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Molecular Identification And Phylogenetic Analysis Of Rotavirus In Children Suffered From Diarrhea Under Five Years Old In Thi-Qar Provience, Of Iraq

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Abstract:

The objective of this study is to determine the frequency of the one most important enteric viruses (rotavirus group A) in stool specimens of children aged less than five years, The age ,sex related distribution, seasonal pattern and clinical symptoms. A total of 100 fecal specimens (including 63 males and 37 females) are collected from infants and children under five years of age in Thi-Qar Province south of Iraq during Five Months (From November 2017 To March2018). According to diarrhea suffered children which revealed that 45% are caused by Rotavirus, the frequency of male children patients with diarrhea caused by rotavirus was higher than their female. The samples are categorized into eight groups according to the age of the children: 1-5 months, 6-10 months, 11-15months, 16-20 months, 21-25 months, 26-30 months, 31-35 months and 36-40 months. Age specific frequency in children patients with diarrhea, caused by rotavirus is high in aged 11-15 months. According to results the percentage of infection with rotavirus show that 16 (35.6%) children are fed on Breast feeding, 22 (48.9%) children are fed on bottle feeding and 7 (15.6%) children are fed on mixed feeding. RT-qPCR is performed for detection of Rotavirus based on VP6 gene. Also RT-PCR technique is performed on some positive isolates in RT-qPCR method that used for Rotavirus genotyping by using DNA sequencing analysis. In Rotavirus phylogenetic tree analysis, results are show that the local Rotavirus isolate (IQ-C1) are closed related to NCBI-Blast Rotavirus (JQ069617.1) (EF472951.1).

Keywords: Rotavirus, RT-qPCR, capsid gene, diarrhea.

1. Introduction:

Diarrheal diseases are a major cause of morbidity and mortality among young children in developing and developed countries (Wilhelmi *et al*., 2003 ; Okitsn- negshi *et al*., 2004). It is established that in Africa, Asia, and Latin America 744 million to one billion cases of acute gastroenteritis and 2.4 to 3.3 million deaths occur annually among children of less than 5 years of age (Okitsn-negshi *et al*., 2004 ; Santos and Hoshino, 2005).

Although at least 25 different bacteria and protozoa can cause childhood diarrhea. More than 75% of cases are caused by viruses, and seven groups of diarrheal viruses (rotavirus, norovirus, sapovirus, adenovirus, astrovirus, parechovirus, and Aichi virus) are considered as the common etiologic agents of acute gastroenteritis in humans (Wilhelmi *et al*., 2003 ; Pham *et al*., 2007). Rotavirus is the main cause of acute viral gastroenteritis in infants and young children worldwide, and in the young animals of a large variety of species (Santos and Hoshino., 2005 ; Parashar *et al*., 2006). Before the early 1970s, no virus had been confirmed as a causative agent of acute gastroenteritis, only bacterial or parasitic etiologic agents could be detected in 10–30% of children with diarrhea. Rotavirus was first described as a human pathogen in 1973 by Bishop and colleagues (Bishop *et al*., 1973). Extensive work on the genetics of these viruses has been performed, and both genotypes and genogroups (which include multiple genotypes) have been established. NoVs can be classified into five genogroups; three of which infect humans, i.e., genogroup I (GI), GII, and GIV (Kroneman *et al*., 2013).

The most commonly found NoVgenogroup is GII with the GII.4 genotype predominating worldwide. Rota Virus classification is currently based on all 11 gene segments of the virus; the genes encoding the spike protein VP4 (P-protease sensitive) and the coat protein VP7 (G-glycoprotein) specify the P and G genotypes, respectively. These proteins are located in the outer capsid of the virus and confer antigenic properties. Group A RVs can be classified into 27 G genotypes and 35 P genotypes which fall within five P genogroups (liu *et al*., 2012). Rotavirus infections spread easily and usually occur in the winter and early spring between about November and April. Rotavirus infection often spread in settings where many children are together such as day care centres (Atchison *et al.,* 2010).

