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Molecular Epidemiology of Group A Rotavirus Diarrhea among Children in Buenos Aires, Argentina, from 1999 to 2003 and Emergence of the Infrequent Genotype G12

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To examine the epidemiology of rotaviruses in Buenos Aires, Argentina, we screened 1,212 stool samples from children with diarrhea in the southern district of Buenos Aires from 1999 to 2003. We identified 187 samples (15.4%) that were positive for group A rotavirus by use of antigen enzyme-linked immunosorbent assay. Among these specimens, 112 were available for typing: 93 (83.0%) were single-type infections, 9 (8.0%) were mixed-type infections with more than one G or P type, and 10 (8.9%) were G and/or P nontypeable. In contrast to the findings in our last study, from 1996 to 1998, genotype P[4], G2 strains were almost completely absent and P[8], G1 and P[8], G4 strains were dominant, representing more than 80% of the G and P types found. Genotypes G2 and G9 were detected in few samples, and type G3 was completely absent. We identified several uncommon genotype G12 strains, representing the first detections outside of Asia and the United States, by sequencing. Using a genotype G12-specific reverse transcription-PCR, we identified eight (6.7%) positive samples for the 1999 to 2003 period. The high degree of sequence identity between recent G12 isolates from Argentina, the United States, and Asian countries suggests a relatively recent introduction(s) of these strains into humans from a common progenitor. The Argentinean G12 strains belonged to genotype P[9], similar to most of the recently described Asian G12 strains. The finding of G12 strains in several other regions of the world raises the possibility that G12 may be emerging globally and suggests that surveillance for this strain should be conducted routinely.

Group A rotavirus, the most common etiologic agent of severe diarrhea in children, causes about 600,000 deaths per year (31). Two outer capsid proteins, VP4 and VP7, define serotypes P (protease-cleaved protein VP4) and G (glycoprotein VP7) (14). Rotavirus serotypes are defined by virus neutralization, but genotypes are more commonly studied since they can be characterized more simply by sequencing or reverse transcription (RT)-PCR. Although 14 G serotypes, 15 G genotypes, 14 P serotypes, and 26 P genotypes are known (14, 28), only five P-G combinations (P[8] with G1, G3, G4, and G9 and P[4] with G2) are prevalent worldwide (25, 34). However, uncommon types and combinations have been observed increasingly, mainly in developing countries (10, 18, 23, 25, 27, 29, 30, 33–35, 38).

Knowledge of genotype prevalence has become more relevant, since new vaccines soon to be licensed will need to protect against the diversity of strains in circulation. Two different approaches have been used to develop a rotavirus vaccine. In one, the modified Jennerian approach, reassortants of bovine rotavirus strain WC3 (Rotateq; Merck), which is naturally attenuated for humans, were constructed with capsid genes of five prevalent human serotypes (G1, G2, G3, G4, and P1A[8]). The reassortants are expected to stimulate homotypic responses and serotype-specific protection to these common rotavirus serotypes. In the other approach, an attenuated human strain of the common serotype P1A[8], G1 (Rotarix; GSK) has been used as a monovalent vaccine to produce both homotypic and heterotypic immune responses and to cross-protect against different serotypes.

It is important for countries considering rotavirus vaccine programs to establish surveillance for the prevalent serotypes of group A rotavirus and to monitor the emergence of rare types not included in candidate vaccines. This information helps define geographic and temporal variations in strain distribution and evolution. The data also help to detect the spread of new strains and delineate the serotypes needed to prepare vaccines that will elicit optimal type-specific protection for different regions of the world.

Although the incidences of rotavirus infection are similar among children in developed and developing countries (6), the fatalities caused by rotavirus diarrhea occur almost entirely in less-developed regions. Rotavirus infections in developing countries also have other noteworthy characteristics, such as the elevated prevalence of unusual serotypes in some settings and a high incidence of mixed-type infections. The higher chance for viral reassortment during mixed infections in children with diarrhea and, in some regions, the closer contact of

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humans with cattle or farm animals could generate the appropriate conditions for gene reassortment and the emergence of novel antigenic strains of human rotaviruses (10, 18, 26, 27, 29, 30, 33, 35). In addition, trials conducted in Brazil and Peru (30) with the rhesus tetravalent vaccine found low vaccine efficacy against serotypes G1 to G4, raising the possibility that the high prevalence of unusual serotypes found in Latin America may contribute to vaccine failure and emphasizing the necessity of continuous surveillance to allow identification and characterization of novel strains.

