Original Article

Molecular detection and phylogenetic analysis of Kenyan human bocavirus isolates

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Abstract

Introduction: The commonly expected causative agents associated with flu-like symptoms in Kenya are the classical viral pathogens identifiable as influenza virus, adenovirus, parainfluenza virus, enteroviruses, respiratory syncytial virus (RSV) and rhinovirus. However, newer agents have been identified globally that present with illnesses clinically indistinguishable from those caused by the classical pathogens; one of them is human bocavirus.

Methodology: A total of 384 specimens were analyzed, primarily to determine if the emerging human bocavirus (HBoV) infections exist in Kenya as coinfections with other respiratory viruses and to describe the genotype of the virus in circulation. In brief, viral nucleic acids were extracted from culture supernatants, amplified by PCR, and sequenced.

Results: HBoV DNA was amplified from 1.8% of screened specimens. Coinfection with parainfluenza virus, adenovirus, and enterovirus was 2.5%, 2%, and 1.4%, respectively. Multiple coinfections consisting of HBoV plus two other viruses were found in 3% of specimens. Isolation occurred in the months of January, March, April, August, and November. Retrospective review of clinical parameters indicated that all the individuals complained of non-specific symptoms, mainly fever, coughs, nasal stuffiness, runny noses, and vomiting. Phylogenetically, the GenBank deposited sequences of this study's isolates cluster closely to the reference strain NC_07455 (HBoV1).

Conclusion: Coinfections with human bocavirus (HBoV1) occur in Kenya, and high incidence might primarily be during the early stages of children's lives.

Key words: human bocavirus; Kenya isolates; respiratory viral infections

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Introduction

Viral upper respiratory infections (URTIs) are frequent and have a significant global health impact, causing between six and nine infection episodes per year in children and between two and four infections per year in adults[1]. Most of these infections are caused by the influenza virus, adenovirus, rhinovirus and parainfluenza viruses. However, new evidence suggests that, occasionally, URTIs are also caused by or may be associated with newer emerging pathogens that include human bocavirus, metapneumovirus, and human coronaviruses (HCoV-229E, HCoV-OC43, SARs-CoV, HCoV-KU1**,** and HCoV-NL63) [2].

Human bocavirus(HBoV) is a novel viral agent that was discovered about seven years ago by Allander *et al.* [3]. It belongs to the family *Parvoviridae* and is structurally similar to the other parvoviruses [4], but molecularly differentiable by the encoded nuclear phosphoprotein (NP1), whose function(s) is unknown [5]. The virus contains a single linear positive-sense and negative-sense single-stranded deoxyribonucleic acid (DNA). Currently, bocaviruses can be classified based on their genetic variability into species 1 to 4 [6]. Initially, only two genotypes were described in the literature as ST1 and ST2 [7]. Accumulating data suggests that the formation of other HBoV variants is likely through recombination events among the species [8,9]. The species HBoV1, which is the focus of this study, is predominantly a respiratory virus, while the rest of the species seem to be more enteric pathogens [10].

A historical review of most survey activities on viral respiratory tract infections in Kenya [11,12] indicates that the focus is always on identifying the classical common viral pathogens (influenza virus, adenovirus, rhinovirus, RSV,andparainfluenza virus).

From these surveys, influenza viruses account for 2% of the cases, adenovirus for 3.6%, RSV for 22.1%, rhinoviruses for 12.2%, herpes simplex virus (HSV) for 13.3%, and parainfluenza virus for 4.3%. Hence, there are few or no studies (especially surveillance programs) specifically examining the presence of newer pathogens associated with upper respiratory infections (especially human bocavirus, metapneumovirus, and human coronaviruses) in East Africa.

The objective of this investigation was to fill in the gap of information about such emerging viral pathogens by isolating and determining the genotype of Kenyan isolates of human bocavirus (HBoV) based on the viral capsid protein gene (VP 1/2).Our focus was on archived supernatants collected from cell cultures that had been exposed to clinical specimens of subjects suspected of having viral acute respiratory infections.

