

ROTAVIRUS SEROTYPES AND ELECTROPHEROTYPES IDENTIFIED AMONG HOSPITALISED CHILDREN IN SÃO LUÍS, MARANHÃO, BRAZIL

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SUMMARY

During June 1997-June 1999 rotavirus infection was screened in infants aged up to 2 years and hospitalised with acute diarrhoea in São Luís, Northeastern Brazil. Altogether, 128 stool samples were collected from diarrhoeic patients and additional 122 faecal specimens from age- and temporal matched inpatients without diarrhoea were obtained; rotavirus positivity rates for these groups were 32.0% (41/128) and 9.8% (12/122), respectively ($p < 0.001$). Both electropherotyping and serotyping could be performed in 42 (79.2%) of the 53 rotavirus-positive stool samples. Long and short electropherotypes were detected at similar rates - 38.1% and 40.5% of specimens, respectively. Overall, a G serotype could be assigned for 35 (83.3%) of specimens, the majority of them (66.7%) bearing G1-serotype specificity. Taking both electropherotypes and serotypes together, G1 rotavirus strains displaying long and short RNA patterns accounted for 30.9% and 19.0% of tested specimens, respectively; all G2 strains had short electropherotype. Rotavirus gastroenteritis was detected year-round and, in 1998, the incidence rates tended to be higher during the second semester than in the first semester: 45.2% and 26.1% ($p = 0.13$), respectively. Rotavirus infections peaked at the second semester of life with frequencies of 30.1% and 13.5% for diarrhoeic children and controls, respectively. While the six rotavirus strains bearing G2-type specificity were circulating throughout the whole study period, G1 serotypes ($n = 27$) emerged as from June 1998 onwards, 20 (74.1%) of which clustering in 1998. These data underscore the importance of rotaviruses in the aetiology of severe infantile gastroenteritis in Northeastern Brazil and sustain the concept that a future vaccine should confer protection against more than one serotype.

KEYWORDS: Rotavirus; Serotypes; Electropherotypes; Children.

INTRODUCTION

Rotavirus gastroenteritis still remains a major cause of morbidity and mortality among infants and young children in both developed and developing countries^{2,19}. It is estimated that 130 million children develop rotavirus-related diarrhoea each year - 18 million of whom experiencing moderate to severe dehydration -, resulting in between 418,000 and 520,000 deaths; of note, 85% of these deaths occur in low-income countries^{18,27,28}. In light of more recent estimates of the global illness and deaths caused by rotavirus disease, however, there are each year approximately 111 million episodes of gastroenteritis requiring only home care, 25 million clinic visits, 2 million hospitalisations, and between 352,000 and 592,000 deaths³¹. Data from several studies conducted throughout the world have shown that rotaviruses account for 15% to 71% (average, 33%) of acute gastroenteritis requiring hospitalisation of infants and young children⁸. In a recent 2-year prospective surveillance for acute diarrhoea in three South American countries rotavirus-associated hospitalisations occurred at rates of 71% (Argentina), 47% (Chile) and 38% (Venezuela)²⁹.

While most epidemiological studies in Brazil have been conducted in North and Southeast regions, data from other areas, particularly the Northeast region, are still scarce. Overall, studies carried out in Brazil have in general focussed on the occurrence of acute-diarrhoea associated with outpatient clinic visits/hospitalisations, yielding average prevalence rates that ranged from 12% to 42% throughout the country^{21,32}.

Rotaviruses form a specific genus of the Reoviridae family. The mature particles are icosahedral, non-enveloped, measure 100 nm in diameter, and consist of a double-stranded RNA (dsRNA) genome of eleven distinct genes surrounded by a triple-layered protein capsid. Studies on the electrophoretic migration patterns of viral genomic dsRNA segments (electropherotyping) have allowed the classification of rotaviruses into two major groups, the long (L) and the short (S) electropherotypes¹⁹. Six of these dsRNA segments encode for six structural proteins (VP1-VP4, VP6 and VP7), whilst five of them encode for five non-structural proteins (NSP1-NSP5). Of importance, structural proteins VP7 (a glycoprotein or G antigen) and VP4 (the protease-

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sensitive protein or P antigen) make up the outermost layer and are known to induce neutralising and protective antibodies, respectively¹⁷. On the basis of antigenic and genetic diversities, 15 G types and 20 P types have been identified to date among rotavirus strains of both human and animal origin. In this context, 10 G types and 11 P types have been recovered in association with human infections, yielding at least three dozen different G/P combinations. Although there is a broad diversity of rotavirus types infecting humans, the majority of isolates from diarrhoeic children fall into the four groups, as follows: P[8]G1, P[4]G2, P[8]G3 and P[8]G4. More recently, however, P[8]G9 and P[6]G9 strains have emerged globally as common G and P type combinations encountered in human infections¹⁷.

