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Research article

Human bocavirus in Saudi Arabia: Molecular epidemiology and Co-infections among children with acute respiratory tract infections during 2014–2016

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ABSTRACT

Respiratory tract infections due to a variety of viruses continue to threaten the human population worldwide, particularly in developing countries. Among the responsible viruses, Human Bocavirus (HBoV), a novel discovered virus, causes respiratory tract and gastroenteritis disorders in young children. In Saudi Arabia, data regarding virus molecular epidemiology and evolution and its implication in respiratory tract infection are scarce. In the current study, genetic diversity and circulation pattern of HBoV-1 among hospitalized children due to acute respiratory tract infection (ARTI) during two consecutive years were charted. We found that 3.44% (2014/2015) and 11.25% (2015/2016) of children hospitalized due to ARTI were infected by HBoV-1. We have shown that HBoV was detected year-round without a marked seasonal peak. HBoV-1 also was co-detected with one or multiple other respiratory viruses. The multisequence analysis showed high sequence identity (~99%) (few point mutation sites) between strains of each genotype and high sequence variation (~79%) between HBoV-1 and the other 3 genotypes. Phylogenetic analysis showed the clustering of the study's isolates in the HBoV-1 subclade. Our data reveal that genetically conserved HBoV-1 was circulating among admitted children during the course of the study. Further epidemiological and molecular characterization of multiple HBoV-1 strains for different years and from all regions of Saudi Arabia are required to understand and monitor the virus evolution.

1. Introduction

Viruses are the most common cause of acute respiratory tract infection (ARTI) which are the major cause of global child morbidity and mortality. Globally, the incidence of ARTI among children under 5 years of age has been estimated to be 12,197.8 new cases per 100,000 children, which was dramatically higher in developing countries [1,2]. Human Bocaviruses 1 (HBoV1), that was recently renamed to *Bocaparvovirus primate* 1 [3], is primarily a respiratory virus found to be present in 21.5% of childhood ARTI annually [4]. This virus species belongs to the family *Parvoviridae*, the subfamily *Parvovirinae*, and the genus *Bocaparvovirus*. The genus includes more than 21 species however only *Bocaparvovirus primate* 1 and Bocaparvovirus *primate 2* include four viruses that infect humans [5]. HBoVs consist of approximately 5.3 kb, single-stranded DNA genome that contains three open reading frames (ORFs) encoding two

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nonstructural proteins NS1 and nuclear phosphoprotein (NP1), and two viral capsid proteins 1 and 2 (VP1 and VP2). Until recently, HBoVs were distinguished into four genotypes (HBoV-1, -2, -3, and -4) [6–9] based on the heterogenicities observed in VP1 sequences and the phylogenetic distances between full genomes. HBoV strains with >8% protein and >10% nucleotide difference in the complete VP1 gene should be considered different species, while those with >1.5% protein and >5% nucleotide difference should be considered different genotypes. Several studies have found that HBoV-1 is associated with ARTI, while HBoV-2, 3, and 4 are linked to gastrointestinal tract infections [10–13].

HBoV-1 was discovered in 17 (3.1%) of 540 respiratory specimens collected from hospitalized children over one year in Sweden in September 2005 [7], Subsequently, HBoV has been reported in respiratory samples from children in various parts of the world including Japan [14], the United States [15], Korea [16], Japan [17], and Australia [18].

Locally, a recent study found HBoV DNA in 193 (1.6%) patients admitted with respiratory symptoms in Riyadh, Saudi Arabia [19]. Another study revealed HBoV in 11.2% of 2266 patients aged 0 to 14 at a Riyadh Tertiary Care Hospital [20]. One study in Al-Taif, a city in Saudi Arabia's western region, reported HBoV-1 in 22.5%% (18/80) of children NPA samples mostly in toddlers aged five months to two years [21]. Furthermore, twenty-one out of three hundred blood samples tested positive for HBoV in another study conducted in Al-Taif, Saudi Arabia [22]. In an Eastern Saudi area, five cases (10.6%) of 47 patients with viremia were positive for HBoV in their serum samples [23]. In a cross-sectional study conducted in southwestern Saudi Arabia, only one patient out of 135 children < 5-year-old was tested positive for HBoV [24]. Although several studies were conducted, data on epidemiology, circulation patterns, and mechanisms used in the diversification and evolution of HBoV and other respiratory viruses in Saudi Arabia are incompletely understood. In the current study, HBoV-1 was detected in NPA samples collected from young children. The full-length VP1/VP2 gene was amplified for sequence and phylogenetic analysis.

