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RESEARCH ARTICLE

Isolation and Identification of a Raccoon Dog Parvovirus and Sequence Analysis of its VP2 Gene

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A B S T R A C T

Raccoon dog parvovirus (RDPV) has spread widely in China, with gradually increasing morbidity and mortality. There is no commercial vaccine specifically for RDPV enteritis in China. Isolation of RDPV is very critical for the vaccine development against RDPV infection. The aim of this study was to isolate and identify RDPVs and analyze the phylogenetic clade of VP2 gene. The rectal swabs from raccoon dogs with severe diarrhea were screened by CPV colloidal gold strip, the positive swabs were treated and cultured in CRFK cells. The viruses in infected CRFK cells were identified by PCR detection, immunofluorescent test and electronic microscope observation. VP2 genes of the isolates were amplified and subjected to sequence. Two-month-old raccoon dogs were inoculated orally with the isolates for the virulence test. The results showed that one RDPV was isolated and named as RDPV HB01 strain. Sequence analysis revealed that the strain shared more than 99% nucleotide homologies with the canine parvovirus (CPV)-2a strains. All experimentally infected animals showed anorexia or depression with diarrhea in various degrees. Observation of histopathological lesions revealed that there were massive necrosis and abscission of villous epithelial cells in small intestine, loose and shedding lymphocyte structure in mesenteric lymph nodes, congestive medullary sinus and decreased lymphocyte of the white medullary lymph follicles in spleen. Therefore, the RDPV HB01 strain is classified as a CPV-2a variant and virulent to raccoon dogs.

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INTRODUCTION

Canine parvovirus (CPV) belongs to the genus parvovirus, family of Parvoviridae. CPV was first reported in healthy dogs in 1967 and named as CPV-1. In 1978, a new type of canine intestinal infectious disease broke out in the United States, and the pathogen was isolated and identified as CPV-2. After 1980s, CPV-2 continuously evolves into CPV-2a and CPV-2b variants, and becomes prevalent all over the world. In the early 21st century, CPV-2c variant was first reported in Italy, as well as in the southern United States, Spain, and Vietnam. It has been reported that the genotype mutant strains have replaced the classical CPV-2 strain at present. Variant viruses infect wild animals such as raccoons, mink, fox and wolf and dogs are the main natural hosts. Recent reports indicate that CPV-2a is prevalent mainly in Australia, India, Hungary, Korea, China and Greece (Mylonakis *et al.,* 2016). CPV-2b is the prevalent variant in the United States, the United Kingdom, and Japan, but with different frequencies (Altman *et al.,* 2017). The CPV-2c is detected mainly in Italy and Argentina, and Uruguay (Truyen, 2006). CPV-2a, CPV-2b, and CPV-2c are currently spreading globally, and their isolation frequencies may be related to the geographic region and time of the sample collection and different commercial flows of dogs imported from foreign countries (Geng *et al.,* 2015).

In the past 40 years, with the continuous emergence of CPV-2 mutant strains, the virus has been transmitted from dogs to other species, such as domestic cats and other domestic or wild carnivores, and CPV-2 has frequently spread across species in carnivores, accompanied by the rapid evolution of multi host coordination (Kumar and Nandi, 2010). In 1984, the viral enteritis in raccoon dog was first reported in Heilongjiang province in China. Three strains of raccoon dog parvovirus (RDPV) from raccoon dogs with enteritis were

isolated from different regions in 1986, confirming the existence of RDPV in China (Xia *et al.,* 1989). Six parvovirus strains were isolated from raccoon dog with suspected viral enteritis from Liaoning and Hebei provinces in 2009. The gene sequence analysis of the isolated parvovirus strains showed that they all belong to CPV-2 type (Yan *et al.,* 2010). Since 2016, RDPV has spread more widely in China, with gradually increasing morbidity and mortality (Ge, 2021; Guo *et al.,* 2021, Xu *et al.,* 2021). The outbreak of RDPV may be related to the mutation of the key amino acid site of VP2 protein (Jia-Yu *et al.,* 2018).

