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Research Paper

Genetic and phylogenetic analysis of canine bufavirus from Anhui Province, Eastern China

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ABSTRACT

Bufavirus is a novel virus associated with canine gastroenteritis. Three strains of bufavirus were first detected in dog feces collected from Anhui province in Eastern China. The near-complete genome sequences were amplified. Sequence alignment showed 98.3–99.5% homology between the three bufavirus strains and reference strains. Phylogenetic analysis showed the distributed viruses forming a cluster of close relationships. Selective pressure analysis of the *VP2* region indicated that the canine bufavirus (CBuV) was mainly subject to negative selection during evolution. The negative selection site was located on the residue of B-cell epitopes, indicating minimal change to the virus's immunogenicity. Since this is the first report of CBuV circulating in Anhui Province, this study will provide further understanding of the phylogenetic and molecular characteristics of CBuV and serve as a reference for prevention and vaccine development.

1. Introduction

Members of the family *Parvoviridae* are common pathogens, which cause a wide range of animal diseases (Lau et al., 2012). The viruses can be divided into the subfamilies *Parvovirinae* and *Densovirinae*. The subfamily *Parvovirinae* can be further divided into *Amdoparvovirus*, *Aveparvovirus*, *Bocaparvovirus*, *Copiparvovirus*, *Dependoparvovirus*, *Erythroparvovirus*, *Protoparvovirus*, and *Tetraparvovirus* (https://talk.ictvonline.org/ictv-reports/ictv_online_report/ssdna-viruses/w/parvoviridae).

Bufaviruses (BuV) are part of the Protoparvovirus genus (Hargitai et al., 2016; Huang et al., 2020). It is a small, non-enveloped, single-stranded DNA virus with a genome size of 4.5–4.8 kb with complex hairpin structures at the 5′ and 3′ ends. BuV also contains two open reading frames (ORFs), ORF1 and ORF2. ORF1 encodes non-structural protein, and ORF2 encodes capsid protein (Sun et al., 2019).

In 2012, it was discovered in the fecal samples of children with diarrhea in Burkina Faso (Phan et al., 2012). Subsequently, BuV was found in wild shrews, megabats, wild rats, pigs, dogs, and cats (Diakoudi et al., 2019; Huang et al., 2020; Martella et al., 2018; Sasaki et al., 2016; Sasaki et al., 2015). In 2018, a virus with a close genetic relationship to the human bufavirus (HuBuV) was detected in dogs with either gastroenteric or respiratory disease in Italy; it was named canine bufavirus (CBuV) (Martella et al., 2018). In China, the CBuV was first detected in Shanghai, causing a high infection rate in dogs (Li et al., 2019).

Presently, the distribution of CBuV has only been reported in Italy and China (Di Martino et al., 2020; Li et al., 2019; Martella et al., 2018; Sun et al., 2019), and its genetic characteristics and pathogenicity are poorly understood. The primary symptom caused by a member of *Protoparvovirus* is diarrhea in carnivore (Chaiyasak et al., 2020; Piegari et al., 2020). Recent studies have shown a positive correlation between CBuV and diarrhea, and CBuV DNA was also detected in the serum sample of dogs with gastroenteritis(Li et al., 2019). In terms of genetic and phylogenetic characteristics, a report has shown that the potential heterogeneity of CBuV and recombination may be a factor in its evolution (Di Martino et al., 2020).

As mentioned, the CBuV was found in Shanghai, China with a hige prevalence. The prevalence of the virus in Anhui province, which has a

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close relationship with Shanghai in terms of trade, is unknown. To this end, fecal samples from different cities in Anhui province, Eastern China were collected in this study to explore the molecular and phylogenetic characteristics of CBuV. The study reveals the epidemic status in Anhui province and related molecular characteristics of CBuV, which provide significant reference for studies on the evolution and epidemiology of CBuV.

