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Changing trends in circulating rotavirus strains in Pune, western India in 2009–2012: Emergence of a rare G9P[4] rotavirus strain

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ABSTRACT

Background: A vast diversity in rotaviruses at inter- and intra-genotypic level underscores the need for monitoring of circulating rotavirus strains. The aim of this study was to update the data on rotavirus disease and strains for the period from January 2009 to December 2012 in Pune, western India which has been one of the sites of the Indian Rotavirus Strain Surveillance Network since November 2005.

Methods: Children aged <5 years admitted for acute gastroenteritis in three different hospitals from Pune city were included in the study. The stool specimens were collected and tested for rotavirus antigen by a commercial enzyme immunoassay. The rotavirus strains were genotyped by multiplex reverse transcription polymerase chain reaction.

Results: During the study period, we found 35.1% of 685 stool specimens contained rotavirus antigen. Frequency of rotavirus detection was greatest (58.5%) among children aged 7–12 months. The G1P[8] (31.4%), G2P[4] (20.2%) and G9P[8] (11.8%) strains were the most common types. We noted predominance of G1P[8] strains (39.6%-46.1%) in all the years of study except 2009 wherein G9P[8] strains scored highest level (15.3%). Subsequent to this, we identified G9P[8] strains at the second highest position in 2010, their sudden decline and rise in G9P[4] strains in 2011–2012. We detected G12 strains in combination with P[6] and P[8] at variable rates (0–10.2%) and highest level (27.1%) of mixed rotavirus infections in 2009 as compared to 2010–2012 (0–3.8%).

Conclusion: The study highlights the huge burden of rotavirus disease and changing profile of circulating rotavirus strains displaying emergence of G9P[4] reassortant strains in Pune, western India and emphasizes the need to analyze the entire genomic constellation of rotavirus strains for better evaluation of the impact of rotavirus.

1. Introduction

Rotavirus is the prime cause of severe gastroenteritis in infants and young children worldwide, but developing countries are the most affected [1]. It is estimated that in India, rotavirus accounts for 22% of the deaths, 30% of the hospitalizations and 8.3% of the outpatient visits occurring globally each year [2]. In order to assess the need for and benefits of currently available rotavirus vaccines in India, the Indian Rotavirus Strain Surveillance Network (IRSN) operated by multiple centers has established foundation of information on clinical, epidemiological and virological features of rotavirus gastroenteritis from India [3]. The IRSN study conducted during November 2005–June 2009 has shown a significant rotavirus disease burden and strain diversity in different geographic regions of the country [4]. During 2005–2009, at the Pune site, we recorded a notable proportion of gastroenteritis infections caused by common (59.2%), uncommon (~10%), emerging[1] (9%) and mixed (15%) G(VP7) and P(VP4) rotavirus genotypes. To better understand the rotavirus strain epidemiology and to explore

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[1] Emerging genotype is the one which is increasing in incidence or has the potential to increase in incidence.

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differences in the profile of rotavirus genotypes over a longer time period, the surveillance study was continued from January 2009 to December 2012 in children <5 years, hospitalized for acute gastroenteritis – the results of which we report here.

2. Methods

2.1. Specimens and testing for rotavirus antigen

Stool specimens were collected from children aged <5 years, hospitalized for acute gastroenteritis in three different hospitals from Pune city, western India. A case of acute gastroenteritis enrolled in the present study was defined as the passage of ≥3 loose or watery stools a day with or without associated symptoms such as vomiting, fever and abdominal pain. All the patients were examined for fever, number of episodes and duration of vomiting and diarrhea, extent of dehydration and treatment for the assessment of severity of disease by 20-point scale of the Vesikari scoring system [5]. The disease condition of each patient was categorized as mild (0–5), moderate (6–10), severe (11–15) and very severe (16–20). Epidemiologic data inclusive of age, dates of diarrhea onset and specimen collection, maximum number of episodes of diarrhea and vomiting in a 24-h period were recorded for all patients. The method employed for specimen collection was approved by the institutional human ethics committee. All the specimens were transported to the laboratory on wet ice and stored at +4 °C until tested.

