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Genotyping and Phylogenic of Norovirus as Main Cause of Children Gastritis in Thi-Qar province

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Abstract:

Norovirus consided as the second cause of viral diarrhea in children.The current study included examination of 200 (115 females and 85 males) stool sample of diarrhea and abdominal pain suffering children, less than 5 years of age. The samples were divided into nine groups according to the age of the children: 1-2, 3-5, 6-8, 9-11, 12-17, 18-23, 24-35, 36-48, 50-55 month. Stool samples were divided for two part, the first part for the molecular diagnosis of virus by real time PCR and then gene sequences of virus and the second part used for ELISA test. Results of real time PCR test revealed that 35 positive sample out of 200 with a percentage of (17.5%).(AccuZo1TM total RNA extraction kit(Bioneer, Korea was used in this test). Real time PCR results showed that Depend on age groups the highly morbidity between 18-23 months (25.8 %) and there was significant differences between age groups on probability ≤0.05. Results showed that high percentage of infection between children depend on artificial nutrition Top value was (21.7%) and the low percentage of infection in children depend on mixed nutrition (breast milk and cereal meals) was (12.6%). ELISA test results included 25 positive samples from total of 200 samples percentage was (12.5%). The sensitivity of this test was 71.4% while specifity was 94% compare to Real time PCR. ELISA test showed that the age group (18-23 months) was (25.8%). with a significant difference at (P>0.05). Artificial feeding is higher than (15.2%) and normal feeding is lower (9.7%). From the positive samples 8 samples sent for gene sequences, four samples were revealed compatibility to GII.2, three samples to GII.4 and one sample compatible with GII.3. depend on base data of NCBI.

Key words: Norovirus, children, Thi-Qar, real time PCR, Phylogenic.

Introduction:

Norovirus (NoV) is one of the most common causes of acute gastroenteritis around the world annually. The number of people infected with NoV is about 20 million in the United States and 15 million in Europe (WHO.2015; (CDC a. n.d. 2016). Norovirus belongs to the Caliciviridae family, which derives its name from the Greek word cup cup calyx, referring to the surface of the virus as the cup (Glass *et al*., 2009 ; Moore, 2016). Norovirus is divided into six genetic groups (GI to GVI) based on the genome sequence and similarities across the genes of the portfolio (Ramani *et al*., 2014 ; Costa *et al*., 2017). GI groups, GII, GIV are known to infect humans. However, the second genetic group GII is the main cause of human neurovirus infection (Green, 2013). Symptoms of the virus are nausea, vomiting, diarrhea and abdominal pain. Children are more likely to develop diarrhea than adults (Sala *et al*., 2014 ; Portal *et al*., 2016). Norovirus has non-developed with dimeter a 27-to-40-nanometer that surrounds a single 7.3- 7.5 kb RNA genome and is divided into three open-reading framesORIFs (Siqueira *et al*., 2017). Infections often occur in close or semi-closed communities, such as long-term care facilities, night camps, hospitals, prisons, schools and cruise ships (Robilotti *et al*., 2015 ; Attia, 2016). The most common routes of transmission of the virus are through stool, mouth, food or water transported from one person to another (Mattioli *et al*., 2015; Moore *et al*., 2015). Methods used to detect Norovirus in clinical specimens include electronic microscopy of stool samples, reverse transcription of real-time RT-PCR, ELISA and rapid immunochromatographic assays (Park *et al*., 2012). The Norovirus season usually begins in the winter and continues until spring between October and March in the Northern Hemisphere (Ahmed *et al*., 2013 ;

Wollants *et al*., 2015). About three quarters of Norovirus cases occur in cold months. Seasonal interpretation of acute gastroenteritis due to Norovirus was unknown, but factors such as precipitation, cold temperatures and the emergence of new variables were expected (Marshall *et al*., 2011; Räsänen, 2016). Previous studies in Iraq wrer used ELISA and RT-PCR assays and chromatography technique as methods for the diagnosis of Norovirus, such as studies in the provinces of Basrah, Mosul, Babil, Najaf, Baghdad and northern Iraq. Aims of the study: Study the spread of the virus as a cause of diarrhea among children and Determining the genetic classification of the Norovirus and DNA sequencing.

Materials and methods:

Case definition:

Acute gastroenteritis has been defined as the occurrence of diarrhea (diarrhea at least three times a day in 27 hours), according to the World Health Organization (Chen *et al*., 2013) Children who are excluded are those over the age of five years.

