

Original Research Article

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Serologic and molecular characterization of rotavirus from children with acute gastroenteritis in Chennai, Tamil Nadu, India

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ABSTRACT

Background: Rotavirus gastroenteritis is the leading cause of diarrhea in infants and young children worldwide. Although, Rotavirus vaccine has been introduced in 2017 in states like Tamil Nadu, there are reports of the role of Rotavirus as one of high disease burden agents with genetic variants arising, especially from low-income countries like India.

Methods: Authors evaluated stool samples from 507 children with acute gastroenteritis Rotavirus A among the hospitalized children (>5 years) to provide baseline information on changing profile in this state. The stool samples were collected and screened for Rotaviral Antigen by Enzyme Immuno Assay and use of semi-multiplex RT PCR technique was conceded out in order to conclude the P and G genotypes of human rotavirus in rotavirus-positive samples from January 2014 to December 2016 in and around Chennai, India.

Results: Of 507 samples collected 213 (42.01%) were positive for rotavirus antigen by Enzyme Immuno Assay (EIA). The maximum positivity (75%) was in the age group of one to two years. Rotavirus positives were subjected to further VP7 and VP4 molecular characterization and the predominant genotypes identified were G9P[4] followed by G9P[8], G1P[8], G3P[8], G2P[4] and mixed types of G2G9 with P[4] and G4P[6][11] with few untypable strains.

Conclusions: This study had demonstrated the Rota Virus Gastro Enteritis (RVGE) is a common disease affecting the pediatric population and G9P[4], G9P[8] circulating types among the gastroenteritis cases reported in the city and its suburban area. This study in comparison to previous ones shows that the dominant serotypes and circulating genotypes changes from time to time within country. The results have reemphasized the need of rotavirus vaccines with broad serotype coverage which may help in decreasing the disease burden in this region of the country.

Keywords: Diarrhea, MDG-IV, Rota virus gastro enteritis, RT-PCR, VP4, VP7

INTRODUCTION

Gastroenteritis is a medical ailment written off as inflammation of the gastrointestinal tract that involves both the stomach and the small intestine resulting in some combinations of diarrhea, vomiting, and abdominal pain and cramping. Universally, most cases in children under five are caused by rotavirus. Less common causes include other bacteria (or their toxins) and parasites. Gastroenteritis primarily affects children in the

developing world.¹ Usually, clinical symptoms of rotavirus illness due to gastroenteritis ranges from mild, watery diarrhea to severe diarrhea associated with vomiting and fever, leading to dehydration with shock, electrolyte imbalance, and death.² Globally, every year, over 2 million children (less than 5 years of age) are hospitalized due to rotavirus infection and accounts for 527,000 (475000-580000) deaths, of which 90% occurred in Africa and Asia. Rotaviruses have been classified into seven heterogenic groups (A to G) based on their genetic

and antigenic properties (VP6 capsid gene); then subtyped into 27G and 35P genotypes based on the variations in VP7 and VP4 proteins.³ Globally, the most important types causing the majority of infections are G1P[8], G2P[4], G3P[8], G4P[8] and G9P[4]. In India, rotaviral associated gastroenteritis cases occur throughout the year, but more commonly reported during the monsoon period. During 1995-1999 and in 2015, an estimation of 1, 00,000 deaths were reported globally, whereas 200,000 deaths were reported in Sub-Saharan African countries. 4 Studies conducted in different parts of India has identified the prevalence which ranged

between 13.2% to 63% and detected G1, G2, G3, G4, G9, G10 and G12 genotypes / P[4], P[6] P[8] and P[11] phenotypes as commonly circulating ones.

In Tamil Nadu, one of the southern states in India, where rotavirus vaccine is not part of the routine immunization schedule during the study, period, however vaccine was being administered by private practitioners, and since 2017, is a part of routine immunization. Few hospital-based studies from south India have revealed the prevalence of rotavirus infections and it ranged from 20% to 63% among the gastroenteritis cases (Table 1).⁵⁻¹²

Table 1: Percentage of hospital-based diarrhea cases due to rotavirus.

