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Research Article

Ungulate bocaparvovirus 4 and rodent bocavirus are different genotypes of the same species of virus

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ABSTRACT

Bocaviruses are associated with many human infectious diseases, such as respiratory tract infections, gastroenteritis, and hepatitis. Rats are known to be reservoirs of bocaviruses, including rodent bocavirus and rat bocavirus. Recently, ungulate bocaparvovirus 4, a known porcine bocavirus, has also been found in rats. Thus, investigating bocaviruses in rats is important for determining the origin of the viruses and preventing and controlling their transmission. To the best of our knowledge, no study to date has investigated bocaviruses in the livers of rats. In this report, a total of 624 rats were trapped in southern China between 2014 and 2017. Liver and serum samples from rats were tested for the prevalence of bocaviruses using PCR. Sequences related to ungulate bocaparvovirus 4 and rodent bocavirus were detected in both liver and serum samples. Interestingly, the prevalence of ungulate bocaparvovirus 4 (reference strain: KJ622366.1) was higher than that of rodent bocavirus (reference strain: KY927868.1) in both liver (2.24% and 0.64%, respectively) and serum samples (2.19% and 0.44%, respectively). The NS1 regions of ungulate bocaparvovirus 4 and rodent bocavirus related sequences displayed over 84% and 88% identity at the nucleic acid and amino acid levels, respectively. Furthermore, these sequences had similar genomic structure, genomic features, and codon usage bias, and shared a common ancestor. These viruses also displayed greater adaptability to rats than pigs. Our results suggested that ungulate bocaparvovirus 4 and rodent bocavirus may originate from rats and may be different genotypes of the same bocavirus species.

1. Introduction

Parvoviruses are small unenveloped viruses with linear single-stranded DNA (ssDNA) genomes. The family *Parvoviridae* comprises two subfamilies, namely, *Densovirinae* and *Parvovirinae*. Members of the *Densovirinae* subfamily infect invertebrates, while those of the *Parvovirinae* subfamily infect vertebrates. Some parvoviruses can infect humans, including parvovirus B19, human bocavirus, adeno-associated virus, human bufavirus, and human parvovirus 4, and are considered to be associated with respiratory tract infections, gastrointestinal infections, hepatitis, and neurological inflammatory disorders (Allander et al., 2005; Arthur et al., 2009; Kapoor et al., 2009, 2010; Matthews et al., 2014; Qiu et al., 2017; Mrzljak and Tabain, 2019; Vilmane et al., 2020).

ssDNA viruses have high substitution and mutation rates, rendering them capable of adapting to new hosts and crossing species barriers (Antia et al., 2003; Shackelton et al., 2005; Parrish et al., 2008; Karuppannan and Opriessnig, 2018; Voorhees et al., 2019). Animal bocaviruses were first isolated in the early 1960s (Manteufel and Truyen, 2008). Owing to technological advances in molecular biology, novel bocaviruses continue to be identified and have been detected in a variety of animals, including pigs, cats, dogs, bats, rabbits, and minks (Lau et al., 2012, 2016; He et al., 2013; Li et al., 2013; Gunn et al., 2015; Lanave et al., 2015; Yoo et al., 2015; Yang et al., 2016). In addition, cross-species transmission has been reported in some of the bocaviruses, including ungulate bocaparvovirus 4, a known porcine bocavirus (Xiong et al., 2019).

The first report of a porcine boca-like virus dates to 2009 (Blomström et al., 2009). Subsequently, different bocaviruses were identified in pigs worldwide. Porcine bocavirus H18 (PBoV-H18), the reference strain of ungulate bocaparvovirus 4, was first reported in 2011 (Shan et al., 2011). Then, in 2015, porcine bocavirus KU14 (PBoV-KU14), another strain of ungulate bocaparvovirus 4, was detected in a pig with diarrhea (Yoo

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et al., 2015). Besides pigs, sequences similar to strains of ungulate bocaparvovirus 4 have been detected in other animals such as minks, shrews, and rats (Wang et al., 2017; Xiong et al., 2019).