Although all four epidemiologically important rotavirus G types are reported to occur throughout Brazil, several recent studies have shown that uncommon strains may account for a significant proportion of isolates in cases of diarrhoea^{6,21,34,40}. While G5 rotavirus may yield prevalence rates of up to 13% in some regions of Brazil²⁰, it is noticeable that strains bearing the G9-type specificity have been detected with increasing frequency in the last several years^{1,36}.

The results from this study extend our knowledge on the epidemiological features of rotavirus infection in the Northeast region of Brazil and provide a preliminary characterisation of strains infecting paediatric patients in the city of São Luís, Brazil as based on RNA migration patterns and serotype-specific monoclonal antibody reactivities.

MATERIALS AND METHODS

Study area and patients: The study was conducted in São Luís, the capital of the state of Maranhão, Northeast Brazil, which is a 988 square kilometre island located near the continent, having a humid tropical climate with heavy downpours during January through April. Temperatures range from 25.2 °C to 30.3 °C and a year-round high air humidity of usually greater than 80% is noted. The population of São Luís is about 870,000 inhabitants and the infant mortality rate is 65/1,000 live births. Between June 1997 and June 1999 a total of 128 stool specimens were collected from children aged < 3 years with acute diarrhoea of less than 14-day duration who were treated for dehydration at the emergency paediatric unit of the university hospital in central São Luís, Maranhão. In addition, stools from 122 temporal- and- age matched infants without diarrhoea, who attended an outpatient unit in the same hospital, were included as controls. This study was approved by an ethical medical committee and written informed consents were obtained from parents or legal guardians of the eligible infants before enrolling them.

Laboratory procedures: Stool samples were obtained from patients and controls for local microbiological examinations. An aliquot of each sample was stored at -20 °C until being transported on ice to *Instituto Evandro Chagas, SVS, Belém, Pará, Brazil*, where rotavirus antigen detection and rotavirus strain characterization were performed. Ten per cent (w/v) faecal extracts were screened for the presence of Group A rotavirus antigen using monoclonal antibodies by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Dakopatts, Denmark). Polyacrylamide gel electrophoresis (PAGE) was carried out on the faecal suspensions using a standard method

which includes extraction of RNA genome by using the glass powder method, essentially as previously described³. The RNA was subsequently electrophoresed in 10% acrylamide gels for 3 h - 4 h at 20 mA at room temperature and segments were visualised by silver staining according to the method of HERRING *et al.*¹⁶. The assay for determination of rotavirus G-serotypes has been described previously by TANIGUCHI *et al.*⁴¹. Briefly, this was a microtitre plate solid-phase assay that uses monoclonal antibodies specific for G types 1, 2, 3, and 4, produced in Sapporo, Japan^{42,43}. ELISA-positive samples which did not react with G1 through G4 monoclonals were further tested for G5-, G6-, G8-, G9- and G10-serotype specificities, as previously described⁹. The latter monoclonal antibodies were kindly donated by Dr. Ruth Bishop (Australia) and Dr. Dennis Lang (USA).

RESULTS

Taking both diarrhoeic- and- control children together rotaviruses were detected in 21.2% of all faecal specimens; the positivity rates in the former and latter groups, however, were 32% (41/128) and 9.8% (12/122), respectively ($p < 0.001$) (data not shown). Both electropherotyping and serotyping could be performed in 42 of the 53 samples. Of the total 53 rotavirus-positive stool samples, 33 (62.2%) showed a clear, characteristic human group A electrophoretic profile, with long and short RNA migration patterns being detected at similar rates - 38.1% and 40.5%, respectively. While three varieties were identified among the short electropherotypes (S1, S2 and S3), strains with long migration patterns exhibited only two distinct profiles (L1 and L2). Figure 1 demonstrates representative patterns obtained from polyacrylamide gel electrophoresis analysis of rotavirus strains excreted from children in São Luís, Maranhão. A G type-specificity could be assigned to 83.3% ($n = 35$) of rotavirus strains when isolates were subjected to serotyping by monoclonal antibodies. Among strains tested for both electropherotypes and serotypes ($n = 42$), 28 (66.7%) could be serotyped as G1, six (14.3%) as G2, and one (2.4%) strain yielded a dual G1/G3 specificity; for the remainder of the samples a multiple reaction could be characterised in one (2.4%) specimen and six (14.3%) could not be typed by the monoclonal antibodies employed in the

