Journal of Medical Virology 84:2003–2007 (2012)

Genotyping of Rotaviruses Detected in Children Admitted to Hospital From Faisalabad Region, Pakistan

Tayyba Iftikhar,[1] Asghar Butt,[2] Khurram Nawaz,[2] Yasra Sarwar,[1] Aamir Ali,[1] Tanveer Mustafa,[1] and Abdul Haque[1]*

[1]*Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan*
[2]*Children Ward, District Headquarters Hospital (DHQ), Faisalabad, Pakistan*

Rotavirus infection is very common in developing countries and occurs at least once in children under the age of 5 years. The rate of detection of rotavirus infection in various age groups (0–5 years) in patients with gastroenteritis admitted to hospital from the Faisalabad region, Pakistan is reported. Out of 300 fecal samples, 189 (57.3%) were positive for rotavirus by immunoassay. Patients aged 7–12 months (35.4%) were infected most commonly followed by the age group 0–6 months (28%). Different genotypes of rotavirus were identified by heminested RT-PCR. The most common genotype was G1P[8] (25.3%), followed by G1P[6] (21.1%). Other genotypes were G1P[9], G2P[6], G9P[10]), G3P[8] (1.5%), and G9P[11] (1%). There were two (1%) cases of mixed G genotype, one patient with two genotypes G1, G10 and another patient with 3 genotypes G1, G10, and G12. There were 6 (3.1%) cases of mixed P genotypes, 3 P[4], P[11] and 3 P[8], P[11]. These results provide an outline of rotavirus infection in this area for the first time. ***J. Med. Virol. 84:2003–2007, 2012.***

KEY WORDS: Group A rotavirus; G-type; P-type; mixed infection

INTRODUCTION

Morbidity and mortality for diarrheal disease are amongst the most critical issues globally and rotavirus is one of the most important causes [Akran et al., 2010]; it is estimated that 125 million cases of rotavirus infection occur annually resulting in 440,000 deaths. Genotyping shows that Group A rotaviruses are the major etiological agents of acute infantile diarrhea. These viruses are the single most important enteropathogen in children less than 5 years of age and responsible for 30–60% diarrheal admissions in hospitals [Kirkwood et al., 2011].

Rotavirus is a double-stranded RNA virus in the family *Reoviridae*. Its genome encodes six structural proteins (VPs) and six non-structural proteins (NSPs). For characterization the most important are VP4 and VP7 proteins which form the outer capsid; both have a role in the entry of the virus into the host cell and in the induction of neutralizing antibodies [Gomez et al., 2011].

Twenty-three G-genotypes and 32 P-genotypes have been described based on nucleotide sequence variation [Zeller et al., 2010]. G1, G2, G3, G4, or G9 in combination with P[4], P[6], or P[8] have been most commonly detected in humans. In recent years, G12 has emerged as an important genotype [Ghosh et al., 2011] and VP7 and VP4 genotype combinations have been identified in humans as the leading cause of severe rotavirus disease: G1P[8], G3P[8], G4P[8] and G9P[8] and G2P[4] [Martella et al., 2011]. In addition to these global rotavirus strains, some new strains have emerged from different regions of the world, which have related, but not identical, genotypes with existing strains [Matthijnssens et al., 2009].

As geopolitically defined by the WHO, mortality remains high in the Eastern Mediterranean Region (EMR) especially in countries such as Pakistan, Afghanistan, Sudan, Yemen, and Somalia [Malek

Grant sponsor: Higher Education Commission (HEC), Pakistan; Grant number: 498.

Conflict of interest: none.

*Correspondence to: Abdul Haque, Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering, P.O. Box 577, Jhang Road, Faisalabad 38000, Pakistan.
E-mail: ahaq_nibge@yahoo.com

Accepted 11 July 2012

DOI 10.1002/jmv.23402
Published online in Wiley Online Library (wileyonlinelibrary.com).

et al., 2010]. As there is no rotavirus surveillance network, Pakistan does not keep any epidemiological record. Many epidemics and hospitalizations are never monitored for rotavirus genotypes [Alam et al., 2009]. Vaccines are available to control this disease and are the most effective approach for the prevention of rotavirus mortality and morbidity. In 2009, WHO recommended the use of vaccines in all countries. Currently, two vaccines are being licensed in European countries, and other vaccines are in early stages of development [Malek et al., 2010]. As Pakistan has high incidence of rotavirus infection, there is need for introduction of vaccination programs.

