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Real-Time Reverse Transcription-PCR for Detection of Norovirus in Children with Acute Gastroenteritis

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ABSTRACT

Norovirus GII is the most commonly identified cause of the outbreaks and the sporadic cases of acute pediatric gastroenteritis, and children's hospitalization is often required. It institutes a significant health problem because of its highly contagious nature and high rate of morbidity. This work describes the development of reverse transcription real-time TaqMan PCR assays for Norovirus GII identification as an etiological agent for acute pediatric gastroenteritis in children less than five years from stool specimens. The result of the RT-PCR assay revealed that Norovirus GII was present in 4(8%) out of 50(100%) feal samples from children with acute gastroenteritis in this study. while detection of Norovirus was 2% in case-control. Severe clinical symptoms associated with Norovirus infection compared with non-viral infection. This research established that the reverse transcription real-time PCR is among the greatest sensitive approaches for Norovirus detection based on the amplification of the DNA from Norovirus RNA, and the highlights needed for Norovirus laboratory investigations.

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1- INTRODUCTION

Enteric viruses are responsible for nearly 70% of the incidences of acute pediatric gastroenteritis. Acute gastroenteritis represents a main reason of morbidity and mortality among children. Diarrheal illnesses killed over 500,000 children worldwide in 2015, accounting for 9% of deaths in children less than 5 years [1]. A variety of viruses, bacteria, and parasites can cause acute gastroenteritis or infectious diarrhea [2]. After the epidemic of diarrhea in 1972, the Norwalk virus becomes the first viral pathogen to be identified as causing diarrhea [3]. Noroviruses are one of the most public causes of the sporadic and the outbreaks of acute gastroenteritis in people of all ages. They start suddenly and are extremely contagious [4]. Globally, After rotavirus, norovirus GII is the second most common cause of viral AGE, causing more than 677 million episodes and 213,500 deaths in children under the age of five each year [5]. Because of the virus's protracted shedding, environmental stability, disinfection resistance, low infectious dosage, and contribution of virus-induced vomiting to the propagation of the infectious agent, Norovirus infections are typically difficult to manage in outbreaks. Because of the specific properties of the virus that improve its propensity to propagate, Norovirus transmission is particularly effective.

The virus is extremely infectious, requiring just a tiny inoculum to produce infection (as little as 10 virions), and it is quite persistent in the environment. It can withstand freezing, heating to 60° C, and chlorine exposure [4]. However, Young children are more susceptible to suffer longer and more severe episodes that require hospitalization, and the disease is becoming recognized as a most important cause of chronic gastroenteritis in immunocompromised people.

Norovirus outbreaks occur in the summer as well, although at a lower incidence and with no pandemic spread to geographically isolated places [5, 6], In Iraq, the peaks of Norovirus infection detection were in November [7].
In real-time reverse transcription PCR, the applicant is recogenaze by a camera that recored fluorescence once evry cycle. This light is emitted either by fluorescent molecules that bindto DNA unspecific way (oftan SYBR green) or by so-called probes that emit light of a certain wavelength when hybridized to the target [8, 9]. Real-time PCR has higher sensitivity and accuracy compared with cPCR. In addition, Because of its relatively high accuracy for particular target DNA/RNA and the ability to quantify viral nucleic acids by CT value, real-time PCR methods for viral genome detection are utilized in the identification of viruses in clinical cases [10].

Diarrhea is the primary sign of acute gastroenteritis. This frequently coexists with infectious diarrhea, combined with a high temperature, nausea, anorexia, and cramping in the abdomen. The presence of viral gastroenteritis was linked to a reduced blood level of C-reactive protein (CRP) and a shorter stay in the hospital, despite the fact that it caused more episodes of vomiting than bacterial gastroenteritis [11, 12].
This study aims to assess the efficiency of detecting Norovirus GII using real-time reverse transcription-PCR in children under five years old. Additionally, C-reactive protein and hematological analyses are employed as markers of inflammation, aiding in the differentiation between viral and bacterial infections.