Reference number	Location	Proportion of RV+ (%)	Age	Year
5	Vietnam	55	<5	2000-2003
6	Japan	19.4	<5	2005-2006
7	Chennai	22.55	0-3	1995-1999
8	Kolkatta, Manipur	4.6 and 89.8	<5	2014
9	Karnataka	11	<5	1995
10	One center in North India and One center in South India	39	<5	2009-2012
11	Tirupathi	25.67	<5	2012-2013
12	Chennai	63.7	<5	2013

Authors are in the process of screening for rotavirus among gastroenteritis subjects from 2014 Till to date. The Chennai city with a population consists of 7.88 million (Census 2011), lies at 30 N latitude.¹³ The climate in Tamil Nadu divided into four; winter (January - February), Pre-monsoon (March - May), Monsoon (June - September) and Post monsoon (October - December). The hottest part of the year is late May to early June with temperature of 35-40 °C and coolest part is January with temperature 19-25 °C the average rainfall is about 140 cm mostly the climate is sunny.¹⁴ Chennai has limited supply of water and gets rain in two spells: irregular spells of conventional rains and southwest monsoon followed by the major spell from the ebbing northeast monsoon beginning in October each year. There is a chance of increased number of enteric viral agents spread due to amplified water breeding sites as water stagnates during monsoon and the cool weather conditions prevalent in the post-monsoon term. During the study period Chennai had received 483 mm rain fall, during the months of November to December, during 2015- and 640 mm during October to December 2014. From September to November it was recorded as 180 mm.¹⁵

In this report, authors present the prevalence of rotavirus and its circulating sub-types among the acute gastroenteritis cases, who had been admitted to tertiary care hospitals catering to the population of Chennai city and suburban areas of Tamil Nadu during the study, period 2014 to 2016.

METHODS

Study area is Stool samples were collected from Acute Gastroenteritis (AGE) subjects, children belonging to 0-5 years of age admitted to four tertiary care centers; three are located in different parts of Chennai city and another in Chengalpattu, a suburban of Chennai (45 kilometers from Chennai). Most of the study population belonged to an economically poor background, and their vaccination status against rotavirus is not known, as it's not part of routine immunization schedule during the study, period.

Case selection

The samples were collected based on the standard World Health Organization (WHO) definition; children with high grade fever (≥ 38 °C), along with occurrence of a three or more loose, liquid, or watery stools or at least one bloody loose stool in a 24-h period and those who got admitted to hospitals.¹⁶ Dehydration levels were also assessed according to the recommendations of the World Health Organization Program for Control of Diarrheal Diseases.¹⁷

Inclusion criteria

An informed consent was obtained from parents or caretaker and obtained basic information (age, sex, clinical symptoms) of the cases. Authors had also got

Institutional Ethical Clearance (IEC) before undertaking the study. The children who were admitted in four centers were from lower socioeconomic status. The children were healthy prior to this episode of diarrhea.

Exclusion criteria

Children with Chronic diarrhea/ chronic diseases and children who had developed diarrhea after hospitalization, children with dysentery, diarrhea of more than 14 days, or diarrhea developing due to any other cause were excluded from this study.

Limitations of the study are authors have taken pediatric children with chronic watery diarrhea among in-patient cases (IP) authors could not enroll adequate number of out-patient (OP) cases which would have reflected on the community burden directly and hence authors have measured the burden of Rota viral diarrhea. In this study authors had collected samples from in-patient subjects from tertiary care centers in Chennai and also from a suburban medical college away from Chennai; this reflects positivity/ prevalence in metropolitan city as well as its suburbs. Since the above-mentioned institutes are referral centres to which cases are referred, the positivity could be higher and does not reflect on the population prevalence precisely.

