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

Sarah Hashem[2], Sahar A. Shoman[1], Aly F. Mohamed[2]
[1]Department of Microbiology, Faculty of Science, Ain Shams University, Cairo, Egypt,
[2]Egyptian Organization for Biological Products and Vaccines (VACSERA Holding Company), Giza, Egypt

ABSTRACT

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 rotavirus types circulating in Egypt. Genotyping by reverse transcription-PCR (RT-PCR) has been widely used to analyse circulating strains. 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) from May 2009 to April 2010. 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 untypeable strains were detected and mixed infections between G1 and G4 in 1.9% (3/158) of the samples were detected. G8, G2 were not detected among collected samples. 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 aid in assessing the most suitable strain candidates used for vaccine production to protect against current circulating and uncommon strains, also for evaluation of cross immunogenicity among variable strains.
Keywords: *Rotavirus; Egyptian infants; Isolation; Virus culture; Genotype*

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 527,000 deaths annually in young children[(1)]**.** Approximately a quarter of these deaths occur in African children, yet the epidemiology of rotavirus infection and the characterization of rotavirus strains in Africa are poorly understood.

In sub-Saharan Africa, one out of every 30 children born will die from diarrhoea before the age of five. Rotavirus contributes 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 childrens' deaths[(2)].

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[(3)]. 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[(4)].

The VP6 inner capsid protein specifies group and subgroup epitopes, while the VP4 outer spike protein and the VP7 outer capsid protein, and carry epitopes specifying neutralizing antibody responses. VP7-specific genotypes are termed G types and VP4-specifc genotypes are termed P types. Among the 24 G types and 33 P types of group A rotaviruses classified so far[(5)], although genotypes G1, G2, G3 and G4 were thought to be of major epidemiological importance due to their worldwide distribution.

Epidemiological studies demonstrated that G1 to G4 types are responsible for most rotavirus infections among children worldwide[(6)]. Other studies have also shown the

increasing importance of the G9 rotaviruses[3]. The G12 rotavirus was first detected in 1990 in the Philippines[7] and has since been found in Asia, Europe, South America, and North America, suggesting that it is possibly emerging all over the world[8,9,10,11]. So far, no cases concerning the G12 rotavirus strain have been reported in northern Africa[12,13].

In a study carried out in Egypt to identify enteropathogens for vaccine development, rotavirus was one of the principle concerns[14]. Therefore, the HRV genotypes circulating in the Egyptian community should be screened. Although severity of disease may differ, rates of rotavirus illness among children in civilized and rural areas 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.

Various techniques have been developed to readily detect rotavirus in stool, 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[15]**.** Relatively simple to perform antigen-detection systems in the form of EIA and latex agglutination (LA) kits have been developed as alternative identification systems[16].

In this study, we have described the distribution and molecular characterization of G genotypes of rotaviruses circulating among infants and children aged <5 years old with diarrhea admitted in three hospitals of Egypt between May 2009 and June 2010.

MATERIALS AND METHODS

1. Study population and fecal specimens

The study methods were designed according to the WHO Protocol for rotavirus hospital-based surveillance[17]. Four hundred and fifty fecal samples were collected from 3 different governorates in Egypt. The majority of the stool samples were 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 and April 2010. They were collected from infants and children below 5 years old attending the out clinic suffering from diarrhea. Stool specimens were stored at -30ºC prior to analysis.

