DOI: 10.7860/NJLM/2020/42555:2378

Original Article

Microbiology Section

Predominance of G12 Genotype in Rotavirus Strains Causing Diarrhoea in Hospitalised Children of a Tertiary Care Centre in Lucknow, Uttar Pradesh, India

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ABSTRACT

Introduction: Currently available vaccines for rotavirus are based on the common genotypes of neutralisation antigens Viral Protein 7 (VP7) and Viral Protein 4 (VP4). Prevalence of uncommon genotypes may result in lower immune response due to homotypic immunity.

Aim: To ascertain the genotypes of VP7 and VP4 in rotavirus strains causing diarrhoea in northern India.

Materials and Methods: The cross-sectional study was conducted from January 2010 to June 2012 at a tertiary care centre in Lucknow, Uttar Pradesh, India. Consecutive stool samples from children under the age of five years hospitalised for acute dehydrating diarrhoea were screened for rotavirus antigen by Enzyme Linked Imunosorbent Assay (ELISA). VP7 and VP4 genes were amplified by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), sequenced (Big-Dye terminator kit using 3130 ABI, Genetic analyser) and genotyped by Rota C software. Molecular Evolutionary Genetics Analysis (MEGA) 5.05 Software was used for phylogenetic analysis.

Results: Of the 260 samples, 58 (22.3%) samples were positive by ELISA. Thus the prevalence of rotavirus infection was 22.3% [95% CI (27.59-17.41)]. Out of these VP7 gene was amplified by RT-PCR from 35 strains of which 32 amplicons were sequenced. Total 14 (43.7%) strains belonged to genotype G12, 3 (9.3%) strains to genotype G9, 7 (21.8 %) to genotype G2 and 8 (25%) were G1. Of the 18 VP4 genes sequenced 7 (38.8%), 6 (33.3%) and 5 (27.7%) strains were genotype P(4), P(6) and P(8) respectively.

Conclusion: Prevalence of rotavirus diarrhoea was 22.3%. Presence of G12 an emerging genotype was the most common VP7 genotype and a rare reassortment strain G9 P(4) was the most significant finding. As G12 genotype is not present in the current vaccines, it could be included in future versions to improve effectiveness.

Keywords: Gene, Molecular epidemiology, Stool sample, Vaccine

INTRODUCTION

Rotaviruses are gastrointestinal pathogens responsible for severe dehydrating diarrhoea in infants and children below five years of age. The mortality among young children attributed to rotavirus is over half a million annually worldwide [1]. Rotaviruses are double stranded RNA viruses belonging to the family Reoviridae.

Mature viral particles are enveloped, 100 nm in diameter with icosahedral symmetry. A triple layered capsid surrounds a genome of 11 segments of double stranded RNA. The genome codes for six structural proteins, viral protein VP1, VP2, VP3, VP4, VP6 and VP7 and six non-structural proteins (NS) NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6. A binary system based on neutralisation antigens G (VP7) and P (VP4) is used to characterise the strains. More than 35 G and 50 P types are currently known to occur in the rotavirus strains infecting human and zoonotic populations [2]. The mainstay for treatment of rotavirus diarrhoea has been to correct dehydration which is mostly severe and death inflicting. But as most patients do not reach hospitals timely many deaths can not be averted. Therefore, the strategy has now shifted from management to prevention. In the past decade two vaccines have been introduced globally as well as in India. These are Rotarix and Rotateq. The former is monovalent human RVA G1P8 strain (GlaxoSmithKline Biologicals, Rixensart, Belgium), while the latter is pentavalent human bovine reassortment vaccine comprising of G1, G2, G3, G4 and P8 strains (Merck Vaccines, Whitehouse Station, NJ, USA). The underlying strategy of these vaccines is immunity against the prevalent genotypes of VP7 and VP4. Both of them have not been very effective in the developing countries with efficacy in the range of 39%-49% [3,4]. In India the seroconversion rates reported are 58.3% and 83% respectively [5]. To increase our understanding of the response to these vaccines we need molecular epidemiological data about common genotypes included in the vaccines as well as some emerging genotypes in both prevaccine and post-vaccine era.

