



Human bocavirus in hospitalized children under 5 years with acute respiratory infection, São Paulo, Brazil, 2010

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Abstract

The aims of this study were to investigate the human bocavirus (HBoV) frequency and genotypes in hospitalized children <5 years presenting acute respiratory infections (ARI) within the São Paulo metropolitan area. Nasopharyngeal samples from 300 patients, previously screened for common respiratory viruses, were tested by qPCR for the *NSP1* and *NP-1* genes. The *VPI/2* gene in positive samples was then amplified by PCR and sequenced. A total of 49 positive HBoV cases (16.3%; mean Ct value of 34.41) were detected with the mean age being 18.1 months (range 1 month to 5 years) and the median age being 1 year of age. Children aged between 0 and 12 months had higher detection rates of HBoV (69.4%; 34/49; mean Ct = 34.45) than children from other age groups (30.6%; 15/49; mean Ct = 34.34). No significant differences were observed between HBoV Ct levels and clinical illness. The occurrence was more frequently associated with fall (38.8%; 19/49) and spring (36.7%; 18/49). All 12 sequenced isolates were identified as HBoV-1, displaying minor genetic variation compared to the Swedish reference strains ST1 and ST2 (99.1–99.7% nt). The sole identification of HBoV-1 supports the hypothesis that this particular genotype is strongly related to ARI, and contributes to the role of this virus in the aetiology of respiratory diseases.

Keywords Human bocavirus · Real-time PCR · Respiratory tract infection · Surveillance · Molecular epidemiology

Human Bocavirus (HBoV) (taxonomically classifiable within the family *Parvoviridae*, subfamily *Parvovirinae*, genus *Bocaparvovirus*), are small, non-enveloped, single-stranded, DNA viruses classifiable into four groups HBoV 1–4 [1, 2]. Studies have supported an association of HBoV-1 with respiratory infections, while HBoV-2 to 4 are most commonly found in fecal specimens, indicating a predisposition and association with gastrointestinal diseases [1–5].

HBoV is increasingly being associated with acute respiratory infection (ARI) with unknown aetiology in young children, displaying global distribution and a prevalence varying from 1.5% to 45.7% [6–9]. In Brazil, HBoV has been detected in children with positive rates ranging from 2.4% to 23% [10–15]; however, its role as a causative agent of ARI diseases is questionable due to its concurrent detection with other respiratory pathogens.

The aim of the present study was to investigate the frequency of HBoV in hospitalized children <5 years presenting ARI. This analysis was performed in nasopharyngeal specimens previously screened for the most common respiratory viruses. Genotyping and phylogenetic analysis of identified HBoV strains was also carried out.

This was a retrospective study conducted with a total of 882 nasopharyngeal aspirates collected in 2010 (January–December) from children aged <5 years, who were admitted with ARI in three hospitals (members of a sentinel surveillance network for influenza) located in the São Paulo metropolitan area, state of São Paulo, Southeast, Brazil. Samples were tested in the Respiratory Diseases

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Laboratory at the Adolfo Lutz Institute, a National Influenza Centre accredited by the World Health Organization. The 882 nasopharyngeal specimens were first screened by indirect immunofluorescence assay (IFA) and/or quantitative (reverse transcription-) polymerase chain reaction (qPCR or qRT-PCR) for human respiratory syncytial virus, human rhinovirus, human influenza viruses A and B, human parainfluenza viruses 1, 2 and 3, and human adenoviruses, according to the standard methods recommended by the Centers of Disease Control and Prevention (CDC). This screening, for the most common respiratory viruses associated with ARI, is part of the Brazilian Influenza-like illness Monitoring Program routine and these data are not included in the present study. A total of 300 negative samples, for all the viruses mentioned above, were subsequently tested for the presence of HBoV.

