Frequency of Rotavirus, Adenovirus and Astrovirus among Patients with Acute Diarrhea by Chromatographic Immunoassay and Enzyme Linked Immunosorbent Assay

تحديد تكرار فيروس الروتا و فيروس الادينو و فيروس الاسترو لدى المرضى المصابين بالأسهال بأستخدام فحص الكروماتوغرافي المناعي وفحص الاليزا

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Abstract

Diarrhea is a major cause of illness and death in children worldwide; however, little information exists about the origin of childhood diarrhea in Iraq. Rotavirus, Adenovirus and Astrovirus are the major causes of sever gastroenteritis in infant and young children, pattern also observed in adult. Confirmation of viral infection by laboratory testing is necessary for reliable surveillance and can be useful in clinical settings to avoid inappropriate use of antimicrobial therapy. Methods: A total of 188 patients their age range from 1-19 (Mean=5.57 ± S.D. = 4.81) years old suffering from diarrhea were included in this study. Stool samples were collected and tested for Rotavirus, Adenovirus and Astrovirus antigens by using the rapid chromatographic test and for Rotavirus and Adenovirus Antigens, ELISA also was done. Rotavirus, Adenovirus and Astrovirus antigens were determined by rapid chromatographic immunoassay in 27 specimens (14.36%), 0 (0%) and 0 (0%) of 188 frozen stool specimens, respectively. Moreover, of these 188 specimens, Rotavirus was found in 35 specimens (18.62%) and Adenovirus in 6 specimens (3.19 %) by using ELISA technique. The present results revealed that Rotaviruses and Adenoviruses have an important role in diarrhea among children especially those less than 5 year's old and viral pathogens should be investigated routinely in diarrhea stool specimens. This study was aimed to determine the frequency of Rotavirus, Adenovirus and Astrovirus in patients with acute gastroenteritis admitted to Al-Emamain Al-Kadhemain Medical City Hospital in Baghdad-Iraq.

Key words: Rotavirus, Adenovirus, Astrovirus, rapid chromatographic test, ELISA.

الملخص

الإسهال هو أحد الأسباب الرئيسية لمرض و وفاة الأطفال في العالم. ومع ذلك، توجد معلومات قليلة حول مسببات إسهال الأطفال في العراق. تعد فيروسات الروتا والأدينو والأسترو من أكثر الأسباب الرئيسية لالتهاب المعدة والأمعاء في الرضع والأطفال الصغار و الكبار. تأكيد الاصابه الفيروسية عن طريق الفحوص المختبرية ضروري و يمكن أن تكون مفيدة في المجال الصحي لتجنب الاستخدام الغير ملائم للمضادات الحيوية في العلاج. شملت الدراسة 188 مريض يعانون من الإسهال تتراوح أعمارهم من1-19 سنة (متوسط = 5.57 ± الانحراف المعياري = 4.81). وتم جمع عينات البراز واجرى لها فحص الكروماتوغرافي المناعي السريع للتحري عن مستضدات كل من فيروس الروتا، الادينو والاسترو بالإضافة إلى فحص الأليزا ELISA للتحري عن مستضدات فيروس الروتا والادينو. حددت مستضدات فيروس الروتا و الادينو والاسترو بواسطة فحص الكروماتوغرافي المناعي وكانت النتائج موجبة في 27 عينه (14.36٪)، 0 (0٪) و 0 (0٪) من عينات البراز المجمدة ال 188، على التوالي. وبأستخدام فحص الاليزا، ومن 188 عينه كانت النتائج موجبة لفيروس الروتا في 35 عينه (18.62٪) والأدينو في 6 عينات (3.19٪). أثبتت الدراسة الحالية أن فيروس الروتا والادينو يلعبان دورا رئيسيا في الإسهال بين الأطفال وخاصة الأطفال اللذين اعمارهم أقل من 5 سنوات. وينبغي التحقيق في مسببات الأمراض الفيروسية بشكل روتيني في عينات البراز المصابين بالاسهال.

