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Acute norovirus gastroenteritis in children in a highly rotavirus-vaccinated population in Northeast Brazil.

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Highlights

- Gastroenteritis is one of the most important causes of morbidity and mortality in children.
- Noroviruses are a leading cause of sporadic cases and outbreaks of acute gastroenteritis.
- Norovirus annual detection rates increased over the study period.
- The detection of norovirus was higher among young children.

Abstract

Background: Gastroenteritis is one of the most important causes of morbidity and mortality in children and an important etiological agent is norovirus.

Objective: We describe the occurrence and characteristics of norovirus diarrhoea in children from Sergipe, Northeast-Brazil, over two consecutive periods of three years following rotavirus vaccine introduction.

Study design: A cross sectional hospital-based survey conducted from October-2006 to September-2009 and from July-2011 to January-2013. Acute diarrhoea cases had a stool sample collected and tested for norovirus by RT-PCR and positive samples were sequenced.

Results: In total 280 (19.6%) of 1432 samples were norovirus positive, including 204 (18.3%) of 1,113 samples collected during the first period and 76 (23.9%) of 318 collected during the second period. The proportion of children with norovirus infection increased significantly through the second study period (χ^2 for trend= 6.7; p=0.009), was more frequent in rotavirus vaccinated and in younger children (p<0.001). Of 280 norovirus-positive specimens, 188 (67.1%) were sequenced. Of these, 12 were genogroup I and 176 genogroup II. The main genotype was GII.4 (149/188, 79.3%), followed by GII.2 (6, 3.2%) and GII.6 (5, 2.6%).

Conclusion: Norovirus annual detection rates increased over the study period. The detection of norovirus was higher among young children.

Key words: norovirus; diarrhoea; children; rotavirus vaccine; Brazil.

1. Background

Gastroenteritis is one of the most important causes of morbidity and mortality in children [1] and an important etiological agent is norovirus (NoV). This single-stranded RNA virus is a common cause of both sporadic and epidemic acute diarrhoea [1,2] and its importance is second only to rotavirus in areas where rotavirus vaccines have not been introduced [2]. Rotavirus vaccines are rapidly being introduced across the world and the relative importance of NoV may increase with the successful reduction of the rotavirus burden. However the epidemiology and burden of NoV has been poorly documented in low and middle income countries introducing rotavirus vaccines [2,3] and it is unclear whether its incidence and severity will remain the same or increase to fill the environmental niche of rotavirus [4]. Although NoV has become the most common cause of severe diarrhoea in industrialized countries with high rotavirus immunization [1,2], few studies have described NoV epidemiology since the introduction of rotavirus vaccines in other settings [5–8]. Brazil was one of the first countries to introduce a monovalent rotavirus vaccine (Rotarix) on a large scale in March 2006 [9], reaching high vaccine coverage levels (>80%) within a year of vaccine introduction and rapidly reducing the incidence of rotavirus-related hospitalizations [10,11].

2. Objective

We have examined the patterns of NoV diarrhoea in children residing in Aracaju, Northeast Brazil over two consecutive periods from October 2006 to September 2009 and from July 2011 to January 2013.

3. Study design

3.1.Study population and sample collection

This was a cross sectional survey conducted from October 2006 to January 2013 aiming to establish the proportion and severity of acute diarrhoea episodes due to rotavirus [11] and NoV. Children <12 years old presenting with acute diarrhoea attending the paediatric emergency service of Sergipe Emergency Hospital (Hospital de Urgência de Sergipe - HUSE) were enrolled consecutively at the time of presentation. HUSE provides 24-hour free medical services and is a reference hospital for Sergipe State (~2 million population). For logistical reasons, only children attending between 8 am and 4 pm from Monday to Friday were included. Due to funding constraints, data for NoV was analysed from October 2006 to September 2009 and from July 2011 to January 2013, but data collection used the same methods and protocols over the study period [10]. After obtaining written parental consent, children were assessed to establish the medical history and clinical presentation and parents were asked for the child vaccination cards. Vaccination cards are routinely brought by parents when they visit health facilities and all vaccinations are recorded. A child was classified as vaccinated for rotavirus if the card had the 2 documented doses of the vaccine. Children with one or no rotavirus vaccines were classified as unvaccinated. Children without vaccination cards were classified as having an unknown vaccination status. Parents were asked to collect one stool specimen from the child before leaving the service and about 60% of parents managed to collect specimens and were included in the study. Stools specimens were stored at 4ºC for a maximum of 24-hours and stored in a -80ºC freezer until processed.

