CHAPTER FOUR

PAPER TWO

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Whole genome analysis of multiple rotavirus strains from a single stool specimen using sequence-independent amplification and 454® pyrosequencing reveals evidence of intergenotype genome segment recombination

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ABSTRACT

Infection of a single host cell with two or more different rotavirus strains creates conditions favourable for evolutionary mechanisms like reassortment and recombination that can generate novel strains. Despite numerous reports describing mixed rotavirus infections, whole genome characterisation of rotavirus strains in a mixed infection case has not been reported. Double-stranded RNA, exhibiting a long electropherotype pattern only, was extracted from a single human stool specimen (RVA/Human-wt/ZAF/2371WC/2008/G9P[8]). Both short and long electropherotype profiles were however detected in the sequence-independent amplified cDNA derived from the dsRNA, suggesting infection with more than one rotavirus strain. 454® pyrosequencing of the amplified cDNA revealed co-infection of at least four strains. Both genotype 1 (Wa-like) and genotype 2 (DS-1-like) were assigned to the consensus sequences obtained from the nine genome segments encoding NSP1–NSP5, VP1–VP3 and VP6. Genotypes assigned to the genome segments encoding VP4 were P[4] (DS-1-like), P[6] (ST3-like) and P[8] (Wa-like) genotypes. Since four distinct genotypes [G2 (DS-1-like), G8, G9 (Wa-like) and G12] were assigned to the four consensus nucleotide sequences obtained for genome segment 9 (VP7), it was concluded that at least four distinct rotaviruses were present in the stool. Intergenotype genome recombination events were observed in genome segments encoding NSP2, NSP4 and VP6. The close similarities of some of the genome segments encoding NSP2, VP6 and VP7 to artiodactyl rotaviruses suggest that some of the infecting strains shared common ancestry with animal strains, or that interspecies transmission occurred previously. The sequence-independent genome amplification technology coupled with 454® pyrosequencing used in this study enabled the characterisation of the whole genomes of multiple rotavirus strains in a single stool specimen that was previously assigned single genotypes, i.e. G9P[8], by sequence-dependent RT-PCR.

1. Introduction

Human rotaviruses are the main cause of severe infant gastroenteritis. Each year 527 000 rotavirus-associated deaths occur, mostly in developing countries (Parashar et al., 2009). Rotaviruses represent a genus in the *Reoviridae* virus family. The mature rotavirus particle contains a 11-segmented double-stranded RNA (dsRNA) genome that is surrounded by three layers of capsid proteins. With the exception of genome segment 11 that encodes two proteins in some group A rotaviruses, each genome segment encodes one protein. There are six structural (VP1–VP4, VP6 and VP7) and six non-structural (NSP1–NSP5) viral proteins. The commonly used dual rotavirus classification system is based on the properties of the two outer capsid proteins, VP4 and VP7 (Estes and Kapikian, 2007). The P and G serotypes/genotypes refer to VP4 (protease-sensitive) and VP7 (glycoprotein), respectively. To date, 35 P and 27 G group A rotavirus genotypes have been defined (Matthijnssens et al., 2011), illustrating the diversity of rotavirus strains.

Point mutations, genome reassortment and recombination are thought to be the main evolutionary forces driving rotavirus strain diversity (Desselberger et al., 2001). Rotaviruses are prone to point mutations as they utilise a viral RNA-dependent RNA polymerase during replication that lacks proof-reading activity (Estes and Kapikian, 2007). One nucleotide mutation is estimated to occur per replication of one full rotavirus genome (Blackhall et al., 1996). Despite increasing the genetic diversity of rotaviruses (Espínola et al., 2008), such nucleotide variations have implications for the characterisation of rotaviruses as it affects serotype reactivity of rotavirus

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strains to monoclonal antibodies (Ciarlet et al., 1997) and primer binding sites used for sequence-dependent genotyping (Martella et al., 2004). The segmented dsRNA genome enables reassortment events between distinct strains infecting a single cell simultaneously (Gouvea and Brantly, 1995). Furthermore, it is believed that zoonotic transmission contribute significantly towards rotavirus strain diversity (Tsugawa and Hoshino, 2008; Ghosh et al., 2010; Martella et al., 2010). Some animal rotavirus strains are thought to be directly introduced into humans via interspecies transmission (Matthijnssens et al., 2006a; Tsugawa and Hoshino, 2008), while others are generated through apparent single or multiple-genome segment reassortment events between human and animal rotaviruses (Matthijnssens et al., 2008b). Several other evolutionary mechanisms like intragenic genome recombination (both inter-lineage and inter-sub-lineage) further expands rotavirus strain diversity (Suzuki et al., 1998; Parra et al., 2007; Phan et al., 2007).

Several approaches have been used to determine the origin and the genetic relationships of rotavirus strains. Recently, the full genome classification scheme based on the nucleotide sequence identity of all 11 rotavirus genome segments has proved to be an excellent tool for identifying and studying the evolution of unusual rotavirus strains (Matthijnssens et al., 2008a). It was shown that the G12 porcine RU172 strain could have been generated through reassortment of human and porcine rotaviruses (Ghosh et al., 2010). Interspecies transmission cases were also confirmed where canine and feline rotavirus strains were directly introduced into humans (Tsugawa and Hoshino, 2008), and Matthijnssens et al. (2006a) demonstrated that a strain RVA/Human-wt/BEL/B4106/2000/G3P[14] that contains an entire lapine genome caused severe disease in humans. Similarly, Matthijnssens et al. (2006b) and Esona et al. (2009) used the whole genome classification scheme to show that G8 human rotaviruses isolated across Africa share ancestral relationships with DS-1-like and animal rotavirus strains, respectively. Furthermore, Matthijnssens et al. (2008a) also illustrated that DS-1- and Wa-like rotaviruses share a common origin with bovine and porcine strains, respectively.

For genome reassortment or recombination to take place, a single host cell must be infected by two or more different rotavirus strains (Estes and Kapikian, 2007). Several molecular epidemiology rotavirus studies suggest that human infection with multiple rotavirus strains, known as mixed infection, is common (Arguelles et al., 2000; Nielsen et al., 2005; Iturriza-Gómara et al., 2009, 2011; Esteban et al., 2010; Han et al., 2010; Mwenda et al., 2010; Potgieter et al., 2010). These mixed infections may create conditions favourable for the generation of novel strains through genome segment reassortment or recombination. Despite numerous reports describing mixed rotavirus infections, no study has yet attempted to characterise the whole genomes of rotavirus strains in a mixed infection. In this study, both short and long electropherotype rotavirus profiles were detected in the cDNA synthesised from the dsRNA of strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8]. Initially a long electropherotype pattern was assigned and sequence-dependent RT-PCR using a cocktail of G1, G2, G3, G8, G9, G12, P[4], P[6] and P[8] genotype-specific primers (Gouvea et al., 1990; Gentsch et al., 1992; Das et al., 1994; Iturizza-Gómara et al., 2004), assigned G9P[8] to the sample. The consensus nucleotide sequences of all 11 genome segments of the multiple rotavirus strains in the sample were generated through sequence-independent genome amplification and 454® pyrosequencing. Sequence analysis suggested the possible origins for the genome segments of the infecting strains, and the role mixed infections could play in influencing evolutionary mechanisms such as genome recombination in the generation of novel rotaviruses.

2. Materials and methods

2.1. Rotavirus strain

Strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8] was obtained from the Viral Gastroenteritis Unit (VGU), National Institute for Communicable Diseases (NICD), South Africa. The stool sample was analysed as part of the routine surveillance activities within VGU which was approved by an ethics committee (protocol number M060449). The sample was collected in 2008 from a 27 month old male child presenting with diarrhoea at Gatesville Hospital, Western Cape Province, South Africa. No additional personal or clinical data were available.

2.2. Rotavirus dsRNA extraction, purification and ligation to oligonucleotides

Approximately 100 µg stool sample was suspended in 200 µl freshly prepared extraction buffer (20 mM Tris–HCl pH 7.4, 10 mM $CaCl_2$ and 0.85% NaCl). TRI-REAGENT–LS (Molecular Research Centre, Ohio, USA) was used to extract the total RNA from the faecal specimen, following the manufacturer's instructions with slight modifications. In brief, the purity of the dsRNA was improved by adding 100 µl DuPont™ Vertrel® XF (DuPont Fluorochemicals, Wilmington, USA) to the sample/TRI-REAGENT suspension. This was followed by the addition of 200 µl chloroform, centrifugation at 4 °C for 15 min at 16,000g, precipitation of dsRNA in isopropanol and centrifugation at room temperature for 30 min at 16,000g. The pellet was re-suspended in 90 µl elution buffer (MinElute gel extraction kit; Qiagen, Hilden, Germany). Contaminating single-stranded RNA (ssRNA) was removed from the sample by adding LiCl (Sigma, St. Louis, USA) to a final concentration of 2 M followed by incubation at 4 °C for 16 h and then centrifugation at 16,000g for 30 min. The integrity of the 11 dsRNA genome segments of rotavirus was evaluated on a 0.8% agarose gel (TBE) containing ethidium bromide. A PC3-T7loop oligonucleotide was ligated to the purified dsRNA as described before (Potgieter et al., 2009). The ligated dsRNA was purified using MinElute Gel extraction columns by following the manufacturer's recommendations (Qiagen, Hilden, Germany).

2.3. Sequence-independent cDNA synthesis and PCR amplification of the rotavirus genome

To synthesise and amplify rotavirus cDNA, the method described by Potgieter et al. (2009) was followed with slight modifications. In brief, 30 mM methyl mercury hydroxide (Alfa Aesar, Massachusetts, USA) was used to denature the purified oligo-ligated dsRNA. Reverse transcription was performed with 10 U Transcriptor High Fidelity Reverse Transcriptase (Roche, Mannheim, Germany), and the excess RNA was removed by adding NaOH (Sigma, St. Louis, USA) to a final concentration of 0.1 M. The cDNA was annealed at 65 °C for 1 h in the presence of 0.1 M Tris/HCl, pH 7.5 (Sigma, St. Louis, USA) and 0.1 M HCl. The cDNA was amplified with the PC2 primer, which is complementary to PC3-T7loop, in a 50 µl PCR reaction mixture that contained 1× Phusion buffer, 0.2 mM dNTPs, 5 µl cDNA and 1 U Phusion High Fidelity DNA polymerase (Finnzymes, Vantaa, Finland). Full-length cDNA was produced by incubating the reaction mixture at 72 °C for 1 min, and subsequent cycling conditions were used as described by Potgieter et al. (2009). Amplified cDNA products were analysed on 1% agarose gels (in TBE buffer) containing ethidium bromide.

2.4. Nucleotide sequencing using GS FLX technology and analysis of the 454® pyrosequence data

The amplified cDNA was purified with a QIAquick® PCR purification kit by following the manufacturer's instructions (Qiagen, Hilden, Germany). A ND-1000 spectrophotometer (NanoDrop Products, Wilmington, USA) was used to quantify the cDNA yield. The 454® pyrosequencing (Margulies et al., 2005) with the GS FLX Titanium (Roche, Mannheim, Germany) technology was performed at Inqaba Biotec (PTY), Pretoria, South Africa. The *GS FLX Titanium general library preparation method manual*, April 2009, and the *GS FLX Titanium sequencing method manual for rapid, paired-end*, and *cDNA rapid libraries (Lib-L), and for small volume emulsions (SV)*, revised January 2010 (Roche, Mannheim, Germany) were used to prepare DNA libraries, emulsion titrations and emulsion-based clonal amplification (emPCR amplification) (Roche, 2009a,b). The study sample was combined in one library with the RVA/Human-tc/USA/DS-1/1976/G2P1B[4] (Mlera et al., 2011) and RVA/Human-wt/MWI/1473/2001/G8P[4] (Jere et al., 2011) strains. These samples were barcoded with multiplex identifier (MID) tags 5, 6 and 7 (Holland et al., 2011), respectively.

Several packages embedded in DNASTAR® Lasergene™ software, version 8.1.2, (www.dnastar.com) were used to generate the consensus nucleotide sequences for each genome segment. In brief, 454® pyrosequence reads were assembled into contigs with SeqMan. The coverage and the orientations of the nucleotide sequence reads were determined in the alignment view of SeqMan. The Trace consensus nucleotide sequences were used. Where necessary, the sequences were edited in both EditSeq and SeqMan. The consensus nucleotide sequences were exported to EditSeq where the deduced amino acid (aa) sequences and the sizes of the functional proteins translated from the ORF of each nucleotide sequence were derived. The name of the consensus sequences of the genome segments was assigned by comparing the generated sequences to NCBI GenBank sequences with the Lasergene™-in-built search engine [basic local alignment sequence tool for nucleotides (BLASTn)]. The nucleotide sequences reported in this study were submitted to the NCBI GenBank under the accession numbers listed in Supplement 1.

2.5. Assignment of genotypes, percentage identities, SimPlot and phylogenetic analyses

All genotypes were assigned by a web-based automated rotavirus genotyping tool, RotaC (http://rotac.regatools.be; Maes et al., 2009). The nucleotide sequences of the reference strains were acquired from GenBank (Accession numbers in Supplement 2). BioEdit (Hall, 1999) was used to align the sequences. The Kimura-2 correction parameter, window size of 200 bp, step size of 20 bp with a consensus threshold of 100% was used for SimPlot analysis (Lole et al., 1999). Phylogenetic and molecular evolutionary analyses were performed using MEGA software, version 4.0 (Tamura et al., 2007). Genetic distances were calculated using the Kimura-2 correction parameter at the nucleotide level. The phylogenetic trees were inferred using the neighbour-joining method with 1000 bootstrap replicates.

3. Results

3.1. Whole genome amplification of strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8]

Strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8] was typed by sequence-dependent RT-PCR as G9P[8] unequivocally. Its dsRNA displayed a long electropherotype pattern (Fig. 1a). When all the 11 genome segments of strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8] were amplified, its cDNA displayed two distinct genome segment 11 profiles that are characteristic of both long and short electropherotype patterns (Fig. 1b) that are commonly associated with Wa- and DS-1-like rotaviruses, respectively (Estes and Kapikian, 2007). This suggested that the child was infected with more than one rotavirus strain. Three additional amplicons (A, B and C) were also observed (Fig. 1b).

3.2. Analyses of the 454® pyrosequence data

A total of 2.7 MB of 454® pyrosequence data containing 8 571 nucleotide sequences of average 400 bases read length was generated. Assembling the sequence reads and comparing the consensus sequences of the generated contigs to NCBI nucleotide sequences with BLASTn revealed that each genome segment was represented by at least 2–4 contigs. The average depth of sequence coverage for the contigs of each genome segment ranged as follows: genome segment 1 (20–40), 2 (9–40), 3 (4–40), 4 (40–69), 5 (40–61), 6 (76–192), 7 (9–119), 8 (21–56), 9 (9–56), 10 (55–71), and 11 (40–213).

3.2.1. Nomenclature and genotypes assigned to all the eleven rotavirus genome segments characterised from the study sample

The study strain was named according to the RCWG guidelines (Matthijnssens et al., 2011). The G and P genotypes in its nomenclature were based on the VP4 and VP7 genotypes initially assigned by sequenced-dependent based RT-PCR assay. To distinguish the consensus nucleotide sequences of the distinct rotavirus populations identified for each genome segment of strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8], the name of the protein that each genome segment encodes and an alphabetical letter was inserted after the common name, 2371WC, assigned to the study sample. For instance, the two consensus nucleotide sequences obtained for genome segment 1 that encode VP1 were designated as RVA/Human-wt/ZAF/2371WCVP1A/2008/G9P[8] and RVA/Human-wt/ZAF/2371WCVP1B/2008/G9P[8].

Two to five distinct consensus sequences for each of the 11 rotavirus genome segments were obtained from the single sample characterised in this study (Supplement 1). RotaC and phylogenetic analyses assigned both Wa- and DS-1-like genotypes to each genome segment of RVA/Human-wt/ZAF/2371WC/2008/G9P[8] (Table 1 and Supplement 3). In summary, both Wa- and DS-1-like genotypes were assigned to genome segments 1–3 (VP1–VP3), 5–8 (NSP1, VP6, NSP2, NSP3), 10 (NSP4) and 11 (NSP5). P[4] (DS-1-like), P[6] and P[8] (Wa-like) genotypes were assigned to genome segment 4 (VP4), while four distinct genotypes (G2, G8, G9 and G12) were assigned to genome segment 9 (VP7). This was unexpected considering that only G9P[8] genotypes were assigned to the sample initially with the nested genotype-specific RT-PCR assay. Based on the four distinct VP7 genotypes, it was concluded that the child was infected with at least four rotavirus strains, of which the other ten additional genome segments were either of Wa- or DS-1-like origin. The technology employed in this study determines simultaneously the consensus nucleotide sequences of all the genome segments of the distinct rotavirus populations present in the sample, which is analogous to the resolution of hetoroplasy in mitochondria DNA mixtures (Holland et al., 2011). It was, however, impossible to assign the unique Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex descriptor to each infecting strain as proposed by the RCWG (Matthijnssens et al., 2011) despite characterising multiple nucleotide sequences for each genome segment, but the genotypes identified suggest that the infecting rotavirus strains belonged to at least two distinct genotypes: Wa- and DS-1-like.

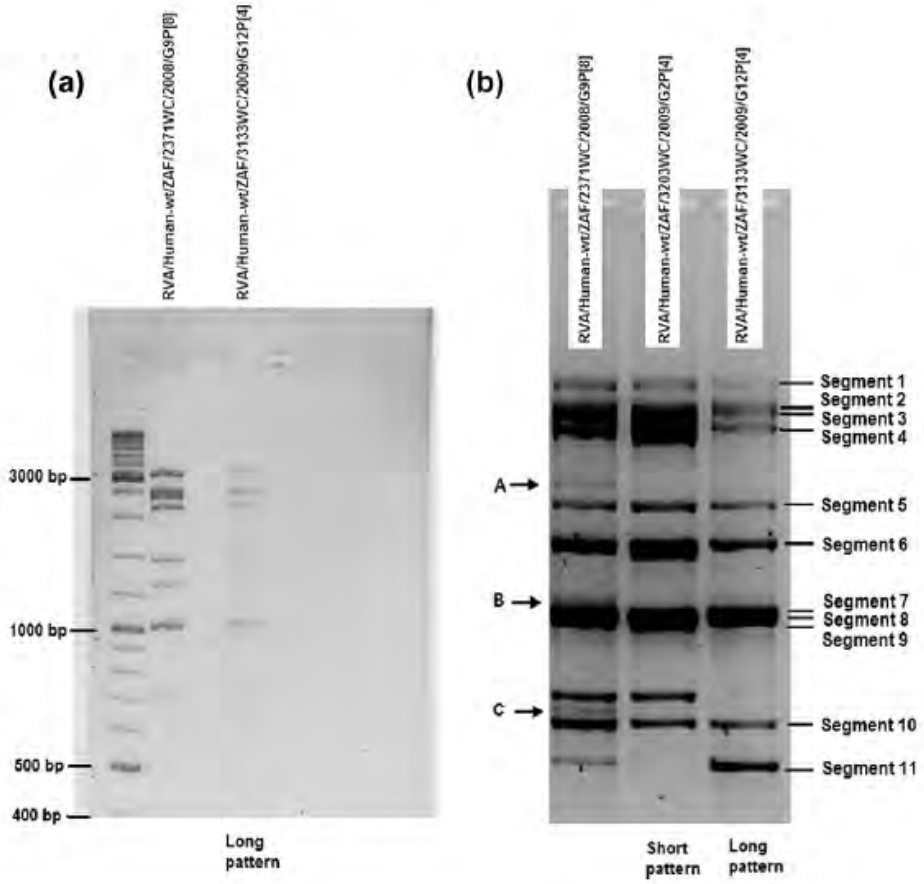

Fig. 1. One percent agarose gels of the purified dsRNA and PCR-amplified cDNA of the study specimen (RVA/Human-wt/ZAF/2371WC/2008/G9P[8]) compared to other reference strains. (a) Purified dsRNA of strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8] extracted directly from a stool sample compared to strain RVA/Human-wt/ZAF/3133WC/2008/G12P[4] that had a long electropherotype profile. (b) PCR-amplified cDNA of the study specimen (RVA/Human-wt/ZAF/2371WC/2008/G9P[8]) analysed with cDNA of two reference rotavirus strains exhibiting short (RVA/Human-wt/ZAF/3203WC/2009/G2P[4]) and long (RVA/Human-wt/ZAF/2371WC/2008/G12P[4]) electropherotype patterns. Additional amplicons to the known eleven rotavirus genome segment observed in the study sample were labelled A, B and C.

3.2.2. Phylogenetic, nucleotide and amino acid sequence analyses of the distinct rotavirus populations obtained for each genome segment from the study specimen

The consensus nucleotide sequences for all 11 genome segments obtained for strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8] that were assigned Wa-like (G9, P[8], I1, R1, C1, M1, A1, N1, T1 and E1) and DS-1-like (G2, P[4], I2, R2, C2, M2, A12 N2, T2 and E2) genotypes (Table 1) (Matthijnssens et al., 2008a) formed phylogenetic clusters with their respective prototype Wa and DS-1 strains (Supplement 3). The consensus nucleotide sequences that were assigned G8, G9 and P[6] genotypes that have not been associated with specific genogroups yet (Matthijnssens et al., 2008a, 2009, 2010), also clustered with the well characterised rotavirus strains that were assigned similar genotypes previously. For instance, both RVA/Human-wt/ZAF/2371WCVP4C/2008/G9P[8] and RVA/Human-wt/ZAF/2371WCVP4D/2008/G9P[8] clustered with P[6] rotavirus strains such as RVA/Human-wt/BGD/Dhaka12-03/2003/G12P[6], RVA/Human-wt/BGD/Matlab13/2003/G12P[6] and RVA/Human-wt/BGD/RV176-00/2000/G12P[6] isolated from Bangladesh (Supplement 3d). RVA/Human-wt/ZAF/2371WCVP7C/2008/G9P[8] clustered with G8 strains like RVA/Human-wt/COD/DRC86/2003/G8P[6], RVA/Human-wt/COD/DRC88/2003/G8P[8] and RVA/Human-wt/MWI/1473/2001/G8P[4] isolated from the Democratic Republic of Congo (DRC) and Malawi, whereas RVA/Human-wt/ZAF/2371WCVP7D/2008/G9P[8] clustered with G12 strains like RVA/Human-wt/BGD/N26/2002/G12P[6], RVA/Human-wt/BGD/RV176-00/2000/G12P[6] and RVA/Human-wt/ZAF/3133WC/2009/G12P[4] isolated from Bangladesh and South Africa (Supplement 3f).

