JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2006, p. 3947–3953 Vol. 44, No. 11
0095-1137/06/$08.00+0 doi:10.1128/JCM.00279-06

Genetic Diversity of Norovirus among Children with Gastroenteritis in São Paulo State, Brazil▽

Juliana Galera Castilho,[1,4] Veridiana Munford,[1] Hugo Reis Resque,[1] Ulysses Fagundes-Neto,[2] Jan Vinjé,[3]† and Maria Lúcia Rácz[1]*

Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo 05508-900, Brazil[1]; UNIFESP-Escola Paulista de Medicina, São Paulo 04023-900, Brazil[2]; Department of Environmental Sciences and Engineering, School of Public Health, University of North Carolina, Chapel Hill, North Carolina[3]; and Instituto Pasteur, São Paulo 01311-000, Brazil[4]

Received 8 February 2006/Returned for modification 15 March 2006/Accepted 21 August 2006

Norovirus (NoV) is one of the most common causes of acute gastroenteritis in children and adults. To study the prevalence and genetic variability of NoV in children with acute gastroenteritis in São Paulo State, Brazil, we examined 234 stool samples from children with or without gastroenteritis during a 5-year period (1995 to 1999). NoV RNA was detected by reverse transcription-PCR and confirmed by DNA sequence analysis. We used two different oligonucleotide primer sets targeting the 3′ end of the RNA polymerase gene (region B), as well a partial capsid region at the 3′ end of the VP1 gene (region D). A total of 78 (33.3%) of the samples tested positive for NoV, and in region B, of the 66 strains sequenced, 4 (6.1%) belonged to GI, 52 (78.7%) belonged to GII, and five samples (7.6%) contained a mixture of the GI and GII genotypes. Phylogenetic analysis showed that the majority (40 of 66 [60.6%]) of the strains belonged to genotype GII.4. The nucleotide sequence identity of three strains was lower than 77.9% compared to a region B reference sequence database but showed 85.3 to 88.8% identity with GII.2 Melksham strain in region D, indicating the circulation of a possible recombinant NoV strain. One sample (GII.3) was sequenced only in region D. In conclusion, we have a total of 67 sequenced strains. This is the first report that describes the predominance of GII.4 NoV strains in children visiting the ambulatory of different hospitals in São Paulo State, Brazil, and we show that mixtures of different strains can be found in individual samples, including some possible new recombinant strains.

Acute gastroenteritis remains a major public health problem worldwide, with more than 700 million estimated cases occurring annually in children less than 5 years of age. The mortality associated with gastroenteritis has been estimated to be 3.5 to 5 million cases per year (14, 32). Many different pathogens have been found in fecal samples of children with gastroenteritis, including parasites, bacteria, and viruses (12). Among the viruses, rotavirus group A, enteric adenovirus, astrovirus, and human caliciviruses (norovirus and sapovirus) are well established as etiologic agents of acute gastroenteritis (1), with norovirus (NoV) as the single most common cause of outbreaks of acute gastroenteritis in all age groups (5, 13). The frequency of NoVs in sporadic cases of gastroenteritis is not yet well defined (1).

NoVs, members of the family *Caliciviridae*, are nonenveloped viruses, 27 to 35 nm in diameter, that possess a positive-sense RNA genome of 7.5 to 7.7 kb. The genome encodes three open reading frames (ORFs), including ORF1 coding for a large polyprotein that after translation is cleaved into nonstructural proteins such as RNA-dependent RNA polymerase (POL), helicase, and protease peptides. ORF2 encodes a major capsid protein (VP1), and ORF3 encodes for a minor capsid protein (VP2) (1, 13).

Human NoVs can be divided into at least two distinct genogroups (GI and GII) which, based on the N-terminal region (region C) of VP1, can be further subdivided into at least 31 different genetic clusters or genotypes (18). When POL sequences are used to classify NoV into genotypes, nucleotide similarities of more than 85% for GI strains or 90% for GII strains belonging to the same genetic cluster have been proposed (37). For NoV diagnostics two different genomic regions are most often used. Traditionally, primers targeting the POL region of the genome (region A) are being used for NoV detection (36, 39), whereas the Centers for Disease Control and Prevention developed an assay targeting the 3′ end of ORF1 (region B 6). To genotype NoV strains, two different regions of the capsid gene have been used (region C [21, 25, 36] and region D [37]) that show similar clustering of strains compared to the complete VP1 protein.