Samples Collection:

A total samples from stool 200 were collected from children under the age of five years with acute gastroenteritis under 5 years of age,The hospital was attended by Al-Shaheed Mohammed Al-Musawi Hospital for children and Bint Al-Huda Hospital for childbirth, children and private clinics in Thi Qar province during 6 month (from November 2016 to April 2017). 20% (v / v for the liquid sample) from each sample prepared in the normal saline solution after mixing strongly, centrifuge the mixture at 8000 rpm for 20 minutes at 4°C and then collect the resulting floating part and place in eppendorf tubes and store in - 20C° so use to extract DNA. Part 2: The stool sample was collected from each child (the same patient) and it was placed in the cup which closed nozzle to preserve the moisture of the sample and not dry it and stored in (-20C °) until used in the ELISA technique.

1. Enzyme link immunosorbant assay (ELISA)

For suspension of fecal samples, add approximately 100-50 mg of stool sample was added to the test tubes with 1ml of Diluent dilution solution to the same test tubes and mixed the mixture in the vortex to allow the suspension for standing to a short period of time (10 minutes) for coarse stool particles to settle and take the floating part In this case, it is centrifuged at 2500 rpm per minute for 5 minutes, The RIDASCREEN® Norovirus 3rd Generation 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, and the rest of the steps that have been conducted according to the manufacturer's instructions.

2.RNA Extraction of Stool

RNA extraction from faecal samples for children was carried out using the AccuZolTM Total RNA extraction kit (Bioneer, korea) and extracted according to the company's instructions.

Determination of RNA extracted from stool samples:

Detection of RNA extracted from stool samples through the spectrophotometer Nanodrop (Thermo.USA) for detecting and measuring the concentration of nucleic acids as RNA was detected by determining the concentration of RNA ng/ml and to measure the purity of the RNA by reading the absorbance with a wavelength of (260/280) Nanometer as the extracted RNA is pure when the absorption ratio is (1.8 -2).

3. Reverse Transcription Real-Time PCR(RT-PCR)

The Real-Time PCR technique was performed using the primers-specific genes of the viral vascular prophylaxis responsible for diagnosing the Norovirus virus and the diagnostic probes were performed according to a method described by(Al-Marsome *et al*., 2016) According to the following steps:

Preparation of an interaction mixture

Prepare the Real-TimePCR reaction mixture was using prepared by RT-qPCR GoTaq® (1-Step RT-qPCR System, Promega. USA) accordancing with the instructions of the company as in the following table:

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Table: (1) RT-qPCR master mix component

RT-PCR master mix	Volume
Total RNA template	5µL
Forward primer (10pmol)	1µL
Reverse primer (10pmol)	1µL
probe (20pmol)	1µL
RT-qPCR Master mix 2X	10
DEPC water	7 µL
Total	25µL

The reaction mixture components were then placed in a PCR tube and inserted into the Real-Time PCR device. All the tubes were then transferred to the Vortex centrifuge (Exispin) and centrifuged at 3000 cycles per minute for three minutes and then placed into the Real-Time PCR device.

Real-Time PCR Thermocycler conditions

Thermocyclers were applied to examine Real-Time PCR by drawing on several q PCR TaqMan kit instructions and the degree of fusion of primers.

Table(2):RT-Real-Time PCR primers and probe

Primer	Sequence		Amplicon
capsid gene Primer	F	ATGCGCTTCCATGATCTGAG	104 bp
	R	TGCCATCCATGTTTGTTGGG	
capsid gene probe	FAM- TGTGGACGGGGGATCGCGAT-BHQ-1		

GenBank: KM246912.1

Statistical Analysis:

The results of the study were statistically analyzed using SPSS version 22. Repeat tests and the Chi sequer test were tested at **P≤ 0.05**.

Results:

1-Percentage of Norovirus infection using Enzyme linked Immuno-sorbent Assay(ELISA):

This technique was used to diagnose Norovirus virus in the current study, The number of positive samples was 25(12.5%) samples out of 200 samples.

Relationship between infection rate and age groups

The study found that the most vulnerable age group (23-18 months) was 25.8%, followed by age groups (6-8 and 24-35 months) was 18.9% and the age group 50-55 (month) There was no infection, a significant difference was observed at the probability level (P≤0.05).

Relationship between infection rate and type of nutrition

The results showed that 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%. There was no significant difference(P>0.05).

2- Percentage of Norovirus infection using Real-Time PCR:

This technique was used to diagnose the percentage of norovirus in the current study samples, the number of positive samples was 35 out of 200 samples (17.5%).

