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RESEARCH ARTICLE

Polymerase Chain Reaction Study of Human Bocavirus in Children with Acute Gastroenteritis

Noha Mostafa Mahmoud[1], Maysaa El Sayed Zaki[2,*], Abdel-Rahman Eid[3], Mai Esam Ahmed[4], Eman Hamdy Mohamed[4] and Ehab Mohamed Fahmy[5]

[1]*Medical Microbiology, and Immunology Department, Mansoura Faculty of Medicine, Mansoura, Egypt*
[2]*Clinical Pathology Department, Mansoura Faculty of Medicine, Mansoura, Egypt*
[3]*Pediatric Department, Genetics Unit, Mansoura Faculty of Medicine, Mansoura, Egypt*
[4]*Clinical Pathology Department, Beni-Suef Faculty of Medicine, Beni-Suef, Egypt*
[5]*Medical Microbiology and Immunology Department, Faculty of Medicine, Aswan University, Aswan, Egypt*

Abstract:

Aim:

The present study aimed to detect Human bocavirus (HBoV) in stool samples from young children below 5 years complaining of acute gastroenteritis (GE) in addition to detection of rotavirus, norovirus, and astrovirus.

Methods:

The study included 90 children below 5 years with acute GE with excluded bacterial pathogens. The determination of the presence of HBoV was performed by nested polymerase chain reaction (PCR) beside determination of astrovirus and norovirus by multiplex PCR and rotavirus antigen by enzyme-linked immunosorbent assay (ELISA).

Results:

The most prevalent virus among the studied viruses was rotavirus (33.3%) detected by ELISA for antigen in the stool. The other three viruses detected by molecular methods were bocavirus (14.4%), astrovirus (13.3%), and norovirus (10%). Mixed viral infection with two or more viruses was detected in 16 children (17.8%). The most common was bocavirus and rotavirus in 6 patients (37.5%). In the study of demographic and clinical presentations of the children with HBoV, the affected children were mainly females, *i.e.*, 10 (76.9%), from rural residence *i.e.*, 11 (84.6%) with the mild classification of GE in 7 children (53.8%) and moderate GE in 6 children (46.2%) and none of them had severe GE. Fever was the most common presenting sign in those children (53.8%**)** followed by vomiting (46.2%).

Conclusion:

The study highlights the existence of HBoV in children with acute GE under the age of five. The infection associated with this virus was either mild or moderate in severity. The combined viral infection was common especially associated with rotavirus. There is a need for further additional study to identify the type of the circulated strain of bocavirus and the confections with other pathogens.

Keywords: Bocavirus, Nested PCR, Norovirus, Astrovirus, Rotavirus, Children, Gastroenteritis.

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1. INTRODUCTION

Acute gastroenteritis (GE) is a global health problem affecting mainly children [1]. The association of viral pathogens is a common etiology of this health problem. The common viral pathogens that have been studied extensively are rotavirus, noroviruses, adenoviruses, and astroviruses [2 - 4]. The association of HBoV with this infection has been documented in several reports in countries, such as Taiwan, *etc* [5]. However, this virus has not been fully studied among children with GE in Egypt [6].

Human bocavirus is a single-stranded DNA non-enveloped

* Address correspondence to this author at the Clinical Pathology Department, Mansoura Faculty of Medicine, Mansoura, Egypt;
E-mail: maysaazaki5@hotmail.com

virus related to the viral family *Parvoviridae* and *Parvovirinae* subfamily. Bocaparvovirus genus has three open reading frames that code for 2 major nonstructural proteins, NS1 and NP1, and 2 structural proteins, VP1 and VP2 [7]. There are 4 genotypes, HBoV 1 to 4. HBoV 1 and 3 are classified within the species primate bocaparvovirus 1 and HBoV 2 and 4 within primate bocaparvovirus 2 [8]. The different genotypes were isolated from different geographical regions and different types of infections, such as HBV1 from respiratory tract infections [7], HBoV2,3,4 were isolated from children with acute GE, and HBoV2 from children with non-polio acute flaccid paralysis [9, 10].

HBoVs are associated with broad-spectrum infections, such as respiratory tract infections and acute gastrointestinal tract infections. It has been isolated from different varieties of samples, such as nasopharyngeal aspirates, fecal, blood, and cerebrospinal fluid [11 - 13].

A previous study from Egypt has detected HBoV from nasopharyngeal swabs from children with acute respiratory tract infections [14].

This study aimed to detect the presence of HBoV in stool samples from young children below 5 years complaining of acute GE in addition to detection of rotavirus, norovirus, and astrovirus.

