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Appendix 1

A. Genome segment 1 (VP1)

	1	2	3	4	5	6	7	8	9	10	11
1	**SA11-N2**										
2	**SA11-N5**	100.00									
3	SA11-H96	100.00	100.00								
4	SA11-Both	99.92	99.92	99.92							
5	SA11-5S	99.99	99.99	99.99	99.90						
6	SA11-5N	99.99	99.99	99.99	99.90	100.00					
7	SA11-30/1A	99.97	99.97	99.97	99.89	99.99	99.99				
8	SA11-30/19	99.96	99.96	99.96	99.88	99.97	99.97	99.99			
9	SA11-tsC	99.99	99.99	99.99	99.90	99.97	99.97	99.96	99.94		
10	DQ457016	99.97	99.97	99.97	99.89	99.99	99.99	100.00	99.99	99.96	
11	AF015955	99.93	99.93	99.93	99.85	99.20	99.20	99.90	99.89	99.20	99.90

B. Genome segment 2 (VP2)

	1	2	3	4	5	6	7	8	9	10	11	12
1	**SA11-N2**											
2	**SA11-N5**	100.00										
3	SA11-H96	100.00	100.00									
4	SA11-Both	99.73	99.73	99.73								
5	SA11-5S	99.96	99.96	99.96	99.70							
6	SA11-5N	99.96	99.96	99.96	99.70	100.00						
7	SA11-30/1A	99.92	99.92	99.92	99.66	99.96	99.96					
8	SA11-30/19	99.96	99.96	99.96	99.70	100.00	100.00	99.96				
9	SA11-R-L	99.96	99.96	99.96	99.70	99.92	99.92	99.89	99.92			
10	SA11-tsF(A)	99.92	99.92	99.92	99.66	99.89	99.89	99.85	99.89	99.89		
11	SA11-tsF(B)	99.92	99.92	99.92	99.66	99.89	99.89	99.85	99.89	99.89	100.00	
12	SA11-C13	99.01	99.01	99.01	99.05	99.97	99.97	99.93	99.97	99.97	99.93	99.93

C. Genome segment 3 (VP3)

	1	2	3	4	5	6	7	8	9	10
1	**SA11-N2**									
2	**SA11-N5**	100.00								
3	SA11-H96	99.96	99.96							
4	SA11-Both	99.84	99.84	99.80						
5	SA11-5S	100.00	100.00	99.96	99.84					
6	SA11-5N	99.96	99.96	99.92	99.80	99.96				
7	SA11-30/1A	99.96	99.96	99.92	99.80	99.96	99.92			
8	SA11-30/10	99.96	99.96	99.92	99.80	99.96	99.92	100.00		
9	SA11-tsB	99.92	99.92	99.88	99.76	99.92	99.88	99.88	99.88	
10	X16062	99.92	99.92	99.88	99.76	99.92	99.88	99.88	99.88	99.84

D. Genome segment 4 (VP4)

	1	2	3	4	5	6	7	8	9	10	11	12
1	**SA11-N2**											
2	**SA11-N5**	100.00										
3	SA11-H96	99.61	99.61									
4	SA11-Both	99.61	99.61	100.00								
5	SA11-5S	65.79	65.79	65.84	65.84							
6	SA11-5N	65.57	65.57	65.62	65.62	99.87						
7	SA11-30/1A	65.48	68.48	65.53	65.53	99.83	99.96					
8	SA11-30/19	65.60	65.60	65.66	65.66	99.91	99.79	99.83				
9	SA11-SEM	99.96	99.96	99.91	99.61	65.69	65.47	65.38	65.50			
10	SA11-L2	99.70	99.70	99.74	99.74	65.57	65.35	65.26	65.38	99.64	99.66	
11	O agent	65.71	65.71	65.77	65.77	99.96	99.91	99.87	99.87	65.61	65.49	
12	D16346	99.96	99.96	99.66	99.66	64.86	65.64	65.55	65.68	99.91	99.74	65.71

E. Genome segment 5 (NSP1)

	1	2	3	4	5	6	7	8	9
1	**SA11-N2**								
2	**SA11-N5**	100.00							
3	SA11-H96 (USA)	99.78	99.78						
4	SA11-H96 (India)	99.72	99.72	99.50					
5	SA11-Both	99.67	99.67	99.89	99.39				
6	SA11-5S	99.67	99.67	99.89	99.39	99.78			
7	SA11-FEM	99.05	99.05	99.28	99.77	99.28	99.17		
8	SA11-4F	99.67	99.67	99.89	99.39	99.78	100.00	99.17	
9	SA11-P-L	99.28	99.28	99.05	99.00	99.05	98.94	98.31	98.94

F. Genome segment 6 (VP6)

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	**SA11-N5**												
2	**SA11-N2**	100.00											
3	SA11-H96 (USA)	100.00	100.00										
4	SA11-H96 (India)	99.83	99.83	99.83									
5	SA11-Both	99.66	99.66	99.66	99.83								
6	SA11-5S	99.92	99.92	99.92	99.75	99.75							
7	SA11-5N	100.00	100.00	100.00	99.83	99.66	99.92						
8	SA11-30/1A	99.92	99.92	99.92	99.75	99.48	99.83	99.92					
9	SA11-30/19	100.00	100.00	100.00	99.83	99.66	99.92	100.00	99.92				
10	SA11-tsG	99.66	99.66	99.66	99.49	99.32	99.48	99.66	99.48	99.66			
11	SA11-R-L	99.92	99.92	99.92	99.75	99.48	99.83	99.92	99.83	99.92	99.48		
12	M27824	99.92	99.92	99.92	99.75	99.48	99.83	99.92	99.83	99.92	99.48	99.83	
13	X00421	100.00	100.00	100.00	99.83	99.66	99.92	100.00	99.92	100.00	99.66	99.92	99.92

G. Genome segment 7 (NSP3)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	**SA11N2**														
2	**SA11-N5**	100.00													
3	SA11-H96 (USA)	100.00	100.00												
4	SA11-H96 (India)	99.79	99.79	99.79											
5	SA11-Both	99.68	99.68	99.68	99.47										
6	SA11-5S	99.89	99.89	99.89	99.68	99.48									
7	SA11-5N	99.89	99.89	99.89	99.68	99.48	99.79								
8	SA11-30/1A	99.89	99.89	99.89	99.68	99.48	99.79	100.00							
9	SA11-30/19	99.89	99.89	99.89	9968.00	99.48	99.79	100.00	100.00						
10	SA11-C14	100.00	100.00	100.00	99.68	99.68	99.89	99.89	99.89	99.89					
11	SA11-4F(A)	99.89	99.89	99.89	99.68	99.48	99.79	100.00	100.00	100.00	99.89				
12	SA11-4F_(B)	99.68	99.68	99.68	99.47	99.48	99.48	99.79	99.79	99.79	99.68	99.79			
13	SA11-L2	99.89	99.89	99.89	99.89	99.48	99.79	99.79	99.79	99.79	99.89	99.79	99.48		
14	SA11-L2Ga613	99.79	99.79	99.79	99.79	99.47	99.68	99.68	99.68	99.68	99.79	99.68	99.47	99.89	
15	SA11-L2Ao123	99.89	99.89	99.89	99.89	99.48	99.79	99.79	99.79	99.79	99.89	99.79	99.48	100.00	99.89

H. Genome segment 8 (NSP2)

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	**SA11-N2**												
2	**SA11-N5**	74.05											
3	SA11-H96 (USA)	74.05	100.00										
4	SA11-H96 (India)	73.74	99.68	99.68									
5	SA11-Both	100.00	74.05	74.05	0.26								
6	SA11-5S	74.05	100.00	100.00	99.68	74.05							
7	SA11-5N	74.05	100.00	100.00	99.68	74.05	100.00						
8	SA11-30/1A	74.05	100.00	100.00	99.68	74.05	100.00	100.00					
9	SA11-30/19	74.05	100.00	100.00	99.68	74.05	100.00	100.00	100.00				
10	SA11-P	73.88	99.89	99.89	99.58	73.88	99.89	99.89	99.89	99.89			
11	SA11-R	74.20	99.89	99.89	99.58	74.20	99.89	99.89	99.89	99.89	99.79		
12	SA11-tsE	73.76	99.68	99.68	99.37	74.76	99.68	99.68	99.68	99.68	99.58	99.58	
13	O agent	100.00	74.05	74.05	73.74	100.00	74.05	74.05	74.05	74.05	73.88	74.20	73.76

I. Genome segment 9 (VP7)

	1	2	3	4	5	6	7	8	9	10	11
1	**SA11-N5**										
2	**SA11-N2**	100.00									
3	SA11-H96	100.00	100.00								
4	SA11-Both	99.80	99.80	99.80							
5	SA11-5S	100.00	100.00	100.00	99.80						
6	SA11-5N	100.00	100.00	100.00	99.80	100.00					
7	SA11-30/1A	100.00	100.00	100.00	99.80	100.00	100.00				
8	SA11-30/10	99.90	99.90	99.90	99.69	99.90	99.90	99.90			
9	X66158	99.80	99.80	99.80	99.80	99.80	99.80	99.80	99.69		
10	K02028	99.08	99.08	99.08	98.87	99.08	99.08	99.08	99.97	98.87	
11	V01546	99.69	99.69	99.69	99.90	99.69	99.69	99.69	99.59	99.69	99.77

J. Genome segment 10 (NSP4)

	1	2	3	4	5	6	7	8	9	10
1	**SA11-N2**									
2	**SA11-N5**	100.00								
3	SA11-H96 (USA)	100.00	100.00							
4	SA11-H96 (India)	99.43	99.43	99.43						
5	SA11-Both	99.62	99.62	99.62	99.06					
6	SA11-5S	100.00	100.00	100.00	99.43	99.62				
7	SA11-5N	100.00	100.00	100.00	99.43	99.62	100.00			
8	SA11-30/1A	100.00	100.00	100.00	99.43	99.62	100.00	100.00		
9	SA11-30/19	100.00	100.00	100.00	99.43	99.62	100.00	100.00	100.00	
10	AF087678	100.00	100.00	100.00	99.43	99.62	100.00	100.00	100.00	100.00

K. Genome segment 11 (NSP5/6)

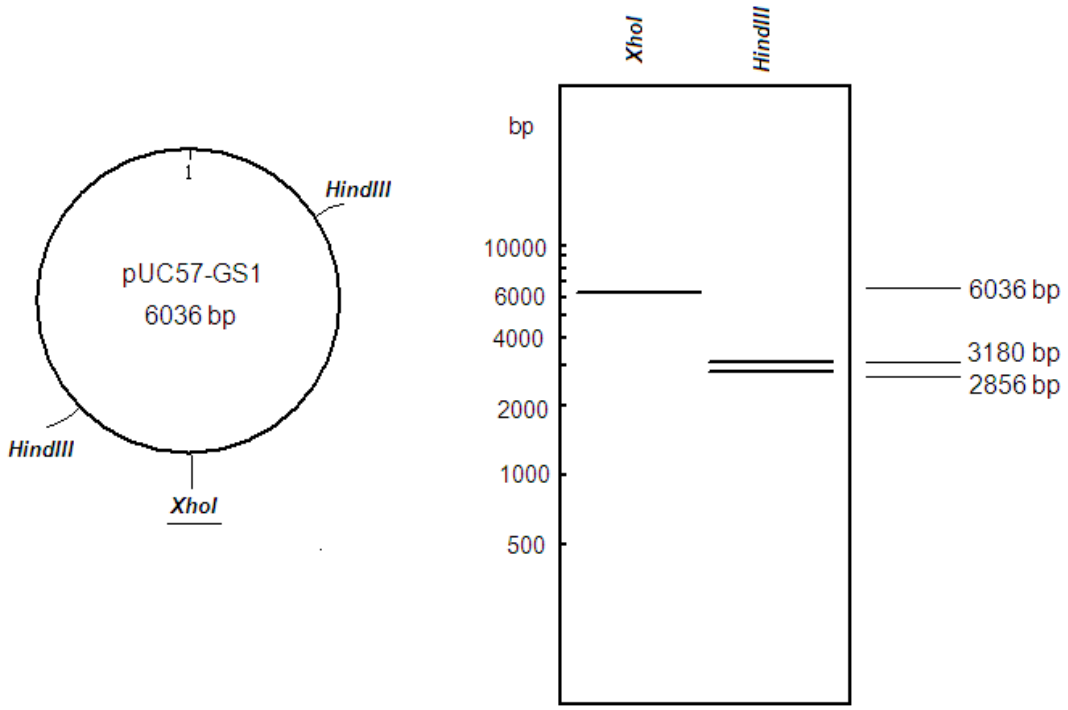
	1	2	3	4	5	6	7	8	9	10	11
1	**SA11-N2**										
2	**SA11-N5**	100.00									
3	SA11-H96 (USA)	100.00	100.00								
4	SA11-H96 (India)	99.83	99.83	99.83							
5	SA11-Both	100.00	100.00	100.00	99.83						
6	SA11-5S	100.00	100.00	100.00	99.83	100.00					
7	SA11-5N	100.00	100.00	100.00	99.83	100.00	100.00				
8	SA11-30/1A	100.00	100.00	100.00	99.83	100.00	100.00	100.00			
9	SA11-30/19	100.00	100.00	100.00	99.83	100.00	100.00	100.00	100.00		
10	AF306493	100.00	100.00	100.00	99.83	100.00	100.00	100.00	100.00	100.00	
11	M28347	100.00	100.00	100.00	99.83	100.00	100.00	100.00	100.00	100.00	100.00

Figure 1. Estimates of evolutionary divergence between the consensus nucleotide sequences of SA11-N2, SA11-N5 and SA11 nucleotide sequences obtained from GenBank. The nucleotide sequences of the bovine rotavirus O agent were used for

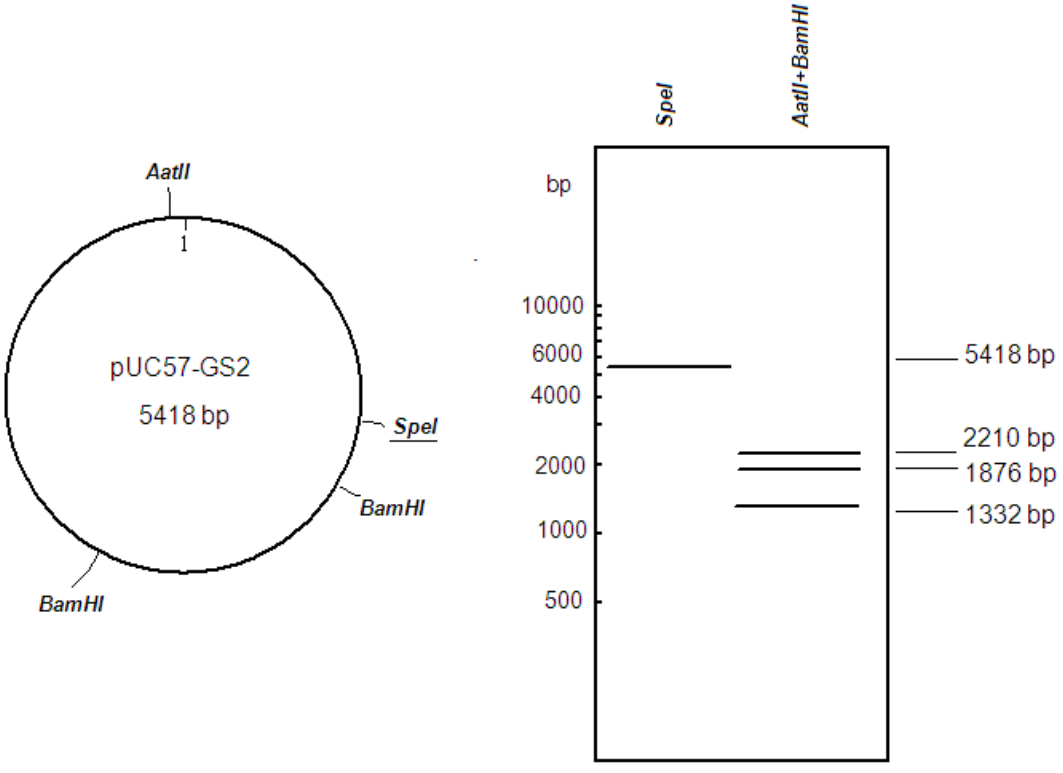
comparison between genome segments 4 (VP4) and 8 (NSP2). Comparisons between SA11-N2, SA11-N5, SA11-H96, SA11-Both and the bovine rotavirus O agent are boxed. The percentage similarities between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004), all results are based on the pairwise analysis and evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). For genome segments 1–4 (VP1–VP4) and 9 (VP7), the SA11-H96 strain was determined by Small and co-workers (2007).

Appendix 2

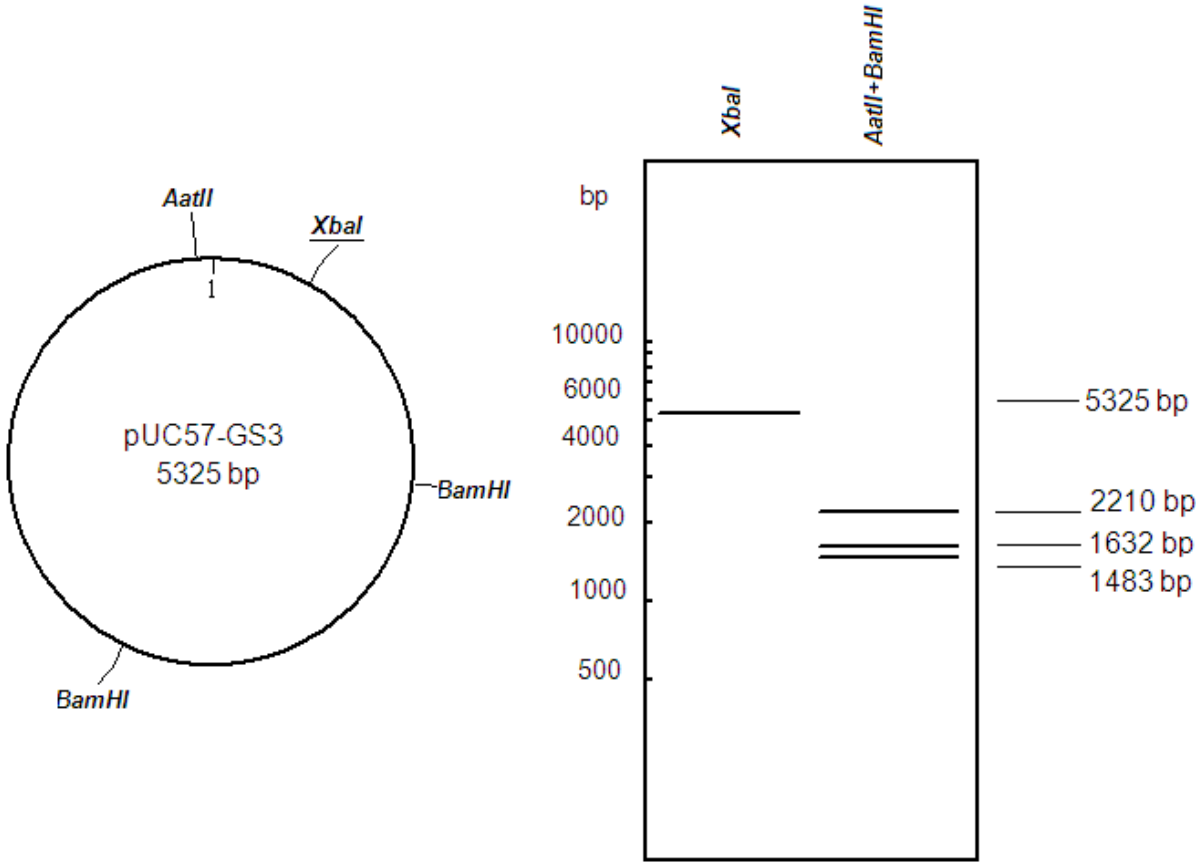
A. pUC57 plasmid with DS-1 genome segment 1 (VP1) consensus cDNA insert



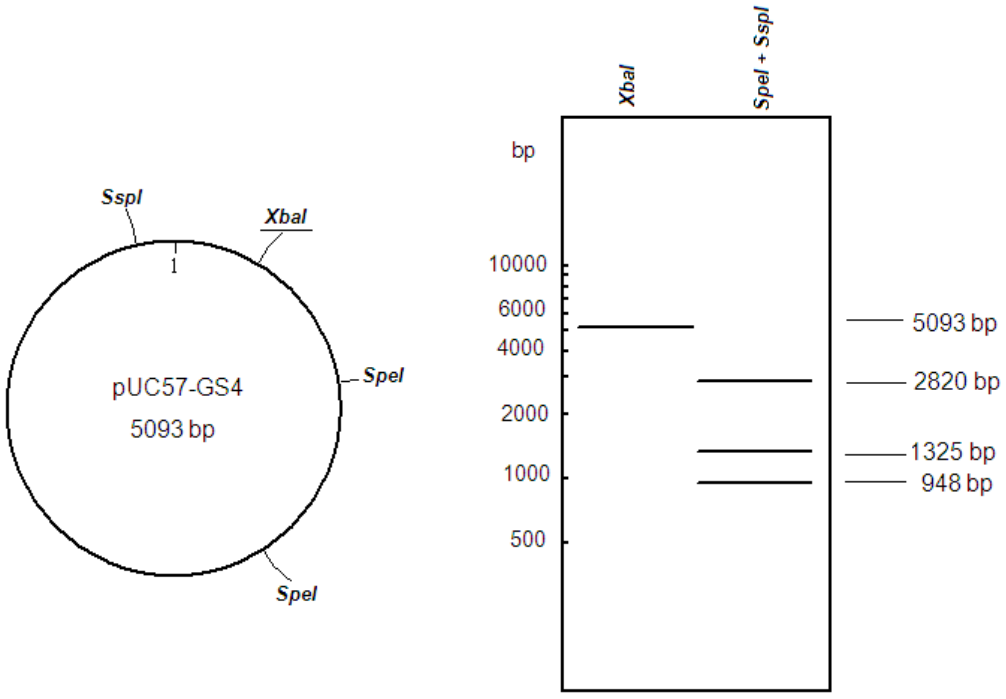
B. pUC57 plasmid with DS-1 genome segment 2 (VP2) consensus cDNA insert



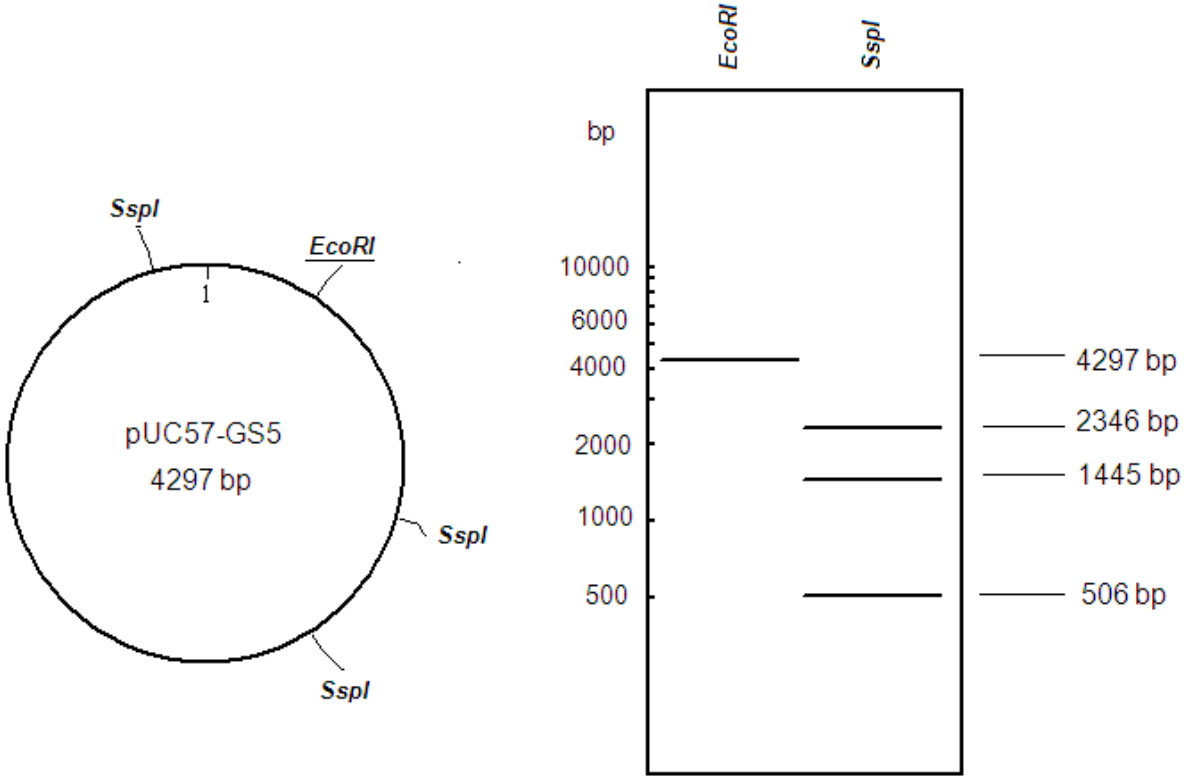
C. pUC57 plasmid with DS-1 genome segment 3 (VP3) consensus cDNA insert



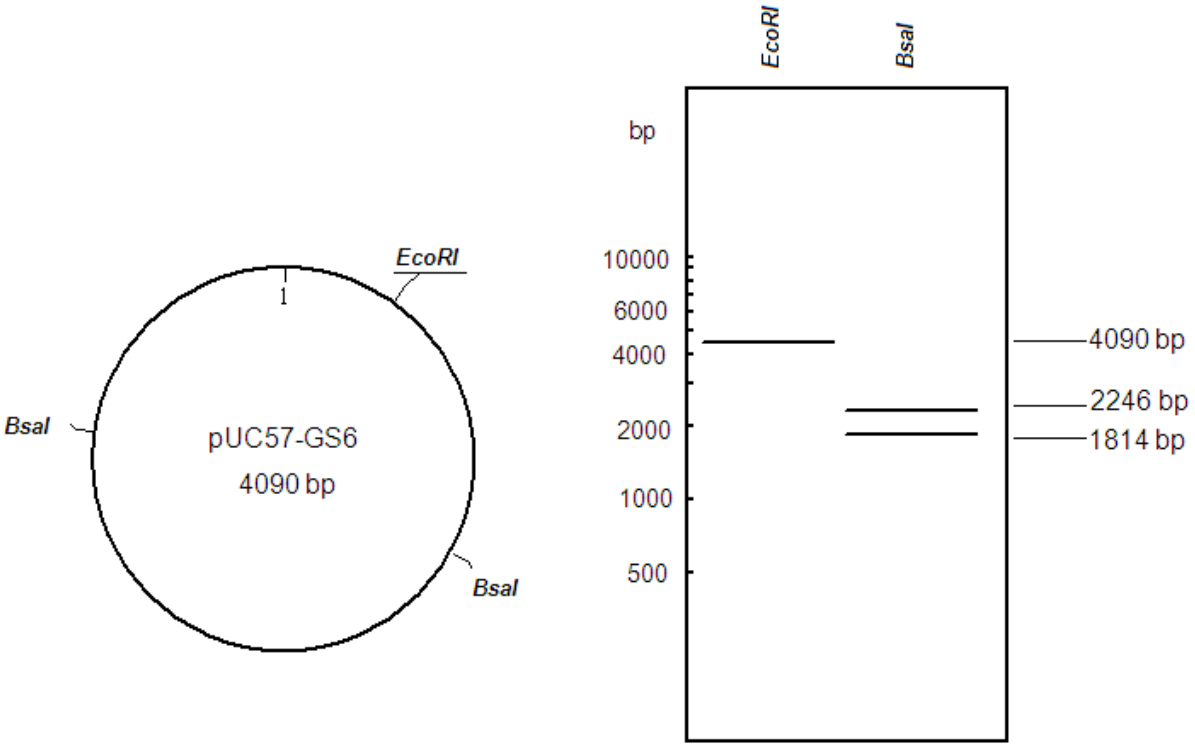
D. pUC57 plasmid with DS-1 genome segment 4 (VP4) consensus cDNA insert



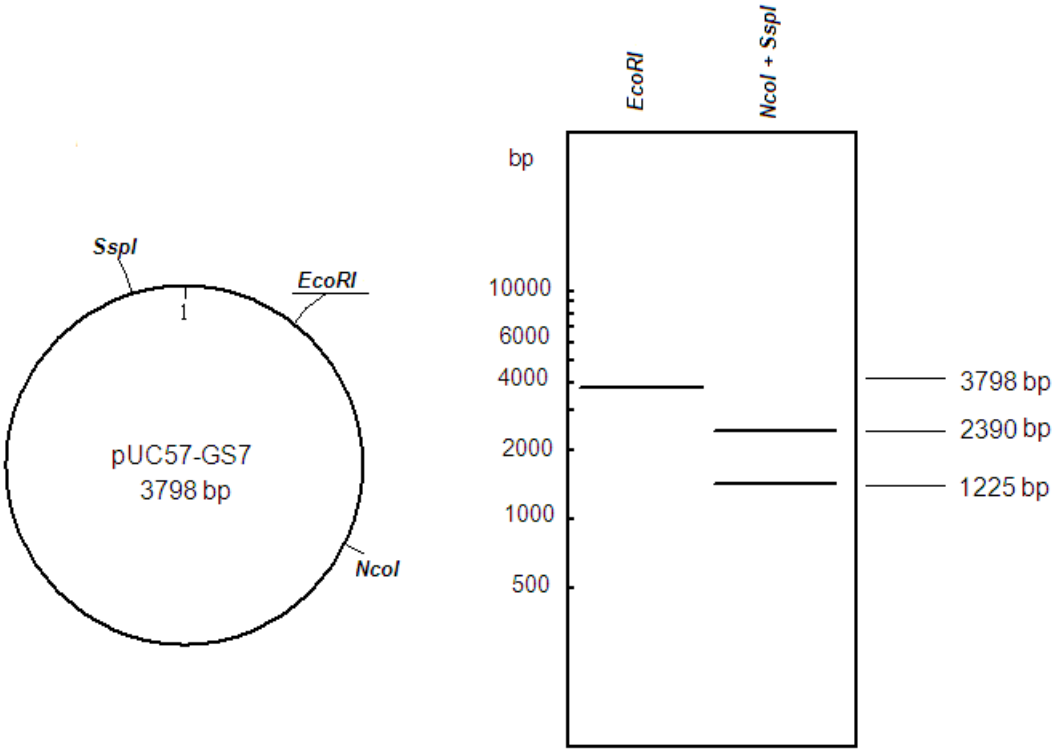
E. pUC57 plasmid with DS-1 genome segment 5 (NSP1) consensus cDNA insert



