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Short communication

First identification of a novel parvovirus distantly related to human bufavirus from diarrheal dogs in China

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ABSTRACT

Bufaviruses are small, nonenveloped, single-stranded DNA viruses belonging to the subfamily *Parvovirinae*. Human bufaviruses were first identified in 2012 in fecal samples from children with diarrhea. A new parvovirus of canines that was first detected in various samples from dogs with enteric and respiratory symptoms in Italy between 2014 and 2018 is closely related to the newly described human bufavirus. To explore the prevalence and genetic diversity of CBuV in Chinese dogs, 540 canine parvovirus (CPV)-positive serum and diarrhea samples were collected in Guangxi Province between 2016 and 2018. Among the samples, 6.25% (5/80) of rectal swabs and 2.5% (5/200) of CPV PCR-positive samples were positive for CBuV. However, the virus was not detected in CPV PCR-negative samples or nasal swabs. Two CBuV isolates were identified from CPV-positive fecal and serum samples by complete sequence analysis, with 99.8%–99.9% NS1 and VP2 protein identity to each another. Sequence analysis indicated that the CBuV GXNN01-2018 isolate VP2 protein shares 99.6% identity with the Italian CBuV ITA/2015/297 isolate and 62.3%–65.5% identity with human bufavirus. Phylogenetic analysis showed that CBuV was significantly distinct from other known bufaviruses and was most closely related to CBuV ITA/2015/297. This is the first report of the existence of CBuV in China, and our findings will strengthen the understanding of the epidemiology of bufaviruses in different animals.

1. Introduction

Parvoviruses are small, nonenveloped viruses with single-stranded DNA genomes. Parvoviruses belong to the family *Parvoviridae* and contain two open reading frames (ORFs) (Decaro and Buonavoglia, 2012; Li et al., 2018b; Streck et al., 2013). ORF1 is located at the 5′ end of the genome and encodes nonstructural (NS) proteins with replicase (REP) activity (Han et al., 2015). ORF2 at the 3′-terminal end encodes a capsid protein located (Filipov et al., 2016). According to the International Committee on the Taxonomy of Viruses, the family *Parvoviridae* comprises the two subfamilies *Parvovirinae* and *Densovirinae* infecting vertebrates and arthropods, respectively (Qiu et al., 2017). The subfamily *Parvovirinae* is further divided into nine genera: *Dependoparvovirus, Copiparvovirus, Bocaparvovirus, Amdoparvovirus, Aveparvovirus, Protoparvovirus, Tetraparvovirus, Erythroparvovirus,* and *Marinoparvovirus* (Filipov et al., 2016).

Bufaviruses are considered to be parvoviruses and comprise a group of viruses that are not restricted to human hosts (Phan et al., 2012); they can also infected nonhuman primates (Sasaki et al., 2015), pigs (Hargitai et al., 2016), rats (Cotmore et al., 2014) and bats (Sasaki et al., 2016). Canine bufavirus (CBuV) was identified in Italy in 2018 from a litter of 3 mixed-breed 5-month-old puppies with canine infectious respiratory disease (CIRD) (Martella et al., 2018). This new canine parvovirus is closely related to the newly described canine bufaviruses detected in various samples from dogs with enteric and respiratory symptoms. Human bufavirus has been detected in diarrheal stools of patients of all ages worldwide (Altay et al., 2015; Vaisanen et al., 2014, 2016; Yahiro et al., 2014). The prevalence of bufaviruses in humans ranges from 0.3% to 4.0%, and there are few data on the clinical significance or association of bufaviruses with enteric or extraenteric diseases (Ayouni et al., 2016; Huang et al., 2015).

However, since the first discovery of CBuV in 2018, the virus has not been reported in Asia. The objectives of this study are to provide more information on the molecular characteristics of the viral genome and to

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determine the phylogenetic and evolutionary diversity of CBuV genome sequences in China.

