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Complete molecular genome analyses of equine rotavirus A strains from different continents reveal several novel genotypes and a largely conserved genotype constellation

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In this study, the complete genome sequences of seven equine group A rotavirus (RVA) strains (RVA/Horse-tc/GBR/L338/1991/G13P[18], RVA/Horse-wt/IRL/03V04954/2003/G3P[12] and RVA/Horse-wt/IRL/04V2024/2004/G14P[12] from Europe; RVA/Horse-wt/ARG/E30/1993/G3P[12], RVA/Horse-wt/ARG/E403/2006/G14P[12] and RVA/Horse-wt/ARG/E4040/2008/G14P[12] from Argentina; and RVA/Horse-wt/ZAF/EqRV-SA1/2006/G14P[12] from South Africa) were determined. Multiple novel genotypes were identified and genotype numbers were assigned by the Rotavirus Classification Working Group: R9 (VP1), C9 (VP2), N9 (NSP2), T12 (NSP3), E14 (NSP4), and H7 and H11 (NSP5). The genotype constellation of L338 was unique: G13-P[18]-I6-R9-C9-M6-A6-N9-T12-E14-H11. The six remaining equine RVA strains showed a largely conserved genotype constellation: G3/G14-P[12]-I2/I6-R2-C2-M3-A10-N2-T3-E2/E12-H7, which is highly divergent from other known non-equine RVA genotype constellations. Phylogenetic analyses revealed that the sequences of these equine RVA strains are related distantly to non-equine RVA strains, and that at least three lineages exist within equine RVA strains. A small number of reassortment events were observed. Interestingly, the three RVA strains from Argentina possessed the E12 genotype, whereas the three RVA strains from Ireland and South Africa possessed the E2 genotype. The unusual E12 genotype has until now only been described in Argentina among RVA strains collected from guanaco, cattle and horses, suggesting geographical isolation of this NSP4 genotype. This conserved genetic configuration of equine RVA strains could be useful for future vaccine development or improvement of currently used equine RVA vaccines.

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INTRODUCTION

Equine group A rotavirus (RVA) strains were first detected in diarrhoeic foals in England in 1975 (Flewett *et al.*, 1975) and are a major cause of dehydrating diarrhoea in young

foals (Browning & Begg, 1996; Frederick *et al.*, 2009; Imagawa *et al.*, 1991; Saif *et al.*, 1994). Serological data from Japan, the USA and France suggest that RVA is a ubiquitous pathogen in horse populations (Conner & Darlington, 1980; Imagawa *et al.*, 1982; Pearson *et al.*, 1982; Takahashi *et al.*, 1979).

RVA strains are icosahedral, non-enveloped viruses possessing a genome of 11 segments of dsRNA. The two outer capsid proteins, VP7 and VP4, elicit neutralizing antibodies independently and are used to differentiate RVA strains into G-types (glycoprotein) and P-types (protease-sensitive), respectively (Ciarlet & Estes, 2002). Currently, 27 G-genotypes and 35 P-genotypes are recognized (Matthijnssens *et al.*, 2011a). A uniform sequence-based genotyping system encompassing all 11 RVA gene segments was developed using the following descriptor to classify RVA strains: Gx-Px-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (Matthijnssens *et al.*, 2008a). This classification system is maintained and updated by the Rotavirus Classification Working Group (RCWG) (Matthijnssens *et al.*, 2008b), which recently also developed a uniform naming scheme for RVAs (Matthijnssens *et al.*, 2011a).

G3 and G14 are the most common VP7 genotypes in horses worldwide and are almost always associated with the P[12] genotype (Ciarlet *et al.*, 1994; Collins *et al.*, 2008; Elschner *et al.*, 2005; Garaicoechea *et al.*, 2011; Iša *et al.*, 1996; Nemoto *et al.*, 2011; Tsunemitsu *et al.*, 2001; van der Heide *et al.*, 2005). Based on their reactivity with mAbs, equine G3 RVA strains can be discriminated into two subtypes, G3A and G3B (Browning *et al.*, 1992). Although G3 RVA strains have been encountered in a wide range of host species, the equine-like G3, G14 and P[12] genotypes have never been encountered in any other host species [although the P[12] genotype has been shown to be related serologically to the human P[10] genotype (Li *et al.*, 1996)]. A highly unusual equine RVA strain, RVA/Horse-tc/GBR/L338/1991/G13P[18], was detected in 1991 in the UK (Browning *et al.*, 1991a). L338 was shown to possess the unique G13 and P[18] genotypes and a highly divergent NSP1 gene sequence, and to be distinct from other human and animal RVA strains by using RNA–RNA hybridization assays (Browning *et al.*, 1991a; Iša & Snodgrass, 1994; Kojima *et al.*, 1996; Taniguchi *et al.*, 1994; Wu *et al.*, 1993). In addition, a limited number of porcine-, bovine- and feline-like RVA strains have been detected in horses. Examples include the G5P[7] RVA strain RVA/Horse-tc/GBR/H-1/1975/G5P[7], which was isolated from a foal in the UK in the mid-1970s, and was shown to be porcine in origin by using RNA–RNA hybridization, serological and sequencing methods (Ciarlet *et al.*, 2000, 2001; Flewett *et al.*, 1975; Hoshino *et al.*, 1983a; Iša & Snodgrass, 1994; Kojima *et al.*, 1996; Taniguchi *et al.*, 1994; Wu *et al.*, 1993). The RVA strain RVA/Horse-tc/GBR/26-94/199X/G8P[1], isolated in the UK, was shown to possess the typical bovine G8P[1] genotypes by using labelled probes (Iša *et al.*, 1996). An additional bovine RVA strain isolated from a foal is strain RVA/Horse-tc/JPN/R-22/1984/G10P[11], which was shown to possess the G10P[11] genotypes and was related closely to bovine RVA strains using RNA–RNA hybridization assays (Imagawa *et al.*, 1991, 1993). An Indian study revealed the presence of unusual bovine-like G6 and G10P6[1] RVA strains in horses in addition to suspected G1 strains between 2003 and 2005, although this paper was retracted later because of misclassification of G6 RVA strains as G16 (Gulati *et al.*, 2007). Recently, an unusual feline-like G3P[3] RVA strain (RVA/Horse-wt/ARG/E3198/2008/G3P[3]) was isolated from a foal in Argentina (Garaicoechea *et al.*, 2011).