Ten percent (w/v) suspension of all of the stool specimens prepared in 0.01 M phosphate buffered saline (PBS) (pH 7.2) were tested for rotavirus A (RVA) antigen using a commercial ELISA kit (Generic Assays, Germany) as per the manufacturer's instructions. The specimens indicating optical density (O.D.) values above the cut off value (0.2 + mean of OD values of negative control wells) were considered positive for rotavirus antigen. All specimens were stored in aliquots at −70 °C for further testing.

2.2. G- and P-typing

The viral nucleic acids were extracted from 30% (w/v) suspensions of all ELISA positive stool specimens using Trizol (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. The VP7 and VP4 genes were genotyped by multiplex reverse transcription (RT)-PCR according to the method described earlier with minor modifications [6]. The viral RNA was subjected to one step RT-PCR (Qiagen, Hilden, Germany) using the sets of outer primers: 9Con1-L/VP7-R deg [7]; Con 3/Con 2 [8] and oligonucleotide primers that could amplify VP7 genotypes G1- G4, G8- G10 and G12 and VP4 genotypes P[4], P[6], P[8], P[9]; P[10] and P[11]. Briefly, 4 μl of ds RNA was denatured at 95 °C for 5 min and then chilled in ice for 2 min. A reaction mix of 46 μl containing 5Xbuffer, dNTPs, RNase-free water, primers 9Con1-L/Con3 and VP7-Rdeg/Con2 and 2 μl of enzyme mix was added to make a final volume of 50 μl. All PCR products were analyzed by electrophoresis using Tris acetate EDTA (TAE) buffer, pH 8.3 on 2% agarose gels, containing ethidium bromide (0.5 μg/ml) and visualized under UV illumination. To determine the VP7 and VP4 genotypes of rotavirus strains non-typeable in multiplex PCR, first round PCR products obtained in agarose gel electrophoresis were sequenced using ABI-PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster city, CA) and a ABI-PRISM 310 Genetic analyzer (Applied Biosystems) after purification on minicolumns (QIAquick: Qiagen, Valencia, CA).

Table 1
Rotavirus positivity in children with acute gastroenteritis in Pune, western India (January 2009–December 2012).

Year	No. positive/No. tested (%)
2009	80/223 (35.8)
2010	35/126 (27.8)[a]
2011	69/200 (34.5)
2012	57/136 (41.9)[b]

a versus b: $P < 0.05$.

2.3. Statistical analysis

A comparison of meteorological data was carried out for different years of the study using paired *t*-test. Two proportions were compared using chi square test. *P*-values <0.05 were considered statistically significant.

3. Results

We collected a total of 685 stool specimens from children hospitalized for acute gastroenteritis during January 2009 to December 2012 in Pune, western India. Of these, 241 (35.1%) were positive for rotavirus antigen by ELISA. Year wise analysis showed significant difference in the rotavirus positivity only between the years 2010 and 2012 ($P < 0.05$) but not in the other years (Table 1).

The mean age (± standard deviation) of children hospitalized with diarrhea was 15.8 ± 12.9 months. The mean age of rotavirus infected children was 13.8 ± 9 months, which was significantly lower ($P < 0.05$) than that of rotavirus uninfected children (16.8 ± 14.4 months). Among the rotavirus infected children, 58.5% were in the age group of 7–12 months, while 14.5% belonged to ≤6 months.

Analysis of the clinical severity scores indicated very severe, severe, moderate and mild disease in 2.8%, 56.5%, 38.7% and 2.3% of the patients suffering for rotavirus gastroenteritis. As against this, 5%, 47%, 38.3% and 7.7% of the patients tested negative for rotavirus experienced very severe, severe, moderate and mild disease, respectively. In general, children with rotavirus diarrhea had significantly less mild and more severe disease than those with rotavirus-negative diarrhea ($P < 0.05$). Rotavirus infected children had more episodes of vomiting than did uninfected children ($P < 0.05$).

