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Genetic and Antigenic Evolution Profiles of G1 Rotaviruses in Córdoba, Argentina, During a 27-Year Period (1980–2006)

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Rotavirus G1 strains represent the most common genotype that causes diarrhea in humans and has been incorporated into both, monovalent and multivalent, rotavirus licensed vaccines. The aim of this study was to determine the evolution profile of G1 rotaviruses in Córdoba, Argentina, over a 27-year period (1980–2006). Intragenotype diversity, represented by lineages within rotavirus circulating strains, was observed. Phylogenetic analysis of the VP7-gene of G1 rotavirus clinical strains showed the circulation of G1 lineage IV and V strains in the 1980s, and co-circulation of lineage I and II strains in the 1990s and 2000–2006. The distribution of G1 in lineages could be linked to multiple nucleotide substitutions distributed across lineages that did not correlate with the emergence of G1 antigenic variants. Moreover, temporal lineage distribution was not linked to significant changes in G1 prevalence. Therefore, the continuous and dominant circulation of G1 over time could not be related to the emergence of antigenic variants in the community. Continuous rotavirus surveillance is necessary to understand rotavirus evolution and to measure how genetic and antigenic changes might affect the effectiveness of vaccines in the future. ***J. Med. Virol. 85:363–369, 2013.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: rotavirus; G1 genotype; phylogeny

INTRODUCTION

Group A rotavirus (RVA) is the most important etiological agent of severe gastroenteritis in infants and young children worldwide [Kapikian and Chanock, 1996; Iturriza-Gómara et al., 2000]. Although almost all children experience RVA infection by the age of 3–5 years [Glass et al., 1996], the majority of an estimated 527,000 rotavirus-associated deaths occur in developing countries [WHO, 2006].

The rotavirus genome consists of 11 double-stranded RNA gene segments that encode six structural (VP1–VP4, VP6 and VP7) and six nonstructural (NSP1–NSP6) proteins [Estes, 2001]. Based on the diversity of the VP7 and VP4 outer capsid proteins, RVA is classified in G (glycoprotein) and P (protease-sensitive) genotypes, respectively. At least 27 G and 35 P genotypes have been identified [Abe et al., 2009; Solberg et al., 2009; Trojnar et al., 2009; Ursu et al., 2009; Collins et al., 2010; Matthijnssens et al., 2011]. Worldwide, the most common genotypes associated with human infection are G1–G4 and G9 associated with P[4] and P[8] [Dennehy, 2008; Ursu et al., 2009]. Both antigens of rotavirus, VP7 and VP4, elicit neutralizing antibodies. However, the antibodies elicited against VP7 protein play a greater role in immunity after natural rotavirus infection as well as after vaccination [Chiba et al., 1986; Kapikian et al., 1996].

The VP7 protein of 326 amino acids (aa) is usually encoded by segment 9 [Estes and Cohen, 1989] and carries nine variable regions (VRs) that are different across the identified serotypes but are highly homologous between the same G type. Four of these regions, VR5, VR7, VR8, and VR9, are regarded as major antigenic regions, namely A (aa 87–100), B (aa

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141–150), C (aa 208–224), and F (aa 235–242), respectively [Coulson and Kirkwood, 1991; Kirkwood et al., 1993; Xin et al., 1993]. Two other antigenic sites have also been described, namely D (aa 291) and E (aa 190) [Dyall-Smith et al., 1986; Kirkwood et al., 1993; Lazdins et al., 1995]. The VP7 sequence analysis of escape mutants selected with G1 serotype-specific monoclonal antibodies (mAbs) has given the information on the amino acids located into the VR5, VR7, VR8, and VR9 involved in virus neutralization [Coulson and Kirkwood, 1991; Xin et al., 1993]. In addition, nucleotide sequence analysis of VP7 of G1 rotaviruses has revealed the existence of at least four G1 genetic lineages based on point mutation across the VP7 gene [Jin et al., 1996; Berois et al., 2003]. More recently, Phan et al. [2007] have proposed a novel nomenclature for G1, in which worldwide rotavirus G1 strains are classified into 11 lineages (I–XI). Of note, lineages I–IX are found only in humans, the porcine rotavirus SW20/21 strain is in lineage X, and lineage XI consists of both bovine rotavirus T449 and porcine rotavirus C60 and C95 strains.

