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The effect of age, gender, type of feeding and receiving a treatment on the distribution of Enterovirus infection among children in Al-Diwaniyah city, Iraq

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The effect of age, gender, type of feeding and receiving a treatment on the distribution of Enterovirus infection among children in Al-Diwaniyah city, Iraq

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ABSTRACT

In developed and developing countries, gastrointestinal infections still account for significant morbidity and mortality rates. The most common type of gastrointestinal infection is 'diarrhea' (rapid production of more or less fluid-like repeated intestinal evacuations). *Rotavirus*, *Astrovirus* and enteric *Adenovirus* is the most common cause of severe diarrhea in children under 5 years old of age. So, this study aimed to detect these viruses in children under five years old who suffered from acute gastrointestinal infections in Al-Diwaniyah city . The Study has been done on 90 fecal samples collected from children with acute gastroenteritis from children < 5 years old. All stool specimens were examined by a real-time polymerase chain reaction (RT-PCR) for *rotavirus*, *astrovirus* and *adenovirus*-positive specimens by using different primers of a specific type. The results showed the presence of viruses genes in 53 samples (58.88) out of a totally 90 samples. Among these positive results, 16.66% was for *Rotavirus*, *Astrovirus* (7.77%) and *Adenovirus* (5.55%) , there was also a mixed infection between (*Rotavirus* & *Astrovirus*) in the rate of (12.22%), (*Rotavirus* & *Adenovirus*) , in the rate of (5.55%) and 6.66 (*adenovirus* & *astrovirus*) . The rate of *Enterovirusrs* was high in the first year of life followed by less than three years of age, the ratios were 60.65% and 75% respectively. Moreover, the infections were observed at 57.14% in males and 61.76% in females. Also, the viruses detected in (59.32%)and (58.06%)samples from children with mixed feeding and breastfeeding, respectively. In addition, the infection rates in children who took treatment and those who did not before diagnosing the type of infection were (53.96%)and (70.37%) sample respectively, as there were no significant differences in the type of feeding and treatment,(p value< 0.05). Regarding the high frequency of infections with *rotavirus*, *astrovirus* and *adenovirus*, continuous monitoring is required to inform, diarrhea prevention programmes, as well as information on new enteroviruses strains. This will help policy-makers make decisions about the introduction of *rotavirus*, *astrovirus* and *adenovirus* vaccines.

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1. Introduction

The gastrointestinal tract is a sensitive organ for infections because contact with the outside is continuous, mostly via the oral path. Gastroenteritis (also known as 'infectious diarrhea') is the inflammation that affects the digestive system's stomach and small intestine. Its symptoms and signs are a combination of diarrhea, vomiting, and abdominal pain. In addition, fever, energy shortages, and dehydration may occur in these cases [1]. Acute gastroenteritis can be caused by viruses, bacteria and parasites [2], and although at least 25% of childhood diarrhea cases are caused by different types of bacteria and parasites, more than 75% are caused by viruses, the most frequently detected viral agents of acute gastroenteritis are *Rotavirus* ,its name derived from the wheel- like type visible in electron microscopy, and is now classified as a genus within the *Reoviridae* family. The particles are 70 nm, icosahedral structures without envelope. An internal and external capsid gives a double sheet, covering a center that includes the viral genome. The double stranded RNA consists of eleven segments, encoding six viral capsid proteins (VP1, 2, 3, 4, 6 and 7) and six non-structural proteins (NSP1–6) [3,4]. *Rotavirus* causes an estimated 2 million hospitalizations and 450,000 deaths among children worldwide each year, with the majority of deaths occurring in Asian and African developing countries[5]. Virtually all children are infected at least once during the first 5 years of life and the highest occurrence is commonly considered as being between the ages of 6 and 24 months[4]. Human *astroviruses* (HAstV) were first identified as a human pathogen in 1975 during an acute gastroenteritis outbreak among infants in the UK[6]. *Astroviruses* were originally known from the feces of infants with gastroenteritis and their name acquire from their distinguished appearance [7,8]. Human *astrovirus* (HAstV) belongs to the *Astroviridae* family, amongst the most common causes of viral gastroenteritis in young children, the first being *Rotavirus*[9]. Positive-sense RNA, the viral nucleic acid is composed of open reading frames (ORFs), including ORF1a, ORF1b and ORF2[10]. ORF1a and ORF1b encode two polyproteins that are non-structural and ORF2 encodes capsid protein (CP) [11]. *Astrovirus* transmission occurs primarily through the fecal – oral route, either by direct contact with infected individuals or through the ingestion of contaminated food and water. HAstV infection usually causes a moderate, watery diarrhea that lasts from 2 to 3 days, along with vomiting, fever, anorexia, and abdominal pain. Vomiting is less frequent in *Astroviruses* than in *Rotaviruses* [12].

