Human Bocavirus in Saudi Arabia: Molecular Epidemiology and Co-Infections Among Children With Acute Respiratory Tract Infections

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Research Article

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Human Bocavirus in Saudi Arabia: Molecular Epidemiology and Co-Infections among children with acute respiratory tract infections

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Abstract

Background

During the last few years since HBoV discovery, the virus has attracted less attention and appeared to be one of the most common acute respiratory tract pathogens in children. However, few studies focused on genetic characterization and epidemiology of HBoV in Saudi Arabia, leading us to evaluate the molecular epidemiology of HBoV strains among children with ARI. This study was designed to study the epidemiological, genetic diversity and circulation pattern of HBoV among hospitalized children (< 7 years) due to acute respiratory tract infection (ARTI) during two consecutive years. Nasopharyngeal aspirate (NPA) samples (n=196) were collected. HBoV and co-infection with other respiratory pathogens were identified using qRT-PCR. Positive samples were subsequently subjected to amplify full-length of VP1/VP2 gene, followed by sequencing and phylogenetic analyses.

Results

Our data showed that 3.4% (2014/2015) and 11.3% (2015/2016) of children hospitalized due to acute respiratory infections (ARI) were infected by HBoV-1. We have shown that HBoV-1 was detected year-round. HBoV-1 also was co-detected with one or multiple other respiratory viruses. Phylogenetic analysis of complete HBoV VP1/2 sequences showed no nucleotide variation and the circulation of HBoV-1. The multisequence analysis showed complete identity between HBoV isolated from 2014/2015 and 2015/2016, suggesting no genetic variability among the local circulating HBoV-1 genotypes. Our data reveal that genetically conserved HBoV-1 is circulating in Saudi Arabia.

Conclusions

The multisequence analysis showed complete identity between HBoV-1 isolated from 2014/2015 and 2015/2016, suggesting no genetic variability among the local circulating HBoV-1 genotypes. Our data reveal that genetically conserved HBoV-1 is circulating year-round in Saudi Arabia.

Keywords: Molecular epidemiology; acute respiratory tract infections; human bocavirus; co-infections; children; genotyping; Saudi Arabia

Introduction

Viral respiratory tract infections (RTIs) are the major (50-90%) infection respiratory syndrome that affects human, particularly infants, children elderly, and the immunocompromised individuals. HBoV infections in infants has been associated with acute respiratory tract infection (ARTI). ARTI among children is a major cause of morbidity and mortality globally [1,2]. HBoV causes more than 21.5% RTIs among children worldwide [2]. HBoV causes lower respiratory tract symptoms includes viral bronchiolitis, wheezing pneumonia and respiratory distress [3,4]. A number of studies revealed that HBoV-1 infection was associated with severe lower respiratory tract diseases, leading to hospitalization and intensive care unit (ICU) admission [2,3,4,5]

HBoV is classified into *Parvoviridae* family, *Parvovirinae* subfamily and the genus *Bocaparvovirus* [6,7]. Human Bocavirus is a 5.3 kb, single stranded DNA genome, icosahedral non-envelope with approximately diameter 18–26 in nanometers [8]. The genome of HBoV contains three open reading frames (ORFs). The first ORF encodes nonstructural proteins (NS), the second ORF encodes (NP1) protein and the third ORF encodes (VP1 and VP2) proteins [9,10,11]. Based on genetic variability of VP1gene, HBoV are grouped into four genotypes (HBoV-1, HBoV-2, HBoV-3 and HBoV-4). Several studies have shown that HBoV1 is associated with respiratory tract infections, while HBoV-2, 3 and 4 are associated with gastrointestinal tract infection [8,9,10].

Although Saudi Arabia is considered a virgin soil for spread and evolution of respiratory viruses, there is substantial lack of data regarding epidemiology, circulation pattern and mechanisms adopted in diversification and evolution of HBoV. In a previous set of studies conducted by our laboratory the prevalence and circulation pattern of several respiratory viruses were studied. HBoV studies in Saudi Arabia have only reported in few studies [19,20,25]. There is no published data on the complete Vp1 and VP2 gene sequences of HBoV-1 in Saudi Arabia. This study was designed to study the epidemiological, genetic diversity, circulation pattern and molecular evolution of bocavirus among children.

