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Human Bocavirus genotypes 1 and 2 detected in younger Amazonian children with acute gastroenteritis or respiratory infections, respectively

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ABSTRACT

Objectives: This study aimed to verify the frequency, genotypes, and etiological role of Human Bocavirus (HBoV) in younger Amazonian children with either acute gastroenteritis (AGE) or respiratory infections (ARI). The influence of Rotarix™ vaccination and co-infection status was also investigated.
Design: HBoV quantitative polymerase chain reaction (qPCR) testing was done on both fecal and saliva (1468 samples) from 734 children < 5 months old living in the Amazon (Brazil, Guyana, and Venezuela). High and median HBoV viral load samples were used for extraction, nested PCR amplification, and sequencing for genotyping. HBoV mRNA detection was done by reverse transcription following DNA amplification.
Results: The overall HBoV frequencies were 14.2% (69/485; AGE) and 14.1% (35/249; ARI) ($p = 0.83$). HBoV exclusively infected 4.5% (22/485; AGE) and 4% (10/249) of the Amazonian children (Odds ratios 1.13, 95% confidence interval= 2.42-0.52). HBoV 1 was mainly detected in feces and saliva from AGE children; and HBoV2, from ARI children. HBoV mRNA was detected only in feces. The Rotarix™ vaccination status did not affect the HBoV frequencies.
Conclusions: We suggest that, after entry into the air/oral pathways, HBoV1 continues infecting toward the intestinal tract causing AGE. HBoV2 can be a causative agent of AGE and ARI in younger Amazonian children.
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Introduction

Despite Human Bocaviruses (HBoV) having been discovered from nasopharyngeal samples from children with an acute respiratory infection (ARI) (Allander et al., 2005), they are currently considered to be emerging viruses that could be associated with cases of acute gastroenteritis (AGE) that have occurred after the introduction of the rotavirus A (RVA) vaccines by National Immunization Programs (NIP) worldwide (Qiu et al. 2017). The HBoV contains a single linear DNA genome organized into 3 ORFs, where ORF1 and ORF2 encode the non-structural proteins NS1 and NP1, and ORF3 encodes the VP1 and VP2 capsid proteins. HBoV is classified as genotype 1 through 4 (Guido et al. 2016).

In this study, the presence of HBoV was investigated in feces and saliva from children presenting either AGE or acute respiratory infection (ARI) living in the Amazon region. The objective of this study was to verify the frequency of HBoV in these two groups with different Rotarix™ (RV1) RVA vaccinal statuses, as well as with and without RVA, norovirus and HAdV co-infections, while also identifying the genotypes infecting AGE or ARI children under 5

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months old. The detection of HBoV DNA and messenger RNAs (mRNA) using a quantitative polymerase chain reaction (qPCR) was also performed to provide data and an argument for HBoV1 infecting the host through both oral and respiratory tracts, reaching the gastrointestinal tract and causing AGE. This phenomenon could explain the cases of HBoV1 that cause AGE in Amazonian children.

Methods

Study population

This study was approved by the Federal University of Roraima Ethical Research Committee (CEP No: 1.333.480 from November 23, 2015). A total of 734 children (430 boys and 304 girls) of under five months of age were enrolled in this study and are divided into two groups: 485 presenting AGE and 249 presenting ARI (non-AGE-symptomatic control samples). All children included here were seen at "Hospital da Criança de Santo Antonio" (HCSA) in the city of Roraima (RR-state, Brazil). All children live in Brazil, Venezuela, or Guyana, including demarcated indigenous areas from the Amazon rainforest (Moraes et al., 2019). Fecal and saliva (containing epithelial cells) samples were collected in parallel from each child after an informed consent form was signed by the parents or guardians and processed according to what was previously published (Carvalho-Costa et al., 2019; Moraes et al., 2019;). Fecal samples had already been tested for RVA, GI and GII norovirus and adenovirus (HAdV) (unpublished results). The Rotarix™ (RV1) RVA vaccine was the only vaccine administrated by the Brazilian NIP (Moraes et al., 2019).

