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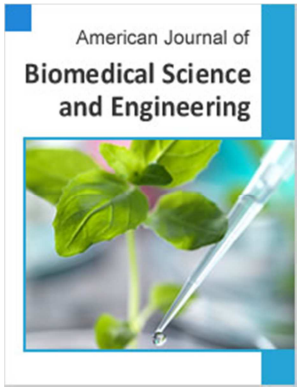

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Comparative Study of Diagnostic Methods for Rotavirus in Children Hospitalized for Diarrheal Disease in Mansoura Pediatric Hospital

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Abstract
Rapid diagnosis assays have the advantage of facilitating the process of large amount of samples in a very short period of time, allowing fast management measure and treatment to reduce disease severity and infection spread. Rotavirus infections are a major cause of diarrhea in children in both developed and developing countries. Rotavirus genetics, patient immunity, and environmental factors are thought to be related to the severity of acute diarrhea due to rotavirus in infants and young children. Diarrhea remains the second most common cause of death among children below 5 years globally. Among various enteric pathogens, rotavirus appears to be the most important etiological agent of acute gastroenteritis in infants and young children. A total of 100 stool samples, this study included 70 children with AGE and 30 healthy control children. They were chosen from Mansoura Pediatric Hospital from November 2015 to March 2017. Rotavirus can be detected in stool samples by electron microscopy (EM), Stool samples were obtained and assayed for rotavirus by the immunochromatography test (ICT), enzyme linked immunosorbent assay (ELISA) and quantitative real time (RT-PCR). 50 out of the 70 patients (71.42%) were positive for qr RT -PCR. Forty-five (64.28%) and 44 (62.85%) were positive for ICT and ELISA, respectively. There was a significant association between the severity of the disease as determined by the Vesikari score and rotavirus infection. The RT-PCR assay evaluated in the study was shown in general to have comparable performance for RVA detection in fecal samples. The real-time PCR assay is excellent tool for diagnostic laboratories, and the RT-PCR assay working in a portable PCR machine is highly sensitive and specific and shows promise for on farm molecular detection of RVA.

1. Introduction

Group A rotaviruses (RVA) are estimated to cause severe gastroenteritis in infants and young children worldwide with approximately 130 million children infected each year. This accounts for approximately 1/3 of all hospital admissions each year for diarrheal disease and is estimated to be responsible for over 500,000 deaths, 2 million

hospitalizations, and 25 million clinic visits each year [1]. The infections range from gastroenteritis, vomiting and fever, followed by watery diarrhea leading to dehydration and potentially death. Oral or intravenous rehydration was the best mortality-reducing treatment before the introduction of rotavirus vaccines [2]. The majority of morbidity and mortality caused by rotavirus gastroenteritis is experienced by children under five in developing countries. Rotavirus causes the deaths of approximately 453,000 children annually, and most of them occur in developing countries in Africa and South Asia [2]. About 40% of patients under five in developing countries are due to rotavirus [3, 4]. The most intractable diarrheas are treated with antibiotics, irrespective of the causative agent. If infection due to rotavirus can be diagnosed early, the misuse of antibiotics can be avoided [5]. Many several techniques have been developed to detect rotavirus in stool samples, including electron microscopy, polyacrylamide gel electrophoresis of viral nucleic acid, various immuno assays, and PCR-based molecular methods [6].

Commercial assays, such as the immunochromatographic test and the latex agglutination test, have been evaluated and compared to other methods, such as ELISA and quantitative real time RT- PCR (qr RT-PCR), and they have shown a wide range of sensitivity and specificity [7]. The molecular techniques are more rapid than the cell culture-based techniques and are more sensitive than EM or EIA. The threshold for detection of RVA by EM is approximately 10^7 viral particles/ml of stool [8].

2. Material and Methods

The aim of this study was to evaluate the sensitivity, specificity and accuracy of different assays for the detection of human group a rotaviruses in stool samples.

This work was conducted in Mansoura Pediatric Hospital from November 2015 to March 2017, Faculty of Medicine, Mansoura University.

A total of 100 stool samples were included in the study. 70 children less than five years old with AGE, who were admitted in to the Pediatric Hospital or treated in the Emergency Department, were included in the study. We obtained the Institutional Review Board's approval for the study before it was initiated. The study included 40 males and 30 females. The exclusion was chronic diarrhea, which was defined as diarrhea that lasted for more than two weeks. Written informed consent for participation was obtained from the parents/guardians of the children.

The control group consisted of 30 healthy children who were free from diarrhea, vomiting, and fever. It included 20 males and 10 females whose ages were less than five. The patient group and the control group were almost matched in age and gender.

