Association Between Norovirus infection and TLR7 Gene Polymorphism in Iraqi Patients with Gastroenteritis

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Abstract
Background: The gastroenteritis disease remains a global public health problem and human Noroviruses persist a cause of gastroenteritis. Noroviruses are highly contagious. They commonly spread through food or water that is contaminated during preparation or through contaminated surfaces. Noroviruses can also spread through close contact with a person who has norovirus infection. Objectives: To determine the rate of human Norovirus infections genome and the role of TLR-7 polymorphism in childhood Iraqi patients with gastroenteritis. Patients and methods: Case control study was carried out on 150 children and infant aged between (6-122 months) with gastroenteritis who attended the middle Euphrates hospitals for Maternity and Children as well as private laboratories, during the period from February 2021 till September 2021. Stool swabs, and blood samples were collected from each participant and stored as frozen at -70 °C to RNA extraction Norovirus genome and TLR-7 genome by using qReal Time PCR and Conventional PCR test, respectively. Results: The mean of age in patients with gastroenteritis was (43.56 months) while the control group (40.6 months) , also we found the infection in male more than female in percentage 58% for male and 42% for female. The rate of human Norovirus infection according to the quantitative real time PCR was 37.6% (29 out of 77) while the negative result was 62.4 % (48 out of 77). The present results indicated the presence of four nucleic acid variants in the investigated samples, namely 56G>A, 147A>T, 231C>T, and 288T>C. Results from the direct nucleic acid translation of the 56G>A indicated that this variant showed a missense effect on the protein, namely p.6S>N. The results of conventional polymerase chain reaction for TLR-7 for detection the relation with the severity of virus demonstrated that only 30% (45 out of 150) was positive for TLR-7 while the negative result was 70% (105 out of 150). The results showed that DNA polymorphism distribution were DNA polymorphism distributions according to CA ; AT ; TA and GA genotypes of TLR-7 polymorphism were respectively 62.2% (28 out of 45 cases) ; 31.1% (14 out of 45 cases); 6.7% (3 out of 45 cases) and 0% (0 out of 45 cases) in the GE patient group and was found GA genotype 100 % (5 out of 5 cases) in the control group. Conclusion: : The genome of the human norovirus genome appears to play a major role in gastroenteritis among infants and children, and TLR-7 has an important role in detecting norovirus severity among infected individuals.
Keywords: Toll like receptor-7; polymorphism; Gastroenteritis ; Real time PCR ; Norovirus

1. Introduction

Norovirus, an RNA virus of the family Caliciviridae, is a human enteric pathogen that causes substantial morbidity across both health care and community settings. *Norovirus* are Member of the Caliciviridae, nonenveloped, icosahedral virus typically 380–400 Å in diameter. The genome consists of a linear, positive-sense, single-stranded RNA of 7.4 to 8.3 kb in size with a covalently linked VPg at the 59 end and a polyadenylated tail at the 39 end [1].Several factors enhance the transmissibility of *Norovirus*, including the small inoculum required to produce infection (100 viral particles), prolonged viral shedding, and its ability to survive in the environment [2]. Human *Norovirus*es (HuNoV) are the main cause of non-bacterial gastroenteritis worldwide, producing high morbidity and mortality rates [3]. *Norovirus*es are a leading cause of epidemic acute gastroenteritis and are also an important cause of sporadic cases of acute gastroenteritis [4]. Because human *Norovirus*es have not been grown in cell culture and there are no convenient animal models in which to evaluate immunity and illness, much of our knowledge about these viruses comes from the study of outbreaks and experimental human infection [5].
NoV transmission typically occurs by the fecal–oral route from contaminated surfaces, food or water, and by person-to-person spread but transmission via droplets, through aerosoliza-tion of HNoV-containing vomitus, can also occur [6,7]. Outbreaks occur in places where people gather (e.g., cruise ships, day-care centers, hospitals). They are facilitated by the low numbers of virions able to cause infections (i.e., low infectious dose) [8,9], high amounts of viral shedding, high environmental stability of HNoV and a relative viral resistance to disinfectants [10]. Infection with NoV typically results

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in a combination of projectile vomiting, non-bloody watery diarrhea, often associated with symptoms such as nausea, chills, headaches, fever, and muscle aches. The infection is usually self-limiting within 2 days of symptom onset [11]. However, dehydration can occur in young children, the elderly and the immunocompromised, which can ultimately lead to death [12].