Materials and Methods

Ethical considerations

The institutional review board at King Saud University- Collage of Medicine University reviewed and approved the study protocol (IRB register number E-14-1326). The parents of the patients participated in this study gave written informed consent before enrollment.

Study design, patients, respiratory samples collection and analysis

Nasopharyngeal aspirate (NPA) samples (n=196), from hospitalized neonates, infants and young children < 7 years of both sexes were collected from King Khalid University Hospital (KKUH) in Riyadh, Saudi Arabia, over two consecutive years (2014/2015 and 2015/2016). ARI cases were defined as an illness that showed one or more of the main symptoms of the clinical manifestations including: cough, fever (temperature, >38 °C), coryza, difficult or shortness of breath, dyspnea, nasal, sneeze and obstruction during admission time. NPA samples were collected from different wards pediatric intensive care unit (PICU), pediatric surgery, cardiology and medical wards). The respiratory samples were placed into 1 mL of universal transport medium (Kit Cat. No. 360c, Copan Italia, Brescia, Italy), transported on ice to the Virology Research Group (VRG) Laboratory, King Saud University, and were mixed via pulse-vortexing and divided into two aliquots. The samples were examined immediately or stored at -80 °C until further test. All methods in this study were preformed in accordance with international guidelines.

HBoV and other respiratory pathogens detection using real-time quantitative polymerase chain reaction

For viral nucleic acid extraction, 140 µl of respiratory clinical samples was used with QIAamp Viral RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer‹s instructions. This kit enables to extract DNA along with the RNA and no step for DNase digestion was included. The extracted viral nucleic acid was eluted in 60 µl of elution buffer and kept aliquoted at -80°C until used in qPCR. During the extraction process an internal RNA virus (extraction control), Equine arteritis virus (EAV), which was included in the

FTD kit (Fast-track Diagnostics, Luxembourg), was added to the lysis buffer for each sample and negative control for monitoring extraction, reverse transcription quality and possible PCR inhibition. The concentration (ng/µl) and purity (260/280) of the RNA were measured using NanoDrop2000 (Thermo- scientific). The extracted nucleic acid samples were directly subjected to multiplex RT-PCR analysis using the FTD® Respiratory Pathogens 21 kit (Fast-track Diagnostics, Junglinster, Luxemburg) using 7500 Applied Biosystem™ real-time machine (ThermoFisher Scientific Inc, MA, USA). This kit able to detect specific viral or bacterial DNA sequences in the reaction through increasing a fluorescence observed from the relevant dual-labelled probe which expressed as a cycle threshold value (Ct). The RT-PCR reaction was performed in a total of 25 µL, containing 12.5 µl Buffer, 1.5µl PPmix, 1µl enzyme mix (Fast-track mastermix) and 10 µl extracted viral nucleic acid. A negative and a positive control involved in the kit were used in all runs to observer the assay performance. Amplification was conducted using 7500 Applied Biosystem™ real-time machine (ThermoFisher Scientific Inc, MA, USA) under the following thermocycling conditions: One cycle of initial reverse transcription at 50 ^{0}C for 15 min, holding at 94 ^{0}C for 1 min, followed by 40 cycles at 94 ^{0}C for 8s s and 60 ^{0}C for 1 min. In each RT-PCR cycles the reading of fluorescence was obtained in the 60 ^{0}C /34 s step and the threshold value (Ct) were determined.

PCR and sequencing of Human Bocavirus (HBoV)

The qPCR-positive samples were used for viral DNA extraction by QIAamp DNA Mini Kit (Qiagen), and subjected to amplify full-length of VP1and VP2 gene. The PCR protocol involved amplification of three fragments that cover the entire VP1 and VP2 genes (Figure 1). Designed oligonucleotide primers used for PCR showed in (Table 1). PCR reaction sequence was amplified using GeneAmp® High-Fidelity PCR Kit (Applied Biosystems, Foster City, CA) according to the manufacturer‹s instructions. The total 50 µl reaction mix consisted of 1 µl GeneAmp PCR buffer II (100 mM Tris_HCl, pH 8.3_500 mM KCl) (Applied Biosystems), 2.5 mM MgCl2, each dNTP at 0.2 mM, 20 pmol each of the primers and 10 µl DNA. The reaction tubes were placed, in GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA), at 95 ^{0}C for 5 min (initial denaturation), followed by 45

cycles of denaturation at 94 ^{0}C for 15 s, annealing at 60 ^{0}C for 1 min and extension at 72 ^{0}C for 1 min, and one cycle at 72 ^{0}C for 10 min (final extension). All PCR products were separated, visualized by agarose gel and were purified with IllustraTM GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). Sequencing of the purified products on both strands was conducted using the specific PCR primers at GTAC Biotech (Cologne, Germany).