The multiplex PCR conducted for genotyping of rotavirus strains showed amplification of VP7 and VP4 genes in 197 (81.7%) and 190 (78.8%) strains respectively and identified genotypes of both genes in 178 (73.8%) strains (Table 2). 32 (13.2%) strains remained untypeable for both genes. We detected infections with mixed rotavirus strains in 18 (10.1%) of the 178 specimens.

Among the strains typed for both VP7 and VP4 genes, G1P[8] strains attained the highest score (31.4%). This was followed by G2P[4] (20.2%); G9P[8] (11.8%); G9P[4] (10.1%); G12P[6] (6.1%); G12P[8] (3.3%); G2P[8] (2.8%); G2P[6] (2.2%); G3P[8] (0.5%); G4P[4] (0.5%) and G1P[4] (0.5%) rotavirus strains. G1P[8] strains continued to remain prevalent in all the years of study except the year 2009 in which G9P[8] strains (15.2%) were predominant. G9P[8] strains remained second highest in the year 2010 and declined markedly

Table 2
Distribution of typed and untyped rotavirus strains detected in children with acute gastroenteritis in Pune, western India (January 2009–December 2012).

Genotype category	No. positive/No. tested (%)
Typed for both G and P	178/241 (73.8)
Typed only for G	19/241 (7.8)
Typed only for P	12/241 (4.9)
Untyped for both	32/241 (13.2)

Table 3
Distribution of G and P typed rotavirus strains in children with acute gastroenteritis in Pune, western India (January 2009–December 2012).

Type	Year (No. tested)				
	2009 $n=59$ No. positive (%positivity)	2010 $n=26$	2011 $n=48$	2012 $n=45$	2009–2012 $n=178$
Common					
G1P[8]	5(8.5)	12(46.1)	19(39.6)	20(44.4)	56(31.4)
G2P[4]	6(10.2)	2(7.7)	14(29.2)	14(31.1)	36(20.2)
G3P[8]	1(1.7)	–	–	–	1(0.5)
G9P[8]	9(15.3)	7(27)	4(8.3)	1(2.2)	21(11.8)
Rare					
G1P[4]	–	1(3.8)	–	–	1(0.5)
G2P[6]	2(3.4)	–	–	2(4.4)	4(2.2)
G2P[8]	5(8.5)	–	–	–	5(2.8)
G4P[4]	1(1.7)	–	–	–	1(0.5)
G9P[4]	2(3.4)	3(11.5)	9(18.7)	4(8.9)	18(10.1)
Emerging					
G12P[8]	6(10.2)	–	–	–	6(3.3)
G12P[6]	6(10.2)	–	1(2)	4(8.9)	11(6.1)
Mixed*	16[a]((27.1)	1[b]((3.8)	1[c](2)	–	18(10.1)

[a] 2009: G9G12P[6]P[8], G1G3P[6], G1G3P[8], G9G12P[6], G2G12P[8], G2P[6]P[8], G2G9P[6], 3 G9G12P[6], 2 G9G12P[8], G9G10P[8], G2G12P[4], 2 G2P[4]P[8].
[b] 2010: G9P[4]P[8].
[c] 2011: G1G9P[8].
* Rotavirus genotypes in mixed infection.

in circulation in 2011–2012. We found higher circulation of G9P[4] strains, an unusual combination of G and P types in 2010–2012 as compared to 2009. Mixed infections were highest (27.1%) in the year 2009 and declined drastically in the following years (Table 3).

4. Discussion

Two rotavirus vaccines, Rotarix™ and RotaTeq® have been licensed in ~90 to 100 countries to use against rotavirus diarrhea. Both vaccines are recommended by the World Health Organization (WHO) in childhood immunization programs conducted globally [9]. Studies report difference in the efficacies of these vaccines against severe rotavirus diarrhea in high and middle income (85–98%) and low income (39–72%) countries [10]. In countries like India, where the vaccine efficacy data is yet to be acquired, monitoring of rotavirus disease and strains is essential to assess the impact of rotavirus vaccines and circulating rotavirus strains on each other. The data obtained in this direction in the present study reaffirm earlier reports (2005–2009) of the characteristics of rotavirus infections, large rotavirus disease burden and strain diversity among children in Pune, western India [3,4]. Our data showed that rotavirus positivity continued to remain significant in each year of the study period (2009–2012) and concurred with recent study reports from India [11]. The frequency of rotavirus infections was markedly higher in 2012 than in 2010 ($P<0.05$). We analyzed these findings with respect to the meteorological data obtained for both years. The mean values obtained for relative humidity and temperature were significantly lower in 2012 (45.9% ± 21.7%, 17.8 °C ± 4.7 °C) than in 2010 (52.9% ± 21.6%, 19.4 °C ± 4.1 °C) ($P=0.004/0.0073$) (Indian Meteorological Department, Government of India, Pune). Our data indicated a deviation of rotavirus infections toward lower humidity and temperature as described previously in eastern India [12].

