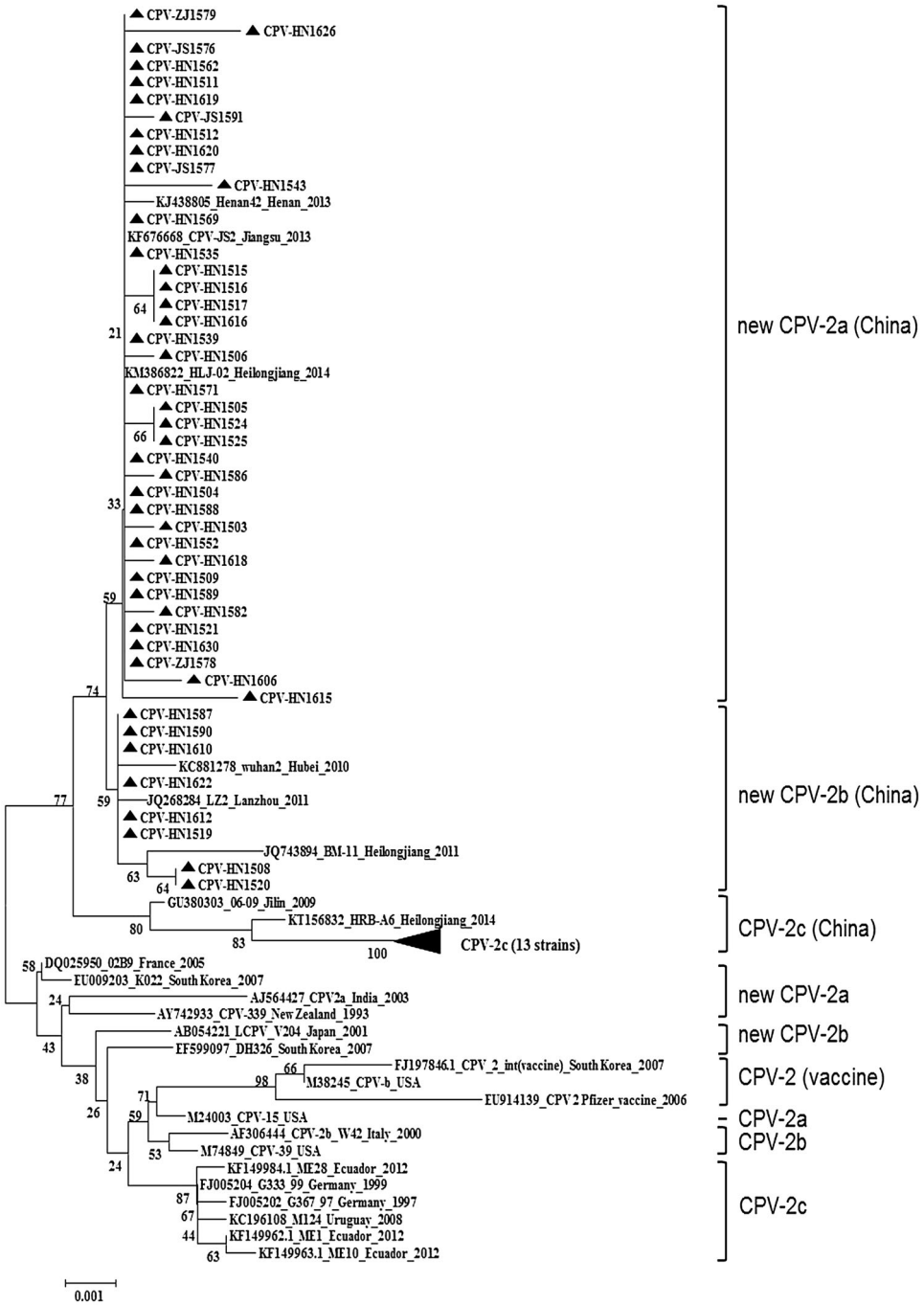

Fig. 1 A phylogenetic tree based on VP2 gene sequences of 59 CPV isolates and 26 reference CPV strains was constructed by neighbor-joining method using the MEGA software, version 5.0 (http://www.megasoftware.net/). Filled triangle indicates the 59 CPV isolates in this study. Horizontal branch lengths are proportional to genetic distances. Scale bars indicate nucleotide substitutions per site. Bootstrap values were calculated based on 1000 replicates

