Detection and Characterization of Group A Rotaviruses (RVA) Detected In Diarrhoeic Children In Madhya Pradesh, Central India

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Abstracts: Background and objectives: Rotavirus is the leading cause of viral gastroenteritis throughout the world and is associated with up to 600000 deaths worldwide every year, of which more than 150,000 occur in India. This study was undertaken to detect and analyze the human rotavirus A (RVA) isolates from Madhya Pradesh, central India, between 2007 and 2008.Methods: Forty diarrhoeic samples from children up to the age of 5 years, admitted or visited the hospital, were screened using RNA-viral electrophoresis (PAGE), reverse transcription (RT)-PCR and selected isolates were further analyzed by sequencing.Results and interpretation: Incidence of RV was 32.5% in children (13/40) and all the isolates showed a typical migration pattern of 4:2:3:2, suggestive of group A RVs. All the PAGE positive samples yielded positive amplification in RT-PCR, confirming them to be human RVA. The VP7 gene sequence analysis of the selected isolates (H-14 and H-16) identified as G1 type revealed that these isolates form a cluster with Indian G1 isolates (mani63-06 and mani 365-07) and strain from Bangladesh (DH378) with sequence identity of more than 97% at amino acid levels. Simplot and boot scan analysis showed no recombination with other G1 strains.Conclusions: The G1 was detected to be the predominant genotype in this area of the country, which is helpful in selecting the vaccine strain. [Chandrashekar K et al NJIRM 2011; 2(4) : 72-76]

Key Words: Rotavirus, RT-PCR, VP7 gene, G1genotypes, Sequence-Phylogenetic analysis.

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Introduction: Diarrhoeal diseases continue to be a significant cause of morbidity and mortality in humans and animals of developing countries. There is a universal agreement that group A rotaviruses (RVA), members of the *Reoviridae* family, are the single most important cause of acute childhood diarrhoea in both developed and developing countries[1]. It has been estimated that up to 600000 deaths are caused by RV worldwide every year, of which more than 150,000 occur in India[2].

Rotavirus (RV) genome consists of 11 segments of dsRNA and encodes six structural (VP1–VP4, VP6, VP7) and six non-structural (NSP1–NSP6) proteins[1]. Analysis of the electrophoretic mobilities of the 11 segments of dsRNA by PAGE yields a pattern, which is both constant and characteristic for a particular RV group. According to the antigenic characteristics of the glycoprotein VP7 of the outer layer, RVA are further classified into different G types[3]. Till date, 25 G (G1-25) types have been described for different RV strains. Some of these genotypes have been found only in certain animal species or in humans and appear to be host-restricted while few of the genotypes have been found in humans and in some animal species both, showing high levels of nucleotide and amino acid homology, suggesting interspecies transmission of RVs[4].

In India, RV is a cause of significant morbidity and mortality among children younger than 5 years of age. The study of RV distribution is important to monitor the dynamics of RV strain and the emergence of novel strains[5, 6]. The perusal of literature revealed that the most common G types detected in India are G1, G2, G4, however, few others have also been detected including G3, G9 and G12. As the RV genome is segmented, it is possible and not uncommon for RVs to undergo reassortment events[4, 7]. Reassortment events result in new strains with variable antigenic properties[3, 4]. The efficiency of RV vaccines has to be followed with particular attention to RV genotypes circulating in the area. Though, different genotypes of RVs have been detected and reported from different parts of India, information is limited from central part of India. During this study, diarrhoeic faecal samples from children were screened using RNA-PAGE and analyzed further at molecular level by RT-PCR and sequencing.

Material and Methods: Collection of clinical material, processing and extraction of viral RNA :Forty diarrhoeic faecal samples were collected from Jabalpur and adjoining areas in Madhya Pradesh. The samples were collected from children below the age of 5 years, who admitted or visited the civil or private hospitals of the region during October 2007 to March 2008. Approximately, 10-20 ml of faecal sample was collected in pre-sterilized zip lock polythene bags. Samples were kept on ice and transported to the laboratory for processing. The 10% (w/v) faecal suspension was made in Phosphate Buffered Saline (0.2mM, pH 7.4; Sigma-Aldrich, St. Louis, USA). The samples were centrifuged at 5000 g for 20 min to remove the coarse particulate matter and the upper aqueous layer was filtered through 0.22 μm pore filter in a fresh tube. The suspensions were archived and stored at -20°C until further use.