Rats are important reservoirs of many pathogens. In addition to ungulate bocaparvovirus 4, other three bocaparvoviruses have been identified in rats, including *Rodent bocaparvovirus 1* (rat bocavirus, RBoV), *Rodent bocaparvovirus 2* (murine bocavirus, MuBoV) (Lau et al., 2017; Williams et al., 2018), and rodent bocavirus (RoBoV) (Zhang et al., 2018). Investigating bocaviruses in rats is important for determining the origin of these viruses as well as preventing and controlling their transmission. Most reports on bocaviruses have focused on fecal or respiratory samples from rats (Lau et al., 2017; Xiong et al., 2018, 2019). Some studies have reported the presence of bocaviruses in rat spleen and lung samples (Lau et al., 2017; Zhang et al., 2018). Due to the link between bocaviruses and hepatitis (MrzljakandTabain, 2019), several studies have investigated bocaviruses in the liver of animals such as canines and marmots (Li et al., 2013; Ao et al., 2017). However, to the best of our knowledge, no study to date has investigated bocaviruses in rat livers.

In this study, liver tissue and serum samples were obtained from urban rats trapped in five cities in China and surveyed for the prevalence of bocaviruses. Interestingly, we found that the prevalence of ungulate bocaparvovirus 4 was higher than that of RoBoV in both liver and serum samples of the animals. Subsequently, we undertook a genomic analysis of the sequences obtained in our study to determine whether ungulate bocaparvovirus 4 and RoBoV are related to the same viral species. We found a high level of similarity among all the near full-length sequences (over 93% at the nucleic acid level) obtained in this study. The *NS1* genes (over 88% at the amino acid level) of the sequences related to ungulate bocaparvovirus 4 and RoBoV also showed a high similarity. Furthermore, sequences related to these two viruses had similar genomic structure, genomic features, and codon usage bias. These findings indicated that ungulate bocaparvovirus 4 and RoBoV may be different genotypes of the same viral species.

2. Materials and methods

2.1. Animal capture and sample collection

Rats were caught from five regions (Malipo County, and Yiyang, Xiamen, Guangzhou and Maoming City) with cage traps near human residences between 2014 and 2017 (Fig. 1 and Table 1). Animals were anesthetized *via* the inhalation of 3% diethyl ether, with the dosage being adjusted according to their heart rate, respiratory frequency, corneal reflection, and extremity muscle tension. Trained personnel wore filtering facepiece respirators, chemical resistant goggles, antistatic uniforms, and chemical resistant gloves as protection against the diethyl ether. Blood was drawn by cardiac puncture and centrifuged to obtain the serum samples. Then the animals were euthanized by cervical dislocation. Liver tissue samples were collected and soaked in RNAlater (Invitrogen, Carlsbad, CA, USA). All the samples were stored at −80 °C before processing. The species of each animal was identified by sequencing the cytochrome *b* (*Cytb*) gene (Arai et al., 2008).

2.2. PCR detection and amplification

Total RNA and DNA were extracted from ~20 mg of liver tissue samples and 200 μL of serum samples using the MiniBEST Viral RNA/DNA Extraction Kit (TaKaRa Biotechnology, Kusatsu, Japan). Bocaviruses in the liver and serum samples were detected by PCR (Lau et al., 2017). All the amplified products were separated on a 1.5% agarose gel. Positive samples were sent to the Beijing Genomics Institute for sequencing. Near full-length genomes were amplified using previously

Fig. 1. Locations of the animal trapping sites in China. Sampling regions are marked with blue stars.

Table 1
Animals trapped in different regions.

Species	Hunan Province	Fujian Province	Yunnan Province	Guangdong Province		Total
	Yiyang City	Xiamen City	Malipo County	Guangzhou City	Maoming City	
Rattus norvegicus	88	35	51	201	86	**461**
Rattus tanezumi	19	24	1	13	7	**64**
Rattus losea	–	98	–	–	–	**98**
Bandicota indica	–	1	–	–	–	**1**
Total	**107**	**158**	**52**	**214**	**93**	**624**

reported primers (Xiong et al., 2019) as well as primers designed in this study (Supplementary Table S1).

2.3. Phylogenetic analysis

The near full-length genomic sequences were aligned with reference sequences obtained from GenBank using the ClustalW multiple sequence alignment program in MEGA (version 6.0). Phylogenetic trees were constructed using MrBayes software (version 3.2; https://www.nbisweden.github.io/MrBayes/) (Ronquist et al., 2012).