Human *Adenoviruses* (HAdVs) are categorized from A to G into the *Adenoviridae* family, the genus *Mastadenovirus*, which includes seven recognized species. Fifty-one serotypes have been characterized by hemagglutination and serum neutralization reactions until now, but new forms of *Adenoviruses*, including some emerging and recombinant viruses, have recently been detected using genomic data. To date, more than 60 types of *Adenoviruses* have been described, grouped into 7 species [13]. Enteric *Adenoviruses* are transmitted directly via the fecal-oral route, as well as indirectly through contact with contaminated surfaces or shared utensils [14]. Diarrhea is one of the clinical characteristics of acute gastroenteritis that occurs across different forms of *Adenoviruses*. In patients with Ad40 and Ad41 infections, the mean length of diarrhea is 6-8 days, and 2-12 days respectively. Prolonged diarrhea was normal, especially in association with Ad41, with symptoms in one-third of children for 2 weeks. Fever and vomiting were mild and had a median duration of 2 days[15,16]. *Adenovirus* causes infection year-round, mostly in young children. The 8–10 day incubation period is longer than in other entry viruses, as is the length of the diarrhea[3]. *Enteric Adenoviruses* in children cause 5-15 per cent of all cases of gastroenteritis. In the warmer months the number of instances increases with *Enteric Adenoviruses*[17]s viruses are fecal samples, where fecal samples should be

collected from infected persons as soon as possible after infection (between the first day to the fourth day of infection) because the virus secretion is high in the acute phase of the disease [2]. Molecular techniques have revolutionized microbiological diagnosis and, because of their speed and high sensitivity and specificity, represent an interesting alternative to traditional techniques despite their high cost. Polymerase chain reaction (PCR) reproduces the physiological mechanism of in vitro replication of DNA in cells, exponentially amplifying a particular double-stranded DNA sequence. Different variants of PCR have been developed since its invention to improve diagnostic yield. PCR is used for the identification and amplification of RNA after a reverse transcription or RT-PCR. The RNA existing in the sample is used as a guide for the synthesis by reverse transcriptase or reverse transcriptase of the complementary DNA (cDNA), and the resulting DNA is amplified by standard PCR. Quantitative PCR (qPCR) or real-time PCR is much faster than traditional models, and gives a continuous and precise quantification of the DNA being produced. To measure the DNA or RNA present in the sample, a regular parallel curve must be executed[18]. The aim of this study is to detect some viral etiologies in children with acute gastroenteritis in Al-Diwaniyah city using molecular diagnostic methods, Real Time Polymerase chain reaction(RT-PCR).

2-Materials and Methods

The present study was performed in Al-Diwaniyah City's Maternity and Pediatric educational hospital over a period of 6 months, from July to December 2019. The research included 90 children and infants aged between 2 months and 5 years with acute gastroenteritis. Cases of bloody diarrhea have been ruled out. The consideration data in this study were age, sex, and type of feeding, in addition to the degree of dehydration and nutritional status that are evaluated during study. Stool samples were collected from every child diagnosed with acute gastroenteritis.

Detection of Rotavirus, Astrovirus and *Adenovirus* in fecal samples

Five ml of fecal samples were collected from every child diagnosed with acute gastroenteritis. Then only 3ml samples were used and 5ml of phosphate buffered saline(PH 7.2) was added to them and then stored at -20 ℃ until used for the enteric viral detection by real-time PCR [19].

Preparation of sample

The 20% of stool suspension was prepared as following steps:

1.The amount 4ml of saline solution and 1 gm (1ml) of stool was transferred into 5 ml tube.

2.The mixture was vortex to get a homogeneous suspension and centrifuged for 3 min to 13000rpm.

3.The supernatant was used for the extraction of the viral DNA/RNA in the next step[20].

Extraction of nucleic acid

The RNA of *rotavirus*, *astrovirus* and the DNA of *adenovirus* in this study were extracted using Viral Gene-spin™ Viral DNA extraction kit (iNtRon Biotechnology/ Korea) from stool samples that collected from children infected with acute gastroenteritis.

Reverse transcription-polymerase chain reaction

The cDNA synthesis was performed for converted viral RNA template into cDNA template by using Accupower® RocketScript™ RT PreMix KIT and done according to company instructions as

following: (10µl) of RNA template and add(1 µl) from Random Hexamer primer 100pmol and then add (9µl) from Nuclease free water. Then the tubes were placed in vortex and briefly spinning down. The RNA converted into cDNA in thermocycler under the following thermocycler conditions(Primer activation, 15℃, 10 min) and then (cDNA synthesis (RT step), 50 ℃, 1 hour) and then (Heat inactivation, 95 ℃, 5 minutes).

