Detection of group A Rota virus and characterization of G type among Egyptian children with diarrhea

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Group A rotaviruses are the most important cause of acute diarrhea in children throughout the world. It is the cause of more than 450,000 deaths per year. There are few data available about rotavirus type circulating in Egypt. Serotyping by ELISA with anti-VP7 serotype-specific monoclonal antibodies and genotyping by reverse transcription-PCR (RT-PCR) have been widely used for typing. The materials of this study comprised 100 stool specimens collected from children less than 5 years old suffering from acute diarrhea, attending Alexandria University Children's Hospital at El-Shatby during the period from January to December 2006. These specimens were subjected to RT-PCR. Rotavirus was detected by RT PCR in 33 (33%) of patients. In the present study, a total of 30 (90.9%) of the 33 rotavirus positive samples were found to be G typeable and the remaining 3 (9.1%) were untypable. Out of the 33 rotavirus positive cases, G4 was the main genotype detected being responsible for 12 cases (36.4%) of rotavirus infections. G1 was the second most common cause and was responsible for 9 cases (27.3%) of the infections. Our study identified G9 in 6 (18.1%) of the positive cases. Mixed G types reflecting dual infections G1+G9 were detected in 3 (3%) of the samples. G2, G3, and G8 were not detected among our cases. These results underline the importance of continued detailed epidemiological and virological studies to identify rotavirus serotypes responsible for severe diarrhea, including characterization of less common and or unusual strains. Knowledge of rotavirus prevalence and strains circulating in the Egyptian community will add in assessing the suitability of candidate vaccines, in order to protect against all currently circulating rotavirus strains.

INTRODUCTION

Group A rotaviruses are the most important cause of acute diarrhea in children throughout the world. It is the cause of more than 450,000 deaths per year (1). In Egypt, during the past decade, numerous studies evaluating diarrheal diseases among children living in the Nile River Delta, northern Egypt revealed that rotavirus is the most commonly identified cause of diarrhea among children seeking medical care for severe illness (2,3,4,5). Development of vaccines against rotavirus infection is considered a high priority in developing countries (6). However, the vaccine efficacy is being challenged by the extensive diversity of the rotaviruses (7-9).

The genome of rotaviruses consists of 11 segments of double stranded RNA (10,11). The rotaviruses are distinguished by the layered proteins forming the capsid, which can be used to differentiate and classify strains, and which are important to the antigenic response. Based on the antigenic properties of the VP6 protein in the inner layer of the capsid, the rotavirus genus is divided into different serogroups (labeled A through to E with two possible additional species F and G) (8,9). The clinically significant rotaviruses belong to group A (12). Further classification of rotaviruses is based on the genetic characteristics or seroreactivity of the VP4 and VP7 viral proteins in the outer layer of the capsid. Each of these proteins induces neutralizing antibodies independently (13). So far, 23 P genotypes and 15 G genotypes (7) have been identified. Ten of the G genotypes and 11 of the P genotypes have been detected in humans (14), but most rotavirus-induced diarrhea in children is caused by a small number of the G genotypes, primarily G1, G2, G3, G4, and the newly emerging strain G9 (15,16,17). However, dominant strains vary between and within geographic regions. Since the rotavirus serotypes (genotypes) circulating in a given region have a direct influence on the predicted efficiency of a potential vaccine for the region, an examination of the G and P type distribution is necessary (18,19).
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 demonstrate the need to incorporate a wider variety of reagents and detection methods in an attempt to identify the full variety of strains in circulation (3,6). Serotyping by ELISA with anti-VP7 serotype-specific monoclonal antibodies and genotyping by reverse transcription-PCR (RT-PCR) have been widely used for typing (20,21,22).

**MATERIAL AND METHODS**

The materials of this study comprised 100 stool specimens collected from children less than 5 years old suffering from acute diarrhea, attending Alexandria University Children's Hospital at El-Shatby during the period from January to December 2006. This study was approved by "Alexandria University Ethical Committee", and an informed consent was obtained from all parents.

All patients were subjected to thorough history taking and clinical examination at the time of specimen collection. An interview questionnaire was designed to obtain data regarding the age, sex, residence, duration of diarrhea and frequency of motions per day, the presence or absence of fever, vomiting and flu –like symptoms, the stool consistency, and the breast feeding.

**Sample collection.** The stool specimens were obtained in a sterile container. About 30 mg of each stool sample were added to 175 ul lysis buffer containing RNase inhibitors supplied in the RNA extraction kit (Promega). The samples were transported the same day to the laboratory as they were stored at - 70°C.