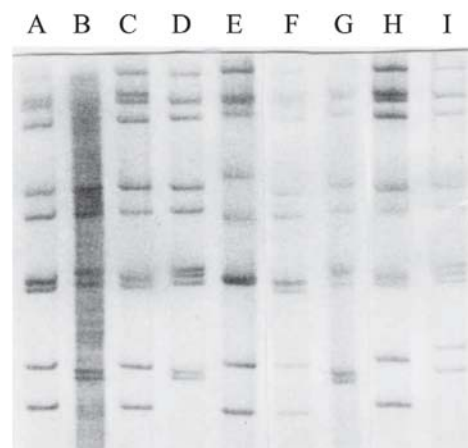


Fig. 1 - Polyacrylamide gel electrophoresis of representative strains from rotaviruses identified in São Luís, Maranhão, Brazil. Lanes A and B display the long (L) and short (S) control RNA profiles, respectively; long study electropherotypes are shown in lanes C (L1), E (L2), F (L1) and H (L1); and short profiles in lanes D (S1), G (S2) and I (S3).

serotyping assays. Taking both electropherotypes and serotypes together, 13 (30.9%) of G1 rotavirus strains showed a long electrophoretic profile, eight (19.0%) had a short electropherotype and in seven (16.7%) the PAGE did not show the RNA profile. All six (14.3%) serotype G2-reactive rotavirus strains exhibited a short electropherotype. A long electrophoretic profile was exhibited by the dual G1/G3-reactive specimen and the strain with multiple serotyping reactions had a short electropherotype. For strains to which one of more serotype(s) could not be determined, long and short electropherotypes were identified at identical rates: 4.8% and 4.8%, respectively (Table 1).

Among diarrhoeic infants, rotavirus was detected from September 1997 to May 1999, with monthly distribution yielding peak incidences during October - December 1997 (> 50%) and August - October 1998 (≥ 60%) (Fig. 2). In children belonging to the control, non-diarrhoeic group there was only one isolate on December 1997, followed by a marked clustering of rotavirus detection during August - November 1998, when incidence rates ranged from 9% to 100% (Fig. 3). For these non-diarrhoeic infants, no rotavirus activity could be observed during the remainder of the study period, that is, June - November,

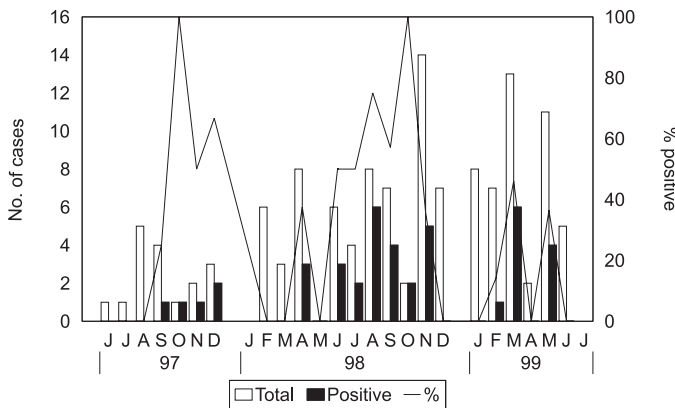


Fig. 2 - Temporal distribution of rotavirus detection in diarrhoeic children attending paediatric wards in São Luís, Maranhão, Brazil.

1997, January - July, 1998, and January - July, 1999. Rotavirus infection was predominant up to 18 months of life, with the highest incidence rates being detected between seven and 18 months of age: 29.3% and 12.5% for diarrhoeic children (Fig. 4) and controls (Fig. 5), respectively.

