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Short communication

Phylogenetic analyses of Norovirus strains detected in Uruguay reveal the circulation of the novel GII.P7/GII.6 recombinant variant

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ABSTRACT

Noroviruses (NoV) are one of the major etiological agent of acute gastroenteritis (AGE) outbreaks worldwide. Distinct NoV genotypes have been associated with different transmission patterns and disease severity in humans. Therefore, it is important to identify genetically different NoV genotypes circulating in a particular region. However, genotyping has become a challenge due to recombination events occurring mainly nearby ORF1/ORF2 junction of NoV genome, leading to distinct genotypes with polymerase and capsid regions derived from parenteral strains. Taking this into account, ORF1/ORF2 sequences were obtained from NoV strains collected from patients with AGE in Uruguay. This study reveals *in silico* evidences of recombination events taking place in four out of six strains analyzed for which its polymerase gene and its capsid region correspond to GII.P7 and to GII.6 genotype, respectively. These results also reveal the circulation of a GII.P7/GII.6 recombinant variant in the natural populations of NoV strains in the northwestern region of Uruguay. As far as we know this is the first report about the circulation of a NoV GII.P7/GII.6 recombinant variant in the Americas.

Noroviruses (NoV), belonging to genus *Norovirus*, family *Caliciviridae*, are a group of related, single-stranded RNA, non-enveloped viruses that cause acute gastroenteritis (AGE) in humans (Green et al., 2000; Xi et al., 1990). NoV are responsible for 50% of all AGE outbreaks, especially in developing countries, were they cause annually one million hospitalizations and 200,000 deaths in children under 5 years old (Patel et al., 2008).

NoV genome is organized into three open reading frames (ORFs). ORF1 is translated as a large polyprotein, which is co and post-translationally cleaved to release at least six mature non-structural (NS) proteins, including the viral RNA-dependent RNA polymerase (RdRp) (Hyde et al., 2009; Hyde and Mackenzie, 2010). ORF2 and ORF3 are translated from a subgenomic RNA, and encode the major and minor capsid proteins, VP1 and VP2, respectively (Thorne and Goodfellow, 2014). Based on their nucleotide capsid VP1 and polymerase sequences, NoV are classified into seven genogroups (GI to GVII) of which GI, GII and GIV have been described in humans, and recently, GVI and GVII were detected in canine and feline species. (Kroneman et al., 2013).

NoV can be further classified into at least 32 different genotypes, which can differ in their ability to cause epidemics, their host range, incidence, virulence and stability in the environment (Fankhauser et al., 2002; Huhti et al., 2011; Kroneman et al., 2008, 2013; Vinjé et al., 2004).

Recombination is one of the main driving forces shaping evolution of viruses (Bull et al., 2007). It provides RNA viruses with an attractive mechanism to adapt to new environmental conditions and hosts, and therefore, to evade the immune system (Van der Walt et al., 2009). It has been experimentally demonstrated the occurrence of a homologous recombination event located at the ORF1/ORF2 overlap, in murine NoV (Mathijs et al., 2010). Several recombination events have been reported worldwide among NoV strains, leading to difficulties in genotyping and molecular epidemiologic studies. It has been well documented that a recombination hotspot is present around the ORF1/ORF2 junction of NoV genome, where a highly conserved sequence of 28 bp-long (5′-GTG AAT GAA GAT GGC GTC KAR YGA CGC Y-3′) and 27 bp-long (5′-GYR AAT GAT GAT GGC GTC KAA RGA CGY-3′) is present for NoV GII and NoV GI, respectively, that seems to play an important role (Bull et al., 2005; Han et al., 2004; Hansman et al., 2004; Hardy et al.,

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1997; Jiang et al., 1999; Katayama et al., 2002, 2004; Oliver et al., 2004).