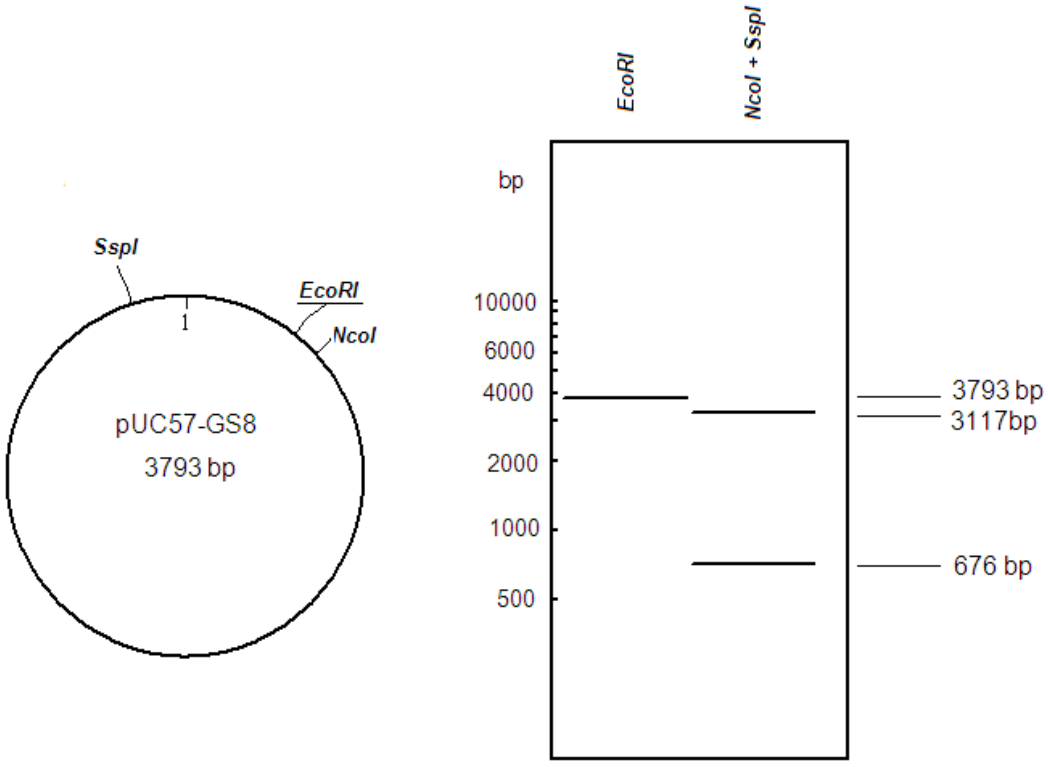
F. pUC57 plasmid with DS-1 genome segment 6 (VP6) consensus cDNA insert



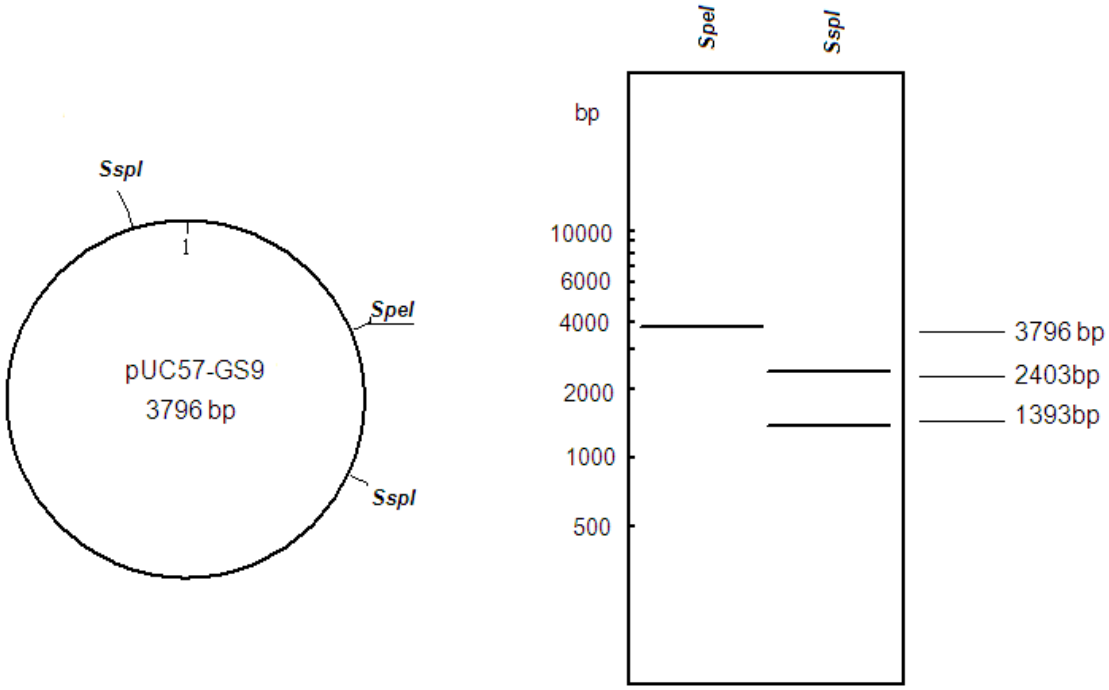
G. pUC57 plasmid with DS-1 genome segment 7 (NSP3) consensus cDNA insert



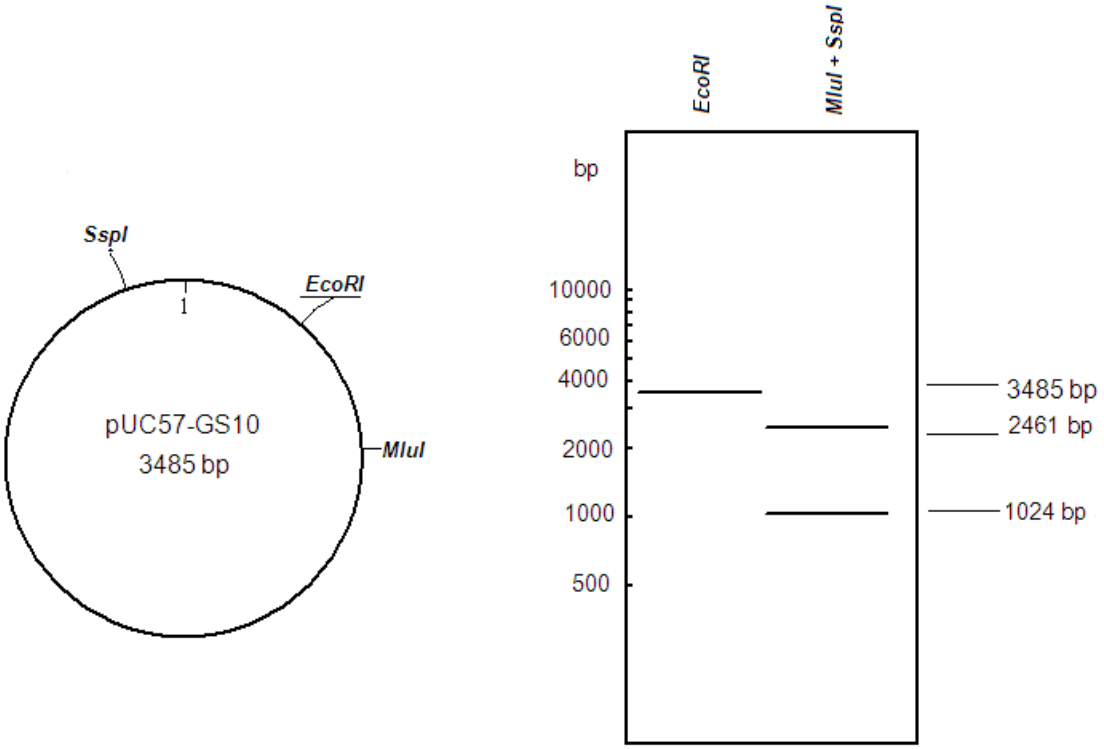
H. pUC57 plasmid with DS-1genome segment 8 (NSP2) consensus cDNA insert



I. pUC57 plasmid with DS-1 genome segment 9 (VP7) consensus cDNA insert



J. pUC57 plasmid with DS-1 genome segment 10 (NSP4) consensus cDNA insert



K. pUC57 plasmid with DS-1 genome segment 11 (NSP5/6) consensus cDNA insert

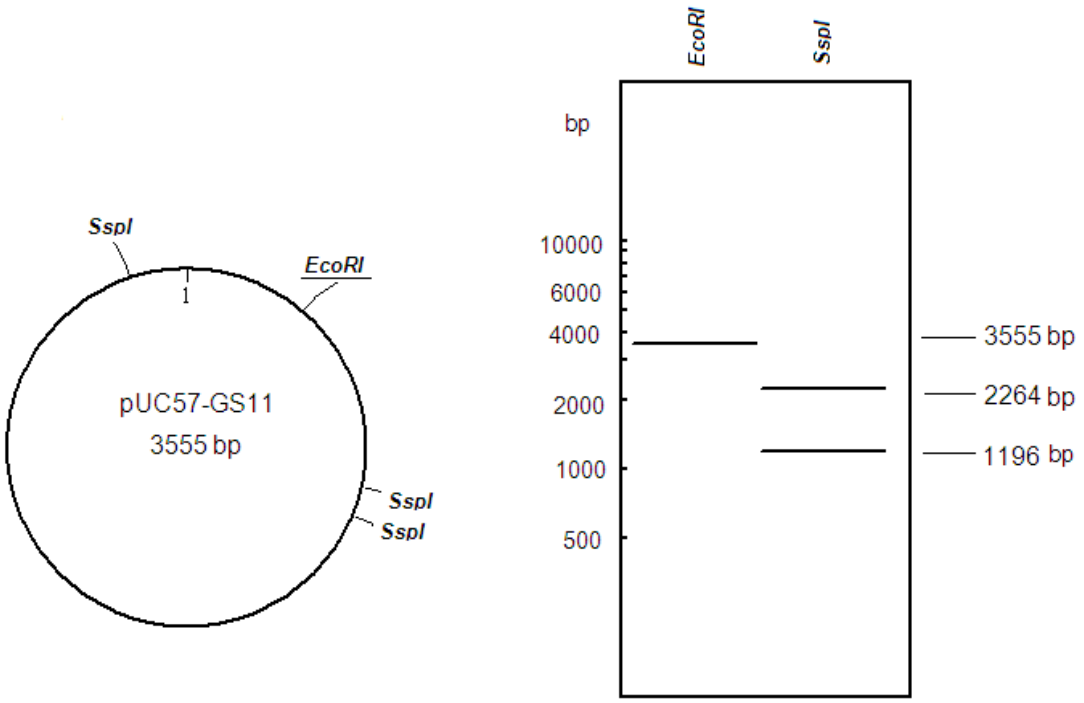


Figure 1. Analysis of *in silico* clones of pUC57 plasmids containing the consensus cDNA of the 11 rotavirus DS-1 genome segment inserts. The *in silico* cloning was performed using DNAMAN software. Each plasmid map shows the size of the plasmid and the location of restriction enzymes used to digest the plasmid. The underlined restriction enzymes were used to linearise the respective plasmid. For each digestion pattern, the restriction enzyme(s) used to digest the plasmid is shown at the top of the lane. The resulting restriction digestion pattern and expected sizes of the generated fragments are shown. For the pUC57 plasmid containing the genome segment 11 cDNA insert **(K)**, one of the fragments obtained from digestion with *SspI* was too small to be shown in the restriction digestion pattern.

Appendix 3

Original article

Luwanika Mlera, Khuzwayo C. Jere, Alberdina A. van Dijk, and Hester G. O'Neill (2011). Determination of the whole-genome consensus sequence of the prototype DS-1 rotavirus using sequence-independent genome amplification and 454® pyrosequencing. *Journal of Virological Methods* **175**, 266–271.

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Journal of Virological Methods 175 (2011) 266–271

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Journal of Virological Methods

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Short communication

Determination of the whole-genome consensus sequence of the prototype DS-1 rotavirus using sequence-independent genome amplification and 454® pyrosequencing

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Article history:
Received 1 March 2011
Received in revised form 20 April 2011
Accepted 3 May 2011
Available online 10 May 2011

Keywords:
dsRNA
DS-1 rotavirus
Sequence-independent genome amplification
454® Pyrosequencing
Consensus sequence

A B S T R A C T

The prototype DS-1 rotavirus strain, is characterised by a short electropherotype and G2P[4] serotype specificity. Following sequence-independent genome amplification and 454® pyrosequencing of genomic cDNA, differences between the newly determined consensus sequence and GenBank sequences were observed in 10 of the 11 genome segments. Only the consensus sequence of genome segment 1 was identical to sequences deposited in GenBank. A novel isoleucine at position 397 in a hydrophobic region of VP4 is described. An additional 7 N-terminal amino acids was found in NSP1. For genome segment 10 the first 34 and last 30 nucleotides of the 5′ and 3′-terminal ends, respectively, were identified. Genome segment 11 was found to be 821 bp long, which is 148 bp longer than the full length genome segment 11 sequence reported previously. This paper reports the first complete consensus genome sequence for the tissue culture adapted DS-1 strain free from cloning bias and the limitations of Sanger sequencing. Sequence differences in previous publications reporting on DS-1 rotavirus genome segment sequencing, were identified and discussed.

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Rotaviruses belong to the *Reoviridae* family and are the most common cause of severe childhood diarrhoea worldwide (Parashar et al., 2006). The triple layered virus particle contains a genome of 11 dsRNA segments. The genome encodes six structural proteins (VP1–VP4, VP6 and VP7) and six non-structural proteins (NSP1–NSP6) (Estes and Kapikian, 2007). Rotavirus genetic diversity is generated through the accumulation of point mutations, genome segment reassortment and intragenic recombination during mixed infections (Parra et al., 2004; Ramig, 1997; Taniguchi and Urasawa, 1995). Rearrangement downstream of the open reading frame (ORF) can occur *in vitro* in tissue culture when rotavirus is propagated at a high multiplicity of infection and also *in vivo* in immune deficient children (Hundley et al., 1987; Pedley et al., 1984). Matthijnssens et al. (2006) proposed that the rearrangement mechanism involves re-entry of the 3′-end of the negative strand into the catalytic core (forming a loop) and the RNA-dependent RNA polymerase making a mistake by switching template.

The DS-1 strain (G2P[4] serotype) is the prototype of DS-1-like strains (subgroup I, short electropherotype). The strain was isolated in 1976 in Washington D.C. (USA) from a gastroenteritis patient (Kalica et al., 1981). Primary adaptation from stool, of the slow-growing strain was performed in MA104 cells without infection of primary cells (Wyatt et al., 1983). Sequence differences exist between DS-1 rotavirus genome segments determined previously. A full genome sequence for the DS-1 strain was reported by Heiman et al. (2008). However, the extreme 5′ and 3′ termini could not be determined directly as genome segment-specific terminal primers were used for RT-PCR amplification. To date, no consensus sequence, which represents the most viable and predominant population of the viral quasispecies (Domingo et al., 2006) has been determined for the DS-1 genome. In this study the complete consensus sequence for the DS-1 genome was determined using sequence-independent amplification and 454® pyrosequencing (Margulies et al., 2005).

The culture-adapted DS-1 virus was provided at 5×10^5 ffu/ml by Dr. Carl Kirkwood (Murdoch Children's Research Institute, Melbourne, Australia) after it had been propagated 10 times in MA104 cells (each propagation at an m.o.i. of < 0.5) following initial adaptation. In this study the virus stock was activated with 10 μg/ml porcine trypsin IX (Sigma) prior to infecting confluent MA104 cells. For dsRNA extraction, confluent MA104 cells in a 75 cm^2 flask were infected at a low m.o.i. of < 1 and propagated in Dulbecco's modified essential medium (Hyclone) containing 1 μg/ml porcine trypsin IX, 1% non essential amino acids (Gibco) and

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doi:10.1016/j.jviromet.2011.05.004

Table 1
Summary of the DS-1 consensus sequence data determined with 454® pyrosequencing in comparison to DS-1 GenBank sequences.

Genome segment	Size (bp)	Amino acids in coding region	Nucleotide differences			Amino acid differences	
			Insertions	Deletions	Point mutations	Insertions	Point mutations
1 (VP1)	3302	1088	None	None	None	None	None
2 (VP2)	2684	879	None	None	3	None	None
3 (VP3)	2591	835	None	None	11	None	None
4 (VP4)	2359	775	None	None	28	None	[a]52S→H, 53W→G, 106S→I, 107S→A, 142T→M, 144T→K, 150N→D, 230R→S, 245K→R, 280I→V, 380T→I, and 397N/D→I
5 (NSP1)	1563	493	1	[b]2	5	1–7 MKSLVEA	[c]137 S→L, 338 I→T, 381E→D, 488R→G
6 (VP6)	1356	397	None	None	1	None	None
7 (NSP3)	1064	313	None	None	1	1–3 MLK	[d]51G→D
8 (NSP2)	1059	317	None	None	1	None	194G→D
9 (VP7)	1062	326	None	None	5	None	None
10 (NSP4)	751	175	None	None	None	None	None
11 (NSP5)	821	200	6	None	6	139–140QE	[e]9G→S and 49N→R

[a] Differences in the translated VP4 amino acid sequence: residues 52–150 occur in VP8* region; residue 150 is a glycosylation site; residue 230 is a trypsin cleavage site; residue 380 occurs in an antigenic region and residue 397 in a hydrophobic region.
[b] Deletion of TA at nucleotides 1553–1554 in the 3′-UTR.
[c] Differences in translated NSP1 amino acid sequence: residue 137 occurs in a region that is important for cytoskeletal localisation while the other changes occur in a region that interacts with interferon regulatory factor 3.
[d] Amino acid difference in translated NSP3 occurs in an RNA-binding region.
[e] The 9G→S difference in the translated NSP5 amino acid sequence occurs in a region that interacts with VP1.

1% antibiotics (penicillin/streptomycin/amphotericin) (Lonza) for 5 days. Viral dsRNA was extracted using Trizol reagent (Invitrogen) and single-stranded RNA precipitated with 2 M LiCl (Sigma) at 4 °C for 16 h followed by centrifugation at 16 000 × *g* for 30 min. The dsRNA in the supernatant was purified using a MinElute kit (Qiagen) following the manufacturer's instructions. To obtain a dataset free from cloning bias, sequence-independent genome amplification was performed as described by Potgieter et al. (2009) with the following modifications: genomic cDNA was synthesised with Transcriptor High Fidelity reverse transcriptase (Roche) and amplified using Phusion High Fidelity DNA polymerase (Finnzymes). Amplified cDNA was purified with a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. The 454® pyrosequencing was performed at Inqaba Biotec™, South Africa, using GS FLX Titanium (Roche).

Pyrosequence data comprised of 10 180 sequence reads of approximately 400 bp each. The Lasergene™ 8.1.2 SeqMan Pro (DNASTAR®) was used for sequence assembly. A total of 36 contigs were obtained and used to determine the complete consensus sequence (Supplementary data S1) for each genome segment. The total size of the consensus genome obtained was 18 612 bp. The average depth of coverage was 204-fold, with genome segment 1 displaying the lowest (22-fold) and genome segment 8 the highest (458-fold) coverage. The identified consensus sequence for each genome segment (GenBank IDs in Supplementary data S2) was compared to all DS-1 rotavirus genome sequences in GenBank (Supplementary data S2).

The termini of the 5′ untranslated regions (UTRs) for all genome segments were the same as described previously for rotaviruses, i.e., 5′-GGC(U/A)$_7$ (Tortorici et al., 2006). The 3′ tetranucleotide terminal sequence GACC-3′ was also obtained as expected (Wentz et al., 1996). The 3′-terminal heptanucleotide sequence, UGUGACC-3′, was present in 9 genome segments, but in genome segments 2 and 10 it was UAUGACC-3′. The UAUGACC-3′ sequence was reported before for the DS-1 strain in genome segment 2 (Matthijnssens et al., 2008). Using the RotaC classification tool (Maes et al., 2009), the full genotype of the DS-1 rotavirus strain was confirmed as G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2.

Differences were observed between the consensus DS-1 sequences and DS-1 sequences in GenBank in 10 of the 11 genome segments. Only the consensus sequence of genome segment 1 (VP1) was identical to the GenBank genome segment 1 sequences. While nucleotide differences were observed in genome segments 2 (VP2), 3 (VP3), 6 (VP6), and 9 (VP7), there were no associated amino acid changes (Table 1). Amino acid differences were observed in deduced amino acid sequences of VP4, NSP1, NSP2, NSP3 and NSP5 (Table 1). In the deduced NSP2 amino acid sequence, a 194G→D change was observed while a 51D→G change was observed in an RNA-binding region of the NSP3 amino acid sequence. A GenBank sequence, EF672579, encoding NSP3 lacks MLK, the first three residues.

The consensus sequence for genome segment 4 (VP4) was compared to four GenBank sequences AB118025, AJ540227, EF672577 and DQ141310. The consensus sequence was identical to DQ141310 (nucleotides 186–678 in the consensus sequence). A 2% variation resulted from a total of 28 nucleotide differences (Supplementary data S3) between all the genome segment 4 sequences. Twelve of these nucleotide differences resulted in amino acid changes. Changes observed in the VP8* region of VP4 were 52S→H, 53W→G, 106S→I, 107S→A, 142T→M, 144T→K, 150N→D and 230R→S. Close scrutiny of the VP8* structure (PDB ID: 2AEN; Monnier et al., 2006) indicated that I106 and A107 are inside the VP8* structure while M142, K144 and D150 are located on the surface of the VP8* structure. A 245K→R change was observed in the region between VP8* and VP5*. Amino acid differences 280I→V and 380T→I were observed in the antigenic domain of VP5*. Furthermore, a novel isoleucine at position 397 (in a hydrophobic region) was observed. Residues V280, I380 and I397 were located on the surface of the predicted VP5* structure (based on PDB ID: 1SLQ; Dormitzer et al., 2004). The novel I397 increases hydrophobicity in the loop region and may increase infectivity since hydrophobicity of the VP5* apex is required for membrane disrup-

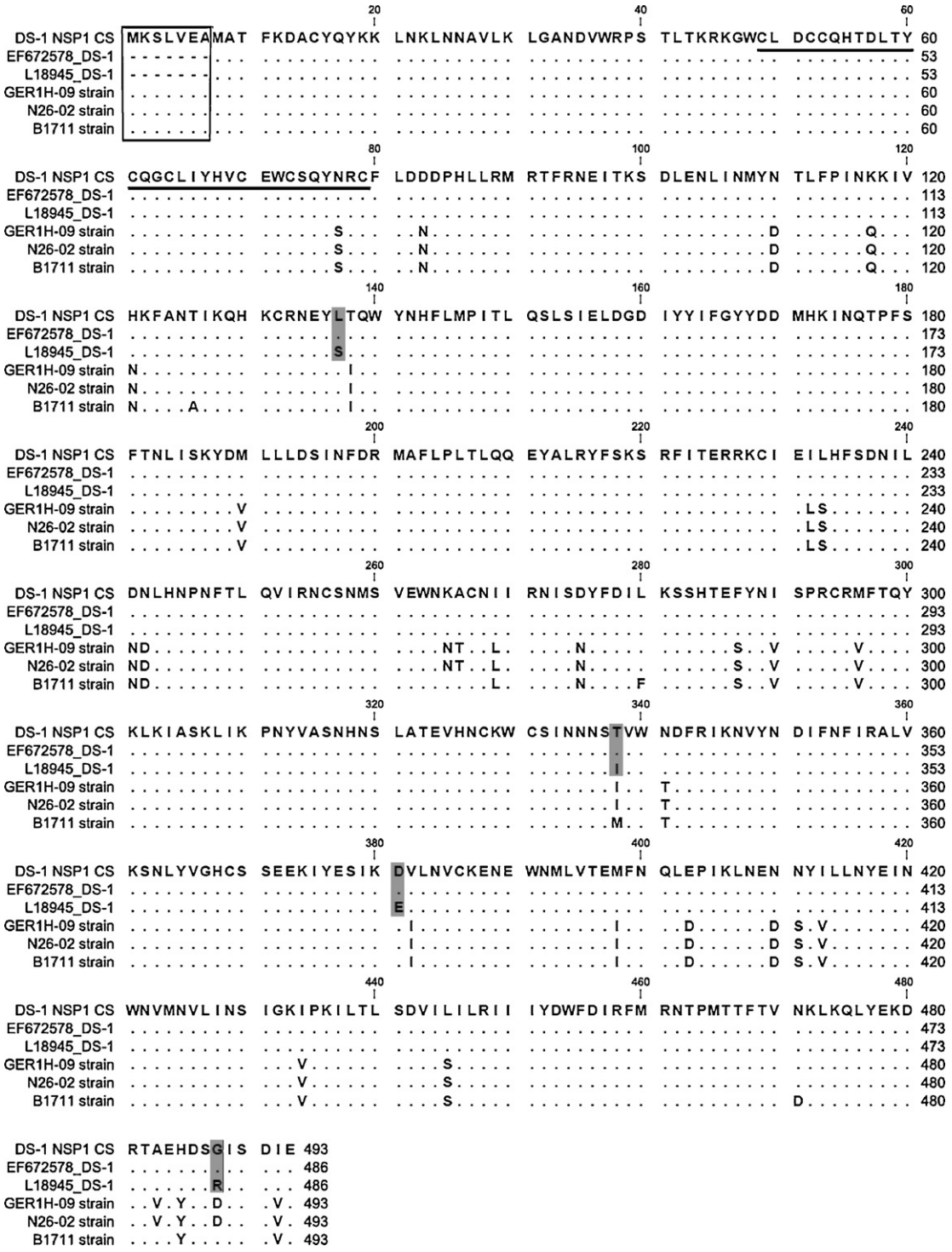

Fig. 1. Alignment of the deduced NSP1 amino acid consensus sequence (DS-1 NSP1 CS) with DS-1 sequences (EF672578 and L18945) and selected DS-1-like sequences (GER1H-09, N26-02 and B1711) in GenBank. The first 7 residues, MKSLVEA, observed at the N-terminal end of the translated consensus sequence and not in GenBank DS-1 sequences but present in the DS-1-like strains B1711, G9P[6]; GER1H-09, G8P[6]; and N26-02, G12P[6] are boxed and differences between the deduced consensus sequence and the DS-1 sequences are shaded. The conserved DS-1 cysteine-rich zinc finger motif is underlined.

tion during rotavirus cell entry (Kim et al., 2010). Pyrosequence data showed that at the corresponding codon position encoding amino acid residue 397, 30% (approximately 150 reads) of sequence reads indicated an AAT codon (Supplementary data S4) encoding an asparagine residue. The 30% translated sequence reads containing N397 suggests that a quasispecies population exists within the analysed DS-1 strain.

VP8* is an important target for neutralising antibodies and P serotype specificity (Dormitzer et al., 2002). The region from amino acids 106–159 is generally a variable region (Gorziglia et al., 1986). The probable biological effect of residues 142 and 144 may be related to antigenicity since these residues are located on the surface of the protein structure in a hypervariable region. According to published sequencing reports, residue 150 is an N-linked glycosy-

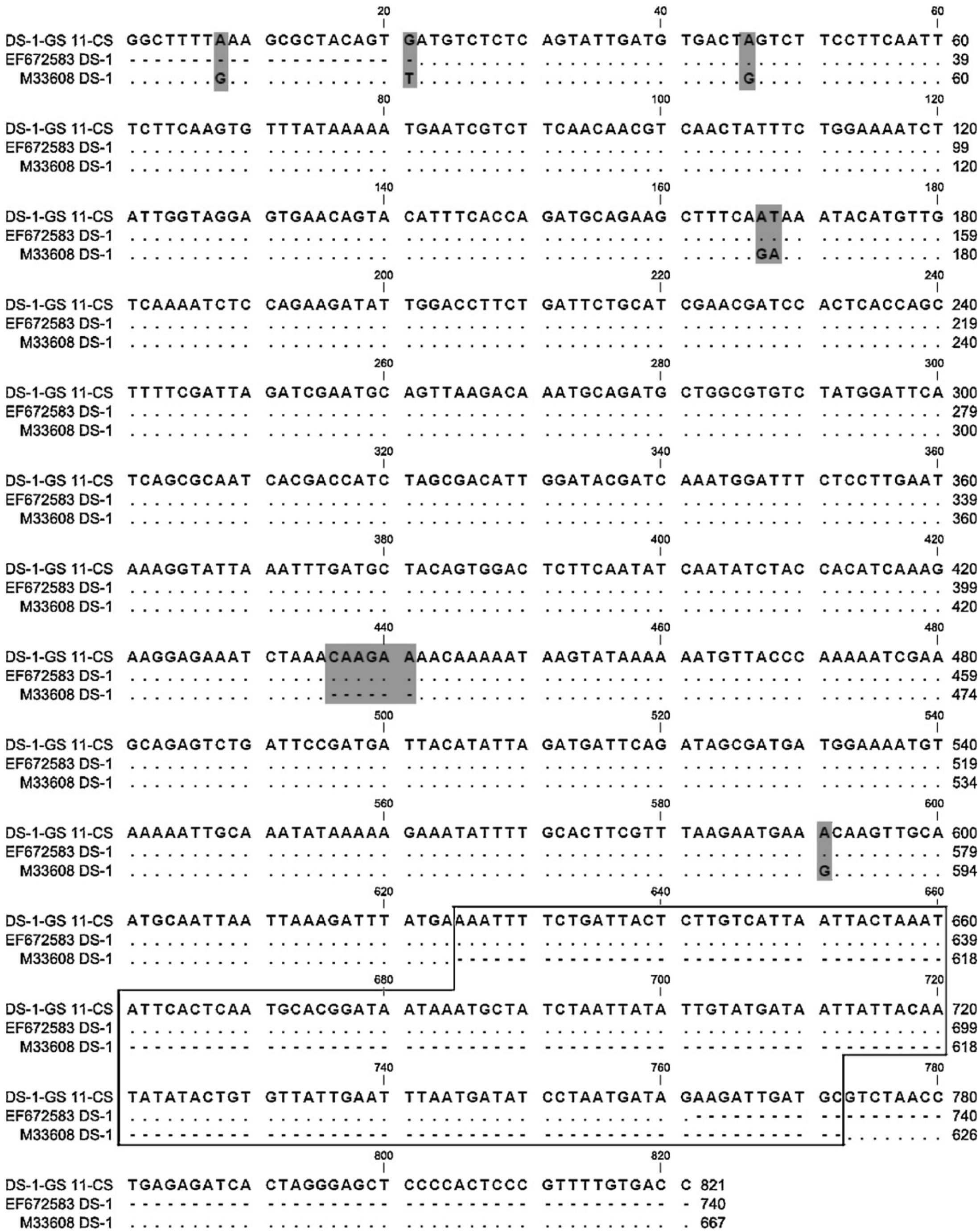

Fig. 2. Alignment of genome segment 11 consensus nucleotide sequence (DS-1-GS 11-CS) with DS-1 genome segment 11 sequences from GenBank (M33608 and EF672583). Boxed sequences (nucleotides 625–772) indicate the 148-nucleotide deletion in M33608. Nucleotide changes and a 6-base deletion in M33608 at positions 436–441 are shaded.

lation site in the DS-1 strain. However, a 150N→D change observed in this study indicates that this site is not glycosylated in the DS-1 strain analysed. This change may affect efficiency of cell entry, antigenicity and virulence (Chattopadhyay et al., 2010) and should be investigated *in vivo*.

Trypsin cleavage of VP4 occurs between R230 and N231 (Arias et al., 1996). However, in this study a 230R→S change was observed. A second trypsin cleavage site present between R246 and A247 was unchanged in the consensus sequence. Cleavage at either or both cleavage sites results in the conformational changes required for entry into cells (Yoder et al., 2009). The cleavage site between R246 and A247 may be preferred for cleavage in the DS-1 strain. Cleavage site preference was observed in the SA11 rotavirus strain where R247 is preferred to R241 (R230 equivalent in DS-1 strain) (Gorziglia et al., 1986). While the biological effect of differences at trypsin cleavage sites is not clear, a second trypsin cleavage site in VP4 has been correlated with virulence (Estes and Cohen, 1989).