This study was undertaken to characterise molecular footprint of the rotavirus strains causing diarrhoea in under five children in the study area. This is the first study to be conducted on rotavirus molecular epidemiology with phylogenetic analysis from this geographical area.

MATERIALS AND METHODS

The present study had cross-sectional study design and was carried out from January 2010 to June 2012 at King George's Medical University, Lucknow, Uttar Pradesh, India. Sample size was calculated according to the formula. $N=p.q\ Z.\alpha/2)^2/d^2$; where N = sample size, p=population proportion, q = 1-p, d=precision (permissible error) of the estimate, z=normal deviate (1.96 for an alpha (type 1 error) of 0.05 at 95% confidence interval). Therefore, taking the following values -$(Z.\alpha/2) = 1.96$, d = .05, p = 19.2% (Mishra V et al., (2009) and q = 80.8%, the sample size (N) was calculated to be 256 [6]. Prior to commencement of the study approval from the Institutional ethics committee was obtained (Ref Code: XLIV ECM/B-P10). Subjects under the study comprised of infants and children under the age of five years admitted to the emergency unit of paediatric department with complains of diarrhoea and dehydration. Written informed consent was taken prior to collection of samples. Diarrhoea is defined as passage of more than 3 loose or watery stools per day. Patients with dysentery (diarrhoea with blood

in stools with or without mucus), persistent diarrhoea (duration more than 2 weeks) and parents not giving consent were excluded from the study. Presence of any unrelated complication was not a factor for rejection. The subjects were consecutively enrolled and stool specimens were collected.

ELISA

Stool samples were tested for rotavirus antigens by a commercial kit using a sandwich format (RIDASCREEN, R- Biopharm,Germany).

RNA Extraction and cDNA construction

The procedure was adapted from Banyayi K et al., [7]. Faecal suspension (10%) in Phosphate buffered saline (2 mM) was centrifuged and the supernatant (250 µL) was subjected to RNA extraction by QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany). Complementary DNA (cDNA) was constructed with random hexamers (2 µM) (Invitrogen, Carlsbad, USA), Moloney murine leukaemia virus (M-MLV) reverse transcriptase (100200U), dntp (0.5µM) and RNAse inhibitor (20U) (SIGMA-Aldrich Corporation, St Louis, USA) in a total reaction mixture of 30 µL. In short RNA (2-5 µL) was mixed with random hexamers and dntps and the 15 µL mixture was incubated at 95°C. The hot mixture was immediately cooled on ice for 5 minutes. Reverse transcriptase and RNAse inhibitor were added next after the mixture was cooled followed by incubation for 10 minute at 25°C succeeded by 60 minutes at 37°C. The cDNA was kept at -20°C.

RT-PCR

VP7 and VP4 gene were amplified by using protocols adapted from Iturriza Gomara M et al., and Gentsch JR et al., [8,9]. The cDNA (2-5 µL) was mixed with 1×reaction buffer, taq polymerase (1.5 U) (DyNAZymeTM; Fisher Scientific UK Ltd., forward and reverse primers (.4 µM), dntps (0.2 µM) made upto a reaction volume of 25 µL. The PCR reaction was put up in a thermal cycler (ABI Veriti, Applied Biosystems, Foster City, USA) under the parameters: Initial denaturation at 94^{0}C for 10 minutes, followed by 35 identical cycles of denaturation for 1 minute, followed by primer annealing at 55°C for 1 minute and extension at 72°C for 1 minute. A final extension step at 72°C for 10 minutes was included. The products of amplification obtained were demonstrated on 1.5% agarose gels embedded with ethidium bromide [Table/Fig-1].

[Table/Fig-1]: Agarose gel electrophoresis showing amplicons of VP7 gene and VP4 gene. Amplicons of base pair size 881 in lane 4, 5 and 6 are of VP7. Amplicons of VP4 gene (876bp) are seen in lane 8 and 9. A 100 base pair molecular marker is run in lane 10. Lane 6 and 9 are of control strain Wa-1.

The primers were obtained from (Imperial Life Sciences Ltd., Gurgaon, India) [8,9] [Table/Fig-2]. The control strains were provided by National Institute of communicable Diseases, Kolkata, West Bengal, India.