Viral DNA was extracted using a QIAamp®Viral DNA Mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The qPCR for NS1 and NP-1 genes was performed according to a previously described protocol [16]. As a control for PCR inhibitors, and to monitor nucleic acid extraction efficiency, each sample was tested by qRT-PCR for the presence of the human ribonuclease (RNase) P gene following the protocol described by Emery et al. [17]. The threshold (Ct) value from this qPCR was used as a proxy measure of HBoV load. The Ct value is inversely proportional to the amount of virus present in the sample, so the lower the Ct value the higher the viral load [18]. HBoV positive samples were subsequently targeted for amplification, by conventional PCR, of a fragment of the VP1/2 capsid gene, as described elsewhere [19]. PCR amplicons were sequenced using the BigDye™ kit v3.1 (Applied Biosystems, Inc., Foster city, CA, USA) with the same primer pairs used for PCR. Dye-labeled products were sequenced using an ABI 3130 sequencer (Applied Biosystems, Inc., Foster city, CA, USA). Sequencing chromatograms were edited manually using Sequencher 4.7 software. Sequences, including a set of reference HBoV sequences from GenBank, were aligned and analyzed using MEGA software version 6.0 [20]. Neighbor-joining (NJ) trees were constructed based on the Kimura two-parameter substitution model. Accession numbers for the sequences are as follows: KY906239-KY90650.

Of the 300 nasopharyngeal samples tested, 49 specimens (16.3%; 24 males and 25 females) were positive for HBoV by qPCR targeting the NS1 and NP-1 genes (mean Ct value of 34.41). The mean age was 18.1 months (range: 1 month to 5 years) while the median age was 1 year of age. Children aged between 0 and 12 months had higher detection rates of HBoV (69.4%; 34/49; mean Ct = 34.45) than children in the other age groups (30.6%; 15/49; mean Ct = 34.34) (Figure 1). To explore a possible association between the amount of virus detected in a positive sample

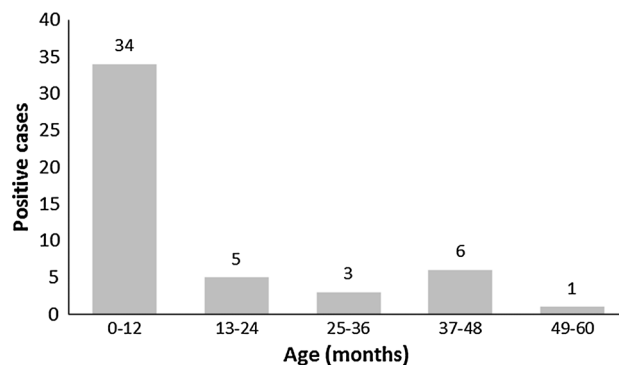


Fig. 1 Age group distribution of human bocavirus positive samples from hospitalized children <5 years of age presenting with ARI in the São Paulo metropolitan area during 2010

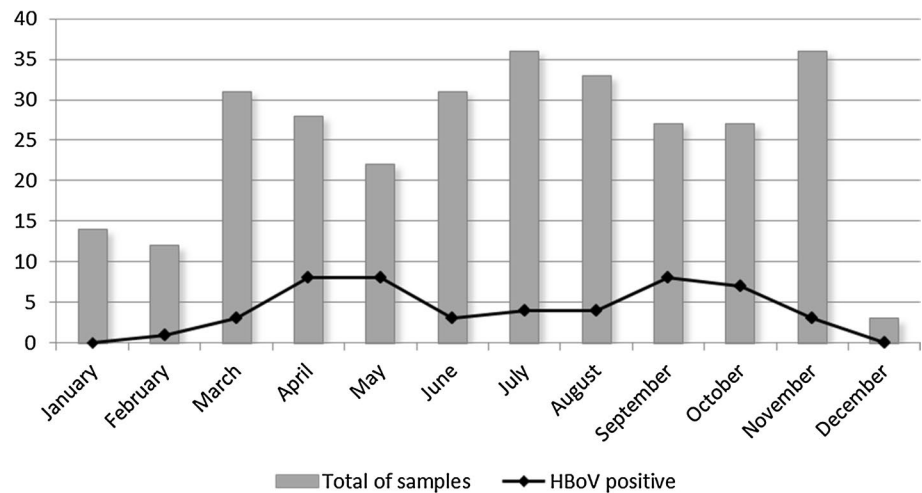
and certain clinical characteristics, an arbitrary Ct value cutoff was used to compare children with higher viral loads (Ct < 35; n=17) and those with lower viral loads (Ct ≥35; n=32). No significant differences were observed between HBoV Ct levels and clinical illness: fever (70.6% low Ct vs. 90.6% high Ct; P = 0.0744), cough (94.1% low Ct vs. 96.9% high Ct; P = 0.6403), sore throat (47.1% low Ct vs. 28.1% high Ct; P = 0.1880), headache (5.9% low Ct vs. 9.4% high Ct; P = 0.6270), runny nose (88.2% low Ct vs. 87.5% high Ct; P = 0.9439), earache (0% low Ct vs. 12.5% high Ct; P = 0.1322), and nasal obstruction (64.7% low Ct vs. 56.2% high Ct; P = 0.5685).

