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RESEARCH ARTICLE

Molecular Study of Norovirus in Pediatric Patients with Gastroenteritis

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Abstract:

Aim:

The aim of the present study was to detect the prevalence of norovirus and genotypes determination by real-time PCR among children below 18 years as an etiology of acute gastroenteritis and to compare rapid detection of norovirus by enzyme-linked immunoassay (EIA) to virus detection by real-time PCR.

Methods:

The research was a cross-sectional study conducted on children below 18 years complaining of community-acquired acute gastroenteritis. A stool sample was subjected to direct-antigen detection by ELISA for norovirus and molecular study by real-time polymerase chain reaction.

Results:

The study included 200 children with acute gastroenteritis with a mean age of 6.7±3.8 years. Norovirus antigen was detected by EIA in 34.5% and by real-time PCR in 30.5% of studied children with genotype GII, the predominant detected genotype (80.97%). Both real-time PCR and antigen detection of norovirus were positive in 43 (70.5%) of the children and negative in 113(81.3%) of the studied children. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy for antigen detection by ELISA were 70.5%, 81.3%, 62.3%, 86.3% and 78%, respectively. Comparison between patients positive for norovirus and those negative for norovirus by real-time PCR revealed non-significant difference as regards age, sex, the season of occurrence and residence.

Conclusion:

The present study highlights that norovirus prevalence is common among pediatric patients with gastroenteritis above 5 years with GII genotype as the prevalent genotype. There was a significant correlation between positive and negative results of antigen detection of norovirus by ELISA and detection of RNA of norovirus by real-time PCR in stool samples. However, the screening for norovirus by ELISA has limited sensitivity and needs to be associated with a molecular method for accurate diagnosis of sporadic cases of gastroenteritis.

Keywords: Norovirus, ELISA, gastroenteritis, children.

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1. INTRODUCTION

Acute diarrhea is a common cause of morbidity in children worldwide. Rotavirus, as an etiology of acute diarrhea, has been decreased after the implementation of vaccine and norovirus has become the most prevalent viral etiology of acute gastroenteritis [1].

Norovirus is a non-enveloped RNA virus associated with acute gastroenteritis transmitted by fecal-oral route in all age groups. Norovirus is divided into 7 genotypes (GI-GVII) and subdivided into 38 genotypes on the basis of the capsid protein sequences [2]. The human etiology of acute gastroenteritis usually belongs to viruses from GI, GII, and GIV with about 95% of outbreaks due to genotype GII [3 - 5]. Rare genotypes of norovirus have emerged as an etiology of acute gastroenteritis in several countries [6, 7].

Diagnostic methods for norovirus include non-culture methods as norovirus cannot be cultivated in cell culture [8]. The diagnostic methods include antigen identification in stool samples by enzyme-linked immunoassay (ELISA). Enzyme-linked immunoassay detects norovirus antigen [illegible] use of

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either monoclonal or polyclonal antibodies with sensitivity less than 70% and high specificity above 90% as reported by previous studies [8, 9]. The accuracy of ELISA depends upon factors such as the number of samples used for each patient and the time of the sample obtaining after infection. The antigen detection by the use of ELISA is considered rapid screening test being useful in the investigation of outbreaks. However, in the diagnosis of sporadic cases, the interpretation of the results must be judged with caution due to the low sensitivity of the test [9]. The specific diagnosis of norovirus has been facilitated in recent years by the use of reverse transcription-polymerase chain reaction PCR (RT-PCR) [10]. Moreover, this method coupled with nucleotide sequencing techniques is able to gather valuable information on the norovirus and provide epidemiological information of norovirus infections in the community. The RT-PCR primers that target the viral RdRp gene in open reading frame 1 (ORF1) or capsid gene in ORF2 have been designed to detect and genotype various norovirus strains [11].

The aim of the present study was to detect the prevalence of norovirus and genotypes determination by real-time PCR among children below 18 years as an etiology of acute gastroenteritis and to compare rapid detection of norovirus by ELISA to virus detection by real-time PCR.