Gene	Primer sequence
VP7 (F)	5'ATGTATGGTATTGAATATACCAC3'
VP7 (R)	5′ AACTTGCCACCATTTTTTCC 3′
VP4(F)	5'TGGCTTCGCCATTTLATAGACA3'
VP4 (R)	5'ATTTCGGACCAT'LTATAACC 3'

[Table/Fig-2]: Forward and reverse primers (and their sequence) of VP7 and VP4 genes.

Sequencing

PCR products were extracted from gel using Pure Link gel extraction kit (Invitrogen Carlsbad, USA). Sequencing was conducted by the dideoxy nucleotide chain termination method with the Big Dye terminator cycle sequencing reaction kit (Applied Biosystems, Foster City, USA) on an automated sequencer (ABI Prism 3100xl, Applied Biosystems, Foster City, USA). The primer set used in sequencing was same as that of amplification.

DNA and Protein Sequence Submission

Searches for nucleotide and protein sequence similarity were done by using the BLAST (Basic Local Alignment Search Tool) on the NCBI (National Center for Biotechnology Information) website http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Genotypes were assigned using Rota C rotavirus genotyping tool on the website http://rotac.regatools.be/according to the recommendations of Rotavirus Classification Working Group (RCWG) [10]. Sequences were submitted to GenBank database using the BanKit v3.0 tool and accession numbers obtained.

STATISTICAL ANALYSIS

Both the protein and nucleic acid sequences were aligned among themselves as well as with prototype strains-Wa, KUN, DS-1, AU-1, N26, RV176 and EW (sourced from Genbank) using CLUSTAL W. Statistical method of UPGMA (unweighted pair group method with arithmetic mean) using Kimura 2- parameter and 1000 bootstrap replicates was used to draw phylogenetic trees by MEGA version 5.05 software [11].

RESULTS

A total of 270 subjects were enrolled for the study. Out of these, stool sample could not be obtained for 10 children. Eventually samples were processed for 260 subjects. Out of 260 stool samples tested with EIA, 58 were positive for rotavirus antigen. Thus the prevalence of rotavirus infection was 22.3% {95% Confidence Interval (27.59-17.41)}

RT-PCR for VP7 and VP4 Gene: VP7 gene by RT-PCR was amplified from 42 samples. VP4 gene was amplified from 25 samples.

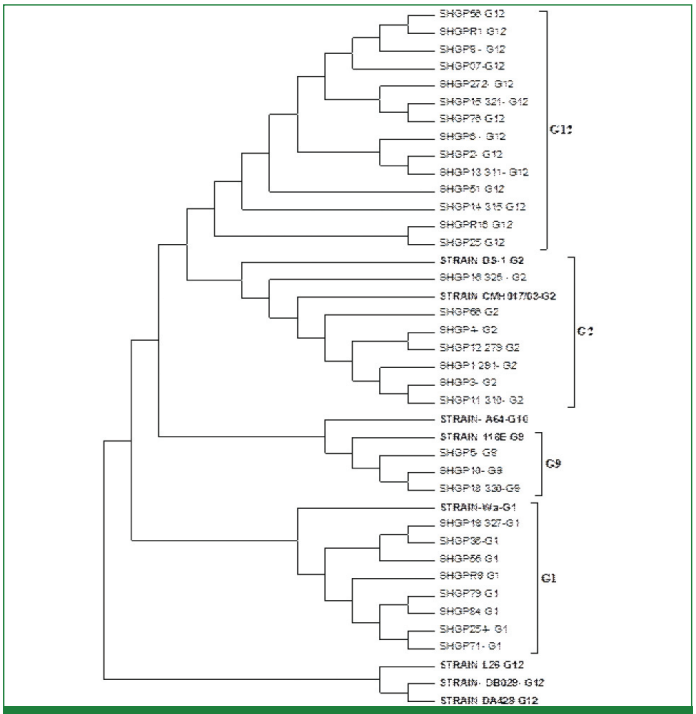
Sequencing: Products showing faint bands or more than one band on gel electrophoresis were excluded and 32 VP7 and 18 VP4 strains were sequenced and subjected to genotyping using the Rota C software [12]. Of the 32 VP7 genes sequenced, total 14 (43.7%) strains belonged to genotype G12, 8 (25.0%) to G1, 7 (21.8%) to genotype G2 and 3 (9.3%) strains were genotype G9. Of the 18 VP4 genes sequenced 7 (38.8%), 6 (33.3%) and 5 (27.7%) strains were genotype P(4), P(6) and P(8) respectively [Table/Fig-3]. Phylogenetic trees for VP7 and VP4 genes are displayed in [Table/Fig-4,5] respectively. The accession numbers of sample strains are: VP7 -: (KF179265 to KF179296) and for VP4 are (KF598830 to KF598847). The accession numbers of the reference strains shown in the tree are: VP7-: {DS-1(G2)-AB118023}; (A64(G10)X63156); (116E(G9)-L14072}; {L26(G12)-M58290}; {DB029(G12)EU179533}; {DA428(G12)- EU179537}. VP4-: {RV5(P4)-M32559}; {Wa(P8)- M96825}; {1076(P6)-M88480}. The sample G12 strains are on a different branch compared to the reference G12 strains.

