



Detection of Human Norovirus among Children with Gastroenteritis in Diyala Governorate

Diyala Valiliğinde Gastroenteritli Çocuklarda İnsan Norovirüsünün Saptanması

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ABSTRACT

Background: Acute gastroenteritis remains a global public health problem and human noroviruses persist a cause of gastroenteritis.

Objectives: To determine the rate of human norovirus infections among children with gastroenteritis in Diyala governorate by enzyme linked immunosorbent assay and immunochromatography, also to assess genogroup 1 (GGI) and genogroup 2 (GGII) by nested polymerase chain reaction among study population.

Patients and methods: Cross sectional study was carried out on 182 children under five years old with acute gastroenteritis who attended the emergency department of pediatrics in Al-Batool Teaching Hospital for Maternity and Children in Baqubah city, during the period from 6th September 2018 till 4th of March 2019. Stool samples were collected from each participant and stored as frozen at -70 °C to use for enzyme linked immunosorbent assay (ELISA) and quick qualitative immunochromatographic test, finally positive results for human norovirus were used for RNA extraction and nested polymerase chain reaction (PCR).

Results: The rate of human norovirus infection was 6.04%, most common among females 6 (54.55%). Age group 1-12 months showed highest frequency 8 (72.73%). The primary education of the mothers was noted in 54.55% followed by 18.18% for each secondary and higher education. Children with artificial milk feeding constituted 81.82% and 18.18% were mixed feeding, while no positive results were recorded among children with breast feeding. The highest infection rate was noticed among patients used filtered and boiled water (54.54%) followed by filtered water (27.28%). Among clinical features caused by virus infection, abdominal pain constituted 11(100%), vomiting 10(90.09%), and nausea was noted in 7(63.63%). The results of nested polymerase chain reaction demonstrated that only one case was positive for human norovirus genogroup 2 (GGII).

Conclusion: Human norovirus genogroup II appear to play a major role in acute gastroenteritis among children.

Keywords: Gastroenteritis, human norovirus, PCR, ELISA

ÖZ

Amaçlar: Akut gastroenterit, küresel bir halk sağlığı sorunu olmaya devam etmektedir ve insan norovirüsleri, gastroenterit nedenidir. Diyala vilayetinde gastroenteritli çocuklar arasında insan norovirüs enfeksiyonlarının oranını enzim bağlantılı immünosorbent testi ve immünokromatografi ile belirlemek, ayrıca çalışma popülasyonunda nested polimeraz zincir reaksiyonu ile genogrup 1 (GGI) ve genogrup 2'yi (GGII) değerlendirmek.

Gereç ve Yöntem: 6 Eylül 2018 - 4 Eylül tarihleri arasında Bakuba ilinde Al-Batool Kadın Doğum ve Çocuk Eğitim Hastanesi pediatri acil servisine başvuran 5 yaş altı 182 çocuk üzerinde kesitsel çalışma yapılmıştır. Her bir katılımcıdan dışkı örnekleri toplandı ve enzime bağlı immünosorbent testi (ELISA) ve hızlı kalitatif immünokromatografik test için kullanılmak üzere -70 °C'de dondurulmuş olarak saklandı, son olarak insan norovirüsü için pozitif sonuçlar RNA ekstraksiyonu ve nested polimeraz zincir reaksiyonu (PCR) için kullanıldı.

Bulgular: İnsan norovirüs enfeksiyonu oranı %6.04, en yaygın kızlarda 6 (%54.55) bulundu. 1-12 aylık yaş grubu en yüksek frekanslı 8 (%72.73) gösterdi. Annelerin eğitim düzeyi ilköğretim %54,55 olarak kaydedildi, ardından orta öğretim %18,18 ve yüksek öğrenim için %18,18 izlendi. Formül mama ile beslenenler çocukların %81,82'sini oluştururken, %18,18'inde karma beslenme belirlenmiş, emzirilen çocuklarda norovirus saptanmamıştır. En yüksek enfeksiyon oranı filtrelenmiş ve kaynatılmış su (%54,54) ve ardından filtrelenmiş su (%27,28) kullanan hastalarda görüldü. Virüs enfeksiyonunun neden olduğu klinik özellikler arasında karın ağrısı 11 (%100), kusma 10 (%90.09) ve bulantı 7 (%63.63) olarak belirlendi. Nested polimeraz zincir reaksiyonunun sonuçları, insan norovirüs genogrup 2 (GGII) için yalnızca bir vakanın pozitif olduğunu gösterdi.