For genome segment 5 (NSP1), the ORF of the consensus sequence was 21 nucleotides longer at the 5′-end, than the ORFs of GenBank sequences L18945 and EF672578. The consensus DS-1 NSP1 amino acid sequence therefore contained 7 additional amino acids (MKSLVEA) at the N-terminal end when compared to L18945 and EF672578 (Fig. 1). This suggests that genome segment 5 might have an alternative start codon. NSP1 is known to vary in length

among different rotavirus strains. For instance, NSP1 of the Wa strain is 486 amino acids long while that of SA11 is 495 amino acids (Nakagomi and Kaga, 1995). The DS-1 NSP1 deduced from the consensus nucleotide sequence was 493 amino acids long. Alignment of the DS-1 consensus sequence to DS-1-like strains such as GER1H-09, N26-02 and B1711 showed that their first 7 N-terminal residues were identical. The full genotype assignment showed that these DS-1-like strains differ only at the P and G genotypes from the DS-1 full genotype. However, published sequences for other DS-1-like strains such as the Tb-Chen strain (G2P[4]) do not have the MKSLVEA sequence at the N-terminal end of NSP1. The most important functional feature of NSP1 is the highly conserved cysteine-rich zinc finger motif (Graff et al., 2007) which was conserved as expected (Fig. 1).

The 34 5′-terminal and 30 3′-terminal nucleotide sequences of genome segment 10 (NSP4) have not been reported previously. These nucleotide sequences were determined in this study as 5′-GGCTTTTAAAAGTTCTGTTCCGAGAGAGCGCGTG-3′ at the 5′ terminus, and 5′-GTTAATGGAAGGAACGGTCTTAATATGACC-3′ at the 3′ terminus.

The consensus sequence for genome segment 11 (NSP5) obtained in this study was 821 bp (Fig. 2) and was compared to a full length DS-1 genome segment 11 sequence (GenBank ID: M33608) of 667 bp. A 148 bp nucleotide sequence (nucleotides 625–772) was not present in the 3′-UTR of M33608, while a GenBank sequence EF672583, a partial DS-1 genome segment 11 sequence, lacks 21 nucleotides at the 5′ terminus and 60 nucleotides at the 3′ terminus (Fig. 2). No internal duplications were observed in the consensus genome segment 11 sequence, indicating the absence of genome segment rearrangement. The 148 bp region may modify the formation of stem loops or cruciform structures (Supplementary data S5) in the dsRNA that have structural or functional roles. Six potentially *cis*-acting 5′-CUUC motifs (Li et al., 2010) were present in M33608 and the consensus sequence at nucleotides 49–52, 53–56, 62–65, 89–92, 206–209, 392–395. A seventh 5′-CUUC motif is present at nucleotides 568–571 in M33608 and at nucleotides 574–577 in the consensus sequence. A potentially *cis*-acting 5′-GGGAGCUCCC palindrome (Li et al., 2010) spanned nucleotides 794–803 in the consensus genome segment 11 sequence and is present at nucleotides 640–649 in M33608 (Supplementary data S5).

In conclusion, the application of the sequence-independent genome amplification technology in combination with 454® pyrosequencing of the DS-1 genome allowed the presentation of a consensus sequence for this strain free from cloning-bias and the limitations of Sanger sequencing. In the process, sequence differences in genome segments 2–11 that were observed during previous sequencing efforts, were addressed. The results suggest the occurrence of quasispecies in some genome segments of the tissue culture-adapted DS-1 strain such as genome segment 4. It is possible that some of the differences reported here resulted from cell culture propagation of the DS-1 strain in different laboratories, introducing point mutations into the genome.

Acknowledgements

The study was supported by a Senior Fellowship in Neglected Tropical Diseases from the European Foundation Initiative for Neglected Tropical Diseases (EFINTD) awarded to HGO (I/84 015). LM is grateful for a PhD bursary (09/41) received from the Poliomyelitis Research Foundation, South Africa. We thank Dr. C. Kirkwood (Murdoch Children's Research Institute, Melbourne, Australia) for kindly providing the DS-1 rotavirus. We also thank Dr. A.C. Potgieter (Deltamune, Pretoria, South Africa) for valuable technical support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jviromet.2011.05.004.

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Appendix 4

Original article

Luwanika Mlera, Hester G. O'Neill, Khuzwayo C. Jere and Alberdina A. van Dijk, (2012). Whole-genome consensus sequence analysis of a South African rotavirus SA11 sample reveals a mixed infection with two close derivatives of the SA11-H96 strain. *Archives of Virology*, Available online 23 December 2012 at http://link.springer.com/article/10.1007/s00705-012-1559-5/fulltext.html.

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Arch Virol
DOI 10.1007/s00705-012-1559-5

ORIGINAL ARTICLE

Whole-genome consensus sequence analysis of a South African rotavirus SA11 sample reveals a mixed infection with two close derivatives of the SA11-H96 strain

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Received: 13 September 2012 / Accepted: 18 October 2012
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Abstract Whole-genome, sequence-independent amplification and 454® pyrosequencing of a rotavirus SA11 cell culture sample with an unknown passage history yielded consensus sequences of twelve complete genome segments. Two distinct sequences for genome segment 8 (encoding NSP2) were present, indicating a mixed infection with two rotavirus SA11 strains. The genotypes of the viruses were G3-P[2]-I2-R2-C5-M5-A5-Nx-T5-E2-H5, where x was either 5 or 2. The strains were named RVA/Simian-tc/ZAF/SA11-N5/1958/G3P[2] and RVA/Simian-tc/ZAF/SA11-N2/1958/G3P[2]. The genotype (N2) and sequence of genome segment 8 of RVA/Simian-tc/ZAF/SA11-N2/1958/G3P[2] were identical to that of the bovine rotavirus O agent. Five novel amino acids were detected in minor population variants of three genome segments. Genome segment 1 (VP1) has a high nucleotide substitution rate, but the substitutions are synonymous. Distance matrices and Bayesian molecular clock phylogenetics showed that SA11-N2 is a reassortant containing genome segment 8 from the O agent, whereas SA11-N5 is a very close derivative of the prototype SA11-H96.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-012-1559-5) contains supplementary material, which is available to authorized users.

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Introduction

Rotavirus, a member of the family *Reoviridae*, is responsible for most cases of severe diarrhoea in children and the young of a variety of animals globally [16, 43]. The virus has a triple-layered capsid that encloses a genome of 11 double-stranded RNA (dsRNA) segments. The genome segments encode six structural proteins, VP1–VP4, VP6 and VP7, and six non-structural proteins, NSP1–NSP6 [16]. The segmented nature of the dsRNA genome facilitates genetic reassortment during mixed infections, which can cause the generation of novel phenotypes [52]. Point mutations, genome segment rearrangement and intergenotype recombination also contribute to rotavirus genome diversity [7, 22, 26, 57].

Simian agent 11 (SA11) was isolated from an overtly healthy monkey in 1958 by Dr. Hubert Malherbe at the National Institute of Virology, Johannesburg, South Africa [33, 34]. Due to its ability to replicate very well in cell culture, rotavirus SA11 became a model for rotavirus biological studies, such as investigating the replication cycle and determining the function of proteins encoded by the genome segments [15, 38]. As a result, the strain was distributed to various laboratories worldwide [15, 30, 44, 47, 54]. Subsequently, genome heterogeneity was described for some of the SA11 genome segments, namely genome segments 4 (VP4), 5 (NSP1), 8 (NSP2) and 7 (NSP3) [30, 46, 54]. Heterogeneity was observed in electrophoretic mobility patterns [46, 47], which was subsequently shown to be the result of nucleotide sequence variations [54]. It is believed that of all the known SA11 derivatives, the strain RVA/Simian-tc/ZAF/SA11-H96/1958/G3P[2], referred to in short as SA11-H96, was derived from the original 1958 isolate [34, 38, 54].

Published online: 23 December 2012

To date, all the nucleotide sequences of SA11 rotavirus strains deposited in GenBank since the early 1980s were determined using Sanger sequencing. The sequences were either generated directly from PCR amplicons of the genome segments or from amplicons that were first cloned into plasmids, followed by sequencing [4, 29, 54]. SA11-H96 is the prototype rotavirus SA11 strain and is considered the most likely representative of a typical simian rotavirus group [38]. However, no consensus sequence, i.e., the sequence of the most viable and predominant genome of the viral population variants [8, 9], has yet been determined for the strain and its variants. Consensus sequence determination is now possible with sequence-independent whole-genome amplification [49] and next-generation sequencing [35, 50, 60]. In this study, the whole-genome consensus sequence of a cell-cultured rotavirus SA11 sample, of which the passage history was unknown, was determined by sequence-independent genome amplification and 454® pyrosequencing. Twelve complete consensus genome segment sequences, indicative of a mixed infection, were obtained from the sample. Bayesian molecular clock evolutionary analysis was applied to further characterise the virus sample by determining the lineage diversification of SA11 derivatives.

Materials and methods

Cells and virus sample

A vial containing a cell-culture-adapted sample labelled rotavirus SA11 was received from the Diarrhoeal Pathogens Research Unit, University of Limpopo (Pretoria, South Africa). The sample was received with an unknown passage history. The cell line in which the virus had been propagated before was also not known. The virus sample was activated with 10 µg/ml porcine trypsin IX (Sigma, St Louis, USA) prior to infecting MA104 cells. MA104 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Utah, USA) supplemented with 1 % non-essential amino acids (Lonza, Maryland, USA), 1 % penicillin/streptomycin/amphotericin (PSA; Lonza). Virus propagation was performed in DMEM supplemented with 1 % non-essential amino acids (NEAA; Lonza, Maryland, USA), 1 % antibiotics and 1 µg/ml porcine trypsin IX (Sigma).

Sequence-independent whole-genome amplification and 454® pyrosequencing

The virus was quantified by plaque assay. Confluent MA104 cells in a 6-well plate (Nunc™) were infected with serially diluted virus for 1 h at room temperature, followed by the addition of 1X MEM overlay containing 0.75 % agarose, 1 % PSA, 1 % NEAA and porcine trypsin IX [1]. The plaque assays were incubated at 37 °C and 5 % CO_2 for up to seven days. A second passage was performed in a 75-cm^2 flask (Nunc™) at a multiplicity of infection of <1, followed by extraction of viral dsRNA using TRIzol Reagent (Invitrogen, California, USA). dsRNA was purified by precipitation of single-stranded RNA (ssRNA) in 2 M LiCl at 4 °C for 16 h. Single-stranded RNA was pelleted by centrifugation at 16,000 × *g* for 30 min at 4 °C. The dsRNA-containing supernatant was further purified with a MinElute kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. To facilitate sequence-independent whole-genome amplification, pure dsRNA was ligated to a primer, PC3-T7 loop, as described previously [49]. cDNA synthesis was performed with AMV reverse transcriptase (Fermentas, St. Leon-Rot, Germany) as described before [49]. Whole-genome amplification was performed using Phusion High Fidelity DNA polymerase (Finnzymes, Vantaa, Finland) and a primer complementary to the PC3-T7-loop, PC2, [49]. Amplified cDNA was purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Pyrosequencing was performed at Inqaba Biotec™ (Pretoria, South Africa), using 454®, GS FLX Titanium technology [35] (Roche, Mannheim, Germany).

Sequence assembly and analysis

Sequence assembly was performed with the SeqMan Pro assembler in Lasergene® v8.1.2 (DNASTAR®, Madison, USA). Determination of the consensus sequence was performed by identifying the majority of nucleotides at each position as described before [25, 40]. Existing SA11 nucleotide sequences were obtained from GenBank using BLAST (NCBI). Alignments were performed with Sequence Viewer v6.4 (CLC Bio) and the *mVISTA* visualisation module [19]. Distance matrix inference of phylogeny was performed using MEGA software v 5.05 [55, 56]. Accession numbers of the consensus sequences of the SA11 genome segments determined in this study and those retrieved from GenBank are listed in Supplementary Table 1.

Bayesian molecular clock evolutionary analysis

The consensus sequence spanning the open reading frame (ORF) of each genome segment and the corresponding ORF nucleotide sequences retrieved from GenBank were used to determine genome segment divergence by molecular clock evolutionary analysis. The ORF sequences were aligned using the BioEdit sequence alignment editor [20]. Bayesian phylogenetic reconstructions were performed by the Markov chain Monte Carlo (MCMC) method in Bayesian Evolutionary Analysis by Sampling Trees (BEAST) software

v1.6.1 [12]. Molecular clock evolutionary analysis in BEAST was performed with the Hasegawa Kishino Yano substitution model [21] with gamma distributed rate variation, uncorrelated lognormal relaxed clock model and a coalescent constant size tree prior. The HKY substitution model was selected by testing with jModelTest v 0.1.1 [48]. Four separate MCMC analytical runs, at 100 million generations per run, were performed for each genome segment. Data from the four runs were combined using LogCombiner v1.6.1. The combined runs were analysed using Tracer v1.5 (http://tree.bio.ed.ac.uk/tracer). Maximum clade trees were annotated using TreeAnnotator v1.6.1 and visualised with FigTree v1.3.1 (http://tree.bio.ed.ac.uk/figtree).

Results and discussion

Determination of the consensus whole-genome sequence of an SA11 sample, assignment of genotype constellation and comparison to other SA11 sequences in GenBank

Pyrosequencing of the sequence-independent amplified rotavirus SA11 genome generated 29,849 reads of approximately 400 bp each, which were assembled into contigs. Twelve full-length consensus genome segment sequences were obtained. For genome segment 8 (NSP2), two distinct consensus sequences were obtained, whereas a single consensus sequence was obtained for the other 10 genome segments. One consensus genome segment 8 sequence was a typical SA11-like sequence and was assigned the N5 genotype. It was identical to that of RVA/Simian-tc/ZAF/SA11-H96/1958/G3P[2]. The second consensus genome segment 8 sequence was DS-1-like [36] and assigned the N2 genotype. Two sets of sequences of the genome segments encoding VP6 and NSP1–NSP5 of RVA/Simian-tc/ZAF/SA11-H96/1958/G3P[2] are available in GenBank. The sequences were determined by two different groups, one in India [14] and the other in the USA [54]. The consensus sequence of genome segment 8 of SA11-N5 was identical to the sequence of RVA/Simian-tc/ZAF/SA11-H96/1958/G3P[2] sequenced in the USA [49]. The N2-genotyped genome segment 8 sequence was identical to that of RVA/Simian-tc/ZAF/SA11-Both/1958/G3P[2]. Strain RVA/Simian-tc/ZAF/SA11-Both/1958/G3P[2] acquired its genome segment 8 from the bovine rotavirus O (Offal) agent (G8P[1]) as a result of reassortment between SA11-H96 and the bovine rotavirus O agent [54]. The bovine rotavirus O agent was isolated from an abattoir in 1965 [34]. Visualisation of the alignment of the two genome segment 8 sequences with *mVISTA* showed that the two sequences were substantially different (Fig. 1). Therefore, it was concluded that the virus sample that was analysed was a mixture of two rotavirus SA11 strains. Traditionally, the detection of mixed infections could be missed with Sanger sequencing. The sequences of the other 10 genome segments of the viruses were identical. Since no additional rotavirus O agent sequences were present, it was hypothesised that if there were any other reassortants between SA11-N5 and the rotavirus O agent, they may have been selected out during propagation in cell culture. The viruses were named RVA/Simian-tc/ZAF/SA11-N2/1958/G3P[2] and RVA/Simian-tc/ZAF/SA11-N5/1958/G3P[2]. The full genotypes assigned using the RotaC v2.0 web tool [32] was G3-P[2]-I2-R2-C5-M5-A5-Nx-T5-E2-H5, where x was either 2 or 5. From here onwards, only the common laboratory names of the SA11 rotavirus derivatives will be used for simplicity. The average depth of pyrosequencing coverage of the genome segments was 367-fold. Genome segment 1 had the lowest coverage of 125-fold, while genome segment 8 of SA11-N2 had the highest coverage of 610-fold. Only genome segments 1 and 3 had an average depth of coverage lower than 200-fold (Table 1).

The 5′-terminal end sequences obtained for all the genome segments of SA11-N5 and SA11-N2 were 5′-GGC(U/A)$_7$-, as expected for group A rotaviruses [58]. The consensus genome segments had various 3′-terminal sequences. The 3′-terminal sequences obtained for the consensus genome segment 1 (VP1), 3 (VP3), 4 (VP4), 6 (VP6), 8 (NSP2) and 9 (VP7) were the typical 3′-terminal end sequences, i.e., -U**G**UG**A**CC-3′ [61]. The consensus genome segment 2 (VP2) contained the sequence -U**A**UG**A**CC-3′ at the 3′-terminal end. This 3′-terminal end sequence was also reported previously for genome segments 2 (VP2) and 10 (NSP4) of the rotavirus DS-1 strain [36, 40]. The atypical 3′-terminal sequences, U**G**UG**AA**CC-3′ and -U**G**UG**G**CC-3′, which were obtained for the consensus genome segments 5 (NSP1) and 7 (NSP3), were identical to the 3′-terminal sequences determined previously for these genome segments in SA11 strains [39, 41, 45, 54]. The atypical terminal sequence, -U**G**UG**AA**CC-3′, was found to reduce the efficiency of dsRNA synthesis and genome segment expression [45].

Nucleotide differences were observed in all genome segments when the consensus genome segment sequences of SA11-N5, SA11-N2 and nucleotide sequences of genome segments of SA11 derivatives available in GenBank were compared to each other. Nucleotide sequence similarity profiling indicated that the closest nucleotide genome segment sequences in GenBank to those of SA11-N5 and SA11-N2 were as follows: With the exception of genome segments 4 (VP4) and 5 (NSP1), the consensus nucleotide sequence of the other nine genome segments of SA11-N5 and SA11-N2 were identical to the sequence of a

Table 1 Comparison of the consensus nucleotide and deduced amino acid sequences of SA11-N5 and SA11-N2 to sequences of SA11-H96 [45], the representative rotavirus SA11 strain

Genome segment	Size (bp)	Amino acids in coding region	Average depth of coverage[a] (-fold)	Nucleotide similarity to rotavirus SA11 derivatives	Molecular clock rate[b] (CV[c])	Nucleotide differences between SA11-N5, SA11 N2 and SA11-H96[d]			Amino acid differences (SA11-H96[c]→SA11-N2/SA11-N5)
						Point variations	Insertions	Deletions	
1 (VP1)	3302	1088	125	SA11-H96 (100 %)	1.3×10^{-4} (1.3)	None	None	None	None
2 (VP2)	2693	882	609	SA11-H96 (100 %)	7.5×10^{-5} (0.5)	None	None	None	None
3 (VP3)	2591	835	156	SA11-5S (100 %)	1.4×10^{-6} (1.1)	2	None	None	650L→H
4 (VP4)	2362	775	358	SA11-Both (99.6 %)	1.4×10^{-5} (0.3)	9	None	None	72T→M, 157P→S, 187A→G, 261F→L, 332Y→S, 366V→M, 388S→P[e]
5 (NSP1)	1614	496	225	SA11-H96 (99.7 %)	4.8×10^{-5} (1.4)	8	None	None	36E→A, 84-86QQL→RTV, 96L→Q, 137K→L, 188E→D
6 (VP6)	1356	397	585	SA11-5 N (100 %)	2.1×10^{-5} (0.6)	3	None	None	None
7 (NSP3)	1105	313	305	SA11-H96 (100 %)	1.3×10^{-4} (0.3)	2	None	None	None
8 $(NSP2)^{N2}$	1059	317	610	SA11-Both (100 %)	ND	205	None	None	45 amino acid differences
8 $(NSP2)^{N5}$	1059	317	265	SA11-H96 (100 %)	1.9×10^{-4} (0.7)	None	None	None	None
9 (VP7)	1063	326	487	SA11-30/1A (100 %)	2.9×10^{-5} (0.4)	1	1	None	29I→T[e], 309V→A[e]
10 (NSP4)	751	175	495	SA11-H96 (100 %)	ND	4	None	None	33F→L[e], 93G→D, 114D→G[e]
11 (NSP5/6)	667	198/93	429	SA11-Both (100 %)	8.5×10^{-5} (0.5)	1	None	None	None

[a] The depth of coverage represents the number of pyrosequence reads that were obtained per specific region of each genome segment

[b] Nucleotide substitution/site/year

[c] Coefficient of variation

[d] Small and co-workers [45]

[e] Novel amino acid residues detected in this study

ND: not determined due to too few nucleotide sequences (genome segment 8) or zero-length interior branches (genome segment 10)

corresponding genome segment of one of five different SA11 derivatives (Table 1). Distance matrices showed that the consensus sequences of eight genome segments of SA11-N5 and SA11-N2 were most similar to those of SA11-H96 (Supplementary Fig. 1). Genome segments 1 (VP1), 2 (VP2), 6 (VP6) 7 (NSP3), 8 (N5 typed NSP2) 9 (VP7) 10 (NSP4) and 11 (NSP5/6) were identical to those of SA11-H96 (Supplementary Fig. 1). Genome segment 3 (VP3) was 100 % identical to that of SA11-5S, while genome segment 4 (VP4) was 99.6 % identical to that of SA11-Both (Table 1; Supplementary Fig. 1, C and D). The nucleotide sequence identities between the genome segments 3 (VP3) and 5 (NSP1) were 99.96 % and 99.78 % identical to the corresponding segments of SA11-H96, respectively (Supplementary Fig. 1, C and E). A 99.61 % nucleotide identity was observed between the consensus genome segment 4 sequence of SA11-N5, SA11-N2 and the corresponding nucleotide sequences of SA11-H96 and other SA11 strains with a P[2] genotype (Fig. 1; Supplementary Fig. 1, D). In contrast, only 77 % nucleotide identity was observed between the consensus genome segment 4 and SA11 strains with a P[1] genotype (Fig. 1). Therefore, genome segment 4 of SA11-N2 and SA11-N5 did not reassort with that of the O agent as is the case for some SA11 P[1] strains such as SA11-5S, SA11-5N and SA11-30-1A.

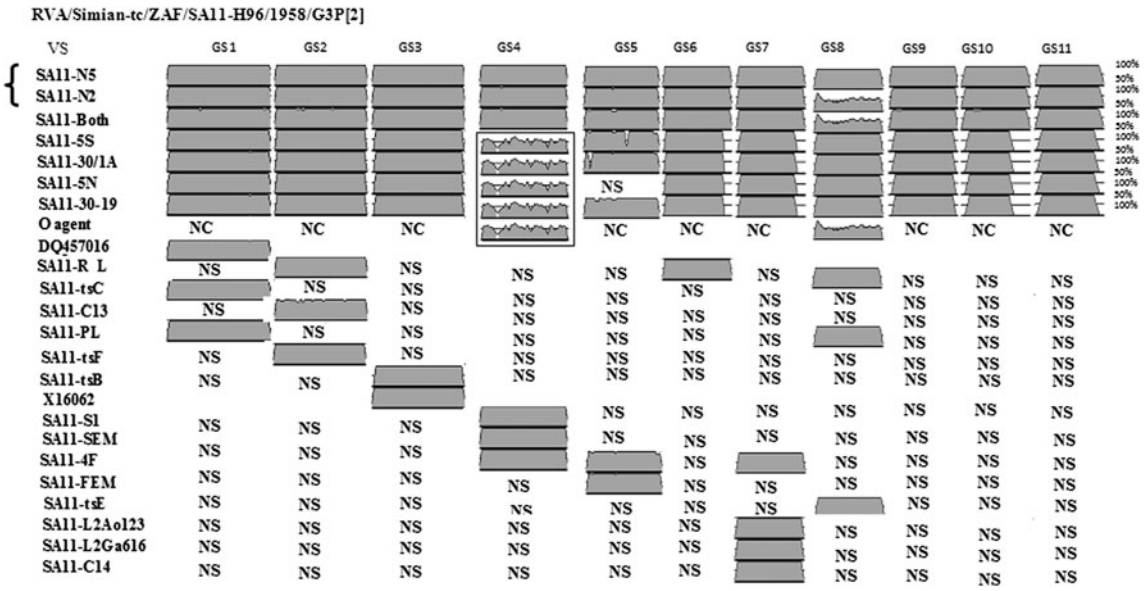

Fig. 1 *mVISTA* visualisation of an alignment comparing the nucleotide sequences of SA11-H96 [54] to the consensus whole-genome nucleotide sequences of SA11-N5, and SA11-N2 and nucleotide sequences of other rotavirus SA11 strains in GenBank. SA11-N5 and SA11-N2 are indicated by the bracket. Common strain names are used for simplicity. "Genome sequence" is abbreviated as GS. Genome segment 4 sequences with a P[1] type are boxed. "NC" represents no comparison performed while "NS" indicates no sequence in GenBank. The GenBank accession numbers are used for some genome segments where no specific strain names were available

The 99.78 % sequence identity of genome segment 5 (NSP1) of SA11-N5 and SA11-N2 with that of SA11-H96 resulted in them grouping to a branch generated by phylogenetic analysis of which genome segment 5 of SA11-H96 has been the sole representative to date (Fig. 2, E), and this confirms a previous observation that genome segment 5 does not seem to co-evolve with the other genome segments of rotavirus SA11 derivatives [38, 54]. Genome segment 6 (VP6) was identical to that of SA11-H96 [54] and SA11-5N (Table 1; Supplementary Fig. 1, F). Genome segment 9 (VP7) was identical to that of SA11-30/1A. Genome segment 11 (NSP5/6) was identical to that of SA11-H96 [54] and SA11-Both (Supplementary Fig. 1, I). Although SA11-N2 obtained genome segment 8 by reassortment between SA11-N5 and the bovine rotavirus O agent like SA11-Both, distance matrices showed that only genome segment 8 (NSP2) and 11 (NSP5/6) of SA11-N2 and SA11-Both were identical (Supplementary Fig. 1, K). Therefore, based on evolutionary divergences observed in distance matrices, it was concluded that SA11-N2 and SA11-N5 are the closest relatives yet identified of the original SA11 isolate of 1958 [54].

Analysis of the amino acid sequences deduced from the pyrosequence data revealed the occurrence of five novel amino acids encoded by the ORFs of three of the genome segments. A 388Pro in the antigenic domain of VP4 (genome segment 4) was observed in 11 % (29/264) of pyrosequence reads that had G, instead of T(U), at nucleotide position 1996 (Table 1; Supplementary Fig. 2A). The nucleotide T(U), in the consensus sequence, results in Ser. This amino acid is located on the surface of the predicted VP5* structure inferred using PDB ID: 1SLQ [10]. It is possible that this change could result in altering of antigenicity, such as conferring an antibody-escape phenotype, a suggestion that needs to be confirmed experimentally. In VP7 (genome segment 9), novel amino acids 29Thr (in the signal peptide located in the N-terminal region) and 309Ala (in the C-terminal region) were also detected. Amino acid 29Thr was a result of 10.6 % (57/537) pyrosequence reads that contained nucleotide C at position 135, while 309Ala resulted from 7.2 % (36/501) pyrosequence reads that contained nucleotide C at position 970 (Supplementary Fig. 2B). The consensus sequence contained nucleotides 135T(U) and 970A, resulting in residues Ile and Val respectively. For genome segment 10 (NSP4), novel amino acids 33Leu and 114Gly were observed in 28.4 % (76/268) pyrosequence reads that indicated nucleotide C at position 138, and 24.5 % (108/333) of pyrosequence reads that indicated nucleotide G at position 382, respectively (Supplementary Fig. 2, C). The consensus NSP4 sequence contained amino acids 33Phe and 114Asp at these respective positions. The detection of these five novel amino acids suggests either the presence of minority variants or sequence differences between SA11-N2 and SA11-N5. However, it was technically not possible to assign these differences to either virus. Therefore, the biological significance of these variations was not clear and should be investigated after the two viruses have been separated by

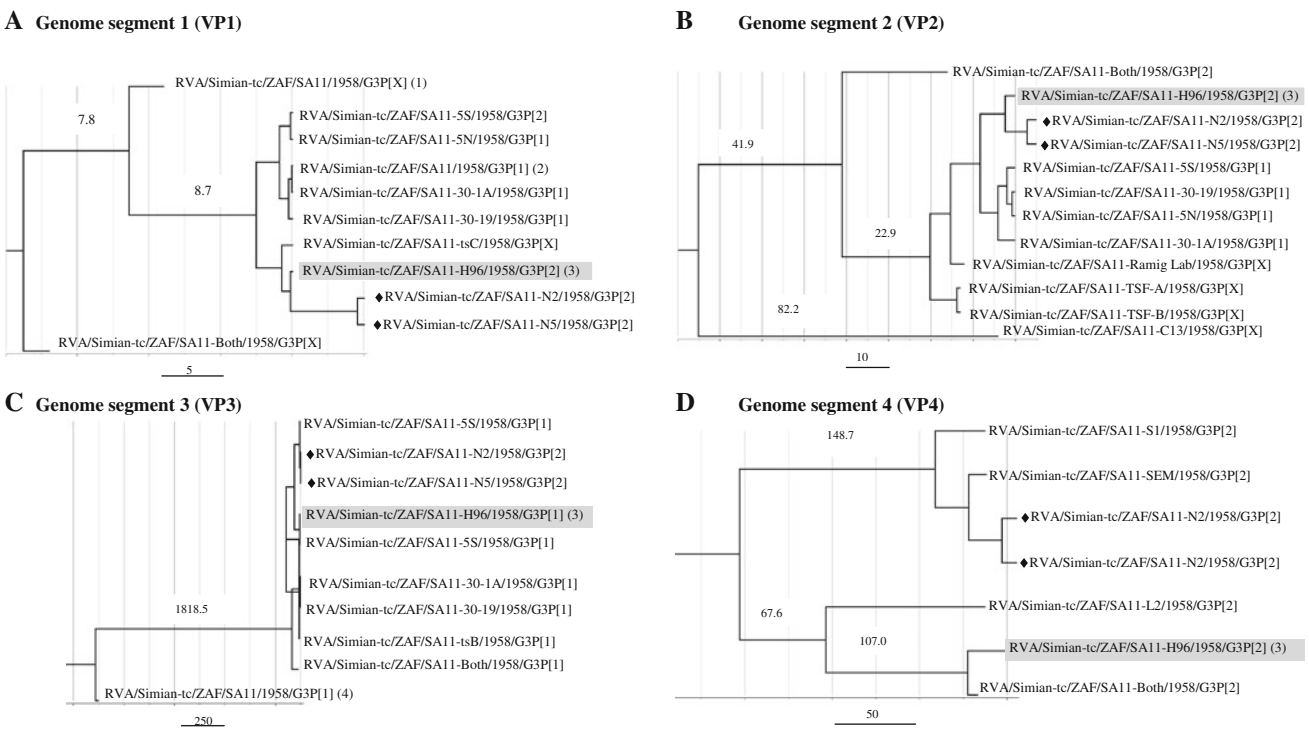

Fig. 2 Maximum clade credibility trees constructed with Bayesian MCMC framework, in BEAST software, to depict the Bayesian molecular clock phylogenetic relationships between SA11-N2, SA11-N5 and rotavirus SA11 sequences obtained from GenBank. Branch times (years) are indicated for some selected branches. The bars below each tree indicate time scales. Rotavirus SA11-N2 and SA11-N5 are indicated by a black diamond. SA11-H96, which is considered the representative of the SA11 strains, is shaded grey. Two SA11-H96 sequences were available in GenBank for genome segments 5 (NSP1), 6 (VP6), 7 (NSP3), 8 (NSP2) and 11 (NSP5/6). Sequences indicated with (3) were sequenced in the USA by Small and co-workers [54], and those indicated with (5) were sequenced in India by Dutta and co-workers [14]. Accession numbers of sequences associated with unspecified strains are as follows: (1): AF015955; (2): DQ457016; (4): X16062; (6): AF290881; (7): AF290883; (8): X00421; (9): GU550506 (10): M87502; (11): AF306493 and (12): M28347

several rounds of plaque purification. No amino acid differences were observed in the deduced protein sequences of NSP2 (SA11-N5) and NSP5/6 (SA11-N2 and SA11-N5) sequences when compared to the respective deduced amino acid sequences of SA11-H96. The above-described results, combined with a lack of passage history, prompted further characterisation of the viruses by the application of molecular clock evolutionary analysis of SA11-N2 and SA11-N5.