Close relationships between four nucleotide sequences identified in this study to those of animal rotavirus strains were also revealed. RVA/Human-wt/ZAF/2371WCVP7C/2008/G9P[8] clustered with G8 bovine strain RVA/Cow-wt/NGA/NGRBg8/XXXX/G8P[X] and human-bovine reassortant strains (RVA/Human-wt/MWI/4103/2000/G8P[8], RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/MWI/MW4097/2000/G8P[8] and RVA/Human-wt/KEN/1290/1991/G8P[X]) that were isolated from Nigeria (Adah et al., 2003) Malawi and Kenya (Page et al., 2010; Jere et al., 2011), respectively (Supplement 3f). These results suggest that the genome segment 9 (VP7) of one of the co-infecting rotavirus strains in the sample that had a G8 genotype shared common ancestry with bovine rotaviruses as was shown previously (Matthijnssens et al., 2006b, 2008a; Esona et al., 2009). RVA/Human-wt/ZAF/2371WCNSP2B/2008/G9P[8] and RVA/Human-wt/ZAF/2371WCNSP2C/2008/G9P[8] clustered with artiodactyl rotavirus strains like RVA/Antelope-wt/ZAF/RC-18-08/G6P[14] and RVA/Sheep-tc/ESP/OVR762/2002/G8P[14] isolated from South Africa (Matthijnssens et al., 2009) and Spain (Ciarlet et al., 2008), respectively (Supplement 3h). This suggested potential previous occurrence of zoonosis or that some of the infecting strains share ancestry with animal rotaviruses. The rest of the other nucleotide sequences characterised in this study were closely related to human rotaviruses isolated elsewhere (Supplement 3).

Table 1
The genotypes and the size of the complete nucleotide and deduced amino acid sequences of all the 11 genome segments in the distinct rotavirus populations obtained for strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8]. The genotypes assigned to each obtained nucleotide sequence shown below does not represent the combinations of the genome segments in the virus particle for each strain.

Sizes of nucleotide sequences generated from sample RVA/Human-wt/ZAF/2371WC/2008/G9P[8] for each rotavirus genome segment

S9(VP7)	S4(VP4)	S6(VP6)	S1(VP1)	S2(VP2)	S3(VP3)	S5(NSP1)	S8(NSP2)	S7(NSP3)	S10(NSP4)	S11(NSP5)
1061[A]	2359[A]	1356[A]	3202[A]	2729[B]	2591[A]	1566[B]	1059[A]	1066[B]	750[A]	664[B,3]
531[B,1]	2359[B]	1356[B]	1363[B,2]	2684[A]	2591[B]	1566[A]	1059[B]	1074[A]	751[B]	816[A,4]
1062[C]	2359[C]	1356[C]			2591[C]		1059[C]		751[C]	
1062[D]	2359[D]	1356[D]							750[D]	
		1356[E]								

Sizes of the deduced amino acid sequences generated from sample RVA/Human-wt/ZAF/2371WC/2008/G9P[8] for each rotavirus genome segment

S9(VP7)	S4(VP4)	S6(VP6)	S1(VP1)	S2(VP2)	S3(VP3)	S5(NSP1)	S8(NSP2)	S7(NSP3)	S10(NSP4)	S11(NSP5)
326[A]	775[A]	397[A]	1088[A]	894[B]	835[A]	493[B]	317[A]	310[B]	175[A]	197[B,3]
159[B,1]	775[B]	397[B]	267[B,2]	879[A]	835[B]	493[A]	317[B]	310[A]	175[B]	200[A,4]
326[C]	775[C]	397[C]			835[C]		317[C]		175[C]	
326[D]	775[D]	397[D]							175[D]	
		397[E]								

Genotypes assigned to each nucleotide sequence generated for each genome segment characterised from sample RVA/Human-wt/ZAF/2371WC/2008/G9P[8]

S9(VP7)	S4(VP4)	S6(VP6)	S1(VP1)	S2(VP2)	S3(VP3)	S5(NSP1)	S8(NSP2)	S7(NSP3)	S10(NSP4)	S11(NSP5)
G9[A]	P[8][A]	I1[C]	R1[A]	C1[B]	M1[C]	A1[B]	N1[C]	T1[B]	E1[A]	H1[B]
G1[B]	P[4][B]	I2[B]	R2[A]	C2[A]	M2[A]	A2[C]	N2[A]	T2[A]	E2[B]	H2[A]
G9[C]	P[6][C]	I1[C]			M1[C]		A1[C]		E2[C]	
G12[D]	P[6][E]	I2[D]							E3[D]	
		I2[E]								

Genotypes assigned to the whole genome of seleceted rotavirus reference strains

Nomenclature of reference strains	S9(VP7)	S4(VP4)	S6(VP6)	S1(VP1)	S2(VP2)	S3(VP3)	S5(NSP1)	S8(NSP2)	S7(NSP3)	S10(NSP4)	S11(NSP5)
RVA/Human-tc/USA/Wa/1974/G1P1A[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/JPN/KU/XXXX/G1P1[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/USA/WI61/1983/G9P1A[8]	G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/BEL/B4633/2003/G12P[8]	G12	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/BGD/Dhaka25-02/2002/G12P[8]	G12	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/USA/DS-1/1976/G2P1B[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/COD/DRC86/2003/G8P[6]	G8	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-tc/IDN/69M/1980/G8P4[10]	G8	P[10]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/COD/DRC88/2003/G8P[8]	G8	P[8]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BGD/RV176-00/2000/G12P[6]	G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E6	H2
RVA/Human-tc/JPN/AU-1/1982/G3P3[9]	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Human-tc/THA/T152/1998/G12P[9]	G12	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Pigeon-tc/JPN/PO-13/1983/G18P[17]	G18	P[17]	I4	R4	C4	M4	A4	N4	T4	E4	H4

[A,B,C,D,E]Represent distinct consensus nucleotide sequences generated for each genome segment obtained in this study.
[1]A partial nucleotide sequence of 531base pairs (from nucleotide position 532–1062) and deduced amino acid sequence of 159 residues (amino acid position 168–326) were obtained for RVA/Human-wt/ZAF/2371WCVP7B/2008/G9P[8].
[2]A partial nucleotide sequence of 1363 base pairs (from nucleotide position 1615–2978) and deduced amino acid sequence of 267 residues (amino acid position 833–979) were obtained for RVA/Human-wt/ZAF/2371WCVP1B/2008/G9P[8].
[3]The short and long out-of-phase ORFs of genome segment 11 for RVA/Human-wt/ZAF/2371WCNSP5B/2008/G9P[8] that encodes NSP6 and NSP5 were translated from nt 22–615 and nt 80–358, respectively. The long electropherotype pattern assigned to the study sample was due to the presence of this genome segment.
[4]The short and long out-of-phase ORFs of the genome segment 11 for RVA/Human-wt/ZAF/2371WCNSP5A/2008/G9P[8] that encodes NSP6 and NSP5 were translated from nt 22–624 and nt 80–358, respectively. The short electropherotype pattern assigned to the study sample was due to the presence of this genome segment.
Colours were added to visualise certain patterns or genome constellations as follows: Green (Wa-like), red (DS-1-like), orange (AU-like), purple (PO-13-like) and blue (some typical animal strains). S, genome segment; VP, viral structural protein; NSP, viral non-structural protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

All conserved amino acids and functional domains of the 11 rotavirus proteins were present in all the amino acid sequences reported in this study as described previously (Estes and Kapikian, 2007; Heiman et al., 2008; Jere et al., 2011). The 23 distinct conserved amino acids in Wa- (genotype 1) and DS-1- (genotype 2) like strains (Heiman et al., 2008) were present in three of the six VP6 sequences of this study (RVA/Human-wt/ZAF/2371WC VP6A/2008/G9P[8], RVA/Human-wt/ZAF/2371WCVP6B/2008/G9P[8] and RVA/Human-wt/ZAF/2371WCVP6E/2008/G9P[8]). For RVA/Human-wt/ZAF/2371WCVP6C/2008/G9P[8] and RVA/Human-wt/ZAF/2371WCVP6D/2008/G9P[8], 8 of the 23 amino acids between residues 61 and 130 (corresponding to nt 203–413) were not conserved. For instance, RVA/Human-wt/ZAF/2371WCVP6D/2008/G9P[8] that was assigned an I2 (DS-1-like) genotype (Table 1) contained residues T, E, I, I, A, V, A and A that are distinct in strains with I1 (Wa-like) genotype instead of N, D, V, V, V, I, S and S that are distinct in I2 (DS-1-like) genotypes at positions 83, 86, 89, 92, 101, 109, 115 and 120, respectively. Similarly, RVA/Human-wt/ZAF/2371WCVP6C/2008/G9P[8] (I1/Wa-like genotype; Table 1) contained residues distinct to I2 (DS-1-like) genotyped strains between aa 61 and 130 (Fig. 2). Since these nucleotide sequences were obtained from completely different contigs and are, therefore, part of different consensus nucleotide sequences, the exchange of different portions of genome segment 6 between different nucleotide sequences was, therefore, suspected.

3.2.3. Evidence of intergenotype genome recombination in genome segments 6 (VP6), 8 (NSP2) and 10 (NSP4)

Multiple nucleotide and amino acid sequence alignments suggested that recombination between the Wa- and DS-1-like genome segment 6, 8 and 10 resulted in progenies with chimeric sequences. Fig. 2 and Supplement 4 demonstrate that RVA/Human-wt/ZAF/2371WCVP6D/2008/G9P[8] was a product of genome recombination between Wa-like (RVA/Human-wt/ZAF/2371WCVP6A/2008/G9P[8]) and DS-1-like (RVA/Human-wt/ZAF/

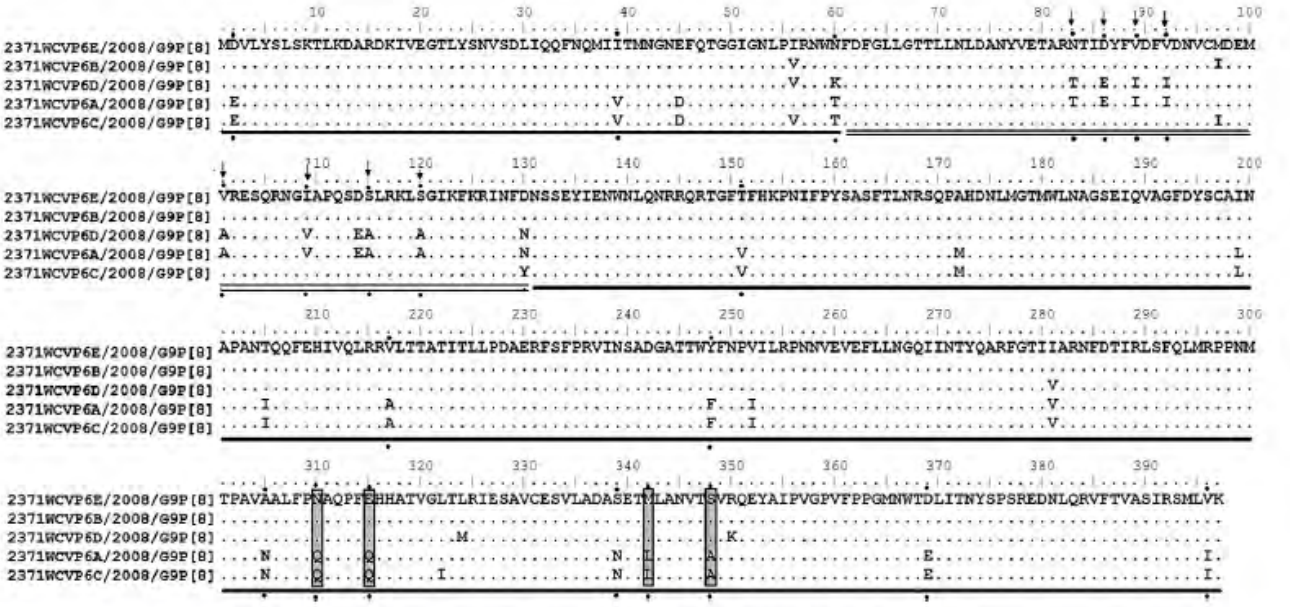

Fig. 2. Five complete amino acid sequences of the rotavirus populations identified for genome segment 6 (VP6). Between aa 1–60 (corresponding to nt 23–202) and 131–397 (corresponding to nt 414–1356) (underlined with a single line), RVA/Human-wt/ZAF/2371WCVP6B/2008/G9P[8], RVA/Human-wt/ZAF/2371WCVP6D/2008/G9P[8] and RVA/Human-wt/ZAF/2371WCVP6E/2008/G9P[8] were similar, while RVA/Human-wt/ZAF/2371WCVP6A/2008/G9P[8] and RVA/Human-wt/ZAF/2371WCVP6C/2008/G9P[8] were almost identical. From amino acid position 61–130 (corresponding to nt 203–413) (underlined with double lines), RVA/Human-wt/ZAF/2371WCVP6D/2008/G9P[8] was identical to RVA/Human-wt/ZAF/2371WCVP6A/2008/G9P[8], whereas RVA/Human-wt/ZAF/2371WCVP6B/2008/G9P[8] and RVA/Human-wt/ZAF/2371WCVP6C/2008/G9P[8] were closely related. The amino acids conserved in Wa-like (I1 genotype) and DS-1-like (I2 genotype) rotavirus strains at positions E2D, V39I, D45E, T60N/S, T83N, E86D, I89V, I92V, A101V, V109I, A115S, A120S, V151T, A217V, F248Y, N305A, Q310N, Q315E, N339S, L342M, A348S, E369D, I396V are shown with dots (.) (Heiman et al., 2008). At amino acid positions 83, 86, 89, 92, 101, 109, 115 and 120, RVA/Human-wt/ZAF/2371WCVP6D/2008/G9P[8] contained residues that are conserved in Wa-like strains, whereas RVA/Human-wt/ZAF/2371WCVP6C/2008/G9P[8] contained residues conserved in DS-1-like rotaviruses, respectively. The shaded residues 310 and 315 represents region D, whereas residues 342 and 348 represent region E involved in subgroup specificities. Dots (.) represent identical amino acid residues to the one appearing along RVA/Human-wt/ZAF/2371WCVP6E/2008/G9P[8] sequence. The prefix RVA/Human-wt/ZAF/was omitted from the name of each nucleotide sequence.

2371WCVP6B/2008/G9P[8]) genome segments. Similarly, RVA/Human-wt/ZAF/2371WCVP6C/2008/G9P[8] appears to be a product of multiple genome recombination between a Wa-like (RVA/Human-wt/ZAF/2371WCVP6A/2008/G9P[8]) and two DS-1-like, (RVA/Human-wt/ZAF/2371WCVP6B/2008/G9P[8] and RVA/Human-wt/ZAF/2371WCVP6E/2008/G9P[8]), genome segments. Intergenotype genome segment recombinations were suspected between nt 202 and 203 (between codons GAA and TTT) and between nt 413 and 414 (between codons GAT and AAT) due to the nucleotide variations and similarities observed between the study sequences. This hypothesis was supported by SimPlot analysis that also revealed that recombination took place within the same regions. The region ranging from nt 203 to 413 (corresponding to aa 61–130), designated as section B in Fig. 3a seems to be introduced in the two progeny nucleotide sequences through genome recombination. Within this region, RVA/Human-wt/ZAF/2371WC VP6C/2008/G9P[8] was closely related to RVA/Human-wt/ZAF/2371WCVP6B/2008/G9P[8] (Fig. 3a.i), whereas RVA/Human-wt/ZAF/2371WCVP6D/2008/G9P[8] was closely related to RVA/Human-wt/ZAF/2371WCVP6A/2008/G9P[8] (Fig 3a.ii). The similarity plots also suggested that intergenotype genome segment recombination occurred between nt 202–203 and 413–414. The nucleotide sequences of the resultant progeny genome segments (RVA/Human-wt/ZAF/2371WCVP6C/2008/G9P[8] and RVA/Human-wt/ZAF/2371WCVP6D/2008/G9P[8]) were different from their putative parental strains. This was also reflected by the separate lineages that the suspected nucleotide sequences for the two progeny genome segment 6 formed within the DS-1- and Wa-like clusters in Supplement 3e.

Multiple sequence alignments (Supplement 5) and SimPlot analyses (Fig. 3b) of the three nucleotide sequences obtained for genome segment 8 (NSP2) showed that RVA/Human-wt/ZAF/2371WCNSP2C/2008/G9P[8] resembled RVA/Human-wt/ZAF/2371 WCNSP2A/2008/G9P[8] from nucleotide position 1–228, and RVA/Human-wt/ZAF/2371WCNSP2B/2008/G9P[8] from nucleotide 500 to 1059. This suggested that RVA/Human-wt/ZAF/2371WCNSP2C/2008/G9P[8] may also have resulted from intergenotype genome segment recombination between RVA/Human-wt/ZAF/2371WCNSP2A/2008/G9P[8] and RVA/Human-wt/ZAF/2371 WCNSP2B/2008/G9P[8], and that genome recombination occurred within the region spanning from nucleotide 229 to 288, designated section B in Fig. 3b.

Upon aligning the four nucleotide and amino acid sequences of genome segment 10 of this study (Supplement 6), RVA/Human-wt/ZAF/2371WCNSP4D/2008/G9P[8] was identical to RVA/Human-wt/ZAF/2371WCNSP4B/2008/G9P[8] and closely related to other DS-1-ike strains from nt 1 to 172. From nt 288 to 750, RVA/Human-wt/ZAF/2371WC NSP4D/2008/G9P[8] was similar to RVA/Human-wt/ZAF/2371WCNSP4A/2008/G9P[8] and other Wa-like strains. SimPlot analysis also suggested that recombination of a DS-1-like (RVA/Human-wt/ZAF/2371WCNSP4B/2008/G9P[8]) and a Wa-like (RVA/Human-wt/ZAF/2371WCNSP4A/2008/G9P[8]) genome segment 10 resulted in RVA/Human-wt/ZAF/2371WCNSP4D/2008/G9P[8] genome segment 10 (Fig. 3c). This may explain why RVA/Human-wt/ZAF/2371WCNSP4D/2008/G9P[8] clustered separately from the rest of the Wa-like strains (Supplement 3j).

3.3. Analysis of the genome segment 4 (VP4) and 9 (VP7) genotype-specific primer binding sites

To investigate why initially the sequence-specific semi-nested RT-PCR only assigned the P[8] genotype to genome segment 4

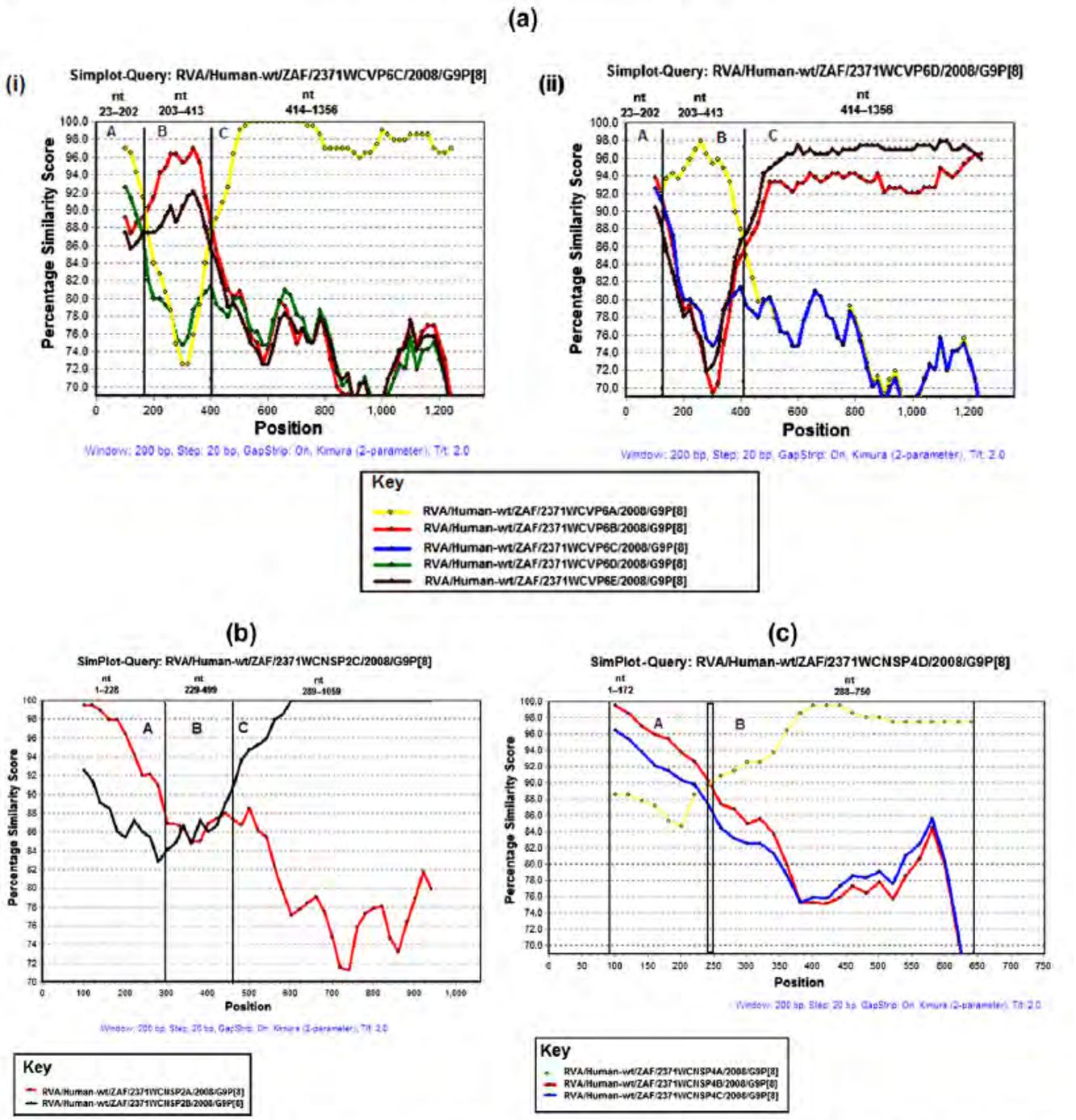

Fig. 3. SimPlot analysis of the nucleotide sequences of genome segment 6 (VP6), 8 (NSP2) and 10 (NSP4) suspected to be involved in recombination. The percentage similarity scores between the nucleotide sequences of the viral populations were generated by SimPlot software (Lole et al., 1999). Each curve indicates the percentage similarities between the query nucleotide sequences (indicated on top of each plot) to the reference nucleotide sequences (indicated in the legend with a distinctive colour). Each point on the curves is the percentage identity centred on the position plotted with sizes between 20 bp. The query sequence is more related to the reference with the highest percent of the mutated tree; (a) in both (i) and (ii) Sections A, B and C represent nt 23–202 (aa 1–60), nt 203–413 (aa 61–130) and nt 414–1356 (aa 131–397), respectively; (a.i) the consensus nucleotide sequence of RVA/Human-wt/ZAF/2371WCVP6C/2008/G9P[8] (query sequence) was closely related to RVA/Human-wt/ZAF/2371WCVP6A/2008/G9P[8] in section A, RVA/Human-wt/ZAF/2371WCVP6B/2008/G9P[8] in section B, and to RVA/Human-wt/ZAF/2371WCVP6A/2008/G9P[8] in section C; (a.ii) the consensus nucleotide sequence of RVA/Human-wt/ZAF/2371WCVP6D/2008/G9P[8] (query sequence) was closely related to RVA/Human-wt/ZAF/2371WCVP6B/2008/G9P[8] in section A, RVA/Human-wt/ZAF/2371WCVP6A/2008/G9P[8] in section B, and to RVA/Human-wt/ZAF/2371WCVP6E/2008/G9P[8] in section C. (b) The nucleotide sequence regions of the genome segment 8 (NSP2) that span from nucleotide position 1–228, 229–499 and 500–1059 were designated by sections A, B and C, respectively. RVA/Human-wt/ZAF/2371WC NSP2C/2008/G9P[8] (query sequence) was similar to RVA/Human-wt/ZAF/2371WCNSP2A/2008/G9P[8] in section A, whereas in section C it was closely related to RVA/Human-wt/ZAF/2371WCNSP2B/2008/G9P[8] up to 100%. The query sequence was less closely related to both reference sequence in section B. (c) The nucleotide sequence regions of the genome segment 10 (NSP4) that spans from nucleotide position 1–172 and 288–750 were designated by sections A and B, respectively. The boxed region represents nt 173–287. RVA/Human-wt/ZAF/2371WCNSP4D/2008/G9P[8] was closely related to RVA/Human-wt/ZAF/2371WCNSP4B/2008/G9P[8] in section A, whereas in section B, it was similar to RVA/Human-wt/ZAF/2371WCNSP4A/2008/G9P[8].

