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Molecular Epidemiology of Group A Rotavirus in Buenos Aires, Argentina 2004–2007: Reemergence of G2P[4] and Emergence of G9P[8] Strains

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Detection and characterization of group A rotavirus in Buenos Aires, Argentina, was conducted on 710 fecal samples from children 0–15 years old collected between 2004 and 2007. Rotavirus was detected in 140 (19.7%) samples with G9P[8] (30.0%) and G2P[4] (21.4%) as the most common genotypes. Mixed (G and/or P) infections accounted for 17.9% of the samples and the emerging G12 strain was detected during 2004 (3.5%) and 2007 (2.5%). Genotype G2 was the most prevalent during 2004 (43.9%) and 2007 (57.5%) and G9 during 2005 (58.0%) and 2006 (61.5%). Analysis of genotype prevalences from studies performed since 1996 in the same area showed striking natural fluctuations in G and P genotype frequencies. In particular, G2P[4] strains disappeared after 1999 and reemerged in 2004 to become the predominant strain by 2007 with a concomitant major decrease in G1P[8] prevalence. The VP7 genes from Argentinian G9 and G2 strains were sequenced and phylogenetic analysis was conducted in order to compare with sequences from strains isolated in regional countries reported previously. Several changes in the deduced amino acid sequence in antigenic regions of the VP7 protein from Argentinian and Brazilian strains were identified compared to vaccine strains. Overall, this study revealed relationships in the circulation of rotavirus strains in South American countries and major replacements in dominant genotypes, including the virtual disappearance of G1P[8] strains in a non-vaccinated population. High numbers of mixed infections speeding up evolution, circulation of rare serotypes, and antigenic drift could, eventually, become challenges for new vaccines. ***J. Med. Virol. 82:1083–1093, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: rotavirus; genotype; diarrhea; vaccine

INTRODUCTION

Group A rotaviruses (RVA) are a major cause of acute gastroenteritis and death in infants and children worldwide, causing an estimated 611,000 deaths in children below 5 years of age. Although the incidence of infection among children in developed and developing countries is similar, outcomes vary widely with about 82% of fatalities occurring in less developed regions [Parashar et al., 2003, 2006]. The ineffectiveness of sanitation measures in reducing the huge burden of rotavirus diarrhea has made this virus a target for vaccine development. In developed countries, effective rotavirus vaccines could reduce the costs of child hospitalizations and clinical visits for acute diarrhea and in developing countries, they could reduce deaths from diarrhea significantly [Glass et al., 2006]. However, the differing epidemiology of rotavirus in developing countries may pose special challenges to the success of the vaccine [Bresee et al., 1999; Castello et al., 2004]. Rotavirus strain surveillance is needed before and after the introduction of new vaccines in order to monitor the prevalence of circulating G and P serotypes and define intratypic diversity by sequencing. In Argentina, two new highly effective rotavirus vaccines: RotaTeq® (Merck and Co., Whitehouse Station, NJ) and Rotarix® (GlaxoSmithKline, Research Triangle Park, NC) have been licensed [Ruiz-Palacios et al., 2006; Vesikari et al., 2006]. However, since neither is yet part of the National

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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Vaccination and Immunization Program, the vaccines are prescribed mainly by private practitioners to a restricted population of children from medium- to high-income families.

Rotaviruses are non-enveloped viruses and their genome consists of 11 double-stranded RNA segments. The electropherotype of a strain is defined by the relative electrophoretic mobility of the RNA segments in polyacrylamide gels. Electropherotyping is often used to identify major strains in particular regions and to monitor their spread within the population [Cunliffe and Nakagomi, 2005]. Mature virus particles possess a triple-layered icosahedral protein capsid. The two outer capsid proteins, VP7 and VP4, form the basis of a dual classification system based on VP7, a glycoprotein (G) and VP4, a protease-cleaved protein (P) types [Estes and Kapikian, 2007]. Twenty G genotypes (15 G serotypes) and 30 P genotypes (14 P serotypes) have been reported thus far [Estes and Kapikian, 2007; Matthijnssens et al., 2008; Schumann et al., 2009; Solberg et al., 2009; Trojnar et al., 2009]. Global epidemiologic surveys have identified G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] as the most common global G–P genotype combinations associated with diarrhea in humans [Glass et al., 2006; Estes and Kapikian, 2007].

In a recent study, G9 strains have been classified in six VP7 gene lineages [Ramachandran et al., 2000; Phan et al., 2007; Martinez-Laso et al., 2008] and virtually all currently detected G9 strains globally belong to a VP7 lineage that is distinct from those isolated in the 1980s [Laird et al., 2003]. Similarly, G2 strains can be divided into four different VP7 gene lineages. The prevalence of both genotype G9 and G2 has increased in neighboring countries like Brazil and Paraguay between 2006 and 2007 [Gurgel et al., 2007; Martinez et al., 2008; Nakagomi et al., 2008; Carvalho-Costa et al., 2009].

In Buenos Aires, rotavirus strain surveillance studies have been conducted annually since 1996 by the same laboratory [Argüelles et al., 2000; Castello et al., 2006]. Here, the results of 4 years of rotavirus surveillance (2004–2007) in the Buenos Aires area are presented along with VP7 gene sequence analysis for representative strains of the prevalent G9 and G2 genotypes circulating during this period and with the electropherotypes of G9 strains. Sequencing of VP7 from G2 strains in 1997 was also performed to compare them with other recent G2 isolates.

MATERIALS AND METHODS

Samples and Detection

Seven hundred ten fecal specimens were collected from children under 15 years of age with acute diarrhea who visited outpatient clinics between January 2004 and October 2007 at the Hospital Materno Infantil in San Francisco Solano, 15 km outside the city of Buenos Aires. This period included four winter seasons in which the peak of rotavirus incidence in Buenos Aires occurs between May and July. Only one sample was collected per patient. Samples were analyzed for the presence of group A rotavirus with an in-house enzyme-linked immunosorbent assay (ELISA) [Argüelles et al., 2000].

RNA Extraction and Typing PCR

Double-stranded RNA was extracted from every rotavirus ELISA-positive stool specimen by the phenol–chloroform method for PAGE analysis [Gouvea et al., 1990] or by the silica powder method for typing RT-PCR [Boom et al., 1990]. To determine both human and animal G and P genotypes, the samples were analyzed by semi-nested, multiplex RT-PCR (OneStep RT-PCR, Qiagen GmbH, Hilden, Germany) using consensus and type-specific primers described previously.

