Table 1
Genotypes of the 11 gene segments of strain P343 compared with those of selected RVA strains with known genomic constellations.

Strain	Genotype										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Pig-tc/THA/P343/1991/G10P[5]	G10	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Human-tc/USA/Wa/1974/G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/USA/DS-1/1976/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-tc/JPN/AU-1/1982/G3P[9]	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Pig-tc/VEN/A131/1988/G3P[7]	G3	P[7]	I5	R1	C2	M1	A1	N1	T1	E1	H1
RVA/Pig-tc/USA/Gottfried/1975/G4P[6]	G4	P[6]	I1	R1	C1	M1	A8	N1	T1	E1	H1
RVA/Pig-tc/USA/OSU/1975/G5P[7]	G5	P[7]	I5	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Cow-tc/USA/NCDV/1967/G6P[1]	G6	P[1]	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-wt/ZAF/1603/2007/G6P[5]	G6	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	**H3**
RVA/Cow-wt/DNK/DK11601/2007/G6P[5][a]	G6	P[5]	**I2**	–	–	–	–	–	–	E2	–
RVA/Cow-tc/GBR/UK/1973/G6P[5]	G6	P[5]	I2	R2	C2	M2	A3	N2	T7	E2	H3
RVA/Cow-tc/USA/WC3/1981/G6P[5]	G6	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Cow-wt/SVN/SI-B17/2004/G6P[11]	G6	P[11]	I2	R2	C2	M2	A3	N2	T6	E2	**H3**
RVA/Human-wt/SVN/SI-R56/2007/G6P[11]	G6	P[11]	I2	R2	C2	M2	A13	N2	T6	**E2**	H3
RVA/Human-tc/ITA/PA169/1988/G6P[14]	G6	P[14]	**I2**	**R2**	**C2**	M2	A3	N2	T6	E2	H3
RVA/Antelope-wt/ZAF/RC-18-08/2008/G6P[14]	G6	P[14]	I2	R2	C2	**M2**	A11	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/Human-tc/KEN/B12/1987/G8P[1]	G8	P[1]	I2	R2	C2	M2	A3	**N2**	T6	E2	H3
RVA/Pig-tc/KOR/PRG9121/2006/G9P[7]	G9	P[7]	I5	R1	C1	M1	A8	N1	T1	E1	H1
RVA/Pig-xx/KOR/PRG942/2006/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1
RVA/Pig-xx/KOR/PRG9235/2006/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1
RVA/Cow-tc/THA/61A/1989/G10P[5]	**G10**	**P[5]**	–	–	–	–	–	–	–	–	–
RVA/Cow-wt/ARG/B2376_D_BA/2003/G10P[5]	G10	P[5]	–	–	–	–	–	–	–	–	–
RVA/Cow-wt/CAN/FMV1077415/2009/G10P[5]	G10	P[5]	–	–	–	–	–	–	–	–	–
RVA/Cow-tc/GER/V1005/1977-1983/G10P[5][a]	G10	P[5]	–	–	–	–	–	–	–	–	–
RVA/Cow-tc/GBR/B223/1983/G10P[11]	G10	P[11]	I2	–	–	–	A13	–	–	E2	–
RVA/Cow-tc/CHN/DQ-75/2008/G10P[11]	G10	P[11]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Giraffe-wt/IRL/GirRV/2008/G10P[11]	G10	P[11]	I2	R2	C2	M2	A3	N2	**T6**	E2	H3
RVA/Human-tc/GBR/A64/1987/G10P[14]	G10	P[14]	I2	R2	C2	M1	A3	N2	T6	E2	H3
RVA/Human-wt/AUS/V585/2011/G10P[14]	G10	P[14]	I2	R2	C2	M2	A11	N2	T6	E2	H3
RVA/Pig-tc/VEN/A253/1988/G11P[7]	G11	P[7]	I5	R1	C2	M1	A1	N1	T1	E1	H1
RVA/Pig-tc/MEX/YM/1983/G11P[7]	G11	P[7]	I5	R1	C1	M1	A8	N1	T1	E1	H1
RVA/Pig-wt/IND/RU172/2002/G12P[7]	G12	P[7]	I5	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Cow-wt/JPN/Azuk-1/2006/G21P[29]	G21	P[29]	I2	R2	C2	M2	A13	N2	T9	E2	H3
RVA/Cow-wt/JPN/Dai-10/2008/G24P[33]	G24	P[33]	I2	R2	C2	M2	A13	N2	T9	E2	H3

Gray shading indicates the gene segments with a genotype identical to that of strain P343.
The gene segments that are most similar to those of strain P343 are highlighted in bold.
"–" indicates that no sequence data were available in the DDBJ and EMBL/GenBank data libraries.
[a] Genotype assignment based on reports by Midgley et al. (2012) (strain DK11601) and Brüssow et al. (1994) (strain V1005). To our knowledge, to date, nucleotide sequence accession numbers for the VP7 and VP4 genes of strain DK11601, and the VP7 gene of strain V1005 are not available in the DDBJ and EMBL/GenBank data libraries.

(G8P[1]), and DQ-75 (G10P[11])), a bovine-like giraffe strain (GirRV (G10P[11])), and bovine-like human strains (PA169 (G6P[14]) and B12 (G8P[1])), although some bovine and bovine-like strains have been found to have other NSP1 (A11 or A13 instead of A3) and NSP3 (T7 or T9 instead of T6) genotypes. Giraffe strain GirRV, and human strains PA169 and B12 have been shown to have bovine backbones and to be likely of bovine origin through their full-genomic analysis (Matthijnssens et al., 2008; Ghosh et al., 2011b; O'Shea et al., 2014). Thus, the genotype constellation of strain P343 was mostly identical to those of bovine and bovine-like strains.

We next constructed phylogenetic trees using the full-genome sequence for each of the 11 gene segments because phylogenetic analysis of RVA nucleotide sequences provides direct evidence of their relatedness to those of other strains, even within the same genotype (Matthijnssens et al., 2008).

The VP7 gene of strain P343 exhibited the maximum nucleotide sequence identity (97.9%) with that of Thai bovine strain 61A (G10P[5]) (Taniguchi et al., 1991) (Table 1), and comparable identities (97.1–97.6%) with Thai bovine strain A44 (G10P[11]) (Taniguchi et al., 1991) and two Indian bovine strains (B75 (G10P[x]) and B69 (G10P[x])) (Varshney et al., 2002). On phylogenetic analysis, strain P343 was found to be closely related with strains 61A and A44, despite their isolation from different species (Fig. 1a).

The VP4 gene of strain P343 showed the highest nucleotide sequence identity (97.7%) with the cognate gene of Thai bovine strain 61A (G10P[5]) (Table 1), and somewhat lower identity (96.8%) with South African bovine strains (1603 (G6P[5]) and 1605 (G6P[5])) (Jere et al., 2012). On phylogenetic analysis, strain P343 was shown to be closely related with strain 61A, despite their isolation from different species (Fig. 1b).

The VP6 gene of strain P343 exhibited the highest nucleotide sequence identity (99.1%) with the VP6 genes of Danish bovine strain DK11601 (G6P[5]) (Midgley et al., 2012) and Italian bovine-like human strain PA169 (G6P[14]) (Gerna et al., 1992) (Table 1). Phylogenetically, strain P343 was found to be closely related with strain DK11601 in a common branch with several bovine and bovine-like human strains (Fig. 1c).

The VP1 gene of strain P343 showed the maximum nucleotide sequence identity (95.3%) with that of Italian

Fig. 1. Phylogenetic trees constructed from the nucleotide sequences of the VP7 (a), VP4 (b), VP6 (c), VP1 (d), VP2 (e), VP3 (f), NSP1 (g), NSP2 (h), NSP3 (i), NSP4 (j), and NSP5 (k) genes of strain P343, and representative RVA strains. In all the trees, the position of strain P343 is shown in red. Bootstrap values of <85% are not shown. Scale bars, 0.02 (h–k), 0.05 (a–f), or 0.1 (g) substitutions per nucleotide.

Fig. 1. (*Continued*).

bovine-like human strain PA169 (G6P[14]) (Table 1), and somewhat lower identity (94.7%) with reference bovine strain UK (G6P[5]). Phylogenetic analysis showed the close relatedness of strains P343 and PA169 in a common branch with a reference strain UK (Fig. 1d).

The VP2 gene of strain P343 showed the highest nucleotide sequence identity (89.7%) with the VP2 gene of

Italian bovine-like human strain PA169 (G6P[14]) (Table 1), and somewhat lower identities (88.9–89.2%) with South African bovine strain 1604 (G8P[1]) (Jere et al., 2012), Irish bovine-like giraffe strain GirRV (G10P[11]) (O'Shea et al., 2014), and Slovene bovine-like human strain SI-R56 (G6P[11]) (Steyer et al., 2013). On phylogenic analysis, strain P343 was found to cluster near these bovine and bovine-like strains (Fig. 1e).

The VP3 gene of strain P343 showed the maximum nucleotide sequence identity (97.4%) with the cognate gene of South African bovine-like antelope strain RC-18-08 (G6P[14]) (Matthijnssens et al., 2009) (Table 1), and comparable similarity (97.1%) with Dutch bovine-like rabbit strain K1130027 (G6P[11]) (Schoondermark-van de Ven et al., 2013). Phylogenetic analysis showed that strain P343 was closely related with strain RC-18-08 in a common branch with strain K1130027 (Fig. 1f).

The NSP1 gene of strain P343 exhibited the highest nucleotide sequence identity (93.2%) with that of reference bovine strain NCDV (G6P[1]) (Table 1). On phylogenic analysis, strain P343 was found to cluster near the clusters formed by several bovine strains and bovine-like strains from a giraffe and humans (Fig. 1g).

The NSP2 gene of strain P343 exhibited the maximum nucleotide identity (97.9%) with that of Kenyan bovine-like human strain B12 (G8P[1]) (Ghosh et al., 2011b) (Table 1), and somewhat lower identity (97.4%) with British bovine-like human strain A64 (G10P[14]) (Matthijnssens et al., 2008). Phylogenic analysis showed strain P343 to be clustered with these and Italian bovine-like human strain 111-05-27 (G6P[14]) (Matthijnssens et al., 2008) (Fig. 1h).