3.2. Detection of Norovirus

RNA was extracted from 140 µl of 10% stool suspensions using the QIAamp Viral RNA extraction kit (QIAGEN, CA, USA), and immediately stored at −80ºC prior NoV detection. Real time RT-PCR was performed on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using primers and probes previously described [12] and the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, CA, USA). Briefly, the assay was carried out in a 25 µl final reaction mixture containing 5 µl of purified RNA with final concentrations of primers and probes of 600 and 300 nM, respectively. The thermal cycling conditions were carried out as follows: a RT step at 55 ºC for 30 min, an initial denaturation step at 95 ºC for 10 min, 45 cycles of PCR amplification at 95 ºC for 15 s, and at 60 ºC for 1 min. A 10-fold serial dilution of a plasmid containing the ORF1/2 junction was used to generate standard curves for virus quantification. Forty cycles were used in the reaction and samples with a cycle threshold <40 were regarded as positive.

NoV-positive samples were genotyped by sequencing the partial 5'-end of ORF2 region (320 nt in length, corresponding to the region C of the NoV genome), as previously described [12,13]. Amplicons were sequenced in both directions using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit® (v. 3.1) and an ABI Prism 3130xl DNA model sequencer (Applied Biosystems).

3.3. Phylogenetic analysis

Phylogenetic analysis was performed only on NoV-positive samples detected between July 2011 and January 2013 at the Rio de Janeiro Oswaldo Cruz Institute. Consensual sequences obtained were aligned and edited using the BioEdit Sequence Alignment Editor (version 7.0.5.3) program, and compared to the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Genotyping Tool available on line (Noronet; http://www.rivm.nl/mpf/norovirus/typingtool) was used to assign the strain to specific genotypes [14]. Phylogenetic analysis used the neighbour-joining

method (Kimura-two parameter model, 2000 bootstrap replicates) in MEGA 6.0 [15].

3.4. Data analysis

Descriptive statistics with 95% confidence intervals (95% CI) were used to describe the characteristics of participants. Proportions were compared using Chi-square (χ^2) and Fisher Exact tests. Variables associated with NoV infection in the bivariate analysis ($p \leq 0.10$) were entered into logistic regressions using backwards stepwise procedures. The severity of the diarrhoea episodes was classified using a standardized score and episodes with scores ≥12 were graded as severe, as previously described [16]. Skewed data, such as age, duration and frequency of diarrhoea and severity score were tested using Mann-Whitney to determinate differences between groups. P values <0.05 were considered statistically significant. Trends by year were explored using Chi-square for trend.

3.5. *Ethics considerations*

The study protocol was approved by the Ethics Research Committees of Sergipe Federal University (CAAE 0839.0.000.107-06 and CAAE 19319413.6.0000.5546) and by the Research Ethics Committee of the Liverpool School of Tropical Medicine. Informed written consent was obtained from all parents or legal guardians of the children.

4. Results

In total 280 (19.6%) of 1,432 samples were positive for NoV. Norovirus was detected less frequently in samples collected from October 2006 to September 2009 than and in samples collected from July 2011 to January 2013 (204 (18.3%) of 1,113 and 76 (23.9%) of 318, respectively, p = 0.03). The proportion of children with NoV ranged from 14.7% (61/416) in 2008 to 27.5% (22/79) in 2011 and increased in successive years through the study period (χ^2 for trend= 6.7; p=0.009). Norovirus was detected all year-round, without clear seasonality and peaks occurring in different months each year (Figure 1A).

Children with NoV were younger than children without NoV [median (IQR); 9 (5-18) vs 14 (6-32) months; p<0.001] and infants (<12 months) were more likely to be infected than older children (167/690 vs113/740; p<0.001). Children with NoV had higher vomiting frequency (1.4 vs 1.2 episodes/day; p= 0.049) and vomiting duration (2.2 vs 1.6 days; p= 0.002) than those without NoV, but fever (55.7% vs 65.3%; p= 0.002) and abdominal pain (48.2% vs 60.3%; p<0.001) were less common among children with than those without NoV (Table 1). One hundred and two (36.4%) of 280 children with Nov had severity scores >12 which was similar to the 412 (35.7%) of 1,152 children without NoV (p = 0.85). The vaccination cards were checked for 1,145 (80.0%) children and 562 (49.1%) had received the rotavirus vaccine. Rotavirus vaccinated children were more likely to have NoV than unvaccinated children [140/562 (24.9%) vs 67/583 (11.5%), p<0.001]. Age (aOR: 1.5, IC95%: 1.2-2.1; p=0.005) and rotavirus vaccination (aOR: 2.5, IC95%: 1.8-3.5; p<0.001) and were independently associated with NoV infection.