الكلمات الدالة: الروتا فيروس، الادينو فيروس، الاسترو فيروس، فحص الكروماتوغرافي المناعي، فحص الأليزا

Introduction

Acute gastroenteritis considers a major cause of morbidity and mortality worldwide. Approximately more than seven hundred million cases of acute diarrhea occurred in children less than five years of age each year. The mortality rate has been reached to 3-5 million cases per year and the majority of this rate occurs in developing countries [1,2]. Viral gastroenteritis is the second most common viral clinical problem after viral upper respiratory tract illness among developed and developing countries [3]. In infant and young children, Rotaviruses was the main causative agents of viral gastroenteritis followed by Adenoviruses, Astroviruses,

Noroviruses and Caliciviruses [3, 4]. By contrast, the role of rotavirus as a pathogen in adults has long been under appreciated. Spread by fecal-oral transmission, rotavirus infection in adults typically manifests with nausea, malaise, headache, abdominal cramping, diarrhoea, and fever. Infection can also be symptomless [5]. Rotavirus was first recognized as a diarrhea agent in 1973 [6]. Rotavirus was derived from the Latin word (Rota) which mean wheel because the viruses have a distinct wheel like shape under electron microscope [7]. Rotaviruses are non-enveloped, icosahedral, double-stranded RNA viruses (dsRNA) comprising a genus within the family Reoviridae. Seven groups of rotavirus (A–G) have been described, with group A rotaviruses being the leading cause of severe dehydrating gastroenteritis in children < 5 years of age worldwide [8, 9]. In developing countries, Rotavirus gastroenteritis responsible for more than 800,000 childhood deaths per year and these may be due to poor nutrition and inadequate health care. Children deaths in the poorest countries reach up to 85% due to Rotavirus infection [1, 10]. Rotavirus diarrhea frequently occurs in the fall and winter in temperate climates, but in tropical settings and in developing countries a defined seasonality has not been observed [6]. Rotaviruses are the most common cause of severe diarrhea during childhood; but also it has been found less commonly in the older children and adults [3]. Adenoviruses are the second cause of severe and acute gastroenteritis in children under five years of age. Adenoviruses are non-enveloped virus, icosahedral symmetry with linear double stranded DNA genome [11]. There were six groups of Adenoviruses symbolized by the letters (A to F) and 51 serotypes, 50% of all Adenoviruses which found in stool samples are belong to types 40 and 41 [9]. Astroviruses are members of the Astroviridae family and considered as a significant reason of gastroenteritis not only in children, but also in the adults. Astroviruses are non-enveloped RNA viruses have an icosahedral symmetry and their genomes are single stranded positive polarity [12]. Astrovirus may cause 2 to 8% of diarrheal episodes in children [13]. Astrovirus infection has been shown to happen in slight outbreaks and mostly during the winter season of the year [3]. Most viruses that cause gastroenteritis cannot be isolated by tissue culture, therefore the direct detection in stool samples using electron microscope is still the main diagnosis method although it was limited to the reference laboratories. So the start using more sensitive method for antigen detection in stool samples based on immunoassay and molecular techniques has amended for the diagnosis of newly known viruses such as Norovirus and Sapovirus [14].

Materials and Methods

2.1. Specimens

In this descriptive cross-sectional study, 188 fecal specimens were collected from patients with diarrhea their ages range from 1-19 years old (Mean=5.57, Standard Deviation [S.D.] = 4.81) admitted to Al Emamain Al-Kadhemain Medical City Hospital in Baghdad - Iraq. From each patient at least 5ml or 5g of feces was collected in a clean, sterile container, labeled accordingly and frozen at (-20)°C until tested for the viral antigens. Samples collections were carried out from 25 May to 25 October 2014. All the participants were aware about the study by using simple language to explain the diarrhea disease and the benefits of this research. An informed consent was obtained from either the mother or the father of the child before included in this study. All parents deny that their children had been vaccinated against Rotavirus.

2.2. Detection of Rotavirus, Adenovirus, and Astrovirus by Rapid Chromatographic Immunoassay

The Rotavirus, Adenovirus, and Astrovirus antigen detection performed on the freezer-preserved stool samples by one step Rotavirus, Adenovirus and Astrovirus COMBO CARD TEST (CerTest Biotec, Spain). This is a ready-to-use kit and the principle of the test based on the use of a nitrocellulose membrane which is pre-coated with mouse monoclonal anti-Rotavirus, anti-Adenovirus and anti-Astrovirus antibodies against specific viral protein on test line region, on the A, B and C wells respectively. The procedure was done following the manufacturer's instructions. Fecal samples were prepared by adding 125mg or 125μl of the fecal sample to the dilution buffer which supplied with the kit and shaking well. Four drops of processed stool samples were dropped into immune-chromatographic well A (Rotavirus strip), B (Adenovirus strip) and C (Astrovirus strip), positive and negative controls were included with each run of the tests. After incubation at room temperature for 10 min, the results were recorded by monitoring of colored development: Negative test; only one green line appears in the control line region which marked with the letter [C]. Positive test: In addition to the green control line, a red color line also appears in the test region which marked with the letter [T]. Invalid: A total absence of the control green colored line regardless the appearance or not of the result lines.