Sample collection

Single fecal specimen from each case was collected within 24 hours after onset of symptoms using a sterile, disposable, wide mouth container for the collection. The samples were transported to the laboratory at King Institute of Preventive Medicine and Research, Chennai, in cold chain, within 24-48 hours of the collection and samples were stored in -20 °C for short period (48 hours) before antigen analysis. Those which were positive for rotaviral antigen were stored at -80 °C until they were tested for further analysis.

Integrated in this study were data of the children admitted to pediatric wards of tertiary care centres with complaints of diarrhoea and authors have collected stool samples from 547 patients and for reasons based upon the exclusion criteria 40 samples were excluded and 507 cases qualified for this study and further analysis as been made for this samples.

Detection of rotavirus specific antigen

The Rotavirus specific antigen was detected by Enzyme Immuno Assay techniques using commercially available EIA kit (Premier TM Rotacclone®, Meridian Biosciences Cincinnati, USA). Samples were diluted with sample diluents (supplemented with kit) and 100µl were added to microtiter well which is coated with group specific rotavirus antigen and incubated for 1 hour. Followed by this, wells were washed for five times with distilled water and 100µl of substrate is added and incubated for 10

minutes. The reaction was terminated with stop solution, and measured the optical density at 450 nm in the ELISA reader. Genotyping by semi-nested multiplex RT-PCR A 10% (w/v) suspension of ELISA positive stool samples was prepared in phosphate-buffered saline (PBS). The fecal suspension was vortexed and centrifuged at 3000 g for 15 minutes. The supernatant was then used for RNA extraction by commercially available virus Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. PCR was performed on ELISA positive samples with three set of primers targeting "Group A Rotavirus (VP6), type VP4 and VP7. Single stranded RNA was used as template for RT-PCR to amplify the VP6 antigen coding gene (VP6-F-{GACGGVGCRACATACATGGT} and VP6-R-{CCAATTCAATNCCTGGTG}) by using Invitrogen one step RT-PCR Kit.¹⁸ By using semi-nested multiplex specific RT-PCR primers G and P genotyping was performed for VP7 and VP4 as described by Gouvea et al.¹⁹ The amplified product was then analyzed on 1.5% agarose gel. Samples which did not react to any of G or P genotype specific primers were considered un-typable.

Statistical analysis

The data were analyzed using SPSS software version 20.0. The significance of differences between proportions of rotavirus positivity in different age group and gender was tested using the chi-squared test p value of (0.05).

RESULTS

Symptoms and age-wise distribution

Among rotavirus positive cases screened, 75% of the positivity was seen in 12-24-month age group which was statistically significant which had a p value of >0.0001, comprising 57% of positivity in less than one year and over 20% of positivity observed in children of 3 to 5 years of age (Table 2).

Males were found to be more affected than females in our study. Statistical significance was observed for the gender distribution that had a p value of 0.01 (Figure 1).

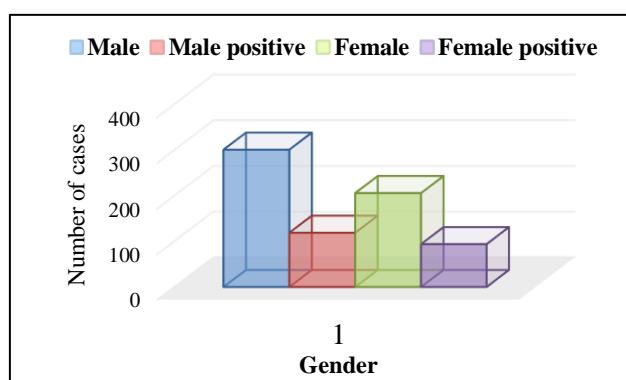


Figure 1: Gender wise distribution of rotaviral gastroenteritis.

Table 2: Age wise distribution of rotaviral gastroenteritis in both genders.