2. 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 both 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. The sensitivity, specificity, and accuracy of LA were calculated from the following equation of Altman and Bland[18].

$$\text{Sensitivity} = \frac{\text{Number of true positives}}{\text{Number of true positives} + \text{number of false negatives}}$$

$$\text{Specificity} = \frac{\text{Number of true negatives}}{\text{Number of true negatives} + \text{number of fapse positives}}$$

$$\text{Accuracy} = \frac{\text{Number of true positives} + \text{number of true negatives}}{\text{Number of true positives} + \text{false positives} + \text{false negatives} + \text{true negatives}}$$

3. Detection of infectious rotavirus by integrated cell culture RT-PCR (ICC-RT-PCR assay)

Twenty ELISA positive samples were selected for adaptation to grow in MA-104 cells. As 0.5 ml Trypsin-treated viral suspension was inoculated on confluent monolayers of MA104 cells and incubated for 90 min at 37°C during which rocking was done every 15 min; the inoculum was then removed without rinsing. Serum-free eagle's medium MEM, supplemented with 5 µg/ml of trypsin was used as the maintenance medium. After an incubation period of 3 to 5 days to permit virus replication, the supernatant was discarded and the viral RNA was extracted from the cell monolayer using Trizol. Extracted nucleic acid was suspended in 20 µl of RNase-free ddH2O and subjected to RT immediately or stored at −80°C.

4. Extraction of viral genome

Rotavirus dsRNA was extracted from 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 200 µl 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 40 µl of RNAse-free water and stored at -20 0C for use in the PCR reactions.

5. Reverse transcription

For reverse transcription (RT), 5 µl of extracted viral genome was added to 0.5 ml of low-bind microcentrifuge tubes containing 1 µl 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 10s to remove the condensation from the walls of the tubes. A reverse transcription mixture (7.5µl) containing 2µl of deoxynucleoside triphosphate mixture (containing 10 Mm each [dATP, dGTP, dCTP, and dTTP]; Fermentas),4 µl of 5x reaction buffer and 1.0 µl RevertAid M-MuLV-Reverse Transcriptase (200U) was then added to each denatured dsRNA sample tube (to give a final reaction volume of 20 µl). The samples were mixed by gentle flicking, centrifuged at 12,000 rpm for 5 s, and subjected to one cycle of reverse transcription (42°C, 60 min) and finally terminating the reaction by heating at 70ºC for 10 min.

6. 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 pool of specific primers that identify the following VP7 genotypes (Table 1): G1, aBT1; G2, aCT2; G3, aET3; G4, aDT4; G8, aAT8; G9, aFT9 and the common primer RVG9 as described previously[19]. PCR products were resolved by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized under UV transilluminator (LUYOR Corporation - CHINA).

Table 1: Oligonucleotide primers for PCR amplification

Primer	*Sequence*	*Position*
Beg9b	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28
End9b	GGTCACATCATACAATTCTAATCTAAG	1062-1036
RVG9	GGTCACATCATACAATTCT	1062-1044
aAT8	GTCACACCATTTGTAAATTCG	178-198
aBT1	CAAGTACTCAAATCAATGATGG	314-335
aCT2	CAATGATATTAACACATTTTCTGTG	411-435
aDT4	CGTTTCTGGTGAGGAGTTG	480-498
aET3	CGTTTGAAGAAGTTGCAACAG	689-709
aFT9	CTAGATGTAACTACAACTAC	757-776

7. Nucleotide sequencing

The G genotypes of group A rotaviruses were determined by direct sequencing of the first PCR products. The PCR products were purified using the PCR purification Kit (fermentas) and sequenced using the same forward (Beg9) primer using a Big Dye terminator cycle sequencing kit (Applied Biosystems, USA).

8. Phylogenetic analysis

Nucleotide sequences of both the genes were aligned in Clustal W and phylogenetic trees were generated using a Kimura two-parameter distance model and Neighbour-Joining (NJ) algorithm with a bootstrap test (**20**).

RESULTS

Identifications of rotaviruses in clinical and laboratory samples

Different methods were used for identification of rotaviruses from clinical

samples. Using a latex agglutination test, rotavirus was detected in 94 of 450 samples. Many samples gave non-interpretable results in which visible agglutination was observed in both control and test latex. This indicated non-specific reaction and unsuitability of the specimen for this test procedure. Specificity and sensitivity of a latex test was lower than in case of using ELISA.

