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PREVALENCE AND MOLECULAR CHARACTERIZATION OF ROTAVIRUS A IN PEDIATRIC PATIENTS WITH ACUTE DIARRHEA.

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ABSTRACT

Rotaviruses are classified in the genus *Rotavirus* and belong to family of Reoviridae, that is a family of viruses that can affect the gastrointestinal system. Reoviridae have genomes consisting of segmented, double-stranded RNA (dsRNA).This study aimed to investigate the prevalence of Rotavirus (RV) among children with acute diarrhea. One hundred and fifty pediatric patients suffering from clinical manifestation of diarrhea, fever, and vomiting were enrolled in this study. All patients underwent ELISA test for stool VP6 protein detection and real time PCR test for VP6 and NSP4 genes detection. Results showed that the frequency rate of Rotavirus infection was 33.3% by ELISA technique. The molecular techniques showed a positivity, of 33.3% for VP6 gene and 34% for NSP4 gene. The ELISA assay represented the more sensitive test in detection of Rotavirus related diarrhea in stool specimens. The results revealed that males tend to be more effected by RV than females and the bottle feeding children were more susceptible to virus infection, comparing to other feeding type. The infection rate had increased with decreasing the age of the children.

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1. Introduction

Rotaviruses are classified in the genus *Rotavirus* and belong to family Reoviridae is a family of viruses that can affect the gastrointestinal system (such as *Rotavirus*). Viruses in the family of Reoviridae have genomes consisting of segmented, double-stranded RNA (dsRNA). Rotavirus can be classified serologically into seven distinct groups named A to G. This classification is according to antigenic groups detectable by a number of serologic tests, such as immunofluorescence, enzyme-linked immunosorbent assay (ELISA) [1].

The antigenic groups are mainly present on the major inner capsid protein VP6. Group A, B, and C rotaviruses are found in both humans and animals, whereas viruses in groups D, E, F, and G have been found only in animals. Group A has been established as the predominant group causing human rotavirus diarrheal disease[2].

There are six NSPs coded by the rotavirus genome, namely NSP1-6. With the exception of NSP4, all of these NSPs bind to the RNA. They have various functions in viral replication, assembly of various proteins and in viral packaging during replication [3].

The NSP4 protein is 175 amino acids in length and is encoded by gene segment 10 (751 bp). It is an endoplasmic reticulum (ER) specific glycoprotein and it is one of the most important non-structural proteins in relation to virus virulence and pathogenesis and is the first multifunctional viral enterotoxin reported. This protein acts as an enterotoxin and is responsible for diarrhea in infants and age-dependent diarrhea in mice. The protein plays a unique role by functioning as an intracellular receptor and converting double-layered viral particles in the cytoplasm into the triple-layered form in the ER [4].

Each year, rotavirus causes approximately 111 million episodes of gastroenteritis with 2 million requiring hospitalizations worldwide. Studies published between 2000 and 2004 indicate that rotavirus causes approximately 39% (range 29% - 45%) of childhood diarrhea hospitalizations, and it is estimated that 611,000 (range 454,000- 705,000) rotavirus-related deaths occur every year, with most cases occurring in the poorest countries. The percentage of rotavirus diarrhea associated with hospitalization is similar in low, middle and high income countries. Furthermore, most of rotavirus infections (75%), occur between 6-24 months of age [5].

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In the recent years, Rotavirus infection are declining worldwide due to the introduction of two vaccination that play a key role in reducing the burden of mortality and hospitalization in children younger than 5 years [6-8].
The aim of this study was to investigate the prevalence of Rotavirus among children with acute diarrhea by ELISA for VP6 protein detection and by molecular methods for detection of human rotavirus vp6 and NSP4 genes.

2. Material and methods

Patients
A total of 150 pediatric patients (81 males and 69 females with an age under five years) attending Bint Al-Huda Teaching Hospital in Thi-Qar province, Iraq, during the period from April 2016 to October 2017 were included in this study. They were all suffering from clinical manifestation of diarrhea, fever, and vomiting.

Sampling and study design
This cross-sectional study was carried on 150 stool samples obtained from children under 5 years old with acute diarrhea. The children who enrolled in the present study had sought medical assistance after several episodes of loose or watery diarrhea. The stool samples were collected using wide-mouthed sterile plastic containers and stored at -20°C until the time of assays. All samples under study were subjected up to only one cycle of thawing and freezing prior to characterization. A questionnaire was performed to collect data for all patients. Patients with the following criteria were excluded from the current study; stool samples with RBCs, Amoebas, flagellates, Eggs, larvae, and cysts.

