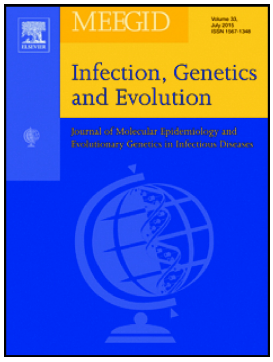

Pre-vaccine rotavirus surveillance in Buenos Aires, Argentina. Characterization of an emergent G1P[8] strain associated to fatal cases in 2014

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Title

Pre-vaccine rotavirus surveillance in Buenos Aires, Argentina. Characterization of an emergent G1P[8] strain associated to fatal cases in 2014.

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1. Introduction

Group A rotaviruses (RVA) are the most important etiological agents of severe gastroenteritis in infants and young children worldwide [WHO, 2013; Estes, 2007]. Two RVA vaccines were licensed since 2006, Rotarix (monovalent, RV1), and RotaTeq (pentavalent, RV5). Within the first years following vaccine introduction, a substantial decline in rotavirus gastroenteritis hospitalizations among children <5 years of age has been observed [Parashar et al., 2013]. Nonetheless, RVA infections are responsible for an estimated 200,000 deaths each year in children under five years of age, with around 90% of these fatalities occurring in poor regions [Parashar et al., 2013, Clark et al., 2017]. Following the recommendations of the World Health Organization [WHO, 2013], Argentina has introduced rotavirus vaccination by incorporating RV1 in the National Immunization Schedule since January 2015.

Rotaviruses are nonenveloped viruses, and their genome consists of 11 double-stranded RNA segments. The two outer capsid proteins, VP7 (**g**lycoprotein) and VP4 (**p**rotease-cleaved protein), defines the G and P types, respectively [Estes, 2007] and have been extensively used in epidemiological studies for strains characterization. In recent years a uniform, sequence-based classification system for the eleven segments was developed, and genotypes were defined for each segment using a letter indicating the coding gene followed by a number indicating the genotype [Matthijnssens et al., 2008]. The majority of all human RVA strains belong to one of two typical genotype constellations: the Wa-like and the DS-1 like. The Wa-like genomes typically show the combination I1-R1-C1-M1-A1-

N1-T1-E1-H1 associated with G1, 3, 4, 9 or 12, and P[8] combinations; while I2-R2-C2-M2-A2-N2-T2-E2-H2 with G2 and P[4] combination characterizing DS-1-like strains.

It is known from studies on strain phylodynamics that new reassortants could spread worldwide in a short time [Matthijnssens et al., 2010] owned to the globalization. This fact is a major concern that warrants continuing surveillance for RVA morbidity, disease severity, and vaccine effectiveness, especially in low-income countries where RVA evolution is highly dynamic and vaccine effectiveness has been suboptimal [Burnett et al., 2016]. In consequence, the molecular epidemiology represents an important tool used globally to study the natural pattern in the strains replacement and, especially, to detect the early emergence of new variants. Based on this type of studies our group has reported the emergence or reemergence of relevant strains on several occasions [Castello et al., 2006; Castello et al., 2009; Esteban et al., 2016; Mandile et al., 2014]. Therefore, the purpose of this study was to analyze the molecular epidemiology of RVA in Buenos Aires from 2012 through 2014 to obtain a global view of RVA strains affecting Argentina before the beginning of massive RV1 vaccination. Furthermore, we decided to emphasize the phylogenetic, epidemiologic, and clinical characterization of a particular G1P[8] strain since it was associated with severe cases of RVA associated gastroenteritis and five fatalities in 2014.

2. Materials and methods

2.1. Sample Collection

The samples were obtained from the Children´s Hospital "Dr. Ricardo Gutierrez" (HNRG), one of the biggest hospitals in Buenos Aires city (CABA), assisting patients from an extended suburban area, named the Greater Buenos Aires (GBA) that includes CABA.

The population of this area is estimated at 13 million people, making it the first urban area of Argentina and the second in South America [https://www.buenosaires.gob.ar/laciudad/ciudad]. A total of 1394 fecal specimens were studied in the mentioned Hospital between January 2012 and December 2014. From these, 296 samples testing positive for RVA were submitted to our laboratory at the Universidad Nacional de Quilmes (UNQ) for further molecular characterization. For an additional study of the strains causing severe cases of gastroenteritis in 2014, two extra sets of samples were obtained from children with acute diarrhea admitted at the Hospital "Dr. Alberto Eurnekian" (HAE) in Ezeiza (30 samples) and the Hospital "Evita" (HE) in Lanús (six samples), located at 30 and 10 kilometers out of CABA respectively. These samples were analyzed at the UNQ for the presence of RVA with an in-house ELISA described elsewhere [Argüelles et al., 2000], resulting in a total of 27 RVA positive samples (21 from HAE and six from HE).