In the study, one RDPV strain was isolated and identified, and the VP2 gene was PCR amplified for sequence analysis. The animal experiment was designed to confirm its pathogenicity to susceptible animals. The isolated RDPV strain may be a candidate strain for inactivated vaccine development for raccoon dog in the future.

MATERIALS AND METHODS

Samples and cells: Thirty-six rectal swabs were collected from raccoon dogs with severe diarrhea from Hebei province during 2018-2019. Crandell Reese Feline Kidney (CRFK) cells were maintained in minimum essential medium (MEM) supplemented with 6% fetal bovine serum (FBS, Hyclone Laboratories Inc, South Logan, UT, USA) at 35℃, with 5% CO2.

Rapid CPV antigen test: A canine parvovirus immunocolloidal gold strip test kit was used for detecting CPV antigen in swab samples. The swab sample were vigorously mixed with the sample diluent to extract the virus antigens. The provided test device was removed from an aluminum foil pouch and placed on a flat and dry surface. The extracted samples were aliquoted using a disposable dropper provided. Four drops of each sample were added into each sample well. The same amount of assay diluent was slowly added drop by drop. As the test reaction began to work, a purple color move across the result window in the center of the test device was observed. The results were interpreted within 5~10 minutes. The presence of only one band ("C") within the result window indicated a negative result. The presence of two colors bands ("T" and "C") within the result window indicated a positive result of canine parvovirus.

Virus isolation: Sterile treated rectal swab samples were 1:20 diluted and synchronously inoculated on the CRFK cells, which is very sensitive to parvovirus isolation. The cells were cultured at 35℃ in 5% CO2 incubator, and the cytopathogenic effect (CPE) was observed daily. When CPE appeared, the cells with CPE were maintained and further passaged, and the virus samples from each passage were harvested and kept at -20℃.

PCR identification and sequencing of VP2 gene: Total DNA of the third-generation virus passaged on CRFK cells was extracted using an EasyPure®Viral DNA/RNA Kit (Transgen Biotech, Beijing) in accordance with the manufacturer's instructions and then screened using a specific PCR assay with PrimerSTAR® Max DNA Polymerase (Takara Bio, Dalian, China) and primers (CPV-2-F and CPV-2-R) targeting on a part of the CPV-2 VP2 gene (Allison *et al.,* 2012). PCR products were analyzed by electrophoresis in a 1% agarose gel and staining with ethidium bromide. The complete VP2 gene sequences were further amplified using two primer pairs (Table 1) and then sequenced by the Sanger method.

Table 1: The primer pairs used in this study

Purpose	Primer pairs	Sequences	Length
CPV2 identification	CPV2-F	5′-ATCACAGCAAACTCAAGCAGACTT-3′	481bp
	CPV2-R	5′-AAATGGTGGTAAGCCCAATGCTC-3′	
VP2 gene amplification	vp2-F	5′-CAACATCAAGACCAACAAAAC-3′	1755bp
	vp2-R	5′-TAACAAACCTTCTAAATCCTA-3′	

Immunofluorescence assay: An indirect immunofluorescence assay was carried out as described previously (Vihinen-Ranta *et al.,* 1998). Briefly, CRFK cell suspension was adjusted in cell counts and placed into the 96-well plates, and the third generation of the virus was inoculated simultaneously. Once CPE appeared in the cell culture after 48 hours, the supernatant was discarded. The cells were fixed with methanol and then incubated with 0.1%Triton 100 for 10 min at room temperature. The diluted anti-CPV monoclonal antibody (VMRD, USA) was added and incubated for 30 min at room temperature. Finally, 200 µl of the diluted rabbit anti-mouse FITC conjugate (containing 0.01% Evans blue) was added and incubated at room temperature for 30 min. After PBS rinsing, the cells were examined under fluorescence microscope.

Electron microscopy observation: The third generation of virus cultured in CRFK cell was harvested and centrifuged at 10000 rpm for 10 min, the supernatant was collected for 0.5% phosphor-tungstic acid staining. The morphology of virus was observed under a transmission electron microscopy after negative staining.

