

Burden and Typing of Rotavirus Group A in Children with Acute Gastroenteritis in Shiraz, Southern Iran

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Abstract

Background: Human Rotavirus is a significant cause of severe gastroenteritis in infants and young children worldwide. In recent years, Rotavirus genotyping by RT-PCR has provided valuable information about the diversity of Rotaviruses circulating worldwide. The purpose of the present study is to monitor the prevalence of the different G types of Rotaviruses circulating in Shiraz, Southern Iran and detect any uncommon or novel types.

Methods: During the period from December 2007 to November 2008, a total of 138 stool samples were collected from children less than 5 years old who were hospitalized for acute gastroenteritis. Rotavirus-associated diarrhea was investigated in fecal specimens with enzyme immunoassays (EIA). Rotavirus-positive specimens were typed by the Nested RT-PCR and by using different types of specific primers.

Results: Out of the 138 collected samples, 34.78% (48 cases) tested positive for Rotavirus. The frequency of G1, G2 and G4 types was 6.25%, 2.08% and 27.08%, respectively. Mixed and non-typeable infections were detected in 33.34% and 31.25% of hospitalized children with acute diarrhea, respectively. This is the first time mixed Rotavirus infections with G1/G3 have been reported in Iran.

Conclusion: The high frequency of Rotavirus detection indicates the severity and the burden of Rotavirus disease may be able to reduce through the implementation of an effective vaccine and continual surveillance for the detection of Rotavirus genotypes circulating in other regions of Iran. Regarding to the noticeable frequency of non-typeable and mixed infections, it is suggested to use the other specific primers and further studies to detection of other novel and unusual types.

Keywords: Human Rotavirus; Diarrhea; Hospitalized children; G types

Introduction

Human *Rotavirus* is the leading cause of severe gastroenteritis in infants and young

children worldwide. An estimated 527,000 children less than five years of age die from *Rotavirus* gastroenteritis each year, with >85% of all *Rotavirus*-related deaths occurring in low-income countries of Asia and sub-Saharan Africa.¹ Recent studies have shown that *Rotavirus* diarrhea causes 39% of childhood diarrhea hospitalizations and 5% of all deaths around the world in children less than 5 years

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Received: 14 Aug 2011 Accepted: 20 Feb 2012

old.² Nearly every child will infect with *Rotavirus* disease by age 5 years, one in five will require a clinic, one in 50 will be hospitalized, and one in 205 will die from this disease.³ The clinical symptoms of *Rotavirus* infection ranges from mild, watery diarrhea of limited duration to acute gastroenteritis diarrhea with fever, vomiting, abdominal pain and dehydration in infants and young children.⁴ Although all children are infected at least once within the first 5 years of life, those between the ages of 6 and 24 months are at greatest risk for developing severe diarrhea from a *Rotavirus* infection.^{5,6} *Rotavirus* is a 70 nm icosahedral virus that belong to the family *Reoviridae*. This virus has significant antigenic specificities including serogroup and genotype, and all *Rotaviruses* are classified accordingly. They are divided into seven serogroups (A to G) on the basis the antigenic properties of the VP6 protein that makes up the middle layer of the triple layered virion.⁷ It is well documented that group A *Rotaviruses* are the most significant causes of acute gastroenteritis in children worldwide.² Within each serogroup, distinct genotypes exist. In group A rotavirus, genotype is specified by two viral structural proteins, VP4 and VP7 that found in the outer capsid.⁷ Genotypes determined by VP7 and VP4 proteins are termed G (for glycoprotein) and P (for protease sensitive) genotypes, respectively. These two proteins are vital factors for vaccine design, because of eliciting the production of neutralizing antibodies to the virus.⁸

Epidemiological studies in different regions of the world have indicated that in temperate climates, *Rotavirus* infection occurs predominantly during the cooler months. But seasonal patterns in tropical climates have

shown rates of *Rotavirus* diarrhea throughout the year with seasonal trends that are less pronounced.⁹ Studies conducted in the different countries around the world have demonstrated that group A *Rotavirus* infection is responsible for 13-45% of all cases of diarrhea in children <5 years old.¹⁰⁻¹⁴ In Iran, very little data on illness caused by *Rotavirus* has been published. These studies indicate that 27-46% of acute diarrhea in children under the age of 5 years is due to *Rotavirus* infection, but epidemiological data are still incomplete.¹⁵⁻¹⁸

