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Original Article

Human bocavirus amongst an all-ages population hospitalised with acute lower respiratory infections in Cambodia

Alicia Arnott, Sirenda Vong, Sareth Rith, Monica Naughtin, Sowath Ly, Bertrand Guillard, Vincent Deubel, Philippe Buchy

Institut Pasteur in Cambodia, Réseau International des Instituts Pasteur, Phnom Penh, Cambodia.
Correspondence: Philippe Buchy, Institut Pasteur in Cambodia, Virology Unit, 5 Monivong blvd, PO Box 983, Phnom Penh, Cambodia.
E-mail: pbuchy@pasteur-kh.org

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Background Human bocavirus (HBoV) is a novel parvovirus that is associated with respiratory and gastrointestinal tract disease.

Objectives To investigate the prevalence and genetic diversity of HBoV amongst hospitalized patients with acute lower respiratory infection (ALRI) in Cambodia.

Study Design Samples were collected from 2773 patients of all ages hospitalised with symptoms of ALRI between 2007 and 2009. All samples were screened by multiplex RT-PCR/PCR for 18 respiratory viruses. All samples positive for HBoV were sequenced and included in this study.

Results Of the samples tested, 43 (1·5%) were positive for HBoV. The incidence of HBoV did not vary between the consecutive seasons investigated, and HBoV infections were detected year-round. The incidence of HBoV infection was highest in patients aged <2 years, with pneumonia or bronchopneumonia the most common clinical diagnosis, regardless of age. A total of 19 patients (44%) were co-infected with HBoV and an additional respiratory pathogen. All isolates were classified as HBoV type 1 (HBoV-1). High conservation between Cambodian NP1 and V1V2 gene sequences was observed.

Conclusions Human bocavirus infection can result in serious illness, however is frequently detected in the context of viral co-infection. Specific studies are required to further understand the true pathogenesis of HBoV in the context of severe respiratory illness.

Keywords Acute lower respiratory tract infection, Cambodia, epidemiology, genetic diversity, human bocavirus, South East Asia.

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Introduction

Human bocavirus (HBoV) is a novel parvovirus that was first identified in pools of nasopharyngeal aspirates obtained from individuals with respiratory tract infections in 2005.[1] The HBoV genome has three open reading frames encoding the 2 non-structural proteins NS1 and NP1, and the structural viral capsid proteins VP1 and VP2.[2,3] There are four closely related HBoV genotypes, HBoV-1, HBoV-2 (consisting of HBoV-2A and HBoV-2B strains), HBoV-3 and HBoV-4, all of which share the same genomic organisation.[3,4] HBoV-1, the genotype initially identified by Schildgen *et al.*[5] in Sweden, has since been detected worldwide. HBoV-2-4 were mainly identified in stool samples.[6,7]

Homology at the protein level between the four HBoV genotypes is high, between 70 and 90%.[3] HBoV-3 is thought by some to be the result of a recombination event between HBoV-1 and HBoV-2, owing to the similarity of the HBoV-3 NS1 and V1V2 genes to those of HBoV-1 and HBoV-2, respectively.[6] Indeed, HBoV-3 is likely detected along with HBoV-1 during routine diagnostic screening, as the conserved NS1 genes regularly targeted for testing are genetically similar.

In patients where HBoV is the only virus detected, the clinical symptoms reported are similar to those occurring as a result of infection with respiratory syncytial virus (RSV) and human metapneumovirus (HMPV), including bronchiolitis, bronchitis, pneumonia and exacerbation of asthma.[5] Predominantly HBoV-1, but also HBoV-2 DNA, has been detected in respiratory samples,[8] whereas all four genotypes have been detected in stool samples, demonstrating that HBoV replication is not limited to the respiratory tract as first thought.[4,8] Indeed, up to 25% of patients with HBoV infection report gastrointestinal symptoms.[8–11]

Hence, the tropism of strains belonging to HBoV genotypes 1–4 remains unknown.

The seasonality of HBoV infection is also unclear, with seasonal[12–14] and year-round[15–17] transmission reported. The clinical significance of HBoV infection is also yet to be fully elucidated, as HBoV is frequently detected in association with additional respiratory pathogens. In the absence of an established cell culture system or animal model, the pathogenicity and replication mechanisms of HBoV remain unclear.[5,18,19] A recent report suggests that HBoV is, however, a true respiratory pathogen capable of causing sometimes severe, and even life-threatening, illness.[20]

Here, we report the findings of the first study investigating the prevalence, seasonality, clinical characteristics and the molecular epidemiology of HBoV in amongst an all-ages population of patients hospitalized for acute lower respiratory illness (ALRI) in Cambodia over 3 consecutive years.

Materials and methods

Sample collection and screening for respiratory virus infection

During April 2007–December 2009, all patients admitted with symptoms of ALRI to Takeo (southern Cambodia) and Kampong Cham (central-north Cambodia) provincial hospitals were recruited into this study.[21] The age-specific criteria used to diagnose ALRI cases and severe ALRI cases were defined previously.[21] Samples were collected, transported and stored as described.[21] Additional clinical specimens and data, including sputum samples for bacteria identification and chest X-rays, were collected from patients where possible.