Sonuç: İnsan norovirüs genogrup II'nin, çocuklar arasında akut gastroenteritte önemli bir rol oynadığı görülmektedir.

Anahtar Kelimeler: Gastroenterit, insan norovirüs, PCR, ELISA

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INTRODUCTION

Acute gastroenteritis remains a global public health concern, causes significant morbidity and mortality among children worldwide.^[1] The causes of acute gastroenteritis in children vary depending on multiple factors such as location, season, and the population studied.^[2] There is a wide range of infections that can cause acute gastroenteritis. These are viruses, bacteria and protozoa.^[3] Viruses are the most frequently implicated pathogens causing pediatric acute gastroenteritis and viral diarrhea in pediatric patients in both outpatient, emergency department, and inpatient settings.^[4] Enteric viruses, particularly human noroviruses affect people of all ages, and are a leading cause of foodborne disease and outbreaks of gastroenteritis worldwide.^[5] Human noroviruses which are small, non-enveloped, positive-stranded RNA viruses belong to Caliciviridae family.^[6] The human norovirus genus can be subdivided in seven genogroups, of which genogroups GI, GII and GIV have been detected in humans, and can be further subdivided into more than 40 genotypes.^[7] Human norovirus causes ~20% of all acute gastroenteritis and ~200,000 deaths per year, primarily in young children.^[8] The transmission of human norovirus occurs primarily via the fecal oral route, including direct person to person contact, consumption of contaminated food or water, or contact with contaminated environmental surfaces.^[9] Common symptoms of illnesses include increase in bowel movement frequency with or without vomiting, fever, abdominal cramping, headache, dehydration and myalgia.^[10] Globally, human norovirus resulted in a total of \$4.2 billion in direct health system costs and \$60.3 billion in societal costs per year.^[11]

In Iraq, several studies have been conducted in various provinces to determine the rate of human norovirus infections by using different techniques while here is no previous study done in Diyala governorate so this study design to determine the rate of human norovirus infections among children with gastroenteritis in Diyala governorate by enzyme linked immunosorbent assay and immunochromatography, also to assess genogroup 1(GGI) and genogroup 2(GGII) by nested polymerase chain reaction among study population.

PATIENTS AND METHODS

A cross sectional study was carried out for patients with acute gastroenteritis who attended to the emergency department of pediatrics in Al-Batool Teaching Hospital for Maternity and Children in Baqubah city. Stool specimens were collected from 182 patients with acute gastroenteritis 115 were males and 67 were females; their ages were ranged from one month to 60 months. The samples were collected and put in Eppendorf tubes then were labeled and stored in deep freeze at -70 °C to use for

enzyme linked immunosorbent assay by use RIDASCREEN Norovirus 3rd generation (Cat No. C1401- Germany) and quick qualitative immunochromatographic test by the RIDA-QUICK Norovirus Test (Cat No. 1402, Germany), finally positive result for human norovirus was used for RNA extraction using a (Mini Kit viral RNA, QIAGEN, Germany) with spin column method according to the manufacturers instruction and the total nucleic acid was recovered in 60µl of nuclease free water and stored at -80°C until analysis, then cDNA synthesis from total RNA template b using (Bioneer, Korea), human norovirus detection was done by using the specific oligonucleotide primer sequences in conventional PCR to detect the presence of human norovirus-G1 and human norovirus-G2 gene.^[12] Nested PCR done with two pairs of primers used to amplify a fragment of the for GI and GII human norovirus gene respectively by a nested polymerase chain reaction assay.^[12] The primers were lyophilized, then dissolved in the sterile nuclease free water to give a final concentration then following same conditions as used for first and second round amplification in a thermocycler.