The performance of the Rotavirus Latex test was evaluated for rotavirus detection in fecal samples of patients with acute gastroenteritis. This assay was compared with the enzyme immunoassay (EIA) ROTASCREEN kit. Four hundred and fifty fecal specimens were analyzed. Ninety four 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, 39/150 (26%) were positive by EIA (**Table 2**). 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 2: Comparison of Latex Agglutination with Enzyme Immunoassay for Detection of Rotavirus in Fecal Specimens.

No. of cases	Techniques				
	Latex assay			ELISA	
	Positive	Negative	indeterminate	Positive	Negative
	94	206	150	158	292
Total	450			450	
Percentage %	20.8	45.7	33.3	35.1	64.889

Detection of rotavirus by integrated cell culture RT-PCR (ICC-RT-PCR assay)

Cytopathogenic effect was detected after 5 passages following a 5-day-incubation period. Viral RNA was extracted from cell monolayers and then genotyping of rotavirus was performed. The specific rotavirus genes could be detected post incubation for 5 days infected with relatively low levels 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 months age group (**Fig. 1)**. The distribution of gender in collected samples was 58% males and 42% females and 57.5 % of the children with rotavirus infection were males. 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.

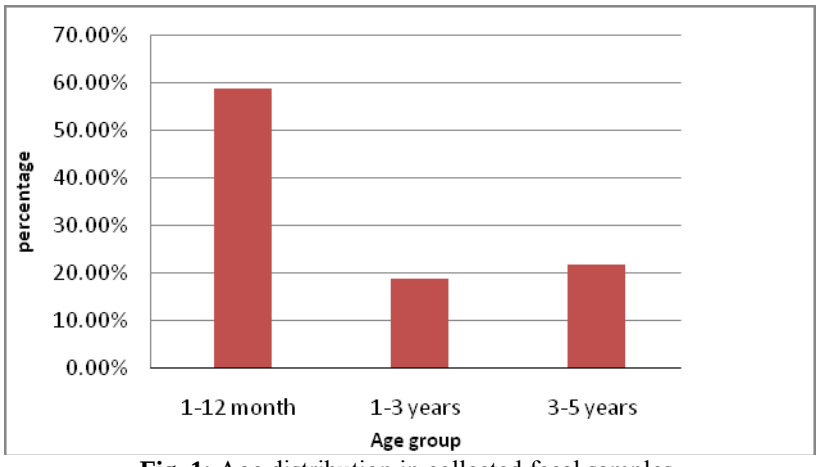

Fig. 1: Age distribution in collected fecal samples.

Seasonal pattern of rotavirus infection.

Samples were collected from May, 2009 to April, 2010 (**Fig. 2A**). The occurrence of rotavirus infection varied according to seasonal temperature. Rotavirus infection was detected continuously from May to April (**Fig. 2B**). Rotavirus was detected most frequently from October to February. The presence of rotavirus remained low from 1.3% to 4.5% in May–September, 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.

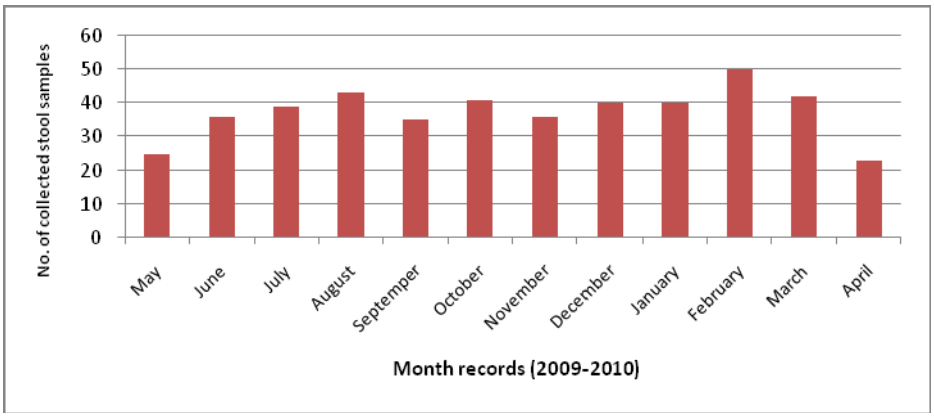

Fig. 2A: Number of stool samples monthly collected from infants and children with acute gastroenteritis during May 2009 to April 2010.