In order to determine the presence of putative recombination events among NoV populations circulating in Uruguay, three diarrheic stool samples and three vomit samples, which were previously diagnosed positive for NoV by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) of the B region (Fankhauser et al., 2002) were studied. These samples were obtained during a surveillance study conducted between May 2011 and May 2012 from Uruguayan patients with AGE in the city of Salto, North-Western Uruguay (for details see Table 1). Voluntary written informed consent were obtained from the parents or legal guardians at enrollment. The study has been accepted by the local health authorities.

Nucleic acids were extracted using the guanidine isothiocyanate-silica method from a 10% fecal suspension in pH 7.2 Tris-calcium buffer (Boom et al., 1990). Complementary DNA (cDNA) was obtained using hexanucleotide random primers (Amersham Bioscience, UK) and the *Superscript II™ reverse transcriptase* (Invitrogen, USA) according to the manufacturer's instructions. In order to investigate the presence of putative recombination events within the analyzed strains, we performed a PCR targeting the overlapping region between ORF1 and ORF2 using primers (Mon431/432; G2SKR/G1SKR) and protocols previously described (Beuret et al., 2002; Fumian et al., 2012; Kojima et al., 2002). An amplicon of 564 bp was obtained and visualized by agarose gel electrophoresis. This amplicon include a 285 bp region present at the 3′ end of ORF1, coding for the RNA polymerase, and 279 bp corresponding to the 5′ end of ORF2, which codes for the capsid protein.

Amplicons were purified using *QIAquick PCR Purification Kit* (Qiagen™), according to manufacturer's instructions. Bidirectional DNA sequencing was performed by the dideoxynucleotide chain termination method, using the *ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 1, v. 3.1* and the ABI Prism 3730 Genetic Analyzer (Applied Biosystems®, Foster City, CA, USA) by the Genomic Platform of DNA sequencing PDTIS/Fiocruz. The sequences obtained in this study were deposited in the GenBank database under accession numbers KJ577799–KJ577802 and KJ865752–KJ865753.

The obtained sequences were first analyzed with the Seqman program implemented in the *DNAStar 5.01 package* (DNASTAR, Madison, USA). Comparable sequences containing ORF1/ORF2 overlap of different NoV genotypes were retrieved from the National Center for Biotechnology Information (NCBI).

Alignment of sequences was performed using the MUSCLE program (Edgar, 2004). Sequences were analyzed with the *Norovirus Genotyping Tool*, v1.0 (Kroneman et al., 2011) in order to determine their genotypes. Due to the possibility of recombination events in the analyzed region, two datasets were generated: one considering the region coding for the RdRp (partial ORF1) and the other including capsid coding region (partial ORF2). Phylogenetic analyses were performed for both datasets that included comparable sequences of different NoV genotypes. The program Modelgenerator (Keane et al., 2006) was used to identify the optimal evolutionary model for both datasets. Akaike Information Criteria and Hierarchical Likelihood Ratio Test indicated that the GTR + Γ model fit the sequence data. Using this model, Maximum Likelihood (ML) trees were constructed using software from the PhyML program (Guindon et al., 2005). As a measure of the robustness of each node, we employed an approximate Likelihood Ratio Test (aLRT) to assesses that the branch being studied provides a significant likelihood gain, in comparison with the null hypothesis that involves collapsing that branch but leaving the rest of the tree topology identical (Anisimova and Gascuel, 2006).

Splits networks were created with SplitsTree 4.13.1 (Huson and Bryant, 2006) using the neighbor-net method (Bryant and Moulton, 2004). Neighbor-net depicts conflicting phylogenic signals in the data that are caused by recombination as cycles within unrooted bifurcating trees. The presence of recombination events was statistically verified after visual identification using the pairwise homoplasy test (PHI). PHI has been shown to powerfully identify the presence/absence of recombination within a wide range of sequence samples with a low false positive rate (Bruen et al., 2006).