Animal study and experimental design: All experimental procedures were performed in accordance with regulatory standards and guidelines approved by local Animal Care & Use Committee, Taizhou, China. Ten healthy 2-month-old raccoon dogs of were randomly divided into two groups. All animals were confirmed to be negative for parvovirus antigen and anti-parvovirus antibody. Raccoon dogs were collectively housed for 3 days prior to exposure to virus, and then individually housed in the isolation units from the time of sample inoculation until the end of the observation course. Raccoon dogs in group 1 (n=5) were orally inoculated with 105.0 TCID50 (50% tissue culture infective doses) of the isolate in a 10 mL volume. The dogs in group 2 (n=5) were orally inoculated with PBS in the same volume. Each group was housed separately in different rooms. After inoculation, animals were monitored twice daily for clinical signs. Clinical signs and food intake were evaluated daily for each experimental animal. On day 8

post infection, the one infected and one control raccoon dogs were euthanized, tissue samples of small intestine, mesenteric lymph nodes and spleens were collected from each animal and processed for histopathological examination following hematoxylin and eosin (H&E) staining, as described previously (Parrish *et al.,* 1991).

RESULTS

Screening of samples by rapid CPV antigen test: The results of CPV antigen detection in raccoon dog rectal swabs by using canine parvovirus immunocolloidal gold strip were shown in Fig 1. Twenty-four samples out of 36 rectal swabs samples showed two colors bands within the result window, indicating a positive result of canine parvovirus.

Virus isolation: The CPEs were observed on the third passage of CRFK cells inoculated with raccoon dog rectal swabs on day 4. The cells in the CPE positive wells showed elongated morphology, enlarged intercellular space, enhanced refraction, and net-like lesions (Fig. 2A). On the contrary, the control CRFK cells (Fig. 2B) grew well with clear edge and normal cell morphology.

PCR identification: The DNA of one sample passaged for three generation in CRFK cells was extracted and amplified. The result showed that a 481-bp specific band appeared in the PCR product, which was consistent with the size of expected target gene, indicating the new isolate belonging to be a member of CPVs (Fig. 3).

Immunofluorescence assay: The isolate at the third passage in CRFK cells were further confirmed using CPV-2 specific indirect immunofluorescent assay. The result showed that specific green fluorescence appeared in the CRFK cells infected with the newly isolated virus (Fig. 4A), which was consistent with the CPV infected cells (Fig. 4B), but no specific green fluorescence appeared in the negative control cells (Fig. 4C) indicating that the isolate belongs to CPV-2.

Electron microscopy observation: The third generation of isolated virus was negative stained and the morphology of virus was observed under transmission electron microscopy, there were a lot of round virus particles without envelope, approximately 20~26nm in diameter, which was consistent with the morphology and size of CPV (Fig. 5). The isolate was named as RDPV HB01.

Sequence and phylogenetic analysis of VP2 gene: The gene sequence analysis of the isolates showed that a full length of VP2 gene of RDPV HB01 strain is 1755 bp and encodes 584 amino acids. Comparison of the VP2 gene of RDPV HB01 to other CPV strains published in Genbank revealed that the nucleotide homology was between 97.1% and 99.7%, and deduced amino acids homology was between 97.7 and 99.5%. The phylogenetic tree created with the sequences of VP2 gene illustrated that RDPV HB01 strain located in the branch of CPV-2a variant (Fig. 6). By comparing the 12 amino acids of VP2 protein between RDPV HB01 and other reference strains, the results showed that 27T, 80K and 87M amino acid sites of the strain were the same as those of feline parvovirus and mink parvovirus. However, the strain retained 93N, 103A, 323N, 426N and 564S, which is the key residues located in antigenic domains of CPV-2 and therefore belong to the CPV-2a variant.

