Diagnosis of Noroviruses in Iraqi children suffering from Gastroenteritis by Enzyme linked assay and conventional PCR

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ABSTRACT

Background: **Noroviruses have been reported to be second to rotaviruses in causing severe childhood gastroenteritis, according to nucleotide sequence analysis of the polymerase or capsid regions, norovirus is classified at least into five genogroups GI–GV, each genogroup is further divided into distinct genotypes. GI, GII, and GIV have been found in humans.**

Amis: **To determine the possible implication of Norovirus in a sample of childhood diarrhea and the efficiency of molecular diagnosis in comparing with ELISA method for the detection of this agent.**

Methods: **Stool samples were collected from 100 patients suffering from diarrhea there age ranged from (6 weeks – 14 years), the stool samples were divided into three portions. The first portion (0.5 mg) was prepared for wet smear preparation, and the second portion was used directly for RNA extraction and directly converted to cDNA and third portion used for ELISA assay, all stored at-20°C.**

Statistical analysis: The statistical analysis of this study was performed using the statistical package for social sciences (SPSS) program version (19) and Microsoft Excel 2010. Categorical data formulated as count and percentage. Chi-square test was used to describe the association of these data. The level of statistical significant difference is below or equal to 0.05.

Results: **the study showed that 28 (28%) samples out of 100 samples were positive for G1 Norovirus with a PCR product size of approximated (380) bp and 72 (72%) samples were negative. All samples (n=100) were negative for G2 Norovirus. The highest incidence of the Norovirus cases was in the age group (2-5) years followed by (< 2 years) age group and then (> 5 years) group and there was no statistical significant difference between age and positive Norovirus cases.**

Conclusions: **Norovirus could be included as one of the most important causes of acute gastroenteritis in Iraq.**

Key words: Norovirus, gastroenteritis, open reading frames (ORFs), cDNA, ELISA.

INTRODUCTION

Human noroviruses are an important cause of epidemic acute viral gastroenteritis. Norovirus symptoms, which typically appear between 12 and 48 h, are generally mild and self-limiting, they can be severe in immunocompromised groups such as infants and the elderly.[1] Viral infection is primarily related to foodborne illness, but person-to-person contact and waterborne outbreaks are also important vehicles for transmission.[2] The Norovirus detection rate in diarrheal patients varies from as high as 31–48% to as low as 3–5%, suggesting that the burden of Norovirus disease may be highly variable geographically.[3] A high titer of shedding by infected persons, a low infectious dose and environmental stability are some of the attributes that facilitate effective norovirus transmission through a variety of modes (person-to-person, food, water, and environment).[4] Viruses are the most frequently

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implicated pathogens causing pediatric acute gastroenteritis (AGE). Rotavirus, Norovirus, Astrovirus, enteric Adenovirus, and Sapovirus have been recognized as the most common agents responsible for viral diarrhea in pediatric patients in both outpatient, emergency department, and inpatient settings.[5] Noroviruses were previously called Norwalk-like viruses. They are a group of non-enveloped, single-stranded RNA viruses that cause acute gastroenteritis. Noroviruses belong to the family Caliciviridae that includes sapoviruses, which also causes acute gastroenteritis.[6] Noroviruses have been reported to be second to rotaviruses in causing severe childhood gastroenteritis, with a genome contains approximately 7.7 kb in length, which is organized into three open reading frames (ORFs) according to nucleotide sequence analysis of the polymerase or capsid regions, norovirus is classified at least into five genogroups GI–GV, each genogroup is further divided into distinct genotypes. GI, GII, and GIV have been found in humans.[7] The goal of this study was to determination the possible implication of Norovirus in a sample of childhood diarrhea and the efficiency of molecular diagnosis in comparing with ELISA method for the detection of this agent.

MATERIAL AND METHODS

Stool samples were collected from 100 patients suffering from diarrhea there age ranged from (6 weeks – 14 years) with a mean age (3.52±3.28). Samples were taken from Al-Emamain Al-Kadhemain Medical City Hospital and Al-Kadhimiya children Hospital in Baghdad. The study conducted through the period from November 2014 to end of April 2015. Stool specimens were collected from each individual included in this study in clean dry containers. These were labeled by case number and name. All specimens were transported to the laboratory for processing and investigations at the same day, stool samples were divided into three portions. The first portion (0.5 mg) was prepared for wet smear preparation, and the second portion was used directly for RNA extraction and directly converted to cDNA and third portion used for ELISA assay, all stored at -20°C.