**RNA extraction:** RNA was extracted from stool by the guanidinium isothiocyanate (GTC) and spin column extraction method using SV total RNA isolation system (Promega, Madison US), according to the manufacturer's instruction.

**RT-PCR** In the present study, we used the PCR assay and primers described by Gouvea et al (23) for the detection and typing of group A rotavirus. The primers specific for gene segment 9, (8, or 7), which encode for the VP7 glycoprotein, are used for the detection of rotavirus. [Beg 9 – End 9] and primers for G typing of rotavirus are displayed in figure 1, and table 1.

**Reverse transcription.** Reverse transcription was performed using Omniscript Reverse Transcriptase kit (Qiagen). The complementary DNA copies (cDNA) from both rotavirus RNA strands were synthesized in 20 ul solutions containing; 2 ul of 10 X enzyme buffer, 0.5 mM of each deoxynucleotide triphosphate, 5 picomoles of each End 9 and Beg 9 primers, 10 ul of the RNA samples and 4 IU of omnireverse transcriptase enzyme. Before the addition of the reverse transcriptase enzyme, the ds RNA of rotaviruses were first denatured by heating the mixture first at 97°C for 5 minutes in the thermocycler (Techne). Then the tubes were transferred quickly to an ice-bath to prevent reannealing of the ds RNA, and the enzyme was added. The tubes were then transferred back to the thermocycler for incubation at 37°C for one hour followed by 94°C for 5 minutes.

**PCR amplification.** Initially, 1,062 bp (full length) gene segment 9, encoding the VP7 glycoprotein in human group A rotavirus, was amplified using primers Beg 9 in the forward direction and primer End 9 in the reverse direction (figure 2). Amplification was performed in a final volume of 50 µl of PCR mixture containing 1µM Of each primer, 10 mM of each deoxynucleotide triphosphate (dATP, dGTP, d TTP, dCTP), 10 mM Tris HCL, 50 mM KCL, 0.1% Triton x-100, 1.5 mM MgCl2, 2.5 units of Taq DNA polymerase (Qiagen), and 10 µl of cDNA. The amplification reaction was applied as follow: denaturation at 94 °C for 1 minute, annealing at 42 °C for 2 minutes and extension at 72 °C for 1 minute (for 25 cycles), followed by a final 7 minutes extension at 72 °C.

**PCR typing.** PCR typing was performed from dsDNA that was obtained from the first amplification of the entire gene 9. In this case, 3ul of the dsDNA product served as the template for this second typing amplification.
The same reaction buffer containing all six serotype specific primers [a AT8, a BT1, a CT2, a DT4, a ET3, a FT9] and RVG9 primer was used but with reduced concentrations of the primer mix 200 nM each primer. The same PCR program was used with only 15 cycles followed by a final extension at 72 °C for seven minutes. The PCR products were resolved by 2% agarose gel electrophoresis and were visualized after ethidium bromide (0.5 ug / ml) staining, using an UV transilluminator and photographed by Polaroid camera.

RESULTS

The present study was carried out in the period from January to December 2006, on 100 stool specimens obtained from children less than 5 years old, who attended the Alexandria University Hospital at el Shatby, and were diagnosed clinically as acute gastroenteritis. The mean age distribution of the patients was 9 ± 7.53 months, ranging from 1 - 35 months. Fifty six percent of the patients were males and 44% were females.

Rotavirus detection by PCR

Rotavirus was detected by RT PCR in 33 (33%) of cases. As shown in figure 2, primers Beg9 and End9 amplified gene segment 9 of rotavirus yielding a PCR product of expected size (1,062 bp)

Rotavirus G-type distribution

In the present study, a total of 30 (90.9%) of the 33 Egyptian rotavirus positive samples were found to be G typed and the remaining 3 (9.1%) were untypable. As shown in table II, out of the 33 rotavirus positive cases, G4 was the main genotype detected being responsible for 12 cases (36.4%) of rotavirus infections. G1 was the second most common cause and was responsible for 9 cases (27.3%) of the infections. Our study identified G9 in 6 (18.1%) of the positive cases. Mixed G types reflecting dual infections G1+G9 were detected in 3 (3%) of the samples. G2, G3, and G8 were not detected among our cases. As shown in figure 2, the six sets of primers used for typing yielded bands of distinct lengths; 749, 583 and 306 bp corresponding to serotype G1, G4 and G9 respectively.