2. Materials and methods

2.1. Sample collection

In total, 120 canine fecal samples were collected from animal hospitals in different cities located in Anhui Province from June to December 2019. Of the total number of samples, 89 were from puppies (<1 year old), and only 31 were from adult dogs (>1 year old). Fifty-two fecal samples (31 adult dogs and 21 puppies) were from healthy dogs, and 68 were from puppies with diarrhea. The fecal samples were collected in sterile centrifuge tubes using rectal swabs and stored at −80 °C until used.

2.2. DNA extraction and PCR amplification

The samples were dissolved in 10% phosphate-buffered saline and mixed in an oscillating manner. The mixture was centrifuged at 10,000 ×*g* for 10 min, and the supernatant was collected. Total DNA was extracted using TIANamp Virus DNA/RNA Kit (TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions. The extracted DNA was stored at −20 °C until use.

CBuV was detected through conventional PCR (cPCR), as previously described (Martella et al., 2018). The primers used for detecting canine parvovirus (CPV) were designed from other sequences in GenBank (accession number: KT382542.1) using Primer Premier 5 software (DNASTAR, Inc., Madison, WI, USA). Reactions comprised 12.5 μL of 2 × Premix Taq® Version 2.0 (TaKaRa Bio Inc., Dalian, China), 1 μL of the DNA templates, 0.4 μM forward and reverse primers, and ddH_2O to obtain a total volume of 25 μL (Table 1). The PCR products were visualized through agarose gel electrophoresis and then verified by sequencing.

2.3. CBuV complete genome amplification

The specific primers used to amplify the near-complete genome were designed from other sequences in GenBank (accession number: MK404087.1) using the Primer Premier 5 software (DNASTAR, Inc.; Table 1). PCR products were purified using a DNA purification kit (TIANGEN Biotech) following the manufacturer's instructions. After ligation of the PCR products into the pMD19-T vector (TaKaRa Bio Inc.), the recombinant plasmids were sent to Sangon Biotech Co Ltd. (Shanghai, China) for sequencing. Each plasmid was sequenced three times. Near-complete genomes of the CBuV strain obtained for this study were splined together using SeqMan software (DNASTAR). ORFs were identified using the NCBI ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/).

2.4. Phylogenetic analysis

Sequence identity was analyzed with the MegAlign 6.0 program (DNASTAR Inc.) by aligning nucleotide and amino acid sequences via the MAFFT method (Katoh and Standley, 2013). Phylogenetic analysis was performed using PhyloSuite software (Zhang et al., 2020). Maximum likelihood phylogenies were inferred using IQ-TREE for 5000 ultrafast bootstraps (Minh et al., 2013; Nguyen et al., 2015) and Shimodaira-Hasegawa-like approximate likelihood-ratio test (Guindon et al., 2010). ModelFinder selected the best substitution model (Kalyaanamoorthy et al., 2017). Phylogenetic trees were visualized using FigTree software (http://tree.bio.ed.ac.uk/software/figtree/). The Recombination Detection Program v.4.39 (RDP 4.39) identified the recombination events.

Table 1
Primers and conditions used for the detection and naer-complete genome sequences amplification of CaBuV.

Primer name	Nucleotide sequence (5′-3′)	Amplicon (bp)	Reaction condition
Detection primer			
CPPV 165F	CTGGTTTAATCCAGCAGACT	645	94 °C for 2 min, 45 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, followed by final extension at 72 °C for 10 min
CPPV 371R	TGAAGACCAAGGTAGTAGGT		
Amplification primer			
BUFA-F1	ATGGCTACATCTACATTCTCTGAC	952	98 °C for 1 min, 40 cycles at 98 °C for 10s, 60 °C for 5 s, and 72 °C for 5 s, followed by final extension at 72 °C for 10 min
BUFA-R1	GCTTTGATGATAGTTTCACCACCTG		
BUFA-F2	ACAGGATGAAGGAAACCACAGTAAC	1183	98 °C for 1 min, 40 cycles at 98 °C for 10s, 58 °C for 5 s, and 72 °C for 5 s, followed by final extension at 72 °C for 10 min
BUFA-R2	AACATTTACCCACAACCCATATAGA		
BUFA-F3	AGCAAGCTACTGTAAACGGTGGGGT	1351	98 °C for 1 min, 40 cycles at 98 °C for 10s, 60 °C for 5 s, and 72 °C for 5 s, followed by final extension at 72 °C for 10 min
BUFA-R3	TGTGTTGTTTCTTCCTGTCCTGTGC		
BUFA-F4	CTCTACCAAACAGACCACGGAC	848	98 °C for 1 min, 40 cycles at 98 °C for 10s, 58 °C for 5 s, and 72 °C for 5 s, followed by final extension at 72 °C for 10 min
BUFA-R4	CTAGATGGTTGGCCTGGATTG		
BUFA-F5	CAATCGGAGGTGAGGGAC	815	98 °C for 1 min, 40 cycles at 98 °C for 10s, 60 °C for 5 s, and 72 °C for 5 s, followed by final extension at 72 °C for 10 min
BUFA-R5	TTATAGAGTAATATTAGGCATAGCT		