2. Materials and methods

2.1. Sample collection and ethics statement

A total of 196 NPAs were collected from neonates, infants, and young children (aged; 0–6 years) of both genders admitted to King Khalid University Hospital (KKUH) in Riyadh, Saudi Arabia with symptoms, including cough, rhinorrhea, dyspnea, and fever during 2014/2015 and 2015/2016. Before enrolling in this study, the patients' parents provided written informed consent. The study was conducted by the declaration of Helsinki and approved by the institutional review board (IRB) of King Khalid University Hospital, Riyadh (Ethics Reference No. 14/4463/IRB 03). Immediately following collection, samples were combined with 2 mL of the virus transport medium (PBS), transported on ice to King Saud University's Virology Research Group (VRG) Laboratory, and stored at −80 °C until analysis.

2.2. Detection of HBoV in clinical samples

Viral DNA was extracted from clinical samples using the QIAamp Viral DNA Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. HBoV1 was detected using multiplex PCR analysis with the FTD® Respiratory Pathogens 21 kit (Fast-track Diagnostics, Junglinster, Luxembourg) on a 7500 Applied BiosystemTM real-time equipment (ThermoFisher Scientific Inc, MA,

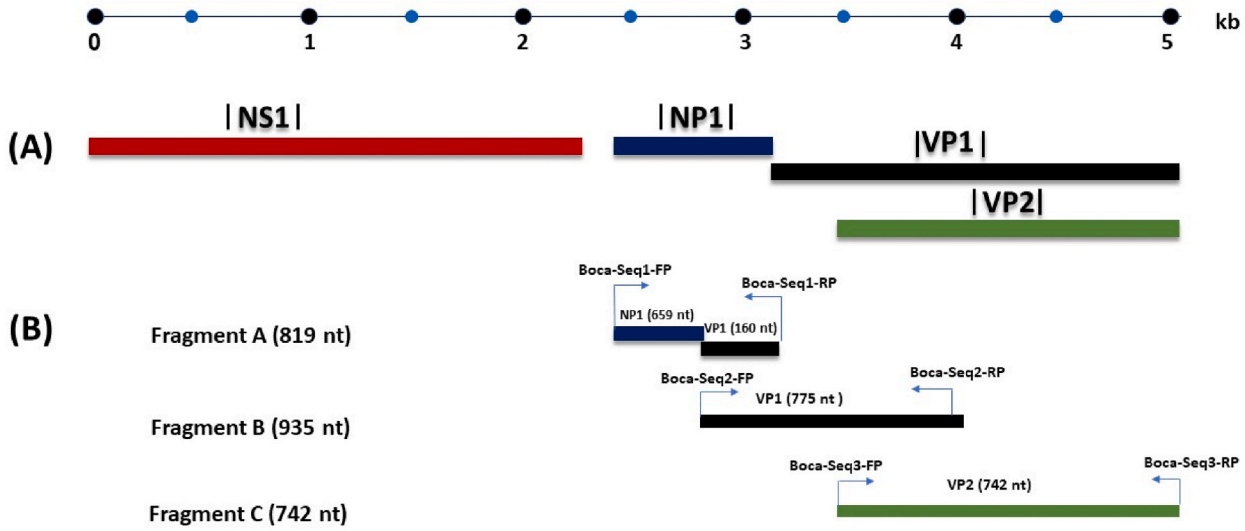

Fig. 1. A) The bocavirus genome is ssDNA, 5.3 Kb with three ORFs encoding two nonstructural proteins NS1 and NP1, and two VP1 and VP2. B) The amplified fragments; Fragment A spans a partial sequence of NP1 and VP1, and Fragment B and C include VP1 and VP2, respectively. The designed primers are indicated above each fragment.

USA) using the following cycling conditions: holding at 94 °C for 1 min, followed by forty cycles at 94 °C for 8s s and 60 °C for 1 min. The fluorescence signal was collected at the 60 °C/34 s stage of each real-time polymerase chain reaction (RT-PCR) cycle, and the threshold value (Ct) was computed.

2.3. Amplification and sequencing of the VP1/VP2 gene

HBoV-1 positive samples were used to amplify the full-length VP1/VP2 gene [Fig. 1A, B] of HBoV-1 in a GeneAmp 9700 thermal cycler using GeneAmp® High-Fidelity PCR Kit (Applied Biosystems, Foster City, CA) and the primer sets (Table 1) according to the kit's guidelines. The reaction was carried out at 95 °C for 5 min (initial denaturation), followed by 45 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, and one cycle of final extension at 72 °C for 10 min. The PCR products were visualized on a 1% ethidium bromide-stained agarose gel and size was discriminated using a 100 bp Plus DNA ladder (Qiagen, Hilden, Germany). The amplified fragments were retrieved from the gel using Illustra™ GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and were sequenced on both strands using GeneArt (Regensburg, Germany). Sequence contigs were edited using the BioEdit program, aversion 7.0.9.1, and assembled by the Edit sequence tool of the MegAlign program, Lasergene software, version 3.18 (DNAStar, Madison, WI). The assembled sequence fragments were deposited in GenBank with accession numbers MH427511 to MH427520.