First round		
Primers	Sequence 3'→5'	Fragment length
COG1F+G1SKR G I	F:5'-CCAACCCARCCATTRTACA-3'	380bp
COG1R+G2SKR G II	R:5'-CCRCNCGCATRHCCRTTTRTACAT -3'	390bp
Second round		
Primers	Sequence 3'→5'	Fragment length
G1SKF+G1SKR G I	F:5'- CTGCCCGAATTYGTAAATGA-3	330bp
G2SKF+G2SKR G II	F:5'-CNTGGGAGGGCGATCGCAA -3'	340bp

STATISTICAL ANALYSIS

All data were analyzed using the statistical package for social sciences (SPSS) and all categorical variables were expressed as frequency and percentage.

RESULTS

The current study revealed that the rate of human norovirus infection was 6.04% (11 out of 182 samples) as shown in **Figure 1**.

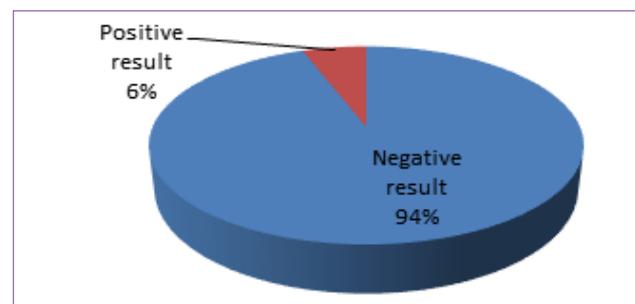


Figure 1. The rate of human norovirus among studied group.



The rate of human norovirus infections among females (54.55 %) was higher than males (45.45%). The age of the patients ranged from 1 day to 60 months. The positive results 8 (72.73%) were in age group 1≤-12 months and 3 (27.27%) their age was 13-24 months, while no positive cases among other ages. Regarding residence the patients who attended to Al-Batool Teaching Hospital from five districts of Diyala governorate; the whole positive results were from Baqubah district. The highest rate of mothers of the patients had primary education level (54.55 %) followed by 18.18 % for each secondary education and higher education as shown in **Table 1**.

Table 1. Distribution of human norovirus according to demographic factors.

Variable factors		No. (%)
Sex	Males	5 (45.45)
	Females	6 (54.55)
Age groups (Months)	1≤-12	8 (72.73)
	13-24	3 (27.27)
Educational level of the mothers	Illiterate	1 (9.09)
	Primary education	6 (54.55)
	Secondary education	2 (18.18)
	High education	2 (18.18)
Total		11 (100)

The distribution of positive human norovirus infection regarding type of feeding showed that 9 (81.82%) were used artificial milk and 2 (18.18%) were mixed feeding that drinking artificial and breast feeding (18.18 %), while there was no positive results recorded among children with breast feeding as shown in **Table 2**.

Table 2. Distribution of positive human norovirus cases according to type of feeding.

Type of feeding	No. (%)
Breast feeding	0 (0)
Artificial feeding	9 (81.82)
Mixed feeding	2 (18.18)
Total	11 (100)

According to the water source the patients with human norovirus were divided into three groups according to different sources of water use, the highest infection rate was noticed among patients who were used filtered and boiled water (54.54 %) followed by filtered water about (27.28 %) and boiled tap water (18.18 %) as shown in **Table 3**.

Table 3. Distribution of positive human norovirus cases according to sours of water.

Water source	No. (%)
Filtered water	3 (27.28)
Filtered and boiled	6 (54.54)
Boiled tap water	2 (18.18)
Total	11 (100)

The most frequent clinical signs among patients with human norovirus infection were fever (45.45%), nausea (63.63%), vomiting (90.09%), weight loss (36.36 %) and dehydration (54.54 %) as well as all patients had abdominal pain (100%) as shown in **Table 4**.

Table 4. Distribution of positive human norovirus cases according to clinical signs.