Age (years)	Total n= 507		Positives (n=213)		Total % of positivity		p value
	Male (301)	Female (206)	Male (119)	Female (94)	Male	Female	
<1 year	57	40	15	16	12.6	17.02	
1-2 year	120	110	67	44	56.3	46.8	
2-3 Year	74	23	20	18	16.8	19.1	
3-4 Year	22	14	9	8	7.56	8.5	
4-5 Year	15	10	6	5	5.04	5.3	
>5 Year	13	9	2	2	1.68	2.1	

The clinical symptoms generally observed among the rotavirus positive subjects were fever (83.82%), vomiting (82.84%), dehydration (96.64%), and diarrhea (94.67%) which were found to be statistically significant (p value>0.001).

Temporal variation

As far as seasonality is concerned, maximum number of cases (Figure 2) and positivity (Figure 3) was seen during the months of October, November and December (66.66%) which happened to be the monsoon months in Chennai, when the North east monsoon sets in.

This was followed by August and September (21.59%), which are the pre-monsoon months in Chennai, with rains on and off.

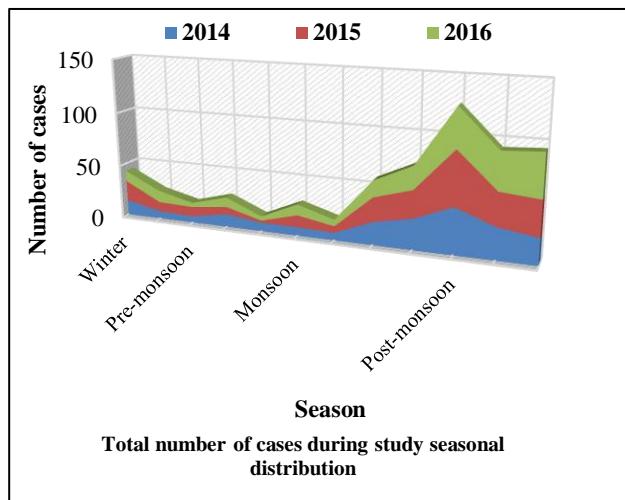


Figure 2: Temporal variation of RVGE cases during study.

Distribution of genotypes

In our study, the commonest genotype seen was G9P[4] (26.8%), followed by G2P[4] (5.63%), G4P[4] (5.63%) and G1P[8] (8.92%). We had identified few uncommon strains G10P[untypable] and G12P[6]

(3.75%) and G2 which showed variations. In this study, few mixed types of G2G9P[4] and G4P[11]P[6] genotypes were identified. Authors had also identified few strains which were untypable for both G and P types by type specific PCR (Figure 4).

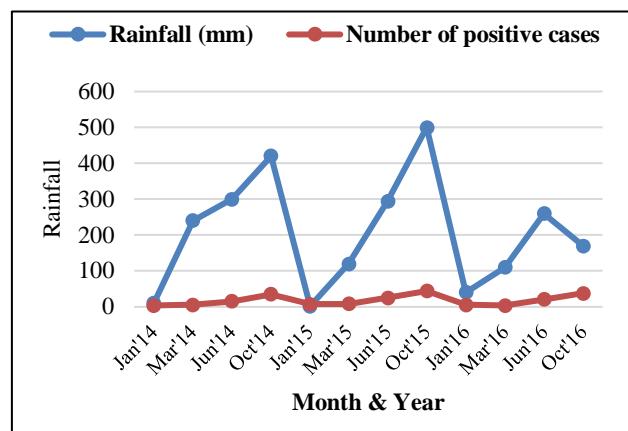


Figure 3: Rainfall in association with RVGE positives observed.