Bayesian molecular clock evolutionary analysis and phylogenetic relationships

Molecular clock phylogenetic analysis was carried out for all 12 consensus genome segment sequences of SA11-N5 and SA11-N2 but failed for 2 of the genome segments. In the case of genome segment 8, there were only three nucleotide sequences available in GenBank for the N2 genotype. It was therefore not possible to perform molecular clock phylogenetic analysis with so few sequences. The genome segment 10 molecular clock results were excluded from the analysis because zero-length interior branches [6] were present in the phylogenetic trees and could not be corrected. For the other ten genome segments, rates of nucleotide substitution were in the range of 1.4×10^{-6}–1.9×10^{-4} nucleotide substitutions/site/year (Table 1). The coefficient of variation for the nucleotide substitution rates was 0.3–1.4 (Table 1). The low CV indicates that significant nucleotide substitution variations occur within the branches. Therefore, the use of a relaxed molecular clock model as opposed to a strict molecular clock was valid [27]. The lowest nucleotide substitution rate was observed for genome segment 3 (VP3), while the highest was observed for genome segment 1 (VP1) (Table 1). High nucleotide substitution rates were also observed for genome segment 8 (NSP2) of SA11-N5 and genome segment 7 (NSP3) of both SA11-N5 and SA11-N2 (Table 1). The high rate of evolution of genome segment 1 was unexpected, since VP1 is a RNA-dependent RNA polymerase (RdRP) which is vital for ensuring viral replication. However, as with other RdRPs, sequence analysis of rotavirus genome segment 1 (VP1) revealed that the region encoding the basic right hand structure motif of RdRPs is highly conserved and that the variations in the

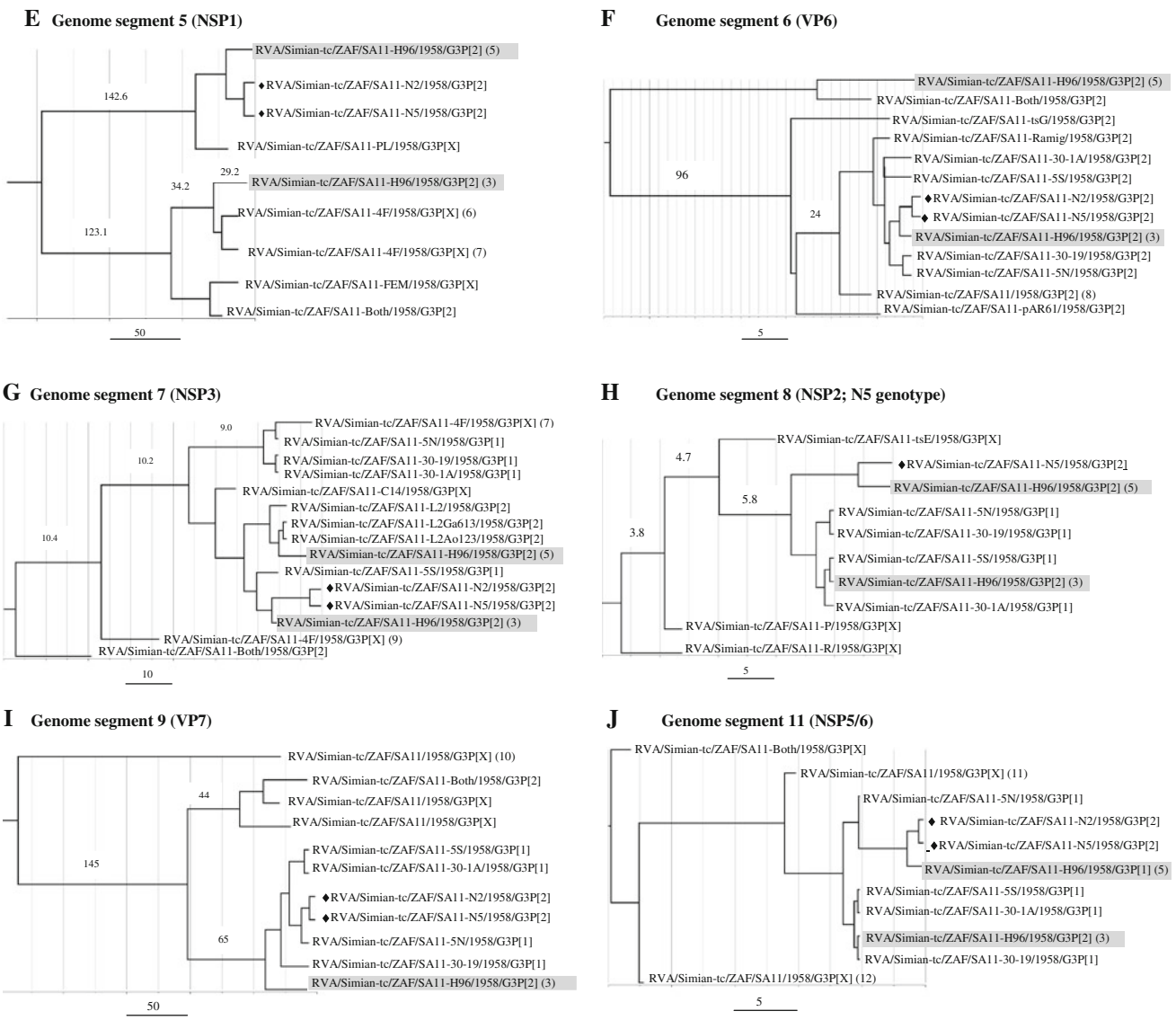

Fig. 2 continued

sequences are generally located close to the C- and N-terminal regions [59]. A report describing an analysis of the evolution of the VP1 of ovine rotaviruses in comparison to other rotavirus group A strains also showed that amino acid variations mainly occurred in the N- and C-terminal regions of the RdRP [5]. Therefore, an analysis of the nucleotide substitution rate for the region spanning nucleotide positions 778–2610, encoding amino acids at positions 260–870 was performed. This part of VP1 contains a unique RdRP region at the N-terminal end, followed by the fingers, palm and thumb regions of the polymerase domain [31, 42, 59]. The nucleotide substitution rate obtained for this polymerase-encoding region was 9.9×10^{-5} nucleotide substitutions/site/year with a CV of 0.6. This nucleotide substitution rate was lower than the overall rate of 1.3×10^{-4} nucleotide substitutions/site/year. However, the Ka/Ks ratio of non-synonymous to synonymous nucleotide substitution rates [23, 63] obtained for the genome segment 1 ORF was 0.6, suggesting that synonymous substitutions were favoured and that genome segment 1 is under pressure to conserve the VP1 sequence. For genome segment 11 (encoding NSP5/6), an overall mutation rate of 5×10^{-5} per replicated base was reported for porcine rotavirus CC86 strain [3]. In this study, we obtained a similar rate of 8.5×10^{-5} for genome segment 11 of the rotavirus SA11 strains. Mutation rates vary across the genome [13], and the difference between the evolution rate of the lowest rate and highest rate observed in this study was ~100-fold, but it is not clear why the difference was so high. However, the general rate of

evolution was comparable to the range of rates of evolution of dsRNA viruses, i.e., $\sim 1 \times 10^{-4}$–1×10^{-6} nucleotide substitutions/site/year [2, 13, 24, 53]. Interestingly, higher nucleotide substation rates of 1.36×10^{-3}–4.78×10^{-3} were reported recently for the genomes of group B rotaviruses [28].

The time-scaled phylogeny and maximum clade credibility (MCC) trees depicted in Fig. 2 confirmed that the most recent common ancestor for genome segments 1 (VP1), 2 (VP2), 6 (VP6) and 7 (NSP3) of SA11-N5 and SA11-N2 were the cognate genome segments from SA11-H96 [54]. For genome segments 5 (NSP1), 8 (NSP2 with genotype N5) and 11 (NSP5/6), the most recent common ancestor for genome segments of SA11-N5 and SA11-N2 genome segments was the SA11-H96 strain characterised in India by Dutta and co-workers [14]. In one of the few reports on molecular clock studies involving rotaviruses, genome segment 4 was found to evolve at a high rate of 0.58×10^{-3} nucleotide substitutions/site/year [24]. Sequences analysed in the report of Jenkins and co-workers [24] were, however, obtained from various field strains. For cell-culture-adapted rotavirus SA11 strains (genotyped P[2]), we determined a slower evolutionary rate of 1.4×10^{-5} nucleotide substitutions/site/year (Table 1). The MCC tree of genome segment 4 indicated that SA11-N2 and SA11-N5 were closely related to SA11-SEM (Fig. 2D).

The evolutionary rate obtained using genome segment 7 open reading frames was 1.3×10^{-4} nucleotide substitutions/site/year, with a coefficient of variation (CV) of 0.3 (Table 1). The substitution rate calculated for the SA11 rotavirus is in accordance with the estimated average rate of 7.9×10^{-4} nucleotide substitutions/site/year in all genome segments, except for segments 1, 3 and 10 for a human group B rotavirus [62]. The MCC tree for genome segment 7 indicated that SA11-N2 and SA11-N5 are closely related to strain SA11-H96 [54] (Fig. 2G). SA11-5S is also closely related to SA11-N2 and SA11-N5 (Fig. 2G). Strain SA11-4F followed a separate and distinct evolutionary path from that of SA11-5S (Fig. 2G).

The rate of evolution for genome segment 9 (VP7) was 2.9×10^{-5} nucleotide substitutions/site/year with a CV of 0.4 (Table 1). The frequency of appearance of monoclonal-antibody-resistant variants *in vitro* has been estimated to be approximately 10^{-5} [57]. The evolutionary rate obtained in this study was lower than the rate estimated for human G9 rotavirus strains [37]. Rates of 1.87×10^{-3} substitutions/site/year for G9 rotaviruses and 1.66×10^{-3} substitutions/site/year for G12 rotaviruses have been reported [37]. Similarly, a nucleotide substitution rate of 1.57×10^{-3} was reported for the VP7-encoding genome segment 9 of group B rotaviruses [51]. Matthijnssens and co-workers attributed the high evolutionary rate they observed for various G9 and G12 rotavirus strains to the immunological pressure on genome segment 9 (VP7) [18, 57]. The same conclusion could also be drawn for the high rate observed for genome segment 4 (VP4) by Jenkins and co-workers [24]. Genome segment 9 (VP7) nucleotide sequences of SA11-N2 and SA11-N5 and those in GenBank are from cell-culture-adapted strains that would only be under the pressure of the cellular innate immunity and culture conditions. This pressure on the genome is presumably less than the total biological pressure exerted during *in vivo* infections and possibly the reason for the relatively lower evolutionary rate observed in this study. The inherent and greater nucleotide sequence diversity between the G9 and G12 strains used in the analyses of Matthijnssens and co-workers is also expected to increase the calculated nucleotide substitution rate. For instance, the genome segment 9 (VP7) of the G9 porcine strain RVA/Pig-tc/KOR/PRG9121/2006/G9P[7] is 93 % identical to that of the human RVA/human-wt/BEL/BE2001/2009/G9P[6] strain which also has a G9 genotype (data not shown), whereas the G3 nucleotide sequences of SA11 derivatives analysed in this study were 99–100 % identical (Supplementary Fig. 1). However, computational estimation of evolutionary rates could result in variations from evolution rates determined experimentally.

Overall, a higher degree of divergence was observed following molecular clock evolutionary analysis in comparison to distance matrices (Fig. 2; Supplementary Fig. 1). The distance matrices indicate very close relationships between the rotavirus SA11 derivatives, but the MCC trees show more divergent relationships among the strains. This difference is attributed to the different approaches of the two methods for reconstructing phylogenetic relationships. The divergences also highlight sequence differences that could have been introduced during cell culture in the many laboratories that sequenced the rotavirus SA11 genome segments. However, Bayesian phylogenetic analysis in MCMC is more statistical (probabilistic), and sampling of trees is in proportion to their phylogenetic likelihood, thereby producing the most credible or consensus tree [11, 12, 17]. On the other hand, distance matrix calculations do not assume a molecular clock in the analysis [17].

In summary, the whole-genome consensus sequences of two rotavirus SA11 strains in a mixed sample were determined by sequence-independent genome amplification and 454® pyrosequencing. Two distinct genome segment 8 (encoding NSP2) nucleotide sequences were present in the same sample. This indicates that the virus sample was a mixture of SA11-N5 and SA11-N2. The SA11-N2 strain acquired a bovine rotavirus genome segment 8 due to reassortment with the bovine rotavirus O agent. The ability to detect the virus mixture in the same sample is another example of the advantage of next-generation sequencing over traditional sequencing methods. Genome segment 4

(VP4) sequences of SA11-N2 and SA11-N5 did not reassort with that of the bovine rotavirus O agent as in other rotavirus SA11 variants. Although SA11-N2 obtained genome segment 8 (NSP2) by reassortment like SA11-Both, SA11-N2 and SA11-Both are not the same. Therefore, the bovine rotavirus O agent must also have co-infected with SA11-N5, resulting in the generation of SA11-N2, as described previously for SA11-Both. However, the absence of additional rotavirus O agent sequences indicates that other reassortants, if any exist, may have been selected out during passage in cell culture or that their concentrations were too low to be detected. The five novel amino acids detected in VP4, VP7 and NSP4 might either reflect the occurrence of minority variants or actual sequence differences between SA11-N2 and SA11-N5.

Based on evolutionary divergences observed in distance matrices and molecular clock phylogenetic analysis, it was concluded that SA11-N2 and SA11-N5 are very close derivatives of the original SA11-H96 isolated in 1958 in South Africa. SA11-N2 is a reassortant carrying genome segment 8 (NSP2) from the O agent on the SA11-H96 backbone, whereas SA11-N5 contains a true SA11-like genome and is the closest derivative of SA11-H96 that has yet been identified.

Acknowledgments This study was funded by a Senior Fellowship awarded to HGO (I/84 015) by the European Foundation Initiative for Neglected Tropical Diseases (EFINTD). HGO also received a postdoctoral fellowship from the North-West University (Potchefstroom, South Africa). LM received a PhD bursary (09/41) from the Poliomyelitis Research Foundation, South Africa. We thank Mrs. I. Peenze, Diarrhoeal Pathogens Research Unit, University of Limpopo (Pretoria, South Africa), for kindly providing the rotavirus SA11 sample, and Dr. M. Coetzee (Department of Genetics, University of Pretoria, South Africa) for assistance with molecular clock analysis.

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Appendix 5

Jere, K.C., **Mlera, L.,** O'Neill, H.G., and van Dijk A.A (2012). Whole genome sequence analyses of three African bovine rotaviruses reveal that they emerged through multiple reassortment events between rotaviruses from different mammalian species. *Veterinary Microbiology*, **159:** 245-250

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Veterinary Microbiology 159 (2012) 245–250

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

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

Short communication

Whole genome sequence analyses of three African bovine rotaviruses reveal that they emerged through multiple reassortment events between rotaviruses from different mammalian species

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ARTICLE INFO

Article history:
Received 28 November 2011
Received in revised form 9 March 2012
Accepted 23 March 2012

Keywords:
Bovine rotavirus
454® Pyrosequencing
Whole genome analysis
Sequence-independent cDNA synthesis and amplification

ABSTRACT

Animal-to-human interspecies transmission is one of the evolutionary mechanisms driving rotavirus strain diversity in humans. Although quite a few studies emanating from Africa revealed evidence of bovine-to-human rotavirus interspecies transmission, whole genome data of African bovine rotavirus strains are not yet available. To gain insight into the complete genome constellation of African bovine rotaviruses, the full genomes of three bovine rotavirus strains were extracted from stool samples collected from calves, amplified using a sequence-independent procedure, followed by 454® pyrosequencing. Strains RVA/Cow-wt/ZAF/1603/2007/G6P[5] and RVA/Cow-wt/ZAF/1605/2007/G6P[5] were both genotyped as G6-P[5]-I2-R2-C2-M2-A3-N2-T6-E2-H3 and were probably two variants of the same rotavirus due to their close nucleotide sequence similarity. The genotype constellation of strain RVA/Cow-wt/ZAF/1604/2007/G8P[1] was G8-P[1]-I2-R2-C2-M2-A3-N2-T6-E2-H3. The genetic relationships and phylogenetic analyses suggested that these three bovine rotavirus strains may have emerged through multiple reassortment events between bovine, giraffe and antelope rotaviruses. Due to the close relatedness of genome segments 1 (encoding VP1), 7 (NSP2), 9 (VP7) and 10 (NSP4) of strain RVA/Cow-wt/ZAF/1604/2007/G8P[1] to those of the corresponding segments of human rotaviruses, RVA strain 1604 may represent bovine strains that were transmitted to humans and possibly reassorted with human rotaviruses previously. The complete nucleotide sequences of the bovine rotavirus strains reported in this study represent the first whole genome data of bovine rotaviruses from Africa.

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1. Introduction

Rotaviruses are some of the primary invaders of the intestinal epithelial of young animals and birds and often cause severe diarrhoea (Mebus et al., 1969; Wani et al., 2003). The mature rotavirus particle consists of a triple layer of capsid proteins that encloses its genomic material. The genome of rotavirus contains 11 segments of double-stranded RNA (dsRNA). Each genome segment encodes a single viral protein, except for segment 11 which encodes two proteins (NSP5 and NSP6). In total, there are six structural (VP) and six non-structural (NSP) proteins (Estes and Kapikian, 2007). Based on the properties of its proteins and genome segments, individual rotaviruses can be classified in several ways. According to immunological epitopes, rotaviruses are subdivided into species A–G

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http://dx.doi.org/10.1016/j.vetmic.2012.03.040

which are commonly named groups that are further classified into serotypes and genotypes (Estes and Kapikian, 2007). A dual classification system that uses genotypes assigned to the genome segments encoding the protease-sensitive VP4 (P types) and the glycoprotein VP7 (G types) is commonly used to distinguish rotavirus strains (Estes and Kapikian, 2007). However, the *Rotavirus Classification Working Group* recommends a whole genome classification system that assigns genotypes to all 11 genome segments (Matthijnssens et al., 2008b). Compared to the dual typing system, the whole genome classification is superior in elucidating the evolutionary mechanisms through which the strain under study has emerged because it uses the genotypes assigned to each genome segment (Ghosh et al., 2011a; Jere et al., 2011a; Matthijnssens et al., 2008a).

A wide genomic and antigenic diversity exists amongst group A rotaviruses. To date, at least 35 G and 27 P genotypes have been identified in both animals and humans (Matthijnssens et al., 2011a). Some rotavirus strains seem to be well adapted in several species, whilst others are species-specific. For instance, strains bearing G1, G2, G3, G4, G9 and G12 in combination with P[4], P[6] or P[8] genotypes are commonly isolated from humans, whilst strains containing G3, G4, G5, G9 and G11 genotypes in association with P[6], P[7], P[13], P[19], P[23], P[26], P[27] are most common in swine, whereas strains with G6, G8 and G10 in association with P[1], P[5], P[11], P[15], and P[21] have been detected in bovine species (Martella et al., 2010). Some studies from Africa have reported on the detection of rotaviruses from cattle (Adah et al., 2002; da Costa Mendes et al., 1993). However, only a few of these studies managed to genotype the circulating bovine strains. One example is the report of Fodha et al. (2005) on the prevalence of G6 and G8 strains. The ability of distinct rotavirus strains to exchange their cognate genome segments through reassortment, and to accumulate point mutations due to lack of proof-reading activity of their RNA-dependent RNA polymerase (RdRp) are the major contributors to the broad rotavirus strain diversity (Desselberger et al., 2001). Animals also play a crucial role in driving rotavirus strain diversity in human communities through animal-to-human interspecies transmission (Martella et al., 2010). This hypothesis is supported by the wide diversity of rotavirus strains characterised from most developing countries (Ahmed et al., 1991; Arguelles et al., 2000; Mwenda et al., 2010) where close proximity exist between human and animal dwellings (Cunliffe et al., 2000) compared to developed countries (Gray et al., 2008).

In order to understand the impact which animal rotaviruses have on the evolution of strains circulation in humans, knowledge of the sequence of the full genome of animal rotaviruses is necessary. However, the genomes of only a few animal rotavirus strains have been fully characterised. Of these, only three strains, RVA/Cat-wt/ITA/BA222/2005/G3P[9] (Martella et al., 2011), RVA/Antelope-wt/ZAF/RC-18-08/G6P[14] (Matthijnssens et al., 2009) and RVA/Horse-wt/ZAF/ EqRV-SA1/2006/G14P[12] (Matthijnssens et al., 2011b) have been sequenced on a mass parallel sequencing platform. Studies on human African rotavirus strains have revealed evidence of bovine-to-human interspecies transmission (Ghosh et al., 2011b; Martella et al., 2011), reassortment between bovine and human strains (Esona et al., 2009; Todd et al., 2010), and a common origin between bovine and some strains circulating in humans, for instance DS-1-like rotaviruses (Jere et al., 2011a; Matthijnssens et al., 2008a). The complete consensus sequence of the genome of bovine rotavirus strains isolated from African countries where several human rotavirus strains are known to share common ancestry with bovine rotaviruses have not yet been reported. Although G6P[5] and G8P[1] strains are often isolated from calves (Martella et al., 2010), Ghosh et al. (2011b) recently isolated a G8P[1] strain in an asymptomatic Kenyan child that revealed evidence for *artiodactyl*-to-human interspecies transmission. In most African communities, reassortment and/or interspecies transmission events between human and animal strains seems to be common due to poor hygienic conditions and close contact between human settlements and livestock (Cunliffe et al., 2000; Esona et al., 2009; Ghosh et al., 2011b; Matthijnssens et al., 2008a). Therefore, determination of the full genome sequence of animal rotavirus strains in this region is important to better understand the genetic diversity and zoonotic aspects of rotavirus strains circulating in humans. These and other mechanisms impact on the global burden of diseases through generation of zoonotic strains (Banyai et al., 2009) or human–animal reassortant strains (Ghosh et al., 2011b; Jere et al., 2011a). In this study, the whole genome of three bovine African rotavirus strains from stools of calves in South Africa were pyrosequenced. The strains were used for no reason other than that they were the only bovine samples available to us at the time. Their genome constellation and relatedness to rotaviruses of human and other mammals were investigated to understand their evolutionary relationships. The genetic diversity in the nine non-G and non-P genome segments of strains carrying similar genome segment 4 (VP4) and 9 (VP7) genotypes (G6P[5]) was also investigated to better understand their genetic relationships.

2. Materials and methods

2.1. Bovine rotavirus samples

Rotavirus strains RVA/Cow-wt/ZAF/1603/2007/G6P[5], RVA/Cow-wt/ZAF/1604/2007/G8P[1] and RVA/Cow-wt/ZAF/1605/2007/G6P[5] were characterised from stools collected in 2007 from three calves presenting with diarrhoea on a farm in Malmesbury, Western Cape Province, South Africa. The stool samples were screened for the presence of rotaviruses at the Onderspoort Veterinary Institute (OVI), South Africa. Making use of the dual RT-PCR based genotyping system (Gentsch et al., 1992; Gouvea et al., 1990), genome segment 4 (VP4) and 9 (VP7) genotypes were assigned to these strains at the Diarrhoeal Pathogens Research Unit (DPRU), South Africa.

2.2. Nucleotide sequencing, genotyping, distance matrices and phylogenetic analyses

The rotavirus dsRNA was extracted from the stool specimens, purified and ligated to the PC3-T7loop oligonucleotide as described previously (Potgieter et al., 2009).

The sequence-independent cDNA synthesis and PCR amplification procedure described by Jere et al. (2011a) was followed to generate and amplify cDNA of the whole genomes of the study bovine rotavirus strains. 454® pyrosequencing (Margulies et al., 2005) with the GS/FLX Titanium (Roche, Mannheim, Germany) technology was performed at Inqaba Biotec (PTY), Pretoria, South Africa. DNASTAR® Lasergene™ software, version 8.1.2 was used to generate consensus nucleotide sequences as described previously (Jere et al., 2011a). The nucleotide sequences reported in this study were submitted to the NCBI GenBank under the accession numbers listed in Supplement 1.

The web-based automated rotavirus genotyping tool, RotaC (http://rotac.regatools.be; Maes et al., 2009) was used to assign genotypes to all 11 genome segments of the study strains. The nucleotide sequences of the selected rotavirus strains characterised elsewhere used for comparison were acquired from GenBank (accession numbers listed in Supplement 2). BioEdit (Hall, 1999) and MEGA software, version 4.0 (Tamura et al., 2007) were used to generate distance matrices and perform phylogenetic and molecular evolutionary analyses.

3. Results

3.1. Genetic constellation and phylogenetic analyses of the genome segments of rotavirus strains RVA/Cow-wt/ZAF/1603/2007/G6P[5] and RVA/Cow-wt/ZAF/1605/2007/G6P[5]

A high sequence coverage rate was achieved for all the genome segments of the three rotaviruses (Supplement 3). The sizes of the complete nucleotide and deduced amino acid sequences for each genome segment of the three bovine study strains are listed in Supplement 4. Based on the genotypes that were assigned to all the nucleotide sequences of the 11 genome segments of RVA strains 1603 and 1605, an identical descriptor, namely G6-P[5]-I2-R2-C2-M2-A3-N2-T6-E2-H3, was assigned to both strains (Fig. 1). This genome constellation suggest that the two strains are typical G6P[5] bovine rotaviruses that are on a genetic backbone comprised of DS-1-like, AU-like and T6 genotypes. These strains may have emerged through multiple reassortment events.

Phylogenetic analysis revealed that all the genome segments of RVA strains 1603 and 1605 were very closely related (Supplement 5). Based on the clusters formed in the phylograms, the closest strains listed in the GenBank to RVA strains 1603 and 1605 are summarised in Table 1. Although genome segments 3 (VP3), 4 (VP4), 5 (NSP1), 6 (VP6), 8 (NSP2) and 9 (VP7) of the G6P[5] study bovine rotavirus strains share a common ancestry with the cognate genome segments of other bovine rotaviruses, their genome segments 1 (VP1) and 2 (VP2) share a common origin with that of a *Hippotragus niger* (sable antelope) RVA strain RC-18-08, genome segments 7 (NSP3) and 11 (NSP5) are most closely related to that of a bovine-like RVA strain UCD identified from a giraffe, whereas segment 10 (NSP4) shares a common origin with that of animal–human reassortant strains such as the human RVA strain PAH136 (De Grazia et al., 2010).

3.2. Genetic constellation and phylogenetic analyses of the genome segments of strain RVA/Cow-wt/ZAF/1604/2007/G8P[1]

Based on genotypes assigned to all the genome segments of RVA strain 1604, it was assigned a unique complete genotype descriptor G8-P[1]-I2-R2-C2-M2-A3-N2-T6-E2-H3. Although RVA strain 1604 has a similar genetic backbone as the two G5P[6] study bovine strains, it contains completely different genome segment 4 (VP4, P[1]) and 9 (VP7, G8) genotypes. Furthermore, there were several nucleotide sequences variations in the genome segments encoding NSP1, NSP2, NSP3, NSP4, NSP5, VP1, VP3, VP3 and VP6 when all the genome segments of the G8P[1] strain were aligned against the other two G6P[5] bovine study strains (data not shown).

Phylogenetic analysis of each of the 11 genome segments of the RVA strain 1604 (Supplement 5) revealed that its genome segments 3 (VP3), 4 (VP4), 5 (NSP1) and 8 (NSP2) were most closely related to those of other bovine rotaviruses. Genome segment 4 (VP4) of the simian RVA strain SA11-4F, which is most closely related to that of RVA strain 1604, is of bovine origin (Mattion and Estes, 1991). The following genetic relationships were observed for the other genome segments: segment 6 (VP6) was most closely related to that of the *H. niger* RVA strain RC-18-08; segments 2 (VP2) and 11 (NSP5) were most closely related to a bovine-like RVA strain UCD characterised from a giraffe; segments 1 (VP1) and 7 (NSP3) were most closely

Rotavirus strain name	Genome constellation										
	S9(VP7)	S4(VP4)	S6(VP6)	S1(VP1)	S2(VP2)	S3(VP3)	S5(NSP1)	S8(NSP2)	S7(NSP3)	S10(NSP4)	S11(NSP5)
RVA/Cow-wt/ZAF/1603/2007/G6P[5]	G6	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Cow-wt/ZAF/1604/2007/G8P[1]	G8	P[1]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Cow-wt/ZAF/1605/2007/G6P[5]	G6	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Cow-tc/FRA/RF/1982/G6P[1]	G6	P[1]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Cow-tc/USA/WC3/1981/G6P[5]	G6	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Cow-tc/GBR/UK/1973/G6P7[5]	G6	P[5]	I2	R2	C2	M2	A3	N2	T7	E2	H3
RVA/Human-tc/USA/DS-1/1976/G2P1B[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-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-tc/KEN/B12/1987/G8P[1]	G8	P[1]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Human-wt/NIC/NIC522/2008/G8P[1]	G8	P[1]	I2	R2	C2	M2	A13	N2	T6	E2	H3
RVA/Human-wt/BGD/RV176-00/2000/G12P[6]	G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E6	H2
RVA/Human-tc/USA/Wa/1974/G1P1A[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-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

Fig. 1. Whole genome classification of the three African bovine rotaviruses analysed in this study compared to well-characterised rotavirus strains listed in the GenBank. Colours were added to visualise patterns of genome constellations as follows: green (Wa-like), red (DS-1-like), orange (AU-like), purple (PO-13-like) and blue (some typical animal rotavirus genotypes). S, genome segment; VP, viral structural protein; NSP, viral non-structural protein.

Table 1
Percentage identities of the most closely related nucleotide sequences of the complete genome segments of rotavirus strains listed in the GenBank compared to the three study bovine strains.