Limited studies have investigated the prevalence and epidemiology of NoV in Brazil, and no studies have been reported on the NoV strain diversity in children with acute gastroenteritis in São Paulo State, Brazil. The knowledge of genomic diversity of NoV in Brazil is limited to two reports: one based in Ceará (27) and one based in Rio de Janeiro (11).

Genetic diversity of RNA viruses can be generated by recombination. For this to occur, different genotypes of NoVs need to coinfect the same cell (34). The presence of different topologies of the same strain when different parts of the genome (e.g., RNA polymerase gene and capsid gene) are analyzed is an indication of recombination (19, 31).

* Corresponding author. Mailing address: Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 1374, São Paulo, São Paulo 05508-900, Brazil. Phone: 55-11-3091-7292. Fax: 55-11-30917354. E-mail: mlracz@usp.br.

† Present address: Centers for Disease Control and Prevention, Atlanta, Georgia.

▽ Published ahead of print on 30 September 2006.

TABLE 1. Oligonucleotide primers used in this study

Region	Name	Sequence (5′–3′)[a]	Sense	Position[b]
B	MON 431	tgg acI agR ggI ccY aaY ca	+	5093–5113
B	MON 432	tgg acI cgY ggI ccY aaY ca	+	5093–5113
B	MON 433	gaa Yct cat cca Yct gaa cat	−	5284–5305
B	MON 434	gaa Scg cat cca Rcg gaa cat	−	5284–5305
D	CapD3	tgY ctY ItI ccH caR caa tgg	+	6432–6453
D	CapC	cct tYc caK Wtc cca Ygg	−	6666–6684

[a] International Union of Biochemistry ambiguity codes: I, inosine; H, (A/C/T); Y, pyrimidine (C/T); R, purine (A/G); S, strong (C/G); K, keto (G-T); W, weak (A-T).
[b] Equivalent position within the NV genomic sequence (M87661).

Jiang et al. (17) have shown that strain Arg320 is a recombinant strain of natural origin. Natural recombination has also been suggested for the strains Snow Mountain (16) and Rotterdam (37).

Due to the tremendous genetic diversity of NoVs, molecular characterization of different genotypes is essential to better understand the epidemiology of strains associated with pediatric gastroenteritis. Therefore, the objective of the present study was to identify and genetically characterize NoV in stools samples from children in São Paulo State, Brazil.

MATERIALS AND METHODS

Specimen collection. A total of 234 fecal specimens were collected from children (<3 years of age) in the State of São Paulo between August 1995 and November 1999; 94 samples were from people with diarrheal symptoms, 45 samples were from children with persistent diarrhea, 55 samples were from controls, and no clinical data were available for 40 samples. Samples were collected at the Hospital São Paulo, the Hospital Municipal de Jundiaí, the Hospital Darci Vargas, and the gastro-pediatric unit of the University Federal of São Paulo. After collection, samples were shipped frozen on dry ice to the virology laboratory and stored at −20°C. All samples had been previously tested for rotavirus group A, enteric adenovirus (30), and astrovirus (28).

Nucleic acid extraction and reverse transcription-PCR (RT-PCR). Fecal suspensions (10%, [wt/vol]) were prepared in phosphate buffered saline (0.01 M, [pH 7.4]). Viral RNA was extracted by using TRIzol (Invitrogen) according to the manufacturers' instructions and stored at −20°C.

RT-PCR specific for NoV was performed by two different assays, targeting regions B (3′ end of ORF1) and region D (3′ end of ORF2) of the viral genome. All samples were first screened by the region B primer set (6), and some genogroup II-positive samples were further tested and characterized by using the region D primer set (38). The oligonucleotide primer sequences are listed in Table 1. All assays were performed with the appropriate positive and negative controls (ultrapure water), and four separate laboratory rooms (for extraction, master mix preparation, adding viral RNA, and post-PCR) were used to avoid cross-contamination.

Nucleotide sequencing and phylogenetic analysis. The 213-bp (region B) and 253-bp (GGII region D) RT-PCR products were purified by using either the concert rapid PCR purification system kit (Gibco-BRL) or the QIAquick PCR purification kit (QIAGEN). Sequencing reactions were carried out in both directions by using the ABI Prism BigDye terminator cycle sequencing ready reaction I kit (Applied Biosystems). The resulting product was precipitated by using the DyeEx 2.0 spin kit (QIAGEN), and the nucleotide sequence was determined by using an automated sequencer (Applied Biosystems ABI Prism 3100 DNA).

