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Isolation and Molecular Genotyping of Group A Rotavirus Strains Circulating Among Egyptian Infants and Children

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Abstract: Purpose: Group A rotaviruses are the most important cause of acute diarrhea in children throughout the world. They are the cause of more than 450,000 annual deaths. There are few data available about rotaviruses type circulating in Egypt. Genotyping by reverse transcription-PCR (RT-PCR) have been widely used. Aim: To identify rotavirus antigens and RNA among stool specimens isolated from Egyptian children and to perform molecular characterization among the detected isolates. Materials and Methods: This study comprised 450 stool specimens collected from children less than 5 years old suffering from acute diarrhea, from three different governorates in Egypt (Cairo, Sharkia and Fayoum) during May 2009 to April 2010. Results: Rotavirus was detected by RT PCR in 158 (35%) of patients stool samples. G1 was the predominant genotype detected in 87/158 cases (55%). G3 was the second most common cause and was responsible for 35 cases (22.2%). whereas G4 represented only 8.2% (13/158) of all strains. Our study identified G9 in 23 (14.5%) of positive cases. No untypable strains were detected and mixed infection between G1and G4 in 1.9% (3/158) of the samples was detected. G8, G2 were not detected among collected samples. Conclusions: These results underline the importance of continued detailed epidemiological and virological studies to identify rotavirus genotypes responsible for severe diarrhea, including characterization of the less common and or unusual strains. Focusing on the more prevalent strains circulating in the Egyptian community will add in assessing the most suitable strain candidates used for vaccine production to protect against current circulating and uncommon strains and for evaluation of cross immunogenicity among variable strains.

Key words: Rotaviruses, diarrhea, RT-PCR, genotyping, infants, children.

INTRODUCTION

Rotavirus infection is associated with acute infantile gastro-enteritis in infants and young children globally. In the developing world, rotavirus is associated with high levels of morbidity and mortality and is estimated to account for 650,000 annual young children deaths. Approximately a quarter of these deaths occur in African children, yet the epidemiology of rotavirus infection and the characterization of rotavirus strains in Africa is poorly understood (Parashar *et al*., 2006). In sub-Saharan Africa, one out of every 30 children born will die from diarrhea before the age of five. Rotaviruses contribute up to 24% of all childhood diarrheal episodes in Africa. Efforts to improve sanitation and provide clean water have not decreased the high mortality due to rotavirus infection in developing countries, focusing the need for an effective rotavirus vaccine. A properly administered vaccine could potentially prevent one in 20 children death (Cunliffe *et al*., 2001).

Rotaviruses are members of the Reoviridae family, and are characterized by their non-enveloped icosahedral structure and 70-nm diameter. When examined under an electron microscope; they have a 'wheel' shape (Kapikian and Chanock, 1996). The capsid consists of three protein layers; the outer capsid is composed of the structural proteins VP7 and VP4, and the inner capsid mainly of VP6. The core is found inside the inner capsid, and encloses the rotavirus genome, composed of 11 segments of double-stranded RNA. Given the segmented nature of the RNA genome, co-infection of cells with two different strains of rotavirus may result in reassortant viruses, with RNA segments from each of the progenitors (Desselberger, 1996).

There are few data available about rotavirus types circulating in Egypt. In spite of the variety of methods used, many untypable strains remained, raising the possibility that additional serotypes may be prevalent in Egypt. This demonstrates the need for a wider variety of reagents and detection methods in an attempt to identify the full variety of circulating strains. Serotyping by ELISA with anti-VP7 serotype-specific monoclonal antibodies and genotyping by (RT-PCR) has been widely used for typing (Naficy *et al*., 1999).

Most rotavirus strains can be grown in cultured cells. However, the procedure is tedious and is restricted to the research laboratory. Various other techniques have been developed to readily detect rotavirus in stool,

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including electron microscopy and polyacrylamide gel electrophoresis (PAGE) of viral nucleic acid and antibody-based assays such as enzyme immunoassay (EIA), immunofluorescence, radioimmunoassay, and solid-phase aggregation of coated erythrocytes. Relatively simple to perform antigen-detection systems in the form of EIA and latex agglutination (LA) kits have been developed as alternative identification systems (Chakravarti *et al*., 1991).