To date, no complete genomes of equine RVA strains have been determined. In this study, the complete genomes of seven equine RVA strains (RVA/Horse-tc/GBR/L338/1991/G13P[18], RVA/Horse-wt/IRL/03V04954/2003/G3P[12] and RVA/Horse-wt/IRL/04V2024/2004/G14P[12] from Europe; RVA/Horse-wt/ARG/E30/1993/G3P[12], RVA/Horse-wt/ARG/E403/2006/G14P[12] and RVA/Horse-wt/ARG/E4040/2008/G14P[12] from Argentina; and RVA/Horse-wt/ZAF/EqRV-SA1/2006/G14P[12] from South Africa) were determined in order to evaluate the genetic relatedness and diversity of equine RVA strains.

RESULTS

Genotype constellations

The VP7, VP6, VP4, VP3 and NSP1 genes of L338 were determined previously (Browning *et al.*, 1991a; Cook & McCrae, 2004; Kojima *et al.*, 1996; Taniguchi *et al.*, 1994) and were shown to be representatives of unique genotypes (G13, I6, P[18], M6 and A6, respectively), related distantly to other known RVA strains (Matthijnssens *et al.*, 2008a). Sequence analyses of the six remaining gene segments (VP1, VP2, NSP2, NSP3, NSP4 and NSP5) of L338 revealed that they also did not belong to any of the established RVA genotypes, according to the guidelines of the RCWG (Matthijnssens *et al.*, 2008b). In addition, the NSP5 gene segments of the six other equine RVA strains were related closely to, but did also not belong to, any of the established NSP5 genotypes. Therefore, the sequences of the potentially new VP1, VP2 and NSP2–5 genotypes were submitted to the RCWG for analysis. Equine RVA strain E30 was assigned as the reference strain for a novel NSP5 genotype H7, and L338 was assigned as the reference RVA strain for the novel R9 (VP1), C9 (VP2), N9 (NSP2), T12 (NSP3), E14 (NSP4) and H11 (NSP5) genotypes.

The complete genotype constellations of the seven equine RVA strains investigated in this study are shown in Table 1. The genome configuration of equine RVA strain L338 is clearly distinct from those of the other six equine RVA strains, only sharing the I6 VP6 genotype with strains E30 and 03V04954. Equine RVA strains E30, E403, E4040, 03V04954, 04V2024 and EqRV-SA1 showed a noticeably conserved genotype constellation, sharing the P[12], R2, C2, M3, A10, N2, T3 and H7 genotypes (Table 1). Equine

Table 1. RVA genotype constellations of the seven equine RVA strains completely sequenced in this study, compared with selected partially sequenced equine RVA reference strains and fully sequenced non-equine RVA strains

RVA strain names are according to new RVA nomenclature rules (Matthijnssens *et al.*, 2011a). Genotypes of equine RVA genotype constellation are colour-coded in blue, orange, yellow and green. An open space refers to genotype not known, as sequencing has not been performed.