Consensus sequences were obtained by using SeqManII (Lasergene; DNAStar, Inc.) software. Multiple sequence alignments and phylogenetic analyses were performed by using the CLUSTAL V method and MegAlign (Lasergene, DNAStar, Inc.) software, respectively.

Reference strains. The following reference strains (with GenBank accession numbers in parentheses) were included in the analysis: GI.1, Norwalk (M87661); GI.2, Southampton (L07418); GI.3, Desert Shield (U04469); GI.3b, Arkansas (AF414405); GI.4, Louisiana (AF414402); GI.5, Florida 1995 (AF414406); GI.6, Hesse (AF093797); GII.1, Hawaii (U07611); GII.1b, 314USA (AF414420); GII.2, Melksham (X81879); GII.3, Toronto (U02030); GII.4, Common Florida (AF080549); GII.4, Bristol (X76716); GII.5, Hillingdon (AJ277607); GII.5, Vermont (AF414423); GII.6, Seacroft (AJ277620); GII.6, Florida 1993 (AF414407); GII.7, Pennsylvania (AF414409); GII.8, Idaho (AY054299); GII.8, Amsterdam (AF195848); GII.9, VA97207 (AY038599.1); Arg320, (AF190817); GII, Swine NoV Sw43/1997/JP, (AB074892); GIII, Jena (AJ011099); and GIV.1 Cruise Ship, (AF414427).

TABLE 2. Genotype classification by sequencing of regions B (POL) and region D (capsid) of 67 strains found in 62 samples from children from São Paulo, Brazil

Region B	Genotype classification in region D (no. of strains)					Total
	GII.2	GII.3	GII.4	GII.6	Not determined	
GI.3b					1	1
GI.4					3	3
GII.4			5		35	40
GII.6/7/8		2		1	2	5
GII.rec[a]		3			1	4
Not determined	3	1			0	4
Mixture					10	10
Total	3	6	5	1	52	67

[a] GII.rec, similar to Arg320 strain.

Nucleotide sequence accession numbers. The nucleotide sequence data of the RNA polymerase gene and capsid gene have been submitted to GenBank and assigned accession numbers DQ386915 to DQ386995.

RESULTS

RT-PCR with region B primers. NoV RNA was detected in 78 (33.3%) of 234 fecal samples. The NoV-positive results were as follows: 34 of 94 (36.2%) acute-diarrhea samples, 12 of 45 persistent-diarrhea samples (26.7%), 20 of 55 (36.4%) control samples, and 12 of 40 (30.0%) samples with no information.

Of these 78 NoV-positive samples, 3 (1.8%), 5 (2.1%), and 26 (11.1%) also tested positive for rotavirus, adenovirus, and astrovirus, respectively (data not shown).

RT-PCR products of 66 NoV strains were sequenced from 61 samples. From the remaining 17 samples we did not have enough fecal material for the complete sequencing reaction and, therefore, the strains could not be genotyped, although some small fragments of the genome were sequenced and were confirmed to be NoV.

We found 58 GII strains: 52 in samples with a single strain and 6 in samples with mixtures of two different strains. Eight GI strains were found: four in samples with single strains and four in mixtures.

Of 52 samples with single GII strains, 40 (76.9%) could be typed as GII.4, 4 (7.7%) had sequences similar to the recombinant Arg320 strain (21), and 5 (9.6%) could not be differentiated in region B (GII.6/7/8 strains), since the nucleotide sequence of this region does not discriminate between these three genotypes (Table 2 and Fig. 1). Three strains—ICB1521, ICB1915, and ICB2230—(5.8%) had less than 77.9% nucleotide sequence similarity than the known GII reference strains, indicating that they may present a new genetic cluster (Fig. 1). Four single GI strains were detected: one GI.3b and three GI.4.