The NSP3 gene of strain P343 showed the highest nucleotide sequence identity (95.3%) with the NSP3 gene of Irish bovine-like giraffe strain GirRV (G10P[11]) (Table 1). On phylogenetic analysis, strain P343 was shown to cluster near the clusters formed by several bovine strains and bovine-like strains from different host species (Fig. 1i).

The NSP4 gene of strain P343 showed the maximum nucleotide sequence similarity (98.8%) with that of Slovene bovine-like human strain SI-R56 (G6P[11]) (Table 1), and comparable similarity (98.4%) with Danish bovine strain DK11601 (G6P[5]). On phylogenetic analysis, strain P343 was found to be closely related with strain DK11601 in a common branch with strain SI-R56, and European bovine strains (CIT-A18 (G6P[11]) and DK11331 (G6P[5])) (Cashman et al., 2010; Midgley et al., 2012) (Fig. 1j).

The NSP5 gene of strain P343 exhibited the highest nucleotide sequence similarity (98.6%) with the cognate genes of Slovene bovine strain SI-B17 (G6P[11]) (Steyer et al., 2013) and Irish giraffe strain GirRV (G10P[11]) (Table 1). On phylogenetic analysis, strain P343 was shown to be clustered with these and South African bovine strains (1603 (G6P[5]) and 1604 (G8P[1])) (Fig. 1k).

In summary, each of the 11 genes of strain P343 was found to be closely related to bovine or bovine-like RVA genes. Therefore, strain P343 has a bovine genetic backbone and was suggested to be of bovine origin.

In the present study, we analyzed the whole genome of an unusual porcine RVA strain P343 with G10P[5] genotypes (RVA/Pig-tc/THA/P343/1991/G10P[5]) from a piglet with diarrhea in Thailand. Strain P343 showed a unique genotype constellation: G10-P[5]-I2-R2-C2-M2-A3-N2-T6-E2-H3, which is commonly found in bovine RVA strains. On phylogenetic analysis, each of the 11 genes of strain P343 appeared to be of bovine origin. Therefore, strain P343 was assumed to be an apparent example of bovine-to-porcine interspecies transmission events of RVAs. Our findings reinforce the increasing evidence that the transmission of RVAs can occur from animal to animal as well as from animal to humans (Martella et al., 2010; Ghosh et al., 2011a). In addition, bovine-like porcine RVA strains have been sporadically detected in pig herds in the Americas, Asia, and Europe, whereas no full genomic sequence data were collected in these studies (Gouvea et al., 1994a and 1994b; Pongsuwanna et al., 1996; Rácz et al., 2000; Martella et al., 2001; Parra et al., 2008). The bovine origin of strain P343 also suggests interspecies transmission due to close proximity of humans to livestock, especially in developing countries where there is intimate contact between humans and livestock. Furthermore, whole genome-based analysis is a reliable tool for studying rare RVA interspecies transmission events.

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Whole genomic analysis of human G12P[6] and G12P[8] rotavirus strains that have emerged in Kenya: Identification of porcine-like NSP4 genes

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Reassortment

ABSTRACT

G12 rotaviruses are globally emerging rotavirus strains causing severe childhood diarrhea. However, the whole genomes of only a few G12 strains have been fully sequenced and analyzed, of which only one G12P[4] and one G12P[6] are from Africa. In this study, we sequenced and characterized the complete genomes of three G12 strains (RVA/Human-tc/KEN/KDH633/2010/G12P[6], RVA/Human-tc/KEN/KDH651/2010/G12P[8], and RVA/Human-tc/KEN/KDH684/2010/G12P[6]) identified in three stool specimens from children with acute diarrhea in Kenya, Africa. On whole genomic analysis, all three Kenyan G12 strains were found to have a Wa-like genetic backbone: G12-P[6]-I1-R1-C1-M1-A1-N1-T1-E1-H1 (strains KDH633 and KDH684) and G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 (strain KDH651). Phylogenetic analysis showed that most genes of the three strains examined in this study were genetically related to globally circulating human G1, G9, and G12 strains. Of note is that the NSP4 genes of strains KDH633 and KDH684 appeared to be of porcine origin, suggesting the occurrence of reassortment between human and porcine strains. Furthermore, strains KDH633 and KDH684 were very closely related to each other in all the 11 gene segments, indicating derivation of the two strains from a common origin. On the other hand, strain KDH651 consistently formed distinct clusters of 10 of the 11 gene segments (VP1-2, VP4, VP6-7, and NSP1-5), indicating a distinct origin of strain KDH651 from that of strains KDH633 and KDH684. To our knowledge, this is the first report on whole genome-based characterization of G12 strains that have emerged in Kenya. Our observations will provide important insights into the evolutionary dynamics of emerging G12 rotaviruses in Africa.

1. Introduction

Group A rotavirus (RVA), a member of the *Reoviridae* family, is the leading etiological agent of severe gastroenteritis in the young of humans and many animal species worldwide. In humans, RVA infections are associated with high morbidity and mortality, being responsible for an estimated annual 453,000 deaths in children <5 years of age (Tate et al., 2012). More than half of these deaths occur in sub-Sahara Africa (Madhi et al., 2010; Mwenda et al., 2010). The RVA virion is a triple-layered, non-enveloped icosahedron enclosing an 11-segment genome of double-stranded (ds)RNA (Estes and Greenberg, 2013). Because of the segmented nature of the genome, reassortment between/within human and animal strains is one of the major processes of genetic evolution of this virus.

RVA has two outer capsid proteins, VP7 and VP4, which are implicated independently in neutralization, and define the G and P genotypes, respectively. To date, RVAs have been classified into at least 27 G and 37 P genotypes (Matthijnssens et al., 2011; Trojnar et al., 2013). Among them, the 5 G (G1-4 and G9) and 3 P (P[4], P[6], and P[8]) genotypes are commonly associated with

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human infections (Santos and Hoshino, 2005; Matthijnssens et al., 2010a). Over the last decade, the worldwide emergence of unusual G12 strains has been a matter of concern, and G12 seems to be the sixth major human G genotype (Rahman et al., 2007; Matthijnssens et al., 2009, 2010a).

The first G12 strain, L26 (G12P[4]), was identified in children with acute diarrhea in the Philippines in 1987 (Taniguchi et al., 1990; Urasawa et al., 1990). A decade later, G12 strains began to emerge globally, predominantly in combination with either the P[6] or P[8] genotype and less commonly with the P[4] and P[9] genotypes (Pongsuwanna et al., 2002; Wakuda et al., 2003; Shinozaki et al., 2004; Samajdar et al., 2006; Rahman et al., 2007; Matthijnssens et al., 2009; Matthijnssens et al., 2010a; Mwenda et al., 2010; Seheri et al., 2014). In animals, G12 strains have been detected in pigs and cattle (Ghosh et al., 2006; Midgley et al., 2012; Ndze et al., 2013a), of which only one porcine G12 strain RU172 (G12P[7]) has been analyzed for whole genome so far (Ghosh et al., 2010). More recently, G12 strains have been increasingly identified in diarrheic children in several African countries (Page et al., 2009, 2014; Cunliffe et al., 2009; Mwenda et al., 2010; Nakagomi et al., 2012; Oluwatoyin Japhet et al., 2012; Enweronu-Laryea et al., 2013; Ndze et al., 2013b; Pukuta et al., 2014; Seheri et al., 2014), indicating the ongoing expansion of G12 strains in Africa. From Kenya, there were no reports of the detection of human G12 strains until 2013, however, the identification of G12 strains from diarrheic children was reported in three independent papers in 2014, whereas no G12 genomic sequence data were collected in these studies (Kiulia et al., 2014; Seheri et al., 2014; Wandera Apondi et al., submitted for publication). In one of these studies, we detected three Kenyan G12 strains, KDH633, KDH651, and KDH684, in stool samples from three diarrheic children (≤3 years old) in the Kiambu area in 2010 (Wandera Apondi et al., submitted for publication). PCR-based G and P genotyping showed that strains KDH633, KDH651, and KDH684 have the G12P[6], G12P[8], and G12P[6] genotypes, respectively.

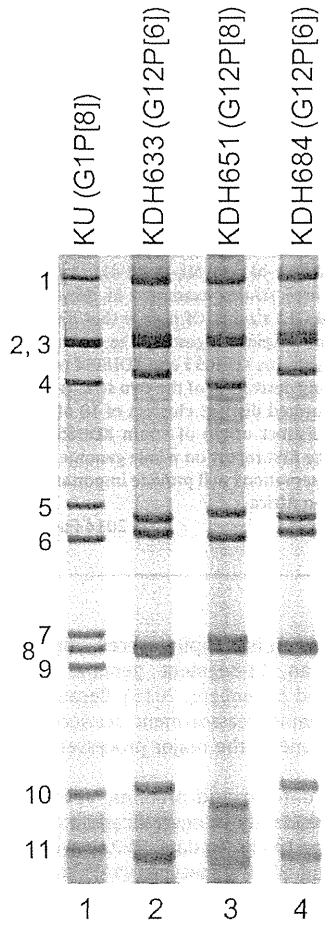

Fig. 1. Genomic dsRNA profiles of strains KDH633, KDH651, and KDH684. Lane 1, dsRNAs of strain KU (G1P[8]) extracted from a cell culture; lanes 2–4, dsRNAs of strains KDH633 (lane 2), KDH651 (lane 3), and KDH684 (lane 4) extracted from cell cultures. The numbers on the left indicate the order of the genomic dsRNA segments of strain KU.