2- METHOD

2.1 Patients and samples collection

Between October 2018 and February 2019, we collected samples of feces as well as 50 samples of blood from children under five years old diagnosed with acute gastroenteritis. This study was conducted at two hospitals: Al-Alawiya Hospital for Children and the Children's Protection Teaching Hospital in Baghdad Medical City, Iraq. Additionally, we collected Fifty blood samples and Fifty stool specimens from children in good health to act as the group serving as a control.The C-reactive protein was determined qualitatively and quantitatively in the serum. And also, the hematological analysis includes WBC count, and differential of blood samples collected from children were measured by using an auto-hematological analyzer (Erma/ Japan).

2.2 Molecular detection of Norovirus by RT-PCR

The Norovirus GII was provided for identification by reverse transcription real-time PCR utilizing an acute gastrointestinal infections screen real kit (Sacace Biotechnologies/ Italy). Two primary procedures are used in the assay: isolation of RNA from fecal samples and reverse transcription Real-Time amplification of RNA for Norovirus. An internal control (IC) is included in the experiment to serve to serve as an amplifying controls of every material that is treated separately and to find any blockage of response. Next, using a Nanodrop spectrophotometer (Bionner, South Korea) to measure the RNA content of the samples in the volume of one microliters to the calculate the concentration of extracted RNA from each sample of the norovirus, and to measure purification and possible the protein contamination.

2.3 Extraction of RNA

The Norovirus RNA for this investigation was retrieved from fecal specimens taken from children under the age of five who had acute gastroenteritis, Using a Ribo-Sorb extraction kit (Sacace Biotechnologies/Italy).

2.4 Procedure for extracting RNA

Norovirus RNA extraction was following the manufacturer's guidelines with slight modifications. Initially, the lysis solution and washing solution were heated to 60-65°C until ice crystals disappeared. Ten microliters of internal control and 450 microliters of lysis fluid were placed into each of the prepared Eppendorf tubes. For every tube, 50 microliters of stool specimen were added to internal control mixture and lysis fluid with mixed will using pipetting, followed by a 5-minute incubation at ambient temperature. Additionally, 50 microliters of C- (negative control from the amplification kit) was added to the designated negative control tube. The tubes were vortexed, then centrifuged for 1 minute at 14,000 rpm, and the supernatant was transferred to new tubes for RNA extraction.
Sorbent reagent (25 microliters) was vigorously vortexed and added to each tube, followed by a 10-second vortex and a 10-minute incubation at room temperature with periodic vortexing. Subsequently, all tubes were centrifuged at 12,000 rpm for 1 minute, and the supernatant was carefully discarded. Each tube received 400 microliters of washing solution, vigorously vortexed, and centrifuged at 12,000 rpm for 1 minute, with the supernatant again discarded. This washing step was repeated twice with 500 microliters of 70% ethanol added to each tube, vortexed vigorously, and centrifuged at 12,000 rpm for 1 minute each time. Then, 400 microliters from the acetone were inserted into every

tube, vortexed forcefully, then centrifuged for 1 minute at 12,000 rpm, followed by the subsequent disposal of the supernatant.
All tubes were incubated with open caps at 60°C for 10 minutes. The resulting pellets were resuspended in 50 microliters of RNA eluent and incubated at 60°C for 10 minutes with vortexing 2-4 times. The tubes were then centrifuged at 12,000 rpm for 2 minutes, yielding viral RNA in the supernatant ready for use in reverse transcription PCR detection or storage at -80°C until further use.

2.5 procedure of Norovirus qualitative RT-PCR
For each clinical specimen, prepare the reaction mix with PCR-mix-1 Norovirus in a new sterile tube labeled as Norovirus. Add the specified components to the tube, vortex it, centrifuge briefly, and then add 15 microliters of the prepared reaction mix to each tube. Next, handle the extracted RNA samples carefully, add 10 microliters of RNA to the reaction mix, and label the positive control. Finally, follow the reverse transcription real-time PCR program for Norovirus detection
The analysis of data for this study was done with SPSS program (licensed materials, version 25). The information was displayed as mean ± SD, or mean plus standard deviation. To compare means and evaluate correlations between the investigated factors linked to infection, we employed the independent-samples T-test and the chi-square test. A p-value of less than 0.05 was deemed to be statistically significant.