2.5. Selection pressure analysis and B-cell epitope prediction

The detection of selection pressure on the *VP2* was performed using three methods, including Single Likelihood Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL), Mixed Effects Model of Evolution (MEME), and Fast Unconstrained Bayesian AppRoximation, for inferring selection (FUBAR) on the DATAMONKEY (http://www.datamonkey.org/). The tertiary structure models of the CBuV VP2 protein were built using the I-TASSER online tool (https://zhanglab.ccmb.med.umich.edu). The viral surface and structure were depicted in PyMOL 2.3.0 (DeLano Scientific, San Carlos, CA, USA). The B-cell epitopes of the CBuV capsid protein were predicted using the online server: (http://

http://sysbio.unl.edu/SVMTriP/) (Yao et al., 2012).

3. Results

3.1. Detection of CBuV in fecal samples

cPCR assay revealed that three fecal samples were positive for CBuV. The overall positive rate was 2.5% (3/120), and the positive rate of the symptomatic animals' group was 4.4% (3/68). Three positive samples were collected from Suzhou, Hefei, and Maanshan in Anhui province. All positive samples were from puppies with diarrhea, and all tested positive for canine parvovirus and negative for canine bocavirus, canine astrovirus, canine kobuvirus, and canine coronavirus.

3.2. Characteristics of the CBuV genomes

The three CBuV strains' nearly-complete sequences were amplified and uploaded to GenBank (accession numbers: MT542982, MT542983, MT577645). All three strains were 4219 nt in length, same as the reference strains. The length of *NS1*, *VP1,* and *VP2* was 1917 nt (320 amino acids), 2133 nt (771 amino acids), and 1707 nt (569 amino acids). The nucleotide similarity between the three strains was 98.9% to 99.5%. Compared with other reference strains (Table 2), the similarity of complete sequences was 98.3–99.5%. The similarity of the NS1, VP1, and VP2 sequences were 98.1–100%, 98.2–99.8% and 98.5–99.5%, respectively. The amino acid similarity of *NS1*, *VP1,* and *VP2* was 99.5–100%, 98.3–99.9%, and 97.9–100%, respectively. In addition, only two non-synonymous substitution sites were found in the *NS1* protein sequences of the three strains. However, 11 non-synonymous substitution sites were identified in the *VP2* region. When compared with canine bocavirus (CBoV) and CPV of the parvovirus genus, the homology was low at 36.6–55.9%.

3.3. Phylogenetic analysis and recombination analysis of CBuV

Phylogenetic analysis was performed based on the near-complete sequences of the three CBuV strains obtained from Anhui Province, other BuVs, and parvovirus from GenBank. Phylogenetic tree analysis (Fig. 1a) shows that HuBuV, CBuV, porcine bufavirus (PoBuV), and megabat bufavirus (MeBuV) formed a large branch. The CBuV branch was more closely related to PoBuV. Phylogenetic analysis of the *VP2* gene revealed a similar topology to the near-complete sequence phylogenetic tree (Fig. 1b). However, in the *VP2* region, the relationship between PoBuV and CBuV was not close. To analyze whether recombination played a role in evolution, CBuVs, CPVs, CBuVs, HuBuVs, and PoBuVs were analyzed by RDP software. However, no recombinant events were found in CBuVs.