Total RNA was extracted from 500 μL faecal suspension using equal volume of TriReag-ent-LS (Sigma-Aldrich, St. Louis, USA) following manufacturer's instructions. The RNA was extracted with Nuclease Free Water (NFW) in a final volume of 25 μL. The isolated RNA was assessed qualitatively and quantitatively using Nanodrop Spectrophotometer (ND-1000, Thermo-Scientific, USA) and stored at -70°C until further use.

Viral RNA electrophoresis (RNA-PAGE) : RNA-PAGE was carried out for the detection and initial screening of samples for RV on the basis of number of genome segments and their typical electrophoretic migration pattern in PAGE. The extracted viral RNA (~250 ng) was dissolved in 2x RNA sample buffer and heated at 56ºC for 5 min to dissolve the pellet. Subsequently, the samples were loaded and electrophorosed at 100 V till the dye came out of the gel using 1x Tris-Glycine buffer. The gel was silver stained and documented.

Reverse-transcription-polymerase chain reaction (RT-PCR): Reverse-transcription for cDNA synthesis from viral RNA was performed using Random Hexamer primer (0.2μg/μl, Fermentas, Lithuania). Initially, 50-100ng of viral RNA, 0.5μL Random

Hexamer primer, 2μL Dimethyl sulphoxide (DMSO) were added in a thin walled PCR tube following incubation of the reaction mixture at 95°C for 5 min for denaturing the RNA strands and immediately snap chilled on ice. Further, 4 μL of 5X RT buffer, 2 μL of 10 mM dNTPs (Fermentas, USA), 40U RNase Inhibitor (Ambion, USA) and 200U MMLV RT (Promega, Madison USA) were added and kept at 37°C in a thermo cycler (GeneAmp®, PCR System 9700, Applied Biosystems). The RT was denatured at 80°C for 5min at the end of the incubation step. The primers of Gouvea et al.[8] Beg9 (5'-3' position 1-28) GGCTTTAAAAGA GAGAATTTCCGTCTGG and End9 (5'-3' position 1062-1039) GGTCACATCATACA ATTCTAATCTAAG were used, for full length amplification of gene segment 9.

The PCR for amplification of the full length VP7 gene was performed by using 3 μL cDNA, 5 μL 10X PCR buffers, 2 mM $MgCl_2$, 2 μL DMSO, 1 μL dNTP, 0.2 μM of forward and reverse generic primers, 1.25 U of DNA polymerase and the volume was made up to 25 μL with NFW. The PCR amplification consisted of initial denaturation at 95°C for 4 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1min, followed by a final incubation at 72°C for 10 min. The PCR amplified products were resolved on 1% agarose gel.

The RT-PCR conditions viz. primer concentration, template concentration, number of cycles and different cyclic temperatures were first optimized with reference RV strains (NCDV, and UK). The positive field samples were detected specifically by using these optimized conditions of RT-PCR for amplification of full-length VP7 gene (1062 bp) of RVA.

Sequencing and Bioinformatics analysis :Selected PCR products of human RVA isolates (H-14 and H-16) were excised from the gel using QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and the gel purified PCR products were sequenced using the Big dye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc, CA, USA) following the manufacturer's instructions on an automated sequencer (Applied Biosystems, 3130 Genetic Analyzer, USA).

The sequence chromatogram was visualized in BioEdit v7.0.5 analysis software (Isis Therapeutics, Carlsbad, CA, USA). Mega Blast was performed with the deduced sequence within the non-redundant nucleotide database to confirm the presence of the gene specific to RVs. Group A rotavirus VP7 sequences from different geographical locations within India and rest of the world were retrieved from the NCBI nucleotide database and assembled into multiple sequence alignment using the Clustal W program of Lasergene 6.0 software (DNASTAR Inc, USA). The evolutionary lineage history was inferred using the Neighbor-Joining method with the bootstrap consensus tree inferred from 1000 replicates being taken to represent the evolutionary history. The recombination was assessed using the SimPlot program as described by Lole et al.[9].

GenBank Submissions: The nucleotide sequence data were submitted to NCBI GenBank with following Accession Number: HM355548 (H-14) and HM 355549 (H-16).

