A Subtype of Human Bocavirus Detected in Rattus Norvegicus Feces in China

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Abstract

Background Bocavirus is a typical zoonotic pathogen with a wide range of hosts. Here, we report the epidemiology of human bocavirus (HBoV) detected in *Rattus norvegicus*.

Methods Between May 2015 and May 2017, 357 *R. norvegicus* were captured in four Chinese provinces. Polymerase chain reaction was used to investigate the prevalence of HBoV in fecal samples. Phylogenetic analysis and sequencing of the entire viral genome were undertaken.

Results HBoV was detected in 0.84% (3/357) of samples. Phylogenetic analysis based on the partial VP1 region and near-full-sequence regions showed that HBoV obtained in *R. norvegicus* was genetically closely related to HBoV-2. One near-full-length HBoV genome (named "GZ533") was acquired, and phylogenetic analysis of the three positive sequences revealed that they shared very high identity in nucleotides and amino acids in the VP1 region (96.0%–99.1%). Comparison of GZ533 and other HBoVs revealed ~100% identity of amino acids in the VP1 region, whereas only 37.5% identity of amino acids when compared with *R. norvegicus* bocavirus.

Conclusion HBoV-2 was detected in *R. norvegicus* in China. *R. norvegicus* may be a carrier of HBoV infection, and its impact on public health merits attention.

1. Introduction

Bocavirus belongs to the genus *Bocaparvovirus* within the family *Parvoviridae*. Its genome is linear single-stranded DNA of length 5.2 kb, and includes three open reading frames (ORFs), which encode four proteins: two non-structural proteins (NS1 and NP), and two capsid proteins (VP1 and VP2) [1]. Bocavirus was first discovered in cattle in the 1960s [2].

Bocavirus is considered to be a typical zoonotic pathogen with a wide range of hosts: humans, gorillas, pigs, dogs, cats, Californian sea lions, bats, and rats [3]. In 2005, a research team based in Sweden were the first to discover human bocavirus (HBoV) in respiratory secretions using polymerase chain reaction (PCR) amplification and high-throughput sequencing [4].

The terminal sequence of HBoV is similar to that of bovine parvovirus and canine parvovirus [5]. HBoV is divided into four genotypes (1–4), and evidence suggests that HBoV-1 is a vital pathogen causing respiratory diseases. Unlike HBoV-1, HBoV-2–4 are detected mainly in stool samples [6], thereby suggesting that HBoV2–4 can cause gastroenteritis [7]. Nevertheless, bocavirus infection may be accompanied by other potential viral diseases and produce co-infections [4, 8].

Due to a lack of animal models and epidemiological studies, whether bocavirus infection is the true cause of diarrhea is not known [9, 10]. In general, disease due to HBoV infection often occurs in winter and spring [11-13]. Children under 5 years of age are considered susceptible to HBoV infection, which is endemic in Europe [14-16], Asia [17], the USA [18], and other countries [19].

Studies have shown reorganization and cross-species transmission of bocavirus. Kumakamba and colleagues discovered bocavirus in the serum, liver, and spleen of monkeys and other non-human primates in the Democratic Republic of Congo. The nucleotide similarity between the detected HBoV-2 and HBoV-3 reached 97%, suggesting that bocavirus can be transmitted between non-human primates and humans [20].

Previously, we showed that bocavirus was detected in rodents and shrews in southern China. Hence, rodents and shrews may be a host of bocavirus, and can spread across species [21]. In the present study, HBoV was detected in the feces of *Rattus norvegicus*. Here, we report the prevalence and genetic characteristics of HBoV in *R. norvegicus* in China.

2. Methods

2.1. Samples

Wild rats were captured close to human residences using cage traps between January 2015 and July 2017 in five Chinese cities: Guangzhou and Maoming in Guangdong Province; Malipo in Yunnan Province; Xiamen in Fujian Province; Yiyang in Hunan Province. Rats were anesthetized with diethyl ether. The species of trapped animals were determined by sequencing the cytochrome-B gene [22]. Fecal samples (~0.2 g per sample) were obtained from each animal, immersed in 700 μL of phosphate-buffered saline (PBS) (0.03% homogenate), and stored at −80°C. Rats were used for other studies after collection of stool samples.