By RT PCR assay rotavirus was detected in children ranging in age from 1 – 23 months, with the mean age value 9 ± 5.12 months. There was no statistical significant association between sex and rotavirus infection, and no statistically significant association with residence (P = 0.322). In the present study, 27 (81.1%) of the rotavirus positive cases did not receive any breast milk during there life, while only 6 (18.2%) were breast fed, which was found to be statistically significant, P= 0.000. As shown in table III, although rotavirus was detected in every season all over the 12 months of the year, a strong significant association between the seasonal trend and the incidence rate of rotavirus infection (P = 0.000) was found as shown in table III. The highest ratio of rotavirus infections was represented in winter (60%), followed by autumn where 50% of the diarrheal cases were due to rotavirus infection. Rotavirus infections in summer represented 33%, and the lowest incidence of rotavirus diarrhea was in spring (10.9%).

Table I: Primers for detection and typing of Rotavirus.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Position</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beg 9b</td>
<td>GGTTTAAAAAGAGAGGAATCGTTTCTGG</td>
<td>1-28</td>
<td></td>
</tr>
<tr>
<td>End 9b</td>
<td>GGTCACATCATACTACTATCTAATCTAAG</td>
<td>1062-1036</td>
<td></td>
</tr>
<tr>
<td>RVG9</td>
<td>GGTCACATCATACTACTATCTAATCTAAG</td>
<td>1062_1044</td>
<td></td>
</tr>
<tr>
<td>aAT8</td>
<td>GTTACACATTGTAAATTCG</td>
<td>178_198</td>
<td>A</td>
</tr>
<tr>
<td>aBT1</td>
<td>CAAGTACTTTCAATGATG</td>
<td>314_335</td>
<td>B</td>
</tr>
<tr>
<td>aCT2</td>
<td>CAAAGGATGTTAAACCAATTTCTGTG</td>
<td>411_435</td>
<td>C</td>
</tr>
<tr>
<td>aDT4</td>
<td>CGTTTCTGGTGAGGTGG</td>
<td>480_498</td>
<td>D</td>
</tr>
<tr>
<td>aET3</td>
<td>CTGTTGAGAGAGATTGCAACAG</td>
<td>689_709</td>
<td>E</td>
</tr>
<tr>
<td>aFT9</td>
<td>CAGGACATGTAACACACACTAC</td>
<td>757_776</td>
<td>F</td>
</tr>
</tbody>
</table>
Fig. 1. Rotavirus gene 9 (or gene 8) which encode the VP7 glycoprotein. Shown are locations of the variable regions and PCR primers and expected length of the amplified segment (23).

Table II: Distribution of Rotavirus genotypes within the Rotavirus positive samples.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Positive cases</th>
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<tbody>
<tr>
<td></td>
<td>No.</td>
</tr>
<tr>
<td>G4</td>
<td>12</td>
</tr>
<tr>
<td>G1</td>
<td>9</td>
</tr>
<tr>
<td>G9</td>
<td>6</td>
</tr>
<tr>
<td>Mixed</td>
<td>3</td>
</tr>
<tr>
<td>Untypable</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
</tr>
</tbody>
</table>

Table III: Relation between season and Rotavirus infection.

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Positive cases</td>
<td>18   60</td>
<td>5   10.9</td>
<td>4   33.3</td>
<td>6   50</td>
<td>33</td>
</tr>
<tr>
<td>Negative cases</td>
<td>12   40</td>
<td>41  89.1</td>
<td>8   66.7</td>
<td>6   50</td>
<td>67</td>
</tr>
<tr>
<td>Total</td>
<td>30   46</td>
<td>46  100</td>
<td>12  100</td>
<td>12  100</td>
<td>100</td>
</tr>
</tbody>
</table>

P  0.001
DISCUSSION

Diarrheal disease remains one of the most important public health challenges worldwide, mainly in developing countries where mortality is still high. Rotaviruses are the leading cause of gastroenteritis in young children. They are responsible for a so-called “universal infection” in young children, as virtually every child will be infected by the age of 5 years (24). Therefore, the huge burden of rotavirus diarrhea has made rotavirus a prime target for global estimation of the prevalence and type distribution of the virus strains all over the world (7,24,25).