The quantitative PCR for human Bocavirus detection in fecal samples and saliva format

A duplex real-time assay was established and formatted for the detection of HBoV and HAdV (HAdV, unpublished results), based on a previously developed qPCR for those viruses (Portes et al., 2017). The method was first tested using samples already previously defined as HBoV, standard curves, and the non-template controls (NTC), available in the RRRL-LVCA, all in duplicate. The specificity of the test was verified comparing the positivity, and the cycle threshold (C_t) values obtained with those already known for the samples tested (Portes et al., 2017). Only sigmoidal curves were considered. The HBoV NTC is named as CI-027 (C_t value 18.02). Primers and probes (FAM-labeled HBoV) used were as previously described by Kantola et al. (2010). To verify unspecific qPCR reactions between HBoV and HAdV, these viruses were tested in a monoplex qPCR format, before proceeding with the duplex format, with parameters defined as described below. The sensitivity of the qPCR was determined using two double-stranded (ds) DNA fragments (gBlock® Gene Fragment, Integrated DNA Technologies, Iowa, USA) designed to contain both viruses' target regions amplified by the primers described above, based on the HBoV1 to -4 genome (VP1-VP2 junction). A 10-fold serial dilution of gBlock® was used to generate a standard curve between log standard concentrations (6×10^7 to 6×10^1 copies per reaction for HAdV and 1×10^6 to 1×10^1 for HBoV) following the formula: Copy number = (DNA amount (ng) $\times$ 6.022 $\times$ 1023 / length (bp) $\times$ 109 216 $\times$ 650). The *Ct* of each standard concentration is already known, making it possible to identify the viral load of each positive sample.

Detection of human Bocavirus in fecal samples and saliva from Amazonian children by quantitative PCR

Total viral nucleic acid from feces was extracted as previously described (Moraes et al., 2019). Five microliters of extracted DNA were used for the duplex qPCR. The reactions were conducted using the QuantiTect® Multiplex PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations in a total volume of 20 μL and with optimized thermal cycling conditions as follows: an enzyme activation step at 95 °C for 15 min, 45 cycles of PCR amplification at 94 °C for one min, and at 60 °C for one min. All qPCR reactions were conducted on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, California, USA). Samples that showed signals crossing the threshold line in both replicas up to a C_t value of 42.00, and presented a characteristic sigmoid curve, were regarded as positives. Samples detected as positives and with a C_t of less than 33 had the total nucleic acids (DNA/RNA) extracted from their respective saliva samples (collected in parallel from the children) via a method described by Moraes et al. (2019) and amplified by qPCR as described above.

Human Bocavirus genotyping by nucleotide sequencing

Preliminary nested PCR was performed according to the C_t values obtained by the duplex qPCR, these being: -Three samples with C_t below 20; - three with C_t between 20 and 30; - three with C_t between 31 and 40. The nested PCR protocol (First and second rounds) applied to the preliminary and final amplification was: Denaturation at 94º for 30 sec. (first round 95 °C during five min), 35 cycles of denaturation at 94º - 15 sec., anneal at 55 °C – 15 sec., and extension at 68 °C – one min. The Platinum® Taq DNA Polymerase High Fidelity enzyme was used according to the manufacturer's recommendations (Invitrogen, Carlsbad, California, USA). Amplicon purification was performed as previously described (Moraes et al., 2019). Sanger nucleotide sequencing was done using primers for the HBoV second round nested PCR as previously described (Chow et al., 2010).

Sequences analysis

For the construction of HBoV phylogenetic trees, VP1 gene nucleotide and amino acid multiple alignments were done using the Mega -Molecular Evolutionary Genetic Analysis Version X software (Kumar et al., 2018; Chow et al., 2010) and the Maximum Likelihood method and Tamura-Nei model (Tamura & Nei, 1993) on results from saliva and fecal samples, and the references sequences obtained from the GenBank database at NCBI for HBoV1, HBoV2, and HBoV3 VP1.