Samples were screened by multiplex RT-PCR/PCR at the Institut Pasteur in Cambodia for 18 respiratory viruses including RSV, human metapneumovirus (HMPV), HBoV, Influenza A and B viruses, coronaviruses OC43, 229E, HKU1 and NL63, severe acute respiratory syndrome-associated coronavirus (SARS-CoV), parainfluenza viruses 1–4, adenoviruses, rhinovirus and enterovirus, as previously reported.[21,22] Samples that tested positive for HBoV were included in this study.

The National Ethics Committee of Cambodia approved this study. All patients/parents of sick children who participated provided written informed consent.

DNA extraction and polymerase chain reaction

Viral DNA from nasopharyngeal samples was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions. DNA was eluted in a final volume of 200 μl Qiagen AE buffer and stored at −80°C until required.

Both HBoV and human Albumin DNA were amplified in parallel from each sample. Amplification of human Albumin DNA was performed as described.[23] To identify strains belonging to HBoV groups 1, 2 and 3, strain-specific primers were used. To identify HBoV-1 strains, a 354-bp fragment of the conserved HBoV-1 NP-1 gene was targeted using published primers.[1] The forward and reverse primers correspond to positions 2281 and 2634 of the NP-1 gene of the HBoV prototype strain St1 (Genbank accession number DQ000495). Thermocycling was performed using Go-Taq Flexi DNA polymerase (Promega, Madisson, WI, USA) under the following conditions: PCR activation at 94°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 59·5°C for 30 seconds, extension at 72°C for 1 minute and a final extension cycle of 72°C for 10 minutes. All samples were also screened by PCR for the presence of HBoV2 and HBoV3 DNA, as described.[8]

For phylogenetic analysis, a 819-bp region of the variable HBoV-1 V1/V2 gene was amplified using published primers.[9] Primers correspond to positions 4370–4387, and 5172–5189 of the HBoV prototype strain St1 genome (Genbank accession number DQ000495). Thermocycling was performed using Go-Taq Flexi DNA polymerase (Promega) under the following conditions: PCR activation at 94°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, extension at 72°C for 2 minutes and a final extension cycle of 72°C for 10 minutes.

Each PCR contained the appropriate positive and negative controls. All PCR products were visualised using ethidium bromide under UV light on a 1·5% agarose gel. When required, PCR products were purified using the QIAquick Gel Extraction protocol of the QIAquick PCR Purification kit (Qiagen) as per the manufacturer's instructions, prior to sequencing. Single-pass sequencing reactions were performed at a contract sequencing facility using the ABI BigDye Terminator Cycle Sequencing kit on an ABI 3730XL automatic DNA Analyser (Macrogen, Seoul, Korea).

Phylogenetic analysis

Consensus sequences were generated using clc main workbench software, version 5·6·1 (http://www.clcbio.com). Nucleotide sequences of reference HBoV strains were obtained from Genbank and used to construct alignments and phylogeny (Table 1). Cambodian and reference HBoV nucleotide sequences were aligned using the clustal w alignment program of mega software, version 4·0.[24] The average pairwise Jukes-Cantor distance was found to be 0·01 for both of the HBoV NP1 and VP1/VP2 alignments, indicating that the data were suitable to generate neighbour-Joining trees.[25] neighbour-Joining trees were constructed using the p-distance nucleotide substitution model, with 1000 bootstrap replicates, using MEGA 4·0 software. Pairwise nucleotide distances (p distance), the proportion of sites at which nucleotide sequences differ divided by the total number of nucleotides compared, were calculated using the

Table 1. Reference human bocavirus (HBoV) strains used in this study to construct phylogeny. Shown are the name of the strain, Genbank accession number, year of isolation, country of isolation and genotype. Whether strains were included in Figure 2 (HBoV NP1 phylogeny), Figure 3 (HBoV VP1/VP2 phylogeny) or Figures 2 and 3 (both HBoV NP1 and VP1/VP2 phylogenies) is indicated