The sequence results of five region B RT-PCR products indicated the presence of mixtures of two different genotypes in each sample. After analyzing the GI and GII region B

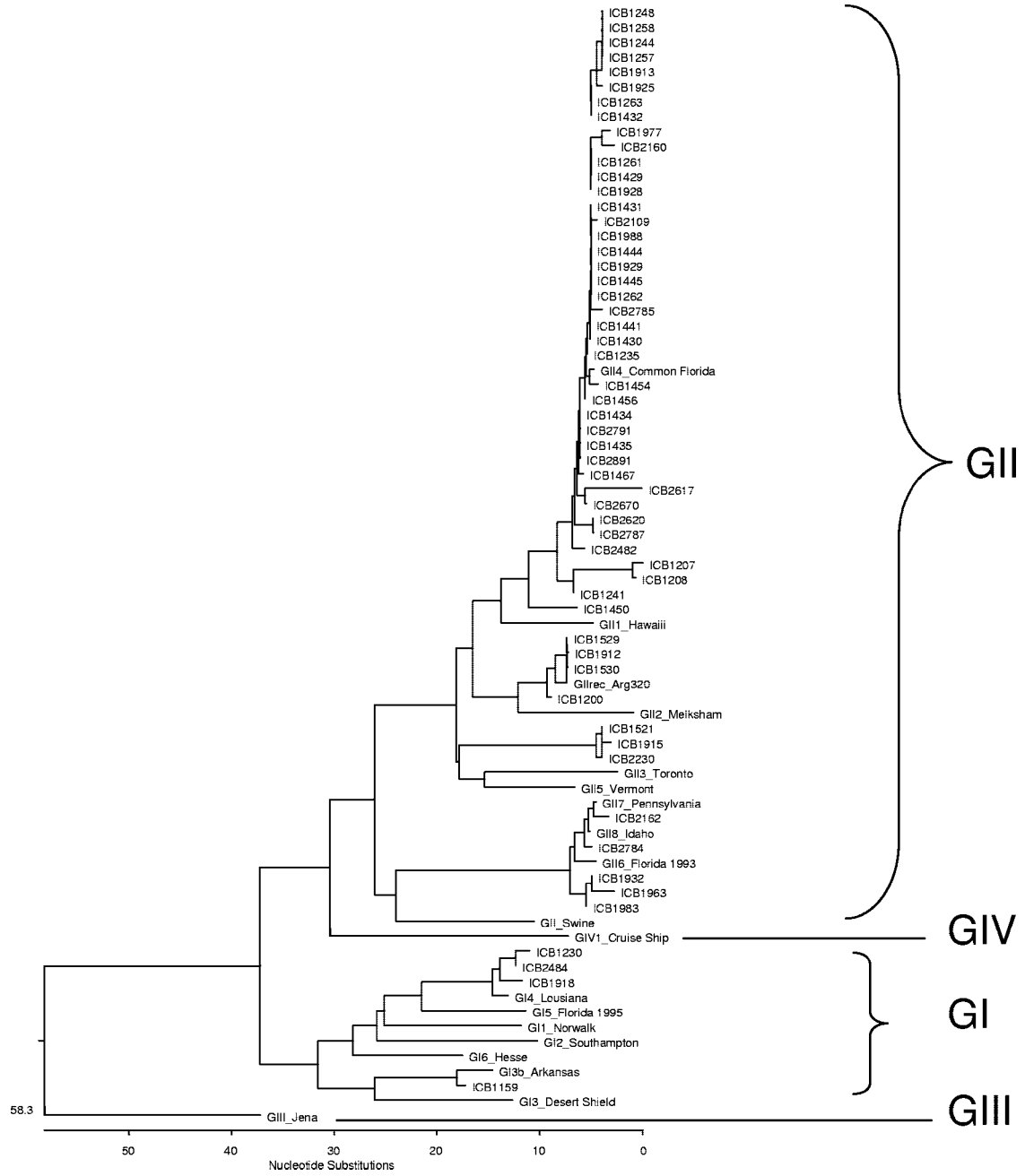

FIG. 1. Phylogenetic tree of region B nucleotide sequences from 56 norovirus samples identified by number detected in children in São Paulo, Brazil, and of norovirus GI and GII reference strains. The lengths of the branches represent the distance between sequence pairs, and the dendrogram was constructed by using the CLUSTAL V method with the MegAlign program (LaserGene/DNAStar). The units at the bottom of the tree indicate the number of substitution events.

sequence reactions separately, we found that two samples contained both GII.4 and GI.3b mixtures, two samples contained GII.4 and GI.4 strains, and one sample contained a GII.4 and an ICB2230-like GII strain (Fig. 2).