2.2. Extraction of nucleic acids and detection using real-time reverse transcription-quantitative polymerase chain reaction (RT-PCR)

RNA and DNA were extracted from the supernatants of fecal samples using the MiniBEST Viral RNA/DNA Extraction Kit according to manufacturer (TaKaRa Biotechnology, Kusatsu, Japan) instructions. Preliminary screening of primers using semi-nested PCR [23] was done for the NS1 region of HBoV and non-human primate bocavirus. For positive samples that had been sequenced, the sequences were aligned with those for BLAST provided by National Center for Biotechnology Information. Those with higher sequence homology to the HBoV sequence were defined tentatively as "positive samples". More primers were designed using the Primer Premier 5 (www.premierbiosoft.com/) to verify the VP1 region (Table 1). With regard to PCR conditions, we used the same methods described by Kapoor and colleagues for the NS1 region [23]. For the VP1 region, the conditions for the first and second rounds of PCR were identical: 94°C for 1 min; 94°C for 30 s, 55°C for 30 s, 72°C for 30 s for 40 cycles; 72°C for 7 min. All PCR products underwent electrophoresis on 1% agarose gels.

2.3. Sequencing of the full viral genome

Ten pairs of primers were designed according to the GenBank reference sequence (accession number: JX257046.1) (data not shown) to amplify the full viral genome. Lasergene SeqMan (DNASTAR, Madison,

WI, USA) was employed for sequence assembly. Finally, one almost full-length HBoV genome was obtained (GenBank accession number: MT012544).

2.4. Phylogenetic analysis

Phylogenetic analysis of bocavirus was done using the neighbor-joining or maximum-likelihood methods in MEGA v6.0 (Oxford Molecular, Oxford, UK). Multiple alignments were undertaken using the "Clustal W" program in MEGA 6.0 for alignment of multiple sequences. Sequences were aligned for homology using DNASTAR. Similarity plots were created using SimPlot 3.5.1 (www.simplot.com/).

3. Results

3.1. Detection of HBoV in *R. norvegicus*

Overall, the percentage of HBoV detected in *R. norvegicus* was 0.84% (3/357). The percentage of HBoV detected in *R. norvegicus* in Guangzhou City, Xiamen City, and Yiyang City was 0.74% (1/135), 5.6% (1/18), and 1.4% (1/69), respectively. HBoV was not detected in Maoming City (0/88) or Malipo (0/47).

3.2. Phylogenetic analysis of HBoV

The nucleotide sequence of the VP1 region of the three positive samples obtained in the present study and other reference sequences from GenBank were used to construct a phylogenetic tree (Figure 1). The positive sequences from Guangzhou City, Xiamen City, and Yiyang City were clustered together with the reference sequence HBoV-2. The similarity of the nucleotide sequences in the VP1 region among these three positive samples was 96.0%–99.1%.

One of the representative positive samples (named "GZ533") was selected for near-full-length sequence amplification. Finally, a near-full-length genomic sequence (5146 nt) closest to the genotype HBoV-2 was obtained (GenBank number: MT012544). Compared with the reference sequence (JX257046.1), GZ533 lacked the first 65 nucleotides and the tail 95 nucleotides. GZ533 had four ORFs (NS1, NP1, VP1, VP2), encoding 640, 215, 667, and 538 amino acids, respectively. A phylogenetic tree was constructed based on the reference sequences of bocavirus from different species, as well as the near-full sequence obtained in this study (Figure 2). GZ533 had the highest nucleotide homology (99.38%) with the reference sequence HBoV-2 strain BJQ435 (JX257046.1). GZ533 was clustered with other HBoV-2 reference sequences. The similarity in nucleotide sequences between GZ533 and other HBoV-2 types was 99.4%–96.1%, and it was clustered together with other HBoV genotypes.