2.4. Genomic analysis

CodonW version 1.4.2 (Xin et al., 2020) was used to analyze the basic nucleotide composition of the *VP2* gene sequences of the bocavirus strains and calculate the relative synonymous codon usage (RSCU) and the effective number of codon (ENC) values. Codons with RSCU values > 1 are used more frequently than expected, while codons with RSCU values < 1 are used less frequently than expected. An RSCU value > 1.6 indicates an overrepresented codon whereas an RSCU value < 0.06 indicates an underrepresented codon (Uddin et al., 2015; Nath Choudhury et al., 2017; Li et al., 2019; Voorhees et al., 2019). The codon adaptation index (CAI) values were calculated using *Rattus* sp. and *Sus* sp. codon usage preferences as references in the CAI calculator (http://genomes.urv.cat/CAIcal/).

Selective pressure for each gene was measured using a free-ratio model in CODEML in the EasyCodeML software (version 1.21) (Gao et al., 2019). An ω-value >1, = 1, or <1 indicated positive selection, neutral evolution, and negative selection, respectively. The site model (M0, M1a, M2a, M3, M7, M8, and M8a) was applied to detect potential selection among sites.

2.5. Statistical analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS), version 13.0 (IBM Corp., Armonk, NY, USA).

2.6. Ethical guidelines

The study protocol was approved by the Animal Ethics and Welfare Committee of the School of Public Health, Southern Medical University, and adhered to the guidelines for the Rules for the Implementation of Laboratory Animal Medicine (1998) from the Ministry of Health, China. All surgical procedures were performed under anesthesia and all efforts were made to minimize animal suffering. No endangered or protected species were used in this study.

3. Results

3.1. Sample collection and bocavirus detection

A total of 624 urban rats were trapped from four provinces (Fig. 1 and Table 1). Total RNA and DNA were obtained from 624 liver tissue samples and 228 serum samples. Bocaviruses were detected in both liver and serum samples of the urban rats (Table 2). A total of 79.17% of the screened sequences related to ungulate bocaparvovirus 4, while 20.83% related to RoBoV. The prevalence of ungulate bocaparvovirus 4 in the liver and serum samples was 2.24% and 2.19%, respectively, while that of RoBoV was 0.64% and 0.44%, respectively (Table 2).

3.2. Phylogenetic and similarity analysis of ungulate bocaparvovirus 4 and RoBoV

Fourteen near full-length genomes were obtained (GenBank accession numbers: MW055885–MW055898). Phylogenetic analysis based on the near full-length genomes showed that 10 near full-length genomic sequences (GZ13, GZ24, GZ30, GZ65, GZ87, GZ91, GZ459, MLP19, MLP27, and MM45) obtained in our study were clustered with a PBoV sequence isolated from rats (MG365883.1), while four (XM86, GZ446, GZ489, and MLP52) were clustered with RoBoV sequences (Fig. 2). Ungulate bocaparvovirus 4 sequences formed a clade with all the sequences obtained in our study, RoBoV sequences, and the PBoV sequence isolated from rats. This clade was distinct from other PBoV strains.

There was a high level of similarity among the near full-length genomic sequences obtained in our study (over 93% at nucleic acid level; Supplementary Table S2). These sequences were also similar to ungulate bocaparvovirus 4 sequence (PBoV-KU14, KJ622366.1), murine-associated porcine bocavirus strains (MuAPBV, MF175076.1 and MG365883.1), and RoBoV strain (KY927868.1) (Supplementary Table S2 and Fig. 3). In the *NS1* genes, a high level of similarity was also found among ungulate bocaparvovirus 4 strains (PBoV-KU14: KJ622366.1 and PBoV-H18: HQ291308.1), MuAPBV sequence (MF175076.1), RoBoV sequence (KY927868.1), and the two randomly selected representative sequences obtained in our study (MM45: MW055885; GZ489: MW055897) at both the nucleotide (>84%) and amino acid (>88%) levels (Table 3).

3.3. Genomic structure analysis of ungulate bocaparvovirus 4, MuAPBV, and RoBoV

A typical genomic structure of *Bocaparvovirus* was found in MM45, GZ489, ungulate bocaparvovirus 4 sequence, MuAPBV sequence, and RoBoV sequence (Fig. 4). Their NS1, VP1, VP2, and NP1 regions were

Table 2
Detection of the ungulate bocaparvovirus 4 and rodent bocavirus in animals.