In this study, the occurrence of rotaviruses in various age groups of patients admitted to a central hospital of Faisalabad, Pakistan which caters to a population of approximately 10 million persons and their distribution according to G and P genotypes based on serological and molecular analysis is reported. This study will be useful as a baseline for efforts to control rotavirus infection in this region including the development of vaccines.

MATERIALS AND METHODS

Collection of Samples

Stool samples from 300 children (0–5 years) suspected of rotavirus infection were collected at the DHQ Hospital, Faisalabad from 4th May 2010 to 15th September 2010. The children with acute gastroenteritis were tested for detection of rotavirus infection. The inclusion criteria for the study were all or any of the following signs and symptoms: stomach pain/cramps; diarrheal stool; high fever/seizures; vomiting; and mucus in the stool. Collection of feces was carried out in glycerol saline buffer (dipotassium phosphate 22.7 mM [3.1 g/L], monopotassium phosphate 7 mM [1 g/L], phenol red 0.003 g/L, and sodium chloride 72 mM [4.2 g/L], pH 7.2). Samples were stored at 4°C and tested within one week. Epidemiological and patient history data including age, sex, social background, duration of illness, clinical diagnosis, and physical appearance of the stool specimen were obtained using questionnaires.

Detection of Rotavirus in Fecal Samples

Stool specimens were screened using a commercial immunoassay kit (Virotect Rota, OD038 Kit, Omega Diagnostic, UK) according to the manufacturer's instructions. Rotavirus positive samples were selected for further studies.

RNA Extraction for Genotyping

Rotavirus dsRNA was extracted from 150 μl of stool sample using viral RNA extraction kit (NucleoSpin, Cat. #740956.250, Macherey-Nagel, Germany) according to the provided protocol. RNA was eluted in 50 μl RNase free water.

cDNA Synthesis and Consensus PCR

Extracted RNA was transcribed to cDNA using Promega AccessQuick™ RT-PCR System (Cat. #A1702) and consensus PCR was performed using G type and P type consensus primers [Gomara et al., 2001; Simmonds et al., 2008]. The concentration of G and P type primers was 10 and 20 μM, respectively. The reaction was carried out with an initial reverse transcription step at 42°C for 1 hr for P types and at 45°C for 45 min for G types. It was followed by a PCR activation step at 94°C for 2 min and 35 cycles of amplification (94°C for 1 min, 52°C for 1 min, and 72°C for 1 min) with a final extension step at 72°C for 7 min.

Multiplex PCR

Rotavirus isolates were further characterized by VP4 and VP7 strain specific multiplex hemi nested PCR. VP7 reverse primer was used in multiplex PCR together with G1, G2, G3, G4, G8, G9, G10, and G12 for G types [Iturriza-Gomara et al., 2004]. For P types VP4F primer was used in combination with P typing primers P [4], P [6], P [8], P [9], P [10], and P [11] [Simmonds et al., 2008].

Statistical Analysis

Statistical analyses were carried out using SPSS version 13. Chi-Square was used to check the significance of differences between proportions and ANOVA to test the significance of differences between means. A P-value of <0.05 was considered as significant.

RESULTS

A total of 300 samples were collected from patients (0–5 years) suffering from gastroenteritis. Rotavirus positive samples (189; 57.3%) were processed further for subtyping.

Age Groups

Patients were divided into 10 groups. Age group 2 (7–12 months) was the group most affected by rotavirus infection (73 [38.6%]), followed by age group 0–6 months (59 [31.2%]) indicating that the disease is most prevalent during first year and accounts for nearly 2/3rd of all cases. Similar pattern was reflected in rotavirus positive samples as well and age group 2 was dominant. As expected, there was a significant decrease in rotavirus positive samples in age groups 12–18 months and 19–24 months but the number was still considerably high (31 [21%]). A sharp decline in rotavirus infection was observed after 2 years of age and total cases between 2 and 5 years were only 40 (13.3%). The proportion of rotavirus positive samples was higher in males (107 [61.1%]) as compared to females (65 [52%]) but no significant association was found between sex and rotavirus infection ($\chi^2 = 2.26$, $P > 0.0$).