G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] are the most common rotavirus strains circulating worldwide. Throughout the study period, G1P[8] rotavirus strains showed highest prevalence, except in the year 2009 where G9P[8] was the predominant strain. Although G2P[4] has been described as the second most predominant strain in other regions of India [4,13], we found variation in its prevalence in comparison with other commonly detected rotavirus strain, G9P[8]. An earlier study from Pune identified the G3P[8] strain once in the year 2005 [3] and was detected only once in this study. Other studies have documented the absence of this strain and the G4P[8] strain indicating that they are uncommon in India.

Earlier rotavirus strain surveillance marked the circulation of unusual combinations of G and P types (G1P[4], G1P[6], G2P[6], G2P[8], G2P[10], G4P[4], G9P[4], G9P[6], G10P[6], G10P[8]) [3,4]. As against this, the present study detected only a limited number of such G-P combinations (G1P[4], G2P[6], G2P[8], G4P[4] and G9P[4]) with a notable contribution of G9P[4] strains. The year 2009 witnessed the highest diversity in circulating rotavirus strains in comparison with the years 2010–2012. Interestingly, the percentage of mixed infections was also highest (27.1%) in 2009 and found to decline to 0% in 2012. Thus, the proportion of mixed infections of rotavirus may correlate with the extent of diversity in rotavirus strains. In the same year, G9P[8] strains which are considered the fifth most common strains, displaced G1P[8] strains known to be predominant globally. Subsequent to this, the prevalence of G9P[8] strains declined after attaining the highest score in the year 2010. This was followed by a marked increase in the circulation of rare G9P[4] strains. It is possible that the occurrence of these strains could be a result of reassortment between G9P[8] and G2P[4] strains. Generation of such a reassortment has been proposed previously [14,15]. It is hypothesized that unusual combinations of G and P types are unfit for survival and hence do not stabilize in the environment [16]. In view of this, the continuous increase in the number of G9P[4] strains vis-a-vis a decrease in G9P[8] strains identified in the present study needs to be monitored further. Nevertheless, the data highlight the prominence of G9 strains in the region and the importance of including the G9 component in the rotavirus vaccine. Such data would also support the development of a designer vaccine for a specific region [17]. G12, known as the emerging genotype worldwide, detected earlier in Pune at a significant level (8.9%) [4] showed variability (0–10.2%) in circulation during the period of present study.

Our study was limited by the data from Pune city only. Hence, the results presented here may not be generalized to the rest of India. Further, G and P-type could not be determined for about 13.2% of rotavirus positive specimens. Point mutations at the primer binding site decrease the affinity of primer binding and may explain the failure to type such strains. This underscores a regular revision of typing primers. Incorporation of VP6 gene RT-PCR

would also help confirm the presence of ELISA reactive untypeable rotavirus strains.

To summarize, this study together with earlier studies that describe rotavirus epidemiology in Pune underlines the heavy burden of rotavirus disease, the predominance of G1P[8] and G2P[4] strains, the continued circulation of G9 strains with the emergence of G9P[4] reassortant and G12 strains in Pune, western India. These findings evoke the need for further analysis of common, rare and emerging strains of rotaviruses at complete genome level to determine intergenogroup reassortments, emergence of unusual lineages, antigenic drift and antigenic shift. Such studies will be useful to understand the mechanisms of rotavirus strain diversity and molecular evolution and most importantly in assessing the efficacy of rotavirus vaccines.

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