Detection of Rotavirus from Hospitalized Diarrheic Children in Uttar Pradesh, India

S. K. Dash · Krishna Kumar · A. Tewari ·
P. Varshney · A. Goel · A. K. Bhatia

Received: 5 July 2011 / Accepted: 4 June 2012 / Published online: 22 June 2012
© Association of Microbiologists of India 2012

Abstract In the present study 220 stool samples collected from diarrheic children admitted to different hospitals and nursing homes of Uttar Pradesh and Uttarakhand were screened for rotavirus. Of 220 diarrheic samples screened 46 samples were found to be positive for rotavirus by RNA PAGE. All the isolates exhibited 4-2-3-2 migration pattern suggesting group A rotavirus. Both long and short electropherotypes were prevalent in these regions. Six different electropherotypes were detected in this study period. Male diarrheic children were found to be more susceptible to rotavirus infection (22.96 %) than that of the female ones (17.64 %). Viral RNA isolated from stool samples again subjected to VP4 gene amplification by RT-PCR using con2 and con3 primer which resulted 876 bp product suggesting group A rotavirus. Besides virus isolation was successfully done using MA104 cell line.

Keywords Human rotavirus · Epidemiological studies · RNA-polyacrylamide gel electrophoresis (RNA-PAGE) · Cell culture · RT-PCR

Introduction

Rotavirus infection is the leading cause of severe acute diarrhea among young children worldwide [7]. An estimated 527,000 children below 5 years of age die from rotavirus induced diarrhea each year, with >85 % of deaths occurring in Africa and Asia [7]. In India, rotavirus induced diarrhea causes an estimated 122,000–153,000 deaths, 457,000–884,000 hospitalizations and 2 million outpatient visits in children below 5 years of age annually [31]. Rotaviruses, are non-enveloped, icosahedral particles consisting of 11 segments of double-stranded RNA (dsRNA) enclosed in a triple-layered protein capsid [12]. Rotaviruses are classified into seven groups (A–G) based on specificity of VP6 inner shell polypeptide. Group A rotaviruses are the leading cause of severe dehydrating gastroenteritis in children below 5 years of age worldwide [12]. Rotaviruses are further classified into electropherotypes on the basis of differences in the relative migration rates of genome segments in polyacrylamide gel electrophoresis (PAGE), thereby creating more opportunities for strain diversification [18]. Broadly, the electropherotype patterns are designated as “long” and “short” based on the fact that short electropherotype strains have a striking reduction in the migration rate of segment 11 due to duplication within the 3′ untranslated region of this segment [22]. Detection by PAGE appears to be promising for the diagnosis of rotavirus infection in animals and human beings. Analysis of electrophoretic mobilities of the segments of dsRNA by PAGE yields a pattern, which is constant, and characteristic for a particular rotavirus isolate [11]. Electrophoretic migration patterns of the rotavirus genomic dsRNA segments has also been extensively used in epidemiological studies of rotavirus in India as well as abroad [2, 6, 8, 9, 23, 24, 29]. Epidemiological studies in different parts of India have

S. K. Dash · K. Kumar · A. Tewari · P. Varshney · A. Goel ·
A. K. Bhatia
Department of Veterinary Microbiology and Immunology,
College of Veterinary Sciences and Animal Husbandry,
DUVASU, Mathura 281001, Uttar Pradesh, India

Present Address:

S. K. Dash (✉)
Division of Virology, Indian Veterinary Research Institute,
Izatnagar, Bareilly 243122, India
e-mail: sandeepkumar.dash@gmail.com

Table 1 Rotavirus detection in children at different places by RNA PAGE

Place	Number of samples screened	+ve	Prevalence (%)
Mathura	96	22	22.91
Vrindavan	71	16	22.53
Agra	15	3	20.00
Aligarh	24	3	12.50
Lucknow	06	0	00.00
Dehradun	08	2	25.00
Total	220	46	20.90

been conducted by different research groups such as Bangalore [2], Mysore [2], New Delhi [6, 8, 9], Bombay [10], Hyderabad [3], Pune [19], Chandigarh [26], and North East India [23]. However, information on this aspect is still not available in Uttar Pradesh, India. Hence, in the present study we have screened different parts of Uttar Pradesh and Uttarakhand (Table 1) for detection of rotavirus in hospitalized diarrheic children.

