Infection, Genetics and Evolution 27 (2014) 402–407

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Short communication

Genomic characterization of a rotavirus G8P[1] detected in a child with diarrhea reveal direct animal-to-human transmission

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ARTICLE INFO

Article history:
Received 13 May 2014
Received in revised form 13 August 2014
Accepted 14 August 2014
Available online 25 August 2014

Keywords:
Rotavirus
Interspecies transmission
Bovine-like rotavirus G8P[1]

ABSTRACT

Group A rotavirus is a major cause of severe gastroenteritis in children and young animals. During a retrospective analysis of samples collected from Paraguayan children under 5 years old with diarrhea, and previously negative for rotavirus and norovirus, we detected the presence of bovine rotavirus sequences by viral metagenomics. Nucleic acid was extracted direct from stool sample and determined to be G8P[1]. The genomic analyzes revealed that the strain presents an Artiodactyl-like genome (G8-P[1]-I2-R2-C2-M1-Ax-N2-T6-E12-H3) suggesting a direct animal-to-human transmission.

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Group A rotaviruses (RVAs) are one of the major pathogens causing diarrhea in children and young animals worldwide. The genome of rotaviruses consists of 11 double-stranded RNA (dsRNA) segments that encode 6 structural (VP1–4, VP6, VP7) and 6 non-structural proteins (NSP1-6). RVA present a triple-layered protein capsid, and the most outermost surface proteins, VP7 and VP4, independently induce neutralizing antibodies. Genetic and antigenic differences within these two proteins have been used to classify rotaviruses in G and P types, respectively (Estes and Greenberg, 2013). Recently, an extended genotyping system based on genetic differences within all 11 segments was established. This new classification system has provided valuable information on the RVA genetic diversity and allowed the precise identification of the genetic relationships among human and animal rotaviruses (Matthijnssens et al., 2008a).

Surveillance of rotavirus has revealed that five strains (G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8]) largely predominate in humans worldwide (Santos and Hoshino, 2005). The detection of uncommon strains in humans has been linked to animal strains, and their increasing frequency has raised concerns for massive vaccination programs (Degiuseppe et al., 2013; Martella et al., 2010; Weinberg et al., 2012). On this regard, G8 strains have been associated with diarrhea in cattle and other species from the Artiodactyl order (Fukai et al., 1999; Okada and Matsumoto, 2002; Parreno et al., 2004), but also have been found circulating in humans at high frequencies in Africa (Cunliffe et al., 1999), and sporadically in Europe, the Americas, and Asia (Ahmed et al., 2013; Banyai et al., 2009b; Delogu et al., 2013; Park et al., 2011; Pietsch et al., 2009). This study describes the detection of a G8P[1] rotavirus strain in stool sample from a Paraguayan child presenting acute gastroenteritis (AGE).

A total of 118 fecal samples collected from children ⩽5 years old with non-bacterial AGE were analyzed by Next Generation Sequencing (NGS) to identify potential new viruses associated with this disease. The patients were admitted as ambulatory or hospitalized patients in a large private hospital from Asuncion, Paraguay between January 2004 and December 2005. The samples were randomly selected from a set of 205 previously tested negative for rotavirus and norovirus. (Amarilla et al., 2007; Galeano et al., 2013; Parra et al., 2005). A total of 140 μl of the stool supernatant was filtered through a 0.45-μm filter (Millipore), and the filtrate was treated with a cocktail of DNases (Turbo DNase from Ambion, Baseline-ZERO from Epicentre, and Benzonase from Novagen) and RNase A (Fermentas) to digest unprotected nucleic acids. Viral nucleic acids were extracted with the QIAamp spin-columns following manufacturer's instructions (Qiagen). The nucleic acids

