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Evaluation of Human Bocavirus (HBoV) as A Cause of Acute Gastroenteritis in Children Under 5 Years: A Case-Control Study

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Abstract

Background Acute gastroenteritis (AGE) is the main cause of infants and children's health problems, in developing countries, high morbidity and mortality rates were reported, Human Bocavirus (HBoV) are enteric viruses that linked to cause AGE.

Objective To estimate the prevalence of HBoV infection in children under five years with AGE and to determine its genotypes by using conventional polymerase chain reaction and Sanger sequence.

Methods This is a case-control study, the sample was collected from 100 children under 5 years of age suffering from AGE (as case group) and from 100 asymptomatic control children (as control group) from Central Teaching Hospital of Pediatrics from December 2021 to April 2022. Each children's stool sample taken was stored at -20°C until they were employed in a conventional polymerase chain reaction to identify the HBoV genotypes.

Results The rate of HBoV infection was found in 10% in AGE case group, while none of control group showed positive result. The genetic analysis showed that six of the investigated samples belonging to the HBoV-2 while four of the investigated samples belonging to the HBoV-3. In HBoV infected children, the age range (1-13 months) had most positive cases 5 (50%) and males had a greater infection rate (80%) than females (20%), however, non-significant association was found in regard to age and sex (P>0.05).

Conclusion A low rate of HBoV DNA was detected in children under five years with AGE suggesting that other pathogen may responsible for infection.

Keywords Human Bocavirus, acute gastroenteritis, children, risk factors, sequencing, phylogenetic tree.

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List of abbreviations: AGE = Acute gastroenteritis, CDC = Centers for disease control, HBoV = Human Bocavirus, NP1 = nuclear phosphoprotein, NS1 = Nonstructural protein 1, PCR = Polymerase chain reaction, RV = Rotavirus

Introduction

Acute gastroenteritis (AGE) is a frequent cause of illness and death in young children and infants, one to five episodes of acute diarrhea may occur in a children under five years every year. Global Health Data Exchange reports that in 2016 diarrhea was fifth leading cause of death among children younger than 5 years, nearly 40% of these deaths are thought to be related to rotavirus (RV), and the majority take place in low-income countries. The most prevalent AGE agents are viruses, the centers for disease control (CDC) estimate that about 446,000 child deaths globally are caused by viral gastroenteritis infections each year [(1)]. Many

human enteric viruses, including human RV, human norovirus, human astrovirus, and Sapovirus, have been linked to cause children diarrhea or AGE, moreover, human Bocavirus (HBoV) is now thought to be a factor in diarrheal gastroenteritis in children [2,3].

HBoV are members of the Parvoviridae family and Parvovirinae subfamily. The HBoV particles are small non-envelopd, icosahedral capsid. A single-stranded DNA, linear, 5 kb genome with two different variants of the nonstructural protein (NS1), a nuclear phosphoprotein (NP1), and two structural capsid proteins (VP1 and VP2) are all encoded by three open reading frames [4]. HBoV are classified into four species, three varieties including HBoV-2, HBoV-3, and HBoV-4, have been reported as enteric viruses that are primarily discharged in diarrheal stools. While HBoV-1 has been found in the respiratory system [5].

Serological and cultural methods for detection the virus have a limited success, so conventional polymerase chain reaction (PCR) and real time PCR considered the main methods of diagnosing HBoV [6].

In Asia, there was a 5.47% frequency of HBoVs in children under the age of 5 who had AGE, 4.07% in Europe, 6.90 % in America, and 29.0 % in Australia [7].

Because to a lack of permissive cell lines and effective animal models, the processes of bocavirus latency, persistence, and reinfection are currently poorly understood. Moreover, the investigation of the pathogenicity of bocaviruses is further complicated by the concurrent infections of HBoV with other viruses [8,9].

The current study aimed to determine the frequency and the genotypes of HBoV in children with acute gastroenteritis and asymptomatic children by conventional PCR and Sanger sequencing.

Methods

Subjects

In this case-control study, samples were taken from 100 children under the age of five years who had AGE (as case group) and from 100 asymptomatic control children (as control group) from Central Teaching Hospital of Pediatrics in Baghdad from December 2021 to April 2022.