2.2. Epidemiological information

From the HNRG Virology Laboratory records, information about the incidence of hospitalized diarrhea cases discriminating between positive and negative samples for RVA, ages, location, and dates was compiled from 2008 through 2014.

2.3. RNA Extraction and genotyping by RT–PCR

Viral RNA was extracted from rotavirus-positive stool specimens by the silica powder method [Boom et al., 1990]. Extracted RNA was denatured at 97°C for 5 min, and the first amplification of VP7, VP4, VP6, or NSP4 segments was performed by RT-PCR with the One-Step RT-PCR kit (QIAGEN GmbH, Germany) with gene-specific consensus primers. For G typing, consensus primers VP7F and VP7R [Iturriza-Gómara et al., 2001] were used in the first round RT-PCR and the VP7R consensus primer with a set of G type-

specific forward primers in the second round PCR [Banerjee et al., 2007; Gouvea et al., 1990, Iturriza-Gómara et al., 2004]. For P typing, consensus primers VP4F and VP4R [Simmonds et al., 2008] were used in the first round RT-PCR and the VP4F primer with a set of P type-specific primers in the second round PCR [Gentsch et al., 1992; Iturriza-Gómara et al., 2000]. Cycle conditions and visualization of PCR products were conducted as reported previously [Esteban et al., 2010].

2.4. Nucleotide Sequence Analysis

Sequence analysis of the genes from G1P[8] strains associated with severe cases in the cold season of 2014 was performed using the RT-PCR amplification products. Briefly, PCR products were gel-purified with the QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany) and sequenced with the automated sequencer ABI3730XL under BigDye terminator cycling conditions (Macrogen Inc., Korea). The nucleotide sequences presented in this article were submitted to the databases at the National Center for Biotechnical Information (NCBI) at the National Library of Medicine and recorded under the GenBank accession numbers: KU145625-KU145654.

2.5. Phylogenetic Analysis

Phylogenetic trees were reconstructed using the neighbor-joining method, and the evolutionary distances between nucleotide sequences were estimated by the Kimura-2 parameter method. The bootstrap probability was calculated for each internal branch of the phylogenetic trees with 1,000 times of resampling. These analyses were conducted using the software MEGA [Tamura et al., 2011].

3. Results

3.1. RVA epidemiology and genotypes circulating during the 2012-2014 period

In the present study, we report for the first time the G and P genotypes for the period 2012-2014. As it can be seen in Table 1, in 2012, approximately even amounts of three G genotypes were seen: 38.2% of G3, 29.4% of G12 and 26.5% of G2; with rates of P[8] and P[4] of 56% and 32%, respectively, as expected for regions with co-circulation of typical G-P combinations of Wa and DS-1 like strains. In 2013, the G12 genotype became sharply dominant (60.9%); meanwhile, G3 and G2 were detected in 21.7% and 8.7% of the samples, respectively. In the following year, 2014, and after nine years of virtual absence [Esteban et al., 2010; Mandile et al., 2014], the G1 genotype re-emerged, representing the 68.8% of the total G-typed strains. Interestingly, these G1P[8] strains were associated with particularly severe cases causing five fatalities and thus were further characterized in this study.

Along with this study, 19 years of continuous RVA genotype surveillance in Argentina [Argüelles et al. 2000; Castello et al., 2006; Castello et al., 2009; Esteban et al., 2010; Mandile et al., 2014] was covered. Thus, in order to contextualize the results obtained for the analyzed 2012-2014 period, the genotypes fluctuations form 1996 to 2014 are depicted in Figure 1.

For the period 2012-2014, 21.2% of the 1394 fecal samples studied for RVA in the HNRG were positive (RVA+). The RVA positivity rates for each year were 20.0% (86 RVA+ from 429 analyzed samples), 18.2% (67 RVA+ from 367 analyzed samples) and 23.9% (143 RVA+ from 598 analyzed samples) in the years 2012, 2013 and 2014, respectively. Notably, the subgroup of RVA positive hospitalized patients was significantly younger than the group testing negative ($p < 0.0001$, t Student test) with a mean of 15.4 months (median: 8.6, SD: 23.2, rank: 0.3-156) and a mean of 28.0 months (median: 12.6, SD: 36.1, rank: 0.1-180), respectively.