RVA strain name	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Horse-tc/GBR/L338/1991/G13P[18]	G13	P[18]	I6	R9	C9	M6	A6	N9	T12	E14	H11
RVA/Horse-wt/ARG/E403/2006/G14P[12]	G14	P[12]	I2	R2	C2	M3	A10	N2	T3	E12	H7
RVA/Horse-wt/ARG/E4040/2008/G14P[12]	G14	P[12]	I2	R2	C2	M3	A10	N2	T3	E12	H7
RVA/Horse-wt/ARG/E30/1993/G3P[12]	G3	P[12]	I6	R2	C2	M3	A10	N2	T3	E12	H7
RVA/Horse-wt/IRL/03V04954/2003/G3P[12]	G3	P[12]	I6	R2	C2	M3	A10	N2	T3	E2	H7
RVA/Horse-wt/IRL/04V2024/2004/G14P[12]	G14	P[12]	I2	R2	C2	M3	A10	N2	T3	E2	H7
RVA/Horse-wt/ZAF/EqRV-SA1/2006/G14P[12]	G14	P[12]	I2	R2	C2	M3	A10	N2	T3	E2	H7
RVA/Horse-tc/USA/FI23/1981/G14P[12]	G14	P[12]	I2				A10			E2	
RVA/Horse-tc/GBR/H-2/1976/G3P[12]	G3	P[12]	I2				A10			E2	
RVA/Horse-tc/USA/FI-14/1981/G3P[12]	G3*	P[12]	I6				A10			E2	
RVA/Horse-tc/JPN/HO-5/1982/G3P[12]	G3	P[12]	I6								
RVA/Human-wt/BEL/B4106/2000/G3P[14]	G3	P[14]	I2	R2	C2	M3	A9	N2	T6	E5	H3
RVA/Simian-tc/USA/RRV/1975/G3P[3]	G3	P[3]	I2	R2	C3	M3	A9	N2	T3	E3	H6
RVA/Human-tc/ISR/Ro1845/1985/G3P[3]	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
RVA/Human-tc/KEN/B12/1987/G8P[1]	G8	P[1]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Human-wt/HUN/BP1062/2004/G8P[14]	G8	P[14]	I2	R2	C2	M2	A11	N2	T6	E2	H3
RVA/Human-tc/USA/DS-1/1976/G2P1B[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-tc/USA/Se584/1998/G6P[9]	G6	P[9]	I2	R2	C2	M2	A3	N2	T1	E2	H3
RVA/Goat-tc/BGD/GO34/1999/G6P[1]	G6	P[1]	I2	R2	C2	M2	A11	N2	T6	E2	H3
RVA/Human-wt/COD/DRC88/2003/G8P[8]	G8	P[8]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BGD/RV176/2000/G12P[6]	G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E6	H2
RVA/Cow-wt/ARG/B383/1998/G15P[11]	G15	P[11]	I2	R5	C2	M2	A13	N2	T6	E12	H3
RVA/Guanaco-wt/ARG/Rio_Negro/1998/G8P[1]	G8	P[1]	I2	R5	C2	M2	A13	N2	T6	E12	H3
RVA/Guanaco-wt/ARG/Chubut/1999/G8P[14]	G8	P[14]	I2	R5	C2	M2	A11	N2	T6	E12	H3
RVA/Sheep-tc/CHN/Lamb-NT/XXXX/G10P[15]	G10	P[15]	I10	R2	C2	M2	A11	N2	T6	E2	H3
RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2]	G3	P[2]	I2	R2	C5	M5	A5	N5	T5	E2	H5
RVA/Human-tc/ITA/PA260-97/1997/G3P[3]	G3	P[3]	I3	R3	C3	M3	A15	N2	T3	E3	H6
RVA/Human-tc/JPN/AU-1/1982/G3P3[9]	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Rhesus-tc/USA/TUCH/2002/G3P[24]	G3	P[24]	I9	R3	C3	M3	A9	N1	T3	E3	H6
RVA/Human-tc/THA/T152/1998/G12P[9]	G12	P[9]	I3	R3	C3	M3	A12	N3	T3	E3	H6
RVA/Human-tc/USA/CC425/1997/G3P3[9]	G3	P[9]								E2	
RVA/Horse-tc/GBR/H1/1975/G5P[7]	G5	P[7]	I5				A8			E1	

*G3 determined by serology only (Hoshino *et al.*, 1983b).

RVA strains E30 and 03V04954 shared the G3 and I6 genotypes, whereas the equine RVA strains E403, E4040, 04V2024 and EqRV-SA1 shared the G14 and I2 genotypes. Interestingly, the three Argentinean equine RVA strains (E30, E403 and E4040) possessed the unusual E12 NSP4 genotype, whereas the remaining equine RVA strains, isolated in Ireland and South Africa, possessed the E2 genotype. The identification of these seven equine RVA genotype constellations suggests at least some level of reassortment among equine RVA strains, implicating the VP7, VP6 and NSP4 genes. The known genotypes of RVA/Horse-tc/USA/FI23/1981/G14P[12], RVA/Horse-tc/GBR/H-2/1976/G3P[12], RVA/Horse-tc/USA/FI-14/1981/G3P[12] and RVA/Horse-tc/JPN/HO-5/1982/G3P[12] are similar to those of the Argentinean, Irish and South African equine RVA strains analysed in this study, showing further evidence for the conserved genotype configuration and the capacity to exchange genotypes in reassortments (Table 1). Upon comparison of the complete genotype constellations of the equine RVA strains with the genotype constellations of other known RVA strains from humans and animals, only a limited relationship could be established; no more than six genotypes were found in common between equine RVA strains and RVA strains from any other host species (Table 1).