Genome segment	RVA/Cow-wt/ZAF/1603/2007/G6P[5]	RVA/Cow-wt/ZAF/1604/2007/G8P[1]	RVA/Cow-wt/ZAF/1605/2007/G6P[5]
1 (VP1)	RVA/Antelope-wt/ZAF/ RC-18-08/G6P[14] (97.5%)	RVA/Human-wt/KEN/B12/1987/G8P[1] (95.4%)	RVA/Antelope-wt/ZAF/ RC-18-08/G6P[14] (96.3%)
2 (VP2)	RVA/Antelope-wt/ZAF/ RC-18-08/G6P[14] (98.5%)	RVA/giraffe-wt/IRL/UCD/2007/ G10P[11] (95.1%)	RVA/Antelope-wt/ZAF/ RC-18-08/G6P[14] (98.4%)
3 (VP3)	RVA/Cow-wt/JPN/Azuk-1/2006/G21P[29] (95.5%)	RVA/Cow-wt/JPN/Azuk-1/2006/G21P[29] (95.4%)	RVA/Cow-wt/JPN/Azuk-1/2006/G21P[29] (95.6%)
4 (VP4)	RVA/Cow-wt/THA/61A/XXXX/G10P[5] (95.7%)	RVA/Vervet monkey-tc/ZAF/SA11-4F/1958/ G3P6[1] (95.4%)[a]	RVA/Cow-wt/THA/61A/XXXX/G10P[5] (95.7%)
5 (NSP1)	RVA/Cow-tc/USA/NCDV/1967/G6P6[1] (97.2%)	RVA/Cow-tc/FRA/RF/1982/G6P[1] (97.5%)	RVA/Cow-wt/ZAF/1603/2007/G6P[5] (97%)
6 (VP6)	RVA/Bovine-tc/JPN/22R/XXXX/GXP[X] (96.5%)	RVA/Antelope-wt/ZAF/ RC-18-08/G6P[14] (98.8%)	RVA/Bovine-tc/JPN/22R/XXXX/GXP[X] (96.5%)
7 (NSP3)	RVA/Giraffe/UCD/IRL/2007/G10P[11] (96.7%)	RVA/Human-wt/ITA/111-05-27/2005/ G6P[14] (96.8%)	RVA/Giraffe/UCD/IRL/2007/G10P[11] (96.6%)
8 (NSP2)	RVA/Cow-lab/USA/UKg9ST3/ 1986/G4P[5] (98%)	RVA/Cow-wt/ARG/B383/1998/G15P[11] (94.9)	RVA/Cow-lab/USA/UKg9ST3/ 1986/G4P[5] (98%)
9 (VP7)	RVA/Cow-tc/GBR/UK/1973/G6P7[5] (95%)	RVA/Human-wt/AUS/MG8/ 1997/G8P[14] (91.7%)	RVA/Cow-tc/GBR/UK/1973/G6P7[5] (95%)
10 (NSP4)	RVA/Human-wt/ITA/PAH136/1996/G3P[9] (94.3%)	RVA/Human-wt/COD/DRC86/2003/G8P[6] (97.3%)	RVA/Human-wt/ITA/PAH136/1996/G3P[9] (94.1%)
11 (NSP5/NSP6)	RVA/giraffe-wt/IRL/UCD/2007/ G10P[11] (98.8%)	RVA/giraffe-wt/IRL/UCD/2007/ G10P[11] (98.2%)	RVA/giraffe-wt/IRL/UCD/2007/ G10P[11] (98.8%)

The table summarises the closest related rotavirus strains to each study bovine strain as shown in the phylograms of all 11 rotavirus genome segments (Supplement 5). The percentages in parenthesis were generated with the basic local alignment search tool (BLAST). Accession numbers of each reference strains are provided in Supplement 2. The strain nomenclature represents the rotavirus group/species of origin/country of identification/common name/ year of identification/G- and P-type. The unique 3-letter abbreviation code used for each country are as listed on the website https://www.cia.gov/library/publications/the-world-factbook/appendix/appendix-d.html (Matthijnssens et al., 2011a,b).

[a] RVA/Vervet monkey-tc/ZAF/SA11-4F/1958/G3P6[1] contains a genome segment 4 (VP4) of bovine origin (Mattion and Estes, 1991).

related to zoonotic-human reassortant rotaviruses like RVA strains B12 and 111-05-27 identified in humans; whereas segments 9 (VP7) and 10 (NSP4) were most closely related to human rotavirus strains that have been suggested to originate from cattle, for instance, RVA strain MG8 collected in Australia (Cooney et al., 2001).

3.3. Distance matrix analyses

Since the nine genome segments encoding VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4 and NSP5 of the three study bovine rotaviruses were assigned identical genotypes (I2-R2-C2-M2-A3-N2-T6-E2-H3), the relationships between their genetic backbones were analysed further. Distance matrix analyses were estimated with MEGA 4 software using the Maximum Composite Likelihood method (Tamura et al., 2007) (Supplement 6). As expected, all the genome segments of RVA strain 1604 were distantly related to those of both RVA strain 1603 [up to 0.12 variance in genome segment 8 (NSP2) and 10 (NSP4)] and RVA strain 1605 [up to 1.64 variance in genome segment 7 (NSP3)]. By contrast, no evolutionary distances (variance of 0.00) was estimated between all the genome segments of the G6P[5] strains. Furthermore, multiple sequence alignment of all the genome segments of the three bovine rotaviruses reported here revealed some nucleotide variations that appeared not to affect the amino acid sequences of the deduced proteins (data not shown). Although the nucleotide sequences of the two G6P[5] rotaviruses were remarkably conserved in general, they did show minor nucleotide variations at the following positions: genome segment 1 (VP1) [3123 G → A]; 2 (VP2) [1445 A → G], 3 (VP3) [1279C → T]; 4 (VP4) [336 G → A]; 5 (NSP1) [T deletion at nt position 11 and TA deletion at nt position 68–69]; and 8 (NSP3) [212–213 AT → TA and 210–212 ACT → CTC] (data not shown).

4. Discussion and conclusion

Previously, the robustness and versatility of the sequence-independent whole genome dsRNA amplification (Potgieter et al., 2009) coupled with next generation sequencing was reported. 454® pyrosequencing (Margulies et al., 2005) and RotaC (Maes et al., 2009) were used to obtain and characterise the consensus nucleotide sequence of the full genome of the prototype rotavirus DS-1 strain (Mlera et al., 2011), African human rotaviruses (Jere et al., 2011a) and to dissect the classification of genome segments of multiple rotavirus strains simultaneously infecting a single patient (Jere et al., 2011b). In this study, the same approach was used to determine the sequences of full genomes of three African bovine rotavirus strains genotyped as G8P[1] and G6P[5]. As often reported for most bovine rotavirus strains (Gerna et al., 1990), both the dsRNA and cDNA of the three study bovine rotavirus strains also displayed long electropherotype profiles. All three study strains had a conserved I2-R2-C2-M2-A3-N2-T6-E2-H3 genetic backbone which is common in some of the well characterised bovine strains (Matthijnssens et al., 2011a).

Numerous studies have illustrated the evidence of animal-to-human interspecies transmission of rotavirus strains (reviewed by Martella et al., 2010). G8P[1] strains RVA/Human-wt/KEN/B12/1987/G8P[1] and RVA/Human-wt/NIC/NIC522/2008/G8P[1] represent two of the few human rotavirus strains that are believed to have been directly transmitted from cattle or other *artiodactyla* species to humans (Banyai et al., 2009; Ghosh et al., 2011b). Comparing the genetic constellation of these only two zoonotic G8P[1] human rotavirus strains to the G8P[1] bovine strain presented in the current study, RVA strain B12 and 1604 shared identical genotype constellations despite nine of their genome segments clustering separately within the phylograms (Supplement 5). This suggests that these two G8P[1] strains were distantly related despite having identical segmental genotypes and genotype constellation. The genotypes assigned to strain RVA/Human-wt/NIC/NIC522/2008/G8P[1] differed only in genome segment 5 (NSP1) to those of the bovine RVA strain 1604 (Fig. 1). Since the nucleotide sequences of strain RVA/Human-wt/NIC/NIC522/2008/G8P[1] were not available from GenBank, the phylogenetic comparisons to the study bovine strains could not be made.

The genetic constellations and the phylogenies of the complete genomes of the three bovine rotavirus strains reported in this study imply that multiple reassortment and interspecies transmission within the *artiodactyla* species most likely led to the emergence of these strains. RVA strains 1603 and 1605 might be the result of multiple reassortment events between bovine, giraffe and antelope rotaviruses. This is hypothesized based on the close similarity of their genome segments to the cognate genome segments of rotaviruses characterised from these *artiodactyla* species. The close similarity between the nucleotide sequences of RVA strains 1603 and 1605 suggests that the two calves carrying them may have been infected by a single rotavirus strain, which then generated quasispecies. Although these isolates have an identical genotype constellation, G6-P[5]-I2-R2-C2-M2-A3-N2-T6-E2-H3, and showed remarkable conservation in five genome segments, some nucleotide variations were obtained in genome segments 1 (VP1), 2 (VP2), 3 (VP3), 4 (VP4), 5 (NSP1) and 7 (NSP3). This may illustrate some minor diversity of the G6P[5] strains that were circulating on the South African farm in 2007. The fact that nucleotide variations were observed between six of the genome segments of the two G6P[5] strains warrants further research to understand whether and how these variations may affect the strain virulence in bovine species or when these strains were transmitted to other species.

RVA strain 1604 potentially emerged through multiple reassortment events between bovine, giraffe and antelope rotaviruses due to the close relationship of its genome segments to the corresponding genome segments of rotaviruses identified from these mammalian species. Evidence suggest that G8 rotaviruses, which are common in cattle, that are frequently detected from infants and young children in Africa potentially originated from or shared a common origin with bovine rotavirus strains (Cunliffe et al., 2000; Esona et al., 2009; Jere et al., 2011a; Matthijnssens et al., 2008a). RVA strain 1604 might be considered an example of such a bovine rotavirus strain due to the close similarity of its genome segments 4 (VP4), 7 (NSP3), 9 (VP7) and 10 (NSP4) to cognate genes of human rotavirus strains. This correlates with the hypothesis made by Esona et al. (2009) and Matthijnssens et al. (2006) that G8 human rotavirus strains detected from several African countries share a common origin with bovine rotavirus strains.

In conclusion, zoonotic studies are hampered by lack of whole genome sequence data of rotavirus strains circulating in animals. Although the full genomes of only two *artiodactyla* rotavirus strains have been characterised from Africa, one from an antelope (Matthijnssens et al., 2009), and one from a horse (Matthijnssens et al., 2011b), no complete nucleotide sequences of the whole genome of African bovine rotavirus strains have been characterised. Accumulation of more sequence data of animal strains using massive parallel next generation sequencing is important to accurately understand the ecology, epidemiology and evolution of rotaviruses. This will also enable deciphering of the true origin of the zoonotic strains circulating in humans. The fact that wide strain diversities have been reported from African countries where interspecies transmission has been implicated as one of the main cause resulting from the close proximity between livestock and human settlement clearly calls for more understanding of rotavirus strains circulating in various animal species from this region.

Competing interests

The author(s) declare that they have no competing interests.

Acknowledgements

We thank the MRC Diarrhoeal Pathogens Research Unit, University of Limpopo, Medunsa Campus, for providing rotavirus strain characterised in this study. We also thank Mr Martin Myaga for determining the electropherotypes of the study strains using polyacrylamide gel electrophoresis (PAGE). We thank Dr. Ulrich Desselberger for critical reading of the manuscript. This study was partially funded by the North-West University, the South African National Research Foundation (grants FA2005031700015 and UID 63427), the Poliomyelitis Research Foundation of South Africa (grant no. 09/34) and Cannon Collins Trust.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2012.03.040.

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Appendix 6

Jere, K.C., **Mlera, L**., Page, N.A., van Dijk A.A., and O'Neill, H.G. (2011). 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. *Infection, Genetics and Evolution*, **11**(8): 2072-2082.

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http://www.sciencedirect.com/science/article/pii/S1567134811003522

Infection, Genetics and Evolution 11 (2011) 2072–2082

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

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

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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ARTICLE INFO

Article history:
Received 22 June 2011
Received in revised form 27 September 2011
Accepted 28 September 2011
Available online 11 October 2011

Keywords:
Rotavirus
Whole genome analysis
Genome recombination
Sequence-independent genome amplification and 454® pyrosequencing

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.

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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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doi:10.1016/j.meegid.2011.09.023

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 16,000*g*, precipitation of dsRNA in isopropanol and centrifugation at room temperature for 30 min at 16,000*g*. 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 16,000*g* 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™-inbuilt 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 heteroplasy 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.

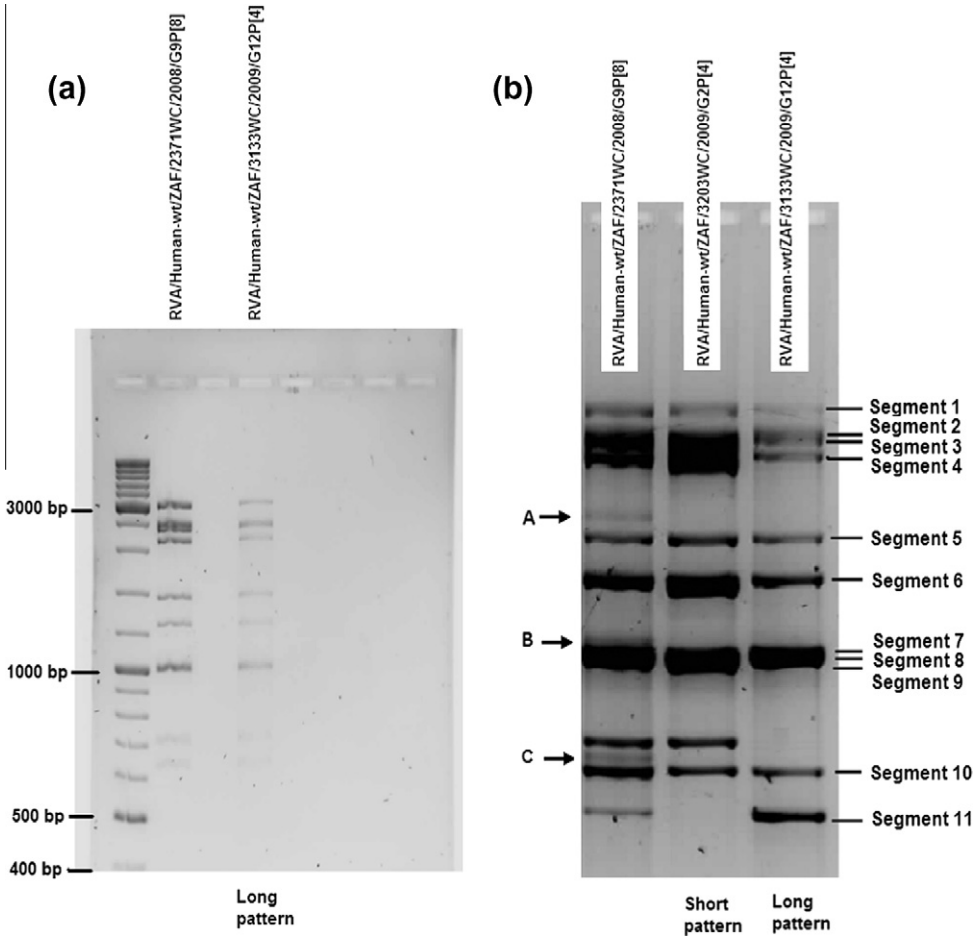

Fig. 1. One percent agarose gels of the purified dsRNA and PCR-amplified cDNA of the study specimen (RVA/Human-wt/ZAF/2371WC/2008/G9P[8]) compared to other reference strains. (a) Purified dsRNA of strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8] extracted directly from a stool sample compared to strain RVA/Human-wt/ZAF/3133WC/2008/G12P[4] 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[A]	R1[A]	C1[B]	M1[A]	A1[B]	N1[A]	T1[B]	E1[A]	H1[B]
G2[B]	P[4][B]	I2[B]	R2[B]	C2[A]	M2[B]	A2[A]	N2[B]	T2[A]	E2[B]	H2[A]
G8[C]	P[6][C]	I1[C]			M1[C]		A1[C]		E2[C]	
G12[D]	P[6][D]	I2[D]							E1[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	R 1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/JPN/KU/XXXX/G1P1[8]	G1	P[8]	I1	R 1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/USA/WI61/1983/G9P1A[8]	G9	P[8]	I1	R 1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/BEL/B4633/2003/G12P[8]	G12	P[8]	I1	R 1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/BGD/Dhaka25-02/2002/G12P[8]	G12	P[8]	I1	R 1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/USA/DS-1/1976/G2P1B[4]	G2	P[4]	I2	R 12	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]	G2	P[4]	I2	R 12	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/COD/DRC86/2003/G8P[6]	G8	P[6]	I2	R 12	C2	M2	A2	N2	T2	E2	H2
RVA/Human-tc/IDN/69M/1980/G8P4[10]	G8	P[10]	I2	R 12	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/COD/DRC88/2003/G8P[8]	G8	P[8]	I2	R 12	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BGD/RV176.00/2000/G12P[6]	G12	P[6]	I2	R 12	C2	M2	A2	N2	T2	E6	H2
RVA/Human-tc/JPN/AU-1/1982/G3P3[9]	G3	P[9]	I3	R 3	C3	M3	A3	N3	T3	E3	H3
RVA/Human-tc/THA/T152/1998/G12P[9]	G12	P[9]	I3	R 3	C3	M3	A3	N3	T3	E3	H3
RVA/Pigeon-tc/JPN/PO-13/1983/G18P[17]	G18	P[17]	I4	R 4	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/

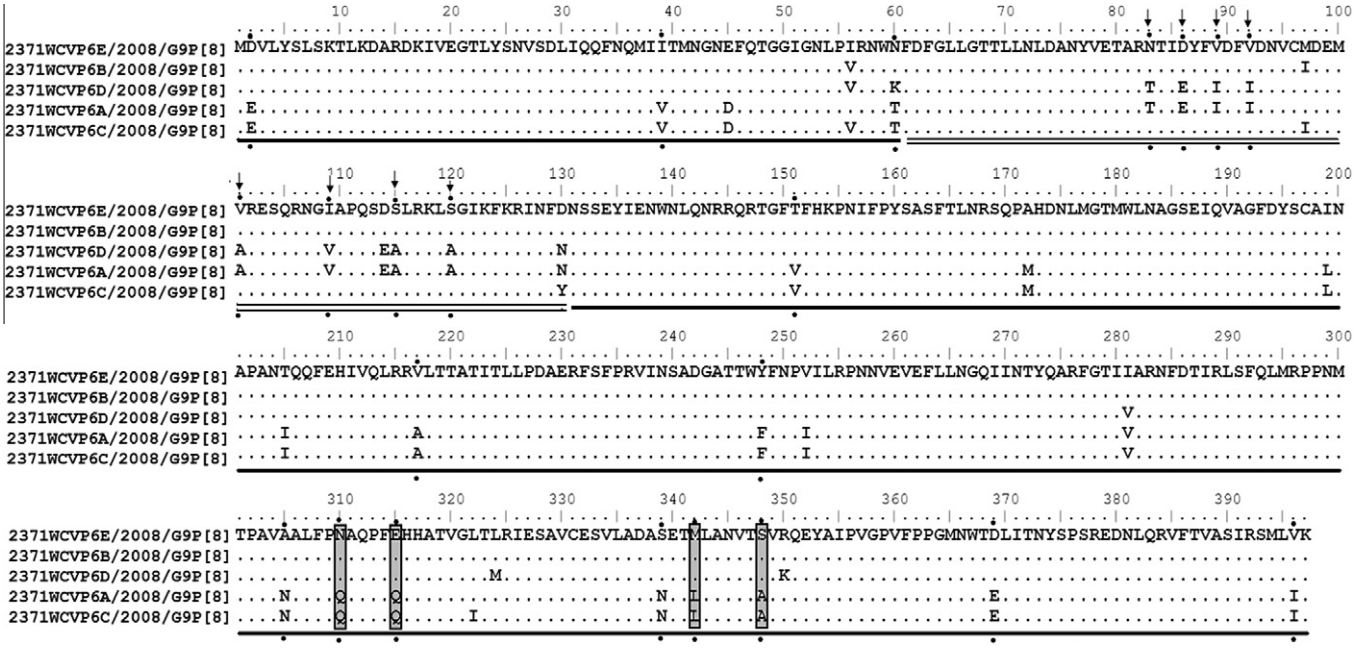

Fig. 2. Five complete amino acid sequences of the rotavirus populations identified for genome segment 6 (VP6). Between aa 1–60 (corresponding to nt 23–202) and 131–397 (corresponding to nt 414–1356) (underlined with a single line), RVA/Human-wt/ZAF/2371WCVP6B/2008/G9P[8], RVA/Human-wt/ZAF/2371WCVP6D/2008/G9P[8] 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

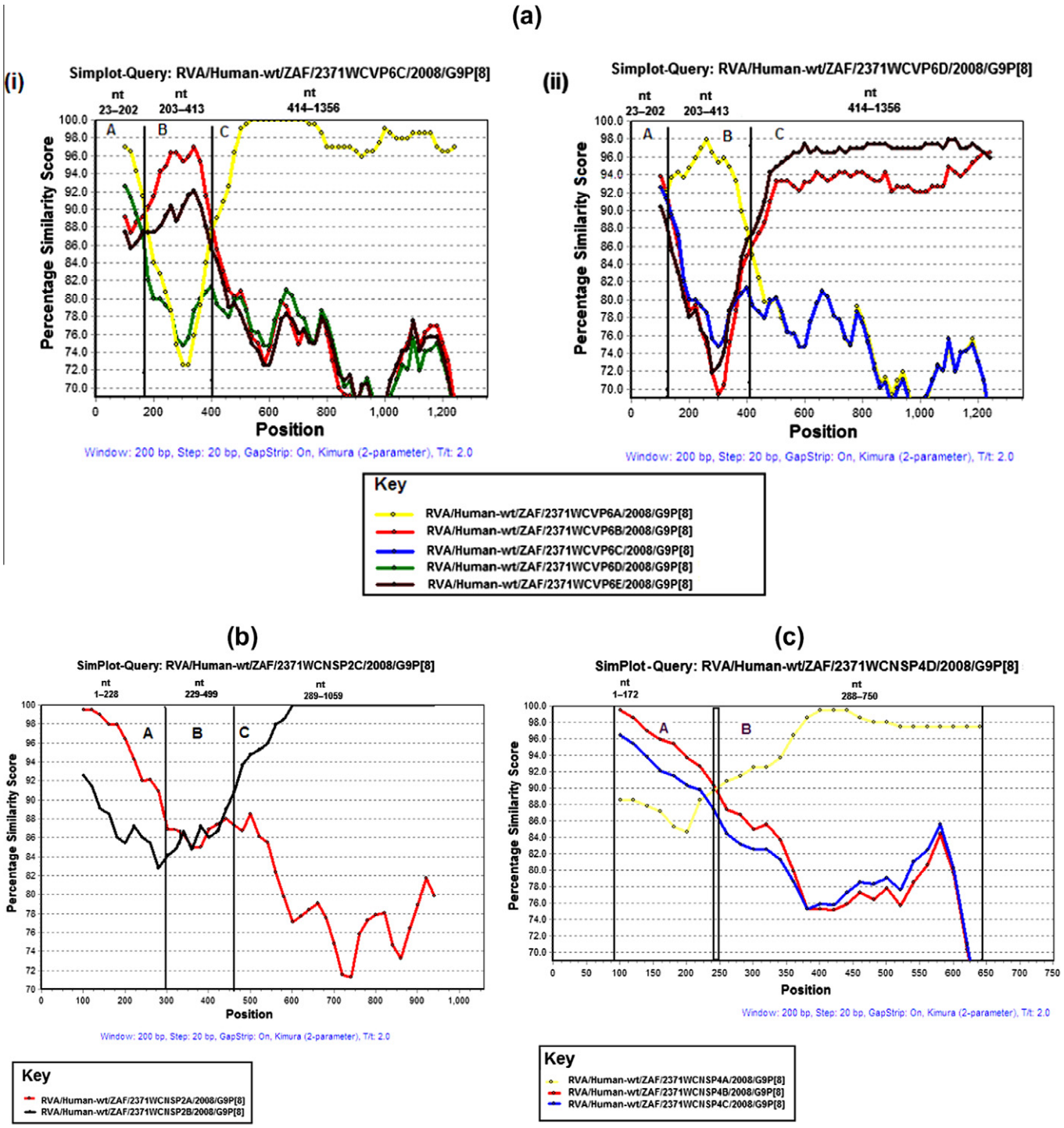

Fig. 3. SimPlot analysis of the nucleotide sequences of genome segment 6 (VP6), 8 (NSP2) and 10 (NSP4) suspected to be involved in recombination. The percentage similarity scores between the nucleotide sequences of the viral populations were generated by SimPlot software (Lole et al., 1999). Each curve 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/2371 WCVP7B/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.

Acknowledgements

We thank the Viral Gastroenteritis Unit, National Institute for Communicable Diseases (NICD) for providing the rotavirus strain characterised in this study. This study was partially funded by the North-West University, the South African National Research Foundation (Grants FA2005031700015 and UID 63427) and the Poliomyelitis Research Foundation of South Africa (Grant No. 09/34).

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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Appendix 7

Jere, K.C., **Mlera, L**., O'Neill, H.G., Potgieter, A.C., Page, N.A., Seheri, M.L., & van Dijk A.A. Whole genome analyses of African G2, G8, G9 and G12 rotavirus strains using sequence-independent amplification and 454® pyrosequencing. *Journal of Medical Virology*, **83**: 2018-2042.

Author guidelines available online at:

http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1096-9071/homepage/ForAuthors.html

Supporting information available online at:

http://onlinelibrary.wiley.com/doi/10.1002/jmv.22207/abstract;jsessionid=53A859AA9306595657C2DEEBF6753F2B.d03t02

Journal of Medical Virology 83:2018–2042 (2011)

Whole Genome Analyses of African G2, G8, G9, and G12 Rotavirus Strains Using Sequence-Independent Amplification and 454® Pyrosequencing

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High mortality rates caused by rotaviruses are associated with several strains such as G2, G8, G9, and G12 rotaviruses. Rotaviruses with G9 and G12 genotypes emerged worldwide in the past two decades. G2 and G8 rotaviruses are however also characterized frequently across Africa. To understand the genetic constellation of African G2, G8, G9, and G12 rotavirus strains and their possible origin, sequence-independent cDNA synthesis, amplification, and 454® pyrosequencing of the whole genomes of five human African rotavirus strains were performed. RotaC and phylogenetic analysis were used to assign and confirm the genotypes of the strains. Strains RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3203WC/2009/G2P[4], RVA/Human-wt/ZAF/3133WC/2009/G12P[4], RVA/Human-wt/ZAF/3176WC/2009/G12P[6], and RVA/Human-wt/ZAF/GR10924/1999/G9P[6] were assigned G8-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2, G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2, G12-P[4]-I1-R1-C1-M1-A1-N1-T1-E1-H1, G12-P[6]-I1-R1-C1-M1-A1-N1-T1-E1-H1, and G9-P[6]-I2-R2-C2-M2-A2-N2-T2-E2-H2 genotypes, respectively. The detection of both Wa- and DS-1-like genotypes in strain RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and Wa-like, DS-1-like and P[6] genotypes in strain RVA/Human-wt/ZAF/GR10924/1999/G9P[6] implies that these two strains were generated through intergenogroup genome reassortment. The close similarity of the genome segments of strain RVA/Human-wt/MWI/1473/2001/G8P[4] to artiodactyl-like, human-bovine reassortant strains and human rotavirus strains suggests that it originated from or shares a common origin with bovine strains. It is therefore possible that this strain might have emerged through interspecies genome reassortment between human and artiodactyl rotaviruses. This study illustrates the swift characterization of all the 11 rotavirus genome segments by using a single set of universal primers for cDNA synthesis followed by 454® pyrosequencing and RotaC analysis. ***J. Med. Virol. 83:2018–2042, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: rotavirus; 454® pyrosequencing; emerging strains; genogroup

INTRODUCTION

Rotaviruses are the leading cause of severe-dehydrating diarrhea. Each year, rotavirus infection is associated with approximately 527,000 deaths among under 5-year olds worldwide. Almost half of these deaths occur in sub-Sahara Africa [Parashar et al., 2009; Mwenda et al., 2010].

Rotaviruses belong to the *Reoviridae* virus family and have a segmented double-stranded RNA (dsRNA) genome composed of 11 segments. The dsRNA segments encode six structural (VP1–VP4, VP6, and

Additional supporting information may be found in the online version of this article.

Grant sponsor: South African National Research Foundation; Grant numbers: FA2005031700015; UID 63427; Grant sponsor: Poliomyelitis Research Foundation of South Africa (partial support); Grant numbers: 09/34. 10/11.

Conflict of interests: The authors declare that they have no conflict of interests.

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Accepted 13 July 2011

DOI 10.1002/jmv.22207
Published online in Wiley Online Library (wileyonlinelibrary.com).

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VP7) and six non-structural (NSP1–NSP6) proteins. The structural VPs assemble around the genomic material into three concentric layers namely, the core (VP1–VP3), inner capsid (VP6), and outer capsid (VP4 and VP7). Seven serogroups (A–G), and at least four subgroups (I, II, I + II, and Non I/II) within group A, have been identified based on the epitopes on the inner capsid protein (VP6). The outer capsid proteins, VP4 and VP7, induce neutralizing antibodies and are used in assigning serotypes [Estes and Kapikian, 2007]. A dual typing system based on the genome segments encoding VP4 (P genotypes) and VP7 (G genotypes) is commonly used. To date, 27 different G- and 35 P-genotypes have been described in both humans and animals [Matthijnssens et al., 2011]. Unlike infections in developed countries, where G1P[8] strains cause almost 70% of the rotavirus diarrhea cases [Gray et al., 2008], wide strain diversity is associated with infections in developing countries and a significant proportion of cases are associated with G2, G8, and G9 rotaviruses [Todd et al., 2010].

Reassortment of the viral genome segments contributes significantly towards rotavirus strain diversity [Estes and Kapikian, 2007]. This process may involve any of the 11 rotavirus genome segments being exchanged between two or more strains during simultaneous infection of one host cell [Greenberg et al., 1981; Matthijnssens et al., 2008a; Tsugawa and Hoshino, 2008]. The dual typing system can not disclose comprehensive details of the molecular evolution and epidemiology of rotaviruses. Whole genome classification of strains may reveal not only certain genetic constellations, such as the common origins of strains, but may also enable identification of distinct rotavirus genotypes following separate evolutionary paths [Matthijnssens et al., 2008b]. Identification of reassortment events [Gentsch et al., 2005] and possible interspecies transmissions occurring within rotavirus populations [Tsugawa and Hoshino, 2008] can also be detected using whole genome analyses. Therefore, whole genome characterization of emerging rotavirus strains could assist in understanding the extent of their genetic relatedness to the current prevailing strains.

Recent advances made with the improvement of the sequence-independent amplification procedure of dsRNA coupled with pyrophosphate-based 454® (GS20/FLX) sequencing, allows cDNA synthesis, amplification and complete nucleotide sequencing of all 11 rotavirus genome segments without any prior knowledge of the viral dsRNA sequence [Potgieter et al., 2009]. Furthermore, Maes et al. [2009] recently developed a web-based tool, RotaC, which can swiftly differentiate the genotypes of all 11 genome segments of group A rotavirus strains. RotaC complies with the guidelines proposed by the *Rotavirus Classification Working Group* (RCWG) in assigning genotypes to nucleotide sequences. Therefore, combining the full genome classification system with sequence-independent amplification techniques, 454® pyrosequencing and RotaC analysis may fast-track the understanding of the role of genome reassortment in rotavirus genome diversity, host range restriction, co-segregation of certain genome segments, and genetic factors that influence adaptation of rotavirus strains to specific host species. In this study, these advances in rotavirus genome characterization were combined in classifying the complete genomes of three strains that emerged in the past two decades (G9P[6], G12P[4], G12P[6]) and one of the prevalent African rotavirus strains (G8P[4]). Since a few studies suggest that the monovalent Rotarix® vaccine currently in use may render lower efficacy to G2P[4] strains [Gurgel et al., 2007; Kirkwood et al., 2011], the whole genome of an African G2P[4] strain was also characterized as it is also detected at high frequencies in most African countries [Sanchez-Padilla et al., 2009].