Samples collection

Stool samples were taken from 100 infants with AGE, 58 of whom were males and 42 of whom were females, and whose ages varied from 1 month to 60 months. Little quantities of diarrheal feces were transferred to Eppendorf tubes using wooden sticks and disposable gloves, then the samples were labeled. Samples were transported to the laboratory after collection at 4˚C, after that they were aliquoted to avoid repeated freezing and thawing then stored at -20˚C until further analysis.

Isolation of viral DNA

DNA was isolated from 200 mg of stool to check for the presence of HBoV nucleic acids. Using the EasyPures Stool DNA Kit for nucleic acid extraction following the manufacturer instructions.

PCR detection of HBoV DNA

A conventional PCR was performed to amplify the HBoV (1-4) VP1/VP2 sequences according to Abdel-Moneim et al. (2016) [10], (453bp) fragment was amplified by using (Pan-Boca forward primer 4808) and (Pan-Boca reverse primer 5241). PCR reaction mix was performed in (25 µl) total volume (5 µl) template DNA, (1 µl) of each forward and reverse primer and (5.5 µl) nuclease-free water, all of these were added to tube contain 12.5 µl of PCR super mix and amplified according to thermal cycling protocol listed in table (1).

Sanger sequencing and phylogenetic tree construction

The reverse termini of the 453 bp resolved PCR amplicons were sequenced commercially according to company's instruction manuals (Macrogen Inc. Geumchen, Seoul, South Korea). Only chromatographs from ABI (Applied Biosystems) that are clear sequence

files were further analyzed, confirming that the changes and annotation are not the result of PCR or sequencing artifacts. The virtual locations and other information of the obtained PCR fragments were determined by comparing the observed DNA sequences of the viral samples with the retrieved DNA sequences of the viral database. All the investigated and analyzed sequences were submitted to the National Center for Biotechnology Information (NCBI) through the web-based GenBank submission tool (BankIt portal) and all the instructions described by the portal were followed as described by the server. Each submitted sequence was provided as nucleic acid sequences and translated to its corresponding reading frame in the NCBI to get unique GenBank accession numbers for the investigated sequences. In this work, a specific comprehensive tree was built using the neighbor-joining methodology. The NCBI Nucleotide Basic Local Alignment Search (BLASTn) service was used to compare the detected variations to their nearby homologous reference sequences. Then, using the Interactive Tree of Life (iTOL) tool suit, a conventional rectangular tree with the observed variation included was constructed using the neighbor-joining technique.

Table 1. Stages and temperature of PCR

Protocol		Temperature	Time	cycle
Stage 1	Initial denaturation	94°C	30 sec	1
Stage 2	Denaturation	94°C	5 sec	40
	Annealing	56°C	30 sec	
	Extension	72°C	30 sec	
Stage 3	Final extension	72°C	600 sec	1

Statistical analysis

Statistical Package for the Social Sciences IBM SPSS statistics version 26 was used to detect the effect of different factors on study parameters. One-way ANOVA and T-test was used to significantly compare between means. Chi-square test was used to significantly compare between percentages (0.05 and 0.01 probability).

Results

Rate of HBoV infection

Based on the result of conventional PCR of this study, ten of the case samples among children with AGE yielded a positive result for HBoV (10 out of 100). But none positive results seen in control group samples (0 out of 100), as shown in table (2) and figure (1). Age group distribution of cases showed (50%) of the positive cases were in age group (1-13) months, (30%) of the positive cases were in age groups (14-26 months) and (20%) were in age group (27-40) months. According to sex distribution, 8 of the 10 cases of HBoV infection in children were male and two were female as shown in table (3).