Phylogenetic analyses

In all of the 11 phylogenetic trees, equine RVA strain L338 clustered distinctly in a separate genotype, with the exception of the VP6 gene segment (Figs 1 and 2). The VP6 gene segment of strain L338 clustered with other equine RVA strains in genotype I6, although it forms a distinct

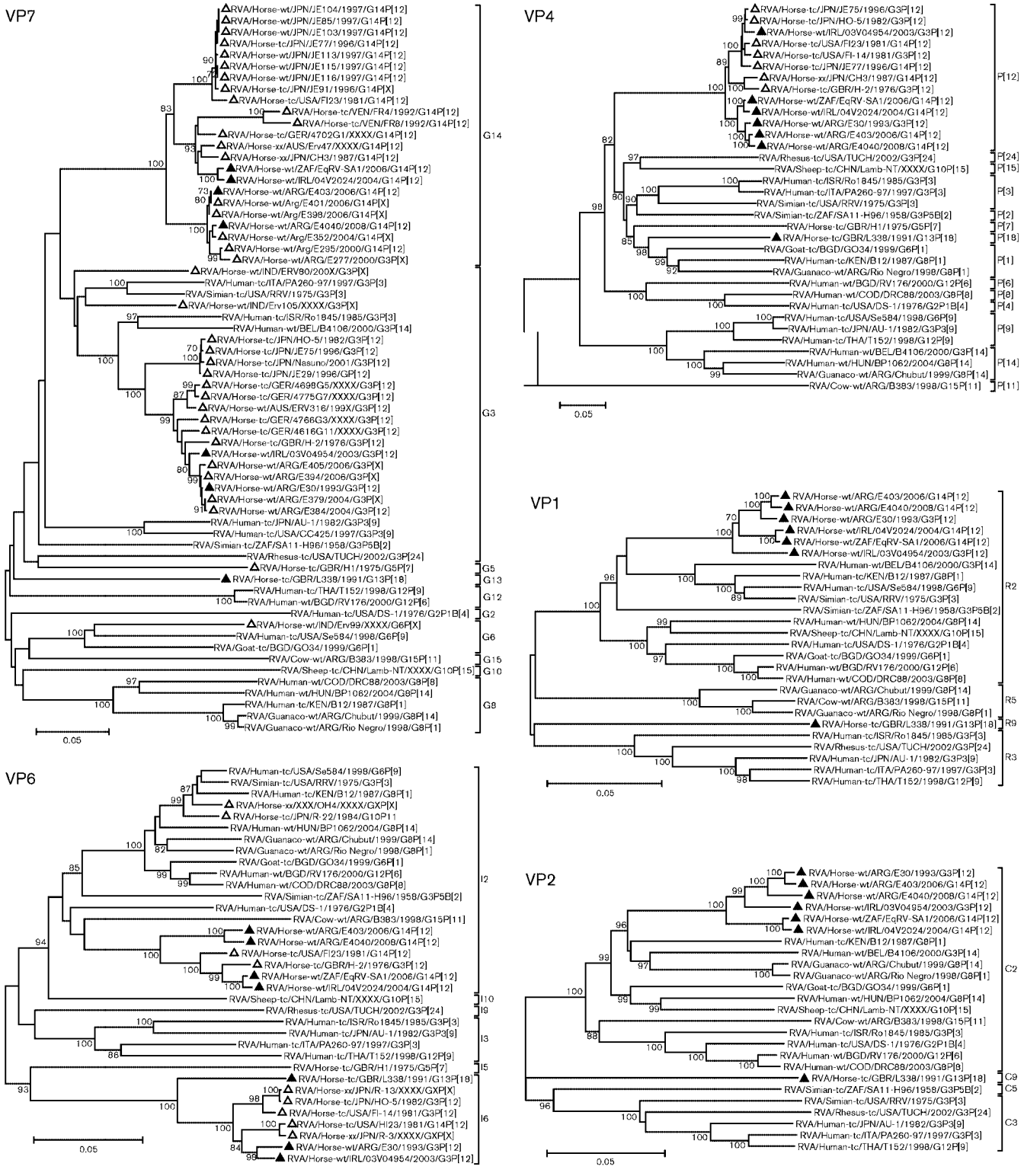

Fig. 1. Phylogenetic trees based on the full-length ORF nucleotide sequences of selected RVA VP1, VP2, VP4, VP6 and VP7 genes. Bootstrap values (1000 replicates) >70 % are shown. The seven equine RVA strains described in this study are marked with ▲. Other equine RVA strains are marked with △. RVA strain names are according to the new RVA nomenclature rules (Matthijnssens *et al.*, 2011a); genotypes are shown on the right. Bars, 0.05 nucleotide substitutions per site.