Methodology

Ethics and study design

This study was within the limits of a descriptive retrospective cross-sectional design and was approved by the Kenyatta National Hospital/University of Nairobi Ethical Review Committee (KNH/UON ERC) and assigned project number P85/3/2010.

Specimens

A total of 384 samples collected from six regions of Kenya (Kisumu, Busia, Nairobi, Mombasa, Kericho, and Kisii) were analyzed. The nasopharyngeal swab specimens were collected from individuals presenting with influenza-like illnesses between January 2007 and June 2009. These specimens were confirmed by immunofluorescence assay to be positive for HSV type 1, RSV, parainfluenza virus, adenovirus, and enterovirus. The clinical specimens were inoculated into the LLC-MK2, HEp-2, rhabdomyosarcoma (RD), and MDCK cell lines and the supernatant was collected by centrifugation within 48 hours after primary inoculation. The processed supernatant was stored at -80^{o}C in 1 mL aliquots containing a mixture of viral transport media and culture media containing Dubelcco's modified eagle medium supplemented with 2% fetal bovine serum and 1% antibiotic.

Clinical parameters

Demographic data, history of illness, and laboratory studies were examined retrospectively from the clinical parameters documented in the database. Cross-tabulation of proportions and frequencies were analyzed by SPSS version 13.0 (SPSS Inc., USA) and EPI info (CDC, Atlanta, USA).

DNA extraction and polymerase chain reaction (PCR)

DNA was extracted from 100-150 µL of the samples using Qiagen QIAmp DNA blood mini kit (Qiagen, Hilden, Germany). Primers used in a semi-nested PCR for HBoV screening originated from a published report by Smuts and Hardie [7], and were designed for the NP1andVP1/VP2 genes (Table 1). These primers are expected to amplify a 368 bp product of NP1 gene, which is confirmed by amplifying a 980 bp product of the VP1/VP2 gene. In brief, a 50 µL PCR reaction was set up containing 1X PCR buffer, 0.2 mM of each primer, 0.2 mM of dNTPs mix, 1.5 mM $MgCl_2$, 2.0 units of Taq polymerase, and 10 µL of DNA. Cycling profile included an initial denaturing at 94°C (10 minutes), followed by 40 cycles of denaturing at 94°C (30 seconds), annealing at 55°C (30 seconds), 72°C (1 minute), a final extension at 72°C (5 minutes), and a hold at 4°C. Two microliters of the primary PCR product were used as a template in separate reaction tubes in the secondary PCR under the same cycling conditions. Ten-µL aliquots of amplicons were loaded on gels and electrophoresis performed in1.5 % (w/v) agarose containing 2 µL of ethidium bromide. The gels were run at 100 mV-150 mV in 1X Tris Borate EDTA buffer pH 8.0. Two markers, a standard 100 bp and 1 Kb DNA ladder (Fermentas, Burlington, Canada) were used as size references, and gels were

Table 1. Oligonucleotide primers for PCR amplification and sequencing of HBoV targeting the viral capsid protein (VP1/2) and nucleoprotein (NP1)

Gene	Forward	Reverse
VP1/2 outer	**VP-A** (5'GCA CTT CTG TAT CAG ATG CCT T-3)	**VP-B** (5'CGT GGT ATG TAG GCG TGT AG-3')
VP1/2 inner	**VP-C** (5'CTT AGA ACT GGT GAG AGC ACT G-3')	**VP-B** (5'CGT GGT ATG TAG GCG TGT AG-3')
NP-1 outer	**NP-A** (5'TAA CTG CTC CAG CAA GTC CTC CA-3')	**NP-B** (5'-GAA GCT CTG TGT TGA CTG AAT-3')
NP-1 inner	**NP-C** (5'CTC ACC TGC GAG CTC TGT AAG TA-3')	**NP-B** (5'-GAA GCT CTG TGT TGAC TGA AT-3')

Adapted from Smuts and Hardie (2006)

photographed on a UVP trans-illuminator. For the NP1 and VP1/2 genes, a positive PCR was one that displayed amplicon of just under 1000 bp and 400 bp, respectively. Figure 1 shows the PCR product representing both the NP1 and the VP1/2 genes. The later fragment was subjected to sequencing as indicated below; this is the most variable region, so it is highly informative for diversity studies.