One of the G serotypes that is considered uncommon, G12, was first identified and serologically characterized in 1990 from strains causing gastroenteritis in children from the Philippines (37, 39). This G specificity was not observed again until 2002, when it appeared in the United States (24), Thailand (32), and, shortly thereafter, India (12) and Japan (36).

In this study, we performed surveillance for group A rotavirus strains among children in a suburban region of Buenos Aires, Argentina. Typing was done by RT-PCR methods specific for human rotavirus genotypes, and nontypeable strains were further characterized by RT-PCR (using primers for animal strains), Southern blotting, and sequencing. In addition, we demonstrated the utility of an RT-PCR method with a G12-specific primer pair for identifying strains of this genotype in stool specimens.

MATERIALS AND METHODS

Surveillance. Between January 1999 and June 2003, we conducted surveillance for children with diarrhea who visited outpatient clinics or who were hospitalized at the Hospital Materno-Infantil de San Francisco Solano (maternity and pediatric hospital) located in the southern suburban area of Buenos Aires Province, 15 km from Buenos Aires. A total of 1,212 children of <15 years of age with diarrhea were enrolled in the study, and fecal specimens were collected from each child and submitted for analysis. Routine testing for bacterial and parasitic pathogens was performed on these samples at the hospital, but no screening was done for viral agents other than rotavirus. Stool suspensions (10% to 20%) were prepared in phosphate-buffered saline, pH 7.2, and stored at −20°C until processed.

Rotavirus detection. All 1,212 specimens were screened for group A rotavirus regardless of status for other pathogens, age of patient, or season collected. Stool suspensions were evaluated with an in-house enzyme-linked immunosorbent assay (ELISA) method specific for group A rotavirus. The ELISA consists of a double-antibody sandwich assay using polyclonal goat antirotavirus antibodies as described previously (1). The estimated sensitivity of this in-house method was 4×10^6 rotavirus particles/ml; thus, it compared favorably with a commercial assay in clinical performance (1).

Typing by multiplex RT-PCR and characterization of nontypeable strains. Clarified stool suspensions from 112 specimens were prepared using Vertrel XF (DuPont Chemicals, Wilmington, DE), and viral RNA was then extracted from each by use of the silica powder method in the presence of 4 M guanidine thiocyanate (5). The double-stranded RNA samples were subjected to multiplex seminested RT-PCR and confirmation by Southern blotting for identification of G and P types by methods described previously (11, 15, 19). Primers for the common human strains were used for initial screening, and nontypeable strains were further investigated using a variety of other type-specific primers (21, 22), a modified 1T-1 primer based on the sequence of the Wa strain, and sequencing (BigDye sequencing kit and ABI Prism 3100 genetic analyzer; PE Applied Biosystems). A specific pair of primers (jrg 226, nucleotides 173 to 190, 5′ TCG TCA TGC TGC CAT TTA 3′ [forward]; jrg 227, nucleotides 327 to 344, 5′ GTC CAG TCG GGA TCA GTT 3′ [reverse]) based on the sequence of strain Se585 from the United States (24) was used to identify additional G12 strains among the G nontypeable strains.

Nucleotide sequence accession number. The complete sequence of the VP7 gene of the Argentine strain Arg720 was obtained and deposited in GenBank under the accession number DQ111868.

TABLE 1. Percentage of rotavirus G and P genotypes detected each year from 1999 to 2003 relative to the total for the period 1996 to 1998

G or P genotype	% of genotype detected in:					
	1996–1998 (n = 100)[a]	1999 (n = 27)	2000 (n = 32)	2001 (n = 26)	2002 (n = 16)	2003 (n = 11)
G1	43.0	42.8	37.5	11.5	68.7	56.2
G2	46.7	3.6	0	0	0	6.2
G4	3.8	21.4	53.1	84.6	18.7	31.2
G9	0	7.1	0	0	0	0
G12	NT[b]	14.3	3.1	3.8	12.5	0
GNT[c]	6.5	10.7	6.2	0	0	6.2
P8	18.5	64.3	90.6	85.2	82.4	83.0
P4	71.8	7.1	0	3.7	5.9	0
P6	0	7.1	0	0	0	17.0
P9	0	7.1	3.1	0	11.8	0
PNT[d]	9.7	14.2	6.2	11.1	0	0

[a] n = number of samples.
[b] NT, not tested.
[c] GNT, G nontypeable.
[d] PNT, P nontypeable.