Animal species	Ungulate bocaparvovirus 4		Rodent bocavirus	
	Liver tissue samples	Serum samples	Liver tissue samples	Serum samples
Rattus norvegicus	2.60 (12/461)	2.35 (4/170)	0.87 (4/461)	0.59 (1/170)
Rattus tanezumi	1.56 (1/64)	5.00 (1/20)	0 (0/64)	0 (0/20)
Rattus losea	1.02 (1/98)	0 (0/38)	0 (0/98)	0 (0/38)
Bandicota indica	0 (0/1)	–	0 (0/1)	–
Total	**2.24 (14/624)**	**2.19 (5/228)**	**0.64 (4/624)**	**0.44 (1/228)**

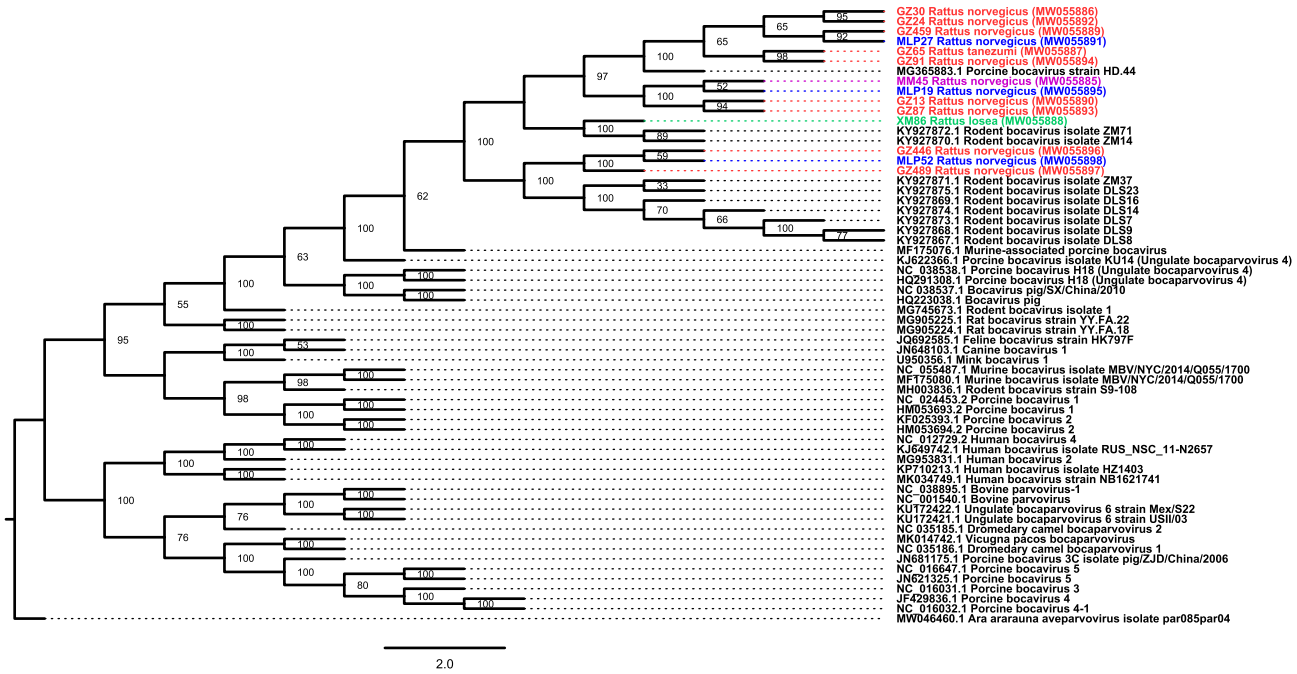

Fig. 2. Phylogenetic tree constructed based on near full-length nucleotide sequences of bocaviruses isolated from rats. Forty-seven representative bocavirus strains derived from rats, pigs, humans, canines, felines, and mink are included for comparison. One aveparvovirus sequence is included as the out-group. The percentages of the posterior probability (PP) values are indicated. Sequences obtained from animals trapped in Guangzhou City are highlighted in red; sequences obtained from animals trapped in Maoming City are highlighted in purple; sequences obtained from animals trapped in Xiamen City are highlighted in green; and sequences obtained from animals trapped in Malipo County are highlighted in blue.

