Detection of rotavirus in fecal samples of infants and young children with acute diarrhea

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ABSTRACT

Rotavirus infection (RV) is the leading cause of severe acute diarrhea among young children worldwide. Rapid and accurate diagnosis of RV infection is crucial for appropriate patient management and infection control. The aim of our study was to compare the diagnostic performance of Lateral flow immuno-chromatography assay (LFICA) as a rapid test for detection of RV antigen in stool specimens collected from Egyptian infants and young children with ELISA, and nested reverse transcription polymerase chain reaction (RT-PCR). Furthermore, to study the frequency of RV infection in Egyptian infants and young children during the summer season 2012 and the effect of certain risk factors including age and gender on the extent and impact of RV infection. The study included 73 infants and young children attending the pediatric Clinic,Al-Zhraa University Hospital in the period from May to October 2012. Their age ranged from six to twenty four months. They were 47 males and 26 females. Stool specimens were collected from all cases. These specimens were processed according to the manufacturer's instructions for RV diagnosis. Out of the 73 tested specimens, 14 (19.2%) and 15 (20.5%) gave positive results for RV antigen by LFIC and ELISA respectively while 18 (24.7%) gave positive results for RV–RNA by nested RT-PCR. Infants <1 year old showed the highest rate of RV infection and male patients were at higher risk than females. In conclusion RV is a common etiological agent of serious diarrhea in infants and young children. RT-PCR is more sensitive than LFIC and ELISA in detecting RV infection. However, LFIC is a rapid and easy test that can aid in the detection of RV in pediatrics helping healthcare provider in making patient management decisions at the same office visit. Negative LFICA results do not rule out the infection with RV so these samples must be tested by another technique like RT-PCR.

Keywords: Rotavirus, Stool Specimen, ELISA, RT-PCR, lateral flow immuno-chromatography, Infants, Young children**.**

INTRODUCTION

Diarrheal diseases remain one of the principal causes of childhood mortality and morbidity. According to the World Health Organization, diarrheal disease is the second leading cause of death in children under five years old worldwide, and is responsible for 1.5 million child deaths every year (Petri *et al,*2008; Tate 2012). Rotavirus infection remains the commonest cause of severe dehydrating diarrhea among children worldwide (Bon *et al* 1999; Black *et al,*2010). Serious conditions have been reported to be associated with RV infection, such as necrotisingenterocolitis (NEC), diffuse intravascular coagulopathy, pneumonia, apnea and seizures (Parashar *et al* 2004; de Villiers &Driessen 2012).

Rotaviruses are non-enveloped double stranded ribonucleic acid (dsRNA) viruses belong to the genus Rotaviruses under the Reoviridae family. The RV genome is composed of eleven dsRNA segments and encodes 6 structural (VP1-VP4, VP6 and VP7) and up to 6 non-structural (NSP1-NSP6) proteins. VP6 is the most conserved protein among the structural proteins of RVs, while VP4 and VP7

possess neutralization antigens and play an important role in virus entry and infection of the target cell (Estes & Cohen, 1989; Kapikian *et al.,*2001). Rotaviruses are grouped into seven different sero-groups (A-G). Of theseseven groups, A-C are known to infect humans, and, group A are more commonly associated with severe, life threatening disease in children worldwide. The virion is composed of an inner core layer which is made up of VP1, VP2, and VP3, an intermediate layer consisting of VP6, and an outer shell composed of VP7 and VP4. (Glass *et al*, 2006) The VP4 and VP7 proteins are important for the development of group A rotavirus vaccine because they are targets for neutralizing antibodies that give genotype specific protection (Hyser and Estes, 2009). There are 23 G genotypes and 32 P-genotypes of group A rotaviruses. The G- types: G1, G2, G3, G4 and G9 together with P-types P4, P6 and P8 are the most common human rotavirus types reported in studies worldwide (Clark &McKendrick 2004).

MATERIAL & METHODS

Patients: Seventy three infants and young children (age range from six to twenty four months). They were 47 males and 26 females. They attended the outpatients Pediatric Clinic,Al-Zahraa University hospital in the period from May to October 2011 suffering from acute diarrhea (≥3 loose stools in 24 hours for ≤ 10 days) were enrolled. Verbal informed consent was obtained from the parents of the children for inclusion in the study. A questionnaire detailing demographic information, the child's medical history and current physical status was completed. All patients were subjected to clinical examination at the time of specimen collection.