2.4. Sequence and phylogenetic analysis

To investigate the evolution of HBoV-1 over time, the assembled sequences of VP1 and VP2 genes of the isolates were aligned against their international counterpart sequences available in the GenBank database. All isolates from this study were aligned with 72 international sequences that represent HBoV genotypes (HBoV-1, 2, 3 and 4) using the Clustal W algorithm of the MegAlign program, Lasergene software, version 3.18 (DNAStar, Madison, WI). Divergence analysis, allocation of mutation sites, and prediction of amino acid changes were accomplished by EditSeq and MegAlign programs, Lasergene. Prediction of potential sites for N- and O-linked glycosylation was assessed using Net-N-glyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc) and Net-*O*-glyc 3.1 (http://www.cbs.dtu. dk/services/NetOGlyc), respectively [25]. A phylogenetic tree based on the complete nucleotide sequence of the VP1/VP2 gene was constructed using the neighbor-joining algorithm method of MEGA 11 [26] with bootstrap values of 1000 replicates. VP1/VP2 gene of HBoV-4 (NC-000883) was used to root the tree.

2.5. Statistical analysis

The Fisher's Exact test was performed to identify significant differences in the prevalence of HBoV-1 isolates across gender and age groups. The posthoc comparison was performed using the Z-test with Bonferroni adjustment. A significance level of $P < 0.05$ was applied. Significant was defined as $P < 0.05$.

3. Results

3.1. Prevalence and epidemiology of HBoV-1

During the two years of this study (2014–2016), we analyzed a total of 196 respiratory samples from hospitalized children (aged; 0–6 years) due to lower respiratory tract infections. Overall, HBoV-1 was detected in 13 samples (6.6%) by RT-PCR. Of these, HBoV-1 was detected in 3.44% (4/116) and 11.25% (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-1 infection was higher in males (69.23%) than in females (30.8%) ($P < 0.05$). Among HBoV-1 -infected children, 84.6% were between 1 and 8 months old, and 15.3%, were between 1 and 6 years ($P < 0.05$) (Table 2).

3.2. HBoV-1 and Co-infections

To study HBoV-1 co-respiratory viral infections, we examined the rate of the major respiratory viruses among the HBoV-1 patients 6.63(13/196). Surprisingly, we found all children with HBoV-1 infection in this study were co-infected with other respiratory viruses

Table 1
PCR primer sets used for amplification of HBoV-1 VP1/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′

Table 2
The demographic and the prevalence of HBoV**-1**.

Seasonal and demographic details		Number of samples	Number of positive samples (%)
Winter season	2014/15	116	4(3.44)
	2015/16	80	9(11.25)
	Total	196	13(6.63)
Age in years	<1 month	10	0
	2–5 months	100	7(53.85)[a]
	6–11 months	45	4(30.77)
	≥12 months	41	2(15.38)
Gender	Male	106	9 (69.23)[b]
	Female	90	4(30.77)
Season	Spring	45	3(23.08)
	Summer	30	3(23.08)
	Autumn	56	3(23.08)
	Winter	65	4(30.76)

Note: Data presented as number (%). Data are displayed as percentages (%). Significantly different ($p < 0.05$) from age groups <2, 6–11, and ≥12 months. [b] Significant difference ($p < 0.05$) to females.

(Table 3), 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-1 plus three other viruses (HRSV, IAV, Adv, and HPIV) was detected in one patient.

3.3. Nucleotide and deduced amino acid sequence analysis

Of the 13 HBoV-1 positive samples, we succeeded only to amplify the sequencing fragments of five samples which were used in sequencing and phylogenetic analysis. The complete sequences of VP1/VP2 gene of HBoV-1 isolates were aligned against different HBoV genotypes from different countries around the world. The nucleotide sequence analysis revealed the presence of a few point mutations among strains of each genotype. A total of 32/2016 mutation sites (1.59%) were recognized as compared to the consensus nucleotide sequence. Seven of these (0.03%) nucleotide substitutions have led to changes in their respective amino acid residues, among which three are unique (A189G, A340V, and N546H) (Table 4). However, high sequence heterogeneity (ranging from 76.6% to 78.9%) was reported in VP1/VP2 proteins between HBoV-1 and the other 3 genotypes (Fig. 2). One sequence gap of three nucleotides was reported in HBoV-1 strains including our isolates at positions 420, 421, and 422. Two sequence gaps were reported in HBoV genotypes 2, 3, and 4; one is at positions 990, 991, 992, and 993 and the second is at positions; 997, 998, 999, 1000, 1001, 1002, 1003, and 1004. One sequence gap at positions 1236, 1237, 1238, 1239, 1240, and 1241 was found in all strains of different genotypes except those of two strains isolated from Russia; (Accession numbers; KJ649741 and KJ649742).

