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Increased frequency of rotavirus G3P[8] and G12P[8] in Argentina during 2008–2009: Whole-genome characterization of emerging G12P[8] strains

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ABSTRACT

Background: Group A rotaviruses are the leading cause of non-bacterial severe diarrhea disease in infants and young children. In humans, the most common genotypes are G1–G4 and G9. Recently, G12 strains have been sporadically reported in several countries, including Argentina, Brazil and Paraguay.
Objectives: To analyze rotavirus strain diversity in Argentina during 2008–2009 and to describe the whole genome-based classification of emerging G12P[8] strains detected in our country.
Study design: Rotavirus positive-samples ($n = 544$) were collected from Argentinean children during 2008–2009, as a part of the National Surveillance Network for Viral Diarrheas. Specimens were genotyped by reverse transcription-polymerase chain reaction (RT-PCR) followed by nested-multiplex PCR. Sequencing of 11 genome segments was performed in 3 randomly selected G12P[8] strains.
Results: G9P[8] was the most frequent strain in 2008, but in 2009 G3P[8] and G12P[8] were the most frequent strains in different geographical regions of the country. The novel emerging G12P[8] strains presented the following combination of genes: G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 (i.e. genotype1, Wa-like strains). The phylogenetic analysis of the VP7 gene of the G12P[8] strains grouped them within lineage III. Previously reported Argentinean G12P[9] strains presented genes from genotype 3 (AU-1-like strains) with a VP7 gene from lineage II.
Conclusions: The emergence of G12P[8] rotaviruses was due to the introduction of a new strain, rather than to a reassortment of the G12P[9] strains previously circulating in our country. This study assesses the temporal and geographical changes in genotypes prevalence as well as the periodic emergence of unusual G genotypes.

1. Background

Group A rotaviruses are the leading cause of non-bacterial severe diarrhea disease in young children worldwide. More than 90% of the deaths related to rotavirus occur in developing countries; however, rotavirus infection causes substantial morbidity and economic impact in developed countries.[1] It has been estimated that in Latin America and the Caribbean, rotavirus causes approximately 10 million diarrhea episodes, 2 million clinic consultations, 75,000 hospital admissions and 15,000 deaths annually.[2]

The rotavirus genome consists of 11 segments of double-stranded RNA (dsRNA) surrounded by a triple-layered capsid: core, inner and outer capsid. Rotaviruses are classified by a dual nomenclature system into G and P genotypes on the basis of the sequence diversity of the VP7 and VP4 genes, respectively.[3] At least 27 G-types and 35 P-types infecting different species have been reported to date,[4] but only five strains (G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8]) have been shown to be the most common in humans.[5] Interestingly, temporal and geographical changes in strain prevalence patterns as well as periodic emergence of unusual G genotypes have been observed.[5–8] Recently, an increasing incidence of G12 strains has been reported in several countries that was associated with the emergence of the two new lineages of the VP7 gene: one (lineage II) associated with P[9] and the other (lineage

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III) with P[4], P[6], and P[8].[9–11] In South America, G12P[9] strains were first sporadically reported in Argentina and Brazil, and then in Paraguay.[12–14] The phylogenetic analyses of the VP7 gene clustered these strains within lineage II, but two G12P[6] strains with lineage III have been recently detected in Brazil,[15] suggesting the introduction of new G12 strains into South America.[13]

Rotarix®, a live attenuated monovalent vaccine, and Rotateq®, a pentavalent, live vaccine, are currently pre-qualified and recommended by the World Health Organization (WHO).[16] In Argentina, both vaccines were licensed in 2006 and, although its use have started in the private health sector; they were not yet implemented in the national vaccination schedule. Continuous monitoring of rotavirus strain diversity during pre- and post-national-vaccination programs will help in the evaluation of the efficacy of current vaccines.

2. Objectives

To analyze the rotavirus strain diversity detected in Argentina during 2008–2009 and the whole genome-based classification of the novel emerging G12P[8] strains to shed light in the introduction of these strains in our country.

3. Study design

3.1. Sample collection

A total of 3581 fecal samples were collected from January 2008 to December 2009 from non-vaccinated children up to 5 years old with acute diarrhea. Immunocompromised patients and/or chronic gastroenteritis were excluded from the study. To ensure that the infecting strains were actually circulating in the community, fecal samples were collected within 24 h of being admitted as out- or in-patients; according to the Argentinean National Surveillance Network for Viral Diarrhea protocol. Samples were collected from large hospitals from the following cities (from North to South): Salta, Tucumán, Catamarca, San Juan, Santa Fé, Buenos Aires, Neuquén and Río Gallegos. Over thirty six percent (1308/3581) of the fecal samples, were found to be positive for rotavirus infection by enzyme immunoassay (IDEIA™ Rotavirus Kit, Oxoid, Ely, Cambridgeshire, UK), and 544 of them (~42%) were randomly chosen for genotyping.