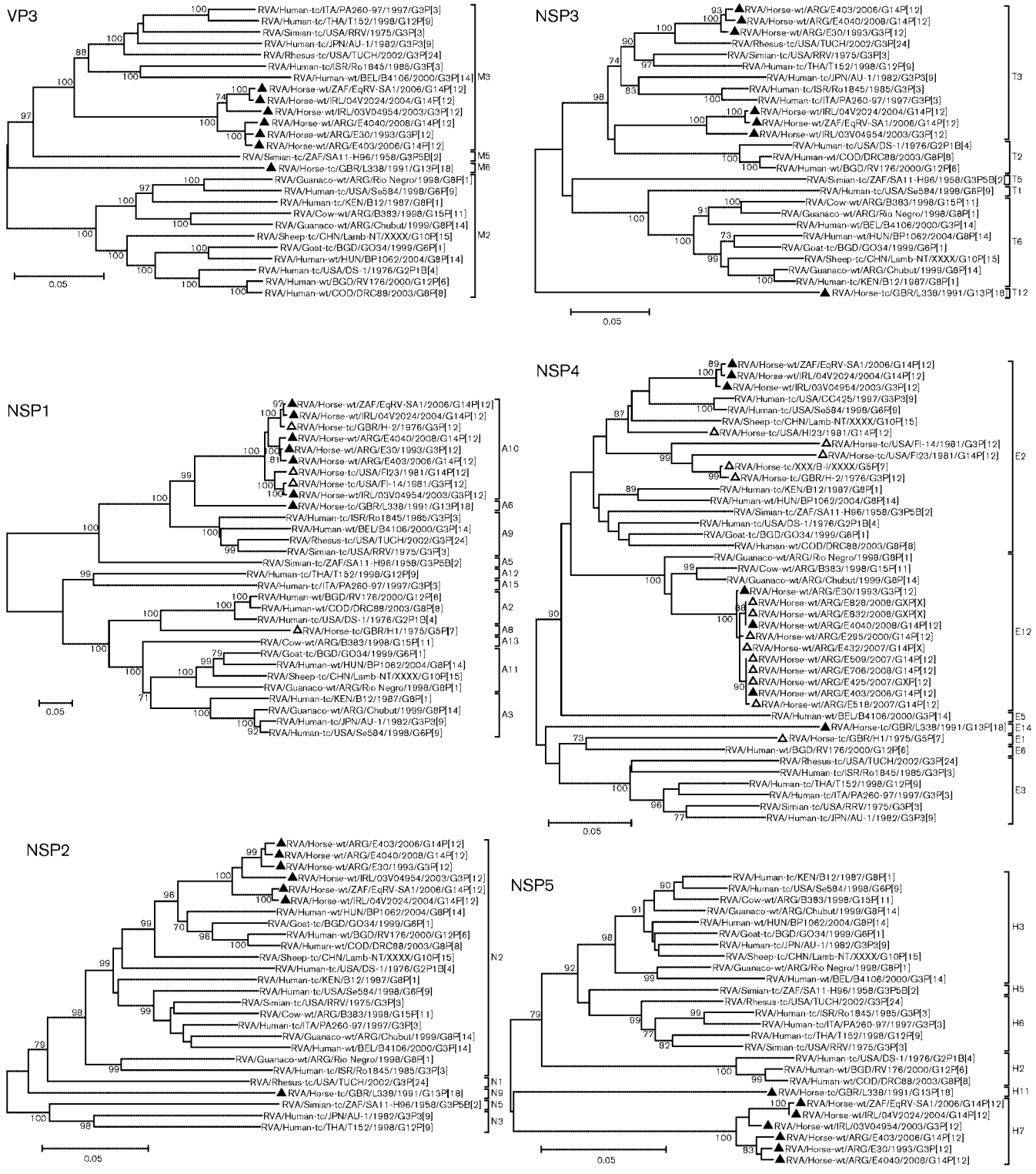

Fig. 2. Phylogenetic trees based on the full-length ORF nucleotide sequences of selected RVA VP3 and NSP1–5 genes. Bootstrap values (1000 replicates) >70 % are shown. The seven equine RVA strains described in this study are marked with ▲. Other equine RVA strains are marked with △. RVA strain names are according to the new RVA nomenclature rules (Matthijnssens *et al.*, 2011a); genotypes are shown on the right. Bars, 0.05 nucleotide substitutions per site.

branch from the other equine RVA strains within this genotype (including Argentinean and Irish equine RVA strains, E30 and 03V04954, respectively). The majority of the equine RVA strains carrying the VP6 I2 genotype form a monophyletic cluster within the I2 genotype; nevertheless, at least two lineages can be distinguished within the equine RVA monophyletic branch, including strains E403 and E4040 (Argentina), and 04V2024 (Ireland) and EqRV-SA1 (South Africa), respectively (Fig. 1). Two other equine RVA strains, OH4 and R-22, with a likely bovine-like origin, clustered in a different subcluster of the I2 genotype (Fig. 1).