RESULTS

Characteristics of rotavirus cases. Of the 1,212 stool specimens screened for rotavirus by ELISA, 187 (15.4%) were positive. Bacteriological testing detected enteropathogenic bacteria in only 10 of the 187 rotavirus-positive cases (four samples were positive for *Aeromonas* spp. and six for *Shigella sonnei*). Of note, all but one of these cases were in children who were more than 3 years old. The rotavirus-positive patients generally presented with vomiting (81%), fever (79%), and mild to moderate dehydration (22%) and had an average of six stools per day. Patients with diarrhea were identified all year long, but the incidence was highest in May, June, and July, the coldest months of the year (data not shown). Patients ranged in age from 0 to 11 years, but most were less than 3 years of age.

Genotyping. Of the 187 rotavirus-positive samples, 112 were adequate for typing. Of these, 93 (83%) had single-type infections, and 9 (8%) had mixed-type infections with more than one G or P type. Ten (9%) specimens were G and/or P nontypeable. Overall, the dominant G types between 1999 and 2003 were G1 (40%) and G4 (44%). Genotype G4 peaked in 2001 with a prevalence of 84.6% but decreased markedly the next year, when G1 became the dominant genotype (Table 1). Genotype P[8], normally associated with G1, G3, G4, or G9, was identified at rates ranging from 64% to 90% during the five seasons; this finding was consistent with the high incidence of G1 and G4. Genotype P[4] (preferentially associated with G2) was detected as a single strain only once. The virtual disappearance of G2 and P[4] types in this period is noteworthy, since this strain was very common from 1996 to 1998 (Table 1). The most prevalent P/G combinations were P[8], G4 (40.2%) and P[8], G1 (37.5%). Genotype G9 was detected in only two samples taken during 1999 (one single-type infection with P[6] and one mixed-type infection with two P types—P[6] and P[4]). A single P[4], G2 strain was detected in 1999, but genotype G2 was identified again in a mixed infection towards the end of the study (Table 2).

TABLE 2. P and G genotype combinations found by RT-PCR and hybridization in 112 rotavirus-positive samples studied from January 1999 to June 2003

P genotype	No. (%) of samples with indicated genotype:							
	G1	G2	G3	G4	G9	G12	Mixed G[a]	GNT[b]
P[8]	42 (37.5)			45 (40.2)			4 (3.6)	
P[4]		1 (0.9)					1 (0.9)	
P[6]					1 (0.9)			
P[9]						4 (3.6)		
Mixed P[c]	1 (0.9)			1 (0.9)	1 (0.9)		1 (0.9)	1 (0.9)
PNT[d]				2 (1.8)		2 (1.8)		5 (4.5)

[a] Includes one sample with G12 plus G1, one sample with G12 plus G4 types, three samples with G1 plus G4, and one sample with G1, G2, and G4.
[b] Nontypeable for G genotype.
[c] Includes one each of P[4+6], P[4+8], and P[8+9] and two of P[6+8].
[d] PNT, nontypeable for P genotype.

Detection of G12 strains. From a total of seven G nontypeable samples in 1999, a cluster of four nontypeable samples from cases that occurred in September and October were further characterized as G12 strains and confirmed by sequencing a fragment of the VP7 gene. One of these strains, which had a long electropherotype (Arg720), was most homologous (97.9%) to the G12 reference strain Se585 from the United States (data not shown). On the basis of these data, a type-specific primer pair that had originally been designed to be homologous to the VP7 gene of strain Se585 was used to screen the rest of the nontypeable samples by RT-PCR. Products were detected from a total of eight samples (6.7%), including four single-type infections with genotype P[9] specificity, two P nontypeable strains, and two mixed-type infections of G1 and G4 strains (Table 2). At least one G12-positive sample was identified each year between 1999 and 2002. Six were detected during spring or summer months, and two in the autumn or winter seasons. The average age of the patients with G12-associated diarrhea was 4.0 years, which was substantially older than the age of children infected with P[8], G1 strains (1.9 years) and P[8], G4 strains (1.3 years). No differences in diarrhea severity were noted between cases caused by G12 isolates and those caused by common strains. A detailed molecular characterization of two Argentine G12 strains and a comparison with other G12 strains will be reported elsewhere (A. A Castello et al., unpublished data).