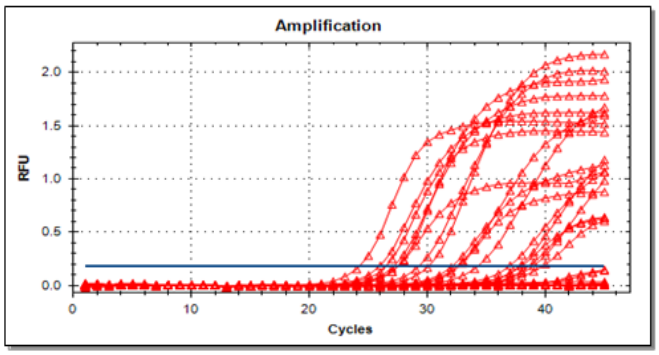

Figure 1: A graph of the amplification products using real time PCR technique, which is shown in the viral vascular genes in Norovirus on the TaqMan probe. Positive amplification samples are exceeded by the number of threshold cycles.

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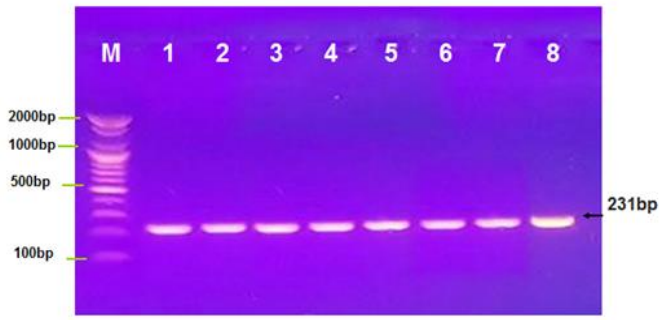

Figure 2: Agarose gel electrophoresis image that show the RT-PCR product analysis of capsid gene in Human Norovirus virus isolates. Where M: marker (2000), lane (1-8) positive Norovirus virus isolates at (231bp) RT-PCR product.

Relationship between infection rate and age groups

The study showed that the most vulnerable age group (18-23 months) was 25.8%, followed by 12-17 months (25.0%). The age group (3-5 months) showed the lowest percentage of 4.0% With a significant difference at a probability level ($P \leq 0.05$).

Relationship between infection rate and type of nutrition

The results of the study showed that children who use artificial milk were more susceptible to infection (21.7%), followed by mixed feeding (21.5%) and normal feeding (12.6%). There were no significant differences at the probability level ($P > 0.05$).

3- DNA sequencing method:

All DNA sequencing results of GII.2, GII.4, GII.3 and viral species were compared with the NCBI gene bank. The results of the present study showed that 8 viral isolates gave a positive result to the DNA sequencing method of the total 10 viral isolates, all species were compared with species in the gene bank. The current study confirmed that six new strains were registered in the genebank. Three new strains of GII.2 were given. Norovirus GII.2 Isolate IQ- No.5, Norovirus GII.2 Isolate IQ-No.6, Norovirus GII.2 Isolate IQ- No.8 With 99% similarity. The results showed a new type of GII.3 and Norovirus GII.2 Isolate IQ-No.2 was given a similarity of 98%. The current study confirmed three GII.4 strains and Norovirus GII.4 Isolate IQ-No.3, Norovirus GII.4 isolate IQ-No.4 by 99% similarity as in Figure (3). Positive samples were recorded in the genebank and became available on the web and the following numbers were given MF 166783، MF 166784، MF 166785، MF 166786، MF 166787، MF 166788 ،MF 166789 ،MF166790.

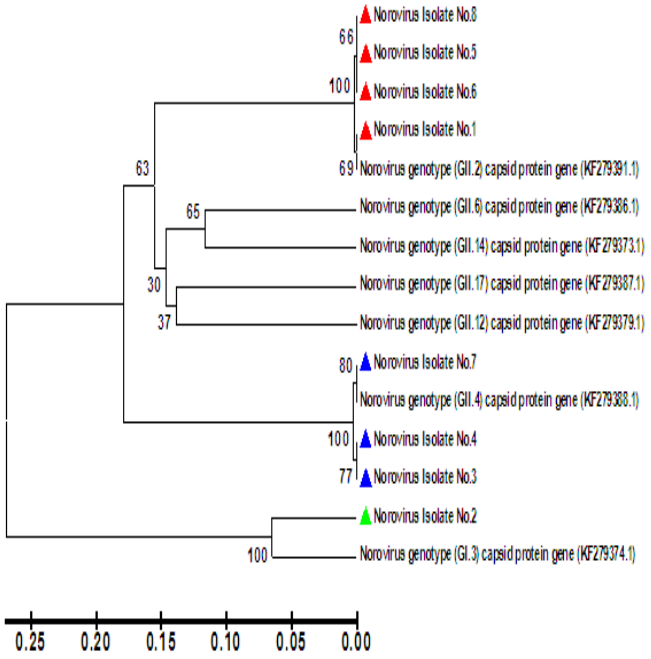

Figure (3): Phylogenetic tree analysis based on capsid protein gene partial sequence that used for Norovirus Genotyping detection from Human stool samples. By comparison GII.2 With KF279391.1، GII.3 With KF279374.1، GII.4 With KF279388.1.