Strain	Accession number	Year	Origin	HBoV genotype	Figure
Humanparvovirus 4	EU874248	Unknown	Africa	–	3
Human parvovirus 5	DQ873391	Unknown	UK	–	3
Human parvovirus B19	FJ429009	2000	Brazil	–	3
Ku1	GQ200737	2010	USA	HBoV-2	2
W298	FJ948860	2001	Australia	HBoV-2	3
UK-648	FJ170280	Unknown	UK	HBoV-2	3
W471	EU918736	2009	Australia	HBoV-3	2, 3
HBoV-4	FJ973561	2010	USA	HBoV-4	2, 3
St1	DQ000495	2003/2004	Sweden	HBoV-1	2, 3
St2	DQ000496	2003/2004	Sweden	HBoV-1	2, 3
HK19	EF450735	2007	Hong Kong	HBoV-1	2, 3
HK4	EF450720	2007	Hong Kong	HBoV-1	3
BJ3064	DQ988933	2006	China	HBoV-1	2
WLL-2	EF441262	2007	China	HBoV-1	2, 3
R3080918004	GQ403983	2008	China	HBoV-1	3
FZ1	GQ455988	2007	China	HBoV-1	3
Lz37	FJ548903	2006/7	China	HBoV-1	2
CU6	EF203920	2007	Thailand	HBoV-1	2, 3
CU74	EF203922	2007	Thailand	HBoV-1	2, 3
TW2717_06	EU984233	2006	Taiwan	HBoV-1	3
Tw2715_06	EU984232	2006	Taiwan	HBoV-1	3
PCH043	FJ767758	Unknown	USA	HBoV-1	2
NZ2005	EF686013	2005	New Zealand	HBoV-1	2
Spain043	EU100023	2005/2006	Spain	HBoV-1	2
MPT5	AM109962	2003/2004	France	HBoV-1	2
REL1/Ba/2007	EU069436	2007	Italy	HBoV-1	3
GE4-IR	GQ906592	2007	Iran	HBoV-1	3

pairwise distance function of mega software version 4·0. Pairwise amino acid distances (p distance), the proportion of sites at which amino acid sequences differ divided by the total number of residues compared, were calculated using the Poisson model in the pairwise distance function of MEGA software version 4·0.

Deduced amino acid sequences and *N*-glycosylation site analysis

Deduced partial amino acid sequences of Cambodian NP1 and VP1/VP2 strains were generated by translating nucleotide sequences with the standard genetic code using MEGA software version 4·0. Potential *N*-glycosylation sites were predicted by the NXT motif, where X is not a proline; potential *O*-glycosylation sites were predicted by the KPX_n motif (where X is any amino acid).[26–29]

Nucleotide sequence accession numbers

The nucleotide sequences of HBoV strains obtained in this study were deposited in GenBank under the accession numbers JQ618248 to JO618263 (NP1 sequences) and JQ618264 to JQ618278 (VP1/VP2 sequences).

Results

A total of 2773 patients presenting with ALRI participated in the study (no refusals). Nasopharyngeal swabs were collected from all patients, of which 43 (1·5%) tested positive for HBoV. HBoV infections were detected year-round, and the annual incidence amongst the study patients did not vary significantly (1·5–1·7%) during 2007–2009 (Figure 1). The median age of the HBoV-infected patients was 1 year (range, 0·3–56 years), and 46·5% were female. The incidence of HBoV infection was highest amongst children aged <2 years (Table 2). Whereas bronchiolitis was only observed in patients aged <2 years, pneumonia or bronchopneumonia was the most common clinical diagnosis, regardless of age (37·2%; Table 2). Only respiratory specimens were available for testing in this study, and no clinical information regarding gastrointestinal symptoms was recorded.

Nineteen (44%) patients were co-infected with an additional respiratory virus (Table 3). Co-infection with HRhV was most frequently detected (21%), followed by parainfluenza virus type 3 (PIV3, 4·6%) and respiratory syncytial

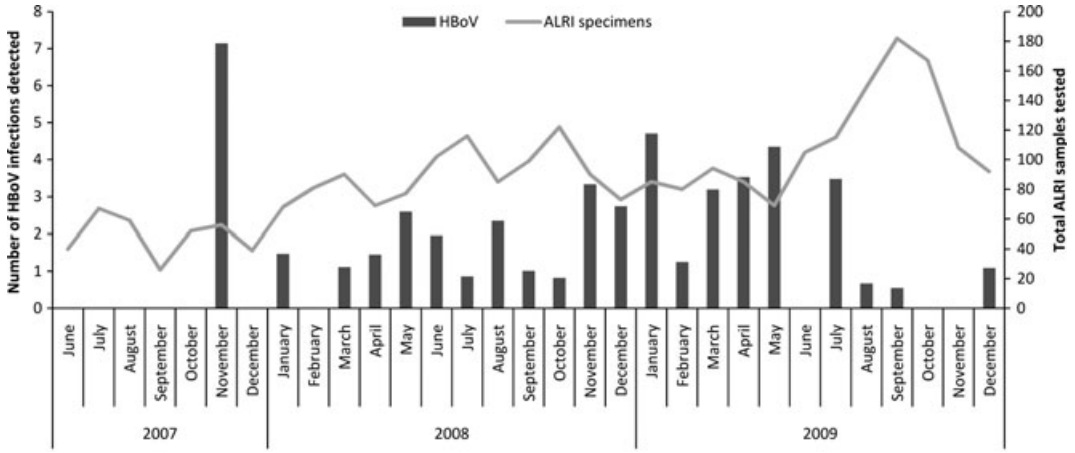

Figure 1. Annual distribution of human bocavirus (HBoV)-positive samples. Data represent the incidence of HBoV infections detected amongst the total number of specimens tested from patients hospitalized for acute lower respiratory infection between June 2007 and December 2009.