Detection of human Bocavirus messenger RNA in fecal and saliva samples genotyped as HBoV1

Fecal and saliva HBoV1 and HBoV2 were tested for the presence of messenger RNA (mRNA). Total nucleic acid extracted was submitted to enzymatic digestion using amplification grade DNAse I (without RNAse activity) (ThermoFisher Scientific, Massachusetts, EUA), according to the manufacturer's instructions. The samples were submitted to reverse transcription following DNA amplification using a SuperScript® III Platinum® One-Step qRT PCR system (Invitrogen, California, EUA) according to manufacturer instructions and using the same primers for qPCR (Portes et al., 2017). The parameters were: Reverse transcription at 50 °C during 30 min., enzyme activation at 95 °C during ten min., 40 cycles of denaturation at 95º - 15 sec., anneal and extension at 60 °C – one min., ending in a 4 °C holding stage. Samples that were submitted to enzymatic digestion with DNAseI were checked for the presence of residual DNA by qPCR as previously described.

Statistical analysis of data

The Statistica 12.6 (December 2014) software was used for all statistical analyses. The statistical tests applied in this study were Pearson Chi-Square (Differences were considered statistically significant at *P* values greater than 0.05) (Pearson, 1900) and odds ratios (OR) (Szumilas, 2010).

Results

The Bocavirus frequencies were similar for the groups of children either with acute gastroenteritis or respiratory infection

The tests with NTCs confirmed their already known C_t values, demonstrating the specificity of the qPCR for HBoV detection in saliva and fecal samples. The qPCR revealed 104 samples positive for HBoV, 10.2% (31/304) from girls, and 17% (73/430) from boys. The HBoV detection rate was: Guyana 20% (1/5); Venezuela 14.3% (6/42); Brazil, state of Roraima 14.1% (95/673), and the state of Amazonas 14.3% (2/14). Table 1A shows the frequency of HBoV being similar in the groups of AGE and ARI children, at 14.2% (69/485) and 14.1% (35/249) (*p*-value = 0.83) respectively. HBoV infection frequency was evaluated exclusively (without considering co-infection with other viruses) or as a co-infection with HAdV, norovirus, and RVA for either AGE or ARI groups. The exclusive HBoV frequency for the AGE group was 4.5% (22/485) and 4.0% (10/249) for the ARI group. HBoV infection frequency was almost double the ARI frequency (1.6%; 4/249) in the AGE group (3.3%; 16/485), when in co-infection with norovirus. On the other hand, for HAdV and RVA in co-infection with HBoV, lower frequencies were shown for AGE (HAdV = 1.2%, 6/485 and RVA 0%) than for ARI (HAdV = 4.8%; 12/249 and RVA = 0.8%; 2/249) (Table 1B). For each infection profile, with HBoV exclusively, HBoV + RVA, HBoV + norovirus, and HBoV + HAdV, the odds ratio (OR) was calculated and was of 1.13, 0, 2.05 and 0.26, respectively. The confidence interval (CI) of 95% for exclusive HBoV was 2.42 – 0.52 (no-significance 1.9), 0 for HBoV + RVA, 6.19 – 0.52 (no-significance value 5.67) for HBoV + norovirus and 0.70 – 0.09 (significance value of 0.61) for HBoV + HAdV. The status of Rotarix™ (RV1) vaccination was considered, and Table 1C shows that no difference could be detected, considering the frequencies for the AGE group in vaccinated children (15.6%; 30/192) was almost equal for the unvaccinated (12.6%; 37/293). For the ARI group, these frequencies were 14.9% (15/101) and 14.2% (21/148) for the vaccinated and unvaccinated groups, respectively (Table 1C). The frequency of HBoV was high in the Boa Vista municipality, state of Roraima, Brazil, corresponding to 49% (51/104) of the HBoV positive fecal samples. The HBoV frequencies detected in feces from children living in Venezuela and Guyana were lower, 3.8% (4/104), and 1% (1/104), respectively. The C_t values detected ranged from 19.9 to 37.0 for 10.6% (11/104) and from 37.0 to 39.4 for 89.4% (93/104) of the HBoV1 positive fecal samples.

