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Phylogenetic analysis of the VP2 gene of canine parvoviruses circulating in China

Renzhou Zhang · Songtao Yang · Wei Zhang · Tao Zhang · Zhijing Xie · Hao Feng · Shujun Wang · Xianzhu Xia

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Abstract Sequences of the full-length gene encoding the main capsid protein VP2 of 22 strains of canine parvovirus (CPV), isolated from domestic dogs in China between 1983 and 2008, were determined and analyzed in comparison with the sequences of 30 other strains of CPV from China and reference CPV isolates retrieved from GenBank. Three types of CPV, including CPV-2, CPV-2a, and CPV-2b, were detected, and CPV-2a (with 297-Ala mutation) was predominant in China. The unique Ile-324 mutation in the VP2 of Chinese CPV isolates was detected, as compared with a Tyr-324 in the VP2 of the reference CPV strains. A phylogenetic tree constructed from the VP2 genes showed that most of the Chinese strains classified in a cluster consisting of Chinese and Korean field isolates, which were distinct from Thai, U.S., and Italian isolates.

Keywords Canine parvovirus · VP2 gene · China · Phylogenetic analysis

R. Zhang · S. Yang · X. Xia (✉)
Laboratory of Virology, Institute of Military Veterinary, Academy of Military Medical Sciences, 130062 Changchun, Jilin Province, China
e-mail: xia_xzh@yahoo.com.cn

R. Zhang
e-mail: zhang_renzhou@yahoo.com

S. Yang
e-mail: yst610223@yahoo.com.cn

W. Zhang · T. Zhang
Institute of Laboratory Animal Sciences, Peking Union Medical College, Chinese Academy of Medical Sciences, 100021 Beijing, China
e-mail: wisherzhw2007@163.com

T. Zhang
e-mail: zhangtao1012450@163.com

Z. Xie
Animal Science and Technology Institute, Shandong Agricultural University, 271018 Taian, Shandong Province, China

H. Feng · S. Wang
Agriculture Division, College of Animal Science and Veterinary Medicine, Jilin University, Changchun, China

Introduction

Canine parvovirus (CPV) is a small, non-enveloped virus, containing a single-stranded DNA (~5 kb). The genome contains two major ORFs, one encoding two non-structural proteins NS1 and NS2, the other, two structural proteins (VP1 and VP2). VP1 and VP2 are translated by alternative splicing from the same primary transcript [1]. Canine parvovirus type 2 was first identified in 1978, and was soon recognized as a major killer of puppies, causing severe haemorrhagic diarrhoea, vomiting, and dehydration. CPV-2 has been shown to be closely related to feline panleukopenia virus (FPLV) and FPLV-like parvoviruses from wild carnivores, such as mink, fox, and red panda.

A variant virus, CPV-2a, with five amino acid (aa) substitutions, emerged in 1979 and replaced CPV-2 worldwide in approximately 1 year [2, 3]. Another antigenic variant, CPV-2b, with a single additional substitution (Asn426Asp) in the VP2 protein, appeared in 1984 and spread globally. Since then, gene sequencing and genetic analyses have been applied frequently and many new CPV variants throughout the world have been detected, such as the "new CPV-2a" and "new CPV-2b" with an additional 297-Ala mutation [4], CPV-2c(a) and CPV-2c(b) with an additional 300-Asp mutation [5], and CPV-2c with an additional 426-Glu mutation [6].

In China, the genetic relationships among parvoviral isolates have not been reported. Here, the VP2 genes of 22 strains of canine parvovirus, isolated from domestic dogs in China between 1983 and 2008, have been sequenced and analyzed in comparison with the sequences of 30 other Chinese strains of CPV (from GenBank) and eight reference CPV isolates.