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http://dx.doi.org/10.1016/j.meegid.2014.08.015
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		NSP2	NSP3	NSP4	NSP5	VP1	VP2	VP3	VP4	VP6	VP7
Human (G1P[8], G2P[4], G4P[6])	RVA/Human-tc/USA/Wa/1974/G1P1A[8]	83.0	78.0	79.7	94.8	76.1	81.5	95.7	63.0	80.2	69.6
	RVA/Human-wt/JPN/KU/1999/G1P[8]	ND	77.5	78.8	94.2	78.5	82.9	85.1	64.0	83.5	69.9
	RVA/Human-tc/USA/DS-1/1976/G2P1B[4]	88.4	78.6	83.3	89.7	84.3	85.0	76.6	64.2	88.4	69.3
	RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]	87.5	82.4	83.1	90.3	83.4	84.3	74.5	64.1	88.4	69.9
	RVA/Human-tc/GBR/ST3/1975/G4P2A[6]	83.6	80.2	79.1	92.9	77.3	82.2	85.1	62.2	78.5	70.0
Human (G8P[4], G8P[6], G8P[8])	RVA/Human-wt/DEU/GER1H-09/2009/G8P[4]	88.4	81.9	83.5	90.3	82.5	82.6	73.4	64.8	83.5	82.1
	RVA/Human-wt/CMR/6782/2000/G8P[6]	ND	80.2	80.6	94.8	76.4	81.9	98.9	63.5	77.7	81.9
	RVA/Human-wt/CMR/6809/2000/G8P[6]	84.2	80.2	81.3	94.8	76.4	81.5	98.9	63.0	85.1	81.7
	RVA/Human-wt/COD/DRC86/2003/G8P[6]	88.8	80.8	82.4	89.7	82.2	84.0	75.5	63.8	85.1	81.5
	RVA/Human-wt/COD/DRC88/2003/G8P[8]	88.4	81.3	82.0	90.3	82.8	84.0	74.5	61.9	86.0	81.4
	RVA/Human-wt/CMR/6780/2000/G8P[8]	83.6	80.2	80.4	94.8	75.2	81.9	98.9	64.2	77.7	82.4
	RVA/Human-wt/CMR/6787/2000/G8P[8]	84.2	80.2	79.7	94.8	75.8	81.5	92.6	64.2	78.5	82.2
	RVA/Human-wt/TUN/6862/2000/G8P[8]	85.1	80.2	79.3	94.8	76.1	81.2	98.9	64.4	78.5	82.2
	RVA/Human-wt/TUN/6854/2002/G8P[8]	84.2	80.2	79.3	94.8	76.1	81.5	98.9	64.4	76.9	82.2
	RVA/Human-wt/CIV/6736/2004/G8P[8]	85.1	79.7	79.3	94.2	75.8	81.9	98.9	64.4	76.9	82.3
	RVA/Human-wt/BRA/R291/2002/G8P[4]	ND	ND	81.9	ND	ND	ND	ND	63.3	ND	82.0
	RVA/Human-wt/ETH/6810/2004/G8P[8]	83.3	80.2	79.3	94.8	76.1	81.2	92.6	64.4	85.1	82.3
Human (G12P[8])	RVA/Human-wt/ARG/Arg7500/2009/G12P[8]	83.9	81.3	80.0	93.5	77.0	81.9	88.3	64.5	80.2	72.2
	RVA/Human-wt/ARG/Arg6653/2008/G12P[8]	83.9	81.3	79.1	93.5	76.1	81.9	87.2	64.5	80.2	72.2
	RVA/Human-wt/ARG/Arg6627/2008/G12P[8]	83.3	80.2	79.3	92.9	77.0	81.9	87.2	64.5	80.2	72.1
Artiodactyla (G6P[1], G6P[5], G6P[14], G8P[1], G8P[14], G15P[11])	RVA/Cow-tc/NGA/NGRBg8/1998/G8P[1]	ND	ND	ND	ND	ND	ND	ND	ND	ND	81.5
	RVA/Cow-tc/USA/NCDV/1967/G6P6[1]	93.3	89.6	82.2	97.4	95.2	87.5	74.5	92.2	87.6	73.0
	RVA/Cow-tc/FRA/RF/1982/G6P[1]	92.7	91.2	82.0	97.4	94.6	87.5	74.5	91.6	86.8	73.2
	RVA/Cow-tc/VEN/BRV033/1990/G6P6[1]	90.6	89.0	79.1	96.1	89.7	83.6	75.5	87.5	81.8	70.7
	RVA/Cow-tc/GBR/UK/1973/G6P7[5]	90.0	81.3	81.5	96.8	78.5	87.1	73.4	64.5	86.8	73.0
	RVA/Cow-tc/USA/WC3/1981/G6P[5]	95.1	91.8	80.6	97.4	94.6	87.1	72.3	63.4	88.4	73.1
	RVA/Cow-wt/ZAF/1603/2007/G6P[5]	90.0	92.9	82.4	98.1	90.6	91.6	75.5	66.0	85.1	84.7
	RVA/Cow-wt/ZAF/1605/2007/G6P[5]	90.0	92.9	82.2	98.1	90.6	91.6	75.5	66.2	86.0	71.7
	RVA/Cow-wt/ZAF/1604/2007/G8P[1]	94.5	84.6	82.0	98.1	92.7	88.2	74.5	90.1	85.1	71.7