Fresh stool samples were obtained within 24 to 48 h of admission. Sterile, wide-necked plastic containers were used to collect and transport the samples.

Macroscopic examination of the samples was conducted as follows: color, consistency (formed/semiformed/liquid), presence of blood, presence of mucus, presence of segments and/or worms. The labeled stool samples were divided into separate aliquots for the detection of rotavirus antigen and nucleic acid and stored at -20°C and -80°C, respectively, until they were assayed.

2.1. Electron Microscopy

Four samples showing discordant or peculiar results were tested by electron microscopy. Direct EM examination of feces after negative staining with phosphotungstic acid is a highly specific method to detect rotavirus particles due to its characteristic morphologic appearance of a triple-layered virus with spikes (see Figure 1). Briefly, fecal samples are suspended in phosphate buffer saline and centrifuged. The final pellet is resuspended in Tris buffer and then negatively stained with phosphotungstic acid on fomvar-coated grids. The grids are then examined under an EM at high magnification (40,000), [9].

2.2. Detection of Rotavirus by Immunochromatography Test ICT

Detection of rotavirus antigen in the stool samples using Atlas Medical (UK) This test is a single-step [10].

Two bands should appear to indicate Rotavirus positive; the red band and the blue band are visible. If only the blue band is visible, it is rotavirus negative. If the blue band is missing, the test is invalid.

2.3. Detection of Rotavirus by ELISA

Detection of rotavirus antigen in stool samples using RIDASCREEN® kit (R-Biopharm, Germany). In this test, monoclonal antibodies against the product of the sixth viral gene (VP6) were used in a sandwich-type method [11].

2.4. qr RT-PCR for Detection of Rotavirus

2.4.1. Extraction of RNA

Preparation of the sample: 0.5-1.0 ml of each stool sample was suspended in up to 5 ml of saline (i.e., up to 1:10 dilution) and mixed by brief vortexing. The solution was clarified by centrifugation for 20 min at 5000 × g. Then, 140 µl of the supernatant was used as the starting material for RNA following the Viral RNA Mini Spin Protocol (Quiagen, USA). RNA was extracted using the QIAamp® Viral RNA Mini Kit, which is commercially available. Extraction was done automatically using the QIAcube instrument according to the manufacturer's instructions. To determine the efficiency of the extraction protocol in removing the high levels of PCR inhibitors present in the stool samples, internal extraction control was introduced to the samples following stool clarification and carried through the preparation, amplification, and detection protocols of the specimen.

2.4.2. Real Time RT-PCR

the one step qr RT-PCR approach was used, which combines the reverse transcription and real-time PCR reaction in a single closed tube. The Primer Design™ genesig Kit for Rotavirus A (Primer Design, Ltd., UK) was used for quantification of Rotavirus A genome using specific primer and probe mix for Rotavirus A that was detected through the FAM channel [12].

3. Results

From the total of 100 stool samples analyzed, the patient group which included 70 patients with AGE and the control group which included 30 healthy children. Rotavirus was detected in 50 (71.42%) out of the 70 patients by qr RT-PCR followed by 45 (64.28%) by ICT and 44 (62.85%) by ELISA. In this study, Table 1 shows that the children within the age group of 6-12 months had the highest rate of rotavirus infection with 27 (54%), while those within the age group of 0-6 months had the least with 2 (4%). However, children within the age group of >24 months had the highest rotavirus negative rate, with 7 (35.0%), and those in the age group of 6 -12 months had the least with 3 (15%). The data were studied statistically, and a statistically significant association was found. However, concerning gender, the incidence of positive tests for rotavirus was similar in males and females. Table 2 shows that there was a significant difference between rotavirus positive patients and rotavirus negative patients regarding vomiting, the duration of diarrhea, the severity of dehydration, and the requirement for IV rehydration. Table 3 shows that 28 (56.0%) of rotavirus positive cases had severe scores, while 16 (32%) had moderate scores, and 6 (12%) had mild scores. However, 5 (25%) of rotavirus negative cases had Mild scores and another 12 (60%) had moderate scores, while only 3 (15%) had severe scores. When these data were studied statistically, we found a statistically significant association between the severity of the illness and rotavirus infection. In other words, cases with rotavirus infection were suffering from more severe illness than rotavirus negative cases.