Table 2. Rate of HBoV infection in case and control samples according to conventional PCR

HBoV DNA	Case	Control	Statistical analysis
Positive	10	0	**P=0.001**
Negative	90	100 (100%)	**Chi-square = 10.52**
Total	100	100 (100%)	

Table 3. Rate of HBoV infection in children with acute gastroenteritis according to age and sex

Parameters		HBoV DNA status Positive (n=10)	Negative (n=90)	Total	Statistical analysis
Age groups (month)	1-13	5 (50%)	46 (51.1%)	51	Chi-square= 0.14 P=0.937
	14-26	3 (30%)	30 (33.3%)	33	
	27-40	2 (20%)	14 (15.5%)	16	
	Total	10(100%)	90 (100%)	100	
Sex	Male	8 (80%)	50 (55.5%)	58	Chi-square = 2.2 P=0.148
	Female	2 (20%)	40 (44.4%)	42	
	Total	10(100%)	90 (100%)	100	

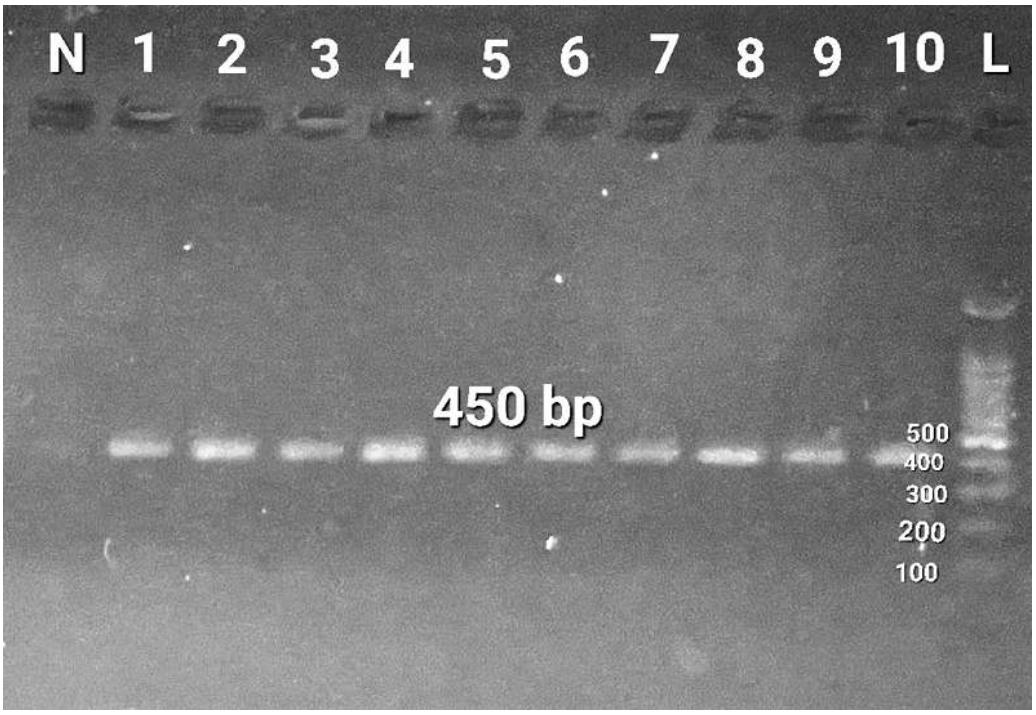

Figure 1. Agarose gel electrophoresis image show positive bands (1, 2, 3, 4, 5, 6,7, 8, 9 & 10) for HBoV in size 450bp, for VP1/VP2 on 1.5% agarose gel, electrical power was 100V, 70A for 30 min. L: DNA ladder (100-1000bp) N: negative control well

Phylogenetic tree

The present work produced an inclusive phylogenetic tree based on the observed nucleic acid sequences and two genotypes were detected in the investigated samples (genotype 2 and 3). This phylogenetic tree contained all six isolates of HBoV 2 sequences (S2, S3, S4, S6, S9, and S10), and the four isolates of HBoV 3 sequences (S1, S5, S7, and S8) alongside other relative reference sequences. Due to the presence of four main genotypes of HBoV, a direct comparison between our samples with the previously known reference genotypes was conducted to find out the accurate genotyping of S1 – S10 samples within the main four groups of these viral particles. Therefore, our viral isolates (S1 – S10) were directly compared with several representative reference samples belonging to the main four viral genotypes, ranging from genotype-1 to genotype-4 within the generated phylogenetic tree. Accordingly, variable representatives for HBoV genotypes 1, 2, 3, and 4 were incorporated within the generated tree based on the VP1/VP2 sequences to get much more accurate insight into the actual phylogenetic distances among them (Figure 2).