Amplicons concentration and purity

The 960 base amplicons for sequencing were purified using the Qiagen QIAquick PCR (Qiagen, Hilden, Germany) purification kit from gels or solutions depending on the level of purity. The concentration of DNA was determined spectrophotometrically using a Thermo Scientific NanoDrop 2000/2000c spectrophotometer (NanoDrop Technologies, Wilmington, USA) on 0.5-2.0 µL of the samples against blanked water controls. The ratio of absorbance at 260 and 280 nm was used to assess the purity of DNA. A ratio of ~1.8 and above was generally accepted as pure for DNA. The eluted DNA was stored at -20°C until analyzed.

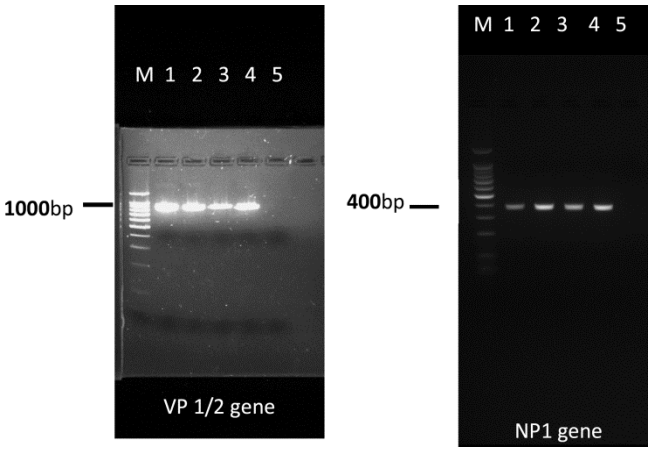
Sequencing

Cycle sequence was performed according to the protocol specified for the Gene Amp 9600 themocycler (Applied Biosytems, Darmstadt, Germany). The primers used for sequencing were similar to those used for the second round of amplification of the VP gene. Excess deoxy terminator was removed from the DNA sequencing products with Centri–Sep™ columns. The purified products were dried in a vacuum centrifuge for 10-15 minutes at -60°C and stored at -20°C. In brief, automated sequencing was performed on the sense and anti-sense strands of HBoV DNA on an ABI Prism 3100 Genetic analyzer version 2 (Applied Biosytems, Darmstadt, Germany) using the ABI Prism BigDyeTerminator cycle ready reaction kit version 2.0 as recommended by the manufacturer. The raw data sequence was analyzed with sequencer software version 4.0.5.

Phylogenetic analysis

The raw ABI sequences of the VP1/2 region were cleaned by trimming off the low quality bases at the end of the chromatogram and assembled into a contig. Base pair errors were corrected using the DNA baser version 2.91.5 sequence assembly software (Heracle Software, Bremen, Germany)**.** The Kenyan isolates were preliminarily compared to known reference sequences of bocavirus HBoV1, HBoV2, HBoV3, HBoV4, parvovirus isolates (canine minute virus and bovine parvovirus), and a few other selected sequences of the virus isolates from South Africa (ZA190/04, ZA2591/04), China (HK5), Europe (Bonn-1), and America (CRD2) via BLASTn [13]. The comparison sequences had accession numbers DQ317556.1 (ZA 2591/04), bovine parvovirus (NC_001540.1), canine minute virus (NC_004442.1), EF450721.1 HK5, NC_014358.1 (bocavirus gorilla), NC_007455.1 (human bocavirus 1), NC_012042.1 (human bocavirus 2), NC_012564.1 (human bocavirus 3), NC_012729.2 (human bocavirus 4), FJ858259.1 (Bonn-1) and (DQ340570.1) (CRD2). The sequences were downloaded as Fasta files from GenBank database and were aligned together with five of this study's sequences labeled KE-4S9/04, KE-9N7/11, KE-10N8/3, KE-48G7/8 and KE-23A8/11 in ClustalW using multiple alignment algorithms. Phylograms were constructed with Mega version 4 [14] using Kimura two-distance-based parameters via neighbor-joining analysis of the amino acid arrangement generated from 1000 bootstrap replicates.