Prevalence of G Types

Among 189 rotavirus positive samples, 172 (91%) were positive with G type specific primers. G1 was the most prevalent genotype (114 cases [60.3%]) followed by G9: 21 (11.1%). Prevalence of other G types in descending order was G10: 15 (7.9%); G2: 9 (4.7%); G12: 5 (2.6%); G3: 3 (1.5%); and G4: 3 (1.5%). As shown in Table I, mixed infection was seen in 2 cases (1%), one showing co-infection with 2 genotypes (G1, G10) and other with 3 genotypes (G1, G10, G12).

Prevalence of P Types

P genotypes were detected in 140 (74%) samples. Most prevalent genotype was P[8]. It was detected in 51 cases (26.9%). Next most frequently detected genotype was P[6] which was found in 48 (25.3%) cases. Prevalence of other P types in descending order was P[10]: 9 (4.7%), P[9]: 6 (3.1%), and P[11]: 2 (1%). There were 6 (3.1%) cases of mixed infection of P[4], P[11], and P[8] (Table I). No P type could be assigned to cDNA prepared from 18 samples (9.5%) using P type consensus primers. These samples were successfully processed for G types using consensus primers. Thirty-two (16.9%) samples were not type able as any P type.

Combination of G and P Types

Different G and P genotype combinations were detected (Table I). Most common was G1P[8] (25.3%), followed by G1P[6] (21.1%), G2P[6] (4.2%), G9P[10] (4.2%), G1P[9] (3.1%), G3P[8] (1.5%), and G9P[11] (1%). There were 2 (1%) cases of mixed G genotype, one with two genotypes G1, G10, and other with three genotypes G1, G10, G12. There were 6 (3.1%) cases of mixed genotypes of P, that is, three with P[4], P[11] and three with P[8], P[11].

TABLE I. G and P Types Combinations Detected in 189 Serotype Positive Samples

G/P combination	No. of strains (%)
G1	114 (60.3)
G1P[8]	48 (25.3)
G1 no P	07 (3.7)
G1P[6]	40 (21.1)
G1P[9]	06 (3.1)
G1 PcDNA	13 (6.8)
G2	09 (4.7)
G2P[6]	08 (4.2)
G2 no P	01 (0.5)
G3	03 (1.5)
G3P[8]	03 (1.5)
G4	03 (1.5)
G4 no P	03 (1.5)
G9	21 (11.1)
G9P[10]	08 (4.2)
G9, P[4], P[11]	03 (1.5)
G9, P[8], P[11]	03 (1.5)
G9P[11]	02 (1.0)
G9 PcDNA	05 (2.6)
G10	15 (7.9)
G10 no P	15 (7.9)
G12	05 (2.6)
G12 no P	05 (2.6)
Mixed	02 (1.0)
G1, G10, no P	01 (0.5)
G1, G10, G12, P[10]	01 (0.5)

DISCUSSION

In this study, the epidemiology of rotavirus infection in infants and young children with gastroenteritis admitted to District Headquarter Hospital, Faisalabad was investigated. It is one of the main hospitals providing services to Faisalabad region, which has a population of more than 10 million and constitutes a major part of central Pakistan. The samples were collected in peak months from May to September in 2010. The data obtained from this study have provided some important features of the epidemiology of rotavirus in this area. However, as data were generated during a small period of 5 months, they may not be representing a broader picture, which might have emerged by a study conducted for several years.

Rotavirus infection occurs between 0 and 23 months of age. As compared to other ages, children aged 7–12 months are found to be more susceptible to rotavirus infection [Gray et al., 2008]. Findings from this study were in accordance with the current report and age group 7–12 months was most affected (73 [38.6%]) followed by age group 0–6 months (59 [31.2%]) indicating that the disease is most prevalent during first year. A sharp decline was observed after 2 years of age and total cases from 2 to 5 years were only 25 (13.3%). Duration of illness was significantly higher in rotavirus positive patients (mean = 3.88 days) as compared to rotavirus negative patients (mean = 2.78 days) ($t = -2.408$, $P < .05$).