Specimens*:* Stool specimen was collected from each case and refrigeratedat 4°C until transported to our laboratory on ice within few hours. In the laboratory,each specimen wasdivided into three aliquots one used immediately for LFICA and one was frozen at -20°C until further testing by ELISA test. The third aliquot was prepared as 10% suspension in 0.1 mol/L phosphate-buffered saline (pH 7.2) and kept at -20°C until tested bynested RT PCR for rotavirus

Methods*:* All samples were screened for RV antigen using,LFICA and ELISA. RV RNA was also investigated by nested RT - PCR

Lateral Flow Immuno-chromatography

The principle: This test utilizes an antigen capture conjugated antibody and reagents that move laterally by chromatography. A positive result appears as a pink or reddish-purple line at the bottom of the test strip when RV antigen is present. A pink to purple control line, also near the bottom of the strip, must be present for any result to be valid.

The procedure: The Stool specimens were tested by one step LFICA supplied by ACON (REF IRO-602, USA) following the manufacturer's instructions.

Antigen Detection Using ELSA Test

The stool specimens were prepared and tested for rotavirus antigen according to instructions of the commercially-available, ProSpecT™ Rotavirus Microplate Assay, (Oxoid Ltd, UK). The assay was validated using the manufacturer's results validation criteria.

The principle: The ProSpecT™ Rotavirus test utilizes a polyclonal antibody in a solid-phase sandwich enzyme immunoassay to detect rotaviruses antigen in stool samples

Nested RT– PCR

Nested polymerase chain reaction amplifying the VP6 region was performed to determine the presence or absence of rotavirus RNA in each stool sample.

RNA Extraction

Viral RNA was extracted from 140 microliter of the preparedstool using the QIAampUltrasens virus kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

RT- PCR was conducted with QiagenOneStep RT-PCR kit (QIAGEN GmbH, Hilden, Germany) The reaction mixture contained 10µl 5 x QiagenOneStep buffer, 1 µl of each primer that was designed to amplify a 379-bp region (nucleotides [nt] 747 to 1126, coding for aa 241 to 367) of the VP6 gene, they were VP6-F (sense) (5′ GAC GGV GCR ACT ACA TGGT 3′) and VP6-R (antisense) (5′ GTC CAA TTC ATN CCT GGT GG 3′),2.0 µl dNTP Mix (containing 10 mM of each dNTP), .2.0 µl QiagenOneStep Enzyme mix, 2.5 µl of RT Enhancer, 2.5 µl of the extracted RNA and up to 50 µl water nuclease-free. The cycling program was 50 ˚C 30 min, 95 ˚C 15 min, followed by 40 cycles of 94 ˚C 30 s, 56 ˚C 30 s, 72 ˚C 60s, and an extra 7 min at 72 ˚C. Twomicroliters of the RT-PCR product of each sample were re-amplified using 1 µ of each internal primers (VP6-NF GCW AGA AATTTT GAT ACA and VP6-NR GATTCACAAACTGCAGA) that amplify 155 bp fragment and 25 µl of PCR reaction mix (DreamTaq DNA polymerase commercial kit supplied by Thermo, Lithuania) containing 2X DreamTaq Green buffer dATP,dCTP,dGTP and dTTP, 0.4 mM each, and 4 mM $MgCl_2$ and up to 50 µl water nuclease-free. The cycling program was one cycle at 95°C for 5 minutes, followed by 35 cycles of 94 ˚C 60 s, 42 ˚C 60 s and 72 ˚C 60s (IturrizaGómara *et al.* 2002). A total of 15µl of nested-PCR products were loaded into 2% agarose gel containing 5 µg/ml ethidium bromide using a 100-bp molecular size marker (Invitrogen) and visualized by transilluminator.

STATISTICAL ANALYSIS

Data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentage). The sensitivity, specificity, positive predictive value, negative predictive value and total agreement of the LFIC and ELISA were calculated relative to RT-PCR as a reference test. Comparison between the study groups was done using Chi-square (X^2) test. A probability value (P value)less than 0.05 was considered statistically significant. All statistical calculations were done using Microsoft Excel version 7 (Microsoft Corporation, NY, and USA) and SPSS (Statistical Package for the Social Science, SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

RESULTS

Out of 73 tested specimens, 18 (24.6%) were positive for RV RNA by nested RT-PCR using a primer pair that amplifies a fragment of a 155 bp in the highly conservative region of the VP6 gene(Figure 1). Fourteen specimens (19.2 %) were positive for RV antigen by LFTCA and 15 (20.5%) were positive for RV antigen by ELISA test with insignificant difference (P>0.05) (Tables 1&2). Compared to RT-PCR as a reference test, the sensitivity, specificity, positive and negative predictive values and total agreements were 77.8%, 100%, 100% 93.2 and 94.5 % for LFICA and 83.3%100%, 100% 49.8 and 95.9 for ELISA respectively(Tables 3 & 4).