Enzyme linked-immunosorbent assay
VP6 protein was detected by ELISA technique (from Genex, USA) by using RIDASCREEN® Rotavirus ELISA kit(R-BIOFARM, Germany).

Viral RNA extraction and real time polymerase chain reaction
Viral RNA was extracted from frozen stool samples by using AccuZol™ viral RNA extraction kit (Bioneer, Korea) according to manufacturer instructions.RT-q PCR was employed for detection of human rotavirus using the primers and TaqMan probe (Table 1) specific for viral protein 6 (VP6) and non-structural protein 4 gene (NSP4) of human rotavirus virus. This technique was carried out according to method described by [9] using Real-Time PCR system (from BioRad, USA).

Primers used for partial length VP6 gene of Group A rotavirus				
Primer	Sequence		Location	Expected amplicon
VP6 primers	VP6-F	GACGGVGCRACTACATGGT	737–755	380 bp
	VP6-R	GTCCAATTCATNCCTGGTG	1116–1098	
Primers used for NSP4 gene of Group A rotavirus				
Primer	Sequence			Product size
NSP4 Primers	F	AAGAAGTGACTGCAGCGATG		137bp
	R	TTGAACCACACGCGATATGG		
NSP4probe	5-FAM-ACTGTCTTCGGAAGCGGCGG-BHQ1-3			

Table 1. Primers and probes used in the study.

Statistical analysis
The statistical calculations were performed using statistical package for the social sciences version 24 (SPSS, IBM Company, Chicago, USA).Chi square test was used to test frequency distribution of categorical variables within groups of the study. Differences were considered statistically significance at p value <0.05.

3. Results

The results of this study showed that out of 150 stool samples of children with acute gastroenteritis, fifty stool samples (33.3%) showed rotavirus infection when tested by ELISA technique.
Results also found that all of RV- positive children (No.=50) were suffering from diarrhea (100%), in addition to vomiting and fever with 31 (43.1%) and 15 (20.8%), respectively ($p<0.01$).
According to sex distribution, males tend to be more effected by RV with 31 (62%) cases in comparison to females with 19 (38%) cases (Table 2). Statistically, gender differences were significant ($p>0.05$).
Regarding to age groups, results showed that the highest RV-positive infection was found in age groups (1-8 months and 9-16 months) with a total of 33 (66%) cases (Table 2; Figure 1), distributed to 19 (38%) and 14 (28%) for both age groups, respectively. On the other hand the lowest infection with rotavirus 4 (8%) was found in age group (3-5 years). This variation of the above results was statistically significant ($p < 0.01$).
Results also revealed that the rate of RV gastroenteritis was the highest in children who used bottle feeding (56%), followed by mixed feeding (26%) and breast feeding (18%), respectively (Table 2). This variation of the above results was statistically significant ($p < 0.01$).

Parameters		ELISA
Sample Size		50 (33.3%)
Symptoms	Diarrhea	50 (100%)
	Vomiting	31 (43.06%)
	Fever	15 (20.83%)
Gender	Male	31 (62%)
	Female	19 (38%)
Feeding Type	Breast Feeding	9 (18%)
	Bottle Feeding	26 (56%)
	Mixed Feeding	13 (26%)
Age	1-8 months	19 (38%)
	9-16 months	14 (28%)
	17-25 months	13 (26%)
	3-5 years	4(8%)

Table 2. Distribution of Rotaviruses (No.= 50) according to study parameters.

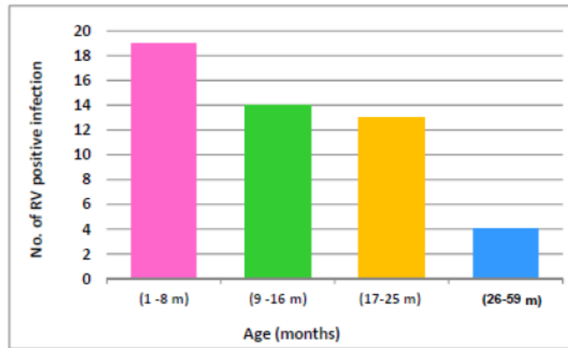

Figure 1. Distribution of pediatric patients with RV positive infection according to age.

Regarding to RT-qPCR, ELISA-negative Rotavirus stool samples (No.=100), were subjected to RT-qPCR for the detection of Rotavirus. ELISA- positive Rotavirus stool samples were also subjected to RT-qPCR as a confirmatory diagnosis of Rotavirus by vp6 gene. Furthermore, these 50 positive samples were subjected to RT-qPCR for further detection of NSP4 in the studied groups.