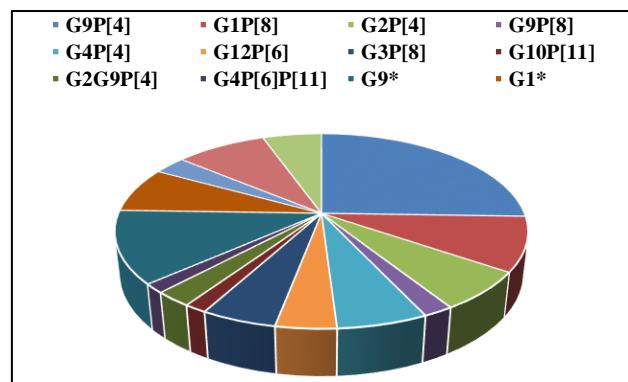


Figure 4: Total genotypes observed during the study period.

Dynamics in genotype distribution

Authors observed there is a fluctuation of rotavirus genotypes during this study period 2014-2016. The overall distribution of the major genotypes identified

among the positives are G9P [4] which was the predominant strain during 2015 (39%) that had declined to (21%) in 2015 and to (19.4%) in 2016. Similarly,

G12P [6] was not detected in 2014 but 6.4% positivity occurred in 2016. G9P [8] was about 3.7% during 2015 and at 1.6% has been identified in 2016 (Table 3).

Table 3: Percentage of genotype distribution of RV cases during 2014-2016.

Genotype	Year			Total number of RV positive cases (n=213 (42.01%))
	2014	2015	2016	
G9P[4]	10	29	12	51 (25.5%)
G1P[8]	11	3	4	18 (9.0%)
G2P[4]	3	5	5	13 (6.5%)
G9P[8]	0	3	1	4 (2.0%)
G4P[4]	5	3	4	12 (6.0%)
G12P[6]	0	4	4	8 (4.0%)
G3P[8]	2	5	3	10 (5.0%)
G10P[11]	0	1	2	3 (1.5%)
G2G9P[4]	0	2	3	5 (2.5%)
G4P[6]P[11]	2	0	1	3 (1.5%)
G9*	8	11	5	24 (12.0%)
G1*	6	5	4	15 (7.5%)
G2*	4	1	1	6 (3.0%)
G4*	6	5	6	17 (8.5%)
UT	2	2	7	11 (5.5%)
Total	58 (35.2%)	80 (41.6%)	62 (41.4%)	200 [#] (39.4%)

* P untypable, # 13 samples did not answer in RT-PCR

Authors also identified few VP7 untypables and VP4 untypables, along with re-emerging and newer strains in his population.

DISCUSSION

The study aims to assess prevalence of rotaviral diarrhea and identify strains among children less than five years among inpatients admitted to different hospitals in and around Chennai during a 3-year period.

The earlier studies which had been conducted from different parts of India, during 1991 to 2003 had shown 13.2% to 63% of prevalence of RV. A study conducted in USA found the positivity ranging between 30 and 50%, which is very similar to our study.²⁰ The predominance in 1-2-year age group is probably because exclusive breast feeding is encouraged up to six months after which weaning is initiated and breast feeding is stopped by one year in the majority of the children. The immune protection from breast feeding protects the infants during early months, and later they become susceptible, especially seventh or eighth month onward due to weaning/mouthing of objects/ increase in mobility. It is to be noted that this was before rotavirus vaccine implementation. This study indicated that there is a decrease in rotaviral infections in the older children (2-5 years). This might be due to the acquired protective immunity developed by the children due to previous

rotavirus infections. This result was similar to studies conducted by Barnes et al, Ford-Jones et al, and Staat et al.²¹⁻²³ In a study from Iraq by Bass et al., boys were more affected than girls, this is similar to this study, in which 63.7% of boys and 35.2% girls respectively were affected boys were more affected, and there could be no specific reason for this.²⁴

The cases were strewn throughout the year, however, during the monsoon period, the premier number of cases were reported, 50 out of 69 positive cases occurred during monsoon and post-monsoon period. During rains, there is stagnation of water and floods resulting in contamination of drinking water causing a spate of diarrheal diseases. In this study in general RV positivity occurred almost all round the year and peaked during post monsoon which is similar to study conducted by Nguyen et al., Maneekaran and Jaing et al.^{25,26}