For G typing, consensus primers Beg9 and End9 [Gouvea et al., 1990] were used in a first-round RT-PCR and three different sets of G type-specific primers (i.e., H1 pool and H2 to detect human G genotypes and A pool to detect animal G genotypes) in a second-round PCR. The H1 pool [Gouvea et al., 1990] containing specific primers for G1–G4, G8, and G9 was used with End9; the H2 pool [Das et al., 1994] containing specific primers for G1–G4 and G9 with 9con1; and the A pool [Gouvea et al., 1994a] containing primers for G5, G6, G8, G10, and G11 with Beg9. A specific pair of primers jrg226 and jrg227 was employed to identify G12 strains [Castello et al., 2006].

For P typing, consensus primers con2 and con3 [Gentsch et al., 1992] were used in a first-round RT-PCR and two different sets of P type-specific primers in a second-round PCR. Primer con3 was used in a second-round PCR with P[4], P[6], P[9], P[10] [Gentsch et al., 1992] and Wa-based P[8] [Castello et al., 2006] specific primers to detect human P genotypes. Primer con2 was employed with P[1], P[5], and P[11] specific primers to detect animal P genotypes [Gouvea et al., 1994b].

PCR products were resolved by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

Nucleotide Sequencing

The PCR products of the gene encoding the VP7 protein of 12 G9 strains isolated in 1999 [Castello et al., 2006], 2005, 2006, and 2007 and four G2 strains isolated in 1997 [Argüelles et al., 2000] and 2007 were amplified with 9con1 and 9con2 primers [Das et al., 1994]. The PCR products were gel purified with the QIAquick gel extraction kit protocol (Qiagen GmbH) and sequenced with the automated sequencer ABI3730XL under Big-Dye™ terminator cycling conditions (Macrogen, Inc., Seoul, Korea).

Nucleotide Sequence Accession Numbers

The nucleotide sequences presented in this article have been submitted to the GenBank database and assigned the following accession numbers: GQ154522–GQ154534 (G9 strains) and GQ154535–GQ154538 (G2 strains).

Sequence Analysis

Sequences were aligned with the Clustal X program [Thompson et al., 1997], and phylogenetic trees were reconstructed with the neighbor-joining method and their evolutionary distance was estimated by Kimura's two-parameter method. The bootstrap probability was calculated for each internal branch of the phylogenetic trees with 1,000 times of re-sampling. These analyses were conducted using MEGA version 4 [Tamura et al., 2007].

Analysis of the dsRNA of Selected Rotavirus Strains by PAGE

Genomic RNA was resolved by PAGE on a 10% polyacrylamide separating gel (acrylamide–bisacrylamide, 29.3:0.7) with a 4% polyacrylamide stacking gel (14 cm × 13 cm × 0.15 cm) in the buffer system of Laemmli [1970]. Electrophoresis was conducted at 18 mA for 16 hr at 4°C using a Hoefer SE600 (Amersham Biosciences, San Francisco, CA) gel apparatus, and the gels were silver stained as previously described [Herring et al., 1982]. The electrophoretic migration patterns of dsRNA of 18 G9 rotavirus strains from San Francisco Solano were analyzed and grouped into different classes.

RESULTS

Rotavirus Detection in Stool Samples

Of the 710 fecal specimens screened by ELISA, 140 (19.7%) were positive for group A rotavirus (Table I). The rotavirus-positive patients from whom complete clinical information was obtained presented generally with vomiting (81.0%), fever (68.0%), and mild-to-moderate dehydration (68.0%) and had an average of six stools per day. Patients ranged from 3 months to 13 years, but most were less than 3 years of age (81.0%).

Prevalence of Rotavirus G and P Genotypes

Among the VP7 genotypes identified, G2 (38.1%) was predominant followed by G9 (36.9%), G1 (12.5%), G4 (6.9%), G3 (3.8%), and G12 (1.9%). Among the VP4 genotypes, P[8] (45.9%) occurred most frequently, followed by P[4] (33.8%) and P[6] (2.0%). Genotypes P[9] and P[10] were rarely identified (Table II). All the samples were G typed, but 16.9% of the samples could not be P typed.

G9P[8] strains were the predominant rotaviruses detected during the period accounting for 30.0% of the total G–P combinations detected, followed by G2P[4] strains (21.4%). Both G–P combinations were detected in samples every year: G9P[8] strains were most common in 2005 and 2006, whereas G2P[4] strains were most common in 2004 and 2007 (Table III). A total of 25 samples (17.9%) had mixed G or P genotypes. Table I presents the number of samples analyzed and the number of mixed infections per year. Many G and P combinations were found in mixed infections (Table III). A notable feature was the detection of the globally emergent genotype G12 in 2004 and 2007.

PAGE Analysis of G9P[8] Strains

Seven different electropherotypes of G9P[8] strains were identified, five with a long pattern and two distinct short patterns, both detected in 2006. Considering that G2P[4] also circulated in that season in elevated frequencies, this suggests that they could be reassortants with the co-circulating G2P[4] strains. A single dominant long electropherotype was present during 2005–2007 and the other six co-circulated at lower frequencies and were detected in only one season each.

Sequence Analysis of G9 Strains

The sequence of the VP7 gene of the 12 G9P[8] strains circulating between 2005 and 2007 was very identical to the G9P[6] strain Arg562 from 1999 (98.5–100% amino acid and 98.8–100% nucleotide identity) and to US1205 strain (98.5–99.3% amino acid and 98.5–99.3% nucleotide identity). They were slightly more divergent (97.1–99.9% nucleotide identities) from regional strains isolated in Brazil and Paraguay.

Phylogenetic analysis indicated that the Argentine G9P[8] and G9P[6] strains belonged to G9 VP7 lineage III (Fig. 1), typical of G9 strains found globally in recent years. Within this lineage, G9P[6] and G9P[8] formed separate clusters. However, Argentine G9P[8] strains isolated in different years clustered in one group. The G9P[6] strain Arg562 had an isoleucine residue at position 171 where most other G9 strains isolated globally contain a threonine residue.

Comparison of Antigenic Regions of G9 Strains

The pattern of amino acid substitutions within VP7 antigenic regions between G9 strains was examined by alignment using reference G9 strain AU32 [proposed to be included in the hexavalent BRV vaccine, Kapikian et al., 2005] as the consensus sequence. The same five amino acid changes were identified in every Argentine

TABLE I. Incidence of Group A Rotavirus (RVA) in Buenos Aires From 2004 to 2007

	Number of positive samples/number of samples tested (%)				
Year	2004	2005	2006	2007	Total
RVA infections	49/250 (19.6)	42/168 (25.0)	13/141 (9.2[a])	36/151 (23.8)	140/710 (19.7)
RVA mixed infections	9/49 (18.4)	7/42 (16.7)	4/13 (30.8)	5/36 (13.9)	25/140 (17.9)

[a]Low detection rate due to over-representation of summer months during sample collection.