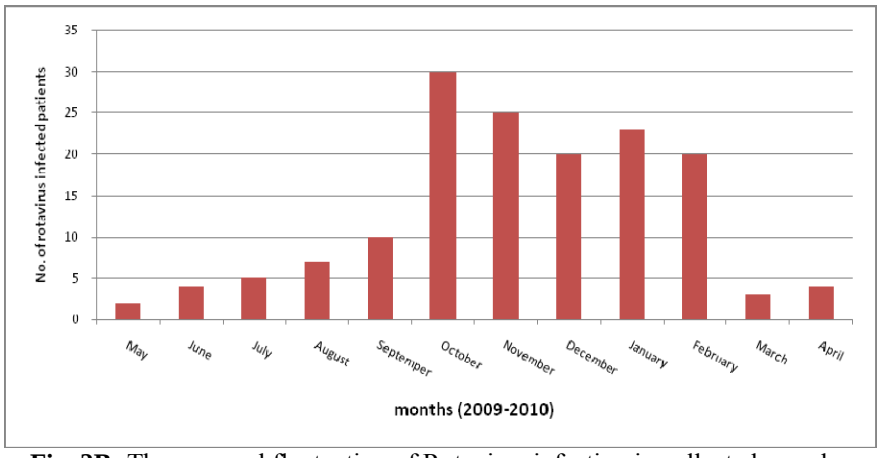

Fig. 2B: The seasonal fluctuation of Rotavirus infection in collected samples

Distribution of G genotypes

Genotype of isolated viruses was determined using RT-PCR (**Fig. 3a & 3b**). 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 3**.

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.

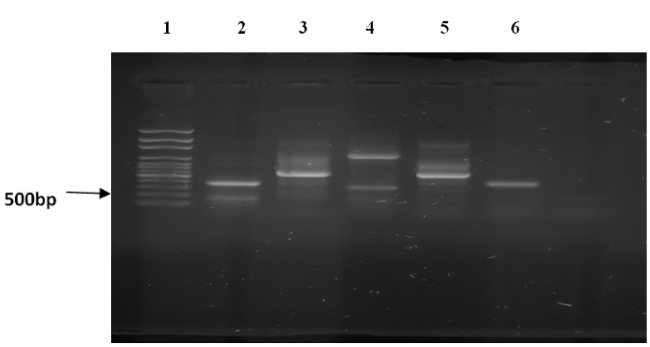

Fig. 3a: Agarose gel electrophoresis showing amplicon sizes for the different G-types, Lane 1 (100bp molecular weight marker), Lane 2 and 6 (G3), Lane 3 (G4), lane 4 (G9), lane 5 (G1+G4 mixed infection,) the arrow shows the 500-bp mark.

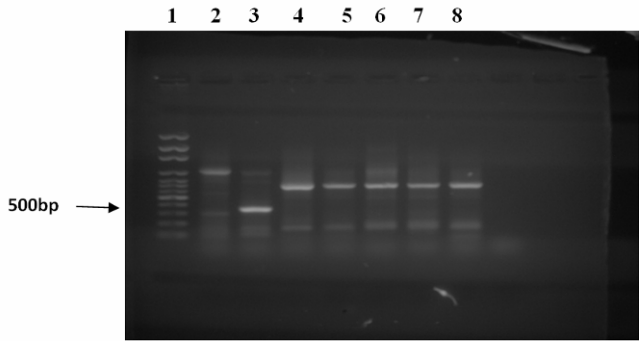

Fig. 3b: Agarose gel electrophoresis showing amplicon sizes for the different G-types, Lane 1 (100bp molecular weight marker), Lane 2 (G9), Lane 3 (G3), Lane 4,5,6,7 and 8 (G1), the arrow shows the 500-bp