Table 2. Clinical diagnosis for human bocavirus (HBoV)-positive patients according to age

Age (years)	Total patients per age group (%)	Clinical diagnosis			
		Bronchiolitis (%)	Bronchitis (%)	Pneumonia/Bronchopneumonia (%)	Other (%)
0 to ≤ 1	20 (46·5)	3 (15·0)	5 (25·0)	8 (40·0)	4 (20·0)
1–2	18 (42)	3 (16·7)	1 (5·5)	7 (38·9)	7 (38·9)
>2–10	2 (4·6)	0	1 (50·0)	0	1 (50·0)
11–20	1 (2·3)	0	1 (100·0)	0	0
≥20	2 (4·6)	0	0	1 (50·0)	1 (50·0)
Total (%)	43	6 (14·0)	8 (18·6)	16 (37·2)	13 (30·2)

Table 3. The number of human bocavirus (HBoV)-positive patients co-infected with an additional virus. The percentage of patients infected with each viral pathogen was calculated relative to the total number (43) of HBoV-positive patients investigated in this study

	Number of cases	Overall (%)
Viral co-infection	19	44·0
Human rhinovirus (HRhV)	9	21·0
Parainfluenza virus type 3	2	4·6
Respiratory syncytial virus (RSV)	2	4·6
Human metapneumovirus	1	2·3
Coronavirus 229E	1	2·3
Adenovirus	1	2·3
Influenza B virus	1	2·3
Coronavirus NL63	1	2·3
RSV + HRhV	1	2·3

virus (RSV, 4·6%). One patient was co-infected with both RSV and HRhV (Table 3).

Molecular epidemiology of HBoV

Detection and distribution of HBoV

Sequences were successfully obtained from 19 (44·2%) samples: 16 NP-1 gene and 15 VP1/VP2 gene sequences. Both NP-1 and VP1/VP2 sequences were successfully amplified from 12 (63%) samples. All Cambodian HBoV strains were classified as HBoV-1 (Figures 2 and 3).

Of the samples from which HBoV-1 sequences were obtained, one was collected in 2007, nine in 2008 and nine in 2009 (Figures 2 and 3). Twelve (63%) samples were collected in Takeo province, and seven (37%) were collected in Kampong Cham province, but the patient recruitment was also higher in Takeo than in Kampong Cham hospital (data not shown).

Analysis of HBoV-1 NP1 gene sequences

Pairwise distance (p distance) analysis revealed very high conservation of NP-1 gene sequences amongst the

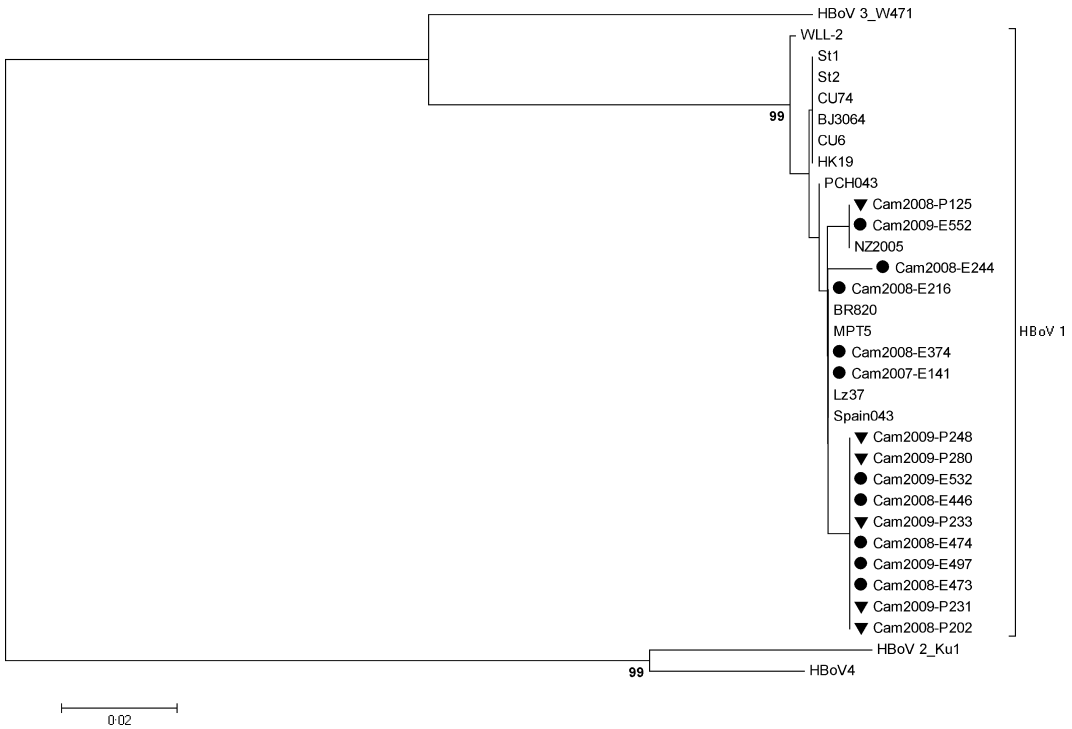

Figure 2. Phylogenetic analysis of partial NP1 gene sequences from Cambodian and reference human bocavirus (HBoV) strains. Phylogeny was constructed using the neighbour-joining method with 1000 bootstrap replicates. Only bootstrap values >70% are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Cambodian strains are indicated by 'Cam' followed by the year of collection. Cambodian strains isolated from Takeo province are indicated by solid triangles; strains collected in Kampong Cham province by solid circles. Genotypes are indicated. HBoV-2, HBoV-3 and HBoV-4 strains were used as outgroups.