Clinical signs	Positive cases - No. (%)	Negative cases - No. (%)
Fever	5 (45.45)	6 (54.55)
Abdominal pain	11 (100)	0 (0)
Nausea	7 (63.63)	4 (36.37)
Vomiting	10 (90.09)	1 (9.90)
Weight loss	4 (36.36)	7 (63.64)
Dehydration	6 (54.54)	5 (45.46)
Total	11 (100)	11 (100)

Nested PCR assay was used for the detection of human norovirus genes, the first round for genogroup 1 and 2 showed negative results from 1 to 8 samples as shown in **Figure 2**, while others (9 and 10), showed positive result for GI and 11 showed negative result as shown in **Figure 3**. Results of second round showed that 1 out of 11(9.10%) samples was positive for G2 human norovirus with a PCR product size of approximated 380 bp and 10 (90.90%) samples were negative. The positive case was boy and in the age group 1-12 months, as shown in **Figure 4**. The second round of PCR was performed to confirm the correct amplification of the first-round product. One of three samples which were positive for the first round were subjected to second round. The result is shown in **Figure 4**. All these samples gave positive results with fragment length of 340bp.

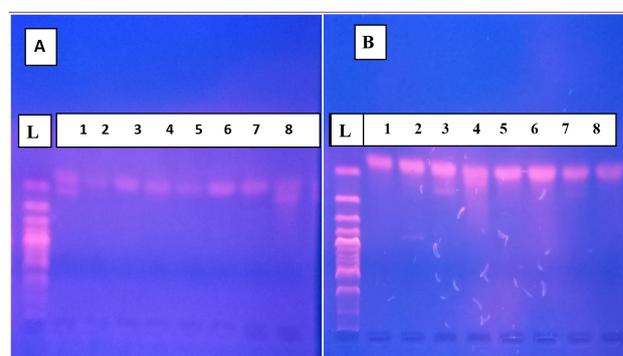


Figure 2. Screening of stool samples of patients (1 to 8) suspected to be infected with human norovirus using nested PCR with primers, produced the target bond 380-390 bp. The 1% agarose gel was electrophoresed and stained with 0.5 mg/ml ethidium bromide, lane L: ladder, lane 1-8 samples A- for genogroup I and B- for genogroup II.

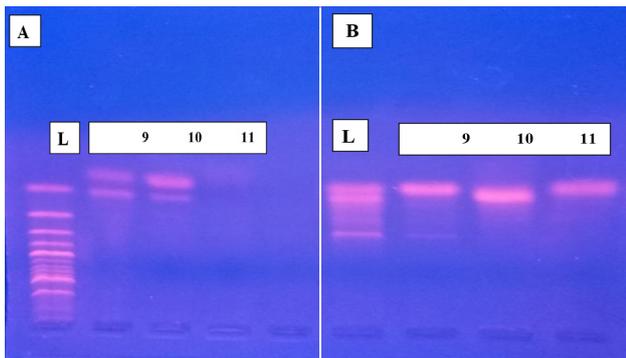


Figure 3. Screening of stool samples of patients (9,10,11) suspected to be infected with human norovirus using nested PCR with primers, produced the target bond 380 - 390 bp. The 1% agarose gel was electrophoresed and stained with 0.5 mg/ml ethidium bromide.

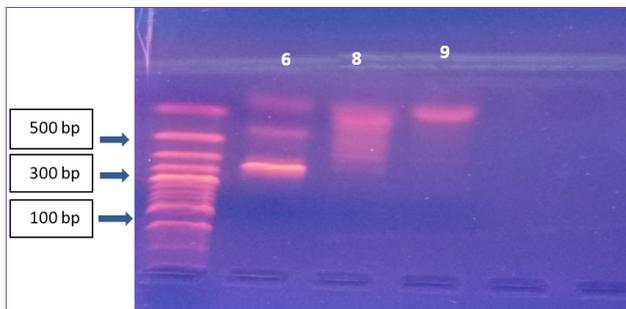


Figure 4. Screening of stool samples of patients (6,9,10) suspected to be infected with human norovirus using nested PCR with primers, produced the target bond 380-390 bp. The 1% agarose gel was electrophoresed and stained with 0.5 mg/ml ethidium bromide. The positive result detected in sample 6.