3.4. Phylogenetic analysis

As shown in Fig. 3A, HBoV-4 was used as an outgroup to root the Neighbor-joining phylogenetic tree. The phylogram shows four main clades and each of which represent one HBoV species. All of the five isolates were clustered with HBoV-1. The distances between the taxa in HBoV-1 is low indicating a close relationship between the 5 isolates and other international sequences. While Fig. 3B is the subtree of HBoV-1 indicating the 5 isolates among HBoV-1 strains.

Table 3
Co-infection with other respiratory viruses among HBoV-1 -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 + IAV
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	12 months	Male	Rhino
13	6 months	Male	HCV-OC43

Table 4
Amino acid changes among strains of the HBoV-1 genotype, dots refer to identical amino acids to the consensus and dash refer to the deletion of the codon.

Strain/Site	17	72	113	150	190	211	281	341	348	410	411	549	593
HBoV-1-2005-(DQ000495)	R	D	K	A	R	N	L	A	Y	–	–	N	T
Riyadh-151-2014	K	.	.	T	.	.	.	V	F	–	–	.	.
Riyadh-03-2016	.	.	.	T	G	.	.	.	.	–	–	.	S
Riyadh-22-2016	.	.	.	T	.	.	.	.	.	–	–	H	S
Riyadh-28-2016	K	.	.	T	.	.	.	.	.	–	–	.	.
Riyadh-63-2016	.	.	.	T	.	.	.	.	.	–	–	H	S
HBoV-1-Fukushima (LC720421)	.	.	.	T	.	.	.	.	.	–	–	.	.
HBoV-1-Fukushima (LC720417)	.	.	.	T	.	.	.	.	.	–	–	.	S
CQ201102 (JN387082)	.	.	.	T	.	.	.	.	.	–	–	.	S
CQ201005 (JN387080)	.	.	.	T	.	.	.	.	.	–	–	.	S
HK1 (EF450717)	.	.	.	T	.	.	.	.	.	–	–	.	S
HK2 (EF450718)	.	.	.	T	.	.	R	.	.	–	–	.	S
SH1 (FJ375127)	.	.	.	T	.	Y	.	.	.	–	–	.	S
SH2 (FJ375128)	.	.	R	T	.	.	.	.	.	–	–	.	S
TUN8977 (HQ585888)	.	.	.	T	.	.	.	.	.	–	–	.	.
TW2739-06 (EU984234)	.	.	.	T	.	.	.	.	.	–	–	.	S
TW2835_06 (EU984235)	.	.	.	T	.	.	.	.	.	–	–	.	S
CU2139UK (GU048665)	.	N	.	G	.	.	.	.	L	–	–	.	N
CU47TH (GU048662)	.	N	.	G	.	.	.	.	L	–	–	.	N
CU54TH (GU048663)	.	N	.	G	.	.	.	.	L	–	–	.	N
HBoV2A(FJ973558)	.	N	.	G	.	.	.	.	L	–	–	.	N
KU1 (GQ200737)	.	N	.	G	.	.	.	.	L	–	–	.	N
SH3 (FJ375129)	.	N	.	G	.	.	.	.	L	–	–	.	N
46-BJ07 (HM132056)	.	N	.	G	.	.	.	.	L	–	–	.	N
BRATO-40 (MG953833)	.	N	.	G	.	.	.	.	L	–	–	.	N
BRATO-57 (MG953832)	.	N	.	G	.	.	.	.	L	–	–	.	N
CU2139UK (GU048665)	.	N	.	G	.	.	.	.	L	–	–	.	N
HBoV3 (JN086998)	.	N	.	G	.	.	.	.	L	–	–	.	N
HBoV3-(NC_012564)	.	N	.	G	.	.	.	.	L	–	–	.	N
LZFB199 (KM624026)	.	N	.	G	.	.	.	.	L	–	–	.	N
CMH-S011-11 (KC461233)	.	N	.	G	.	T	.	.	L	N	P	.	N
IM10 (GQ867667)	.	N	.	G	.	.	.	.	L	–	–	.	N
LZFB08 (KM624027)	.	N	.	G	.	T	.	.	L	N	P	.	N
MC8 (GQ867666)	.	N	.	G	.	.	.	.	L	–	–	.	N
RUS_NSC_11-N2655 (KJ649741)	.	N	.	G	.	.	.	.	L	N	P	.	N
RUS_NSC_11-N2657 (KJ649742)	.	N	.	G	.	T	.	.	L	N	P	.	N