TABLE II. Distribution and Frequency of G and P Genotypes of Group A Rotavirus Detected in Buenos Aires From 2004 to 2007

	2004		2005		2006		2007			
Genotype	n	%	n	%	n	%	n	%	Total n	% over the 4-year period
G1	17	29.8	1	2.0	0	0.0	2	5.0	20	12.5
G2	25	43.9	8	16.0	5	38.5	23	57.5	61	38.1
G3	2	3.5	4	8.0	0	0.0	0	0.0	6	3.8
G4	3	5.3	8	16.0	0	0.0	0	0.0	11	6.9
G9	8	14.0	29	58.0	8	61.5	14	35.0	59	36.9
G12	2	3.5	0	0.0	0	0.0	1	2.5	3	1.9
Total	57	100.0	50	100.0	13	100.0	40	100.0	160	100.0
P[8]	13	26.0	36	81.8	11	64.7	8	21.6	68	45.9
P[4]	18	36.0	5	11.4	5	29.4	22	59.5	50	33.8
P[6]	3	6.0	0	0.0	0	0.0	0	0.0	3	2.0
P[9]	1	2.0	0	0.0	0	0.0	0	0.0	1	0.7
P[10]	1	2.0	0	0.0	0	0.0	0	0.0	1	0.7
PNT	14	28.0	3	6.8	1	5.9	7	18.9	25	16.9
Total	50	100.0	44	100.0	17	100.0	37	100.0	148	100.0

PNT, P non-typeable.

TABLE III. Distribution and Frequency of G and P Genotype Combinations of Group A Rotavirus Detected in Buenos Aires From 2004 to 2007

	2004		2005		2006		2007			
Strain	n	%	n	%	n	%	n	%	Total n	% over the 4-year period
G1P[4]	5	10.2					2	5.6	7	5.0
G1P[6]	1	2.0							1	0.7
G1P[8]	3	6.1	1	2.4					4	2.9
G1P[10]	1	2.0							1	0.7
G1PNT	4	8.2							4	2.9
G2P[4]	10	20.4	3	7.1	1	7.7	16	44.4	30	21.4
G2P[8]	1	2.0							1	0.7
G2PNT	8	16.3			1	7.7	3	8.3	12	8.6
G3P[8]			1	2.4					1	0.7
G4P[8]	1	2.0	1	2.4					2	1.4
G4PNT			2	4.8					2	1.4
G9P[4]	1	2.0					1	2.8	2	1.4
G9P[6]	1	2.0							1	0.7
G9P[8]	2	4.1	26	61.9	7	53.8	7	19.4	42	30.0
G9PNT	1		1	2.4			2	5.6	4	2.9
G12P[9]	1								1	0.7
G1 + 2P[4]	1								1	0.7
G1 + 2P[8]	2	4.1							2	1.4
G2 + 4P[8]	1	2.0							1	0.7
G2 + 4PNT	1	2.0							1	0.7
G2 + 9P[4]							1	2.8	1	0.7
G2 + 9P[8]			1	2.4					1	0.7
G2 + 9PNT							2	5.6	2	1.4
G2P[4 + 8]					3	23.1	1	2.8	4	2.9
G2 + 3 + 4P[8]			2	4.8					2	1.4
G2 + 3P[4 + 8]			1	2.4					1	0.7
G2 + 4P[4 + 8]			1	2.4					1	0.7
G3 + 9P[8]	2	4.1							2	1.4
G4 + 9P[8]			1	2.4					1	0.7
G4P[4 + 8]			1	2.4					1	0.7
G9P[4 + 8]					1	7.7			1	0.7
G9P[6 + 8]	1	2.0							1	0.7
G12 + 2P[4]	1	2.0							1	0.7
G12 + 9P[4]							1	2.8	1	0.7
Total	49	100.0	42	100.0	13	100.0	36	100.0	140	100.0

PNT, P non-typeable.
The most prevalent strains of the period are highlighted.