Since data from HNRG was available for every year between 2008 and 2014, we decided to explore the particularities of this last year when G1P[8] strains reemerged. Significant differences in the number of hospitalized patients and their ages could be appreciated between the mean values of the 2008-2013 period and the year 2014. During the cold season of this year (May-August), a total of 130 patients were hospitalized in HNRG due to RVA associated diarrhea, a number significantly higher ($p<0.0001$, z test) than the mean for the period 2008-2013 (61.8, SD 28.3) as can be graphically appreciated in the figure 2. Additionally, the mean age of patients suffering RVA associated gastroenteritis during 2014 was 12.4 months (median: 9.1, SD: 9.8) which was significantly higher ($p< 0.01$, t Student test) than the mean of 9.9 months (median: 8.0 months, SD: 7.9 months) for the period 2008-2013.

3.2. VP7 phylogeny from the G1P[8] strains

A phylogenetic study of the VP7 gene, including most of the available sequences of Argentine and regional strains and also representative global G1 isolates, is depicted in figure 3. As can be appreciated different lineages and sublineages related to several global or regional G1 strains have circulated in our country during the last thirty years. In the present work, six samples from the year 2014, and one from 2013 were successfully sequenced. Sequences analysis showed that all belong to the lineage II, a lineage that has never been reported previously in Argentina. These sequences clustered together and were closely related (99% nucleotide identity) to a group of Thai, Vietnamese, and Russian strains circulating between 2012 and 2014. This group of sequences is a little more distantly related to European and South African strains from 2009 and 2011, and to the subgroup of DS-1 like G1P[8] strains circulating in Asia, Africa, and Brazil during 2012 and 2013 [Jere et al., 2017; Komoto et al., 2016; Luchs et al., 2019; Nakagomi et al., 2017].

Furthermore, from the deduced aminoacidic sequences of VP7 protein (data not shown), we have detected a remarkable change in the 2013-2014 strains when compared to those previously characterized. The amino acid N or S in the position 147 is replaced by a D, exchanging a polar non-charged amino acid by a charged one within the antigenic region B. Interestingly, this change is only present in the Argentine strains from 2013 and 2014 and those circulating in Vietnam, Russia and Thailand during 2012 and 2013. Although this finding does not allow us to ensure the association of the mutation with the severity of the cases, it prompts us to continue with these studies to understand whether or not this association exists.

3.3. VP4 phylogeny from the G1P[8] strains

In the present work, 15 VP4 genes were partially sequenced from samples characterized as P[8] genotype, seven in combination with G1 (four from 2014 and three from 2008), and eight in combination with G12 or G4. As can be observed in figure 4, all these Argentine sequences belong to the lineage III, but clustering in five markedly different groups. In particular, sequences associated with the G1 genotype are included in three of those clusters. The phylogram shows that the sequences from 2008 are heterogeneous, appearing in two groups; meanwhile, the sequences circulating during 2014 are much more homogeneous grouping in a single cluster. Consistent with the findings showed above based on the VP7 gene, the sequences of the VP4 gene from the G1P[8] strains circulating in 2014 are related to Asian strains circulating from 2012 through 2014, but not related to G1P[8] DS-1-like strains.

3.4. NSP4 phylogeny from the G1P[8] strains

The NSP4 gene from seven G1P[8] strains was sequenced, five from the year 2014, and two from 2008. All these sequences belong to the E1 genotype, lineage E1a, but they

cluster in two different groups in accordance with the year of isolation (figure 5). This phylogenetic study further confirms the results described earlier about the close relationship of the Argentine strains from 2014 with the strains circulating in Vietnam in 2012 and 2013. Meanwhile, the NSP4 genes of the Argentine G1P[8] strains from 2008 have close relationships with strains circulating in Europe and America between 2001 and 2008.

3.5. VP6 phylogeny from the G1P[8] strains

The VP6 gene from six G1P[8] strains was sequenced, four were from the year 2014, and two from 2008. These sequences belong to the I1 genotype, lineage 1, but they cluster in two different groups in accordance with the year of isolation (figure 6). This phylogenetic study, again, confirms the relationships described above for the 2014 Argentine strains.