(VP4) and G9 genotype to genome segment 9 (VP7) of RVA/Human-wt/ZAF/2371WC/2008/G9P[8] when several genotypes were detected by sequence-independent amplification coupled with 454® pyrosequencing, the primer binding regions within the nucleotide sequences of these genome segments were aligned against the genotype-specific oligonucleotides that were used. Nucleotide mismatches were not observed between the primer binding regions of the nucleotide sequence obtained in this study that were assigned G9, P[6] and P[8] genotypes when compared against their respective mG9, 3T-1 and 1T-1 primers (Gentsch et al., 1992; Iturizza-Gómara et al., 2004) that were part of the cocktail used in genotyping the study strain previously (Supplement 7). There were no matching sequences between the P[4]-specific 2T-1 primer (CTATTGTTAGAGGTTAGA GTC) (Gentsch et al., 1992) and the P[4] RVA/Human-wt/ZAF/2371WCVP4B/2008/G9P[8] nucleotide sequence (data not shown). Three nucleotide mismatches were observed between the G8-specific primer aAT8 and the G8 RVA/Human-wt/ZAF/2371WCVP7C/2008/G9P[8] nucleotide sequence at positions 189 (G → C), 193(T → C), and 195(G → A). Five mismatches were observed between the G12-specific primer mG12 and the G12 RVA/Human-wt/ZAF/2371WCVP7D/2008/G9P[8] nucleotide sequence at positions 549 (A → G), 555 (T → C), 556 (A → G), 561 (C → G) and 556 (T → G). Surprisingly, the mG12 primer sequence was identical to a region (nt 547–566) within the G9 RVA/Human-wt/ZAF/2371WCVP7A/2008/G9P[8] nucleotide sequence. It was not possible to carry out similar comparisons within the G2 nucleotide sequence as only a partial G2 nucleotide sequence was available (from nt position 532 to 1062), and the G2 genotype-specific primers that were used bind between nucleotide positions 411–435 (Gouvea et al., 1990) and 262–281 (Das et al., 1994).

4. Discussion

The specimen characterised in this study was part of a surveillance study of rotavirus strains circulating in the Western Cape Province of South Africa. Sequence-dependent RT-PCR that used genotype-specific primers only assigned the P[8] and G9 genotypes to the genome segments 4 and 9 of the study strain, respectively. In addition, the dsRNA profile had only a long electropherotype profile. Upon synthesising and amplifying the cDNA from the dsRNA extracted from the stool sample with the sequence-independent procedure (Potgieter et al., 2009), both long and short electropherotype profiles were observed (Fig. 1). Whole genome characterisation studies classify rotavirus G2 genotype (commonly associated with short electropherotype) as a DS-1-like genotypes, whereas G1, G3, G4 and G9 genotypes (commonly associated with long electropherotype) as Wa-like genotypes (Matthijnssens et al., 2008a). Based on the detection of both short and long electropherotypes in the cDNA synthesised from the dsRNA extracted from the study stool sample, it was suspected that the 27 month old child was infected with more than one rotavirus strain. Identification of both Wa- and DS-1-like genotypes in each genome segment of RVA/Human-wt/ZAF/2371WC/2008/G9P[8] was further evidence of mixed rotavirus strain infection.

The origin of the three additional amplicons (A, B and C in Fig. 1b) could only be speculated upon. The sequence-independent synthesis of cDNA employed in this study can amplify any dsRNA present in the sample (Maan et al., 2007). In addition to rotavirus nucleotide sequences, non-specific nucleotide sequences that ranged from 187 to 603 bp were also generated (data not shown). Most of these non-specific sequence reads were closely related to organisms like the Cryptosporidium dsRNA virus (Accession number: EU183404). Based on the approximate sizes of the additional amplicons observed on agarose gel, only amplicons C could be linked to these non-specific sequence reads. Analysis of all the generated nucleotide sequences did not reveal any evidence that the additional amplicons in this study resulted from genome recombination or rearrangement as reported previously (Kojima et al., 2000; Cao et al., 2008) as the sizes of the study chimeric nucleotide sequences correlated with other rotavirus genome segment known to exhibit normal electropherotype profiles (Heiman et al., 2008). Some studies described that rotavirus dsRNA genomes isolated from chronically infected immunodeficient children tend to display atypical dsRNA profiles (Hundley et al., 1987). Missing profiles or various bands that are probably concatemeric forms of dsRNA are sometimes observed in such cases (Pedley et al., 1984; Desselberger, 1996). Although the child in this study had severe diarrhoea, his immune status was unknown and as such no correlation could be made between the presence of atypical bands and the immune status.

Data generated through 454® pyrosequence has been used in forensic sciences recently to resolve heteroplasy and determine variants in mitochondria DNA by utilising MID sequences to differentiate mixtures of pooled samples being run together on the same pyrosequencing plate (Holland et al., 2011; Zaragoza et al., 2010). However, deconvolution of all the eleven genome segments of rotavirus strains in mixed rotavirus infection has not been reported to date. In this study, the possibility that characterisation of multiple genotypes in all 11 genome segment was due to contamination was investigated. Since the study specimen was solicited from the VGU (NICD), the nucleotide sequences reported in this study were compared to the nucleotide sequences of the other rotavirus strains present in the NWU and VGU laboratories. No homology at both nucleotide and amino acid level was observed except between nucleotide sequences of genome segment 9 for strain RVA/Human-wt/ZAF/3203/2009/G2P[4] and RVA/Human-wt/ZAF/2371WCVP7B/2008/G9P[8] (data not shown). Since the other two samples that were combined in one library with the study sample had each a single rotavirus population, it was unlikely that the identification of multiple rotavirus genotypes in the study sample resulted from contamination during sample handling. Considering (i) the age of the child who was likely to play with fomites, (ii) the relatively poor sanitary conditions of the surrounding areas of Gatesville hospital (Cape flats, residence of the majority of the patients) where the child was admitted, and the close proximity between animal and human dwellings in this areas, and (iii) the high environmental stability of rotaviruses, the possibility that the child was infected with multiple rotavirus strains was high.

Infection of a single host cell by different/heterotypic rotavirus strains increases the possibility of generating antigenic variants through evolutionary mechanisms like reassortment (Gouvea and Brantly, 1995; Matthijnssens et al., 2006a, 2008a,b; Esona et al., 2009; Ghosh et al., 2010) and recombination (Suzuki et al., 1998; Parra et al., 2004; Phan et al., 2007). Despite evidence of intragenic recombination in rotaviruses, first reported by Suzuki et al. (1998), it is still unclear how this process occurs. However, homologous recombination and template switching have been suggested as the major molecular mechanism in other RNA viruses (Kirkegaard and Baltimore, 1986). Recently, a number of reports have illustrated recombination within the rotavirus genome segments encoding VP7 (Parra et al., 2004; Phan et al., 2007; Martínez-Laso et al., 2009), NSPs (Cao et al., 2008; Donker et al., 2011), and intragenic recombination in other dsRNA viruses, for instance, bluetongue virus (He et al., 2010). In this study, analysis of 454® pyrosequence-generated data revealed evidence of intergenotype rotavirus genome recombination in the genome segments 6 (VP6), 8 (NSP2) and 10 (NSP4).

The approach used to characterise strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8] in this study might be useful as a quality control tool for the sequence-dependent based dual genotyping

system commonly used in assigning rotavirus genotypes. Sequence-dependent RT-PCR assigned only G9 and P[8] genotypes to genome segments 9 (VP7) and 4 (VP4), respectively. However, analysing the 454® pyrosequence-generated data generated for strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8] revealed also the presence of G2, G8, G12, P[4] and P[6] genotypes. These genotypes represent some of the frequently characterised rotavirus genotypes in South Africa in addition to the G1 genotypes (Potgieter et al., 2010; Mwenda et al., 2010; Seheri et al., 2010). Iturriza-Gómara et al. (2000) observed that changes within the first three 3′ nucleotides of the primer binding sites affect both the product yield and priming efficiency of the oligonucleotides, while Sommer and Tautz (1989) showed that such mismatches prevent PCR-amplification of the templates. Lack of complementarity between the 2-T1 primer and the P[4] genotyped sequence, and between the mG12 and the G12 genotyped sequence reported in this study may explain why these genotypes were not assigned. The nucleotide mismatches within the aAT8 primer binding site of RVA/Human-wt/ZAF/2371WCVP7C/2008/G9P[8] may explain why G8 was not detected. What prevented the detection of the other genotypes can only be speculated at this point. One possible reason could be that the concentrations of these viral subpopulations in the sample were too low. During assembly of the 454® pyrosequence reads, the P[8] sequence reads were more abundant than the P[6] reads (P[6] = 90 and P[8] = 694). This argument was also true for genome segment 9 (VP7), as the number of sequence reads for G2 and G12 were lower than for G8 and G9 (G2 = 15, G8 = 515, G9 = 527, G12 = 54). The fact that four distinct consensus nucleotide sequences were obtained for genome segments 4 and 9 (Table 1) suggested that the child was co-infected with four different rotavirus strains.

The reason why only two consensus nucleotide sequences were generated for genome segments 1 (VP1), 2 (VP2), 5 (NSP1), 7 (NSP3), and 11 (NSP5/6) could be due to conservation between the nucleotide sequences of genotype 1 (Wa-like) and 2 (DS-1-like) in these genome segments (Heiman et al., 2008). Since the majority of the human rotavirus strains with G2, G8, G9 and G12 VP7 genotypes have either a Wa- or DS-1-like genetic backbone (Heiman et al., 2008; Matthijnssens et al., 2008a, 2011; Bányai et al., 2011), it is likely that the strains involved in the mixed infection in this study possessed either a Wa- or DS-1-like genetic backbone. Therefore, single complete consensus nucleotide sequences might have been generated from 454® pyrosequence reads derived from different rotavirus populations with the same genotype. Perhaps more depth of coverage will allow resolution of the rotavirus populations with the same genotype within each genome segment. In case of genome segments 6 (VP6), 8 (NSP2) and 10 (NSP4), identification of more than one Wa- or DS-1-like genotype could be explained by the presence of intergenotype chimeric genome segments. One of the shortfalls of using sequence-independent amplification to characterise rotavirus strains from mixed infection cases is that it does not permit checking for reassortment events as the complete descriptor of Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, proposed by the *Rotavirus Classification Working Group* (RCWG) (Matthijnssens et al., 2008a, 2011), may not be assigned to each strain that is present in the sample despite characterising multiple nucleotide sequences for each genome segment (Table 1).

Previously, evidence of interspecies or zoonotic transmission has been based on close relationships of one or more of the 11 genome segments of human rotaviruses to animal strains (Tsugawa and Hoshino, 2008; Martella et al., 2010). In this study, one of the nucleotide sequences of genome segment 9 (RVA/Human-wt/ZAF/2371WCVP7C/2008/G9P[8]) was more closely related to a bovine strain RVA/Cow-wt/NGA/NGRBg8/XXXX/G8P[X] than human rotaviruses (Supplement 3e). This also applied to one of the nucleotide sequence of genome segment 6 (RVA/Human-wt/ZAF/2371WCVP6A/2008/G9P[8]) that was more closely related to rotaviruses isolated from the sable antelope and ovine species (Supplement 3d). Two nucleotide sequences of genome segment 8 (RVA/Human-wt/ZAF/2371WCNSP2B/2008/G9P[8] and RVA/Human-wt/ZAF/2371WCNSP2C/2008/G9P[8]) were closely related to artiodactyl rotavirus strains (Matthijnssens et al., 2009) (Supplement 3h). This suggested that some of the rotavirus strains that were present in the stool sample, characterised in this study, could be human–animal reassortant rotaviruses or were introduced into humans from animals. It is possible that the child was infected with both human and animal rotaviruses simultaneously, or that the infecting strains were generated through previous reassortment events between human and animal strains and are now circulating in the human population. If direct interspecies transmission occurred, the data suggests that evolutionary events like reassortment and recombination are most likely and children may provide a suitable mixing vessel, similar to porcine hosts for influenza viruses (Khamrin et al., 2007).

In conclusion, combining sequence-independent, 454® pyrosequencing and RotaC genotyping allows characterisation of the full genomes of multiple strains present in a wild type sample. For the first time, this study has illustrated how infection with multiple rotavirus strains of different genotypes may result in progeny novel rotaviruses through genome recombinations. The increased numbers of mixed infection cases reported from developing countries may be one of the attributes that widen the rotavirus strain diversity reported from these regions (Ahmed et al., 1991; Leite et al., 1996; Arguelles et al., 2000; Nielsen et al., 2005; Mwenda et al., 2010; Potgieter et al., 2010). Therefore, full genome characterisation of multiple rotavirus strains infecting a single host coupled with ultra-deep sequencing with more depth of coverage may assist in further understanding the possible evolutionary mechanisms followed by the infecting rotavirus strains. Finally, strain analysis using the sequence-independent RT-PCR approach should be encouraged on selected specimens, especially those reported as untypable strains and mixed infections. This may assist in understanding the complete epidemiology of rotavirus strains as well as the evolutionary mechanisms used to generate rotavirus strain diversity.

5. Authors' contributions

KCJ was involved in the design of the study, laboratory assays, data analysis and manuscript writing. NAP was involved in the collection of study specimen and manuscript writing. LM was involved in data analysis and manuscript writing. HGO and AAvD were involved in the study design, data analysis and manuscript writing. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.09.023.

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Supplementary Data

Supplement 1. GenBank accession numbers of all nucleotide sequences generated in this study for each rotavirus genome segment.

	GenBank accession numbers										
Study RV Strain	S1(VP1)	S2(VP2)	S3(VP3)	S4(VP4)	S6(VP6)	S9(VP7)	S5(NSP1)	S8(NSP2)	S7(NSP3)	S10(NSP4)	S11(NSP5)
RVA/Human-wt/ZAF/2371WC/2008/G9P[8]	JN013987 JN013988	JN013989 JN013990	JN013991 JN013992 JN013993	JN013994 JN013995 JN013996 JN013997	JN014002 JN014003 JN014004 JN014005 JN014006	JN013998 JN013999 JN014000 JN014001	JN013974 JN013975	JN013976 JN013977 JN013978	JN013979 JN013980	JN013981 JN013982 JN013983 JN013984	JN013985 JN013986

Supplement 2. GenBank accession numbers of nucleotide sequences used to construct phylogenetic trees for genome segments encoding VP1, VP2, VP3, VP4, VP6 and VP7, NSP1, NSP2, NSP3, NSP4 and NSP5.

Genome segment 1 (VP1):

RVA/Human-wt/USA/LB2719/2006/G1P[8]: HM467922; RVA/Human-wt/BGD/Dhaka16/ 2003/G1P[8]: DQ492669; RVA/Human-wt/BEL/B4633/2003/G12P[8]: DQ146638; RVA/Human-wt/USA/LB2771/2006/G1P[8]: HM467924; RVA/Human-tc/GBR/ST3/1975/ G4P2A[6]: EF583045; RVA/Human-wt/USA/LB2758/2006/G1P[8]: HM467923; RVA/Human-tc/USA/WI61/1983/G9P1A[8]: EF583049; RVA/Human-wt/JPN/KU/1974/ G1P1[8]: AB022765; RVA/Macaque-tc/USA/ PTRV/1990/G8P[1]: FJ422131; RVA/Human-wt/HUN/BP1879/2003/G6P[14]: FN665677; RVA/Human-wt/USA/LB2764/2006/G2P[4]: HM467926; RVA/Human-wt/BGD/MMC88/2005/G2P[4]: HQ641364; RVA/Human-wt/BGD/ MMC6/2005/G2P[4]: HQ641355; RVA/Human-wt/USA/LB2744/2006/G2P[4]; HM467925; RVA/Human-wt/USA/LB2772/2006/G2P[4]; HM467927; RVA/Human-wt/COD/DRC86/2003/G8P[6]: DQ005125; RVA/Pigeon-tc/JPN/PO-13/1983/G18P[17]: AB009629; RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]: AY787653; RVA/Human-tc/CHN/R479/2004/G4P[6]: GU189551; RVA/Human-wt/BGD/Dhaka6/2001/G11P[25]: EF560705; RVA/Human-tc/JPN/YO/1977/G3P1A[8]: DQ870497; RVA/Human/ JPN/Hosokawa/1983/G4P1A[8]: DQ870489; RVA/Human-tc/PHL/L26/1987/ G12P[4]: DQ146693; RVA/Sheep-tc/CHN/Lamb-NT/XXXX/G10P[15]: FJ031024; RVA/Human-wt/COD/DRC88/2003/G8P[8]: DQ005114; RVA/Human-wt/USA/CH5446/1991/G3P[8]: FJ947428; RVA/Human-tc/IND/0613158-CA/2006/G1P[8]: EU984103; RVA/Human-wt/BEL/B3458/ 2003/G9P[8]: DQ870501; RVA/Human-wt/ZAF/GR10924/ 1999/G9P[6]: FJ183353; RVA/Human-tc/THA/T152/1998/G12P[9]: DQ146699; RVA/Cow-wt/JPN/Azuk-1/2006/G21P[29]: AB573079; RVA/Cow-tc/JPN/Dai-10/2007/G24P[33]: AB573070; RVA/Cow-tc/FRA/RF/1982/G6P[1]: J04346; RVA/Simian-tc/USA/RRV/ 1975/G3P[3]: EU636924; RVA/Pig-tc/USA/Gottfried/1983/G4P[6]: M32805; RVA/Human-tc/USA/DS-1/1976/G2P1B[4]: DQ870505; RVA/Cow-tc/USA/NCDV/ 1967/G6P6[1]: DQ870493 ; RVA/Human-tc/JPN/YO/1977/G3P1A[8]: DQ870497; RVA/Human-tc/JPN/S2/1980/G2P[4]: DQ870485; RVA/Pig-tc/VEN/A131/1988/G3P9[7]: EF560618; RVA/Human-tc/USA/Wa/1974/G1P1A[8]: DQ490539; RVA/Pig-tc/VEN/A253/1988/ G11P9[7]: EF560621; RVA/Human-tc/ITA/PA169/1988/G6P[14]: EF554126; RVA/Human-wt/HUN/Hun5/1997/G6P[14]: EF554104; RVA/Human-tc/AUS/MG6/1993/G6P[14]: EF554093; RVA/Goat-tc/BGD/GO34/1999/G6P[1]: GU937877; RVA/Human-wt/BGD/RV176-00/2000/G12P[6]: DQ490551; RVA/Human-wt/BGD/RV161/2000/ G12P[6]: DQ490545; RVA/Human-tc/IDN/69M/1980/G8P4[10]: EF576937; RVA/Human-wt/BEL/B10925-97/1997/G6P[14]: EF554015; RVA/Human-wt/ITA/PAH136/1996/G3P[9]: GU296420.