Immunological detection of Norovirus by ELISA test:

This test was done using Norovirus ELISA kits Biopharm/ Germany briefly as follow. 1 ml samples dilution buffer diluent were added to labeled test tube and to make a suspension with a stool sample, 100 mg of stool sample was added with a spatula to the labeled test tube, homogenize the stool suspension by using vortex mixer, let the suspension stand a short period of time (10 minutes) for the coarse stool particles to settle, and then centrifuged the sample at 5000 rpm for 5 minutes to the supernatant particle-free. The Norovirus ELISA test employs specific monoclonal antibodies in a sandwich-type method. The well surface of the microwell plate is coated with specific antibodies to the antigens of several different genotypes. A pipette is used to place a suspension of the stool sample to be examined as well as the controls in the well of the microwell plate, the rest of the steps performed according to the manufacturer's instructions.

Molecular detection of Norovirus:

Viral RNA extraction and detection. Viral RNA was extracted by using 10% fecal supernatant using the total RNA extraction reagent purification kit (Bioneer/Korea) to purify RNA For RT, the isolated RNA Norovirus were converted into the first strand cDNA by using Wizard Go Scrpit Reverse Transcriptase kit (Promega/ USA) according to the manufacturer's instructions. Norovirus detection was performed by using the specific oligonucleotide primer

sequences in conventional PCR to detect the presence of Norovirus-G1 and Norovirus-G2 gene was taken from [8] and human Beta-globin primers was taken from [9] used as an experimental internal control during protocols of PCR a positive control for confirming the acceptability of the extracted DNA to template, those genes synthesized in Alpha DNA® (Canada) shown in (**Table-1**). DNA template was prepared. The primers were diluted by adding nuclease free water according to the manufacturer instructions. The master mix contents were thawed at room temperature before use, and the PCR master mix was made on a separate biohazard safety cabinet with wearing hand gloves at all times to avoid contamination. For each reaction within single pre-mixed PCR reaction tube, 2µl from forward primer and reverse primer for Norovirus- G1 were added. Five microliter of DNA template was added for each reaction tube. Twelve and a half microliters of GoTaq® Green Master Mix was added for each reaction tube, the volume was completed to 25µl with Deionized Nuclease –Free and tubes were then spun down with a mini centrifuge to ensure adequate mixing of the reaction components. The same reaction was repeated for Norovirus- G2. PCR mixture without DNA template (non-template negative control) were used as negative control. The tubes were placed on the PCR machine (Cleaver Scientific Thermal Cycler TC32/80)and the PCR program, with the right cycling conditions pre-installed, Amplification for Norovirus genes was as follows: 94°C for 3 min followed by 40 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 60 sec, terminating in 72°C for 7 min, 10 µl of each PCR product was subjected to 1% (wt/vol) agarose gel electrophoresis with ethidium bromide (0.5 µg /ml; Sigma) Five microliters of the 100bp DNA ladder were mixed with one microliter of blue/orange 6X loading dye and subjected to electrophoresis in a single lane. Served as marker during PCR products electrophoresis Amplicon visualization was performed using an UV light transilluminator.

Table 1. Primers sequences of Norovirus gene and Beta-globin gene with their relevant product size

		Nucleotide sequences	**GenBankAccession No**	**Products**
Genes		**(5' ---------------- ►3')**		**bP**
	F	CGYTGGATGCGNTTYCATGA	AB597369	
GI	R	CTTAGACGCCATCATCATTYAC		380-390
	F	CARGARBCNATGTTYAGRTGGATGAG		
GII	R	TCGACGCCATCTTCATTCACA	AB597520.	380-390
β-globin	F R	GAAGAGCCAAGGACAGGTAC GGAAAATAGACCAATAGGCAG	Saiki *et al,* 1988 (9)	408

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RESULTS

Demographic information of the patients:

The total of 100 patients suffering from diarrhea were enrolled in this study, 67 (67%) were boys and 33 (33%) were girls with a sex ratio of 2:1 Figure (1). The studied patients were divided into three age groups; ≤ 2 years, 2-5 years and > 5 years Figure (2).

