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Short communication

Phylogenetic analysis of porcine rotavirus in Argentina: Increasing diversity of G4 strains and evidence of interspecies transmission

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Abstract

Group A rotaviruses are one of the most frequently detected viral agents associated with neonatal diarrhea in piglets. In order to characterize rotavirus (RV) strains circulating in Argentinean swine, four porcine production farms located in Buenos Aires were studied. RV strains genotyped as P[6]G4, P[6]G8 and P[1]G6 were found in piglets under 30 days of age, without diarrhea. Phylogenetic and sequence analysis of the *VP7* gene from G4 strains available in databases, reveals five porcine new lineages (III–VII) and three sublineages (VIIa–VIIc). The G4 porcine Argentinean strains were grouped with a porcine RV strain isolated in Brazil and another RV strain isolated from a child with diarrhea in Mexico, constituting an American lineage (VII). On the other hand, porcine G6 and G8 were closely related to RV's circulating in Argentinean cattle and South-American camelids, respectively. The fact that G4 porcine lineages were epidemiologically related to human strains, and G6 and G8 Argentinean porcine strains were found related to bovine and South-American camelids, respectively, suggests that pigs might play a crucial role as reservoir and generator of newly adapted emerging RV strains for human and other species.

Keywords: Porcine rotavirus; Genetic diversity; G genotype; Argentina

1. Main text

Neonatal diarrhea is considered a significant sanitary problem in the porcine production industry, causing a highly-negative economic impact. Group A rotaviruses are one of the most frequently detected viral agents associated to diarrhea affecting piglets

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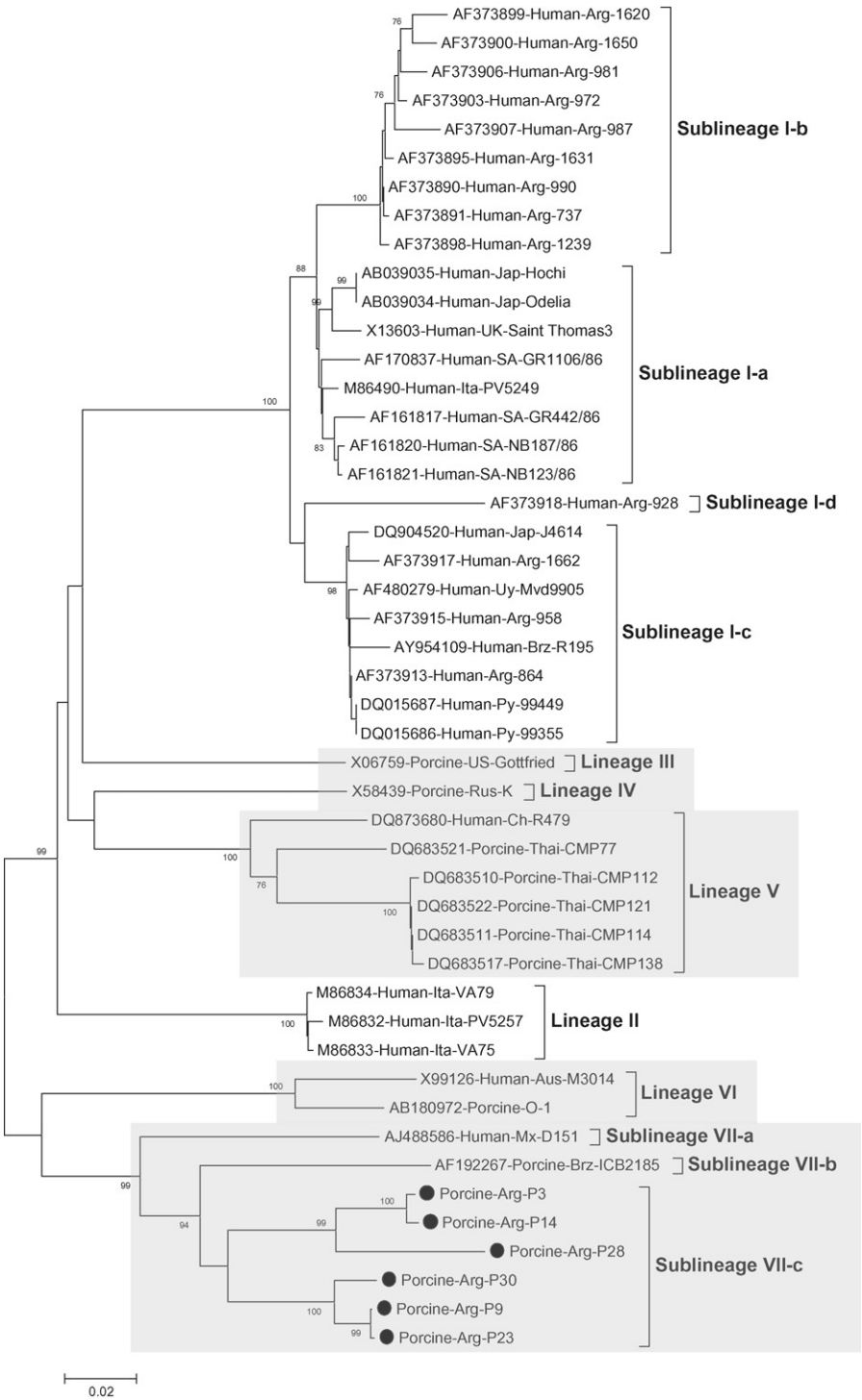