Figure 1. A representative agarose gel (1.5 % [w/v]) showing PCR results of NP1 and VP1.



Lane M (100 bp ladder markers). Lanes 1 and 2 are VP gene PCR products of 980bp, while lanes 3 and 4 show NP1 gene PCR products of 398 bp from two specimens (KE-4S9/04) and (KE-10N8/03). 5 = negative control. PCR-amplified products of 980 bp and 398 bp are shown with arrows.

Results

Number of isolates and demographics

Half of all the specimens assayed in this study (51.3%) were from children between one and two years of age, 29% were from children below one year of age, and 18.5% were from subjects older than three but younger than seven years, while 0.9% were from adults.

Table 2. Summary of clinical and demographic data on seven individuals positive for HBoV DNA

HBOV-POSITIVE SUBJECTS (n = 7)							
Demographics							
Subject no.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Age in months	22	22	13	8	13	14	8
Sex	M	M	F	F	F	F	F
Geo-location	Busia	Nairobi	Kisumu	Kisumu	Kisumu	Kisumu	Kisii
Period of isolation	Nov 08	Aug 07	Jan 07	Oct 07	Nov 07	Mar 08	Apr 09
Clinical Presentation							
Temp ≥ 38°C	√	√	√	√	√	√	√
Cough, nasal stuffiness, runny nose	√	√	√	√	√	√	√
Breathing difficulty	√	√	-	-	-	-	-
Diarrhea	-	-	-	-	-	-	-
Vomiting	-	-	√	√	√	√	√
Fatigue, malaise, poor appetite	√	-	-	-	-	-	-
Neurological - restlessness	-	√	-	-	-	-	-
Coinfections	Para 3	hAdv	Ent	hAdv	Ent/hAdv	HSV/Ent	Para 3

Para: parainfluenza 3; hAdv: human adenovirus; Ent: enteroviruses; HSV: herpes simplex virus; √: symptom present; -:symptomabsent; M: male;F: female; , 1: KE-23A8/11; 2: KE-48G7/8; 3:KE-38N7/01; 4: KE-9N7/10; 5: 9N7/11; 6: KE-10N8/3; 7: KE-4S9/04; Bus: Busia; Nai: Nairobi; Kis: Kisumu; Kss: Kisii

There was a slight predominance of males (56%) in comparison to the females (44%). HBoV DNA was amplified from seven samples out of 384 screened specimens (1.8%); this was from five females and two males.

A retrospective review of records for age revealed that two (28.5%) of the seven positive subjects were children less than 12 months of age (8 months precisely) and the other five were children between one and two years of age (71.5%).

Co-pathogens

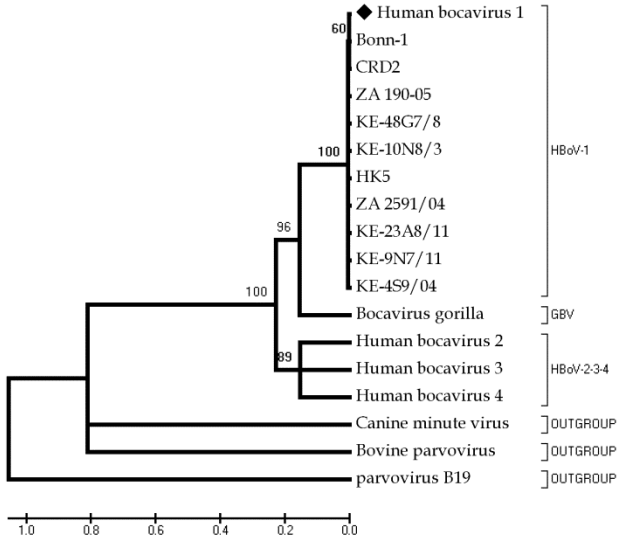
Among the HBoV-positive samples, coinfections with a single virus were observed in two with parainfluenza 3 only (2.5%), two with adenovirus only (2%), and one with enterovirus only (1.4%). Multiple infections consisting of HBoV plus two other viruses, which collectively accounted for 3%, were observed when enteroviruswas isolated together with adenovirus, and also separately with HSV-1. HBoV was isolated in all the three years represented by the samples screened.