2. Materials and methods

2.1. Sample collection

A total of 540 samples from domestic dogs were collected in Guangxi Province, China, between 2016 and 2018. Two hundred dog serum samples from 10 counties in Guangxi Province were Canine parvovirus (CPV) positive by PCR; another two hundred dog serum samples from the same location were CPV negative by PCR. Eighty diarrheal stool samples from urban pet dogs in Guangxi Province were positive for other diseases (including canine distemper, n = 39, and canine parvovirus disease, n = 41). Sixty nasal swab samples from urban pet dogs with unknown disease were collected.

The experimental procedures were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Health Animal Care and Use Committee of the Guangxi Science and Technology Department.

2.2. DNA extraction

Viral DNA was extracted from supernatants using TaKaRa MinBEST Viral DNA Extraction Kit Ver. 4.0 (Takara Co. Dalian, China).

2.3. Detection and complete genome amplification of CBuV

PCR was performed for the detection of CBuV conserved regions using two primer sets (CBuV-P1/CBuV-P2 and CBuV-P3/CBuV-P4) previously described by Martella et al(Martella et al., 2018). The nested PCR product was separated by 1% agarose gel electrophoresis, purified using AxyPrep DNA Gel Extraction Kit (AxyGene, Hangzhou, China), cloned into the pMD18-T vector and sequenced (Takara Co. Dalian, China).

PCR-positive samples were selected for amplifying complete CBuV genomes. The five pairs of primers designed for this purpose are shown in Table 1. Each PCR mixture consisted of 5 μL template DNA, 1 μL primers (25 μM each primer), 2.5 U TransStart® FastPfu Fly DNA Polymerase (TransGen Biotech, Beijing, China), 5 μL of 5× TransStart® FastPfu Fly DNA buffer (TransGen Biotech, Beijing, China), and ddH_2O to a final volume of 25 μL. Amplification was initiated by an initial denaturation for 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 65 °C, and 30 s at 72 °C, and a final extension for 10 min at 72 °C. The amplified product was purified with an AxyPrep DNA Gel Extraction Kit (AxyGene, Hangzhou, China) and cloned into pMD18-T (Takara Co. Dalian, China) according to the manufacturer's instructions. The cloned products were then sequenced (Takara Co. Dalian, China).

Table 1
Specific primers used in this study.

Primer name	Primer sequence (5'–3')	Size (bp)
CBV-P1	TATGTCATCTGGTTGGTAATGTTGGTTG	536
CBV-P2	TTCTTTTGAATTTGTTTCGTGTGGTTCT	
CBV-P3	GAGACAAAACCTGAACATAAGCAACCAA	620
CBV-P4	GAGGAACCCAACCTAAAAGATAGAGAAA	
CBV-1F	ATGGCTACATCTACATTCTCTGACA	560
CBV-560R	TAGAAATGTATTGTCTTTTGGTG	
CBV-484F	TTACTAACTGGTGCAGTGCCTTTACATA	1083
CBV-1567R	TTTTGAGTTCAATTCTGACACATCTGTC	
CBV-1464F	AATGACCACAAATGAGGACATTACAAAA	1189
CBV-2653R	ATGGAACTCAGTACGGTTATTAAAAGCG	
CBV-2584F	AGGTGGAGGTGGAGGTGGAAGCGGTGTT	1171
CBV-3755R	CCTGGTGTGGATTTTTTGGAGTGTAGTC	
CBV-3700R	AGGAAACCAACAAATGACAGGACAA	519
CBV-4219R	TTATAGAGTAATATTAGGCATAGCT	

2.4. Analysis of CBuV sequences

Nucleotide and deduced amino acid sequences were compiled and edited using the DNAMAN8 program and aligned with the Lasergene sequence analysis software package (MegAlign) using Clustal W. Phylogenetic trees were generated by the neighbor-joining (NJ) method with the p-distance model and 1000 bootstrap replicates using the MEGA package version 5.0.

2.5. Nucleotide sequence accession numbers

The complete genomic sequences of the 2 CBuV isolates identified in this study have been deposited in the GenBank database. For genetic analysis, 27 bufavirus genomes were retrieved from GenBank. The general information and accession numbers of all 27 bufavirus isolates are listed in Table S1.