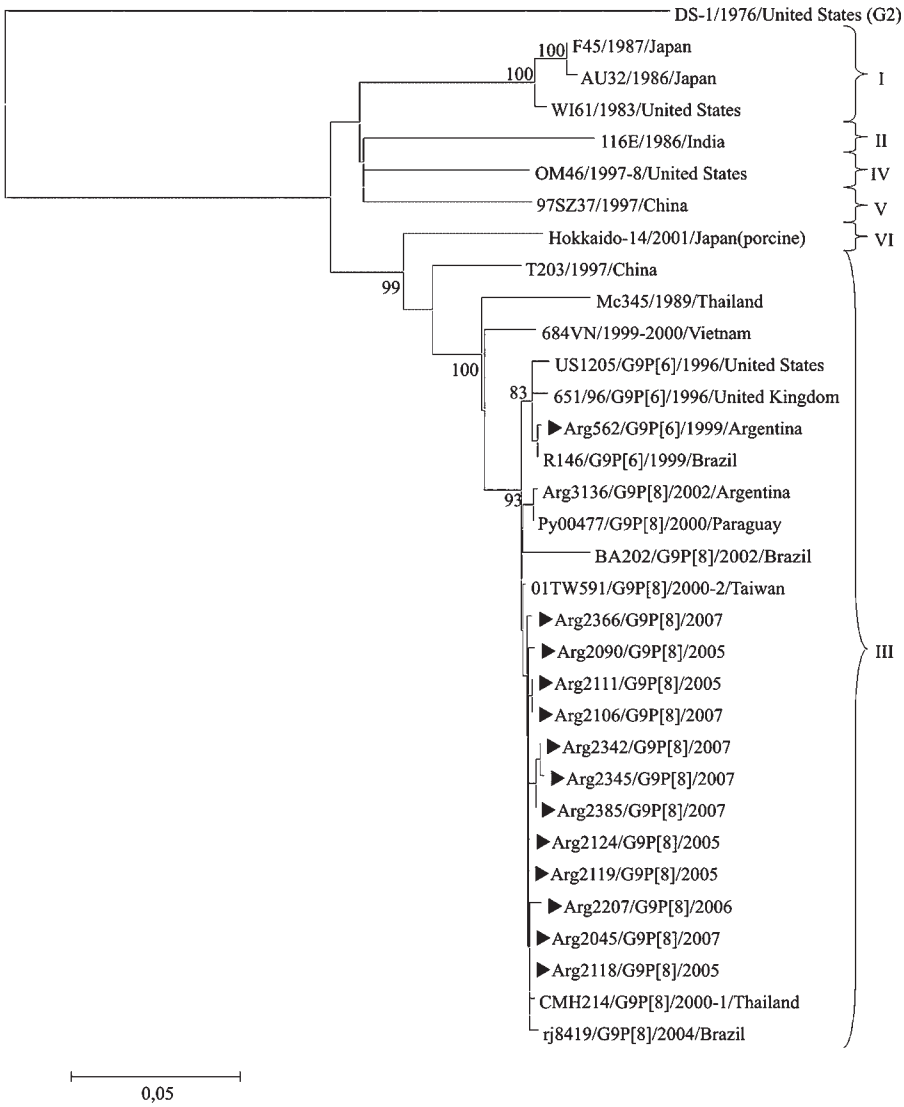

Fig. 1. Phylogenetic analysis of G9 strains. The tree was constructed from nucleotide sequences of VP7 genes of G9 rotavirus strains obtained from the GenBank database, using the Kimura two-parameter and neighbor-joining methods using MEGA4. The lineages are indicated on the right and the bootstrap values are shown at the branch nodes (values <80% not shown). The Argentine strains isolated between 2004 and 2007 are marked with a filled triangle. For each strain, its name/year of isolation/country of origin are indicated, and in the case of lineage III strains, the associated P-type was added before the year of isolation. DS-1 prototype strain (G2 genotype) was included as an outgroup.

G9 strain (region A: position 87 (A → T), region B: position 149 (K → E), region C: position 208 (T → I), position 220 (A → T), and region F: position 242 (T → N)) except for a sample isolated in 2007 that only contained four of these amino acid substitutions (Table IV). The amino acid substitution at position 208 is characteristic of lineages other than III. In this case, however, it is the result of a nucleotide change (ATT → ACT) different from the one coding threonine in other lineages (ACA).

Sequence Analysis of G2 Strains

The sequences of G2 strains isolated in 1997 and 2007 shared great nucleotide (96.5–100%) and deduced amino acid (97.6–100%) identity. Compared to the prototype DS-1 strain, however, Argentine G2 strains are divergent by 13.6–14.3% and 3.5–5.1% in nucleotide and amino acid composition, respectively. Nucleotide sequences from the G2 strains isolated in 1997 were more closely related to each other than to the G2 strains isolated in 2007. When comparing the deduced amino acid sequences, six substitutions were identified at positions 36 (K → R), 37 (F → L), 44 (I → M), 55 (M → I), 132 (Q → R), and 213 (N → D) from 1997 to 2007 strains, respectively.

Phylogenetic analysis indicated that the Argentine G2P[4] strains belong to VP7 G2 lineage II (Fig. 2), while other local strains isolated in Brazil in 2002 belong to lineage III. More recent sequence information from regional G2P[4] strains was not available from the

TABLE IV. Comparison of the Antigenic Regions A, B, C, and F of the VP7 Gene of G9 and G2 Strains

VP7 Antigenic Regions

Sequence	A (87 - 101)														
	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101
AU32	A	E	A	S	T	Q	I	G	D	T	E	W	K	D	T
Arg/562/G9P[6]/1999	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg2045/G9P[8]/2005	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg2090/G9P[8]/2005	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg2106/G9P[8]/2005	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg2111/G9P[8]/2005	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg2118/G9P[8]/2005	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg2119/G9P[8]/2005	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg2124/G9P[8]/2005	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg2207/G9P[8]/2006	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg2342/G9P[8]/2007	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg2345/G9P[8]/2007	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg2366/G9P[8]/2007	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg2385/G9P[8]/2007	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R143/G9P[6]/1999/Brazil	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R160/G9P[8]/1999/Brazil	T	.	.	L	.	.	.	.	.	.	.	.	.	.	.
rj8207/G9P[8]/2004/Brazil	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
rj8419/G9P[8]/2004/Brazil	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BA206/G9P[8]/1999/Brazil	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BA201/G9P[8]/2000/Brazil	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BA202/G9P[8]/2002/Brazil	T	.	.	.	.	.	.	.	.	.	.	.	.	N	.
Py00477/G9P[8]/2000/Paraguay	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DS-1	A	E	A	K	N	E	I	S	D	D	E	W	E	N	T
Arg0083/1997/Argentina	T	.	.	.	.	.	.	.	.	N	.	.	.	.	.
Arg0044/1997/Argentina	T	.	.	.	.	.	.	.	.	N	.	.	.	.	.
Arg2389/2007/Argentina	T	.	.	.	.	.	.	.	.	N	.	.	.	.	.
Arg2395/2007/Argentina	T	.	.	.	.	.	.	.	.	N	.	.	.	.	.
rj5619/2002/Brazil	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
rj5323/2002/Brazil	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SA356PT/1996/South Africa	T	.	.	.	.	.	.	.	.	N	.	.	.	.	.
GH1803/1999/Ghana	T	.	.	.	.	.	.	.	.	N	.	.	.	.	.
TN1529/1999/Tunisia	T	.	.	.	.	.	.	.	.	N	.	.	.	.	.
SA514GR/1987/South Africa	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KY3303/1999/Kenya	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TW6/1981/Taiwan	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
95A/1995/Australia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SA1831GR/1993/SouthAfrica	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Sequence	B (142 - 152)										
	142	143	144	145	146	147	148	149	150	151	152
AU32	M	K	Y	D	S	T	L	K	L	D	M
Arg/562/G9P[6]/1999	.	.	.	.	.	.	.	E	.	.	.
Arg2045/G9P[8]/2005	.	.	.	.	.	.	.	E	.	.	.
Arg2090/G9P[8]/2005	.	.	.	.	.	.	.	E	.	.	.
Arg2106/G9P[8]/2005	.	.	.	.	.	.	.	E	.	.	.
Arg2111/G9P[8]/2005	.	.	.	.	.	.	.	E	.	.	.
Arg2118/G9P[8]/2005	.	.	.	.	.	.	.	E	.	.	.
Arg2119/G9P[8]/2005	.	.	.	.	.	.	.	E	.	.	.
Arg2124/G9P[8]/2005	.	.	.	.	.	.	.	E	.	.	.
Arg2207/G9P[8]/2006	.	.	.	.	.	.	.	E	.	.	.
Arg2342/G9P[8]/2007	.	.	.	.	.	.	.	E	.	.	.
Arg2345/G9P[8]/2007	.	.	.	.	.	.	.	E	.	.	.
Arg2366/G9P[8]/2007	.	.	.	.	.	.	.	E	.	.	.
Arg2385/G9P[8]/2007	.	.	.	.	.	.	.	E	.	.	.
R143/G9P[6]/1999/Brazil	.	.	.	.	.	.	.	E	.	.	.
R160/G9P[8]/1999/Brazil	.	.	.	.	.	.	.	E	.	.	.
rj8207/G9P[8]/2004/Brazil	.	.	.	.	.	.	.	E	.	.	.
rj8419/G9P[8]/2004/Brazil	.	.	.	.	.	.	.	E	.	.	.
BA206/G9P[8]/1999/Brazil	V	.	.	.	.	P	.	E	.	.	.
BA201/G9P[8]/2000/Brazil	.	.	.	.	.	.	.	E	.	.	.
BA202/G9P[8]/2002/Brazil	.	.	.	.	.	P	.	E	.	.	.
Py00477/G9P[8]/2000/Paraguay	.	.	.	.	.	.	.	E	.	.	.
DS-1	M	R	Y	D	N	T	S	E	L	D	A
Arg0083/1997/Argentina	.	.	.	.	.	.	.	.	.	.	.
Arg0044/1997/Argentina	.	.	.	.	.	.	.	.	.	.	.
Arg2389/2007/Argentina	.	.	.	.	.	.	.	.	.	.	.
Arg2395/2007/Argentina	.	.	.	.	.	.	.	.	.	.	.
rj5619/2002/Brazil	.	.	.	.	.	.	.	.	.	.	.
rj5323/2002/Brazil	.	.	.	.	.	.	.	.	.	.	.
SA356PT/1996/South Africa	.	.	.	.	.	.	.	.	.	.	.
GH1803/1999/Ghana	.	.	.	.	.	.	.	.	.	.	.
TN1529/1999/Tunisia	.	.	.	.	.	.	.	.	.	.	.
SA514GR/1987/South Africa	.	.	.	.	.	.	.	.	.	.	.
KY3303/1999/Kenya	.	.	.	.	.	.	.	.	.	.	.
TW6/1981/Taiwan	.	.	.	.	.	.	.	.	.	.	.
95A/1995/Australia	.	.	.	.	.	.	.	.	.	.	.
SA1831GR/1993/SouthAfrica	.	.	.	.	.	A	.	.	.	.	P