In a study done in Egypt by Wierzba et al in 2006 to identify enteropathogens for vaccine development, rotavirus was of principle concern, followed by ETEC, Shigella, and Campylobacter (5). Therefore, the HRV serotypes circulating in the Egyptian community should be determined. 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 the present study, Rotaviruses were identified by RT-PCR assays in 33 (33%) of the 100 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. Our
results were similar to findings from 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 (29). Shukry et al (1989) (30), Radwan et al (1997)(6) and El-Mougi et al (1998)(31), detected the antigen of rotavirus in 33%, 35.6% and 40%, of stool samples obtained from children with acute diarrhea respectively, using the ELISA technique. 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(32). Our results show remarkable agreement with the results of other investigators in Venezuela, Spain and Dhaka (33,34,35). However, in other settings (36,37), rotavirus was detected with a higher percentage rates than found in our study which confirm the huge disease burden over the world, and the variability of its prevalence from a region to another.

As mention before, Rotavirus diarrhea is widely distributed allover the world. The disease usually presents with similar epidemiological and clinical pattern within developed and developing countries. The age of the children with rotavirus diarrhea detected in this study ranged between one and twenty three months. The age group that experienced the highest incidence of rotavirus diarrhea ranged from six to twelve months, with the median age 9 months. These findings were in agreement with the previous studies done in Egypt (3, 7, 38). In addition, other investigators in different countries (39, 40) recorded that the highest rate of rotavirus isolation was found among children in their first year of life. These findings confirm the role of the immune system in prevention of the disease, which helps decline of rotavirus diarrhea with age.

In the present study, rotavirus presented with a marked seasonal peak during the cold months of the year (December – February), similar to those observed in England (41), Finland (42), Spain (34) and Tunisia (39). Our results were similar to a previous cohort study conducted in Bilbeis (Egypt), in which the rate of rotavirus isolation was predominant in the colder months (November – April). However, the seasonal peak of rotavirus infection in Egypt tends to shift over consecutive years (43).

Rotavirus strain surveillance has proven to continue as a world wide effort, in order to determine the prevalent strains in each country and to apply a rotavirus vaccination program that provide a serotype specific immunity to every child. Previous epidemiological studies conducted in many countries from different continents such as Tunisia (36), Nigeria (44) and Spain (34) reported that G1 rotaviruses predominated as a cause of severe rotavirus diarrhea, with G2, G3, and G4 strains responsible for the majority of the residual diarrhea. However, the results presented here reveal predominance of G4 (36.4%) as the most common cause of diarrhea followed by G1 (27.3%) genotype.

Reports of the distribution and epidemiology of human rotavirus G types in Egypt are limited in number. In a study done by Radwan et al (7), enzyme immunoassay (EIA) has been used to detect, for the first time in Egypt, the relative frequency and temporal distribution of HRV G serotypes 1 to 4 among the community of neonates and infants with and without acute diarrhea. Serotypes G1 and G4 predominated in all age groups. Mixed (G1 plus G4) and nontypeable specimens represented 16.1 and 38.7% of the total number serotyped, respectively. Recently, a study in Cairo, Egypt was done on raw sewage samples taken from three sewage treatment plants based on G typing. The predominated type for rotavirus identified in that study was found to be G1 (69.9%), followed by G3 (13.0%), G4 (8.7%), and G9 (8.7%). The results presented in this study revealed the occurrence of G9 strains in clinical specimens in Egypt for the first time, although this does not necessarily imply emergence of new strains, since this serotype was not investigated in previous studies (45). The absence of G2 and G3 genotypes in our study might reflect the absence of these strains in Egypt as have been published by
Villena et al 2003 (45), where no G2 rotavirus genotypes were detected within the sewage samples. A more reasonable explanation is the low level circulation of such strains in our community, which has been reported by Radwan et al 1997 (7), where G2 was represented with 3.2% and G3 6.5% of rotavirus positive samples. These findings confirm the great diversity within rotavirus strains circulating in humans which make the prevalence of rotavirus genotypes varies according to location and time.