MATERIALS AND METHODS

Study Population and Fecal Specimens:

The study methods were designed according to the WHO Generic Protocol for rotavirus hospital-based surveillance (WHO, 2002). 450 fecal samples were collected from 3 different governorates in Egypt, Most of the stool samples collected from Cairo (El-Demerdash hospital) 200/450, followed by Fayoum (Fayoum general hospital) (150/450) and Sharkia governorate (Belbes general hospital) (100/450) during May 2009 to April 2010 from infants and children below 5 years old attended to the out clinic suffering from diarrhea. Stool specimens were stored at -30^{o}C until use.

Rotavirus Antigen Detection by Latex and ELISA:

Latex agglutination (LA) was performed using the commercial latex agglutination kit (Remel, USA). The test was considered positive for rotavirus if distinct agglutination was observed with test latex but not with control latex and indeterminate if agglutination was observed in test and control latex. All fecal samples were screened for rotavirus group A-specific antigen by enzyme immunoassay (EIA) (Rotascreen ELISA kit, UK). The assay was performed according to the manufacturer's instructions.

Detection of Infectious Rotavirus by ICC-RT-PCR Assay:

Twenty ELISA positive samples were selected for adaptation to grow in MA-104 cell-line. As 0.5 ml Trypsin-treated viral suspension was inoculated on confluent sheet of MA104 cells and incubated for 90 min at 37°C rocking every 15 min; then the inoculum was removed without rinsing. Serum-free eagle's medium MEM, supplemented with 5µg/ml of Trypsin was used as the maintenance medium. After a period of incubation (1 to 5 days) for virus replication, the supernatant was discarded and the viral RNA was extracted from the cell monolayer using Trizol. The nucleic acid was suspended in 20µl of RNase-free ddH_2O and subjected to RT immediately or stored at - 80°C.

Extraction of Viral Genome:

Rotavirus dsRNA was extracted from tested ELISA positive samples. Fecal specimens were thawed, diluted with Phosphate buffer saline to 10% suspensions, and centrifuged at 5,000 rpm for 15 min. Viral RNA was extracted from 200µl of the supernatant using a spin column technique (Viral RNA Miniprep Kit, Axygen Biosciences, USA) according to the manufacturer's instructions. The extracted dsRNA was suspended in 40µl of RNAse-free water and stored at -20^{o}C for use in the PCR reactions.

Reverse Transcription:

For reverse transcription (RT), 5µl of extracted viral genome was added to 0.5 ml of low-bind microcentrifuge tubes containing 1µl of each VP7 consensus primer (20 pmol). RNase-free water was added to a final volume of 12.5µl, and the samples were mixed and denatured at 97°C for 5 min in a thermal cycler (Eppendorf, Germany).

Samples were cooled on ice for 5 min and centrifuged at 12,000 rpm for 10 sec. to remove the condensation from the walls of the tubes. A reverse transcription mixture (7.5µl) containing 2µl of deoxynucleoside triphosphate mixture (containing 10Mm each [dATP, dGTP, dCTP and dTTP]; Fermentas), 4µl of 5x reaction buffer and 1.0µl RevertAid M-MuLV-Reverse Transcriptase (200u) was then added to each denatured dsRNA sample tube (to give a final reaction volume of 20µl). The samples were mixed by gentle flicking, centrifuged at 12,000 rpm for 5 sec. and subjected to one cycle of reverse transcription (42°C, 60 min) and terminate the reaction by heating at 70 ^{0}C for 10 min.

Rotavirus Genotyping:

To determine the G types of rotavirus isolates, PCR assay was performed. Initially, 1,062-bp (full-length) gene segment 9, encoding the VP7 glycoprotein in human group A rotaviruses, was amplified using primer Beg9 (5'-GGC TTT AAA AGA GAG AAT TTC CGT CTG G-3') in the forward direction and primer End9 (5'-GGT CAC ATC ATA CAA TTC TAA TCT AAG-3') in the reverse direction. This was followed by multiplex heminested PCR using a cocktail of specific primers that identify the following VP7 genotypes: G1, aBT1; G2, aCT2; G3, aET3; G4, aDT4; G8, aAT8; G9, aFT9 and the common primer RVG9 (Gouvea *et al*., 1990). PCR

products were resolved by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized under UV transilluminator (Uvitec, EU).

Results:

Identifications of Rotaviruses in Clinical and Laboratory Samples:

Different methods were used for identification of rotaviruses from clinical samples. Using latex agglutination test, rotavirus was detected in 94 of 450 samples, many samples give non-interpretable result in which visible agglutination was observed in both control and test latex which indicate non-specific reaction and unsuitability of the specimen for this test procedure. Specificity and sensitivity of latex test was lower than in case of using ELISA.

