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ORIGINAL ARTICLE

Types of Rotavirus Causing Acute Diarrhea among Children in Western India, their Demographic Pattern and Disease Severity

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ABSTRACT

Background: Rotavirus infection is a major cause of severe acute gastroenteritis among infants and children all over the world[1] with winter out-breaks of diarrhea in temperate and cooler parts almost round the year. However, this varies in different part of India.[2-6] Diarrhea is a major cause of under-5 mortality, contributing to approximately over 1,50,000 infant deaths in our country per year.[15,16] Different genotypes have been identified and many more are emerging by way of mutation, genetic shift and genetic drifts. Rotavirus are classified antigenically as A (Most common), B, C, D, E by ELISA and genotypically as G (1through 12) and P (1 through 8) by Reverse Transcriptase PCR, in combinations.

Materials and methods: Stool samples of 110 infants and children from 6 to 60 months of age, with suspected viral diarrhea over one year period were studied for serotypes and genotypes; and compared for their respective disease severity.

Results: Thirty-four percent were found positive for Rotavirus-A by ELISA. Of the positive, 33.4% were found to be of G9 genotype, much higher than reported from other parts of the country. On the other hand, merely 13.6% of G1 and G4 each were detected, contrary to high prevalence elsewhere. On electropherotyping, the long-arm types were associated with more severe disease (64.6% showing moderate to severe dehydration) than their short-arm types (Only 16.6% showed moderate dehydration only) $p < 0.009$. No difference in incidence of severe dehydration between AD positive for Rotavirus (11.7%) and those found negative (11.8%), presumably due to other viruses, after excluding invasive diarrhea.

Conclusion: Emergence of diverse strains, i.e. more of G9 and G12 genotypes than earlier reports of G1 and G2 types indicate considerable genetic shift in the region. Such trend could have significant implication on degree of seroconversion from currently used live vaccines, using G1 or bovine reassortant G1-3 strains only, seen in recent studies from Africa and Malayi.[29] Contrary to claims that Rotavirus diarrhea usually threatened severe diarrhea, no significant difference in incidence of severe diarrhea was observed between Rotavirus positive and Rotavirus negative acute diarrhea.

Keywords: Diarrhea, Dehydration, Rotavirus, ELISA, Reverse Transcriptase PCR, RNA PAGE (Electropherotyping).

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INTRODUCTION

Diarrhea is an important public health problem in the tropical countries, especially in developing countries like India. It is a major cause of infantile morbidity and mortality world-wide.[1-6] Factors, such as humidity, temperature, climate, sanitation and socioeconomic conditions, contribute to this in a major way.[7,8] Acute diarrhea may be caused by bacteria, virus or parasites. A great majority of cases are due to viruses (Rotavirus 10-35%, Norovirus 2-20%, Astrovirus, Adenovirus 2-10%, Calcivirus, Corona virus, Norwalk virus, etc). Viruses are responsible for more than half of all diarrheas during infancy.[9] In another 45 to 60% cases, no causal agent is detected, possibly due to untypable viruses.

The name Rotavirus is coined from the Latin word Rota (Meaning wheel), because the virus has a distinct wheel like shape. The genome of rotavirus consists of 11 segments of double stranded linear molecules of RNA which are 18,555 nucleoside base pairs. The RNA is surrounded by a double icosahedral protein capsid. The viral particle measures 60 to 80 nm in diameter and is not enveloped.

One of the unique features of rotavirus is its genetic, antigenic and geographical diversity, making it difficult to design a universal effective vaccine. But a sound knowledge regarding types and subtypes of rotaviruses circulating in different regions of India would help in understanding the basis for efficacy or nonefficacy of vaccines which have been designed against this virus.[10]

Reassortment of various rotavirus strains is an important mechanism for generation of their novel and unusual strains. A significant number of children also have mixed rotavirus

infections. There are very few studies in terms of different antigenic and genetic variants from various regions of India so far.

MATERIALS AND METHODS

A total of 110 stool samples were collected in a sterile container from children having acute diarrhea (As defined by WHO) aged between 6 and 60 months, attending pediatrics outpatient Department at MGM Hospital, Kalamboli, Navi Mumbai from 1st July 2009 to 31st December 2011. Presence of tenesmus, frank blood in stool or pus cells in sheets or more than 10 per high power field on microscopy were excluded, presuming these to be of bacterial etiology.