TLRs are pattern recognition receptors (PRRs) of the innate immune system that have been shown to exhibit tissue or mucosa-specific expression patterns. Each TLR has its own agonist (or set of agonists), known as pathogen associated molecular patterns (PAMP). These TLR agonists, or PAMP, include bacterial ligands, virus-specific ribonucleotide motifs (i.e., dsRNA), and imidazoquinoline compounds and all are currently studied as adjuvants [13]. Toll-like receptor-7(TLR7) is a forms from family of intracellular nucleic acid sensors maintained under strong Purifying selection, which attests of their essential role in host survival to viral infection [14].TLR7 localizes to the endosomal compartment, where it binds microbial or self-derived single-stranded RNA ligands [15].In plasmacytoid dendritic cells (pDCs),TLR7engagement elicits Strong type-I interferon(IFN) production and is critical to the induction no f antiviral immune responses [16].TLR7is also an essential component of antibody-mediated immunosurveillance against there-activation of endogenous retro viruses [17].

2. Experiments

Group of study

The study included the infected group with gastroenteritis (n= 150) and healthy non-infected as control group (n=50). The infected group represents people who were confirmed to be infected with the gastroenteritis depending on the symptoms after detection by medicinal and their results were positive. As for the control group, they are healthy volunteers.

Experimental design

This study was conducted during the period from February 2021 to September 2021. one hundred fifty participants aged 6–120 months were taken from general hospitals as well as many private clinical in Middle Euphrates provinces -Iraq .

Sample types and collection

Stool swabs as well as blood from each study group of infants and children patients suffering from gastroenteritis should be enrolled, that classify into One hundred – fifty stool swabs as well as blood specimens from infants and children patients suffering from gastroenteritis, and Fifty stool swabs and blood specimens of apparently healthy persons as control group.

The samples collection included Endometrium and\or cervical swabs ; fetal fluids swab were in 3 ml liquid viral transport media tube (UTM),each specimen was aliquot into three cryotube containing 1000 µl of the sample which stored at (-20°c) until genome extraction. After that, required part of specimens were taken and centrifuged at 10000 g/min for 5 minutes, discarded the supernatant except 100 µl of the solution was left to be used in re-suspension of the pellet for RNA/DNA extraction.

3. Methods

Extraction the viral nucleic acid from gastroenteritis patients specimens

Principle

By using specific viral DNA/RNA extraction kit (Intron/Korea); the viral genomic was extracted ,purifying and migrated using agarose gel from the blood, stool and fetal fluid as a first step to amplify the target *Norovirus* RNA.

Detection of Norovirus (NoV) by Real Time Polymerase Chain Reaction (RT-PCR).

Principle

Real-time PCR (qPCR) is based on two major processes: Firstly, isolation of viral genome (DNA\ RNA) from specimens, and Secondly, Real time amplification for each sample . In real-time PCR, the accumulating amplified product can be detected at each cycle with fluorescent dyes. This increasing signal allows to achieve sensitive detection and quantification of pathogens.

Procedure

GoTaq 1-Step RT-qPCR System(a,b) – (A6020/ Promega \ USA) combines GoScript™ Reverse Transcriptase and go Taq-qPCR master mix in a sigle step real time amplification reaction. the system which is optimized for RT-qPCR contains a propriety fluorescent DNA-binding dye, BRYT Green Dye.The system enables detection of RNA expression levels using aone-step RT-qPCR method, combining Go Script™ Reverse Transcriptase and GoTaq- qPCR Master Mix in a single step real-time amplification reaction.

The *Norovirus* genome that was targeted by real time PCR involve two primers , the *Norovirus* –GI primer (IDT/USA) with nucleotide sequence ,Forward primer (5″-3″) CTGCCCGAATTYGTAAATGA and Reverse primer with sequence (5″-3″) CCAACCCARCCATTRTACA, while the *Norovirus* –GII primer (IDT/USA) with nucleotide sequence ,Forward primer (5″-3″) CNTGGGAGGGCGATCGCAA and Reverse primer with sequence (5″-3″) CCRCCNGCATRHCCRTTRTACAT.

Detection of TLR-7 Genes Polymorphism By Polymerase Chain Reaction (PCR)

Procedure

Reactions were placed in a thermal cycler (Biometra-Germany) that had been preheated to 94°C and beforehand set up to the desired cyclic conditions.

The target regions of TLR-7 genome , by using specific TLR-7 primer (IDT/USA) with product size (408bp) and nucleotide sequence ,Forward primer (5"-3") ACTGACAAATACAGTCATGGGGTT and Reverse primer with sequence (5"-3") GGGAGATGTCTGGTATGTGGTT.