MATERIALS AND METHODS

Rotavirus Strains and Ethical Approval

Selected strains were obtained from the existing stool sample collections of the National Institute for Communicable Diseases (NICD) and the University of Limpopo (Medunsa Campus). Ethical approval was granted from NICD (protocol number M060449) and the Medunsa Research Ethics committees (protocol number MR58-2003) prior to collection of these samples. The selection criteria for the study strains were based on: (i) the emerging rotavirus G genotypes (G9 and G12); (ii) common G genotype in the sub-Saharan African region (G8 and G9); (iii) G genotype speculated to be less protected by Rotarix® vaccine [Gurgel et al., 2007; Kirkwood et al., 2011], but detected frequently in Africa (G2) [Sanchez-Padilla et al., 2009]; and (iv) P genotypes that are commonly associated with G2, G8, G9, and G12 during rotavirus infection (P[4], P[6], and P[8]) [Estes and Kapikian, 2007]. Therefore, five human rotavirus genomes were selected (Table I).

Extraction and Purification of the Rotavirus dsRNA

Either 100 μg stool sample was suspended in 200 μl freshly prepared extraction buffer (containing 20 mM Tris–HCl, pH 7.4, 10 mM $CaCl_2$ and 0.85% NaOH) or 150 μl liquid stool sample was mixed with 150 μl extraction buffer. TRI-REAGENT–LS (Molecular Research Centre, Cincinnati, OH) was used for total RNA extraction from the fecal specimens, following the manufacturer's instructions with slight modifications. DuPont™ Vertrel® XF (DuPont Fluorochemicals, Wilmington, DE) was added to each sample to improve the purity of the extracted dsRNA. TRI-REAGENT–LS and DuPont™ Vertrel® XF were added in ratios of 3:1 and 1:3 to the suspended stool specimens, respectively. This was followed by the addition of 200 μl chloroform, centrifugation at 4°C for 15 min at 16,000 × *g*, precipitation of RNA in isopropanol

TABLE I. The 454® Pyrosequence Data Generated From the Rotavirus Strains Used in This Study

Rotavirus strain[a]	Genotype[b]	Yield[c] (μg)	Raw data generated (MB)	Nt sequences generated[d]
RVA/Human-wt/MWI/1473/2001/G8P[4][e]	G8P[4]	14.07	3.5	11,326
RVA/Human-wt/ZAF/3133WC/2009/G12P[4]	G12P[6/8][f]	5.5	3.7	10,992
RVA/Human-wt/ZAF/3176WC/2009/G12P[6]	G12P[6]	6.08	3.6	10,924
RVA/Human-wt/ZAF/3203WC/2009/G2P[4]	G2P[4]	8.13	3.4	10,304
RVA/Human-wt/ZAF/GR10924/1999/G9P[6][g]	G9P[6]	—	—	8,571

Nt, nucleotide.
[a]The sample names are based on the laboratory numbers that were assigned by Medunsa and NICD.
[b]Genotyping was assigned previously at Medunsa and NICD by RT-PCR with G-specific (Gouvea et al., 1990; Das et al., 1994; Cunliffe et al., 1999) and P-specific (Iturriza-Gómara et al., 2004) primers.
[c]Purified rotavirus PCR products prepared for 454® pyrosequencing by pooling amplicons of 5–10 PCR prepared from a single cDNA preparation for each strain.
[d]GS/FLX Titanium 454® pyrosequencing technology was used; the average read length was 400 bases.
[e]RVA/Human-wt/MWI/1473/2001/G8P[4] was collected in Malawi, while the rest of the study strains were collected in South Africa.
[f]Strain RVA/Human-wt/ZAF/3133WC/2009/G12P[4] was assigned mixed P[6]/P[8] VP4 genotypes by sequence-dependent PCR previously. RotaC assigned a P[4] genotype to the complete nucleotide sequence of the genome segment 4 generated through 454® pyrosequencing of the cDNA synthesized with sequence-independent amplification PCR. Therefore, strain RVA/Human-wt/ZAF/3133WC/2009/G12P[4] was re-assigned a P[4] genotype (also depicted in Table IV).
[g]RVA/Human-wt/ZAF/GR10924/1999/G9P[6] was sequenced previously (Potgieter et al., 2009).

and centrifugation at room temperature for 30 min at 16,000 × *g*. The pellet was re-suspended in 90 μl elution buffer (MinElute gel extraction kit; Qiagen, Hilden, Germany). Single-stranded RNA (ssRNA) was removed through precipitation with 2 M LiCl (Sigma, St. Louis, MO) at 4°C for 16 hr followed by centrifugation at 16,000 × *g* for 30 min. The extracted dsRNA was purified from the resulting supernatant with a MinElute gel extraction kit (Qiagen), following the manufacturer's instructions. The integrity of dsRNA was evaluated on a 0.8% TBE agarose gel stained with ethidium bromide.

Oligonucleotides Used and Oligo-Ligation

An "anchor primer," PC3-T7loop, and its complementary primer, PC2, described by Potgieter et al. [2009] were used in the RT-PCR amplification reactions. The primers were synthesized by TIB MOLBIOL, Berlin, Germany. Ligation of PC3-T7loop to dsRNA was carried out as described before [Potgieter et al., 2009] for 16 hr at 37°C. Ligated dsRNA was purified using MinElute Gel extraction columns following the manufacturer's recommendations (Qiagen).

Sequence-Independent cDNA Synthesis and PCR Amplification of the Rotavirus Genome

Denaturation of the purified ligated dsRNA was achieved by adding methyl mercury hydroxide (Alfa Aesar, Haverhill, MA) to a final concentration of 30 mM. Reverse transcription was carried out as described by Potgieter et al. [2009] with the modification that 10 U Transcriptor High Fidelity Reverse Transcriptase (Roche, Mannheim, Germany) was used. Following cDNA synthesis, the excess RNA was removed through the addition of NaOH (Sigma) to a final concentration of 0.1 M and incubation in a thermal cycler at 65°C for 30 min. Before cDNA annealing, Tris–HCl, pH 7.5 (Sigma), was added to a final concentration of 0.1 M followed by the addition of HCl (Sigma) to a final concentration of 0.1 M. The cDNA was annealed at 65°C for 1 hr.

The primer PC2 was used to amplify the rotavirus cDNA. The 50 μl PCR mixture contained 1× Phusion buffer, 0.2 mM dNTPs, 5 μl cDNA and 1 U Phusion High Fidelity DNA polymerase (Finnzymes, Vantaa, Finland). The first step during cycling was incubation at 72°C for 1 min to fill incomplete cDNA ends to produce intact cDNA. Cycling conditions were used as described before [Potgieter et al., 2009]. At least five reactions were set up per sample to obtain the required yield for pyrosequencing. Amplified cDNA was analyzed on 1% TBE agarose gels containing ethidium bromide.

Nucleotide Sequencing Using GS FLX Technology

Amplified cDNA was purified using a QIA®quick PCR purification kit according to the manufacturer's instructions (Qiagen). The cDNA concentrations were determined using a ND-1000 Spectrophotometer (NanoDrop Products, Wilmington, DE). The preparation of DNA libraries, titrations, emPCR™ and sequencing with the GS FLX Titanium (Roche) technology were performed at Inqaba Biotec, Pretoria, South Africa. The whole genomes of the study strains were pyrosequenced by combining three tagged samples in 100 μl reaction for each lane on the Pico Titre Plate (PTP).

Analysis of 454® Pyrosequenced Data

SeqMan within the DNASTAR® Lasergene™ software package, version 8.1.2, was used to assemble the 454® pyrosequence reads into a number of contigs of

which each corresponded to a specific rotavirus genome segment. These contigs constituted a varied number of sequence reads ranging from 48 to 4,000. The coverage and the orientation of each read were evaluated in the alignment view. The Trace consensus sequence was used as it judges both the peak quality as well as the consensus base at any given point. The consensus sequences were exported to MegAlign and manually checked. Where ambiguity codes were detected, the sequences were manually edited in the alignment view, SeqMan. The consensus nucleotide sequences were subsequently compared to NCBI GenBank sequences by using BLASTn. The deduced amino acid sequences and the sizes of the translated proteins were derived using EditSeq. Genotypes were assigned to the sequences of the genome segments depending on the percentage identities (>95%) revealed after BLASTn and BLASTp searches. All multiple nucleotide and amino acid sequence alignments and analysis between the study strains and reference strains from the GenBank (http://www.ncbi.nlm.nih.gov/genbank) were performed with BioEdit software [Hall, 1999]. The nucleotide sequence data for the complete genomes of the rotavirus strains reported in this study were submitted to the NCBI GenBank under the accession numbers listed in Table II.

Assignment of Genotypes and Phylogenetic Analysis

A web-based tool, RotaC version 1.0 (http://rotac.regatools.be.) [Maes et al., 2009] was used to assign genotypes to all 11 genome segments of the study strains. The nucleotide sequences of the reference strains were acquired from GenBank (Supplementary Data 1). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 software [Tamura et al., 2007]. Genetic distances were calculated using the Kimura 2 correction parameter at the nucleotide level, and the phylogenetic trees were constructed using the Neighbor-Joining method with 1,000 bootstrap replicates.

RESULTS

Assignment of Genotypes and Whole Genome Classification of the Study Strains

All 11 genome segments of each of the four African rotavirus strains (RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3203WC/2009/G2P[4], RVA/Human-wt/ZAF/3133WC/2009/G12P[4], RVA/Human-wt/ZAF/3176WC/2009/G12P[6]) were amplified from stool samples and pyrosequenced successfully. The whole genome of strain RVA/Human-wt/ZAF/GR10924/1999/G9P[6], also analyzed in this study, was amplified and pyrosequenced previously [Potgieter et al., 2009] (Table I). The GS FLX Titanium 454® pyrosequence data generated in this study ranged from 2.7 to 3.7 MB per strain, with at least 4,186,000–4,589,600

TABLE II. GenBank Accession Numbers of All the Rotavirus Genome Segments of Each of the Study Strains

Study strains	GenBank Accession numbers										
	S1 (VP1)	S2 (VP2)	S3 (VP3)	S4 (VP4)	S6 (VP6)	S9 (VP7)	S5(NSP1)	S8(NSP2)	S7(NSP3)	S10(NSP4)	S11(NSP5)
RVA/Human-wtfMWI/1473/2001/G8P[4]	HQ657133	HQ657134	HQ657135	HQ657136	HQ657137	HQ657138	HQ657139	HQ657140	HQ657141	HQ657142	HQ657143
RVA/Human-wtfZAF/3133WC/2009/G12P[4]	HQ657144	HQ657145	HQ657146	HQ657147	HQ657148	HQ657149	HQ657150	HQ657151	HQ657152	HQ657153	HQ657154
RVAfHuman-wt/ZAF/3176WC/2009/G12P[6]	HQ657155	HQ657156	HQ657157	HQ657158	HQ657159	HQ657160	HQ657161	HQ657162	HQ657163	HQ657164	HQ657165
RVA/Human-wt/ZAF/3203WC/2009/G2P[4]	HQ657166	HQ657167	HQ657168	HQ657169	HQ657170	HQ657171	HQ657172	HQ657173	HQ657174	HQ657175	HQ657176

VP, viral structural protein; NSP, viral non-structural protein; and S, genome segment.

sequences and average read lengths of approximately 400 bp. This was greater than the 95,1275 sequences generated for strain RVA/Human-wt/ZAF/GR10924/1999/G9P[6] on the GS20 genome sequencing platform which generated average read lengths of 105 bp in a previous study [Potgieter et al., 2009] (Table I). The sizes of the complete nucleotide and deduced amino acid sequences for all the strains analyzed in this study are summarized in Table III. The percentage similarity of the nucleotide sequences of each study strain to reference sequences in GenBank was above the proposed ± 3% cut-off values [Matthijnssens et al., 2008b]. The genome constellations determined for the analyzed strains are summarized in Table IV. In summary, the genetic nature and constellations of the study strains were as follows: strain RVA/Human-wt/MWI/1473/2001/G8P[4] is a DS-1-like strain with a G8 VP7, G8-P[4]-I2-R2-C2-M2-A2-N2-T2-E2; strain RVA/Human-wt/ZAF/3133WC/2009/G12P[4] is an intergenogroup reassortant G12P[4] strain on a Wa-like genetic backbone, G12-P[4]-I1-R1-C1-M1-A1-N1-T1-E1-H1; strain RVA/Human-wt/ZAF/3176WC/2009/G12P[6] is a G12P[6] strain on a Wa-like genetic backbone, G12-P[6]-I1-R1-C1-M1-A1-N1-T1-E1-H1; strain RVA/Human-wt/ZAF/3203WC/2009/G2P[4] is a pure DS-1 like strain, G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2; and strain RVA/Human-wt/ZAF/GR10924/1999/G9P[6] is an intergenogroup reassortant G9P[6] strain on a DS-1-like genetic backbone.

Sequence Analysis of the Individual Genome Segments of the Study Rotavirus Strains

Genome segment 1 (VP1). Based on the distance matrices analysis, RotaC and phylogenetic analysis, the genome segment 1 of strains RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6] were of Wa-like origin, whereas those of RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3203WC/2009/G2P[4], and RVA/Human-wt/ZAF/GR10924/1999/G9P[6] were of DS-1-like origin (Fig. 1A and Table IV). Phylogenetic analysis showed that genome segment 1 of the Wa-like study strains were closely related by grouping distinctly within the Wa-like cluster. The genome segment 1 of DS-1-like study strains formed separate clusters with strains isolated from United States of America (USA)(RVA/Human-wt/USA/LB2744/2006/G2P[4] and RVA/Human-wt/USA/LB2772/2006/G2P[4]), Democratic Republic of Congo (DRC)(RVA/Human-wt/COD/DRC86/2003/G8P[6] and RVA/Human-wt/COD/DRC88/2003/G8P[8]) and the Philippines (RVA/Human-tc/PHL/L26/1987/G12P[4]). Genome segment 1 of strain RVA/Human-wt/MWI/1473/2001/G8P[4] clustered with that of G12P[4] strain RVA/Human-tc/PHL/L26/1987/G12P[4]. Both these strains clustered near the artiodactyl-like human strain RVA/Human-wt/HUN/Hun5/1997/G6P[14], RVA/Human-wt/HUN/BP1879/2003/G6P[14] and a multi-reassortant bovine-feline/canine-human reassortant strain RVA/Human-

TABLE III. Size of the Complete Nucleotide and Deduced Amino Acid Sequences of the Study Strains

	Genome segments										
Study strains	S1(VP1)	S2(VP2)	S3(VP3)	S4(VP4)	S6(VP6)	S9(VP7)	S5 (NSP1)	S8 (NSP2)	S7 (NSP3)	S10 (NSP4)	S11 (NSP5)	S11 (NSP6)
Nucleotides (bp)												
RVA/Human-wt/MWI/1473/2001/G8P[4][a]	3,202	2,484	2,591	2,359	1,356	1,062	1,566	1,059	1,066	751	816	816
RVA/Human-wt/ZAF/3133WC/2009/G12P[4][b]	3,202	2,729	2,591	2,359	1,356	1,062	1,566	1,059	1,074	750	664	664
RVA/Human-wt/ZAF/3176WC/2009/G12P[6][b]	3,202	2,729	2,591	2,359	1,356	1,062	1,566	1,059	1,074	750	664	664
RVA/Human-wt/ZAF/3203WC/2009/G2P[4][a]	3,202	2,484	2,591	2,359	1,356	1,062	1,566	1,059	1,066	751	816	816
RVA/Human-wt/ZAF/GR10924/1999/G9P[6][a]	3,202	2,484	2,591	2,359	1,356	1,061	1,566	1,059	1,066	751	816	816
Deduced amino acids (aa)												
RVA/Human-wt/MWI/1473/2001/G8P[4][a]	1,088	879	835	775	397	326	493	317	310	175	200	92
RVA/Human-wt/ZAF/3133WC/2009/G12P[4][b]	1,088	894	835	775	397	326	493	317	310	175	197	92
RVA/Human-wt/ZAF/3176WC/2009/G12P[6][b]	1,088	894	835	775	397	326	493	317	310	175	197	92
RVA/Human-wt/ZAF/3203WC/2009/G2P[4][a]	1,088	879	835	775	397	326	493	317	310	175	200	92
RVA/Human-wt/ZAF/GR10924/1999/G9P[6][a]	1,088	879	835	775	397	326	493	317	310	175	200	92

Aa, amino acid; bp,base pairs, VP, viral structural protein; NSP, viral non-structural protein; and S, genome segment.
[a]Study strains on a DS-1-like genetic backbone. The short and long out-of-phase ORFs of the genome segment 11 for DS-1-like study strains were translated from nt 22–615 and nt 80–358 for NSP6 and NSP5, respectively.
[b]Study strains on a Wa-like genetic backbone. The short and long out-of-phase ORFs of segment 11 for Wa-like study strains were translated from nt 80–358 and nt 22–624 for NSP6 and NSP5, respectively.

TABLE IV. The Whole Genome Classification of the Rotavirus Strains Characterized in this Study

	Genome constellations										
	S9(VP7)	S4(VP4)	S6(VP6)	S1(VP1)	S2(VP2)	S3(VP3)	S5(NSP1)	S8(NSP2)	S7(NSP3)	S10(NSP4)	S11(NSP5)
Study strains											
RVA/Human-wt/MWI/1473/2001/G8P[4][a]	G8(98.1)	P[4](95)	C2(99.2)	R2(96.4)	C2(98.7)	M2(97.4)	A2(97.9)	N2 (98.3)	T2(98.7)	E2(98.3)	H2(100)
RVA/Human-wt/ZAF/3133WC/2009/G12P[4][b]	G12(99)	P[4](96.1)	C1(98.2)	R1(99.3)	C1(99)	M1(98.7	A1(99)	N1(98.7)	T1(99.3)	E1(98.9)	H1(99.7)
RVA/Human-wt/ZAF/3176WC/2009/G12P[6][b]	G12(99)	P[6](98.8)	C1(97.3)	R1(99.3)	C1(99)	M1(98.7)	A1(99)	N1(98.8)	T1(99.3)	E1(98.9)	H1(99.7)
RVA/Human-wt/ZAF/3203WC/2009/G2P[4][a]	G2(96.4)	P[4](96.1)	C2(98.2)	R2(97.9)	C2(97.7)	M2(96.9)	A2(97.6)	N2(97.4)	T2(98)	E2(96.8)	H2(99.7)
RVA/Human-wt/ZAF/GR10924/1999/G9P[6][a]	G9(99.2)	P[6](99)	C2(99)	R2(98.8)	C2(98.8)	M2(98.8)	A2(98.4)	N2(99.4)	T2(98.7)	E2(98.4)	H2(99.3)
Reference strains											
RVA/Human-tc/USA/Wa/1974/G1P1A[8]	G1	P[8]	C1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/JPN/KU/XXXX/G1P1[8]	G1	P[8]	C1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/BGD/Dhaka16/2003/G1P[8]	G1	P[8]	C1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/USA/D/1974/G1P1A[8]	G1	P[8]	C1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/USA/DS-1/1976/G2P1B[4]	G2	P[4]	C2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]	G2	P[4]	C2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-tc/USA/P/1974/G3P1A[8]	G3	P[8]	C1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/GBR/ST3/1975/G4P2A[6]	G4	P[6]	C1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Pig-tc/USA/Gottfried/1983/G4P[6]	G4	P[6]	C1	R1	C1	M1	A8	N1	T1	E1	H1
RVA/Human-tc/BRA/IAL28/1992/G5P[8]	G5	P[8]	C1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/COD/DRC86/2003/G8P[6]	G8	P[6]	C2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-tc/IDN/69M/1980/G8P4[10]	G8	P[10]	C2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-tc/USA/WI61/1983/G9P1A[8]	G9	P[8]	C1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/BGD/Matlab13/2003/G12P[6]	G12	P[6]	C1	R1	C1	M1	A1	N1	T2	E1	H1
RVA/Human-wt/BGD/RV161/2000/G12P[6]	G12	P[6]	C2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-tc/JPN/AU-1/1982/G3P3[9]	G3	P[9]	C3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Human-tc/THA/T152/1998/G12P[9]	G12	P[9]	C3	R3	C3	M3	A12	N3	T3	E3	H16
RVA/Pigeon-tc/JPN/PO-13/1983/G18P[17]	G18	P[17]	C4	R4	C4	M4	A4	N4	T4	E4	H4

Table appears in color in the online version of the journal.
The percentage similarity of each study nucleotide sequence to reference sequences in GenBank was above the proposed ± 3% cut-off values (indicated in brackets). The Wa- or DS-1-like genogroups were assigned to the study human rotavirus strains if at least seven genome segments belonged to the respective Wa- or DS-1-like genotype (Matthijnssens et al., 2008b). Colors were added to visualize certain patterns or genome constellations as follows: Green (Wa-like), red (DS-1-like), orange (AU-like), yellow (PO-13-like), and blue (some typical animal strains).
VP, viral structural protein; NSP, viral non-structural protein.
[a]Study strains on a DS-1-like genetic backbone.
[b]Study strains on a Wa-like genetic backbone.

wt/ITA/PAH136/1996/G3P[9] [Matthijnssens et al., 2009]. This suggests that the genome segment 1 of strain RVA/Human-wt/MWI/1473/2001/G8P[4] originated from or shares a common origin with artiodactyl strains (Fig. 1A).

VP1 of all the study strains contained the conserved four putative RNA-dependent RNA polymerase motifs at residues 512–527, 582–608, 626–636, and 690–702 [Bruenn, 1991]. As described by Heiman et al. [2008], the deduced VP1 of the Wa- (R1 genotype) and DS-1-(R2 genotype) like study strains also contained the conserved amino acid S at position 512 and 514 (Supplementary Data 2).

Genome segment 2 (VP2). Genome segment 2 of the study strains was of Wa- (RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6]) and DS-1- (RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3203WC/2009/G2P[4], and RVA/Human-wt/ZAF/GR10924/1999/G9P[6]) like origin (Fig. 1B; Table IV). Genome segment 2 of the Wa-like study strains showed close resemblance and clustered with Wa-like G12P[6] strain (RVA/Human-wt/BGD/Dhaka12-03/2003/G12P[6]) isolated from Bangladesh and the G1/P[8] strain (RVA/Human-wt/USA/2007719825/2007/G1P[8]) from USA. The genome segment 2 of RVA/Human-wt/ZAF/GR10924/1999/G9P[6] clustered with DS-1-like human strains isolated from Bangladesh, whereas RVA/Human-wt/ZAF/3203WC/2009/G2P[4] did not cluster with any strain. Of interest was RVA/Human-wt/MWI/1473/2001/G8P[4] that clustered with an unusual G6P[6] rotavirus human strain RVA/Human-wt/BEL/B1711/2002/G6P[6] that acquired its genome segments 3 (VP3) and 9 (VP7) from bovine rotaviruses through reassortment [Matthijnssens et al., 2008a] (Fig. 1B).

Similar to strain RVA/Human-wt/USA/LB2719/2006/G1P[8] [Bányai et al., 2011], the VP2 of the DS-1-like study strains were 15 amino acids shorter than that of the Wa-like strains. The VP2 of the Wa-like study strains contained up to 12 amino acid (MENKNKNKNNNR) insertions following residue 32 (Supplementary Data 3). As Ito et al. [2001] reported, high amino acid variations were also observed within the RNA-binding domain of VP2 of all the study strains (data not shown). The amino acid variations observed between the two putative conserved leucine zipper motifs (aa 526–567 and 655–696) [Kumar et al., 1989; Mitchell and Both, 1990] of the VP2 of Wa- and DS-1-like study strains were consistent with findings of Heiman et al. [2008]. In addition to numerous amino acid differences between Wa- and DS-1-like strains observed previously by Heiman et al. [2008], three new variations (A613T, A662S, and D712E) were also observed in this study (Supplementary Data 3).

Genome segment 3 (VP3). Genome segment 3 of the study strains also segregated into Wa- (RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6]) and DS-1- (RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3203WC/2009/G2P[4], and RVA/Human-wt/ZAF/GR10924/1999/G9P[6]) like genotypes (Fig. 1C and Table IV). The VP3 encoding genome segments of the DS-1-like and Wa-like study strains exhibited nucleotide similarities of 97.7–99% with their respective prototype strains. Genome segment 3 of both the Wa-like study strains were closely related to that of strain RVA/Human-wt/USA/LB2719/2006/G1P[8] that was recently isolated from the USA [Bányai et al., 2011]. Genome segment 3 of the DS-1-like study strains did not cluster with the prototype DS-1 strain, but with M2B strains isolated from Bangladesh, DRC, and USA (Fig. 1C).

Genome segment 4 (VP4). Genome segment 4 of strains RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3133WC/2009/G12P[4], and RVA/Human-wt/ZAF/3203WC/2009/G2P[4] was of DS-1-like (P[4]) origin, whereas those of RVA/Human-wt/ZAF/3176WC/2009/G12P[6] and RVA/Human-wt/ZAF/GR10924/1999/G9P[6] were of human (P[6]) origin (Fig. 1D and Table IV). Phylogenetically, strains RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3203WC/2009/G2P[4] demonstrated close resemblance by grouping together within a cluster consisting of P[4] strains isolated from Germany (RVA/Human-wt/DEU/GER1H-09/2009/G8P[4]), Japan (RVA/Human-wt/JPN/KO-2/XXXX/G2P[4]), and USA (RVA/Human-wt/USA/LB2772/2006/G2P[4] and RVA/Human-wt/USA/LB2744/2006/G2P[4]). Strain RVA/Human-wt/MWI/1473/2001/G8P[4] clustered with bovine-human reassortant strain RVA/Human-wt/MWI/MW333/XXXX/G8P[4] which was also collected from Malawi [Cunliffe et al., 2000]. Strains RVA/Human-wt/ZAF/3176WC/2009/G12P[6] and RVA/Human-wt/ZAF/GR10924/1999/G9P[6] clustered with P[6]-I human strains within the P[6]-Ia lineage (Fig. 1D). All the study strains contained the potential trypsin cleavage sites (arginine) at positions 230, 240, and 581 [Estes and Kapikian, 2007]. In addition, other potential trypsin cleavage sites (lysine) described by Crawford et al. [2001] at residues 257 and 466 (data not shown) were also observed.

Genome segment 6 (VP6). Genome segment 6 of the study strains was of Wa- (RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6]) and DS-1- (RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3203WC/2009/G2P[4], and RVA/Human-wt/ZAF/GR10924/1999/G9P[6]) like origin (Fig. 1E and Table IV). Genome segment 6 of the Wa-like study strains were closely related, and clustered with RVA/Human-wt/THA/CMH185-01/XXXX/G3P[8] and RVA/Human-wt/KOR/CAU164/XXXX/G1P[8] strains isolated from Thailand and South Korea, respectively. Genome segment 6 of the DS-1-like study strains formed distinct clusters with 12 reference strains isolated from Bangladesh, India, Belgium, and USA. As was observed for genome segment 2 of strain RVA/Human-wt/MWI/1473/2001/G8P[4], its genome segment 6 was also closely related to that of strain RVA/Human-wt/BEL/B1711/2002/G6P[6] (Fig. 1E).

Fig. 1. 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**: Phylograms for genome segments 1–4, 6 and 9 (VP1–VP4, VP6, and VP7), respectively. **H**–**K**: Phylograms for genome segments 5, 7, 10, and 11 (NSP1–NSP5), respectively. The nomenclature of all the rotavirus strains indicates the rotavirus group, species where the strain was isolated, name of the country where the strain was originally isolated, the common name, year of isolation, and the genotypes for genome segment 4 and 9 as proposed by the RCWG [Matthijnssens et al., 2011]. Accession numbers of all the reference strains are listed in Supplementary Data 1. The names of the study strains are enclosed in boxes. The strains enclosed in boxes with dashed lines in phylograms of genome segments encoding VP1 and VP7 indicates strains sharing common origin with artiodactyls-like rotaviruses, whereas in the VP6 dendrogram, it shows strains with a common origin to the porcine Gottfried strain. The horizontal branch lengths are proportional to the genetic distance calculated by the Neighbor-Joining method. The numbers adjacent to the node represents the bootstrap value of 1,000 replicates, and values <70% are not shown. The scale bar shows the genetic distance expressed as nucleotide substitution per rate of the nucleotide sequences.