Among the common genotypes, G1 specificity alone contributes >50% of rotavirus infections and is thus identified as a common cause of rotavirus disease worldwide [Gentsch et al., 2005; Santos and Hoshino, 2005]. In Córdoba, Argentina, G1 strains have been described as a predominant genotype in human rotavirus infections during 1980–2006 [Barril et al., 2006, 2010]. Two rotavirus vaccines, G1P[8] RVA vaccine (Rotarix®, GlaxoSmithKline Biologicals, Rixensart, Belgium) and a pentavalent G1–G4 and P[8] RVA vaccine (Rotateq®, Merck and Sanofi Pasteur MSD, NJ) were licensed in 2006, but have not yet been incorporated to the Argentina Vaccination Schedule. In view of the introduction of rotavirus vaccines, the aim of the present study was to evaluate the natural evolution profile of rotavirus G1 strains in Córdoba, Argentina, during 1980–2006 (named as the pre-vaccination era). The results obtained are baseline data to evaluate G1 evolution dynamics under vaccine-induced immune pressure once a rotavirus vaccine is widely introduced in a community.

MATERIALS AND METHODS

Samples

From 1980 to 2006, 317 G1 strains were detected in stool specimens from children aged <3 years that presented with acute gastroenteritis at different hospitals in Córdoba city. Out of the 317 G1 strains, 165 corresponded to G1 single infections and 152 to co-infections with other G types. From the 165 G1 single infections, 0–46 G1 samples were isolated in each year. To obtain an overview of G1 RVA evolution, a total of 32 G1P[8] strains were selected, involving one sample isolated from each of the following years: 1982 and 1987; two samples in the years: 1980, 1984, 1986, 1989, 1996, 1999–2003, 2005, and 2006; and three samples in the years 1997 and 1998. During 1990–1995 only three rotavirus-positive samples were characterized as G1 genotype and none of them were associated with P[8] genotype. Therefore, they were not included in the present study.

Nucleic Acids Extraction

Viral double-stranded (ds) RNA was extracted from the stool samples by the phenol-chlorophorm method, followed by alcohol precipitation according to standard procedures [Perry et al., 1972]. After drying, the pellets were diluted in 20 μl of sterile distilled water.

RT-PCR

Extracted dsRNA was first reverse-transcribed into gene 9 (VP7) full-length cDNA with the generic primers Beg9/End9 [Gouvea et al., 1990]. Then, in a second step, the cDNA product was used as a template for VP7 amplification with the same Beg9/End9 pair of primers.

G Genotyping

The VP7 full-length PCR products were used as templates in combination with two cocktails of type-specific forward primers and the generic reverse primer End9 for G genotyping [Gouvea et al., 1990]. The cocktails were: G1 (aBT1), G2 (aCT2), and G3 (aET3) in one mixture; and G4 (aDT4), G8 (aAT8), and G9 (aFT9) in the second. The amplicons were analyzed by electrophoresis on 10% polyacrylamide gels and visualized after silver staining, as described elsewhere [Herring et al., 1982], to achieve high resolution of the products obtained.

Nucleotide Sequencing and Phylogenetic Analysis

The VP7 gene of G1 strains was examined using comprehensive sequence analysis. The PCR amplicons were purified and sequenced in both directions with primers Beg9 and End9 using the dideoxy-nucleotide chain terminator method with Big Dye TM terminator version 3.1 cycling conditions on an automated sequencer (model 3730xl; Applied Biosystems, Foster City, CA) by Macrogen Inc. (Seoul, South Korea). Sequences were assembled and analyzed with the BioEdit version 7.0.5.2 alignment editor [Hall, 1999] and were compared against the GenBank database using BLAST program. The resultant gene and deduced amino acid sequences were aligned using the CLUSTAL X program [Thompson et al., 1997]. Phylogenetic analysis was carried out with the MEGA software, version 4.0 [Tamura et al., 2007], with the Kimura two-parameter [Kimura, 1980] as a model of substitution and the neighbor-joining method to construct the phylogenetic tree. The statistical significance of the inferred phylogenies was estimated by bootstrap analysis with 1,000 pseudoreplicate data sets.