3.2. G and P typing

Viral dsRNA was extracted using a MagMAX™-96 Viral RNA isolation Kit (Ambion, Austin, TX, US) according to the manufacturer's instructions. Rotavirus-positive specimens were genotyped by reverse transcription-polymerase chain reaction (RT-PCR) followed by nested-multiplex PCR with consensus and type-specific primers as described previously.[17–19] The first-round amplicons of untyped G-types were further analyzed by nested PCR using different sets of G5, G8 and G12 type-specific primers for the VP7 gene.[18,20,21] All of these strains were found to bear the G12 genotype. To confirm these results, we randomly selected 10% of these strains for VP7 gene partial nucleotide sequencing (data not shown). Each of these VP7 sequences was confirmed as G12 by using the BLAST program.[22]

3.3. Whole-genome sequencing of G12P[8] strains

To analyze the G12P[8] strains according to the Rotavirus Classification Working Group (RCWG),[23] sequencing of 11 genome segments was performed in three randomly selected strains. Viral dsRNA was extracted as described previously and the 11 segments were amplified by RT-PCR using Qiagen One Step RT-PCR kit (Qiagen, Valencia, CA, US) according to the manufacturer's instructions. PCR products were purified with a QIAQuick PCR purification kit (Qiagen) and the sequencing was performed using the dideoxynucleotide chain termination method with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, US) on a 3500Dx Genetic Analyzer automated sequencer (Applied Biosystems). Primers targeting the conserved 5′- and 3′-end regions as well as internal primers were used for RT-PCR and sequence reactions (primer sequences are available upon request).

3.4. Sequence and phylogenetic analyses

The nucleotide sequences reported in this study were submitted to the GenBank database under the numbers: JN088419–JN088451. The alignments were carried out using the BioEdit v7.0.1.[24] The aligned sequences were analyzed using MEGA v5.[25] The phylogenetic trees were constructed using neighbor-joining and Kimura 2-parameter as a nucleotide substitution model to compare with previously published analyses.[23]

4. Results

4.1. G and P typing

A total of 544 rotavirus-positive samples collected were genotyped in the National Reference Laboratory of Argentina. The overall data shows that G3P[8] strains were the most frequently detected (39%), followed by G12P[8] (24.8%), G9P[8] (17.5%), and G1P[8] (11.6%) (Table 1). As previously described, seasonal and geographic variations in the predominant strains can be detected in Argentina.[26,27] Thus, during the first year, G9P[8] was the most frequently detected genotype (82/206; 39.8%), followed by G1P[8] (36/206; 17.5%), G12P[8] (35/206; 17.0%) and G3P[8] (29/206;

Table 1
G and P type of rotavirus strains circulating in Argentina from 2008 to 2009.

Location	RV + analyzed samples	G and P type								
		G1P[8]	G2P[4]	G3P[8]	G3P[NT]	G4P[8]	G9P[8]	G12P[8]	Mixed	NT
Salta[a]	15	1	0	3	1	0	0	9	1	0
Tucumán	75	15	4	4	0	0	16	34	1	1
San Juan	132	23	0	47	1	0	61	0	0	0
Catamarca[a]	41	0	0	8	1	0	0	32	0	0
Santa Fe	70	11	5	44	4	6	0	0	0	0
Buenos Aires	115	12	3	72	2	0	4	17	3	2
Neuquén[a]	15	1	0	14	0	0	0	0	0	0
Río Gallegos	81	0	0	20	1	0	14	43	2	1
Total	544	63 (11.6)[b]	12 (2.2)	212 (39.0)	10 (1.8)	6 (1.1)	95 (17.5)	135 (24.8)	7 (1.3)	4 (0.7)

[a] No samples were collected in 2008.
[b] Percentage from total samples in parenthesis.