Cambodian HBoV-1 strains. Homology at the nucleotide (nt) level varied between 98·9 and 100%. Identity at the amino acid (aa) level was slightly lower, between 96·7 and 100%. The NP-1 gene is highly conserved between strains, and however, we identified 5 different non-synonymous mutations present in Cambodian NP-1 sequences relative to the prototype HBoV-1 strain St1 (Figure 4). The observed nt mutations resulted in the following 5 aa substitutions: Ser^{92} (100% conservation amongst Cambodian NP-1 sequences), Asn^{44} (65%), Asn^{59} (12%), Ser^{47} (6%), Arg^{53} (6%) (Figure 4). We observed 1 potential *N*-glycosylation site, starting at amino acid position 86, which was conserved amongst all reference and Cambodian HBoV strains analysed (Figure 4).

Analysis of HBoV-1 VP1/VP2 gene sequences

Homology at the nt level was also very high amongst the partial VP1/VP2 gene sequences, between 97·1 and 100%. Homology at the aa level was slightly lower, between 96·6 and 100%.

Present on the surface of the virion, the VP1/VP2 protein is potentially subjected to strong selective pressure from the host immune response. Relative to the reference strains St1 and St2, 8 non-synonymous nt mutations resulting in the following aa substitutions were observed amongst Cambodian VP1/VP2 gene sequences: His^{546} (66%), Leu^{631} (26%), Tyr^{540} (26%), Thr^{650} (13%), Ser^{466} (6%), Pro^{490} (6%), Gln^{493} (6%) and Lys^{545} (6%) (Figure 5). One additional nt mutation resulting in the aa substitution Ser^{590}, present in reference strain St2 but not St1, was conserved amongst all of the Cambodian VP1/VP2 sequences investigated (Figure 5). A stop codon was present at position 672 in all reference and Cambodian VP1/VP2 sequences included in this study (Figure 5).

Potential N- and O-glycosylation sites amongst HBoV-1 VP1/VP2 gene sequences

Two potential *N*-glycosylation sites, located at amino acid positions 519 and 638, were conserved amongst the reference isolates and all of the Cambodian VP1/VP2 sequences investigated (Figure 5). One potential *O*-glycosylation site was identified, located at amino acid position 552, which was also conserved amongst the reference isolates and all of the Cambodian VP1/VP2 sequences analysed (Figure 5).

Discussion

Here, we report the results of the first study to investigate the incidence and genetic diversity of HBoV amongst