Materials and methods

Virus isolation

Twenty-two PCR-positive fecal samples from clinical cases, collected since the early 1980s in various areas of China, had been stored at −80°C. For virus isolation, the samples were filtered through a 0.22-μm Millipore filter. Cat kidney F81 cells (obtained from the China Institute of Veterinary Drugs Control) were inoculated with the filtrates and cultured in Eagle's Minimal Essential Medium (MEM) containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulfate). The cells were incubated at 37°C and observed daily for 4 to 5 days for cytopathic effects (CPE). Five blind passages were carried out for each sample.

VP2 gene amplification and sequencing

Viral DNA was extracted using the AxyPrep^{TM} viral DNA miniprep kit (Axygen Scientific, USA) from viral isolates, according to the manufacturer's instructions. PCR amplification was performed using ExTaq polymerase (TaKaRa Biotechnology Co., Dalian, China) and primers P-forward: 5′-GGATCCCCAATGAGTGATGGAGCAGTTCAACCA GAC-3′ (located on the CPV-d VP1 gene sequence at position 499 to 528), and P-reverse: 5′-CTCGAGTTAA TATAATTTTCTAGGTGCTAGTTGA-3′ (located at position 2,229 to 2,257), designed according to the DNA sequence of CPV-d in GenBank (M23255).

Following purification, the DNA amplicons were cloned into the pMD18-T vector (TaKaRa Biotechnology Co.) and their sequences determined by Sangon Biological Engineering Technology & Service Co., Shanghai, China.

Phylogenetic analysis

Fifty-two VP2 sequences [the 22 sequences determined here and the 30 from GenBank (Table 1)] were compared with the VP2 sequences of 8 reference strains of CPV (CPV-b, CPV-15, CPV-V120, CPV-39, CPV-Taichung, LCPV-V139, LCPV-V203, and CPV-G7/97) retrieved from the GenBank database. Nucleotide sequences and deduced aa sequences were aligned with the MegAlign program of DNASTAR (DNASTAR, Madison, WI, USA). Phylogenetic analysis was performed using the MEGA program, version 4.0 (http://www.megasoftware.net/). The neighbor-joining method was chosen to draw a nucleotide phylogenetic tree. The reliability of the phylogenetic tree obtained for the VP2 region was evaluated by running 1,000 replicates in the bootstrap test.

Table 1 China-isolated CPV sequences retrieved from GenBank and analyzed in this study

Strain	Origin	GenBank No.	Year isolated	Genetic type
CPV-GN	Nanjing	DQ120515	1986	CPV-2b
CPV-HN-3	Guangdong	DQ177497	2000	CPV-2b
CPV-B-2004	Beijing	EF011664	2004	CPV-2a
RPPV	Yunnan	DQ354068	2004	CPV-2a
CPV-NJ-04	Nanjing	EU095252	2004	CPV-2a
CPV-06-11-NJ	Nanjing	FJ432716	2006	CPV-2a
CPV/nj01/06	Nanjing	EU310373	2006	CPV-2a
CPV-WH02/06	Wuhan	EU377537	2006	CPV-2a
CPV/BJ004/07	Beijing	EF666059	2007	CPV-2a
CPV/BJ010/07	Beijing	EF666062	2007	CPV-2a
CPV/BJ044/07	Beijing	EU145954	2007	CPV-2b
CPV/BJ069/07	Beijing	EU145958	2007	CPV-2a
CPV/BJ077/07	Beijing	EU145959	2007	CPV-2a
CPV/BJ082/07	Beijing	EU145960	2007	CPV-2a
CPV-SHZ	Shihezi	EU170352	2007	CPV-2a
CPV-HLJ-JQ	Heilongjiang	EU697385	2007	CPV-2a
CPV/BJ005/07	Beijing	EU666060	2007	CPV-2a
CPV-APD1	NA	EU213074	2007	CPV-2a
CPV-BD2	NA	EU213079	2007	CPV-2a
CPV-BD4	NA	EU213081	2007	CPV-2a
CPV-HT	NA	EU213077	2007	CPV-2a
CPV-HZ0761	NA	EU213073	2007	CPV-2a
CPV-JB1	NA	EU213076	2007	CPV-2a
CPV-KT2	NA	EU213083	2007	CPV-2a
CPV-JB-3	NA	EU483510	2007	CPV-2b
CPV-ZD-11	NA	EU483514	2007	CPV-2a
CPV-ZD-30	NA	EU483516	2007	CPV-2a
CPV-ZD-35	NA	EU483517	2007	CPV-2b
CPV-ZD-7	NA	EU483513	2007	CPV-2a
CPV-08-5-WH	Wuhan	FJ432717	2008	CPV-2a