3.5. Glycosylation profiles of VP1/VP2 proteins

The glycosylation profiles of this study's HBoV-1 isolates (VP1/VP2) were determined and compared to the international strains of different genotypes. The number of O-linked glycosylation sites in the HBoV-1 genotype varied from 15 to 20 sites in recently detected strains from Fukushima, Japan (G-score 0.5–0.95). Some O-linked glycosylation sites are mostly conserved among the different HBoV genotypes (residues; 111, 118, 127, 131). Some residues are specific to the genotype HBoV-1 and -2 (residues; 133, 142, 149, 150, 154, 156, 442, and 491). Among the HBoV-1 strains, the 5 isolates lack O-lined glycosylation sites at residues 156, 410, 413, 421, 442, 443, 468, and 491). HBoV-4 strains showed fewer O-linked glycosylation sites ranging from 12 in strain Rus-Nsc10-N751 (accession number: JQ964115) to 13 in strain MC8 (accession number: GQ867666). HBoV genotypes other than HBoV-1 have unique glycosylation residues at 141, 155, 407, 473, and 488. On the contrary, only four N-linked glycosylation sites were predicted in the HBoV-1 genotype including our isolates (residues 296, 339, 407, 504, and 638). In other genotypes, the number and location of N-linked glycosylation varies from 2 (residues; 297 and 516) in HBoV-3 to 4 in HBoV-2 (296, 339, 407, and 504) (Fig. 2).

4. Discussion

In the present study, we studied the prevalence and epidemiology of HBoV among children (aged; 0–6 years) in Saudi Arabia, as well as major respiratory viral co-infections of circulating HBoV-1 genotype. Overall, HBoV-1 was detected in 3.44% (4/116) and 11.25% (9/80) of respiratory samples collected during 2014/2015 and 2015/2016, respectively. Of the children infected with HBoV-1, 84.6% were between the ages of 1 and 8 months, and 15.3% were between the ages of 1 and 6 years. An early study published in 2016 found a 1.8% prevalence of HBoV in children with a mean age of 2.1 months [27]. Another prior study found that the frequency of HBoV infection is often higher in children aged 3–6 months [28]. Further, in one study conducted in Al-Taif, Saudi Arabia, HBoV was identified in 22% (18/80), mainly in ages from 2 months to 10 years [22]. Furthermore, according to a recent study, HBoV was found in 10.0% of children aged <2 years old in Ningxia, China [29]. According to a meta-analysis using a random-effects model, the overall prevalence of HBoV in children under the age of two years was 13% [30]. These findings back up prior research that found HBoV in bronchiolitis samples regularly, sometimes as the second or third most prevalent viral agent [31]. In various countries, the prevalence

Fig. 2. Deduced amino acid alignments of complete VP1/VP2 proteins of HBoV-1. Representative strains from different genotypes were selected, and the alignment was done by the Clustal W method running within the MegAlign program (DNAstar). Alignments are shown in comparison with the consensus sequences of the first discovered HBoV-1 isolate (Accession number: DQ000495). Dots represent the identical amino acid residues. Genotype-specific amino acid residues are highlighted in red and predicted O-glycosylation sites are indicated by small filled circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