HBoV1 was mainly detected in AGE Amazonian children

Twenty-seven fecal and saliva paired samples were selected for genotyping, defined by C_t values below 20 and between 20 and 30. 63.1% (12/19) of the amplicons obtained from samples of AGE children and 87.5% (7/8) from samples of ARI children were sequenced, and the HBoV genotype was defined with success (Table 2). 68.4% (13/19) of genotyped HBoV were from children without co-infection, and 31.6% (6/19) were from children co-infected with HAdV (2 AGE children and 4 ARI children) (Table 2). The HBoV1 genotype was predominantly detected in fecal and saliva samples from AGE children, with 75% (9/12) detection in feces and 100% (11/11) in saliva. The HBoV2 genotype was detected in 25% (3/12) of the remaining fecal samples from AGE children. The HBoV2 genotypes were predominant and detected in 71.4% (5/7) of samples from ARI children. Only one sample of the HBoV3 and HBoV1 genotypes was detected in children presenting ARI, the detection rate was 14.3% (1/7) for each one. Table 2 summarizes these results.

Table 1
Frequency of Human Bocavirus (HBoV) detected in the Amazon region in two different groups of children, these being acute gastroenteritis (AGE) and acute respiratory infection (ARI): **A.** HBoV positivity without considering HBoV with other enteropathogens; **B.** HBoV co-infection with norovirus and human adenovirus (HAdV).

A

Group of children (734)	HBoV positivity 104 (%)	p^* value
AGE (485)	69 (14.2%)	p = 0.83171
ARI (249)	35 (14.1%)	

Note:* - The value of significance (p-value) was found comparing both groups of children presenting their clinical symptoms.

B

Virus (es) detection rate	Groups of children n = 734 (100%)		
	AGE n = 485 (100%)	**ARI n = 249 (100%)**	**OR**
HBoV only	22 (4.5%)	10 (4.0%)	1.13
HBoV + RVA	0 (0.0%)	2 (0.8%)	0
HBoV + norovirus	16 (3.3%)	4 (1.6%)	2.05
HBoV + HAdV	6 (1.2%)	12 (4.8%)	0.26

Note: * - The odds ratio (OR) was calculated for each infection profile, these being: HBoV exclusively (1.13); HBoV + RVA (0.0); HBoV + norovirus (2.05); HBoV+HAdV (0.26). The confidence interval (CI) of 95% for HBoV alone was 2.42 – 0.52 (no-significance 1.9), 0 for co-infection by RVA and HBoV, 6.19 – 0.67 (no-significance value 5.67) for co-infection by HBoV and norovirus and, for co-infection by HBoV and HAdV, 0.70 – 0.09 (significance value of 0.61).

C

Groups of children (n = 734)	HBoV positivity and vaccination status	
AGE n = 485	**Vaccinated n = 192** 30 (15,6%) p * = 0, 35218	**Unvaccinated n= 293** 37(12,6%)
ARI n = 249	**Vaccinated n = 101** 15 (14,9%) p = 0, 88412	**Unvaccinated n= 148** 21 (14,2%)

Note: * The Pearson Chi-Square [19] test was applied to define the level of marginal significance (Valor p) in comparison to the positive and negative HBoV results found in each AGE and ARI group.

Table 2
HBoV genotype defined by sequencing nucleotides from DNA amplicons obtained from fecal and saliva samples. The samples also had their DNA digested and were submitted to mRNA amplification. **A.** Group of children presenting acute gastroenteritis (AGE); **B.** Group of children presenting acute respiratory infection (ARI).