Genome segment 2 (VP2):

RVA/Human-wt/USA/LB2719/2006/G1P[8]: HM467928; RVA/Human-wt/USA/ LB2771/ 2006/G1P[8]: HM467930; RVA/Human-wt/USA/LB2758/2006/G1P[8]: HM467929; RVA/Human-tc/USA/Wa/1974/G1P1A[8]: X14942; RVA/Human-wt/USA/LB2772/2006/ G2P[4]: HM467933; RVA/Human-wt/USA/LB2744/2006/G2P[4]: HM467925; RVA/ Human-wt/DEU/GER1H-09/2009/G8P[4]: GQ414541; RVA/Human-wt/USA/ LB2764/ 2006/G2P[4]: HM467932; RVA/Human-wt/BGD/MMC88/2005/G2P[4]: HQ641365; RVA/Human-wt/BGD/MMC6/2005/G2P[4]: HQ641356; RVA/Human-wt/COD/DRC86/ 2003/G8P[8]: DQ005124; RVA/Human-wt/COD/DRC88/2003/G8P[8]: DQ005124; RVA/ Human-tc/USA/WI61/1983/G9P1A[8]: EF583050; RVA/Human-tc/GBR/ST3/1975/ G4P2A[6]: EF583046; RVA/Human-tc/USA/Se584/1998/G6P[9]: EF583042; RVA/Human-tc/USA/P/1974/G3P1A[8]: EF583038; RVA/Human-tc/USA/DS-1/1976/G2P1B[4]: EF583026; RVA/Pigeon-tc/JPN/PO-13/1983/G18P[17]: AB009630; RVA/Human-wt/JPN/KU/1974/G1P1[8]: AB022766; RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]: AY787652; RVA/Human-tc/JPN/YO/1977/G3P1A[8]: DQ870498; RVA/Human/JPN/Hosokawa/1983/ G4P1A[8]: DQ870490; RVA/Human-wt/BEL/B4633/2003/G12P[8]: DQ146639; RVA/ Human-wt/BGD/Dhaka12-03/2003/G12P[6: DQ146661; RVA/Simian-tc/USA/RRV/1975/ G3P[3]: EU636925; RVA/Human-wt/ZAF/GR10924/1999/G9P[6]: FJ183354; RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7[5]: GU565053; RVA/Vaccine/USA/ RotaTeq-SC2-9/1992/G2P7[5]: GU565064; RVA/Vaccine/USA/RotaTeq-WI78-8/1992/ G3P7[5]: GU565075; RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]: GU565042; RVA/Pig-tc/USA/Gottfried/1983/G4P[6]: GU199487; RVA/Human-tc/CHN/R479/2004/ G4P[6]: GU189552; RVA/Pig-tc/USA/OSU/1977/G5P9[7]: GU199515; RVA/Cow-wt/JPN/Azuk-1/2006/G21P[29]: AB573080; RVA/Cow-tc/JPN/Dai-10/2007/G24P[33]: AB573071; RVA/Cat-tc/AUS/Cat2/1984/G3P[9]: EU708957; RVA/Cat-tc/AUS/Cat97/ 1984/G3P[3]: EU708946; RVA/Cow-tc/USA/NCDV/1967/G6P6[1]: DQ870494; RVA/Dog-tc/USA/CU-1/1982/G3P[3]: EU708913; RVA/Human-tc/USA/HCR3A/1984/G3P[3]: EU708902; RVA/Dog-tc/USA/A79-10/XXXX/G3P[3]: EU708935; RVA/Human-wt/DEU/GER1H-09/2009/G8P[4]: GQ414541; RVA/Human-wt/COD/DRC86/2003/G8P[6]; DQ005124; RVA/Human-wt/BGD/N26/2002/G12P[6]: DQ146683; RVA/Human-wt/BGD/RV161/2000/G12P[6]: DQ490546; RVA/Human-wt/BEL/B1711/2002/G6P[6]: EF554083; RVA/Human-wt/USA/DC1359/1980/G4P[8]: HM773866; RVA/Human-wt/BGD/Dhaka16/2003/G1P[8]: DQ492670; RVA/Vaccine/USA/RotaTeq-BrB-9/1996/ G4P7[5]: GU565086.

Genome segment 3 (VP3):

RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]: GU565043; RVA/Human-wt/USA/LB2719/2006/G1P[8]: HM467935; RVA/Human-tc/USA/P/1974/G3P1A[8]: EF583039; RVA/Human-wt/BGD/Dhaka16/2003/G1P[8]: DQ492671; RVA/Human-wt/JPN/KU/1974/G1P1[8]: AB022767; RVA/Human-wt/USA/LB2771/2006/G1P[8]: HM467939; RVA/ Human-wt/USA/LB2758/2006/G1P[8]: HM467937; RVA/Human-wt/BGD/MMC88/2005/ G2P[4]: HQ641366; RVA/Sheep-tc/ESP/OVR762/2002/G8P[14] : EF554150; RVA /Human-wt/USA/LB2772/2006/G2P[4]: HM46793;4 RVA/Human-wt/USA/LB2744/2006/ G2P[4]: HM467936; RVA/Human-wt/BGD/N26/2002/G12P[6]: DQ146684; RVA/Pigeon-tc/JPN/PO-

13/1983/G18P[17]: AB009631; RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]: AY787654; RVA/Human-tc/CHN/R479/2004/G4P[6]: GU189553; RVA/Human-tc/GBR/ ST3/1975/G4P2A[6]: AY277919; RVA/Human-wt/BGD/Dhaka6/2001/G11P[25]: EF560706; RVA/Human-tc/USA/DS-1/1976/G2P1B[4]: AY277914; RVA/Human-tc/JPN/YO/1977/G3P1A[8]: DQ870499: RVA/Human-tc/JPN/S2/1980/G2P[4]: DQ870487; RVA/Human/JPN/Hosokawa/1983/G4P1A[8]: DQ870491; RVA/Human-tc/ITA/PA169/ 1988/G6P[14]: EF554128; RVA/Human-tc/GBR/A64/1987/G10P11[14]: AY277920; RVA/Human-tc/USA/WI61/1983/G9P1A[8]: AY277917; RVA/Guanaco-wt/ARG/ RioNegro/1998/G8P[1]: FJ347124; RVA/Human-tc/THA/T152/1998/G12P[9]: DQ146701; RVA/Human-wt/USA/CH5446/1991/G3P[8]: FJ947905; RVA/Human-wt/ZAF/GR10924/ 1999/G9P[6]: FJ183355; RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7[5]: GU565054; RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7[5]: GU565065; RVA/Vaccine/USA/ RotaTeq-WI78-8/1992/G3P7[5]: GU565076; RVA/Cow-wt/JPN/Azuk-1/2006/G21P[29]: AB573081; RVA/Cow-tc/JPN/Dai-10/2007/G24P[33]: AB573072; RVA/Human-tc/USA/P/1974/G3P1A[8]: EF583039; EF583051; RVA/Pig-tc/USA/Gottfried/1983/G4P[6]: GU199488; RVA/Sheep-tc/CHN/Lamb-NT/XXXX/G10P[15]: FJ031026; OVR762: EF554150; RVA/Cow-tc/CHN/DQ-75/2008/G10P[11]: GU384193; RVA/Human-wt/BGD /N26/2002/G12P[6]: DQ146684; RVA/Human-wt/BGD/RV176-00/2000/G12P[6]: DQ490553 ; RVA/Human-wt/COD/DRC88/2003/G8P[8]: DQ005112; RVA/Goat-tc/BGD/GO34/1999/G6P[1]: GU937879; RVA/Human-tc/IDN/69M/1980/G8P4[10]: EF576915; RVA/Cow-tc/FRA/RF/1982/G6P[1]: AY116592.

Genome segment 4 (VP4):

RVA/Human-wt/IND/APO6/2006/G1P[8]: HM467807; RVA/Human-wt/USA/LB2719/ 2006/G1P[8]: HM467940; RVA/Human-wt/USA/LB2771/2006/G1P[8]: HM467944; RVA/Human-wt/JPN/KU/1974/G1P1[8]: AB222784; RVA/Human-wt/USA/LB2758/ 2006/G1P[8]: HM467942; RVA/Human-wt/USA/LB2772/2006/G2P[4]: HM467945; RVA/Human-wt/USA/LB2744/2006/G2P[4]: HM467941; RVA/Human-wt/USA/LB2764/ 2006/G2P[4]: HM467943; RVA/Human-wt/COD/DRC86/2003/G8P[6]: DQ005122; RVA/Pigeon-tc/JPN/PO-13/1983/G18P[17]: AB009632; RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]: AY787644; RVA/Human-tc/CHN/R479/2004/G4P[6]: GU189554; RVA/Human-tc/USA/Wa/1974/G1P1A[8]: L34161; RVA/Human-tc/JPN/YO/1977/ G3P1A[8]: AB008279; RVA/Human-tc/IND/IS-2/XXXX/G2P[4]: X82323; RVA/Human-wt/KOR/CAU202/200X/G9P[8]: EF059923; RVA/Human-wt/COD/DRC88/2003/G8P[8]: DQ005111; RVA/Human-wt/BGD/RV176-00/2000/G12P[6]: DQ490554; RVA/Human-wt/IND/SC185/XXXX/GXP[4]: AJ299459; RVA/Human-wt/IND/0613158-CA/2006/ G1P[8]: EU984107; RVA/Human-wt/BGD/MMC6/2005/G2P[4]: EU839950; RVA/Human-tc/IND/107E1B/XXXX/G3P[4]: U07753; RVA/Human-wt/ZAF/GR10924/1999/G9P[6]: FJ183356; RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]: GU565044; RVA/Human-wt/BGD/DH392/2004/G2P[4]: EU839949; RVA/Human-tc/PHL/L26/1987/G12P[4]: EF672591; RVA/Human-wt/MWI/MW333/XXXX/G8P[4]: AJ278256; RVA/Human-wt/BGD/SK423/2005/G12P[6]: EU839946; RVA/Human-wt/BGD/SK277/2005/G12P[6]:

EU839948; RVA/Human-wt/BGD/Dhaka12-03/2003/G12P[6]: DQ146663; RVA/Pig-tc/USA/Gottfried/1983/G4P[6]: M33516; RVA/Macaque-tc/USA/PTRV/1990/G8P[1]: FJ422134; RVA/Cat-tc/AUS/Cat2/1984/G3P[9]: EU708959; RVA/Human-wt/HUN/Hun5/ 1997/G6P[14]: EF554106; RVA/Human-tc/IND/107E1B/XXXX/G3P[4]: U07753; RVA/Human-wt/BGD/Dhaka25-02/2002/G12P[8]: DQ146652; RVA/Human-wt/JPN/KO-2/XXXX/G2P[4]: AF401755; RVA/Human-wt/DEU/GER1H-09/2009/G8P[4]: GQ414543; RVA/Human-tc/IND/NRI/XXXX/GXP[4]: AF531909; RVA/Human-wt/CHN/XJ00-486/2000/G2P[6]: DQ321492; RVA/Human-tc/KOR/CAU195/200X/G12P[6]: EF059920; RVA/Human-wt/USA/US1205/XXXX/G9P[6]: AF079356; RVA/Human-wt/BGD/ Matlab13/2003/G12P[6]: DQ146674; RVA/Human-tc/GBR/ST3/1975/G4P2A[6]: EF672612; RVA/Human-wt/USA/DC2266/1976/G3P[8]: FJ947884; RVA/Human-tc/BRA/IAL28/1992/G5P[8]: EF672584; RVA/Human-tc/USA/WI61/1983/G9P1A[8]: EF672619; RVA/Human-wt/ JPN/Kagawa-90-544/XXXX/G4P[8]: AB039939.

Genome segment 6 (VP6):

RVA/Human-wt/USA/DC4613/1980/G4P[8]: HM773914; RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P7[5]: GU565078; RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7[5]: GU565067; RVA/Human-tc/IDN/69M/1980/G8P4[10]: EF576916; RVA/Human-wt/USA/ LB2764/2006/G2P[4]: HM467949; RVA/Human-wt/USA/LB2772/2006/G2P[4]: HM467951; RVA/Human-wt/USA/LB2744/2006/G2P[4]: HM467947; RVA/Human-wt/COD/DRC88/2003/G8P[8]: DQ005110; RVA/Pigeon-tc/JPN/PO-13/1983/G18P[17]: D16329; RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]: AY787645; RVA/Human-tc/ JPN/YO/1977/G3P1A[8]: DQ870500; RVA/Human-tc/JPN/S2/1980/G2P[4]: DQ870488; RVA/Human/JPN/Hosokawa/1983/G4P1A[8]: DQ870492; RVA/Human-wt/IND/TK119/ XXXX/GXP[X]: AY456527; RVA/Human-wt/USA/US9828/XXXX/G9P[8]: EF426139; RVA/Human-wt/IND/ISO92/XXXX/G9P[X]: EF472947; RVA/Human-wt/BEL/B10925-97/1997/G6P[14]: EF554119; RVA/Human-wt/ZAF/GR10924/1999/G9P[6]: FJ183358; RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7[5]: GU565056; RVA/Vaccine/USA/ RotaTeq-BrB-9/1996/G4P7[5]: GU565089; RVA/Vaccine/USA/RotaTeq-WI79-4/1992/ G6P1A[8]: GU565045; RVA/Macaque-tc/USA/PTRV/1990/G8P[1]: FJ422136; RVA/Pig-tc/USA/Gottfried/1983/G4P[6]: EU372799; RVA/Sheep-tc/ESP/OVR762/2002/G8P[14]: EF554152; RVA/Human-tc/ITA/PA169/1988/G6P[14]: EF554130; RVA/Cow-tc/ FRA/RF/1982/G6P[1]: K02254; RVA/Human-tc/PHL/L26/1987/G12P[4]: DQ146695; RVA/Human-tc/GBR/ST3/1975/G4P2A[6]: EF583048; RVA/Human-wt/THA/CMH185-01/XXXX/G3P[8]: EU372749; RVA/Human-wt/KOR/CAU164/XXXX/G1P[8]: EU679386; RVA/Human-wt/BGD/Matlab36-02/2002/G11P[8]: GU199507; RVA/Human-wt/IND/

ISO13/XXXX/G12P[X]: EF472944; RVA/Human-wt/BGD/Dhaka6/2001/ G11P[25]: EF560707; RVA/Human-wt/BGD/SK423/2005/G12P[6]: EU839965; RVA/Human-tc/AUS/RV3/1977/G3P2A[6]: U04741; RVA/Human-wt/BGD/MMC38/ 2005/G9PB[8]: EU979380; RVA/Human-wt/USA/ US8922/XXXX/G2P[4]: EF426132; RVA/Human-wt/THA/CMHO54/2005/G2P[4]: GU288640; RVA/Human-wt/BEL/B1711/ 2002/G6P[6]: EF554086; RVA/Human-wt/BGD/RV176-00/2000/G12P[6]: DQ490555; RVA/Human-wt/BGD/RV161/2000/G12P[6]: DQ490549; RVA/Human-wt/BGD/N26/2002/ G12P[6]: DQ146686; RVA/Human-wt/DEU/GER1H-09/2009/G8P[4]: GQ414544; RVA/Human-wt/IND/ISO97/XXXX/G9P[4]: EF472949; RVA/Antelope-wt/ZAF/RC-18-08/G6P[14]: FJ495131; RVA/Human-wt/USA/LB2758/2006/G1P[8]: HM467948; RVA/Human-wt/USA/LB2719/2006/G1P[8]: HM467946; RVA/Human-wt/USA/ LB2771/2006/G1P[8]: HM467950.

Genome segment 9 (VP7):

RVA/pig-wt/JPN/JP3-6/XXXX/G9P[6]: AB176684; RVA/Human-tc/IDN/69M/1980/ G8P4[10]: EF672560; RVA/Human-tc/USA/Wa/1974/G1P1A[8]: EF672616; RVA/ Human-wt/COD/DRC88/2003/G8P[8]: DQ005109; RVA/Pigeon-tc/JPN/PO-13/1983/ G18P[17]: D82979; RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]: AY787646; RVA/Human-wt/KEN/KY3103/1999/G2P[4]: AY261349; RVA/Human-tc/JPN/YO/1977/ G3P1A[8]: D86284; RVA/Cow-wt/NGA/NGRBg8/XXXX/G8P[X]: AF361439; RVA/cow-tc/JPN/Tokushima9503/XXXX/G8P[X]: AB044293; RVA/Human-wt/THA/ MS064/ XXXX/G12P[X]: AB436813; RVA/Human-wt/THA/MS040/2007/G12P[X]: AB436817; RVA/Human-wt/BGD/DH408/2005/G2P[4]: EU839928; RVA/Human-wt/BGD/SK299/ 2005/G2P[4]: EU839926; RVA/Human-wt/KEN/KY6950/2002/G8P[6]: FJ386446; RVA/Human-wt/MWI/MW1479/2001/G8P[4]: FJ386441; RVA/Human-wt/THA/CMH020/ 2005/G9P[8]: GQ149704; RVA/Human-wt/THA/NK002-01/XXXX/ G9P[X]: AB436824; RVA/Human-wt/THA/MS038/2007/G12P[X]: AB436816; RVA/Human-wt/BGD/SK423/ 2005/G12P[6]: EU839934; RVA/Human-wt/ZAF/GR10924/ 1999/G9P[6]: FJ183360; RVA/Human-wt/JPN/AU32/1995/G12P[X]: AB045372; RVA/Human-wt/USA/AU32/ XXXX/G9P[X]: AB180970; RVA/Human-tc/USA/WI61/ 1983/G9P1A[8]: AB180969; RVA/Human-tc/IND/116E/1985/G9P[11]: L14072; RVA/Human-wt/IND/RMC321/ 1990/G9P[19]: AF501578; RVA/pig-wt/JPN/ Mc345/XXXX/G9P[19]: D38055; RVA/Human-wt/THA/CMP003/XXXX/G9P[19]: AY707787; RVA/Human-tc/PHL/L26/ 1987/G12P[4]: M58290; RVA/Human-wt/JPN/ CP727/XXXX/ G12P[9]: AB125852; RVA/Human-

wt/BRA/HC91/XXXX/G12P[X]: AY855065; RVA/Human-wt/KOR/Kor588/ 2002/G12P[9]: EU496259; RVA/Human-tc/THA/T152/ 1998/G12P[9]: AB071404; RVA/Human-wt/ARG/Arg721/1999/G12P[9]: EU496254; RVA/Pig-wt/IND/RU172/2002/ G12P[7]: DQ204743; RVA/Cat-tc/AUS/Cat97/1984/G3P[3]: EU708950; RVA/Macaque-tc/USA/PTRV/1990/G8P[1]: FJ422138; RVA/Sheep-tc/ESP/OVR762/2002/G8P[14]: EF554153; RVA/Human-wt/AUS/95A/XXXX/G2P[X]: U73947; RVA/Human-wt/CHN/ T79/XXXX/G2P[X]: AF450292; RVA/Human-wt/ZAF/ 64SB/1996/G2P[4]: AY261341; RVA/pig-wt/JPN/JP3-6/XXXX/G9P[6]: AB176678; RVA/pig-wt/JPN/JP29-6/XXXX/ G9P[6]: AB176681; RVA/pig-wt/JPN/99-TK2082VP7/ 1999/G9P[X]: AB091755; RVA/pig-wt/JPN/99-TK2091VP7/1999/G9P[X]: AB091756; RVA/Pig-wt/JPN/Hokkaido-14/XXXX/ G9P[23]: AB091756; RVA/Human-wt/ZAF/UP30/ XXXX/G8P[X]: AF143690; RVA/Human-wt/ZAF/I8197LC98/1998/G9P[6]: AF529868; RVA/Human-wt/MWI/ MW4103/2000/G8P[8]: FJ386443; RVA/Human-wt/KEN/KY6914/ 2002/G8P[4]: FJ386445; Si/KY1646/1999/G8P[6]: FJ386444; RVA/Human-wt/MWI/ MW4097/2000/G8P[8]: FJ386442; RVA/Human-wt/TZA/TN1529/1999/G2P[4]: AY261357; RVA/Human-wt/BFA/BF3767/1999/G2P[6]; AY261355; RVA/Human-wt/BGD/RV176-00/2000/G12P[6: DQ490556; RVA/Human-wt/KEN/1290/1991/G8P[X]: EU488721; RVA/Human-wt/BGD/N26/2002/G12P[6]: DQ146687; RVA/Human-wt/IND/ISO16/ XXXX/G12P[6]: DQ099751; RVA/Human-wt/BGD/MMC29/2005/G12P[6]: EU839935; RVA/Human-wt/USA/LB2744/2006/G2P[4]: HM467953; RVA/Human-wt/USA/LB2772/ 2006/G2P[4]: HM467981; RVA/Human-wt/USA/LB2764/2006/G2P[4]: HM467955.

Genome segment 5 (NSP1):

RVA/Human-wt/USA/LB2771/2006/G1P[8]: HM467960; RVA/Human-wt/USA/LB2758/ 2006/G1P[8]: HM467959; RVA/Human-wt/USA/LB2719/2006/G1P[8]: HM467958; RVA/Human-wt/USA/LB2772/2006/G2P[4]: HM467921; RVA/Human-wt/USA/LB2744/ 2006/G2P[4]: HM467961; RVA/Human-wt/USA/ LB2764/2006/G2P[4]: HM467962; RVA/Human-tc/USA/Wa/1974/G1P1A[8]: AF306494; RVA/Human-tc/USA/DS-1/1976/ G2P1B[4]: EF672578; RVA/Human-wt/COD/DRC88/2003/G8P[8]: DQ005108; RVA/ Human-wt/COD/DRC86/2003/G8P[6]: DQ005119; RVA/Human-tc/ITA/PA169/1988/ G6P[14]: EF554132; RVA/Human-wt/ZAF/GR10924/1999/G9P[6]: FJ183357; RVA/ Human-wt/BGD/RV176-00/2000/G12P[6]: DQ490557; RVA/Human-wt/BGD/Matlab13/ 2003/G12P[6]: DQ146677; RVA/Cat-tc/AUS/Cat2/1984/G3P[9]: FRU23727; RVA/ Pigeon-tc/JPN/PO-13/1983/G18P[17]: AB009633; RVA/Human-wt/CHN/TB-Chen/1996/ G2P[4]: AY787647; RVA/Human-tc/GBR/ST3/1975/G4P2A[6]: U11492; RVA/Human-

wt/KOR/KJ172/XXXX/G8P[7]: FJ206215; RVA/Human-wt/BGD/RV161/2000/G12P[6]: DQ490540; RVA/Human-wt/BGD/Dhaka25-02/2002/G12P[8]: DQ146655; RVA/Human-wt/BEL/B3458/2003/G9P[8]: EF990709; RVA/Human-wt/JPN/IGV-80-3/XXXX/G1P[X]: X59297; RVA/Human-wt/DEU/GER1H-09/2009/G8P[4]: GQ414546; RVA/Pig-tc/USA/ OSU/1977/G5P9[7]: U08432; RVA/Pig-tc/USA/Gottfried/1983/G4P[6]: U08431; RVA/Human-wt/BGD/SK423/2005/G12P[6]: EU839966; RVA/Human-wt/BGD/N26/ 2002/G12P[6]: DQ146688; RVA/Human-tc/IDN/69M/1980/G8P4[10]: EF672557.