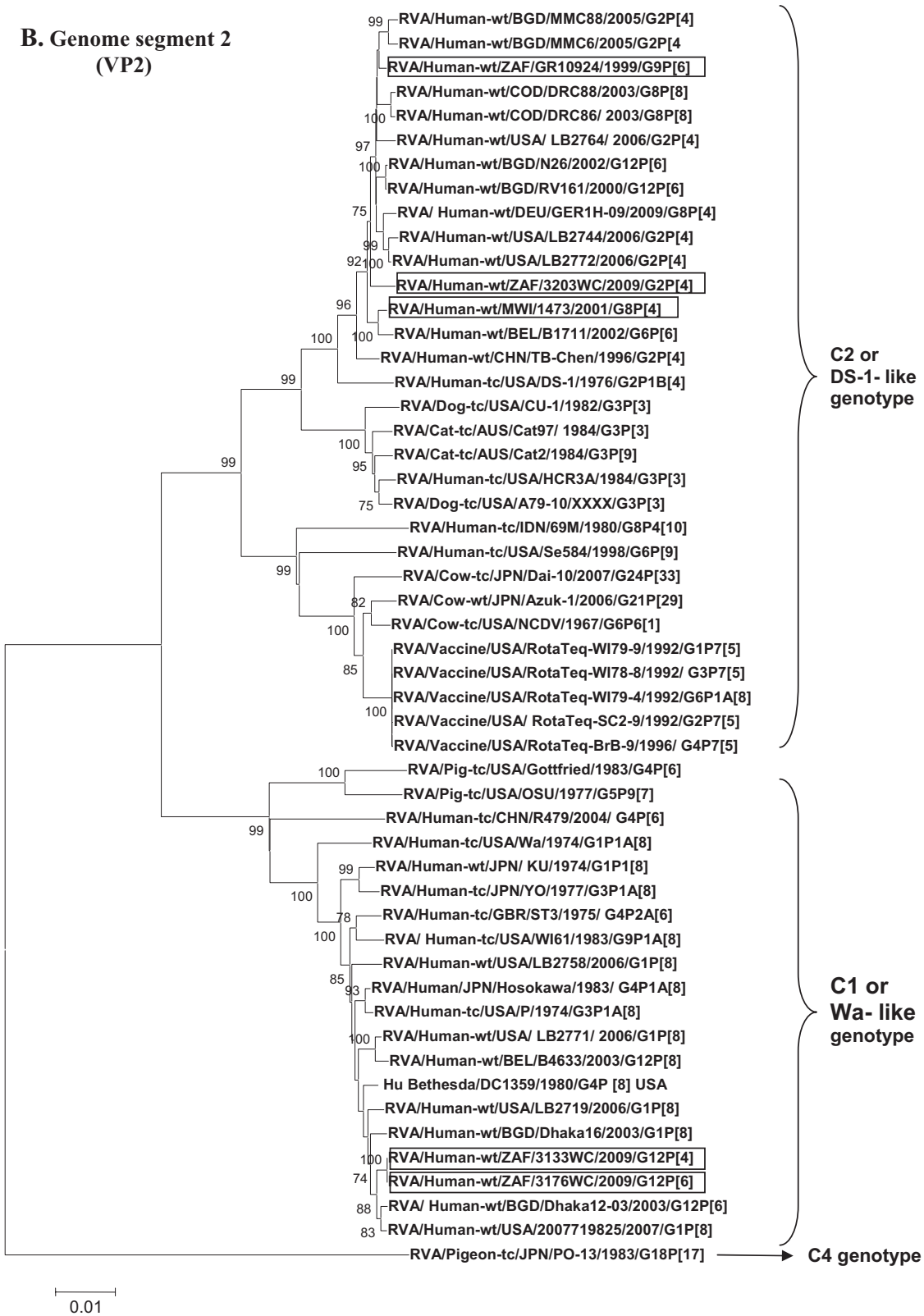


Fig. 1. (*Continued*)

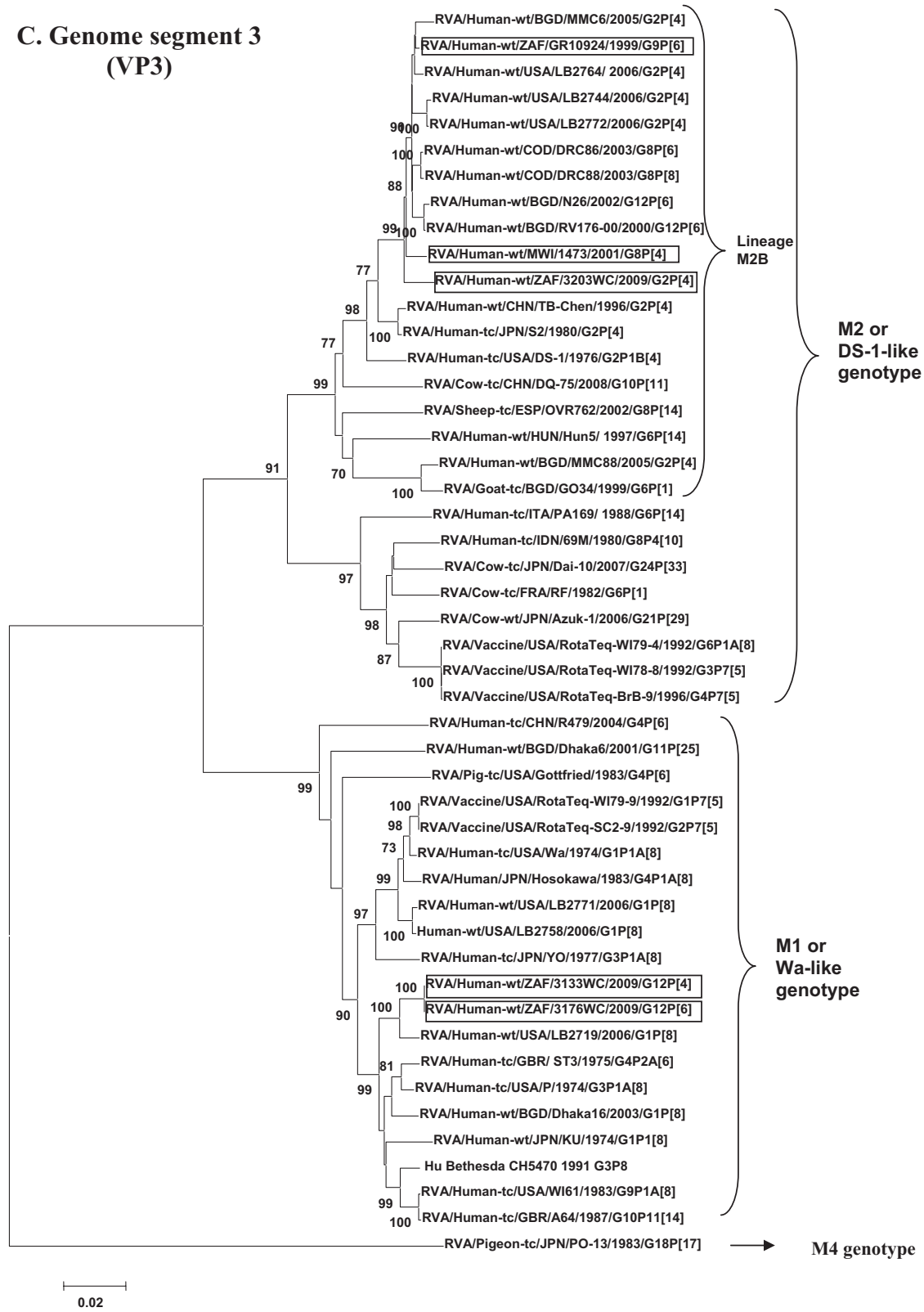


Fig. 1. (*Continued*)

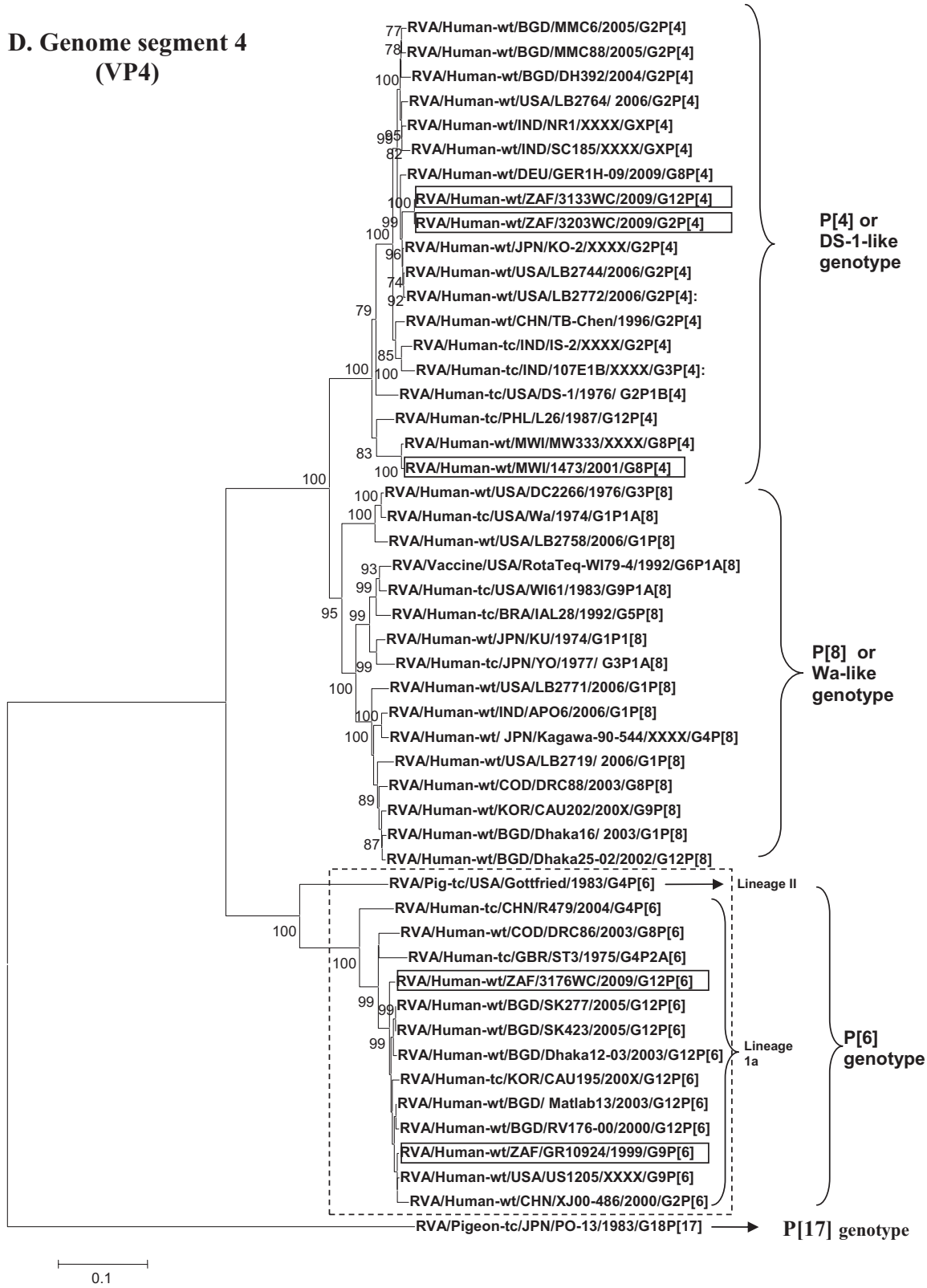
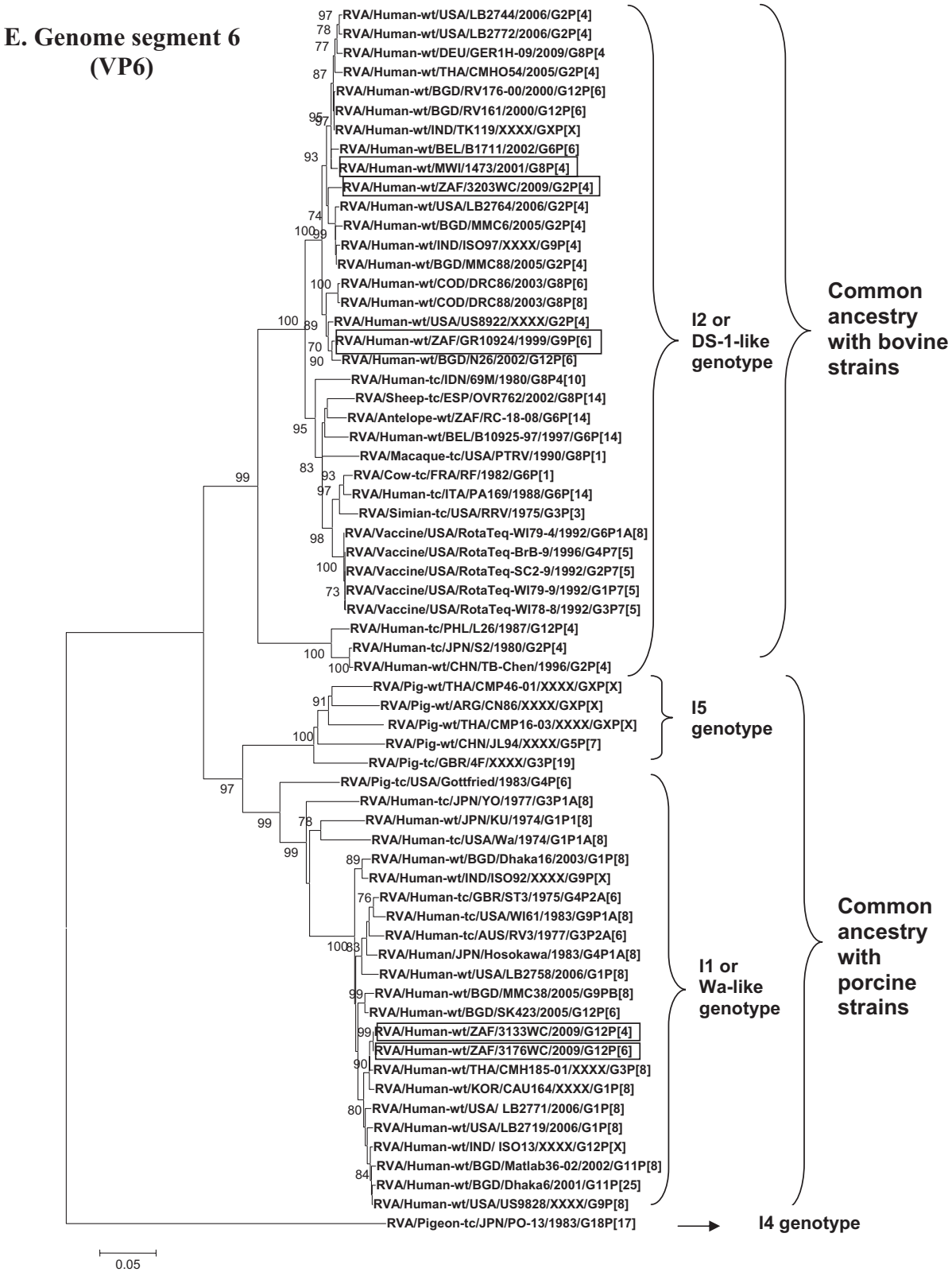


Fig. 1. (*Continued*)



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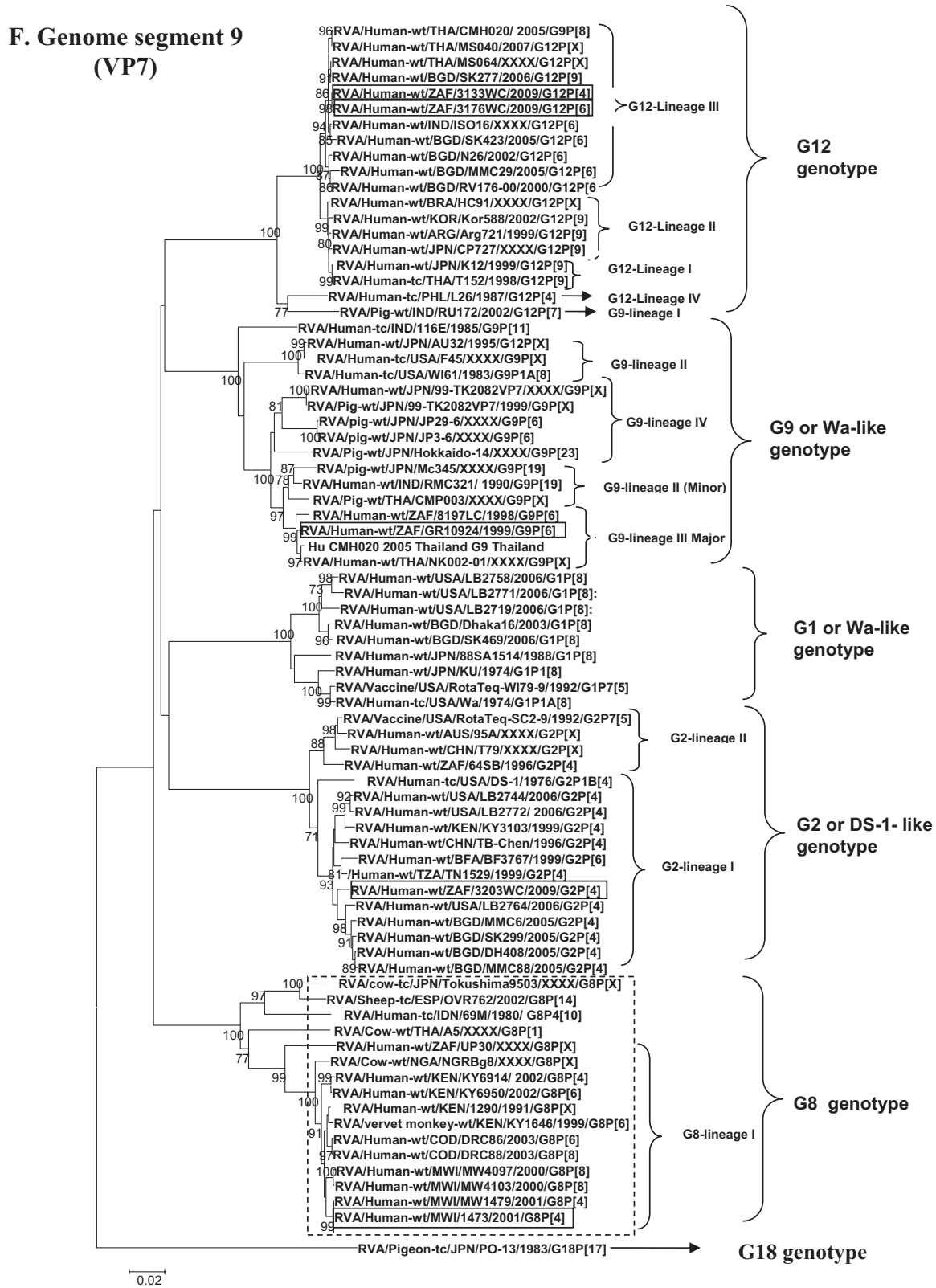
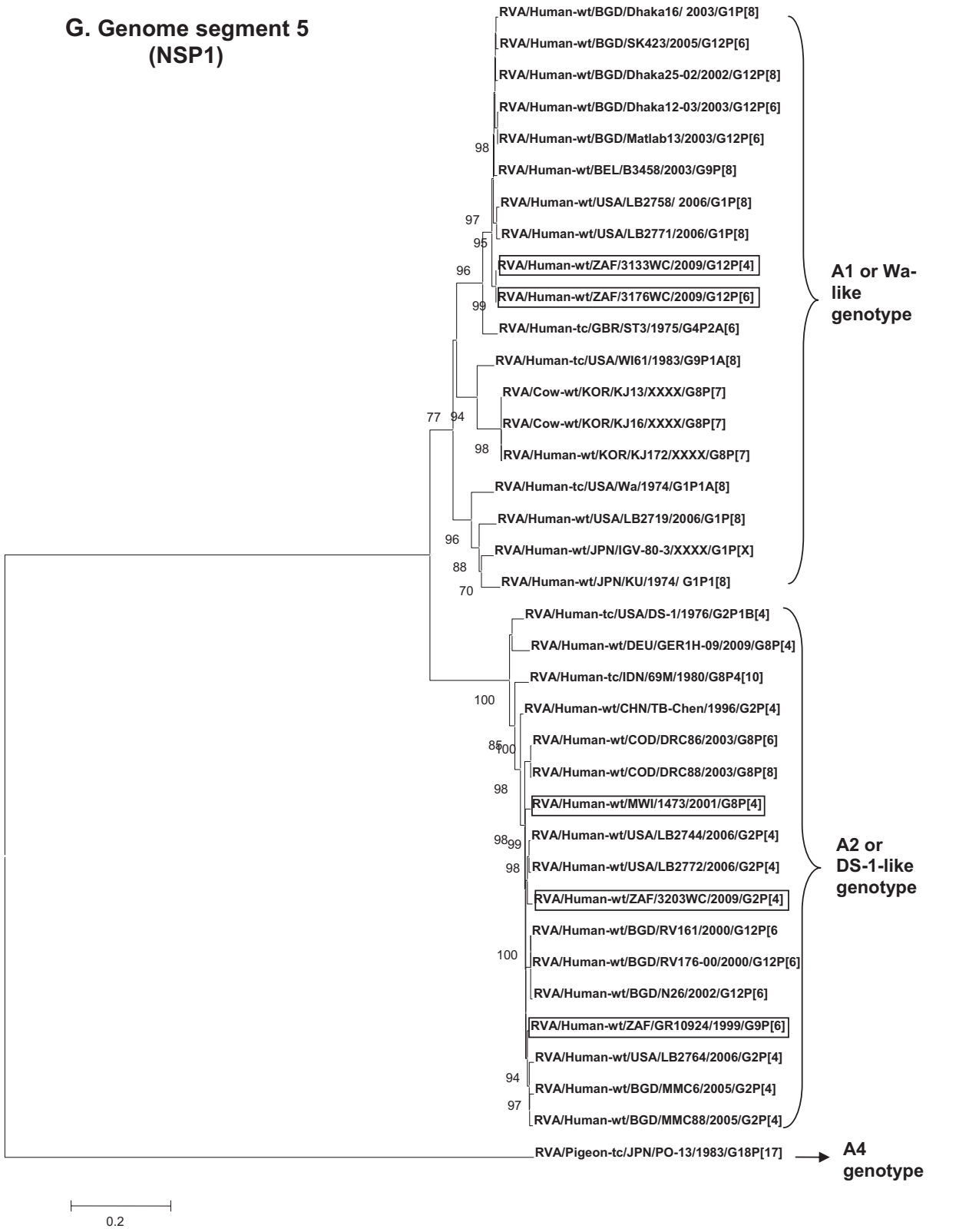


Fig. 1. (*Continued*)



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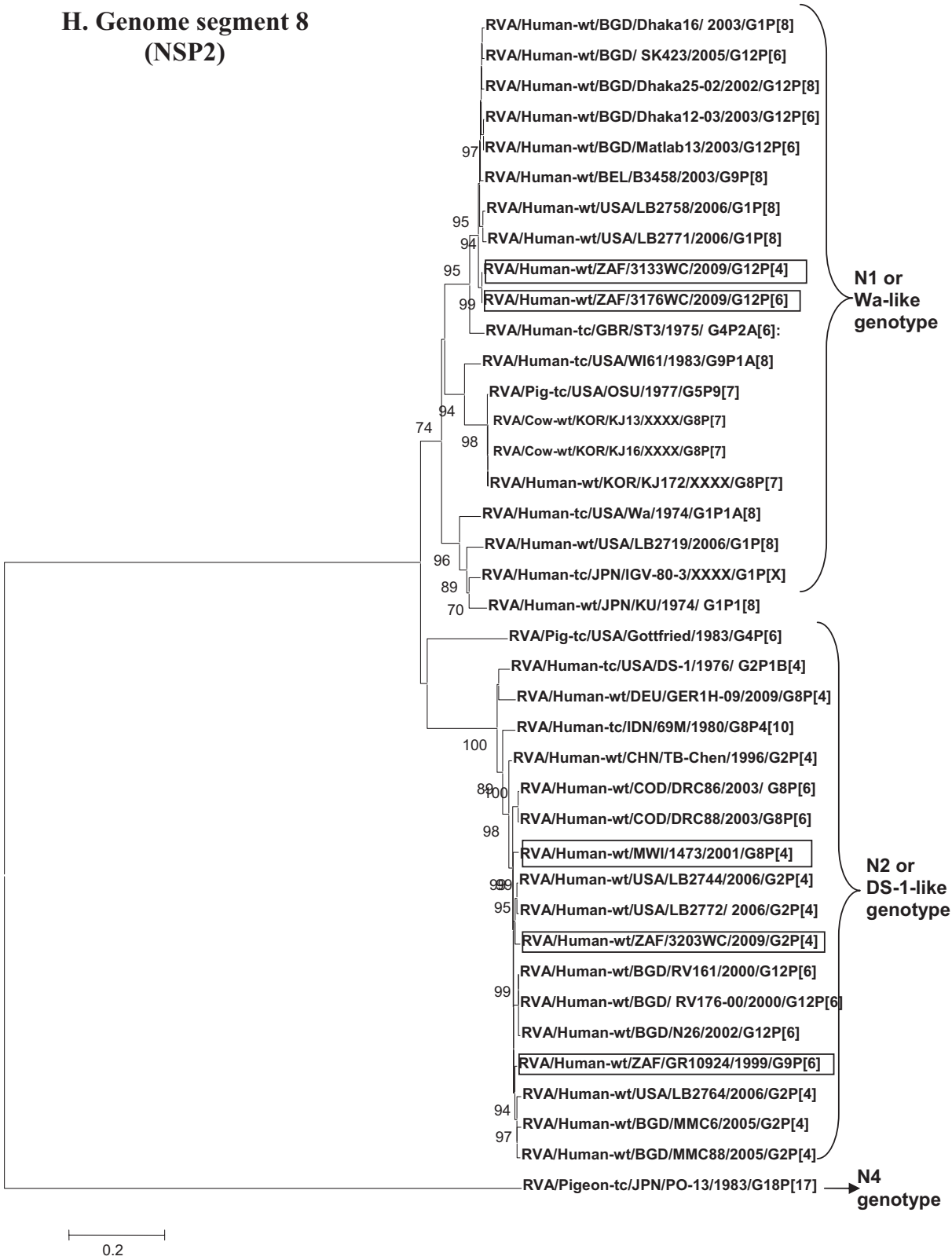

Fig. 1. (*Continued*)

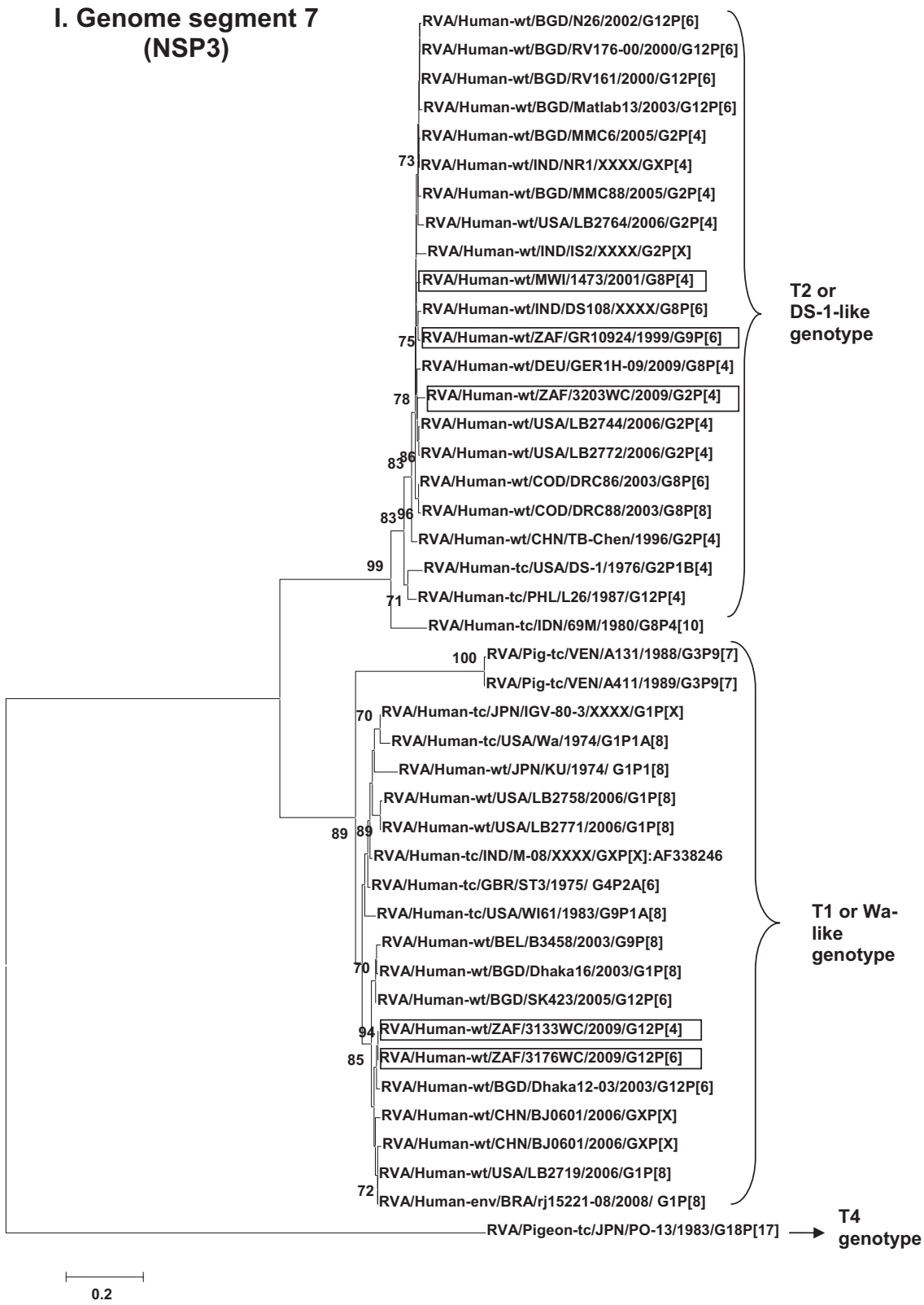

Fig. 1. (*Continued*)

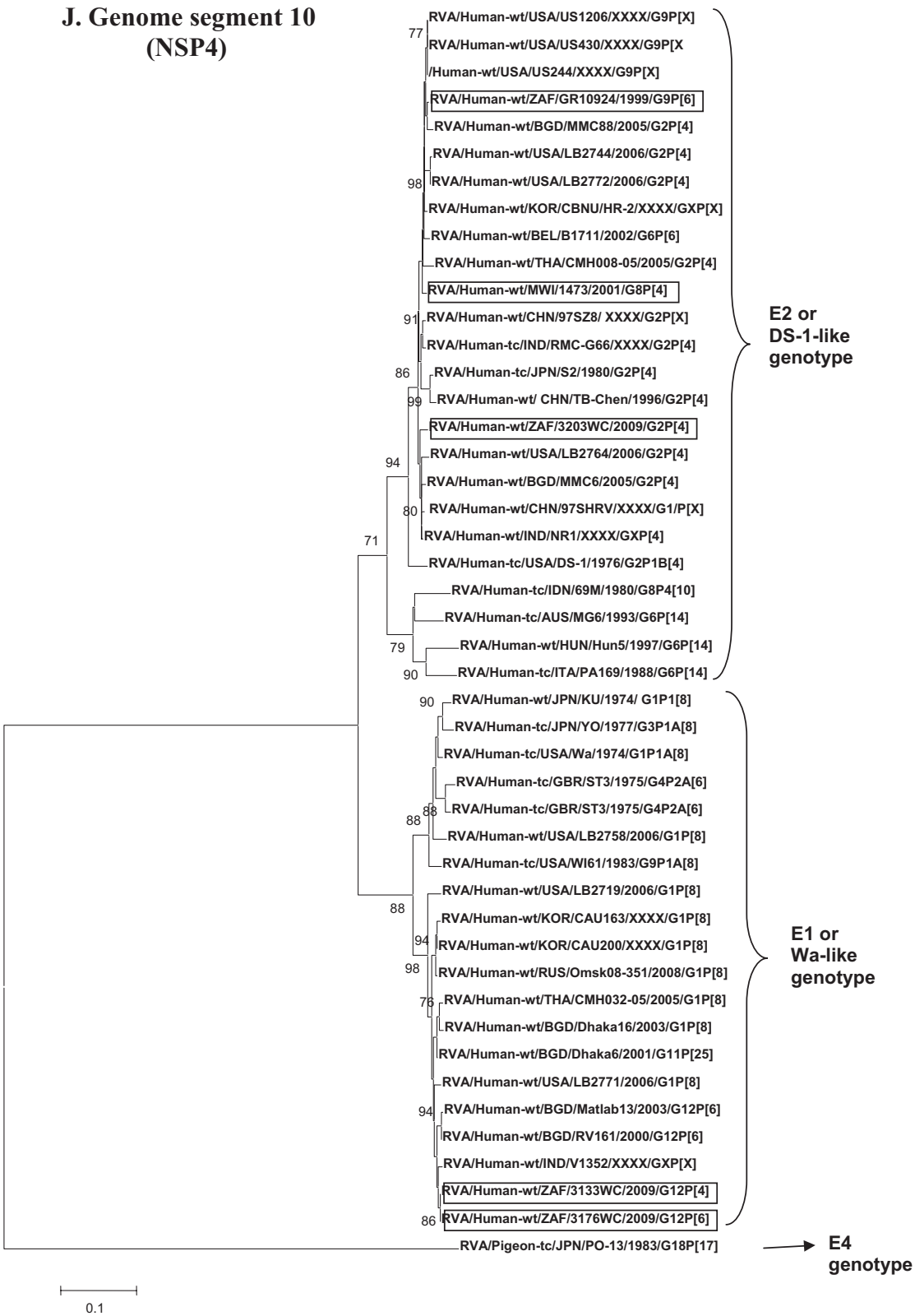

Fig. 1. (*Continued*)