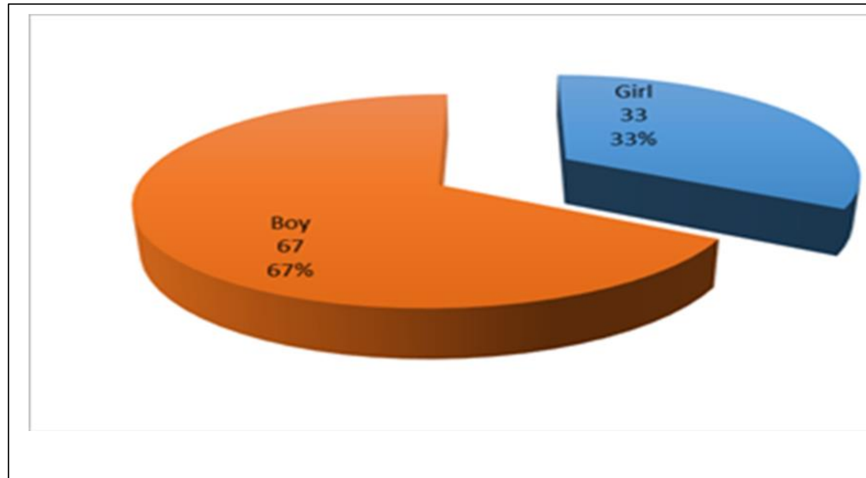

Fig 1. Gender distribution of the patients.

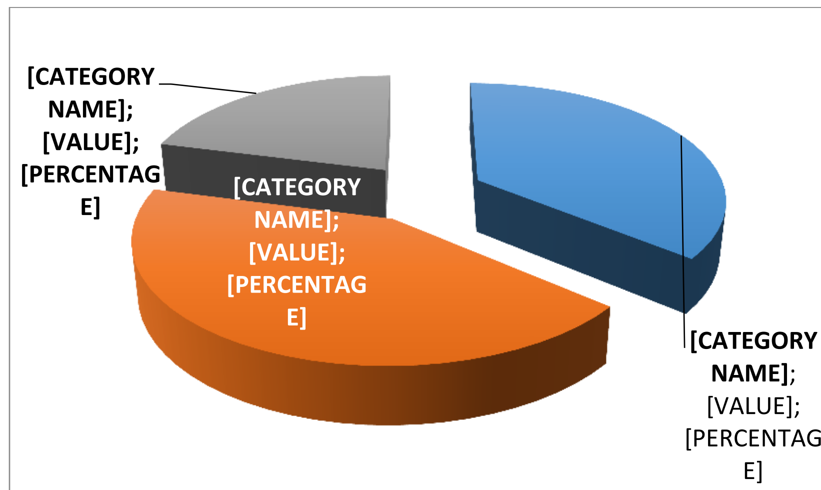

Fig 2. Age groups classification of the patients.

Among 28 (28%) patients proved to be PCR positive for Norovirus (19) were boys and (9) were girls and there was no statistical significant difference between them (P > 0.05). The highest incidence of the Norovirus cases was in the age group (2-5) years followed by (< 2 years) age group and then (> 5 years) group and there was no statistical significant difference between age and positive Norovirus cases (P > 0.05), (Table-2).

Table 2. Distribution of Norovirus cases among different age group of studied patients.

			Age groups			P-value
			< 2 years	2-5 years	> 5 years	
Norovirus PCR	Positive		9	14	5	0.675
		%	25.0%	32.6%	23.8%	
	Negative		27	29	16	
		%	75.0%	67.4%	76.2%	
	Total		36	43	21	
		%	**100.0%**	**100.0%**	**100.0%**	

Detection of cDNA of GI and GII genes for *Norovirus* using conventional PCR:

Conventional PCR assay was used for the detections of Norovirus genes, results showed that 28 (28%) samples out of 100 samples were positive for G1 Norovirus with a PCR product size of approximated (380) bp and 72 (72%) samples were negative (Figure-3) .All samples (n=100) were negative for G2 Norovirus