4. Discussion

In the present work, we analyzed the circulation of RVA genotypes in the period 2012-2014 in GBA, completing a panoramic view of the pre-vaccine era along 19 years by revisiting our previous reports [Argüelles et al., 2000; Castello et al., 2006; Esteban et al., 2010; Mandile et al., 2014]. During the last year of this surveillance, we detected an emerging rotavirus G1P[8] strain causing severe cases of gastroenteritis and five fatalities. Thus, we explore further this emerging strain by sequencing of four genes and examining some of their epidemiological characteristics.

In the analyzed period 2012-2014, we found that 21.2% of the fecal samples tested in the HNRG resulted positive for RVA, with the mean age of the patients of 15.4 months. Interestingly, significantly more cases of RVA associated gastroenteritis were hospitalized in the HNRG, and the patients were significantly older, during the cold season (May

through August) of 2014 when compared with the previous six cold seasons (2008-2013). These facts could be attributed to an epidemic outbreak of RVA dominated by a more virulent strain or, most probably, considering the older ages of the patients, due to a strain that has not been circulating in our population for a long time. Deficient immunity toward a less prevalent genotype could be a factor involved in this high incidence, as was previously argued for G2 genotype in gastroenteritis outbreaks among school-aged children and adults [Griffin et al., 2002, Mikami et al., 2004]. A closer analysis of the patients' age distribution for the 2014 cold season shows that a large number of RVA cases were in the range of 16-24 months of age. At this lifetime, the probability of previous first contact with RVA is high, suggesting low heterotypic protection from previous infection/s. From an epidemiological point of view, the low detection rate of the G1 genotype between 2005 and 2013 in our area may be a factor determining this incomplete or inefficient immunity against the emerging G1P[8] strains among the general population.

The particular epidemiologic characteristics of the year 2014, including the death of five children associated with RVA infection, prompt us to investigate further the dominant G1P[8] strain. The high genetic homogeneity found for the VP7 gene and the fact that, to the best of our knowledge, they belong to a lineage that has not been previously detected in Argentina, suggests a recent introduction. Strikingly, the only G1 strain that could be sequenced in 2013 presents a highly related VP7sequence, a fact that would indicate the possibility that the strains causing the epidemic outbreak would have been circulating since 2013 at a low level. However, this cannot be asserted based on a single gene, but, unfortunately, we were unable to amplify additional genes from this sample from 2013.

The phylogenetic study of the VP7 gene of the five G1P[8] isolates from 2014, and the one from 2013 demonstrated that these Argentine strains are very closely related to a

group of Thai, Vietnamese, and Russian human strains and a group of Vietnamese porcine strains circulating since 2012 [Chieochansin et al., 2016; Phan et al., 2016]. This relationship is supported by the sequence study of the other three genes, VP4, VP6, and NSP4. At the same time, the absence of reported Brazilian strains, or of some other neighboring country, in the same group indicates a new continental introduction through Argentina, most probably from Asian countries, from where a previous similar introduction was described for G12P[9] strains that circulated in Buenos Aires between the years 1999 and 2003 [Castello et al., 2006; Castello et al., 2009]. In line with this idea, the phylogenetic study also indicated that these Argentine G1P[8] strains were not related to the DS-1-like G1P[8] strains that emerged in 2012-2013 in Asia [Nakagomi et al., 2017] and were circulating in Brazil during 2013 [Luchs et al., 2019].

With this study, we completed 12 years of continuous surveillance of RVA strains in the Greater Buenos Aires before the RV1 vaccine was massively implemented in January 2015. So, this work gives us a whole panorama of the pre-vaccine era of the RVA molecular epidemiology in the most populated region of Argentina. In this sense, this information represents an important starting point to study and evaluate the impact of the vaccine introduction on the RVA epidemiology/ecology in Argentina. In particular, analyze the vaccine performance, and to detect emerging strains escaping the vaccine.

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Contributors

Marcelo G. Mandile: Conceptualization, Formal Analysis, Investigation, Writing – Original draft preparation. **Marcelo H. Argüelles:** Writing – Review & editing, Data curation. **Carlos F. Temprana:** Writing – Review & editing, Visualization. **Estefanía S. Peri Ibáñez:** Visualization. **Dalila Silvestre:** Visualization. **Alejandra Musto:** Resources, Visualization. **Alberto Rodríguez Pérez:** Resources, Visualization. **Alicia Mistchenko:** Resources, Data curation, Visualization. **Graciela Glikmann:** Supervision, Funding acquisition, Writing – Review & editing. **Alejandro A. Castello:** Conceptualization, Supervision, Writing – Original draft preparation, Funding acquisition.

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Declarations of interest

None.

Ethical approval

Not required.