	RVA/Cow-tc/THA/A5-13/1988/G8P[1]	ND	ND	ND	ND	ND	ND	ND	76.9	ND	82.4
	RVA/Cow-wt/ARG/B383/1998/G15P[11]	95.4	87.9	95.3	100.0	78.9	82.9	71.3	49.4	84.3	70.7
	RVA/Goat-tc/BGD/GO34/1999/G6P[1]	88.4	90.1	83.5	98.1	82.2	86.4	71.3	78.4	81.8	74.9
	RVA/Goat-tc/BGD/GO100/1999/G6P[1]	ND	ND	83.5	98.1	ND	ND	ND	78.4	81.8	74.9
	RVA/Goat-tc/BGD/GO102/1999/G6P[1]	ND	ND	83.5	98.1	ND	ND	ND	78.4	81.8	74.7
	RVA/Antelope-wt/ZAF/RC-18-08/G6P[14]	91.2	87.4	82.6	97.4	90.0	92.0	73.4	61.1	87.6	73.0
	RVA/Guanaco-wt/ARG/Rio_Negro/1998/G8P[1]	87.2	87.4	92.9	97.4	79.2	90.2	72.3	79.5	86.0	87.0
	RVA/Guanaco-wt/ARG/Chubut/1999/G8P[14]	90.6	92.3	98.2	98.7	78.9	90.9	70.2	63.7	87.6	86.7
	RVA/Vicuna-wt/ARG/C75/2010/G8P[14]	90.3	92.9	71.9	ND	95.2	91.3	70.2	63.5	88.4	86.5
	RVA/Sheep-tc/ESP/OVR762/2002/G8P[14]	89.7	90.1	83.5	97.4	85.8	89.9	71.3	62.0	87.6	86.2
	RVA/Rhesus-tc/USA/PTRV/1990/G8P[1]	94.5	90.7	81.7	98.1	95.5	87.5	70.2	91.6	84.3	87.0
Human infected Artiodactyla-like rotaviruses	RVA/Human-tc/ITA/PA169/1988/G6P[14]	89.4	92.9	83.3	98.1	88.8	88.2	78.7	62.6	86.8	72.8
	RVA/Human-tc/AUS/MG6/1993/G6P[14]	90.6	89.6	82.9	98.1	94.6	86.8	75.5	62.7	87.6	73.5
	RVA/Human-wt/HUN/Hun5/1997/G6P[14]	89.1	90.1	82.2	97.4	83.1	90.2	72.3	62.6	85.1	73.8
	RVA/Human-wt/BEL/B10925/1997/G6P[14]	89.1	90.1	83.5	98.1	84.6	89.5	70.2	63.0	86.8	73.2
	RVA/Human-wt/ITA/111-05-27/2005/G6P[14]	91.8	90.1	83.5	98.1	85.2	89.5	74.5	63.1	86.8	73.1
	RVA/Human-tc/KEN/B12/1987/G8P[1]	91.2	93.4	81.3	98.7	93.4	96.5	75.5	79.2	88.4	86.7
	RVA/Human-tc/IND/69M/1980/G8P4[10]	91.2	80.2	82.2	94.8	83.7	90.6	77.7	68.6	83.5	85.1
	RVA/Human-wt/HUN/BP1062/2004/G8P[14]	88.4	92.3	83.5	98.1	82.8	85.7	75.5	62.7	90.1	81.4
	RVA/Human-wt/BRA/R239/200X/G10P[9]	ND	ND	97.6	ND	ND	ND	ND	62.0	ND	74.6
	RVA/Human-wt/ARG/Arg4605/2006/G4P[6]	86.0	79.1	79.5	94.8	81.0	80.8	86.2	62.7	78.5	69.9
	RVA/Human-wt/ARG/Arg4671/2006/G4P[6]	86.0	78.0	80.0	95.5	79.2	81.5	86.2	62.9	78.5	68.6
	RVA/Human-tc/NGA/HMG035/2000/G8P[1]	ND	ND	ND	ND	ND	ND	ND	81.7	ND	81.4
	RVA/Pig-tc/USA/OSU/1977/G5P9[7]	84.8	79.7	80.2	94.8	79.5	81.5	87.2	71.8	83.5	74.7
Perissodactyla (G3P[12], G13P[18], G14P[12])	RVA/Horse-wt/ARG/E30/1993/G3P[12]	87.8	84.1	92.4	87.7	84.9	88.9	80.9	68.9	78.5	73.4
	RVA/Horse-wt/IRL/03V04954/2003/G3P[12]	87.2	80.2	82.0	87.7	83.7	89.5	80.9	68.9	80.2	73.7
	RVA/Horse-tc/GBR/L338/1991/G13P[18]	83.3	74.2	77.7	91.0	78.2	78.4	79.8	73.9	74.4	71.0
	RVA/Horse-wt/IRL/04V2024/2004/G14P[12]	88.1	80.2	81.7	87.1	83.4	87.5	79.8	68.9	77.7	73.3
	RVA/Horse-wt/ARG/E403/2006/G14P[12]	88.4	81.9	92.2	87.7	84.3	88.9	80.9	69.3	81.8	74.8
	RVA/Horse-wt/ZAF/EqRV-SA1/2006/G14P[12]	87.8	79.7	81.7	87.1	83.7	88.2	79.8	69.5	78.5	72.8
	RVA/Horse-wt/ARG/E4040/2008/G14P[12]	88.8	82.4	92.0	87.1	84.3	87.5	81.9	69.1	82.6	74.7