Sequence	C (208 - 221)													
	208	209	210	211	212	213	214	215	216	217	218	219	220	221
AU32	T	T	T	N	T	A	T	F	E	E	V	A	A	S
Arg/562/G9P[6]/1999	I	.	.	.	.	.	.	.	.	.	.	.	T	.
Arg2045/G9P[8]/2005	I	.	.	.	.	.	.	.	.	.	.	.	T	.
Arg2090/G9P[8]/2005	I	.	.	.	.	.	.	.	.	.	.	.	T	.
Arg2106/G9P[8]/2005	I	.	.	.	.	.	.	.	.	.	.	.	T	.
Arg2111/G9P[8]/2005	I	.	.	.	.	.	.	.	.	.	.	.	T	.
Arg2118/G9P[8]/2005	I	.	.	.	.	.	.	.	.	.	.	.	T	.
Arg2119/G9P[8]/2005	I	.	.	.	.	.	.	.	.	.	.	.	T	.
Arg2124/G9P[8]/2005	I	.	.	.	.	.	.	.	.	.	.	.	T	.
Arg2207/G9P[8]/2006	I	.	.	.	.	.	.	.	.	.	.	.	T	.
Arg2342/G9P[8]/2007	I	.	.	.	.	.	.	.	.	.	.	.	T	.
Arg2345/G9P[8]/2007	I	.	.	.	.	.	.	.	.	.	.	.	T	.
Arg2366/G9P[8]/2007	.	.	.	.	.	.	.	.	.	.	.	.	T	.
Arg2385/G9P[8]/2007	I	.	.	.	.	.	.	.	.	.	.	.	T	.
R143/G9P[6]/1999/Brazil	I	.	.	.	.	.	.	.	.	.	.	.	T	.
R160/G9P[8]/1999/Brazil	I	.	.	.	.	.	.	.	.	.	.	.	T	.
rj8207/G9P[8]/2004/Brazil	I	.	.	.	.	.	.	.	.	.	.	.	T	.
rj8419/G9P[8]/2004/Brazil	I	.	.	.	.	V	.	.	.	.	.	.	T	.
BA206/G9P[8]/1999/Brazil	I	.	.	.	.	.	.	.	.	.	.	.	T	.
BA201/G9P[8]/2000/Brazil	I	.	.	.	.	.	.	.	.	.	.	.	T	.
BA202/G9P[8]/2002/Brazil	I	.	.	.	.	.	.	.	.	.	.	.	T	.
Py00477/G9P[8]/2000/Paraguay	I	.	.	.	.	.	.	.	.	.	.	.	T	.
DS-1	K	T	T	D	V	N	T	F	E	I	V	A	S	S
Arg0083/1997/Argentina	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg0044/1997/Argentina	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Arg2389/2007/Argentina	.	.	.	.	.	D	.	.	.	.	.	.	.	.
Arg2395/2007/Argentina	.	.	.	.	.	D	.	.	.	.	.	.	.	.
rj5619/2002/Brazil	.	.	.	.	.	.	.	.	.	.	.	.	.	.
rj5323/2002/Brazil	.	.	.	.	.	S	.	.	.	.	.	.	.	.
SA356PT/1996/South Africa	.	.	.	.	.	D	.	.	.	.	.	.	.	.
GH1803/1999/Ghana	.	.	.	.	.	D	.	.	.	.	.	.	.	.
TN1529/1999/Tunisia	.	.	.	.	.	D	.	.	.	.	.	.	.	.
SA514GR/1987/South Africa	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KY3303/1999/Kenya	.	.	.	.	.	D	.	.	.	.	.	.	.	.
TW6/1981/Taiwan	.	.	.	.	.	.	.	.	.	.	.	.	.	.
95A/1995/Australia	.	.	.	.	.	S	.	.	.	.	.	.	.	.
SA1831GR/1993/SouthAfrica	.	.	.	.	.	S	.	.	.	T	.	S	.	.

