Phylogenetic Analysis of Human Bocavirus Isolated from Children with Lower Respiratory Tract Infections in Baghdad, Iraq

Duraid Ali Hasan1*, Areej Atiyah Hussein1, Qasim Sharhan Al-Mayah2 and Iman M Aufi3

1Department of Microbiology, College of Medicine, University of Diyala - Iraq.
2Medical Research Unit, College of Medicine, Al-Nahrain University - Iraq.
3National Influenza Center - Central Public Health Laboratory - Iraq.

*Corresponding author.
Duraid Ali Hasan (duraidali860@hotmail.com)

Abstract

Acute respiratory infection is the major cause of morbidity and mortality worldwide, several types of viruses associated with this disease. This study aims to determine the infection rate of human bocavirus in children with lower respiratory tract infection and identify the genotyping among study population. Cross sectional study which consists of 122 children under five years old suffering from lower respiratory tract infection (75 males and 47 females); their aged from range 1 month to 60 months. They were attending respiratory wards in Fatima Al-Zahraa Hospital, Al-Elwiya Pediatrics Hospital, Ibn Al-Baladi Hospital and Pediatrics Protection Hospital in Baghdad, during the period from December 2017 to February 2018. Nasopharyngeal samples were collected from each participant, then used for DNA extraction and amplification with specific primers. Out of all 122 samples, eight samples were positive for HBoV (6.6%). Most infection were recorded in males 5(62.5%) and patients in age group 1-30 months 7(87.5%). The results of phylogenetic analysis for HBoV DNA isolated from nasopharyngeal swabs revealed all local isolates (8 isolates) are with HBoV type 1, half of the local isolates (4) were closed to Iranian isolation and the other four were closed to isolates from different regions (Tunisia, Italy and Argentina isolation). Some variant in amino acid noticed after alignment to the local isolates with some Gene Bank isolates and deletion of amino acid occur in isolates number one and five in more than two position but the substitution revealed in isolate number six in three position. In conclusion, infection rate of HBoV-1 comparable with result of neighboring countries, local isolate of HBoV-1 have their characteristic genetic structure which differentiation them from other international isolate.

Key words: Acute respiratory infection, human bocavirus, molecular detection, viral sequence.


Introduction

Respiratory tract infections (RTI) are the most important causes of morbidity and mortality among children especially in developing countries [1]. The medical costs estimated at almost $1 billion in the United States [2]. Viruses cause the majority of respiratory tract infections (81%) in children particularly those under the age of five [3]. All HBoV strains package negative single strand DNA while only a minority packages the positive strand [4].Several evidences indicate that human bocavirus genomes can exist and/or persist as closed circular DNA molecules [5]. Viral genome is consisting of three open reading frames, the first of which encodes the first two the non-structural protein 1 (NS1) and nuclear phosphoprotein (NP1), and the third one encodes the viral capsid proteins 1 and 2 (VP1 and VP2) [6]. Four types of human bocavirus (HBoV1-4) were reported worldwide according to genomic structure and amino acid sequence similarity [7]. Human bocavirus-1 is associated with RTI [1]. In contrast to HBoV1, types 2-4 seem to be more associated with gastrointestinal symptoms, although their prevalence among
enteric pathogens is limited [8]. Human bocavirus1 transmitted most likely by the respiratory route; it causes respiratory illness, and it can be detected in very high loads in the airways during the acute phase, after which it may persist at low viral loads for months [9]. It is presence in feces suggests that this virus is transmitted by the fecal-oral route [10]. In children HBoV may cause more severe clinical conditions such as encephalitis and life-threatening complications [11]. Human bocavirus1, the fourth most prevalent respiratory virus, was detected worldwide with epidemiology range 2-33% of children less than two years of age with respiratory tract infections [12]. The infection rate of HBoV1 mostly is higher in winter and spring than other seasons in different countries and regions [9]. Many studies of HBoV in neighboring countries revealed different percent of infection vary 6-22 as, Turkey, 6.7% [13], Jordan 9.1% [14], Iran, 10.7% [15] and Saudi Arabia, 22.5% [16]. A recent study done in Iraq by Atyah et al (2017) revealed an infection rate by using real time PCR is 24.6% [17]. This study design to determine the infection rate of human bocavirus in children with lower respiratory tract infection and identify the genotyping among study population.