Child identification	HBoV genotype	
	Feces	Saliva
A		
Child 1	HBoV1	HBoV1
Child 2	HBoV1	HBoV1
Child 3	*	HBoV1
Child 4	HBoV2	*
Child 5	HBoV1	HBoV1
Child 6	HBoV1	HBoV1
Child 7	HBoV1	HBoV1
Child 8	HBoV1	HBoV1
Child 9	HBoV2	HBoV1
Child 10	HBoV1	HBoV1
Child 11	HBoV1	HBoV1
Child 12	HBoV1	HBoV1
B		
Child 13	HBoV3	*
Child 14	HBoV2	*
Child 15	HBoV2	HBoV1
Child 16	HBoV2	*
Child 17	HBoV2	HBoV1
Child 18	HBoV1	HBoV1
Child 19	HBoV2	HBoV2

Notes: * - The DNA amplicons obtained from these saliva samples was not of good enough quality to provide comprehensive nucleotide sequences.
Bold and underlined samples had their mRNA amplified.
AGE children 4, 11, and ARI children 13, 15, 18, and 19 were HAdV co-infected presenting Ct values of 31.8, 40.2, 36.53, 36.12, 37.15, and 42.91, respectively.

A phylogenetic tree (Fig. 1A) shows the evolutionary proximity between HBoV1, HBoV2, and HBoV3 detected in feces and saliva and others that have been identified in children living in the Amazon region (states of Acre, Amazonas, Pará, Roraima, and Tocantins), Rio de Janeiro and Goiás. HBoV1 nucleotide sequences detected from the AGE children 1, 5, 6, 7, 8, 10, 11, 12 share equal similarities between them, regardless of whether they were recovered from fecal or saliva samples. Children 18 and 19, from the ARI group and genotyped respectively as HBoV1 and HBoV2, also share equal similarity between fecal and saliva samples. All these children were clustered in the same clade in the phylogenetic tree, together with reference sequences from the Amazon region and other Brazilian states (Rio de Janeiro, Goiás), except for nucleotide sequences recovered from child 12 (feces and saliva) clustered in a separated clade and with a bootstrap of 85%. HBoV3 nucleotide sequences were recovered from the feces of ARI child 13 living in Mucajaí (Fig. 1B) and presented a 99% similarity with the reference sequence MH 003678.1 from the Amazonas state (Fig. 1A and B). The quality of the DNA recovered from child 13's saliva was not adequate for defining the HBoV genotype. The nucleotide sequence from child 19 shares equal similarity between fecal and saliva samples and was HBoV2. The nucleotide sequence recovered from the feces of children 9, 15, and 17 were similarly HBoV2; however, these sequences recovered from saliva were similar to HBoV1. The DNA recovered from feces of children 4, 14, and 16 were similar to HBoV2, and the saliva from these children did not provide nucleotide sequences adequate enough to determine the HBoV genotype. The HBoV2 genotyped samples from children enrolled in this study were clustered together with reference sequences from the states of Goias, Rio de Janeiro (child 9), Tocantins, and Amazonas (child 15). The remaining (children 4, 14, 16, 17, and 19) were clustered in a separate clade.

Messenger RNA was detected only in feces but not in saliva

Successfully genotyped HBoV positive fecal and saliva samples were used to verify the presence of mRNA (Table 2). The detection rate of mRNA was 21.0% (4/19) and only on fecal samples. Messenger RNA was not detected in any saliva samples. Three HBoV1 mRNA positive samples were from AGE children 5, 11, and 12; one HBoV2 mRNA positive sample was from ARI child 19. Children 11 and 19 were HAdV co-infected (Table 2).