Fig. 1. Phylogenetic tree constructed using *VP7* sequences of G4 strains using Kimura 2 parameter as a model of nucleotide substitution and Neighbor-Joining for tree reconstruction. Only bootstrap values above 75 and representative strains from human lineages and sublineages are

Table 1
Genotyping of group A RV circulating among Argentinean porcine production farms

Farm	Management	Group A RV (ELISA)	G-P types combination			Details
			G4P[6]	G8P[6]	G6P[1]	
A	Intensive	16	1			P9: pre-weaning piglet, 30 days-old, ND
B	Extensive	6	3		1	P3; P14 and P28: pre-weaning piglet 10, 23 and 16 days-old; ND
C	Intensive	4	2			P23 and P30 pre-weaning piglet 15 and 21 days-old; ND
D	Extensive	2		1		P24: pre-weaning piglet 25 days-old; ND
Total		30	6	1	1	

ND: no diarrhea.

between 1 and 8 weeks of age (Saif et al., 1994). However, the virus is also often detected in nondiarrheic piglets, especially during the pre-weaning period (Lecce and King, 1978).

The virus contains a genome with 11 segments of double-stranded RNA (dsRNA), surrounded by a triple-layered protein shell, consisting of a core, an inner capsid and an outer capsid. Rotaviruses are classified into G and P types, according to the genetic and antigenic diversity of the two outer capsid proteins, VP7 and VP4, respectively. At least 16 G types and more than 27 different P types have been identified in humans and animals so far (Estes, 1996; Gulati et al., 2006; Fukai et al., 2007; Khamrin et al., 2007; Martella et al., 2007; Steyer et al., 2007).

Four common porcine G types have been detected worldwide: G3, G4, G5 and G11, associated to common P types, P[6] and P[7] (Gouvea et al., 1994a). Moreover, P types P[13], P[19], P[23], P[26] and P[27] have also been described as common genotypes in swine. Furthermore, typical human G and P types (G1, G2, G9, M37-like type P[6] and P[8]) and bovine G and P types (G6, G8, G10, P[1], P[5] and P[11]) have also been described in swine (Gouvea et al., 1994b,a; Ciarlet et al., 1995; Martella et al., 2005, 2006, 2007). Genomic characterization of RV strains is essential to study the diversity of circulating porcine strains and their relationship with human and other heterologous strains, as swine might play a role as reservoirs for human RV strains (Santos et al., 1999; Palombo, 2002). The porcine RV strains circulating in Argentina have only been antigenically characterized describing the presence of G1, G2, G3 and G5 (Bellinzoni et al., 1987, 1990; Mattion et al., 1989). Only strains typed as G1 and G5 were further studied by sequence analysis and confirmed to be P[7]G1 and P[7]G5 (Ciarlet et al., 1995). However, no additional surveys or typing of porcine RV had been conducted in Argentina since then.

To characterize group A rotaviruses circulating in swine during 1999, 906 stool samples were collected (901 from pigs without diarrhea and 5 from pigs with diarrhea) from 4 porcine production farms located in Buenos Aires, Argentina. The number of samples obtained represented 15% of the pre-weaning piglet population of each farm. Diarrheic and nondiarrheic piglets under 45 days of age were sampled. Farms A and C were under intensive management, while farms B and D applied extensive management (Table 1) (Vidales et al., 2002).