Table I. The general nested PCR primer pairs for HBoV-1 VP1and VP2 gene.

Primer name	Primer sequence
Boca-Seq1-FP	5′-GAA GAC GAG GGA GAG TAC ATC-3′
Boca-Seq1-RP	5′-CCT CCA ATA CTT CCT GTT CCT C-3′
Boca-Seq2-FP	5′-GTC TGA CAC TGA CAT TCA AGA CC-3′
Boca-Seq2-RP	5′-GTT GGT GCC AGA CAT CCG CTT G-3′
Boca-Seq3-FP	5′-GGA CCA CAG TCA TCA GAC-3′
Boca-Seq3-RP	5′-CCA CTA CCA TCG GGC TG-3′

Phylogenetic and alignment analysis. The HBoV complete VP1 and VP2 gene sequence were downloaded from GenBank and edited and assembled using Bioedit program, version 7.0 (Ibis Biosciences, Carlsbad, CA). Phylogenetic analyses were performed using MEGA software. Briefly, HBoV sequences were aligned using Clustal W algorithm. A phylogenetic tree was constructed with the neighbor-joining algorithm with 1000 bootstrap resampling.

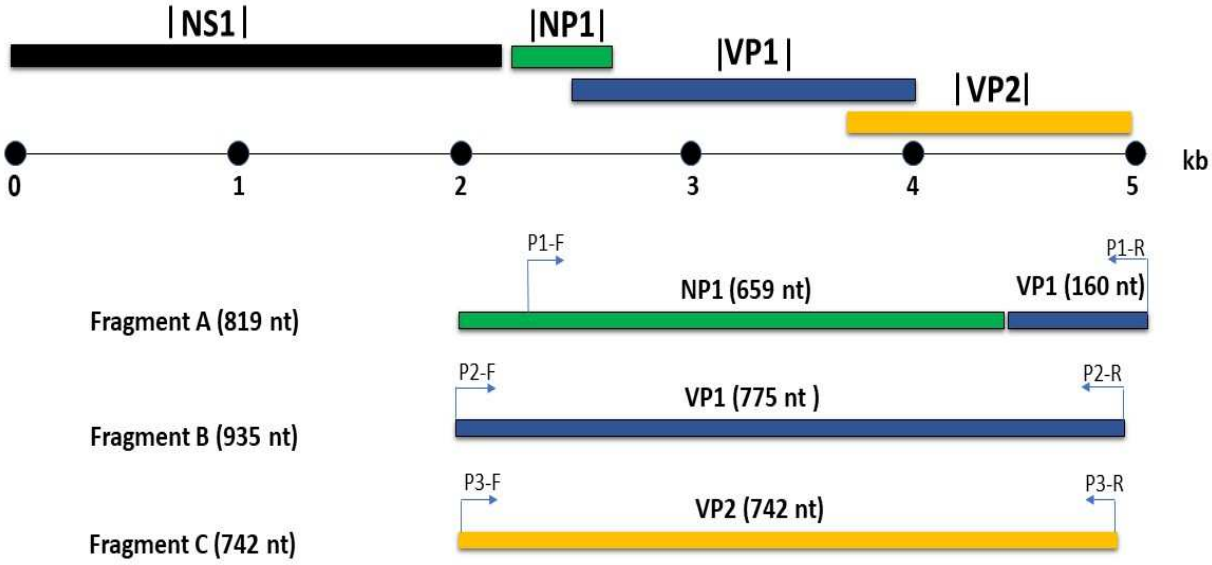

Figure 1. shows the primers design and overlapped amplified area of the HBoV-1 VP1 and VP2 genes that was used for PCR and sequencing.

