Detecting and Genotyping of Human Bocavirus among Children with Gastroenteritis in Diyala Governorate

Ghasaq Mundher Ali¹*, Areej Atiyah Hussein², Jalil Ibrahim Kadim³, Iman M Aufr⁴

¹BSc, Department of Microbiology, College of Medicine, University of Diyala, Iraq
²Ph.D, Department of Microbiology, College of Medicine, University of Diyala, Iraq
³FICMS, Department of pediatrics, College of Medicine, University of Diyala, Iraq
⁴MSc, National Influenza Center - Central Public Health Laboratory, Iraq

*Correspondence: Ghasaq Mundher Ali, Email: ghasaq094@gmail.com

Abstract

Human Bocavirus a newly detected parvovirus initially described as a respiratory pathogen, to be a possible causative agent of gastroenteritis in children. This cross-sectional study was carried out for patients with acute gastroenteritis who present in the Emergency Department of Pediatrics in Al-Batool Teaching Hospital for Maternity and Children in Baqubah city, during the period from July 2019 to March 2020. The study population consists of 100 children with diarrhea collected from children less than five years old (58 males, 42 females) to detect human Bocavirus by conventional polymerase chain reaction. Human Bocavirus was detected in 9 samples (9%), high infection observed in males (66.66%) and in age group 6-12 months (55.55%). Most infection occur in children whose mothers were illiterates and primary education with equal percentage (44.44%). The genetic analysis of the sequence of four samples (4 out of 9 positive case) showed three of them belong to HBoV-3, whereas the fourth belong to HBoV-2.

Keywords: Acute gastroenteritis, human Bocavirus, molecular detection


Introduction

Acute gastroenteritis (AGE) is the leading cause of morbidity worldwide especially among children under five years old and elderly population. Diarrheal diseases remain the second cause of death among children <5 years old, mostly in low- and middle-income countries. According to the Centers for Disease Control (CDC), viral gastroenteritis infections can account for over 446,000 deaths of children per year worldwide [1]. Human enteric viruses are considered to be the major cause of acute diarrhoea in young and belonging to different taxonomic groups. Many viruses such as rotavirus, adenovirus, human norovirus, human astroviruses, and Sapovirus have been known to associate with these diseases and also, human Bocavirus (HBoV) has been considered as agent associated with diarrhea in humans [2-3]. Human bocaviruses belong to the family Parvovirinae, subfamily Parvovirinae. The viral particles are small non-envelop, icosahedra capsid. Genome of HBoV consists of an approximately 5.5 kb linear single-stranded DNA, possesses three open reading frames (ORFs): ORF1 encoding non-structural protein NS1, ORF2 encodes nuclear phosphoprotein NP1 and ORF3 encodes viral capsid proteins VP1/VP2 [4]. Human Bocavirus is the second human pathogen parvovirus and divided into four species; HBoV-1 were predominantly identified in respiratory samples whereas the three other types, HBoV-2, HBoV-3 and HBoV-4, have been detected in fecal samples and described as enteric viruses [5]. Human Bocavirus can be transmitted through direct contact with infected faces (faecal-oral-route), ingestion of contaminated food/water, person-to-person contact and poor personal hygiene [6]. The main methods of diagnosing the human Bocavirus by conventional PCR and real time PCR may be due to the little success of the serological and cultural techniques of the virus [7]. Very limited studies have been conducted in Iraq focusing on the relationship between human Bocavirus and respiratory tract infections such as [8-10] and no one about related the virus with gastrointestinal tract so the current study design to detecting and genotyping of human Bocavirus in children under five years old with gastroenteritis in Diyala governorate.
Materials and methods

Study design
Cross-sectional study was carried out for children under five years with acute gastroenteritis who present in the Emergency Department of Pediatrics in Al-Batool Teaching Hospital for Maternity and Children in Baqubah city, during the period from July 2019 to March 2020.