Studies of the genotyping in different regions of the world have indicated that G1-G4 and G9 types are the most common G types detected in children with *Rotavirus* gastroenteritis.^{13, 19, 20} But in recent years, other rare or uncommon *Rotavirus* G types, such as G5, G8, G10, G11 and G12, have been reported in many countries.^{6, 14, 21-24} In other diarrheal diseases, improvement of hygiene and sanitation may reduce incidences, but these measures are unlikely to be sufficient for *Rotavirus* control. Regarding to the high burden of *Rotavirus* infection, an effective *Rotavirus* vaccine program will reduce the morbidity associated with severe *Rotavirus* diarrhea. Before that program can be implemented, information is needed on the current burden of *Rotavirus* disease and the distribution and frequency of *Rotavirus* strains circulating in different regions of the country. The objectives of this study were to describe epidemiology of *Rotavirus* disease and determine the G types of *Rotavirus* circulating in children aged <5 years old with acute gastroenteritis in Shiraz, Iran.

Materials and Methods

Specimen collection

In this study a verbal consent was taken from either parent of the enrolled child prior to the interview and collection of stool samples. From December 2007 to November 2008, a total of 138 stool specimens were collected during the course of treatment from children under 5 years of age who were hospitalized with acute gastroenteritis in Shahid Dastgheib and Nemazee hospitals in Shiraz, Southern Iran. All the fecal specimens were transported to the infectious disease unit laboratory and stored at -70°C until the time of assay. All samples underwent only one cycle of thawing and freezing prior to characterization. A standard structured questionnaire was used to obtain the information regarding the age, sex, duration of hospital stay, severity of clinical symptoms and type of feeding (as breast/bottle feeding) for each case.

Rotavirus detection

All samples were screened for group A *Rotavirus* by enzyme immunoassay (EIA) (Rotavirus Ag ELISA, DRG, Germany), according to the manufacturer's instructions.

Viral RNA extraction

Genomic RNA was extracted with a commercially available mixture of phenol and guanidine thiocyanate (RNX-Plus kit,

CinnaGen, Tehran, Iran). Isolation of whole RNA was performed according to the manufacture's protocol. Briefly, 500 µl RNX-Plus solution was mixed with a 20% stool suspension in phosphate buffer saline (PBS) at a pH of 7.2 at a volume ratio of 3 to 1. After complete dissociation of nucleoprotein complexes, chloroform was added to the mixture followed by vigorous shaking and centrifugation at 12,000 g for 15 min at 4°C. The upper aqueous phase was transferred to a fresh tube and the RNA precipitated by mixing it with isopropanol. The supernatant was removed and the RNA pellet was washed once with 75% ethanol. The RNA pellet was then briefly air dried and dissolved in diethyl pyrocarbonate (DEPC) treated water.

Reverse transcription-polymerase chain reaction

Briefly, 5 µl of dsRNA was added to a mix of DMSO, 5X RT buffer, dNTPs, primers Beg9, End9,²⁵ and DW, denatured at 97°C for 5 min. Then RT enzyme and RNase inhibitor were added to make a final volume of 20 µl. The RT-PCR reaction was carried out for 60 min at 42°C to produce the complementary (cDNA) used for PCR amplification *Rotavirus*.

Nested multiplex PCR for G genotyping

The G-typing was performed according to *Rotavirus* detection and typing protocol provided by WHO.²⁵ Briefly, the first round VP7 consensus PCR was carried out with 10 µl of cDNA in 40 µl of the VP7 reagent

mixtures. The cycling parameters used were: 30 cycles at 94°C for 1 min, 42°C for 2 min, 72°C for 2 min, and a final extension at 72°C for 5 min. The second round VP7 multiplex PCR was carried out with 5 µl of first round VP7 amplicons in 40 µl of the second round VP7 reagent mixtures. Cycling was done with 20 cycles of the same cycling profile as the first reaction. The PCR mixtures contained 10x PCR buffer, MgCl₂ (50 mM), deoxynucleoside triphosphates (10 mM), primers (10 pmol), and Taq DNA polymerase (1U). The amplified product was visualized by gel electrophoresis using 2% agarose gel containing ethidium bromide (10 µg/mL). The 100 bp DNA ladder (GeneRuler™, Fermentas

life science) was used as a molecular weight standard. Primer sequences are shown in *Table 1*.²⁵

Data analysis

Data was statistically analyzed by SPSS version 15 (SPSS Inc., Chicago, IL, USA). Statistical analysis χ^2 test was used to analyze the data obtained to the age group, sex, G types and seasonal distribution of the group A *Rotavirus* and also type of feeding. Fisher's exact test was used to analyze the clinical symptoms. P value <0.05 was considered statistically significant.