3. Results

3.1. Detection of CBuV in clinical samples

In the present study, the overall prevalence of CBuV was 1.85% (10/540) among domestic dogs in Guangxi Province. Of the 200 CPV PCR-positive samples, 5 were CBuV positive (positivity rate, 2.5%); among the 80 diarrheal stool samples, 5 (6.25%) were CBuV positive. Two hundred CPV PCR-negative samples and 60 nasal swabs were also CBuV negative. The CBuV prevalence rate among the samples in this study was lower than that of the samples obtained in Italy. According to PCR, ten CBuV-positive dogs were also coinfected with CPV (Zhang et al., 2010), but no coinfections with CDV were identified by RT-PCR (Amude et al., 2006). Coinfection with CBuV and CPV was not observed in the Italian study.

3.2. Sequence analysis of CBuV genomes

Two complete genomes of approximately 4219 bp were obtained from CPV-positive fecal and serum samples. BLAST alignment results confirmed the genomes to be CBuV, and they were designated as strains GXNN01-2018 and GXNN02-2018 (GenBank accession Nos. MK404086 and MK404087, respectively) (Table S1).

Sequence analysis demonstrated that the CBuV genome contains two main ORFs; these genes located at the 5′-end and 3′-end encode the putative nonstructural protein (NS1) and viral capsid protein (Cap), respectively. The left ORF, generating NS1, encodes 638 aa with 2 conserved replication initiator motifs, ^{131}GLHFHVLLQ^{139} and ^{217}IVRYFLTKQP^{226}. The Walker loop ^{405}GPASTGKS ^{412}[GXXXXGK(T/S)], which is an ATP- or GTP-binding motif, is present in NS1. The right ORF encoding VP1 and VP2 is 2316 bp. The VP1 gene consists of 2133 bp and encodes 710 aa with a conserved phospholipase A2 (PLA2) motif similar to the VP1 protein of the family *Parvovirinae*. The VP2 gene is 1707 long bp and encodes 568 aa with a glycine-rich sequence (GGGGGGGSGVG) at the N-terminus. The conserved motifs of the Ca^{2+}-binding loop (YXGXR) and the catalytic center (HDXXY) of the putative secretory phospholipase A2 (PLA2) domain were identified in the VP1 gene (Fig. 2). Furthermore, the conserved motif of the Ca^{2+}-binding loop of PLA2 is "YXGXG" in CBuV rather than "YXGXR" or "YXGXF" found in human bufavirus (Fig. 2).

3.3. Comparative genomic sequence analysis

Sequence similarity between the two complete genomes ranges from 99.3% and from 99.0% to 99.4% compared with the CBuV ITA/2015/297 strain, suggesting no obvious geographical differences among these strains. Table 2 presents the amino acid sequence identities of the NS1, VP1 and VP2 proteins of CBuV GXNN01-2018, bufaviruses and related animal parvoviruses (Table 2). The GXNN01-2018 strain exhibits

Table 2
CBuV GXNN01-2018 strain nucleotide sequence similarities for sequences derived from different bufavirus strains.

Isolate	NS1		VP1		VP2	
	Length (aa)	Identity (%)	Length (aa)	Identity (%)	Length (aa)	Identity (%)
CBuV ITA/2015/297	638	97.2	710	99.6	568	99.8
Bufavirus-1 strain BF.96	671	55.2	707	64.6	569	65.5
Bufavirus-2 strain BF.39	673	55.9	707	62	569	62.5
Bufavirus-3 AHP-178	673	56.1	710	61.9	572	62.3
WUHARV parvovirus	665	58.7	737	67.7	569	69.0
Porcine bufavirus strain 61	639	67.4	728	53.2	587	69.2
Rat bufavirus	644	57.8	726	53.0	585	53.5
Bat Mr-PV/Shaanxi 2011	493	60.1	668	59.9	531	58.8
Eulipotyphla protoparvovirus	639	57.1	728	54.1	587	54.6
Porcine parvovirus	613	40.4	729	35.3	579	33.8

55.2–56.1% and 62.3–65.5% aa sequence identity with the NS1 and VP2 proteins, respectively, of human bufaviruses. Interestingly, porcine bufavirus strain 61 and bat Mr-PV/Shaanxi2011, which were previously reported as bufaviruses, present 60.1–67.4% aa sequence identity with the NS1 protein of the GXNN01-2018 strain.