Genome segment 8 (NSP2):

RVA/Human-wt/USA/LB2758/2006/G1P[8]: HM467965; RVA/Human-wt/USA/LB2772/ 2006/G2P[4]: HM467969; RVA/Human-wt/USA/LB2744/2006/G2P[4]: HM467967; RVA/Human-wt/USA/ LB2764/2006/G2P[4]: HM467968; RVA/Human-wt/USA/ LB2771/2006/G1P[8]: HM467966; RVA/Human-wt/USA/LB2719/2006/G1P[8]: HM467964; RVA/Human-tc/USA/Wa/1974/G1P1A[8]: L04534; RVA/Human-wt/COD/ DRC88/2003/G8P[6]: DQ005107; RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]: AY787648; RVA/Human-wt/BGD/Dhaka6/2001/G11P[25]: EF560709; RVA/Sheep-tc/ESP/OVR762/2002/G8P[14]: EF554155; RVA/Human-wt/BEL/B1711/2002/G6P[6]: EF554089; RVA/Human-wt/BEL/B1711/2002/G6P[6]: EF554089; RVA/Sheep-tc/CHN/Lamb-NT/XXXX/G10P[15]: FJ031020; RVA/Macaque-tc/USA/PTRV/1990/ G8P[1]: FJ422139; RVA/Human-wt/BGD/Dhaka12-03/2003/G12P[6]: DQ146667; RVA/Human-wt/BGD/Dhaka25-02/2002/G12P[8]: DQ146656; RVA/Human-wt/BGD/ SK423/2005/G12P[6]: EU839967; RVA/Human-wt/ITA/PAH136/1996/G3P[9]: GU296412; RVA/Human-tc/USA/Wa variant Virwa/1974/G1P1A[8]: FJ423120; RVA/Human-wt/IND/ RMC321/1990/G9P[19]: AF506293; RVA/Pigeon-tc/JPN/PO-13/1983/G18P[17]: AB009625; RVA/Human-wt/ZAF/GR10924/1999/G9P[6]: FJ183361; GU565081; RVA/Cow-wt/JPN/Azuk-1/2006/G21P[29]: AB573084; RVA/Cow-tc/JPN/Dai-10/2007/ G24P[33]: AB573075; RVA/Macaque-tc/USA/PTRV/1990/G8P[1]: FJ422137; RVA/Cat-tc/AUS/Cat2/1984/G3P[9]: EU708963; RVA/Human-wt/BGD/RV161/2000/G12P[6]: DQ490541; RVA/Human-wt/IND/NR1/XXXX/GXP[X]: AF506018; RVA/Human-wt/BGD/ RV176-00/2000/G12P[6: DQ490558; RVA/Human-wt/BGD/Matlab36-02/2002/G11P[8]: GU199510; RVA/Human-wt/USA/2007719825/2007/G1P[8]: HM773751; RVA/Human-wt/DEU/GER126-08/2008/G3G12P[8]: FJ747621; RVA/Pig-tc/USA/Gottfried/1983/G4P[6]: GU199489; RVA/Pig-wt/IND/RU172/2002/G12P[7]: GU199195; RVA/Human-tc/CHN/ R479/2004/G4P[6]:

GU189556; RVA/Antelope-wt/ZAF/RC-18-08/G6P[14]: FJ495134; RVA/Sheep-tc/ESP/OVR762/2002/G8P[14]: EF554155.

Genome segment 7 (NSP3):

RVA/Human-tc/IND/M-08/XXXX/GXP[X]:AF338246; RVA/Human-wt/USA/LB2719/2006/G1P[8]: HM467970; RVA/Human-wt/USA/ LB2764/2006/G2P[4]: HM467974; RVA/Human-wt/USA/LB2744/2006/G2P[4]: HM467973; RVA/Human-wt/USA/LB2772/2006/G2P[4]: HM467975; RVA/Human-wt/USA/LB2758/2006/G1P[8]: HM467971; RVA/Human-wt/USA/LB2771/2006/G1P[8]: HM467972; RVA/Human-tc/USA/Wa/1974/G1P1A[8]; X81434; RVA/Human-tc/ITA/PA169/1988/G6P[14]: EF554134; RVA/ Human-tc/AUS/MG6/1993/G6P[14]: EF554101; RVA/Human-tc/USA/DS-1/1976/ G2P1B[4]: EF136660; RVA/Human-wt/ZAF/GR10924/1999/G9P[6]: FJ183359; RVA/Human-wt/BEL/B4633/2003/G12P[8]: DQ146646; RVA/Human-wt/BGD/Dhaka12-03/2003/G12P[6]: DQ146668; RVA/Human-wt/BGD/N26/2002/G12P[6]: DQ146690; RVA/Human-wt/COD/DRC88/2003/G8P[8]: DQ005106; RVA/Pigeon-tc/JPN/PO-13/1983/G18P[17]: AB009626; RVA/Human-wt/BEL/B3458/2003/G9P[8]: EF990711; RVA/Human-tc/PHL/L26/1987/G12P[4]: DQ146697; RVA/Human-wt/COD/DRC86/2003/ G8P[6]: DQ005117; RVA/Human-tc/JPN/IGV-80-3/XXXX/G1P[X]: AF190170; RVA/Human-wt/BGD/SK423/2005/G12P[6]: EU839968; RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]: AY787649; RVA/Cat-tc/AUS/Cat2/1984/G3P[9]: EU708964; RVA/Cat-tc/AUS/Cat97/1984/G3P[3]: EU708953; RVA/Human-wt/BGD/Matlab13/2003/G12P[6]: DQ146679; RVA/Human-wt/BGD/RV176-00/2000/G12P[6]: DQ490559; RVA/Human-wt/BGD/RV161/2000/G12P[6]: DQ490542; RVA/Human-env/BRA/rj15221-08/2008/ G1P[8]: GU831596; RVA/Human-wt/CHN/BJ0601/2006/GXP[X]: EU868888; RVA/Human-wt/IND/IS2/XXXX/G2P[X]: RVA/Human-wt/IND/DS108/XXXX/G8P[6]; RVA/Human-wt/IND/DS108/XXXX/G8P[6]: FJ861656; RVA/Human-wt/DEU/GER1H-09/2009/G8P[4]: GQ414548; RVA/Human-wt/IND/NR1/XXXX/GXP[X]: AF506019.

Genome segment 10 (NSP4):

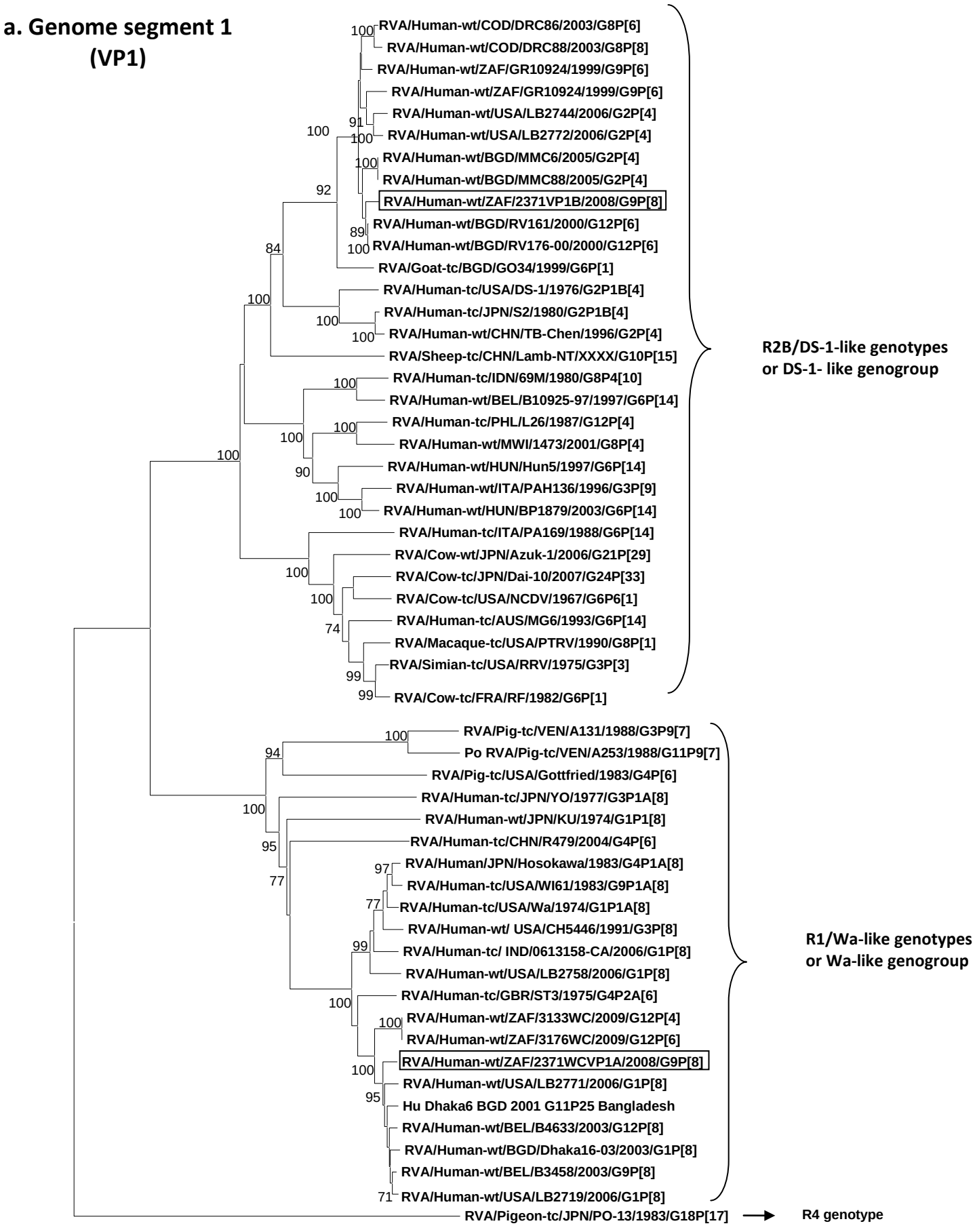
RVA/Human-wt/BEL/B10925-97/1997/G6P[14]:EF554124; RVA/Human-wt/USA/LB2771/2006/G1P[8]: HM467978; RVA/Human-wt/USA/LB2758/2006/G1P[8]: HM467977; RVA/Human-wt/USA/LB2772/2006/G2P[4]: HM467981; RVA/Human-wt/USA/LB2744/2006/G2P[4]: HM467979; RVA/Human-wt/USA/ LB2764/2006/G2P[4]: HM467980; RVA/Human-tc/USA/Wa/1974/G1P1A[8]: AF093199; RVA/Human-wt/

CHN/TB-Chen/1996/G2P[4]: AY787650; RVA/Human-wt/BGD/Dhaka6/2001/G11P[25]; RVA/Human-tc/GBR/ST3/1975/G4P2A[6]: U59110; RVA/Sheep-tc/ESP/OVR762/2002/ G8P[14]: EF554157; RVA/Cow-tc/FRA/RF/1982/G6P[1]: AY116593; RVA/Human-tc/AUS/MG6/1993/G6P[14]: EF554102; RVA/Human-tc/ITA/PA169/1988/G6P[14]: EF554135; RVA/Human-wt/HUN/Hun5/1997/G6P[14]: EF554113; RVA/Human-tc/JPN/S2/1980/G2P[4]: U59104; RVA/Human-tc/JPN/YO/1977/G3P1A[8]: AB008236; RVA/Human-wt/USA/US1206/XXXX/G9P[X]: AJ400638; RVA/Human-wt/BEL/B1711/ 2002/G6P[6]: EF554091; RVA/Human-wt/THA/CMH032-05/2005/G1P[8]: GU288646; RVA/Human-wt/KOR/CAU200/XXXX/G1P[8]: EU679381; RVA/Human-wt/KOR/ CBNU/HR-2/XXXX/GXP[X]: AF469677; RVA/Human-wt/ZAF/GR10924/1999/G9P[6]: FJ183363; RVA/Macaque-tc/USA/PTRV/1990/G8P[1]: FJ422140; RVA/Sheep-tc/ESP/ OVR762/2002/G8P[14]: EF554157; RVA/Human-wt/USA/US244/XXXX/G9P[X]: AJ400640; RVA/Human-wt/USA/US430/XXXX/G9P[X]: AJ400644; RVA/Human-wt/THA/CMH008-05/2005/G2P[4]: GU288642; RVA/Human-wt/IND/V1352/XXXX/ GXP[X]: AB196959; RVA/Human-wt/RUS/Omsk08-351/2008/G1P[8]: GQ465022; RVA/Human-wt/KOR/CAU163/XXXX/G1P[8]: EU679379; RVA/Human-wt/BGD/ Matlab13/2003/G12P[6]: DQ146680; RVA/Human-wt/BGD/RV161/2000/ G12P[6]: DQ490543; RVA/Human-tc/IND/RMC-G66/XXXX/G2P[4]: AY601545; RVA/Human-wt/IND/NR1/XXXX/GXP[X]: AF506291; RVA/Human-wt/CHN/97SZ8/ XXXX/G2P[X]: AY159649.

Genome segment 11 (NSP5):

RVA/Human-wt/USA/LB2719/2006/G1P[8]: HM467916; RVA/Human-wt/USA/LB2771/ 2006/G1P[8]: HM467918; RVA/Human-wt/USA/LB2758/2006/G1P[8]: HM467917; RVA/Human-wt/USA/LB2764/2006/G2P[4]: HM467920; RVA/Human-tc/USA/DS-1/ 1976/G2P1B[4]: EF672583; RVA/Human-wt/USA/LB2772/2006/G2P[4]: HM467921; RVA/Human-wt/USA/LB2744/2006/G2P[4]: HM467919; RVA/Human-tc/USA/Wa/ 1974/G1P1A[8]: AF306494; RVA/Pigeon-tc/JPN/PO-13/1983/G18P[17]: AB009628; RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]: AY787651; RVA/Human-wt/BGD/Dhaka6/ 2001/G11P[25]: EF560712; RVA/Sheep-tc/ESP/OVR762/2002/G8P[14]: EF554158; RVA/Human-tc/ITA/PA169/1988/G6P[14]: EF554136; RVA/Human-wt/HUN/Hun5/ 1997/G6P[14]: EF554114; RVA/Human-wt/BEL/B1711/2002/G6P[6]: EF554092; RVA/Human-wt/BRA/rj11149/2005/G9P[8]: FJ794021; RVA/Human-wt/THA/CMHO54 /2005/G2P[4]: GU288657; RVA/Human-tc/IND/RMC-G66/XXXX/G2P[4]: AY769694; RVA/Human-wt/COD/DRC86/2003/G8P[6]: DQ005115; RVA/Human-wt/BRA/rj11149/ 1998/G9P[8]: FJ794019; RVA/Human-tc/PHL/L26/1987/G12P[4]: DQ146698; RVA/ Human-wt/ZAF/GR10924/1999/G9P[6]: FJ183362; RVA/Sheep-tc/ESP/OVR762/2002/ G8P[14]:

EF554158; RVA/Cat-tc/AUS/Cat2/1984/G3P[9]: EU708966; RVA/Cat-tc/AUS/Cat97/1984/G3P[3]: EU708955; RVA/Human-wt/BGD/N26/2002/G12P[6]: DQ146692; RVA/Human-wt/BGD/Dhaka116-00/2000/G2P[4]: DQ492666; RVA/Human-tc/FRA/S79/XXXX/GXP[X]: EF590985; RVA/Human-wt/BGD/SK277/2005/G12P[6]: EU839976; RVA/Human-wt/COD/DRC88/2003/G8P[8]: DQ005104; RVA/Human-tc/FRA/S79/XXXX/GXP[X]: EF590985.


a. Genome segment 1 (VP1)
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RVA/Human-wt/USA/LB2744/2006/G2P[4]
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RVA/Human-wt/ZAF/2371VP1B/2008/G9P[8]
RVA/Human-wt/BGD/RV161/2000/G12P[6]
RVA/Human-wt/BGD/RV176-00/2000/G12P[6]
RVA/Goat-tc/BGD/GO34/1999/G6P[1]
RVA/Human-tc/USA/DS-1/1976/G2P1B[4]
RVA/Human-tc/JPN/S2/1980/G2P1B[4]
RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]
RVA/Sheep-tc/CHN/Lamb-NT/XXXX/G10P[15]
RVA/Human-tc/IDN/69M/1980/G8P4[10]
RVA/Human-wt/BEL/B10925-97/1997/G6P[14]
RVA/Human-tc/PHL/L26/1987/G12P[4]
RVA/Human-wt/MWI/1473/2001/G8P[4]
RVA/Human-wt/HUN/Hun5/1997/G6P[14]
RVA/Human-wt/ITA/PAH136/1996/G3P[9]
RVA/Human-wt/HUN/BP1879/2003/G6P[14]
RVA/Human-tc/ITA/PA169/1988/G6P[14]
RVA/Cow-wt/JPN/Azuk-1/2006/G21P[29]
RVA/Cow-tc/JPN/Dai-10/2007/G24P[33]
RVA/Cow-tc/USA/NCDV/1967/G6P6[1]
RVA/Human-tc/AUS/MG6/1993/G6P[14]
RVA/Macaque-tc/USA/PTRV/1990/G8P[1]
RVA/Simian-tc/USA/RRV/1975/G3P[3]
RVA/Cow-tc/FRA/RF/1982/G6P[1]
R2B/DS-1-like genotypes or DS-1- like genogroup
RVA/Pig-tc/VEN/A131/1988/G3P9[7]
Po RVA/Pig-tc/VEN/A253/1988/G11P9[7]
RVA/Pig-tc/USA/Gottfried/1983/G4P[6]
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RVA/Human-tc/CHN/R479/2004/G4P[6]
RVA/Human/JPN/Hosokawa/1983/G4P1A[8]
RVA/Human-tc/USA/WI61/1983/G9P1A[8]
RVA/Human-tc/USA/Wa/1974/G1P1A[8]
RVA/Human-wt/ USA/CH5446/1991/G3P[8]
RVA/Human-tc/ IND/0613158-CA/2006/G1P[8]
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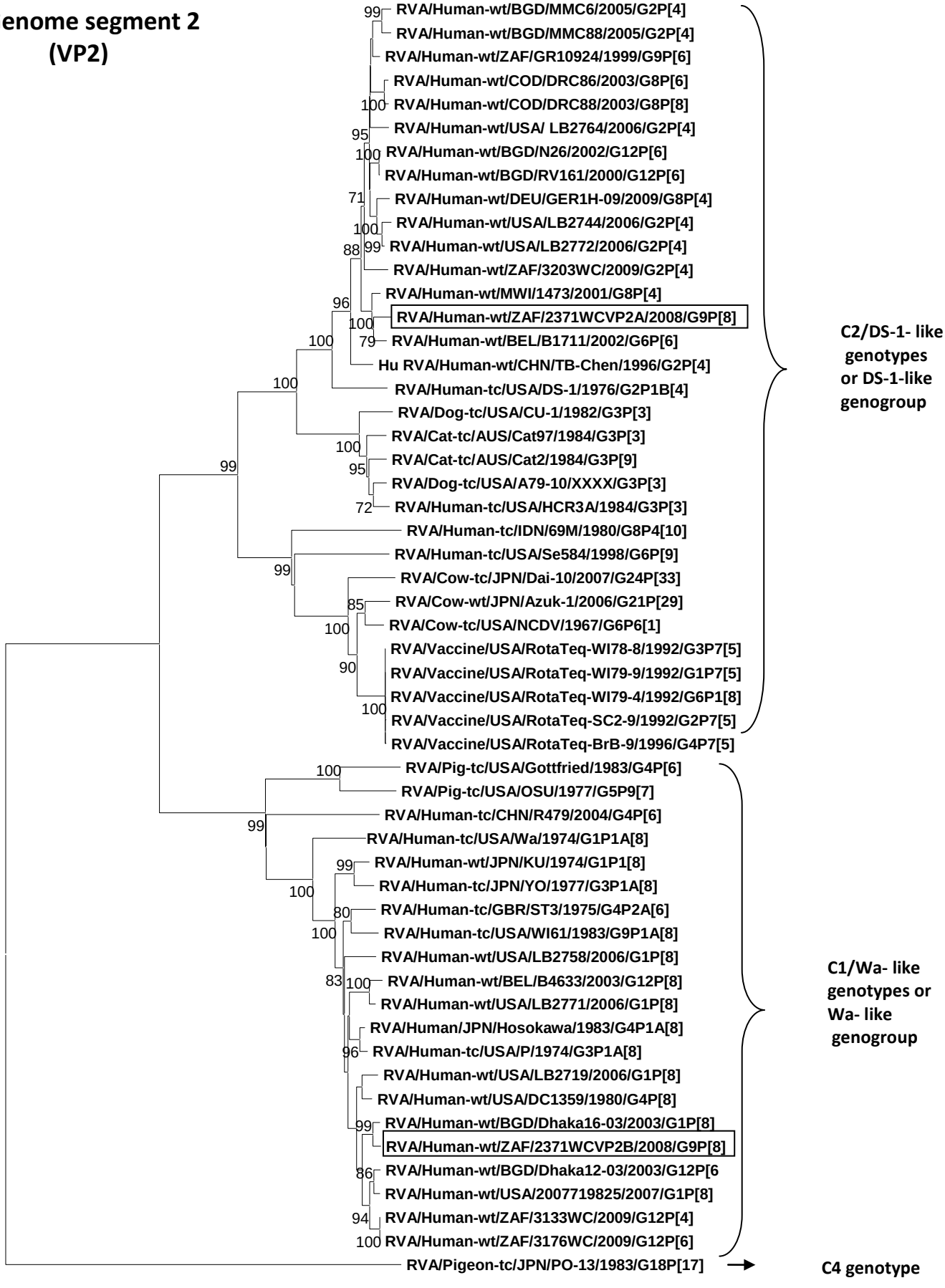
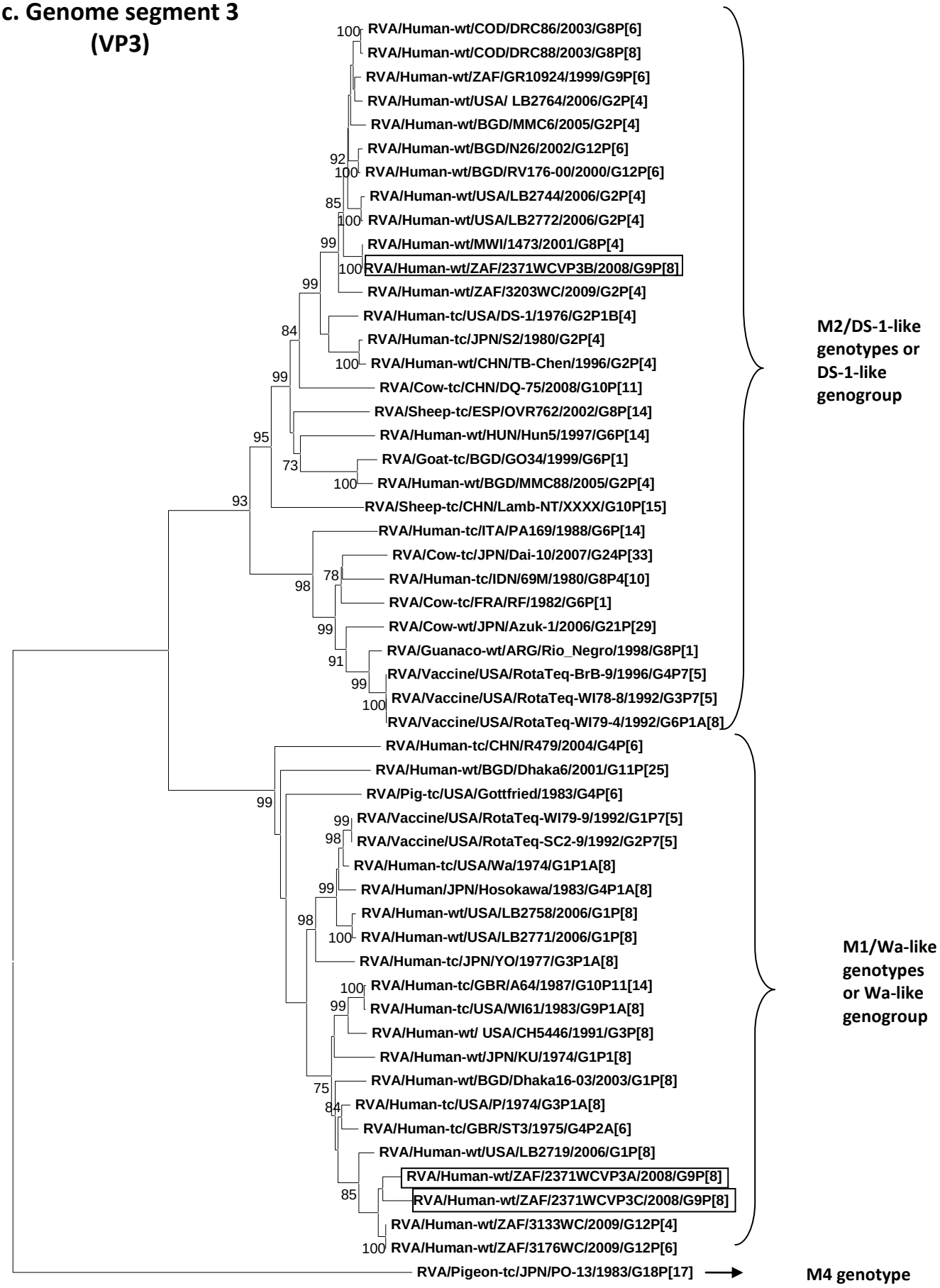
RVA/Human-tc/GBR/ST3/1975/G4P2A[6]
RVA/Human-wt/ZAF/3133WC/2009/G12P[4]
RVA/Human-wt/ZAF/3176WC/2009/G12P[6]
RVA/Human-wt/ZAF/2371WCVP1A/2008/G9P[8]
RVA/Human-wt/USA/LB2771/2006/G1P[8]
Hu Dhaka6 BGD 2001 G11P25 Bangladesh
RVA/Human-wt/BEL/B4633/2003/G12P[8]
RVA/Human-wt/BGD/Dhaka16-03/2003/G1P[8]
RVA/Human-wt/BEL/B3458/2003/G9P[8]
RVA/Human-wt/USA/LB2719/2006/G1P[8]
R1/Wa-like genotypes or Wa-like genogroup
RVA/Pigeon-tc/JPN/PO-13/1983/G18P[17]
R4 genotype
0.02