Results

Prevalence and epidemiology of HBoV

During the two-year period of this study (2014–2016), we analysed a total of 196 respiratory samples from hospitalized children (age; 1 month to 7 years) due to lower respiratory tract infections. Overall, HBoV was detected in 13 samples (6.6%) by RT-PCR. Of these, HBoV was detected in 3.4% (4/116) and in 11.3% (9/80) of samples collected during 2014/2015 and 2015/2016, respectively. We have shown that HBoV was detected year-round without a marked seasonality. HBoV infection was higher in males (69.2%) than in females (30.8%). Among HBoV-infected children, 84.6% were between 1 and 8 months old and 15.3%, between 1 and 6 years.

HBoV and Co-Infections

In order to study HBoV co- respiratory viral infections, we examined the rate of the major respiratory viruses among the HBoV patients (n = 196). Surprisingly, we found all children with HBoV infection in this study were co-infected with other respiratory viruses (Table 2), including 3.6% patients with Rhinovirus, 3.1% with human respiratory syncytial virus (HRSV), 2.6 % with parainfluenza virus (PIV), 1.5% with adenovirus (Adv), 1.0% with influenza A virus (IAV), 0.5% with coronavirus OC43 (HCoV-OC43) and 0.5% with metapneumovirus (HMPV). Multiple infection consisting of HBoV plus three other viruses (HRSV, IAV, Adv, and HPIV) was detected in one patient.

Table 2. Co-infection with other respiratory viruses among HBoV positive children.

Patient No	Age	Gender	Co-infection with other respiratory viruses
1	8 months	Male	HMPV
2	5 months	Male	HRSV+Rhino+HPIV-3
3	5 months	Male	HRSV
4	5 months	Female	HRSV+Rhino+Entero
5	6 months	Female	Rhino+ Influenza A virus

6	4 months	Female	HRSV+Adv+IAV+HPIV-3+
7	19 months	Female	Rhino+ Adv+ HPIV-3
8	5 months	Male	Rhino+ Adv
9	7 months	Male	Adv+ HPIV-3
10	4 months	Male	Rhino+HPIV-1
11	4 months	Male	HRSV
12	7 months	Male	Rhino
13	6 months	Male	HCoV-OC43

HBoV molecular characterization

We successfully sequenced all HBoV positive samples. The results of sequences were compared with HBoV reference sequences registered in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). Phylogenetic tree constructed showed that HBoV-positive specimens of this study clustered around HBoV-1 genotype sharing 97-100% identity with HBoV-1 reference sequences (Figure 2). We detected only HBoV-1 genotype, our results suggest that a single genetic lineage of HBoV-1 is circulating and causing respiratory infections among children in Saudi Arabia. Ten complete sequence of HBoV-VP1/VP2 genes were deposited in GenBank Accession number (MH427520, MH427519, MH427518, MH427517, MH427516, MH427515, MH427514, MH427513, MH427512 and MH427511).

