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Research article

Molecular characterization of equine rotaviruses circulating in Argentinean foals during a 17-year surveillance period (1992–2008)

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ABSTRACT

P[12]G3 and P[12]G14 equine rotaviruses (ERVs) are epidemiologically important in horses. In Argentina, the prevalent ERV strains have been historically P[12]G3. The aim of this study was the detection and characterization of ERV strains circulating in foals in Argentina during a 17-year study (1992–2008). Additionally, the gene sequences of VP7, VP4 and NSP4 encoding genes of representative Argentinean ERV strains were determined and phylogenetic analyses were performed to elucidate the evolutionary relationships of the ERV strains in Argentina. ERVs were detected in 165 (21%) out of 771 diarrheic stool samples, which corresponded to 45 (39%) of 116 outbreaks from the surveyed thoroughbred horse farms. From the positive cases, 51% ($n = 23$) were G3, 33% ($n = 15$) were G14, 4% ($n = 2$) represented a G3 + G14 mixed infection and 11% ($n = 5$) of the cases could not be characterized. G3 ERV was detected during the entire period, while G14 ERV was first detected in 2000 and increased its incidence specially in 2006 and 2007. All the analyzed strains belonged to the VP4 P[12] genotype, except for one G3 case which belonged to the P[3] genotype, constituting the first report of a P[3]G3 ERV strain. Phylogenetic analysis of VP7 protein revealed that the G3 Argentinean ERV strains clustered with ERVs from Ireland, while the G14 Argentinean ERV strains formed a distinct cluster within the G14 genotype. The VP4 of the P[12] ERV strains clustered with P[12] strains from Ireland and France. The NSP4 of the Argentinean ERV strains clustered with the NSP4 genotype E12, along with those of guanaco and bovine strains from Argentina, suggesting the a close evolutionary relationship among these Argentinean strains. The results of this study showed changes in the incidence of G3 and G14 during the studied period. The increase in the frequency of G14 ERV, not included in the vaccine, in the second half of the period, may have implications for vaccine design.

1. Introduction

Since the initial detection of group A equine rotavirus (ERV) in diarrheic foals in England in 1975 (Flewett et al., 1975), ERVs have been detected in several countries showing a worldwide distribution. Currently, ERV is considered the major cause of dehydrating diarrhea in foals younger than 3 months of age (Saif et al., 1994). The two rotavirus outer capsid proteins VP7 (glycoprotein) and VP4 (protease-sensitive) are the basis for a widely used dual classification system defining the G types and P types, respectively (Matthijnssens et al., 2008a). Currently, 23 G genotypes and 31 P genotypes have been described in humans and animals (Abe et al., 2009; Hoshino et al., 2002; Matthijnssens et al., 2008a; Schumann et al., 2009; Solberg et al., 2009; Trojnar et al., 2009; Ursu et al., 2009).

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At least 7 G types (G3, G5, G6, G8, G10, G13 and G14) and 5 P types (P[1], P[7], P[11], P[12] and P[18]) have been described among ERVs (Browning et al., 1991a,b). Several surveys of ERV diarrheic field samples in England, Ireland, Australia, USA, Venezuela, Japan, Germany, and The Netherlands have suggested that the ERV population consists mainly of G3 and G14 types associated with a single P type: P[12] (Browning and Begg, 1996; Browning et al., 1992a,b; Ciarlet et al., 1994; Collins et al., 2008; Elschner et al., 2005; Fukai et al., 2006; Hardy et al., 1991; Imagawa et al., 1993; Isa et al., 1996; Nakagomi et al., 2003; Tsunemitsu et al., 2001; van der Heide et al., 2005). ERV G3 type has been shown to be predominant worldwide, with two associated subtypes G3A and G3B, while the G14 type has emerged as the second most common ERV G type. The G3A subtype has been detected in foals from England (Isa et al., 1996), Ireland (Collins et al., 2008), Australia (Browning and Begg, 1996) United States (Browning et al., 1992a) and Germany (Elschner et al., 2005), while the G3B subtype has been detected in Japan (Tsunemitsu et al., 2001) and United States (Browning et al., 1992a). Additional G/P combinations have been detected in horses, each consisting of a single isolate, H-1, P[7]G5 (Hoshino et al., 1983a), L338, P[18]G13 (Browning et al., 1991a), Eq/R-22, P[11]G10 (Imagawa et al., 1994), Eq/26/94, P[1]G8 (Isa et al., 1996), possibly representing examples of inter-species transmission from pigs, sheep or cows to horses.

A few ERV inactivated vaccines for the prevention of rotavirus diarrhea in foals have been developed. Pregnant mares are vaccinated in the last third of pregnancy to provide passive transfer of antibodies to foals against ERV. A vaccine developed in USA using the ERV strain H2 (P[12]G3) (Fort Dodge Animal Health, USA) was initially licensed in the USA, Great Britain and Ireland and now is commercially available in several countries (Powell et al., 1997). Another vaccine, formulated with the ERV strain HO-5 (P[12]G3), was licensed in Japan in 2000 and tested in a field study (Imagawa et al., 2005).

In Argentina, ERV associated with diarrhea in foals was first described in 1985 (Valle et al., 1990).

The Virology Institute of INTA has been studying ERVs since 1992 confirming a high incidence of rotavirus disease in thoroughbred breeding farms of Argentina, which results in important economic looses associated to the treatment, and occasionally, the death of the affected foals. The prevalent ERV strains were antigenically characterized as G3. To control the disease, an inactivated vaccine, Rota Coli Equina® (Instituto San Jorge Bargó, Buenos Aires, Argentina) with the prototype ERV H2 (P[12]G3), simian rotavirus (SRV) SA11 (P[2]G3), and bovine rotavirus (BRV) NCDV-Lincoln (P[1]G6) strains, formulated in aqueous adjuvant (Alumina gel), was developed (Barrandeguy et al., 1998). The systematic application of this vaccine to the pregnant mares, since 1996, has significantly reduced the incidence of ERV diarrhea in foals in the most important thoroughbred horse farms of the country. However, in the last three years of this study, the ERV morbidity increased considerably. Therefore, the aims of the present study were to evaluate the incidence and G types of ERV circulating in thoroughbred foals in Argentina from 1992 to 2008. Additionally, phylogenetic analyses of the VP7, VP4 and NSP4 encoding genes of representative ERV Argentinean strains were conducted.