RT-PCR with region D primers. Six samples that could not be assigned to a defined genotype, three samples showing GII sequences in region B with identity with the recombinant strain Arg320, one sample not sequenced in region B, and five GII.4 strains were analyzed by region D RT-PCR. Of the three unresolved (GII6/7/8) region B strains, one (ICB1963) showed 91.8% nucleotide identity with the GII.6 reference strain (Seacroft) and two showed high sequence identity with the GII.3 reference strain (Toronto) in region D (Table 2 and Fig. 3). Three strains (ICB1529, ICB1912, and ICB1200) showing region B identity to the recombinant Arg320 strain clustered with GII3 prototype strain Toronto in region D.

The three (ICB1521, ICB1915, and ICB2230) unresolved region B sequences that showed <77.9% nucleotide sequence

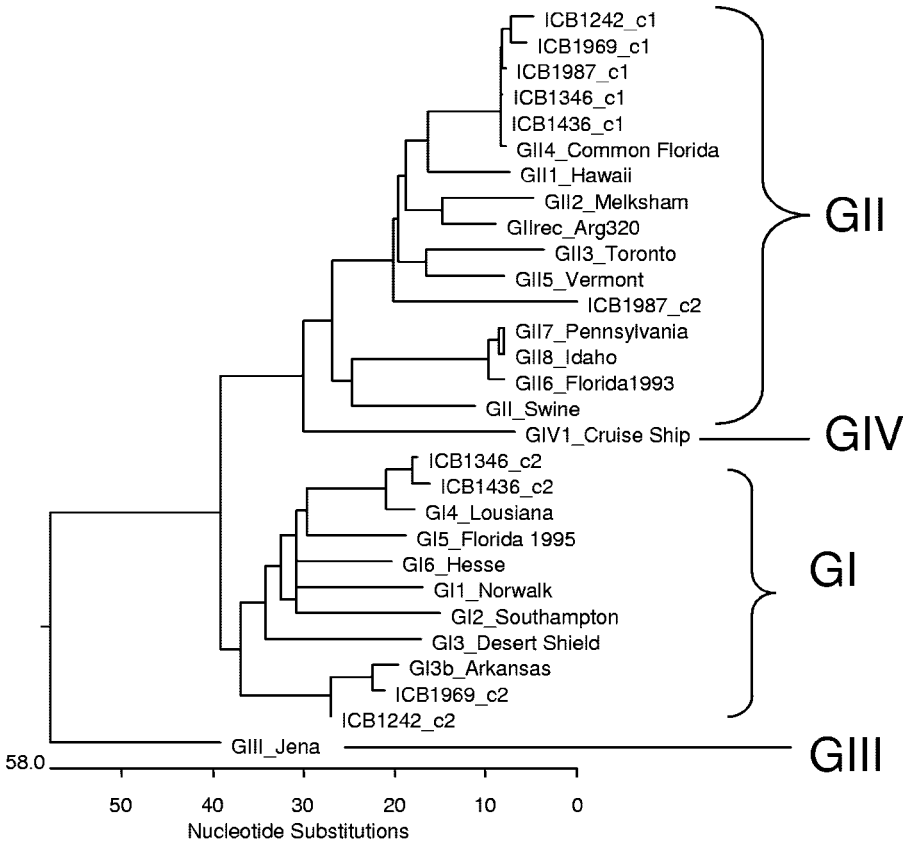

FIG. 2. Genetic relatedness of the region B nucleotide sequences from five samples (ICB1242, ICB1969, ICB1987, ICB1346, and ICB1436) containing a mixture (c1 and c2) of norovirus genotypes and norovirus GI and GII reference strains. The lengths of the branches represent the distance between sequence pairs, and the dendrogram was constructed by using the CLUSTAL V method with the MegAlign program (LaserGene/DNAStar). The units at the bottom of the tree indicate the number of substitution events.

identity compared to the region B sequence database at the Centers for Disease Control and Prevention showed 85.3 to 88.8% nucleotide sequence identity and 92.9 to 100.0% amino acid sequence identity with GII.2 prototype strain Melksham in region D (Fig. 3). One sample (ICB1443), for which no region B sequence was obtained, was identified as GII.3.

DISCUSSION

RT-PCR has become a routine diagnostic method for the detection of NoV RNA in clinical laboratories (1). Application of RT-PCR-based methods to screen stool specimens has shown not only that the overwhelming majority of outbreaks of acute gastroenteritis are attributable to NoVs but also that these viruses are the cause of numerous cases of sporadic gastroenteritis (2, 3, 4, 7–9, 15, 20, 22, 26, 27, 33, 35).