Assessment of Severity

Proper history taking and detailed clinical examination was carried out. The severity of diarrhea was assessed with Vesikari scoring system,[11] based on duration of diarrhea, maximum number of stools passed per day, vomiting, grade of fever, severity of dehydration, altered sensorium and requirement of intravenous fluids. Since, accurate temperature measurements were not possible in the field, it was recorded as normal, low-grade or high-grade fever in the history, as reported by the caregivers. An episode was considered mild for a score of ≤ 5, moderately severe for a score of >5 to 10, and severe for a score of >10. Case of severe dehydration got hospitalized and managed.

Collection of Samples

All stool samples collected in the hospitals were transported within 2 hours to the testing laboratory. Detection of VP6 antigen in diarrheal stool sample was carried out by using 'IVD Research Inc.' Rotavirus antigen was detected by using ELISA. Out of 110 samples, 34 (30.9%) were positive for VP6 antigen suggestive of Rotavirus-A.

RNA PAGE (Electropherotyping)

Rotavirus double-stranded RNA was extracted from stool of infected stool samples by using Trizol-LS reagent (Life Technologies, Rockville, Md). All fecal specimens were analyzed by polyacrylamide gel electrophoresis (PAGE) to confirm Rotavirus group A and for presence of group B or C rotavirus double-stranded RNA (dsRNA), if any. The methods have been described in detail elsewhere.[12]

RNA Extraction using QIAamp Viral RNA Mini Kit

Phosphate buffer saline suspensions of fecal samples 10% were clarified by centrifugation at 10,000 gm for 10 minutes. Genomic RNA was extracted from 140 µl of 10% stool suspensions using a spin column technique according to the manufacturer's instructions. (QIAamp Viral RNA mini kit from QIAGEN GmbH, Hilden, Germany).

Reverse Transcription-PCR (RT-PCR) Genotyping

In view of high cost involved in genotyping, stool samples, found positive for rotavirus either by ELISA or by electropherotyping were subjected to genotyping by RT PCR. Prior standardization was done for amplification of Rotavirus RNA, identifying the specific G and P genotypes. All the RT-PCRs were performed with viral RNA extracted from reference samples as the positive controls and water as the negative control.

Rotavirus G Genotyping

Reverse transcription was used to synthesize the cDNA corresponding to the genomic segment encoding VP7, and the characterization of G genotypes was performed with specific oligonucleotide primers according to a previously described system.[13,14] The cocktail of primers used for typing was the common primer 9con1 and the G type-specific primers G1 (9T1-1), G2 (9T1-2), G3 (9T-3P), G4 (9T-4) and G9 (9T-9) and G8 (MW-8). The sizes of the G type-specific PCR products were 110 bp (G9), 160 bp (G1), 246 bp (G2), 466 bp (G4) and 405 bp (G3). The reaction was carried out with an initial reverse transcription step at 45°C for 1 hour, followed by 40 cycles of amplification (30 sec at 94°C, 45 sec at 45°C, 1 min at 72°C), and a final extension of 10 min at 72°C in a thermal cycler (peqLAB).

Rotavirus P Genotyping

A semi nested multiplex type specific PCR was used for P typing. In the first round of PCR, consensus primers Con3 and Con2 (Complementary to conserved region of VP4 gene) were used to amplify an 877 bp region. The consensus Con3 and the P type specific primers for P4 (2T-1), P6 (3T-1), P8 (1T-1)[13] were used during the second round of PCR. The PCR mixture composition and thermal conditions for the first and second rounds of amplification were the same for the G typing, except for the primers used for amplification.

Agarose Gel Electrophoresis

All amplified PCR products after the first and second rounds of PCR were subjected to electrophoresis on 2% agarose gel containing 0.5 mg/ml of ethidium bromide and observed under ultraviolet light. Specific segment sizes for different G and P genotypes were observed.

Statistical Methods

Sample size was calculated using the incidence rate in the community (20%), with a power 80% and alpha error of 0.05. Data was analyzed using Students t-test and Chi-square test. Virus phenotypes and genotypes were compared with their respective disease severity in terms of vasikari scores on clinical parameters recorded on pretested proforma for each case.