The presence of group A RV in the samples was assessed using PAGE and a RV antigen capture ELISA, as previously described (Laemmli, 1970; Cornaglia et al., 1989). RV was only detected in 30 stool samples (3.3%) by one or both techniques, corresponding to piglets from 1 to 45 days of age. Only five of them presented diarrhea while the remaining were shedding the virus asymptomatically.

Genotyping (G and P) was carried out using a nested multiplex reverse transcription (RT)-PCR

shown. The newly described lineages and sublineages are shown shaded. Argentinean porcine strains are marked with a dot. The GenBank accession numbers, host species and countries of origin are shown where available. The abbreviations for countries are as follows: Arg: Argentina; Aus: Australia; Brz: Brazil; Ch: China; Ita: Italy; Jap: Japan; Mx: Mexico; Py: Paraguay; Rus: Russia; SA: South Africa; Thai: Thailand; UK: United Kingdon; US: United States; Uy: Uruguay.

method, using primers specific for human, bovine and porcine genotypes (Gouvea et al., 1990, 1994b,a; Gentsch et al., 1992; Das et al., 1994; Garaicoechea et al., 2006). Sequencing was carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABIPrism 377 DNA sequencer (Applied Biosystems, Foster City, CA). The sequences of *VP7* gene corresponding to Argentinean porcine RV strains were deposited in GenBank database under accession numbers AY115858, AY115859, AY115860, AY115861, AY115862 and AY115863 (G4 strains), EF474079 (G6 strain) and EF474080 (G8 strain). The phylogenetic analyses were performed using Neighbor-Joining and Kimura 2 parameter as a model of nucleotide substitution (Kimura, 1980; Saitou and Nei, 1987). The statistical significance of phylogenies was calculated by bootstrap analysis with 1000 pseudoreplicate data sets. The phylogenetic relationship among these sequences was also confirmed by parsimony analysis (Swofford, 2002).

Eight out of 30 RV-positive samples were successfully characterized. Two of them were typed as G6 and G8 by using the nested PCR designed for genotyping while six remaining samples failed to generate a nested PCR product on any attempt with the primers used. Further sequence analysis of partial *VP7* gene sequence confirmed the two previously typed samples as G6 and G8, while the untypeable samples showed the highest percentage of identity with sequences corresponding to G4 RV strains. Thus, the genotype G4 represented 75% (6/8) of the successfully characterized strains, and it was distributed in three out of four farms studied (farms A, B and C), indicating a broad circulation of this strain in the population under study. In addition, the co-circulation of G4 and G6 was detected in Farm B, while G8 type was the only RV found in Farm D (Table 1). Both, G4 and G8 samples were associated to P[6], a common P type found in pigs, while G6 strain was combined with P[1], previously described in bovines.

Ninety-two out of 142 *VP7* gene sequences retrieved from the GenBank database were used for the phylogenetic analysis of G4 genotype, since those contained a matching region when compared to the Argentinean porcine strains. In addition, 34 and 23 *VP7* gene sequences were used for the phylogenetic analysis of the G6 and G8 strains. The alignments are available from the authors upon request.

The G4 phylogenetic tree revealed two human lineages (I and II) and four sublineages (Ia–Id) that had been previously described (Bok et al., 2002). Besides, five additional not previously reported porcine lineages were identified (III, IV, V, VI and VII) (Fig. 1). Each of the porcine lineages III, IV and VI are represented by only one porcine strain: Gottfried, originally isolated from the intestinal content of a sucking piglet with diarrhea in USA (Bohl et al., 1984); K, isolated from a 12-day old piglet with diarrhea in Russia (Akopian et al., 1992) and O-1, isolated in USA, respectively. Meanwhile, lineage V grouped Thai porcine strains with a human strain R479 detected in China (Wang et al., 2007), and lineage VII grouped Argentinean (P3 P9, P14, P23, P28 and P30), a Brazilian porcine strain ICB2185, and a human strain D151 detected in Mexico (Fig. 1) (Racz et al., 2000; Laird et al., 2003). It is noticeable that the human strain M3014 detected in Australia (Palombo et al., 1997) was closely related (94.6% of nucleotide identity) with the porcine O-1 strain from USA (Fig. 1). All strains from lineage VII were detected in combination with P[6], thus, corresponding exclusively to P[6]G4 strains isolated in the Americas. The same epidemiological pattern seems to be observed with the lineage V, where the Thai porcine strains were grouped with the human R479 strain detected in China, both Asiatic countries (Fig. 1).