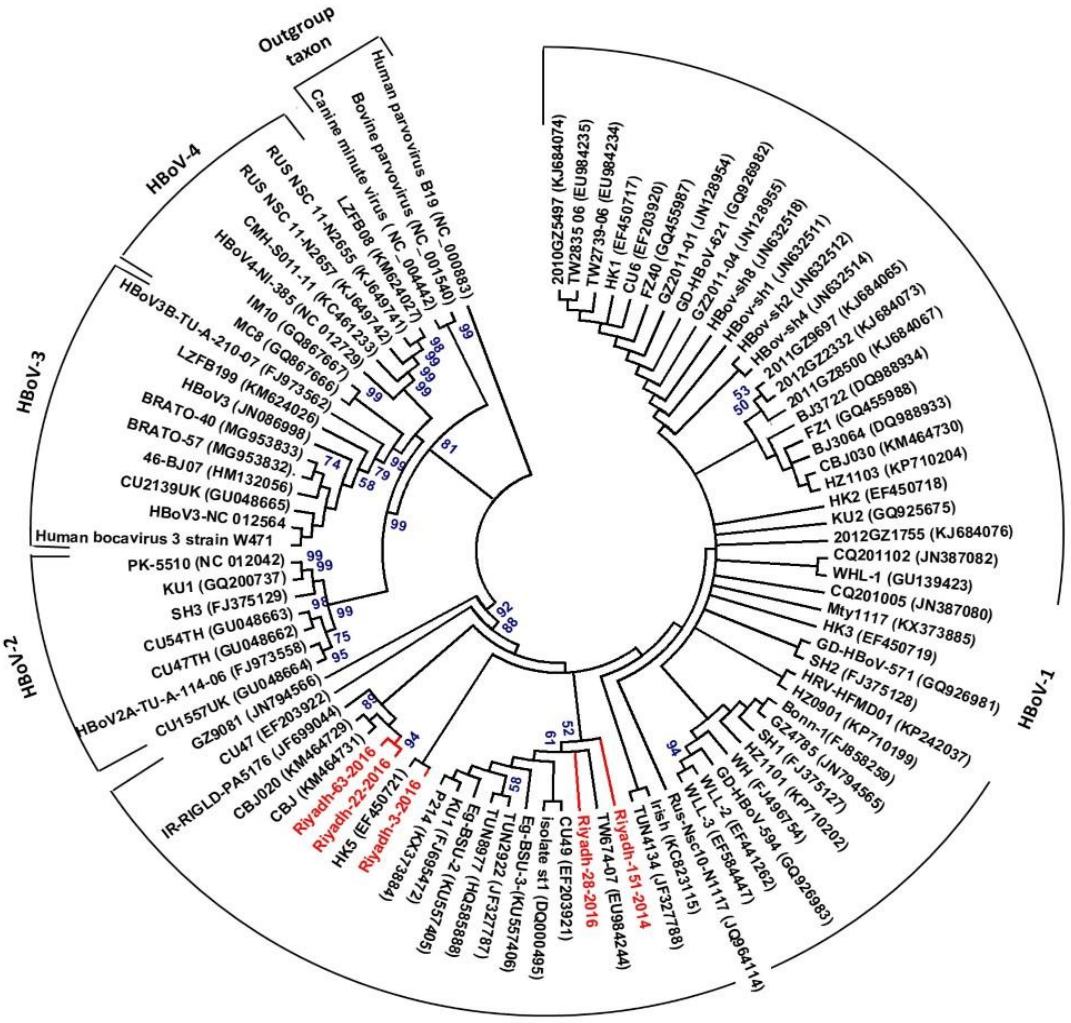

Figure 2. Phylogenetic tree of HBoV-1 positive samples from children with acute respiratory tract infections from Saudi Arabia. Nucleotide-sequence-based phylogenetic analysis of complete HBoV-1 VP1 and VP2 genes (shown in red) and reference HBoV strains from the GenBank database. A neighbor-joining tree of complete VP1 and VP2 nucleotide sequences was conducted using MEGA software. The accession number, country and year of each strain are shown. Phylogenetic trees were constructed with the neighbor-joining method. Our HBoV strains were in the same cluster as the reference isolates and they belonged to HBoV-1.

Discussion

During the last few years since HBoV discovery, HBoV is appeared to be one of the most common acute respiratory tract pathogens in children (1). In the present study, we studied the prevalence and epidemiology of HBoV among children up to 6 years old in Saudi Arabia, as well as major respiratory viral co-infections and the characterization of circulating HBoV genotypes. Overall, HBoV was detected in 3.4% (4/116) and 11.3% (9/80) of respiratory samples collected during 2014/2015 and 2015/2016, respectively. Interestingly, we detected HBoV in one case age 41 days-old child, supporting the possibility that HBoV can infect a very young child. We have shown that HBoV-1 was detected year-round without a marked seasonality. Besides, co-infections with one or multiple respiratory viruses were detected in all HBoV-positive samples. Interestingly, all HBoV1 infected cases were associated with high rates of other respiratory viral co-infections. Several studies revealed that rates of detection of HBoV in acute respiratory infection (ARI) is about 3-19.0% [13,14]. In the present study, the overall detection rate of HBoV in among hospitalized children due to LRTI was 6.63% which was similar to other countries of the world.