RESULTS

110 children between 6 and 60 months (M 68, F 42) with suspected cases of acute viral diarrhea were screened by ELISA after excluding invasive (Bacterial) diarrhea by history of presentation and stool examination. 34 (30.9%) of them came positive for Rotavirus group.

Age, Seasonal Predilection and Duration

58.8% of Rotavirus positive cases were in the age group of 6 to 12 months, 23.5% in 13 to 24 months and 14.7% in 25 to 60 months. Rotavirus diarrhea was seen round the year but 67.6% occurred during colder months (November-February), 23.5% during March-May and 8.8% during July-October in this study. In majority of Rotavirus positive cases, 2.9% lasted only for 1 day, 8.8% for 2 days, 17.6% for 3 days, 47% for 4 days and 20.53% for 5 days.

Disease Severity

Of 34 cases of diarrhea positive for Rotavirus A on ELISA, 14.7% were having no dehydration, 38.2% had mild, 35.2% moderate and 11.7% severe dehydration. Among children whose stool samples were negative for Rotavirus, 47.36% had no dehydration, 22.36% had mild, 18.42 moderate and 11.8% severe dehydration. Loose, watery motion was the most common complaint (85.2%) followed by vomiting (61.76%) among Rotavirus positive cases. Fever was seen in 35.29% and respiratory symptoms in 8.82% of rotavirus positive cases. In rotavirus negative cases, watery stool was in 78.9% cases, vomiting in 34.2%, fever in 30.26% and respiratory symptoms in 3.9% cases.

RNA PAGE Electropherotyping

Out of 34 samples, 32 showed typical Rota-A type bands. Rest 2 showed Rota-B type bands in addition, suggesting mixed infection. None of ELISA negative samples were found positive either for Rotavirus-A or B in electropherotyping, demonstrating its reliability at par ELISA.

Long and Short Electropherotypes (Fig. 1)

Of 39 positive for purely Rotavirus-A, 17 were found to be of Long-electropherotype (Long-E) type (53.1%) and the rest were of short-electropherotype (Short-E) types (46.9%). The association of electropherosubtypes with clinical disease severity was assessed. Long-E types were found to be associated with more severe disease. Eleven, i.e. 64.6% of all Long E type caused moderate and severe dehydration as compared to only 2, i.e. 13.3% of all Short E type causing moderate dehydration only, but no severe dehydration. This was highly significant ($p < 0.009$).

Genotyping

Out of 34 stool samples positive for Rotavirus, 15 were subjected to RT-PCR by computer generated randomization. Three different G types were detected: G1, G4 and G9. Of these, G9 was the most prevalent genotype (26.66% alone and 6.6% mixed with G1), followed by G1 and G4 (13.3% each). Rests were untypable (40%) by the set of primers used. Nine could be genotyped for VP4 and 4 could be genotyped for VP7. The P types found were P(4) 20% and P(6) 6.7%. Others were untypable. The only G-P combinations seen was G1P(4) 26%. Although G9 was the commonest genotype in our study, its P type could not be typed in most. There was only one fully genotyped strain of G1P (4) in our study (Table 1).

DISCUSSION

Age, Seasonal Predilection and Duration

In the US and Europe, Rotavirus infection occurs primarily during the winter. Some studies from India suggested the disease occurs year-round there. The peak of infection occurs during the winter[16] while another study found 2 peaks per year.[17] In two other Indian studies, one from north and the other from south (Kerala), no seasonal pattern was found.[17,18] The temporal distribution of Rotavirus incidence was observed in this study throughout the year, with higher incidence during colder season (November-February).