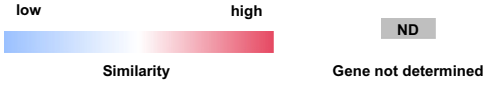

Fig. 1. Heat map showing the genetic differences between the Paraguayan G8P[1] strain and other rotavirus strains detected in various animals and humans. The nomenclature suggested by the rotavirus classification-working group (RCWG) was used for each sample. Information on host-specie, genotype, place and date of isolation are described in their names. Maximum and minimum values of similarities were selected for each gene for the heat map. Color legend is indicated at the bottom of the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

from five fecal samples were pooled together. Viral metagenome library was prepared by ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicentre) and run on the Miseq Illumina platform. Illumina reads and assembled contigs >100-bp were compared to the GenBank protein databases using BLASTx. A stringent *E* value of 10^{-10} was used as the cut-off for highly significant sequence similarity to known viruses. In one of the pools nine sequences showed significant BLASTx matches with bovine rotaviruses. The pool also presented multiple reads from human norovirus and enteric adenoviruses. Of note is that initial screening of rotaviruses was done by detection of characteristic viral dsRNA in polyacrylamide gel electrophoresis (PAGE), while for noroviruses a set of generic primers targeting the RNApol gene (primers Mon431/Mon432 and Mon433/Mon434) were used (Amarilla et al., 2007; Galeano et al., 2013). To further investigate the presence of bovine rotavirus and human noroviruses in those samples, RNA was extracted from a 10% fecal suspension from each of the five samples using Boom's method (Boom et al., 1990), and analyzed by