Discussion

The similar frequency detected of 14% both for the AGE and ARI groups of children could be explained by HBoV having been associated with both the clinical conditions (Qiu et al. 2017). The global HBoV prevalence in both children and adults was estimated from 2005 to 2016 (Guido et al., 2016), including studies evaluating AGE and ARI, for HBoV exclusively: 5.9% (95% CI: 5.7-6.1) for AGE cases and 6.3% (95% CI: 6.2-6.4) for ARI cases. This prevalence was very similar to our study. For co-infections, the prevalence was of 46.7% (95% CI: 44.2-49.2) for AGE cases, and ranged from 8.3% (95% CI: 0.0-19.4) to 100% for ARI cases. These authors also presented the prevalence for Brazil: 4.8% (95% CI: 4.0-5.7; HBoV exclusively) and 15.1% (95% CI: 8.7-21.6; co-infection). For ARI cases, 10.8% (95% CI: 9.6-11.9; HBoV exclusively) and 90.1% (95% CI: 86.7-93.5; co-infection). In our study, the frequency was similar to that reported for Brazil in 2016, considering HBoV infection exclusively or in co-infection in AGE children. Otherwise, the frequency reported for ARI children in the 2016 study (Guido et al., 2016) was very different from that detected in our study, considering either HBoV exclusively or in co-infection, as we detected low frequencies ranging from 0.8% to 4.0% at maximum, even considering the co-infection. The number of samples collected in one year from Roraima, Brazil was very representative (n = 673), and children presenting AGE living in Roraima state were mainly HBoV infected rather than presenting ARI (AGE 59.6%; 62/104; ARI 40.4%; 42/104).

Recently Soares et al. (2019) reported an HBoV detection rate of 24.0 % (54/225) in samples from AGE children under 5 years old living in Brazil in the Amazon region, where most of the samples were collected from children living in the states of Acre, Amazonas, and Para, and only one sample was from Roraima.

In our study, RVA and norovirus co-infections could be excluded from considering HBoV as the causal viral agent of AGE. RVA and noroviruses are the two primary etiological agents of acute gastroenteritis (AGE) in children under 5 years old (Tate et al., 2016; Pires et al., 2015; Ahmed et al., 2014). HAdV is a significant viral agent of ARI (Ison and Hayden, 2016), and could be excluded for the ARI group, for HBoV to be an eligible causal viral agent of ARI. 4.5% (22/485) of AGE children were infected only by HBoV, and 3.3% (15/435) were infected by both HBoV and norovirus. 4.0% (10/249) of ARI children were infected only by HBoV, and 4.8% (12/249) were infected by both HBoV and HAdV. The similar frequencies detected in both AGE and ARI for HBoV exclusively give this virus the status of a viral causal agent for both infections, but of low frequency in the Amazon region. Concerning HBoV co-infection with RVA, no HBoV positive fecal sample was detected in the AGE group of children. In Brazil, HBoV and RVA co-infection causing AGE was detected in higher frequencies just after the introduction of the RV1 vaccine, with rates of 64.3% (9/14) (Sousa et al., 2012) and 21.4% (3/14) (Albuquerque et al., 2007). In 2017, Portes et al. (2017) conducted a study involving 200 HIV-1 and 125 non-HIV positive AGE children, and the frequency of HBoV and RVA co-infection was low, corresponding to 1.5% and 0.8% for the two groups, respectively. The RV1 vaccinal status can affect the frequencies of viruses other than RVA, as what happened with norovirus (Ahmed et al., 2014). However, for the children enrolled