The results revealed that all ELISA- negative Rotavirus stool samples (No. =100), were negative in RT-qPCR for vp6 gene, while ELISA-positive Rotavirus stool samples (No.50, 33.3%) were positive by RT-qPCR technique for the same gene (Figure 2). Results of RT-qPCR for detection the presences of NSP4 in 50 stool samples obtained from children with a cute gastroenteritis, found that 17 samples (34%) were NSP4- positive.

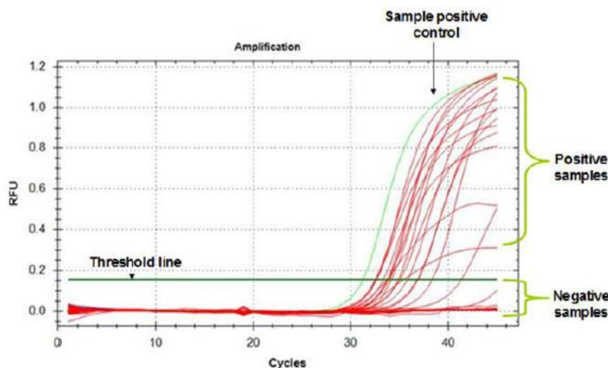

Figure 2. The graph of positive RV obtained by RT-q PCR Thermocycler.

4. Discussion and conclusions

Diarrhea is a leading cause of death in children under five years of age globally, with an estimated 1.5 million child deaths per year [10]. Rotavirus infection remains the commonest cause of severe dehydrating diarrhea among children worldwide [7].

ELISA test kits for detection of viral antigens is commonly used in many laboratories as a highly sensitive method of rotavirus diagnostic worldwide to detect the virus directly in stool samples.

Rotavirus can be detected by reactive antibodies against epitopes of VP6 shared among group A rotavirus. Monoclonal antibodies (MAbs) are also used for the determination of subgroups within group A rotavirus [11].

A sudden onset of symptoms typically manifests in children 1 to 2 days after infection with RV. The clinical picture of RV- gastroenteritis is characterized by 4 to 7 days of acute febrile illness, vomiting, and watery, non-bloody diarrhea. This combination can lead to rapid dehydration without appropriate intervention. Secondary infection with RV are clinically milder or asymptomatic [12]. The results showed that diarrhea was the predominate symptom among RV-infected children included in this study. These results were slightly different from those obtained by Al-Jabiry [13] who found that the incidence rates of vomiting and diarrhea of pediatric patients were 60% and 40%, respectively. On other hand, our finding was similar to a research conducted in Iran [14].

The results of the present study showed a lower prevalence rate of RV (33.3%) (by ELISA method) compared with other similar studies (that used the same serological diagnostic tool) conducted in Basrah [15], Erbil [16], and Babylon [17] with RV- positive cases of 43.3%, 37%, and 45.76%, respectively.

The male predominance in the present study was also confirmed by several authors in Iraq and some regional neighboring countries [1, 7,13, 18,19].

Regarding to the distribution of infected children according to age, rotaviruses infect nearly fewest 4 (8%) of the children by the age of 3–5 years and are globally the leading cause of severe, dehydrating diarrhea in children aged <5 years. In low income countries, the median age at the primary rotavirus infection ranges from 6 to 9 months (80% occur among infants <1 year old) whereas in high income countries, the first episode may occasionally be delayed until the age of 2–5 years, though the majority still occur in infancy (65% occur among infants <1 year old) [6,20].

The result of the present study according to age distribution was in consistent with other survey studies in Iraq [15,18]. The severity of rotavirus infection is age-dependent. Although the disease can occur at any age, clinically significant incidents most commonly occur in young infants and children [21].

Regarding to the distribution of infected children according to type of feeding, a study found a similar results with a predominate RV- positive infection in children with bottle feeding [18]. On the other hand, other studies showed similar infection distribution according to the feeding type [22]. The breastfeeding, according to recent evidences, could also protect against intussusception that could complicate a gastrointestinal infection in children under 5 years old [23].

Qadri *et al*. [24] supposed that there were sufficient evidences displaying a relationship between breast feeding and the prevention of diarrheal diseases with bacterial origin, such as diarrhea caused by *Vibrio cholerae, Escherichia coli,* were breast feeding has proven to be important in protection. However, the link between viral diarrhea and breast feeding is less clear.