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Figure captions

Figure 1. Prevalence of G and P genotypes in the greater Buenos Aires.

A. Prevalence of G genotypes in the greater Buenos Aires between 1996 and 2014. B. Prevalence of P genotypes in the greater Buenos Aires between 1996 and 2014. Percentages were calculated on the basis of the total number of genotypes detected.

Figure 2. Number of cases of RVA infections from 2008 to 2014.

Number of patients below five years old hospitalized with RVA associated diarrhea on a monthly basis between 2008 and 2014 in the Children´s Hospital Ricardo Gutierrez, CABA, Argentina.

Figure 3. Phylogenetic analysis of the VP7 gene from G1 strains.

Nucleotide sequences were obtained from the GenBank database or produced for this work. The lineages are indicated on the right and the bootstrap values are shown at the branch nodes (values <65% are not shown). The Argentine strains analyzed in this study are shown in boldface. Strains are named according to the nomenclature proposed by the Rotavirus Classification Working Group.

Figure 4. Phylogenetic analysis of the VP4 gene from G1 strains.

Nucleotide sequences were obtained from the GenBank database or produced for this work. The lineages are indicated on the right and the bootstrap values are shown at the branch nodes (values <65% are not shown). The Argentine strains analyzed in this study are shown in boldface. Strains are named according to the nomenclature proposed by the Rotavirus Classification Working Group.

Figure 5. Phylogenetic analysis of the NSP4 gene from G1 strains.

Nucleotide sequences were obtained from the GenBank database or produced for this work. The lineages are indicated on the right and the bootstrap values are shown at the

branch nodes (values <65% are not shown). The Argentine strains analyzed in this study are shown in boldface. Strains are named according to the nomenclature proposed by the Rotavirus Classification Working Group.

Figure 6. Phylogenetic analysis of the VP6 gene from G1 strains.

Nucleotide sequences were obtained from the GenBank database or produced for this work. The lineages are indicated on the right and the bootstrap values are shown at the branch nodes (values <65% are not shown). The Argentine strains analyzed in this study are shown in boldface. Strains are named according to the nomenclature proposed by the Rotavirus Classification Working Group.

Contributors

Marcelo G. Mandile: Conceptualization, Formal Analysis, Investigation, Writing – Original draft preparation. **Marcelo H. Argüelles:** Writing – Review & editing, Data curation. **Carlos F. Temprana:** Writing – Review & editing, Visualization. **Estefanía S. Peri Ibáñez:** Visualization. **Dalila Silvestre:** Visualization. **Alejandra Musto:** Resources, Visualization. **Alberto Rodríguez Pérez:** Resources, Visualization. **Alicia Mistchenko:** Resources, Data curation, Visualization. **Graciela Glikmann:** Supervision, Funding acquisition, Writing – Review & editing. **Alejandro A. Castelan:** Conceptualization, Supervision, Writing – Original draft preparation, Funding acquisition.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Table 1. Distribution and frequency of G and P genotypes of group A rotavirus from 2012 through 2014 detected in Buenos Aires

Genotype	2012		2013		2014		Total no.	% Over the 3-year period
	no. of samples	%	no. of samples	%	no. of samples	%		
G1	0	0.0	1	4.3	22	68.8	23	25.8
G2	9	26.5	2	8.7	3	9.4	14	15.7
G3	13	38.2	5	21.7	0	0.0	18	20.2
G4	1	2.9	0	0.0	0	0.0	1	1.1
G10	1	2.9	0	0.0	0	0.0	1	1.1
G12	10	29.4	14	60.9	6	18.8	30	33.7
GNT	0	0.0	1	4.3	1	3.1	2	2.2
Total	34	100.0	23	100.0	32	100.0	89	100.0
P[4]	8	31.0	2	9.1	3	10.0	13	16.9
P[8]	14	56.0	18	81.8	25	83.3	57	[illegible]4.0
PNT	3	12.0	2	9.1	2	6.7	7	[illegible]
Total	25	100.0	22	100.0	30	100.0	77	[illegible]0

PNT, P not typeable.
GNT, G not typeable.

Highlights

- RVA genotype fluctuation along 19 years in the pre-vaccine era was reviewed
- Increased incidence of severe RVA associated diarrhoeas during 2014 in Buenos Aires
- The reemergence of G1P[8] strains detected in 2014 was associated with severe cases
- 2014 RVA associated gastroenteritis patients were older than those from last years
- Phylogenetic analyses suggest an extra continental introduction of G1 strains