Materials and Methods

Collection of Specimens

Two hundred and twenty fecal samples were collected from diarrheic children admitted to different Government and private hospitals/nursing homes i.e. Vatshalya Hospital, Vrindavan; Ashok Hospital, Mathura; Deep Hospital, Mathura; Arun Hospital, Agra; Manocha Clinic, Agra; Bansal Nursing Home, Aligarh; Gomti Nursing Home, Lucknow; Doon Govt. Hospital, Dehradun (Table 1) during study period 2007–2009. The stool samples were collected in sterilized plastic containers and transported under ice to the Department of Veterinary Microbiology, DUVASU, Mathura and stored at -20°C till further processing. All the further steps were carried out in the virology laboratory and cell culture laboratory, Department of Veterinary Microbiology, DUVASU, Mathura.

Preparation of Fecal Suspension

10 % Fecal suspension was prepared in phosphate buffer saline (PBS, pH 7.2) and centrifuged at 10,000 rpm for 30 min. 1 ml of this fecal supernatant was used for viral RNA extraction and rest was stored at -70°C .

Extraction of Viral RNA from Fecal Supernatant

Viral RNA extraction was done using phenol:chloroform method [14]. The pellet was suspended in 2X RNA sample

buffer for RNA PAGE analysis and stored at -20°C until required.

Screening by RNA-PAGE

Electrophoresis of extracted RNA was performed as per the method described by Laemmli [21] and Herring et al. [14] in 7.5 % resolving and 5 % stacking gel. The silver staining of the gel was done as described by Herring et al. [14]. The stained gel was photographed and stored in 10 % ethanol.

Classification of Rotaviruses into Sub groups

Classification of the electropherotypes was performed as per the method described by Tam et al. [29] and Rasool et al. [24] with slight modifications. The classification is as follows: I—short pattern, II—long pattern; A—when all the segments were resolved, B—when 2 and 3 co-migrated, C—when 7 and 8 co-migrated, D—when 8 and 9 co-migrated, G—when 7, 8 and 9 co-migrated, E—when 2, 3 co-migrated along with 7 and 8, F—when 2, 3 co-migrated along with 7, 8 and 9 (We adopted).

Isolation of Virus

Epithelial line derived from rhesus monkey kidney (MA-104) cell line was used for isolation of human rotaviruses from fecal suspensions. Confluent monolayer of MA-104 cells were grown in cell culture flasks using DMEM [16] growth medium supplemented with 10 % fetal calf serum. The methodology of Sato et al. [25] was followed with minor modifications. RNA-PAGE positive supernatant were trypsinized with 20 $\mu\text{g/ml}$ trypsin for pre-activation at 37°C for 1 h and finally filtered through 0.2 μ membrane filters. The confluent cells were seeded with 1 ml of pre-activated supernatant and incubated for 1 h at 37°C for adsorption of virus, then decanted and washed with PBS. Serum free maintenance medium containing 1 $\mu\text{g/ml}$ trypsin, 100 IU/ml penicillin and 100 $\mu\text{g/ml}$ of streptomycin was added. The inoculated cultures were then incubated for 5–7 days. 2–3 blind passages were done depending upon the development of cytopathic effects (CPE). CPE was characterized by cell rounding, cell detachment and lytic foci [20]. The virus was harvested from the cells, by three cycles of freezing and thawing, when they showed 75 % CPE. The virus was concentrated and purified [30]. The virus was aliquoted in 1 ml fraction and used for extraction of viral RNA and subjected to RNA-PAGE as described earlier.

RT-PCR Assay

Viral RNA was extracted from 200 μl of the 10 % rotavirus positive stool suspension in balanced salt solution,

Table 2 Detection of rotavirus in children of different age and sex groups

Age (months)	Fecal samples screened by PAGE for rotavirus					
	Male children	+ve	%	Female children	+ve	%
1 day–4 months	35	08	22.85	15	03	20.00
4–8	44	13	29.54	27	06	22.22
8–12	41	07	17.07	30	04	13.33
≥12	15	03	20.00	13	02	15.38
Total	135	31	22.96	85	15	17.64

using guanidine isothiocyanate silica [4]. Then it was eluted in 50 µl of RNase free water containing RNasin (RNase inhibitor). The extracted nucleic acid was then reverse transcribed using random primers (hexamers) [15]. The resultant cDNA was then used as template for PCR amplification using primer pair con2 (5'ATTTTCGGACCA TTTATAACC3') and con3 (5'TGGCTTCGCCATTTTA-TAGACA3') for VP4 gene [13] targeting an 876 bp product.