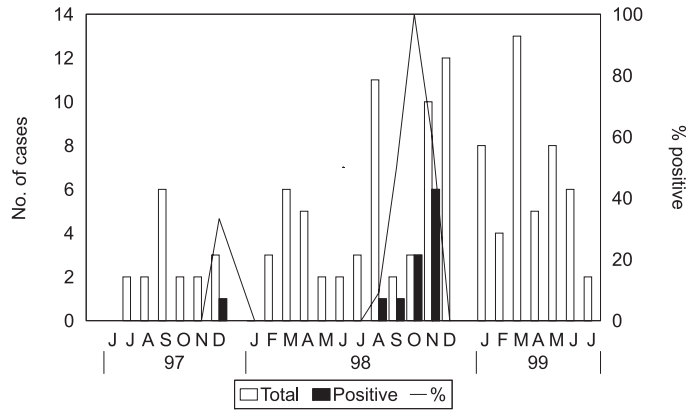


Fig. 3 - Temporal distribution of rotavirus detection in non-diarrhoeic children (controls) attending paediatric wards in São Luís, Maranhão, Brazil.

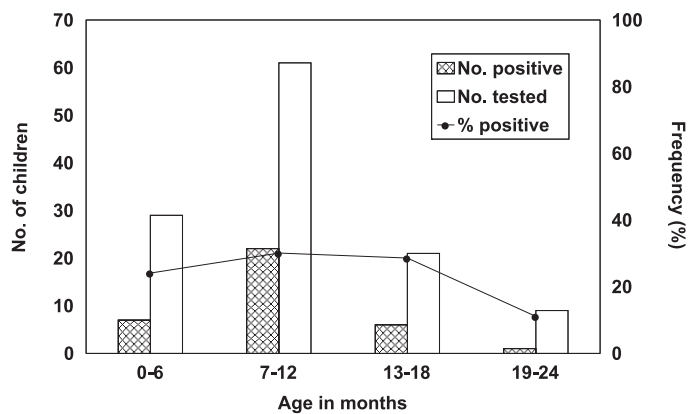


Fig. 4 - Age-distribution of rotavirus infections in 120 diarrhoeic children attending paediatric wards in São Luís, Maranhão, Brazil.

Table 1
Frequency of rotavirus serotypes and electropherotypes in children from São Luís, Maranhão

Electropherotypes	Serotypes					Total
	G1	G2	G1+G3	MR*	ND†	
Long	13 (30.9)§		1 (2.4)		2 (4.8)	16 (38.1)
Short	8 (19.0)	6 (14.3)		1 (2.4)	2 (4.8)	17 (40.5)
Negative	7 (16.7)				1 (2.4)	8 (19.0)
ND†					1 (2.4)	1 (2.4)
Total	28 (66.7)	6 (14.3)	1 (2.4)	1 (2.4)	6 (14.3)	42 (100.0)

* Multiple reaction; † Not determined; § Percentage

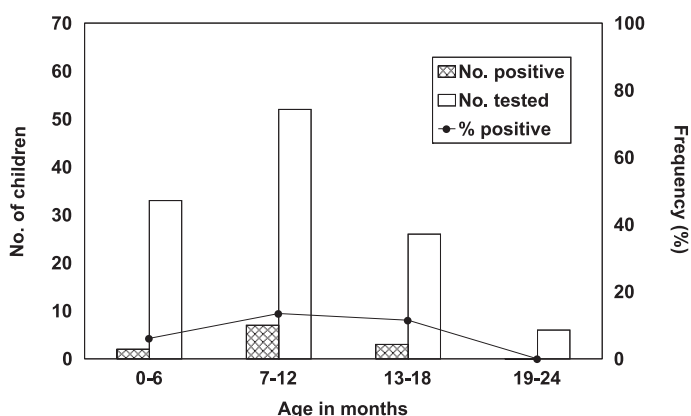


Fig. 5 - Age-distribution of rotavirus infections in 117 non-diarrhoeic children attending paediatric wards in São Luís, Maranhão, Brazil.

The temporal distribution of circulating rotavirus serotypes in São Luís, Maranhão from June 1997 to July 1999 is represented in Figure 6. It has been demonstrated that whereas the six infections caused by rotavirus strains bearing G2-serotype specificity occurred throughout the whole study period, G1 types (n = 27) circulated as from June 1998 onwards; of these, 20 (74.1%) clustered from June to November 1998. There were six untypeable rotavirus strains, five of which being detected during July - October 1998. Of nine rotavirus isolates from controls in which serotyping was performed, eight belonged to G1 type and one strain resulted untypeable.