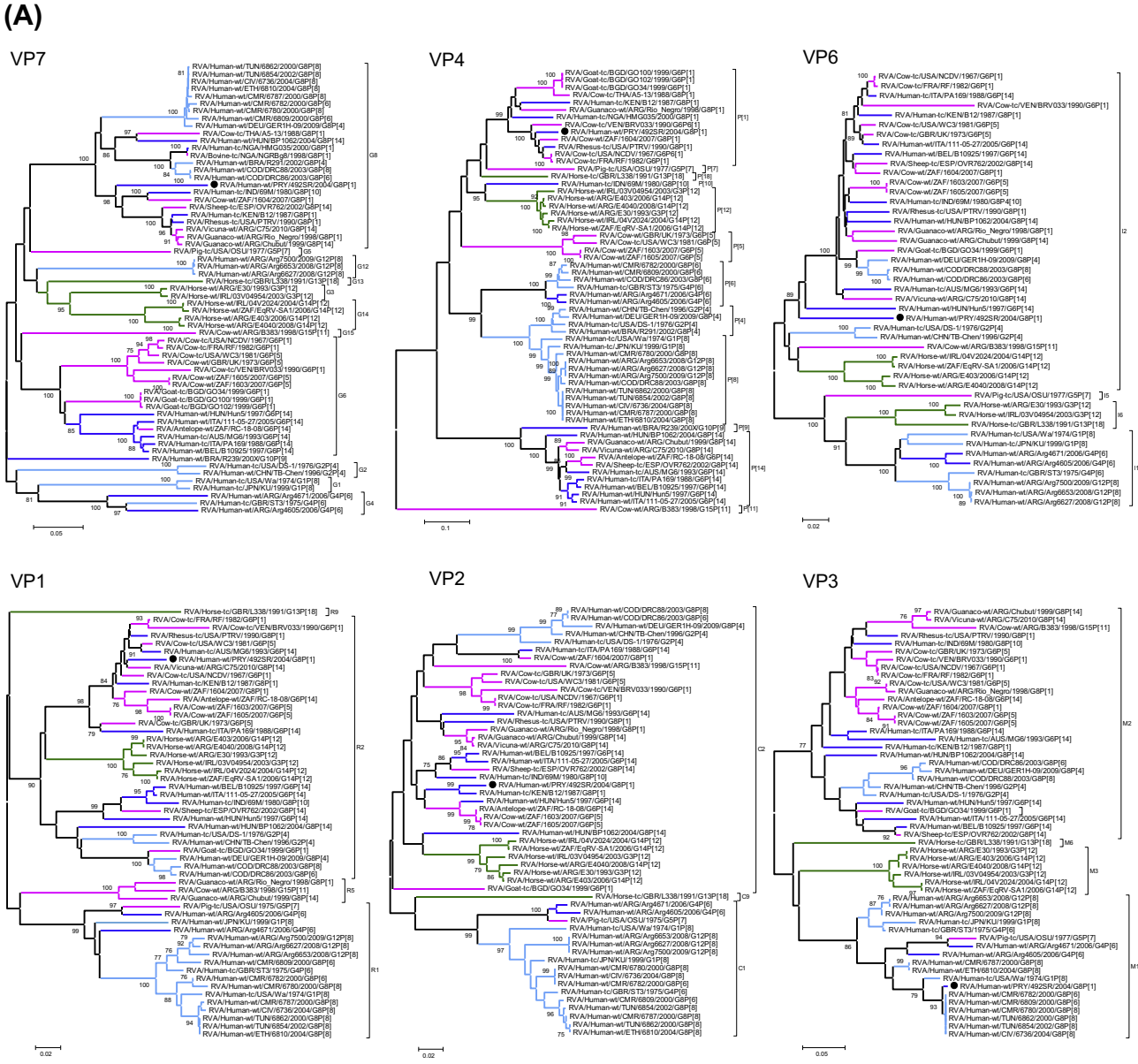

Fig. 2. Phylogenetic trees of genes of G8P[1] rotavirus. The phylogenetic trees from the genes segment of structural proteins (A) and non-structural proteins (B) were constructed using the Neighbor-Joining method and Kimura 2-parameter as a model of nucleotide substitution using the software MEGA v5.2. The statistical significance was estimated by bootstrap method (1000 pseudo-replicates). The Paraguayan G8P[1] strain is indicated by a black square. Branches of the trees were color-coded according to the host: human (light blue), horses (green), different species from the Artiodactyl order (pink), Artiodactyl-like strains detected in humans (dark blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

RT-PCR using primers for the NSP5 gene from rotaviruses (Martinez et al., 2010) and a set of primers to detect the RNA polymerase (RNApol) gene from caliciviruses (Jiang et al., 1999; Rohayem et al., 2004). One sample (492SR) was positive for both rotavirus and norovirus (data not shown). G and P typing of the rotavirus-positive-sample was performed using a heminested multiplex PCR (Malik et al., 2012), revealing the presence of a G8P[1] strain. The sample 492SR was collected from a 9-month-old child with AGE during the rotavirus seasonal peak of 2004. No other strain was detected bearing this genotype in this year (Parra et al., 2007).

Ten gene segments were successfully amplified using primers that target conserved regions of the 5′- or 3′-end from each segment (primers sequences available upon request). Despite of multiple attempts using different set of primers, previously described (Degiuseppe et al., 2013; Fujii et al., 2012; Matthijnssens et al., 2006a) and newly designed (available upon request), we have no success amplifying the NSP1 gene. Amplicons were purified from agarose gels using QIAquick Gel Extraction Kit (Qiagen), and sequencing was performed by Macrogen Inc. (Seoul, Korea). The phylogenetic trees were constructed using the Neighbor-Joining method and Kimura 2-parameter as a model of nucleotide substitution using the software MEGA v5.05. The statistical significance was estimated by bootstrap method (1000 pseudo-replicates). The nucleotide sequences have been deposited in GenBank under accession numbers KJ803831–KJ803840.