SimPlot version 3.5.1 (Lole et al., 1999) was used to perform the recombination breakpoint analyses. SimPlot analysis was performed by setting the window width and the step size to 200 bp and 20 bp, respectively. We also used different methods implemented in the Recombination Detection Program v.4.16 (RDP4) (Martin et al., 2010), such as Bootscan/Recscan (Martin et al., 2005), RDP (Martin and Rybicki, 2000), and MaxChi (Smith, 1992). Uruguayan sequences KJ577799 to KJ577802 were included as queries, while genotype GII.6 (AB039778) and GII.7 (AB258331) sequences were used as putative parental sequences.

The results of the analysis performed using the *Norovirus Genotyping Tool* suggests that two of the Uruguayan strains belong to genotypes GII.1 and GII.4 (KJ865752 and KJ865753, respectively). Surprisingly, the analysis of the other four Uruguayan strains (KJ577799–KJ577802) indicated incongruence between their genotype classification based on ORF1 and ORF2 regions, which grouped them into GII.P7 and GII.6, respectively (Supplementary Table 1).

In order to confirm the results obtained using the *Norovirus Genotyping Tool*, ML phylogenetic trees were constructed (Fig. 1). Again, a close genetic relation among Uruguayan KJ865752 and KJ865753 strains with previously described GII.1 and GII.4 genotypes, respectively, was found. The other four Uruguayan strains (KJ577799–KJ577802) were assigned to clusters composed of previously described GII.P7 genotype, respectively, for ORF1 (RdRp) sequences (see Fig. 1A). Nevertheless, when the same analysis was performed using ORF2 (VP1) sequences, a clear genetic relation among these four Uruguayan strains and GII.6 genotype was found (see Fig. 1B).

With the objective to analyze the possible recombination events occurring among the Uruguayan strains, the 564 bp alignment containing the ORF1/ORF2 junction was explored using neighbor-net method implemented in SplitsTree 4.13.1. A reticulate phylogenetic network was evident, due to the presence of conflicting phylogenetic signals within the sequences (Supplementary Fig. 1). We statistically verified the presence of recombination events identified visually in phylogenetic graphs using the PHI test,

Table 1
Characteristics of the samples analyzed.

Strain number	Type of sample	Sex	Age	Isolation date	Genotype	Accession number
70	Feces	F	NA	05/2011	GII.7/GII.6	KJ577799
93	Feces	M	NA	06/2011	GII.7/GII.6	KJ577800
234	Feces	M	6 years	04/2012	GII.7/GII.6	KJ577801
243	Vomit	F	25 years	05/2012	GII.7/GII.6	KJ577802
178	Vomit	F	9 years	03/2012	GII.4	KJ865752
229	Vomit	M	4 years	04/2012	GII.1	KJ865753

NA: not available.