In the present study, rotavirus genotype G9 was responsible for 18.1% of the rotavirus infections. This percentage confirm the need for continued surveillance to identify the persistence of G9 strain in our community, as the emergence of such pathogen necessitates the urgent consideration of G9 moiety in rotavirus vaccines considered for use in Egypt. Since the introduction of RT–PCR for rotavirus genotyping, many epidemiological surveillance studies have been conducted, and new data has been collected to understand this complex epidemiology(3,37,44). However, there is considerable number of strains all over the world that remain untypable by PCR using the current primers. In the present study, 9.1% of the rotavirus positive samples could not be G genotyped. However, the percentage of the untypable rotavirus stains detected in sewage in Cairo (30%) was higher than that found in the current study. In addition, 38.7% of rotavirus positive specimens collected from infants in Cairo by Radwan et al (6), were untypable. In other settings all over the world, the proportion of rotavirus strains that remain untypable exists with different ratios (37,46,47). It was suggested that failure of the currently used RT-PCR assay to characterize all rotavirus genotypes may be due to either failure of the laboratory to use the full range of primers required to detect uncommon strains, or the strains are novel enough that they must be sequenced so that new and special primers can be applied (47).

In recent years, it was reported that accumulation of point mutation in VP7 genes was the main cause of failure of rotavirus G typing. It was suggested that the use of modified or degenerated primers or changing the primer binding site allow strains that were previously untypable to be completely typed through avoiding the mismatch between the primers used and VP7 genes (48-49). Moreover, following new typing methodologies like microarray procedures could be successful in characterization of all the existing rotavirus strains (50).

Finally, our results underline the importance of continued detailed epidemiological and virological studies to identify rotavirus serotypes responsible for severe gastroenteritis, including characterization of less common and or unusual strains. Knowledge of rotavirus prevalence and strains circulating in our community will help in assessing the suitability of candidate vaccines, in order to protect against all currently circulating rotavirus strains.

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الكشف عن فيروس الروتا المجموعه الأولي وتصنيف نوع في حالات الأطفال المصريين المصابين بالاسهال

يعد فيروس روتا من أهم المسببات للنزلات المعدية لأطفالنا على مستوى العالم. ويعتبر أطباء التعليم، ضد هذا فيروس كأطراف الفعالية للسيطرة عليه والحد من انتشاره الواسع. لهذا السبب، كان المسح الشامل لأنواع فيروس روتا من أهم الجهود المبذولة عالمياً لتحديث مدى إمكانية تطبيق النظام الخاص بالتعليم ضد هذا الفيروس في كل بلد، حيث أن هذا أخطأ بمدى الطفل بالمناخ الخاصة لكل نوع من أنواع الفيروس على حدود. وقد أجري هذا البحث على 100 من الأطفال للفيروس ببالأسهال الحاد، والمترددين على مستشفى الشاطبي الجامعي، والأطفال بзванة الإسكندرية خلال مدة قدرها 12 شهرًا اعتبارًا من يناير إلى ديسمبر 2007. حيث تم فحص عينات البراز المأخوذة من هؤلاء الأطفال عن طريق التفاعل البوليمرزى المتسلسل باستخدام إنزيم الإنتساخ الإنكاسكي للكشف عن الحمض النووي الخاص فيروس روتا. وقد وجد أن 33% من العينات كانت إيجابية لهذا الفيروس. وقد تم الكشف عن الجين الخاص بإظهار بروتين VP7 لتحديد أنواع فيروس روتا ضمن العينات الإيجابية، وذلك عن طريق استخدام التفاعل البوليمرزى المتسلسل. ووجد أن G4 هو النوع السائد في الفيروس ضمن هذا البحث حيث يمثل نسبة تصل إلى G3 36.4%، يليه G1 الذي تصل نسبته إلى 27.3%، ولم يتم الكشف عن أي من G2 أو G8. وقد تم الكشف عن G9 بنسبة 18.1%، وهو من أنواع فيروس روتا المنتشرة حديثًا على مستوى العالم. هذا بالإضافية إلى توصيف 9.1% من العينات الإيجابية بالإصابة المختلطة والتي تشمل نوعين من فيروس روتا هما G1+G9 معاً. أما النسبة المتبقية من العينات الإيجابية وهي 1.9% فلم يمكن تحديد نوع الفيروس بها. وقد وجد في هذا البحث أن أكثر الإصابات بفيروس روتا كانت في الشهر الباردة من العام (ديسمبر إلى فبراير)، وأن معظم الأطفال المصابين بلغ عمرهم من 6 إلى 12 شهراً. بالإضافة إلى ذلك، فقد وجد أن حالات الإسهال الناجمة عن الإصابة بفيروس روتا مصطلحة بالقى والارتفاع درجة الحرارة والبراز السائل. هذه النتائج توضح أهمية استمرارية الدراسة المستفيضة من الناحية العلاجية والفيروسولوجية للتعرف على الأنواع البروتينية لفيروس روتا.