2. MATERIALS AND METHODS

The research was a cross-sectional study conducted on children below 18 years complaining of community-acquired acute gastroenteritis recruited from Mansoura University Children Hospital outpatient clinics, Egypt from January 2018 till May 2019. The inclusion criteria were children below 18 years complaining of acute gastroenteritis with the passage of soft stool more than 3 times per day with or without vomiting with no antibiotics therapy in the past three days and bacterial and parasite infestations were excluded by laboratory methods. The exclusion of parasite infestation was performed by light microscopy examination within approximately one hour from the time of collection by Ridley-Allen modified formol-ether concentration method [12]. Bacterial infections were excluded by negative stool culture for *Salmonella*, *Shigella* and *Campylobacter* [13]. Children with chronic diseases such as hepatic, renal or autoimmune disorders were excluded from the study. The study was approved by the Mansoura Ethical committee and approval was obtained from the parents of each participant.

Each child was subjected to full medical history taking including age, sex, residence, presence of symptoms and signs such as fever, bloody diarrhea and vomiting besides complete clinical examination.

3. LABORATORY METHOD

3.1. Sample

A stool sample was collected from each child in a sterile container and transported to the laboratory within 30 minutes as subjected to macroscopic examination including the consistency, presence of mucus and blood. Then, each stool was divided into two aliquots: one for direct -antigen detection by ELISA (ELISA-Ridascreen, Germany) for norovirus and molecular study for norovirus by real-time polymerase chain reaction (PCR).

3.2. Norovirus detection by antigen ELISA

The kit detects norovirus genotypes I and II in stool specimens by a specific monoclonal antibody bound to microwell surfaces. At first, a stool sample was diluted with diluent buffer 1:6 and mixed, then left 10 minutes. One hundred microns of the supernatant was used and transferred to well with incubation for 60 minutes at room temperature. After incubation, the wells were emptied and washed 5 times by 300 microns of wash buffer. Then, 50 microns of enzyme conjugate was added and incubated for 30 minutes then washed as described and 50 microns of chromogen was added. The absorbance was measured at 450 nm. The calculated cut off was determined by adding 0.15 to the absorption value of negative control.

3.3. Extraction of RNA

The stool suspension was prepared with sterile water in a concentration of 10% and viral RNA was extracted from 140 microns suspensions QIAamp viral RNA extraction kit (Qiagen-Germany), according to the instructions of the manufacturer. RNA was eluted with 60 microns of diethyl pyro carbonate-treated water and stored at −80 °C until use in real-time PCR.

3.3.1. Real-time quantitative RT-PCR.

3.3.1.1. Reverse transcriptase

The extracted RNA was used in reverse transcriptase reaction for cDNA formation. At first, extracted RNA was incubated with DNase I then added to the reaction mixture of 2.5 microns with 150mM Tris -HCL buffer, pH 8.3, 225 mM KCl, 9 mM $MgCl_2$) and 1 U of RQ1 DNase (Promega Madison, Wis.). The reaction mixture was incubated at 37°C for 30 minutes to digest DNA and then at 75°C for 5 minutes to inactivate the enzyme. Then, reverse transcription was carried out by the use of 15 microliters of treated RNA added to mixture containing 100 mM Tris-HCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$, a 1 mM concentration of each deoxynucleotide triphosphate, 10 mM dithiothreitol, 75 pmol of random hexamers (Amersham Pharmacia Biotech, Piscataway, N.J.), 30 U of RNasin (Promega), and 200 U of SuperScript II RNase H (−) reverse transcriptase (Gibco BRL, Gaithersburg, Md.). RT was performed at 42°C for 2 hours, and the enzyme was inactivated at 70°C for 15 minutes.

3.3.2. Real-Time PCR

Real-time PCR was carried out by the use of primers and probes summarized in Table (**1**) [14]. The reaction mixture was 50 microliters with 5 microliters of cDNA. Universal PCR master mix was used (Applied Biosystems- Waltham, Massachusetts, USA) that contains 25 µl of TaqMan and each primer was added with the concentration of 400 nM and specific probes were added for GI 15 pmol and for GII 5 pmol. The amplification process was performed by the use of one-

step real-time PCR (Applied Biosystems) with the following steps incubation at 50ºC for 2 minutes, then denaturation at 95°C for 10 minutes followed by 45 cycles of amplification (denaturation at 95°C for 15 seconds, annealing and extension at 56°C for 1 minute). Amplification data were collected and analyzed with software version 1.6 (Applied Biosystems-Waltham, Massachusetts, USA). Positive controls were plasmid containing amplified products by the use of primer sets G1FF-G1SKR and G2FB-G2SKR of the ORF1-ORF2 junction from strains SzUG1 and U201 [14].