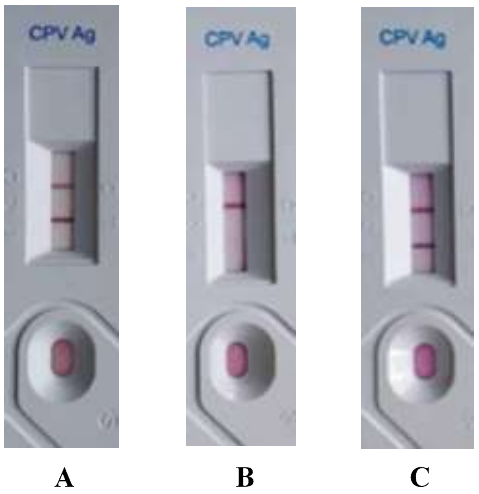

Fig. 1: Rapid detection of CPV antigen from swab samples. Canine parvovirus immunocolloidal gold strip test kits were used for detecting CPV antigen in rectal swab sample (A). The presence of only one band within the result window was indicated as a negative result of CPV (B). The presence of two colors bands within the result window was indicated as a positive result of CPV (C).

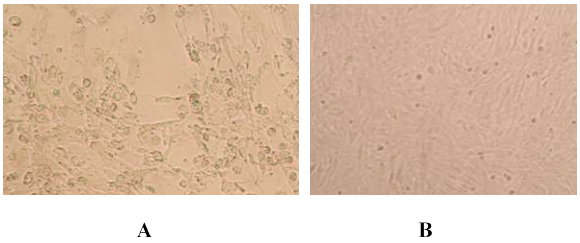

Fig. 2: Cytopathogenic effect induced by the virus in CRFK cells. The rectal swab samples were treated aseptically and passaged on CRFK cells, the CPE was observed daily. A: Infected cells, B: Control cells.

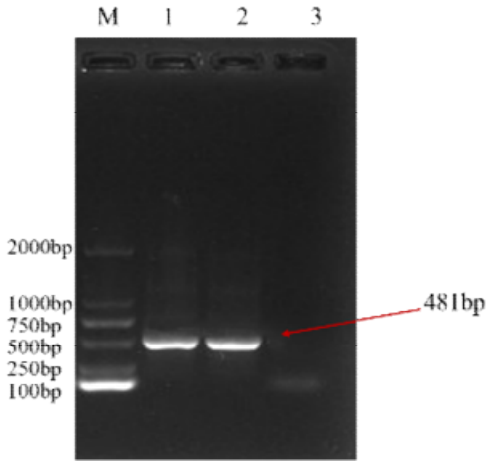

Fig. 3: PCR identification for RDPV**.** The total DNA of infected CRFK cells was extracted and subjected to PCR amplified. The PCR product was analyzed by agarose gel electrophoresis. M: DNA Marker (DL2000), 1: The third-generation virus passaged on CRFK cells, 2: Positive control, 3: Negative control.

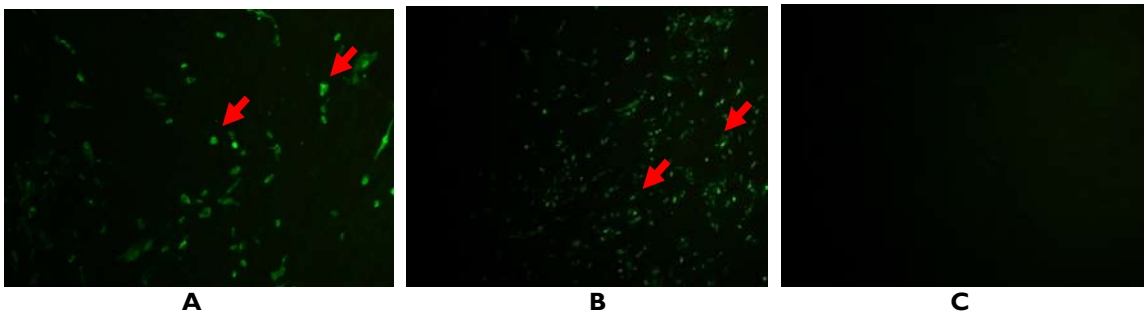

Fig. 4: Immunofluorescence detection of RDPV in CRFK cells. The third generation of RAPV was inoculated into CRFK cells and stained with anti-CPV monoclonal antibody. A: RDPV infected cells, B: Positive control, C: Negative control.