Stool samples collection and DNA extraction
Stool samples were collected from 100 children suffering from acute gastroenteritis; their ages were ranged from 1 month to 60 months. The stool samples were collected using disposable gloves and wooden sticks to transfer small amounts of diarrhea to Eppendorf tubes and labeled it. These were putted in deep freeze (-20 to -80°C) for store. The DNA was extracted from stool samples using Presto™ stool DNA extraction kit (Geneaid, Taiwan).

Gene amplification by conventional PCR
The sequence of primers listed in Table (1) was used in this study to amplify 495-nt fragment within the ORF of NS1 of Human Bocavirus 1-4 [11, 12].

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence 5′-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBoV2-sf2</td>
<td>(5′-TGCTTCAACAGGCAAAACAAG-3′)</td>
</tr>
<tr>
<td>HBoV2-sr2</td>
<td>(5′-TCCAAGAGGAATGAGTTTGG-3′)</td>
</tr>
</tbody>
</table>

Primer preparation
All primers were supplied by (XXIDT company- Belgium) in lyophilized form. Primers were dissolved in PCR water (for 100μM from forward and reverses primers added 747μl, 659μl respectively) to prepare a stock solution of a final concentration (100 pmol/μl) and the stock was kept at (-20). A working solution concentration (10 pmol/μl) was prepared by adding 10μl of stock solution to 90μl of PCR water.

Protocol of conventional PCR
A conventional PCR was performed to amplify the NS1 gene; a (495bp) fragment was amplified by using forward and reverse primer. PCR reaction mix was performed in (25μl) total volume, (3μl) template DNA, (1μl) of each forward and reverse primer and (15μl) PCR water, all of these were added to tube contain 5μl of PCR Pre Mix according to protocol kit (Accu Power® PCR Pre Mix kit, V2/2017, Bioneer, Korea) then thermal cycling was listed in Table (2).

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Denaturation</td>
<td>94 °C</td>
<td>5 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>30 second</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>58 °C</td>
<td>30 second</td>
<td>40 cycles</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>30 second</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72 °C</td>
<td>5 minutes</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

Sequencing of PCR products and data analysis
Successful amplification of the target regions (NS1) of human Bocavirus 1-4 were done by conventional PCR, (25 μl) of product together with primers, were sent to Macrogen in South Korea for direct sequencing. Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov) and Bio Edit program.

Statistical analysis
The statistical package for social sciences (SPSS), version 22 was used for statistical analysis of all data. Analyzed categorical variables were expressed as frequency and percentage.

Results
Relating to the result of conventional PCR, there are 9 (9%) samples which give positive result (9 out of 100) for human Bocavirus among children with acute gastroenteritis as show in Figure (1) after electrophoresis on the agarose gel as show in Figure (2).
In the study population, 9(9%) specimens were positive for HBoV, according to type of gender 6 (6 out of 9 positive samples) were males and 3 were females. High infection rate (55.55%) among children in the age group (6-12 months), followed by (22.22%) of them were for each age group (0-5 months) and (13-24 months), and no infection were noticed for children in the age group (25-36 months). During this study, the mothers of infected children with illiteracy and primary education with an equal percentage (44.44%) were the highest, (11.11%) with secondary education, while no infection were observed for children whose mothers were in higher education.