A whole genome-based genotyping system was recently proposed for RVAs based on the assignment to all the 11 gene segments (i.e., G/P and non-G/P genes) (Matthijnssens et al., 2008). In the new genotyping system, the acronym Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, where x is an integer, defines the genotype of the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 genes of a given RVA strain. The Wa-like strains are characterized by non-G/P genotypes (I1-R1-C1-M1-A1-N1-T1-E1-H1), and tend to have G/P genotypes G1P[8], G3P[8], G4P[8], or G9P[8] (Dennis et al., 2014). In contrast, the DS-1-like strains are characterized by non-G/P genotypes (I2-R2-C2-M2-A2-N2-T2-E2-H2), and tend to have G/P genotype G2P[4]. The third minor AU-1-like strains are characterized by non-G/P genotypes (I3-R3-C3-M3-A3-N3-T3-E3-H3), and tend to have G/P genotype G3P[9]. Whole genome-based analysis is a reliable method for obtaining conclusive data on the origin of an RVA strain, and for tracing its evolutionary pattern (Matthijnssens et al., 2008; Ghosh and Kobayashi, 2011). To date, the whole genome sequences of only a few G12 strains, including strains 3133WC (G12P[4]) and 3176WC (G12P[6]), from Africa have been fully sequenced and characterized, providing evidence of the Wa-like genotype backbone of these African G12 strains (Jere et al., 2011). As strains 3133WC and 3176WC were identified in South Africa, whole genomic analysis of Kenyan (east African) strains KDH633, KDH651, and KDH684 might be useful for obtaining more precise understanding of the evolutionary patterns of emerging G12 strains in Africa. In the present study, we analyzed the whole genomes of three G12 strains that have emerged in Kenya.

2. Materials and methods

2.1. Virus strains

The full-genomic sequences were determined for strains KDH633, KDH651, and KDH684, which were identified as the sole pathogens causing diarrhea in three stool specimens from children with acute diarrhea during the RVA strain surveillance in the Kiambu district, Kenya in 2010 enrolling a total of 68 RVA-positive fecal samples (Wandera Apondi et al., submitted for publication). Stool samples containing strains KDH633, KDH651, and KDH684 were kept at −30 °C until use. The study was approved by the Kenya Medical Research Institute Ethics Review Committee (KEMRI/RES/7/3/1).

2.2. Virus isolation

Stool samples suspended in PBS containing 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$ were inoculated onto monkey kidney cell line MA104 for virus isolation (Komoto et al., 2013), and the cultures were serially passaged two more times in MA104 cells. The viral dsRNAs were extracted from the culture fluids using TRI Reagent LS (Molecular Research Center) according to the manufacturer's

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Table 1
Genotype natures of the 11 gene segments of three Kenyan G12 strains, KDH633, KDH651, and KDH684, with those of selected human and porcine strains.

Strain	Genotype										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Human-tc/KEN/KDH633/2010/G12P[6]	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/KEN/KDH651/2010/G12P[8]	G12	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/KEN/KDH684/2010/G12P[6]	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/ZAF/3133WC/2009/G12P[4]	G12	P[4]	I1	R1	C1	M1	A1	N1[b,c]	T1[a,c]	E1	H1
RVA/Human-wt/ZAF/3176WC/2009/G12P[6]	G12	P[6]	I1	R1	C1	M1	A1	N1[b,c]	T1[a,c]	E1	H1
RVA/Human-tc/USA/Wa/1974/G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/USA/VU05-06-69/2005/G1P[8]	G1	P[8]	I1	R1[a,c]	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/BEL/BE00017/2006/G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1[b]	T1	E1	H1
RVA/Human-wt/USA/VU06-07-27/2006/G1P[8]	G1	P[8]	I1	R1[a,c]	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/USA/USA2007719739/2007/G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1[b]	H1
RVA/Human-wt/USA/USA2007719825/2007/G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1[b]	T1	E1	H1[a,c]
RVA/Human-wt/AUS/CK00083/2008/G1P[8]	G1	P[8]	I1	R1	C1[a,c]	M1	A1	N1[b]	T1	E1	H1
RVA/Human-wt/BEL/BE00112/2009/G1P[8]	G1	P[8]	I1[a,c]	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/AUS/CK20043/2010/G1P[8]	G1	P[8]	I1	R1[b]	C1	M1	A1[b]	N1	T1	E1	H1[b]
RVA/Human-tc/USA/DS-1/1976/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-tc/JPN/AU-1/1982/G3P[9]	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Human-wt/NPL/KTM368/2004/G11P[25]	G11	P[25]	I12	R1	C1	M1	A1[b,c]	N1	T1	E1	H1
RVA/Human-tc/PHL/L26/1987/G12P[4]	G12	P[4]	I2	R2	C2	M1M2	A2	N1	T2	E2	H1
RVA/Human-wt/RGD RV161/2000/G12P[6]	G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E1	H2
RVA/Human-wt/RGD RV176/2000/G12P[6]	G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E6	H2
RVA/Human-wt/RGD N26/2002/G12P[6]	G12	P[6]	I2	R2	C2	M2	A2	N1	T2	E6	H2
RVA/Human-wt/BGD Dhaka12-03/2003/G12P[6]	G12	P[6]	I1	R1	C1	M1[a,b,c]	A1[a,c]	N1	T1[a,c]	E1	H1
RVA/Human-wt/BGD Matlab13-03 2003/G12P[6]	G12	P[6]	I1	R1	C1	M1	A1[a,c]	N1	T2	E1	H1
RVA/Human-tc/BGD/SK277/2005/G12P[6]	G12	P[6][a,c]	I1	–	–	–	A1	N1	T1	E1	H1
RVA/Human-tc/BGD/SK423/2005/G12P[6]	G12	P[6][a,c]	I1	–	–	–	A1	N1	T1	E1	H1
RVA/Human-wt/MWI/KCH1124/2005-2007 G12P[6]	G12[a,c]	P[6]	–	–	–	–	–	–	–	–	–
RVA/Human-tc/KOR/CAU195/2006/G12P[6]	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/KOR/CAU214/2006/G12P[6]	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-tc/MWI/MAL88/2007/G12P[6]	G12	P[6][a,c]	I2	–	–	–	–	–	–	E2	–
RVA/Human-wt/TUA CU331-NR/2008/G12P[6]	G12	P[6]	I1	R1	C1	M1[a,b,c]	A1	N1	T1	E1	H1
RVA/Human-wt/UGA/MRC-DPRU3713/2010/G12P[6]	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1[a,c]	H1
RVA/Pig-wt/IND/RU172/2002/G12P[7]	G12	P[7]	I5	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/BGD Dhaka25-02/2002/G12P[8]	G12	P[8]	I1	R1[a,c]	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/BEL/B4633/2003/G12P[8]	G12	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/LKA/05SIC009/2005/G12P[8]	G12[b]	P[8]	–	–	–	–	–	–	–	–	–
RVA/Human-wt/IND/ISO125/2005/G12P[8]	G12[b]	P[8]	–	–	–	–	–	–	–	–	–
RVA/Human-wt/USA/VU05-06-72/2005/G12P[8]	G12	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/USA/VU05-06-74/2005/G12P[8]	G12	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/ARG/Arg6627/2008/G12P[8]	G12	P[8][b]	I1[b]	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Human-wt/USA/VU08-09-6/2008/G12P[8]	G12	P[8]	I1	R1	C1	M1	A1	N1	T1[b]	E1	H1
RVA/Human-wt/USA/VU08-09-39/2008/G12P[8]	G12	P[8][b]	I1	R1	C1[b]	M1	A1	N1	T1	E1	H1
RVA/Human-wt/USA/VU08-09-40/2008/G12P[8]	G12	P[8]	I1	R1	C1[b]	M1	A1	N1	T1	E1	H1H2
RVA/Human-wt/ARG/Arg7500/2009/G12P[8]	G12	P[8][b]	I1	R1	C1	M1	A1	N1	T1[b]	E1	H1
RVA/Human-wt/THA/CU460-KK/2009/G12P[8]	G12	P[8]	I1	R1	C1	M1[a,b,c]	A1	N1	T1	E1	H1
RVA/Human-tc/THA/T152/1998/G12P[9]	G12	P[9]	I3	R3	C3	M3	A12	N3	T3	E3	H6

Strains KDH633, KDH651, and KDH684 are shown in red.
Gray indicates the 10 gene segments (VP7, VP6, VP1-3, and NSP1-5) with genotypes identical to those of strains KDH633, KDH651, and KDH684.
Blue indicates the VP4 gene segment with a P[6] genotype identical to those of strains KDH633 and KDH684.
Green indicates the VP4 gene segments with a P[8] genotype identical to that of strain KDH651.
"–" indicates that no sequence data were available in the DDBJ and EMBL/GenBank data libraries.
[a]The gene segments that are most similar to those of strain KDH633.
[b]The gene segments that are most similar to those of strain KDH651.
[c]The gene segments that are most similar to those of strain KDH684.

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instructions. The extracted dsRNAs were used for (i) polyacrylamide gel electrophoresis (PAGE) analysis, and (ii) whole genomic analysis. For PAGE analysis, the dsRNAs were electrophoresed in a 10% polyacrylamide gel for 16 h at 20 mA at room temperature, followed by silver staining (Komoto et al., 2006) to determine the genomic dsRNA profiles. For whole genomic analysis, viral dsRNAs were subjected to RT-PCR and dideoxynucleotide sequencing as described below.

2.3. RT-PCR and dideoxynucleotide sequencing

Full-length nucleotide sequences excluding the 5′- and 3′- end primer sequences of all 11 segments of the three strains were determined from amplified cDNA products by RT-PCR with specific primers. The primers used for cDNA amplification of individual genes are listed in Supplementary Table S1. The RT-PCR was performed with ReverTra Ace (Toyobo) and Ex Taq HS (Takara Bio). The RT-PCR products obtained were cloned into the pCR4-TOPO vector using a TOPO TA cloning kit for sequencing (Invitrogen). Three clones for each gene were sequenced using an ABI PRISM 3730 DNA Analyzer (Life Technologies/Applied Biosystems). The sequencing was performed with the universal M13FW(-20) and M13RV primers annealing to the pCR4-TOPO vector. Primer walking sequencing was performed to cover the complete sequences of the VP1-4 genes. The nucleotide sequences were aligned and then translated into amino acid sequences using GENETYX Ver. 11 (GENETYX).

2.4. Determination of RVA genotypes

The genotype of each of the 11 gene segments of the three strains was determined using the RotaC v2.0 automated genotyping tool (http://rotac.regatools.be/) (Maes et al., 2009) according to the guidelines proposed by the Rotavirus Classification Working Group (RCWG).