Table 3: 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	G1+G4	Total
Cairo	34	10	7	4	2	57
Sharkia Governorate	27	16	8	3	1	55
Fayoum Governorate	22	13	6	4	1	46
Total	83	35	23	13	4	158

Nucleotide sequencing and phylogenetic analysis

Nucleotide sequence of G1 strains showed 90–94% identity with reference strain Wa. However, their identities were higher (99.2–100%) compared to the Egypt-7 and Egypt-8 strains. The Egyptian G1 strains also exhibited 97% identity to G1 strain isolated in Israel (Israel-56). The isolated G3 and G4 sequences showed 95-97% and 97–98% nucleotide identity with reference strains YO and Hochi from Japan, respectively.

Egyptian G9 strain exhibited the least homology (86%) to Indian strain 116E and only 78.7% identity with prototype G9 strain WI61.

DISCUSSION

Rotavirus is one of the most common causes of non-bacterial gastroenteritis, not only in the developing countries but also in developed countries like Japan, Korea and Germany[21, 22, 6].

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[23,14].

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 positive reactions. However, samples producing weakly positive or indeterminate reactions should be retested in a more specific and sensitive assay, such as EIA.

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. We identified most of the globally common rotavirus types (G1, G3, G4, and G9) in our study. G1 was identified with a very high prevalence (55%) and followed by G3 (22.2%), G9 (14.5%), and G4 (8.2%). These results are consistent with previous findings on rotavirus prevalence in Ireland 2001-2004 which indicate the emergence of G3- and G9-serotypes as epidemiologically important rotavirus strains, and the low prevalence of the previously common G2 and G9 strains[24, 25].

In a study conducted by Halloran et al., G8 strains were detected as mixed infection with G2 strains.G8 strains were first detected in Egypt from the stool of children participating in a 3 year study of rotavirus epidemiology[26]. In contrast to this study, G2 and G8 could not be detected.

Our results were similar to findings of an active surveillance studies conducted in Egypt's hospitals over the past decade. A one year study on the bacterial and viral etiology of patients with acute gastroenteritis in Cairo revealed that rotavirus was responsible for 57.4% of diarrheal illnesses in which G1 and G2 were the most predominant rotavirus strains and unusual G12 strain was first detected[27]. **Shukry et al (28**), **Radwan et al.**[29] and **El-Mougi et al.**[30], detected rotavirus antigen in 33%, 35.6% and 40%, f stool samples obtained from children with acute diarrhea respectively, using ELISA. 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[31]. Our results showed remarkable agreement with the results of other investigators in Venezuela, Spain and Dhaka[32,33,34] 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 age for infection. 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[35].

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[22]. Three samples with a G1/G4 mixed infection are suggestive of an on-going natural reassortment between genotypes in the community.

During the past decade, the numbers of countries that have reported the detection of rotavirus G9 strains have increased dramatically. Apparently G9 rotaviruses are expanding on a global scale; currently, the G9 genotype is considered to be the fifth most common type worldwide. Genotype G9 strains have been isolated in various African countries, including South Africa, Botswana, Malawi, Kenya, Cameroon, Nigeria, Ghana, Ivory Coast, Guinea-Bissau and Libya[36].

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[37] were also detected in this study. Both developed and developing countries are also increasingly detecting the G9 genotype e.g. UK, USA, Australia and Bangladesh[38,39,40,41]. This suggests a continued evolution of rotaviruses. Therefore vaccines under development should consider targeting G9 and include it as one of the common genotypes.

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 present study confirms the current burden of rotavirus gastroenteritis in younger children, especially small infants, and highlights the diversity of rotavirus strains circulating 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. Continuous prospective monitoring of circulating strains of rotavirus is desirable to detect any changes in their distribution promptly and to assess the effectiveness of active immunization program.

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