The lineages and sublineages defined above were supported by nucleotide and amino acid distances. The nucleotide distances among sublineages and lineages ranged from 4.1 to 10.8% and 12.4 to 20.6%, respectively. However, strains from sublineage VIIa and VIIb showed nucleotide differences of 12.9 and 12.6%, respectively, when compared to the Argentinean strains from sublineage VIIc (Table 2). Thus, in contrast to the observed nucleotide distances range within most lineages (0.5–5.5%), lineage VII appeared as a more heterogeneous group, presenting a 9% distance within it. The distance value was also high if only Argentinean porcine strains were considered (6.7%), also showing increased heterogeneity among strains from the same geographical origin. Therefore, despite the high percentage of nucleotide distances, lineage VII was further sub-divided into three sublineages (i.e. VIIa–VIIc), based on phylogenetic and epidemiological data (Fig. 1).

Table 2
Percentages of nucleotide (lower-left) and amino acid (upper-right); distances between lineages and sublineages of genotype G4

	Sublineage-Ia	Sublineage-Ib	Sublineage-Ic	Sublineage-Id	Lineage-II	Lineage-III	Lineage-IV	Lineage-V	Lineage-VI	Sublineage-VIIa	Sublineage-VIIb	Sublineage-VIIc
Sublineage-Ia		4.7[1,2]	3.4	7.9	7.8	6.2	9.1	11.2	12.5	11.7	12.9	13.6
Sublineage-Ib	4.1		4.2	7.6	7.6	6.3	8.4	11	12.5	11.4	12.8	13.7
Sublineage-Ic	4.2	5.1		7.5	7.6	6.2	8.1	10.5	12	11.3	12.3	13.3
Sublineage-Id	8.5	8.4	6.5		10.4	6.8	11.3	12.5	14.6	13.6	14.1	15.5
Lineage-II	14	13.8	14.3	15.4		5.9	8.1	8.8	12	11.3	10.9	11
Lineage-III	13.7	13.8	13.1	15.2	12.4		7.3	8.7	11.5	11.3	10.4	11.6
Lineage-IV	14.3	14.4	13.7	16.2	14	13.8		6.2	12.9	9.5	8.6	10.3
Lineag-V	15.3	15.5	15.5	18.2	13.9	14.8	13.8		11.6	10.8	8.9	10.3
Lineage-VI	17.8	18	17	19.8	16.8	17.2	18.4	17.1		10.6	11.8	11.2
Sublineage-VIIa	17.5	17.6	17	19.6	16.2	17.5	17.5	17.1	16.4		5.1	5.9
Sublineage-VIIb	18.8	18.2	18.6	20.6	16.1	17.2	16.6	17.1	17.6	12.9		5.9
Sublineage-VIIc	18	17.9	17.6	20	16.7	18.1	17.6	18.2	16.9	12.6	10.8	

[1]Percentages of distances are expressed as mean values.
[2]Distances between sublineages are presented shaded.

Amino acid sequences ranged from 5.9 to 15.5% distance values among lineages and 3.4 to 5.9% among sublineages. RV strain Arg-928 from sublineage Id showed increased amino acid differences ranging from 7.5 to 7.9% when compared to the remaining human sublineages. Amino acid sequences of the Argentinean porcine strains differed by at least 10.3% when compared to the other strains (Table 2). The low degree of amino acid difference (6.2%) found between strains from lineage IV and V could be explained by the geographical proximity and commercial relationship among the countries of origin of these lineages (i.e. Russia, China and Thailand; all of them Euro-Asiatic countries).

Argentinean porcine strains presented a total of 45 amino acid changes within the region of the VP7 analyzed when compared to reference human strain ST3 (data not shown). Fifteen of them were located within the gene's variable antigenic regions A, B and C, suggesting antigenic differences between human and porcine strains.