2.5. Phylogenetic analyses

Multiple alignment of each viral gene was performed using CLUSTAL W (Thompson et al., 1994). Phylogenetic trees were con-

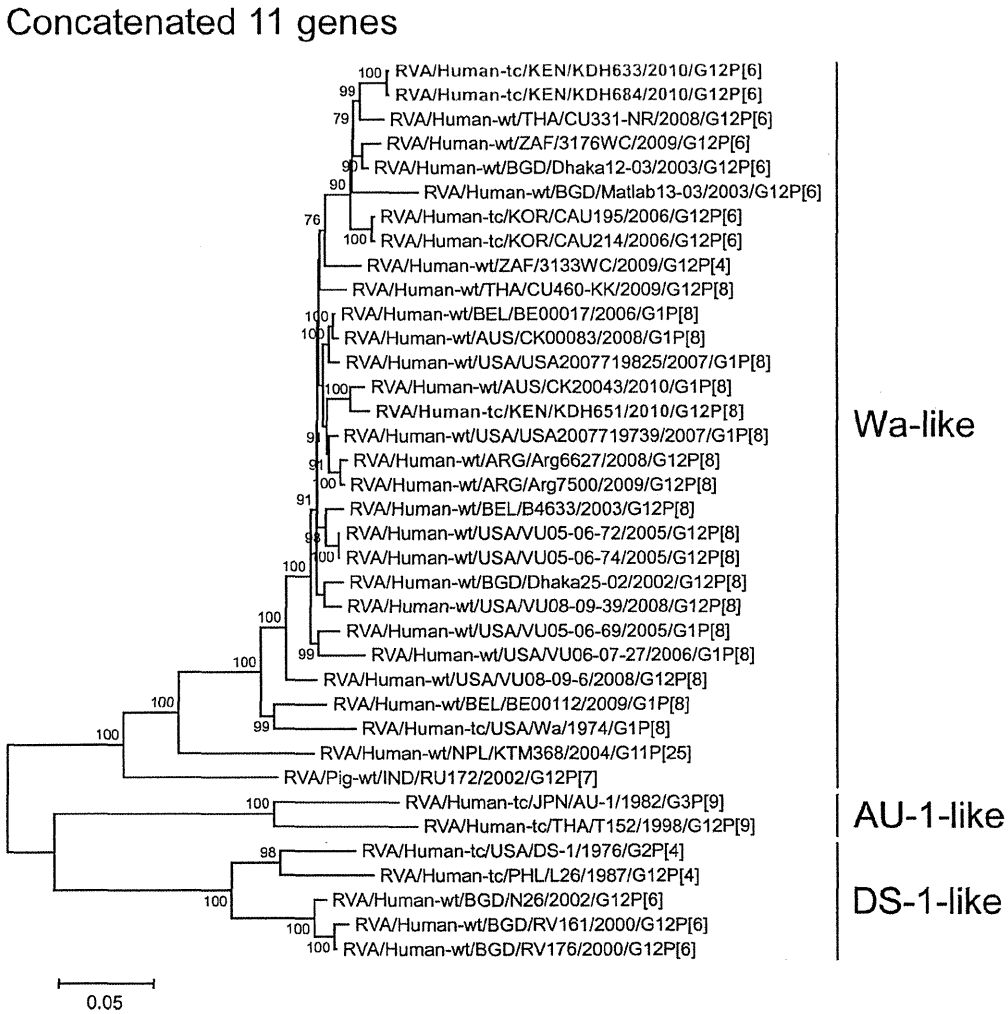

Fig. 2. Phylogenetic tree constructed from the concatenated nucleotide sequences of all the 11 genes of strains KDH633, KDH651, KDH684, and selected human and porcine RVA strains. In the concatenated tree, the positions of strains KDH633, KDH651, and KDH684 are shown in red. Bootstrap values of <75% are not shown. Scale bars, 0.05 substitutions per nucleotide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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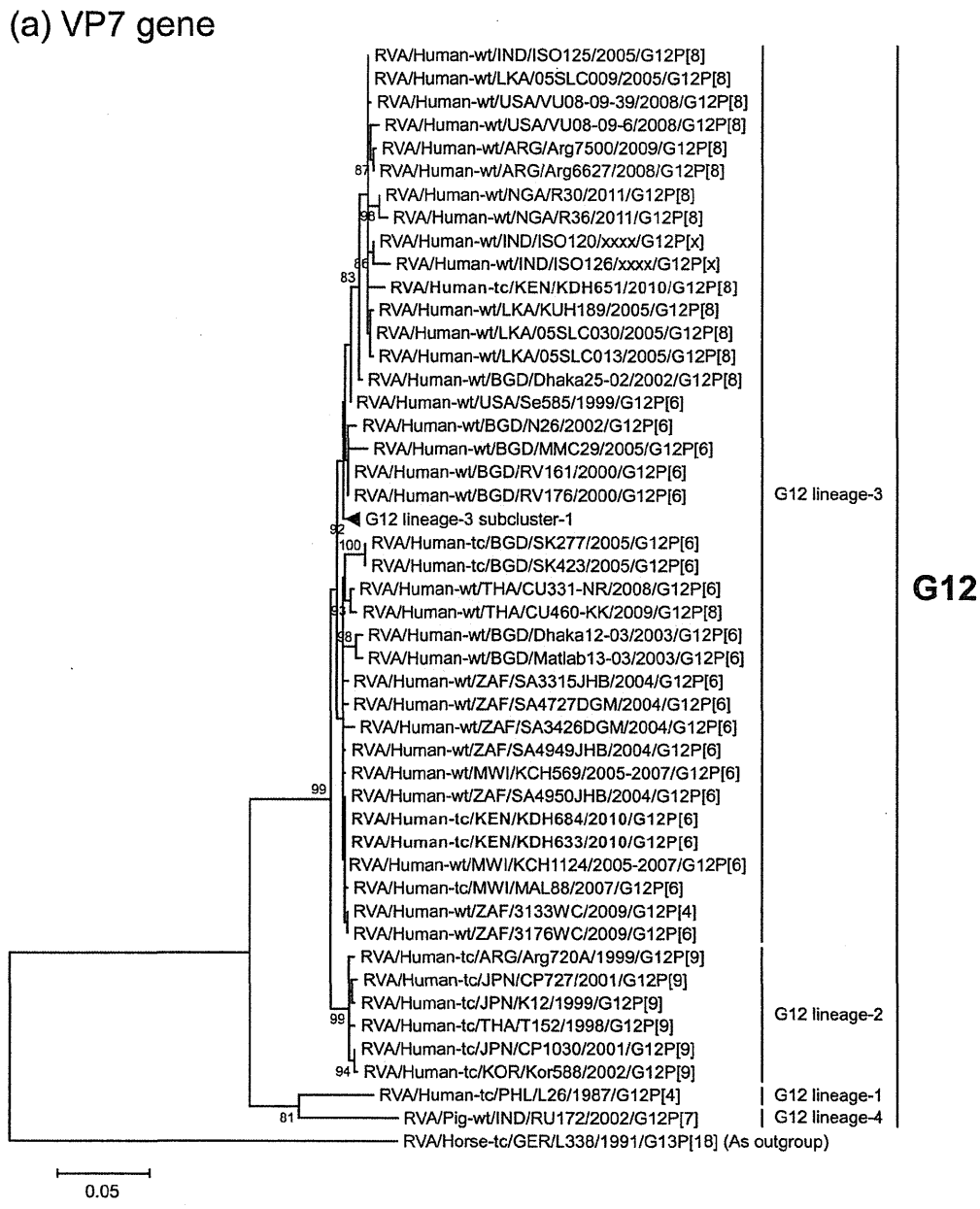

Fig. 3. Phylogenetic trees constructed from the nucleotide sequences of the VP7 (a), VP4 (b), VP6 (c), VP1 (d), VP2 (e), VP3 (f), NSP1 (g), NSP2 (h), NSP3 (i), NSP4 (j), and NSP5 (k) genes of strains KDH633, KDH651, KDH684, and representative RVA strains. In all the trees, the positions of strains KDH633, KDH651, and KDH684 are shown in red. Bootstrap values of <75% are not shown. Scale bars, 0.02 (e, h, j, and k) or 0.05 (a–d, f, g, and i) substitutions per nucleotide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

structed using the maximum likelihood method and the Hasegawa-Kishino-Yano substitution model using MEGA6.06 (Tamura et al., 2013). The reliability of the branching order was estimated from 1000 bootstrap replicates (Felsenstein, 1985). The results of phylogenetic analyses were validated using several other genetic distance models, such as Jukes-Cantor, Tamura 3-parameter, Tamura-Nei, and Kimura 2-parameter (data not shown).

2.6. Nucleotide sequence accession numbers

The nucleotide sequence data presented in this paper have been deposited in the DDBJ and EMBL/GenBank data libraries. The accession numbers for the nucleotide sequences of VP1-4, VP6-7, and NSP1-5 of strains KDH633, KDH651, and KDH684 are AB861945–AB861955, AB861956–AB861966, and AB861967–AB861977, respectively.