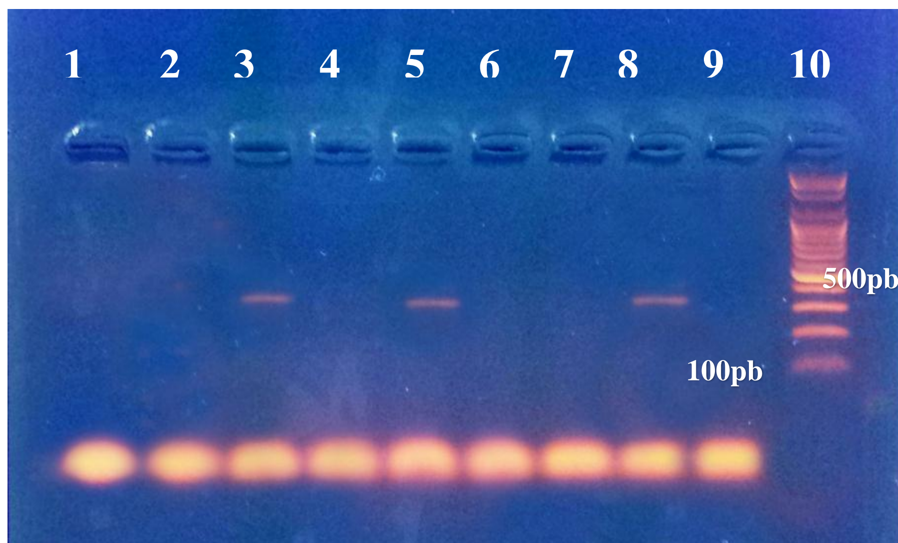

Fig 3. Gel electrophoresis (1% agarose, 7v/cm2, and 1.5hrs) of the PCR products Lane 10 100bp DNA ladder. Lane (3, 5, 8) Positive sample for GI genes of Norovirus (380) bp

Immunological detection of *Norovirus*:

Twenty six samples (26%) were ELISA positive and the remainder 74 (74%) were ELISA negative **(Table-3)**. Out of (28) were positive by PCR, three of them were ELISA negative and (25) samples were positive in both PCR and ELISA, One sample out (26) positive by ELISA were negative in PCR. The sensitivity and specificity of ELISA compared with PCR in current study were 89.29% and 98.61% respectively with Positive Predictive value 96.15% and Negative Predictive value 95.95% and kappa index was 0.899 table (3).

Table 3. Sensitivity and specificity of ELISA Vs PCR in detection of noroviruses

Norovirus			PCR		Total
			Positive	**Negative**	
ELISA	**Positive**		25	1	26
		%	89.3%	1.4%	26.0%
	Negative		3	71	74
		%	10.7%	98.6%	74.0%
Total			28	72	100
		%	100.0%	100.0%	100.0%
Sensitivity			89.29%		
Specificity			98.61%		
Positive Predictive Value			96.15%		
Negative Predictive Value			95.95%		
kappa index			0.899		

DISCUSSION

This study revealed that there is no statistical significant differences between the infection with *Norovirus* and gender, boys were (19) (28.4%) out (67) (67%) and girls were (9) (27.3%) out (33) (33%). This result was in agreement with another study by Li-Juan *et al*, (2010) [10] that showed the infectivity rate of *Norovirus* was approximately equal between male (55.1%) and female (44.9%). The current study, showed that children of all age groups infected with Norovirus there was no significant different between them, the highest infection in age (2-5) years (32.6%) followed by (<2 years) (25%) and then (> 5 years) (23.8%). This results was in accordance with other study by Li-Juan *et al*, (2010)[10] who mentioned that there is no significant difference in the distribution of age was reported in Norovirus infection. While in disagreement with the study of Lintao Sai *et al* (2013) [11] who observed highest infection in children under 2 years of age. Also disagreement with other study by Nguyen V. Trang *et al*, (2012) [12] observed highest rates of Norovirus infection in children less than 2 years. This may be because the large numbers of samples taken in these studies. The present study showed that the PCR method was more sensitive and specific than ELISA method, the results showed (28) out (100) samples were positive with Norovirus in PCR method, while in ELISA method there were (26) out (100) samples infected with Norovirus, three samples positive in PCR method were negative in ELISA method, and this finding confirmed by Beatrix Kele (2011) [13] who found that PCR method is more sensitive and specific than ELISA method. This result quite accord with study done by De Bruin *et al* who found that PCR positive samples were negative by the ELISA may be due to the fact that detection of Norovirus in stool samples by ELISA is based on the detection of viral antigens and may thus be hampered by the antigenic diversity of Noroviruses. [14] Our results showed that all cases (n=28) of Norovirus by using RT-conventional PCR was genogroup 1 (G1) this is in agreement with Linda V. *et al* (2015) [15] and disagreement with Nguyen V *et al* (2012) [16] who found that G11 is the most dominant genotype for noroviruses, the possible explanations for this discrimination in such results is that genogroup-specific differences have been reported with regard to environmental persistence,[17] sensitivity to removal, [18] and binding to receptors. [19] These biological differences may underpin strain-specific epidemiologic patterns, suggesting a

potentially useful approach for norovirus attribution such an approach was recently developed in a norovirus attribution study, which showed that the proportion of foodborne and person-to-person outbreaks differed between genotypes.[20]

CONCLUSIONS

Norovirus could be included as one of the most important causes of acute gastroenteritis in IraqELISA method is useful in diagnoses of Norovirus and will offer a p

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Conflict of interest

The author declares that they have no competing interests.

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