The performance of the Rotavirus Latex test (Remel, UK) was evaluated for rotavirus detection in fecal samples of patients with acute gastroenteritis. This assay was compared with the enzyme immunoassay (EIA) ROTASCREEN (Microgen, UK). 450 fecal specimens were analyzed. 94 samples (20.8%) were reactive, 206/450 (45.7%) were nonreactive, and 150/450 (33.3%) were indeterminate by LA. All LA-positive samples were positive by EIA, and 25 LA-negative samples were positive by EIA. Of specimens indeterminate by LA, 55/150 (36.6%) were positive by EIA. The sensitivity, specificity, and accuracy of LA were 79%, 100% and 91%, respectively. Positive predictive value was 100% and negative predictive value was 87.8%.

Table 1: Comparison of Latex Agglutination with Enzyme Immunoassay for Detection of Rotavirus in Fecal Specimens**.**

Latex agglutination	Enzyme Immunoassay	
	Positive	Negative
Positive	94	0
Negative	25	181

Detection of Rotavirus by ICC-RT-PCR Assay:

CPE was detected after 5 passages with 5-day-incubation period. Viral RNA was extracted from cell monolayer, genotyping of rotavirus was done. The specific rotaviruses gene could be detected post incubation for 5 days infected with relatively low level of rotavirus.

Distribution of Age and Gender:

Rotavirus was detected in 158 of 450 (35.1%) of stool samples by ELISA. The lowest age in collected samples was 40 days; the highest age was 5 years. Most of the patients (59%) were in the 1-12 month age group. The distribution of gender in collected samples was 58% male and 42% female and 57.5 % of the children with rotavirus infection were male. Rotavirus was detected in children of all age groups, but the highest detection rates were observed in children aged 6-24 months, accounting for 69% of all rotavirus cases.

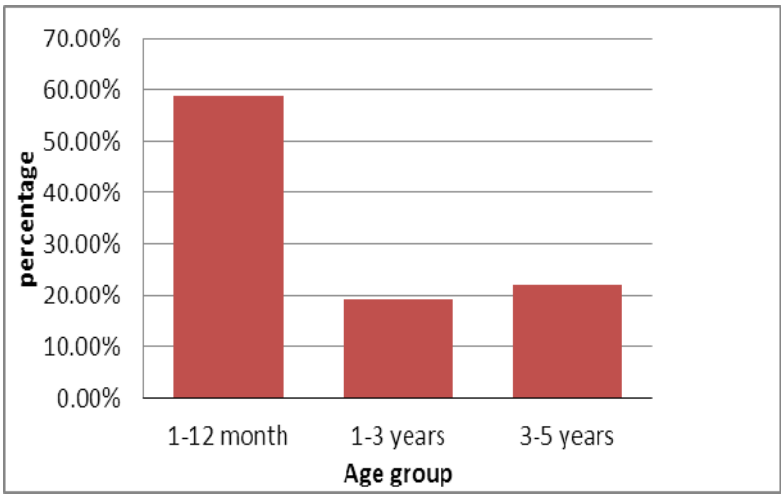

Fig. 1: Age distribution in collected fecal samples.

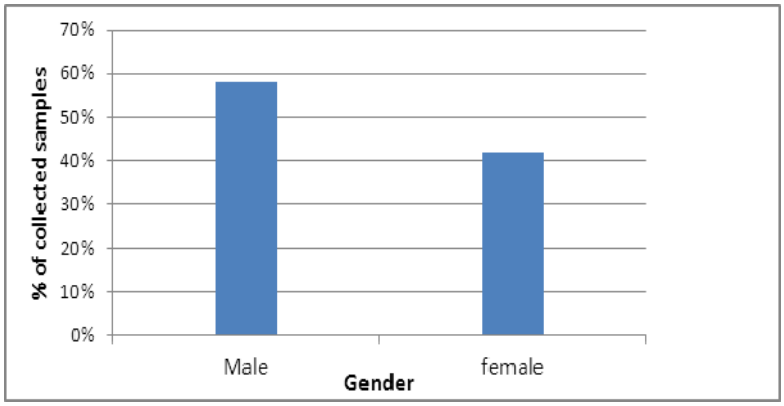

Fig. 2: Gender distribution in collected fecal samples.