3. Results

3.1. Isolation of strains KDH633, KDH651, and KDH684 in cell culture

For molecular characterization of the emerging G12 strains in Kenya, we primarily attempted to isolate strains KDH633,

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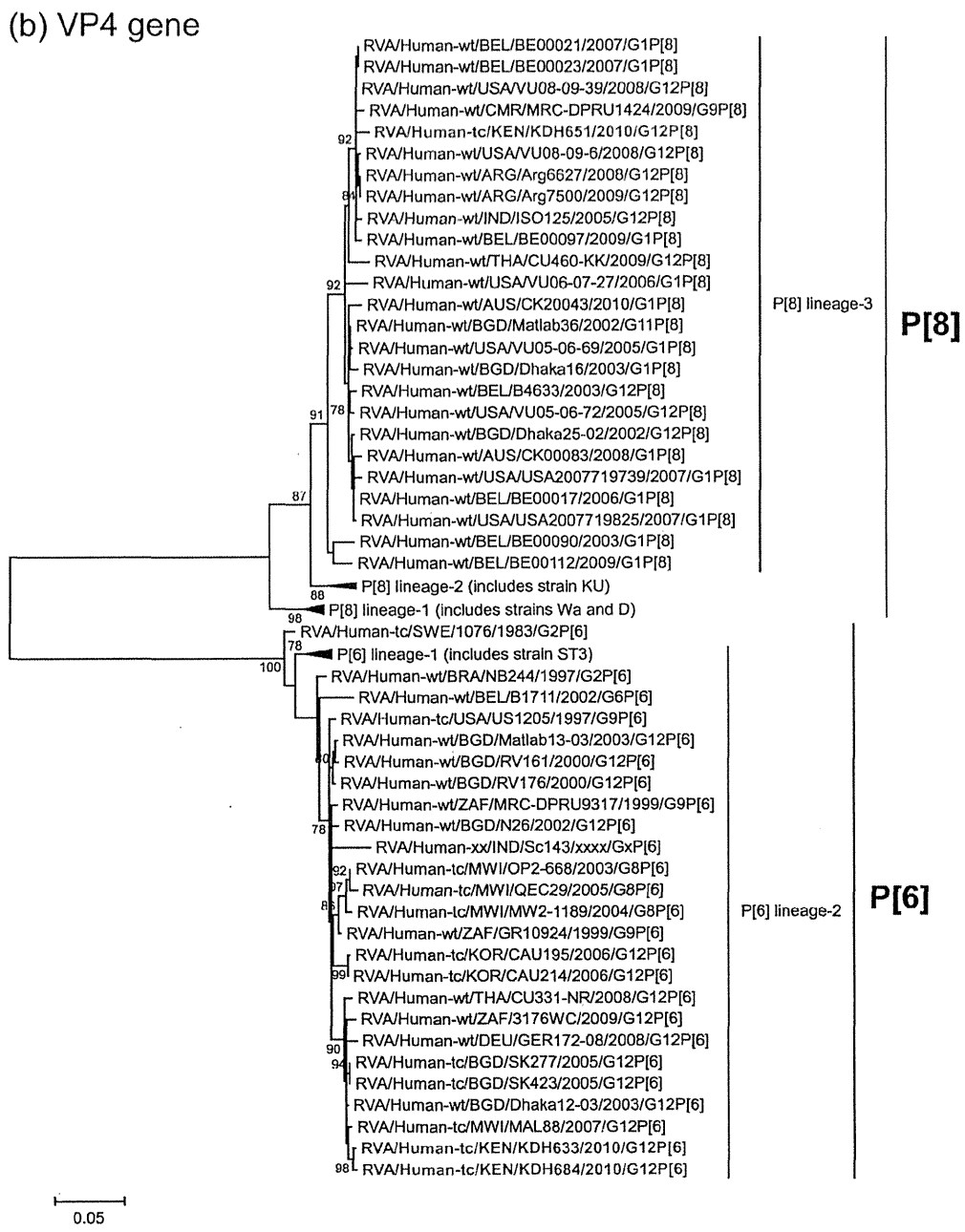

Fig. 3 (*continued*)

KDH651, and KDH684 using the MA104 cell line; all the three strains could be cell culture-adapted. Virion dsRNAs were extracted and then analyzed by PAGE. Fig. 1 shows the profiles of viral dsRNAs from human strain KU (G1P[8]) as a reference (lane 1), and strains KDH633 (lane 2), KDH651 (lane 3), and KDH684 (lane 4) from the cell cultures. They all showed a long electropherotype. Cell culture-adapted strains KDH633, KDH651, and KDH684 were named RVA/Human-tc/KEN/KDH633/2010/G12P[6], RVA/Human-tc/KEN/KDH651/2010/G12P[8], and RVA/Human-tc/KEN/KDH684/2010/G12P[6], respectively, according to the guidelines for the uniformity of RVAs proposed by the RCWG. Of note was that strains KDH633 and KDH684 showed an almost identical electropherotype, suggesting a close genetic relatedness between the two strains.

3.2. Nucleotide sequencing and whole-genome-based genotyping of strains KDH633, KDH651, and KDH684

In order to gain an insight into the genetic variability among strains KDH633, KDH651, and KDH684, and the genetic relatedness with other RVA strains worldwide, the full-length nucleotide sequences excluding the 5′- and 3′- end primer sequences

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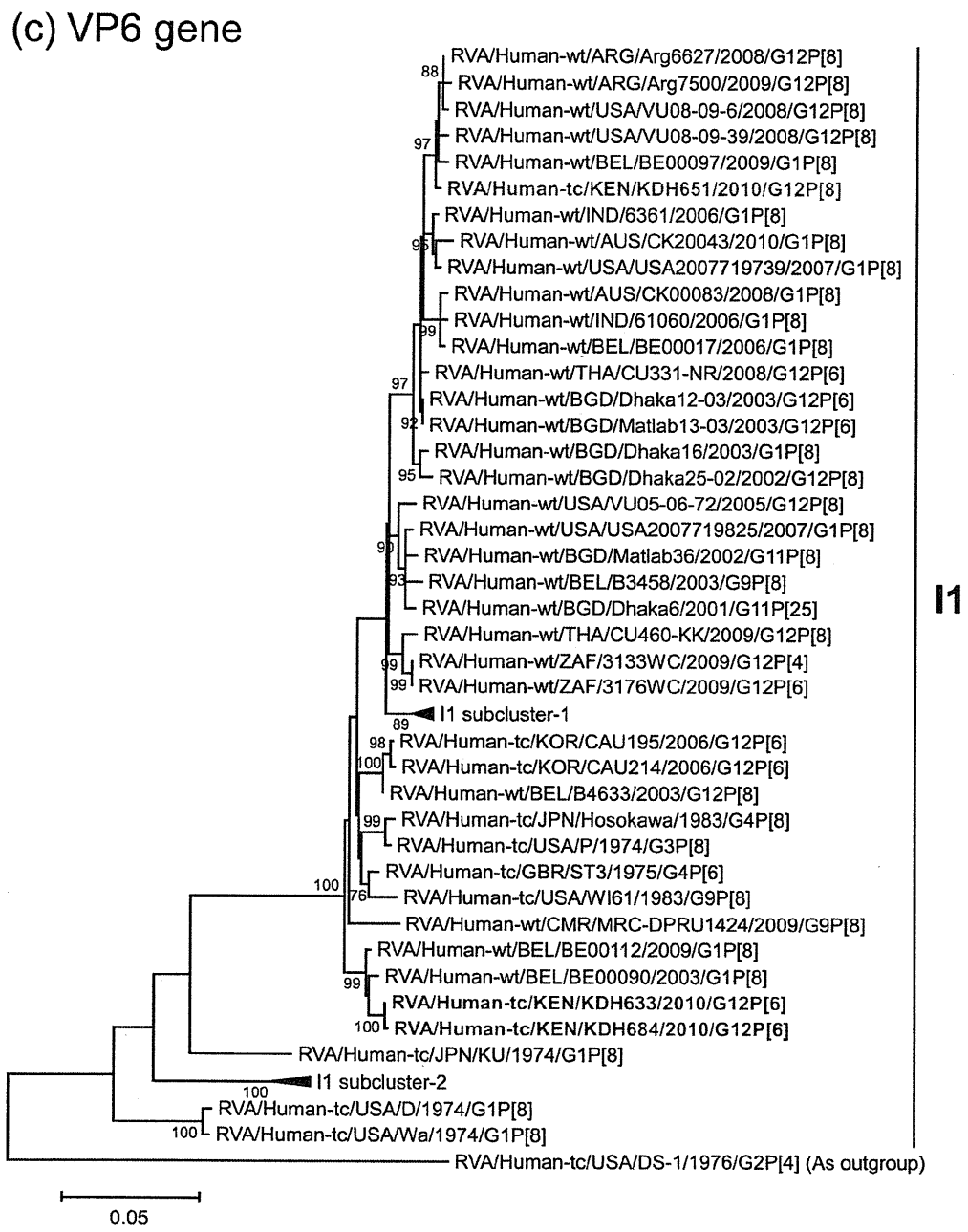

Fig. 3 (*continued*)

of all the 11 segments of these three strains were determined and genotyped. The 11 genes of strains KDH633, KDH651, and KDH684 were assigned as G12-P[6]-I1-R1-C1-M1-A1-N1-T1-E1-H1, G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1, and G12-P[6]-I1-R1-C1-M1-A1-N1-T1-E1-H1, respectively (Table 1). Strains KDH633, KDH651, and KDH684 were confirmed to have the G12P[6], G12P[8], and G12P[6] genotypes, respectively, as determined by PCR-based genotyping (Wandera Apondi et al., submitted for publication). Comparison of the complete genotype constellations of strains KDH633, KDH651, and KDH684 with those of other G12 and non-G12 strains is shown in Table 1. All the three Kenyan G12 strains exhibited typical Wa-like genotype constellations, which are commonly found in the G12 strains recently detected worldwide (Rahman et al., 2007). Furthermore, as suggested by the genomic dsRNA profiles observed on PAGE analysis (Fig. 1), strains KDH633 and KDH684 exhibited extremely high nucleotide sequence identities (99.6–100%) to each other for all the 11 gene segments (Supplementary Table S2). On the other hand, the nucleotide sequence similarities of the 11 gene segments of strain KDH651 to those of strains KDH633 and KDH684 were comparatively low (75.1–98.6%) (Supplementary Table S2).