Result & Discussion: RNA electrophoresis has been used increasingly to study the molecular epidemiology of RV infection throughout the world. In the present study, RVs genome was detected in 13 diarrhoeic faecal samples, using RNA electrophoresis. Silver impregnated RNA-PAGE revealed classical 11 segments with 4:2:3:2 migration patterns, which is indicative of group A (RVA). The segment 3 & 4 and 7 & 8 were co-migratory (Fig 1). All samples showed long electropherotypes (based on migration of 10th and 11th genome segments) with exception of two human samples, which showed short electropherotypic pattern (H22 & H24) (Fig. 1). In this region, two types of electropherotypes were found to be circulating, which is in accordance with electropherotypes found in Chandigarh[10]. In contrast to our finding, six, ten and nine different electropherotypes were found in Manipur, Bangalore and Indonesia, respectively[11,12]. Variation in the sequence leads to change in the migration pattern of viral genome segments leading to appearance of different electropherotypes within same group of RVs[13].
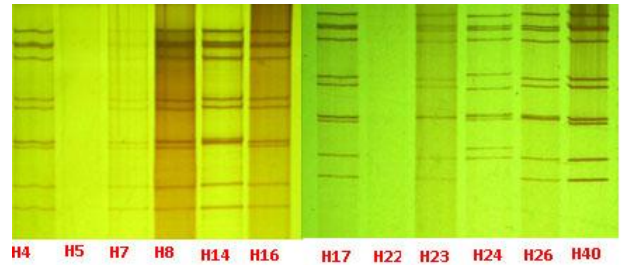

Fig. 1. RNA-PAGE of diarrhoeic faecal samples from children up to the age of 5 years. The gel was silver impregnated.

Of the 40 diarrhoeic human faecal samples, 13 children were found positive for the RV infection, with the overall incidence of 32.5%. Of the 15 male and 25 female diarrhoeic children, 05 and 08 children were found positive for RV infection with 33.3% and 32% occurrence, respectively. Below one year of age, 10 children were found positive for the virus out of 33 diarrhoeic children with prevalence of 30.3%, while 3 out of 4 children between 1 to 2 yrs of age were found positive for the RV with prevalence of 75%. Rotavirus infection in human beings is well documented with a variable degree of incidence. Our findings are in contrast to the report of Aijaz et al.[11] (1996) in which they reported prevalence of 21.6% and 11.2%, in Bangalore and Mysore, respectively. Similar lower prevalence was reported from Chennai (22.55%) and Vellore (18%)[14,15].

All PAGE positive human RVA samples were further tested in RT-PCR with full length amplification of VP7 gene (1062 bp) from all 13 samples (Fig. 2). Randomly, VP7 gene sequences from two isolates viz. H-14 (898 bp) and H-16 (232 bp) were selected and analyzed using automated RV genotyping tool (RotaC) which confirmed G1 genotypes detection in this part of central India. The phylogram clustered all G1 isolates in two groups (Fig.3a). The H-14 and H-16 RVA isolates clustered with G1 isolates from Bangladesh (DH378), Thailand (CMH036-04, Thai-2104), South Africa (SA4799DGM), South Korea (Seoul-697) and Indian RV strains from Manipur (mani140-06, mani63-06 and mani365-07).
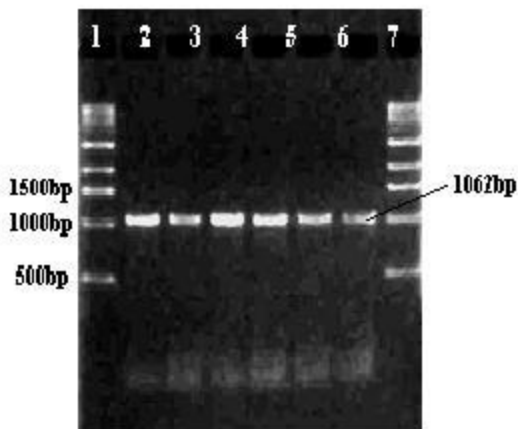
Fig. 2: Reverse transcription-PCR amplifying full length (1062 bp) VP7 gene of human rotaviruses. Arrow indicates – 1062 bp VP7 gene specific product. Lane indicates – 1 and 7: 1kb DNA ladder (Fermentas); 2 to 6 field RV isolates with amplicons of VP7 gene.
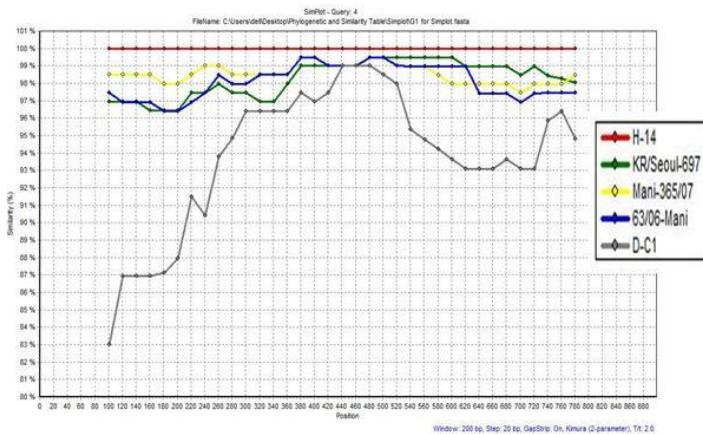