Nucleotide Sequence Accession Numbers

The nucleotide sequences obtained in this study were deposited in NCBI GenBank under accession numbers JQ710662–JQ710677 and JX458953–JX458968. The referenced sequences in the GenBank database are as follows: Wa (K02033), D (AB118022), KU (D16343), K2 (D16323), 88H249 (AB081795), VN-281 (DQ508167), Thai-1604 (DQ512981), G192B (AF043678), HOU8697 (U88717), RV-4 (M64666), AU007 (AB081799), 421 (D16326), 417 (D16328), Mvd9812 (AF480289), PA10/90 (DQ377587), PA5/90 (DQ377573), Kor-64 (U26378), C95porcine (L24165), T449bovine (M92651).

Statistical Analysis

The chi square test was used to compare the detection frequencies of rotavirus G1 genotype and the temporal rotavirus G1 lineage distribution. Resultant *P*-values less than 0.05 were considered as statistically significant.

RESULTS

The nucleotide and deduced amino acid sequences of the 32 Argentinean VP7-G1 strains were determined and compared with G1 strains available in the GenBank database. The local VP7 gene sequences revealed high nucleotide and amino acid identities between them, ranging from 92.2% to 100% and from 93.2% to 100%, respectively. The phylogenetic analysis unveiled that all the G1 strains isolated in Córdoba in the 1980s grouped into VP7-G1 lineages IV and V; and G1 strains isolated in the 1990s and 2000–2006 belonged to lineages I and II. No G1-lineage III strains were detected (Fig. 1).

Several synonymous and non-synonymous interlineage substitutions were observed. The amino acid substitutions between strains of different lineages were revealed in variable and conserved regions of the VP7 protein. The amino acid detailed analysis was referred to the previously described antigenic regions (Fig. 2). In the antigenic region A (aa 87–100) some point mutations were observed between strains of the same lineage and also between different lineage. Thus, two strains of lineage I (year 1996) and lineage II (1997) revealed the point mutation 87-Thr → Asn. Four strains of lineage I (strains that circulated in 1999, 2001, and 2005), all the strains of lineage II (strains of years 1997, 2000, 2002, 2003, and 2006) and lineage V (strains of years 1980, 1984–1989) showed the mutation 94-Ser → Asn. Also, two lineage IV strains (strains that circulated in 1980 and 1984) presented the point mutation 96-Gly → Val. Amino acid mutations were also observed in the antigenic region B (aa 141–150). Four lineage II strains (strains of years 2000, 2002, 2003, and 2006) showed the mutation 147-Asn → Ser. Numerous point mutations were observed in antigenic region C (aa 208–224). Six lineage I strains (from years 1996, 1998, 1999, and 2001) revealed the mutation 212-Val → Ile. Two lineage II strains (year 1997) and all lineage I strains (strains that circulated in the 1990s and 2000s) unveiled the amino acid change 217-Met → Ile → Thr, compared with the earlier strains that circulated in Córdoba in the 1980s (lineage IV and V), but the other five lineage II strains (that circulated in the years 1997, 2000, 2002, 2003, and 2006) did not revealed the amino acid change in this position. Also, one strain of lineage I (strain that circulated in 2003) revealed the mutation 219-Ala → Thr. Lastly, in the antigenic region D (aa 291), lineage I and IV strains showed the mutation 291-Lys → Arg.