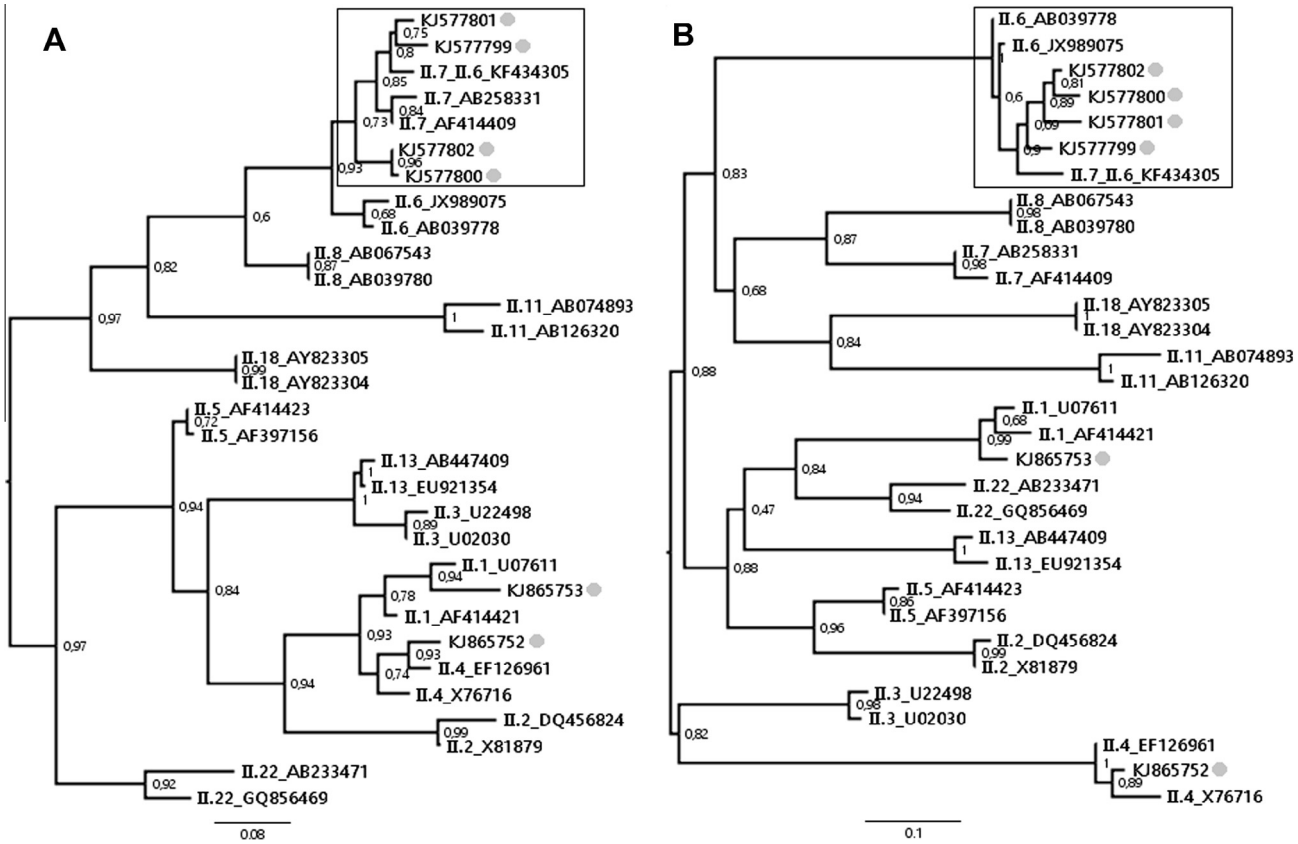

Fig. 1. Maximum likelihood phylogenetic tree analysis of NoV strains isolated in Uruguay. Strains previously reported are shown by their genotype followed by their accession numbers. Strains isolated in Uruguay are shown by their accession numbers highlighted in gray. The clusters where Uruguayan strains have been assigned are indicated by a frame. Numbers at the branches show aLRT values. Bars at the bottom of the trees indicate nucleotide Distance. The results obtained using partial ORF1 and ORF2 regions are shown in (A) and (B), respectively.

which confirmed that these conflicting signals are due to recombination (statistic-value < 0.001).

In order to identify the precise location of the putative recombination breakpoint that were suggested by the results of the previous phylogenetic analyses, we employed different methods implemented in SimPlot and RDP4 programs to analyze the ORF1/ORF2 junction for the four potentially recombinant samples involved in this study. The results of these analyzes are shown in Fig. 2.

As expected, SimPlot analysis revealed the presence of recombination break-points at positions 208–214 of Uruguayan NoV KJ577799, KJ577800, KJ577801 and KJ577802 (see Fig. 2A). These positions correspond to nucleotides 5027–5033 respective to reference strain Lordsdale (accession number X86557).

Finally, to validate SimPlot results, Bootscan/Recscan analysis, implemented in RDP4 program, was employed. As it shown in Fig. 2B, similar results were found using both approaches; since recombination break-points were detected at positions 215 and 220 of the same four Uruguayan NoV strains (positions 5034–5039 relative to reference strain Lordsdale, accession number X86557). The recombination points determined by other methods implemented in RDP4 program (RDP and MaxChi), showed similar results (data not shown).