Overall, most of the genes from the strain RVA/Human-wt/PRY/492SR/2004/G8P[1] showed a higher degree of similarity (average of 85.6%) with strains detected in different species from the Artiodactyl order (e.g., cow, goat, antelope, guanaco, and vicuna; Fig. 1) than for human strains (average of 80.1%); except for segment 3, which showed more similarity with human VP3 (88.5%) than an

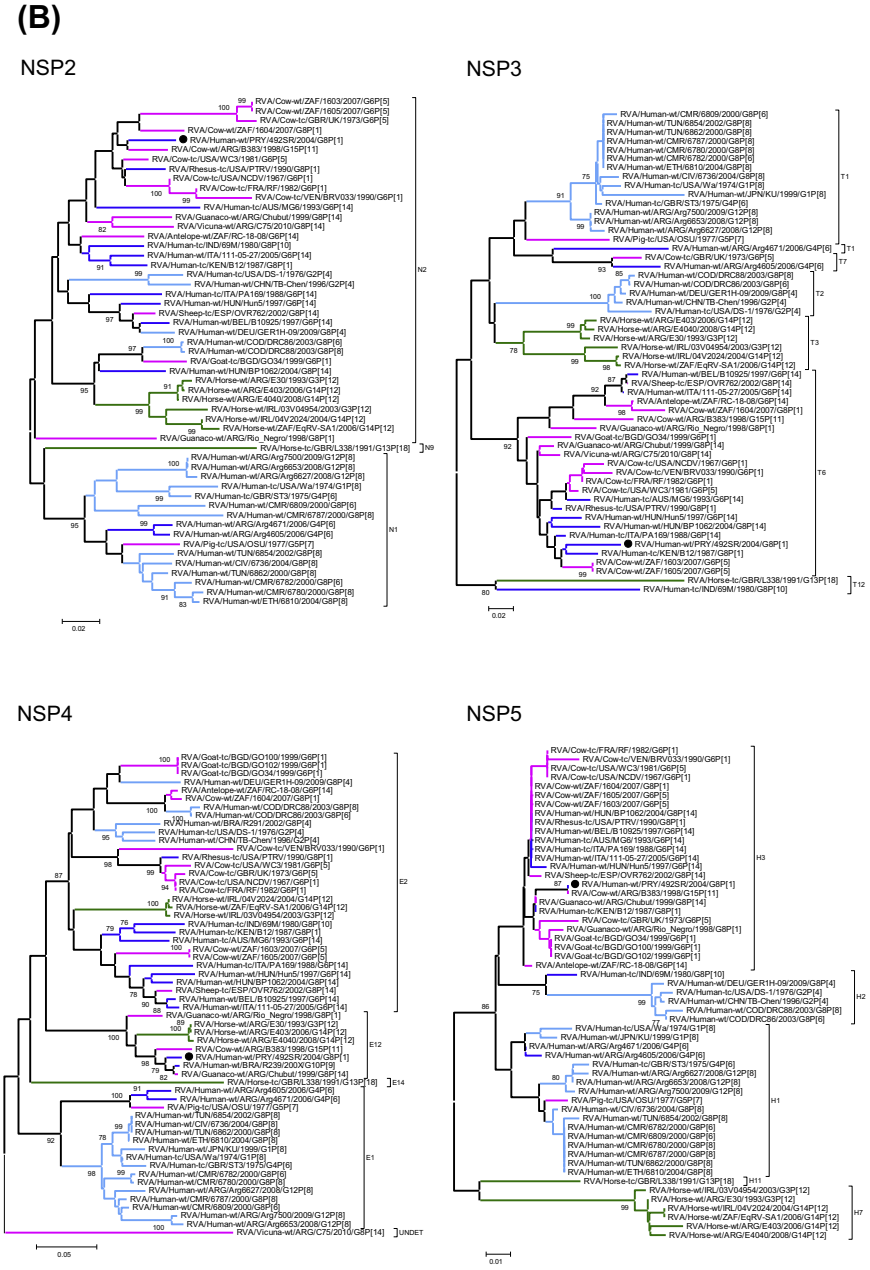

Fig. 2 (*continued*)

Artiodactyl-like gene (75%). Phylogenetic analyzes showed that the G8P[1] Paraguayan strain presented a G8-P[1]-I2-R2-C2-M1-Ax-N2-T6-E12-H3 genomic constellation (Fig. 2), which was similar to the ones described for Artiodactyls species (Matthijnssens et al., 2008a), and mainly composed by DS-1 gene segments.