3.4. Phylogenetic relationships

Two phylogenetic trees were constructed on the basis of the full-length genomes and VP1 of other bufaviruses. According to N-J tree analysis of the full-length genome, the two isolates cluster with the CBuV ITA/2015/297 strain (Fig. 1). Another N-J phylogenetic tree based on VP1 from other bufaviruses in this study confirmed the result of the tree based on the complete genome sequences (Fig. S1).

4. Discussion

In recent years, several new bufaviruses have been detected, mostly due to the development of next-generation sequencing (NGS) and metagenomics. CBuV was newly discovered and identified for the first time in Italy and Hungary (Martella et al., 2018). In that study, 18/58 (31.0%) of animals with canine infectious respiratory disease (CIRD) tested positive for CBuV, whereas the virus was not detected in respiratory samples from 90 animals without clinical signs. Molecular screening by qPCR revealed CBuV DNA in 32.1% (26/81) of stool samples or rectal swabs from animals with signs of gastroenteritis and 15/78 (19.2%) of samples from animals without clinical signs, though these differences were not statistically significant. The authors suggested that CBuV is a common component of the canine fecal virome (Martella et al., 2018).

In the present study, 10 samples were positive for CBuV, which indicates that CBuV may be common in China. The CBuV prevalence rate in our samples was lower than that of the samples obtained in Italy. Elina Väisänen et al. (Vaisanen et al., 2018) observed major differences in the seroprevalence of bufavirus in different countries. The CBuV prevalence is reportedly similar to that of human bufavirus (Huang

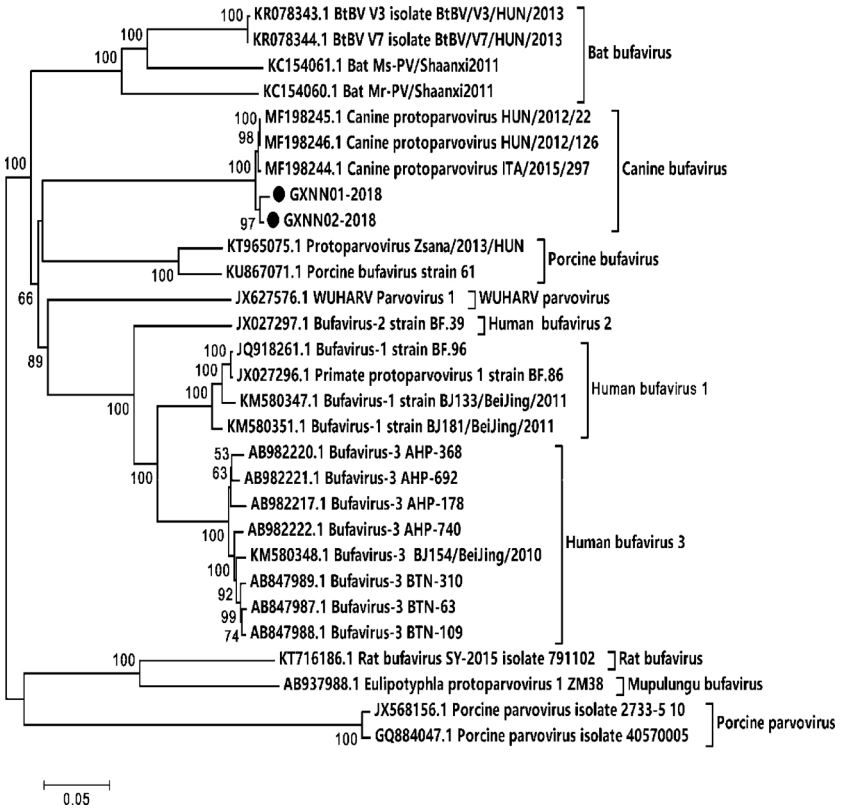

Fig. 1. Phylogenetic analyses of CBuV based on the full-length genome using the neighbor-joining algorithm and 1000 bootstrap replications in a heuristic search with MEGA 5.