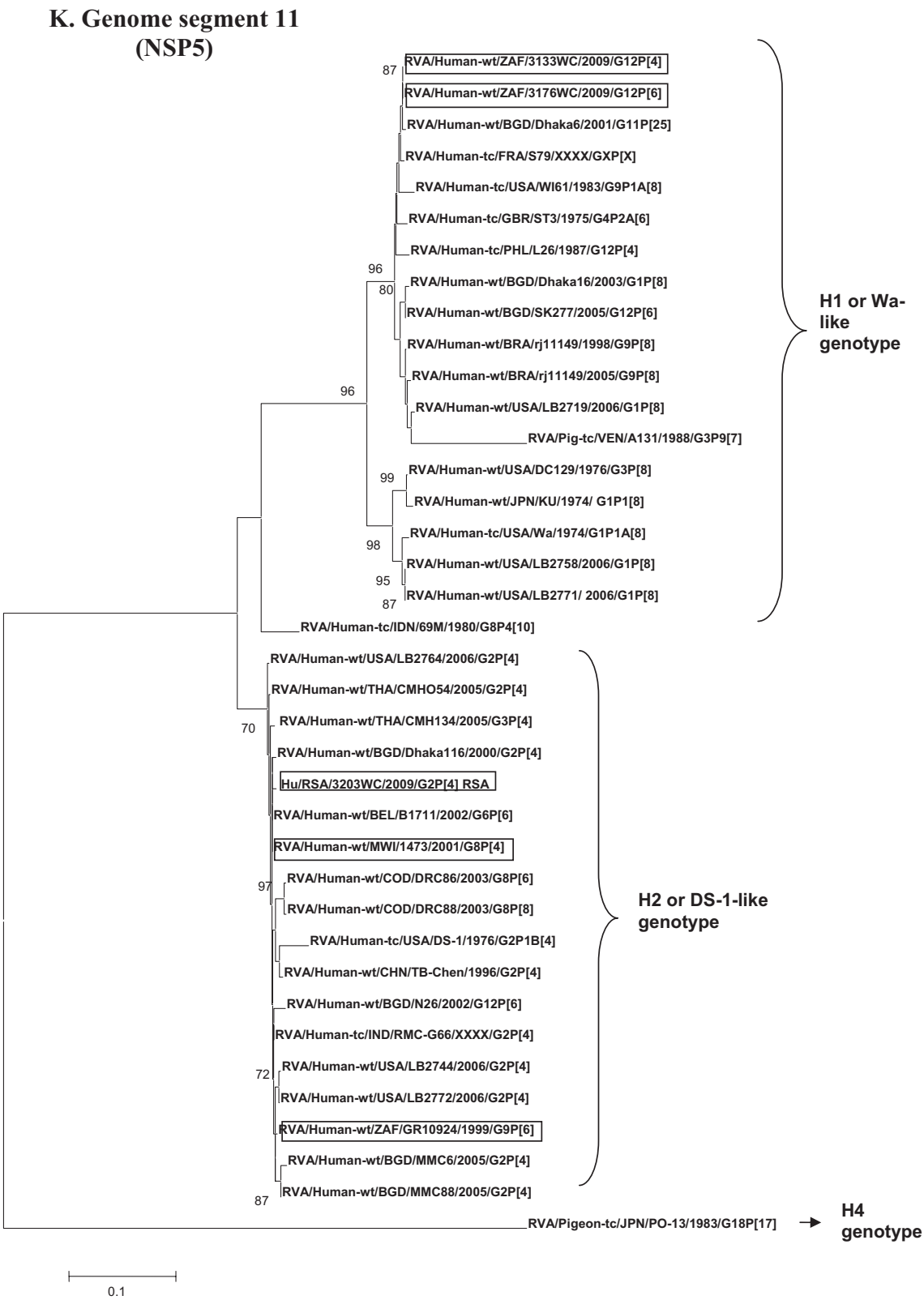

Fig. 1. (*Continued*)

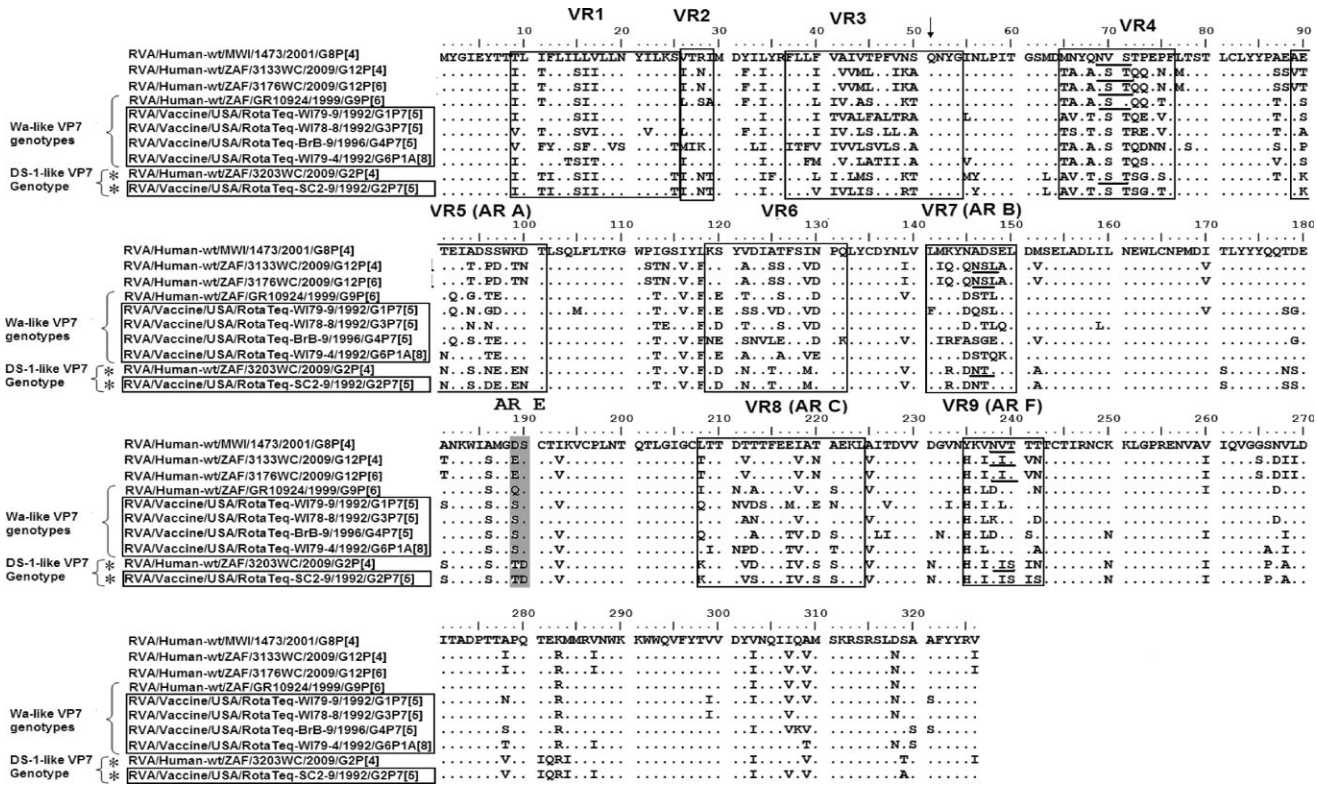

Fig. 2. Comparison of the variable (VR) and antigenic regions (AR) of VP7 of the study strains to the bovine-human reassortant RotaTeq® strains. The conserved trypsin cleavage site (51Q) is indicated with an arrow. Potential N-linked glycosylation sites are underlined. The nine VRs (VR1–VR9), identified in VP7 are boxed (aa positions 91–25, 25–29, 37–54, 65–76, 89–101, 119–132, 141–150, 208–224, and 235–242, respectively) (Dyall-Smith et al. 1986; Ciarlet et al., 1997). VRs 5, 7, 8, and 9 include the antigenic epitopes that define serotypes, and correspond to ARs A, B, C, and F, respectively. Antigenic regions D and E occur at aa 291 and 189–190, respectively (Dyall-Smith et al., 1986; Ciarlet et al., 1994, 1997). Amino acids in AR E where changes seem to be related to the genogroup of the strains are highlighted in grey. Strains with similar G2 genotypes are indicated with an asterisks (*). A period (.) represents residues similar to amino acids in strain RVA/Human-wt/MWI/1473/2001/G8P[4] at any given position. The names of the rotavirus strains incorporated in the RotaTeq® vaccine are boxed. VR, variable region; AR, antigenic region.

VP6 contains subgroup-specific epitopes that are used to classify group A rotaviruses into subgroups I, II, both I and II, or non-I and non-II. Subgroup I is defined by region A (aa 45 and 46) and region C (aa 114 and 120), while subgroup II is defined by region B (aa 83, 86, 89, and 92), D (aa 312 or 314, 317, or 319) and E (aa 341 or 343, 350 or 352) [Gorziglia et al., 1988]. The amino acid positions of regions A–C of the study strains were consistent with the findings of Gorziglia et al. [1988]. However, region D was at position 310 and 315, instead of position 312 and 314 as reported previously. Region E was localized at residues 342 and 348. Furthermore, all the amino acid variation between the Wa- and DS-1-like study strains correlated with that reported by Heiman et al. [2008] (Supplementary Data 4).

Genome segment 9 (VP7). Genome segment 9 of strains RVA/Human-wt/ZAF/3203WC/2009/G2P[4] and RVA/Human-wt/ZAF/GR10924/1999/G9P[6] were of DS-1- (G2 genotype) and Wa-like (G9 genotype) origin, respectively. The G8 and G12 genotypes assigned to strains RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3133WC/2009/G12P[4], and RVA/Human-wt/ZAF/3176WC/2009/G12P[6] were not assigned to a specific genogroup as the full genome rotavirus classification scheme does not classify G8 and G12 genotypes into specific genogroups (Table IV) [Matthijnssens et al., 2008b; Esona et al., 2009; Martella et al., 2010].

Phylogenetically, the genome segment 9 of strain RVA/Human-wt/MWI/1473/2001/G8P[4] clustered within the G8-lineage I reference strains isolated from African countries, and it was closely related to strain RVA/Human-wt/MWI/MW1479/2001/G8P[4] which was also collected from Malawi in 2001. The phylogram for genome segment 9 (Fig. 1F) showed that strain RVA/Human-wt/MWI/1473/2001/G8P[4] shares a common origin with artiodactyl strains like RVA/cow-tc/JPN/Tokushima9503/XXXX/G8P[X], RVA/Cow-wt/THA/A5/XXXX/G8P[1] and RVA/Sheep-tc/ESP/OVR762/2002/G8P[14]. Strain Hu/RSA/3203WC/2009/G2P[4] showed close similarity to the DS-1-like strain RVA/Human-wt/USA/LB2764/2006/G2P[4] isolated from the USA and strains characterized from Bangladesh within lineage I of G2 genotype. Strain RVA/Human-wt/ZAF/GR10924/1999/G9P[6]

was closely related to strains isolated from Thailand (RVA/Human-wt/THA/CMH020/2005/G9P[8] and RVA/Human-wt/THA/NK002-01/XXXX/G9P[X]) and South Africa (RVA/Human-wt/ZAF/I8197LC98/1998/G9P[6]) that forms lineage III (major) within the G9 Wa-like genotype. Strains RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6] were closely related and clustered with G12 strains isolated from Asian countries within lineage III. Since all the study strains were isolated from hospitalized children, the clusters are in agreement with the report of Matthijnssens et al. [2010] that, of recent, the majority of severe diarrhea cases caused by G9 and G12 rotaviruses are associated with sub-lineage III of either genotype.

All the VP7 proteins of the study strains contained the conserved proline and cysteine residues identified previously [Ciarlet et al., 2002] as well as a conserved trypsin cleavage site the glutamine residue at position 51Q (Fig. 2) [Stirzaker et al., 1987]. The VP7 proteins of RVA/Human-wt/ZAF/3203WC/2009/G2P[4] and the emerging G12 (RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6]) strains possessed three potential N-linked glycosylation sites at positions 69, 146, and 238, which were similar to the VP7 of the G2 (strain RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7[5]) component of RotaTeq®. Similar observations were made in other reference strains with G2 and G12 genotypes. Strain RVA/Human-wt/ZAF/GR10924/1999/G9P[6] and other G9 reference strains possessed only one potential glycosylation site at position 69 which is similar to the VP7 of the G3 (strain RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P7[5]) and G4 (strain RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P7[5]) components of RotaTeq®. The similarities may be partially explained by the classification of G3, G4, and G9 strains into Wa-like genogroup [Matthijnssens et al., 2008b], whereas the G2 study strain and strain RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7[5] have the same VP7 genotype. Strain RVA/Human-wt/MWI/1473/2001/G8P[4] possessed two potential glycosylation sites at positions 69 and 238, which are similar to the G1 (strain RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7[5]) and P1A[8] (strain RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]) components of RotaTeq® (Fig. 2).

VP7 contains the major neutralizing sites targeted by the cytotoxic T-lymphocytes, leading to production of neutralizing antibodies by B cells [Dyall-Smith et al., 1986]. At least nine VP7 variable regions (VR1–VR9) are known. Six antigenic regions (AR) have been described before and are defined as A (aa 87–101), B (aa 143–152), C (aa 208–224), D (aa 291), E (aa 189), and F (aa 235–242). Antigenic regions A, B, C, and F correspond to VR5, VR7, VR8, and VR9, respectively [Dyall-Smith et al., 1986; Ciarlet et al., 1994]. As expected, the VP7 of RVA/Human-wt/ZAF/3203WC/2009/G2P[4] and the G2 (strain RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7[5]) component of RotaTeq® were similar, although some amino acid substitutions were observed within VR3 (L40V, A42V, M44I, and K49R), VR4 (S75T), VR5 (N96D), and VR9 (N242) (Fig. 2). There were no similarities between the antigenic regions of the study rotavirus strains to VP7 components of the human-bovine reassortant strains of the RotaTeq® vaccine with dissimilar genotypes, which was expected. This raises the question as to whether cross-protection against these emerging strains would be achieved by RotaTeq®. Interestingly, amino acid changes within the AR E seem to be related to the genogroup of the strains. At aa 189–190, all the Wa-like VP7 components of RotaTeq® (G1, G3, G4, and P1A[8] genotypes) had SS, strains with DS-1-like VP7 genotype (bovine-human reassortant strain RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7[5] and RVA/Human-wt/ZAF/3203WC/2009/G2P[4]) had TD, whereas the emerging G9 and G12 had ES and QS amino acids, respectively (Fig. 2).

Genome segment 5 (NSP1). Genome segment 5 of the study strains was of Wa- (RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6]) and DS-1- (RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3203WC/2009/G2P[4], and RVA/Human-wt/ZAF/GR10924/1999/G9P[6]) like origin (Fig. 1G and Table IV). The phylogenetic analysis showed that the genome segment 5 of the Wa-like study strains were closely related to the G1P[8] strains (RVA/Human-wt/USA/LB2758/2006/G1P[8] and RVA/Human-wt/USA/LB2771/2006/G1P[8]) recently isolated from USA [Bányai et al., 2011]. Genome segment 5 of the DS-1-like study strains formed separate clusters with DS-1-like strains isolated from USA, Bangladesh and DRC, respectively (Fig. 1G).

The conserved cysteine-rich motif C–X_2–C–X_8–C–X_2–C–X_3–H–X–C–X_2–C–X_5–C that spans from aa 42–72, which is believed to be a zinc- and virus-specific RNA-binding domain [Hua et al., 1993], was present in all the NSP1s of the study strains. However, it was located from aa 49–79. The amino acids Q64E and G/D70S that segregate depending on Wa- and DS-1-like genotypes [Heiman et al., 2008] were conserved within the cysteine-rich motifs of the study strains. However, they occurred at amino acid position 71 and 77, respectively. Other variations between Wa- and DS-1-like strains were also observed at amino acid position Q62R, T65L, and M66I within the cysteine-rich motif region (Supplementary Data 5).

Genome segment 8 (NSP2). Genome segment 8 of the study strains were of Wa- (RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6]) and DS-1- (RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3203WC/2009/G2P[4], and RVA/Human-wt/ZAF/GR10924/1999/G9P[6]) like origin (Fig. 1H and Table IV). Similar to genome segment 5, phylogenetic analysis revealed a close relationship between genome segment 8 of the two Wa-like study strains, which were related to strains with N1 genotypes isolated

from USA, Bangladesh, and Belgium. The genome segment 8 of DS-1-like study strains clustered with strains with N2 genotypes isolated from USA, Bangladesh, and DRC (Fig. 1H). The NSP2s of all the study strains contained the putative RNA-binding domain that stretches from aa 205–241 as described by Patton et al. [1993].

Genome segment 7 (NSP3). Genome segment 7 of the study strains was of Wa- (RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6]) and DS-1- (RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3203WC/2009/G2P[4], and RVA/Human-wt/ZAF/GR10924/1999/G9P[6]) like origin (Fig. 1I and Table IV). Phylogenetically, genome segment 7 of the Wa-like study strains clustered with T1 genotyped strains and were closely related to G12P[6] strains (RVA/Human-wt/BGD/SK423/2005/G12P[6] and RVA/Human-wt/BGD/Dhaka12-03/2003/G12P[6]) isolated from Bangladesh. The DS-1-like study strains clustered with T2 genotyped strains where strains RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3203WC/2009/G2P[4], and RVA/Human-wt/ZAF/GR10924/1999/G9P[6] were closely related to RVA/Human-wt/IND/IS2/XXXX/G2P[X], RVA/Human-wt/DEU/GER1H-09/2009/G8P[4], and RVA/Human-wt/IND/DS108/XXXX/G8P[6] strains isolated from India, Germany, and USA, respectively (Fig. 1I).

The basic (aa 81–150), acidic (aa 151–169), and hydrophobic heptads repeat regions (aa 174–229) described by Mattion et al. [1992] were also present in the study strains. The DS-1-like NSP3 proteins were different from that of the Wa-like strains within the basic regions at amino acid positions R96K, L106M, L107T, V129I, and E145D. One multiple (AFIE157–160SYVD) amino acid substitution was observed within the acidic region. The hydrophobic heptads region ranged from residues 181 to 237 in all the study strains. The hydrophobic residues were conserved in both DS-1- and Wa-like amino acid sequences at positions 181 (F), 188 (V), 195 (W), 202 (V), 209 (L), 223 (L), and 230 (L). However, the hydrophobic residues at position 216 and 237 were replaced with polar residues (Q and K, respectively) in both the study and other reference strains (Supplementary Data 6).

Genome segment 10 (NSP4). Genome segment 10 of the study strains was also of Wa- (RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6]) and DS-1- (RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3203WC/2009/G2P[4], and RVA/Human-wt/ZAF/GR10924/1999/G9P[6]) like origin (Fig. 1J and Table IV). Phylogenetically, genome segment 10 of the two Wa-like study strains clustered closely to strain RVA/Human-wt/IND/V1352/XXXX/GXP[X] from India and G12 strains (RVA/Human-wt/BGD/RV161/2000/G12P[6] and RVA/Human-wt/BGD/Matlab13/2003/G12P[6]) from Bangladesh. The DS-1-like study strains clustered with E2 genotyped reference strains. Strain RVA/Human-wt/ZAF/3203WC/2009/G2P[4] clustered with strains isolated from Asia, although a close relationship with the RVA/Human-wt/USA/LB2764/2006/G2P[4] strain isolated from USA was also observed. RVA/Human-wt/ZAF/GR10924/1999/G9P[6] was closely related to the RVA/Human-wt/BGD/MMC88/2005/G2P[4] strain isolated from Bangladesh, while RVA/Human-wt/MWI/1473/2001/G8P[4] was closely related to RVA/Human-wt/THA/CMH008-05/2005/G2P[4] and RVA/Human-wt/BEL/B1711/2002/G6P[6] strains isolated from Thailand and Belgium, respectively (Fig. 1J).

The five putative functional domains of NSP4 (the oligomerization-associated, hydrophobic, VP4-binding, single-stranded particle-binding, toxic peptide region, and the pathogenesis-associated region) were present in genome segment 10 of all the study strains and were conserved with respect to Wa- and DS-1-like genotypes as described by Heiman et al. [2008] (Supplementary Data 7).

Genome segment 11 (NSP5 and NSP6). Genome segment 11 of the study strains were also of Wa- (RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6]) and DS-1- (RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/3203WC/2009/G2P[4], and RVA/Human-wt/ZAF/GR10924/1999/G9P[6]) like origin (Fig. 1K and Table IV). The phylogram of genome segment 11 showed close relationships between the two Wa-like study strains to several strains with H1 genotypes isolated from different countries, of which RVA/Human-wt/BGD/Dhaka6/2001/G11P[25] was the closest. RVA/Human-wt/ZAF/GR10924/1999/G9P[6] was closely related to USA (RVA/Human-wt/USA/LB2744/2006/G2P[4] and RVA/Human-wt/USA/LB2772/2006/G2P[4]) and Bangladesh (RVA/Human-wt/BGD/MMC6/2005/G2P[4] and RVA/Human-wt/BGD/MMC88/2005/G2P[4]) strains, while RVA/Human-wt/MWI/1473/2001/G8P[4] and RVA/Human-wt/ZAF/3203WC/2009/G2P[4] were closely related to RVA/Human-wt/BEL/B1711/2002/G6P[6] and RVA/Human-wt/BGD/Dhaka116-00/2000/G2P[4] strains isolated from Belgium and Bangladesh (Fig. 1K).

DISCUSSION

According to the whole genome rotavirus classification scheme, a rotavirus strain is classified as belonging to Wa-, DS-1-, or AU-like genogroups if the genotypes of at least seven of its genome segments correlate with prototype strains of these genogroups [Matthijnssens et al., 2008b]. The strains characterized in this study were genogrouped without the use of RNA–RNA hybridization experiments that are often laborious and lengthy. Instead, sequence-independent cDNA synthesis and genome amplification, 454® pyrosequencing of the amplified genome segments and RotaC were used to assign genotypes. The study strains were classified into Wa- (Hu/RSA/3133WC/2009/G12P[4] and Hu/RSA/3176WC/2009/G12P[6]) and DS-1- (RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/

3203WC/2009/G2P[4], and the RVA/Human-wt/ZAF/GR10924/1999/G9P[6]) like genogroups based on the genotypes assigned to each genome segment.

Phylogenetic analysis and inferences were conducted to trace the origin of the strains characterized in this study. The genome segment 1 (VP1) of RVA/Human-wt/MWI/1473/2001/G8P[4] clustered near the artiodactyl-like human strain RVA/Human-wt/HUN/Hun5/1997/G6P[14] [Matthijnssens et al., 2009] and the bovine-feline/canine-human reassortant strain RVA/Human-wt/ITA/PAH136/1996/G3P[9] [De Grazia et al., 2010]. The nine genome segments encoding VP2–VP4, VP6, NSP1–NSP5 of strain RVA/Human-wt/MWI/1473/2001/G8P[4] were closely related to human DS-1-like strains, whereas the genome segment encoding VP7 was closely related to strains isolated from Malawi (like RVA/Human-wt/MWI/MW1479/2001/G8P[4]) which are bovine-human reassortants or share common ancestry with bovine rotaviruses [Cunliffe et al., 2000]. Phylogenetic analysis also suggests that the genome segment 9 of strain RVA/Human-wt/MWI/1473/2001/G8P[4] shares common ancestry with bovine rotaviruses. Considering that the G8 genotype that was assigned to its VP7 is common in cattle, these observations suggest that strain RVA/Human-wt/MWI/MW1479/2001/G8P[4] originated from or shares a common origin with artiodactyl strains. It is, therefore, possible that strain RVA/Human-wt/MWI/MW1479/2001/G8P[4] emerged through interspecies reassortment between human and artiodactyl rotaviruses.

All the genome segments of strain RVA/Human-wt/ZAF/GR10924/1999/G9P[6] were closely related to human rotavirus strains. The genome segments encoding VP1–VP3, VP6, NSP1–NSP5 were assigned DS-1-like genotypes, whereas genome segments encoding VP4 and VP7 were P[4] and G9 (Wa-like), respectively (G9-P[6]-I2-R2-C2-M2-A2-N2-T2-E2-H2). To date, most G9 strains that were characterized fully have a Wa-like genetic backbone [Heiman et al., 2008; Mukherjee et al., 2009; Mijatovic-Rustempasic et al., 2011]. Therefore, RVA/Human-wt/ZAF/GR10924/1999/G9P[6] may represent the first human rotavirus strain to be completely characterized with G9 VP7 and P[6] VP4 genotypes on a DS-1-like genetic backbone. Strain RVA/Human-wt/ZAF/GR10924/1999/G9P[6] might have emerged through multiple genome reassortment events between Wa-, DS-1, and human P[6] rotaviruses.

RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6] represent the first completely molecularly characterized emerging G12 rotavirus strains from Africa. The Wa-like genetic constellation of the G12 study strains was similar to G12 strains isolated from Bangladesh (RVA/Human-wt/BGD/Dhaka12-03/2003/G12P[6] and RVA/Human-wt/BGD/Dhaka25-02/2002/G12P[8]) and Belgium (RVA/Human-wt/BEL/B4633/2003/G12P[8]) [Rahman et al., 2007]. Strain RVA/Human-wt/ZAF/3133WC/2009/G12P[4] contained a DS-1-like P[4] encoding genome segment 4. It is, therefore, most likely that this strain emerged through intergenogroup genome reassortment events between human Wa- and human DS-1-like rotavirus strains. RVA/Human-wt/ZAF/3176WC/2009/G12P[6] is a human strain with a P[6] VP4 in a human Wa-like genetic backbone. Since it has been hypothesized that Wa-like strains and porcine strains have a common origin [Matthijnssens et al., 2008b], strain RVA/Human-wt/ZAF/3176WC/2009/G12P[6] might share a common origin with porcine strains.

All the genome segments of strain RVA/Human-wt/ZAF/3203WC/2009/G2P[4] were closely related to DS-1-like human strains (Table IV), hence it was classified into DS-1-like genogroup. Most of the full genomes of the African DS-1-like strains that have been characterized to date have a G8 VP7 genotype [Matthijnssens et al., 2006; Esona et al., 2009]. Therefore, RVA/Human-wt/ZAF/3203WC/2009/G2P[4] may represent the first pure member of the DS-1-like genogroup to be characterized from Africa. Although rotaviruses with G2 genotypes are considered as one of the predominant strains worldwide [Santos and Hoshino, 2005], studies reporting on their full genome characterization are limited [Heiman et al., 2008; Bányai et al., 2011]. The increased predominance of G2P[4] strains amongst the Rotarix® vaccinated population in Brazil from 5% to 95% [Gurgel et al., 2007], and the continued prevalence of G2P[4] rotaviruses in Australian states where Rotarix® was introduced [Kirkwood et al., 2011] seems to suggest that G2P[4] rotaviruses are less protected by the live-attenuated G1P[8] monovalent Rotarix® vaccine. Since G2 rotaviruses are detected frequently across Africa [Sanchez-Padilla et al., 2009], characterization of the full genomes of more G2 rotavirus is important to understand the possible genetic reasons that may lead to these varied vaccination efficacies.

Use of data generated through whole genome sequence-independent amplification and 454® pyrosequencing in classifying rotaviruses offers a number of advantages over the dual classification system that uses sequence-specific primers complementary to genome segment 4 and 9 nucleotide sequences. A typical example is strain RVA/Human-wt/ZAF/3133WC/2009/G12P[4] which was incorrectly genotyped as P[6/8] by genotype-specific RT-PCR. RotaC and phylogenetic analysis of the sequence-independent generated nucleotide sequences revealed that strain RVA/Human-wt/ZAF/3133WC/2009/G12P[4] possessed a P[4] genotype. The complete correlation in genotypes assigned by whole genome classification that utilized sequence-independent RT-PCR amplification to phylogenetic classification highlights the shortfalls of the exclusive use of RT-PCR based on genotype-specific primers in assigning rotavirus genotypes. This may lead to the occasional misreporting of results due to oligonucleotide mispriming. Therefore, a full genome classification system coupled with sequence-independent genome amplification and 454® pyrosequencing could

be used for quality control purposes in verifying the accuracy or reliability of the genotype-specific RT-PCR-based methods.

One of the limitations of the full genome classification scheme [Matthijnssens et al., 2008b] is its inability to determine exactly when reassortment or interspecies transmission events took place [Esona et al., 2009]. For this reason, the time when the evolutionary mechanisms leading to the emergence of the strains characterized in the present study occurred, could not be determined. Complementing such analysis with phylodynamic studies [Matthijnssens et al., 2010] may be useful to elucidate this.

Although the amino acid variations between the Wa- (genotype 1) and DS-1- (genotype 2) proteins of the study strains were consistent with the findings of Heiman et al. [2008], a few additional differences were observed for VP2, VP6, VP7, NSP1, NSP3, and NSP4. In VP2: amino acid sequences of the Wa-like study strains were 15 residues longer than that of the DS-1-like strains due to amino acid insertions at positions 21 (E), 32 (MENKNKNKNNNR), and 347 (EK). This was also observed in some Wa-like reference strains isolated from Bangladesh (RVA/Human-wt/BGD/Dhaka12-03/2003/G12P[6] and RVA/Human-wt/BGD/Dhaka16-03/2003/G1P[8]) and USA (RVA/Human-wt/USA/2007719825/2007/G1P[8]. In the current study, the Wa-like study strains and some reference strains contained 12 amino acid insertions in the N terminal region. Previously only 4, 8, and 10 amino acid insertions were observed for strains RVA/Human-wt/USA/LB2719/2006/G1P[8] (894 aa), RVA/Human-wt/USA/LB2758/2006/G1P[8] (898 aa), and RVA/Human-wt/USA/LB2771/2006/G1P[8] (900 aa), respectively, resulting in different lengths of VP2 [Bányai et al., 2011]. In VP6, region D that defines subgroup II specificity of rotaviruses was localized at position 310 and 315 in all the study strains, instead of position 312 and 314 as reported previously [Gorziglia et al., 1988]. Since Wa- and DS-1-like strains are associated with subgroup II and I, respectively, this finding may correlate with site-mutagenesis studies by Tang et al. [1997] that indicated that a single amino acid mutation at residue 315 is sufficient to change subgroup specificity of a strain. In NSP1, the conserved cysteine-rich motif ranged from aa 49 to 79 in all the study strains, instead of aa 42–72 as reported previously [Hua et al., 1993]. In NSP3, several variations were observed in the acidic region, while residues in the hydrophobic heptads region at position 216 and 237 were replaced with polar residues (Q and K, respectively). In NSP4, the VP4-binding (aa 112–148) and the pathogenesis-associated domains (aa 114–135) [Ball et al., 1996] of Wa-like and DS-1-like strains were different due to several amino acid substitutions that occurred within these regions. Whether these amino acid variations may affect the virulence of rotavirus strains could not be determined in this study. In VP7, several amino acid variations were observed between the antigenic regions of the outer capsid proteins of the study strains and the VP7 of the bovine-human RotaTeq® strains, which was expected. There were also some differences between the antigenic regions of strain RVA/Human-wt/ZAF/3203WC/2009/G2P[4] and the VP7 of the G2 component of RotaTeq® (strain RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7[5]), which was unexpected as both have a G2 genotype. This may affect the level of cross-protection that can be rendered by the vaccine to some of these strains.

As evidence is now emerging that correlates rotavirus vaccine efficacy with specific rotavirus genotypes [Ruiz-Palacios et al., 2006; Gurgel et al., 2007; WHO, 2008], further full genome characterization studies should be encouraged to understand the complete genomic constellations of regionally prevalent strains, such as G8 rotaviruses [Santos and Hoshino, 2005], and emerging genotypes, such as G12 [Le et al., 2008]. This information can eventually be used to formulate regional rotavirus vaccines. Since most rotavirus mortalities are caused by rotaviruses that belong to either Wa- or DS-1-like genogroups, combining genotypes from these two genogroups in the formulation of the next generation of rotavirus vaccines might improve rotavirus vaccine efficacy.

In conclusion, the findings highlight the role of rotavirus intergenogroup and interspecies genome reassortment in generating rotavirus strain diversity. Furthermore, the study illustrates how sequence-independent amplification coupled with 454® pyrosequencing of the full rotavirus genome can be used to understand the evolutionary mechanisms of rotaviruses swiftly.

ACKNOWLEDGMENTS

We thank the Viral Gastroenteritis Unit (NICD), and the Medical Research Council/Diarrheal Pathogens Research Unit, Department of Virology, University of Limpopo (Medunsa Campus) for donating the rotavirus strains characterized in this study.

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