The demographic and clinical symptoms of RV positive cases are illustrated in Table (5).Infants <12 months old showed the highest rate of RV infection where 55.5% (10/18) were positive for RV-RNA, then the rate of infection decreased to 44.4% (8/18) in children with age ranged from 12-24 months(Table 5),but the difference was statistical insignificant (P>0.05) The percentage of male children

positive for RV was higher than that of females (48.84%) versus (41.38%). but the difference did not reach statistical significance (P>0.05).Ten out of 18 (55.5%) patientshad fever while all RV positive cases 18/18 (100%) had diarrhea and vomiting(Table 5).

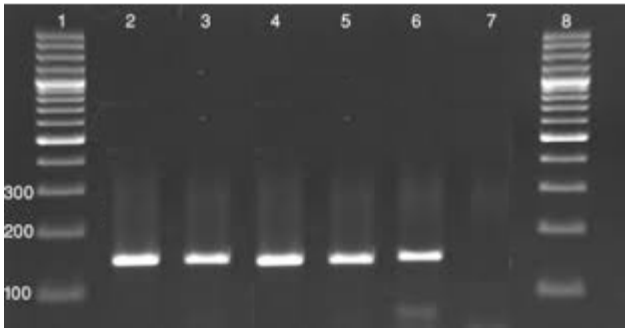

Fig. 1: Ethidium bromide stained 2% agarose gel electrophoresis for RV nested-PCR products: lane 1 & lane 8 represent 100 bp DNA molecular size ladder, Lane 2, 3, 4, 5 and 6, positive amplification of a 155 bp fragment of RV VP6 gene sequence. Lane 7 negative control

Table 1. Comparison between nested RT-PCR & LFICA for RV Detection.

Nested RT-PCR	LFICA		Total	P value
	Positive	Negative		
Positive	14	4	18	>0.05
Negative	0	55	55	
Total	14	59	73	

Table 2. Comparison between nested RT-PCR & ELISA for RV Detection

Nested RT-PCR	ELISA		Total	P value
	Positive	Negative		
Positive	15	3	18	>0.05
Negative	0	55	55	
Total	15	58	73	

Table 3. Performance Parameters of LFICA in Comparison with RT-PCR.

PCR results		LFICA				
		Sensitivity	Specificity	*PPV	**NPV	Total agreement
Positive	18	14/18 (77.8 %)	14/14 (100 %)	14/14 (100%)	55/59 (93.22 %)	69/73 (94.5%)
Negative	55					
Total	73					

*PPV= positive predictive value **NPV= Negative predictive value

Table 4. Performance Parameters of ELISA in Comparison with RT-PCR.

PCR results		ELISA				
		Sensitivity	Specificity	*PPV	**NPV	Total agreement
Positive	18	15/18 (83. 33%)	15/15 (100 %)	15/15 (100 %)	55/58 (94.8 %)	70/73 (95.9%)
Negative	55					
Total	73					

*PPV= positive predictive value **NPV= Negative predictive value

Table 5. The demographic and clinical features of children with rotavirusinfection

Variable		Rotavirus Positive (Total 18) N (%)
Sex	Male	14 (77.8%)
	Female	4 (22.2%)
Age	≤ 12 months	10 (55.5%)
	>12 months	8 (44.5%)
Fever	Present	10 (55.5 %)
	Absent	8 (44.5%)
Vomiting	Present	18 (100 %)
	Absent	0 (0%)
Loos stool	Present	18 (100 %)
	Absent	0 (0%)
Dehydration	Present	3 (16.7 %)
	Absent	15 (83.3%)