HBoV was isolated twice, consistently in November and March, in two different years and from two different regions. Clinical characteristics of subjects with HBoV DNA positive specimen alongside the month and place of isolation are outlined in Table 2. Most of the isolates were from the samples from the western and Nyanza regions of Kenya.

Sequence analysis

The VP1/2 (980 bp) gene region amplified corresponded to nucleotide positions 4102-5002 of the published sequence of the prototype strain. A search for homologies between this sequence and sequences in the public database indicated that the Kenyan sequences had 99% homology to human bocaviruses. The phylogenetic tree indicated two levels of separation. At the first level, HBoV2, 3, and 4 were found to cluster together and were separated from other HBoV isolates and the GBV (Figure 2). Interestingly, the data suggests that HBoV1 is more closely related to the gorilla bocavirus than it is to HBoV2, 3, and 4.

Figure 2. Phylogenetic analysis of five isolates of human bocavirus originating from Kenya



Sequences retrieved from GenBank for comparison are labeled as HK5, GBV, CRD2, ZA, and Bonn-1. The reference DNA sequences for bovine parvovirus and canine minute virus served as the out-group sequences.

At the second level of separation, the gorilla bocavirus was separated from the rest of the isolates, which were found to cluster together. The latter comprised the five isolates from Kenya and CRD2, Bonn-1, HK5, CRD2, ZA 190-05, ZA2591/04, and human bocavirus 1. The clustering of the tree was supported by high bootstrap values of 61%-100%**.**

Accession numbers

Five Kenyan sequences of the HBoV-VP1/VP2 gene representing DNA of five samples were deposited in GenBank and have been assigned accession numbers HQ288041 (KE-9N7/11), HQ288042 (KE-23A8/11), HQ288043 (KE-10N8/3), HQ326235 (KE- 48G7/8) and HQ326236 (KE-4S9/04).

Discussion

The first comprehensive investigation of the role of viruses in causing acute respiratory infections in Kenya dates back to the mid-1980s, when Hazlett *et al.* [12] isolated a large proportion of viral agents, many of which were considered less important then. Currently, that trend has greatly changed due to a complex interaction of viruses with other factors in the human population and environment resulting in the resurgence and emergence of new viral pathogens. In the Kenya Demographic Health Survey (KDHS) report of 2009, it was estimated that countrywide, 56% of all cases of outpatient clinic consultations in children below five years of age were due to repeated episodes of acute respiratory tract infections. These children generally present with complaints of cough accompanied by congestion and difficulty in breathing or fever. These signs perhaps are often erroneously attributed to influenza virus infections, but nevertheless rarely warrant initiation of an investigation of the etiological agent responsible. It is more likely that some of these respiratory infections are caused by the newer emerging pathogens that include human bocavirus, metapneumovirus, and human coronaviruses. Globally, as reviewed by Allander [15], human bocavirus has variably been detected in 1.5%-19% of individuals with acute respiratory illness, with a noted frequency of detection of between 5.0%-5.5% in most of the studies.