Results and Discussion

In this study we selected electropherotyping (RNA-PAGE) as the primary identifier of rotavirus strains in feces of diarrheic children due to the following reasons. Each rotavirus strain reveals a single distinct electropherotype upon PAGE. Analysis of the electrophoretic mobility of the 11 segments of dsRNA by PAGE yields a pattern which is both constant and characteristic for a particular rotavirus isolate [11]. Besides it is easier to collect fecal samples in comparison to serum samples. Again RNA PAGE has been used to track down the individual strains in epidemiological studies of rotavirus infection which may include circulation of single or multiple strains in the same epidemic season, geographical area and appearance or disappearance of different strains [6, 26, 28]. All samples positive for rotavirus were subjected to PAGE thrice to confirm the reproducibility of the migration pattern of the genome. In this investigation samples from 220 diarrheic children of below 5 years of age were analyzed by RNA PAGE and 46 samples (20.9 %) were found positive (Table 1). However, in other studies rotavirus prevalence of 5–71, 13.48, 16.19, 23.4 % have been reported from different parts of the country [3, 5, 8, 17]. All the RNA PAGE positive samples exhibited 4-2-3-2 migration pattern (Zone I, II, III, IV) suggesting group A rotavirus [1]. The results showed that 31 of 135 (22.97 %) male children were found positive whereas rotavirus was detected in 15 of 85 (17.64 %) samples of female children (Table 2). Higher susceptibility of male children to rotavirus infection has also been

reported by previous research groups [5, 19]. Age wise susceptibility was also evaluated. Children below 1 year of age (89.13 %) were found to be more susceptible to rotavirus infection (Table 2). Similar results were recorded by other research groups, [10, 19] where children between 6 and 24 months were found to be more susceptible.

Electrophoretic pattern of the rotavirus positive samples obtained were compared with the reference strain SA11 (Fig. 1, Lane 4). Long pattern predominated over the short as out of 46 rotavirus positive samples 33 (71.74 %) exhibited long and 13 (28.26 %) showed short pattern. Predominance of long pattern over the short has also been reported [6, 8]. Contrary to our findings Phukan et al. [23] have found 48.48 % long pattern in North East India where short electropherotype predominated.

In this investigation segmented genome profiles were further analyzed on the basis of migration and co-migration of different segments within zone I, II and III. Migration and co migration based analysis of genome profile revealed that in one sample all the segments were resolved and distinct and it was of long pattern so designated as IIA subgroup (Fig. 1; Lane 9). In two samples segments 7, 8 of zone III co migrated and were of long pattern so grouped as IIC (Fig. 1; Lane 1). In twenty four samples segments 7, 8 and 9 of zone III co migrated and belonged to long pattern so sub grouped as IIG (Figs. 1, 2; Lane 3, 5, 6, 7, 10, 11, 13). Genome profile of six samples exhibited co migration of segments 2 and 3 of zone I and 7, 8 and 9 of zone III; it was of long pattern so designated as IIF (Fig. 2; Lane 12, 14). In one sample segments 8 and 9 of zone III co migrated but it was of short pattern so sub grouped as ID (Fig. 1; Lane 8). In twelve samples segments 2, 3 of zone I and segments 7, 8, 9 of zone III co migrated and were of short pattern so sub grouped as IF (Figs. 1, 2; Lane 2, 15). In the present study we have reported six different electropherotypes. However, 2, 5, 6 and 10 different electropherotypes have been reported from different parts of India by different research groups [2, 5, 6, 10].