Table 2 shows the homology in amino acids between the putative NS1/NP/VP1/VP2 amino-acid sequence of GZ533 and that of the reference sequence. GZ533 and HBoV-2 had the highest similarity in amino acids (>90%), and the similarity in amino acids in VP1/VP2 reached 100%. We suspected that GZ533 and bocavirus (JX257046.1) were from the same strain. Compared with other types of HBoV, the similarity in amino acids of GZ533 was 68.8%–90.9%. According to the International Committee of Taxonomy (http://ictv.global), if the sequence of amino acids of the NS1 region is <85% similar to the

representative strain, it can be regarded as a new bocaparvovirus. The similarity of amino acids in the NS1 region of GZ533 was 90.5% compared with that of HBoV-2, so it should be classified into the same category. GZ533 was derived from rat feces, but the similarity of amino acids of each part of GZ533 was ≤41% compared with that of bocavirus in *R. norvegicus*. The similarity of amino acids in the VP1 region was 37.5%, whereas it was 37% in the NS1 region, so it can be considered a different type of bocavirus.

3.3. Similarity plots

Similarity plots for sequences were created using SimPlot 3.5.1 to characterize the recombination of GZ533(Figure 3). Analyses of similarity plots showed that the sequence of GZ533 had high similarity with HBoV-2 (FJ375129.1). The line chart was located at a similar rate (>0.95) and was almost parallel to the x-axis, indicating the absence of a recombination signal. GZ533 had low homology with bocavirus from *R. norvegicus* (KY927869.1), and the similarity was <0.6. There was no cross between the two polylines, which also indicated no recombination of the sequence.

4. Discussion

We detected, for the first time, HBoV in stool samples collected from *R. norvegicus*. HBoV is found in mainly in the stool and respiratory specimens of children [24, 25] and infants [24, 26]. Also, we obtained a nearly-full-length nucleotide sequence (5146 nt). Previously, we undertook a metagenomics study focusing on rats and shrews, and found bocavirus to be present in their feces. In the present study, HBoV appeared to be spread across species. According to phylogenetic analysis, HBoV detected from stool samples in our study was HBoV-2. This finding is in accordance with a review indicating that HBoV-2 DNA is present mainly in fecal samples [27].

The genomic sequence of GZ533 and HBoV were clustered together on the phylogenetic tree. The similarity between the NS1 protein of GZ533 and HBoV has been reported to be >85% [1] but the similarity with murine bocavirus is low. We used two reference sequences from GenBank (HBoV (FJ375129.1) and murine bocavirus (KY927869.1)) in similarity plots for sequences to analyze recombination in the GZ533 sequence. The sequence obtained in the present study showed a high degree of consistency with HBoV at various nucleotide positions. Whether a recombination phenomenon occurred is not known.

There is no clear evidence for recombination, but bocavirus shows genetic diversity between hosts, and bocavirus recombination in humans and pigs is common [28, 29]. Cross-species transmission of bocavirus has also occurred in bats and pigs [30], and some studies have shown that the mutation rate of parvoviruses is close to that of single-stranded RNA viruses [31, 32]. Thus, the evolution of the murine bocavirus may be rapid. Although the positive sequence in this study did not recombine, the spread of different types of Bocavirus through the species barrier has occurred based on the above evidence. The threat to livestock and humans is unknown, so additional research is needed.

The three positive samples detected in our study were HBoV-2 and the pathogenicity of HBoV-2 is incompletely understood [33]. Experimental studies based on cell lines and animals are needed to identify

the pathogenicity of HBoV subtypes.