Patients and methods

Cross sectional study which consists of 122 children under five years old suffering from lower respiratory tract infection after examined by a practitioner physician (75 males and 47 females); their aged from range 1 month to 60 months. They were attending respiratory wards in Fatima Al-Zahraa Hospital, Al-Elwiya Pediatrics Hospital, Ibn Al-Baladi Hospital and Pediatrics Protection Hospital in Baghdad, during the period from December 2017 to February 2018. Nasopharyngeal samples were collected from each participant by specific swabs from Sigma Virocult Company in UK with viral transport medium and stored as frozen at -70 °C until use. The DNA was extracted from nasopharyngeal swab specimens using commercial kit (Instant virus DNA/RNA, Cat. No. 845-KS-4500250, German). Three of primers listed in Table (1) were used in the present study to amplify the fragments of human bocavirus VPI/2 capsid gene [18]. And more recently by Kenmoe in Cameroon (2017) and were found to be very efficient for detection of HBoV1-4 [19]. Primers were dissolved in TBE buffer to prepare a stock solution of a final concentration of (100 pmol/μl) and the stock was kept at (-20). A working solution (10 pmol/μl) concentration was prepared by adding 10 μl of the stock solution to 90μl of the distilled water.

Table (1): Sequence of primers utilized in the present study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5′-3′</th>
<th>TM  °C</th>
<th>GC %</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP-A</td>
<td>5′-GCACCTCTCTGTATCAGATGCCTT-3′</td>
<td>57.5</td>
<td>45.5</td>
<td>904bp</td>
</tr>
<tr>
<td>VP-B</td>
<td>5′-CGTGGTATGTAGCCGTGTAG-3′</td>
<td>56.9</td>
<td>55.0</td>
<td></td>
</tr>
<tr>
<td>VP-C</td>
<td>5′-CTTAGAAGCTGGTGAGGACACTG-3′</td>
<td>57.5</td>
<td>50.0</td>
<td>850bp</td>
</tr>
<tr>
<td>VP-B</td>
<td>5′-CGTGGTATGTAGCCGTGTAG-3′</td>
<td>56.9</td>
<td>55.0</td>
<td></td>
</tr>
</tbody>
</table>

A semi-nested PCR was performed to amplify the VPI/2 gene. For the first round PCR, a (904bp) fragment was amplified by using forward primer (VP-A) and reverse primer (VP-B). PCR amplification mixture was performed in (50μl) final volume, (8μl) of template DNA, (2μl) of each primer and (38μl) distilled water was added to master mix according to protocol then thermal cycling was done as shown in Table (2). A second round PCR was performed to improve the sensitivity, in which (2μl) of reverse primer (VP-B) and (2μl) of the other forward primer (VP-C) and (4μl) of primary PCR product were mixed with (42μl) distilled water to performed (50μl) final volume according to the previous protocol [20].

©Annals of Tropical Medicine & Public Health S418
After successful amplification of the target regions of HBoV1-4 by using primers, (25μl) of PCR product along with primers, were sent abroad to Macrogen Company in South Korea for direct sequencing.

Results
Over 60% of the study population are males, the most predominate age group is 1-12 months (53.2%). Statistical analysis not revealed any significant differences as shown in Table (3).

Table (3): Distribution of children with LRTI according to gender and age.

<table>
<thead>
<tr>
<th>Variable factors</th>
<th>Samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender type</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>75 (61.4%)</td>
</tr>
<tr>
<td>Female</td>
<td>47 (38.6%)</td>
</tr>
<tr>
<td><strong>Age groups</strong></td>
<td></td>
</tr>
<tr>
<td>(1-12) months</td>
<td>65 (53.2%)</td>
</tr>
<tr>
<td>(13-24) months</td>
<td>38 (31.1%)</td>
</tr>
<tr>
<td>(25-36) months</td>
<td>6 (4.9%)</td>
</tr>
<tr>
<td>(37-48) months</td>
<td>8 (9.7%)</td>
</tr>
<tr>
<td>(49-60) months</td>
<td>5 (6.1%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>122 (100%)</td>
</tr>
</tbody>
</table>

According to result of seminested-PCR, there are 8 (6.6%) samples which gave positive result (figure 1) for the first round PCR with a fragment length of 980bp as shown in Figure (2).
**Figure (1):** Positive and negative result of HBoV according to seminested-PCR in children with LRTI.

**Figure (2):** Gel electrophoresis of the first round PCR with product size 980bp stained with ethidium bromide and illustrated under UV light.

A second round of PCR was performed to confirm the correct amplification of the first round product. All samples positive for the first round were subjected to second round. The result is shown in Figure (3). All these samples gave positive results with fragment length of 850bp.

**Figure (3):** Gel electrophoresis of the second round PCR of HBoV with product size 850bp stained with ethidium bromide and illustrated under UV light.
On studying the demographic factors between positive and negative groups as shown in Table (4), the present study had found that the gender groups did not differ significantly between positive and negative groups, 62.55% of HBoV-positive males compare to 61.4% among HBoV-negative males. Similarly, 1-30 months almost similar proportion between positive and negative group at the same age group (87.5% and 84.2%) respectively.

Table (4): Human bocavirus infections rate among children according to gender and age.