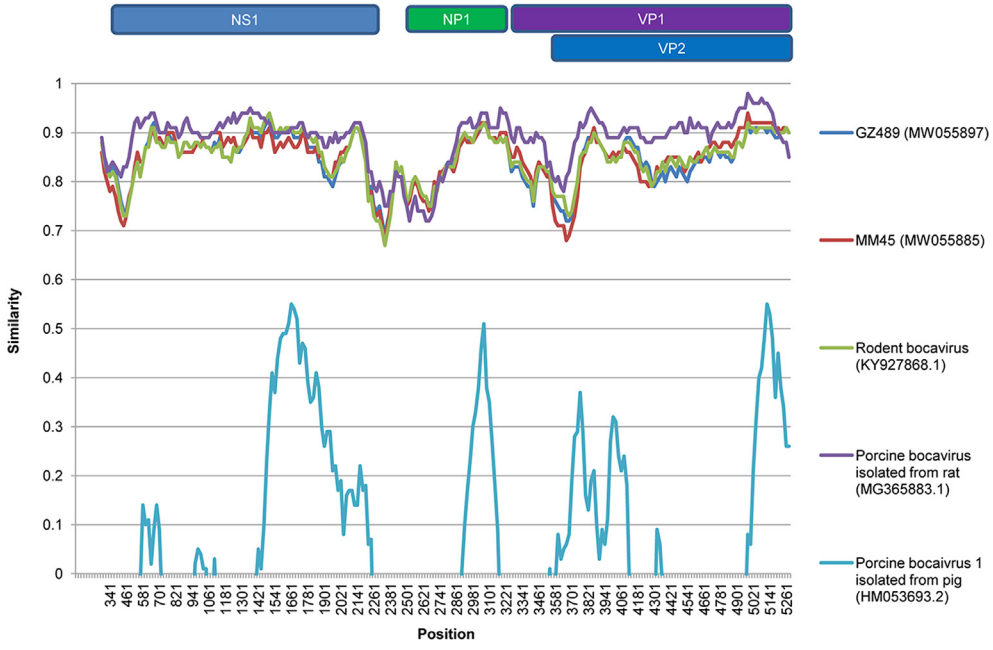

Fig. 3. Similarities among different bocaviruses at the nucleic acid level (using ungulate bocaparvovirus 4 strain KJ622366.1 as the reference genome).

found to be shorter than those of the other two porcine bocavirus strains (HM053693.2 and NC_016031.1).

3.4. Nucleotide composition and codon usage preference analysis based on VP2 gene sequences

The nucleotide composition of the VP2 region was found to be similar among MM45 (MW055885), GZ489 (MW055897), ungulate bocaparvovirus 4 sequence (KJ622366.1), MuAPBV sequence (MF175076.1), and the RoBoV sequence (KY927868.1) (Supplementary Table S3), including a compositional bias in favor of A and G and a significantly higher abundance of A3s (62.25%) and C3s (27.18%) than other porcine bocavirus strains. These sequences had lower ENC values (38.52–41.60) when compared with other porcine bocavirus strains. Among the codons of the VP2-encoding gene (Supplementary Table S4), AGA was the only over-represented codon in all sequences (an RSCU value > 1.6). Interestingly, MM45, GZ489, ungulate bocaparvovirus 4 sequence, MuAPBV sequence, and RoBoV sequence had a strong preference for A-ended

Table 3
Nucleotide acid and amino acid identity for the NS1 regions of bocaviruses.