Globally, rotavirus infection is present all the year around which suggests that low level transmission could maintain the chain of transmission. Before rotavirus is discovered, the disease was called ‘winter diarrhoea and winter gastroenteritis (Pitzer *et al*., 2009).

2. Materials and Methods:

2.1 Stool Samples Collection

A total of 100 fecal specimens were collected from infants and children under five years of age in Thi-Qar Province south of Iraq during Five Months (From November 2017 To March2018), stool specimens (60) were collected from the city of Nasiriyah (including Mohammed Al Mousawi children's Hospital, Bint Al-huda Teaching Hospital and Private Clincs) and 40 stool specimens were collected from different cities in Thi-Qar Province (including 63 males and 37 females) suffering from acute gastroenteritis. Stool suspension from 10% to 50% of each specimen was prepared in 1 ml NaCl after vigorously mixing. The stool suspension was clarified by centrifugation at 8000 rpm for 15 min at 4 °C. The resulting supernatants were collected and stored at -20 °C until use for nucleic acid extraction.

2.2 Reverse Transcription Real-Time PCR (qPCR)

RT-qPCR was performed for detection of Rotavirus based on VP6 gene. Viral RNA was extracted from stool samples by using AccuZol^{TM} Total RNA extraction kit (Bioneer, Korea), the extracted RNA from stool samples were estimated by using Nanodrop spectrophotometer that were used to measurement the RNA concentration and purity at absorbance 260/280 nm at ratio 1.8 as pure RNA. All primers used in detection VP6 gene were designed by using NCBI Gene-Bank and Primer one online and provided by (Bioneer Company, Korea). Forward primer (5′ACGCTCCAGCAAATATTCAGC3′), Reverse primer (5′AACCATGTAGTTGCGCCATC3′) and Rotavirus A VP6 gene Probe FAM-TGTCCAGCTAAGGCGTGCGC-BHQ-1. RT-qPCR was performed with 5µL of template RNA in a total reaction volume of 20 µl consisting of 1µL of Forward Primer, 1µL Reverse Primer, Probe 1µL RT-qPCR Master mix 2X (10) and DEPC water (2 µL). The RT-qPCR program consisted of cDNA step (95 °C 15 min), Pre-Denaturation (95 °C 5 min), 45 cycles of denaturation (95ºC for 20 sec.), annealing (60ºC for 30 sec.), and detection (Scan) to amplified VP6 gene. RT-qPCR data analysis was performed by calculation the threshold cycle number (CT) value that presented the positive amplification in Real-Time PCR cycle number.

2.3 RT-PCR

RT-PCR technique was performed on some positive isolates in RT-qPCR method that used for Rotavirus genotyping by using DNA sequencing analysis. PCR was performed with 5µL of template DNA in a total reaction volume of 20 µl consisting of 1.5µL of Forward Primer,1.5µL Reverse Primer and PCR water 12 µL. The PCR program consisted of cDNA synthesis step (50˚C for 1 min), Initial Denaturation (95˚C for 5 min), and 30 cycles of denaturation (95ºC for 30 sec.), annealing (55ºC for 30 sec.), and extension (72ºC for 30 sec) and a final extension step at 72°C for 5 min and hold for 4 min to amplified VP6 gene. The positive result of the VP6 gene was confirmed by 2% agarose gel electrophoresis, then electric current was performed at 100 volt and 80 AM for 1hour. PCR products were visualized by using UV Transilluminator (Kim *et al.*, 2012).

DNA sequencing method was performed for confirmative detection and genetic relationship analysis Rotavirus A by using NCBI- BLAST and phylogenetic tree analysis. The sequencing of the Rotavirus A RT-PCR product of VP6 genes was purified from agarose gel by using (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada).

4. Results

3.1 Sex Distribution of Rotavirus-Positive in Diarrheal Group of Children under 5 years old

According to diarrhea suffered children revealed that 45% were caused by Rotavirus. Table (1) demonstrate of rotavirus-positive in diarrheal group of children according to gender. The study found that from one hundred children with diarrhea, the percentage of male is higher than female. The patient group by rotavirus shows that (68.9%) of them are males and (31.1%) are females This result shows there is statistical significant differences between male and female ($P<0.01$).