2.3. Detection of Rotavirus and Adenovirus by Sandwich ELISA:

All (188) samples which tested for Rotavirus, Adenovirus, and Astrovirus by Rapid Chromatographic Immunoassay were retested also for Rotavirus and Adenovirus Antigen by sandwich ELISA (RIDASCREEN® Rotavirus, Germany) and (DIAGNOSTIC AUTOMATION, INC, USA), respectively. In Rotavirus test, monoclonal antibodies were used in a sandwich ELISA to capture the Rotavirus capsid protein of gene 6 (VP6) from stool supernatant. Then second monoclonal antibodies, which were conjugated with horseradish peroxidase. This reaction was visualized by the addition of the substrate. While in Adenovirus test, polyclonal antibodies were used in a sandwich-type method to capture the Adenovirus Antigen from stool supernatant. Then second monoclonal antibodies are applied, which binds the complex. This reaction was visualized by the addition of the conjugate and a blue color developed following the addition of the chromogen and peroxide. The procedure was done following manufacturer's instructions. For sample preparation, fecal samples were diluted by adding 100mg or 100µl of the fecal sample to test tube containing 1 ml RIDASCREEN® sample dilution buffer which supplied with the Rotavirus kit. While the fecal sample was diluted by adding 1 gram or 1 ml of stool sample to test tube containing 4 ml of diluted wash buffer supplied with the Adenovirus kit. After mixing of stool suspension in a vortex mixer. Samples were leafed for a short time to settle down then centrifuged at 5000 rpm for 5 minutes. Then the clarified supernatant of the stool suspension used directly in the test following manufacturer's instructions. Positive and Negative control were included each time the Kits were run. The optical density (O.D.) was obtained at the wave length (450 nm and reference filter 630 nm) and zeros the reader on air. For Rotavirus detection, the cut-off value was calculated by adding 0.15 extinction units to the measured extinction for the negative control. The net O. D. of each sample was considered positive if their extinction is more than 10 % above the calculated cut-off. While the samples were considered equivocal and repeated if their extinction is within ± 10 % of the cut-off. The samples with extinctions more than 10 % below the calculated cut-off were considered negative. For Adenovirus detection, the O. D. of each sample was considered positive if their absorbance reading of 0.15 and above. Samples were considered negative if their absorbance reading less than 0.15 according to manufacturer's instructions.

2.4. Statistical Analysis

The data were analyzed using SPSS program (Statistical Package for the Social Sciences), versions IBM SPSS Statistics, 2015. The data were presented as percentages.

Results and Discussion

Among the total of 188 stool specimens, Rotaviruses were identified in 27/188 specimens (14.36%) of patients with diarrhea and 0 (0%) were due to Adenovirus and Astroviruses by COMBO CARD TEST. While by ELISA out of the 188 cases of diarrhea, 35 specimens (18.62%) were due to Rotaviruses, 6 specimens (3.19 %) were due to Adenoviruses and unfortunately there was no available ELISA Kit for Astroviruses, as shown in Table (1). COMBO CARD TEST is a rapid diagnostic method but a negative result may occur because the antigen level lower than the detection limits of this assay. ELISA tests have been shown to be a sensitive and specific method for the determination of viral pathogen, large sample quantities can also be processed in a short time [15]. The utilization of rapid and simple technique for diagnosis of viruses causing gastroenteritis can improve patient care by reducing unnecessary treatments with antibiotic, this make the physicians more capable to take an accurate treatment decisions and isolate suspected patients to reduce nosocomial spread [14]. The prevalence of viral pathogens using immunochromatographic in Kirkuk city-Iraq was (33.3%) for Rotavirus, (6.6%) for Adenovirus and (33.3%) for co-infection by both [9]. In Turkey was (16.7%) for Rotavirus, (1.0%) for adenovirus and (0.4%) for co-infection by both [16] which is disagreed with the results of this study. Al-Badani and Nafi, [10, 17] reported that these differences in results due to using fresh or frozen stool specimens, the collection of samples were done in different seasons, the age of patients, duration of the study, the methods used in detecting, using different kits for the same method and social habits of the population, e.g. personal hygiene, and/or environmental variations.

Table (1): Frequency of Rotaviruses, Adenoviruses and Astrovirus among the study group using COMBO CARD TEST and ELISA.