	HM053693.2	HM053694.2	NC_016031.1	JF429836.1	NC_016647.1	KJ622366.1	MF175076.1	HQ291308.1	KY927868.1	MM45	GZ489
HM053693.2	ID										
HM053694.2	0.941 (0.933)	ID									
NC_016031.1	0.443 (0.376)	0.450 (0.375)	ID								
JF429836.1	0.439 (0.377)	0.445 (0.375)	0.970 (0.989)	ID							
NC_016647.1	0.439 (0.376)	0.447 (0.377)	0.814 (0.787)	0.811 (0.784)	ID						
KJ622366.1	0.463 (0.418)	0.463 (0.430)	0.454 (0.374)	0.457 (0.374)	0.460 (0.362)	ID					
MF175076.1	0.477 (0.424)	0.481 (0.437)	0.451 (0.374)	0.455 (0.374)	0.457 (0.365)	0.909 (0.943)	ID				
HQ291308.1	0.459 (0.413)	0.460 (0.424)	0.453 (0.377)	0.455 (0.377)	0.463 (0.365)	0.943 (0.951)	0.877 (0.903)	ID			
KY927868.1	0.480 (0.420)	0.484 (0.433)	0.453 (0.375)	0.459 (0.375)	0.462 (0.368)	0.879 (0.921)	0.915 (0.955)	0.848 (0.886)	ID		
MM45 (MW055885)	0.478 (0.421)	0.484 (0.434)	0.454 (0.375)	0.459 (0.375)	0.458 (0.368)	0.876 (0.925)	0.914 (0.960)	0.848 (0.889)	0.956 (0.981)	ID	
GZ489 (MW055897)	0.480 (0.420)	0.484 (0.433)	0.452 (0.375)	0.458 (0.375)	0.461 (0.368)	0.88 (0.922)	0.916 (0.957)	0.850 (0.888)	0.991 (0.995)	0.956 (0.984)	ID

Amino acid identity is indicated in brackets.
Porcine bocavirus: HM053693.2, HM053694.2, NC 016031.1, JF429836.1, NC 016647.1, KJ622366.1, HQ291308.1.
Murine-associated porcine bocavirus: MF175076.1.
Rodent bocavirus: KY927868.1.
Sequences obtained in this study: MM45 (MW055885), GZ489 (MW055897).