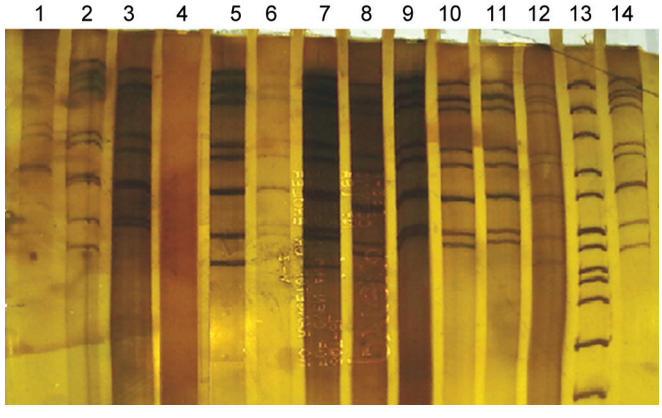

Fig. 1: PAGE of Rotaviruses showing the long and short electropherotyping [Lane 3, 6, 9, 10-13: long type; Lane 1, 2, 5, 12 and 14: shorter type; Lane 7, 8: untypable (mixed infection, also mimicking Rotavirus-B type)]

Table 1: G(P) genotypes of Rotavirus in fecal samples from symptomatic infants and children

No. of patients	*ELISA*	*Electro-pherotyping*	*G-Genotype*	*P-Genotype*
1	Positive	Long	G1 + G9	Untypable
2	Positive	Long	Untypable	P4
3	Positive	Long	G1	P4
4	Positive	Short	G9	Untypable
5	Positive	Short	G1	Untypable
6	Positive	Short	G4	Negative
7	Positive	Short	G9	Untypable
8	Positive	Short	G4	Negative
9	Positive	Short	Untypable	P4
10	Positive	Long	G9	Negative
11	Positive	Short	G9	Untypable
12	Positive	Long	Untypable	P6
13	Positive	Long	Untypable	Untypable
14	Positive	Long	Untypable	Untypable*
15	Positive	Long	Untypable	Untypable*

*An unusual pattern in electropherotyping

Incidence

In this study, Rotavirus was found positive in approximately 31% of clinically suspected viral diarrhea which compares well with findings of Mathew et al from Kerala (35.8%),[18] Kelkar et al from Pune (28-30%)[19] and in studies reported from Delhi (33.33%).[20] The incidence at Navi Mumbai in this study seems to be much higher than reported from other centers in India such as Chandigarh (19%), Vellore (18%) and Chennai[21] (22%) and less as compared to studies from Manipur (41%).

Age

In the present study, the highest (58.8%) incidence of Rotavirus diarrhea was seen among 6 to 12 months of the age which is comparable to reports from India and abroad. In our study, it was least common during late infancy which might be due to continued protection of maternal antibodies transferred transplacentally as well as from breastfeeding.

Seasonal Predilection

In this study, (67.6%) cases occurred during colder months November to February, 23.5% during March to May and 8.8% during July to October. Our results correlate well with the study of Kelkar et al (Pune, about 200 km from our location), who got 61.8% positive cases in winter and 9.8% in rainy season.

Symptomatology

Fever, vomiting and respiratory symptoms were present in 35.29, 61.76 and 8.82% respectively of all children having diarrhea whose stool samples were positive for Rotavirus in our study. Shariff et al[22] in their study recorded fever in 26% cases. In our study, vomiting was present in 61.76% of cases corresponding well with Sheriff et al (56%) but is lower than reported by Nafi et al (86.4%). Mathew et al from Kerala[18] recently reported mild fever in 32.8%, moderate in 37.1% and >38.7°C in 46.7% of all fever cases.

Degree of Dehydration

Of Rotavirus positive diarrhea cases in our study, 14.7% had no dehydration. Mild dehydration was present in 38.2%, moderate in 35.2% and severe dehydration in 11.7% of cases. Our results are slightly higher than Shariff et al and Nafi et al who recorded dehydration in 76% cases and 72.3% cases respectively on admission.[22,23] In their study only 11 (7.4%) presented with severe dehydration, while the remaining 96 (64.9%) were considered to have mild dehydration. Patients with dehydration in the current study were significantly younger than those without dehydration. Among the non-Rotavirus diarrhea cases, 46.36% had no dehydration, 22.36% had mild dehydration, 14.42% had moderate dehydration and 11.8% had severe dehydration; just similar to Rotavirus positive cases of diarrhea. This drove away the myth that Rotavirus diarrhea is often associated with severe dehydration while other forms were not. However, Mathew et al[18] studying Rotavirus positive hospitalized children only reported no dehydration in 32.2%, some dehydration in 45.3% and severe dehydration in 44.4% of cases. This could be a sample bias as their study was conducted on hospitalized children who were obviously admitted for treatment of severe diarrhea, needing parenteral fluid therapy.