Virus	COMBO CARD TEST		ELISA	
	No.	%	No.	%
Rotavirus	27	14.36	35	18.62
Adenovirus	0	0	6	3.19
Astrovirus	0	0	*N/A	*N/A
Negative	161	85.64	147	78.19
Total	188	100	188	100

*N/A=Not available

The prevalence of Rotavirus infection in infants and young children was 40% in Kuwait [10], 37% in Turkey and Erbil, Iraqi Kurdistan [18, 19], 24% in Basrah, Iraq [20], 33% in Jordan, 31% in Oman [10] and 29.5% in Iran [11]. Hamkar *et.al.* [3] reported that the Rotavirus, Adenovirus and Astrovirus were detected in 62%, 2.3%, and 3% of patients, respectively in Northern Iran. In this study the prevalence of Rotavirus by ELISA was 18.62% and this low percentage than other studies might be due to the vaccination against Rotavirus, although we are excluded the vaccinated children but most parents unaware that their children were have been vaccinated against Rotavirus. In the first life of year, Rotavirus vaccine can prevent almost all 85% to 98% of Rotavirus illness episodes [21]. In 2009, the World Health Organization (WHO) recommended to included Rotavirus vaccination in all national immunization programs. In Iraq, Rotavirus vaccine used later of 2013 that supposed decrease gastroenteritis caused by this virus [22]. The introduction of Rotavirus vaccines can hopefully protect children against numerous Rotavirus serotypes [14]. In Iraq, it is very important to know that one of acute diarrhea etiology was viral pathogens in order to plan for viral diarrhea disease control strategies.

In this study the prevalence of Adenovirus by ELISA was 3.19% which disagreement with Grimwood *et. al.*, [23] who mention that the prevalence of Adenovirus infection range from 2.6% to 14% and with Samarbaf-Zadeh *et. al.* [11] who reported that the prevalence of Adenovirus by ELISA test was 2.5% in Iran, while in Thailand 9%, Korea 2.8%, Bangladesh 14%, Australia 3.1%, Italy 2.6% and USA 4.8% [24]. One study found that an association between Rotavirus which affects cells covered in microvilli and Adenovirus which affects cells that are dividing to generate new cells with microvilli and that may represent an interaction between pathogens to cause more severe diarrhea [25]. This study showed that the prevalence of Astrovirus using COMBO CARD TEST was 0 (0%), while the rate of detection of Astrovirus varies from 2% to 13% using monoclonal antibodies in enzyme immunoassays [14].

Other causes of diarrhea in children include bacterial and parasitic diarrhea disease [1]. Also, the differences in season can explain these differences in viral prevalence. In Northern Iran, Hamkar *et. al.* [3] found that 78% of Rotavirus incidence was occurred during winter and only 2% of this infection was occurred during summer, in addition Hamkar *et. al*. [3] mention that the winter predominant of Astrovirus was documented. In contrast Al-Badani *et. al*. [10] found that Rotavirus infection was occurred throughout the year with higher frequency of infection in summer than winter season in Taiz -Yemen. The Seasonal differences were noted especially in temperate climates in which the infections increase during winter, while in tropical climates the infections occur throughout the year [17].

As shown in figure (1), out of 188 patients 84 (44.68%) were female and 104 (55.32) were male.
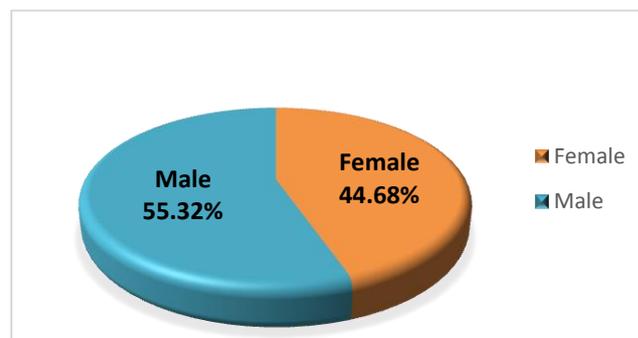

Fig. (1): Gender distribution of study group (Total No. = 188)

According to Table (2), 35 Rotavirus positive samples, fourteen (40%) specimens belonged to female and twenty one (60%) to male patients. In addition, of 6 Adenovirus positive samples, two (33.33%) specimens belonged to female and four (66.67%) to male patients. In my opinion the explanation of this male predominance may be due to social factors rather than a higher rate of infection because boys are more in touch with environment than girls in Arab society. A study in North Iran by Hamkar *et. al.* [3] found that no differences in the detection rate between male and female patients, while in contrast a study in China and another one in Bahrain found that the majority of Rotavirus infections occurred in males [26,27]. Zaman *et. al.*, [9] reported that Rotavirus was detected In Kirkuk city by latex agglutination test in (63.3%) males and (36.3%) females.