Real Time PCR

Real Time PCR was performed for detection *Rotavirus* and *Astrovirus* by cDNA template and *Adenovirus* by DNA that isolated from stool samples according to the method carried out by [21,22]. The qPCR master mix reaction components were added into Real-Time PCR strips tubes ,then all strips tubes mixed by vortex and centrifuged for 3000rpm for 3 minutes in Exispin centrifuge, after that transferred into Real-Time PCR thermocycler. Real Time PCR thermocycler conditions was set according to primer annealing temperature and qPCR TaqMan kit instructions as in the following conditions of qPCR thermocycler: Pre-Denaturation in 95°C 5 min by 1 Cycle , Denaturation in 95°C 20 sec by 45 Cycle and then Annealing/Extension in (50-55°C^{1} 1min), (50-55°C^{2} 1min) ,(60°C^{3} 1min) by 45 Cycle then Detection (Scan).

Real-Time PCR Data analysis:

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 as well as real time PCR standard curve used for measured Real Time PCR efficient.

3-Results and Discussion

In this study ,we performed examination, which is more accurate, sensitive and specific in terms of detection of microbes, it is a RT-PCR for 90 fecal samples from children with acute gastroenteritis to detect *rotavirus*, *adenovirus* & *astrovirus*.

Real-time PCR examination showed that the percentage of single *Rotavirus* (16.66%) in 15 samples out of a total of 90 samples. *Rotavirus* were also shown in mixed infections with *Astrovirus* in the rate of (12.22%) in 11 samples, and also present with mixed infection with *Adenovirus* in the rate of (5.55%) in 5 samples (Table 1).

The results of RT-PCR also detect the presence of Astrovirus in the stool samples which are examined previously by chromatographic immunoassay . The viral infection appeared as a single with the rate of 7.77% in 7 samples, and it was also present in mixed infections (Rotavirus - Astrovirus) in 11 samples (12.12%) and (Astrovirus-Adenovirus) in the rate of (66.6%) in 6 samples.

Finally, the results of the Real-Time PCR examination showed the occurrence of infection with Adenovirus, where it was shown alone in 10% in 9 samples, also, Adenovirus was shown with mixed infections, as follows: (Adenovirus - Rotavirus) with rates of 5.55%, and (Adenovirus – Astrovirus) with rates of 66.6%, and these results are more clarified in table(1). We obtained from the results of the current study that Rotavirus is the most prevalent virus in the positive samples . This percentage was comparable and consistent with the result of a study conducted by Al-Janabi [23] in Baghdad where the proportion of Rotavirus was the highest among the viral intestinal infections which is about (34%), and this percentage is a combination of single and mixed infections with other viruses. These results also approached the results of the Lekana-Douki study, as it showed that Rotavirus had the

highest incidence among other enteroviruses(27%) [24]. Also, the results of our study are similar in terms of the sequence of intestinal virus infections with Shaheen's results in Egypt, where most infections were positive for Rotavirus, followed by Astrovirus and finally Adenovirus[25].

Table (1): Distribution of Enteric virus's infection between the Patients by RT-PCR

Total No	Positive Cases No (%) 53(58.88)						X2	P value
	Single Infection			Co-infection				
	Rota	*Astro*	*Adeno*	(*Rota*+ *Astro*)	(*Rota*+ *Adeno*)	(*Adeno*+ *Astro*)		
90	15 (16.66)	7 (7.77)	9 (10)	11 (12.22)	5 (5.55)	6 (6.66)	8.32	0.138 (NS)
	31(58.49)			22(41.5)				
X2	2.16							
P value	0.141(NS)							

X2:Chi-Square test , **p** :probability

Real-time PCR test showed the rates of viral intestinal infections for the age groups, that most cases of infection with Enteroviruses occur in children with ages ranging between (2-3) years with ratio(75%) and the percentage of infection falls at the age of (4-5) years and this finding may be due to the less number of patients in this group. While, the results showed that the age groups of less than a year and (24-36) months are more common than other age groups, which represent 60-65% and 75% of the total cases of acute gastroenteritis, respectively. Also, the statistical analysis revealed that there are no significant differences in the distribution between different age groups, as shown in Table (2).