3.4. Statistical Analysis

Statistical analysis was performed by the use of SPPS24. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy were calculated by medCalc software.

4. RESULTS

The study included 200 children with acute gastroenteritis with a mean age of 6.7±3.8 years; they were 56.5% males and 43.5% females. The children were mainly from rural residence 60.5% with vomiting (80%), fever in 52.5% and abdominal pain (38%). The study was conducted mainly during winter (40%) seasons. Norovirus antigen was detected by ELISA in 34.5% and by real-time PCR in 30.5% of the studied children with genotype GII, the predominant detected genotype (80.97%), Table (**2**).

Both real-time PCR and antigen detection of norovirus were positive in 43 (70.5%) of the children and negative in 113(81.3%) of the studied children, data not shown. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy for antigen detection by ELISA were 70.5%, 81.3%, 62.3%, 86.3% and 78% respectively, Table (**3**).

Comparison between patients positive for norovirus and those negative for norovirus by real-time PCR revealed non-significant difference as regards age, sex, the season of occurrence and residence. There were also insignificant differences as regards clinical symptoms, Table (**4**).

5. DISCUSSION

Norovirus was identified as a key cause of diarrhea in children especially after the introduction of a rotavirus vaccine. There is speculation that norovirus is a common etiology of gastroenteritis in all age groups [15]. In the present study, the mean age of the studied children was older than previous studies from the same region of Egypt, which demonstrates the prevalence of norovirus in children with gastroenteritis below 5 years [16, 17]. Norovirus antigen was detected by ELISA in 34.5% and by real-time PCR in 30.5% of the studied children. The prevalence was more or less similar to previous studies [16, 17]. This finding supports the hypothesis that norovirus is a wide-spread virus that affects both young children and adolescents in developing countries [18].

Table 1. Primers and probes sequences used for norovirus detection by real-time PCR.

Norovirus Genotypes	Sequences of the Primers and Probes
G1 Probe RING1(a)-TP RING1(b)-TP	5′-CGYTGGATGCGNTTYCATGA-/3 5′-CTTAGACGCCATCATCATTYAC-/3 FAM-AGATYGCGATCYCCTGTCCA-TAMRA FAM-AGATCGCGGTCTCCTGTCCA-TAMRA
GII Probe RING2-TP	5′-CARGARBCNATGTTYAGRTGGATGAG-/3 5′-TCGACGCCATCTTCATTCACA-/3 FAM-TGGGAGGGCGATCGCAATCT-TAMRA

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

Parameter	
Sex Male (No.-%) Female (No.-%)	 113 56.5% 87 43.5%
Age (mean± SD) years	6.7± 3.8
Residence Rural (No.-%) Urban (No.-%)	 121 60.5% 79 39.5%
Abdominal pain (No.-%)	76 38%
vomiting (No.-%)	160 80%
Fever (No.-%)	105 52.5%
Season Summer (No.-%) Autumn (No.-%) Winter (No.-%) Spring (No.-%)	 46 23% 40 20% 80 40% 34 17%
Norovirus positive by ELISA(No.-%)	69 34.5%

(Table 2) contd.....

Parameter	
Norovirus Positive by Real time PCR(No.-%)	
Genotypes of Norovirus	61 30.5%
GII(No.-%)	50 81.97%
GI(No.-%)	11 18.03%

Table 3. Diagnostic value of antigen detection of norovirus by ELISA as compared to real time PCR.

	Sensitivity		Specificity		Positive Predictive value		Negative Predictive Value		Accuracy	
	%	95%CI	%	95%CI	%	95%CI	%	95%CI	%	95%CI
Norovirus Detection by ELISA	70.5%	57.4%-81.5%	81.3%	73.8%-87.4%	62.3%	53.01%-70.8%	86.3%	80.9%-90.3%	78%	71.6%-83.5%

Table 4. Comparison between demographic and clinical data of children positive for norovirus by real-time PCR as compared to children negative for norovirus by PCR.