Fig.3a. Phylogenetic and percent sequence identity of Human G1 RV isolates (H-14 and H-16) from MP, central India with other RV G1 isolates. The H-14 and H-16 are highlighted in green dots. The phylogenetic analysis was completed with MEGA4 software that used a neighbor-joining algorithm and absolute distances and that followed 1,000 bootstrap replicates. The tree is based on the partial sequence of the VP7 gene.

The H-14 isolates showed highest sequence homology of 99.3% at amino acid level with RV G1 isolates from Bangladesh (DH378) followed by 99% with Thai-2104 strain from Thailand (Fig.3a). The H-14 isolate showed minimum sequence homology of 92.6% with G1 strain from Pakistan. The another RVA isolate, H-16, showed highest sequence homology of 97.4% at amino acid level with G1 isolates from Bangladesh (DH378), Thailand (CMH036-04), South African (SA4799DGM), and mani365-07 strain from Manipur, India. The H-16 isolates showed minimum sequence homology of 77.9% with G1 isolate from Vietnam (NT173) (Fig.3a). Similarity plot (Simplot) and boot scan analysis showed no recombination with other strains (Fig.3b). The initial part of the VP7 gene sequence showed higher similarity with Manipuri strain (mani 63-06 and mani365-07) and later part with South Korean (Seoul-697) strain (Fig.3b).
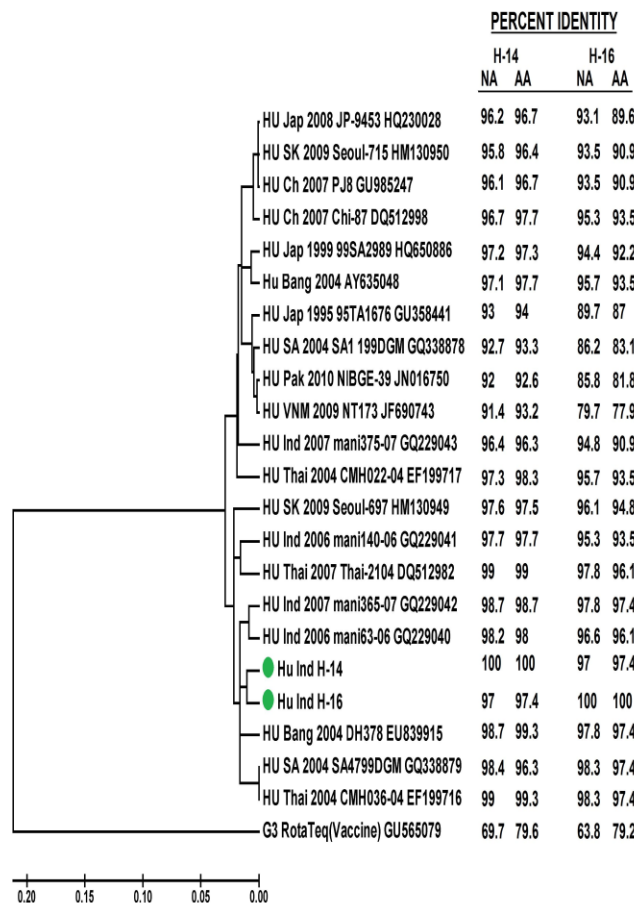
3b. Similarity plot showing relatedness of various G1 isolates VP7 gene sequences.

Conclusion: the data on the molecular epidemiology of RVs in this part of the country has shown the higher incidence of human RVA with predominance of G1 genotype and implementation of the use of vaccine carrying G1 RVA type for preventing the diarrhoea in children.