Table (2): Gender distribution of patients those excreting Rotavirus VP6 antigen and/or Adenovirus antigen detected by ELISA.

Gender	Viral Pathogens		
	Rotavirus (%)	Adenovirus (%)	Total viruses (%)
Female	14 (40)	2 (33.33)	16 (39.02)
Male	21 (60)	4 (66.67)	25 (60.98)
Total viruses	35 (100)	6 (100)	41 (100)

Age of the study group range from 1 to 19 years (Mean = 5.57, S.D. = 4.81). As shown in figure (2) the age distribution of study group into six categories were: 12/188; 6.4% within the category ≤ 1 year (newborn and infant), 33/188; 17.6% within the category ≤ 2 years (toddler), 88/188; 46.8% within the category ≤ 5 years (preschooler child), 21/188; 11.2% within the category < 10 years (school aged kids), 26/188; 13.8% % within the category range from10 to 17 years (pre-teen and teenager) and 8/188; 4.3% within the category 18+ years (adult).
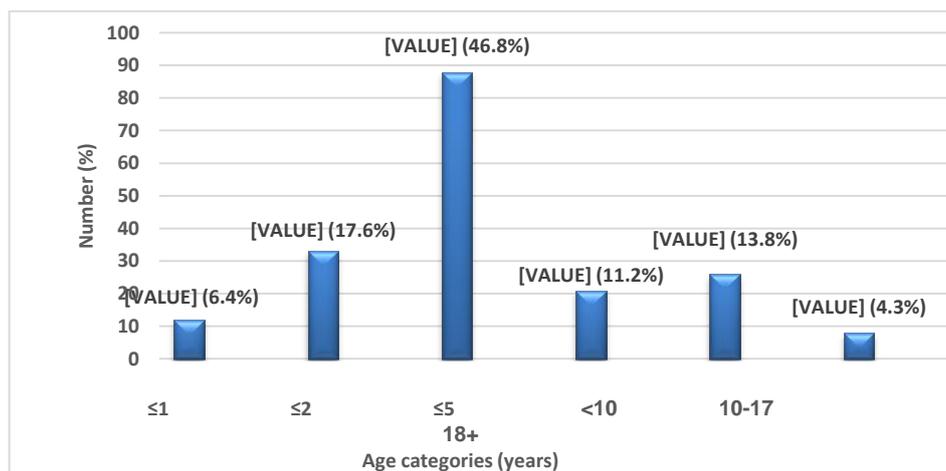

Fig. (2): Age distribution of study group (Total No. = 188)

This study showed that the most of viral infections were detected among children less than 5 years of age according to Table (3). The age group ≤ 5 years shows the highest prevalence of Rotavirus (48.57%) and Adenovirus (50%), respectively. This finding is in agreement with a study conducted in Northern Iran [3] and in [1] which found the majority of enteric Adenovirus infections were detected in children from 0 to 5 years old. Although Hamkar *et. al.* [3] found that the majority of Rotavirus infection (61.1%) was among children aged <1 year but this confirmed my opinion about the introduction of rotavirus vaccine later of 2013 in Iraqi newborn and infant, those aged less than one year, which explain that this study showed the higher prevalence of Rotavirus in the age group ≤ 5 years followed by age group ≤ 2. Meqdam and Thwiny, [14] reported that the Rotavirus group A was common in the first two years of life in Saudi children. Another studies reported that the peak of Rotavirus infection in children was between 6 and 12 months of age [9, 10, 28], some of them explained that the infection significantly less frequent in breastfeeding than among bottle-feeding babies [10]. Astrovirus and Adenovirus were detected predominantly in the 2 to 5 year old age group of children, with a prevalence of 8.3% and 3.5%, respectively [3]. Hamkar *et. al.* [3] found that 92% of Astrovirus infection was in children <5 years old. The poor nutrition, overcrowding, lack of water, and inadequate sanitation made this population especially vulnerable to diarrhea.

Table (3): Age distribution of patients those excreting Rotavirus VP6 antigen and/or Adenovirus antigen detected by ELISA in 188 sample.

Age group (years)	Viral Pathogens		
	Rotavirus (%)	Adenovirus (%)	Total viruses (%)
≤ 1	3 (8.57)	0 (0)	3 (7.32)
≤ 2	6 (17.14)	2 (33.33)	8 (19.51)
≤ 5	17 (48.57)	3 (50)	20 (48.78)
< 10	4 (11.43)	0 (0)	4 (9.76)
10-17	4 (11.43)	1 (16.67)	5 (12.19)
18 +	1 (2.86)	0 (0)	1 (2.44)
Total viruses	35 (100)	6 (100)	41 (100)

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