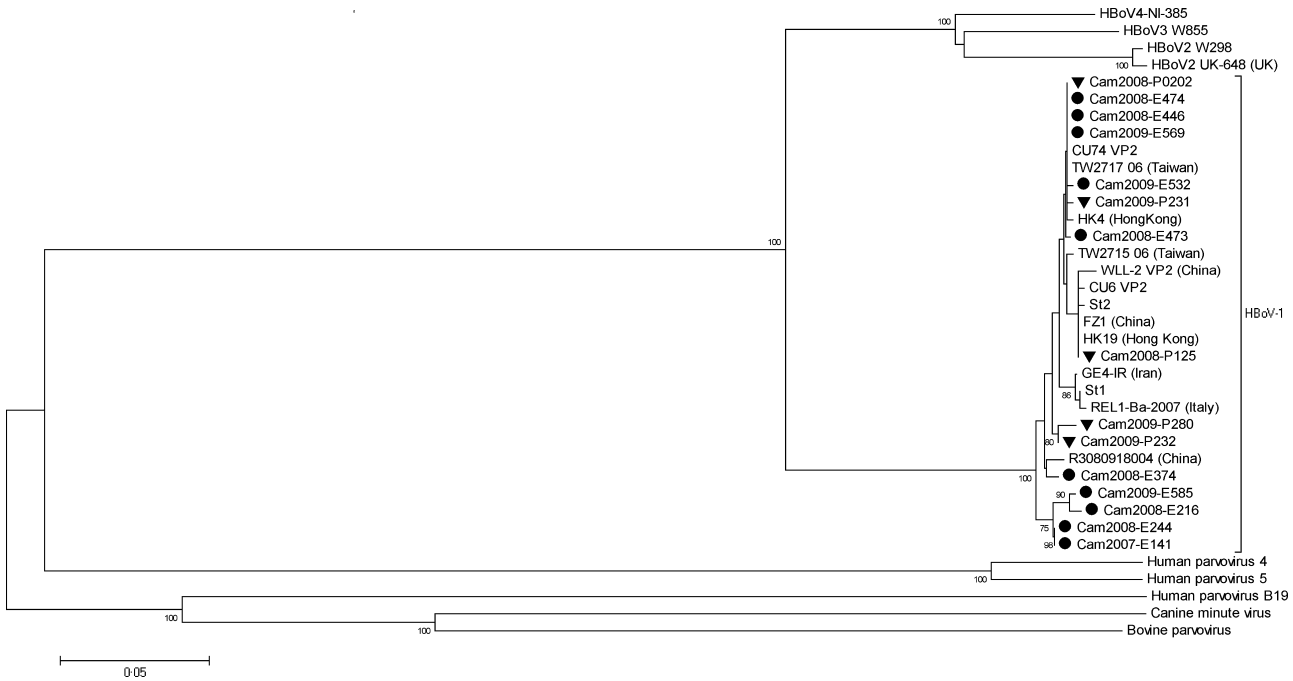

Figure 3. Phylogenetic analysis of partial VP1/VP2 gene sequences from Cambodian and reference human bocavirus (HBoV) strains. Phylogeny was constructed using the neighbour-joining method with 1000 bootstrap replicates. Only bootstrap values >70% are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Cambodian strains are indicated by 'Cam' followed by the year of collection. Cambodian strains isolated from Takeo province are indicated by solid triangles; strains collected in Kampong Cham province by solid circles. Genotypes are indicated. Human parvovirus 4, Human parvovirus 5 and Human parvovirus B19 strains were used as outgroups.

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                10        20        30        40        50        60        70        80        90
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
St1   GNMKDKHRSYKRKGSPERGERKRHWQTTHHRSRSRSPIRHSGERGSGSYHQEHPISHLSSCTASKTSDQVMKTRESTSGKKDNRTNPYTV
St2   ..........................................................................................NRT.....
Cam2008-E244  ..........................................S.....R.....................................NRT...S.
Cam2009-P280  ..........................................N.....................................NRT...S.
Cam2008-E374  ..........................................................................NRT...S.
Cam2009-E532  ..........................................N.....................................NRT...S.
Cam2009-E552  ..........................................................N.....................NRT...S.
Cam2008-P125  ..........................................................N.....................NRT...S.
Cam2008-P202  ..........................................N.....................................NRT...S.
Cam2009-P231  ..........................................N.....................................NRT...S.
Cam2009-P233  ..........................................N.....................................NRT...S.
Cam2009-P248  ..........................................N.....................................NRT...S.
Cam2009-E497  ..........................................N.....................................NRT...S.
Cam2007-E141  ..........................................................................NRT...S.
Cam2008-E216  ..........................................................................NRT...S.
Cam2008-E446  ..........................................N.....................................NRT...S.
Cam2008-E473  ..........................................N.....................................NRT...S.
Cam2008-E474  ..........................................N.....................................NRT...S.
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Figure 4. Partial amino acid sequences of the NP1 protein from Cambodian HBoV-1 strains. The alignment is shown relative to the prototype HBoV-1 strain St1 (Genbank accession number DQ000495). Numbers indicate the position of residues relative to amino acid residues 1–93 of the NP1 gene (corresponding to nt positions 2419–2691 of the St1 genome). Dots indicate identical residues. Potential *N*-glycosylation sites (NXT motifs, where X is not a proline) are underlined.

hospitalised patients in Cambodia. Seasonality of HBoV infection was not observed in this study, confirming the results of Buecher *et al.*,[22] who previously investigated viral causes of influenza-like illness (ILI) amongst Cambodian out-patients.

Globally, the incidence of HBoV infection has been reported to range from 1·5 to 19%.[30] Overall, the incidence of HBoV infection amongst the all-ages population of Cambodian patients hospitalised with ALRI was 1·5%, which did not vary annually and was lower than that reported regionally.[31–34] Amongst the same population, the incidence of HBoV was similar to that of HMPV (1·7%),[21] but lower than RSV (8·2%).[35] A number of factors can influence incidence estimates, including disease severity