2. Materials and methods

2.1. Fecal samples

Seven hundred seventy one (771) stool samples from diarrheic foals collected from 116 diarrhea outbreaks registered in 35 thoroughbred horse farms located in the Buenos Aires Province were analyzed in this study. For data analysis purposes, when several samples were submitted by a farm at different times during the same foaling season, all the samples were considered part of the same outbreak of diarrhea. When only one sample was submitted, it was considered as a single or separate case of diarrhea.

2.2. Reference virus strains

Prototype ERV H-1 (P[7]G5) and H2 (P[12]G3), SRV SA11 (P[2]G3), and BRV INDIANA (IND) (P[5]G6), B223 (P[11]G10), NCDV-Cody (P[1]G8), NCDV-Lincoln (P[1]G6) strains, kindly provided by Linda J. Saif, Food Animal Health Research Program, Ohio State University, USA, were cultured in MA104 cells as described (Theil et al., 1977) and used as controls in the G-typing assay standardization.

2.3. Rotavirus diagnosis

All fecal samples were initially screened for the presence of group A rotavirus antigen by enzyme-linked immunoassay (ELISA) as described previously (Cornaglia et al., 1989). ELISA-positive samples were further analyzed by rotavirus genotyping and serotyping assays.

2.4. RNA extraction

RNA was extracted from 10% fecal suspensions using TRIzol® (Life Technologies Inc., Frederick, Maryland, USA), following the manufacturer's instructions.

2.5. RT-PCR-hemi-nested multiplex for G typing

The VP7 (G) gene was amplified using RT-PCR assay with consensus primers Gra-5 and Gra-3 (Chang et al., 1996), using standard PCR procedure (Parreno et al., 2004).

The G-typing hemi-nested multiplex PCR assay was carried out using the reverse consensus primer Gra-3, a forward typing primer specific for G14 (G14D) (Tsunemitsu et al., 2001), and G3Arg (5′ GGA TGT TTG ACC ACC GAT 3′), a forward G3 typing primer designed in our laboratory based on alignment of Argentinean G3 ERV sequences and G14, G6, G8 and G10 sequences, that anneals from nucleotide 660–680 in VP7 gene.

The hemi-nested Multiplex PCR was run under the following cycling conditions: 94 °C for 2 min, then 30 cycles of 94 °C for 30 s, 44 °C for 30 s and 72 °C for 45 s; then a final extension of 72 °C for 7 min. First and second round PCR products were run and visualized in a 1.8% molecular biology grade agarose gel, containing 4 μg/ml ethidium

bromide. G-typing results were confirmed by sequencing of the amplified VP7 product.

2.6. G-typing monoclonal antibody (MAb) capture ELISA

To elucidate whether the Argentinean G3 ERV strains belonged to the antigenic G3A subtype, samples were tested by a MAb capture ELISA as described (Hurtado et al., 1995). The presence of VP7 in the sample was detected using a broadly reactive MAb (Common 60) directed to a VP7 non-neutralizing epitope. MAbs 4F8 (G3A-specific), IC3 (G6-specific) and E7 (G10-specific) were used to determine the serotype of the ERV strains. G14-specific MAbs are not available. All ELISA reagents were kindly provided by Linda J. Saif, Food Animal Health Research Program, Ohio State University, USA.

2.7. Amplification of VP7, VP8* and NSP4 genes for sequence analysis

Amplification of VP7 gene (1062 base pairs [bp]) was carried out as previously described for the first round of the G-typing PCR. For P type determination, amplification of VP8* segment (879 bp) of gene 4 was carried out using Con-2 and Con-3 consensus primers (Gouvea et al., 1994). Amplification of the NSP4 gene (751 bp) was carried out using published oligonucleotides NSP4F and NSP4R (Ciarlet et al., 2000).

2.8. Nucleotide sequencing

VP7, VP8* and NSP4 amplified products were purified from agarose gel using the Qiaquick Gel Purification Kit (Qiagen, Gmbh, Hilden, Germany). Samples purified in sufficient quantity were sequenced in both senses using the same primers used for the amplification of the PCR product. The PCR products were submitted for sequencing procedures to Macrogen Inc., Korea (http://macrogen.com).

2.9. VP7 nucleotide sequence of prototype strain H2

The ERV strain H2 (P[12],G3), kindly supplied by Y. Hoshino (National Institutes of Health, Bethesda, Maryland), was isolated in 1976 from a foal with diarrhea in the United Kingdom, and adapted to growth in primary African green monkey kidney (AGMK) (Hoshino et al., 1983b). ERV H2 was plaque-purified three times and was cultivated in African green monkey embryonic kidney (MA104) cells (Microbiological Associates, Bethesda, Maryland) as described (Ciarlet et al., 2000).

Single-stranded RNA transcripts were prepared from purified double-layered H2 virus particles as described (Ciarlet et al., 1994). The cognate genome segment 9, encoding the major outer capsid glycoprotein VP7, of ERV H2 was amplified by PCR to generate a fragment of 1062 base pairs (bp). Briefly, reverse transcriptase was used to generate gene 9 complementary (c) DNA using a primer complementary to the 3′ end of gene (5′ GGT CAC ATC ATA CAA TTC TAA TCT AAG 3′) of the simian rotavirus strain SA11 strain. Immediately after synthesis of the first cDNA strand, PCR of gene 9 was achieved with the corresponding 3′ end primer and a primer complementary to the 5′ end of SA11's gene 9 (5′ GGC TTT AAA AGA GAG AAT TTC CGT CTG G 3′). Amplified DNA was cloned into TA3pCR2.1 vector (Invitrogen Corp., San Diego, CA) according to the manufacturer's instructions.

For accuracy in sequence determination, two VP7 clones of ERV H2 from individual PCR reactions were sequenced by the dideoxynucleotide chain termination method, using the T7 sequencing kit (USB, Cleveland, OH). Confirmation of the DNA sequence was performed by sequencing both DNA strands of each of the different clones using the M13 forward and reverse primers. The names, nucleotide (nt) positions on the VP7 gene, polarities (plus or minus sense), and sequences (5′–3′) of additional primers used were: p706 (gene 9, nt 805–824 from bovine rotavirus strain NCDV, minus sense), GCT ACG TTT TCT CTT GGT CC; and pMC9A (gene 9, nt 314–335 from human rotavirus strain Wa, positive sense), CAA GTA CTC AAA TCA ATG ATG G. The nucleotide sequence of the H2 ERV strain was deposited in GenBank under the accession number: HM160096.