<table>
<thead>
<tr>
<th>Variable factors</th>
<th>Positive (8)</th>
<th>Negative (114)</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
<td>5(62.5%)</td>
<td>70(61.4%)</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>3 (37.5%)</td>
<td>43(29.82%)</td>
<td>47</td>
</tr>
<tr>
<td>Age groups</td>
<td>(1-30) m</td>
<td>7(87.5%)</td>
<td>96 (84.21%)</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>(31-60)m</td>
<td>1(12.5%)</td>
<td>18 (15.78%)</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Urban</td>
<td>6 (75%)</td>
<td>70 (61.40%)</td>
<td>76</td>
</tr>
</tbody>
</table>

The genetic relatedness of the local isolate were analyzed with MEGA 6 software through constriction phylogenetic tree (Figure 4). All local isolates are with HBoV type 1 as indicted from the figure and HBoV type 2, 3 and 4 clustered away from the local and reference isolates belonging to type 1. The result of this analysis confirmed that is of p-distance. Isolate 1, 5, 6 and 7 clustered with JX944710.1 (HBoV type 1 Iranian isolation). Isolate 3 and 8 clustered with AB481075.1, EU698026.1, JF327789.1. However, they are closest to JF327789.1 (HBoV type 1 Tunisia isolate). Isolate 2 clustered with KR014471.1 (HBoV type 1 Italy isolation) While isolate 4 clustered with KC544968.1 (HBoV type 1 Argentina isolation).
Figure (4): Phylogenetic tree for vp1/vp2 genes (HBoV) constructed by the neighbor joining method for 8 local isolates from nasopharyngeal swabs and 18 reference isolate from Gene Bank. Phylogenetic distances were measured by the kimura two-parameter, model of the tree was statistically support by bootstrapping with 1000 replicates. Bootstrap values below 50% are not shown. Current isolates are indicated with Black Square.

As vp1 and vp2 genes are protein-coding genes, the nucleotide sequences of these genes were translated into the corresponding amino acid by using EMBOSS Transeq available online at https://www.ebi.ac.uk/TOOLS/emboss.transeq/. As well, the reference sequences were who translated by this program. Bio Edit software (version 6) was used for align and compare the different amino acid sequences between local isolate and three reference sequences. According to the results of the alignment in the current study, deletion and substitution revealed in amino acid as shown in Table (5).

Table (5): Diversity of HBoV capsid protein (vp1/2) amino acid in local isolates.

<table>
<thead>
<tr>
<th>No. of isolate</th>
<th>Variation</th>
<th>Position of amino acid</th>
<th>Diversity in amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Deletion</td>
<td>1413, 1463, 1513, 1563</td>
<td>Y (Tyrosin), F (Phenylalanine), Q (Glutamine), M (Methionine)</td>
</tr>
<tr>
<td>5</td>
<td>Deletion</td>
<td>14591, 1558</td>
<td>F (Phenylalanine), D (Aspartic acid)</td>
</tr>
<tr>
<td>6</td>
<td>Substitution</td>
<td>1609, 1610, 1617</td>
<td>A (Alanine)→R (Arginine), T (Threonine)→N (Asparagine), R (Arginine)→G (Glycine)</td>
</tr>
<tr>
<td>1, 5, 6 and 7</td>
<td>Substitution</td>
<td>1583</td>
<td>S (Serine)</td>
</tr>
<tr>
<td>2, 3, 4 and 8</td>
<td>Substitution</td>
<td>1583</td>
<td>Y (Tyrosin)</td>
</tr>
</tbody>
</table>
Discussion