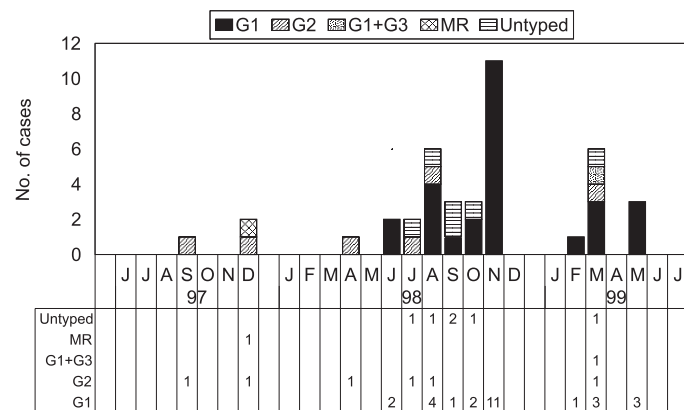


Fig. 6 - Monthly distribution of rotavirus serotypes in children attending paediatric wards in São Luís, Maranhão, Brazil. Note: collection date was not available from one patient excreting G1 rotavirus. MR, Multiple reaction.

DISCUSSION

During almost three decades of rotavirus studies in Brazil it can be observed that a large amount of meaningful data were gathered from numerous hospital-based surveys across the country^{21,25}. It is noticeable, however, that epidemiological data on rotavirus infection still remain scarce for the north-eastern region, where poverty and poor sanitation conditions largely prevail among both peri-urban and rural communities. This study added several data to the knowledge on the epidemiology of rotavirus infection in one of the country's poorest

regions, particularly with regards to the prevalence of different G (or VP7) serotypes. Overall, results presented herein confirm previous observations from different parts of Brazil and elsewhere in the world that rotavirus in general accounts for a significant proportion of both infants and young children who are hospitalised due to acute gastroenteritis^{24,31}. At least 25 hospital-based surveys have been conducted across Brazil during the past two decades, showing that rotavirus may account for up to 66.0% of cases of acute gastroenteritis among children aged less than five years²⁵. Although a wide range in the prevalence rates of rotavirus-related gastroenteritis has been observed throughout the country, our data are in agreement with those from the majority of studies conducted in Brazil, where this enteropathogen was found to cause around one-third of diarrhoeal episodes requiring treatment at the hospital^{21,26}. Furthermore, our results are comparable to those from previous observations in São Luís, Brazil, which showed that rotavirus occurs at frequency of approximately 25% among infants hospitalised for acute diarrhoea³⁹. Interestingly, our data show that an unusual high proportion (~ 10%) of hospitalised infants without diarrhoea were found to excrete rotavirus, a prevalence rate that in general exceeds those frequencies reported for control, non-diarrhoeic children in previous studies carried out in Northern regions of Brazil. In the course of an earlier, two-year, hospital-based surveillance study conducted in São Luís, Brazil, for example, an average of only 6% rotavirus-positivity was found among children assigned to the control group³⁹. For some as yet unexplained reason rotaviruses appeared to have spread efficiently within paediatric wards during August - November 1998, thus causing asymptomatic infection in a significant proportion of children who were assigned to the control group. It seems therefore likely that such a marked peak incidence of (symptom-free) rotavirus infection has accounted for the overall unusual high prevalence rate among non-diarrhoeic children in our study.

The variability in strain prevalence in São Luís, Brazil was assessed through the characterisation of electrophoretic profiles and serotype analysis. Unlike several other studies conducted to date in Brazil and elsewhere, long and short electropherotypes were found to circulate at similar rates throughout the study period^{19,21}. In general it would be expected a predominance of rotavirus strains with long electropherotype, as classically this RNA migration pattern correlates with the most common Wa genogroup which comprises most rotavirus strains of serotypes G1, G3, G4 and G9. Conversely, rotavirus strains displaying short electropherotype are usually included in the apparently world-wide less prevalent DS-1 genogroup, which is composed mainly of G2 strains¹⁹. It is noticeable from our data that a lower variation of electropherotypes could be identified in São Luís, as compared to other studies carried out in Brazil and elsewhere, where over 15 distinct RNA migration patterns were reported to be co-circulating within the same community^{11,12,15}. It should be pointed out in this regard that a great diversity of genomic variants in the same locality, as indicated by the large number of electropherotypes, may reflect the potential for active genetic re-assortment between different viral strains¹⁹. The occurrence of eight (19.0%) rotavirus-positive samples that yielded negative results by PAGE was possibly due to an insufficient RNA concentration, therefore below the limit of 3-4 ng following silver staining¹⁶.