2.10. VP7, VP4 and NSP4 sequence analyses

Sequence analyses were performed using 24 sequences of VP7, 11 of VP8*, 9 sequences of NSP4 obtained in this study, and sequences representing the other genotypes obtained from the GenBank database. Sequences were edited with the BioEdit 7.0.5.3 sequence Alignment Editor (Hall, 1999) and aligned using default parameters of ClustalX (Thompson et al., 2002). For phylogenetic analyses, the gap treatments were applied (Jones et al., 2004). Briefly, when gaps were located at any of the extremes of the alignment they were considered as missing data. When gaps were located in middle of the alignment, they were treated as a fifth state, since they represented insertion–deletion events. The Modeltest program (Posada and Crandall, 1998) together with PAUP* (Swofford, 1998) were used to infer the sequences evolutionary model. The best fit model according to the Akaike Information Criterion (AIC) was GTR+G. Phylogenetic analyses were conducted using the distance method Neighbor-Joining (NJ) (Saitou and Nei, 1987), performed with PAUP*. Bootstrap with 1000 resample matrices were performed in order to assess the support for the identified groups (Felsenstein, 1985). Trees were edited and drawn with TreeDyn (http://www.treedyn.org).

The nucleotide sequence data reported in this paper were deposited in GenBank under the following accession numbers: VP7: GU373916–GU373939, VP4: GU373940–GU373950, NSP4: GU373951–GU373959.

3. Results

3.1. Incidence of rotavirus diarrhea in Argentinean thoroughbred horses

A total of 771 stool samples from diarrheic foals were analyzed. The samples corresponded to 116 single cases or outbreaks of diarrhea registered from 1992 to 2008 in thoroughbred horse farms located in Buenos Aires,

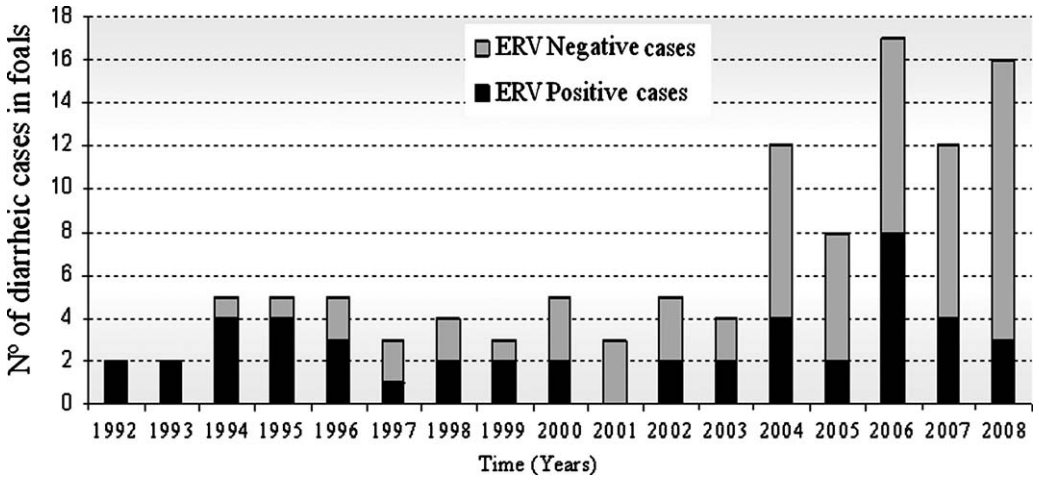

Fig. 1. Number of positive and negative cases of group A ERV detected by year during the surveillance period. Dark grey sections represent positive cases; light grey sections represent negative cases.

Argentina. ERV was detected in 165 (21%) out of 771 samples, which corresponded to 45 (39%) out of 116 outbreaks of diarrhea analyzed during the 17-year period. Fig. 1 depicts the number of cases of ERV infection detected each year during the surveillance period.

3.2. Rotavirus G genotyping and serotyping

Initially, the Argentinean ERV strains could not be properly typed with the "hemi-nested Multiplex RT-PCR" technique described by Tsunemitsu (Tsunemitsu et al., 2001) since the G3 primer used could not distinguish between G3, G6, G8 and G10 genotypes. Therefore the G3-specific primer was redesigned taking into account local G3 ERV strains and to avoid mistyping with G6, G8 and G10 genotypes. The new G3-specific primer, G3Arg, did not amplify ERVs belonging to genotypes G5, G6, G8 and G10 (data not shown).

The G and P types of the ERV strains circulating in a total of 45 rotavirus-outbreaks (18 horse farms) are summarized in Table 1. The G typing revealed that during the period evaluated (1992–2008), 23 (51%) out of 45 rotavirus-positive outbreaks were G3, 15 (33%) were G14 and 2 (4%) represented a G3 and G14 mixed infection in the same sample. A total of 5 (11%) could not be typed. In addition, all G3 ERV strains reacted with MAb 4F8, specific for the G3A subtype, while none of the G14 ERV strains reacted with any of the MAbs (specific for G3A, G6 or G10).

G3 ERVs were detected during the entire surveillance period and was the prevalent type of ERV from 1992 to

Table 1
G types of ERV cases circulating in Argentinean foals from 1992 to 2008.

	G type of the outbreaks of ERV detected each year			
Year	G3[a]	G14[b]	G3 + G14	Not-typeable
1992	2			
1993	1 (E30)		1	
1994	4 (E68)			
1995	3 (E137, E152)			1
1996	1			2
1997	1			
1998	1		1	
1999	2			
2000		2 (E277, E295)		
2001				
2002				
2003	2			
2004	2 (E379, E384)	1 (E352)		1
2005	1			1
2006	2 (E394, E405)	6 (E398, E401, E403, E412, E415)		
2007		4 (E419, E427, E432, E504, E505, E509, E518)		
2008	1[c]	2 (E706)		
Total	23	15	2	5

[a] Out of the 23 G3 ERV outbreaks detected, only 8 were confirmed by sequencing. The names of the 8 G3 ERV strains sequenced are shown in parentheses.
[b] Out of the 15 G14 ERV outbreaks detected, 13 were confirmed by sequence analysis. When more than one diarrhea episode occurred in the same haras in different times during a year, they were considered as one outbreak but those outbreaks were confirmed by sequence analysis of more than one strain, representative of each diarrhea episode. The names of the 16 G3 ERV strains sequenced are shown in parentheses.
[c] This strain was an atypical case of ERV that belonged to P[3]G3 genotype.