Frequently, rats are involved in different aspects of human life, and may cause fecal−oral transmission through contaminated water and food. The nucleotide sequence obtained in the present study showed a high degree of homology with viral nucleotides detected in the stool of a child with acute diarrhea in Beijing (China), which demonstrates the wide geographic spread of HBoV-2. Differences in the prevalence of detection of HBoV infection in different regions in the present study were probably due to variations in geographic and climatic conditions. Previously, we showed that murine bocavirus and porcine bocavirus were detected in a sample (named YY-26), and HBoV was detected in stool samples from *R. norvegicus* in the present study. Compared with the VP1 regions of different types of bocavirus detected in YY-26, the similarity of nucleotides and amino acids between HBoV and murine bocavirus was 58.6% and 11.9%, respectively. The similarity in nucleotides and amino acids between HBoV and porcine bocavirus was 40.9% and 14.1%, respectively.

Our study had three main limitations. First, precise information on the age of the wild rats and diarrhea symptoms were not available. Thus, additional epidemiology studies are required to investigate the prevalence of bocavirus in rats and its association with diarrhea. Second, whether the HBoV identified in the present study was alive and passed through only the intestinal tract with food is not known. Therefore, it is essential to ascertain if rats serve as a host for HBoV. Third, experiments using cell lines or animals are needed to determine if HBoV can reproduce in rat cells.

5. Conclusion

We identified, for the first time, HBoV from fecal samples in rats. More detailed analyses, including epidemiological and experimental investigations, are required to ascertain the pathogenicity, genetic diversity, and phylogenetic relationships.

Declarations

Funding

Conflicts of interest

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

Availability of data and material

All data generated or analyzed during this study are included in this published article. Access to raw data can be acquired by contacting the corresponding author via email.

Code availability

Not applicable

Authors' contributions

Conceptualization: F.F.Y., M.Y.Z. Methodology: F.W. Formal analysis: M.Y.Z. Investigation: Q.S.L., F.W. Writing (original draft preparation): F.F.Y. Writing (review and editing): Q.C. Supervision: Q.C.

Ethics approval

The study protocol was approved by the Animal Ethics and Welfare Committee of the School of Public Health within Southern Medical University (Guangzhou, China) and adhered to the guidelines for the Rules for the Implementation of Laboratory Animal Medicine (1998) from the Ministry of Health (Beijing, China). Endangered or protected species were not involved in this study.

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Tables

Table 1 Primers for the detection of Human bocavirus.

name	5'-3'	Length(bp)	Targetedregion
PANBOV-F1	TAATGCAYCARGAYTGGGTIGANCC	290	NS1
PANBOV-F2	GCAYCARGAYTGGGTIGANCCWGC		
PANBOV-R	GTACAGTCRTAYTCRTTRAARCACCA		
HBoV-VP1-F1	CGCCGTGGCTCCTGCTCT	570	VP1
HBoV-VP1-R1	TGTTCGCCATCACAAAAGATGTG		
HBoV-VP1-F2	GGCTCCTGCTCTAGGAAATAAAGAG		
HBoV-VP1-R2	CCTGCTGTTAGGTCGTTGTTGTATGT		

Table 2 Comparison of putative amino acid similarity between GZ533 and Bocavirus from different species (%)