In our study the use of a panel of monoclonal antibodies to antigenically type the VP7 of rotavirus isolates proved highly sensitive,

in that over 80% of strains could be clearly assigned to a specific G serotype. This seems to be in contrast with previous studies indicating that the use of serotype-specific monoclonal antibodies by ELISA may leave over 50% of rotavirus strains in stool specimens, untyped^{13,23,41}. Of note, failure in serotyping by ELISA has been mainly attributable to either possible intratypic strain variations or the presence in stool samples of poorly antigenic VP7 molecule detached from the virion. It would seem plausible to postulate that such conditions apparently did not occur when serotyping rotavirus isolates directly from stool samples in São Luís, Brazil.

Rotaviruses bearing VP7 G1 serotype-specificity were found to be predominant in São Luís, Brazil, accounting for approximately 70% of typed rotavirus isolates. In a country-wide - and even global context -, G1 rotavirus strains have been largely predominant, being even regarded as a prime target of current vaccination strategies^{10,21}. Although accounting for only 14% of rotavirus isolates, rotaviruses bearing G2 serotype-specificity were the second most prevalent strains identified throughout the study period, in a pattern similar to that previously described for Latin America and Brazil^{7,24}. It is worth mentioning that, unlike G1 strains, rotaviruses belonging to G2 serotype appear to display a cyclic pattern of occurrence, as it has been demonstrated in North Brazil and South Africa^{15,24,30}. Continuous monitoring of circulating rotavirus strains is therefore needed in São Luís, Brazil and elsewhere, since a major emergence of this (G2) DS-1 genogroup strain may possibly pose a challenge to current (G1) Wa genogroup-based vaccine strategies^{10,14}. In addition to this, the fluctuation in the relative frequencies of rotavirus strains over time can be evidenced by the fact that a previous survey conducted in this same setting had demonstrated a serotype G3 "season"^{7,40}. It was interesting that we did not detect any "uncommon" strains in the present study, in spite of using a panel of VP-7 serotype-specific monoclonal antibodies that would allow the detection of at least nine distinct rotavirus antigenic types. This seems to be in contrast with previous several studies in Brazil showing that unusual serotypes such as G5, G8 and G10 are detectable at rates that may be as high as 25%^{1,20,24,35,37}. It is also noticeable in this context that G9 - currently regarded as the fifth epidemiologically important serotype - could not be identified among rotaviruses isolated from 1997 to 1999 in São Luís, Brazil, probably due to its recent emergence worldwide^{7,25,33}. Also of interest was the finding that mixed G-type infections among study children were detectable at surprisingly too low rates (one G1+G3 infection only), if compared with studies conducted in several other regions in Brazil where as high as 30% of paediatric patients may be concurrently infected with more than one serotype^{7,20,24,34}. Overall, such apparently divergent results, as compared to those from several other studies throughout the country, may be due to the fact that, in São Luís, Brazil, the vast majority of rotaviruses belonged to a single serotype.

Taking both serotypes and electropherotypes together, it is particularly intriguing the detection of eight rotavirus strains with G1-specificity that displayed a short RNA pattern. Such unusual combinations represent interesting phenotypes which are likely to have emerged from naturally occurring viral reassortments in the study community and should be further characterised through the determination of complete VP7 genes.

An unusual pattern of temporal distribution of rotavirus serotypes

could be noted in São Luís, Brazil from June 1997 to July 1999. While G2 strains were detected at low incidence rates throughout the entire study period, rotaviruses bearing G1 serotype-specificity have emerged only as from June 1998 onwards, with most of isolates (> 70%) clustering from June to November 1998. These data are in contrast with most of studies conducted in Brazil to date, where G1 types are in general maintained over time, whereas G2 rotaviruses - which are generally regarded as highly susceptible to selection pressure - tend to follow a cyclic circulation pattern in the community^{19,24}. An interesting finding was that rotaviruses infecting children who belonged to the non-diarrhoeic, control group clustered during the second half of 1998 and belonged to serotype G1. This is in clear temporal correlation with the overall peak incidence of rotavirus strains bearing G1-type specificity, as from June to November 1998.