In this study, co-infections with one or multiple respiratory viruses were detected all HBoV-positive samples. Several studies, have shown that HBoV-infected children were more likely to be co-infected with other respiratory viruses [12,15,16]. The higher rate of HBoV co-infection is in line with a higher HBoV co-infection which reported from different countries around the world [17,18,19]. In addition, previous study from Saudi Arabia found only one child diagnosed with HBoV as a single virus entity while most of children (17/18) showed coinfection with other viral pathogens. The most commonly detected viruses were HRSV (72.2%), IAV (66.66%), adenovirus (33.33%) and HPIV-3 (5.5%) [20]. Likewise, we found 3.6% patients co-infected with Rhinovirus, (3.1%) with human respiratory syncytial virus (HRSV), 2.6 % with parainfluenza virus (PIV), 1.5% with adenovirus (Adv), 1.0% with influenza A virus (IAV), 0.5% with coronavirus OC43 (HCoV-OC43) and 0.5% with metapneumovirus (HMPV). Multiple infection consisting

of HBoV plus three other viruses (HRSV, IAV, Adv, and HPIV) was detected in one patient.

Seasonal distribution of HBoV is not well defined, and might be affected by geographic areas. Some studies showed that high prevalence of HBoV infections occurred in the winter and spring [21,22,23,24], while other studies showed high prevalence in the early summer and late spring [23,24,25]. However, clear seasonal activity was not observed in some studies. In Saudi Arabia, the seasonality of HBoV infection has not been clearly reported. In one study conducted in Eastern province Saudi Arabia, HBoV was detected in August, October and December [19,25]. Nevertheless, seasonal variations of respiratory viruses detected from children with respiratory tract infections was studied. Respiratory viruses were detected year-round with a peak in winter (December to March) in Saudi Arabia [19,25]. In this study, we have shown that HBoV was detected year-round without a marked seasonality, thus, we cannot draw any conclusions about seasonality based on our data. HBoV seasonality should be investigated carefully, an overlapping in seasonality of HBoV may be due to the HBoV- coinfection and different respiratory viruses co-circulating at the same time. Establishing the national surveillance system is needed to identify a clear picture of HBoV seasonality in Saudi Arabia.

Based on Vp1/ and VP2 complete gene sequences, all HBoV strains isolated in this study were identified as HBoV-1. Although no variability exists in HBoV-1 genomic region encoding Vp1/ and VP2, all our HBoV strains were in the same cluster as the reference isolates and they belonged to HBoV-1. The multisequence analysis showed complete identity between HBoV isolated from 2014/2015 and 2015/2016, suggesting minor genetic variability among the local circulating HBoV-1 strains over short period of time. Together with several studies from different geographical regions [20,21,22,23]. our results provide support evidence for a global circulation of HBoV-1 among children causing ARTI. Several HBoV-1 molecular epidemiology studies have been conducted previously in Saudi Arabia [20,25]. however, the complete viral sequences published were infrequent and inadequate to draw any chronological or spatial characteristics. Molecular characterization

of multiple HBoV-1 strains from all regions of Saudi Arabia might help in understanding the evolution of this virus. In a previous set of studies conducted by our laboratory (Virology research group), the prevalence and circulation pattern of several respiratory viruses were studied. Although the results were promising, tracking the epidemiology and genetic variation among Saudi isolates for an extended period is strongly recommended.

In conclusion, over a period of two years (2014–2016), we detected HBoV in 3.4% (4/116) and 11.3% (9/80) of samples collected in 2014/2015 and 2015/2016, respectively. We detected HBoV year-round without a marked seasonality with high rates of viral co-infections. Our data reveal that genetically conserved HBoV-1 is circulating in the Saudi Arabia. Further epidemiological and molecular characterization are required of multiple HBoV-1 strains from all regions of Saudi Arabia are required to understand the evolution of this virus.

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Declarations

Ethics approval and consent to participate

The institutional review board at King Saud University- Collage of Medicine reviewed and approved the study protocol (IRB register number E-14-1326). The parents of the patients participated in this study gave written informed consent before enrollment.