For VP4 and NSP1, the equine RVA strains analysed in our study clustered together with other known equine RVA strains, in genotypes containing only equine RVA strains, P[12] and A10, respectively. The NSP5 gene segments of the six P[12] RVA strains investigated in this study are, to date, the only representatives of the novel H7 genotype. For these three gene segments, a very similar clustering pattern was observed, with the three Argentinean strains clustering closely together, the two G14P[12] strains from Ireland and South Africa clustering together and the Irish G3P[12] strain clustering more distantly (Figs 1 and 2). For the VP1-, VP2-, VP3- and NSP2-encoding gene segments, a very similar clustering pattern was observed as for VP4, NSP1 and NSP5. Although the respective R2, C2, M3 and N2 genotypes, in which these equine RVA strains clustered, are shared with RVA strains from many other host species, the equine RVA strains formed a distinct monophyletic branch within each of the R2, C2, M3 and N2 genotypes (Figs 1 and 2). In addition, within this monophyletic branch, a similar clustering pattern of the equine RVA strains to that described above was observed, with the three Argentinean strains, the G14P[12] strains from Ireland and South Africa and the G3P[12] strains from Ireland forming distinct lineages (Figs 1 and 2). For NSP3, the Irish and South African RVA strains formed a distinct monophyletic cluster within the T3 genotype, with the two G14P[12] strains again clustering very closely together. On the other hand, the three Argentinean equine RVA strains clustered closely together in a distinct branch within the T3 genotype (Fig. 2), in close proximity to the NSP3 of RVA/Rhesus-tc/USA/TUCH/2002/G3P[24] (93.2–93.5 % identity at the nucleotide level), but distantly from the Irish and South African equine RVA strains (85.2–86.0 % identity at the nucleotide level).

The VP7 G14 genotype formed a monophyletic cluster and contained only equine RVA strains (Fig. 1). Within the G14 genotype, a few lineages were obvious, with the G14 Argentinean strains clustering closely with other equine RVA G14 strains isolated in Argentina between 2000 and 2006. The Irish and South African G14 equine RVA strains clustered in a different lineage, along with equine RVA strains isolated in Japan, Germany, Australia and Venezuela (Fig. 1). The vast majority of the equine RVA G3 strains formed a monophyletic cluster within the G3 genotype. Equine G3 strains E30 (Argentina) and 03V04954 (Ireland) clustered closely together with a number of equine RVA strains isolated in Argentina, England, Australia and Germany (Fig. 1) across at least three decades. Two unusual equine G3 RVA strains isolated in India (RVA/Horse-wt/IND/ERV80/200X/G3P[X] and RVA/Horse-wt/IND/Erv105/XXXX/G3P[X]) clustered distantly from other RVA strains isolated from horses or other host species (Fig. 1). The NSP4 gene segments of the equine RVA strains from Ireland and South Africa clustered very closely together in a distinct branch within the E2 genotype, whilst the NSP4 of other equine RVA strains that belong to genotype E2 clustered separately (Fig. 2). Interestingly, the Argentinean equine RVA strains E30, E403 and E4040 clustered very closely together with other Argentinean RVA strains isolated between 2000 and 2008 within the E12 genotype. The only currently published non-equine RVA strains possessing the E12 NSP4 genotype belong to RVA isolated from cattle and llama guanaco (Matthijnssens *et al.*, 2009b) – all isolated in Argentina.

Equine RVA lineages

Based on the phylogenetic analyses, at least three lineages became apparent among the equine RVA strains studied, as shown in Table 2. Except for the VP7 (G3) and VP6 (I6) gene segments of RVA strain E30, the three Argentinean equine RVA strains (E30, E403 and E4040) were closely

Table 2. RVA genotype constellation of seven completely sequenced equine RVA strains, colour-coded according to lineages found in the phylogenetic analyses

RVA strain names are according to new RVA nomenclature rules (Matthijnssens *et al.*, 2011a). Different phylogenetic lineages are colour-coded in blue, orange, and shades of green and magenta.

RVA strain name	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Horse-tc/GBR/L338/1991/G13P[18]	G13	P[18]	I6	R9	C9	M6	A6	N9	T12	E14	H11
RVA/Horse-wt/ARG/E403/2006/G14P[12]	G14	P[12]	I2	R2	C2	M3	A10	N2	T3	E12	H7
RVA/Horse-wt/ARG/E4040/2008/G14P[12]	G14	P[12]	I2	R2	C2	M3	A10	N2	T3	E12	H7
RVA/Horse-wt/ARG/E30/1993/G3P[12]	G3	P[12]	I6	R2	C2	M3	A10	N2	T3	E12	H7
RVA/Horse-wt/IRL/03V04954/2003/G3P[12]	G3	P[12]	I6	R2	C2	M3	A10	N2	T3	E2	H7
RVA/Horse-wt/IRL/04V2024/2004/G14P[12]	G14	P[12]	I2	R2	C2	M3	A10	N2	T3	E2	H7
RVA/Horse-wt/ZAF/EqRV-SA1/2006/G14P[12]	G14	P[12]	I2	R2	C2	M3	A10	N2	T3	E2	H7

related to each other (97.3–99.6 % nucleotide identity among homologous gene segments, indicated in green in Table 2), suggesting a clonal origin combined with a recent reassortment event involving VP7 and VP6. A second lineage contained the Irish and South African G14P[12] RVA strains (04V2024 and EqRV-SA1), which showed high nucleotide identities (99.2–99.8 %) among all their respective 11 gene segments (shown in blue in Table 2), suggesting a recent common ancestor irrespective of the large geographical distance (>6000 miles/9600 km) between their places of detection. The NSP4 gene segment of the equine RVA strain 03V04954 was also related closely to the NSP4 gene segments of equine RVA strains 04V2024 and EqRV-SA1 (99.2 %), suggesting a recent common origin. The third lineage is currently only represented by the Irish equine RVA strain 03V04954 (magenta in Table 2). The genotypes G3 and I6 of strain 03V04954 were also related closely to the G3 and I6 genotypes of the equine RVA strain E30 (98.7 and 98.0 % identity at the nucleotide level, respectively).