Table(1): Sex Distribution of Rotavirus-Positive in Diarrheal Group of Children under 5 years old.

Variable	Diarrheal Group	
Sex groups	No. of positive cases	% of positive cases
Male	31	68.9
Female	14	31.1
Total	45	100

Chi-square=14.27, df=1 chi=3.84, significant (P<0.01)

3.2 Distribution of Patients with Rotavirus According to the Feeding Type:

In table (2) we can see the distribution of patients with rotavirus according to the feeding types, show 16 (35.6%) child are fed on Breast feeding, 22 (48.9%) child are fed on bottle feeding and 7 (15.6%) child are feeding on mixed feeding. There are statistical significant differences between three types of feeding (p<0.01).

Table (2): Showing Distribution of Patients with Rotavirus According to the Feeding Type.

Feeding Type			Total %
Breast feeding	Bottle feeding	Mixed feeding	
No. of positive cases (%)	No. of positive cases (%)	No. of positive cases (%)	
16 (35.6)	22 (48.9)	7 (15.6)	100

Chi-square=16.89, DF=2, chi-square table=5.99, significance (p<0.01)

3.3 Age Distribution of Rotavirus-Positive in Diarrheal Group of Children under 5 Years Old

Table (3) shows that more age groups were between (11-15 months) 13 cases, the percentage (28.9%) of the patients infected with rotavirus followed (21-25 months) 10 cases, percentage (22.2%) while the age groups (36-40 months) The less percentage of the patients infected with rotavirus were 1 case, percentage (2.2%) there are statistical significant differences between the age groups (P<0.01).

Table(3) Age Distribution of Rotavirus-Positive in Diarrheal Group of Children under 5 Years Old.

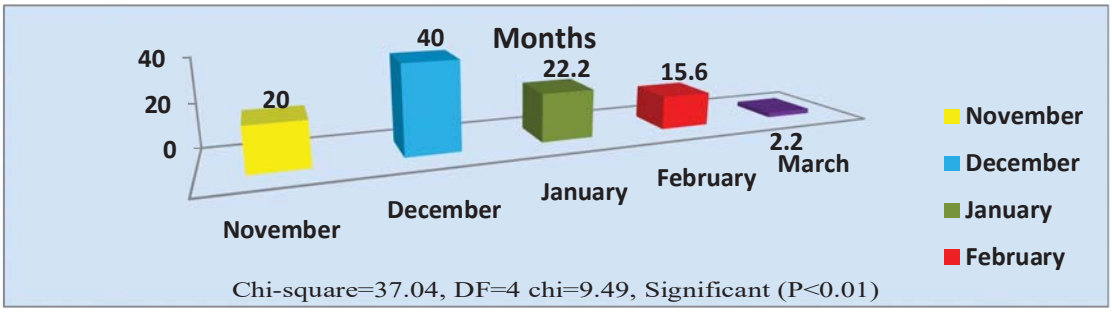
Variable	Diarrheal Group	
Age groups (months)	No. of positive cases	% of positive cases
1-5	5	11.1
6-10	4	8.9
11-15	13	28.9
16-20	6	13.3
21-25	10	22.2

26-30	3	6.7
31-35	3	6.7
36-40	1	2.2
Total	45	100

Chi-square=26.37, df=7, chi=14.07, Significant (P<0.01)

3.4 Distribution of Rotavirus in Diarrheal Group of Children under 5 Years According to the Months of the Year.

According to the months of the year distribution, it has been observed that rotavirus infection is detected throughout the 5 months, the months of the year distribution of rotavirus infection is as follows: in December (18 cases) the percentage 40%, in January, (10 cases) the percentage 22.2%, in November (9 cases) the percentage 20%,in February (7cases) the percentage 15.6% and (1case) 2.2% in March, (Figure 1). The highest rate of detection of rotavirus is founded in December (18 cases, 40%) and the lowest in the month march (1cases, 2.2%) there are statistical significant differences between the months of the year