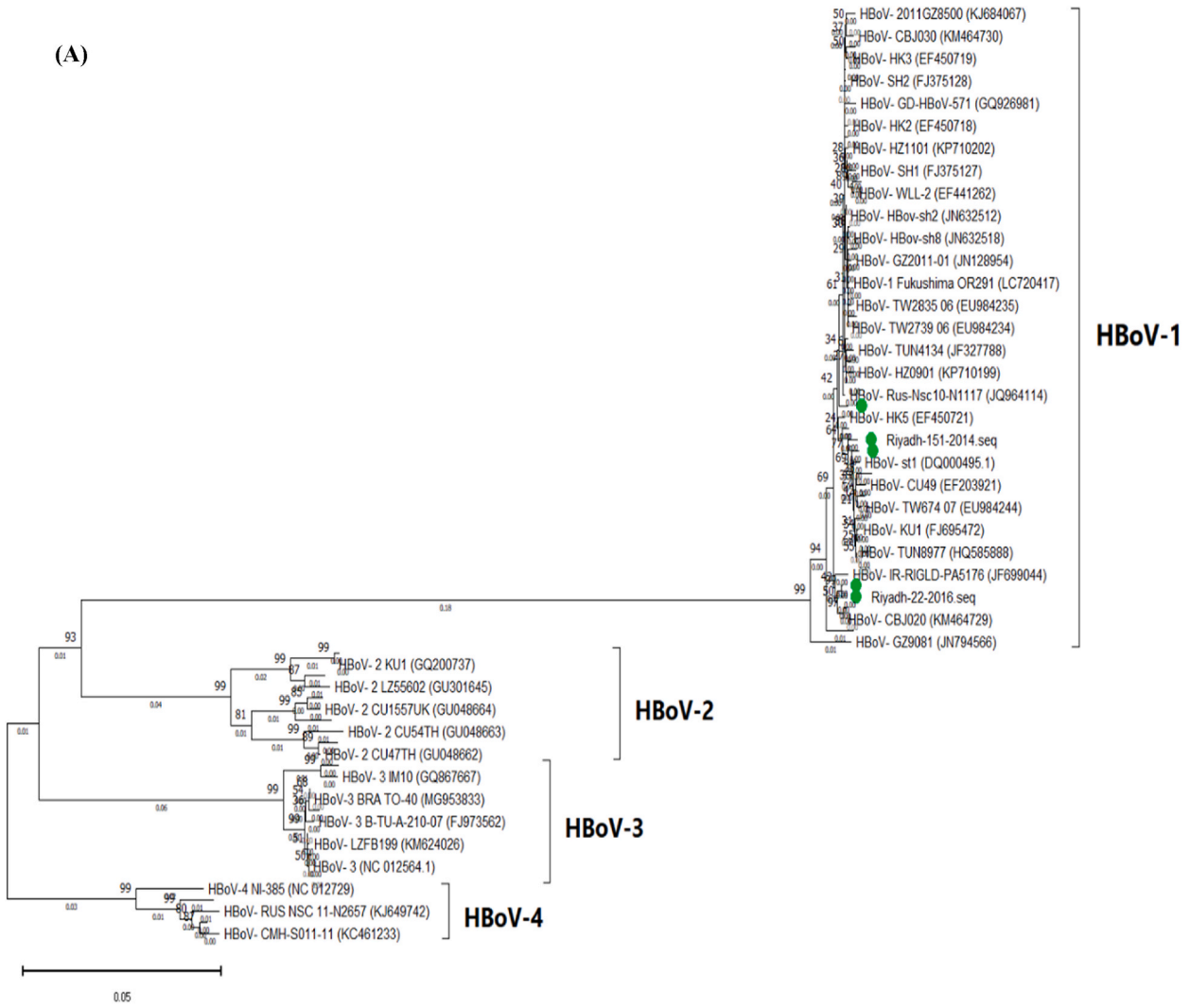

Fig. 3. The Neighbor-joining analysis of the VP1 and VP2 regions of the five HBoV-1 isolates against 72 sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drown to scale with branch lenghts in the same units of the evolutionary distances used to infer the phylogenetic tree. The evloutionary distances were computed using kimura 2-parameter method. (A) shows the distribution of the 5 isolates against all 4 genotypes of HBoV labelled with green circles and (B) is the subtree of HBoV-1 indicating the 5 isolates among this-1 genotypes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of HBoV infection in children with bronchiolitis ranges from 1.8% to 37% [27,32]. Other studies also found that HBoV infection is more common in children under the age of two, and is only rarely found in adults and the elderly [33,34]. Whereas, other studies revealed that the rate of detection of HBoV in ARTI is about 3–19.0% [7,35]. Cangiano et al. (1.8%) in Italy and Macao et al. (37.1%) in Portugal had the lowest and highest HBoV frequencies among the research reviewed here. This suggests that geographic location may be one of the variables contributing to the observed heterogeneity among the evaluated research. In the present study, the overall detection rate of HBoV-1 among hospitalized children due to ARTI was 6.63% which was similar to other countries of the world. Together with several studies from different geographical regions [21,36–38], our results provide supportive evidence for the global circulation of HBoV-1 among children causing ARTI.

Interestingly, we detected HBoV in one case of a 41-day-old child, supporting the possibility that HBoV can infect a very young child. Although HBoV rarely crosses the placental barrier to the fetus, an early investigation reported that HBoV DNA was frequently present in 43 (25%) of pregnant women. Six of the 43 samples were from placenta tissue, 37 were aborted tissue products of conception, and 10 included histologically proven embryonic tissue sections [39]. Further, Kesebir and colleagues reported three newborns (14% of 22) with suspected nosocomial HBoV infection [15]. The infected infants were 1, 4, and 6 months old when their NPAs were collected, and they had been hospitalized from birth. Two of the three patients developed HBoV-positive NPAs within four