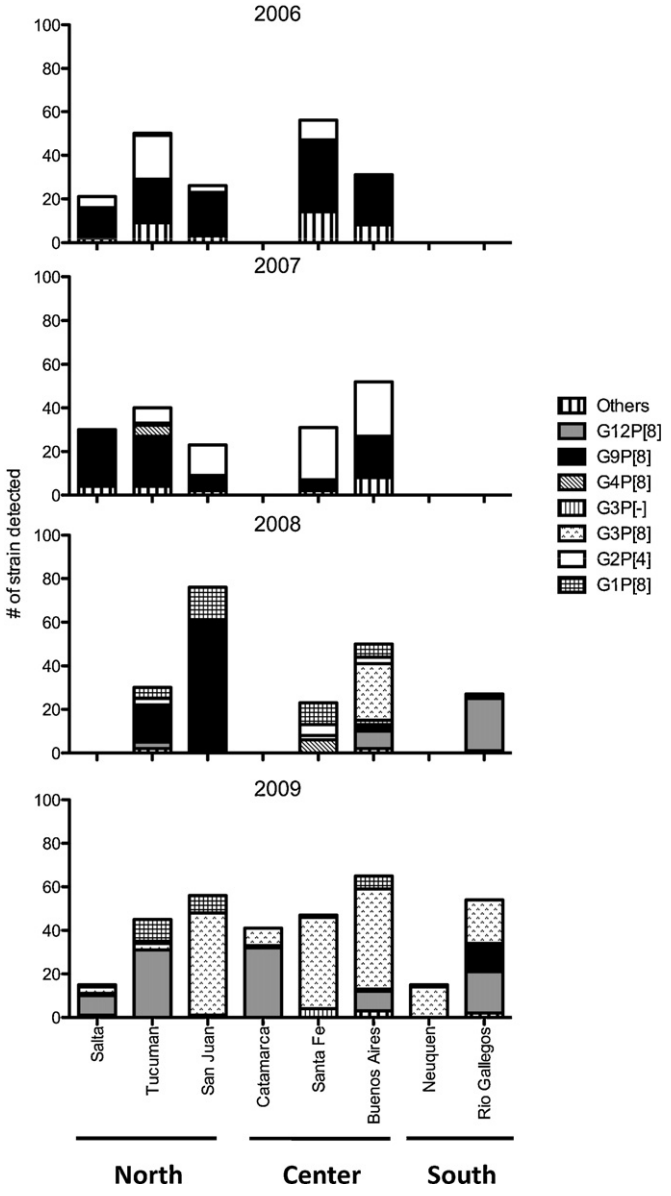

Fig. 1. Yearly distribution of rotavirus genotypes in different cities from Argentina from 2006 to 2009. The data from the first two years of surveillance (2006–2007) were obtained from Stupka et al.[27]

14.1%). In 2009, G3P[8] was the most frequently detected genotype (183/338; 54.1%) followed by G12P[8] (100/338; 29.6%) and G1P[8] (27/338; 8%) (Fig. 1). These variations showed to be more evident in 2008 than in 2009. For example, G9P[8] strains predominated in Tucumán and San Juan during 2008, shifting to G12P[8] and G3P[8] during 2009, respectively (Fig. 1). Interestingly, in Buenos Aires, G3P[8] strains largely predominated during both years. Of note is that no samples were collected in three sentinel laboratories during 2008, and therefore changes in the predominance of strains cannot be assessed.

4.2. Sequence analyses

To understand the phylogenetic relationships and the epidemiological dynamics of the emerging G12P[8] strains, we sequenced and analyzed the 11 genome segments from three strains that were randomly selected. Over 93% of the whole genome was covered for each strain (sequences available at GenBank database). Using sequence and phylogenetic analyses and the following the classification system recently described by the RCWG, the G12P[8] strains presented the following combination of genes: G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 (Fig. 2). Thus, the G12P[8] strains can be considered from genotype 1 (i.e. Wa-like strains). All gene segments, but NSP4, showed a high degree of similarity (≥99.5%). Segment NSP4 showed a percentage of identity of 94.6%, which was due to differences found in the strain Arg6627 (Fig. 2). Regardless of these differences, the three Argentinean strains grouped within the E1 genotype for NSP4. The phylogenetic analysis of the VP7 gene of the G12P[8] strains grouped them within lineage III, while the previously reported G12P[9] Argentinean strains were grouped within lineage II. The Argentinean G12P[8] strains grouped together with a German strain detected in 2009 and several Asian strains (Fig. 3).[10] It is worth mentioning that the Argentinean G12P[9] strains had insufficient sequence data available for a definitive classification according to the mentioned guidelines. However, the available sequences of VP7, VP4, NSP1, NSP4 and NSP5 from G12P[9] strains circulating in South America showed that they are related to the T152 strain, which belongs to genogroup III (i.e. AU-1-like strains) (Figs. 2 and 3).