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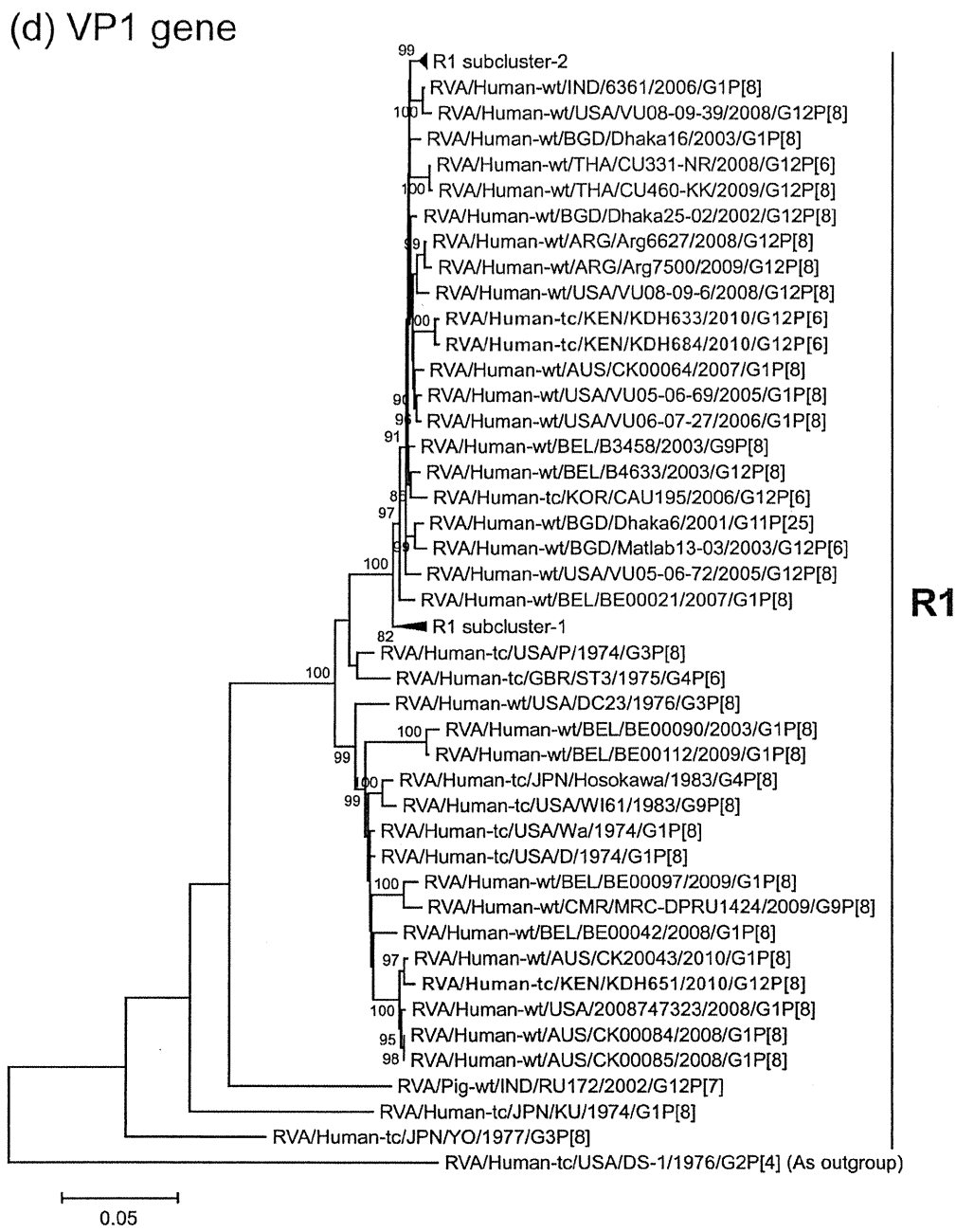

Fig. 3 (*continued*)

3.3. Phylogenetic analyses

In order to understand the whole genomic relatedness of strains KDH633, KDH651, and KDH684, and other representative RVA strains for which the whole genome data are available, phylogenetic tree was constructed using the concatenated open reading frame nucleotide sequences for each strain (Fig. 2). In the concatenated tree, strains KDH633, KDH651, and KDH684 were found to locate within the Wa-like subcluster. Specifically, strains KDH633 and KDH684 were closely related with Thai human strain CU331-NR (G12P[6]) (Khananurak et al., 2010) in a common branch with several human G12P[6] strains. In contrast, strain KDH651 was closely related with Australian human strain CK20043 (G1P[8]) in a common branch with several human G1 and G12 strains.

We next constructed phylogenetic trees using the full-genome sequence for each of the 11 gene segments because phylogenetic analysis of RVA nucleotide sequences provides direct evidence of their relatedness to those of other strains, even within the same genotype (Matthijnssens et al., 2008).

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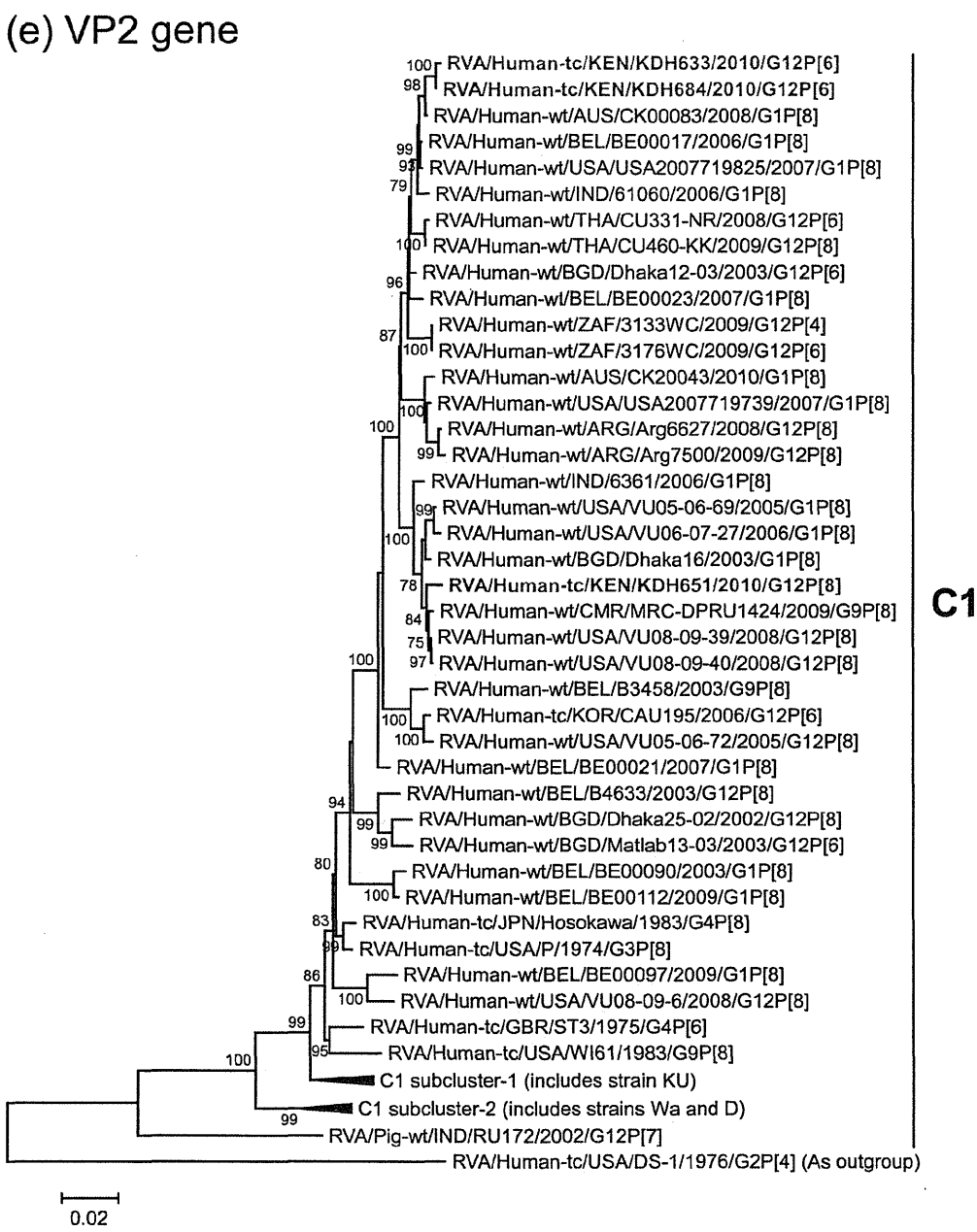

Fig. 3 (*continued*)

The VP7 genes of strains KDH633 and KDH684 exhibited the maximum nucleotide sequence identity (99.7%) with that of Malawian human strain KCH1124 (G12P[6]) (Cunliffe et al., 2009) (Table 1). On phylogenetic analysis, strains KDH633 and KDH684 clustered with strain KCH1124 and several G12P[6] strains from Africa in G12 lineage-3, in which the majority of globally circulating G12 strains cluster (Rahman et al., 2007; Matthijnssens et al., 2010a) (Fig. 3a). On the other hand, the VP7 gene of strain KDH651 showed the highest nucleotide sequence similarity (99.1%) with Sri Lankan human strain 05SLC009 (G12P[8]) (Ahmed et al., 2010) and Indian human strain ISO125 (G12P[8]) (Samajdar et al., 2008) (Table 1). Phylogenetically, strain KDH651 was found to form clusters near these and several G12P[8] strains from Asia in G12 lineage-3 (Fig. 3a).

The VP4 genes of strains KDH633 and KDH684 showed the highest nucleotide sequence similarities (99.1–99.2% and 99.0%, respectively) with the cognate genes of Malawian human strain MAL88 (G12P[6]) (Nakagomi et al., 2012), and Bangladeshi human strains (SK277 (G12P[6]) and SK423 (G12P[6])) (Paul et al., 2008) (Table 1). On phylogenetic analysis, strains KDH633 and KDH684 clustered near these and several human G12 strains from different countries of the world in P[6] lineage-2 (Fig. 3b). In contrast, the