Currently, 42 different combinations of G and P genotypes are reported; globally there are five genotypes most prevalent in humans (G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8]). These genotypes cause >90% of human rotavirus cases detected worldwide [Shim et al., 2010].

Over the last decades, G1 rotavirus has been responsible for acute gastroenteritis in children [Khamrin et al., 2007]. In this study, seven G types (Table I) were detected. As expected, G1 was the most prevalent (114 cases [60.3%]) G type. It was followed by G9 (21 [11.1%]) and G10: 15 (7.9%). Five P serotypes were detected in 144 (74%) samples (Table I). P[8] was the most prevalent (51 cases ([26.9%]) followed by P[6] (48[25.3%]). There were 6 (3.1%) cases of mixed infection of P[4], P[11], and P[8].

Among the five most common genotypes, G1P[8] is the most predominant in children of both developed and developing countries. This genotype is responsible for 50–65% rotavirus infections in children [Arora and Chitambar, 2011]. G1P[8] was also reported as the most dominant genotype in studies conducted in Karachi, Pakistan during 1990–1997 and 2005–2009 [Qazi et al., 2009].

Although G1P[8] is the predominant genotype circulating globally, G2 has also emerged intermittently and randomly as the major cause of rotavirus gastroenteritis especially in Brazil and Korea. Although G2 is usually associated with P[4], there have been recent outbreaks of G2P[6] [Han et al., 2010; Clark et al., 2011]. The dynamism of rotavirus infections is further reflected by the observation that G3P[8] was the second most important genotype in Africa between 1982 and 2001 and 1989 and 2004 [Esona et al., 2010]. In this study, G4 was detected in 1.5% cases with non-typeable P type. The prevalence of G4 was reported as 8.4% in a previous study (1990–1997) from Karachi, Pakistan [Qazi et al., 2009].

The findings in this study had a unique pattern. Although G1P[8] was most common as expected (25.3%), G1P[6] (21.1%) was not lagging far behind. These genotypes were followed by G2P[6] and G9P[10] (4.2%), G1P[9] (3.1%), and G9P[11] (1%). It is worth mentioning that G1P[9], G2P[6], and G9P[10] have not been commonly reported, though G1P[9] was detected in Korea recently [Han et al., 2010]. G3P[8], and G9P[11] genotypes (Table I) were detected with lower frequency as compared with existing reports.

This study shows various combinations of G9 with P types. Human rotavirus G9 strains were first reported in children with gastroenteritis in Philadelphia in 1983, but they disappeared during the next season [Clark et al., 2004]. However G9 has since emerged as the fifth most common type and has been reported throughout the world though varying in its prevalence. It is important to note that G9 has higher susceptibility to genetic change than G1–G4 [Zuridah et al., 2010]. Our results are not consistent with a study conducted in Karachi, Pakistan during 2005–2007 in which G9P[8] was the most dominant genotype followed by G1P[8] [Qazi et al., 2009].

G12 was detected for the first time in Pakistan in 2009 [Alam et al., 2009]. In this study G12 was detected in 2.6% cases with non-typeable P type.

In current study, there were 2 (1%) cases of mixed G genotype, one with 2 genotypes G1, G10 and other with 3 genotypes G1, G10, and G12. There were 6 (3.1%) cases of mixed genotypes of P, that is, 3 each of P[4], P[11] and P[8], P[11]. In a study (2005–2009) conducted in Karachi, Pakistan the prevalance of mixed genotypes was lower [Qazi et al., 2009]. Mixed genotypes are found when many genotypes circulate at the same time. This phenomenon is more common in developing countries in which a greater variation of different genotype combinations is usually detected [Ribas Mde et al., 2011]. The similarity between rotavirus strains from different animal species may be responsible for interspecies transmission and this may occur by genetic reassortment or as a whole virion [Armah et al., 2010]. Keeping domestic animals is very common in Pakistan, and humans and animals usually live closely and share same water reservoirs in rural settings. In this situation mixed infections can occur easily as reported in this study.