DISCUSSION

Diarrhoea is one of the most common medical problems of newborn foals, and RVA is the most common cause of foal enteritis worldwide (Browning *et al.*, 1991b; Collins *et al.*, 2008; Dwyer, 2007; Frederick *et al.*, 2009; Garaicoechea *et al.*, 2011; Nemoto *et al.*, 2011). Three different inactivated parenteral equine RVA vaccines have been developed and tested, and are being used in several countries worldwide. All three vaccines, developed in the USA, Japan and Argentina, contain a G3P[12] equine RVA strain (H-2 or HO-5) (Barrandeguy *et al.*, 1998; Garaicoechea *et al.*, 2011; Imagawa *et al.*, 2005; Powell *et al.*, 1997). In addition, the Argentinean vaccine contains the simian RVA strain SA11 (G3P[2]) and the bovine RVA strain NCDV-Lincoln (G6P[1]) (Barrandeguy *et al.*, 1998). No complete genomic information of equine RVA strains is currently available and, due to the importance of this information for future vaccine development or improvement, we determined the complete genome sequence of seven equine RVA strains with three different G/P-genotype combinations (G3P[12], G14P[12] and G13P[18]) isolated from three different continents (South America, Africa and Europe).

This report extends and confirms the unusual nature of equine RVA strain L338, a single isolate detected in 1991 from a diarrhoeic foal in Great Britain (Wu *et al.*, 1993). Epidemiological RVA surveys in equine populations in subsequent years in different countries have failed to detect any additional RVA strains with properties similar to those of L338. Complete genomic L338 RVA characterization revealed little or no relation to other RVA strains, which makes it difficult to speculate about its origin. Perhaps strain L338 may represent another example of interspecies transmission from an unknown species to horses (Ciarlet *et al.*, 2001).

The other six equine RVA strains analysed in this study revealed a great level of conservation in their genotype constellations, with at least eight gene segments possessing the same genotype. Only the VP7, VP6 and NSP4 gene segments showed the presence of two different genotypes: G3/G14, I2/I6 and E2/E12, respectively. Although only seven complete equine RVA genomes are currently known, the current analysis shows evidence of reassortment events, as has been shown for RVA strains from many other host species (Iturriza-Gómara *et al.*, 2011; Martella *et al.*, 2010; Matthijnssens *et al.*, 2011b; McDonald *et al.*, 2009; Schumann *et al.*, 2009). However, within the conserved equine genotype constellations, a number of different lineages could be distinguished. These lineages are provisional and designated rather arbitrarily, and more complete equine RVA genome data will be needed before the relationships of these lineages can be firmly established. Imagawa *et al.* (1994) also described the presence of different levels of genetic relatedness among co-circulating equine RVA strains by using RNA–RNA hybridization assays; however, whether the difference that they observed and the different lineages found in our current study can be correlated cannot yet be confirmed, and further sequence analyses of these strains need to be done to address this question. Nonetheless, at the lineage level, reassortment is an important mechanism for the generation of genetic diversity.

Along with earlier surveillance studies, our data indicate that epidemiologically important strains in horses carry either G3 or G14 VP7 and P[12] VP4 genotypes. However, previous detection of porcine-like RVA genes in the equine RVA strain H-1, typical bovine-like neutralization antigens in British, Indian and Japanese equine RVA strains, feline-like neutralization antigens in an Argentinean equine RVA strain and the enigmatic origin of L338 suggest that the genetic diversity of equine RVA strains may be increased by interspecies transmission events. Further studies are needed to determine whether any of these heterologous RVA strains could become important in horses and thus would represent additional equine RVA genogroups.

A striking observation was the presence of the E12 NSP4 genotype in the three Argentinean equine RVA strains, and not in the other equine RVA strains investigated. This E12 genotype was only recently described for the first time in two RVA strains (RVA/Guanaco-wt/ARG/Chubut/1999/G8P[14] and RVA/Guanaco-wt/ARG/Rio_Negro/1998/G8P[1]) isolated from guanacos, and the unusual G15P[11] bovine RVA strain RVA/Cow-wt/ARG/B383/1998/G15P[11], also in Argentina (Matthijnssens *et al.*, 2009b). A recent epidemiological study of RVA strains in foals in Argentina revealed the presence of this unusual E12 genotype in all analysed equine RVA strains from 1993 to 2008 (Garaicoechea *et al.*, 2011). Unpublished data showed that the E12 NSP4 genotype has also been detected in other Argentinean bovine and goat RVA strains (A. Badaracco, unpublished data). Within the limits of the small number of equine RVA strains studied, our finding that the NSP4 E12 genotype appears to be associated only with equine RVA strains

isolated in Argentina, and not with RVA strains isolated in Europe and Africa, is highly surprising, given that horses are transported frequently across all continents. Although this observation could be just a sampling artefact, the data might suggest that quarantine measures involved with horse (product) transportation might be successful in confining these E12-carrying RVA strains to South America. However, this geographical boundary appears not to be present between Europe and Africa, as two nearly identical G14P[12] RVA strains (04V2024 and EqRV-SA1) have been detected in Ireland and South Africa. In order to determine whether this apparent geographical dichotomy is absolute, analysis of additional equine RVA strains is necessary.