2. MATERIALS AND METHODS

The study included 90 children below 5 years with acute GE recruited from out-patient clinics of the Mansoura University Children Hospital, Egypt from January 2019 till March 2020. The children were complaining of acute GE, as defined by WHO as diarrhea with three or more watery or loose stools within 24 hours for less than fourteen days [15]. The exclusion criteria were children aged above 5 years, and children with manifestations of gastrointestinal disturbance attributed to other etiologies, such as drug reactions, renal diseases, and liver diseases. The study was approved by the Mansoura Faculty of Medicine Ethical Committee (R.21.04.1297) and consent was obtained from their parents.

Every child was subjected to history taking and clinical examination. The clinical severity of the disease was evaluated by child examination and parents/guardians interview by the study pediatrician using the 20-point scale of Vesikari [16] based on the frequency and severity of diarrhea, episodes of vomiting, associated fever, and dehydration. Vesikari score was considered mild if below 7, moderate from 7-10, and severe if ≥11.

A stool sample was obtained from each child in a clean, leak-proof screw-capped container and transported within 30 minutes to the laboratory.

2.1. Stool Examination

Stool samples were evaluated for the presence of visible mucus and blood. The microbiological culture was performed for possible bacterial pathogens with culture on sheep's blood, MacConkey, sorbitol-MacConkey, and *Salmonella-Shigella* agar after inoculation into Selenite F broth (BBL; Becton Dickinson). All cultures were incubated at 35°C for 24 hours. These techniques are adequate to isolate *E. coli* O157:H7, *Pleisiomonas shigelloides*, *Aeromonas* species, *Salmonella,* and *Shigella* species [17].

2.2. Detection of Rotavirus by ELISA Testing

The antigen detection for rotavirus was performed immediately after the transference of samples to the laboratory. The ELISA kit (RIDASCREEN® kit (R-Biopharm, Germany) uses a sandwich technique with the use of monoclonal antibodies to the sixth viral antigen VP6 coated to microwells. as per manufacturer's instructions 0.1 gram of stool samples were diluted in phosphate buffer saline (1:10) and centrifuged at 7,000 rpm for ten minutes. The suspension prepared from stool samples and control samples was applied to the wells of the microtiter plate with monoclonal anti-rotavirus antibodies and incubated at room temperature for one hour. It was washed trice, and streptavidin poly-peroxidase conjugate was added and incubated for 30 minutes. If rotavirus antigen was present in stool samples, a sandwich complex was formed consisting of immobilized antibodies, the rotavirus antigens, and the conjugated antibodies with the biotin-streptavidin-peroxidase. Then, it was washed again to remove the free streptavidin poly-peroxidase conjugate. The substrate was added that convert the colorless reaction in the wells of the microtiter plate to blue if the test is positive. After 15 minutes of incubation, a stop reagent was added that changed the color from blue to yellow. The extinction is proportional to the concentration of rotaviruses found in the specimen.

2.3. Nucleic Acid Extraction

The nucleic acid extraction was performed immediately after the transference of samples to the laboratory and the extracted nucleic acid was stored at -20°C until further amplification. Fecal samples were subjected to DNA extraction for bocavirus detection by nested PCR and RNA extraction for multiplex PCR detection of norovirus and astrovirus.

For DNA, commercially available QIAamp Stool Mini Kit 51604(Qiagen, 19300 Germantown Road Germantown, MD 20874, USA) was used according to the manufacturer's instructions. DNA binds specifically to the QIAamp silica-gel membrane while contaminants pass through it. For extraction of viral RNA, QIAamp® Viral RNA Mini Kit (Qiagen, Hilden Germany) was used according to the manufacturer's instructions.

2.4. Multiplex Reverse Transcriptase (RT) PCR of Norovirus and Astrovirus

Both RT step and PCR were performed in the same tube using 3mL of extracted RNA. The protocol of the amplification was followed according to Rohayem *et al*. The used primers are summarized in Table **1** [18].

2.5. Nested PCR Amplification of Bocaviruses

HBoV DNA was amplified by nested PCR with primers, as given in Table **1**. In the first amplification procedure, the amplification volume was 50 microliters supplied from the Qiagen amplification mixture. The used extracted DNA was 5 microliters with 0.5μmol/L of HBoV NS1 primers. The

amplification procedure was as follows: denaturation at 94°C for three minutes, thirty-five cycles of amplification (40 seconds at 94°C, 30 seconds at 62°C, 65 seconds at 72°C), and final extension at 72°C for five minutes. This amplification produced a 960-bp of NS1 coding for a non-structural protein [19].