3- RESULTS AND DISCUSSION

3.1 Estimation of RNA concentration and purity
Using a nanodrop instrument (Bionner, South Korea), both the purity and concentration of extracted Norovirus GII RNA were measured. The findings indicated that the RNA extracted concentrations varied between 94-561 ng/ml, with purity ranged between 1.7-2.2.

3.2 Detection of Norovirus GII by RT-PCR
Norovirus GII was distinguished on the JOE (red) canal together with an internal control tube that included internal control and PCR-mix-1 Norovirus.

The specimeis considered positive for Norovirus GII when the Ct value of the red line was lower than the boundary value. (Figure 1).

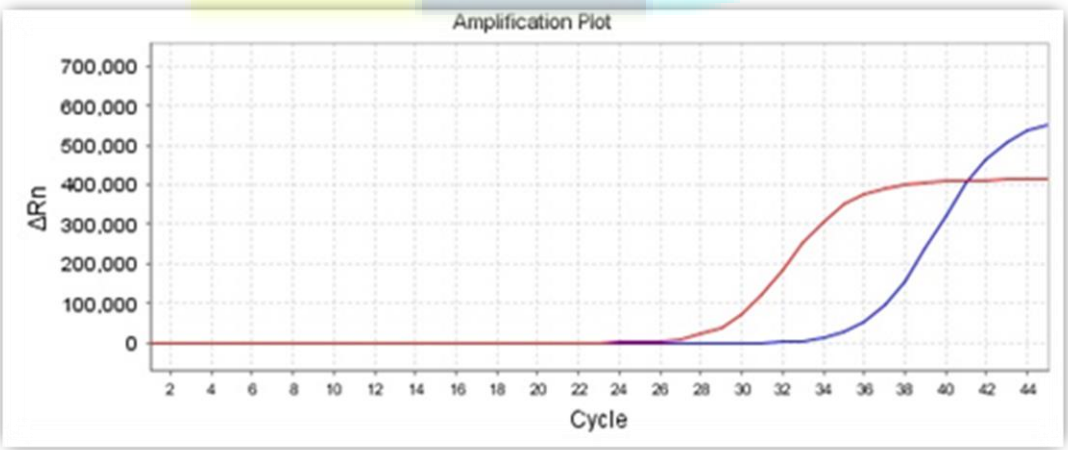

Figure -1 The reverse transcription real-time PCR multiplex amplification of internal control and Norovirus GII. The value being expressed is shown by blue and red curves of internal control (32.1 Ct) and Norovirus GII (29.9 Ct) respectively. The Norovirus GII was discovered in 4 (8%) of 50 (100%) stool specimanes from children uder five years with acute gastroenteritis infections, according to the results of reverse transcription real-time PCR multiplex detection. This result matched to Study in Diyala, Iraq showed that the Norovirus infection was detected in 6.04% of children with gastroenteritis symptomatic [13], also closed to other studies state that 10.5% of samples were positive for Norovirus, also the overall Norovirus prevalence was 12.3% [14] and 11% had Norovirus detection by Daniel-Wayman et al., 2018 [15]. In contrast, other studies showed that the Norovirus was detected in 17.0% of viral

gastroenteritis, the Norovirus infection combined with severe gastroenteritis, and the children with Norovirus infection had typical clinical symptoms [1].

In the current study, the hematological parameters were measured by using of autohematological analyzer, and the results revealed that the total leucocyte was ranged from 6.400-13.600 cell/cm in children with acute gastroenteritis infection. also, the result of the present study revealed that the sluggish high lymphocytosis averages were observed in the children infected with Norovirus compared with the case-control group [16]. Moreover, serum CRP levels were lower in viral gastroenteritis than in non-viral gastroenteritis cases. The quantity of C-reactive protein did not significantly differ amongst the enteric virus infections [1].

The result shows that the enteric virus infection caused diarrhea with a watery or loose stool consistency in all of the positive samples, according to the general stool inspection. There was no blood in the stool samples of virus infection, which could mean that the virus causes non-bloody diarrhea. These findings are consistent with the concept that if the stool is bloody, the reason is more likely to be bacterial or E. histolytica [17, 18].