Seasonal Pattern of Rotavirus Infection:

The occurrence of rotavirus infection varied according to seasonal temperature. Rotavirus infection was detected continuously from May to April (Fig. 3B). Rotavirus was detected most frequently from October to February. The presence of rotavirus remained low from 1.3% to 4.5% in May–September and in March and April as well. From this study, it is clear that peaks of infection were detected in the cold months. Rotavirus infection is common in autumn and winter seasons in Egypt. The highest prevalence of rotavirus infection was found during October (30/158) followed by November (25/158) and January (23/158) respectively.

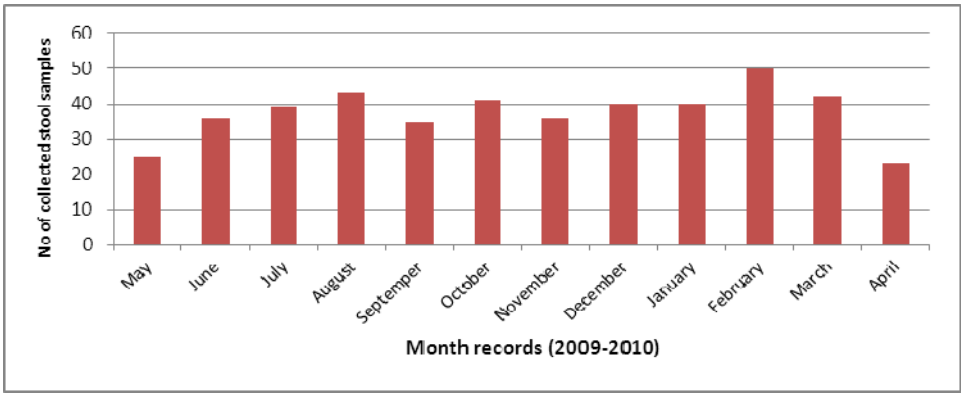

Fig. 3A: Number of stool samples monthly collected from infants and children with acute gastroenteritis during June 2009 to April 2010.

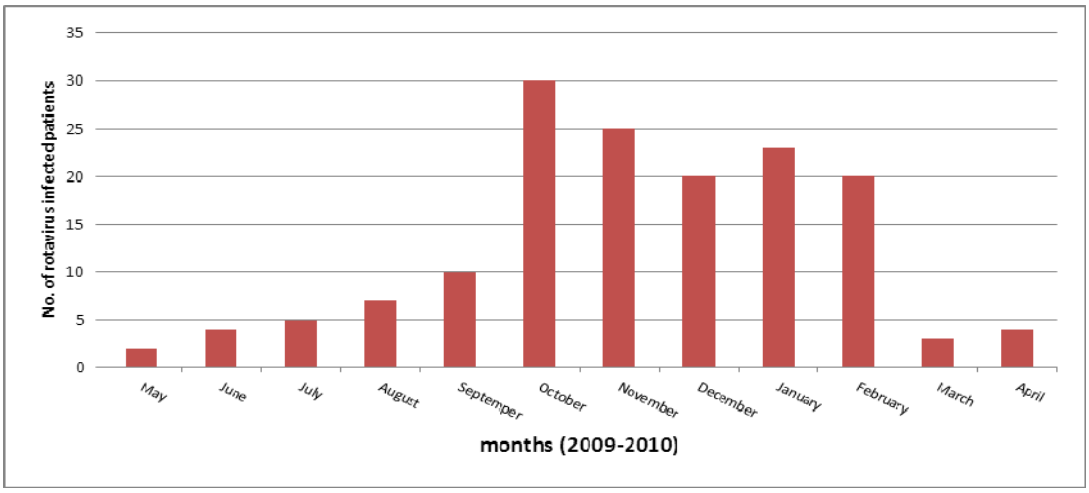

Fig. 3B: The seasonal fluctuation of Rotavirus infection in collected samples.

Distribution of G Genotypes:

A total of 158 rotavirus-positive specimens were characterized for G genotypes. The distribution of group A rotavirus genotypes during the study period is shown in Table (1). It was detected that G1 genotype was the most prevalent, 55% (87/158). Other globally common genotypes (G3, and G4) were less frequently identified in Egypt; G3 accounted for 22.2% (35/158), whereas G4 represented only 8.2% (13/158) of all strains during the study period.

While G9 strain represented as 14.5% (23/158). No untypable strains were detected and mixed infection between G1and G4 was detected in 3 of the samples. G8, G2 were not detected among isolates.