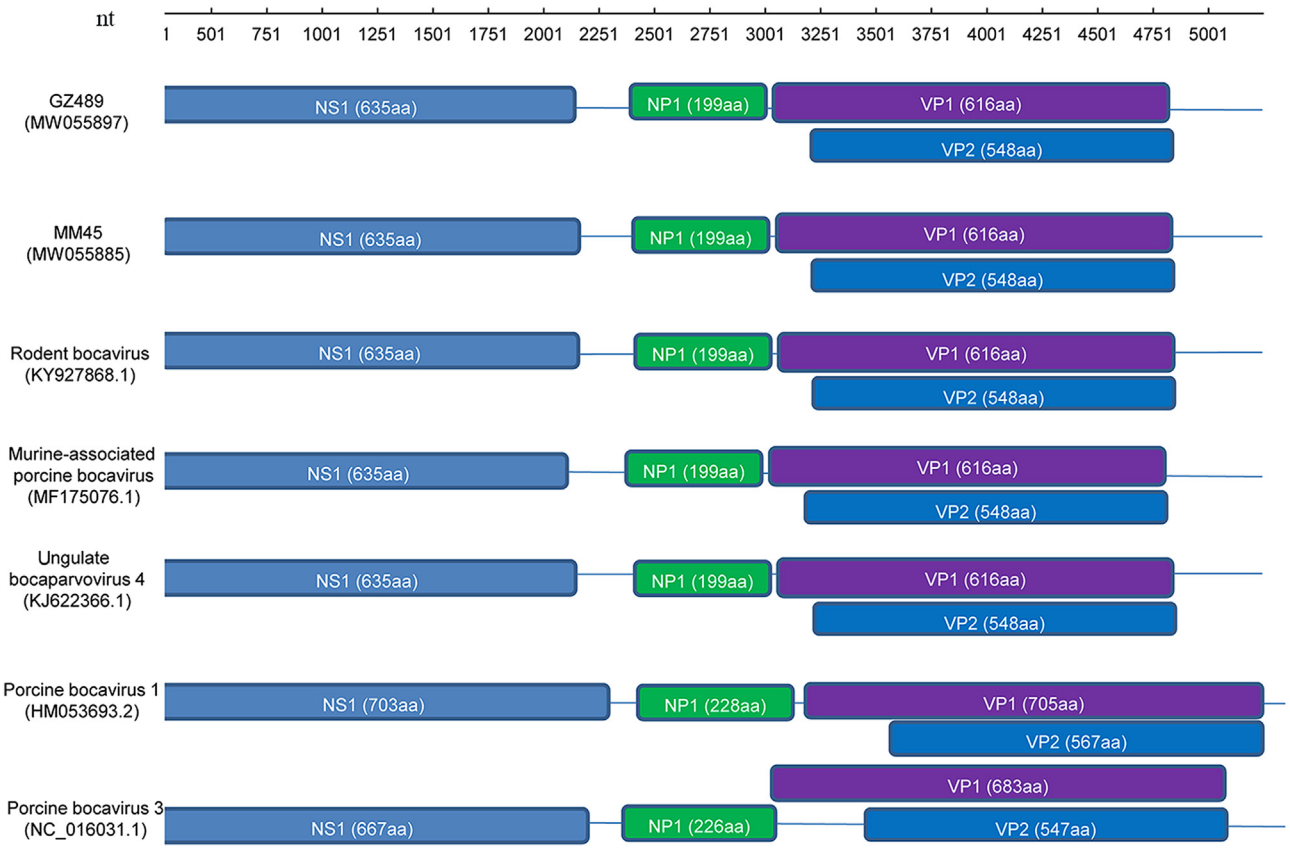

Fig. 4. Genomic features of different bocaviruses.

codons (GCA [Ala], ACA [Thr], CCA [Pro], UCA [Ser], GAA [Glu], AUA [Ile], AAA [Lys], CUA [Leu], and CAA [Gln]). In addition, the CAI values of the VP2-encoding genes within these sequences varied between 0.658 and 0.675 (mean: 0.665) when using *Sus* sp. codon usage as the reference, and between 0.666 and 0.682 (mean: 0.670) when using *Rattus* sp. codon usage as the reference.

3.5. Selective pressure analysis based on NS1, NP1, VP1, and VP2 genes

Selective pressure analysis was undertaken to investigate the evolution of the bocaviruses found in rats and porcine. Although the ω-values for four genes (*NS1*, *NP1*, *VP1*, and *VP2* genes) varied, all were <1 (ω = 0.1257, 0.1350, 0.1027, and 0.0951, respectively), suggesting that all these genes were under negative selection pressure. No positive selection signal was found in the *NS1* and *VP1* genes; however, positive selection signals were identified in the *NP1* and *VP2* genes (Fig. 5). In the *NP1* gene, a positive selection signal (ω = 14.0336) was found in the clade containing sequences related to RoBoV and ungulate bocaparvovirus 4. In the *VP2* gene, we also found a positive selection signal (ω = 26.3222) in the clade containing RoBoV sequences and an ungulate bocaparvovirus 4 sequence (PBoV-KU14). Site model analysis showed no aa sites with positive selection signals in all genes tested.

Fig. 5. Selective pressure analyses based on the *NS1* (**A**), *NP1* (**B**), *VP1* (**C**), and *VP2* (**D**) genes of rat bocaviruses, murine bocaviruses, rodent bocaviruses, ungulate bocaparvovirus 4, and other porcine bocaviruses. The *dS* values are shown and the ω-values (*dN*/*dS*) are given in parenthesis.

4. Discussion

This is the first study to report the detection of bocaviruses in liver tissue samples from rats. The prevalence and genomic characteristics of bocaviruses in rats in southern China were revealed. The detection of bocaviruses in rat liver tissue samples suggested that bocavirus infection might also cause liver diseases in rats, as happens in humans (Kainulainen et al., 2008). Except for Yiyang City, bocavirus-infected rats were found in all regions included in our study, indicating that bocaviruses are widely distributed in rats from southern China. Four *Rattus norvegicus* and one *Rattus tanezumi* were positive for bocaviruses in both liver tissue and serum samples. The detection of bocavirus in serum samples suggested that bocaviruses can cause viremia in rats. Other members of our research group have previously found bocaviruses in throat swab, fecal, and serum samples from rats (Xiong et al., 2018, 2019), indicating that bocaviruses has a wide tissue tropism.