During the study period, HBoV infections in patients with influenza-like illness occurred throughout the year. However, the occurrence of HBoV cases fell more frequently in fall (38.8%; 19/49) and spring (36.7%; 18/49) (March-May and September-November). Despite the small number of cases, it is noteworthy that fewer cases (22.4%; 11/49) were also detected during the winter months in this region (Figure 2).

Targeting the 49 HBoV positive samples, DNA representing the VP1/2 capsid region could be amplified, sequenced and analyzed from 12 samples (24.5%). All 12 sequences clustered with HBoV-1 reference sequences, while HBoV-2, HBoV-3, and HBoV-4 genotypes were not identified. Nucleotide sequences among the 12 isolates showed high identity ranging from 99.3 to 100 % (100% aa), with similar values also observed following comparison with other HBoV-1 strains (99.1–99.7% nt, 99.3–100% aa) identified in Brazil and worldwide. Comparison with the Swedish reference sequences ST1 and ST2, showed nt and aa similarities of 99.1–99.7% and 99.3–100%, respectively (Figure 3).

The HBoV single-infection frequency detected (16.3%) was similar to that observed in another study carried out in the pediatric population (<5 years old) in Northeast Brazil (15.3%, 41/268) [13]. However, the prevalence of single-infections was lower when compared to China (20.2%, 78/386) [9], and higher when compared to other studies in

Fig. 2 Temporal distribution of human bocavirus positive samples from hospitalized children <5 years of age presenting with ARI in the São Paulo metropolitan area during 2010



South Brazil (0.2%, 1/455 and 1.1%, 5/433) [12, 15], Southeast Brazil (2.0%, 5/262) [10], Northeast Brazil (3.0%, 2/66) [11], Mexico (0.4%, 6/1404) [21], Argentina (3.7%, 18/488) [22], and India (6.6%, 20/305) [23]. This observed discrepancy may be explained by differences in the study design, recruitment criteria, settings, diagnostic methods or regional and temporal differences [8, 24]. Our study suggests that HBoV might have an epidemiological impact (16.3% positivity) as a sole causative agent of disease among patients <5 years, since co-infection with the most common respiratory viral pathogens (HRSV, HRV, Flu A, Flu B, HPIV-1 to 3, and HAdV) was previously conducted. The presence of bacterial pathogens, or other unknown human viruses (i.e. coronavirus and enterovirus), can however not be excluded in these HBoV mono-infections. It is important to note that the present study included a limited number of patients from only three health care facilities in a specific area of São Paulo city, and is, therefore, not representative of the entire country, and also does not allow definitive conclusions to be made about the clinical impact of this agent. In order to better understand the epidemiological pattern of HBoV infections and to analyse its clinical relevance, further studies on larger groups of patients using standardised detection methods are necessary.