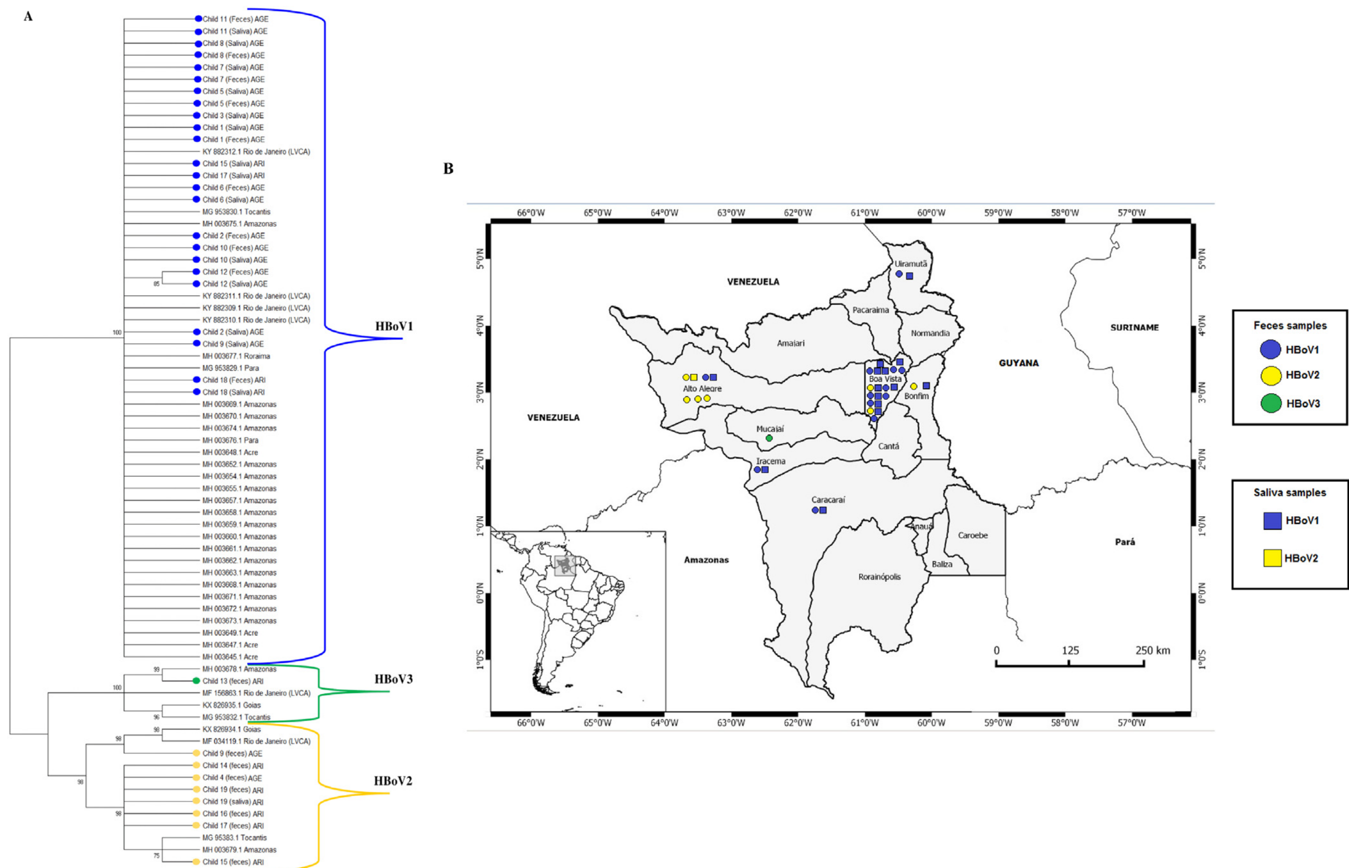

Fig. 1. A. Phylogenetic tree encompassing the genotyped Bocavirus 1 (HBoV1), 2 (HBoV2), and 3 (HBoV3) detected in feces and saliva samples from Amazonian children. Each child was identified by numbers. References GenBank samples were included and accessed according to their reference numbers. The analysis was inferred by using the Maximum Likelihood method and the Tamura-Nei model [1]. All bootstrap-calculated values were above 70%, as estimated with 2,000 pseudo-replicate data sets at each node. **B.** Map of the state of Roraima showing the municipalities where the children whose samples were collected live, as well as the Brazilian states and countries with which it borders. Adapted from Landscape Metrics Lab, Department of Geography - Federal University of Roraima (http://ufrr.br/mepa/). The HBoV 1, 2, and 3 are represented by colored circles or squares, respectively, if they were detected in the feces or saliva.

in this study, the RV1 vaccinal status did not affect the HBoV detection rates because the frequencies were similar for the AGE and ARI groups in vaccinated and unvaccinated groups. Studies have shown the presence of HBoV in association with other potential pathogens for both AGE and ARI children, which led to the hypothesis that the virus may be a "harmless passenger" (Schildgen et al., 2008). In our study, the detection rate of HBoV was also low, excluding co-infections with norovirus. The C_t criterion up to 30.0 selected mainly HBoV positive fecal samples without co-infection.