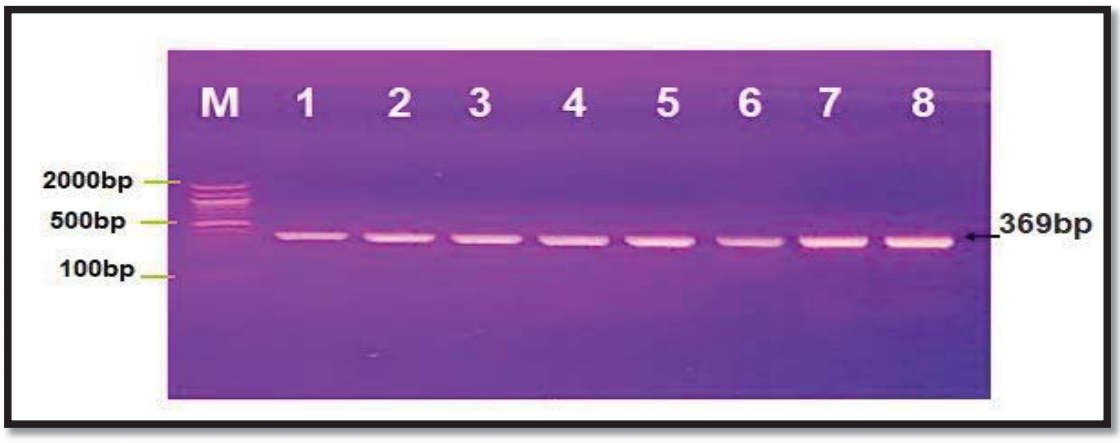


(P<0.01).

Figure (1): Distribution of Rotavirus in Diarrheal Group of Children under 5 Years According to the Months of the Year.

3.5 Result of RT-qPCR

The Graph of Positive Rotavirus Obtained by RT-qPCR Thermo Cycler Template Samples Arise in Fluorescence of the Signal of Each Cycle Indicates Amplification as the figure (2).



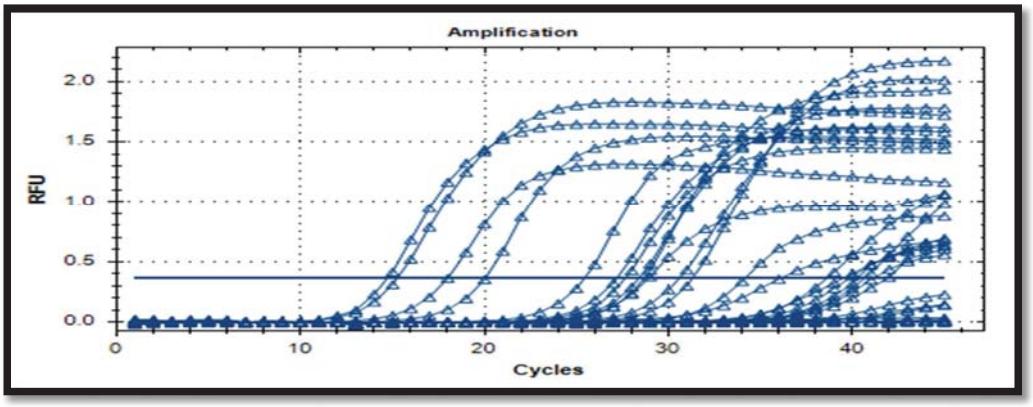


Figure (2): the threshold cycle number (CT) value.

3.6 Purification of Reaction Products RT-PCR

The RNA extracted from the sample is amplified by the results of Real Time RT-PCR technique. The previously mentioned results are amplified. The amplification of the gene is carried out with a PCR results, and its presence is detected by Gel-Electrolysis of PCR products on Gel agarose at concentration(2%) When the base pair size is 369bp as the figure (2).

Figure (3): Agarose Gel Electrophoresis Image That Show the RT-PCR Product Analysis of VP6 Gene Rotavirus from in RNA Extracted from Stool Patients Samples, Where M: Ladder (100-2000bp), lane (1-8) Some Positive Samples at (369bp) PCR Product Size.