Of 280 NoV-positive specimens, 188 (67.1%) were sequenced. Of these, 176 were genogroup II and 12 genogroup I. The main genotype was GII.4 (149/188, 79.3%), followed by GII.2 (6, 3.2%) which emerged periodically over the study period as shown in table 2. Children with GII.4 NoV had a median (IQR) age of 8 (5-15) months compared to a median age of 11 (4-28) months for non-GII.4 (p=0.07). There was no difference in the diarrhoea severity of children with GII.4 and non-GII.4 strains, with a median score of 9.

There was a predominance of GII.4 over time, with genotype GII.2 emerging periodically and the circulation of small proportions of other genotypes. Phylogenetic analysis demonstrated the predominance of GII.4 New Orleans variant from January 2011 to May 2012 and its replacement with GII.4 Sydney from May to August 2012 (Figures 1B and 2).

5. Discussion

This study detected NoV in 19.2% of children hospitalized with acute gastroenteritis with an

annual range between 14.7% and 27.8% over the study period. Globally, hospital-based series in children with diarrhoea report a median of 14%, which seems lower than the proportions detected in our study [17]. Although our data suggests an annual increase in the proportion of cases, similar detection rates had been reported from Chile, India and Nicaragua prior to rotavirus vaccine introduction [18–20]. This relative increase may also be due to the decrease of number of rotavirus-related hospitalizations [10,11] and all-cause diarrhoea cases due to the effectiveness of the rotavirus vaccine [21] and the herd effect of the vaccine that has resulted in an overall and not a real increase in the overall number of NoV cases. The increasing trend reported here however is rare and could be an early indication of changes in the epidemiology of the pathogen in a population with a high rotavirus vaccination coverage. However, as the study was conducted after the introduction of the rotavirus vaccines, data of a completely unvaccinated population were unavailable.

The seasonality pattern of NoV in geographical areas close to the Equator is poorly described. In temperate regions NoV has a higher frequency during the winter and spring [22,23], but seasonal patterns become less well established in tropical areas [24]. This blurred pattern was also present in this study, as infections were detected all year round. Norovirus is detected more frequency in the community than in hospital studies. This is due to norovirus causing less severe diarrhoea than rotavirus and parents tend not to seek medical services [25,26]. In our study, the severity of diarrhoea episodes was similar in children with and without NoV and similar high detection rates had been reported from Chile, India and Nicaragua at that time when the vaccines had not been introduced [18–20].

Although rotavirus vaccinated children were more likely to have NoV than unvaccinated children, vaccinated children were younger and NoV infections were more common in younger children. Furthermore, as the severity of diarrhoea episodes was similar in vaccinated and unvaccinated children with and without NoV, these differences are likely an

artefact due to the confounding effects of other variables.

There were many NoV genotypes circulating over the years, and GII.4 was the predominant genotype detected, which is in agreement with others [27,28]. In Brazil, GII.4 NoV is most frequently reported from emergency departments [5,6,29,30]. The presence of a variety of genotypes in Sergipe and the periodic shift of GII.4 variants (i.e., GII.4 New-Orleans_2009 and GII.4 Sydney_2012) reflects the emergence worldwide of GII.4 variants [29,31–36], confirming that the NoV genotypes have a wide geographical distribution. The increased detection of GII.4 could be explained by GII.4 theoretically causing more severe disease than other genotypes [31,37], but GII.4 diarrhoea severity in our patients was similar to other genotypes and we do not have evidence of an increased severity.

A limited analysis of the children's home addresses (data not shown) did not establish geographical associations between children with similar genotypes. Studies investigating the transmission of norovirus should consider collecting detailed descriptive data and analyse different NoV genotypes separately to improve our understanding of the transmission routes of norovirus within the community [38].

This study has a number of strengths including its onset at the time of the introduction of the rotavirus vaccine and the standard approach of recruitment and examination of children over the years. However, it also has significant limitations, as we do not have data prior to the introduction of the vaccines, data on the aetiology of non-NoV episodes, nor data on healthy asymptomatic controls. Annual surveillance is an aggregation of cases occurring over twelve months and there were significant variations between the months, from no cases over a six months' period to over 30% over thirteen months. The increasing trend detected therefore might be an artefact of aggregation of small outbreaks over time. The apparent increase in norovirus infection can also be an artefactual consequence of the reduction in in the burden of rotavirus and all-cause diarrhoea brought about by the rotavirus vaccination program [10,11].

Furthermore, only participants who provide a stool specimen were included in the analysis, which is likely to select children who stayed longer in the rehydration units and to introduce a bias towards higher disease severity. Finally, the descriptive study design does not offer mechanistic insights as to why norovirus infections might have increased.