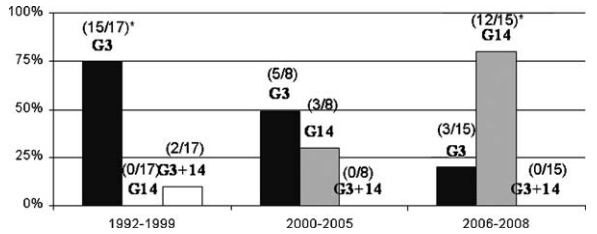

Fig. 2. Prevalence of G3 and G14 ERVs detected during the entire surveillance period. Black, grey and white bars represent the percentage of G3, G14 and G3 + G14 outbreaks in each indicated time frame.

1999. In 2000, G14 ERV was first detected as the only ERV causing diarrhea in foals in a farm that did not use vaccination, but this genotype became the most frequent ERV in the last three years (2006–2008) of the surveillance period (Fig. 2). All typing results were confirmed by sequence analysis.

3.3. Sequence and phylogenetic analyses of the VP7, VP8* and NSP4 genes of Argentinean ERV strains

A total of 8 G3 and 16 G14 VP7 genes were sequenced (three G14 outbreaks were represented by more than one sequence). Phylogenetic analyses of the VP7 of the G3

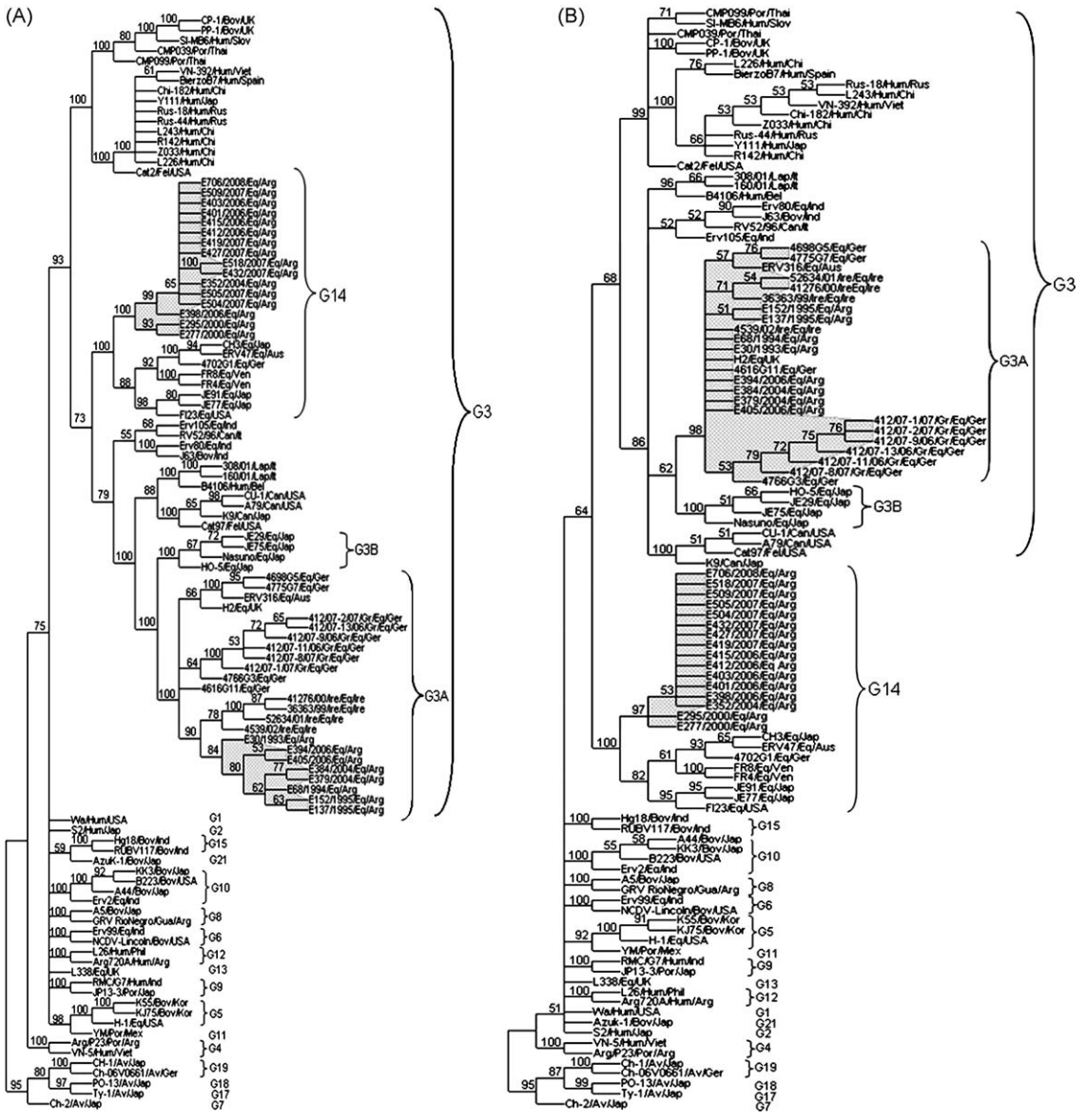

Fig. 3. Phylogenetic analyses of nucleotide (A) and amino acid (B) sequences of VP7 genes from Argentinean ERV. Bootstraps with 1000 resample matrices were performed. Branches with bootstrap value under 50 were collapsed. Abbreviations: Av: avian, Eq: equine, Por: porcine, Bov: bovine, Hum: human, Gua: guanaco, Lap: Lapine, Can: canine, Fel: feline, Ind: India, USA: United States, UK: United Kingdom, Slov: Eslovenia, Jap: Japan, Thai: Thailand, Viet: Vietnam, Chi: China, Rus: Rusia, Aus: Australia, Mex: Mexico, Ven: Venezuela, Arg: Argentina, Ger: Germany, It: Italy, Ire: Ireland, Phil: Philippines, Kor: Korea.