Phylogenetic Tree Analysis Based on VP6 Gene Partial Sequence That Used for Rotavirus A Confirmative Detection and Genetic Relationship Analysis . The Phylogenetic Tree is Constructed Using Un Weighted Pair Group Method with Arithmetic Mean (UPGMA Tree) in (MEGA 6.0 Version) as the figure (4).

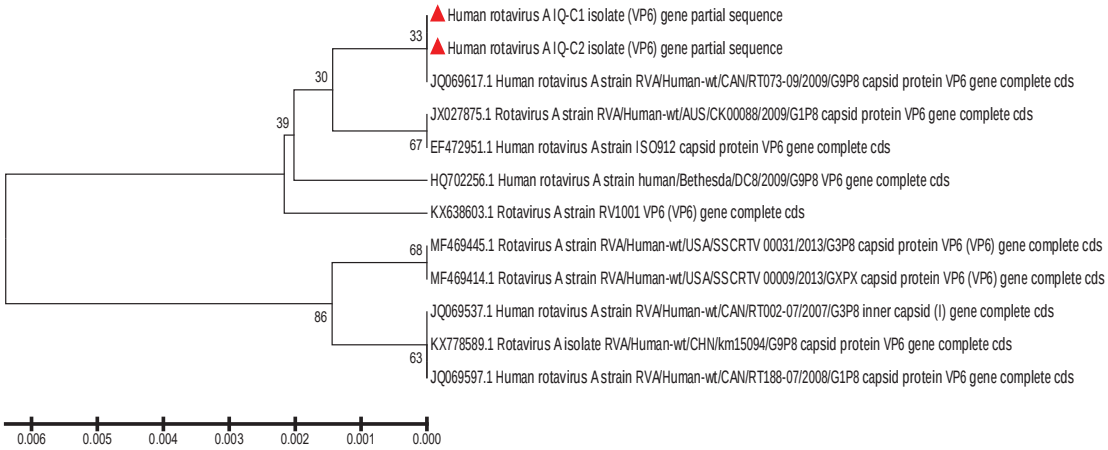


Figure (4): The Local Rotavirus A Isolate (IQ-C1 And IQ-C2) Were Show Closed Related to NCBI-Blast Rotavirus A (JQ069617.1) Genotypeg9 p8, Whereas, NCBI-Blast Rotavirus A are Shown Less Different and Out of Tree.

4. Discussion

The current study was performed to evaluate the prevalence of rotavirus, among the children with acute gastroenteritis, and the association with demographic and some parameters including age, sex, feeding type in children under five years of age in Thi-Qar province.

The prevalence of rotavirus in the present study among males 31 (68.9%) are higher than those in females 14 (31.1%) within children under 5 years old with significant differences between them. This finding corresponds with other previous studies by (Nasab *et al*., 2016). A higher rate of rotavirus infection is detected in males more than females. Also these results in line with another study by (Moyo *et al*., 2014) who was found male infected with rotavirus was (61.2%) higher than in female was (38.8%), and by (Center,2013). Of the 421 Rotavirus cases seen at the hospital, 235/421 (56%) were males and 186/421 (44%) were females (Rasanen, 2016). The overall proportion of rotavirus infection was comparable between males and females. More male children (53%) experienced diarrheal diseases compared to female children (47%) and are more susceptible to rotavirus infection. This suggests that boys become more frequently sick than girls (Seheri, 2013). The reason for the gender difference is not determined, but the of this study results are consistent with the findings that had been reported previously by (Nguyen *et al.,* 2004; Kim *et al*., 2005).Also the results are matched with the results in Taiwan by (Yang *et al*., 2010) who was show the mean of male infected was 56.1% was higher than those in female.

During the study, of the relationship between the incidence of rotavirus and the pattern of nutrition shows the rate of rotavirus gastroenteritis is the highest in children who were used bottle feeding with 22 (48.9%) children, followed by breast feeding and mixed feeding with 16 (35.6%) and 7 (15.6%), respectively. This variation of the above results is statistically significant ($p < 0.01$). Another similar local study found a similar infection with a predominate RV- positive infection in children with Bottle feeding (Ali *et al*., 2010). On the other hand, other studies showed different results according to the feeding type (Nakawesi *et al*., 2010) .The present study results showed that bottle feeding children tend to be more susceptible to face RV diarrhea, comparing with infants who are breast-fed and mix-feed. This is due to the ease of transmission of viruses in contaminated feeding bottles .in another a local study carried out in Kurdistan, Iraq, (Rashid and Sharif, 2006) 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.