DISCUSSION

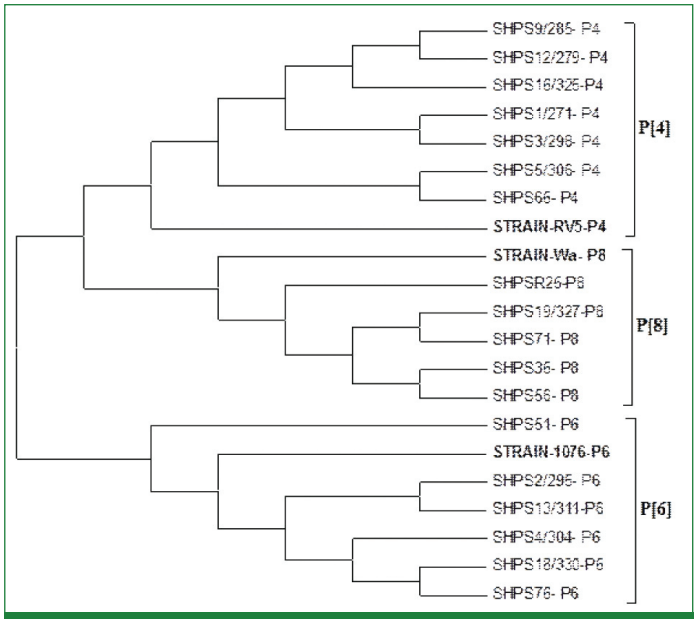
Globally the most common genotypes of rotavirus strains causing diarhoea are G1, G2, G3 and G9 [13,14]. G5 genotype, previously found only in pigs and horses, was detected in Brazilian children in 1983 and is now commonly reported from Brazil at high rates [15] Similarly, G8 rotaviruses are found predominantly in the African continent especially in Malawi [16]. The most common serotype combinations globally have been G1P(8) and G2P(4) with G3, G9 and G12 types occurring in lesser percentages.

No	ISOLATE	VP7 Genotype	VP4 Genotype
1	LKO/R1	G12	-
2	LKO/36	G1	P(8)
3	LKO/R9	G1	-
4	LKO/51	G12	P(6)
5	LKO/55	G1	-
6	LKO/56	G12	P(8)
7	LKO/R25	G12	P(8)
8	LKO/71	G1	P(8)
9	LKO/R16	G12	-
10	LKO/84	G1	-
11	LKO/66	G2	P(4)
12	LKO/76	G12	P(6)
13	LKO/79	G1	-
14	LKO/254	G1	-
15	LKO/272	G12	-
16	LKO1/271	G2	P(4)
17	LKO2/295	G12	P(6)
18	LKO3/298	G2	P(4)
19	LKO4/304	G2	P(6)
20	LKO5/306	G9	P(4)
21	LKO6/310	G12	-
22	LKO9/285	G12	P(4)
23	LKO10/308	G9	-
24	LKO11/310	G2	-
25	LKO12/279	G2	P(4)
26	LKO13/311	G12	P(6)
27	LK014/315	G12	-
28	LKO15/321	G12	-
29	LKO16/325	G2	P(4)
30	LKO18/330	G9	P(6)
31	LKO19/327	G1	P(8)
32	LKO/07	G12	-

[Table/Fig-3]: Rotavirus isolates sequenced and genotyped.