Any seasonal distribution for HBoV is debatable as it seems to vary according to the geographic area. Some studies have demonstrated that HBoV infections occurred with high prevalence in the winter and spring [25, 26], while other reports indicate prevalence in the late spring and early summer [27, 28]. On the other hand, some authors did not observe any obvious seasonal activity [6]. In Brazil, the seasonality of HBoV infection has been reported to occur in April and early autumn in the Southeast region [10], and in spring and fall in the Northeast region [13], supporting the data described here. Nevertheless, Pilger et al [12] showed that HBoV infection peaks in winter in the Southern region.

HBoV seasonality should be analyzed carefully; associated co-infections may lead to an overlap in seasonality with different respiratory viral pathogens circulating at the same time. Continued surveillance across the whole country is necessary in order to develop a clear understanding on the seasonality of HBoV in Brazil.

In this study, HBoV was found mainly in children <1 year (69.4%), an age at which children still have maternal antibody protection. This phenomenon suggests that maternal antibody against HBoV might not provide complete protection to children during early infancy. Our data is in line with the observation reported in previous studies that infants and toddlers are generally more prone to HBoV infection, with the virus being commonly identified in children below 24–48 months of age [12, 22, 23, 29]. Future studies concerning HBoV antibody seroprevalence in distinct age groups are required in order to shed light on this issue [29]. Data obtained from a comparison of Ct values may be of help in understanding HBoV infection dynamics. Our results suggested that neither viral load nor age range are directly associated to clinical manifestations and disease severity among HBoV infections in children <5 years of age. Nevertheless, a direct relationship between disease severity and HBoV load has been reported in previous studies [30–32]. Unfortunately, our study design and sample size did not allow us to draw conclusions to confirm or refute these findings.

HBoV-1 was the sole genotype detected here. Nonetheless, molecular typing of all HBoV positive samples (n=49) is not available. Thus, the identification of HBoV genotypes, other than HBoV-1, associated with ARI might be lost. Together with previous reports from Asia [9, 23], Europe [7], South America [10], Central America [33] and North America [34], our findings provide further evidence for a global distribution of HBoV-1, and its high association with respiratory infections. The genetic analysis of HBoV-1 strains showed minimal genomic variability (0.3–0.9% nt,