**Table (3):** Distribution of human Bocavirus according to demographic factors

<table>
<thead>
<tr>
<th>Variable factors</th>
<th>Negative No. (%)</th>
<th>Positive No. (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>52(57.14%)</td>
<td>6(66.66%)</td>
<td>58</td>
</tr>
<tr>
<td>Females</td>
<td>39(42.85%)</td>
<td>3(33.33%)</td>
<td>42</td>
</tr>
<tr>
<td>Age group (month)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5</td>
<td>41(45.05%)</td>
<td>2(22.22%)</td>
<td>43</td>
</tr>
<tr>
<td>6-12</td>
<td>35(38.46%)</td>
<td>5(55.55%)</td>
<td>40</td>
</tr>
<tr>
<td>13-24</td>
<td>12(13.18%)</td>
<td>2(22.22%)</td>
<td>14</td>
</tr>
<tr>
<td>25-36</td>
<td>3(3.29%)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Mother educational level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Illiterates</td>
<td>40(43.95%)</td>
<td>4(44.44%)</td>
<td>44</td>
</tr>
<tr>
<td>Primary education</td>
<td>29(31.86%)</td>
<td>4(44.44%)</td>
<td>33</td>
</tr>
<tr>
<td>Secondary education</td>
<td>11(12.08%)</td>
<td>1(11.11%)</td>
<td>12</td>
</tr>
<tr>
<td>High education</td>
<td>11(12.08%)</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>91(100%)</td>
<td>9(100%)</td>
<td>100</td>
</tr>
</tbody>
</table>

**Genetic analysis and phylogenetic tree**

The genetic relatedness of the local isolates was analyzed with MEGA X software through constriction phylogenetic tree (Figure 3). The four local isolates under sequence ID: MT461694.1, MT461695.1, MT461696.1, MT461697.1 are with two type of HBoV, isolates 1,2,3 related to HBoV type 3, while isolate 4 related to HBoV type 2 as indicted from the figure and HBoV type 1 clustered away from the local and reference isolates belonging to type 2,3. The result of this analysis confirmed that is of p-distance. Isolate 1, 2, 3 clustered with GQ867666.1 (HBoV type 3Brazilian isolation). Isolate 4 clustered withKX962154.1 (HBoV type 2Egyptian isolate).
Figure 3: Phylogenetic tree for NS1 gene (HBoV) constructed by Neighbor-Joining method, involved 19 nucleotide sequences (15 sequences reference from Gene Bank). The evolutionary distances were computed using the Maximum Composite Likelihood method. Evolutionary analyses were conducted in MEGA X.

Discussion

The rate of human Bocavirus according to the result of conventional PCR was 9% (9/100 cases). This percentage is close to that of its prevalence in many countries, such as in Iran [13], Taiwan [14], Turkey [15] and Albania [16], where the rates were 8%, 8.5%, 8.7%, 9.1% respectively, while this percentage was higher than in other countries such as Russia [17], Egypt [18], South Africa [19], India [20], Bangladesh [21] and Beijing- China [22]. This wide variation among studies could be attributed to the time and period of samples collection, number of samples, the age of the study population, the season, the techniques are used to diagnose the virus, variations in habits, geographical differences, climate changes and the immunological and nutritional situations of individuals, all of these factors may contribute to the difference in the prevalence rates of the virus between studies. In the present study, the rate of human Bocavirus was higher in males (66.66%) than females (33.33%). This result agreement with (Alam et al., 2015) in Pakistan [23] and (Sharif et al., 2020) in Bangladesh [21]. While Rikhotso et al., (2019) in South Africa reported that the distribution of HBoV was higher in females than in males [24]. On the other hand (El-Mosallamy et al., 2015) in Egypt [18] and (Mohammadi et al., 2020) in Iran [25] found that the proportion of HBoV positive specimens was equally distributed between the two genders. We suggest the result of this study may be due to the number of male samples collected is higher than the number of female samples, in addition to the superiority of males over females in terms of physical activity in the stages of breastfeeding and childhood. The distribution of human Bocavirus in the current study showing most infection (55.55%) was observed in infant among age group (6-12) months, following by equal percentage (22.22%) for each age group 0-5 months and 13-24 months, while no infection noticed in age group (25-36). This is in agreement with studies carried out in Egypt [18], India [20], South Africa [24] and Brazil [26]. The higher risks at ages 6-12 months and 13-23 months, may be due to diminish of immunity, as the amount of trance-placental antibodies of the child starts dwindling after 6 months of age in addition could be attributed to the fact that children at these age are either crawling or walking and can easily pick dirt or other contaminated objects for playing or eating [27]. The cause of decreased infections in the first six months of birth may be the result of breastfeeding and acquired immunity transmitted to the fetus from the mother through the placenta. While the cause of less infection at the age of above 24 months may be due to previous infections that give the child immunity to the disease [28], according to educational level of mothers of the children infection with human Bocavirus. The results of this study showed an equal percentage (44.44%) for each of the mothers who are illiterate and with primary education. In addition to (11.11%) of the mothers with secondary education, while no infection with mothers which have high education. These results corresponded with the study conducted by (Alaa et al., 2014) in Baghdad, where they explained that the Parents with low levels of education are more likely to have poor nutritional knowledge. It’s found that maternal nutritional knowledge is crucial for decreasing the illness [29]. Uneducated mothers or those with primary education are less knowledgeable about the causes of diarrhea and ways to deal with it, as well as lack of knowledge of the risks of diarrhea for infants and children that may lead to death. The result was a lack of knowledge and education that some of the mothers of these children were using the cow’s milk powder full-fat (not intended for infants ex: Al-Mudhish, and some of them used baby milk, but it was not appropriate for the age of the infant, in addition to use the pacifier for the purpose of silencing the child from crying without wash or sterilization as well as the lack of awareness and poor hygiene. According to the results of phylogenetic analysis for human Bocavirus isolate from stool samples revealed 4 isolates (Sequence ID: MT461694.1, MT461695.1, MT461696.1, MT461697.1). These local isolates sequence alignment with 15