The PCR amplification procedure was make according to the conditions , Initial denaturation / 95C^0 /5 min , Denaturation / 95C0 / 30 sec, Annealing/58 C0 /30 sec, Extension 72 C0 /30 sec, Final extension/ 72 C0 /10min, and Number of cycles /40

Statistical analysis

All data were analyzed using one-way ANOVA, and means were compared using the Duncan test. The significance levels were $P<0.01$ & $P<0.05$ [18].

4. Results

I.Distribution of Patients with Gastroenteritis (GE) and Apparently Healthy Control (AHC) Groups According to Their Age.

Table (1)shows the mean age groups of the study population , where the mean age of the patients with GE was (43.56±8.31 months) was more than the mean age of the AHC (40.6± 10.96 months). there are non-significant statistical differences (p=0.42) between ARTI and AHC.

Table 1 : Distribution of Patients with GE and AHC according to Their Age.

(P-value)	Range		S. E	S. D	Mean of age(Months)	No.	Study groups
	Maximum	Minimum					
P=0.42N S(P>0.05)	120 months	6 months	2.304	8.31	43.56	150	GE
	120 months	8months	4.59	10.96	40.6	50	AHC
						200	Total

Distribution of Patients with GE and AHC According to Their Gender.

Gender distribution is represented in Figure (1). Where the Fifty-eight percent (58%) (116 out of 200 cases) of the study population were male, while female represented 42% (84 out of 200 cases).

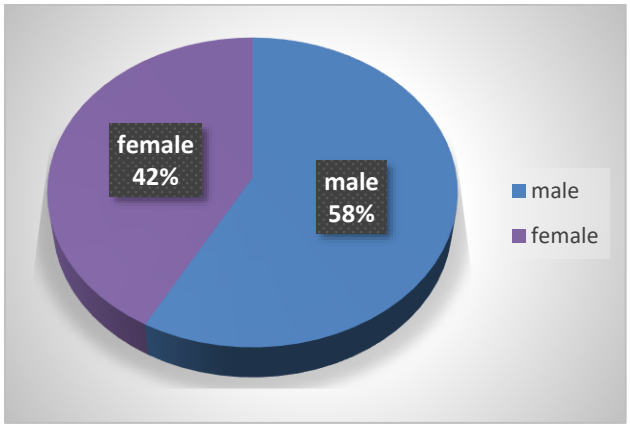

Figure 1: Gender Distribution of the Study Population.

Detection and Quantatiative of Norovirus Genotypes By qRT.PCR

The total positive result of *Norovirus* genome according to qRT-PCR shows 37.6% (29 out of 77 cases) as positive while less than 62.4% (48 out of 77 cases) as negative, as shown in Table -2 as well as Figures (2 A & B) . Statistically significant differences (p = 0.03) among patients group.

Table 2: Percentage of *Norovirus* Genotypes Positive Signals in Patients with GE by Using qRT.PCR Technique

NoV genotypes	No.	%	P value
Positive	29	37.6%	P=0.03 Sign >0.05
Negative	48	62.4 %	
Total	77	100	

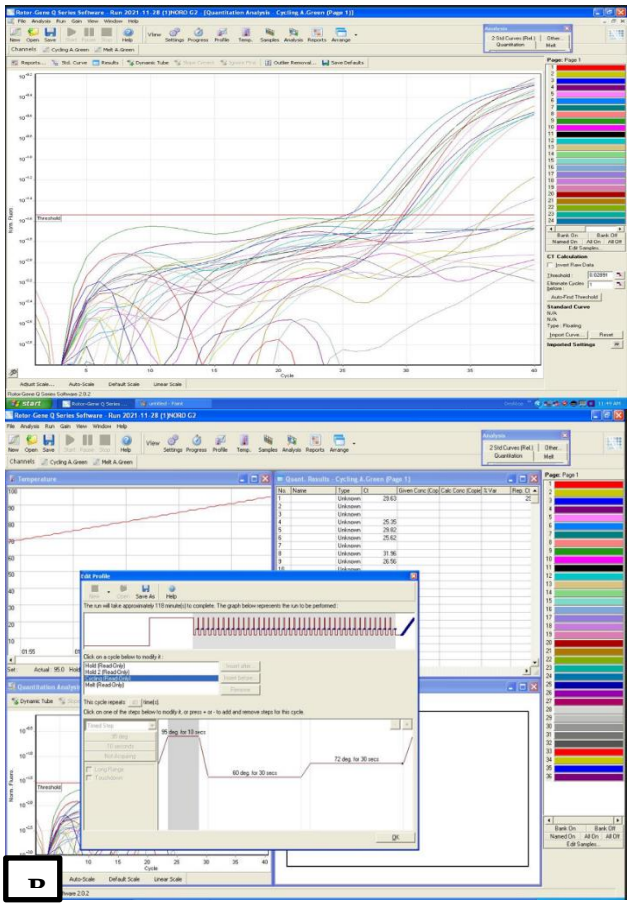

Figure 2: Detection the Norovirus genome by real time quantitative PCR

Cycle threshold of *Norovirus* genome that showing in many colored lines after 45 cycle in real time PCR