NA not available

Results

Sequence comparisons showed nucleotide identities of 98.3–100% among the CPVs isolated in China since the early 1980s. Nucleotide sequences were translated into aa

sequences to identify the isolates as CPV-2, CPV-2a, or other types. Critical positions of the CPV VP2 gene products of isolates sequenced in this study are summarized in Table 2.

As shown, all CPV-2a/2b strains, except one original CPV-2a (without the 297-Ala mutation) collected in 1986, were identified as "new CPV-2a" or "new CPV-2b", all retaining the aa changes of the variants from the original CPV-2 (Met87Leu, Ile101Thr, Ala301Gly, Asp305Tyr) and displaying the mutation Ser → Ala at site 297 of the VP2 protein. CPV-2 type viruses were identified in one isolate of 1983 and three collected in 2008. There were

Table 2 Amino acid substitutions in the VP2 gene of CPV isolates analyzed in this study

Strain	Origin	Year	Genetic type	Amino acid at position											
				80	87	93	101	267	297	300	301	305	324	426	564
Reference strains															
CPV-b	U.S.	1978	CPV-2	Arg	Met	Asn	Ile	Phe	Ser	Ala	Thr	Asp	Tyr	Asn	Ser
Intervet/vaccine	N.A.	N.A.	CPV-2	–	–	–	–	–	–	–	–	–	–	–	–
CPV-15	U.S.	1984	CPV-2a	–	Leu	–	Thr	–	–	Gly	–	Tyr	–	–	–
CPV-V120	Vietnam	2000	New CPV-2a	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	–	–	–
CPV-39	U.S.	1984	CPV-2b	–	Leu	–	Thr	–	–	Gly	–	Tyr	–	Asp	–
CPV-Taichung	Taiwan	2004	New CPV-2b	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	–	Asp	–
CPV-V139	Vietnam	2000	CPV-2c(a)	–	Leu	–	Thr	–	Ala	Asp	–	Tyr	–	–	–
CPV-V203	Vietnam	2000	CPV-2c(b)	–	Leu	–	Thr	–	Ala	Asp	–	Tyr	–	Asp	–
CPV-G7/97	Germany	1997	CPV-2c	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	–	Glu	–
Chinese isolates															
YB8301	Yanbian	1983	CPV-2	–	–	–	–	–	–	Asp	Ile	–	–	–	–
CC8601	Changchun	1986	CPV-2a	–	Leu	–	Thr	–	–	Gly	–	Tyr	–	–	–
BJ9901	Beijing	1999	New CPV-2a	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	–	–	–
QB0101	Changchun	2001	New CPV-2a	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	–	–	–
GZ0202	Guizhou	2002	New CPV-2b	–	Leu	–	Thr	Tyr	Ala	Gly	–	Tyr	–	Asp	–
YN0201	Yunnan	2002	New CPV-2b	–	Leu	–	Thr	Tyr	Ala	Gly	–	Tyr	–	Asp	–
YN0202	Yunnan	2002	New CPV-2a	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	–	–	–
YN0203	Yunnan	2002	New CPV-2b	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	–	Asp	–
SD0201	Shandong	2002	New CPV-2a	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	–	–	–
JL0202	Jilin	2002	New CPV-2a	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	–	–	Asn
GZ0201	Guizhou	2002	New CPV-2b	–	Leu	–	Thr	Tyr	Ala	Gly	–	Tyr	–	Asp	–
JL0201	Jilin	2002	New CPV-2a	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	–	–	–
SCDN	Yaan	2005	New CPV-2a	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	–	–	–
SCJM	Yaan	2005	New CPV-2b	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	–	Asp	–
CN080402	Sichuan	2007	New CPV-2a	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	Ile	–	–
CN080405	Sichuan	2007	New CPV-2a	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	Ile	–	–
CNJL0804	Jilin	2007	New CPV-2a	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	Ile	–	–
CN080401	Sichuan	2008	CPV-2	–	–	Lys	–	–	–	–	–	–	–	–	–
CN080403	Sichuan	2008	CPV-2	–	–	Lys	–	–	–	–	–	–	–	–	–
CN080404	Sichuan	2008	New CPV-2a	–	Leu	–	Thr	–	Ala	Gly	–	Tyr	Ile	–	–
CN080406	Sichuan	2008	New CPV-2a	Ile	Leu	–	Thr	–	Ala	Gly	–	Tyr	Ile	–	–
CN080407	Sichuan	2008	CPV-2	–	–	Lys	–	–	–	–	–	–	–	–	–