http://doi.org/10.36295/ASRO.2020.231437
references isolates software Mega X, the first three isolates were found to be related to HBoV type 3 while the last isolate related to HBoV type 2. The three isolates A6, B7, C8 cluster with human Bocavirus type 3 strain MC8 from Brazil with identity 99%, the nucleotide sequence of A6 and C8 isolates contains two substitutions (two transition) whereas the B7 isolate contain four substitutions (two transition and two transfusion). While GAJ6 isolate cluster with HBoV-2 strain BI-8 from Egypt with identity 99% and the nucleotide sequence contain one substitution (transition). The phylogenetic analysis of HBoV isolates has demonstrated a high similarity between the nucleotide sequences of the isolates belonging to the same Bocavirus genotype but originating from different geographic regions. This may be elated with studying the same part of the NS1 gene and suggests multiple origins of HBoV in Iraq. They were probably introduced into the country at different times, from different places. Babkin et al., 2013 suggests a high potential of HBoV distribution over the globe, taking into account a high rate of genetic variation displayed by this virus [9]. The distribution of human Bocavirus genotype in children with acute gastroenteritis has been shown to vary by country, study period, type of specimen and study population [20]. Also, it may be the result of the difference in the studied gene region. In this study, we did not obtain isolates for human Bocavirus type 1. Although it is closely related to respiratory diseases, it has been detected in fecal samples in some studies and suggestion that HBoV-1 may be a pneumoenteric virus, with infection starting on respiratory surfaces before spreading to the intestines [19, 30]. The number of human Bocavirus type 3 isolates was higher than human Bocavirus type 2 in this present study and this corresponds to [19] compared to previous studies that described HBoV type 2 more common [31, 32]. The widespread distribution of HBoV3 in comparison to HBoV2 could presumably be due to differences in pathogenesis that may influence their transmission route and ability to establish persistence [33]. Two isolates (one of HBoV type 3 and one of HBoV type 2) were suffering from chest inflammation with a cough before starting the symptoms of gastroenteritis; we suggest that it infect the respiratory system and then move to the intestine. In particular, the two types were detected in other studies in respiratory samples. We were unable to detect HBoV4 in this study. This was agreement with other studies [34-35], which makes the role of HBoV4 genotype unclear.

Conclusions
The rate of human Bocavirus infection was (9%) in children with acute gastroenteritis in Diyala governorate. Most infection rate in males, among ages 6-12 months and most of the mothers of the infected children are uneducated or with primary education. The genetic analysis of the sequence of four samples (4 out of 9 positive cases) showed three of them belong to HBoV-3, whereas the fourth belong to HBoV-2.

References