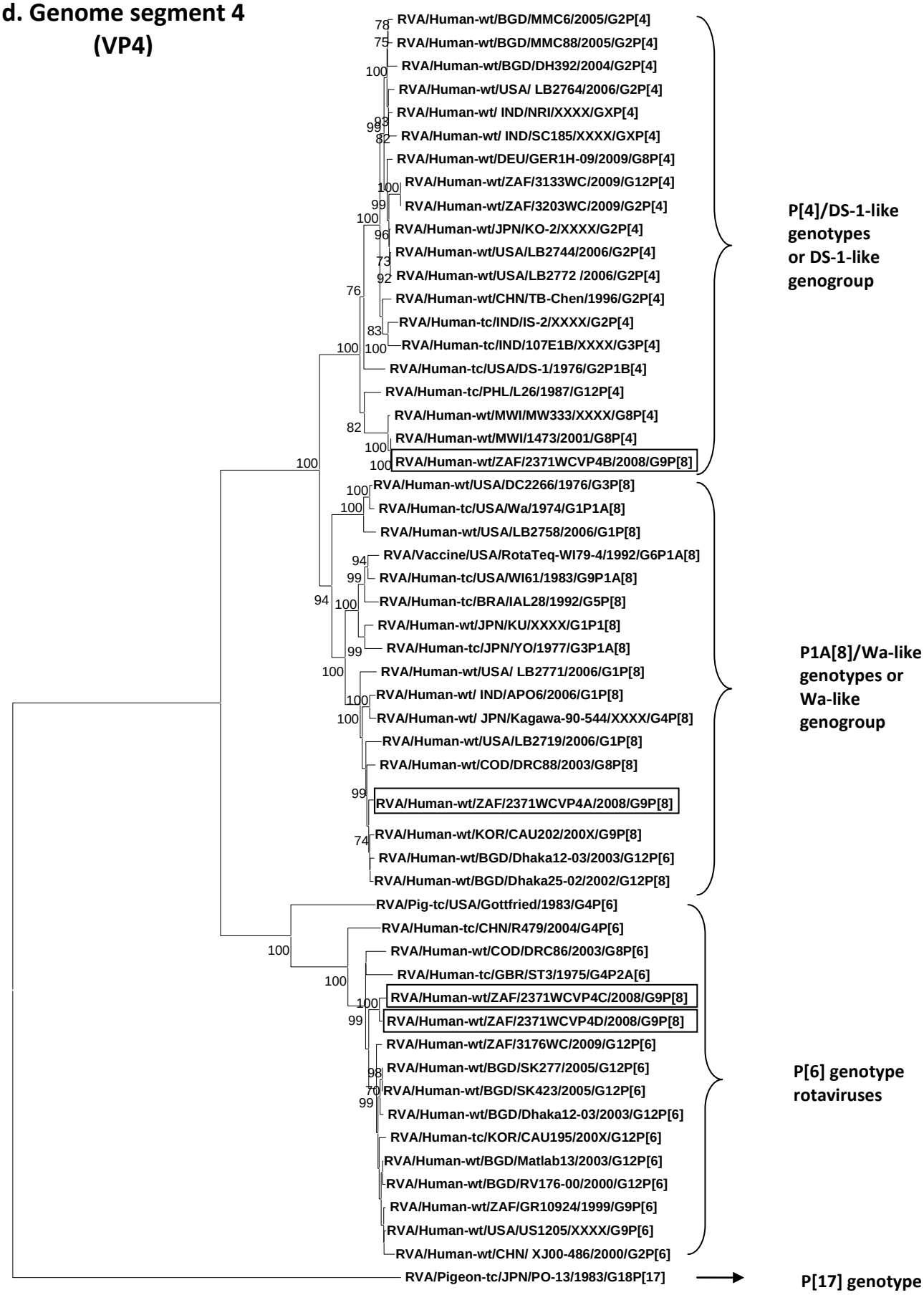
b. Genome segment 2 (VP2)



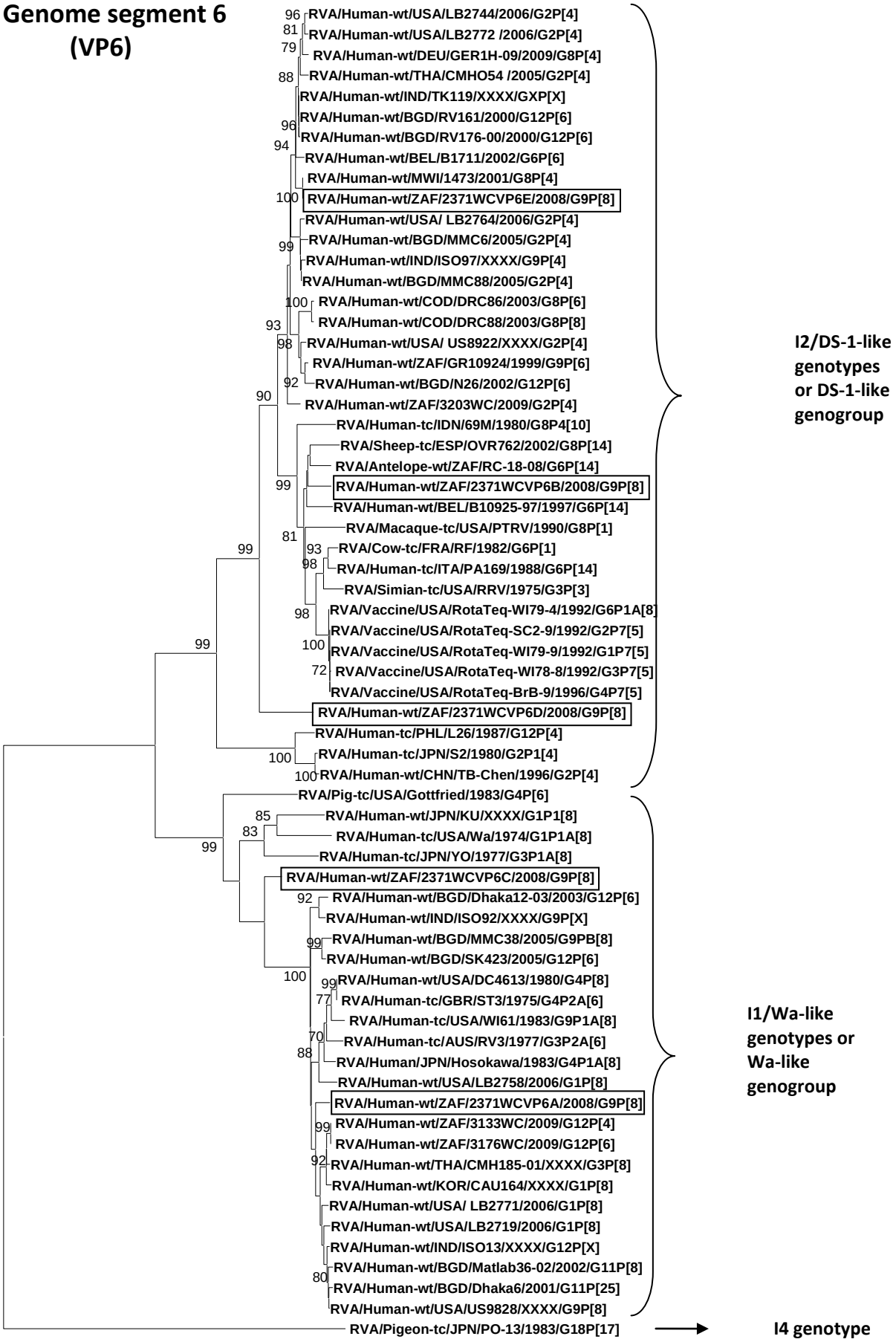
c. Genome segment 3 (VP3)



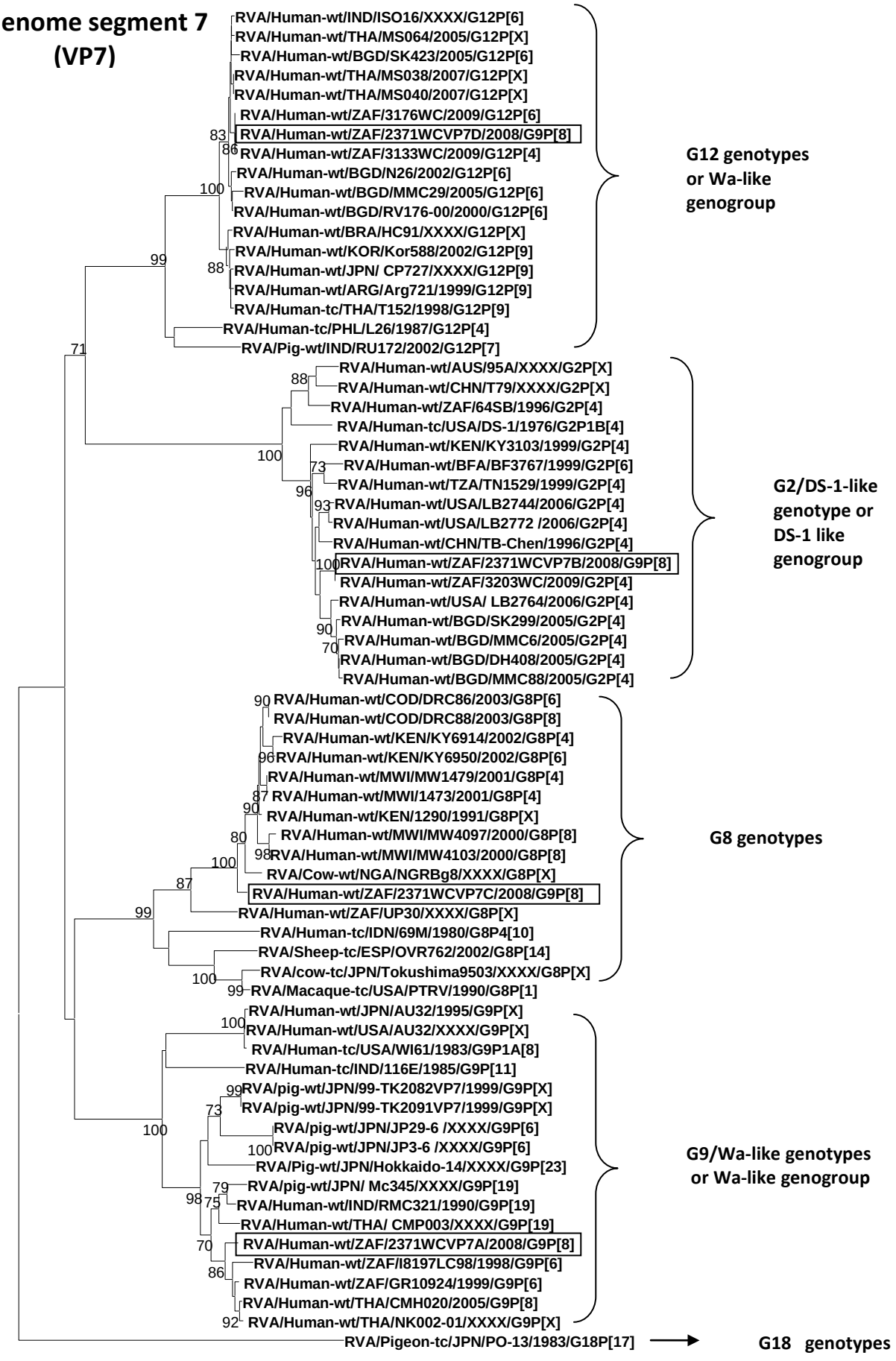
d. Genome segment 4 (VP4)



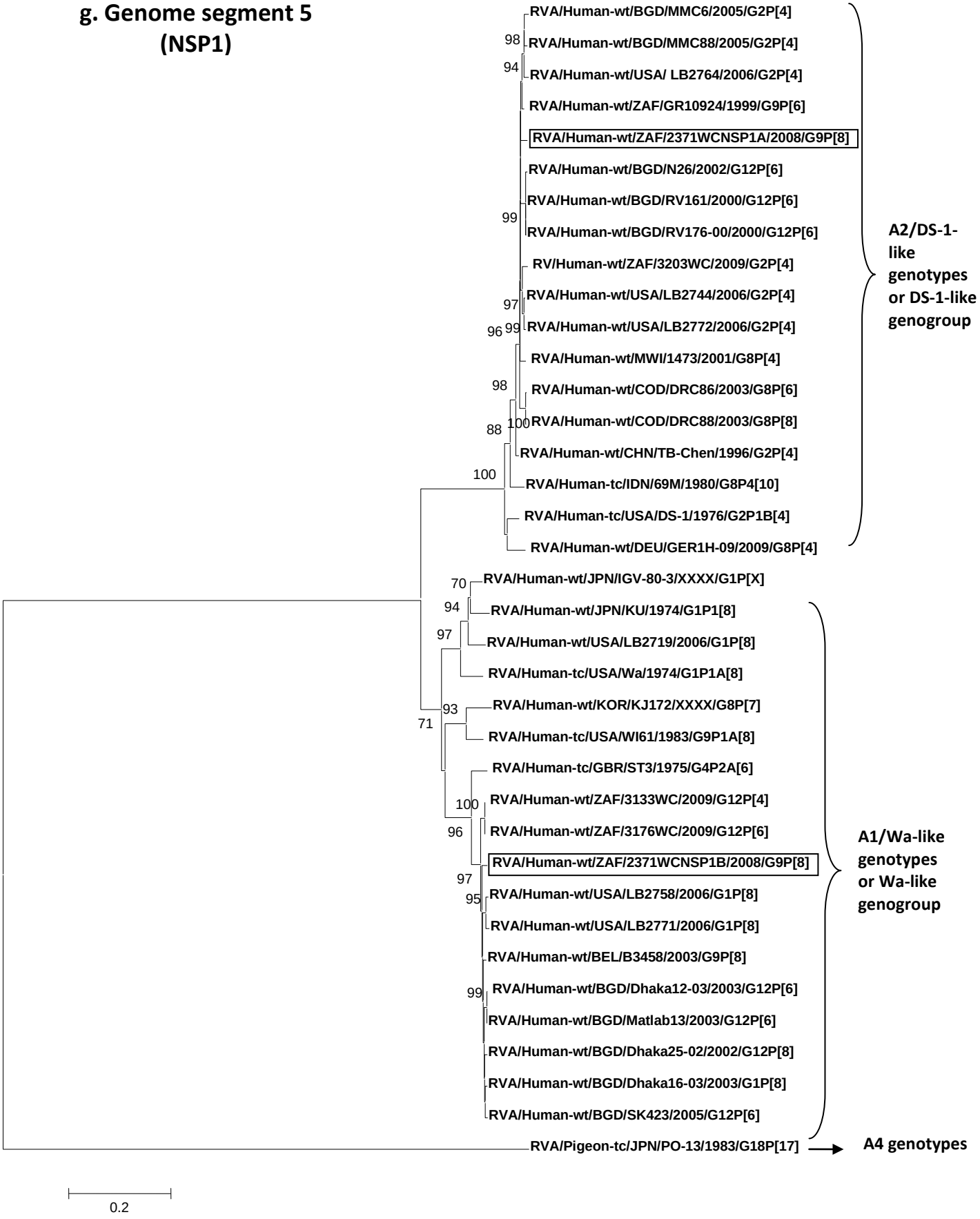
e. Genome segment 6 (VP6)



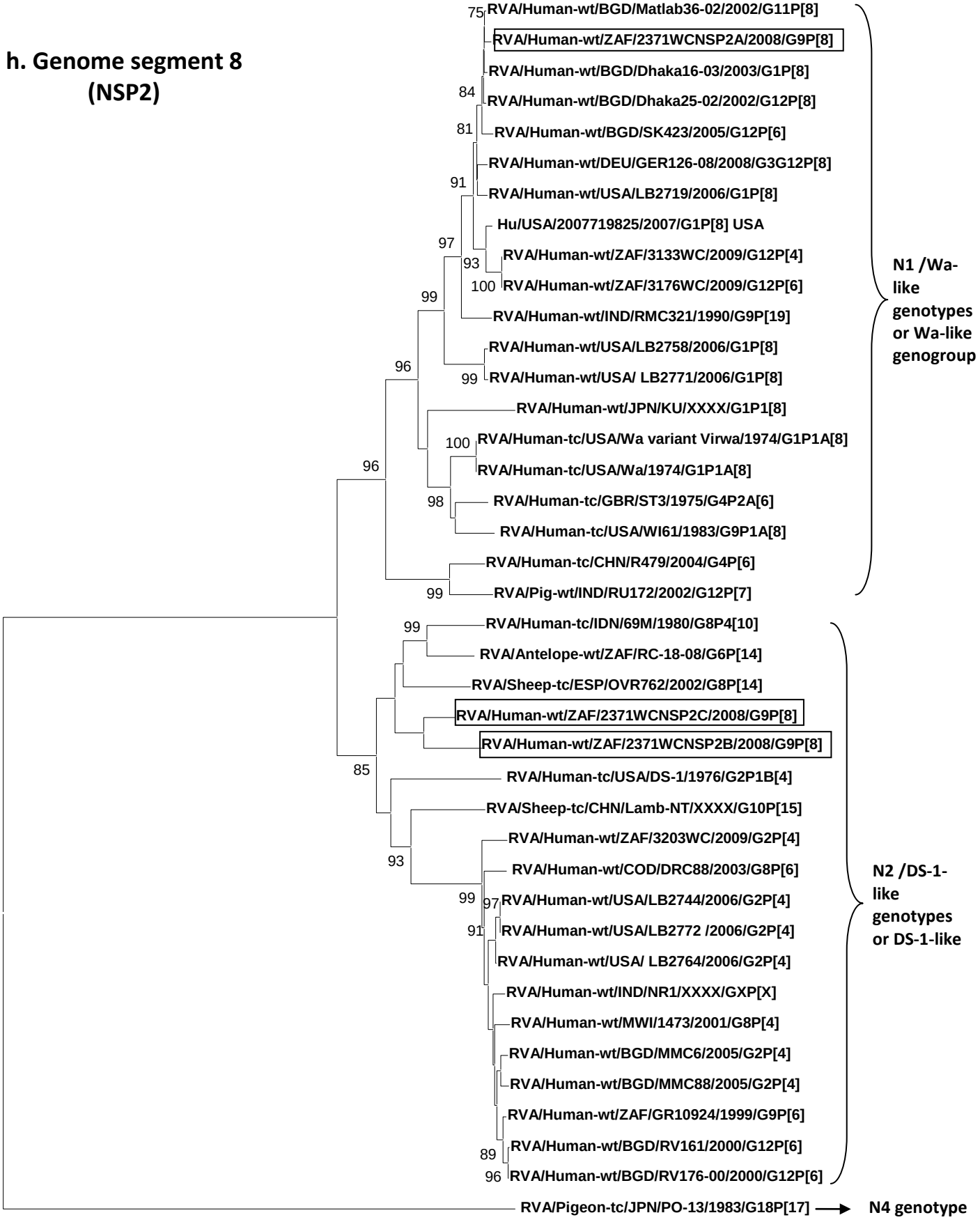
f. Genome segment 7 (VP7)