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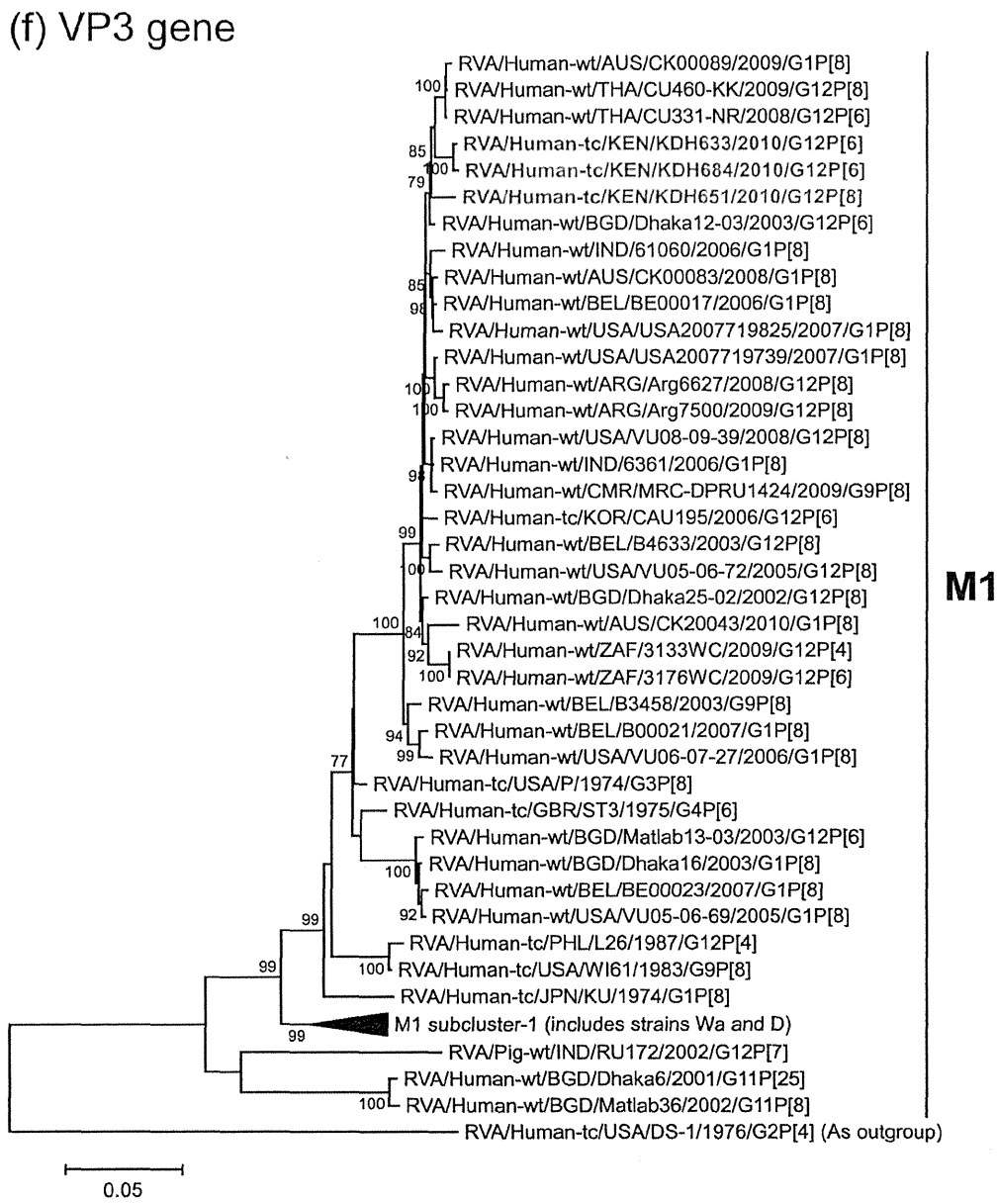

Fig. 3 (*continued*)

VP4 gene of strain KDH651 exhibited the maximum nucleotide sequence identity (98.9%) with American human strain VU08-09-39 (G12P[8]) (McDonald et al., 2012), and Argentinean human strains (Arg6627 (G12P[8]) and Arg7500 (G12P[8])) (Stupka et al., 2012) (Table 1). Phylogenetically, strain KDH651 was found to be clustered near these, and several human G1, G9, and G12 strains from different parts of the world in P[8] lineage-3 (Fig. 3b).

The VP6 genes of strains KDH633 and KDH684 exhibited the highest nucleotide sequence identities (99.1% and 99.0%, respectively) with the VP6 gene of Belgian human strain BE00112 (G1P[8]) (Table 1). On phylogenetic analysis, strains KDH633 and KDH684 were found to be closely related with strain BE00112 and Belgian human strain BE00090 (G1P[8]) (Fig. 3c). On the other hand, the VP6 gene of strain KDH651 showed the maximum nucleotide similarity (99.5%) with Argentinean human strain Arg6627 (G12P[8]) (Table 1). Phylogenetically, strain KDH651 was clustered with strain Arg6627, and several human G1 and G12 strains (Fig. 3c).

The VP1 genes of strains KDH633 and KDH684 showed the maximum nucleotide sequence identities (98.6% and 98.5–98.6%, respectively) with the cognate genes of American human strains (VU05-06-69 (G1P[8]) and VU06-07-27 (G1P[8])) (McDonald et al., 2012), and Bangladeshi human strain Dhaka25-02 (G12P[8]) (Rahman et al., 2007) (Table 1). On phylogenetic analysis, strains KDH633 and KDH684 were found to be clustered near these, and several human G1 and G12 strains (Fig. 3d). On

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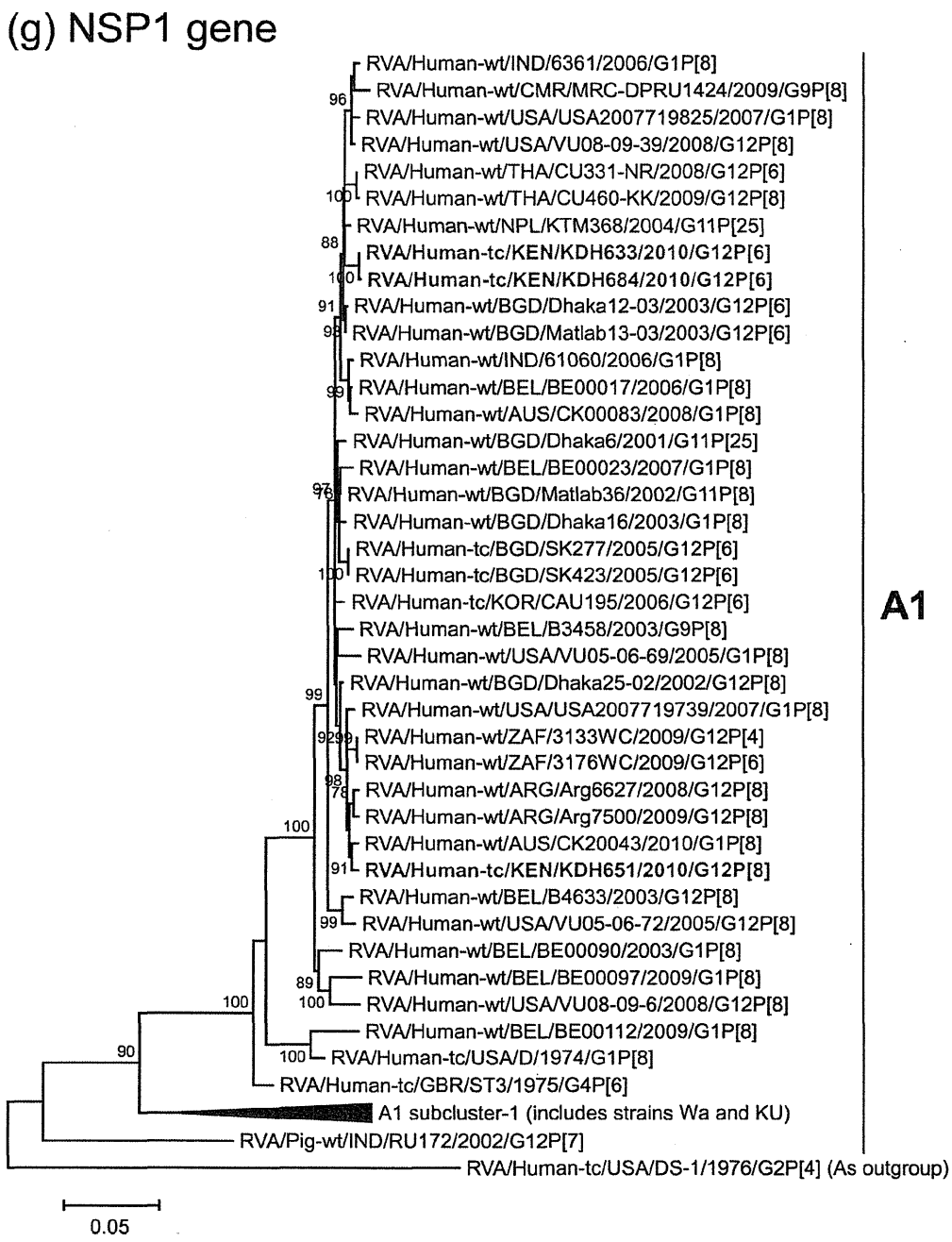

Fig. 3 (*continued*)

the other hand, the VP1 gene of strain KDH651 exhibited the highest nucleotide sequence similarity (99.4%) with Australian human strain CK20043 (G1P[8]) (Table 1). Phylogenetically, strain KDH651was closely related with strain CK20043 in a common branch with Australian human strains (CK00084 (G1P[8]) and CK00085 (G1P[8])), and American human strain 2008747323 (G1P[8]) (Fig. 3d).

The VP2 genes of strains KDH633 and KDH684 showed the highest nucleotide sequence similarities (99.4 and 99.5%, respectively) with the VP2 gene of Australian human strain CK00083 (G1P[8]) (Table 1). On phylogenetic analysis, strains KDH633 and KDH684 were found to be closely related with strain CK00083 in a common branch with several human G1 strains (Fig. 3e). In contrast, the VP2 gene of strain KDH651 exhibited the maximum nucleotide sequence identity (99.2%) with American human strains VU08-09-39 (G12P[8]) and VU08-09-40 (G12P[8]) (McDonald et al., 2012) (Table 1). On phylogenetic analysis, strain KDH651 was found to be clustered with these strains and Cameroonian human strain MRC-DPRU1424 (G9P[8]) (Nyaga et al., 2013) (Fig. 3e).