Table 2: Distribution of group A rotavirus G strains among infants and children with diarrhea in Egyptian Governorates between May 2009 and April 2010.

Governorates Genotype	G1	G3	G9	G4	Total
Cairo	36	10	7	4	57
Sharkia Governorate	28	16	8	3	55
Fayoum Governorate	23	13	6	4	46
Total	87	35	23	13	158

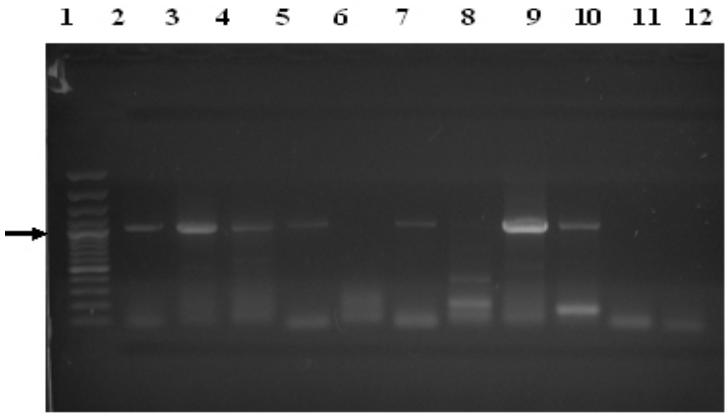

Fig. 4A: Agarose gel electrophoresis of specific RT-PCR products.
(Lanes 1 (100-bp molecular marker), Lanes 2,3,4,5,7,9 and 10 (1062bp full-length VP7 gene), lane 6,8,11 and 12 (negative samples). The arrow shows the 1000-bp mark.)

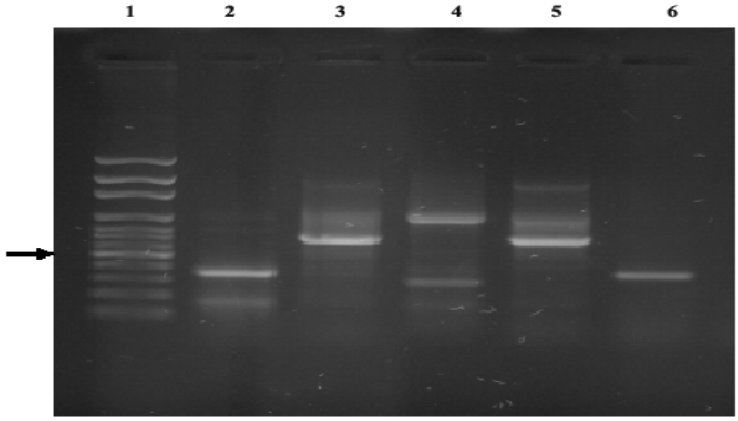

Fig. 4B: Agarose gel electrophoresis of specific RT-PCR products.
(Lane 1 (100-bp molecular weight marker), Lane 2,4 and 6 (G9), Lane 3,5 (G1). The arrow shows the 500-bp mark.)

Discussion:

RAV is of the most common cause of non-bacterial gastroenteritis, not only in the developing countries but also in developed countries like Japan, Korea and Germany (Parashar *et al.*, 2006). In Egypt, during the past decade, numerous studies evaluating diarrheal diseases among children living in the Nile River Delta, Northern Egypt revealed that rotavirus was the most commonly identified cause of diarrhea among children seeking medical care for severe illness (Naficy *et al*., 1999 and Wierzba *et al*., 2006). Typically large amounts of virus particles are present in feces during the peak of infection, thus facilitating the diagnosis of the viral infection.

However; as few as 10 virus particles are sufficient for the infection of small children. To minimize the risk of Rotavirus infection, an increase in sensitivity of the rotavirus detection assay is therefore desirable. Conventional ELISA detection of rotavirus antigen for all known human Rotavirus goes along with typical limits of detection of ≈ 1x 10^5 particles/ml. Several approaches to the establishment of RT-PCR assay of the viral RNA have been described, and the limit of detection was found to be ≈ 100 particles/ml (Adler *et al*., 2005).