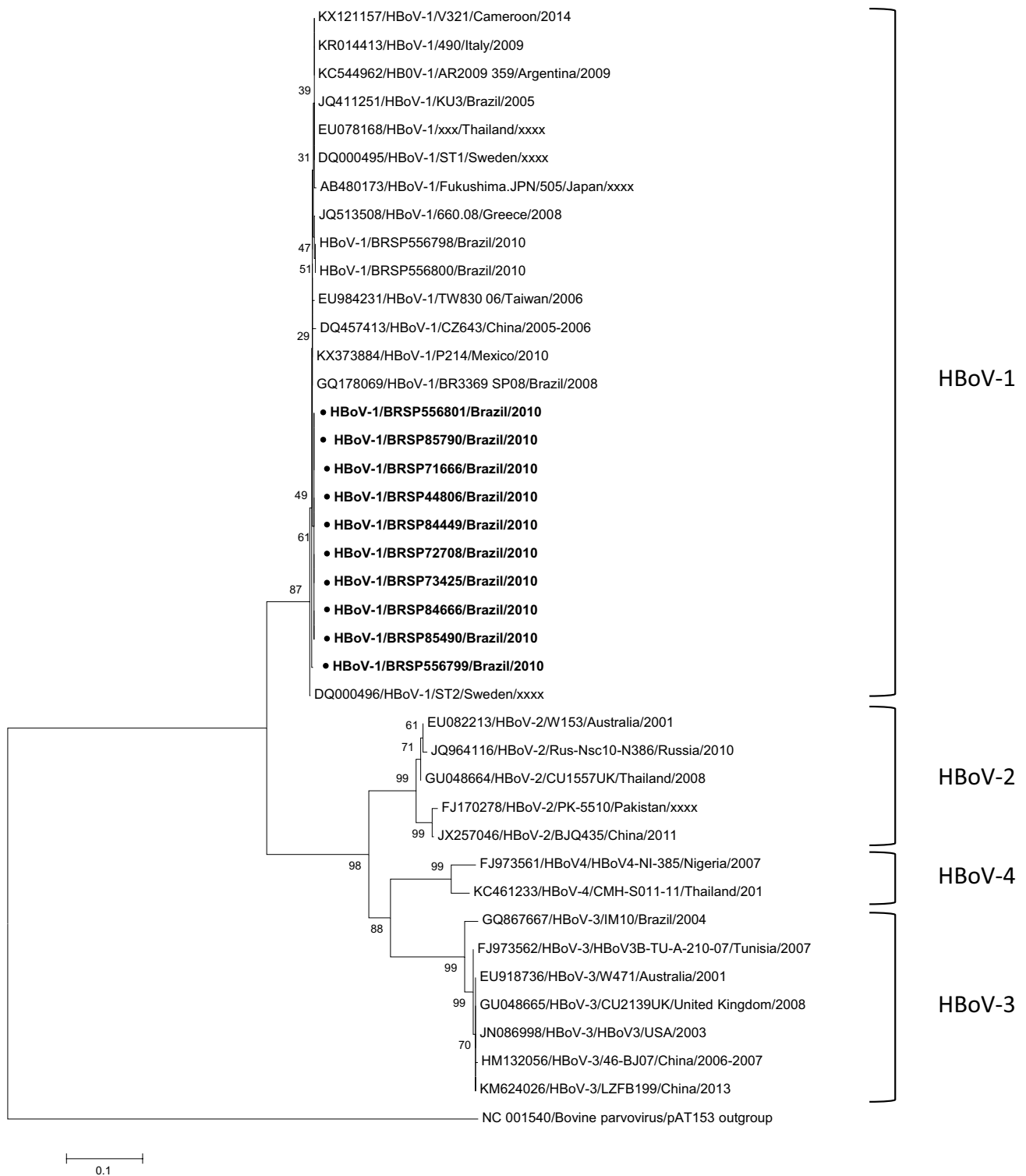


Fig. 3 Nucleotide-sequence-based phylogenetic analysis of the Brazilian HBoV-1 VP1/2 genes (indicated in bold) and other selected human HBoV strains. A neighbor-joining tree of partial VP1/2 nucleotide sequences was generated using MEGA 6.0 software. Reference HBoV strains were obtained from the GenBank database. The acces-

sion number, isolates, country and year of each strain are indicated. HBoV-1 to -4 represents the 4 different and previously described genotypes. The scale indicates the number of divergent nucleotide residues. Bootstrap values are shown as percentages at the branch nodes

0–0.7% aa), similar to data reported previously [2, 35], suggesting minor genetic variability among HBoV-1 strains. It is possible that this low genetic diversity is due to the lack of HBoV-1 sequences available from different regions [33]. Molecular epidemiology of HBoV-1 has been conducted previously in Brazil [10]; however, the viral sequences acquired were rare and insufficient to reveal any temporal or spatial characteristics. Molecular characterization of multiple HBoV-1 strains from Brazil, and other Latin America countries, could help to improve understanding of the evolution of this virus.

In conclusion, this study suggests that HBoV-1 is an important and frequent pathogen in respiratory tract infections in young children, in particular in the metropolitan area of São Paulo, and especially during late spring and summer seasons. In addition, only HBoV-1 was identified, supporting the hypothesis that this particular genotype is strongly associated with respiratory tract infections. Information from this study will contribute to a growing database on the molecular diversity of circulating HBoV-1 worldwide, and provide clues about the role of this virus in the aetiology of respiratory diseases.

Compliance with ethical standards

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Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval Previous ethics committee approval was granted by Adolfo Lutz Institute (CEPIAL) (no. 048/2011). This was an anonymous unlinked study and informed consent was not required according to the resolution 466/12 concerning research involving humans beings -Conselho Nacional de Saúde (CNS)/Ministério da Saúde (MS), Brasília, 2012.

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