Phylogenetic analysis showed that the *VP7* gene of Argentinean porcine strain P[1]G6 was closely related to the bovine RV reference strain, IND (P[5]G6; 98.6% of nucleotide identity), and was grouped in the same lineage with the bovine P[5]G6 strains, which represented the most prevalent genotype in Argentinean cattle during 1994–2003 (Fig. 2a) (Garaicoechea et al., 2006). It is noteworthy that the percentage of nucleotide differences between the Argentinean porcine strain P22: P[1]G6 and the P[5]G6 (lineage IV; NCDV-like) Argentinean bovine strains ranged from 7 to 8.2%, while it differed from 18.5 to 19.5% with the Argentinean bovine strains P[11]G6 (lineage III; Hun-4-like). This genotype was previously described in pigs from Brazil (Racz et al., 2000), while the combination P[5]G6 was also detected in pigs from Italy (Martella et al., 2001). Unfortunately, the sequences of those strains were not available in the public databases for phylogenetic analysis. Interestingly P[1] genotype, commonly found in bovines, was detected in Argentinean guanacos (Parreno et al., 2004) and pigs (this study), but not in Argentinean cattle, in the same time period (Garaicoechea et al., 2006).

The Argentinean porcine G8 clustered together with G8 RV strains found in South-American camelids (*guanaco or Lama guanicoe*), from the Patagonia

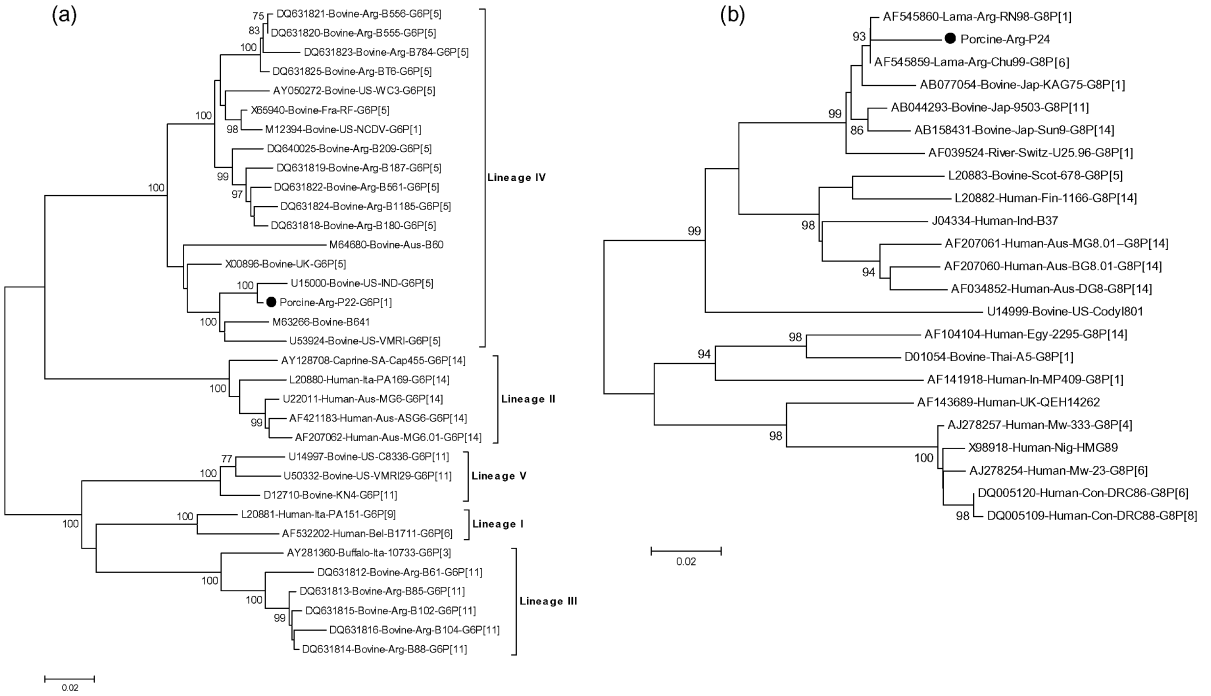

Fig. 2. Phylogenetic tree constructed using *VP7* sequences of G6 (a) and G8 (b) strains using Kimura 2 parameter as a model of nucleotide substitution and Neighbor-Joining for tree reconstruction. Only bootstrap values above 75 are shown. Argentinean porcine strains are marked with a dot. The GenBank accession numbers, host species, countries of origin, G and P types are shown where available. The abbreviations for countries are as follows: Arg: Argentina; Aus: Australia; Brz: Brazil; Bel: Belgium; Con: Congo; Ch: China; Egy: Egypt; Fra: France; Fin: Finland; In: India; Ind: Indonesia; Ita: Italy; Jap: Japan; Mw: Malawi; Nig: Nigeria; Scot: Scotland; Switz: Switzerland; SA: South Africa; Thai: Thailand; UK: United Kingdom; US: United States.