Previous studies have reported the detection of sequences related to ungulate bocaparvovirus 4 in rats (Williams et al., 2018; Xiong et al., 2019). In addition, an ungulate bocaparvovirus 4 related sequence has been detected in minks (Wang et al., 2017). These findings suggested that ungulate bocaparvovirus 4 can infect different animals. In our study, the detection rates of ungulate bocaparvovirus 4 were higher than those of RoBoV in both liver tissue and serum samples. In Guangzhou City, the rats were trapped near human residences, and there was no pig farm nearby. Surprisingly, the detection rate of ungulate bocaparvovirus 4 (4.67%) was still higher than that of RoBoV (1.40%). In addition, most of the bocavirus sequences detected in pigs in China related to other species of PBoV instead of ungulate bocaparvovirus 4 (Liu et al., 2014; Meng et al., 2018; Zhou et al., 2018; Zheng et al., 2021).

In our study, fourteen highly similar (over 93% similarity at the nucleotide level) near full-length genomes were amplified. Ten of them related to ungulate bocaparvovirus 4 and four to RoBoV. Phylogenetic analysis showed that the sequences obtained in our study, ungulate bocaparvovirus 4 sequences, RoBoV sequences, and MuAPBV sequence clustered in a clade that was distinct from other PBoVs, suggesting that these viruses have cross-species transmission potential and might have originated from a common ancestor. According to the current criteria of the International Committee on Taxonomy of Viruses (ICTV) (https://ictvonline.org/virusTaxonomy.asp), a novel bocavirus species is defined as one that shares <85.0% amino acid identity in the *NS1* gene with other species (Cotmore et al., 2014). The amino acid identity of the *NS1* gene among ungulate bocaparvovirus 4 sequences, rodent bocavirus sequence, MuAPBV sequence, and the sequences detected in our study was over 88%, implying that all these sequences belonged to the same species of virus.

In addition, we found that MM45, GZ489, ungulate bocaparvovirus 4 sequence, MuAPBV sequence, and RoBoV sequence shared similar genomic structures and features, which was consistent with the results of a previous study (Zhang et al., 2018). The bocavirus VP2 capsid protein plays an important role in viral pathogenicity and the determination of host range. Codon usage bias can affect protein structure and function (Xin et al., 2020). In this study, we also found similar nucleotide composition and codon usage bias in the *VP2* genes within these sequences. The CAI values of the *VP2* genes indicated that all these viruses had higher adaptability to rats than pigs, suggesting that they may have originated from rats.

To determine the evolutionary path of these viruses, we performed selective pressure analyses. All four bocavirus genes were under negative selection, especially the *NP1* gene, which encodes a nonstructural protein important for the expression of viral capsid proteins (Zou et al., 2016). This result is consistent with that obtained for human bocavirus (Lu et al., 2015).

PBoV-H18 and PBoV-KU14, strains of ungulate bocaparvovirus 4, were first detected in pigs in 2011 and 2015, respectively (Shan et al., 2011; Yoo et al., 2015). However, in these studies, RoBoV sequences were not included in the analyses as RoBoV had not yet been identified. The authors classified PBoV-H18 and PBoV-KU14 as novel species of PBoV because they were found in pigs (Shan et al., 2011; Yoo et al., 2015; Zhang et al., 2018). In 2018, a novel rodent bocavirus was discovered, and the similarities between ungulate bocaparvovirus 4 and RoBoV sequences was reported (Zhang et al., 2018). The results of our study indicated that ungulate bocaparvovirus 4 and RoBoV may be two genotypes within the same viral species and may have originated from rats. Further studies are needed to verify this possibility.

Data availability

The datasets supporting the conclusions of this article are included within the article and its additional files. Representative sequences of the near full-length genomes of the viruses were uploaded to NCBI (accession numbers: MW055885–MW055898).

Ethics statement

The study protocol was approved by the Animal Ethics and Welfare Committee of the School of Public Health, Southern Medical University, and adhered to the guidelines for the Rules for the Implementation of Laboratory Animal Medicine (1998) from the Ministry of Health, China. All institutional and national guidelines for the care and use of laboratory animals were followed. All surgical procedures were performed under anesthesia and all efforts were made to minimize the suffering of the animals. No endangered or protected species were used in this study.

Author contributions

Wenqiao He: Methodology, investigation, formal analysis, and writing (original draft). Yuhan Gao: Investigation and resources. Yuqi Wen and Xuemei Ke: Resources. Zejin Ou and Mingji Cheng: Visualization. Jiaqi Fu and Yun Mo: Validation. Qing Chen: Conceptualization, project administration, funding acquisition, and writing (review and editing).

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.02.002.

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