Table 1. *Comparison between rotavirus positive and negative results (by qrRT-PCR).*

Age (months)	Rotavirus negative No=20	Rotavirus positive No=50	X^2	P
0-6	5 (25.0%)	2 (4.0%)	Fisher exact	0.08
6-12	3 (15.0%)	27 (54.0%)	5.37	0.02
12-24	5 (25.0%)	15 (30.0%)	Fisher exact	0.53
>24	7 (35.0%)	6 (12.0%)	Fisher exact	0.16

Table 2. *The clinical manifestations among rotavirus positive and negative results.*

Clinical manifestations		Rotavirus negative patients (n=20)	Rotavirus positive patients (n=50)	t/X^2	P
Fever		12 (60.0%)	42 (84.0%)	0.13	0.72
Vomiting		11 (55.0%)	46 (92.0%)	3.73	0.054
Duration of diarrhea (days)		4.6±0.9	5.5±1.5	2.15	0.04
Frequency of diarrhea (per day)		4.9±0.9	5.3±1.3	1.32	0.19
Degree of Dehydration	No dehydration	7	12	1.1	0.15
	Mild to Moderate	7	32	2.3	0.045
	Severe	2	6	2.5	0.04
Treatment received	Oral rehydration	12	22	0.8	0.65
	IV rehydration	3	28	3.2	0.03

Table 3. *Relation between rotavirus infection and Vesikari score.*

Severity (Vesikari scale)	Rotavirus negative (n=20)	Rotavirus positive (n=50)	X^2	P
Mild	5 (25.0%)	6 (12.0%)		
Moderate	12 (60.0%)	16 (32.0%)	0.04	0.02
Sever	3 (15.0%)	28 (56.0%)		

The severity of diarrhea, as determined by the Vesikari score, was significantly and negatively associated with the PCR Ct value ($P < 0.05$), indicating that the children with severe diarrhea excreted more virus than children with less severe disease. According to the results in Table 4, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using qr RT -PCR as a standard. Regarding ICT, these values were found to be 90, 100, 100, and 75%, respectively. For ELISA, these values were 88, 100, 100, and 71%, respectively. The results of EM for rotavirus detection are shown in Figure 1.

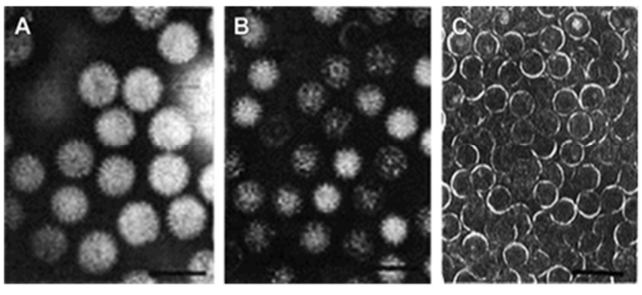

Figure 1. *Rotavirus under EM.*

Particle-3 forms of typical rotavirus particles as seen under the EM after negative staining (A–C). (A) Complete infectious rotavirus particle, triple layered particle, double-shelled, 70-nm in diameter with wheel-shape appearance. (B) Double layered particle, single-shelled, 55-nm in diameter with a circular bristled appearance. (C) Single-layered core particle, 37-nm in diameter and appears hexagonal under EM. The results of ICT for rotavirus detection are shown in Figure 2, The strip to the right shows red and blue bands (rotavirus positive). The strip to the left shows only the blue band (rotavirus negative).

The results of ELISA test for rotavirus antigen detection are shown in Figure 3, ELISA plate showing the following: Well number 1A is the negative control, well number IB is the positive control, wells with colored reaction are positive for rotavirus and wells with colorless reaction are negative for rotavirus.

The results of the qr RT-PCR are shown in Figure 4. The intensity of the fluorescence was plotted against the PCR cycle number, and the intersection between the amplification curve and the threshold line is the Ct (threshold cycle), which is a measure of the concentration of the target rotavirus in the PCR reaction. Different colors represent different samples with different concentrations of nucleic acid. The CT value was plotted against the rotavirus copy number (quantity). Guided by the known copy number of the standards, the copy number of rotavirus in the unknown samples were determined.

Table 4. *Comparison of the results showed by qrRT-PCR, ICT and ELISA.*

Method		qr RT-PCR		Total
		Positive	Negative	
ICT	Positive	45	5	50
	Negative	5	15	20
ELISA	Positive	44	5	49
	Negative	6	15	21