Our results show that NoVs are commonly found in sporadic cases of gastroenteritis, as well as in controls, in children in São Paulo State, Brazil. The overall frequency (33.3%) of NoV found in our study is substantially higher than in other studies, which report a prevalence in the range of 6 to 19% (3, 4, 7, 8, 15, 20, 22, 27). The circulation of more pathogenic strains, different requirements for hospital admission, and the very low income of the population under study could perhaps explain the high frequency of NoVs. In addition, the presence of NoVs as opportunistic agents in infections with other agents are other conditions that may require further study. Another striking finding is the high proportion (36.4%) of NoV positives in children without diarrheal symptoms, which might be due to the fact that NoV RNA can be detected up to 3 weeks after the onset of illness (29). Also, children could have been infected with different genotypes of norovirus in the last 6 to 12 months, which offers immunological protection against disease but not infection. Asymptomatic infection is common in children under 5 years of age.

The predominance of NoVs GII strains detected in the present study is in agreement with previous reports of a higher prevalence of GII strains over GI in outbreaks, as well as studies on sporadic gastroenteritis (3, 4, 8, 9, 11, 20, 23, 24, 25, 26, 33, 35). The reason for this is unknown, although differences in biological properties, such as virulence, routes of transmission, or stability of the virus in the environment, are possible explanations (4).

Genotyping of the NoV strains detected in our study showed that 40 of 67 (59.7%) of the strains belonged to GII.4 genotype. Viruses of this genotype caused 60 outbreaks in geographically distant locations within the United States and were identified, by sequence comparisons, in another seven countries on five continents during a same period (25). Strains of this genotype were detected in the United States in April 1995;