Amino acids identical to those in CPV-b are indicated by *dashes*. *Boxes* indicate a novel amino acid substitution. Deduced amino acid sequences of the reference VP2 gene were obtained from GenBank: CPV-b (accession number: M38245), Intervet/vaccine (Nobivac DHPPI, GQ169552), CPV-15 (M24003), CPV-V120 (AB054215), CPV-39 (M74849), CPV-Taichung (AY869724), CPV-V139 (AB054222), CPV-V203 (AB054224), CPV-G7/97 (FJ005196). Sequences of Chinese isolates have been deposited in GenBank: SCDN (accession number: DQ903936), SCJM (EF028071), CN080402 (FJ435343), CN080405 (FJ435346), CN080401 (FJ435342), CN080403 (FJ435344), CN080404 (FJ435345), CN080406 (FJ435347), CN080407 (FJ435348), CNJL0804 (GU569936), GZ0202 (GU569937), YN0201 (GU569938), YN0202 (GU569939), YN0203 (GU569940), SD0201 (GU569941), JL0201 (GU569942), YB8301 (GU569943), GZ0201 (GU569944), QB0101 (GU569945), JL0202 (GU569946), BJ9901 (GU569947), CC8601 (GU569948)

12 isolates of "new CPV-2a", indicating that this has been the predominant CPV in China since the late 1990s, while "new CPV-2b" was isolated with a lower frequency (five isolates). Neither CPV-2c(a), CPV-2c(b) (with 300Asp mutation) nor CPV-2c (with 426Glu mutation) was found.

A mutation Phe → Tyr was encountered at position 267 in CPV-GZ0201, CPV-YN0201, CPV-GZ0202, CPV/WH02/06, CPV-JB1, CPV-JB3 and CPV-08-05-WH. There was a unique mutation within the VP2 of Chinese and Korean strains of "New CPV-2a" at Tyr324Ile. Interestingly, only a single isolate of this variant (CPV-B-2004; Table 1) was detected among all viruses collected before 2005, while 27 of the 33 CPVs collected since 2006 have the mutation Tyr324Ile, caused by mutation TAT → ATT at nt 970–972 of the VP2 gene.

To examine the phylogenetic relationships of the Chinese isolates with representative CPVs, a tree based on the nucleotide sequence from 1 to 1,750 of the VP2 gene was constructed. As shown in Fig. 1, most of the CPVs isolated in China formed a major monophyletic cluster, with one Korean isolate also clustering in this group. A group of four Korean isolates clustered close to the Chinese isolates, indicating a close relationship between the isolates from China and Korea. Eleven of the 13 CPVs collected before 2002 in China fell into a cluster consisting of CPVs isolated from Italy, Vietnam, Thailand, and USA.