This result coincided with the result of the researcher, AL-Jeboury [26] in Babylon Governorate, where the highest incidence of intestinal viruses was recorded among children aged (2-3 years). It also coincided with the results of the researcher ,Allayeh *et al*. [27] in Egypt, who found in their results that the highest incidence of viral intestinal infection in relation to (Rota, Astro and Adenovirus) was in the age group of less than one year, as well as consistent with the results of the researcher in Thi Qar, where the highest incidence of injury in the age group was less than two years, which was estimated at (60%) [28].

While in Taiwan, the researchers mentioned that the age group 3-5 years is more susceptible to infection with Enteroviruses, as the percentage of infection in this category reached 44% [29], and as Temu and his group explained in 2012 that children in Tanzania who are 3 to 4 years old are at greater risk of developing Enteroviruses, at 50% [30].

Table (2): Distribution of enteric virus's infection between the age groups by RT-PCR

Age Group (Months)	Total No. (%)	No. of positive Cases (%)	Positive Cases(Viral Causes) No (%)						X^2	P value
			Single Infection			Co-infection				
			Rota	*Astro*	*Adeno*	*Rota-Astro*	*Rota-Adeno*	*Astro-Aden*		
1-12	61 (67.77)	37 (60.65)	12 (19.67)	5 (8.19)	5 (8.19)	7 (11.47)	4 (6.55)	4 (6.55)	8.449	0.133 (NS)
13-24	20 (22.22)	11(55)	1(5)	2(10)	4(20)	1(5)	1(5)	2(10)	4.103	0.535 (NS)
25-36	4(4.44)	3(75)	1(25)	0(0)	0(0)	2(50)	0(0)	0(0)	8	0.156 (NS)
37-48	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0	1 (NS)
≤ 49-61	5(5.5)	2(40)	1(20)	0(0)	0(0)	1(20)	0(0)	0(0)	4.286	0.509 (NS)
Total	90(10)	53(58.88)	15(16.66)	7(7.77)	9(10)	11(12.22)	5(5.55)	6(6.66)		
X2	176.5	1.369	2.597	0.912	3.443	6.607	0.658	1.001		
P value	0 (HS)	0.713 (NS)	0.458 (NS)	0.823 (NS)	0.328 (NS)	0.086 (NS)	0.883 (NS)	0.801 (NS)		

NS: No significant differences (P>0.05)

HS: Highly significant difference (P <0.01)

There was a slight difference in the distribution of males and females in terms of the total intestinal virus infection .In general, female infection was higher than the infection rate in males, as the total viral intestinal infections in males reached 57.14% in 32 samples, while the female infection rate was 61.76% in 21 samples. On the other hand, the results of the statistical analysis of our current study showed that there are no significant differences between the ratio of male infection to the ratio of female infection in the case of infection with Rotavirus, as well as for Astrovirus and Adenovirus, That is mean both sexes are susceptible to intestinal viruses .

This result corresponds to the researcher's results, Allayeh *et al.* [27] and this is in line with the results of many studies conducted inside Iraq and outside it, which confirms that the female infection rate is higher than the male infection rate. Temu *et al*. [30] indicated that the female infection rate is 23%, which is slightly higher than the male infection rate which reached 19%. However, through statistical analysis, it was found that there were no significant differences between the rates of injuries.

While the results of our current study were in disagreement with to the results of researchers in Baghdad ,Ali *et al*. [31] where his results showed that the percentage of male infection is higher than that of female infection.

Table (3): Distribution of enteric virus's infection between the gender groups by using RT-PCR

Gender No(%)	Total No.(%)	No. of positive & %	Positive Cases(Viral Causes) No (%)						X^2	P value
			Single Infection			Co-infection				
			Rota	Astro	Adeno	Rota+Adeno	Astro+Adeno	Rota+Adeno		
Male	56(62.22)	32(57.14)	8(14.28)	4(7.14)	5(8.92)	6(10.71)	5(8.92)	4(7.14)	2.349	0.799(NS)
Female	34(37.77)	21(61.76)	7 (20.58)	3(8.82)	4(11.76)	5 (14.70)	1(2.94)	1(2.94)	8.759	0.119(NS)
Total	90(100)	53(58.88)	15(16.66)	7(7.77)	9(10)	11(12.22)	6(6.66)	5(5.55)		
X^2	10.7	0.187	0.605	0.083	0.189	0.314	1.219	0.712		
P value	0.001(HS)	0.666(NS)	0.437(NS)	0.773(NS)	0.664(NS)	0.575(NS)	0.270(NS)	0.399(NS)		

The results of our current study found that intestinal viruses are more prevalent among children who are fed through mixed feeding than children with breastfeeding. As the results recorded the total number of positive infections with enteroviruses in children with mixed feeding, at a rate of about (59.32%), while the total infections of children with breastfeeding was about (58.06%), The statistical analysis showed that there are high significant differences between the total infants of children with breastfeeding and children with mixed feeding ($p > 0.002$).