DISCUSSION

In this study the rate of human norovirus infection obtained was 6.04% (11 out of 182) among children with acute gastroenteritis who attended to Al-Batool Teaching Hospital for Maternity and Children in Baqubah city. The current study supports the previous findings of many other studies concerning this subject such as ^[13] in Lebanon with the rate (6.32%), ^[14] in Basrah who get a rate of 8% and ^[15] in Tunisia who get a rate 8.99%. On the other hand, the rate of infection in this study was higher than that reported by ^[16] in Saudi Arabia with the rate 3.56%. Respect to other reports, the rate of this study is lower than that reported by ^[17] in Thi-Qar Province with rate 17.5%, in other two reports that done by Mohammed et al. ^[18] and ^[19] in Mousl and Baghdad city, the rate was 28%. The positive rate was 30% among children as a study done by ^[20] in Kurdistan region and the rate was 37.9% in a study done by ^[21] in Al-Najaf. The variation in detection rates can be attributed to the differences between populations, the variations in time of study or to the laboratory tests used. ^[22] The low level of hygiene and sanitation in these areas, as well as the low access to potable water facilitated the spread of human norovirus strains. ^[23] This could be related to population differences in terms of lifestyle, awareness, hygiene, number of

infected people in the same family and several subjects. According to the result of this study, the rate of infection was higher among females (54.55%) than that in males (45.45%). The obtained results are in agreement with the finding done in Iraq (in Basrah) and China. ^[14,24] However, in comparison with a study done by, ^[25] concerning the sex, a study revealed a high number of male's patients than females' patients in Diyala. So, several studies reported that there are no significant differences between sex and human norovirus infection like the study done in Baghdad by ^[26] they interpret the result by males and females exposed to chance of infection due to all of them were living under the same conditions and climates of disease. ^[18] This study demonstrated that the highest frequency of infection was found among infants between 1≤-12 months with rate 72.73%, and about 27.27% in the age group 13 to 24 months. This result seems to be like other studies revealed that human norovirus infection was more among children. Within same range of age viruses cause about 70% of episodes of infectious diarrhea in the pediatric age group and children could have been infected with different genotypes of norovirus in 6 to 12 months. The asymptomatic infection is common in children under the age of five years of age. ^[27,28] In the current study the educational level of the mothers of children was divided into four categories, the highest rate of infection (54.55%) was noticed among children whose their mother's level of education was primary, followed by those within both secondary and high level of education (18.18% for each level), and the lowest rate of infection (9.09%) was reported illiterate level of education. The result agrees with those reported by ^[29] several studies showed that children from mothers who had no education or primary education only, had a higher chance of developing infections than children from more educated mothers (secondary education and above). This is probably due to the fact that children spend more time with their mothers, whose educational level will determine the quality of care and many social and environmental factors that may expose the child to different infections. ^[30,31] Based on type of feeding, this study was recorded that 81.82% of children consume artificial milk and bottle feeding, 18.18% were mixed feeding since they drank artificial and breast feeding. No positive results were noticed among children with breast feeding. A study investigated the relationship between the pediatric feeding type and the percentage of infection, revealed that the highly incidence with viruses' human norovirus GGII which reached 26.19% was reported in infant using bottle-feed in comparing with infants who were breast-fed and mix-feed. The association between bottle-fed and viral infection statically was highly significant ($p \leq 0.01$). ^[18] Other study done in Al-Najaf showed an increase was explained as resulting from using solid food beside or instead of breast feeding, therefore exclusively depending on breast feeding belonged to the passive immunity that infants receiving