Table 1. The sequences of the used primers to detect viruses and the amplified base pair (bp).

Virus	Sequences of the Primers	Base Pair
Norovirus I	5′-ATGGTGATGATGAAATAGTGTC-′3 5′-ATTTCGGGCAGAAGATTG-′3	490 bp
Norovirus II	5′-GCACACTGTGTTACACTTCC--′3 5′-50ACATTGGCTCTTGTCTGG-′3	822 bp
Astrovirus	5′-CGTCATTGTTTGTTGTCATACT-′3 5′-0ACATGTGCTGCTGTTACTATG--′3	347 bp
HBoV NS1	5′-GGACGTGGT**S**CGTGGGAAC-′3 5′GTCCTGTGAATG**W**GTAGGACAAAGG-′3	960 bp
HBoV2ndNS1	5′-CC**W**GTAATTAT**W**TCCACTAACCA-′3 5′-AGAGTACA**K**TCGTACTCATT**R**AA-′3	200 bp

In the second amplification, HBoV NS1 2nd primers were used to detect all HBoVs genotypes. The reaction volume was 50 microliters containing two microliters of the 1st PCR product and 0.5µmol/L of HBoV NS1 2nd primers. The amplification procedure was: three minutes at 94°C, thirty cycles of amplification (30 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C), and at 72°C for 5 minutes.

PCR products were detected by electrophoresis in 1.5% gel for 30 minutes and visualized by ultraviolet light.

2.6. Statistical Analysis

The data of the study was analyzed by SPPS 22. The numerical data were expressed as mean and standard deviation (SD), median, minimum and maximum. The numerical data were compared by T-test, and the P-value was considered significant if it was less than 0.05. The qualitative data were expressed as numbers and percentages. The comparison between qualitative data was made by the use of chi-square and the P-value was considered significant if it was less than 0.05.

3. RESULTS

The study included 90 children with acute GE with ages from 6 months up to 50 months. The included children had no bacterial pathogens associated with GE. There were 41 males and 49 females. The main presenting symptoms were fever (55.6%), followed by abdominal pain (37.8%). The prevalence of acute GE was slightly higher in the autumn season (31.1%) and summer season (28.9%).

The severity of the GE was mainly mild (47.8%). The children were mainly from rural residences (57.8%). At least one virus was detected in 47 of the studied children (52.2%) (Table **2**).

Table 2. Demographic and clinical data of the studied children.

Clinical and Demographic Data	No. of Patients (%)
Age (Months)	
Mean ±SD	20.22± 11.23
Median	16.5
Minimum	6.00
Maximum	50.0
Quartile	
Percentile 25	12.0
Percentile 50	16.5
Percentile 75	24.0
Sex	
Male	41 45.6 %
Female	49 54.4 %
Season	
Summer	26 28.9 %
Spring	16 17.8 %
Winter	20 22.2 %
Autumn	28 31.1 %
Abdominal pain	34 37.8 %
Fever	50 55.6 %
Vomiting	25 27.8 %
Vesikari classification	
Mild	43 47.8 %
Moderate	33 36.7 %
Severe	14 15.6 %
Residence	
Rural	52 57.8 %
Urban	38 42.2 %
Detection of viral pathogens	4752.2 %

Table 3. The presence of bocavirus, rotavirus, astrovirus, and norovirus among children.

Virus	No. %
Bocavirus	13 14.4%
Rotavirus	30 33.3%
Astrovirus	12 13.3%
Norovirus	9 10%
Coinfection with two or more viruses	16 17.8%
Total	90 100%

The most prevalent virus among the studied children was rotavirus (33.3%) detected by ELISA for antigen in the stool. The other three viruses detected by molecular methods were bocavirus (14.4%), astrovirus (13.3%), and norovirus (10%). From the 13 positive samples for HBoV, 7 were of mixed infections, and 6 included single pathogen (Table **3**).

Mixed viral infection with two or more viruses was detected in 16 children (17.8%) (Table **3**). The most common was bocavirus and rotavirus in 6 patients (37.5%), followed by rotavirus and astrovirus in 5 patients (31.2%), rotavirus and norovirus in 4 patients (25.0%), and bocavirus and astrovirus in 1 patient (6.2%).

In the study of demographic and clinical presentations of the children with bocavirus detected in the stool by nested PCR, the affected children were mainly females, *i.e*., 10 (76.9%), from rural residence *i.e*., 11 (84.6%) with the mild classification of GE in 7 children (53.8%) and moderate GE in 6 children (46.2%) and none of them had severe GE. Fever was the most common presenting sign in those children (53.8**%),** followed by vomiting (46.2%). As regards the association with other viruses, more than half of the detected bocavirus was associated with other viruses (53.8%) with a common association with rotavirus in 6 patients (46.2%) (Table **4**).