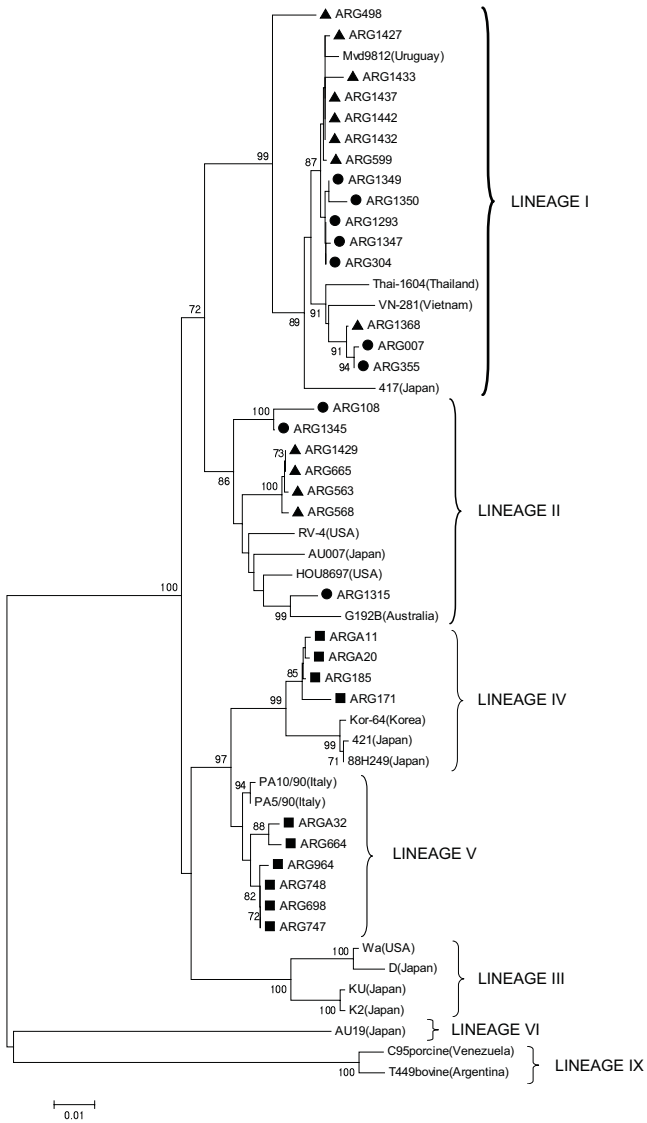

Fig. 1. Phylogenetic tree based on VP7-gene nucleotide sequences of G1 rotavirus strains. The tree was constructed by the neighbor-joining method and the Kimura two-parameter model. Bootstrap values above 70% are given at branch nodes. The scale bar represents 1% genetic distance. G1 rotavirus lineages are shown. The strains isolated in Córdoba, Argentina, are indicated by squares (1980s), circles (1990s), and triangles (2000s).

Lineage	Strain	Region A (aa 87–100)	Region B (aa 141–150)	Region E (aa 190)	Region C (aa 208–224)	Region F (aa 235–242)	Region D (aa 291)
		90 100	150	190	210 220	240	291
		...| ....|....|	....|....|	|	..| ....|....|	|....| ..	.
III	Wa(USA)	TEAS TQINDGDWKD	LMKYDQSLEL	S	QTT NVDSFEMIAE NEKL	HKINLT TT	K
IV	ARGA11	....S.VE...	......N...	.	... .V..	....	R
IV	ARGA20	....S..E...	......N...	.	... .V..	....	R
IV	ARG171	....S.VE...	......N...	.	... .V..	....	R
IV	ARG185	....S..E...	......N...	.	... .V..	....	R
V	ARGA32	....E...	......N...	.	... .V..	....	.
V	ARG664	....E...	......N...	.	... .V..	....	.
V	ARG698	....E...	......N...	.	... .V..	....	.
V	ARG747	....E...	......N...	.	... .V..	....	.
V	ARG748	....E...	......N...	.	... .V..	....	.
V	ARG964	....E...	......N...	.	... .V..	....	.
II	ARG1315	N... ......E...	......N...	.	... .V..	....	.
II	ARG108	....E...	......N...	.	... .IV..	....	.
II	ARG1345	....E...	......N...	.	... .IV..	....	.
II	ARG563	....E...	.....	.	... .V..	....	.
II	ARG1429	....E...	.....	.	... .V..	....	.
II	ARG568	....E...	.....	.	... .V..	....	.
II	ARG665	....E...	.....	.	... .V..	....	.
I	ARG1293	....S..E...	......N...	.	... .I....TV..	....	R
I	ARG007	N... ...S..E...	......N...	.	... .TV..	....	R
I	ARG1347	....S..E...	......N...	.	... .I....TV..	....	R
I	ARG304	....S..E...	......N...	.	... .I....TV..	....	R
I	ARG355	....S..E...	......N...	.	... .TV..	....	R
I	ARG1349	....E...	......N...	.	... .I....TV..	....	R
I	ARG1350	....E...	......N...	.	... .I....TV..	....	R
I	ARG1368	....S..E...	......N...	.	... .TV..	....	R
I	ARG1427	....S..E...	......N...	.	... .TV..	....	R
I	ARG498	....E...	......N...	.	... .I....TV..	....	R
I	ARG1432	....S..E...	......N...	.	... .TV..	....	R
I	ARG1433	....S..E...	......N...	.	... .TVT.	....	R
I	ARG1437	....S..E...	......N...	.	... .TV..	....	R
I	ARG599	....E...	......N...	.	... .TV..	....	R
I	ARG1442	....S..E...	......N...	.	... .TV..	....	R

Fig. 2. Alignment of antigenic regions of VP7 deduced amino acid sequences of G1 strains isolated in Córdoba, Argentina, during the period 1980–2006, compared to reference strain Wa. Conserved amino acid residues are indicated by dots.