In summary, the proportion diarrhoea cases due to NoV has increased over the years following the introduction of rotavirus vaccine. These effects could be an artefact due to the reduction of the burden of rotavirus. GII.4 was the most common genotype identified over the years, with temporal clusters of GII.4 variants. NoV GII.4 were similar to variants reported elsewhere and were not associated with increased disease severity. Further studies to monitor changes in the epidemiology of norovirus in this highly rotavirus-vaccinated population are warranted and a longer period of surveillance is needed.

Conflicts of interest

The authors have declared they don't have competing interests.

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List of figures

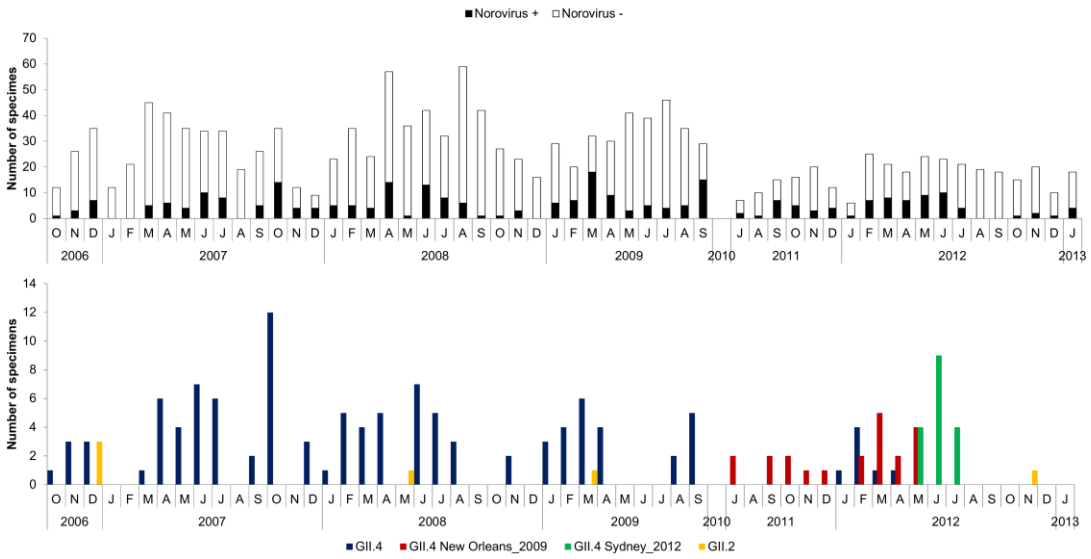

Figure 1. A. Number of samples positive and negative for norovirus by month. B. The bars represent the norovirus specimens most frequently genotyped during the study period.

Figure 2. Phylogenetic dendrogram based on partial capsid nucleotide sequences of noroviruses strains detected from October 2006 to January 2013. NoV prototypes obtained from GenBank and genotypes and accession numbers shown on the right.

Tables

Table 1. Characteristics of the children with acute diarrhoea.

Variables	Norovirus		p-value
	Negative N=1152	Positive N=280	
Sex, male, n (%)	605 (52.6)	163 (58.2)	0.08
Age in months, median (IQR)	14 (6-32)	9 (5-18)	<0.001
Age group, months			
<12	525 (45.5)	167 (59.6)	<0.001
12-<24	238 (20.7)	68 (24.3)	0.18
≥24	389 (33.8)	45 (16.1)	<0.001
Fever, n (%)	752 (65.3)	156 (55.7)	0.002
Abdominal pain, n (%)	694 (60.3)	135 (48.2)	<0.001
Severe dehydration, n (%)	63 (5.5)	16 (5.7)	0.87
Diarrhoea			
Duration in days, median (IQR)	3 (2-5)	3 (2-5)	0.25
Frequency per day, median (IQR)	4 (3-6)	4 (3-6)	0.60
Vomiting			
Present, n (%)	656 (57.0)	172 (61.4)	0.17
Duration in days, mean (SD)	1.6	2.2	0.002
Frequency per day, mean (SD)	1.2	1.4	0.049
Severity score			
Median (IQR)	10 (7-12)	10 (7-12)	0.95
Score ≥12, n (%)	412 (35.7)	102 (36.4)	0.85

Table 2. Distribution of norovirus capsid genotypes.

Genotypes	2006	2007	2008	2009	2011	2012	2013	Total
GII.4	7	41	32	24	8	37		149
GII.2	3		1	1		1		6
GI.4			5					5
GII.6			3		1			4
GI.14		3						3
GII.13		2						2
GII.9			2					2
GII.8							2	2
GII.12				1				1
GI.3		1						1
GI.7		1						1
GI.12		1						1
GI.10				1				1
GII.3				1				1
GII.14				1				1
GII.7							1	1
GII.NT		1		6				7
Total	10	50	43	35	9	38	3	188