Consent for publication

Not applicable

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Complete sequence of HBoV-VP1/VP2 genes deposited in GenBank from this study, Accession numbers:

1. MH427520: https://www.ncbi.nlm.nih.gov/nuccore/MH427520.1
2. MH427519: https://www.ncbi.nlm.nih.gov/nuccore/MH427519.1
3. MH427518: https://www.ncbi.nlm.nih.gov/nuccore/MH427518.1
4. MH427517: https://www.ncbi.nlm.nih.gov/nuccore/MH427517.1
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9. MH427512: https://www.ncbi.nlm.nih.gov/nuccore/MH427512.1
10. MH427511: https://www.ncbi.nlm.nih.gov/nuccore/MH427511.1

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Study design, respiratory samples collection and performed the experiments: Maaweya E Hamed and Haitham M Amer. Molecular analysis: Mohamed A Farrag. Clinical data collection and manuscript revision: Fahad N Almajhdi, Ibrahim M. Aziz and Rauf Bhat. Wrote the paper: Maaweya E Hamed. All authors read and approved the final manuscript.

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Figures

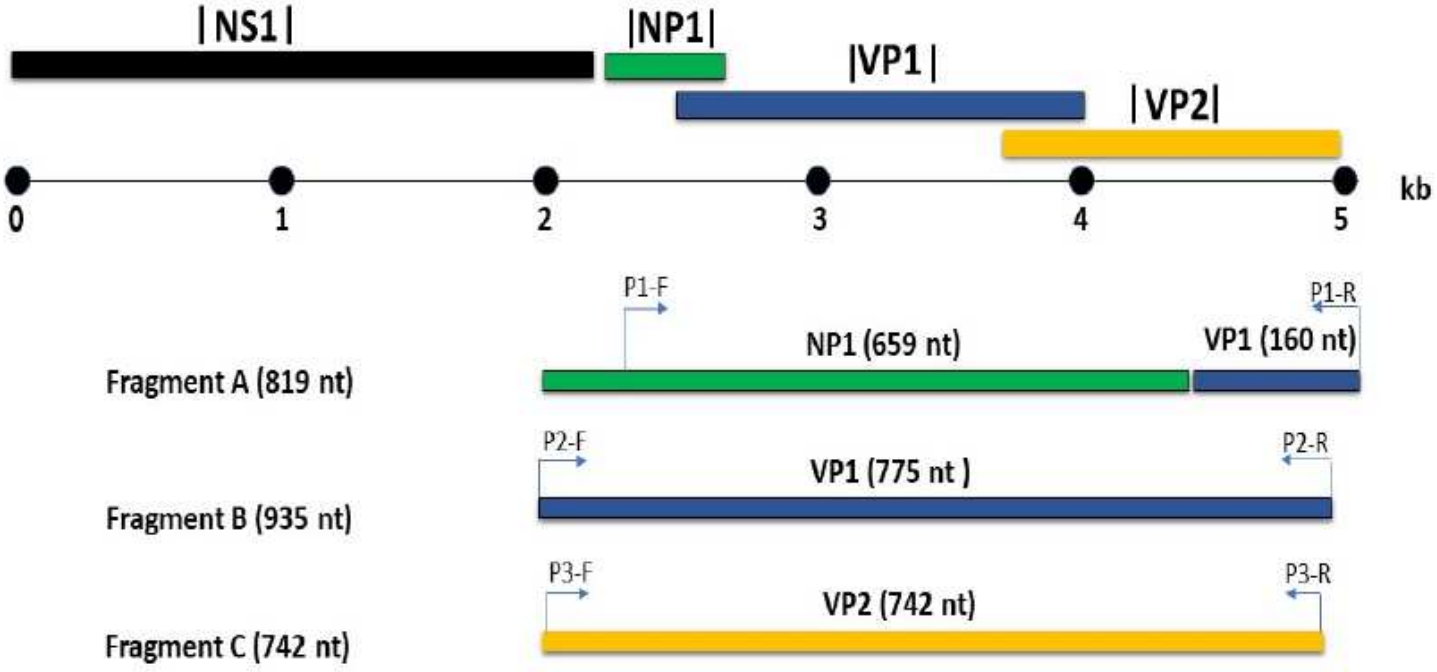

Figure 1

shows the primers design and overlapped amplified area of the HBoV-1 VP1 and VP2 genes that was used for PCR and sequencing.