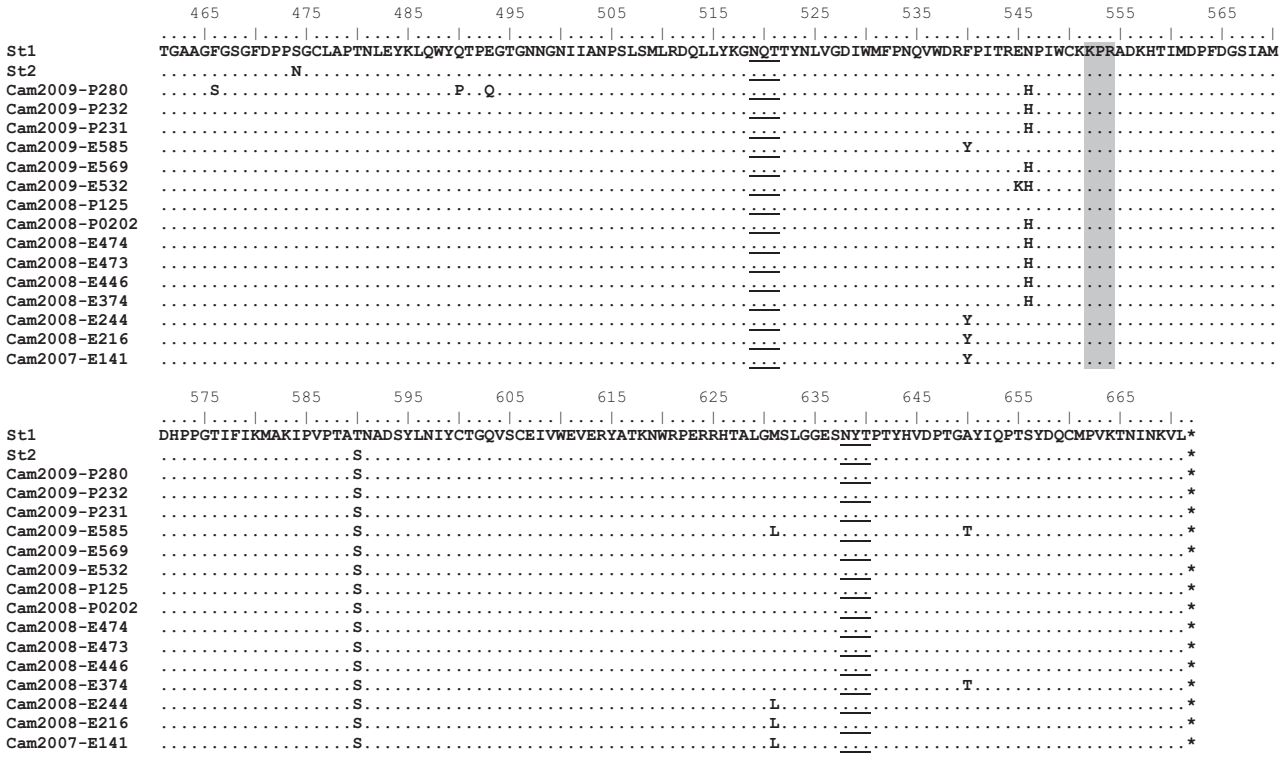

Figure 5. Partial amino acid sequences of the VP1/VP2 protein from Cambodian HBoV-1 strains. The alignment is shown relative to the prototype HBoV-1 strain St1 (Genbank accession number DQ000495). Numbers indicate the position of residues relative to amino acid residues 461–672 of the NP1 gene (corresponding to nt positions 4366–5001 of the St1 genome). Dots indicate identical residues; stop codons are indicated by an asterisk. Potential *N*-glycosylation sites (NXT motifs, where X is not a proline) are underlined; a potential *O*-glycosylation site (KPX where X is any amino acid) is indicated by shading.

amongst the population investigated, study design, testing protocols and sensitivity of diagnostic tests used, especially as low viral loads are common in HBoV infections.[5] In this study, ALRI patient samples were screened for HBoV infection using a highly sensitive multiplex PCR assay previously shown to have a lower limit of detection of 4 copies of HBoV DNA/μl of viral transport medium.[22] Therefore, the low incidence of HBoV infection observed amongst the Cambodian ALRI population was not a result of limited sensitivity of the diagnostic test used.

The proportion of HBoV infections detected amongst hospitalised Cambodian ALRI patients was significantly higher compared to that of Cambodian outpatients with ILI (1·5% versus 0·4%, $P = 0{\cdot}02$).[22] The higher incidence of HBoV infection amongst ALRI patients was not thought to be a result of a high carriage rate within the population. In parallel, the incidence of HBoV infection amongst Cambodian ILI outpatients and asymptomatic controls was investigated previously, with only one asymptomatic individual testing positive for HBoV DNA.[22] However, persistent and prolonged shedding is a characteristic of parvoviruses, and it is thought that low-level asymptomatic shedding can persist following HBoV infection.[20,36,37] Two independent studies reported the HBoV carriage rate to be 43% amongst asymptomatic children,[38] and that the incidence of HBoV amongst asymptomatic children was higher than amongst symptomatic children.[39] However, these two studies were conducted amongst children aged <2 years. Hence, the high carriage rate may have been biased towards the very young age of the sample population with HBoV incidence highest amongst children aged <2 years.[1,9,40] Amongst a population of slightly older children, up to 5 years of age, Brieu *et al.*[36] reported that HBoV DNA was not detected following testing of asymptomatic children. In the absence of an established continuous culture system, it is currently unclear to what extent shedding occurs following HBoV infection, whether shedding and carriage rates amongst children and adults are equivalent, and whether shedding is innocuous or infectious. However, the higher incidence of HBoV infection amongst hospitalised Cambodian ALRI patients observed in this study relative to outpatients with ILI suggests that HBoV infection can result in severe illness.