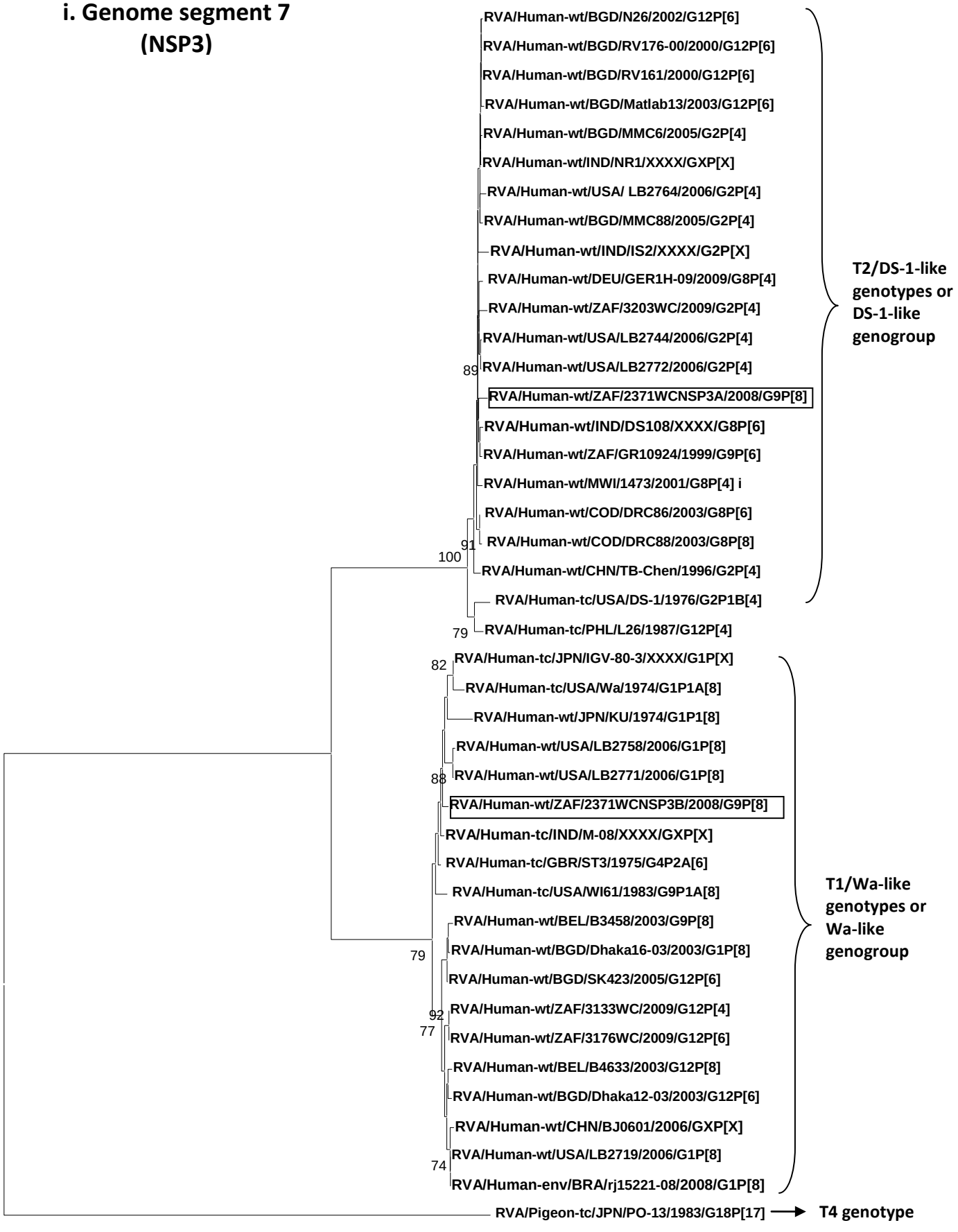
g. Genome segment 5 (NSP1)



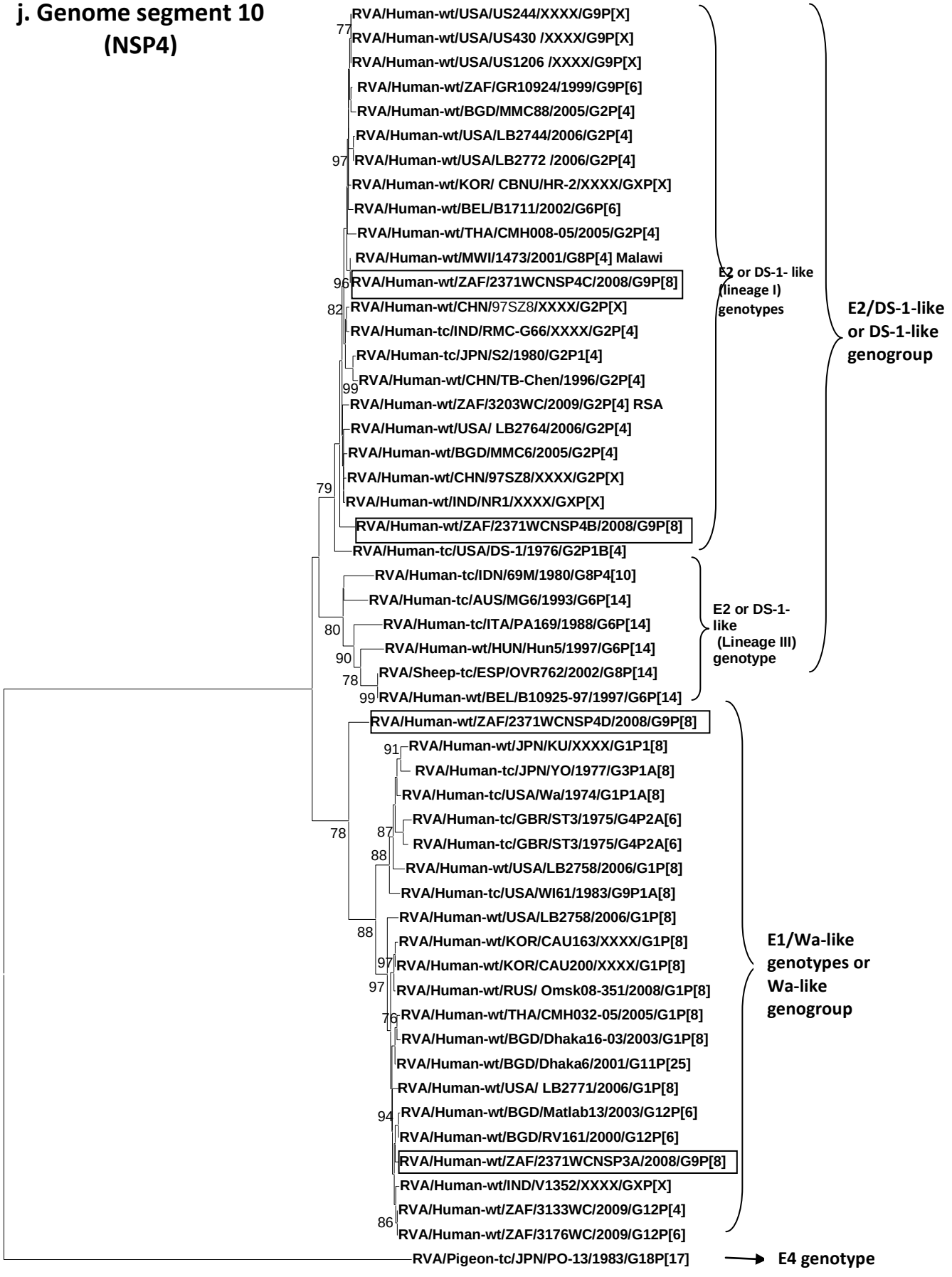
h. Genome segment 8 (NSP2)



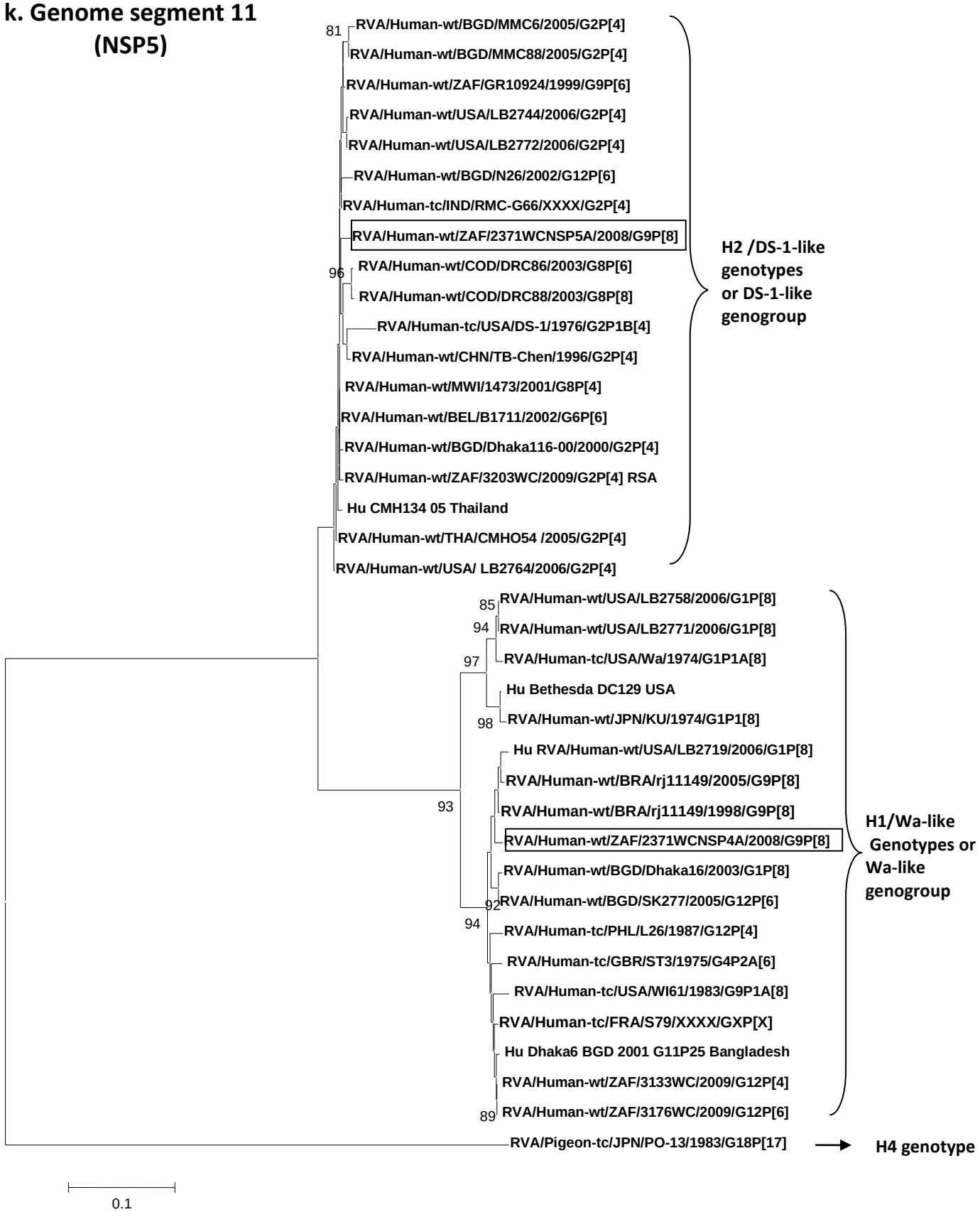
i. Genome segment 7 (NSP3)



j. Genome segment 10 (NSP4)

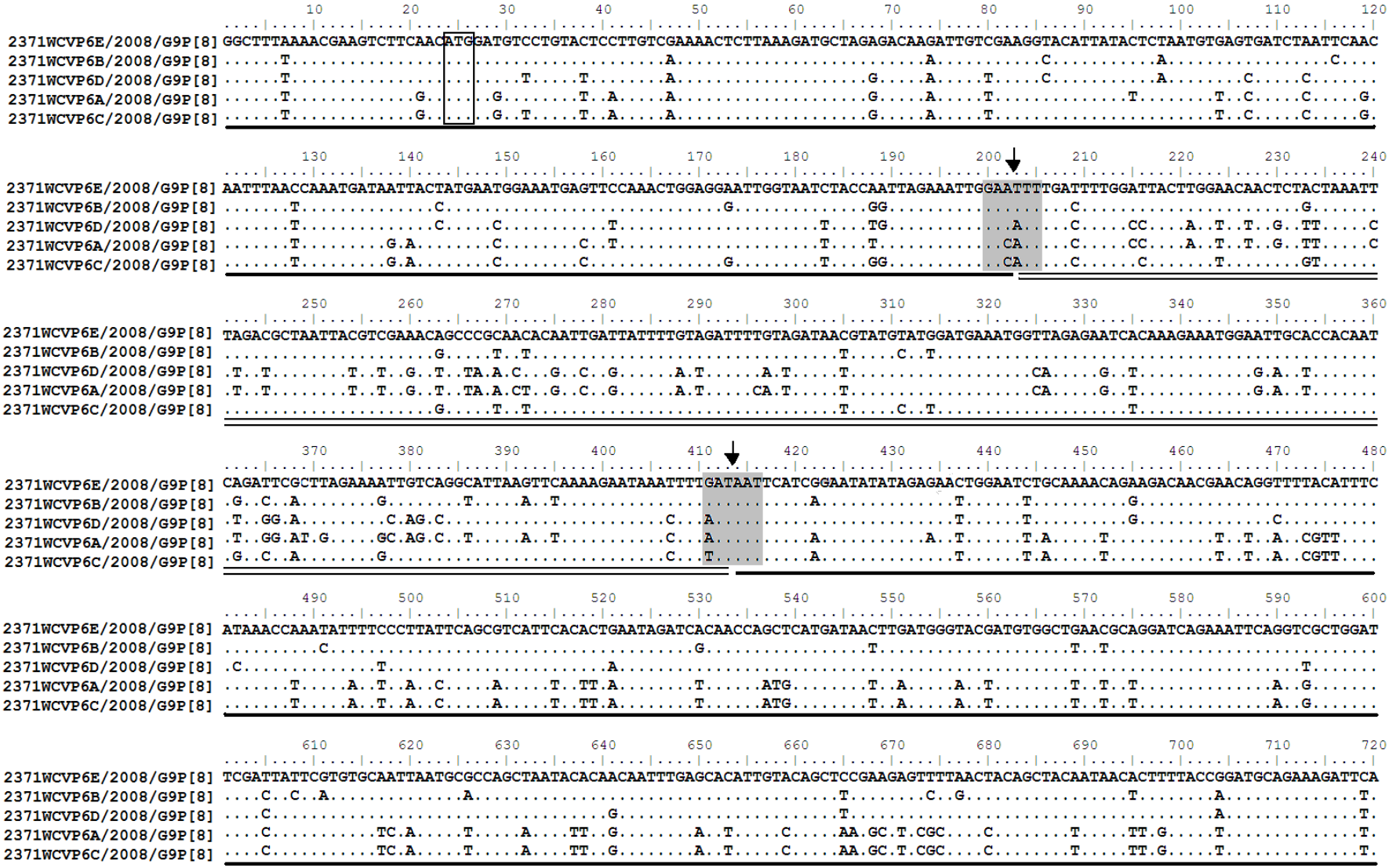


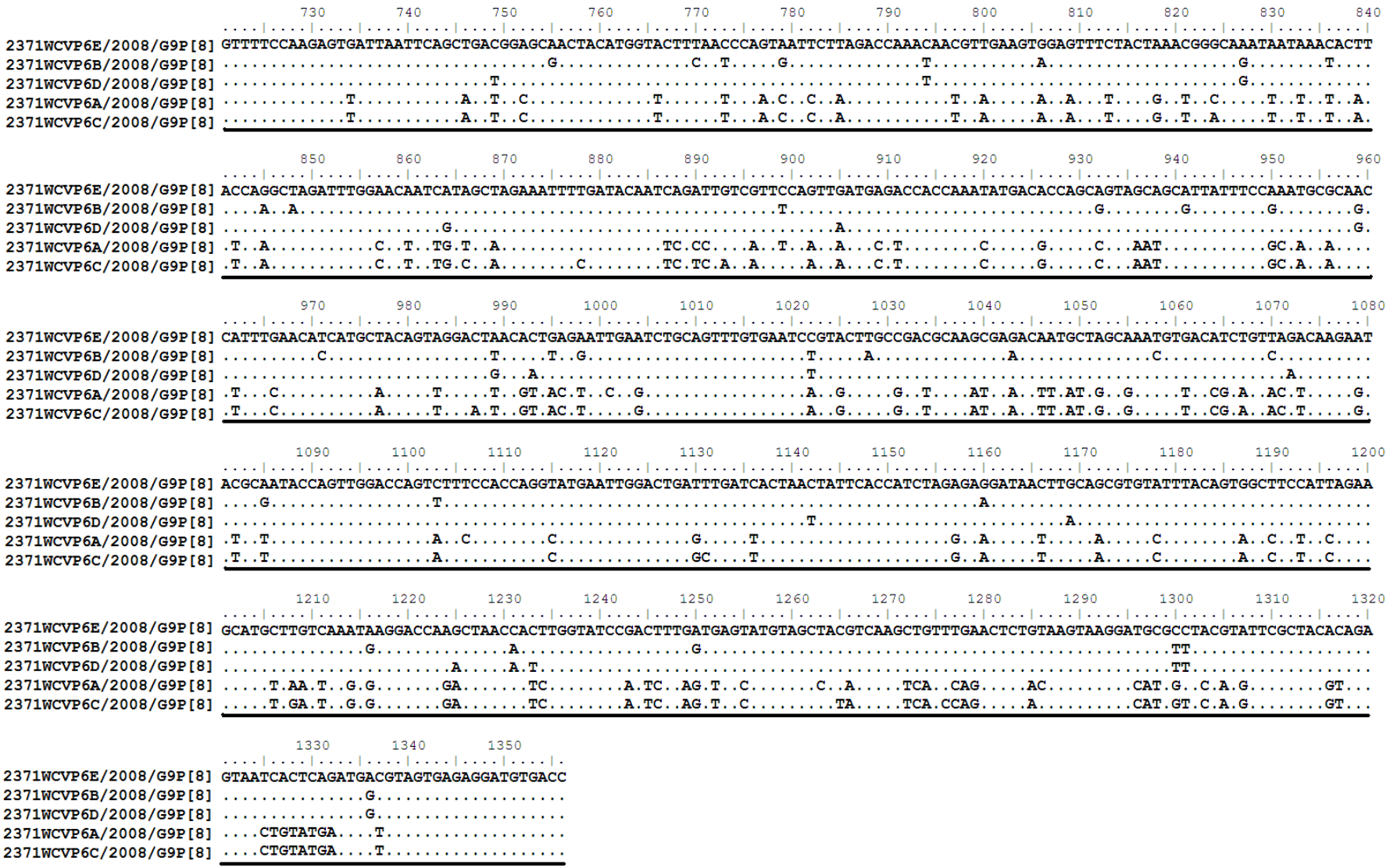
k. Genome segment 11 (NSP5)



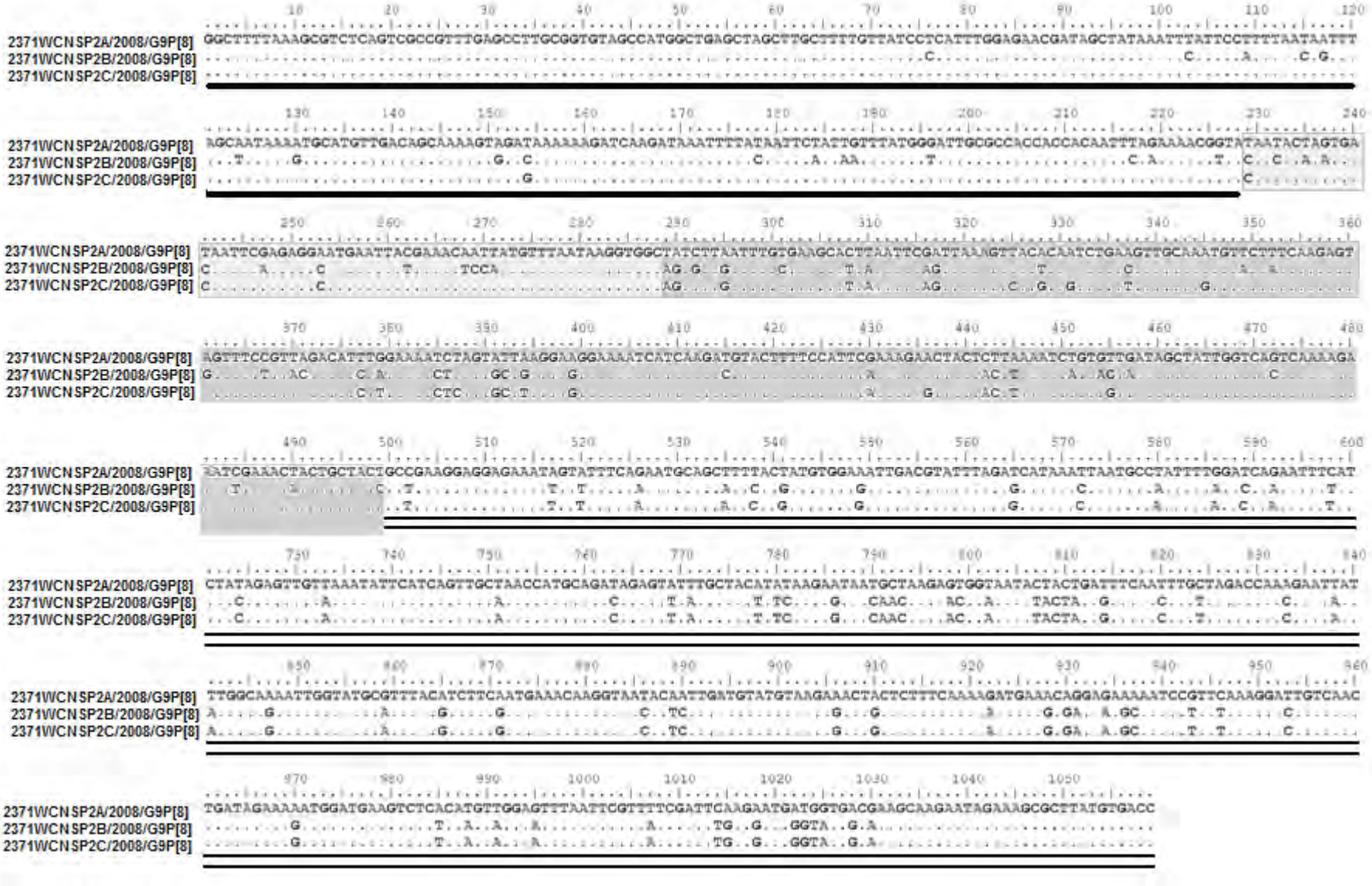
F

Supplement 3. Phylograms based on the full-length nucleotide sequences of rotavirus genome segments encoding structural (VP1–VP4, VP6 and VP7) and non-structural (NSP1–NSP5) proteins. A–F represents the phylograms for genome segments 1–4, 6 and 9 (VP1–VP4, VP6 and VP7), respectively. The nomenclature of all the rotavirus strains included in the phylograms indicates the rotavirus group/species of origin-(wild type or tissue culture sample)/country of isolation (unique 3-letter abbreviation code for each country)/common name of the sample/year of isolation/G- and P-type of each strain as proposed by the RCWG (Matthijnssens et al., 2011). Accession numbers of all the reference strains are listed in Supplement 2. The study strains are boxed. The horizontal branch lengths are proportional to the genetic distance calculated by the Neighbour-Joining method. The numbers adjacent to the nodes represent the bootstrap value of 1,000 replicates, and values less than 70% were not shown. The scale bars represent nucleotide substitutions per site. For genome segment 4 (VP4) and 9 (VP7), the nucleotide sequence assigned with specific genotypes were designated as follows: VP4A= P[8], VP4B= P[4], VP4C= P[6], VP4D= P[6], VP7A= G9, VP7B= G2, VP7C= G8, and VP7D= G12.