References:

1. Estes M. and Kapikian A. Rotaviruses. In: Knipe DM., Howley PM., Griffin DE., Lamb RA., Martin MA., Roizman B and Straus SE. (eds), Fields Virology, 5th edition, 2007, Kluwer Health/Lippincott, Williams and Wilkins; Philadelphia; P. 1917-1974.

2. Parashar UD., Gibson CJ., Bresse JS., Glass RI. Rotavirus and severe childhood diarrhea. Emerging Infectious Diseases. 2006; 12: 304–306.

3. Matthijnssens J, Ciarlet M., McDonald SM., Attoui H., Banyai K., Brister JR., Buesa J., Esona MD., Estes MK., Gentsh JR., Iturriza-Gomara M., Johne R., Kirkwood CD., Martella V., Mertens PPC., Nakagomi O., Parreno V., Rahman M., Ruggeri FM., Saif LJ., Santos N., Steyer A., Taniguchi K., Patton JT., Desselberger U. and Van Ranst M. Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). Archives of Virology. 2011 Online, 20 May 2011. DOI 10.1007/s00705-011-1006-z.

4. Gentsch JR., Laird AR., Bielfelt B., Griffin DD., Banyai K., Ramachandran M., Jain V., Cunliffe N A., Nakagomi O., Kirkwood CD., Fischer TK., Parashar UD., Bresee JS., Jiang B and Glass RI. Serotype diversity and reassortment between human and animal rotavirus strains: implications for rotavirus vaccine programs. Journal of Infectious Disease. 2005; 192(S1):146-159.

5. Jain V, Das BK., Bhan MK., Glass RI., Gentsch JR. The Indian Strain Surveillance Collaborating Laboratories. Great Diversity of group A rotavirus strains and high prevalence of mixed rotavirus infections in India. Journal of Clinical Microbiology. 2001; 39:3524-3529.

6. Kelkar SD, Purohit SG., Simha KV. Prevalence of rotavirus diarrhoea among hospitalized children in Pune, India. Indian Journal of Medical Research. 1999; 109: 131-135.

7. Iturriza-Gómara M., Wong C., Blome S., Desselberger U and Gray J. Molecular characterization of VP6 genes of human rotavirus isolates: correlation of genogroups with subgroups and evidence of independent segregation. Journal of Virology. 2002; 76: 6596–6601.

8. Gouvea V, Glass RI., Woods P., Taniguchi K., Clark HF., Rorrester B. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. Journal of Clinical Microbiology. 1990; 28(2):276-282.

9. Lole KS, Bollinger RC., Paranjape RS., Gadkari D., Kulkarni SS., Novak NG., Ingersoll R., Sheppard HW., Ray SC. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. Journal of Virology. 1999; 73(1):152-160

10. Singh V, Broor S., Mehta S., Mehta SK. Molecular epidemiology of human rotavirus infections in Chandigarh (India). Indian Journal of Medical Research. 1990; 91: 9-14.

11. Aijaz S, Gowda K., Jagannath HV., Reddy RR., Maiya PP., Ward RL. Epidemiology of symptomatic human rotaviruses in Bangalore and Mysore, India, from 1988 to 1994 as determined by electropherotype, subgroup and serotype analysis. Archives of Virology. 1996; 141:1-12.

12. Albert MJ, Soenarto Y., Bishop RF. Epidemiology of Rotavirus Diarrhea in Yogyakarta, Indonesia, as Revealed by Electrophoresis of Genome RNA. Journal of Clinical Microbiology. 1982; 16(4): 731-733.

13. Sukumaran M, Gowda K., Maiya PP., Srinivas TP., Kumar MS., Aijaz S. Exclusive asymptomatic neonatal infections by human rotavirus strains having subgroup I specificity and "long" RNA electropherotype. Archives of Virology. 1992; 126: 239-251.

14. Brown DWG, Mathan MM., Mathew M., Martin R., Beards GM., Mathan VI. Rotavirus Epidemiology in Vellore, South India: Group, Subgroup, Serotype, and Electropherotype. Journal of Clinical Microbiology. 1988; 26(11): 2410-2414.

15. Saravanan P, Ananthan S., Ananthasubramanian M. Rotavirus infection among infants and young children in Chennai, South India. Indian Journal of Medical Microbiology. 2004; 22(4): 212-221.