Primer	Sequence (5'→3')	Position	Type
Beg9	GGC TTT AAA AGA GAG AAT TTC CGT CTG G	nt 1-28	-
End9	GGT CAC ATC ATA CAA TTC TAA TCT AAG	nt 1062-1036	-
aBT1	CAA GTA CTC AAA TCA ATG ATG G	nt 314-335	G1
aCT2	CAA TGA TAT TAA CAC ATT TTC TGT G	nt 411-435	G2
aET3	CGT TTG AAG AAG TTG CAA CAG	nt 689-709	G3
aDT4	CGT TTC TGG TGA GGA GTT G	nt 480-498	G4
aAT8	GTC ACA CCA TTT GTA AAT TCG	nt 178-198	G8
aFT9	CTA GAT GTA ACT ACA ACT AC	nt 757-776	G9

Table 1: Primer sequences and positions used for genotyping of VP7 gene in Rotavirus strains.²⁵

Results

Rotavirus detection

A total of 48 (34.78%) diarrheic fecal specimens were confirmed as *Rotavirus* positive using EIA assay.

Rotavirus and demographic data

More males (68.75%) were infected than females (31.25%), and the male-to-female ratio of *Rotavirus* infection was 2.2:1 ($P=0.026$). The age of infected children ranged from 1 to 59 months (*Figure 1*),

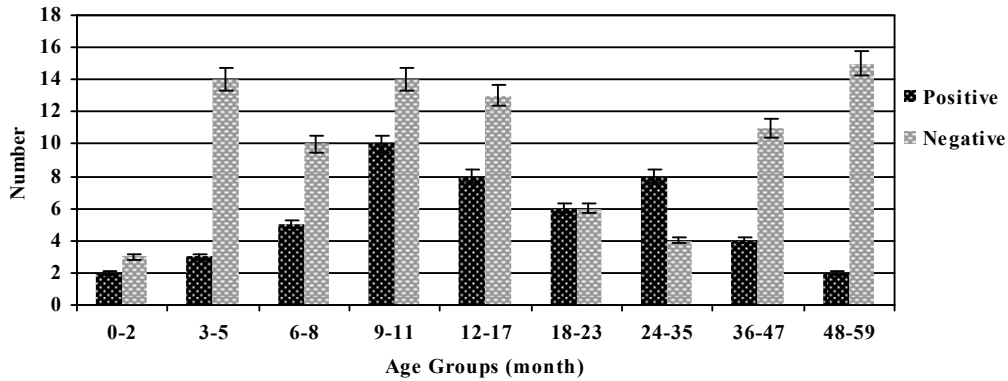


Figure 1: Age distribution of human Rotaviruses from December 2007 to November 2008

and the median age was 7.9 months. Children less than 24 months of age accounted for 70.83% of the overall *Rotavirus*-positive cases with those between 9 and 11 months of age being the most affected ($P= 0.052$). The survey of clinical symptoms in *Rotavirus* gastroenteritis cases showed that children with infection had diarrhea (97.92%), vomiting (77.08%), fever (52.08%) and convulsion (6.25%). Also there was a relationship between *Rotavirus* infection diarrhea ($P= 0.001$) and convulsion ($P= 0.049$) symptoms. According to the season distribution, the

highest prevalence of infection was identified in autumn (45.83%), followed by winter (33.34%),

Summer (12.50%) and spring (8.33%), respectively ($P= 0.012$). *Rotavirus* was detected continuously in the 8-month period lasting from September to April. *Rotavirus* was detected most frequently in December–February. The presence of *Rotavirus* remained low in May–August in which no G type was detected ($P= 0.199$) (Figure 2).