3.4. Selection pressure analysis and B-cell epitopes prediction based on VP2

Selection pressure analysis of the *VP2* of all CBuV strains revealed positively selected codon site and 14 negative sites. Of the 14 negative sites, 11 were detected by several methods (Table 2). Codon 255 was a potential positively selected codon site detected by the FUBAR method. Codons 313, 380, 566, 411, 240, 223, 352, 158, 465, and 366 were potential negatively selected codon sites detected by FUBAR and SLAC. Codons 313 and 411 were detected by FUBAR, SLAC, MEME, and FEL.

To further understand the effect of selection pressure on the CaBoV, B-cell epitope analysis of three *VP2* sequences was performed. The *VP2* dominant epitopes of AH-001 and AH-002 strains were located on AA239-258, 99–118, 42–61, 301–320, and 373–392. The epitopes of AH-003 were located on AA99-118, 42–61, 301–320, 373–392, and 504–523 (Table 3). Interestingly, a comprehensive analysis of pressure selected B-cell epitopes revealed that codons 240, 313, and 380 were located on the predicted B-cell epitopes (Fig. 2). (See Table 4.)

4. Discussion

Bufavirus is a potential enteric pathogen that causes diarrhea in children (Altay et al., 2015; Chieochansin et al., 2015). CBuV was first found in canines with gastrointestinal and respiratory diseases (Martella et al., 2018). In Italy, the positive rate was 7.7% (16/207) (Di Martino et al., 2020). In China, CBuV has been found in Shanghai, Guangxi province and Henan province, and the positive rates were 42.15% (51/121), 2.5% (5/200), and 1.74% (2/115), respectively (Li et al., 2019; Shao et al., 2020; Sun et al., 2019). In this study, three positive samples were detected, and the positive rate was 2.5% (3/120) in Anhui province. Positive rates in other parts of China are significantly lower than those in Shanghai, indicating that CBuV prevalence may have regional differences.Further studies are needed to confirm the significance of this difference.

In previous reports from China and Italy, the presence of CBuV was found in dogs both healthy and with diarrhea (Di Martino et al., 2020; Sun et al., 2019). All three dogs positive for CBuV in this study were co-infected with CPV but were negative for canine bocavirus, canine

Table 2
General information and GenBank accession numbers of reference strains used in this study.

Strains	Organism	GenBank accseeion no.	District of origin	Nucleotides	Host
CBuV-88	Canine bufavirus	MH645362.1	China	4249	Dog
GXNN01-2018	Canine bufavirus	MK404086.1	China	4219	Dog
GXNN02-2018	Canine bufavirus	MK404087.1	China	4219	Dog
ITA/2015/297	Canine bufavirus	MF198244.1	Italy	4219	Dog
HUN/2012/126	Canine bufavirus	MF198246.1	Hungary	4219	Dog
MAG12-57	Megabat bufavirus	LC085675.1	Indonesia	4765	*Pteropus vampyrus*
BTN-109	Human bufavirus	AB847988.1	Bhutan	4734	Human
BTN-310	Human bufavirus	AB847989.1	Bhutan	4766	Human
BF.96	Human bufavirus	JQ918261.1	Burkina Faso	4912	Human
BF.39	Human bufavirus	JX027297	Burkina Faso	4562	Human
BJ133	Human bufavirus	KM580347.1	China	4882	Human
61	Porcine bufavirus	KU867071.1	Austria	4189	swine
GX018	Porcine bufavirus	MK279312.1	China	4189	swine
FJNP2018	Porcine bufavirus	MK279316.1	China	4189	swine
SY-2015	Rat bufavirus	KT716186.1	China	4634	wild rat
Y1	Canine parvovirus	D26079.1	Japan	5075	Dog
CPV-SH1516	Canine parvovirus	MG013488.1	China	5059	Dog
CPV-AHhf1	Canine parvovirus	MT010564.1	China	5061	Dog
5 MGL	Canine parvovirus	MH660909.1	Mongolia	5075	Dog
UFMT	Canine parvovirus	KY073269.1	Brazil	5318	Dog
CPV-SH14	Canine parvovirus	KT382542.1	China	5062	Dog