A review of the literature indicates that there has been no study specifically investigating the presence of HBoV in respiratory infections in Kenya. The main objective of this report was to demonstrate the presence of HBoV in Kenya as a co-infecting agent, to describe the genotype in circulation, and to establish its phylogenetic relationship with isolates from other regions. Here we report the presence of HBoV in Kenya among patients with upper respiratory infections. Our findings suggest that HBoV1-like lineage (St2) is the main strain circulating in Kenya. In this study, the total number of HBoV isolated was 1.8%, and it is unclear as to whether or not this statistic implies that HBoV plays a relatively unimportant role in acute respiratory infections in Kenya. However, the findings show that the virus is actually circulating within the population. Characteristically, all of the seven individuals in whom the virus DNA was isolated ranged in age from a few months to less than two years, and were also infected with one of the other regular respiratory viruses. This observation is consistent with the observations of many other authors [16-18]. One explanation that is consistently put forward to account for these observations is that maternal antibodies provide protection to children younger than nine months of age, meaning that, as the concentration of maternal IgG declines, the incidence will increase as the children grow older [19-21].

In regards to the clinical presentation of infections involving HBoV, it seems that the general symptoms that usher in influenza-like illnesses are common to a wide variety of viral infections, and thus there is no one single sign that can be said to be pathognomonic for HBoV infections. The presence of vomiting in some of the individuals suggested gastric discomfort, even though there was no record of diarrhea episodes. These symptoms of gastroenteritis have been highlighted in other studies. For example, one of the reasons for seeking health care services for acute respiratory infections in 10% of the children in whom HBoV was isolated in Italy was gastroenteritis [22]**.** Perhaps HBoV is a pneumoenteric virus, meaning that under certain settings, HBoV infection might be initiated at the respiratory site before it extends to involve the intestinal sites. However, examining whether HBoV was the cause of vomiting in individuals whose samples we analyzed in the current study was not within the scope of this project. It is important to note that we isolated the virus in respiratory-associated specimens even though the virus has also been associated with diarrhea in humans [23], which is also a feature common in animal parvovirus infections [24]. These unique characteristics reflect the many aspects of virus infection that are yet to be explored, especially in the developing world where gastroenteritis and diarrhea cases are high.

In immunologic investigations by many other authors, it is apparent that up to 96% of healthy adults have past immunity to HBoV [19,21,25]. We speculate that the explanation for such high exposure could be that respiratory HBoV infections may provide efficient means by which the virus is perpetuated in the community throughout the years, and this is in turn serves to sustain a high level of seropositvity among populations in those communities with high seroprevalence. In Africa, there are no documented investigations of the seroprevalence status in humans.

Epidemiologically, the true origin of human bocaviruses is unknown, which makes it difficult to characterize the sources and risk factors for the transmission of the virus. It is worth mentioning that the results of phylogenetic analysis in our study showed that HBoV1 is clustered closer to the gorilla bocavirus. This raises the possibility that human bocavirus (especially HBoV1) has its origins in non-human primates. Sharp *et al*. explored the potential role of non-primates in HBoV infections [26]; in their seroprevalence study, it is evident that there is widespread infection of non-primates with parvoviruses that are not only genetically but also antigenically similar to three parvoviruses that infect humans (PARV4, HBoV, and B19 virus). A human behavior that promotes transmission of viral diseases to man from animals is close interaction with animals harboring the pathogens. Perhaps such an event may have occurred in some of the cases included in this study, considering that many of our HBoV isolates emerged from sites in the western region of Kenya where human-primate interaction is common.

In conclusion, even though the diagnosis of HBoV will not result in specific antiviral interventions at the moment, the current lack of common epidemiological knowledge – especially when investigating emerging pathogens – leaves us unable to predict the effect and magnitude a novel human pathogen may or may not have. The virus unknowably may cause symptoms similar to common colds, other upper respiratory tract infections, probably pneumonia, and possibly gastroenteritis. As evident, several aspects about infection with the virus remain to be assessed in East Africa, such as seroprevalence and the virus' role in gastroenteritis. Most importantly, this study effort has provided supplemental information about other plausible causative agents of upper respiratory tract infections other than, or in addition to, the principal and classical viral targets; this should add to the national and international collation of data about newly recognized or rare infectious agents in Kenya.

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