The data presented in this study suggest strongly that the vast majority of the currently circulating equine RVA strains are highly conserved, with only limited genetic diversity. Whether or not this high genetic conservation is suggestive of slow genetic diversification, which would be surprising given the natural history of RVA infection and the role of reassortment events that contribute greatly to the genetic diversification of RVA strains in nature, this serves as a solid basis for the development of effective vaccines against epidemiologically major RVA strains causing disease in horses. However, continued monitoring of circulating RVA strains will be crucial, as the possible genetic pressure of vaccines could itself influence the distribution of equine RVA genotypes, as has been suggested for equine and human RVA strains (Garaicoechea *et al.*, 2011; Matthijnssens *et al.*, 2009a).

METHODS

RVA strain collection. The unusual RVA strain RVA/Horse-tc/GBR/L338/1991/G13P[18] was isolated in the UK (Browning *et al.*, 1991a) and was kindly supplied by Dr Koki Taniguchi (Department of Virology and Parasitology, School of Medicine, Fujita Health University, Toyoake, Aichi, Japan). RVA strains RVA/Horse-wt/ARG/E403/2006/G14P[12], RVA/Horse-wt/ARG/E4040/2008/G14P[12] and RVA/Horse-wt/ARG/E30/1993/G3P[12] were isolated in Argentina during a large RVA-surveillance programme in horses (Garaicoechea *et al.*, 2011). RVA strains RVA/Horse-wt/IRL/03V04954/2003/G3P[12] and RVA/Horse-wt/IRL/04V2024/2004/G14P[12] were isolated during a retrospective passive surveillance study from diarrhoeic foals in Ireland (Collins *et al.*, 2008). RVA/Horse-wt/ZAF/EqRV-SA1/2006/G14P[12] was isolated in Brits, North West Province, South Africa, in July 2006.

Sequencing. Entire genome sequences, including the 5′- and 3′-end sequences of RVA strains L338, E30, E403 and E4040, were determined as described previously (Matthijnssens *et al.*, 2011b). The complete genome of RVA strain EqRV-SA1 was determined using a sequence-independent method described previously (Potgieter *et al.*, 2002), with significant improvements (Potgieter *et al.*, 2009). The complete genomes of the Irish equine RVA strains 03V04954 and 04V2024 were sequenced by using the primer-walking method. The 5′ and 3′ ends were determined by using a strategy described elsewhere (Matthijnssens *et al.*, 2006).

Phylogenetic analysis. Multiple sequence alignments were constructed in CLUSTAL_X v. 2 (Larkin *et al.*, 2007) and subsequently edited in MEGA5 (Tamura *et al.*, 2011). Phylogenetic and molecular evolutionary analyses were conducted using MEGA5 (Tamura *et al.*, 2011). Genetic distances were calculated using the Kimura two-parameter correction at the nucleotide level, and phylogenetic trees were constructed by using the neighbour-joining method with 1000 bootstrap replicates.

Genotyping. The genotypes of each of the 11 genome segments for all the RVA strains under investigation were determined according to the genotyping recommendations of the RCWG (Matthijnssens *et al.*, 2008b), using the RotaC online classification tool (http://rotac.regatools.be/) (Maes *et al.*, 2009). The sequences of the VP1, VP2, NSP2, NSP3, NSP4 and NSP5 genome segments of strain L338, and the NSP5 sequence of RVA strain E30 did not belong to any of the established RVA genotypes, and were submitted to the RCWG for appropriate genotype assignment.

GenBank accession numbers. GenBank accession numbers (VP1–VP4, VP6, VP7, NSP1–NSP5) for each individual genome segment are: JF712555–JF712565 (RVA/Horse-tc/GBR/L338/1991/G13P[18]), JF712566–JF712576 (RVA/Horse-wt/ARG/E30/1993/G3P[12]), JF712577–JF712587 (RVA/Horse-wt/ARG/E403/2006/G14P[12]), JN872865–JN872875 (RVA/Horse-wt/ARG/E4040/2008/G14P[12]), JQ345489–JQ345499 (RVA/Horse-wt/ZAF/EqRV-SA1/2006/G14P[12]) and JN903507–JN903528 for RVA/Horse-wt/IRL/03V04954/2003/G3P[12] and RVA/Horse-wt/IRL/04V2024/2004/G14P[12].

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