Argentinean ERVs revealed a close genetic relationship to subtype A G3 ERV strains isolated in Ireland, while the VP7 of the G14 Argentinean ERVs formed a distinct cluster within the G14 genotype, separated from other G14 ERV reference strains isolated in Japan, Venezuela, Germany, and Australia (Fig. 3A). When analyzing the G3 genotype, the topology of the tree indicated that at the nucleotide level G3 was not a monophyletic group, because the G14 strains constituted a subcluster within the G3 genotype (Fig. 3A). However, at the amino acid (aa) level, the G14

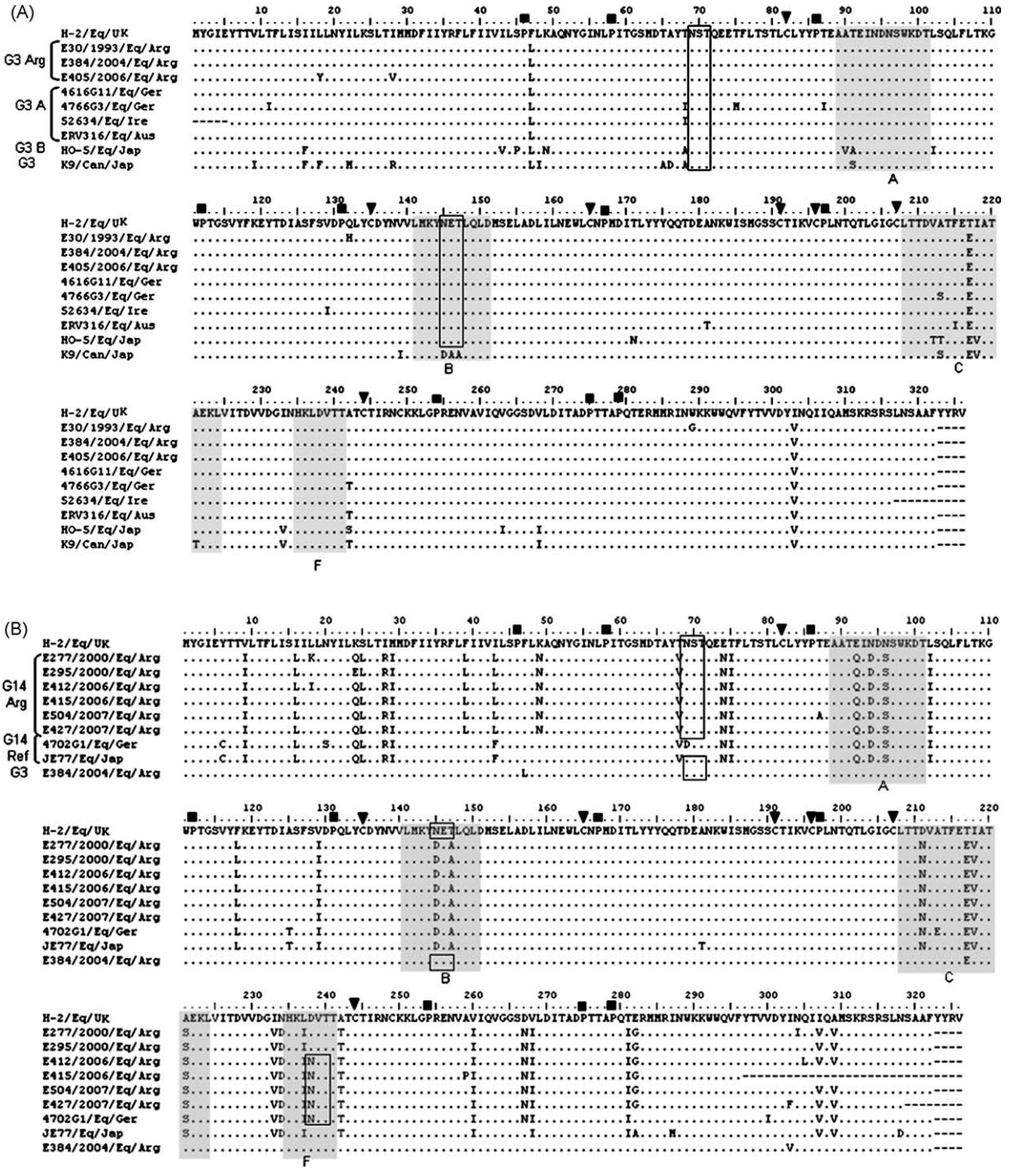

Fig. 4. Comparison of the deduced amino acid sequences of the outer capsid VP7 of G3 Argentinean ERV strains (A) and the G14 Argentinean ERV strains (B) with reference strain H2 included in the vaccine and other representative G3 and G14 VP7 sequences. Variable regions A, B, C and F (aminoacids 89–101, 141–151, 208–224 and 235–242, respectively) (Estes, 2001) are shown in shadow. Potential N-linked glycosylation sites are shown in boxes. Conserved cysteine (▼) and proline (■) residues are indicated. Abbreviations: Eq: Equine, Can: Canine, UK: United Kingdom, Arg: Argentine, Ger: Germany, Ire: Ireland, Aus: Australia, Jap: Japan.

strains formed a separate cluster from the G3 strains (Fig. 3B).

The G3 Argentinean strains showed conservation of the ten prolines, the seven cysteines and the two glycosylation sites (aa 69 and 145) present in H2 ERV strain, included in the vaccine. No aa substitutions were observed within the antigenic regions A, B and F. Only one mutation was detected in the region C (aa 217) of the Argentinean G3 strains (Fig. 4A).

All the ERV G14 strains included in Fig. 4B, lack one of the glycosylation sites present in H2 strain (aa 145). A new potential glycosylation site was observed at amino acid 238 in the G14 strains detected in Argentina in 2006 and 2007 as well as in the 4702G1 strain from Germany, but not in the G14 strains detected in Argentina in 2000. A total of nine aa substitutions were observed in the antigenic regions A, B, C and F in comparison with H2 strain, this observation is related with the antigenic difference between G3 and G14 serotypes (Fig. 4B).

Phylogenetic analyses of the nucleotide sequences of the VP8* portion of eleven Argentinean ERV strains revealed that all ERV Argentinean strains analyzed clustered close to P[12] ERVs isolated from Ireland and France (Fig. 5). However, one G3 ERV strain detected in Argentina in 2008, LoSa/2008/Arg, was shown to belong to the P[3] genotype. The phylogenetic analysis of the complete genome of this strain is under progress.