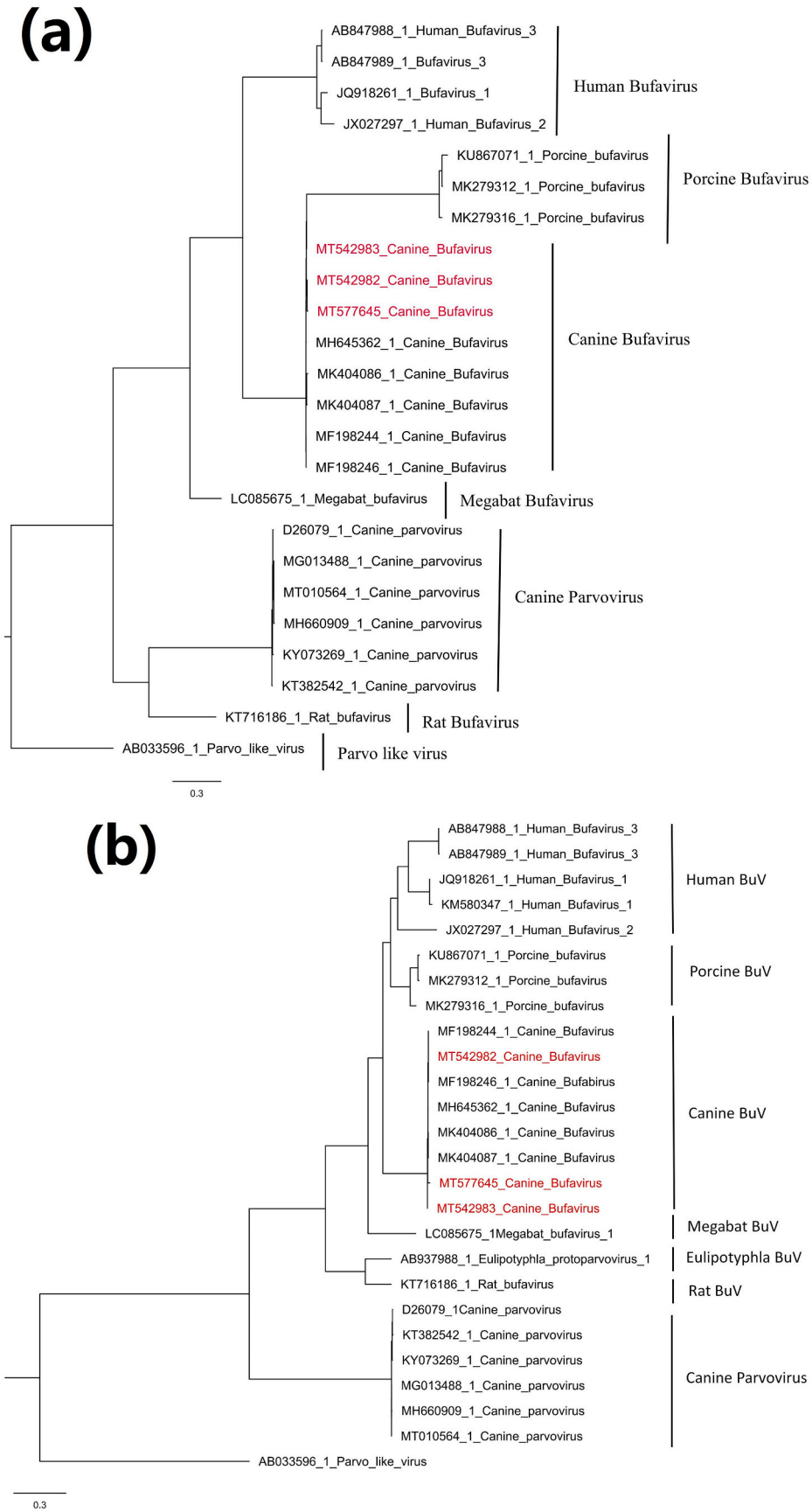

Fig. 1. Phylogenetic trees based on the near-complete genome sequences and VP2 of canine bufavirus (CBuVs) and canine parvovirus. (a) Near-complete gene sequences ($n = 26$), (b) VP2 gene ($n = 29$). Strains from this study are marked with black spots. Parvo-like virus was taken as the outgroup. The tree was constructed using the Maximum Likelihood method with 1000 bootstrap replicates. The best substitution model was all TVM + G4 + F selected by ModelFinder.

Table 3
The selection Sites of VP2 gene by three method.

Method	Selection sites		Threshold
	Positive/diversifying	Negative/purifying	