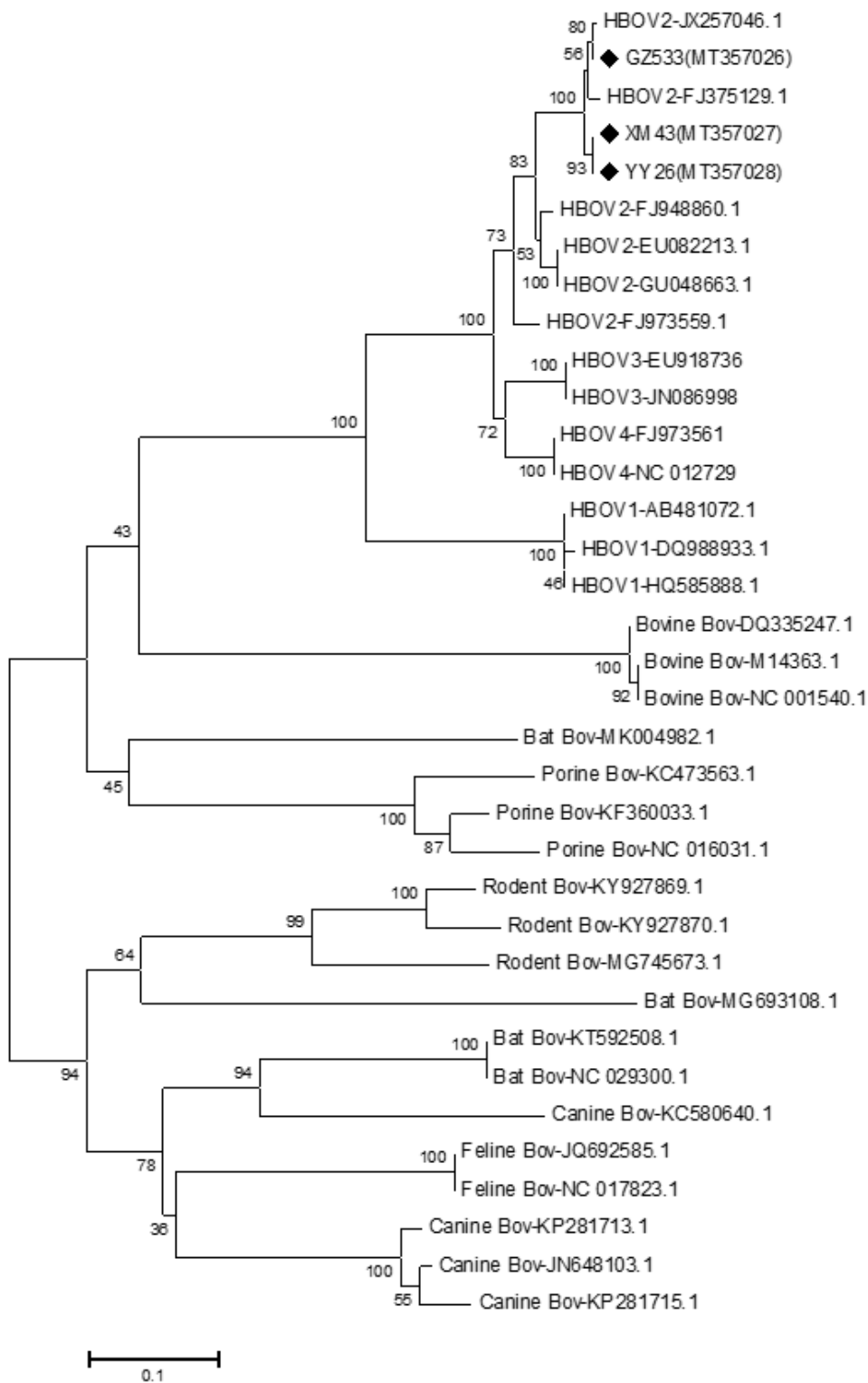| GZ533	NS1	NP1	VP1	VP2
HBoV-1(DQ988933.1)	70.9	69.8	80.3	78.2
HBoV-2(JX257046.1)	90.5	98.6	100	100
HBoV-3(EU918736)	70.6	68.8	91	89.8
HBoV-4(NC012729)	83.1	83.2	90.9	89.2
Rodent Bocavirus(KY927869.1)	37	41	37.5	40.5
Bat Bocavirus(KT592508.1)	40.3	44.3	46.7	44.5
Canine Bocavirus(JN648103.1)	42.4	46.6	45.8	43.4
Feline Bocavirus(NC_017823.1)	38.3	40.5	47.4	46.1
Bovine Bocavirus(DQ335247.1)	35.2	47.9	46.8	44.8
Porcine Bocavirus(KC473563.1）	37.5	37.4	43.6	41.1