[Table/Fig-4]: Phylogenetic tree for VP7 Gene: The evolutionary history was inferred using the Neighbor-Joining method. Reference strains are shown in bold. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.



[Table/Fig-5]: Phylogenetic tree for VP4 Gene: The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree was inferred from 500 replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Reference strains are shown in bold.

In Europe, the European rotavirus surveillance network, EuroRotaNet has also conveyed similar findings. Most of the strains isolated were G1P(8), G2P(4), G3P(8), G4P(8), and G9P(8). G1P(8) remained the most prevalent genotype in Europe also with about 50% strains belonging to it. There was also a significant diversity of cocirculating strains [17]. Some of the representative studies done globally on genotypes and strains prevalent are summarised in [Table/Fig-6] [18-30]. Some of these studies have reported presence of emerging genotype G12 in higher numbers [20,21,24].

In India rotavirus isolates show a lot of genetic variation. The most common genotypes found in the strains causing diarrhoea are G1P(8), G2P(4) and G9P(6) [27-33]. A few studies from northern India (New Delhi and Manipur) have also reported isolation of G12 strains [34,35]. Giri S et al., in a pan-India study (2019) [28] reported a 12.1% prevalence of G12 genotype while in study by George S et al., from Kerala reported only G1 and G9 strains [30].

Contrary to the global and national trends, in this study the predominant VP7 genotype was G12 with 43.75% strains belonging to it, 21.8% typed to genotype G2 and 25% were G1. Only 9.6% strains were G9. G12 was found in combination with P(6) and P(8), G1 with P(8) and G2 with P(4). G9 combined with both P(6) and P(4).

G12 rotaviruses were first reported from the Philippines in 1987 [36] after which there was a period of quiescence of a decade after which they reappeared and were subsequently found in Thailand [37] and United States [38]. Subsequently they were reported from India [39], Japan [40], Nepal [41], Bangladesh [42], Hungary [43]. Uchida R et al., from Nepal reported a 20% prevalence of G12 [23]. In another Nepalese study reported by Ansari S et al., the G12 prevalence has increased to 55.9%. Thus, the G12 strains have advanced their footprint across the planet in the first two decades of this century. Nepal, and northern India seem to be have an emergence of G12 as a dominant strain.

The G12 genotypes detected globally have been highly homologous in their sequences indicating a common ancestor. However, the occurrence of different VP4 types in combination with G12 rotavirus, similar to G9 strains within the Indian subcontinent, has been due to reassortment with other gene segments [38,39]. Rahman M et al., made an interesting observation that the first G12 rotavirus prototype strain, L26, has porcine-like NSP2 and NSP5 genes, indicative of G12 having an animal origin [42].