Quantitative & melting curve for Norovirus genome

Detection the TLR-7 Gene in patients with gastrointestinal

Figure (3) shows the TLR-7 gene using specific primer, where the revealed that the presence a single band (408 bp) of the target sequence of TLR-

7 gene in agarose gel .

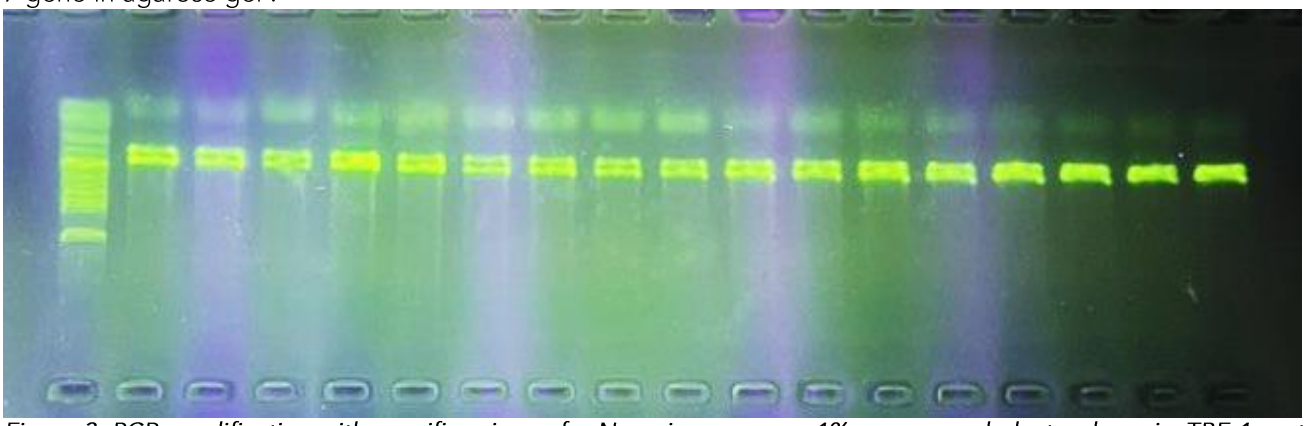

Figure 3: PCR amplification with specific primers for Norovirus gene on 1% agarose gel electrophoresis, TBE 1×, at voltage 75 for 60 min (A) Lane 1 =1 kb DNA ladder, whileLane 2 -18 =PCR product of TLR-7 gene at 408 bp.

Table 3 shows the percentage of a single band (408 bp) of the target sequence of TLR-7 gene. The positive result, according to PCR amplification of a single band (408 bp) of TLR-7 gene in women patients with GE and AHC were 30% (45 of 150 cases) and 10% (5 of 50 cases), respectively . While, the negative results were in patients with GE and AHC were 70% (105 of 150 cases) and 90% (45 of 50 cases), respectively .

Table 3: Percentage of TLR-7 signals in patients with GE and AHC groups by PCR technique		
TLR-7 gene band	GE No. (%)	AHC No. (%)
Positive	45 (30%)	5 (10%)
Negative	105 (70%)	45 (90%)
Total	150 (100%)	50 (100%)

V.Genotyping of TLR-7 Among Study Groups.

The results showed that DNA polymorphism distribution were DNA polymorphism distributions according to CA ; AT ; TA and GA genotypes of TLR-7 polymorphism were respectively 62.2% (28 out of 45 cases) ; 31.1% (14 out of 45 cases); 6.7% (3 out of 45 cases) and 0% (0 out of 45 cases) in the GE patient group and was found GA genotype 100 % (5 out of 5 cases) in the control group. In addition , was found just transversion mutation in GE patients , while in control group just transition mutation in TLR-7 gene Table (4).