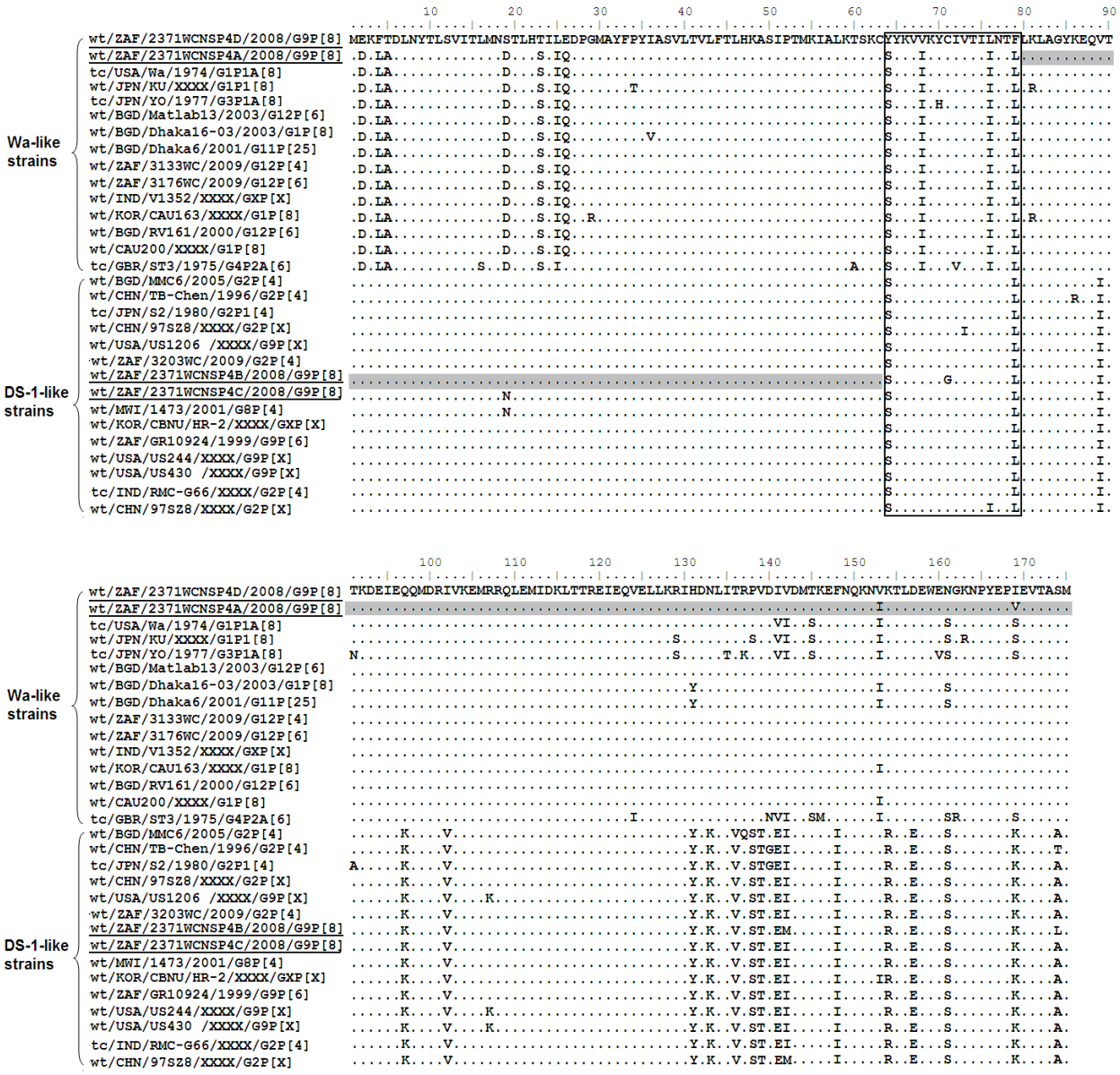

2371WCVP6E/2008/G9P[8]
2371WCVP6B/2008/G9P[8]
2371WCVP6D/2008/G9P[8]
2371WCVP6A/2008/G9P[8]
2371WCVP6C/2008/G9P[8]


730 740 750 760 770 780 790 800 810 820 830 840
2371WCVP6E/2008/G9P[8] GTTTTCCAAGAGTGATTAATTCAGCTGACGGAGCAACTACATGGTACTTTAACCCAGTAATTCTTAGACCAAACAACGTTGAAGTGGAGTTTCTACTAAACGGGCAAATAATAAACACTT
2371WCVP6B/2008/G9P[8] .....G.....C..T.....G.....T.....A.....G.....T....
2371WCVP6D/2008/G9P[8] .....T.....T.....G.....
2371WCVP6A/2008/G9P[8] .....T.....A..T..C.....T.....T...A.C..C..A.....T..A.....A..A...T....G..T..C.....T..T..T..A.
2371WCVP6C/2008/G9P[8] .....T.....A..T..C.....T.....T...A.C..C..A.....T..A.....A..A...T....G..T..A.....T..T..T..A.
850 860 870 880 890 900 910 920 930 940 950 960
2371WCVP6E/2008/G9P[8] ACCAGGCTAGATTTGGAACAATCATAGCTAGAAATTTTGATACAATCAGATTGTCGTTCCAGTTGATGAGACCACCAAATATGACACCAGCAGTAGCAGCATTATTTCCAAATGCGCAAC
2371WCVP6B/2008/G9P[8] ....A..A.....T.....G.....G.....G.....G.
2371WCVP6D/2008/G9P[8] .....G.....A.....G.
2371WCVP6A/2008/G9P[8] .T..A.....C..T..TG.T..A.....TC.CC....A..T..A..A...C.T.....C.....G.....C...AAT.....GC.A..A....
2371WCVP6C/2008/G9P[8] .T..A.....C..T..TG.C..A.....C.....TC.TC.A..A.....A..A...C.T.....C.....G.....C...AAT.....GC.A..A....
970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080
2371WCVP6E/2008/G9P[8] CATTTGAACATCATGCTACAGTAGGACTAACACTGAGAATTGAATCTGCAGTTTGTGAATCCGTACTTGCCGACGCAAGCGAGACAATGCTAGCAAATGTGACATCTGTTAGACAAGAAT
2371WCVP6B/2008/G9P[8] .....C.....T.....T..G.....T.....A.....A.....C.....C.....
2371WCVP6D/2008/G9P[8] .....G...A.....T.....A.....
2371WCVP6A/2008/G9P[8] .T...C.....A.....T.....T..GT.AC.T..C..G.....A..G.....G..T....AT..A..TT.AT.G..G.....T..CG.A..AC.T.....G.
2371WCVP6C/2008/G9P[8] .T...C.....A.....T...A.T..GT.AC.T.....G.....A..G.....G..T....AT..A..TT.AT.G..G.....T..CG.A..AC.T.....G.
1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
2371WCVP6E/2008/G9P[8] ACGCAATACCAGTTGGACCAGTCTTTCCACCAGGTATGAATTGGACTGATTTGATCACTAACTATTCACCATCTAGAGAGGATAACTTGCAGCGTGTATTTACAGTGGCTTCCATTAGAA
2371WCVP6B/2008/G9P[8] ....G.....T.....A.....
2371WCVP6D/2008/G9P[8] .....T.....A.....
2371WCVP6A/2008/G9P[8] .T..T.....A..C.....C.....G.....T.....G..A.....T.....A.....C.....A..C..T..C....
2371WCVP6C/2008/G9P[8] .T..T.....A.....C.....GC....T.....G..A.....T.....A.....C.....A..C..T..C....
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320
2371WCVP6E/2008/G9P[8] GCATGCTTGTCAAATAAGGACCAAGCTAACCACTTGGTATCCGACTTTGATGAGTATGTAGCTACGTCAAGCTGTTTGAACTCTGTAAGTAAGGATGCGCCTACGTATTCGCTACACAGA
2371WCVP6B/2008/G9P[8] .....G.....A.....G.....TT.....
2371WCVP6D/2008/G9P[8] .....A.....A.T.....TT.....
2371WCVP6A/2008/G9P[8] .....T.AA.T..G.G.....GA.....TC.....A.TC..AG.T..C.....C..A.....TCA..CAG.....AC.....CAT.G..C.A.G.....GT...
2371WCVP6C/2008/G9P[8] .....T.GA.T..G.G.....GA.....TC.....A.TC..AG.T..C.....TA.....TCA.CCAG.....A.....CAT.GT.C.A.G.....GT...
1330 1340 1350
2371WCVP6E/2008/G9P[8] GTAATCACTCAGATGACGTAGTGAGAGGATGTGACC
2371WCVP6B/2008/G9P[8] .....G.....
2371WCVP6D/2008/G9P[8] .....G.....
2371WCVP6A/2008/G9P[8] ....CTGTATGA....T.....
2371WCVP6C/2008/G9P[8] ....CTGTATGA....T.....

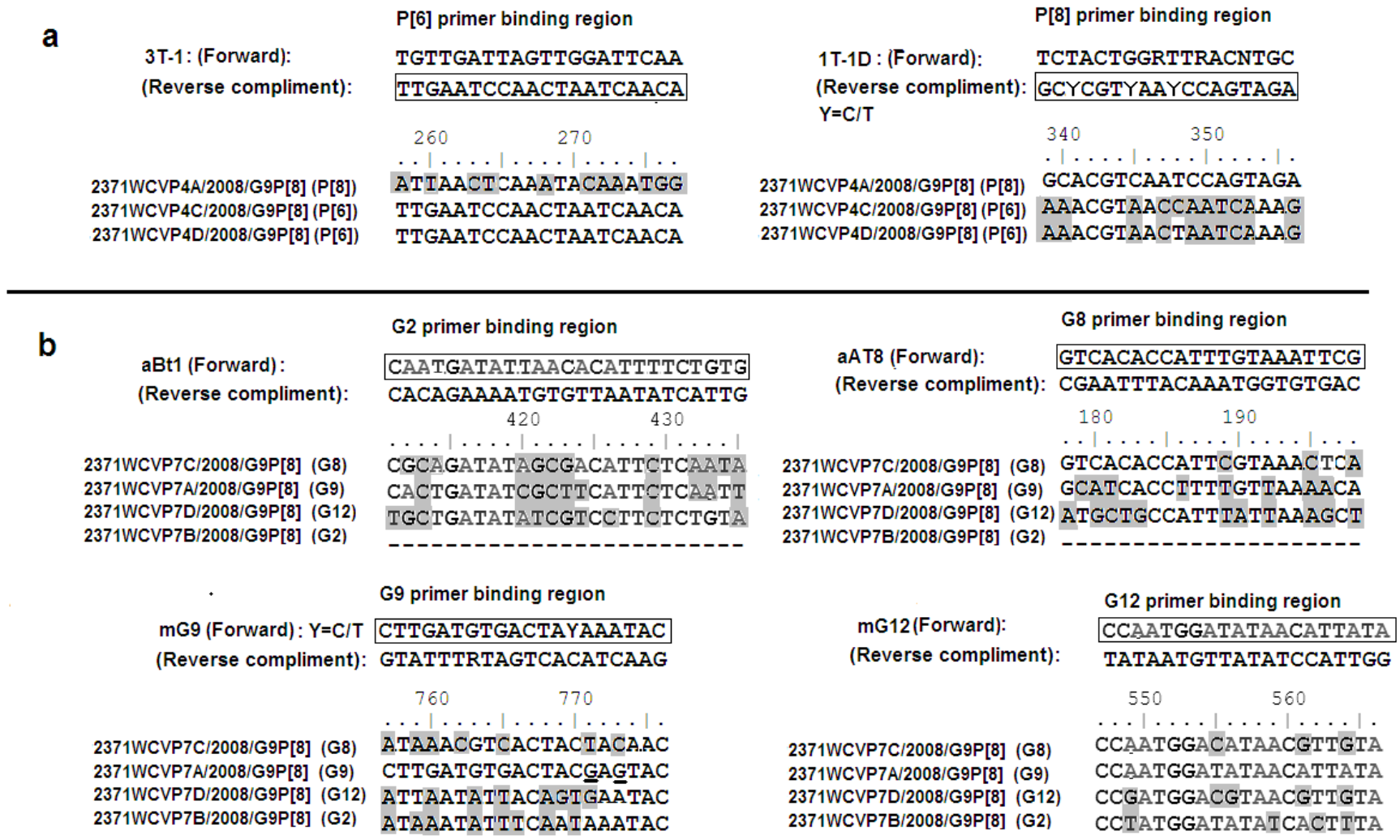
Supplement 4. An alignment of five distinct complete consensus nucleotide sequences identified in this study for genome segment 6 (VP6). In the region between nucleotide 1–202 and 414–1356 (underlined with a single line) RVA/Human-wt/ZAF/ 2371WCVP6A/2008/G9P[8] and RVA/Human-wt/ZAF/2371WCVP6C/2008/G9P[8] were very similar, whereas RVA/Human-wt/ZAF/2371WCVP6B/2008/G9P[8] and RVA/ Human-wt/ZAF/2371WCVP6D/2008/G9P[8] resembled each other. From nucleotide position 203–413 (underlined with double lines), RVA/ Human-wt/ZAF/2371WCVP6A/2008/G9P[8] was almost identical to RVA/Human-wt/ZAF/2371WCVP6D/2008/G9P[8], whereas RVA/ Human-wt/ZAF/2371WCVP6B/2008/G9P[8], RVA/Human-wt/ZAF/2371WCVP6C/2008/G9P[8] and RVA/Human-wt/ZAF/2371WCVP6E/ 2008/G9P[8] were closely related. The break points where recombination was suspected are shown with arrows between nt 202–203 (between codons GAA and TTT) and 413–414 (between codons GAT and AAT) (shaded in grey). The ATG translation start point is boxed. Dots (.) represent identical nucleotide to the one appearing along RVA/Human-wt/ZAF/2371WCVP6E/2008/G9P[8] sequence. The prefix RVA/Human-wt/ZAF/ was omitted from the name of each nucleotide sequence.


2371WCNSP2A/2008/G9P[8]
2371WCNSP2B/2008/G9P[8]
2371WCNSP2C/2008/G9P[8]

Supplement 5. Alignment of the complete nucleotide sequences of the genome segment 8 (NSP2) obtained in this study. RVA/Human-wt/ZAF/ 2371WCNSP2C/2008/G9P[8] resembled RVA/Human-wt/ZAF/2371WCNSP2A/2008/G9P[8] from nucleotide position 1–228 (underlined with a single line) and RVA/Human-wt/ZAF/2371WCNSP2B/2008/G9P[8] from nucleotide 500–1059 (underline with double lines). Nucleotide variations were observed between 229–499 in all the three sequences (shaded in grey). However, the nucleotide sequence of RVA/Human-wt/ZAF/2371WCNSP2C/2008/G9P[8] partly resembled RVA/Human-wt/ZAF/2371WCNSP2A/2008/G9P[8] from 229–288 (shaded in light grey), whereas from 289–499 (shaded dark grey), it resembled RVA/Human-wt/ZAF/ 2371WCNSP2B/2008/G9P[8]. Dots (.) represent identical nucleotide to the one appearing along RVA/Human-wt/ZAF/2371WCNSP2A/2008/G9P[8] sequence. The prefix RVA/Human-wt/ZAF/ was omitted in all the names of nucleotide sequences.



Supplement 6. Alignments of amino acid sequences for NSP4 obtained in this study for strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8] against other reference strains. From aa 1–63 (corresponding to nt 1–172) RVA/Human-wt/ZAF/2371WCNSP4D/ 2008/G9P[8] was identical to RVA/Human-wt/ZAF/2371WC NSP4B/2008/G9P[8] (shaded in grey) and closely related to other DS-1-ike strains. From aa 80–175 (corresponding to nt 288–750), RVA/Human-wt/ZAF/2371WCNSP4D/2008/G9P[8] was similar to RVA/Human-wt/ZAF/2371WCNSP4A/ 2008/G9P[8 (shaded in grey) and other genomes segments 10 of other Wa-like strains. RVA/Human-wt/ZAF/2371WCNSP4D/2008/G9P[8] was not closely related to either Wa- or DS-1-like rotavirus strains from 64–79 aa (corresponding to nt 173–287) (boxed regions). Dots (.) represent identical nucleotide to the one appearing along RVA/Human-wt/ZAF/2371WCNSP4D/ 2008/G9P[8] sequence. All the strains were isolated from humans and the prefix RVA/Human-wt/ZAF/ was omitted in all the names of nucleotide sequences.


a
P[6] primer binding region
3T-1 : (Forward):
TGTTGATTAGTTGGATTCAA
(Reverse compliment):
TTGAATCCAACTAATCAACA
260
270
2371WCVP4A/2008/G9P[8] (P[8])
ATTAACTCAAATACAAATGG
2371WCVP4C/2008/G9P[8] (P[6])
TTGAATCCAACTAATCAACA
2371WCVP4D/2008/G9P[8] (P[6])
TTGAATCCAACTAATCAACA
P[8] primer binding region
1T-1D : (Forward):
TCTACTGGRTTRACNTGC
(Reverse compliment):
GCYCGTYAAYCCAGTAGA
Y=C/T
340
350
2371WCVP4A/2008/G9P[8] (P[8])
GCACGTCAATCCAGTAGA
2371WCVP4C/2008/G9P[8] (P[6])
AAACGTAACCAATCAAAG
2371WCVP4D/2008/G9P[8] (P[6])
AAACGTAACTAATCAAAG
b
G2 primer binding region
aBt1 (Forward) :
CAATGATATTAACACATTTTCTGTG
(Reverse compliment):
CACAGAAAATGTGTTAATATCATTG
420
430
2371WCVP7C/2008/G9P[8] (G8)
CGCAGATATAGCGACATTCTCAATA
2371WCVP7A/2008/G9P[8] (G9)
CACTGATATCGCTTCATTCTCAATT
2371WCVP7D/2008/G9P[8] (G12)
TGCTGATATATCGTCCTTCTCTGTA
2371WCVP7B/2008/G9P[8] (G2)
G8 primer binding region
aAT8 (Forward):
GTCACACCATTTGTAAATTCG
(Reverse compliment):
CGAATTTACAAATGGTGTGAC
180
190
2371WCVP7C/2008/G9P[8] (G8)
GTCACACCATTCGTAAACTCA
2371WCVP7A/2008/G9P[8] (G9)
GCATCACCTTTTGTTAAAACA
2371WCVP7D/2008/G9P[8] (G12)
ATGCTGCCATTTATTAAAGCT
2371WCVP7B/2008/G9P[8] (G2)
G9 primer binding region
mG9 (Forward) : Y=C/T
CTTGATGTGACTAYAAATAC
(Reverse compliment):
GTATTTRTAGTCACATCAAG
760
770
2371WCVP7C/2008/G9P[8] (G8)
ATAAACGTCACTACTACAAC
2371WCVP7A/2008/G9P[8] (G9)
CTTGATGTGACTACGAGTAC
2371WCVP7D/2008/G9P[8] (G12)
ATTAATATTACAGTGAATAC
2371WCVP7B/2008/G9P[8] (G2)
ATAAATATTTCAATAAATAC
G12 primer binding region
mG12 (Forward):
CCAATGGATATAACATTATA
(Reverse compliment):
TATAATGTTATATCCATTGG
550
560
2371WCVP7C/2008/G9P[8] (G8)
CCAATGGACATAACGTTGTA
2371WCVP7A/2008/G9P[8] (G9)
CCAATGGATATAACATTATA
2371WCVP7D/2008/G9P[8] (G12)
CCGATGGACGTAACGTTGTA
2371WCVP7B/2008/G9P[8] (G2)
CCTATGGATATATCACTTTA

Supplement 7. Primer binding sites for genome segment 4 (VP4) and 9 (VP7) genotype-specific oligonucleotides. a) The P[6] (3T-1) and P[8] (1T-1D) primer binding region within the identified nucleotide sequences of the genome segment 4 (VP4) (Gentsch et al., 1992); b) the G2 (aBt1), G8 (aAT8), G12 (mG12) and G9 (mG9) primer binding region within the obtained nucleotide sequences of the genome segment 9 (VP7) (Gouvea et al., 1990; Das et al., 1994). Mismatches between the study nucleotide sequences to the oligonucleotides are shaded in grey. The mismatches between the Hu/RSA/2371WCVP7A/2008/G9P[8] (G9) study nucleotide sequence to the G9 primer sequence at nucleotide positions 771 and 773 (underlined) was a result of normal variation that occurs between different rotavirus strains as the Human-wt/ZAF/2371WCVP7A/2008/G9P[8] was isolated from strain RVA-Human-wt/ZAF/2371WC/2008/G9P[8], whereas the primer mG9 was designed based on the nucleotide sequence of strain RVA/Human-tc/USA/WI61/1983/G9P1A[8]. The matching nucleotides between the forward and complementary sequences of the primers to the study sequences are boxed. The prefix RVA/Human-wt/ZAF/ was omitted from the name of each nucleotide sequence.