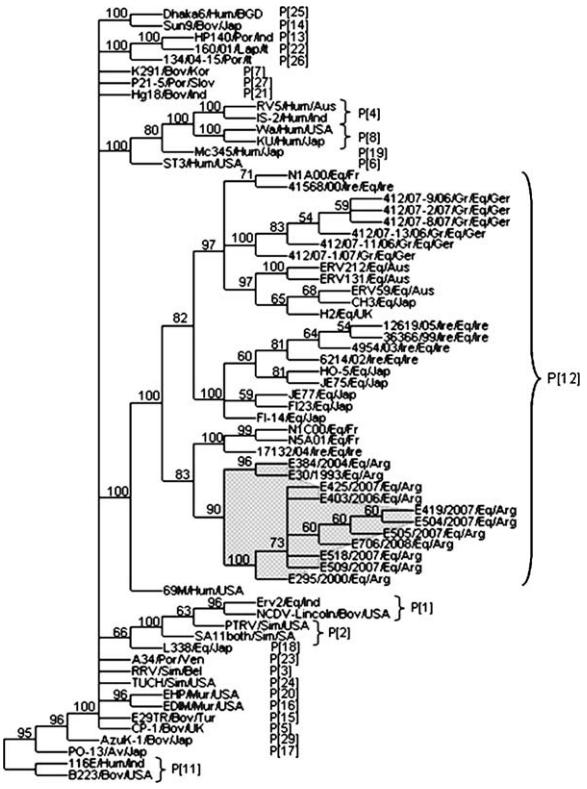

Fig. 5. Phylogenetic analysis of nucleotide sequences of the VP8* genes from selected Argentinean ERVs. Bootstraps with 1000 resample matrices were performed. Branches with bootstrap value under 50 were collapsed. Abbreviations: Av: avian, Eq: equine, Por: porcine, Bov: bovine, Hum: human, Lap: lapine, Sim: Simian, Mur: Murine, Bgd: Bangladesh, Ind: India, USA: United States, UK: United Kingdom, Slov: Eslovenia, Jap: Japan, Aus: Australia, Ven: Venezuela, Arg: Argentina, Ger: Germany, It: Italy, Ire: Ireland, Kor: Korea, Fr: France, Bel: Belgium, Tur: Turkey.

The VP8* aa alignment is depicted in Fig. 6 and it showed that the three potential trypsin cleavage sites are conserved, as well as the cysteines and prolines present in H2 strain. One proline residue was present in the conserved region flanking the trypsin cleavage site in all the P[12] sequences of the Argentinean ERV strains that belonged to G14 genotype. Six aa changes were observed in the variable region of P[12] sequences of Argentinean G3 ERVs, while 9-10 aa substitutions were observed in the variable region of P[12] sequences of Argentinean G14 ERVs.

The phylogenetic analysis of nine NSP4 sequences of the Argentinean ERV strains showed that they clustered closely with those of the BRV and guanaco rotavirus strains, isolated in Argentina, belonging to the recently described E12 NSP4 genotype (Matthijnssens et al., 2009) (Fig. 7).

The E12 Argentinean ERV strains showed conservation of the two potential glycosylation sites present in NSP4 sequence of H2 strain. cysteines and prolines were also conserved, however two proline residues were observed in all E12 strains and not in H2 (aa 139 and 168). One of these prolines is located in the double layer particle binding site (aa 161–175) and the other one in the "inter-species variable domain" (aa 135–141). Eleven to twelve aa substitutions were observed within the enterotoxigenic region (aa 112–175) of E12 ERV sequence compared with NSP4 from H2 ERV strain. From these twelve substitutions, six were located in the "inter-specie variable domain" (aa 135–141), which length is seven aa (Fig. 8).

4. Discussion

Rotavirus diarrhea in foals is a very important illness in thoroughbred farms worldwide (Dwyer, 1993). In Argentina, outbreaks of diarrhea due to ERV in thoroughbred breeding farms occur every foaling season during the months of September–October, in concordance with the highest density of susceptible animals. In some horse farms, the outbreaks may affect up to 100% of foals. In this report, ERV was detected in 39% of the diarrhea outbreaks registered in 35 thoroughbred horse farms during a 17-year surveillance period (1992–2008), indicating a high circulation of ERVs in the Argentinean horse farms.

The characterized Argentinean strains were shown to belong to genotypes G3 (51%) or G14 (33%); no other G type was detected. In addition, 4% represented mixed infection with G3 and G14 strains, while 11% of the samples were untypable by the methodology used. All G3 strains were shown to belong to the G3A serotype using a monoclonal antibody capture ELISA, as well as by phylogenetic analysis. The VP4 of all ERV strains belonged to P[12] genotype, except for one G3 ERV strain LoSa/2008/Arg, detected in a horse farm from Buenos Aires province, in 2008, whose VP4 type was P[3]. This finding constituted the first report of a P[3]G3 ERV strain and the complete genome analysis of this P[3]G3 ERV strain is under