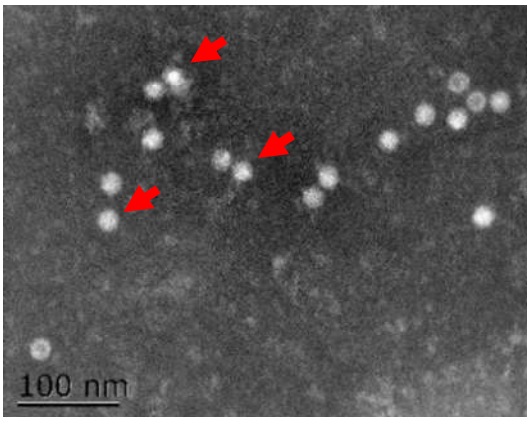

Fig. 5: Morphology observation of the isolated virus under electron microscopy. The third generation of virus cultured in CRFK cell was harvested and the morphology of virus particle was observed under a transmission electron microscopy after negative staining. The virus particles were indicated by arrows.

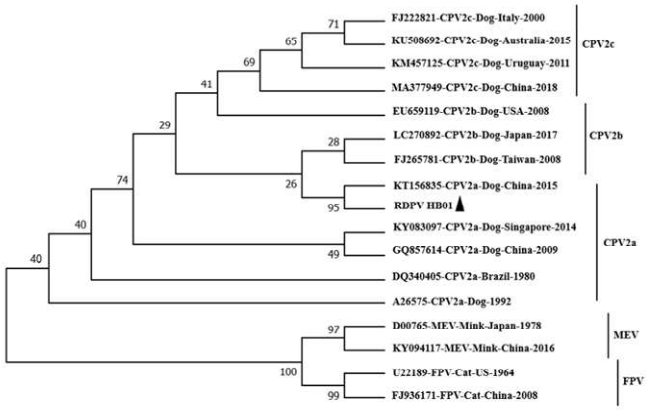

Fig. 6: Phylogenetic tree based on VP2 gene. The phylogenetic tree was created based on the sequences of VP2 gene from RDPV HB01 (▲) and other reference strains deposited in Genbank.