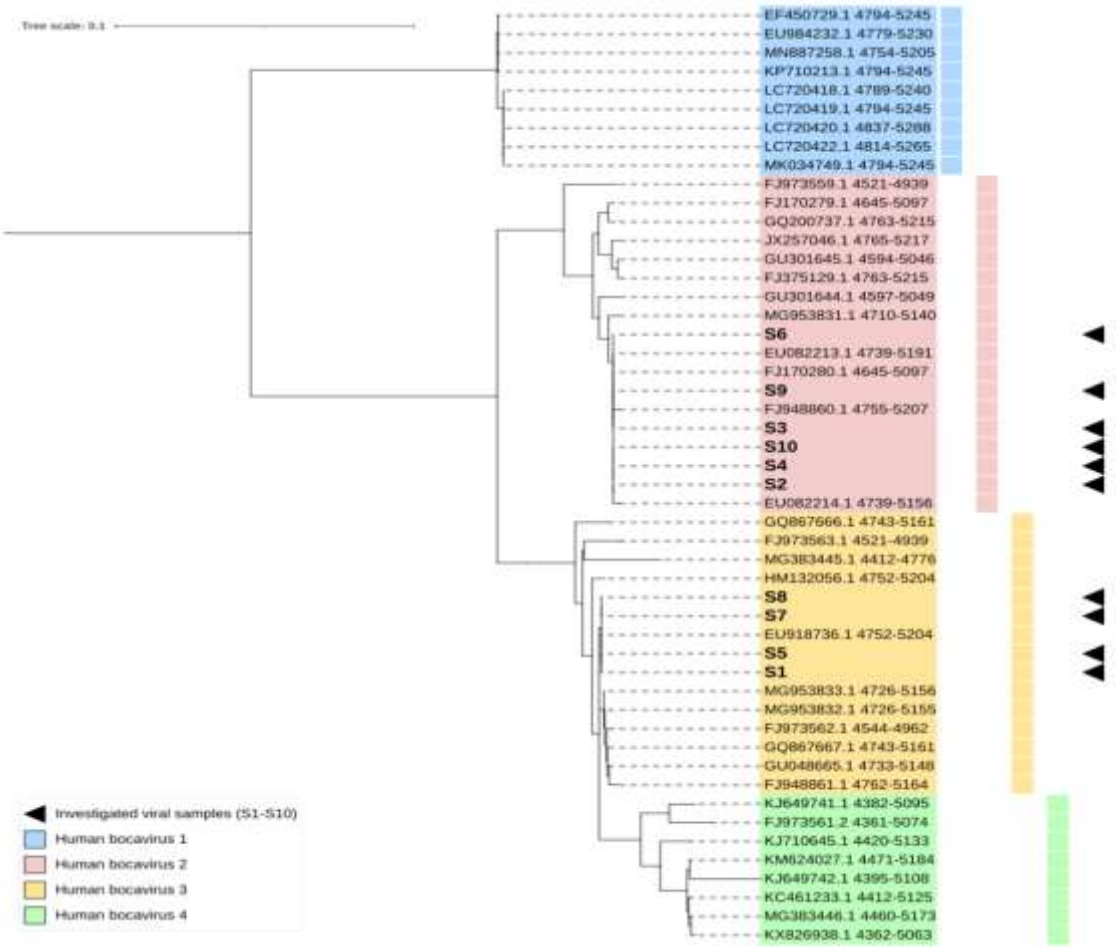

Figure 2. The detailed rectangle phylogenetic tree of the VP1/VP2 locus genetic variations in the local 10 human bocavirus isolates. The different-colored numbers represent the different genotypes that were used. The degree of phylogenetic locations among the tree-categorized viral species is shown by the scale of the left half of the tree. The letter "S" stands for the sample codes under investigation