Following a pattern similar to that generally reported for tropical regions, rotaviruses were detected year-round in São Luís, Brazil; however, a trend for higher prevalence rate could be observed during the second semester of 1998, in connection with the emergence of strains bearing G1 type-specificity^{19,32,39}. In regards to the age distribution, our data seem to corroborate those from previous surveillance studies conducted in Brazil and elsewhere, indicating that a higher susceptibility to rotavirus disease occurs after the first 4-6 months of age^{19,22,24,39}. It would therefore be advisable that future vaccine strategies should comply with an immunisation schema for completion within the first semester of life²¹.

Although the present study has added epidemiological data on rotavirus infections in the more northern tropical areas of Brazil, a further characterisation of the strains recovered from hospitalised children in São Luís is worth to be pursued. This would comprise the use of reverse transcription polymerase chain reaction for identification of VP4 (P) and VP7 (G) rotavirus genotypes, as well as the oligonucleotide sequencing applied to a subset of selected strains that could not be antigenically typed or were found to be unusual when taking serotype and electropherotype together.

Despite the large amount of data gathered from numerous rotavirus surveys conducted in Brazil, results are often not comparable, since methodologies used were different among studies. In view of this, a country-wide surveillance system for rotavirus gastroenteritis is needed using standardised procedures, in a way similar to those recently implemented in Africa and Asia^{4,5,38}. Besides determining the burden of rotavirus disease, this nation-wide surveillance system would allow monitoring of the diversity of circulating rotavirus strains. This is particularly important in a current scenario where at least two rotavirus candidate vaccines are successfully approaching completion of phase III trials^{10,14}.

RESUMO

Sorotipos e eletroferotipos de rotavírus identificados entre crianças hospitalizadas em São Luís, Maranhão, Brasil

De junho de 1997 a junho de 1999, pesquisou-se a infecção por rotavírus entre crianças até 2 anos de idade internadas com quadro diarréico agudo em São Luís, nordeste do Brasil. Coletaram-se 128 espécimes fecais oriundos de pacientes diarréicos. Paralelamente,

obtiveram-se 122 amostras de um contingente caracterizado como controle, comparável ao anterior no tocante às idades e distribuição temporal. As frequências de positividade para rotavírus alcançaram 32,0% (41/128) e 9,8% (12/122), respectivamente ($p < 0,001$). Procedeu-se à determinação dos sorotipos e eletroferotipos dos rotavírus em 42 (79,2%) das 53 amostras reativas para rotavírus. Identificaram-se eletroferotipos “longo” e “curto” em frequências similares - 38,1% e 40,5%, respectivamente. De um modo geral, caracterizou-se o sorotipo G em 35 (83,3%) das amostras positivas, a maioria, revelando especificidade para o tipo G1. Considerando o conjunto dos eletroferotipos e sorotipos, rotavírus classificados como G1 exibiram padrões eletroforéticos “longo” e “curto” nas frequências de 30,9% e 19%, respectivamente. Todos os rotavírus do tipo G2 apresentaram eletroferotipo de configuração “curta”. No tocante ao perfil temporal, observou-se que as gastroenterites por rotavírus naquela região ocorrem ao longo de todo o ano, denotando-se tendência quanto à mais expressiva concentração no segundo semestre de vida das crianças, se comparado ao primeiro; em síntese, 45,2% e 26,1% ($p = 0,13$), respectivamente. As infecções por rotavírus configuraram picos quanto à distribuição durante o segundo semestre de vida, com frequências de 30,1% e 13,5%, respectivamente. Aqueles do tipo G2 circularam durante todo o período de estudo, enquanto o sorotipo G1 ($n = 27$) emergiu a partir de junho de 1998. Aliás, detectaram-se 20 (74,1%) das amostras virais com essa última especificidade ao longo de 1998.

Os dados acima sustentam a importância dos rotavírus na etiologia das gastroenterites graves no nordeste brasileiro e consubstanciam o conceito de que uma futura vacina contra esses enteropatógenos necessariamente deve conferir proteção frente aos múltiplos sorotipos circulantes.

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