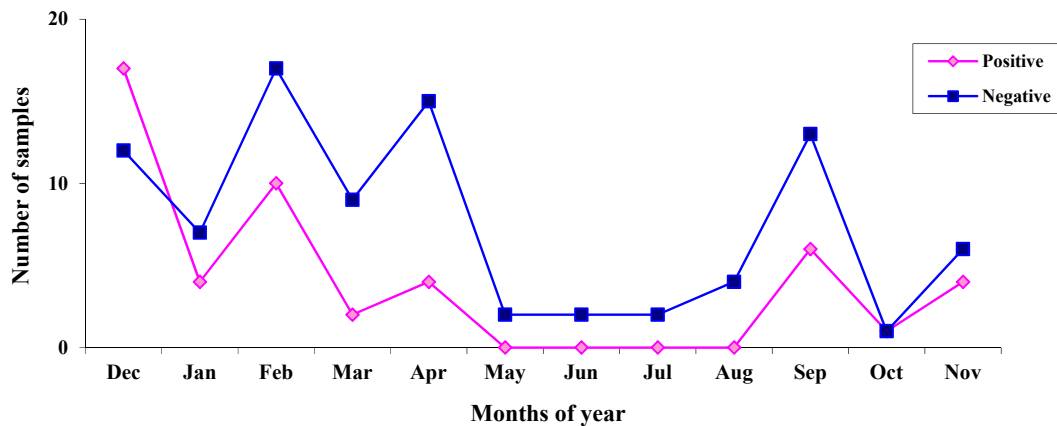


Figure 2. Monthly distribution of human Rotaviruses from December 2007 to November 2008

Overall, 62.50 % of the children with acute gastroenteritis were not breastfed and 37.50 % were breastfeeding at the time of presentation of *Rotavirus* infection ($P= 0.236$).

Rotavirus genotyping

Genotyping was performed on 48 *Rotavirus* positive stool samples by using Nested RT-PCR (Figure 3). The most common circulating G type in the population under surveillance was mixed types, that being identified in 16 strains out of 48 samples (33.34%), followed by non-typeable (15/48, 31.25%), G4 (13/48, 27.08%), G1 (3/48, 6.25%) and G2 (1/48, 2.08%), respectively. The most prevalent mixed G types were G1/G4 (37.50%), G1/G8 (25%), G1/G3 (18.75%), G2/G8 (12.50%) and G3/G4 (6.25%), consecutively. The G types G3, G8 and G9 were not individually detected during the study. The most frequent detected type was mixed G types in females (53.33%) and G4 in males (33.33%) ($P= 0.169$). The most prevalent *Rotavirus* type reported was non-typeable and mixed infection (each 50%) in spring, non-typeable and G1 (each 33.33%) in summer, non-typeable and G4 in the autumn (each 36.36%) and mixed infection in the winter (50%) seasons ($P= 0.049$).

Discussion

Rotavirus is a significant cause of diarrhea in developed and under developed countries in children under five years of age, and it is essential to determine the circulating genotypes and their temporal and geographical variations. From 2007 to 2008, constant surveillance of *Rotavirus* diarrhea in Shiraz, Iran, showed a prevalence of 34.78% in children less than 5 years of age with gastroenteritis. This result is comparable to the disease burden of *Rotavirus* seen in other

studies in Iran and different countries which has shown to be between 13 to 40% of all cases of gastroenteritis.^{10,12,14,16-18} Extensive studies in different regions of the world have indicated that in temperate climates, *Rotavirus* infection occurs predominantly during the cooler months.^{9,26,27} On the other hand, seasonal patterns in tropical climates have shown rates of *Rotavirus* diarrhea throughout the year with seasonal trends that are less pronounced.^{6,9,12,20,21} This study demonstrated that there was a significant correlation between the seasonal distribution and *Rotavirus*-positive cases. *Rotavirus* gastroenteritis occurred throughout the year, with more cases occurring in the winter with a seasonal peak observed in the months of December to February. These findings are similar to those reported in countries with temperate climates such as: Iran,^{15,16} China,²⁶ Burkina Faso,²⁸ and Spain.²⁹ Similar surveillances for prolonged time period are needed in order to ascertain accurately the seasonality associated with *Rotavirus* infection in the studied area. The occurrence of the group A *Rotavirus* was cumulatively observed in the first 24 months of life (70.83%) more than in the older age groups, as was found in previous investigations.^{11,16,26,28,30} *Rotavirus* age distribution was related to the peak incidence of infection, decline in maternal antibodies, and immaturity of new passive immune responses. The high frequency of *Rotavirus* gastroenteritis in this age group highlights the need for a vaccine to offer optimal protection against acute *Rotavirus* infection in children <2 years old. During this study, diarrhea was the symptom most commonly reported that is associated with *Rotavirus* infection, followed by vomiting,