The cDNA prepared from 18 samples could not be amplified for any positive results for P type although processing for G types was successful. For another 32 seropositive samples, no cDNA was obtained. Various other researchers had similar failure with some specimens. Failure in genotyping may be caused by primer mismatch due to the high diversity leading to genomic reassortment, mixed infections, or interspecies transmission [Banerjee et al., 2008; Zuridah et al., 2010].

Rotavirus vaccine is not included in extended program of immunization (EPI) in Pakistan. For an effective vaccine, understanding of rotavirus circulating strains is important. This information is especially important in countries like Pakistan with high diarrheal mortality rate. Unfortunately, no epidemiological record regarding the status of rotavirus genotypes in Pakistan is available [Alam et al., 2009]. There are only three published reports on rotavirus genotyping in Pakistan originating from Karachi and Rawalpindi.

This is the first study outlining the molecular diversity of rotaviruses in the Faisalabad region, an area with a population greater than many countries. It was found that G1P[8] and G1P[6] were predominant in this area, with several other unique findings as reported above. This report may also be helpful as a reference for vaccine preparation for local needs.

ACKNOWLEDGMENTS

We are grateful to National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad for providing the facilities for this study.

The study was approved by Ethical Committees of both National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad and District Headquarters Hospital (DHQ), Faisalabad. Informed consent was obtained from parents/guardians of all children.

REFERENCES

Akran V, Peenze I, Akoua-Koffi C, Kette H, de Beer MC, Dosso M, Steele AD. 2010. Molecular characterization and genotyping of human rotavirus strains in Abidjan, Cote d'Ivoire. J Infect Dis 202:S220–S224.

Alam MM, Malik SA, Shaukat S, Naeem A, Sharif S, Angez M, Rana MS, Khurshid A, Zaidi SZ. 2009. Genetic characterization of rotavirus subtypes in Pakistan-first report of G12 genotype from Pakistan under WHO-Eastern Mediterranean region. Virus Res 144:280–284.

Armah GE, Hoshino Y, Santos N, Binka F, Damanka S, Adjei R, Honma S, Tatsumi M, Manful T, Anto F. 2010. The global spread of rotavirus G10 strains: Detection in Ghanaian children hospitalized with diarrhea. J Infect Dis 202:S231–S238.

Arora R, Chitambar SD. 2011. Full genomic analysis of Indian G1P[8] rotavirus strains. Infect Genet Evol 11:504–511.

Banerjee I, Primrose Gladstone B, Iturriza-Gomara M, Gray JJ, Brown DW, Kang G. 2008. Evidence of intrafamilial transmission of rotavirus in a birth cohort in South India. J Med Virol 80:1858–1863.

Clark HF, Lawley DA, Schaffer A, Patacsil JM, Marcello AE, Glass RI, Jain V, Gentsch J. 2004. Assessment of the epidemic potential of a new strain of rotavirus associated with the novel G9 serotype which caused an outbreak in the United States for the first time in the 1995–1996 season. J Clin Microbio 42:1434–1438.

Clark HF, Lawley D, DiStefano D, Maliga M, Kilby B, Kulnis G, Mallette L, DiNubile MJ. 2011. An unusual outbreak of rotavirus genotype G2P[6] during the 2005–2006 epidemic season in Philadelphia. Diagn Micr Infec Dis 70:218–222.

Esona MD, Armah GE, Steele AD. 2010. Rotavirus VP4 and VP7 genotypes circulating in Cameroon: Identification of unusual types. J Infect Dis 202:205–211.

Ghosh S, Gatheru Z, Nyangao J, Adachi N, Urushibara N, Kobayashi N. 2011. Full genomic analysis of a G8P[1] rotavirus strain isolated from an asymptomatic infant in Kenya provides evidence for an artiodactyl-to-human interspecies transmission event. J Med Virol 83:367–376.

Gomara MI, Cubitt D, Desselberger U, Gray J. 2001. Amino acid substitution within the VP7 protein of G2 rotavirus strains associated with failure to serotype. J Clin Microbiol 39:3796–3798.