In the study, G and P type analysis showed that G9P[4] (26.8%), followed by G2P[4] (5.63%), G4P[4] (5.63%) and G1P[8] (8.92%) were seen in this order. Close by were mixed G types of G2G9 with P[4] and G4P[11]P[6] genotypes. In a study by Mullik S et al, they had detected G1P[8] as the most common strain (32%) followed by G9P[8] (16.9%), G2P[4] (13.5%) and G9P4 (10.75%).¹⁶ This correlates with this current study and marginally similar to this previous study done on samples from suburban Chennai (rural) during the year 2009-2010 in

which there was prevalence of G1P[8] and G9P[8].[918] G9 is recognized as the most common emerging type of rotavirus that has been seen globally from the middle of 1990s. Strains with mixed genotype specificities and emergence of newer strains with unusual genotypes {G1P[4], G2P[8], G4P[4], G10P[11], G9P[11], G9P[6]} were also reported from recent studies in India, and Bangladesh.¹⁹

Globally, viruses carrying either G1, G3, G4, G9, in association with P[8], and G2 in association with P[4], are the most common strains of rotavirus. In our region (Tamil Nadu) Rotavac vaccine, which is live attenuated, monovalent vaccine containing G9P[11] was not administered during the study period as routine immunization, was given sparsely, that too only by private practitioners, and all the subjects from whom samples were collected in this study were not vaccinated according to the information from parents or guardian. Rotavac, an oral vaccine developed by Bharat Biotech is administered to infants in a three-dose course at the ages of 6, 10 and 14 weeks. In Tamil Nadu, this vaccine has been introduced in the routine immunization from June 2017.²⁷ Another neonatal strain G10P[11] was reported from Vellore, which was originally considered exclusive to neonates by some and as an agent causing asymptomatic infection, has also been reported from older children in our center.²⁴ Authors have had two cases in 3-4 age group and one from 4-5 years of age indicating the emergence of newer strains mostly among the non-neonatal age group. From a study during 2009-2010 samples from suburban areas of Chennai were screened for rotavirus, [18] G1P[8] was predominant followed by G2P[4], G9P[8], G9P[4]. In this current study, which has been performed in children attending the tertiary care hospitals from Chennai during 2014-2016 showed G9P[4] as being the most common circulating strain followed by G2P[4], G3P[8], G4P[8], G9P[8].

This shows a noticeable shift of circulating strains in Chennai city, which warrants requisite of surveillance and continuous monitoring of circulating strains, in view of vaccine against Rotavirus being introduced and this is of utmost importance. It's worthy to note that G4P[11], P[6] being zoonotic reassorted strain, which was detected in this study, showing the possibility of zoonotic transmission, and reflecting the complex epidemiology of group A rotaviruses in India. The occurrence of rotaviral infections and emergence of newer strains such as G12P[6], with such variations may be expedited by high density population, poor and unhygienic conditions and lack of safe drinking water.

The lacunae in this study being that authors have not screened for other bacterial or protozoan agents responsible for diarrhea and for other diarrheal viruses to rule out co-infections and thus authors have planned to take up later. Authors had not done further monitor of the admitted subjects to comment upon morbidity and mortality. Authors could detect the causative agent in

42.01%, the remaining 57.99% goes unidentified which could be taken up later. Auxiliary follow up on mortality could not be done by us. These are some of our limitations.

CONCLUSION

In conclusion, 42.01% of rotavirus infection among acute gastroenteritis in under-five was evaluated, this being a public health issue, particularly in view of its significant association with severe forms of diarrhoea with its associated complications. There should be a national level intense, state wise surveillance of rotavirus and its circulating types among the gastroenteritis cases with vaccine being introduced in routine immunization to keep an eye on effective prevention. Meanwhile circulating genotypes change from time to time within country which could emphasize the impact on vaccination.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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