Rotavirus G1 detection rate during the studied period (1980–2006) and the temporal rotavirus G1 lineage distribution was not linked to significant changes in G1 circulation prevalence in the settled population ($P > 0.05$; Table I). G1 prevalence was high almost all over the studied period, independently of the G1 lineage detected. Significant changes in G1 prevalence were only observed among periods that revealed the same G1 lineage circulation (i.e., years 1996–2002, media G1-I prevalence 57.1%, vs. years 2003–2006, media G1-I prevalence 28.1%, $P < 0.05$), meanwhile the change of G1 lineage did not have repercussions in G1 prevalence (i.e., year 1996, G1-I prevalence 54.5%, year 1997, G1-II prevalence 50.0%, $P > 0.05$; and year 1998, G1-I prevalence 62.7%, $P > 0.05$).

DISCUSSION

In view of the imminent incorporation of rotavirus vaccination into the Argentina childhood immunization schedule, and the dominance and persistence of G1 genotype in Córdoba throughout the years [Barril et al., 2006], an investigation of the heterogeneity and natural evolution of local G1 strains became of interest.

G1 strains that circulated in Córdoba during the 27-year period were different from the G1 reference strain Wa, at both nucleotide and amino acid levels. Reference strain Wa was obtained in the 1970s in USA and classified as lineage III [Jin et al., 1996]. Our result supports findings from other studies that suggest the extinction of lineage III G1 strains circulation worldwide [Diwakarla and Palombo, 1999; Arista et al., 2006; Rodriguez-Castillo et al., 2006; Le et al., 2010].

A study of the genetic evolution profile of G1 strains in Córdoba, Argentina, during the period 1980–2006 showed that G1 strains grouped into four lineages (I, II, IV, and V), alternating cycles of circulation, co-circulation, emergence, disappearance, and/or persistence of intragenotypic G1 variants. This result provided evidence of genetic variation over the years. During the 1980s, lineage IV and V G1 strains circulated in Córdoba, which is in agreement with other studies reporting lineage IV and V circulation in different countries during the same period [Jin et al., 1996; Arista et al., 2006].

In 1996, lineage I G1 strains emerged in Córdoba and circulated for at least 11 years (1996–2006), alternating cycles of appearance and disappearance. The first report of the circulation of lineage I strains was

TABLE I. Lineage Distribution of G1 Rotavirus Strains Circulating in Córdoba, Argentina, During the Period 1980–2006

Year	Rotavirus G1 prevalence[a] (%)	G1 lineage	Reference
1980	8/12 (66.7)	IV, V	Barril et al. [2006]
1982	2/5 (40.0)	IV	
1984	9/16 (56.2)	IV, V	
1986	12/20 (60.0)	IV, V	
1987	4/9 (44.4)	V	
1989	10/14 (71.4)	V	
1996	6/11 (54.5)	I	
1997	67/134 (50.0)	II	
1998	89/142 (62.7)	I	
1999	4/7 (57.1)	I	
2000	24/36 (66.7)	I, II	
2001	22/46 (47.8)	I	
2002	16/23 (69.6)	I, II	
2003	4/12 (33.3)	I, II	
2005	11/39 (28.2)	I	Data not published
2006	19/50 (38.0)	I, II	Barril et al. [2010]

[a]Rotavirus G1 prevalence = G1 rotavirus positive samples/total rotavirus positive samples.

in 1986 in Italy, where lineage I strains were sporadically detected during a 19-year period [Arista et al., 2006]. Since then, lineage I strains were detected as predominant in many countries, such as Bangladesh, Malawi, Brazil, and Japan [Cunliffe et al., 2001; Araujo et al., 2007; Phan et al., 2007; Ahmed et al., 2010]. In 1997, the circulation of lineage II G1 strains was observed in Córdoba. Circulation of lineage I and/or lineage II G1 strains during the 1990s–2000s was also revealed in neighboring countries, such as Uruguay, Paraguay, and Brazil [Berois et al., 2003; Parra et al., 2005; Araujo et al., 2007]. In fact, Maunula and von Bonsdorff [1998] have suggested that the lineages might predominate in different parts of the world concurrently.