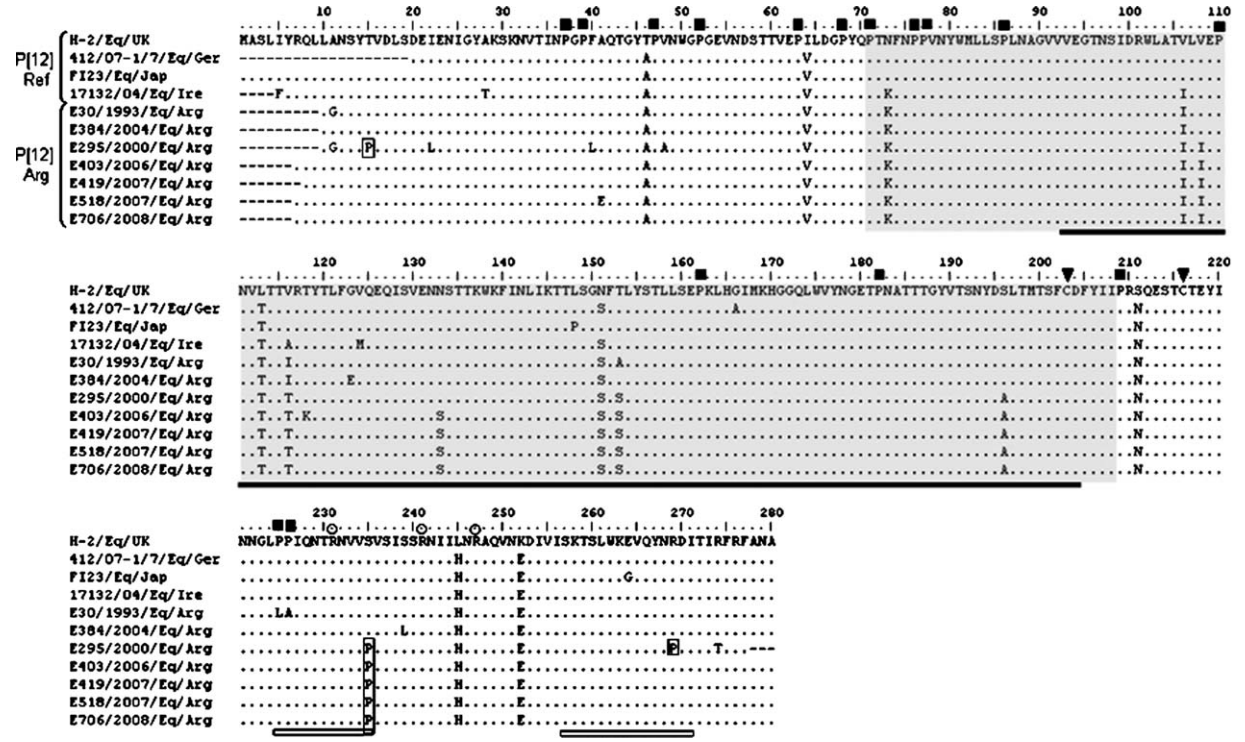

Fig. 6. Comparison of the deduced amino acid sequence of the outer capsid VP8* trypsin cleavage product of VP4 of the Argentinean ERV strains with reference strain H2 included in the vaccine and other representative P[12] representative sequences. Variable region (aa 71–204) is shown in shadow. Hemaglutination domain (aa 93–208) is underlined (▬). Conserved trypsin cleavage sites (○), cysteine (▼) and proline (■) residues are indicated. A proline residue at aa 235 only present in the Argentinean P[12] strains associated to G14 genotype is shown in box. Conserved regions flanking the cleavage sites are underlined (▭).

progress to elucidate its relationship with other ERV and other animal rotavirus strains.

Early studies of prevalence of G3 ERVs in Argentina (Parreño, unpublished data), prompted generation of an inactivated vaccine, Rota Coli Equina® (Instituto San Jorge Bagó), formulated with the ERV H2 (P[12]G3), SRV SA11 (P[2]G3) and BRV NCDV-Lincoln (P[1]G6) strains manufactured in Argentina. This vaccine was experimentally evaluated from 1994 by INTA and was commercially available in 1996. Argentina was the first country with a licensed vaccine to prevent ERV diarrhea in foals in the local market (Barrandeguy et al., 1998). Since the vaccine has been widely applied to pregnant mares in polo and thoroughbred horse farms, the incidence of diarrhea outbreaks for ERV decreased, and no ERV was detected in farms that submitted samples to the service during the years 2001 and 2002. However, in the years 2006 and 2007,an increase in diarrhea cases positive for ERV coincided with a change in the prevalent genotype, from G3 to G14, which was not covered by the Argentinean ERV vaccine. This finding could be due to a cyclic or emerging pattern of G14. To elucidate this issue ERV detected in the following years will be analyzed.

Similar results of temporal changes in the circulating genotypes, after introduction of P[12]G3 inactivated vaccine were observed in Japan (Tsunemitsu et al., 2001) and in Ireland (Collins et al., 2008). These results suggest that the ERV vaccine should be updated according to the increase in the circulation of G14 strains. Changing profiles of the G or P types circulating in human and bovine RV have been previously described (Cashman et al., 2010; Lennon et al., 2008; Reidy et al., 2005)

The P[12]G3 and P[12]G14 combinations detected in Argentinean foals are characteristic of equine in other countries (Browning and Begg, 1996; Browning et al., 1991a, 1992a; Ciarlet et al., 1994; Collins et al., 2008; Elschner et al., 2005; Hardy et al., 1991; Imagawa et al., 1991, 1993, 1994; Isa et al., 1996; Takagi et al., 1994; van der Heide et al., 2005). The G3 genotype is widely distributed in nature worldwide and affects several animal species, including humans. The G3 G type in ERV was divided into two subtypes: G3A subtype has been detected in foals from England (Isa et al., 1996), Ireland (Collins et al., 2008), Australia (Browning and Begg, 1996) United States (Browning et al., 1992a) and Germany (Elschner et al., 2005), while G3B has been detected in Japan (Tsunemitsu et al., 2001) and United States (Browning et al., 1992a). The Argentinean ERV strains with G3 genotype, reacted with 4F8 Mab (Browning et al., 1992b) indicating that they belonged to G3A subtype. This result was in concordance with the phylogenetic analysis of the VP7 gene of these strains.

The G14 genotype is restricted to ERV and according to phylogenetic analysis based on nucleotide sequences it appears to constitute a cluster within the G3 genotype, with percentages of identity higher than 80%, the cut off

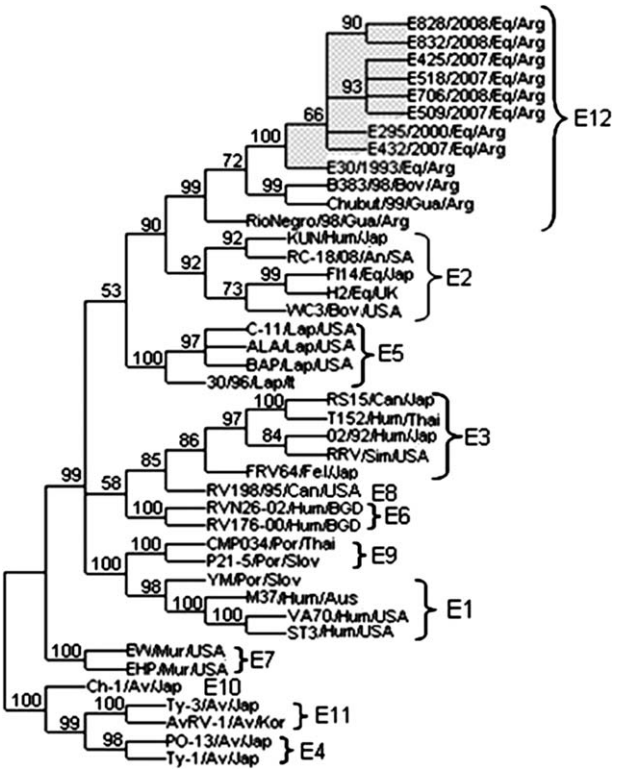

Fig. 7. Phylogenetic analysis of nucleotide sequences of the NSP4 genes from selected Argentinean ERVs. Bootstraps with 1000 resample matrices were performed. Branches with bootstrap value under 50 were collapsed. Abbreviations: Av: avian, Eq: equine, Por: porcine, Bov: bovine, Hum: human, Lap: lapine, Sim: Simian, Mur: Murine, Fel: feline, An: antelope, Can: canine, Gua: guanaco, BGD: Bangladesh, USA: United States, UK: United Kingdom, Slov: Eslovenia, Jap: Japan, Aus: Australia, Arg: Argentina, It: Italy, Thai: Thailand, SA: South Africa, Kor: Korea.