Gomez MM, de Mendonca MC, Volotao Ede M, Tort LF, da Silva MF, Cristina J, Leite JP. 2011. Rotavirus A genotype P[4] G2: Genetic diversity and reassortment events among strains circulating in Brazil between 2005 and 2009. J Med Virol 83:1093–1106.

Gray J, Vesikari T, Van Damme P, Giaquinto C, Mrukowicz J, Guarino A, Dagan R, Szajewska H, Usonis V. 2008. Rotavirus. J Pediatr Gastroenterol Nutr 46:S24–S31.

Han TH, Kim CH, Chung JY, Park SH, Hwang ES. 2010. Genetic characterization of rotavirus in children in South Korea from 2007 to 2009. Arch Virol 155:1663–1673.

Iturriza-Gomara M, Kang G, Gray J. 2004. Rotavirus genotyping: Keeping up with an evolving population of human rotaviruses. J Clin Microbiol 31:259–265.

Khamrin P, Peerakome S, Tonusin S, Malasao R, Okitsu S, Mizuguchi M, Ushijima H, Maneekarn N. 2007. Changing pattern of rotavirus G genotype distribution in Chiang Mai, Thailand from 2002 to 2004: Decline of G9 and reemergence of G1 and G2. J Med Virol 79:1775–1782.

Kirkwood CD, Boniface K, Barnes GL, Bishop RF. 2011. Distribution of rotavirus genotypes after introduction of rotavirus vaccines, Rotarix® and RotaTeq®, into the National Immunization Program of Australia. Pediatr Infect Dis J 30:S48–S53.

Malek MA, Teleb N, Abu-Elyazeed R, Riddle MS, Sherif ME, Steele AD, Glass RI, Bresee JS. 2010. The epidemiology of rotavirus diarrhea in countries in the Eastern Mediterranean Region. J Infect Dis 202:S12–S22.

Martella V, Potgieter AC, Lorusso E, De Grazia S, Giammanco GM, Matthijnssens J, Banyai K, Ciarlet M, Lavazza A, Decaro N, Buonavoglia C. 2011. A feline rotavirus G3P[9] carries traces of multiple reassortment events and resembles rare human G3P[9] rotaviruses. J Gen Virol 92:1214–1221.

Matthijnssens J, Bilcke J, Ciarlet M, Martella V, Banyai K, Rahman M, Zeller M, Beutels P, Van Damme P, Van Ranst M. 2009. Rotavirus disease and vaccination: Impact on genotype diversity. Future Microbiol 4:1303–1316.

Qazi R, Sultana S, Sundar S, Warraich H, un-Nisa T, Rais A, Zaidi AK. 2009. Population-based surveillance for severe rotavirus gastroenteritis in children in Karachi, Pakistan. Vaccine 27:F25–F30.

Ribas Mde L, Nagashima S, Calzado A, Acosta G, Tejero Y, Cordero Y, Piedra D, Kobayashi N. 2011. Emergence of G9 as a predominant genotype of human rotaviruses in Cuba. J Med Virol 83: 738–744.

Shim SY, Jung YC, Le VP, Son DW, Ryoo E, Shim JO, Lim I, Kim W. 2010. Genetic variation of G4P[6] rotaviruses: Evidence for novel strains circulating between the hospital and community. J Med Virol 82:700–706.

Simmonds MK, Armah G, Asmah R, Banerjee I, Damanka S, Esona M, Gentsch JR, Gray JJ, Kirkwood C, Page N, Iturriza-Gomara M. 2008. New oligonucleotide primers for P-typing of rotavirus strains: Strategies for typing previously untypeable strains. J Clin Virol 42:368–373.

Zeller M, Rahman M, Heylen E, De Coster S, De Vos S, Arijs I, Novo L, Verstappen N, Van Ranst M, Matthijnssens J. 2010. Rotavirus incidence and genotype distribution before and after national rotavirus vaccine introduction in Belgium. Vaccine 28:7507–7513.

Zuridah H, Kirkwood CD, Bogdanovic-Sakran N, Bishop RF, Yap KL. 2010. Circulating human group A rotavirus genotypes in Malaysia. J Med Virol 82:707–711.