The results of present study reveal that are significant differences between the age groups and the percentage of rotavirus-positive patients with highest value is (28.9%) at age group of (11-15) months, and the lowest one is (2.2%) at age group of (36-40) months. This result is in line with the data obtained in Tehran by (Nasab *et al*.,2016) which scored that the highest value of rotavirus positive in children was 46.9% between (13-24) months, whereas the lowest value was (6.1%) at age group of (37-60) months. However, there are other results indicat to the percentage of rotavirus-positive patients with highest value is (35.5%) at age group of (12-23) months, and the lowest one is (4.0%) at age group of (0-5) months (Yang *et al*.,2010). Most of the children (233/421, 55%) were between 6 and 24 months of age, and 149/421 (35%) were between 12 and 24 months of age (Rasanen, 2016).

In South Africa, acute diarrheal diseases were ranked as the third major cause of childhood mortality in children less than 5 years of age (Bradshaw *et al*., 2003) where the majority of deaths were among black and colored children <5years. An estimated 160-200 children die each day (Westaway and Viljoen, 2000) with a diarrheal morbidity at 1.5 million

cases per annum in children less than 5 years of age (von Schirnding *et al.*, 1993). As observed in the other parts of the world, the burden of rotavirus disease is predominantly in children less than 2 years of age (Parashar *et al*., 2009)

In this study, (13/45) of rotaviral diarrheas are identified in the age group of less than 2 years (11-15) months. This finding is in agreement with previous studies done in Turkey which showed a 63% rate of rotavirus in diarrheic patients who were 2 year of age or less (Karadag *et al*., 2005). In a review of studies, de Zoysa and Feachem, (1985) estimated that rotavirus-associated disease worldwide accounts for 20-70% of all hospital cases of diarrhea as well as 20% of diarrheal deaths in children less than 5 years of age. Rotavirus is the leading cause of gastroenteritis in infants and younger child with the findings of most studies, consistent demonstrating that rotavirus mainly affects younger children aged less than 5 years and the proportion of rotavirus infections among younger children was higher than that in the older age groups.(Wu *et al*., 2008).In temperate climates, RV AGE seasons typically occur during the winter months, beginning in late autumn or winter and lasting until the spring (Than *et al*., 2013). This seasonal pattern is seen in both the northern and southern hemispheres, most clearly in Europe, North America and Oceania, the RV seasonal peak occurring in the winter months of each. Each virus has its own seasonal distribution; i.e. in the United States, rotavirus infections occur during the cooler months of the year whereas adenovirus infections occur throughout the year (Gibson *et al*., 2011). In tropical countries, the seasonal pattern is not seen or is not as definable. On the other hand, there are countries of temperate climate with a year-round occurrence of RV AGE and tropical countries with RV seasonality (Patel *et al*., 2013). The reason for seasonality is not completely understood: there is probably no single explaining factor, but the seasonality is the result of multiple co-predictors including the temperature, humidity and yearly rainfall, altitude, population density and behavioral factors, and perhaps the economic income of the country (Patel *et al*.,2013). The economic status of countries does not explain the seasonal or year-round occurrence of AGE because in countries with similar income levels, seasonality exists or is lacking, depending on the country's distance to the equator (Patel *et al*.,2013). rotavirus infection in South Africa occurs throughout the year with marked seasonal changes (Seheri, 2013). We witnessed a peaked incidence during the winter months with low prevalence during summer months. The peak incidence rates of rotavirus infections observed are 40%, 22.2% and 20% during December, January and November, respectively, among children less than 5 years of age . these results in corresponding with other in developed countries with temperate climatic conditions (Nakagomi *et al*., 2005; Gleizes *et al*., 2006)**.**

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