S no	Author	Year	Geographical area	Most prevalent strains/genotypes	Reference
1	Kheyami AM et al.,	2004-05	Saudi arabia	G1P(8)(44%),G2P (4)(20%)	[18]
2	Tian Y et al.,	2011-16	Bejing China	G9 (64.4%), P (8) (87.0%)	[19]
3	Udeani TK et al.,	2013-14	Nigeria	G3P(6)(19.4%), G12P(8)(16.6%), G1P(6) (13.8%) G12 (30.5%), G1(25%),G3(27%)	[20]
4	João ED et al.,	2012-13	Mozambique	G2 (32.4%), G12 (28.0%), P(4) (41.4%) and P(6) (22.9%); G2P(4) (42.3%	[21]
5	Bone-cisse C et al.,	2010-13	Côte d'Ivoire	G12 (46.6%), G1 (13.1%), P(8) (49.8%). G12P(8) (26.6%)	[22]
6	Uchida R et al.,	2003-04	Nepal	G1P (8) (70%), G12 strains with P(8) or P(6) were 20%	[23]
7	Ansari S et al.,	2011	Nepal	G12 (55.9%) with either P(6),P (4) or P(8).	[24]
8	Nokes DJ et al.,	2002-04	Kenya	P(8)G1 (42%), P(8)G9 (15%), P(4)G8 (7%), P(6)G8 (6%), and P(8)G8 (4%)	[25]
9	Nyango J et al.,	2000-02	Kenya	G1 (21%), G2(12%), G3 (0.9%) and G9 (7.4%),	[26]
10	Saravaran P et al.,	1995-99	Chennai, India	G2 (66.1%), G4 (13.6%), G1 (9.3%) ,G3 (1.7%).	[27]
11	Giri S et al.,	2012-16	India	Southern India : G1P(8)(56.3%), G2P(4)(9.1%), G9P(4)(7.6%), G9P(8) (4.2%), and G12P(6)(3.7%) d Northern India: G1P(8) (36%), G9P(4)(11.4%), G2P(4)(11.2%), G12P(6) (8.4%), and G3P(8)(5.9%).	[28]
12	Sharma A et al.,	2010-11	India	20% prevalence rate for rotaviruses	[29]
13	George S et al.,	2013	India	G1 (80.95%) and G9 (19.04%). G1P8-predominant strain.	[30]

[Table/Fig-6]: Prevalence of different genotypes of VP4 and VP7 gene reported globally [18-30].

In our study too, the G12 sample strains were homologous but did not appear to be phylogenetically close to the reference G12 strains DA428 and the original L26 strain isolated from Phillipines in 1987 [36]. This finding was significant as it denotes evolutionary diversification of the G12 strains circulating in this geographical area.

Compared to the VP7 gene we were not very successful in amplifying the VP4 gene. Reason could be point mutations at the primer binding sites which can result in failures of PCR detection and genotyping [44]. No uncommon genotype was found in the VP4 gene.

Two oral vaccines have been recommended by WHO (World Health Organisation) and are being used by paediatricians. They are Rotarix™ (GlaxoSmithKline Biologicals, Rixensart, Belgium) and RotaTeq™ (RV5, Merck, Whitehouse Station, NJ, USA). Rotarix™ contains a monovalent live attenuated human G1P(8) strain, whereas RotaTeq contains 5 reassortant RVA strains, on a bovine framework with a human RVA VP7 (G1-G4) or VP4 (P(8)) gene segment. Both the vaccines have not been as effective as compared to the west [3,45,46]. It has been argued that genotypic diversity of rotavirus in developing world is responsible for this phenomenon. Both these vaccines don't have a G9 and G12 component. Type specific immunity for protection is considered important by many workers and this could be one of the reasons. A study from National Institute of Virology, Pune on diarrhoea samples has also reported the presence of uncommon genotype G12 and a rare reassortment strain G9P [4,47]. We have also found G9P [4] in a single strain. Therefore molecular surveillance for presence of uncommon genotypes is the need of the hour. As this study reports the presence of G12 genotype in good numbers which is not common in other parts of the world, it can be argued that this genotype should be included in future versions of existing vaccines.

LIMITATION

As the cDNA of the sample was limited in quantity, we could not repeat RT-PCR and sequencing for the strains which could not be typed.

CONCLUSION

Diarrhoea due to rotavirus infection had a prevalence of 22.3%. G12 was the most common VP7 genotype while P(4) was the most common VP4 genotype of the rotavirus strains. Surveillance for other emerging genotypes should be done routinely to assess the molecular epidemiology of rotavirus infection.

Funding

Indian Council of Medical Research has provided financial support in the form of fellowship and contingency to MD- PhD scholar Dr Shilpi Srivastava and funding for the Project 'To develop a grade 1 diagnostic virology laboratory in Uttar Pradesh, India.

ACKNOWLEDGEMENTS

We would like to acknowledge Indian Council of Medical Research for their financial support and National Institute of Communicable Diseases Kolkata for providing control strains.

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FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **Jul 31, 2019**
Date of Peer Review: **Aug 25, 2019**
Date of Acceptance: **Oct 25, 2019**
Date of Publishing: **Jan 01, 2020**