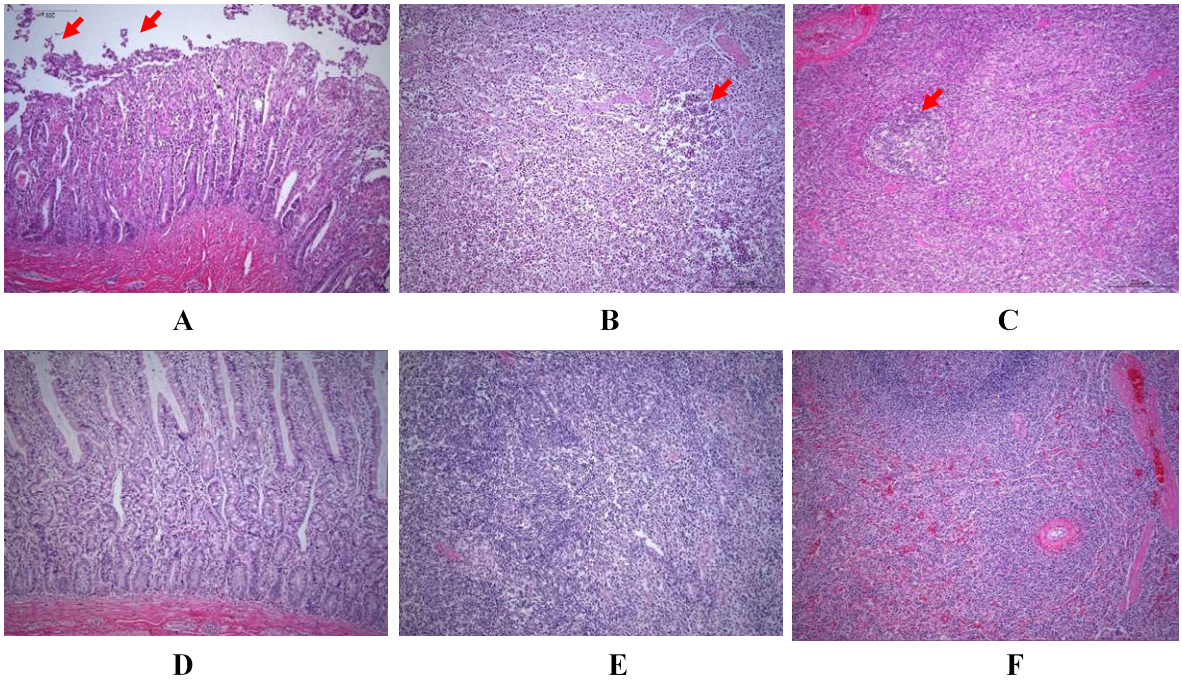

Fig. 7: Histological examination of raccoon dogs after RDPV challenge. The small intestine, mesenteric lymph nodes and spleens of infected or control raccoon dogs were collected for histopathological examination following hematoxylin and eosin (H&E) staining. Abscission of villous epithelial cells in small intestine (A), loose and shedding of lymphocyte structure in mesenteric lymph node (B), and lymphocytic decrease in the white medullary lymph follicles of spleen (C) from infected raccoon dogs were indicated by arrows. Small intestine (D), mesenteric lymph node (E), and spleen (F) from normal raccoon dogs were set as controls.

Clinical signs observations and histological examination post infection: All raccoon dogs inoculated with RDPV HB01 strain showed diarrhea in various degrees, mainly with loose stool and watery feces, accompanied by anorexia or depression. Three out of five raccoon dogs died of severe enteritis. Conversely, no clinical signs were observed in the control group. For histological examination, massive necrosis and abscission of villous epithelial cells were observed in small intestine in the RDPV-inoculated raccoon dogs (Fig. 7A); the lymphocyte structure was loose and shedding in mesenteric lymph nodes (Fig. 7B); there was congestion in the medullary sinus and lymphocytic decrease in the white medullary lymph follicles of spleen (Fig. 7C). In contrast, all the small intestine, mesenteric lymph nodes and spleens from control raccoon dogs exhibited normal tissue and structures (Fig. 7D, 7E, 7F).

DISCUSSION

Parvovirus belongs to the genus Parvovirinae, a member of the Parvoviridae family, which is the single strand DNA virus without envelope. It could infect a variety of mammals. Most parvoviruses are species-specific and can proliferate on specific host cells. It is generally believed that RDPV first emerged by CPV-2 cross-species transmission in raccoon dogs. It is a closely related to FPLV, MEV and other carnivore parvoviruses and considered to be a branch of viruses causing feline panleukopenia syndrome.

In this study, one RDPV was isolated from a raccoon dog farm in Hebei province. The isolated virus was confirmed as an RDPV by observing the CPE on CRFK cell culture, PCR identification, morphology of viral particles, and immunofluorescence assay. It is reported that CPV-2 has been isolated from lungs, spleen, liver, kidneys, and myocardium. The samples including liver, spleen, brain, and rectal swabs collected from diseased raccoon dogs for virus identification, rapid CPV antigen test showed that there were 66.7% (24/36) positive ratio in rectal swabs, suggesting that the viruses are shed from rectum in higher ratio and rectal swabs can be collected for virus isolation. Clinically, the diseased raccoon dogs in the farm showed depress, anorexia, bloody diarrhea and even death. After necropsy, the intestinal folds, thin intestinal wall and enlarged mesenteric lymph nodes were observed. Our experimental infection showed similar clinical symptom and histopathologic lesion, suggesting that RDPV HB01 strain has already adapted to raccoon dogs and cause serious disease. Previous study showed that the RDPV isolates were belonged to CPV-2 (Yan *et al.,* 2010; Lu *et al.,* 2020), CPV-2a variant was rarely isolated. Our study showed that the RDPV HB01 strain was belonged to CPV-2a branch, which contribute to the study of RDPV evolution.