SLAC	/	**313,380,566,411,240,223,352,158,465,366**	$P < 0.1$
FEL	/	**313,414**	
MEME	/	**313,414**	
FUBAR	205	**313,414,566,380,240,411,352,465,158,366**,161,230,555,552,**223**	Posterior probability of 0.9

Note: The bold were predicted by more than one method.

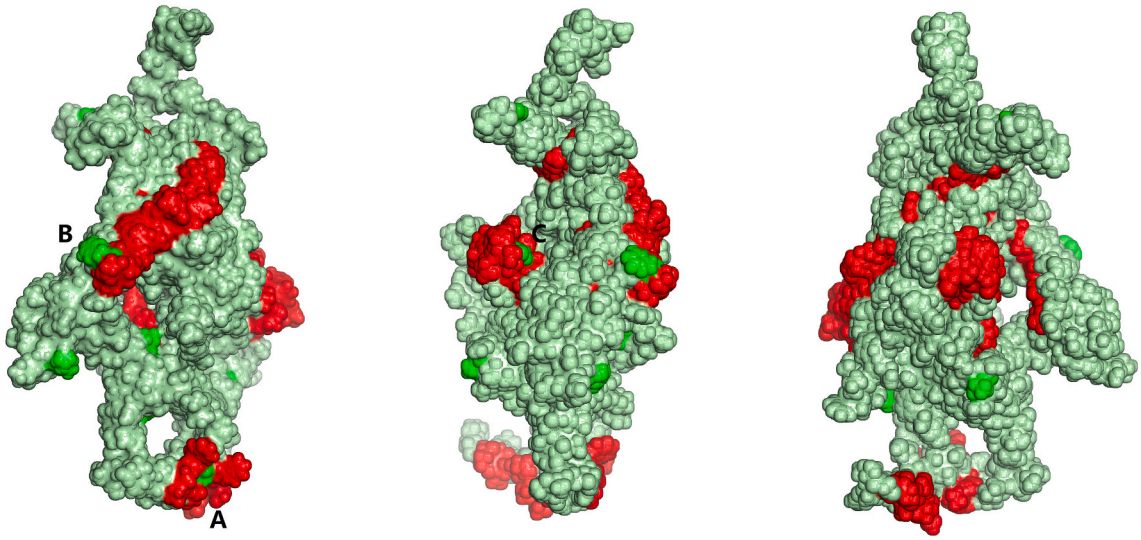

Fig. 2. Map of B-cell epitopes prediction and pressure selection site of VP2 gene. The predicted epitopes in the VP2 protein is indicated in red. The selective pressure sites are indicated in green. Codons 240 (A), 313 (B), and 380 (C) were located on the predicted B-cell epitopes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
The results of B-cell epitope prediction.