Discussion

This study shows that 10% infection rate with HBoV after testing 100 stool samples of children under 5 years of age suffering from AGE from Central Teaching Hospital of Pediatrics (Baghdad) by using conventional PCR, the results were consistent with other research performed globally like 9% in Diyala-Iraq [11], 8.7% in Turkey [12], 8.5% in Taiwan [13] and 8.1% in Germany [14]. This research's infection rate is lower than that obtained in 14.4% in Iran [15], 13.92% in Italy [16] and 13% in Pakistan [17]. Limited investigations have been conducted on the HBoV in Iraq and the other Arab nations and many of these studies focused on researching the virus and how it can affect the respiratory system like (Atyah et al., 2017) [18], (Rasheed et al., 2019) [19] and (Hasan et al., 2020) [20], (Al-Shuwaikh, 2021) [9] in Iraq, (Essa et al., 2015) [21] in Kuwait, (Bubshait et al., 2015) [22] in Saudi Arabia and (Al-Rousan et al., 2011) [23] in Jordan. The methods used to identify the virus may have a significant impact on how differently prevalent of HBoV in different research, Real-time PCR has been used in several research to diagnose the human bocavirus such as (Amr et al., 2017) [24] (Abdel-Moneim et al., 2018) [25] while the conventional-PCR was used in this study. The season of the year when samples were collected may also have an effect on the risk of HBoV. Samples of this study were collected during the winter season (from December 2021 to April 2022). While different finding in other research; the spring months are the peak of HBoV prevalence [26] and in another study the peak of HBoV was in summer [27].

Although this study showed no significant association between HBoV infection and age or sex of children with AGE, the results of the current study showed that 50% of infections were found in children aged 1 to 13 months, with three cases found in children aged 1 to 5 months and two cases found in children aged 6 to 12 months (6-12 months), three cases between (14-26 months) and only two cases showed between (27-40 months). These results are similar to the results of many investigations such as Rikhotso et al., (2020) in South Africa [28], El-Mosallamy et al., (2015) in Egypt [29], Lasure and Gopalkrishna, (2017) in India [30] and Soares et al., (2019) in Brazil [5].The prevalence of HBoV in this investigation revealed that males (80%) had more infections than females (20%), these results are agreed with the studies in Pakistan (Alam et al., 2015) [17] and in Bangladesh (Sharif et al., 2020) [31].

The investigated samples of VP1/VP2 sequences were deposited in the NCBI web server, and six unique accession numbers were obtained for six analyzed sequences, GenBank OQ079962, OQ079963, OQ079964, OQ079965, OQ079966, and OQ079967 were deposited to represent the S2, S3, S4, S6, S9, and S10 samples, respectively of the analyzed HBoV 2 samples. Whereas four unique accession numbers were obtained for six analyzed sequences, GenBank OQ079968, OQ079969, OQ079970, and OQ079971 were deposited to represent the S1, S5, S7, and S8 samples, respectively of the analyzed HBoV 3 samples. In this investigation, there were more HBoV 2 isolates than human bocavirus 3 and this was disagreed with (Netshikweta et al., 2020) that was found the number of human bocavirus 3 was higher than HBoV 2 [32]. Based on the VP1/VP2 amplicons, it was inferred from this tree that the currently identified clinical isolates were positioned within two genotypes, genotype-2 and genotype-3. This study was unable to detect HBoV 4. This was in agreement with other studies such as study of (Risku et al., 2012) and (Rikhotso et al., 2020), which say the role of HBoV 4 genotype is unclear [28,33].

In conclusions, the current investigation found HBoV infection in children with AGE while the control group was negative, indicating a role for HBoV in the pathogenesis of AGE in children under the age of five. However, more research is needed to determine whether HBoV is a mono infection or a co-infection in AGE.

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Author contribution

Mohammad: performed the laboratory works and wrote the draft of this paper. Dr. Al-Shuwaikh designed, supervised and interpreted the results of this work, Dr. Al-Hamdani contributed to sample collection and clinical aspects. The final version of this manuscript was read and approved by all the authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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