fever and convulsion. These findings are in keeping with studies conducted in Iran and other countries.^{10,28,30-33} In the present study, evaluation of the breastfeeding status of infants less than 12 months of age with severe diarrhea showed that *Rotavirus*-positive cases were infrequent among those being breastfed at the time of acute gastroenteritis. This result suggests that breastfeeding may be a protective factor against *Rotavirus* infection, as reported in other studies.^{28,30,31} Worldwide genetic diversity of circulating *Rotavirus* strains is associated with the presentation of new emerging strains, causing variability in the geographical distribution of the virus. The most common circulating G type in our study was mixed types with two different *Rotavirus* G types. The proportion of mixed infections (33.34%) reported in this study is substantially lower than what was reported in Guinea-Bissau (59%),³⁴ and Iran (60%),³⁵ and higher than studies conducted in Ireland,³⁶ Indonesia,³⁷ Africa,³⁸ India,³⁹ with the prevalence of 28.5%, 23%, 21.4% and 21%, respectively. In the current survey, we documented the first case of G1/G3 mixed infection in Iran. This G type has been reported in young children with gastroenteritis in Mexico.⁴⁰ Some of the mixed types detected in the present investigation are similar with those identified in other studies.^{10,20,38,40} The high prevalence of mixed infections may reflect the observed diversity of strains. Patients harboring multiple *Rotaviruses* may offer a unique environment for the re-assortment process, facilitating generation of novel *Rotavirus* strains and the maintenance of the observed diversity.^{15,34,39} Separately, the high mixed infection with different *Rotavirus* strains may be explained by greater

environmental contamination with *Rotavirus*, for example water resources, coupled with greater contact between children and the environment around them.³⁹ Therefore, the frequency of mixed infection of *Rotaviruses* and its effect on the development of *Rotavirus* vaccine should be thoroughly investigated. During this study, 31.25% of the evaluated samples were G untypeable that might be related to the presence of novel strains, the failure of the genotyping due to the presence of the other genes was not investigated in this survey; for example, rare G types such as G5, G6, G10, G12, and failure in RT-PCR technique.²⁵ Non-typeable *Rotavirus* strains are rarely reported in children with acute diarrhea in Iran and other countries.^{15,16,19,37,41} In recent years G4 strain has been detected at relatively high frequency from Iran,^{15,42} to Italy,¹⁹ to South Korea,³² and Brazil.⁴¹ The present study revealed G4 type as the prevalent G type individually in 27.08% of all *Rotavirus*-positive cases. Numerous molecular epidemiological studies have shown that G1 is the most common circulating G type worldwide.^{6,14,15,37,43} However the G1 type was observed only in 6.25% of all children with *Rotavirus* diarrhea. We detected the G2 type in the specimens of only 2.08% of patients with acute diarrhea, which is in contrast with studies conducted in Italy, Sierra Leone and India, which demonstrated that the G2 type is one of the most prevalent *Rotavirus* types.^{10,19,20,44} In the current study, neither the G3 nor the G8 types were detected individually. These findings are distinct from those results observed in Sierra Leone,¹⁰ Turkey,¹³ Malawi,²¹ and China,⁴⁵ where these G types have been identified as the most common types in children. In recent years

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there has been an increase in research of the importance of G9 type in many countries including Latin America,¹² Iran,¹⁶ Albania,¹⁹ Brazil,³⁷ Cuba,⁴⁶ and Tanzania.⁴⁷ However, the G9 type was not detected during the study. Investigations in developing and industrialized countries have demonstrated the need for new generations of *Rotavirus* vaccines to include G9 strains due to the increasing emergence of this type of group A *Rotavirus*. In conclusion this study provides information on the epidemiology and description of the *Rotavirus* G types circulating among Iranian children with acute diarrhea. Our results indicate that gastroenteritis caused by *Rotavirus* in the country is a significant health problem, particularly among children less than 2 years of age and during the cold season. These data will be useful for making an informed decision about the introduction of *Rotavirus* vaccine in Iran and provides a baseline data for future vaccine studies.

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Financial support

None declared.

Conflict of interest

None declared.

Acknowledgment

Our special thanks and appreciation go to the Islamic Azad University, Jahrom Branch, who provided executive support of this Project. We also thank the thoughtful of Mehdi Kargar and Dr. Ramin Yaghobi who assisted us in performing this project.

Author Contribution

KM carried out the design of the study, coordination and scientific consultation at Islamic Azad University of Jahrom. JT and NA participated in sampling and practical methods. All authors read and approved the final manuscript.

- [<http://dx.doi.org/10.1002/jmv.21999>]
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