Compared with feline and mink parvovirus, the VP2 gene of RDPV HB01 strain shared higher nucleotide homology (99.7%) and amino acid homology (99.5%) with CPV-2a. Phylogenetic tree analysis showed that RDPV HB01 strain was clustered into the branch of CPV-2a. CPV-2a was approximately discovered in 1980 in the USA, and CPV-2b and 2c were identified in 1984 and 2000 in the USA and Italy. The typing of CPV-2 variants is commonly based on the residue 426 (Asn in 2a, Asp in 2b, and Glu in 2c) in the VP2 protein of the parvovirus (Truyen *et al.,* 1996). In this study, residue 426 of RDPV HB01 strain was Asn (N), in accordance with the character of the CPV-2a subtype. In fact, CPV-2a, 2b and 2c have reappeared in the host range for cats (Decaro and Buonavoglia, 2012) and have increased their own pathogenicity, causing more severe disease with a shorter incubation period. Moreover, the new virus variants are shed in the faeces at much higher titers, and a lower virus dose seems to be required for efficient infection (Clark *et al.,* 2018). Epidemiological survey indicates that the newest type CPV-2c is becoming prevalent in different geographic regions and is often associated to severe disease in adult dogs (Woolford *et al.,* 2017). Phylogenetic models indicated that CPV-2a likely emerged in Australia between 1973 and 1988, while CPV-2b likely emerged between 1985 and 1998 (Zhang *et al.,* 2010). In recent years, the prevalence of raccoon dog parvovirus enteritis has become increasing. There is no commercial vaccine specifically for RDPV enteritis in China. Mostly, mink or canine parvovirus enteritis inactivated vaccine was applied for preventing RDPV enteritis. However, the efficacy of cross protection needs to be further studied. Therefore, it is of great practical significance to isolate RDPV, screen out vaccine candidate strain with strong specificity, good immunogenicity and prepare RDPV vaccine for effective control of RDPV epidemic (Li *et al.,* 2018).

Since raccoon dogs are kept in captivity for a short time and kept in the wild for a long time, they are the natural host for parvovirus. It has been confirmed that the 80, 87, 103 and 323 residues in VP2 protein play an important role in host adaptation. The residues 80 and 87 of RDPV HB01 strain were Lys (K) and Met (M), which are consistent with FPLV and MEV, while residues 103 and 323 of RDPV HB01 strain were Ala (A) and Asn (N), which are consistent with CPV2 (Aldaz *et al.,* 2013). In addition, it is reported that the residue 300 is an important site related to the host range of CPV (Allison *et al.*, 2015), and residue 300 of RDPV HB01 strain is Ala (A), which is consistent with CPV-2. The parvovirus might undergo intragenic or intergenic recombination in raccoon dogs, thus forming a new virus different from other carnivore parvovirus, or the intermediate stage in the process of cross species evolution of carnivore parvovirus (Decaro and Buonavoglia, 2012). Our data suggests that RDPV HB01 strain may be in the intermediate stage of the evolution of FPLV and CPV-2, or an adapted variant for the new host (raccoon dog).

Competing interests: The authors declare that they have no competing interests.

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Authors contribution: WS and DP conceived and designed the study. HZ, YH and LW executed the

experiment and detected the clinical samples. ZL and XY analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

REFERENCES

Aldaz J, Garcia-Diaz J, Calleros L, *et al.,* 2013. High local genetic diversity of canine parvovirus from Ecuador. Vet Microbiol 166:214-9.

Allison AB, Harbison CE, Pagan I, *et al.,* 2012. Role of multiple hosts in the cross-species transmission and emergence of a pandemic parvovirus. J Virol 86:865-72.