ELISA and Electropherotyping

In our study, out of the 32 samples which were positive for Rotavirus by PAGE (Electropherotyping), 53.1% showed long electropherotypes and 46.9% showed short electropherotypes (*see* Fig. 1). A study from Vellore[24] found long type predominance over shorter type. Out of 117 samples, 75 (64.1%) showed long patterns and 25 (21%) short patterns. Das et al[25] also found a predominance of long electropherotypes. In contrast to our findings, the study of from Chennai[26] found a predominance of Short E types as compared to Long E types. This variation needs to be elucidated further by larger studies. A strong association of Short and Long E types with subgroups I and II respectively have been observed by some workers. Studies have correlated the presence of severe gastroenteritis and found subgroup II to be associated with more severe disease. In our study, the Long E types were associated with more severe illness in majority of the cases as compared to the Short E types. 64.63% cases of Long E type had severe disease (moderate to severe dehydration) in

contrast to 13.36% of Short E type causing severe disease. The difference was highly significant (p = 0.009) not reported so far.

Genotyping

Out of 15 ELISA and electropherotype positive samples randomized from 34 by taking alternate samples, RT PCR failed to detect any G-type in 4 samples and no P-type in 9 samples. This could be due to numerous factors including small sample volume picked up for analysis due to cost constraints, time of sampling, inhibitors in the samples that could have masked PCR reaction and storage time before analysis.

Out of 15 samples tested, 5 were positive for G9 (33.33%). Such higher prevalence of G9 not been reported from any part of India earlier except Kerala[18] although larger sample size required to confirm this. One sample positive for Rotavirus genotype G9, also exhibited G1 genotype, suggesting ongoing genetic shift between 2 different genotypes undergoing genetic reshuffle while infecting the same host cell. A study conducted in Kerala in the corresponding period[18] had also shown higher G9 genotypes (29%) with G1 P(8) 49.7%, G9 P(8) 26.4%, G2 P(4) 5.5%, G9 P(4) 2.6%, G12 P(6) 1.3%, G1 P(6) 0.8%, G12 P(8) 0.8%, G1 P(4) 0.2%, G1 P(Untypable) 0.2%, G9 P(Untypable) 2.4% and others, mixed to be 9.2%.

All recent studies confirm the diversity of Rotavirus strains much greater than previously recognized as their epidemiology is changing rapidly. Specific genotypes, such as G9 and G12, are emerging in various parts of the world, particularly in developing countries where G1 or G2 were the only genotypes earlier. In a review article by Broor et al[27] on molecular epidemiology of Rotaviruses in India, much genetic and antigenic diversity was highlighted. Electropherotyping demonstrated multiple electropherotypes co-circulating at a given time in a particular community, leading to extensive genomic variation and appearance of new strains**.**

In India, the most common G types are G1 and G2 and P types were of P(4) and P(8). Of late, G9 is being reported in isolated manner as an emerging strain besides P(6) strains of Bovine population. Our study has shown predominance of G9 strain (33.33% of positive samples) followed by G4 and G1. One sample was positive for P(6) strain. One strain had multiple G types. Though G9 was the commonest genotype in our study, its P type could not be detected in any. There was only one fully genotyped strain, G1 P(4) type. The findings are suggestive of re-emergence of diverse strain in the region. Besides pointing toward wide variation of Rotavirus serotypes, the present study raised the possibility of several novel strains circulating in Navi Mumbai region, including the emerging G9 and P6 types. Larger sample size needs to be studied before drawing important conclusions for the region.

The diversity of Rotavirus strains and its high incidence emphasize that vaccines need to be redesigned against a broad range of strains. Currently one monovalent (G1) vaccine (Rotarix, Glaxo) and one polyvalent human-bovine reassortant with G1, 2, 3 4 serotypes (Rotateq, MSD) oral attenuated vaccines are available. These vaccines, which were earlier showing protective efficacy of over 85% in European and American population with G1-3 strains in the eighties, recently showed a mere 35 to 46% efficacy in the Middle East, South Africa, Malawi and other low-income countries; where G9 and G12 had emerged as predominant strains.[28,29] The manufacturers still drive these vaccines with claim of 'Cross protection' and 'Herd immunity' on basis of few observational studies, but not without conflict of interests. Such claims are strictly not consistent with scientific concept of cross protection in viral infections. No prospective seroconversion study is available from our country for confirmation of such claims.