Figures

Figure 1

Phylogenetic tree based on the VP1 region nucleotide sequence of Human bocaviruses (Neighbor-joining method)
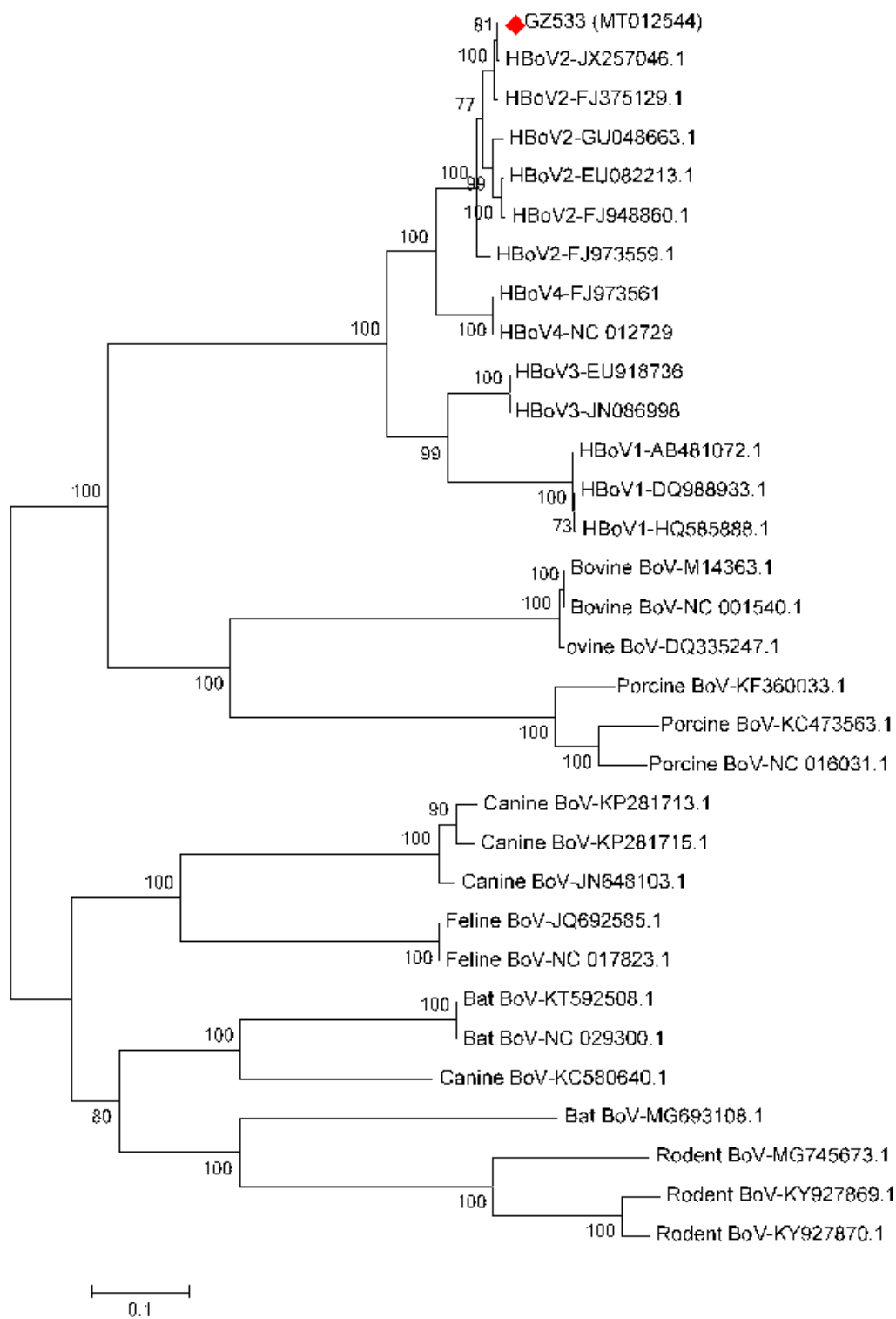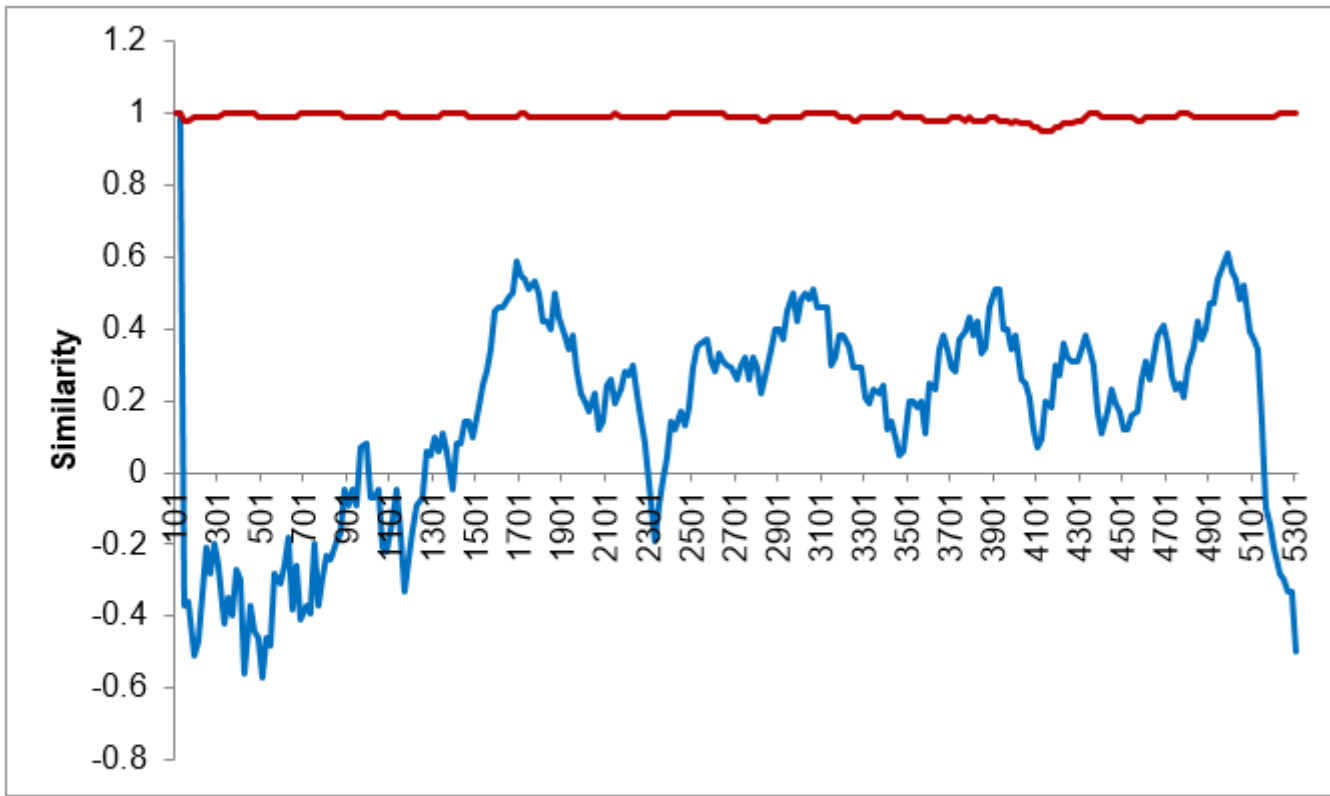
Figure 2

Phylogenetic tree based on the near-full Bocavirus genome nucleotide sequence (Maximum likelihood method)

Windows: 200bp. Step: 20bp. GapStrip: On. Kimura(2-parameter). T/t: 2.0

Figure 3

Similarity plots of the near-full genomes of GZ533 Query: GZ533; blue: Rodent Bocavirus (KY927869.1); red: HBoV-2 (FJ375129.1)