In this study, we explored the genetic variability of NoV strains circulating in the northwest region of Uruguay, through sequence analysis of their ORF1/ORF2 junction region. We could appreciate the presence of conflicting phylogenetic signals among four of the six strains analyzed, indicating that RdRp and capsid genes could have derived from different ancestral strains. This fact suggested that the strains analyzed may have emerged as a result of a recombination event between GII.7 and GII.6 strains. This hypothesis was further investigated through different approaches. The results of these studies revealed recombination break-points between positions 5027–5039 of the NoV genome (relative to reference strain Lordsdale) in four out of six NoV strains collected in Uruguay. This is in agreement with previous reports describing NoV recombinant strains, where the recombination breakpoints were estimated between nucleotides 4981 and 5117 (Bull et al., 2005; Fumian et al., 2012; Hansman et al., 2004; Hardy et al., 1997; Jiang et al., 1999; Katayama et al., 2002).

The presence of a recombination hotspot around ORF1/ORF2 junction of NoV genome seems to be related with the presence of a highly conserved sequence in this region, which is also present at the 5′ end of ORF1 (Bull et al., 2005). A conserved sequence of 28 bp (5′-GTG AAT GAA GAT GGC GTC GAA TGA CGC Y-3′) was observed in all Uruguayan strains. This sequence has been suggested to form a stem loop structure that acts as a RNA promoter sequence, playing a significant role for the recombination events to occur (Bull et al., 2005; Kao et al., 2001; Miller et al., 1985; Morales et al., 2004; Pletneva et al., 2001).

The results of these studies highlight the importance of carrying out a deeper NoV surveillance studies in order to perform appropriate epidemiological analyses and constitute the first evidence about the circulation of this specific genetic variant in the Americas. More studies will be needed to address the extent and the role of recombination in NoV populations circulating in the South American region. The only GII.P7/GII.6 recombinant strain reported to date was recently detected in Burkina Faso (accession number KF434305) (Huynen et al., 2013). This strain was also analyzed in the present work and presented high similarity to Uruguayan strains, both in its phylogenetic signal and in its recombination pattern. However, taking into account the high frequency of recombination in this genomic region of NoV, as well as the