5. Discussion

Diarrheal diseases represent a high burden to the Argentinean government, being the second cause of morbidity in children under 5 years of age. In 2009, the National Health Surveillance System reported almost one million cases of acute diarrhea, half of which occurred in children under 5 years of age, and of which rotavirus represents the most prevalent enteropathogen.[28] To prevent and control viral diarrhea in Latin America, a great effort from the Pan American Health Organization (PAHO) has resulted in the inclusion of Rotavirus vaccines in the national immunization programs of fourteen countries. Although still controversial, the diversity of circulating strains could potentially impair the effectiveness of such vaccines.[29–31] Therefore, continuous monitoring of rotavirus diversity is important to assess the pre- and post-vaccination scenarios. In this study, we present the molecular characterization and diversity of the rotavirus strains detected in non-vaccinated children during the second two-year period of the National Surveillance Network for Viral Diarrheas in Argentina.

Rotavirus G9P[8] has been shown to be one of the predominant strains in South America over the last decade.[6,32] In Argentina, G9P[8] strains were sporadically detected in 2004,[33] but during 2006–2007 it was the most frequent strain through the country (Fig. 1).[26,27] Although G9P[8] was the predominant strain during 2008, it was not detected with the same prevalence in all sentinel laboratories. Thus, G9P[8] strains predominated in two locations, while G1P[8], G3P[8] and G12P[8] strains predominated in Santa Fe, Buenos Aires and Río Gallegos, respectively. Noteworthy, the frequency of G9P[8] strains decreased drastically in 2009, which coincided with the increased numbers of G3P[8] and G12P[8] strains. In Argentina, G3P[8] strains were sporadically detected,[27,34] but since 2008 its prevalence has increased, reaching more that half of the samples characterized in 2009. Although the frequency of detection of G2P[4] strains during 2006–2007 showed an increasing trend, the frequency during 2008–2009 decreased below 10%. This phenomenon of periodic fluctuation of G2P[4] strains has also been seen in other countries of South America, Asia and Australia in the last few years.[35,36] Thus, co-circulation and/or differences in the predominant strain depending on the location where the survey was carried out underscore the importance of national surveillance networks that allow regional as well as global analyses.

The prevalence of group A rotavirus circulating strains in Latin America and the Caribbean has been previously assessed by