There was an insignificant difference in the demographic and clinical data among children infected with bocavirus and non-infected children except for the rural residence (OR 4.82-95% CI: 1.003-23.25, P=0.034) and the presence of other viruses with bocavirus as 53.8% of infected children with bocavirus had other viruses compared to 10.4% of non-infected children (OR 10.26-95%CI:2.70-37.42, P=0.001) (Table **4**).

In the comparison of demographic and clinical findings between children with single bocavirus with other children, the vomiting had a statistically significant increase in children with bocavirus as a single virus detected (66.7%, P=0.003) compared to other children (25%). The severity of the gastroenteritis was distributed as mild and moderate (50% for each) with no severe manifestation associated with bocavirus infection (Table **5**).

Table 4. Comparison of demographic, clinical, and virological findings between children with and without bocavirus.

-	Children with bocavirus (n=13) No. %	Children without bocavirus (n=77) No. %	P-value	Odds Ratio (OR)	95%CI
Sex Male Female	 3 23.08% 10 76.9%	 38 49.4% 39 50.6%	0.079	0.31	0.079-1.20
Age (mean ±SD) months	16.8 ±12.85	20.79±10.9	0.24	-	-
Abdominal pain	5 38.5%	29 37.7%	0.96	1.03	0.3-3.46
Vomiting	6 46.2%	19 24.7%	0.11	2.16	0.78-8.75
Fever	7 53.8%	43 55.8%	0.89	0.92	0.28-3.00
Vesikari classification Mild Moderate Severe	7 53.8% 6 46.2% 0 0%	36 46.8% 27 35.1% 14 18.2%	0.24		
Residence Rural Urban	 11 84.6% 2 15.4%	 41 53.2% 36 46.8%	0.034	4.82	1.003-23.25
Season Summer Spring Winter Autumn	 3 23.1% 4 30.8% 5 38.5% 1 7.7%	 23 29.9% 12 15.6% 15 19.5% 27 35.1%	0.107		
Rotavirus	6 46.2%	24 31.2%	0.289	1.89	0.57-6.23
Astrovirus	1 7.7%	11 14.3%	0.52	0.5	0.059-4.23
Norovirus	1 7.7%	8 10.4%	0.764	0.072	0.082-6.27
Mixed	7 53.8%	8 10.4%	0.001	10.26	2.70-37.42

Table 5. Comparison of demographic and clinical findings between children with single bocavirus and other children.

	Children with bocavirus (n=6) No. %	Children without bocavirus (n=84) No. %	P-value	Odds Ratio (OR)	95% CI
Sex Male Female	 2 33.3% 4 66.7%	 39 46.4% 45 53.6%	0.7	0.75	0..13-4.4
Age (mean± SD) months	64.0± 36.9	73.7± 50.3	0.6		
Abdominal pain	1 16.7%	33 39.2±	0.3	0.31	0.35-2.8
Vomiting	4 66.7%	21 25%	0.03	6.0	1.02-36.1
Fever	2 33.3±	48 57.1±	0.26	0.37	0.6-2.2
Vesikari classification Mild Moderate Severe	3 50% 3 50%	40 47.6% 30 35.7% 14 16.7%	0.0001		
Residence Rural Urban	4 66.7% 2 33.3%	48 57.1% 36 42.9%	0.64	1.5	0.26-8.6
Season Summer Spring Winter Autumn	0 0% 3 50% 2 33.3% 1 16.7%	26 30.9% 13 15.5% 18 21.4% 27 32.1%	0.094		

4. DISCUSSION

Acute GE in children associated with viral infections remains an important cause of morbidity and mortality. It is mandatory to define the possible viral pathogens associated with diarrhea for the implementation of effective interventions and avoid the use of unnecessary antibiotics [20, 21].

The study demonstrated a slightly higher occurrence of acute GE in the autumn season (31.1%) and the summer season (28.9%). In tropical and subtropical regions, such as in Egypt, acute GE can be detected due to viral pathogens in all seasons [22, 23].

The children were mainly from rural residences. This reflects the need for a safe water supply, proper sewage disposal, and insurance of proper encouragement of breastfeeding to prevent this infection [24].

In the present study, at least one virus was detected in 47 of the studied children (52.2%). A similar result (36.7%) was reported previously [25]. The most prevalent virus among the studied viruses was rotavirus (33.3%) detected by ELISA for antigen in the stool. This finding was in agreement with the previous reports from Egypt (31%) and other geographical regions in children below 5 years old [25 - 27]. The result supports the need for the routine practice of rotavirus vaccination in children to decrease this infection rate.