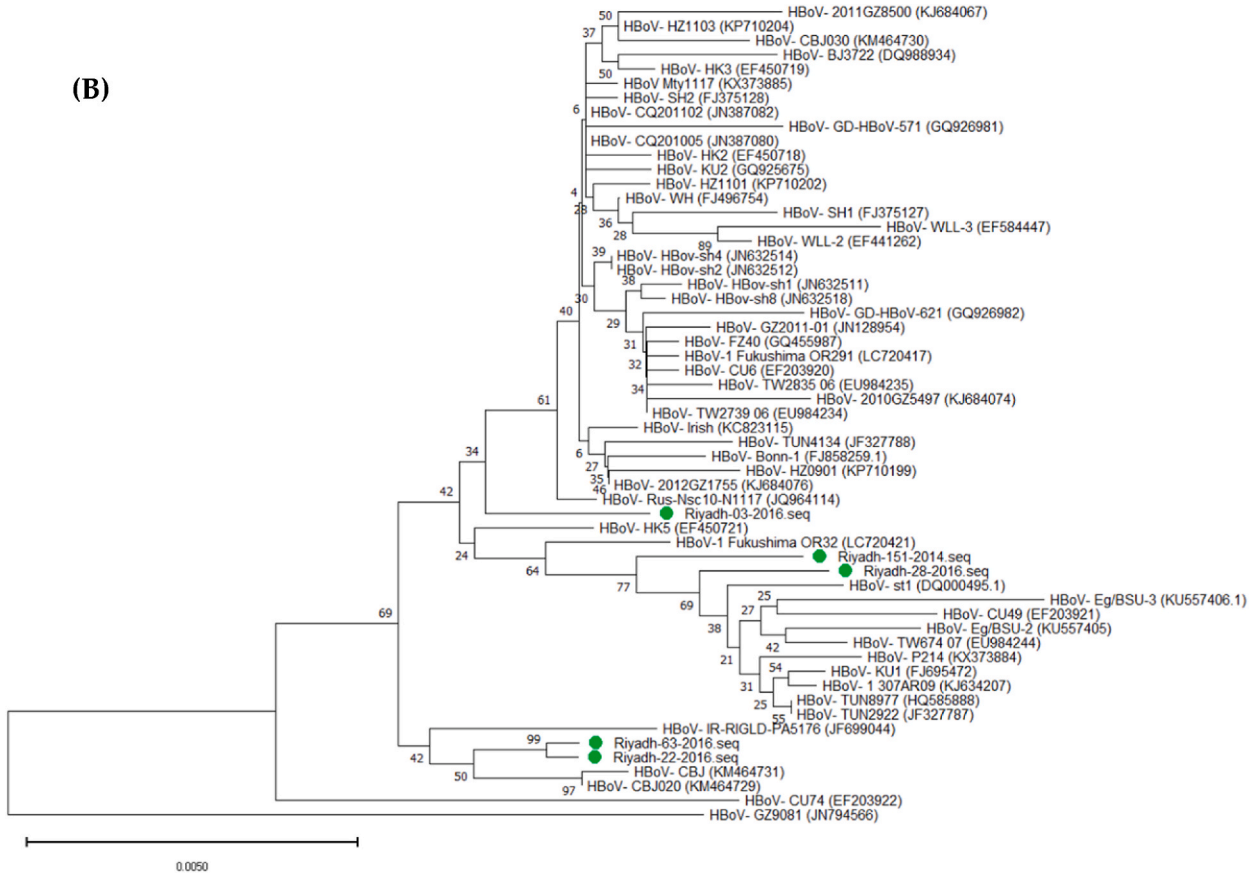

Fig. 3. (*continued*).

days of each other.

HBoV co-infection with other viruses is extremely prevalent [9,40]. Besides, co-infections with one or multiple respiratory viruses were detected in all HBoV-positive samples. Interestingly, all HBoV-1 infected cases were associated with high rates of other respiratory viral co-infections. In HBoV-positive patients, co-infection with HRSV was prevalent (65.6%). HBoV is frequently seen in Iranian children with ARTI or gastrointestinal symptoms, and it is frequently found in co-infections with RSV and rotavirus, respectively [40]. A recent study in Riyadh discovered that HBoV DNA was present in 193 (1.6%) of all patients (11,709) [19]. In this study, co-infections with one or more respiratory viruses were discovered in all HBoV-positive samples. Several studies have found that children infected with HBoV are more likely to be infected with other respiratory viruses [34,41,42]. The higher rate of HBoV co-infection is in line with a higher HBoV co-infection reported from different countries around the world [10,43,44]. In addition, a previous study from Saudi Arabia found only one child diagnosed with HBoV as a single virus entity while most of the children (17/18) showed coinfection with other viral pathogens. The most commonly detected viruses were HRSV (72.2%), IAV (66.66%), Adv (33.33%), and HPIV-3 (5.5%) [38]. Likewise, we found 3.6% of patients co-infected with Rhinovirus, (3.1%) with HRSV, 2.6 % with PIV, 1.5% with Adv, 1.0% with IAV, 0.5% with coronavirus OC43 (HCoV-OC43) and 0.5% with HMPV. Multiple infection consisting of HBoV plus three other viruses (HRSV, IAV, Adv, and HPIV) was detected in one patient. Co-infection is expected to exacerbate the disease outcome. This is attributed to the combination effect of the viruses included in the confection state. The pathogenic role of HBoV-1 as a primary causative agent of respiratory infections remains unknown due to high co-infection rates and prolonged virus shedding. Several studies suggest that HBoV-1 may be connected to severe respiratory disorders. This virus has been reported in children hospitalized for bronchiolitis [45], asthma exacerbations, and first-time wheezing episodes [36,46]. In one prospective investigation that compared 16 respiratory viruses in children admitted with respiratory infection, wheezing was observed in more than half of the children for whom HBoV-1 was identified as the primary cause. Surprisingly, children who had coinfections had less wheezing [47]. A recent study reported that the clinical features of HBoV-1 mono-infections did not differ from those of HBoV-1 co-infections [48].