Sequence	F (233 - 242)									
	233	234	235	236	237	238	239	240	241	242
AU32	V	N	H	K	L	D	V	T	T	T
Arg/562/G9P[6]/1999	.	.	.	.	.	.	.	.	.	N
Arg2045/G9P[8]/2005	.	.	.	.	.	.	.	.	.	N
Arg2090/G9P[8]/2005	.	.	.	.	.	.	.	.	.	N
Arg2106/G9P[8]/2005	.	.	.	.	.	.	.	.	.	N
Arg2111/G9P[8]/2005	.	.	.	.	.	.	.	.	.	N
Arg2118/G9P[8]/2005	.	.	.	.	.	.	.	.	.	N
Arg2119/G9P[8]/2005	.	.	.	.	.	.	.	.	.	N
Arg2124/G9P[8]/2005	.	.	.	.	.	.	.	.	.	N
Arg2207/G9P[8]/2006	.	.	.	.	.	.	.	.	.	N
Arg2342/G9P[8]/2007	.	.	.	.	.	.	.	.	.	N
Arg2345/G9P[8]/2007	.	.	.	.	.	.	.	.	.	N
Arg2366/G9P[8]/2007	.	.	.	.	.	.	.	.	.	N
Arg2385/G9P[8]/2007	.	.	.	.	.	.	.	.	.	N
R143/G9P[6]/1999/Brazil	.	.	.	.	.	.	.	.	.	N
R160/G9P[8]/1999/Brazil	.	.	.	.	.	.	.	.	I	N
rj8207/G9P[8]/2004/Brazil	.	.	.	.	.	.	.	.	.	N
rj8419/G9P[8]/2004/Brazil	.	.	.	.	.	.	.	.	.	N
BA206/G9P[8]/1999/Brazil	.	.	.	.	.	.	.	.	I	N
BA201/G9P[8]/2000/Brazil	.	.	.	.	.	.	.	.	.	N
BA202/G9P[8]/2002/Brazil	.	.	.	.	.	.	.	.	.	N
Py00477/G9P[8]/2000/Paraguay	.	.	.	.	.	.	.	.	.	N
DS-1	V	N	H	K	I	N	I	S	I	N
Arg0083/1997/Argentina	.	.	.	.	.	.	.	.	.	S
Arg0044/1997/Argentina	.	.	.	.	.	.	.	.	.	S
Arg2389/2007/Argentina	.	.	.	.	.	.	.	.	.	S
Arg2395/2007/Argentina	.	.	.	.	.	.	.	.	.	S
rj5619/2002/Brazil	.	.	.	.	.	.	.	.	V	S
rj5323/2002/Brazil	.	.	.	.	.	.	.	.	V	S
SA356PT/1996/South Africa	.	.	.	N	.	.	.	.	.	S
GH1803/1999/Ghana	.	.	.	E	.	.	.	.	.	S
TN1529/1999/Tunisia	.	.	.	.	.	.	.	.	.	S
SA514GR/1987/South Africa	.	.	.	.	.	.	V	.	.	.
KY3303/1999/Kenya	.	.	.	.	.	.	V	.	.	S
TW6/1981/Taiwan	A	.	.	.	.	.	.	.	.	.
95A/1995/Australia	.	.	.	.	.	.	.	.	V	S
SA1831GR/1993/SouthAfrica	I	.	.	.	.	.	.	.	.	S

The deduced amino acid sequences of antigenic regions of G9 strains are compared with prototype strain AU32 and in the case of G2 strains, with prototype strain DS-1. Strains from the same lineage isolated worldwide are used for comparison. Residues that match sequences of antigenic regions are denoted by dots.

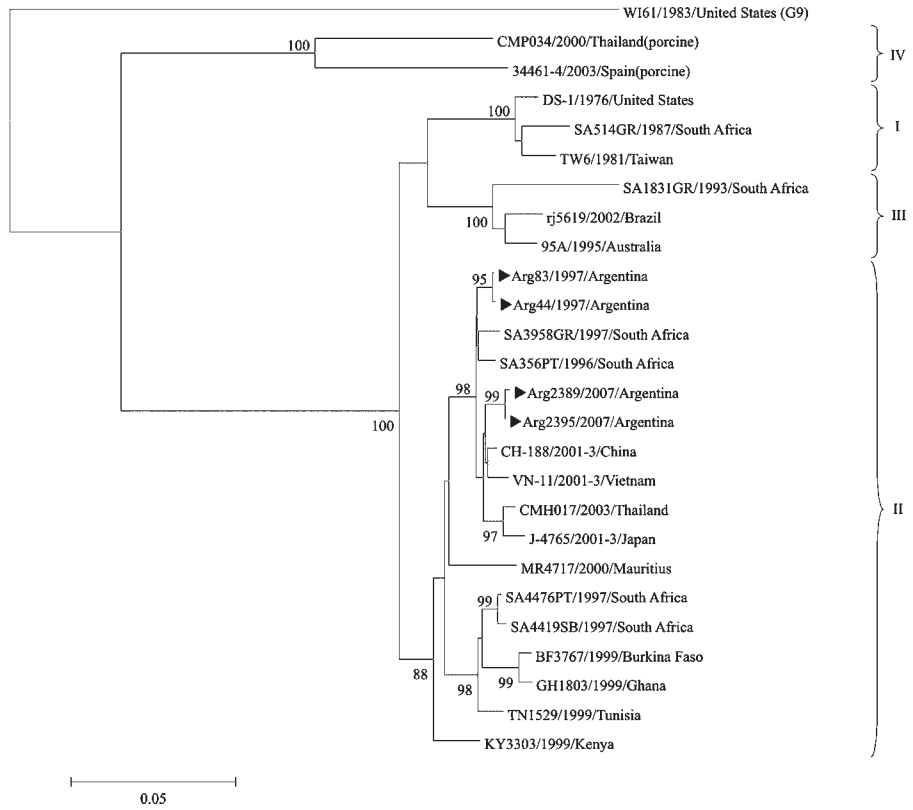

Fig. 2. Phylogenetic analysis of G2 strains. The tree was constructed from nucleotide sequences of VP7 genes of G2 rotavirus strains obtained from the GenBank database, using the Kimura two-parameter and neighbor-joining methods using MEGA4. The lineages are indicated on the right and the bootstrap values are shown at the branch nodes (values <80% not shown). The Argentine strains isolated in 1997 and 2007 are marked with a filled triangle. For each strain, its name/year of isolation/country is indicated. WI61 prototype strain (G9 genotype) was included as an outgroup.

GenBank database. Argentine strains formed separate clusters according to the year of isolation.