Phylogenetic analyzes classified the VP1 genes from the Paraguayan G8P[1] closely related to other rotavirus strains detected in a South American camelid and clustered only with other Artiodactyl or Artiodactyl-like strains (Fig. 2A). VP2 and VP6 genes grouped with bovine-like strains detected in humans but relatively distant from other South American Artiodactyl strains (Fig. 2B). In contrast, the VP3 gene clustered with typical human strains and presented the genotype 1, suggesting an adaptation to the new host. The NSP2 gene also showed genotype 2 (N2), closely related to a bovine strain detected in Argentina. The NSP5 gene was the only segment that belonged to genotype 3 (H3), and the G8P[1] Paraguayan strain clustered with South American bovine strain bearing the genotypes G15P[11]. The combination of genes belonging to genotype 2 and 3, is a common feature of the genomes from rotavirus strains detected in Artiodactyl species (Matthijnssens et al., 2008a). The NSP3 gene was found to belong to the T6 genotype, which is phylogenetically distant from T2 and T3, but is in a cluster typically associated with rotavirus strains detected in artiodactyl animals (Fig. 2B). The NSP4 gene clustered within the genotype E12 (Fig. 2B), closely related to the G10P[9] Brazilian strain (R239) detected in humans, and the strains B383 (G15P[11]) and Chubut (G8P[14]) detected in cows and guanacos from Argentina. The E12 genotype was first described in Guanaco and Bovine rotavirus in Argentina (Matthijnssens et al., 2009), and recently has been characterized as part of a large conserved genome constellation in Argentinean horses (Fig. 2B) (Garaicoechea et al., 2011; Matthijnssens et al., 2012). Thus, these

findings expand the number of countries where strains bearing the E12 genotype were detected, and also reinforce the notion that this is a signature genotype for ungulates from South America.

The genotype P[1] is commonly found in cattle and caprine (Ciarlet et al., 1997; Fukai et al., 1999; Ghosh et al., 2010; Okada and Matsumoto, 2002), and in South America was described in camelids (Parreno et al., 2004) and porcine (Parra et al., 2008). The VP4 from the Paraguayan G8P[1] clustered with high bootstraps values (100%) with bovine rotavirus strains presenting G8 and G6 genotypes and detected in three different continents. (Fig. 2A). The gene from the VP7 of G8 strains is divided in three lineages (Fukai et al., 2004). Interestingly, the Paraguayan G8P[1] was clustered in the root of lineage I, which also group the G8 strains detected in Argentinean camelids (Fig. 2A), reinforcing the geographic clustering of the strains analyzed.

G8 strains causing AGE have been detected at high frequencies in the African continent, specially associated with DS1- and WA-like genes (Esona et al., 2009; Matthijnssens et al., 2006b). G8P[1] rotaviruses were mainly found in cattle (Ciarlet et al., 1997; Fukai et al., 1999; Okada and Matsumoto, 2002), and rarely reported in other species (Ghosh et al., 2010; Louge Uriarte et al., 2014). Only five cases of G8P[1] strains, all with constellation of genes more similar to animals, were reported in humans, being only three of them detected in children with diarrhea (Banyai et al., 2009a; Ghosh et al., 2011). The sequence and phylogenetic analyzes of the genes analyzed from the 492SR rotavirus-positive sample provides evidence for a direct interspecies transmission animal-to-human, with a potential reassortment with human strains as evidenced by the human-like VP3. Hoshino et al. proposed the role of VP3 in host range restriction (Hoshino et al., 1995), and reassortment between bovine and human strain yielding viable virus has been previously reported (Matthijnssens et al., 2008b, 2010). Thus, the introduction of human VP3 into a bovine backbone could enhance the infectivity of this strain in a human host. However, because we also detected norovirus in this sample it is difficult to determine the real cause of diarrhea in this child. Thus, continue monitoring of circulating rotavirus strains in humans and animals will be required to shed light into the mechanism by which rotaviruses transmit from different species.

Acknowledgment

This work was partially supported by a fellowship from Consejo Nacional de Ciencia y Tecnología (CONACYT), Paraguay to M.M.

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