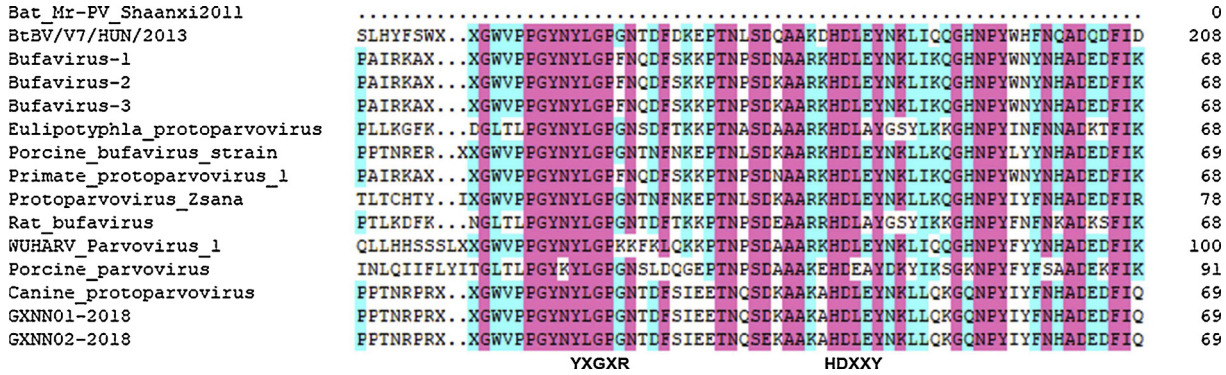

Fig. 2. Sequence alignment of the putative phospholipase A2 motif of CBuV with other bufaviruses. The conserved amino acids of the Ca^{2+}-binding loop (YXGXR) and the catalytic residues (HDXXY) are indicated at the bottom of the alignment.

et al., 2015; Vaisanen et al., 2016; Yahiro et al., 2014). In the present study, two CBuV isolates were detected in canine fecal samples and serum samples, and genome comparison revealed differences in only 11 aa between them. Furthermore, pairwise comparison of CBuV GXNN01-2018 VP2 aa sequences showed the highest identity (69.2%) with porcine bufavirus strain 61, followed by WUHARV parvovirus (69.0%) and human bufavirus (62.3–65.5%) (Table 2). Phylogenetic analysis based on the full-length genome demonstrated that two isolates form a special cluster with CBuV.

Of interest, bufaviruses have also been detected in the sera and spleens of monkeys and in the spleens of shrews (Sasaki et al., 2015). This is the first study to detect CBuV in dog sera, suggesting the possibility of systemic infection or viremia after infection. To date, many viruses have been detected in the serum of dogs, such as canine distemper virus (Li et al., 2018a), canine parvovirus 2 (Li et al., 2017; Zhao et al., 2017, 2016), torque teno canis virus (Lan et al., 2011; Sun et al., 2017), canine bocaparvovirus (Choi et al., 2016), and canine circovirus (Dowgier et al., 2017; Sun et al., 2019). Therefore, virus detection in serum from dogs can quickly help in the diagnosis of diseases.

Currently, the role of bufaviruses in the development of clinical disease is still uncertain, and information regarding viral pathogenicity is scarce (Hargitai et al., 2016; Vaisanen et al., 2018). The CBuV-infected dogs presented nasal discharge, coughing, and respiratory distress, but they completely recovered from the disease after 2 weeks; such clinical signs of infection can be easily ignored. Overall, the pathogenic potential of CBuV in dogs remains unknown. This study may be attributable to an inadequate description of the clinical manifestations of CBuV infections.

In summary, this is the first study to detect CBuV in dog sera in China, to analyze the prevalence of CBuV and to obtain the complete CBuV genomic sequence. In addition, we found evidence for CBuV and CPV coinfection. The findings may provide new insight for future studies to determine the role of CBuV in dog diseases.

Conflict of interest statement

The authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.03.020.

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