Comparison of Antigenic Regions of G2 Strains

The pattern of amino acid substitutions within VP7 antigenic regions between G2 strains was examined through an alignment using the VP7 of reference G2 strain DS-1 as the consensus sequence. Compared to DS-1, three amino acid changes were identified in regions A and F of Argentine G2 strains isolated in 1997 (region A: position 87 (A → T), position 96 (D → N) and region F: position 242 (N → S)), and an additional amino acid substitution was detected in region C position 213 (N → D), in the case of G2 strains isolated in 2007 (Table IV). Thus, only one amino acid change was detected in VP7 antigenic regions of Argentine G2 strains isolated in 1997 compared to 2007.

Group A Rotavirus G and P Genotype Frequencies During 1996–2007

The present results along with previous findings [Argüelles et al., 2000; Castello et al., 2006] complete more than 10 years of continuous genotype surveillance. This body of information allows the establishment of a natural pattern of fluctuations in genotype frequencies in the same area (Fig. 3). Two remarkable features are evident from this analysis: first, strains belonging to the globally emergent G9P[8] genotype not previously detected in Buenos Aires were the predominant strain during each year between 2004 and 2007 and second, G2P[4] strains that had not been detected after 1999 reappeared in 2004, reaching a prevalence of 57.5% in 2007. During 1998 and 1999, G9P[6] strains were detected in Argentina [Bok et al., 2001; Castello et al., 2006] and since 2004 the genotype G9 reappeared at much higher frequencies but now as G9P[8] strains (this study). Several other features can be highlighted regarding the distribution of G and P genotypes over time for the studied area in the context of Argentinian investigations: (i) G3 strains have not been detected at high frequencies in Argentina since the first samples evaluated from 1983 [Castello et al., 2003; Barril et al., 2006; present study], (ii) G4 was the most common strain from 2000 through 2001 but this genotype became less common over time and was not detected in 2006 and 2007, (iii) a similar pattern was observed for G1, which was most common during 2002 and 2003 but diminished constantly until 2006 when it was not detected at all, (iv) low frequencies of novel G12 strains were detected in Buenos Aires between 1999 and 2002 [Castello et al., 2006] and in 2004 and 2007 (present

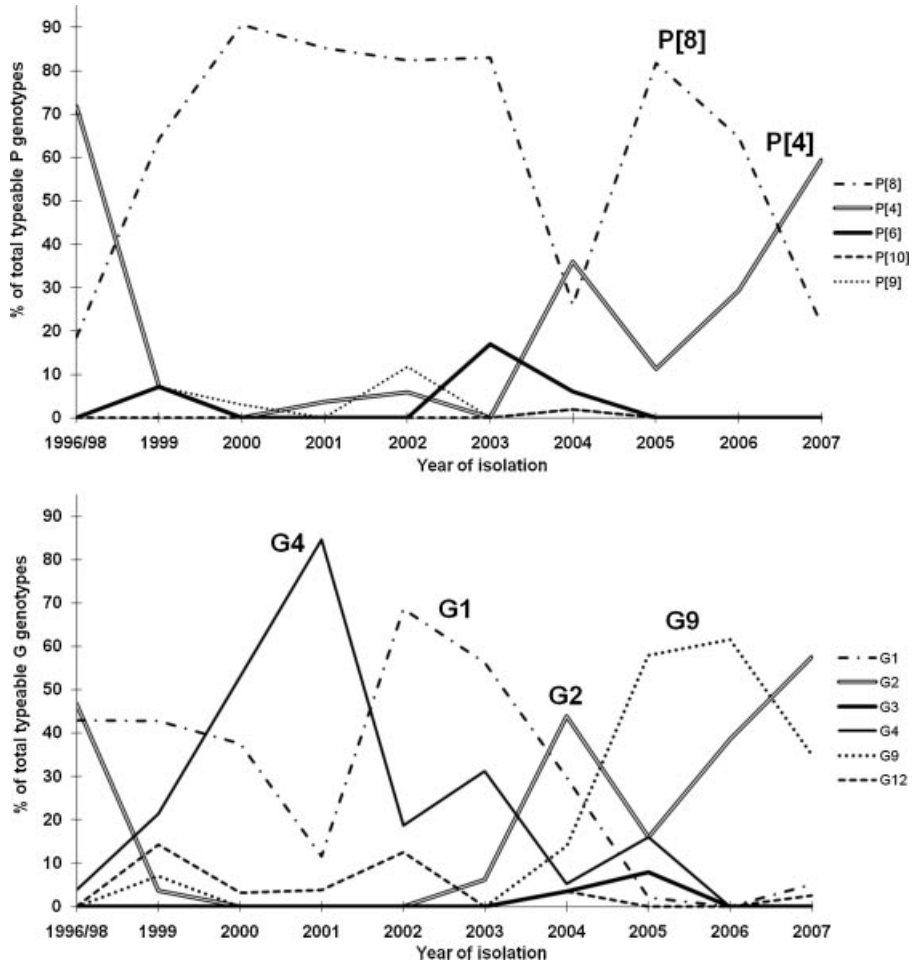

Fig. 3. Genotypes frequency fluctuation over an 11-year period in Buenos Aires, Argentina. The percentage is expressed in relation to the total typeable genotypes per year. Data represented in the graphs originated from surveillance studies conducted between 1996 and 1998 [Argüelles et al., 2000], between 1999 and 2003 [Castello et al., 2006], and between 2004 and 2007 (this study). Top: G genotypes; bottom: P genotypes.

study), (v) the distribution of P types followed typical patterns with high rates of P genotype [8] associated with G1 and G4 strains from 1999 to 2003 and then combined with G9 in 2005 and 2006; similarly, genotype P[4] was prevalent during 1996–1998 and 2004–2007 in its classical combination with G2, (vi) the unusual genotype P[10] and the large number of P non-typeable samples detected during 2004–2007 require further investigation which is underway. Interestingly, 73.0% of the P non-typeable strains were identified in patients above 3 years old (data not shown).

DISCUSSION

Monitoring the diversity of rotavirus strains in a single community over time provides insights into the introduction, evolution, and dispersion of new strains and the influence that the introduction of a new vaccine can have on these natural patterns. The VP7 (G genotype) and VP4 (P genotype) gene segments of rotavirus strains circulating in a non-vaccinated population in Buenos Aires from 2004 to 2007 were characterized in this study. These results along with previous findings [Argüelles et al., 2000; Castello et al., 2006] provide more than 10 years of continuous strain surveillance which allowed the natural pattern of fluctuations in genotype frequencies for the same area to be examined.