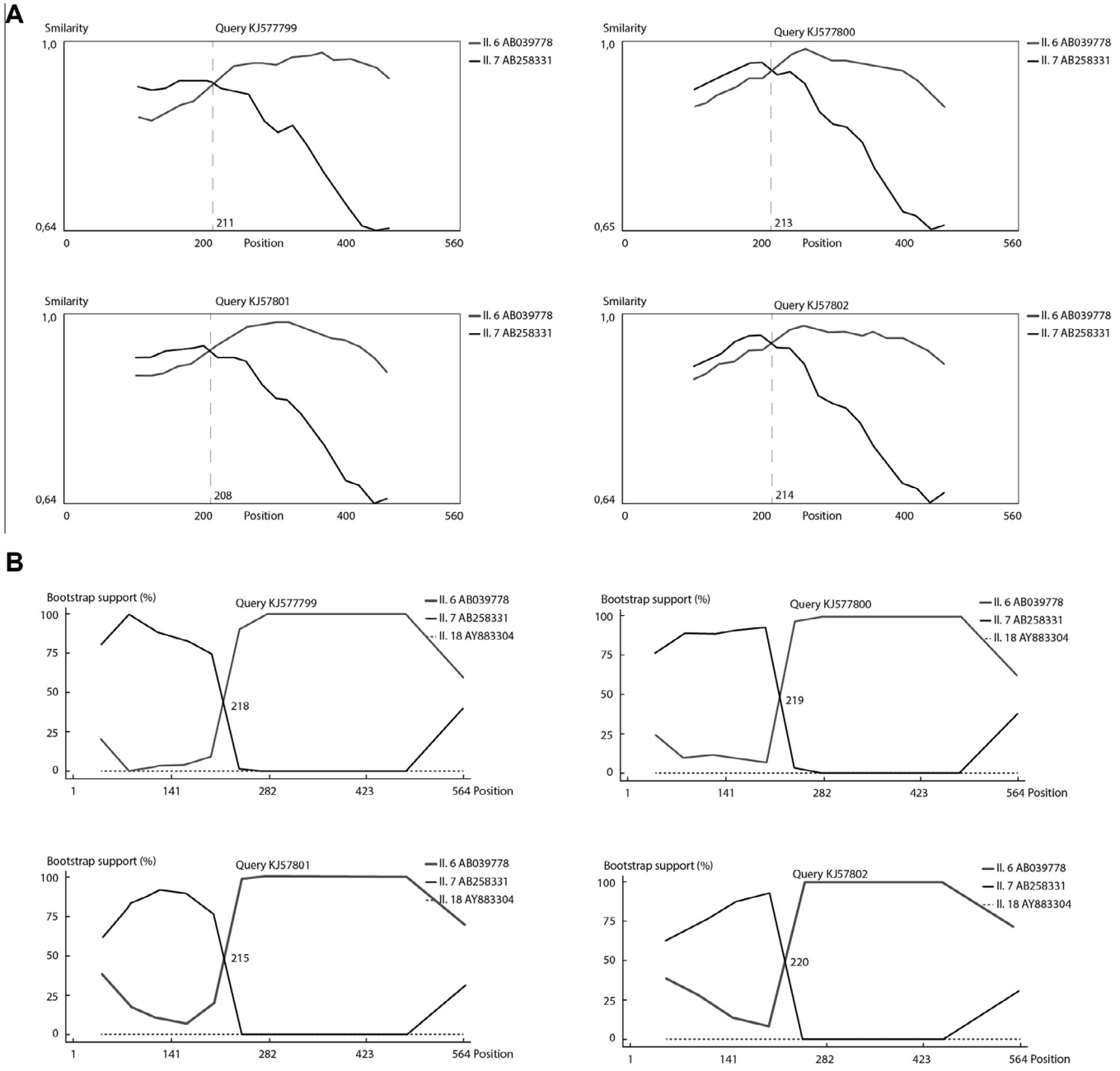

Fig. 2. Determination of recombination breakpoints. (A) SimPlot analysis results. The *y*-axis gives the percentage of identity within a sliding window of 200 bp wide, with a step size between plots of 20 bp. Comparison of the 4 Uruguayan strains with their putative parental NoV strains of genotypes GII.6 (AB039778) and GII.7 (AB258331) is indicated by gray and black lines, respectively. The vertical dashed lines indicate the recombination break-point site. (B) Bootscan/Recscan analysis results. The *y*-axis gives the percentage of bootstrap support values of permutated trees using a sliding window of 200 bp wide with a step size between plots of 20 bp. Uruguayan sequences were analyzed with strains genotype GII.6 (AB039778) and genotype GII.7 (AB258331), as putative parental strains, and a GII.18 strain (AY883304) as an outlier sequence, indicated in gray, black and dotted lines, respectively. The recombination break-point position for each case is indicated.

geographical distance between the strains considered, a direct relation between these recombinant events seems unlikely. A more feasible scenario could be a consequence of the circulation of both GII.6 and GII.7 genotypes in a particular area. Co-circulation of different NoV genotypes may lead to co-infection, increasing the possibility of recombination events and giving rise to the circulation of new recombinant strains (Jiang et al., 1999; Vidal et al., 2006). Therefore, deeper research concerning NoV genotypes circulating in northwestern region of Uruguay and surrounding areas, are essential in order to understand the origin and dynamics of the reported recombinants. Furthermore, we report the circulation of the globally detected GII.1 and GII.4, based on its identification in two of the analyzed strains. However, the relative distribution of these genotypes and their prevalence will require more intensive molecular epidemiologic studies in Uruguay. This studies, together with reports of this nature in neighbor countries (Vidal et al., 2006; Fioretti et al., 2011, 2004; Gomes et al., 2008), will provide us a better understanding of the behavior and spread of NoV genotypes in the Latin American region.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.10.026.

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