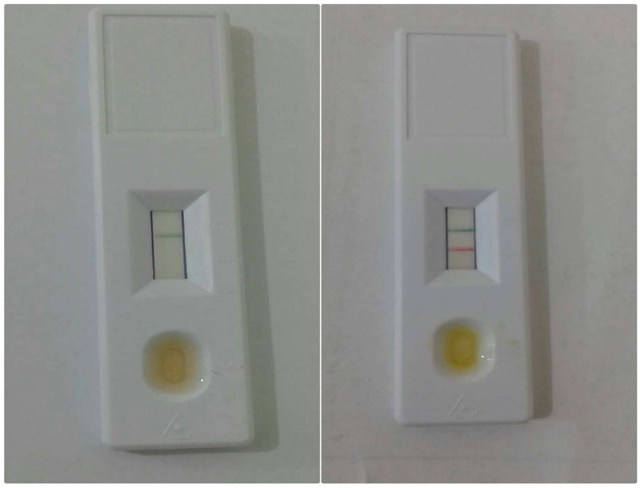

Figure 2. *ICT for rotavirus detection.*

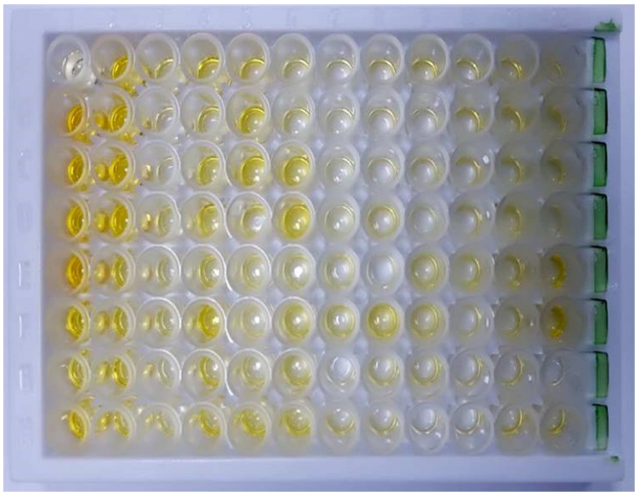

Figure 3. *ELISA test for rotavirus antigen detection.*

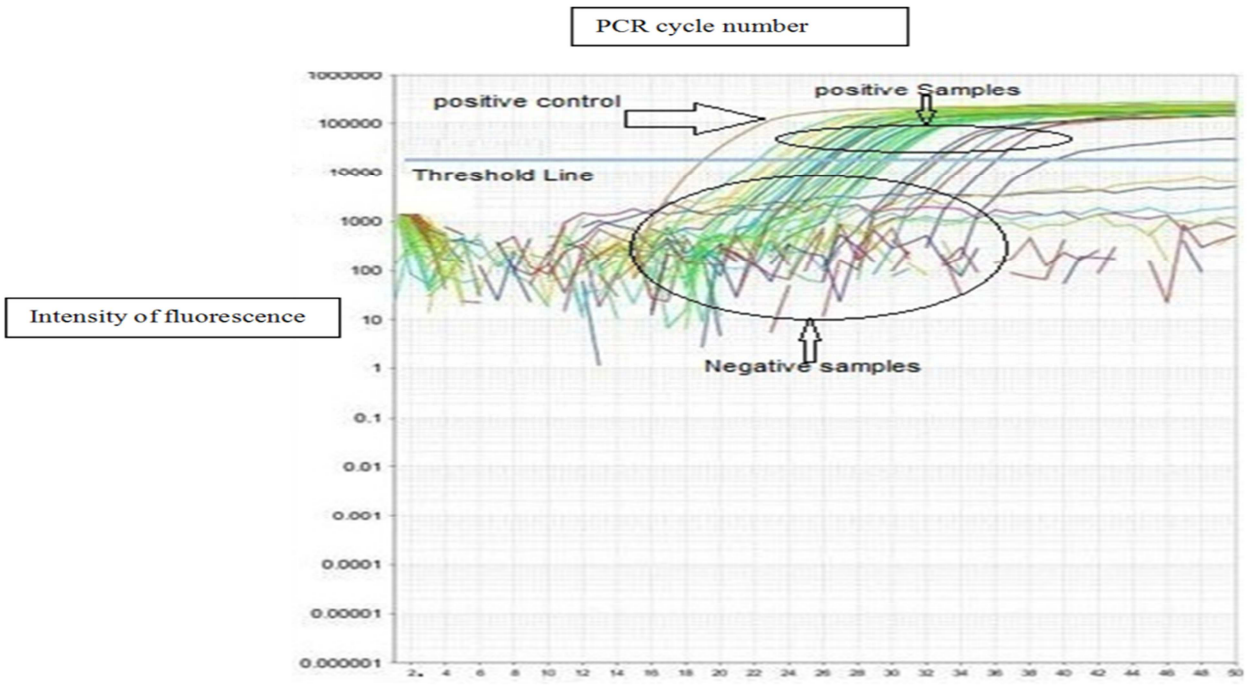

Figure 4. *Amplification Plot (Intensity of fluorescence vs. Cycle) for rotavirus.*