from his mother during this period, gave him protecting against the common infectious disease like the viruses infections, and decreases of the possible transmission from contaminated food.^[21] The results showed in Thi-Qar; children who used artificial milk were 15.2%, followed by mixed feeding with 10.6%, while normal feeding had the lowest percentage of 9.7% of infection but without significant difference ($p > 0.05$).^[17] This is due to the ease of transmission of viruses in contaminated feeding bottles, careless of parents involving using of contaminated water sources, careless food handling and low level of hygiene. Other a study found a link between breast-feeding and low rates of human norovirus infection, possibly because of the protective role of maternal antibodies or the presence of Fucosylated glycans in the mother's breast milk.^[32] The water considered as a way of this human norovirus transmission, about 54.54% from infected children were drink or use the filtered boiled water in their feeding, followed by filtered water about 27.28 % this rate may because the filtered water is only used for filtration not for sterilization, and boiled tap water about 18.18%. The spread of the virus in children with artificial or mixed feeding may be due to the use of untreated water for the preparation of milk, which is one of the important ways to transport norovirus.^[33] Also, this related with human noroviruses are a leading cause of recreational waterborne illnesses and responsible for the majority of viral-associated gastrointestinal illnesses nationwide.^[34] The results of the current study may be explained by the method of boiling the water was not accurate less than 100 °C or the milk bottle that used after boiling process was not very clean or contaminated, the filter used for filtration was old or expired as well as maybe related with the information's that were collected from the mothers were not accurate.

Most clinical features were considered in this study as followings, abdominal pain (100%) was generally more widespread among children with gastroenteritis followed by vomiting (90.09%), nausea (63.63%), dehydration (54.54 %), fever 5(45.45%) and weight loss (36.36%). This result is compared with the result of a study was done in Basrah, showed that the most common clinical symptoms beside diarrhea observed among norovirus infected children were vomiting (94%), fever (69%), dehydration (50%) and abdominal pain (31%).^[14] The disease often begins with vomiting, followed by abdominal cramps, fever (in 37 to 45% of the cases), watery diarrhea, and other constitutional symptoms such as headache, chills, and myalgias. The illness normally lasts only 2 to 3 days but can last longer (i.e., 4 to 6 days) in nosocomial outbreaks and among children younger than 11 years of age.^[35] Other study done by^[36] in Shanghai during human norovirus outbreak in 2017 showed that diarrhea is not a dominant symptom of norovirus infection and implied that age-specific host factors and genotype-specific viral factors play roles in the pathology of norovirus infection.

Among 11 cases were positive to human norovirus, only one case proved positive for GII human norovirus by nested PCR belonged to a male in the age group 1≤12 months. This result in agreed with the result of study done by^[26] in Baghdad which showed that most positive samples results were GII among five genotypes: GII.4, GII.2, GII.17, GII.21 and GI.3, and with^[18] in Mosul city, who found human norovirus genotype II was the most frequent virus identified in 24 cases (57.14%) than genotype I in 4 cases (9.52%). The result of current study demonstrated that the high infection rate with the both viruses in age group under 1years. Other studies done in different areas such as in Turkey by^[37] determined the genogroups GI and GIV were the most common. In India one study done by^[38] found infected children were positive for genogroup II (GII) was 98.80 compared to only two samples (1.20%) being positive for genogroup I (GI). In Indonesia^[39] recorded that the majority ($n = 69/75$, 92%) of the human noroviruses identified belonged to genogroup II, and several genotypes were identified by sequencing a subset of samples. The result of current study was disagreeing with the result of^[19] who found that the most human norovirus infections in Baghdad belong to GI by using conventional PCR technique. The number of human norovirus positive results by PCR technique showed fewer number results compare to was lower than that by ELISA technique, this could be related to the genetic variations of RNA viruses that exhibit highly mutation rates, with ratio rate of 10⁻⁴ to 10⁻⁶ errors per nucleotide which is equivalent to approximately one mutation per genome generated during each replication cycle within a population.^[40] Human norovirus genogroup II appear to be plays a major role as a cause of acute gastroenteritis among children especially less than 1 year old.

ETHICAL CONSIDERATIONS

Ethics Committee Approval: The study was approved by Ethics committee at college of Medicine, University of Diyala.

Informed Consent: Written informed consent was obtained from all participants who participated in this study.

Status of Peer-review: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.



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