Although HBoV infection is diagnosed throughout the year, it is most common in the winter and spring [49]. Nonetheless, the seasonal prevalence of HBoV is still a source of conflict, with mounting evidence pointing to increased rates of viral infection during the colder months of the year, particularly January and February [50]. The seasonal peaks of HBoV infections vary among countries and might be affected by climatic, geographic factors, and population enrolled. In this study, we have shown that HBoV was detected year-round without a marked seasonality. A recent study found that HBoV activity peaked in the winter, followed by autumn [29]. In

contrast, the seasonal prevalence of HBoV peaked in the summer. Some studies showed that a high prevalence of HBoV infections occurred in the winter and spring [36,37,51], while other studies showed a high prevalence in the early summer and late spring [36,51,52]. As a result, sampling time may be a factor contributing to study heterogeneity. The seasonality of HBoV infection in Saudi Arabia has not been fully reported. In one investigation, HBoV was found in an Eastern Saudi area in August, October, and December [23]. As a result, sampling time may be a factor contributing to study heterogeneity. This can be a source of bias in these investigations. However, clear seasonal activity was not observed in some studies [53,54]. Nevertheless, seasonal variations of respiratory viruses detected in children with respiratory tract infections were studied. Establishing the national surveillance system is needed to identify a clear picture of HBoV seasonality in Saudi Arabia.

Although few HBoV-1 molecular epidemiology studies have been conducted previously in Saudi Arabia [22,23,55], 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 more HBoV1 isolated from different parts of Saudi Arabia for extended periods is strongly recommended. In the current study, phylogenetic analysis based on VP1/VP2 complete gene sequences, all HBoV isolates in this study were identified as HBoV-1.

At the nucleotide sequence level of the VP1/VP2 gene, the multisequence analysis showed a high sequence identity between strains of each HBoV genotype and high sequence heterogeneity between HBoV-1 and the other 3 HBoV genotypes. This indicates the ability of the VP1/VP3 gene to differentiate between different HBoV genotypes. The few numbers of mutation sites detected among isolates from 2014/2015 and 2015/2016 suggest minor genetic variability among the local circulating HBoV-1 isolates over a short period. This also suggests another way for virus mutation which is thought to be through genetic recombination [56]. Glycoproteins acquire several physicochemical and biological characteristics through the glycosylation process. Such a process affects protein folding, conformational stabilization, resistance to proteolysis, and regulation of intracellular traffic and localization [57]. In addition, glycosylation affects the virus infectivity and antigenicity. For example, glycosylation may mask epitopes and hence enable the virus to escape the preexisting immunity [58]. In our study, several amino acid residues in the VP1/VP2 protein were predicted as potential sites for O-linked glycosylation, and very few N-linked glycosylation sites. In addition, amino acid residues specified for O- and N-glycosylation varied among different HBoV genotypes which could contribute to the virus survival and adaptation in the respiratory tract (HBoV-1) and gastrointestinal tract (HBoV-2, -3, and -4).

The small sample size and cross-sectional nature of this study limited its scope. This study cannot suggest any cause for recurring illnesses. More extensive studies with larger sample sizes encompassing diverse locations of Saudi Arabia over consecutive epidemic seasons are needed to understand the evolution of this virus.

5. Conclusions

In conclusion, over two years (2014–2016), we detected HBoV in 3.44% (4/116) and 11.25% (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 Saudi Arabia. Further epidemiological and molecular characterization of multiple HBoV-1 strains from all regions of Saudi Arabia are required to understand the evolution of this virus.

Ethics approval and consent to participate

The study was conducted by the Declaration of Helsinki and approved by the institutional review board (IRB) of King Khalid University Hospital, Riyadh (Ethics Reference No. 14/4463/IRB 03).

Data availability

All the data is provided.

CRediT authorship contribution statement

Mohamed A. Farrag: Writing – review & editing, Writing – original draft, Software, Methodology, Funding acquisition, Data curation, Conceptualization. **Ibrahim M. Aziz:** Writing – review & editing, Writing – original draft, Software, Methodology. **Asma N. Alsaleh:** Visualization, Validation. **Fahad N. Almajhdi:** Validation, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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