It is possible that breast feeding may only be protective if it is practiced with intensity and frequency that allows continuous high level protection of the intestinal mucosa rather than sporadic or low volume feeds [23, 25]. On the other hand, in a local study carried out in Kurdistan, Iraq, Rashid and Sharif [26] found that only 3 breast feeding in 30 children were RV-positive. The researchers proposed that breast feeding reduces the risk of rotavirus, and they notice that 10% of breast feeding infants showed less severe signs. Since the RV positive samples may be considered not much enough.

So assessing a final conclusion for the role of feeding type in rotavirus diarrhea may be unconvincing in addition to that we think that some mothers not gave us the true history about their feeding type in which some of them may be afraid from not using breast feeding as a feeding type [26].

Reverse transcription polymerase chain reaction (RT-PCR), which is highly sensitive in detecting small concentration of rotavirus in stool specimens, is also used for strain identification and further differentiation [27].

The only molecular detection local study founded in Iraq, that of [16], which used RT-PCR for detection of eight RV genotypes.

In the present study, RT-qPCR technique was conducted by targeting the non-structural protein-4 gene (NSP4) as a universal gene to detect the presence of RV infection. The mechanism of rotavirus induced diarrhea is not fully understood, but it is caused in part by the first described viral enterotoxin-rotavirus non-structural protein 4 (NSP4) [28]. NSP4 has pleiotropic properties in cells related to its involvement in both rotavirus pathogenesis and morphogenesis [4].

The present study faced a problem of financing both serological and molecular techniques used in the study. This matter obligated the authors to make some limitations such as the identical number of the tested samples in both techniques.

Wittwer [29]was the first who began to develop Real-Time polymerase chain reaction (RT-qPCR) machine. This type of instrument has an integrated fluorimeter to allow in-tube real-time analysis of DNA template samples. Arise in fluorescence of the signal of each cycle indicates amplification. The ability to monitor reaction progress has a number of advantages over end point analysis. As a result, Real-Time PCR has proven to be a powerful tool for genetic analysis [3].

DNA detection simultaneous to amplification is preferentially achieved by the use of target sequence-specific oligonucleotides linked to two different molecules, a fluorescent reporter molecule and a quenching molecule [31]. These probes bind the target cDNA between the two PCR primers and are degraded or released by the DNA polymerase during DNA synthesis. In case of degradation the reporter and quencher molecules are released and separated, which results in the emission of an increased fluorescence signal from the reporter. Different variation of this principle of reporter and quencher are used by the different commercially available assays. The fluorescence signal, intensified during each round of amplification, is proportional to the amount of RNA in the starting sample. Quantification in absolute numbers is achieved by comparing the kinetics of the target amplification with the amplification kinetics of an internal control of a defined initial control of a defined initial concentration [32].

Sensitivity, specificity, and speed should be the driving factors behind choosing a microbial detection method, and Real-Time PCR applications can address all these needs.

It has become common to relegate viral culture to specialized virology laboratories rather than include it in the routine diagnostic laboratory, where the focus is on rapid results turnaround [32]. The sensitivity of PCR- based methods is 100,000 more than that of PAGE [33].Accurate information on the burden of rotavirus gastroenteritis are crucial to guide recommendation for rotavirus vaccination [34]. Most epidemiological data have been based in survey studies data, thus, as the distribution of circulating rotavirus serotypes may vary dynamically, prospective studies are needed to help the health authorities in planning effective immunization strategies, and for new up-to-date rotavirus vaccines [35].

Peritoneal injection or luminal administration of NSP4 into 6 to 10 days-old mice causes diarrhea within hours after incubation. This diarrhea is due to extra cellular NSP4 stimulating a transient, receptor-mediated phospholipase C activation, leading to elevated intracellular calcium levels and subsequent chloride secretion [36]. During virus morphogenesis, intra-cellular NSP4 (iNSP4) serves as an intracellular receptor for the badding of nescent, immature double-layered particles into the lumen of endoplasmic reticulum (ER), where the virus maturation occurs [37]. Inhibition of iNSP4 expression using small interfering RNA alters the cellular distribution of other viral proteins, including the formation of viroplasms, where virus replication occurs, and also modulates viral mRNA synthesis, suggesting an additional role for NSP4 in virus replication [38].

5. Conclusions

ELISA assay was more sensitive in detection of Rotavirus related diarrhea in stool specimens. The prevalence of Rotavirus infection in pediatric patients in this study was 33.3% by ELISA method. On the other hand, the molecular prevalence of Rotavirus infection was 33.3%, and 34%, for VP6 and NSP4 genes, respectively.

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