DISCUSSION

Considering the seriousness of rotavirus infections, the development of rapid and sensitive diagnostic assays is of the utmost importance for diagnosing and monitoring RV and consequently reduces hospital stays, the cost of hospital care, antimicrobial use and complications. The efficiency of diagnosis also allows for proper precautions to be taken to prevent or minimize RV spread. Initially, electron microscopy (EM) was used as a diagnostic method of rotaviruses since the discovery of the virus in 1973. EM has traditionally been used as a "gold standard" in evaluations of rotavirus detection assays. However, classical EM is highly specific and rapid but is not suitable for testing large numbers of specimens. It requires an electron microscope and a skillful operator, which may make the method unsuitable for small laboratories. EM is of low sensitivity as the specimens should contain approximately 10^6 viral particles/mL to be detected (Madeley& Cosgrove 1975; Mijatovic-Rustempasic*et al.,* 2013). Various immunoassays such as latex agglutination (LA) tests and enzyme immunosorbent assays (ELISA) are commonly used as an alternative to EM for diagnosis of rotavirus infection. ELISA technique has been adopted by the World Health Organization as the standard method for the detection of rotavirus antigen in stools. ELISA have the advantage of giving numerical results which can be objectively interpreted but they require multiple steps in processing and usually are not cost effective for testing

small numbers of specimens (Beards*et al* 1984 & Thomas *et al* 1988). It appears that degradation of VP7 antigen by proteolytic enzymes during freezing and thawing was a major factor in the loss of typing ability by ELISA.

Virus isolation is considered the 'gold standard' method for RV diagnosis. However, it requires fully equipped laboratories with skilled professionals and has a long turnaround time. Additionally, RV tends to be labile and loss of infectivity can occur during transport. Many studies have reported that nucleic acid amplification techniques are more sensitive than viral culture for detecting RV in clinical samples (Mijatovic-Rustempasic*et al*., 2013). This may be explained at least in part by non-viability of viral particles in the specimens that can be detected by RT-PCR while virus isolation requires the presence of viable viral particles to achieve a positive result. Consequently, in our study RT-PCR was used as the gold standard for RV detection. Samples positive by this method were considered true positives to evaluate LFICA that have been developed as a rapid test for direct qualitative detection of RV antigen in stool specimens in comparison to ELISA test. The results showed that 24.65 % of the stool specimens were positive for RV-RNA by RT-PCR while 19.2 % were positive for RV antigen by LFICA with sensitivity and specificity of 77.8% and 100%, respectively. Positive and negative predictive values were 100% and 93.22% respectively. While 20.5 % were positive for rotavirus detection by ELISA technique with sensitivity and specificity of 83.3 % and 100 % respectively. Positive and negative predictive values were 100% and 94.92% respectively. Our results was in accordance with results obtained by other studies.Maes,*et al*., (2003),compared LFICA for detection of RV in human diarrhea samples with electron microscopy and found that the LFICA had a sensitivity and specificity of 94 and 100%, respectively. However,Levidiotou*et al.* (2009),investigated the role of enteric viruses as a cause of gastroenteritis in 4604 hospitalized children in north-west Greece. They found that RV was detected in 21.35%, by ELISA and the rate of detection was increased by10 % using RT-PCR. Also they noticed that rotavirus was the leading cause of viral gastroenteritis that is usually associated with severe illness.

Regarding the frequency of RV infection rate our results are in agreement withMatson *et al* (2010),who detectedRV antigen by ELISA in 259/1026 (25.2%) rectal swabs collected in 2000-2002 during hospital-based surveillance from children < 5 years of age presenting with diarrhea as the primary complain at one of 2 hospitals in Egypt: Abu Homos District Hospital, the main referral hospital for a rural district in the Nile Delta, and Benha Fever Hospital, the main referral center for a periurban area north of Cairo. As well as (EL-Mohamady*et al,* 2006), who detected RV infection in 21% of children aged ≤ 6 months and living in the Tamiya District of the Fayum governorate located in Southern Egypt.

Several studies were conducted in Egypt and their results showed higher or lower frequencies' of rotavirus detection. Hashem*et al* (2012), detected RV infection using RT-PCR in 158/450 (35.1%) stool samples collected from children with acute gastroenteritis, who attended the outpatient clinic in El-Demerdash hospital, El-Fayoum general hospital and Belbes general hospital in Egypt. As well as Amer *et al* (2007), identified RV by RT-PCR in 33% of the stool specimens collected from children with acute gastroenteritis, who attended the outpatient clinic in EL-Shatby hospital in Alexandria, over a 12 months period from January to December 2006 with a marked seasonal peak during the cold months of the