Allison AB, Organtini LJ, Zhang S, *et al.,* 2015. Single Mutations in the VP2 300 Loop Region of the Three-Fold Spike of the Carnivore Parvovirus Capsid Can Determine Host Range. J Virol 90:753-67.

Altman KD, Kelman M and Ward MP, 2017. Are vaccine strain, type or administration protocol risk factors for canine parvovirus vaccine failure? Vet Microbiol 210:8-16.

Clark NJ, Seddon JM, Kyaw-Tanner M, *et al.,* 2018. Emergence of canine parvovirus subtype 2b (CPV-2b) infections in Australian dogs. Infect Genet Evol 58:50-5.

Decaro N and Buonavoglia C, 2012. Canine parvovirus--a review of epidemiological and diagnostic aspects, with emphasis on type 2c. Vet Microbiol 155:1-12.

Geng Y, Guo D, Li C, *et al.,* 2015. Co-Circulation of the Rare CPV-2c with Unique Gln370Arg Substitution, New CPV-2b with Unique Thr440Ala Substitution, and New CPV-2a with High Prevalence and Variation in Heilongjiang Province, Northeast China. PLoS One 10:e0137288.

Ge X. 2021. Isolation and identification of Raccoon Dog Parvovirus and Sequence Analysis on Its VP2 Gene. China Animal Health Inspection 38:120-3.

Guo Z, Li H, Jia A, *et al.,* 2021. Epidemic analysis of parvovirus enteritis in special fur animals in China from 2013 to 2020. Breeding Feed 12:28-31

Jia-Yu Y, Qian Z, Fei-Fe, D, *et al.,* 2018. Emergence of novel canine parvovirus type 2 and its pathogenesis in raccoon dogs. Vet Microbiol 216:7-12.

Kumar M and Nandi S, 2010. Molecular typing of canine parvovirus variants by polymerase chain reaction and restriction enzyme analysis. Transbound Emerg Dis 57:458-63.

Li X, Wu H, Wang L, *et al.,* 2018. Genetic characterization of parvoviruses in domestic cats in Henan province, China. Transbound Emerg Dis 65:1429-35.

Lu R, Yu Y, Zhu X, *et al.,* 2020. Molecular characteristics of the capsid protein VP2 gene of canine parvovirus type 2 amplified from raccoon dogs in Hebei province, China. Arch Virol 165:2453-9.

Mylonakis ME, Kalli I and Rallis TS, 2016. Canine parvoviral enteritis: an update on the clinical diagnosis, treatment, and prevention. Vet Med (Auckl) 7:91-100.

Parrish CR, Aquadro CF, Strassheim ML, *et al.,* 1991. Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. J Virol 65:6544-52.

Truyen U, 2006. Evolution of canine parvovirus--a need for new vaccines? Vet Microbiol 117:9-13.

Truyen U, Evermann JF, Vieler E, *et al.,* 1996. Evolution of canine parvovirus involved loss and gain of feline host range. Virology 215:186-9.

Vihinen-Ranta M, Kalela A, Makinen P, *et al.,* 1998. Intracellular route of canine parvovirus entry. J Virol 72:802-6.

Woolford L, Crocker P, Bobrowski H, *et al.,* 2017. Detection of the Canine Parvovirus 2c Subtype in Australian Dogs. Viral Immunol 30:371-6.

Xia X, Ye J, Fan Q, *et al.,* 1989. Isolation, identification and immunization of natural attenuated strain of canine parvovirus. Chinese Livestock Poult Inf Dis 06:6-10.

Xu L, Ge P, Wang J, *et al.,* 2021. Isolation and identification of a canine parvovirus CPV-CL01 strain from Raccoon Dog. Shandong Anim Husb Vet 42:8-10.

Yan X, Zhang L and Chai X, 2010. Canine parvovirus type 2 was found in foxes and raccoons in China. Special Wild Econ Anim Plant Res 01:79

Zhang R, Yang S, Zhang W, *et al.,* 2010. Phylogenetic analysis of the VP2 gene of canine parvoviruses circulating in China. Virus Genes 40: 397-402.