Discussion:

The current study found that the rate of infection with the virus using RT-PCR was 17.5%, which is higher than the percentage reached (Thwiny and Hasony, 2013). In the province of Basra was (8%). Studies were carried out in northern Iraq and Baghdad province and recorded a higher percentage than the percentage in the present study were 30% and 32.27%, respectively (Al Mashhadani *et al*., 2008; Mohamed *et al*., 2015). The rate of infection was lower than that of Burkina Faso (21%). The low level of hygiene and sanitation in these areas, as well as the low access to potable water, facilitated the spread of Norovirus

strains(Huynen *et al*., 2013). In another study, the rate of infection was 6.5% and revealed that the difference in rates from other studies may be due to the criteria of selection of patients in the study where the selected patients of all age groups, unlike other studies (Kittigul *et al*., 2009). The ELISA test was 12.5%, and this ratio is close to a study in Najaf province, with an infection rate of 12% (Al-Ameedi and Al-Amar, 2015). The reason for the detection rates may be due to differences in population, study time, patient selection criteria and sample collection time contribute to different outcomes(Lennon *et al*., 2014). The current study showed may a significant difference, with the highest incidence in the age group (18-23 month) at 25.8%. The results of current study at the local level coincide with a study in Basra province by (Thwiny and Hasony, 2013), it was found that the infection was among children under the age of two years. Because this age group of children are turning to nutrition after weaning that may be contaminated with the virus. The results of the study were compared to a study conducted in Baghdad province, where the age groups did not differ significantly and reached the highest age (2-5) years, according to a study by (Liu *et al*., 2010) with no significant difference in age distribution. The results of current study showed that there were no significant differences. The highest incidence was in infants with 21.7% using RT-PCR (22.5%) and ELISA (15.2%). This percentage was lower than that in Mosul, where artificial milk was the highest and reached 26.19% due to the ease of transmission of viruses in the bottle of contaminated nutrition (Mohamed et al., 2016). The spread of the virus in children with artificial or mixed feeding be due to the use of untreated water for the preparation of milk, which is one of the important ways to transport Norovirus, as well as proteins in milk powder can reduce the amount of antibodies in breast milk, these types of antibodies Play a protective role against infectious agents (Al-Ameedi and Al-Amar, 2015). A study found a link between breast-feeding and low rates of NoV infection, possibly because of the protective role of maternal antibodies or the presence of Fucosylated glycans in the mother's breast milk (Makita *et al*., 2006). The process of naming and classifying the Norovirus virus depends on the analysis of the nucleotides of the ORF1 (ORF2) regions, both or one (Kroneman *et al*., 2013). The genetic profiling of the virus GII.4 is one of the most common types of Norovirus. In a study conducted at the University of California, United States, 85% of the cases after the analysis of the nucleotide sequence of the gene responsible for the formation of the viral portfolio were caused by GII.4 (Holzknecht *et al*., 2015). So far there have been 47 changes in ORF1 and 37 in ORF2 as well as when both regions are labeled (Kroneman *et al*., 2013). The gene of the study was used to study the nucleotide sequence and compare it with the gene bank to compare whether the species in Thi-Qar province have different changes or correspond to the known ones in the bank. Previous studies of this gene indicate that there are mutations in the sequence of nucleotides except some changes in the order of amino acids reaching from 125-152 (Chan *et al*., 2015; Cuevas *et al*., 2016). In the analysis of the nucleotide sequence of genes in the Norovirus virus, several sub-branches of this genotype have emerged, the most important of which are GII.1, GII.2, GII.3, GII.4, GII.6, GII.12, GII.17 (Lu et al. 2015). The results obtained from the current study show that four isolates are GII.2, three GII.4 and one isolated GII.3. The present study is consistent with a study of GII.4 and GII.3 patterns in children (Cheng et al., 2010; Tran et al., 2013). Another study found the Norovirus epidemic in children caused by GII.4 (Puustinen *et al*., 2012). A recent study found that few cases of Norovirus outbreaks were associated with GII.4 (Huynen *et al*., 2013).

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