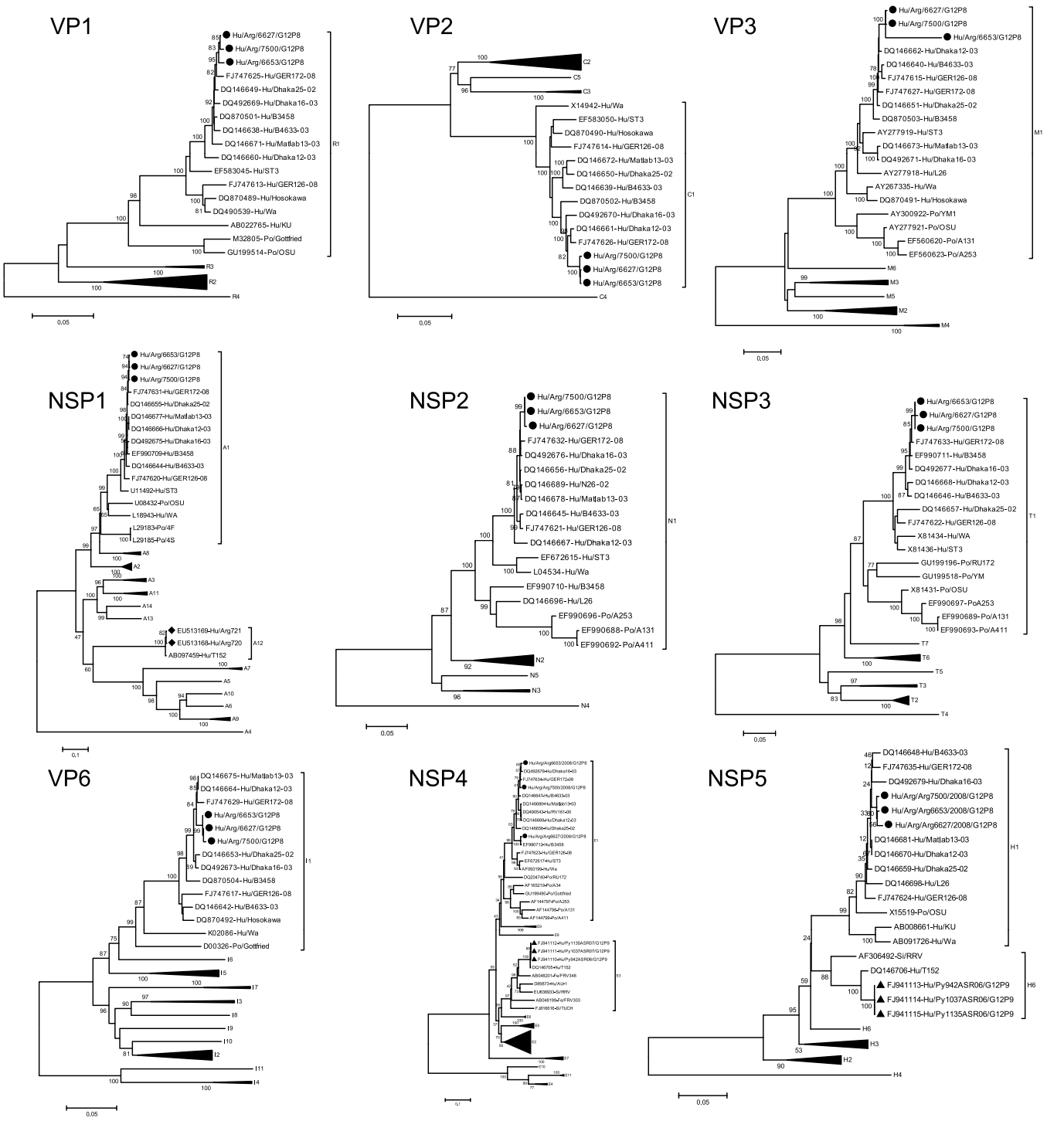

Fig. 2. Phylogenetic analysis of non-structural (NSP1-5) and structural (VP1-3, VP6) rotavirus genes from Argentinean G12P[8] strains and rotavirus strains detected worldwide. The scale bar is proportional to the phylogenetic distance. The Argentinean G12P[8] and G12P[9] strains are marked with a filled circle and diamond, respectively. G12 strains detected in neighboring countries to Argentina are marked with a filled triangle. For each strain (where available), the GenBank accession numbers and host species are shown. Arg, Argentina; Hu, Human; Po, Porcine; Si, Simian; Fe, Feline.

meta-analysis.[32] The most prevalent strains in the continent were found to be G1P[8], G2P[4] and G9P[8]; however, G12P[9] was sporadically detected in Brazil[14] and Argentina,[37] yielding a global prevalence of 5.4% among diarrheic patients. Of note is that G12P[9] strains have been recently reported to be circulating in Paraguay,[13] confirming the widespread distribution of this strain throughout the South Cone. G12P[8] strains have been shown to be circulating in different continents;[38–40] however, they had not been previously detected in South America.[32] During this survey, G12P[8] strains were detected at high prevalence rates in different regions of the country, being the predominant strain in three locations during 2009. The molecular characterization of Argentinean G12P[9] strains has shown that they are related to the T152 strain, which belongs to genotype 3 (i.e. AU-1-like strains),[37] and the phylogenetic analysis of the VP7 gene grouped them within lineage II.[10,13] The whole-genome sequencing analyses of Argentinean G12P[8] strains showed that they belong to genotype 1 (i.e. Wa-like strains). The fact that the Argentinean G12P[8] strains were