year (December – February). A similar finding were also, reported by Shukry*et al* (1986), Radwan *et al* (1997) and El-Mougi e*t al.* (1998) who detected rotavirus antigen in 33%, 35.6% and 40%, of stool samples obtained from children with acute diarrhea respectively, using the ELISA technique. However, a five years study on the bacterial and viral etiology of infantile diarrhea in Alexandria revealed that rotavirus was responsible for 15.8% of diarrheal illnesses in infants and children attended the outpatient clinic in EL-Shatby children hospital, during the period of 1982-1987 (Massoud*et al* 1989). This differences can be interpreted by the seasonal fluctuation and the different periods of sample collection as our samples were collected during summer months only while in the other studies samples collected all over the year and the results showed marked seasonal peak of RV diarrhea during the colder months of autumn and/or winter (Hashem*et al*., 2012; Amer*et al*, 2007). Also, previous cohort study conducted in Bilbeis (Egypt) by Zaki*et al* (1986), whofound that the rate of rotavirus isolation was predominant in the colder months (November – April) and a similar finding was reported by other studies (Ryan *et al* 1996,Vesikari*et al*, 1999, Sanchez-Fauquier *et al*, 2004,Trabelsi*et al*, 2000).Also, the seasonal peak of rotavirus infection tends to shift over consecutive years (Zaki*et al* 1986, Patel*et al* 2013; Sarkar*et al*., 2013).

Our results also, show remarkable agreement with the results of other investigators in Venezuela, Spain and Dhaka (Salinas *et al* 2004, Sánchez-Fauquier 2004, and Rahman *et al* 2005). However, in other settings (Guardado*et al* 2004 and Sánchez-Fauquier *et al* 2006), rotavirus was detected with a higher percentage rates which confirm the huge disease burden over the world, and the variability of its prevalence from a region to another.

According to the WHO scientific working group (1980), most cases of RV infection are in children between 6-24 months with a peak incidence at 9-12 months which was observed in our study where the age group that experienced the highest incidence of RV diarrhea ranged from six to twelve months, with median age 10 months. These findings were in agreement with the earlier studies done in Egypt (Zaghloul *et al*., 2013; Hashem*et al*., 2012; Amer *et al*., 2007; Naficy *et al*., 1999; Reves *et al*., 1989). In addition, other investigators in different countries recorded that the highest rate of rotavirus isolation was found among children in their first year of life (Shetty *et al* 2014,Veeravigrom*et al* 2004, Trabelsi *et al* 2000, Singh *et al* 1989). These findings confirm the role of the immune system in prevention of the disease, which helps decline of rotavirus diarrhea with age.

Regarding the gender as a risk factor, our results delineate that male patients are at higher risk than female patients where 77.7% of the total positive for RV were males versus 22.3% females. This is in agreement with Hashem*et al* (2012), who found that 57.5 % of the Egyptian children with RV infection were males.

In our study out of 18 positive RV cases, 10 patients (55.5%) had fever either at the time of examination or fever was present 2-3 days prior to asking medical care. Vomiting was the first symptom in 11 cases of RV infection which later progressed to acute diarrhea. All RV positive cases (100%) had diarrhea and vomiting. The same observation was reported byShetty*et al*. (2014) as they found that out of 10 positive RV cases, nine patients (90%) had fever either at the time of examination or fever was present 2-3 days prior to hospitalization. Vomiting was the first symptom in some cases of RV infection which later progressed to acute diarrhea. All

Rotavirus positive cases (100%) had diarrhea and vomiting. Similar finding were reported in earlier study by Singh *et al*., (1989) who found that 72.1% cases of RV had complaints of vomiting and diarrhea.

Three cases in our study were dehydrated (16.7 %).Nakawesi*et al*. (2010), found that Children with dehydration were about two times more likely to have rotavirus diarrhea. Several studies have reported a similar finding (Binka*et al*, 2003,Nafic*yet al* 1999). This finding is not surprising as gastroenteritis was more severe in rotavirus-positive than in rotavirus-negative children (Albano*et al*2007&Binka *et al*, 2003)

IN CONCLUSION: Rotavirus is a common etiological agent of serious diarrhea in infants and young children. RT-PCR is more sensitive than ELISA and LFIC in detecting RV infection but RT-PCR is relatively expensive and labor intensive so it is not suitable as a routine rotavirus detection test. However, LFIC is a rapid and easy test that can aid in the detection of RV in pediatrics helping healthcare provider in making patient management decisions at the same office visit. Negative LFICA results do not rule out the infection with RV so these samples must be tested by another technique like RT-PCR.Also, our results underline the importance of continued detailed epidemiological and virological studies to identify rotavirus prevalence and strains circulating in our community to help in assessing the suitability of candidate vaccines, in order to protect against all currently circulating rotavirus strains.

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