Table 4: Comparison between patient with GE and HC on percentages of TLR-7 expressed gene polymorphism.								
95% C.I for OR [Patients]		P value	OR [Control]	OR [Patients]	Study group		Type of Mutation	Polymorphism of TLR-7 gene
					GE NO. (150)	HCNO. (50)		
Upper	*lower*							
0.95	0.79	0.08	1.1	0.7	62.2%	0.0%	Transversion	C\A
0.95	0.85	0.001	1.3	0.8	31.1%	0.0%	Transversion	A\T
0.95	0.79	0.08	1.1	0.7	6.7%	0.0%	Transversion	T\A
0.99	0.90	0.01	1.2	0.7	0.00%	100%	Transtion	G\ A

5. Discussion

The host innate response is the first line of defense following infection, and the outcome of infection is governed largely by an interplay between the virus and host antiviral defenses. To efficiently combat Human *Norovirus* disease, it is necessary to understand the viral life cycle and whether viral replication is affected by host restrictions [19].

In the current study we found the mean of age in patients with gastrointestinal at (43.56 months) , these results are compatible with Benninga *et al* [20].which indicated that infants and most young children have low immunity against bacterial and viral infections associated with the digestive system, such as E. coli and *Norovirus*, and therefore the average age of infection is close to what we obtained in our results above.

Our results showed that the average age of infection with gastroenteritis at age 3 year and less was higher than the age after 3 years. This may be due to the fact that the digestive system may be fully developed and functional, but the immature anatomical and functional features of the early intestine predispose to it abnormal bacterial (bacterial or viral) colonization in the intestine, the latter may interfere with function, the immune and neurological development of the digestive system, which makes microorganisms capable of causing disease in abundance at this age specifically [21].

The rate of human *Norovirus* infection obtained was 37.6% % (29 out of 77) among children with gastroenteritis who attended to many Hospitals Middle Euphrates provinces for Maternity and

Children , while some studies makes in Baqubah city / Diyala /Iraq [22] in addition to many other studies concerning this subject such as [23] in Lebanon with the rate (6.32%),[24] in Basrah who get a rate of 8% and [25] in Tunisia who get a rate 8.99%. 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 [26].

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. This could be related to population differences in terms of lifestyle, awareness, hygiene, number of infected people in the same family and several subjects [27].

The current result, was found that the rate of infection in males was higher than females, reaching 58% for males, while it was 42% for females. While a study was conducted on gastroenteritis patients, researcher Obili *et al* [28] who explained that females have a higher infection than males due to the different composition of the digestive system and the proximity of the stool opening from the urine opening, which may increase the incidence of infectious diseases that may be caused by bacteria or viruses, although According to the result of this study, the rate of infection was higher among males than females , these finding consistent with a study done by Hussein *et al* [29] concerning the sex, a study revealed a high number of male's patients than females' patients in Diyala, However, a few recent studies however have shown that there is a slight decline in these gender differences in the recent years, this is may be depending on the type infections that them get it in this age [30] while other studies have been conducted in Iraq (in Basrah) and China shows that the infection in female more than male [31,32] , However few recent studies however have shown that there is a slight decline in these gender differences in the recent years, this is may be depending on the type infections that them get it in this age [33].

In this study, we evaluated the presence of *Norovirus* genes Using RT-qPCR in Samples blood, stool and fetal fluid where HuNoV was diagnosed in 37.6% while negative samples were 62.4% (Table 3 and Figure 3). Therefore, although this percentage is somewhat small, but there are some factors that may reduce the rate of viral genome diagnosis, including what the researchers, Kim *et al* [34] and Schrader *et al* [35], referred to when diagnosing the virus with this technique, which is the presence of some ions and salts in the samples that contain the virus, as is the case with samples stool and other fetal fluids, which are potential inhibitors of RT-qPCR, especially that viral genes of the type RNA, which are more affected by the presence of substances that affect the diagnosis.

Small-molecule agonists of TLRs are currently used for detection the severity of many viruses since have important role against viruses [36]. One important example has been the use of TLR7 and TLR9 agonists in combination with detection severity of many viruses and also have theraputic role used in a treatment for HIV- patients [37,38].

TLR-7 as diagnostic tool for detection the severity of norovirus and our finding shows increasing in the concentration of TLR-7 at patients group in contrast to control group , so our selection of this immunological parameter came in agreement with many previous studies on the effects of TLR against RNA virus infection, as it has an important role in increasing the immune response through increasing the immune response and increasing the production of proinflammatory cytokines. that lead to inhibition of viral replication [39,40].

Receptor-like receptors (TLRs) including TLR3, TLR7 and TLR8 are essential for activation of the antiviral response upon viral infection, and can sense ssRNA or dsRNA in the cytosol [41]. Studies have shown an increased viral infection of *Norovirus* in children with TLR7 [42].

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