Although temporal substitution of G1 lineages in a region is important as a viral evolutionary marker, it is more important to determine if those substitutions correlate with antigenic changes in the strains. This could explain the persistence and predominance of G1 strains throughout the years.

Antigenicity assays with mAbs have shown that the presence of Asn at the amino acid residue 94 (antigenic region A) is related to virus neutralization and viruses with the substitution 94-Asn → Ser/Thr are not neutralized [Coulson and Kirkwood, 1991; Diwakarla and Palombo, 1999]. In addition, the amino acid residue 94 is considered to be essential for discrimination of lineage I and II G1 strains; lineage II strains contain Asn at this position while lineage I strains contain Ser/Thr [Diwakarla and Palombo, 1999]. The analysis of the amino acid residue 94 was not enough to discriminate all local G1 strains in lineage I or lineage II. This result suggests that complete VP7 gene analysis is needed to assign a strain to a lineage. The Ser to Asn substitution at amino acid position 147, present in almost all the strains that circulated in Córdoba, affects a critical component of neutralization epitope identified in previous studies [Dyall-Smith et al., 1986; Coulson and Kirkwood, 1991].

Genetic variation within antigenic region C was also observed, involving the amino acid residue 217. It has been reported that the amino acid change 217-Met → Thr/Ile does not correlate with reactivity alteration to mAbs, but it could be considered as an evolutionary marker [Diwakarla and Palombo, 1999]. In agreement with this hypothesis, the most recent strains isolated in Córdoba corresponding to lineage I and some lineage II strains (years 1990s and 2000s) revealed the amino acid substitution 217-Met → Thr/Ile, compared with the earlier G1 isolates from the 1980s. However, most of the recent lineage II strains (years 1990s and 2000s) did not revealed the mutation. Thus, it was not possible to differentiate older strains from the most recent strains by analyzing the amino acid residue 217.

Lineage I and IV strains that circulated in Córdoba showed the substitution 291-Lys → Arg in the antigenic site D. This amino acid change affects a hydrophobic and highly conserved region that triggers rotavirus binding to MA104 cells [Dyall-Smith et al., 1986; Frenchick et al., 1988]. Studies with mAbs have demonstrated the selection of neutralization resistant variants with point mutations at this antigenic site [Coulson and Kirkwood, 1991].

Other point substitutions within antigenic regions were observed (87-Thr → Asn, 96-Gly → Val, 212-Val → Ile, 219-Ala → Thr) but the implications of these changes are still unknown.

The results presented above revealed that genetic variation of G1 strains detected in Córdoba had led to the replacement and/or co-circulation of different G1 lineages over the years. However, significant changes in G1 prevalence throughout the studied period did not correlate with G1 lineages changes in the community. This could be explained because lineage changes were associated with multiple interlineage nucleotide

substitutions, but did not correlate with the emergence of G1 antigenic variants. This result contrasts with the correlation observed by Arista et al. [2006] between the peaks of G1 infections and the various VP7 lineages and sublineages observed over a 19-year period (1986–2004). Thus, the results of this study highlight that the persistence and dominance of G1 rotavirus strains would not be linked to a dominant amino acid profile in the A–F antigenic regions. It is possible that the persistence of G1 genotype in nature over the years could be explained by a more stable VP7–VP4 combination, where certain lineages of P[8] associate with G1 specificity in a more suitable way [Araujo et al., 2007].

The present study, although performed with a limited number of samples analyzed, revealed that antigenic changes in RVA could not be inferred from the phylogenetic analysis of G1 strains.

Because rotavirus vaccines have not been introduced to the Argentina vaccination schedule yet, the results obtained in the present study show the evolutionary dynamics of G1 strains in Córdoba, in the absence of vaccine-induced immunity. The investigation of the genetic and antigenic heterogeneity of the circulating strains is important to anticipate antigenic changes that could affect the effectiveness of vaccines in the future.

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