Strains	Location	Eptitope residue	Sore
AH-001 (MT542982)/AH-002 (MT542983)	239–258	KFDDIQFITVENCVPIELLR	1.000
	99–118	NDSYHAKVETPWSLLHANCW	0.845
	42–61	NRTEFHYHNGEVTIVCHART	0.775
	301–320	GEGQNFEMVNTWQWGDRDTP	0.762
	373–392	ASEKAVFDYAHGEMSPNEKD	0.724
AH-003 (MT577645)	99–118	NDSYHAKVETPWSLLHANCW	1.000
	42–61	NRTEFHYHNGEVTIVCHATR	0.918
	301–320	GEGQNFEMVNTWQWGDRDTP	0.902
	373–392	ASEKAVFDYAHGEMSPNEKD	0.857
	504–523	IVTYATFWWSGTLVFKGKLR	0.689

astrovirus, canine kobuvirus, canine coronavirus, and canine distemper virus. Therefore, the role of CBuV in diarrhea and whether co-infection would increase the mortality rate could not be confirmed in this study. More pathogenicity studies are required to clarify whether CBuV plays a major role in diarrhea in dogs.

CBuV homology was compared with the references, and the results showed a high homology. Phylogenetic analysis, based on the nearly complete sequencing of the *VP2*, indicated that CBuV formed a unique cluster, and the three isolated strains were closely related to other reference strains. Compared with HuBuV with three distinct branches, CBuV was conservative in evolution (Yahiro et al., 2014). As a member of the genus *Protoparvovirus*, CBuV was not closely related to CPV in the phylogenetic tree, which is consistent with the homology comparison between CPV and CBuV mentioned above. In addition, genetic heterogeneity has been recently reported to be found in the region downstream, where there may be recombination. This finding suggests that recombination may play a role in the evolution of CBuV. In this study, no significant recombination event was found, which may be due to geographical differences or limited genetic information. Hence, the role of recombination in CBuV evolution needs more attention.

In amino acid alignment, we found relatively higher amino acid mutations in *VP2* than in *NS1*. To elucidate whether external selective pressure was associated with these mutations, selection pressure analysis was performed by analyzing existing sequences. The results showed a lower rate of positive selection in the *VP2* but higher rates of negative selection. Negative selection pressure means that the gene will not increase in variation under pressure from the external environment, and that there is a tendency for gene sequence conservation (Miller et al., 2009). Therefore, external selection pressure may not be significantly associated with the presence of mutations. Due to the lack of available genetic information, further analysis is limited.

Our concern was regarding whether external selection pressure affects the immune response to the virus. Interestingly, we found that the positively selected codon site did not coincide with the B-cell epitope. However, negative selected codon sites 240, 313, and 380 were located on the predicted B-cell epitopes (Fig. 2). This suggests that selective pressure does not significantly change the B-cell epitopes of CBuV. In parvoviruses, the VP2 region contains major epitopes (Lopez de Turiso et al., 1991). As is known to all, B-cell epitope is a group of residues on the surface of an antigen which recognized by either a particular B-cell receptor (BCR) or a particular antibody molecule of the immune system and determine the immunogenicity of viruse (Zhang et al., 2016). Therefore, the conservatism of epitope residues means that the immunogenicity of the CBuV may not significantly alter, which indicates that new serotypes of the CBuV may not easily produced. This is beneficial for virus prevention and vaccine development.

Herein, three of 120 fecal samples were positive for CBuV, providing molecular evidence for the presence of the CBuV in Anhui Province. Phylogenetic analysis and sequence alignment showed a high homology with other reference strains, indicating that CBuV was relatively conservative. In addition, CBuVs were subject to negative selection, which helped maintain the conservation of viral genes. And the negative selection codons were located on B-cell epitopes, it did not affect the immunogenicity of CBuVs. This study provides a reference for further understanding of the epidemic and molecular characteristics of the virus in China.

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Author statement

Xu Guo, Da Zhang, and Wei Li were involved in performing experiments. Wei Li and Ziteng Fu conducted a sample collection. Guangqing Liu and Jianfei Sun were involved in data analysis, experimental design. Yong Wang, Shudong Jiang and Yongdong Li wrote the manuscript. Xu Guo and Yong Wang revised and examined the manuscript. All authors read and approved the final manuscript. All authors have declared that no competing interests exist.

Declaration of Competing Interest

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