Among this viral etiology, bocavirus is a potential pathogen responsible for this infection. Therefore, this study was performed to study the existence of HBoV in stool samples from children with acute GE.

Nested PCR was used to detect bocavirus in the fecal samples, and HBoV was detected in 14.4% of the samples. A previous study reported a lower rate of bocavirus in Bangladesh (7.24%) [28]. However, this rate was still higher than the rates reported in earlier studies in Africa including one by El-Mosallamy *et al*. [29] that found HBoV in 2% of children less than two years in Egypt and a study by Lekana-Douki *et al*. [30] that reported 2.2% rate in children less than five years in Gabon. A recent study from Egypt detected HBoV in 58% of examined cases below 5 years. This higher prevalence can be attributed to the recruitment of children from different locations in Egypt, namely Giza [31].

Previous reports of HBoV frequency around the world showed a variation from 1.5% to 19% in South Africa [32]. In a meta-analysis study about the prevalence of HBoV in European countries, the prevalence differed from 2.0% up to 45.69% with a pooled estimate of 9.57% (95%CI7.66-11.91%) [33]. In Brazil, HBoV was detected in 12.4%(n = 110) of stool samples in children with GE [34].

The difference in the prevalence rates of HBoV may be due to the difference in the age of the included children, studied sample size, and geographical regions.

Mixed viral infection with two or more viruses was detected in 16 children with the most common combined viral infection being bocavirus and rotavirus in 6 patients (37.5%). Previous reports supported this finding regarding the co-infection with other enteric viruses with bocavirus [35, 36]. HBoV mixed infections were found to be high in our study, with 6 cases of mono-infection with bocavirus in children. It has been suggested that the high rate of mixed infection is because bocavirus is a helper virus, facilitating the replication of other pathogens or it may need a helper virus to facilitate a productive infection [37].

Meanwhile, the presence of HBoV as a single pathogen in 6 samples might indicate its role as a true pathogen in acute GE in children; however, further research needs to be carried out to validate these findings. They may also reflect the presence of other undetected pathogens in those children that needs further investigations [28].

The infected children with HBoV were mainly females from rural residences. Fever was the most common presenting sign in those children followed by vomiting. This is contradictory to previous studies that reported fever and vomiting as the less frequent presenting sign [32].

Numerous studies have evaluated the significance of bocavirus presence in diarrheal diseases with either supporting or objecting to its significance as a pathogen in acute GE [37, 38]. In the present study, no statistically significant association was found between bocavirus detection and any gastrointestinal symptoms compared to the children with the absence of bocavirus in the stool samples. Moreover, there was no patient with HBoV that suffered from severe GE as measured by the Vesikari score system.

There was a statistically insignificant difference between the mean age of children with bocavirus infection and those without bocavirus infection. The infection with bocavirus under the age of 5 years is variable as it is thought to increase after the age of 6 months due to a decline in the protection of maternal antibodies by this age and then decline after the age of 24 months due to repeated exposure [39, 40]. However, this was not supported by the findings of the present study nor the previous study conducted by Netshikweta *et al*. [32].

In the present study, astrovirus was detected in 13.3% of children, and norovirus was detected in 10% of children. The prevalence rates of astrovirus and norovirus in acute GE in children ranged from 0% to 30% and from 7.8% to 15%, respectively, in previous studies [41 - 43].

There is a need for the implementation of a wide-scale surveillance system for laboratory diagnosis of viral GE to define the types of common viruses associated with this infection.

There were some limitations in the present study. The genotype of the bocavirus was not performed. Furthermore, the study needs to be expanded to include more children with GE.

CONCLUSION

The study highlights the presence of bocavirus in children with acute GE under the age of five years. The infection associated with this virus was either mild or moderate in severity. The combined viral infection was common, especially associated with rotavirus.

LIMITATIONS

There is a need for further additional study to identify the type of circulated strain of bocavirus and the coinfections with other pathogens.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study has been approved by the institutional review board of the Mansoura Faculty of Medicine ethical committee (registration number: R.21.04.1297).

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or research committee and with the 1975 Declaration of Helsinki, as revised in 2013.

CONSENT OF PUBLICATION

Informed consent was obtained from parents for conducting this study.

STANDARDS OF REPORTING

STROBE guidelines and methodologies were followed in this study.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available on the given link: https://zenodo.org/record/5642609#.YYJUw3qxXTA.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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