Norovirus-infected children were a high percentage of clinical symptoms, along with a study in Iraq by Thwiny 2015 [7]. Apart from diarrhea, the most prevalent clinical symptoms among Norovirus-infected children were the vomiting (94 %), the fever (69 %), the dehydration (50 %), and the stomach discomfort (31 %). In addition, the results exhibited that most children infected with acute gastroenteritis were found to suffer from frequent bowel motion more than 5 times per day. Norovirus was the leading highest severe gastroenteritis-causing agent in children with rotavirus vaccine [19]. Children aged less than two years were more at risk of hospitalization In addition, the results of this study revealed that there was a highly statistically significant difference (p-value < 0.001) in the clinical symptoms with and without enteric viral infection between children with acute gastroenteritis and control groups.

The results obtained in this study employing RT-PCR detection are consistent with those from other research that were reflexed the real-time reverse transcription PCR assay for the detection of numerous varieties of viruses in clinical situations should be established on the use of suitable specimens, extraction of nucleic acids based on viral characteristics, and Specificity and sensitivity are utilized to select diagnostic procedures. In addition, The performance of the test procedure must be evaluated by assessing sensitivity, specificity, precision, and reproducibility when using real-time reverse transcription PCR. The accuracy and sensitivity of real-time PCR are greater [20, 21]. RT-PCR is a straightforward method that is also reliable, quick, adaptable, and sensitive for simultaneous detection of pathogenic viruses in a given specimen. It is important to offer the true pathogen spectrum, even in cases when the pathogens exhibit the same symptoms along with various indicators [22, 23].

4- CONCLUSION

The conclusions are drawn:

1- The real-time PCR technique of reverse transcription can serve as a dependable and precise instrument for the detection and distinction of several viral causes of acute gastroenteritis.

2-This study exhibits that the Norovirus important viral etiology of the acute gastroenteritis disease incidence in children, aged less than five years, and highlights the need to routinely implement laboratory investigations.

3-The results showed severe clinical symptoms associated with Norovirus infection compared with the non-viral infection.

4-The estimation of CRP level, WBCs count, and deferential WBCs can be represented as a weak tool for early identification of childhood viral acute gastroenteritis.

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النسخ العكسي في الوقت الحقيقي لتفاعل البوليميراز المتسلسل للكشف عن نوروفيروس لدى الأطفال المصابين بالتهاب المعدة والأمعاء الحاد

الخلاصة

نوروفيرس النمط الجيني الثاني يعد السبب الأكثر شيوعًا لتفشي أمراض التهاب الأمعاء الحاد لدى الأطفال، وغالبًا ما يلزم دخول الأطفال إلى المستشفى. يتسبب في مشكلة صحية كبيرة بسبب طبيعته شديدة العدوى وارتفاع معدل الإصابة بالأمراض. يصف هذا البحث استخدام تحليل النسخ العكسي (تاك مان) ذو الوقت الحقيقي لتفاعل البوليميراز المتسلسل لتحديد نوروفيرس كعامل مسبب لالتهاب الأمعاء الحاد عند الأطفال الذين تقل أعمارهم عن خمس سنوات من عينات البراز. أظهرت نتيجة النسخ العكسي ذو الوقت الحقيقي لتفاعل البوليميراز المتسلسل أن نوروفيرس النمط الجيني الثاني كان موجودًا في 4 (8٪) من 50 (100٪) عينة براز من أطفال مصابين بالتهاب الأمعاء الحاد في هذه الدراسة. بينما كان الكشف عن نوروفيروس في2٪ من الحالات الغير مصابة. كما اثبت النتائج وجود الأعراض السريرية الشديدة المصاحبة لعدوى نوروفيروس مقارنة بالعدوى غير الفيروسية. أثبت هذا البحث أن النسخ العكسي ذو الوقت الحقيقي لتفاعل البوليميراز المتسلسل هو من بين أعظم الأساليب الحساسة للكشف عن نوروفيروس بناءً على تضخيم الحمض النووي من الحمض النووي الريبي الفيروسي ،كما اثبتت ان الفحص المختبري عن النوروفيروس من الضروريات المهمه لصحة الاطفال.