The present study was also focused on isolation of rotavirus using MA104 cell line from stool supernatant found positive in RNA PAGE. Eight RNA PAGE positive

Fig. 1 Electrophoretic migration patterns of different rotavirus isolates belonged to group A. Lane 4 SA11 reference strain (R), Lane 1 subgroup IIC, Lane 2 subgroup IF, Lane 3, 5, 6, 7, 10 and 11 subgroup IIG, Lane 8 subgroup ID, Lane 9 subgroup IIA

Fig. 2 Electrophoretic migration patterns of different rotavirus isolates belonged to group A. Lane 12, 14 subgroup IIF, Lane 13 subgroup IIG, Lane 15 subgroup IF

fecal supernatant were randomly selected and used for virus isolation using MA 104 cell line. One sample got adapted in cell line and produced characteristic CPE at 48 h incubation followed by maximum at 72 h. CPE was characterized by cell rounding, lytic foci and cell detachment. The genome pattern of cell culture adapted strain was compared with the corresponding original human rotavirus from the stool suspension by polyacrylamide gel electrophoresis. Co-electrophoresis revealed that in both cases all the corresponding RNA segments migrated identically (Fig. 3, Lane 1, 2). Similar findings were also previously observed [30]. On the other hand changes in the arrangement of segments in culture adapted group A rotaviruses has also been reported [27]. Faecal supernatants found positive for rotavirus by RNA PAGE when analyzed by RT PCR method for VP4 gene an 876 bp product was

Fig. 3 Comparison of PAGE of RNA obtained from rotavirus grown in cell culture and corresponding stool sample RNA by co-electrophoresis. Lane 1 stool sample RNA (subgroup IIF), Lane 2 culture adapted rotavirus from same stool sample (subgroup IIF), Lane 3 SA11 reference strain (R)

obtained (Fig. 4, Lane 1, 2) which further confirmed group A rotaviruses.

In conclusion RNA PAGE method is a useful technique to get important epidemiological data on rotavirus disease outbreaks. The results clearly indicate that human rotaviruses belonging to group A are circulating among children of Uttar Pradesh, India. Further studies are required to determine the genotypes of the isolates.

Fig. 4 RT-PCR assay of rotavirus positive stool supernatants. Lane 1, 2 stool supernatant from two samples (product size 876 bp), Lane 3 negative control, M ladder (1 kb plus-Fermentas)

Acknowledgments We are grateful to all the doctors, medical/paramedical staffs for their cooperation during sample collection. The authors express their sincere gratitude to Dr. Gagandeep Kang, CMC, Vellore, India for providing SA11 reference strain of rotavirus. We thank NCCS, Pune, for providing MA-104 cell line. Authors have no conflicts of interest.