DISCUSSION

Rotavirus is a major cause of morbidity and mortality in developed and developing countries, and the introduction of an effective vaccine is considered crucial to reduce deaths, hospitalizations, and the disease burden associated with this pathogen (8). Several candidate vaccines have been formulated to match the most-prevalent serotypes, but there is a need to define the currently circulating serotypes and their temporal and geographic variations. Such studies will be important to identify changes in the prevalence of circulating strains and the emergence of novel strains that may impact the efficacy of vaccines. Here, we describe the first detection of G12 strains in Argentina, representing the possible emergence of a new strain in Latin America, consistent with recent findings in the United States and several Asian countries.

In 1996 we established group A rotavirus strain surveillance in a densely populated suburban area of southern greater Buenos Aires and found that the most-prevalent genotypes were P[4] and G2, present in 71% and 50% of the samples, respectively, for the 1996 to 1998 period (1). In the present study, covering the 1999 to 2003 period, we documented the virtual disappearance of these previously common genotypes. Concomitantly, we observed a large increase in the incidence of G4 strains compared to only 4% of the total in the 1996 to 1998 period (1). The other important G type that was continually present throughout the study was G1 (Table 1). The fluctuating nature of serotype prevalences raises the possibility that serotype G2 could represent a major circulating strain in the future. Since G2 strains share neither P nor G serotype nor overall genome constellation (DS-1 genogroup) with one of the current vaccine strains (Rotarix, P1A[8], G1 genotype and Wa genogroup), it will be important to determine whether the Rotarix vaccine provides adequate protection against G2.

The rise in the frequency of G4 strains could reflect a nationwide tendency, since a surveillance network in Argentina reported incidences for G4 of 5% for the first year (1996 to 1997) and 31% for the second (1997 to 1998) (2). A phylogenetic study of VP7 genes from 28 G4 strains identified three different genetic sublineages, and the authors speculated that the high prevalence of this genotype could be related to the sequential appearance of these genetically distinct G4 strains (3).

Several other features were noted in the distribution of strains. First, genotype G3 has been consistently absent during the previous and present studies, which is in accordance with the low level of G3 detection in other surveys (2, 7, 13, 17). Second, we did not detect serotype G5, which is endemic in some neighboring areas in Brazil (20) and Paraguay (9) and has been detected at lower rates in the northern provinces of Argentina (22). Third, serotype G9 was uncommon (1.7%) and associated with genotype P[6], the same combination that was previously detected in southern Argentina in 1998 and 1999 (4). A nationwide surveillance network studied samples from 1996 until June 1999 (2, 4) and over the last year detected a rise in the frequencies of G9, but mainly in southern regions of the country. For the area closest to our surveillance location, this network reported four G9 strains in 15 samples tested (26.7%) in 1999, and we detected two G9 strains in 27 samples (7.4%) in that year. Since the two locations are about 60 km apart, it is not surprising that the incidences of G9 detected are different. Last, we found the rare G12 strains, the only human group A rotavirus type that had not been detected in animals until recently (16). This serotype was originally described for samples from the Philippines collected during 1987 and 1988 (37) and was not reported again until 2002, when it was identified among samples collected in the latter half of the 1990s in the United States and Thailand (32). More recently, G12 has been found in India (12) and Japan (36).

In studies in the Philippines, North America, Thailand, and India, the G12 strains were isolated from children less than 4 years of age, whereas in the Japanese study, the two isolates came from an infant (1 year) and an adult (45 years). In the

present study, eight G12 samples came from individuals 6 months to 11 years old, with a mean age of 4 years. This mean age was substantially higher than that of children infected with G1 and G4 strains (mean ages, 1.93 and 1.27 years, respectively). Although this difference was not statistically significant, these data raise the possibility that P[9], G12 strains may be able to infect older children due to a failure of cross-protection induced by previous infections with common strains.

This study represents the first detection of P[9], G12 strains in Latin America and, as far as we are aware, the first report of G12 outside Asia and the United States. The finding that these strains were present in patients with diarrhea in 4 of the 5 years of study, together with other recent reports of closely related strains in Asia and the United States, raises questions about the possible emergence of G12 as a new global genotype. It is also noteworthy that G12 strains have now been detected in a variety of gene constellations, including long and short electropherotypes, and in association with three different P genotypes and two different genogroups (12, 24, 32, 36, 37). This raises questions about the potential of this strain to spread efficiently in the population through reassortment.

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