region of Argentina during the same period (99% of nucleotide identity, Fig. 2b) (Parreno et al., 2004). Genotype G8 is the third most common G type found in cattle associated to P[1] and P[5]. This G type was not found in a 10-year-survey of calves with diarrhea recently conducted in Argentina (Garaicoechea et al., 2006), but it was detected in combination with P[1] and P[14] in newborn guanacos with severe diarrhea from Rio Negro and Chubut province in the Patagonia region (Parreno et al., 2004). The finding of a P[6]G8 strain circulating in pigs from farms located in Buenos Aires suggests that G8 must be present in cattle or other domestic livestock in Argentina. The circulation of P[6]G8 in pigs might suggest reassortment of these G8 VP7 of unknown origin with the most prevalent P type found in this study (i.e. P[6] type). Furthermore, both phylogenetic analyses are supported by the high degree of similarity between the porcine strains compared to bovine (G6) and South-American camelid strains (G8). Therefore, the close phylogenetic relationship and the high degree of similarity found among porcine RV strains and bovine or guanaco strains, might suggest that these strains could have been originated through interspecies transmission.

The high percentage of samples that were positive by ELISA and/or PAGE but failed to give a PCR product (22/30), in both RT-PCR step and genotyping step, could be due to several reasons: (i) very low amount of virus in the sample, (ii) PCR inhibitors in the sample, since all of them (8/25) belonged to asymptomatic pre-weaning piglets with detectable maternal IgG antibodies in feces (Vidales et al., 2002), (iii) samples were stored at $-20\ ^{\circ}C$ and virus/RNA deterioration can also be expected, (iv) the circulation of RV strains with genotypes not included in the pool of primers or (v) the accumulation of point mutations in the primer binding sites of the *VP4* and *VP7* genes.

	(5' - 3')					
Primer aDT4	CGT	TTC	TGG	TGA	GGA	GTT G
Arg-P9	TA.	.G.	...	A..	A..	... A
Arg-P3	TA.	CG.	...	A..	A..	... A
Arg-P14	TA.	CG.	...	A..	A..	... A
Arg-P23	TA.	.G.	...	A..	A..	... A
Arg-P28	TA.	CG.	...	A..	A..	... A
Arg-P30	TA.	.G.	C..	A..	A..	... A

	(5' - 3')*					
Primer 1T-4	GGG	TCG	ATG	GAA	AAT	TCT
Arg-P9	..A	..A	..T	...	...	...
Arg-P3	..A	..T	..T	...	...	..C
Arg-P14	..A	..T	..T	...	...	..C
Arg-P23	..A	..A	..T	...	...	...
Arg-P28	..A	..T	..T	...	..C	..C
Arg-P30	..A	..A	..T	...	...	...

* reverse complement sequences

Fig. 3. Nucleotide identity of Argentinean porcine strains compared to aDT4 and 1T-4 primer sequences, used for genotyping (Gouvea et al., 1990; Das et al., 1994).

In this regard, the G4 Argentinean porcine strains presented 6 and 7 mismatches with the primer aDT4 (19 nt of length) and 3–5 mismatches with the primer 1T-4 (18 nt long), used in the PCR for G4 typing (Fig. 3). Therefore, the accumulation of point mutations in the binding site of the primers could be one of the reasons for the genotyping failure of the G4 strains detected in this study.

Finally, this study has limitations: (i) only four farms located in the same province were surveyed, (ii) a low frequency of group A RV was detected and (iii) a low number of samples were typed; but to the author's knowledge, this is the first report of circulation of P[6]G4; P[6]G8 and P[1]G6, in Argentinean swine. The high diversity of RV found in such limited study with samples derived from newborn piglets without diarrhea highlights the importance of conducting a larger survey to better define the RV strains circulating in the porcine population. A better understanding of the RV circulating in porcine worldwide will contribute to increase our knowledge of the complex epidemiology of rotaviruses. In addition, the fact that G4 porcine lineages were epidemiologically related with human strains, and G6 and G8 Argentinean porcine strains were found related to bovine and South-American camelids, respectively, suggests that pigs might play a crucial role as reservoir and generator of newly adapted emerging RV strains, for human and other species.

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