	Positive PCR for Norovirus (n=61)	Negative PCR for Norovirus (n=139)	P
Sex Male (No.-%) Female (No.-%)	 33 54.1% 28 45.9%	 80 57.6% 59 42.4%	P=0.4
Age (mean± SD)	6.9± 3.5	6.7± 3.7	P=0.4
Abdominal pain (No.-%)	24 39.3%	52 37.4%	P=0.5
vomiting (No.-%)	61 100%	99 71.2%	P=0.03
Fever (No.-%)	29 47.5%	75 53.9%	P=0.4
Residence Rural (No.-%)	 36 59.01%	 85 61.2%	P=0.8
Season Summer (No.-%) Autumn (No.-%) Winter (No.-%) Spring (No.-%)	 17 27.9% 13 21.3% 22 36.1% 9 14.8%	 29 20.9% 27 19.4% 58 41.7% 25 17.9%	P=0.7

calculated with chi-square test

The predominant genotype was GII detected in 80.97%. Globally, the prevalence of GII accounts for an average of 96.0% while GI with an average of 3.6%, and mixed infections of GI and GII with an average of 0.4% (14). There are seven genotypes of norovirus (GI to GVII) with GI, GII and GIV that are associated with human gastroenteritis [19]. There is sub-classification of genotypes to 14 ORF1-based and 9 ORF2-based genotypes that have been described for GI and 27 ORF1-based and 22 ORF2-based genotypes for GII [19]. The predominance of GII in gastroenteritis can be attributed to several factors higher rates of evolution and progressive accumulation of mutations that lead to evasion of the host immune responses [20, 21]. Moreover, pandemic GII.4 variants can broadly bind to a wide set of human blood group antigens, which supports the norovirus transmission by providing a large genetically susceptible population to infection [22].

The main clinical features of the affected children were vomiting, fever and abdominal pain. This is generally the common clinical features of norovirus gastroenteritis as mentioned previously [23]. There was a statistically insignificant difference between patients with norovirus positive real time PCR and those negative by PCR as regards demographics, clinical symptoms and season of occurrence. The clinical symptoms are common presenting symptoms of gastroenteritis due to different etiology [24]. The infection due to norovirus is predicted throughout the year [25].

The diagnosis of norovirus depends mainly upon laboratory detection of RNA of the virus in the stool samples by molecular techniques such as film array for gastrointestinal pathogens panel [26 - 28]. However, there are some limitations to these methods including the availability and the expense of the used equipment for molecular tests. Other used methods include the detection of the virus particle by electron microscope and the detection of viral antigen by enzyme immunoassay, and immunochromatographic lateral flow assays, although these tests are limited by moderate sensitivity or high cost [29].

In the present study, the comparison of ELISA and real-time PCR for the detection of norovirus in the stool of patients revealed agreement of the detection of positive stool for norovirus was 70.5% and higher agreement, 82.2% (30). The discrepancy between antigen detection of norovirus by ELISA and the detection of RNA by real-time PCR could be attributed to low detection limits of ELISA as compared to higher detection limits by real-time PCR [30]. The specificity of ELISA was 81.3% as compared to real-time PCR and this may be due to cross-reactivity with other microorganisms, interference, and non-specific reactivity of monoclonal antibodies used in the kit [30]. Therefore, there was apparent inconsis-

tency in the results of both tests and the results of ELISA alone cannot be used as a single diagnostic test for the detection of norovirus infection.

CONCLUSION

The present study highlights that norovirus prevalence is common among pediatric patients with gastroenteritis above 5 years with GII genotype as the prevalent genotype. There was a significant correlation between positive and negative results of antigen detection of norovirus by EIA and the detection of RNA of norovirus by real-time PCR in the stool samples. However, the screening for norovirus by ELIA has limited sensitivity and needs to be associated with a molecular method for accurate diagnosis of sporadic cases of gastroenteritis.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

~~Not Applicable~~.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not Applicable.

AVAILABILITY OF DATA AND MATERIALS

Not Applicable

FUNDING

None.

CONFLICT OF INTEREST

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

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Declared none.

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