References

- Adah MI, Wade A, Taniguchi K (2001) Molecular epidemiology of rotaviruses in Nigeria: detection of unusual strains with G2P [6] and G8P [1] specificities. *J Clin Microbiol* 39:3969–3975
- Aijaz S, Gowda K, Jagannath HV, Reddy RR, Maiya PP, Ward RL et al (1996) Epidemiology of symptomatic human rotaviruses in Bangalore and Mysore, India, from 1988 to 1994 as determined by electropherotype, subgroup and serotype analysis. *Arch Virol* 141:715–726
- Anand T, Raju TA, Rao MV, Rao LV, Sharma G (2000) Symptomatic human rotavirus subgroups, serotypes & electropherotypes in Hyderabad, India. *Indian J Med Res* 112:1–4
- Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J (1990) Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 28:495–503
- Broor S, Ghosh D, Mathur P (2003) Molecular epidemiology of rotaviruses in India. *Indian J Med Res* 118:59–67
- Broor S, Husain M, Chatterjee B, Chakraborty A, Seth P (1993) Temporal variation in the distribution of rotavirus electropherotypes in Delhi, India. *J Diarrhoeal Dis Res* 11:14–18
- Centers for Disease Control and Prevention (2008) Rotavirus surveillance—worldwide, 2001–2008. *MMWR Morb Mortal Wkly Rep* 57:1255–1257
- Chakravarti A, Rawat D, Chakravarti A (2004) Molecular epidemiology of rotavirus in Delhi. *Indian J Pathol Microbiol* 47:90–93
- Chatterjee B, Husain M, Kavita SP, Broor S (1996) Diversity of rotavirus strains infecting pediatric patients in New Delhi, India. *J Trop Pediatr* 42:207–210
- Desai HS, Banker DD (1993) Rotavirus infection among children in Bombay. *Indian J Med Sci* 47:27–33
- Estes MK, Graham DY, Dimitrov DH (1984) The molecular epidemiology of rotavirus gastroenteritis. *Prog Med Virol* 29:1–22
- Estes MK, Kapikian AZ (2006) Rotaviruses. In: Knipe DM, Howley PM (eds) *Fields virology*, 5th edn. Lippincott Williams & Wilkins, Philadelphia, pp 1917–1974
- Gentsch JR, Glass RI, Woods P et al (1992) Identification of group A rotavirus gene 4 types by polymerase chain reaction. *J Clin Microbiol* 30:1365–1373
- Herring AJ, Inglis NF, Ojeh CK, Snodgrass DR, Menzies JD (1982) Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. *J Clin Microbiol* 16:473–477
- Iturriza-Gomara M, Green J, Brown DW, Desselberger U, Gray JJ (1999) Comparison of specific and random priming in the reverse transcriptase polymerase chain reaction for genotyping group A rotaviruses. *J Virol Methods* 78:93–103
- Ji S, Bi Y, Yang H, Yang F, Song J, Tao X, Cui X (2002) Cultivation and serial propagation of a new rotavirus causing adult diarrhoea in primary human embryo kidney cells. *Zhonghua Yi Xue Za Zhi (Natl Med J China)* 82:14–18 (in Chinese; English Abstract)
- Kang G, Kelkar SD, Chitambar SD, Ray P, Naik T (2005) Epidemiological profile of rotaviral infection in India: challenges for the 21st century. *J Infect Dis* 192(Suppl):S120–S126
- Kapikian AZ, Hoshino Y, Chanock RM (2001) Rotaviruses, fields virology, 4th edn. Lippincott Williams and Wilkins, London, pp 1787–1833
- Kelkar SD, Purohit SG, Simha KV (1999) Prevalence of rotavirus diarrhoea among hospitalized children in Pune, India. *Indian J Med Res* 109:131–135
- Kutsuzawa T, Konno T, Suzuki H, Kapikian AZ, Ebina T, Ishida N (1982) Isolation of human rotavirus subgroups 1 and 2 in cell culture. *J Clin Microbiol* 16:727–730
- Laemmli UK (1970) Cleavage of non-structural proteins during the assembly of the head of the bacteriophage. *Nature (London)* 227:680–685
- Matsui SM, Mackow ER, Matsuno S, Paul PS, Greenberg HB (1990) Sequence analysis of gene 11 equivalents from “short” and “supershort” strains of rotavirus. *J Virol* 64:120–124
- Phukan AC, Patgiri DK, Mahanta J (2003) Rotavirus associated acute diarrhoea in hospitalized children in Dibrugarh, northeast India. *Indian J Pathol Microbiol* 46:274–278
- Rasool N, Othman RY, Adenan MI, Hamzah M (1989) Temporal variation of Malaysian rotavirus electropherotypes. *J Clin Microbiol* 27:785–787
- Sato K, Inaba Y, Shinozaki T et al (1981) Isolation of human rotavirus in cell cultures. *Arch Virol* 69:155–160
- Singh V, Broor S, Mehta S, Mehta SK (1990) Molecular epidemiology of human rotavirus infections in Chandigarh, India. *Indian J Med Res* 91:9–14
- Smitalova R, Rodak L, Psikal I, Smid B (2006) Isolation, immunochemical demonstration of field strains of porcine group A rotaviruses and electrophoretic analysis of RNA segments of group A and C rotaviruses. *Vet Med Czech* 51: 288–295
- Svensson L, Uhnöo I, Grandien M, Wadell G (1986) Molecular epidemiology of rotavirus infection in Uppsala, Sweden, 1981; disappearance of a predominant electropherotype. *J Med Virol* 18:101–111

29. Tam JS, Kum WW, Lam B, Yeng CY, Ng MH (1986) Molecular epidemiology of human rotavirus infection in Hong Kong. *J Clin Microbiol* 23:660–664
30. Taniguchi K, Urasawa S, Urasawa T (1982) Electrophoretic analysis of RNA segments of human rotaviruses cultivated in cell culture. *J Gen Virol* 60:171
31. Tate JE, Chitambar S, Esposito DH et al (2009) Disease and economic burden of rotavirus diarrhoea in India. *Vaccine* 27:S18–S24