4. Discussion

There is an urgent need for the development of a rapid, sensitive test for the identification of viruses and classification of viral strains. In this study, we compared AGE patients and children in a control group regarding the detection of rotavirus by different techniques, as Electron microscopy, ICT, ELISA, and qr RT-PCR,. 50 cases from a total of 70 cases (71.4%) had rotavirus in their stools by qr RT-PCR.. These results were agree with the results of Vainio et al. [13], who showed that 72% of the children with diarrhea enrolled in their study from March 2006 to February 2008 had rotavirus in their stools by RT-PCR. Like that, in a study conducted by Manjula [12], rotavirus accounted for 64% of the hospitalizations of children with acute diarrheal illness. However, a lower percentage was obtained by Ahmed et al. [14].

A study extended from January 2004 through April 2007. However, they reported higher frequencies (65%) in children who were less than one year old than in older children. Furthermore, rotavirus-associated diarrhea peaked from the late summer (September) to the late fall (November), similar to the trend reported in a population-based cohort study conducted by Naficy et al. [15] in Egypt during 1995-1998 period. In this study 90% of rotavirus diarrheal episodes occurred between July and November. However, in a much earlier study conducted in Bab El-Sha'reya University Hospital in Cairo, Egypt, by El-Mougi et al. [16], rotavirus infection peaked from September to March. The high percent reported in our study may be explained by the fact that it was conducted from November 2015 to March 2017, and these months fall in the period of high prevalence of rotavirus. However, in a systematic review of studies conducted by Malek et al. [17] concerning the epidemiology of rotavirus diarrhea in countries in the eastern Mediterranean region, the studies demonstrated occurrences of rotavirus diarrheal episodes year-round and showed that seasonal fluctuations were less significant in some countries, such as Egypt and Iran. This difference in seasonality was explained by Mwenda et al. [18] by the difference in the climatic conditions. There were no positive cases detected among the control group by any of the tested methods. However, the detection of rotavirus in the control group varies widely between studies [19-20]. Nevertheless, because the above studies were from industrialized countries in which rotavirus vaccinations are routine and vaccine virus was detected in some of the healthy controls, these findings might not apply to developing countries in which the severity of infection, rates of asymptomatic viral shedding, and performance of the EIA may differ [20]. A Ct cut-off value of 24 was proposed by Phillips et al. [21] by comparing real-time PCR results to ELISA results in cases and asymptomatic controls for interpretation of real-time PCR results and relation to clinical symptoms. Relating the Ct-value, as a quantification of viral load, to the severity of clinical symptoms to set up useful cut-off values seems to be an adequate approach to improve the interpretation of real-time PCR results. This assay should solve an emerging clinical problem in the area of increased use of molecular diagnostic tools. However, the cut-off values observed above are not directly applicable to local real- time PCR results, because every PCR assay has different performance. The local distribution and quantification of viruses can alter cut-off values [22]. In this study, children within the age group of 6-12 months had the highest rate of rotavirus infection. Similar results were reported by Enweronu-Laryea et al. [23] and Manjula. However, in this study, children within the age group of 0-6 months had the lowest rate of rotavirus infection. This was in agreement with the results of Junaid et al. [24]. This low incidence can be attributed to passive immunity acquired by the infants from their mothers, which wanes after six months. It is also possible that the higher rate of breast feeding in this age group protects the infants via passing of IgA anti-rotavirus antibodies to the infants. In this study, there was no statistically significant relation between gender and the occurrence of rotavirus infection. These results were in a good agreement with the results of Manjula. However, Nguyen et al. [25]. Findings indicated that males were affected predominantly, and Junaid et al. [24] found that males were twice as susceptible as females. In the current study, the infants with rotavirus infections had infections that were more severe than the children who were rotavirus negative. These results were compared to the results of Forster et al. [26], who found that AGE was more severe in rotavirus-positive subjects. In this study, there was a statistically significant negative relation between the Vesikari score and PCR Ct value ($P < 0.05$), similar to the results of other studies [27, 28], indicating that infants with severe disease excrete more virus than children with less severe disease. Regarding to the comparison between results obtained by qr RT-PCR, ICT, and ELISA, these results agreed with those of other research [29]. The authors concluded that ICT kits could be used as an alternative method for the rapid screening of “group A” rotavirus in fecal specimens, especially during acute outbreaks of gastroenteritis.

5. Conclusions

The RT-PCR assay used in this study was shown to be highly sensitive and specific for a broad spectrum of RVA genotypes, with many advantages over previously published. This RT-PCR assay may be very vital for testing other clinical or environmental samples. Finally, the assay is a true quantitative tool for determination of RVA load in stool samples.

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