These results were consistent and agreed with the results of researchers in many of the previous studies and abroad, such as a study by Sharifi-Rad *et al.* [32] in Iran which recorded the rate of infection of children with mixed feeding by about (62.19%)in (51sample) while the infection rate of children with breastfeeding by about (37.8%) in (31 sample).

Table (4): Distribution of enteric virus's infection according to the type of feeding by using RT-PCR.

Type of Feeding	No. (%)	No. of positive & %	Positive Cases(Viral Causes) No.(%)						X^2	P value
			Single Infection			Co-infection				
			Rota	*Astro*	*Adeno*	*Rota+ Astro*	*Rota+ Adeno*	*Astro+ Adeno*		
Breast	31(34.44)	18(58.06)	3(9.67)	1(3.22)	4(12.9)	6(19.35)	2(6.45)	2(6.45)	5.905	0.316(NS)
Mixed	59(65.55)	35(59.32)	12 (20.33)	6 (10.16)	5 (8.47)	5 (8.47)	3 (5.13)	4 (6.77)	9.670	0.085(NS)
Total	90(30)	53	15(16.66)	7(7.77)	9(10)	11(12.22)	5(5.55)	6(6.66)		
X^2	1.620	0.13	1.663	1.366	0.443	2.242	0.072	0.004		
P value	0.551(NS)	0.908(NS)	0.197(NS)	0.242(NS)	0.506(NS)	0.134(NS)	0.788(NS)	0.953(NS)		

The results of our current study also showed that the percentage of children infection who took treatment before diagnosing an infection (either it was viral or bacterial infection) equal and there is no difference between it and the percentage of child infection who did not take any treatment at all, As in Table(5).

Table (5): Distribution of Enterovirus infection among children receiving treatment and those who are not by using RT- PCR

Treatment	No. of Cases %	No. of positive cases &(%)	Positive Cases(Viral Causes) No. (%)						X2	P value
			Single Infection			Co-infection				
			Rota	*Astro*	*Adeno*	*Rota+Astro*	Rota+*Adeno*	*AStro+Adeno*		
With	63 (70)	34 (53.96)	10 (15.87)	4 (6.34)	5 (7.93)	7 (11.11)	3 (4.76)	5 (7.93)	6.076	0.299 (NS)
Without	27 (30)	19 (70.37)	5 (18.51)	3 (11.11)	4 (14.81)	4 (14.81)	2 (7.40)	1 (3.7)	3.876	0.567 (NS)
Total	90 (100)	53 (58.88)	15 (16.66)	7 (7.77)	9 (10)	11 (12.22)	5 (5.55)	6 (6.66)		
X^2	28.8	2.1	0.095	0.597	0.994	0.242	0.252	0.544		
P value	0 (HS)	0.147 (NS)	0.758 (NS)	0.440 (NS)	0.319 (NS)	0.623 (NS)	0.616 (NS)	0.461 (NS)		

The results showed that the total percentage of children infection with Enteroviruses who took treatment before diagnosing a viral infection at 53.96% in 34 positive samples out of 90 samples. While the total percentage of of children with intestinal viruses who did not take any treatment before diagnosing the type of infection at 70.37% in 19 samples, positive out of 90 samples.

We also noted in the results of the current study that the rates of infection with rotavirus for children with treatment 15.87% while without treatment 18.15%, as well as Astrovirus in children with treatment 6.34% and without treatment 11.11%, and finally the percentage of Adenovirus infection in childern with treatment was 7. 39% and without treatment was (14.18%). Also, the results showed that in cases of mixed injuries, there are also no significant differences between the rates of injuries, whether individual or mixed.

where the results showed that there is no treatment that might be antibiotics or the like affecting the infection of the virus or limiting its spread, and we found that giving (antibiotic) treatment to children who suffer acute gastroenteritis has no effect on viral infection if the condition is not diagnosed as a viral or bacterial infection ,and as we mentioned before antibiotics are only recommended for acute bloody Diarrhea / dysentery, understanding the pattern and type of antibiotic and its use in cases of acute diarrhea is important for determining a health promotion program, therapeutic intervention and the correct use of antimicrobials, thus reducing the cost of treatment to the patient as well as stabilizing his health condition [33].

4-Conclusions

The current study demonstrated that Rotavirus, Astrovirus and Adenovirus play a major role in acute gastroenteritis among children, especially children whose age is less than five years. Viral pathogens should be investigated routinely in stool samples of people with diarrhea and gastroenteritis.

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