Discussion

The analysis has indicated that "new CPV-2a" and "new CPV-2b" are co-circulating in China, with the former being isolated with a much higher frequency. "New CPV-2a/2b" appear to have replaced the prototype CPV-2a/2b strains and become the predominant types in many countries [4, 7–10]. It has been reported that mutation Ala297 does not change the viral antigenic type [11], and so the Ala297 variant cannot be distinguished serologically. However, the emergence and spread of this variant indicates that the Ala297 mutation potentially has had a marked influence on the process of continuing host adaptation, and previous research has shown that site 297 is under strong positive selection [12].

This study detected one site mutation among the CPV-2a/2b isolates in China: Tyr → Ile at position 324. This mutation was first detected in China in 2004 and also reported in three isolates from Korea in the same year [13]. Twenty-seven of the 30 CPV-2a/2b isolates collected after 2006 contained this mutation, which was not found in the 17 isolates collected before 2002. Since our study consisted of a limited number of isolates, however, it is not possible to state with certainty that this mutation did not exist in CPVs in China before 2000. The substitution at residue 324 has not been previously identified. Previous studies have shown that residue 324 is subject to strong positive selection in all parvoviruses of carnivores [14]. Residue 324 is adjacent to residue 323, which affects canine transferrin receptor (TfR) binding and, together with residue 93 [15–19], the canine host range. The 324 mutation is likely to have had an effect on the parvovirus host range.

In our study, three isolates collected in 2008 were found to be CPV-2 type, which have a close relationship with three most commonly used vaccine strains indicated by the phylogenetic tree. The vaccination histories of the three puppy hosts are unavailable. Since CPV-2 viruses are no longer considered to circulate in dog populations worldwide, it is likely that the virus detected in the puppies was CPV-2 vaccine virus [20]. Reversion of the CPV vaccine strains to virulence is unlikely, as the attenuation of virulence has proved to be highly stable [21, 22]. When a vaccine strain was detected in a diarrhoeic fecal sample, it was generally present together with CPV field strain that infects the dog shortly before or after the vaccine administration, or with other pathogens commonly associated with enteritis in dogs [23].

As the phylogenetic tree shows, most of the viruses isolated in China formed a large cluster, while some strains clustered together with viruses from regions outside China. Most of the CPVs isolated in China formed a specific cluster and certain mutations detected in Chinese CPVs probably arose during the process of local adaptation, as indicated by previous surveys [12, 24].

There are evidences to suggest that complete immunity may not be provided to pups if CPV2 vaccines are used, considering that the CPV2 vaccine appears to provide a comparatively lower and shorter immunity against heterologous CPVs [4, 25, 26]. In many countries in Europe, such as United Kingdom, Germany, and Italy, CPV-2a has been overtaken by CPV-2b or, recently appeared, CPV-2c, many researches have been conducted to evaluate the immunity conferred by CPV-2 vaccine against the CPV-2b type [26–28]. In Europe, CPV2b-based vaccines have been developed and are available now. Nevertheless, our study showed that CPV-2a is most predominant in China, and the vaccines available in China are CPV-2 type vaccine. The effectiveness of CPV-2 vaccine against CPV-2a type has not been evaluated in China.

In summary, "new CPV-2a" is the prominent type of CPV in China. Most Chinese isolates contained the mutation 324Ile, which probably arose around 2004 when this variant was first detected in China and Korea [13]. CPV-2c, which has displayed an exceptional ability to spread rapidly through the canine population in Italy [8, 29] and other European countries [30], Asia [10] and America [31–33], has not been