With G9 and other non-G1, G2, G3 serotypes now emerging in certain parts of our country as was seen in the Middle East, Latin America and Africa, similar antigenic shifts are certainly a possibility in other parts of the country as well by mutation, genetic shifts, drifts, due to fast population dynamics and climate change. Larger Multicentric, double-blind and prospective studies are desirable, besides looking for role of other enteric viruses responsible for causation of diarrhea in the Rotavirus negative samples. Our population generally behaved differently to oral vaccines (e.g. OPV) with heavily loaded gut-microbiota as well as high parasite load as compared to the Westerners. Moreover, individual variation in susceptibility to viral infections needs to be taken into consideration, than blindly banking upon mass scale vaccination at exorbitant cost. In outbreak studies of a GII-3 and a GII-4 Norovirus strain, association between HBGA phenotypes and viral infection was established.[30] Such hypothesis was extrapolated to show human susceptibility and resistance to Norwalk virus infection.[31] Persons carrying more than one functional FUT2 allele, expressing al2 fucosyl-transferase2, were termed as secretors and can express the A and B blood group antigens as well as H-type 1 and Lewis b (Leb) antigens on mucosa as well as secretions.[34] Same needs to be explored in case of Rotavirus and other enteric viruses which have not been studied so far.

The newer indigenous vaccine from India, using bovine re-assortant neonatal strain 116E, of G9P(11) type, initially detected at the AIIMS, New Delhi, was developed by the

department of Biotechnology (Government of India), cleared by the US FDA and under manufacture by Bharat Biotech is scheduled for a phase-IV study. Promised at a cost of mere 1$ per dose, has shown a slightly better efficacy of 53.6% (95% CI 35.0-66.9; $p < 0.001$) against severe Rotavirus acute gastroenteritis and good tolerability in Phase-III study.[32,33] There is a genuine need to develop cheaper but effective vaccine selectively against locally prevalent strains in the region, revised from time to time, as in case of Influenza. May be, an injectable form that could be integrated with other UIP vaccines is a convenient option for the resource scarce countries.

SUMMARY AND CONCLUSION

Rotavirus affected children mainly below 2 years (82.3%) in the suburban region of Navi Mumbai, India. The highest (58.8%) incidence was seen in 6 to 12 months of the age. Peak incidence was found in December (19.05%), followed by January (17.8%). Maximum Rotavirus positive samples (67.6%) were isolated in winter months. Average duration of Rotavirus diarrhea was 3 to 5 days, 80% resolved within 4 days. Predominant associated symptoms in Rotavirus diarrhea were vomiting (61.76%), fever (35.29%) and respiratory symptoms (8.82%), in that order. Mild dehydration was present in 38.2%, moderate in 35.2% and severe in 11.7% of cases of confirmed rotavirus diarrhea in this study. Of 110 cases of diarrhea in the age group of 6 months to 5 years, 34 (30.90%) were positive for Rotavirus-A. Of them, 2 were of mixed A and B serotypes.

Of 29 Rota-A confirmed by electropherotype, 53.1% were of long type and 46.9% short electropherotype. Long ones were associated with more severe dehydration as compared to short ones. The difference was highly significant ($p < 0.009$). G9 was found to be most prevalent genotype (33.33%), followed by G1 (20%) and G4 (13.3%). It suggested emergence of diverse strains in the region. One was positive both for G1 as well as G9, showing antigenic shift by ongoing genetic shuffle between 2 genotypes, infecting the same host cell. Of P types identified, P(4) were 20%, P(6) 6.7% and rest were untypable. The only G-P combinations seen was G1P(4) (26%). There is a genuine need to develop cheaper, effective and indigenous but effective vaccines, directed against the country specific strains. With the right policy prioritization at national level, this will not remain as a distant dream to include it in the national immunization program free, delivering it at grass-root levels, where it is needed the most.

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