Twenty-four (56%) of the HBoV-infected individuals tested positive for infection with HBoV only, of which 14 patients were classified as severe respiratory infection, including three patients aged <1 year who presented with

severe pneumonia (data not shown). Indeed, pneumonia was the most common clinical diagnosis amongst the ALRI patients investigated, regardless of age. This finding was consistent with the results of similar studies in which HBoV-1 infection has been associated with severe respiratory illness, including pneumonia,[20,32,40,41,42] bronchiolitis or bronchitis.[36,38–40] Amongst a study of children aged <2 years hospitalised for acute asthma, HBoV was the predominant virus detected.[43] Recently, a severe case of HBoV infection resulting in acute respiratory failure was reported, in which HBoV was the only pathogen detected.[20] The possibility that clinical illness amongst the Cambodian HBoV-infected patients was exacerbated because of bacterial co-infection cannot, however, be excluded as testing for bacterial co-infection could not be performed because of a lack of suitable specimens obtained from young participants.

Whether HBoV represents a true pathogen, or an opportunistic co-pathogen, has been the source of much debate.[2,5] A primary reason that the role of HBoV as a sole pathogenic agent is questioned is that HBoV is commonly detected as a co-infection. Globally, the frequency of HBoV-1 co-infection with other respiratory viruses amongst children with lower respiratory tract infection is reported to be as high as 83%.[4,5,44] Furthermore, it has been reported that HBoV co-infection may be higher amongst hospitalised individuals compared with those with mild illness as shedding is potentially enhanced by airway inflammation resulting from co-infection with an additional respiratory pathogen, or that HBoV may play a role in enhancing or aggravating symptoms of existing infections.[5] Mechanisms proposed to explain the high rate of co-infection with other respiratory viruses include that HBoV is either a helper virus, facilitating replication of other respiratory pathogens or that HBoV may require a helper virus to facilitate productive infection.[45] It must also be considered that the more recent widespread use of highly sensitive multiplex assays enabling simultaneous detection of multiple pathogens in a single patient sample may also contribute to the high rate of co-infection with HBoV and additional respiratory pathogens detected. In this study, 44% of patients were co-infected with HBoV and an additional respiratory virus. Regionally, the reported rate of HBoV co-infections varies and does not appear to be particularly associated with hospitalisation: 42% amongst hospitalized children in Singapore,[32] 90% amongst out-patient children aged <5 years in rural Thailand,[37] 9% amongst hospitalised children in Vietnam.[31] In this study, co-infection with HBoV and HRhV was most frequently detected (Table 3), analogous to the findings of a similar study conducted amongst paediatric patients in rural Thailand.[37] Whether the high frequency of HBoV and HRhV co-infection is a reflection of the high incidence of HRhV circulating in the general population[2] or whether HRhV co-infection is beneficial to HBoV replication and pathogenesis remains unknown. In the absence of an established cell culture system or animal model, much remains unclear regarding the pathogenesis of HBoV.

All of the HBoV strains obtained from respiratory specimens for analysis in this study were classified as HBoV-1. Globally, HBoV-1 strains have been reported to show low nucleotide and protein diversity; mean genetic diversity <1% at nt and <0·5% at aa levels.[44] Comparatively, the diversity of HBoV-2, -3 and -4 strains, all identified in stool species, is far greater.[8] The low level of diversity reported worldwide amongst HBoV-1 strains has resulted in speculation that HBoV-1 evolved recently from the enteric bocavirus species, acquiring tropism for cells of the respiratory tract.[8,46] The organisation of the HBoV genome is similar to that of other Parvoviruses, namely that the non-structural proteins are encoded by conserved genomic regions, whereas diversity is concentrated in regions encoding the capsid proteins.[45] Analysis of amino acid sequences obtained from Cambodian HBoV-1 strains revealed 0–2·9% nt diversity and 0–3·4% aa diversity amongst VP1/VP2 sequences. Greater variation amongst sequences encoding the VP1/VP2 capsid proteins was anticipated as VP1/VP2 is under pressure from host immune responses. The level of diversity amongst Cambodian NP1 sequences was 0–1·1% at the nt level, which is similar to that recently reported by Kapoor *et al.*[46] Surprisingly, the level of diversity at the aa level amongst NP1 sequences was equivalent to that observed for VP1/VP2 sequences, at 0–3·3%. Five amino acid substitutions were observed amongst Cambodian NP1 aa sequences, including Asn^{599} that has been reported previously by Ma *et al.*,[47] following analysis of HBoV-1 NP1 sequences obtained from Japanese children with ALRI. The observed diversity amongst Cambodian NP-1 sequences may have occurred as a result of immune pressure, as it was recently reported that the HBoV non-structural proteins, including NP1, are targeted by humoral responses.[30] The potential impact, if any, of non-synonymous mutations within this conserved region of the HBoV-1 genome remains unclear and warrants further investigation.

To the best of our knowledge, we present the results of the first study to investigate the circulation and diversity of HBoV infection amongst hospitalised patients in Cambodia. We demonstrate that similar to the findings of other studies, HBoV infection can result in serious illness, however is frequently detected in the context of viral co-infection. Ongoing studies are required to further understand the true pathogenesis of HBoV-1 in the context of severe respiratory illness.

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