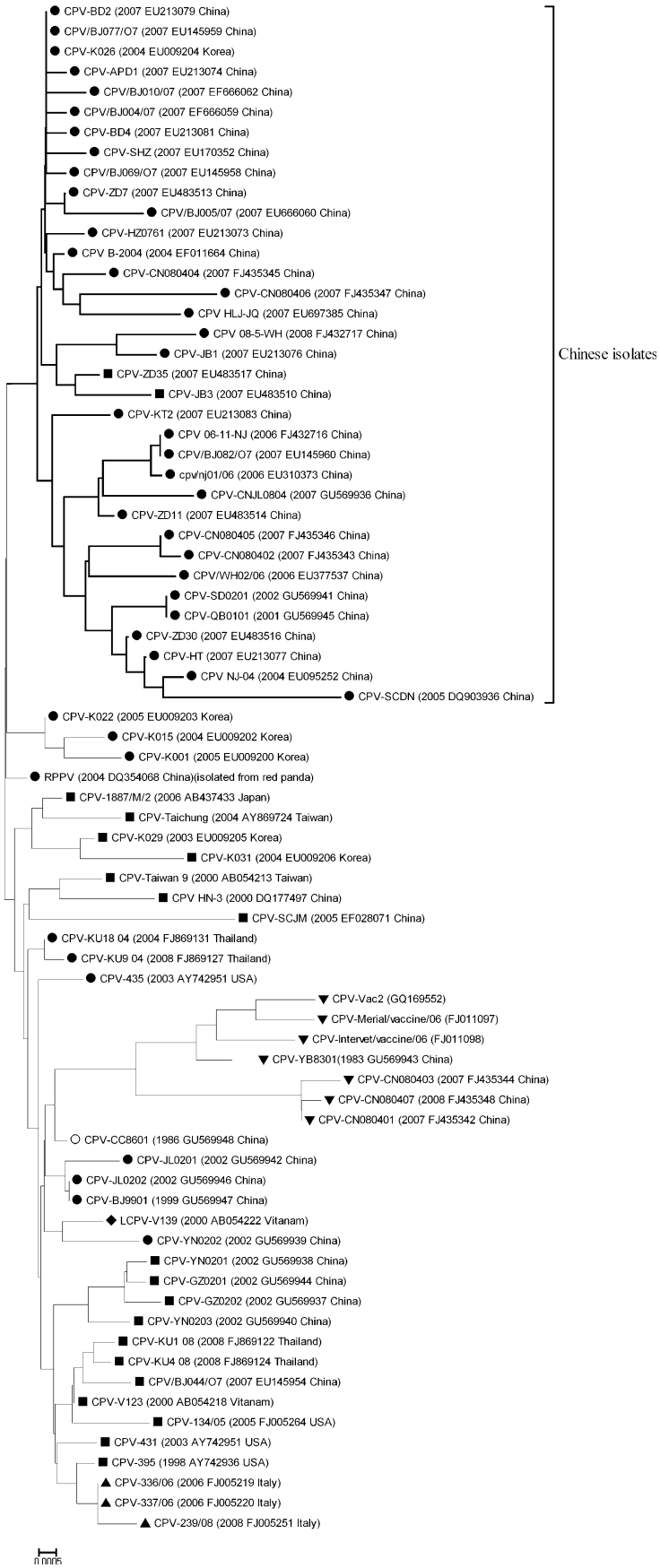

Fig. 1 Phylogenetic tree based on the VP2 gene sequence (1,750 nucleotides) of the parvoviruses. The last five nucleotides of the VP2 sequences were deleted from the VP2 gene alignment, as some of the sequences were incomplete, resulting in an alignment of 1,750 nt. The sequences from regions outside China used to construct phylogenetic trees were retrieved from GenBank. CPV type 2 isolates: *inverted triangle*; CPV types 2a and 2b isolates (with 297Ala mutation): *filled circle* and *filled square*, respectively; CPV type 2c isolates (with 426Glu mutation): *filled triangle*; CPV-2c(a) with 300Ala mutation: *filled rhombus*; the original CPV-2a type (without 297Ala mutation): *open circle*. Statistical support was provided by bootstrapping over 1,000 replicates and bootstrap values >75% are indicated at the correspondent nodes. *Horizontal branch lengths* are drawn to scale (nucleotide substitutions per site). Following the names of sequences, in *parentheses*, are year of isolation, GenBank accession number and the locations. *NA* not available

detected in China. We conclude that due to the continuing evolution of CPV, monitoring of field CPV isolates, and detection of genetic mutation and antigenic changes, will be necessary to control CPV infection in China.

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