In the present study, Rotaviruses positive samples were identified using RT-PCR in 158/450 stool samples (35.1%). Samples collected from children with acute gastroenteritis, who attended the outpatient clinic in three different Hospitals in Egypt. The obtained results are similar to the findings of an active surveillance studies conducted in Egypt over the past decade. 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). More recently, Rotavirus was detected in 17% of diarrheal cases within 356 children aged ≤ 6 months, in children living in the Tamiya District of the Fayoum governorate located in Southern Egypt, between August and September 2003 (EL-Mohamady *et al*., 2006). The results within hand show remarkable agreement with those obtained by (Salinas *et al*. 2004 and Sanchez *et al*., 2004) respectively in Venezuela and Spain which confirm the huge disease burden over the world and the variability of its prevalence from a region to another. The majority of the cases occurred in children younger than 2 years, which is the most susceptible target age group. In slightly older children, rotavirus infection can be asymptomatic, probably because they have some degree of protection from clinical disease owing to previous infection with this agent (Bos *et al*., 1995).

The latex agglutination (LA) assay for rotavirus diagnosis is rapid and inexpensive. We observed that LA kit used was a highly specific and rapid method. While it may be useful in rapid screening during outbreaks, its low sensitivity compared to ELISA makes it unsuitable for use in routine clinical practice. In the present study, 33.3% of test samples had indeterminate results using LA and were confirmed using ELISA. The data revealed that the degree of positivity of the LA test showed a roughly linear relationship with the degree of EIA optical density, and the positive predictive value of the test was high. Thus, the simple and inexpensive LA test would be useful as a screening procedure to detect rotaviruses in stools of children with diarrhea. Therefore, it is possible to conclude that strongly positive reactions with the LA test may be regarded as true-positive reactions. However, samples producing weakly positive or indeterminate reactions should be retested in a more specific and sensitive assay, such as EIA.

Rotavirus replication starts as soon as the RNA enters the nucleus, and the transcription of the VP7 gene is known to persist throughout the entire infection. The specific primers in this study were designed in the conservative sequence of the VP7 gene, and target for several strains of rotaviruses. Therefore, the primers can detect various serotypes of rotaviruses. After 7 days incubation, viral RNA could also be extracted from supernatant using miniprep extraction kit suggesting that a complete lytic cycle may take more than 5 days with low level of infection (Page *et al*., 2002). All of the commonly occurring G genotypes in the African region (G1–G4) were detected except G2. The VP7 genotype G1 viral strain was more predominant, a trend that has been observed globally (Gentsch *et al*., 1996). Three samples with a G1/G4 mixed specificity are suggestive of an on-going natural reassortment between serotypes in the community.

During the past decade, the number of countries that have reported the detection of rotavirus G9 strains has increased dramatically. Apparently G9 rotaviruses are expanding on a global scale; currently, the G9 serotype is considered to be the fifth most common type worldwide. First identified in the United States in 1983, serotype G9 human rotavirus strains are currently being detected globally. Serotype G9 strains have been isolated in various African countries, including South Africa, Botswana, Malawi, Kenya, Cameroon, Nigeria, Ghana, Ivory Coast, Guinea-Bissau and Libya (Cunliffe *et al*., 2001).

Generally the results obtained from the G-typing in this study were similar to those in other African countries (G1–G4). It was found that genotype G9 strains isolated in various African countries [16] were also detected in this study. Other developed and developing countries are also increasingly detecting the G9 serotype e.g. UK, USA and Australia (Cubbit *et al*., 2000; Griffin *et al*., 2000 and Kirkwood *et al*., 2002). This suggests a continued evolution of rotaviruses. Therefore vaccines under development should consider targeting G9 and include it as one of the common serotypes.

A study carried out in Egypt to identify enteropathogens for vaccine development, rotavirus was of principle concern (Wierzba *et al*., 2006**)**. Therefore, the HRV serotypes circulating in the Egyptian community should be screened. Although severity of disease may differ, rates of rotavirus illness among children in civilized and rural area are similar, indicating that clean water supplies and good hygiene have little effect on virus transmission, and further improvements in water or hygiene are unlikely to prevent the disease. The present study was an attempt to participate in the research efforts made in Egypt for the estimation of the frequency and type distribution of rotaviruses, using the molecular technique RT-PCR.

In conclusion, the findings of this study have confirmed the occurrence of group A human rotavirus in stool samples of children with gastroenteritis. We also confirmed some of the circulating VP7 genotypes in Egypt.

This study demonstrated that rotavirus G9 persists in Egypt but has not become a predominant strain and that G1, G3 and G4 genotypes are still circulating in the community of collected samples. Other uncommon strains may have been overlooked because of low prevalence, and lack of routine testing in the hospital settings.

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