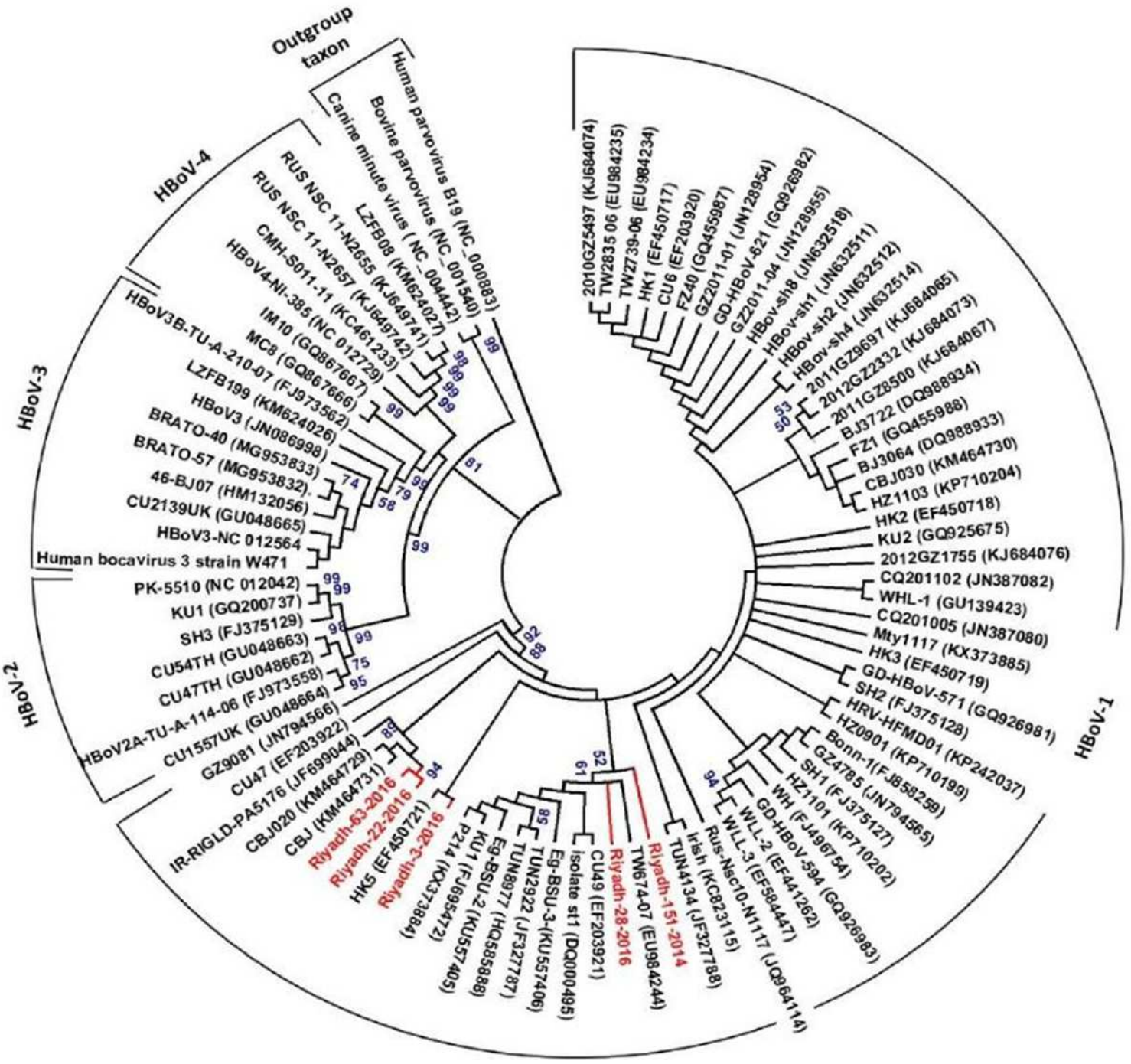

Figure 2

Phylogenetic tree of HBoV-1 positive samples from children with acute respiratory tract infections from Saudi Arabia. Nucleotide-sequence-based phylogenetic analysis of complete HBoV-1 VP1 and VP2 genes (shown in red) and reference HBoV strains from the GenBank database. A neighbor-joining tree of complete VP1 and VP2 nucleotide sequences was conducted using MEGA software. The accession number, country and year of each strain are shown. Phylogenetic trees were constructed with the neighbor-joining method. Our HBoV strains were in the same cluster as the reference isolates and they belonged to HBoV-1.