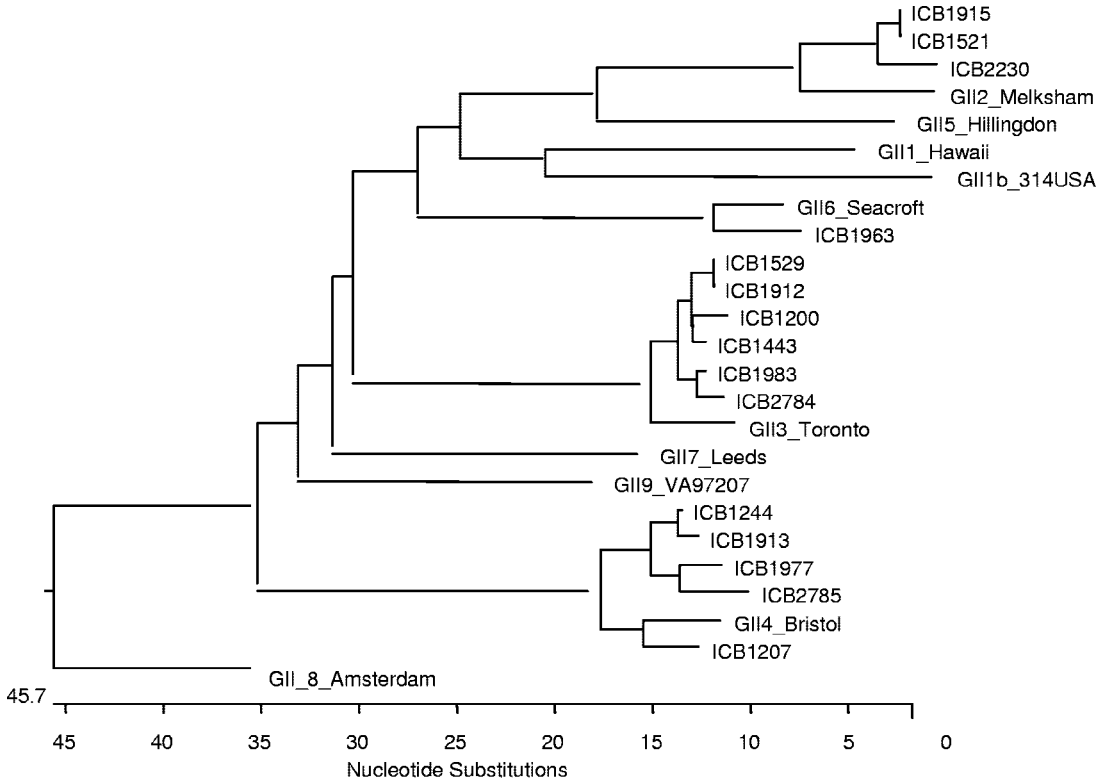

FIG. 3. Phylogenetic tree of region D nucleotide sequences from 15 norovirus strains detected in the present study (ICB1200, ICB1207, ICB1244, ICB1443, ICB1521, ICB1529, ICB1912, ICB1913, ICB1915, ICB1963, ICB1977, ICB1983, ICB2230, ICB2784, and ICB2785) and GI and GII norovirus reference strains. The lengths of branches represent the distance between sequence pairs, and the dendrogram was constructed by the CLUSTAL V method with the MegAlign program (LaserGene/DNAStar). The units at the bottom of the tree indicate the number of substitution events.

in Brazil, Canada, Australia, and The Netherlands in late 1995; in Australia, The Netherlands, and China in 1996; and in Germany in 1997, suggesting that the strain was circulating globally during this time period (25). Recent studies on sporadic cases of pediatric acute gastroenteritis have also shown the predominance of the GII.4 genotype in several countries (2, 4, 10, 15, 20, 22). In Ceará (northeastern region), Brazil, strains similar to the GII.4 Lordsdale strain have been reported as early as 1990 (27). Gallimore et al. (11) examined outbreaks of acute gastroenteritis in a children's day care facility in Rio de Janeiro (Southeast Region) from 1996 to 1998 and showed that the majority of strains belonged to GII.4. Our study confirms previous findings in our country that GII.4 are the most prevalent NoV strains in this time period.

Because first-generation NoV RT-PCR assays targeted a small region of the POL gene, many reports on NoV genotyping have been based upon sequences amplified from this region (38). However, because VP1 is responsible for the differences in antigenicity, the complete VP1 protein has been proposed as the gold standard for classification of NoVs into genogroups and genotypes (37, 40). Amplification of a small region of the capsid gene (region C or D) has been shown to accurately classify NoVs comparable to the complete VP1 (38). Therefore, testing fecal specimens for NoV by conventional RT-PCR using region B primers (6), followed by genotyping of strains by region C or D, seems to be an adequate strategy for the sensitive detection and reliable characterization of NoV strains (38). We detected four strains that had POL and capsid sequences similar to the Arg320 recombinant strain (17) Similar strains have been identified in Argentina in 1995 (17) and were also identified in children with diarrhea in Argentina in the same time our samples were collected (23).

Region B primers used in the present study do not discriminate between GII.6, GII.7, and GII.8 genotypes because the genomic region targeted by these primers is conserved (6). Three of the five samples identified as GII.6/7/8 in region could be classified as GII.6 (one strain) and GII.3 (two strains). Differences in tree topology when different regions of the genome are analyzed may be an indication of a recombinant strain with most likely the conserved ORF1-ORF2 junction region as the crossover site (37). The fact that we found several samples that contained genetically different strains illustrates that the conditions for recombination between strains exist in the studied population.

In region B, three strains showed less than 77.9% identity with representative strains of all known GI and GII strains; however, region D sequences of these strains showed nucleotide sequence identities from 85.3 to 88.8% and amino acid identities from 92.9 to 100% with the GII.2 Melksham strain. These results discard the possibility of a new genotype but reinforce the possibility of a new recombinant containing the GII.2 genotype capsid protein and a novel polymerase sequence of a unknown parent strain.

Mixed infections with different genotypes of NoVs were

previously described in outbreaks associated with contaminated oysters or water (34) but, to our knowledge, mixed infections have not been previously described in sporadic cases of NoV gastroenteritis. In five of our samples, two different GI and GII genotypes were found using all four region B primers in separate sequencing reactions. All genotypes that were detected in the mixtures were also found as individual strains in other fecal samples in the present study.

In conclusion, we found a high frequency of NoV strains in stool samples collected from children in São Paulo State, Brazil with or without acute gastroenteritis. GII.4 strains was the predominant strain detected, along with different potential recombinant strains, as well as mixed infections, that are required to generate such strains.

ACKNOWLEDGMENTS

This study was supported by FAPESP grants 2001/07298-8 and 2004/01757-9. J.G.C. and M.L.R. have CNPq scholarships.

We thank Paulo Brandão for critical review of the manuscript.

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