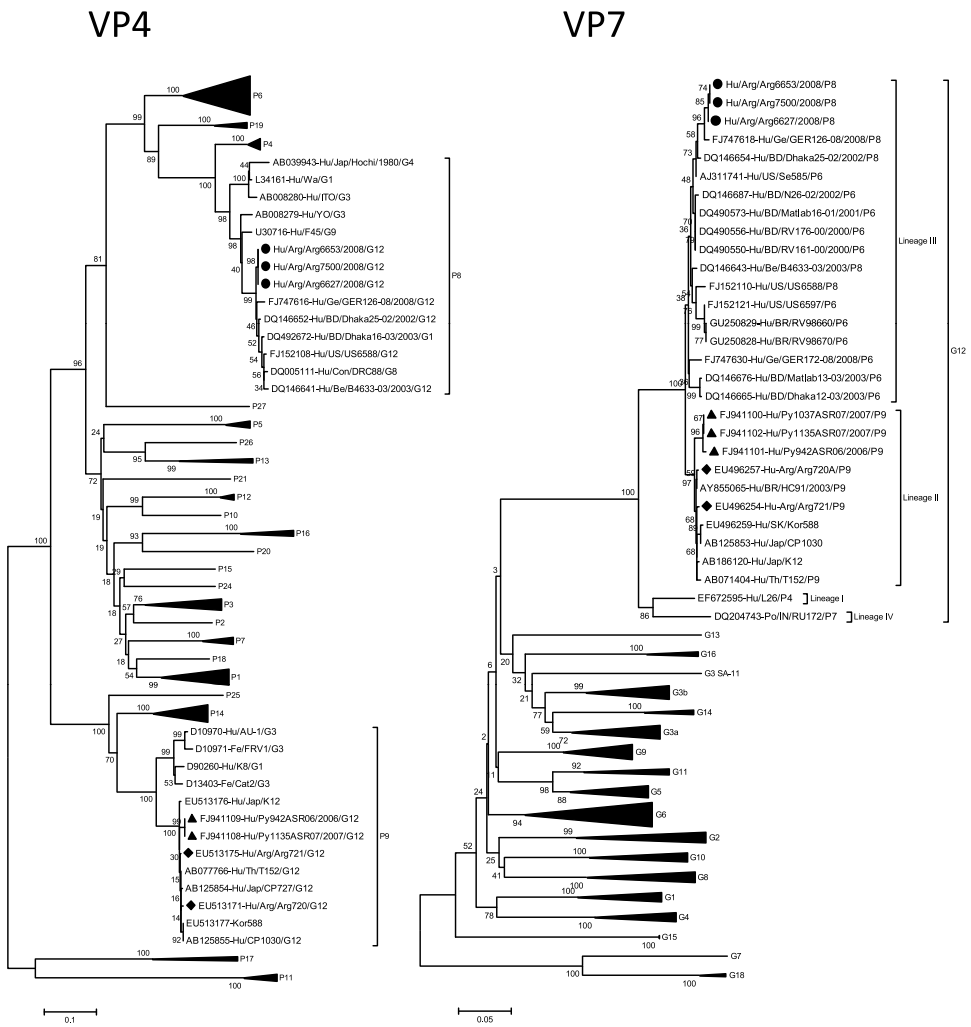

Fig. 3. Phylogenetic analysis of the VP4 and VP7 genes from Argentinean G12P[8] strains and rotavirus strains detected worldwide. The tree was constructed using Kimura 2-parameter as a nucleotide substitution model and neighbor-joining for tree reconstruction. The scale bar is proportional to the phylogenetic distance. The Argentinean G12P[8] and G12P[9] strains are marked with a filled circle and diamond, respectively. G12 strains detected in neighboring countries to Argentina are marked with a filled triangle. For each strain (where available), the GenBank accession numbers and host species and the country of origin are shown. Abbreviations for locations: Arg, Argentina; Be, Belgium; BD, Bangladesh; BR, Brazil; Con, Democratic Republic of Congo; Ge, Germany; IN, India; Jap, Japan; Py, Paraguay; SK, Sri Lanka; Th, Thailand; US, United States. Fe, Feline; Hu, Human; Po, Porcine.

phylogenetically clustered within lineage III of the VP7 gene, together with the whole-genome analyses, suggests that the G12P[8] emergence in Argentina is due to newly introduced strains, rather than being reassortants from the G12P[9] strains previously circulating in our country. The efficient spread nationwide of the strains reported in our study, in contrast with the previously sporadically detected G12P[9] strains, reinforces the evidence that some preferred genome constellations (genotypes 1 and 2) are more fit than AU-1-like strains in the human population.[41] Before being considered one of the predominant strains worldwide, G9 exhibited a high reassortment activity that resulted in a variety of strains (e.g. G9P[4], G9P[6], G9P[8], G9P[11]). Thus, because G12 strains have been detected with a variety of gene constellations and P-types (P[4], P[6], P[8], and P[9]),[10,42,43] it seems that G12 is mimicking the evolutionary events that lead G9 to be included as a major human rotavirus genotype.[10]

The complex dynamics of rotavirus epidemiology underscores the importance of National Surveillance Networks to assess the role of rotavirus in diarrheal diseases as well as the effect of vaccination on rotavirus evolution and diversity. Finally, this report summarizes four years of national surveillance of rotavirus diversity in Argentina, and, to the best of our knowledge, the first whole-genome data of rotavirus strains detected in humans in South America that will help further studies in addressing the evolution of rotavirus in this region.

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Competing interests

None declared.

Ethical approval

Not required.

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