Europe (Italy, Bulgaria, England, Germany, Greece, Portugal, Sweden, and Spain, Tirana), Africa (Tunisia), Asia (India, Vietnam), Oceania (Australia), and the Americas (United States, Argentina, and Uruguay) [12]. In China, CPV-2c was first detected by PCR–RFLP and sequence analysis in Jilin province in 2010 [18]. Subsequently, 18 of 43 samples belonged to CPV-2c were reported in Beijing in 2014 [15]. However, it was not reported until this study that CPV-2c has spread to Henan, Guangxi and Jiangsu provinces.

Parvovirus VP2 protein, the major capsid protein, is highly antigenic and plays an important role in determining viral host range and tissue tropism [6]. Mutations affecting important residues of capsid protein have been recognized, suggesting that CPV is still evolving [8]. The antigenic differences between CPV genotypes are a consequence of amino acid substitutions in the viral capsid protein VP2. Either residue 93 or residue 300 in VP2 protein of CPV binds to the cellular transferrin receptor (TfR) determines CPV infectivity. The mutation in the residue 297, not

detected in "old CPV-2" strain, is predominant in the variants 2a and 2b detected in different countries [1, 8]. After 2001, a CPV-2a variant, with an amino acid change at position 555 within CPV VP2 protein was reported. This new variant has a Val-555 replacing of Ile-555 and represents a reversion of the sequence of original type CPV-2 [8]. With the subsequent introduction and spread of new 2a variant, the Ile-555 of old CPV-2a is no longer detected in the field [3, 20].

Two mutations (nt 800 and 970, corresponding to residues 267 and 324, respectively) occurred frequently in new CPV-2a isolated in this study and in other Chinese strains (GenBank no: GU380303, JQ268284, JQ743894, KC881278, KF676668, KJ438805, KM386822 and KT156832). Residues 267 and 324 mutations may represent an adaptation of CPV circulating in China. Residue 267 is located in a minor antigenic site which is close to epitope B. However, a mutation to residue 267 may not influence the antigenicity of CPV because it is not exposed on the capsid surface, as previously reported for CPV-2a from China and Thailand and CPV-2b from Vietnam [9]. In addition it has been speculated that this substitution might play an important role in transmission and infection [16]. Residue 324 is subject to positive selection and may influence the amino acid sequences at residue 323, which is known to be involved in host range via binding with the canine transferrin receptor [7]. The Tyr324Ile mutation in CPV-2a strains may also lead to a stronger receptor binding which has been previously reported in China [6, 16, 17] and other countries [9].

Consistent with previous reports, Ala5Gly mutation in CPV-2c was observed in this study and its potential functional consequence remains to be determined [15]. The Gln370Arg point mutation of all CPV-2c in this study was also reported in domestic dogs and a giant panda in China [5, 15]. This alteration could be involved in a conformational change in VP2 protein which is required during virus replication cycle, or it may mediate an effect on receptor binding through neighboring residues. In any event, this alteration is likely to affect the host range of virus [5]. Asn419Ser in CPV-JS1591 in this study, a new CPV-2a isolate, was first reported and the significance of this mutation is unclear and warrants further investigations. Thr440Ala mutation was frequently reported in new CPV-2a and new CPV-2b variants. Residue 440 is located on the top of the three-fold spike which works as the main antigenic site of CPV [14]. Thus, the substitutions at 440 in the new CPV strains could play an important role in transmission and infection and might provide a selective advantage in the process of local adaptation [16].

In conclusion, a CPV epidemiological survey in domestic dogs was conducted in four Chinese provinces and the results showed that new CPV-2a is the prominent genotype of CPV from 2015 to 2016. Noticeably, CPV-2c with Ala5Gly and Gln370Arg mutations was first detected in Henan, Guangxi and Jiangsu provinces. The phylogenetic analysis revealed that Chinese CPV strains formed a distinct cluster which separated from vaccine strains as well as isolate from other countries. The high incidence of amino acid point mutations in VP2 genes indicated constant evolution of CPV in China.

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