According to the results of nasopharyngeal swabs, the current study revealed 6.6% prevalence of HBoV infection in pediatric children with LRTI; this percentage lower than reported by Atyah et al. (2017) found HBoV infection is (24%) in children under 15 years old [17]. And higher than reported by several researchers they found that 0.80%, 1.5% and 3.1% respectively [21][14][22]. These variations in prevalence of HBoV in different studies can be attributed to several factors, first of all is the detection method. In the current study, the molecular detection conducted by conventional-PCR, while, several above studies, particularly those with high prevalence, real-time PCR was used. There is general agreement that qPCR is more sensitive. The second important reason and associated with the first one is low viral load of HBoV already existed. In Chinese study conducted on NPS from children with severe LRTI, the authors found 48% specimen were with low viral load[23]. Therefore, the conventional PCR may not detect all positive samples because of relatively low viral load. The other explanation for disposition among result is season of sampling which may also affect in prevalence. Most available studies showed high prevalence of HBoV in cooler months than hotter months. Some studies revealed high frequency of the virus during autumn and spring in Iran and Argentina [24][25]. While Smuts and Hardie (2006) It has been shown previously that HBoV infection occurs predominantly during the winter season [16]. And about 80% of these infections occurred between November and March [26]. While in study done by Zheng et al., (2010) found that did not observed any seasonal fluctuation in children infected with human bocavirus [27]. Other reasons that may affect the prevalence are the number of samples and age groups. The prevalence of HBoV was 10.7% in a large number of NPS samples (662) on Korean children with respiratory infection [28]. Brieu et al. (2008), they detect on human bocavirus in (10.8%) 55 of the 507 hospitalized children under five years of age [26]. While 29 (7.1%) HBoV positive cases out of 406 children in study done by [27]. The age groups are crucial factors affecting the prevalence of HBoV, because many studies revealed high prevalence of HBoV in children under two years old, so most children age in the current study was less than two years old. A study conducted in Brazil, the prevalence in children aged under 12 months revealed high rate of HBoV [29]. Other study revealed, the prevalence of HBoV on Panamanian children with respiratory illness were under two years old respectively [30]. Based on statistical analysis, there was no significant association between HBoV infection with gender and age. This may ascribed to the limited number of samples and very few positive results. These results are compatible with many other international studies [13][31]. According to gender, it has been found that the infection in males more than females seems to be similar with those participated in other studies such as [32][33]. While current study inconsistent with study conducted by Bubshait et al., (2015) revealed the gender females (60%) was higher than males among Saudi patients [34]. Concerning the age group, many studies reported a significant association. In a Korean study involved 1528 children with acute RTI, there were 178 patient positive for HBoV. The mean age of the patients was 24 months [35]. According to phylogenetic analysis of HBoV, to the best of our knowledge; the current study is the first study in Iraq so the results of phylogenetic analysis for HBoV DNA isolated from NPS revealed 8 isolates. After the alignment of these local isolates sequence with 18 references isolates by software mega 6 software, all these isolates were found to be related to HBoV type 1. Many studies in neighboring countries showed similar results with the current study. In 80 children with RTI from Saudi Arabia reported that HBoV infections was only type 1[16]. Another study in Iran involved 140 children with acute
RTI less than two years old during fall and winter, was the same result [15]. Globally, the result of HBoV study in Cameroonian children with acute respiratory infection was only type 1 [36]. This indicates that HBoV1 may be one of the common pathogens responsible for the hospitalization of children with acute lower respiratory tract infection with symptoms of wheezing. According to phylogenetic analysis, all 8 local isolates was not identical to any references isolate but the compatible was 99% with different isolates references. The reason for not full identity may because of the current study was the first study in Iraq by sequences. Therefore, recent study revealed less than 100% identity between reference isolates from Gene Bank and Korean HBoV isolates, the similarity was 98.77-99.5% [37]. Another study revealed the identity sequence between Iranian HBoV isolates and the Gene Bank isolates were 98.95-99.88% [24]. In a group1 (four local isolates) were close to Iranian isolates, these isolates were existence in 50% from all local isolates. The reason for close to Iranian isolates may be due to the closeness geographical between our country and Iran and the volume of visitor during trade exchange or during religious tourism. Group2 (two local isolate), group3 (one local isolate) and group4 (one local isolate) were close to Tunisian, Italian and Argentinian isolates respectively. Existence of these isolates in Iraq may be due to foreign visitors during tourism or may be all these isolates are international existence. In the current study, all 8 local isolates sequence alignment with 3 isolates reference sequence by using EMBOSS Transeq to corresponding amino acid sequence between local and reference isolates, the sequence of amino acid was varies in sequence of amino acid especially in isolates 1, 5 and 6. Isolate 1 had four deletion of amino acid and isolate 5 had two deletion of amino acid and isolate 6 had 3 substitutions. Likewise, another variant in sequence of the local isolates separate these isolates into two groups (each one four isolate), both of them had one substitution of amino acid in the same position but all group had different amino acid. Of note, the last two substitutions exist in different references isolates. In regard to substitution and deletion of amino acid in the current study, many studies had diversity in the nucleotide and amino acid sequence. Chinese study conducted by screening 993 respiratory samples for HBoV by PCR revealed NS1 exhibited evolution substitutions. The nucleotide deletions and substitutions occurred in NP1 and VP1 represented novel molecular signatures enabling subtype differentiation between HBoVs [38]. Another Italian study on unhealthy children with respiratory disease, seven of the 13 strains had mutation in one of the sites of vp1/2 gene [39]. Some studies revealed the relationship between viral load and mutation of amino acid, study done by Hao et al.,(2013) revealed that few nucleotide changes were correlated with a lower viral load [40]. Otherwise, another study revealed a double mutant was observed in samples with a significantly higher viral load [39]. In conclusion, infection rate of HBoV-1 comparable with result of neighboring countries, local isolate of HBoV-1 have their characteristic genetic structure which differentiation them from other international isolate. Most local isolates are close to Iranian isolates. Further studies could be occurred to determine the diversity in the prevalence of HBoV and define the local strain.

References