value for a new genotype, according to the RCWG (Matthijnssens et al., 2008a,b). However, when the phylogenetic analysis was performed with the deduced aa sequences, the G14 ERV strains clearly clustered as an independent G type. In fact, the alignment of the aa sequences showed that the genotype G3 of the Argentinean ERVs strains is well represented by the VP7 protein of H2 (G3 subtype A), the strain included in the vaccine, while

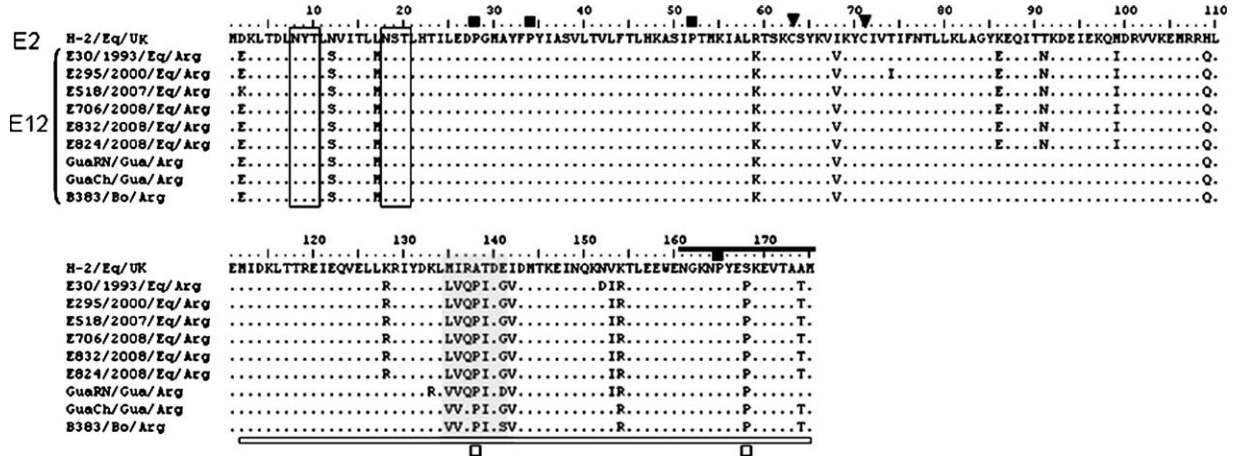

Fig. 8. Comparison of the deduced amino acid sequence of the Argentinean ERV strains NSP4 proteins (E12 genotype) with reference strain H2 (E2 genotype), included in the vaccine and other representative E12 representative sequences. Inter-specie variable domain (aa 135–141) is shown in shadow. Potential N-linked glycosylation sites are shown in boxes. Enterotoxigenic region (aa 93–208) is underlined (▭) and double layer particle binding region is indicated (▬). Conserved cysteine (▼) and proline (■) residues are also indicated. Two proline residues at aa 235 and present in the Argentinean E12 strains but not in H2 are shown (□).

the G14 strains showed nine mutations within the antigenic regions, including the generation of new glycosylation sites, compared with that of the H2 strain. This observation is in concordance with cross-neutralization assay results previously published (Ciarlet et al., 1994) that established G14 as a different serotype from G3, but serologically more related than the other serotypes.

The sequence analysis of VP8 showed that P[12] was the prevalent genotype in ERVs from Argentina. Only one case with a different P-type was detected in the 17-year survey. In concordance with VP7 G3 results, Argentinean ERV strains clustered close to P[12] ERVs isolated from Ireland (Collins et al., 2008).

All G14 Argentinean ERV strains possess a proline residue in the conserved region flanking one of the trypsin cleavage sites. The effect of this aa substitution should be further investigated because it might cause a conformational change that could modify the availability of the cleavage site, and therefore, could affect the virulence and tissue culture-adaptation of these strains. The high conservation of the P type of the ERV, suggest that the development of a recombinant subunit vaccine based on P[12] main antigenic region, would be an excellent prevention strategy to avoid the breaking of the immune barrier originated for the cyclic switch in the G types, when using inactivated vaccines. These studies are in progress in our laboratory using a recently reported approach with a subunit vaccine VP8*-*Brucella* spp. lumazine synthase (BLS), where BLS works as a an antigen delivery system (Bellido et al., 2009).

The NSP4 enterotoxin sequence were also analyzed, and they showed a geographical relationship, given that all sequences that corresponded to Argentinean strains constituted E12 genotype, independently of the host species. The strong geographical relationship is also supported by the ''inter-species variable domain'', which is supposed to vary according to the species of origin of the RV strain. The inter-species variable domain sequences of ERV Argentinean strains are more related to the GuaRioNegro strain isolated from a guanaco of region of Río Negro, Argentina, than with that of the H2 ERV strain.

The ERVs detected in this study were genetically homogeneous compared to human RV, porcine RV or bovine RV. The ERV strains belonged to sport horses, which in turn are a homogeneous population with almost no contact with other animals. Although the sport equine population showed a high rate of international movement, this movement did not induce contact with other animal species. Furthermore, horse farmers do not produce other animal species in the same farm. If equine population would have contact with other animal species, it might be infected by more heterogeneous RV population.

Changes in ERV epidemiology, as observed in this study, could respond to systematic vaccination strategies to control ERV-related diarrhea. Given that the current vaccine for ERV in Argentina does not include the G14 genotype, and G14 ERVs were detected in most of the cases in the last 3 years of the 17-year surveillance period, the results of this study suggest that inclusion of G14 in commercial vaccines to prevent ERV diarrhea in Argentinean foals may be warranted.

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