In our study, the HBoV1 genotype was predominantly detected in feces and saliva samples from AGE children, with 75% (9/12) detected in feces and 100% (11/11) in saliva. The HBoV2 genotype was detected in 25% (3/12) of the remaining fecal samples from AGE children. The HBoV2 genotypes were predominant and detected in 71.4% (5/7) of samples from ARI children. The prevalence of HBoV1 under HBoV2 (respectively 94.8% and 2.6%) from AGE samples was also reported by Soares et al. (2019). Unfortunately, scarce data concerning HBoV genotype frequency is available for Brazil. HBoV1 is mainly associated with ARI, despite having also been detected in samples from AGE children (Guido et al., 2016; Schildgen, 2013; Chow & Esper, 2009). HBoV2 and HBoV3 are rarely detected in respiratory secretions, but frequently so in fecal samples from children with AGE (Paloniemi et al., 2014; Chow et al., 2010; Arthur et al., 2009). HBoV4, although rarer, has already been found in fecal samples (Kapoor et al., 2010, 2009).

Since samples from ARI children served as non-AGE children-symptomatic control samples, we could infer that HBoV1 is a causative agent of AGE since we detected mRNA in fecal samples only from AGE children that were infected only by this agent without co-infection (two samples).

HBoV1 and HBoV2 mRNA (one from AGE and one from ARI children respectively) were detected in two HBoV and HAdV co-infected fecal samples; however, the C_t values were very high for HAdV (40.2 and 42.91, respectively). The C_t values that reflect the viral load could be very complicated for HBoV detection and association of this agent as causative of disease. The HBoV viral load usually is low and also could persist on the tissues such as the nasopharynx and tonsils (Ivaska et al., 2019), not reflecting real infection. The HBoV1 infection cannot be diagnosed with standard DNA PCR; quantitative PCR and serology are better diagnostic approaches. Because of their high clinical specificity, HBoV1 mRNA, and antigen detections have shown promising results (Christensen et al., 2019).

The phylogenetic analysis showed, for most of the HBoV nucleotide sequences, a similarity between HBoV genomes detected from feces and saliva, reflecting both the persistence of this virus and its probable migration from the respiratory to the intestinal tract. This similarity also indicates that even for ARI children, there is shedding towards the feces. Children living in the Amazon region mainly in the state of Roraima have been infected by the same HBoV1, HBoV2, and HBoV3 that are circulating in other Amazon regions, because the nucleotide sequencing showed more

similarity to samples from the Amazon region than samples from Rio de Janeiro and Goias. However, the HBoV1 recovered from child 12 was notably different (bootstrap of 85).

An explanation for the detection of HBoV1 causing AGE more often than ARI could be that there is no physical separation of children presenting either AGE or ARI at HCSA. This condition could benefit this genotype in infecting the intestinal tract.

The detected genotypes HBoV1 and HBoV2 were mainly from children living respectively in Boa Vista (the capital of Roraima) and Alto Alegre (indigenous rainforest). There are a lot of factors contributing to the diversity of HBoV samples detected infecting children living in the Amazon region from families presenting a strong indigenous ethnicity, including genetic and cultural aspects.

Conclusions

The similar HBoV frequencies detected either in AGE or ARI children from the Amazon region show that the genotype 1 is probably the causative agent of AGE, infecting children eventually by the oral or air pathways towards to the intestinal tract, being eliminated in feces (HBoV1 mRNA detection). Despite HBoV2 having been frequently associated with AGE, in our study, this genotype could be an etiological viral agent causative of ARI in children < 5 months old.

Conflict of interest

The authors are unaware of any conflict of interest.

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