On the surface, the distribution of strains in Buenos Aires appears to be similar to what has been found elsewhere: the five strains found most common globally, G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] [Gentsch et al., 2005; Santos and Hoshino, 2005] were also the most common strains found in Buenos Aires. However, further characterization of strains has identified a few unusual variants present in low prevalence (e.g., G12, P[9], P[6], and P[10]), a higher than expected rate of mixed infections, and a high prevalence of the globally emergent G9 strain and antigenically distinct G2 strains in the most recent 4 years. These results provide some interesting insights into current discussions about the potential effectiveness of new vaccines being

introduced and the possible replacement of strains that this might encourage. A high frequency of G2P[4] rotaviruses was detected in Buenos Aires and this strain is quite distinct from most of the other common serotypes in circulation. Rotavirus strains have been classified into genogroups based upon hybridization of probes made from mRNA of one strain with RNA from the 11 segments of rotavirus of a different strain. These genogroups reflect differences in the origin of the strain and the antigenicity of the virus. The G2 serotype belongs to a phylogenetically distinct genogroup (the DS-1 genogroup) [Matthijnssens et al., 2008] and probes made from the gene segments of this strain do not cross-react with RNA from the other common rotavirus strains of the more common Wa genogroup [Flores et al., 1985]. In a meta-analysis including the results of one phase 3 and two phase 2 studies from Finland and Latin America, the monovalent Rotarix® vaccine, which belongs to the Wa genogroup, was less effective in protecting against G2 strains (i.e., the DS-1 genogroup) than against the remaining common strains in the Wa genogroup [Ruiz-Palacios et al., 2006].

In Brazil, Rotarix® was introduced into the routine program of childhood immunization in March 2006 and subsequent surveillance indicated an increased prevalence of genotype G2P[4] strains circulating following this national program. Several investigators interpreted this to mean that, as a result of vaccination, an immunological pressure was exerted which resulted in the selection of strains for which the vaccine is less effective [Gurgel et al., 2007; Nakagomi et al., 2008; Carvalho-Costa et al., 2009]. The present data and data from other South American countries that border Brazil indicate that G2 strains have been extremely common in recent years even in countries where Rotarix® had not been introduced [Patel et al., 2008]. In the case of G2 strains, Argentina and Brazil proved to have a similar fluctuation pattern given its prevalence during 1996–1998 in Rio de Janeiro (34%) [Araujo et al., 2002] and Buenos Aires (43%) [Argüelles et al., 2000] and the present reemergence in both countries. Taking into account that G2 has also become the most commonly detected genotype in neighboring Paraguay [Martinez et al., 2008], this could represent a regional phenomenon. Consequently, the high rates of G2 strains found in Brazil may well represent natural background circulation of these strains unrelated to the impact of the vaccine. Additionally, the virtual disappearance of G1P[8] strains was observed in this study during 2006 and 2007 which is a particularly noticeable event since this is the most common strain in humans and was found rarely at frequencies below 10% in Latin America between 1995 and 2004 [Castello et al., 2004]. The pronounced lowering in G1P[8] strains has also been observed in Brazil and Paraguay since before 2006 [Carvalho-Costa et al., 2009] and again, not influenced by the population vaccination status with Rotarix®.

Strains of the unusual serotype G12P[9] were also identified in this study and, as was reported recently, they belong to the uncommon human genogroup AU-1 [Castello et al., 2009]. These strains have been detected at low frequencies in Buenos Aires since 1999 and more recently in Brazil and Paraguay [Castello et al., 2006; Pietruchinski et al., 2006; Martinez et al., 2008]. The G12P[9] shares no antigens with the Rotarix® vaccine and has no overlap with the other licensed rotavirus vaccine, Rotateq®. How well this new generation of rotavirus vaccines protects against this novel strain will have to be monitored as greater experience is gained with the vaccines. The Rotateq® vaccine has proven to be less than 50% protective against hospitalizations of children in Nicaragua [Patel et al., 2009], suggesting that differences in serotype-specific protection will have to be assessed carefully.

Sequence analysis provides further insights into the introduction, circulation, and evolution of rotaviruses in a given setting. The present study and previously reported data on genotyping and sequencing show a high degree of similarity among G9 strains circulating in South American countries since 1999 including G9P[6] and G9P[8] strains [Santos et al., 2001, 2005; Parra et al., 2005, 2007; Castello et al., 2006; Volotão et al., 2006; Araujo et al., 2007; Stupka et al., 2007]. Of note is a replacement at position 171 (T → I) that was previously described for Argentine G9P[6] strains [Bok et al., 2001] and is also noted in Brazilian G9P[6] strains from the same year [Santos et al., 2001]. Conversely, the only two sequences from regional G2 strains available in GenBank [Rio de Janeiro, Brazil, 2002, Araujo et al., 2007] belong to a different lineage from the Argentine G2 strains from 1997 and 2007 sequenced in this work suggesting that, unlike G9 strains, G2 strains from Argentina could have a different origin than those from Brazil. On comparing the deduced amino acid sequences it could be appreciated that the VP7 genes from Buenos Aires G2 isolates collected in 1997 and 2007 had few differences. Similarly, the deduced amino acid sequences of the VP7 antigenic regions of G9 strains in the current collection are, with one exception, identical to strains examined in 1999 and to Brazilian strains isolated in 1999, 2000, and 2004 [Santos et al., 2001, 2005; Araujo et al., 2007]. These strains do have several amino acid changes in antigenically relevant regions when compared with prototype G2 (DS-1) and G9 (AU-32) strains and such differences might in the future relate to changing efficacy of these live oral vaccines.

The high prevalence of mixed infections (18%) during the latest surveillance period 2004–2007 is of interest because these mixed infections provide a clear way for the virus to evolve over time. Reassortment of segmented viruses like rotavirus is a recognized means for a virus to acquire new gene segments and differentiate over time. Globally, this has been documented better in populations living in very poor settings where enteric infections are more common rather than more industrialized settings where rotavirus infections have been mixed rarely [Gentsch et al., 2005]. Rates up to 44% with an average rate of 12% of mixed infections were calculated for Latin America [Castello et al., 2004] raising the prospect that novel reassortants might be

suspected as surveillance activities continue. To detect better these reassortants, more sensitive methods to identify the origin of each gene segment will be required in order to characterize fully the virus rather than a simple identification of the G and P components.

As rotavirus vaccines are introduced into Argentina and other countries, the need for ongoing surveillance of strains will become particularly important to monitor instances where vaccine fails and children who have been fully immunized become hospitalized for rotavirus infections. The emergence and circulation of novel serotypes in the region may provide other insights into the stability of these strains and their regional circulation over time.

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