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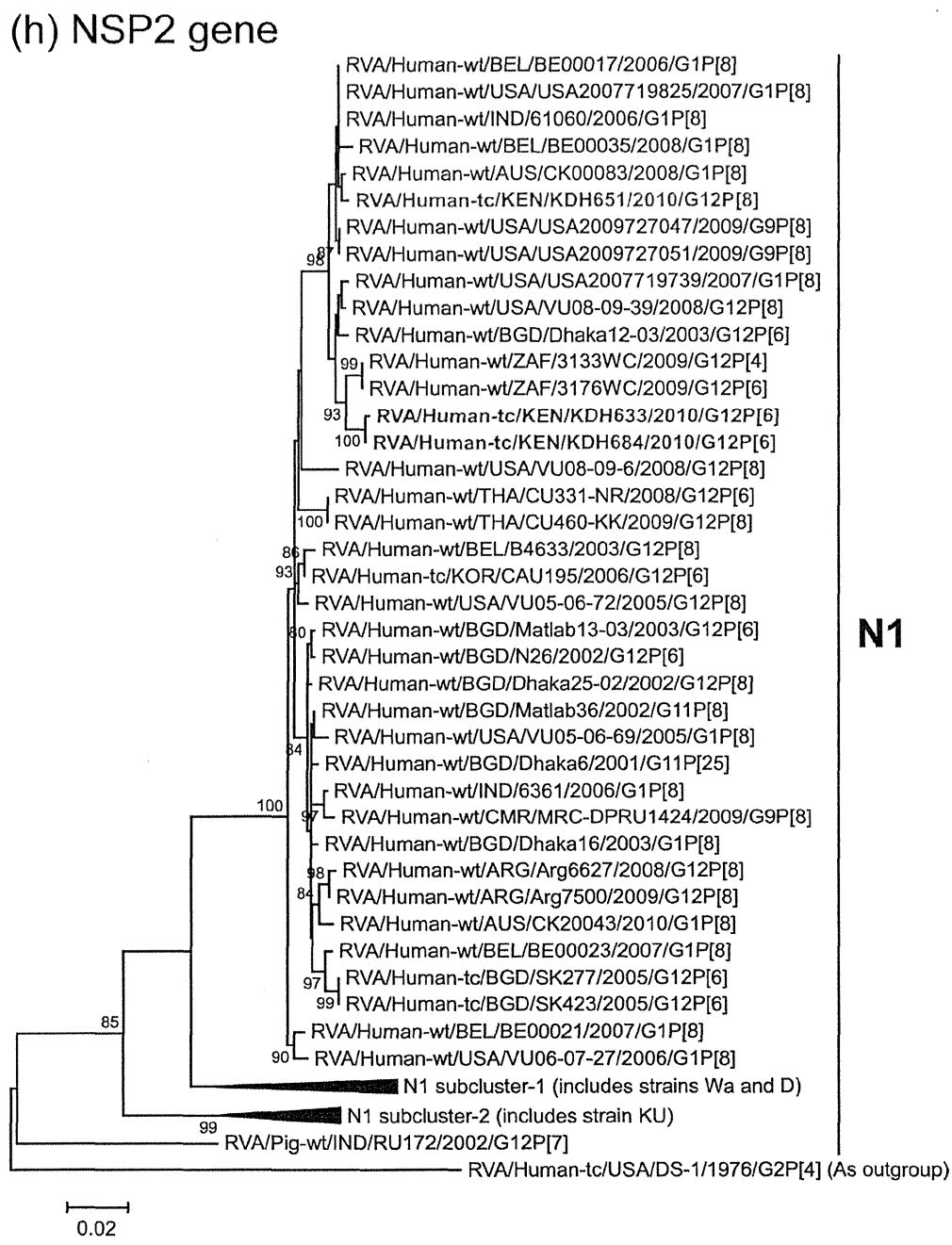

Fig. 3 (*continued*)

All of the VP3 genes of strains KDH633, KDH651, and KDH684 showed the maximum nucleotide sequence identities (98.6%, 98.5–98.6%, and 98.5%, respectively) with those of Bangladeshi human strain Dhaka12-03 (G12P[6]) (Rahman et al., 2007), and Thai human strains (CU331-NR (G12P[6]) and CU460-KK (G12P[8]) (Khananurak et al., 2010)) (Table 1). On phylogenetic analysis, strains KDH633, KDH651, and KDH684 were found to form a cluster with these strains, and a very close relationship was observed between strains KDH633 and KDH684 (Fig. 3f).

The NSP1 genes of strains KDH633 and KDH684 exhibited the highest nucleotide sequence identities (98.9–99.0% and 98.9%, respectively) with the cognate genes of Bangladeshi human strains (Matlab13-03 (G12P[6]) (Rahman et al., 2007) and Dhaka12-03 (G12P[6])), and Nepalese human strain KTM368 (G11P[25]) (Matthijnssens et al., 2010b) (Table 1). On phylogenetic analysis, strains KDH633 and KDH684 were found to be clustered near these strains (Fig. 3g). On the other hand, the NSP1 gene of strain KDH651 showed the maximum nucleotide sequence similarity

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Fig. 3 (*continued*)

(99.3%) with Australian human strain CK20043 (G1P[8]) (Table 1). Phylogenetically, strain KDH651 was closely related with strain CK20043 in a common branch with Argentinean human strains Arg6627 (G12P[8]) and Arg7500 (G12P[8]) (Fig. 3g).

The NSP2 genes of strains KDH633 and KDH684 exhibited the maximum nucleotide identities (98.7–98.8% and 98.8–98.9%, respectively) with those of South African human strains 3133WC (G12P[4]) and 3176WC (G12P[6]) (Table 1). On phylogenetic analysis, strains KDH633 and KDH684 were found to be closely related with these strains (Fig. 3h). On the other hand, the NSP2 gene of strain KDH651 showed the highest nucleotide sequence identity (99.7%) with Australian human strain CK00083 (G1P[8]), Belgian human strain BE00017 (G1P[8]), and American human strain USA2007719825 (G1P[8]) (Table 1). Phylogenetically, strain KDH651 was found to be clustered with these, and several human G1 and G9 strains (Fig. 3h).

The NSP3 genes of strains KDH633 and KDH684 showed the highest nucleotide sequence identities (99.0% and 98.7%, respectively) with the NSP3 genes of South African human strains (3133WC (G12P[4]) and 3176WC (G12P[6])), and Bangladeshi human strain Dhaka12-03 (G12P[6]) (Table 1). On phylogenetic analysis, strains KDH633 and KDH684 were found to be closely related with strains 3133WC and 3176WC in a common branch with strain Dhaka12-03 (Fig. 3i). In contrast, the NSP3 gene of strain KDH651 exhibited the maximum nucleotide sequence identity (99.3%) with American human strain VU08-09-6 (G12P[8]) and Argentinean human strain Arg7500 (G12P[8]) (Table 1). On phylogenetic analysis, strain KDH651 was found to cluster near these and several human G1 strains (Fig. 3i).

The NSP4 genes of strains KDH633 and KDH684 showed the maximum nucleotide sequence similarity (99.7%) with the cognate gene of Ugandan human strain MRC-DPRU3713 (G12P[6]) (Table 1),

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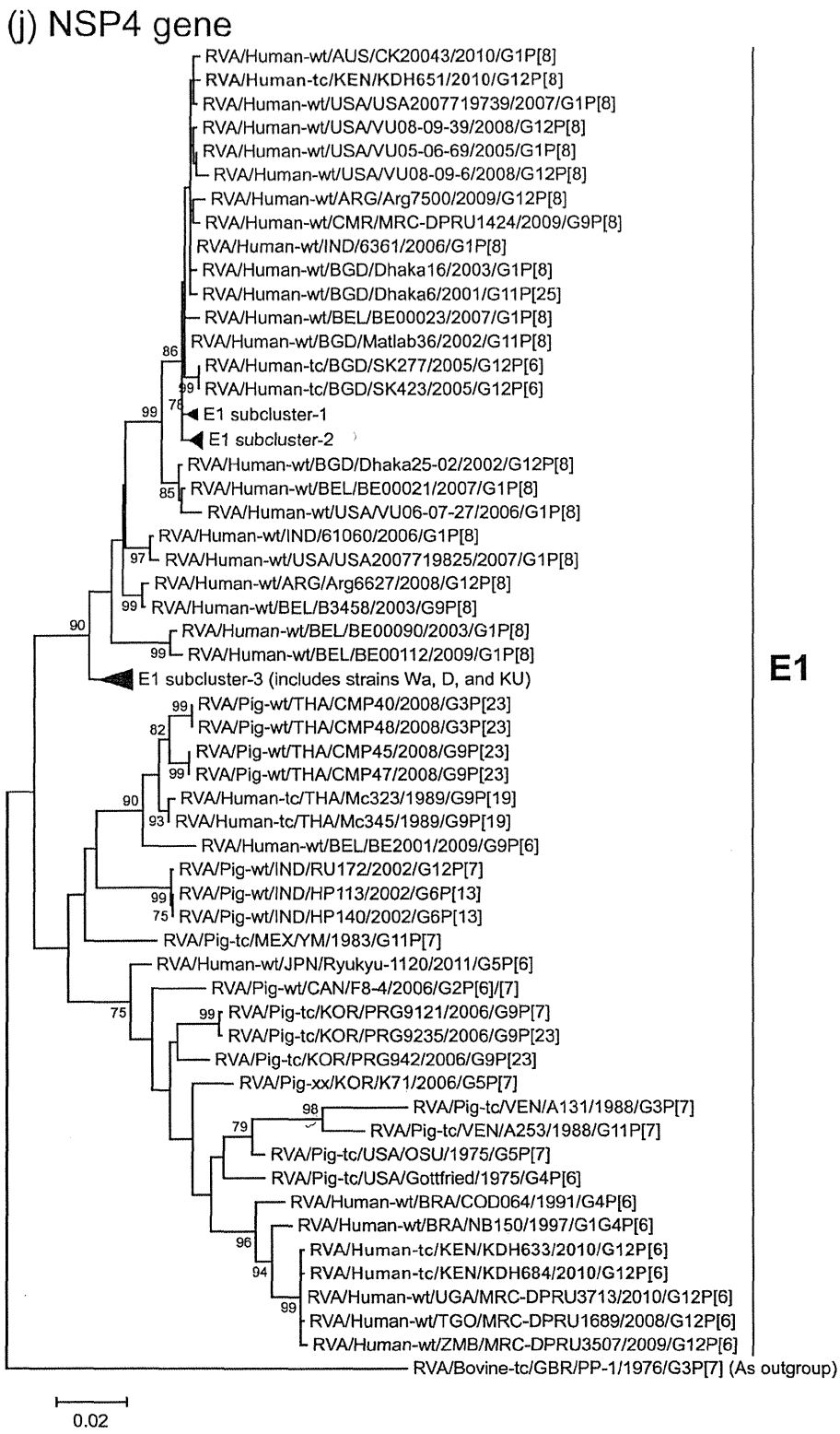

Fig. 3 (*continued*)

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Fig. 3 (*continued*)

and comparable similarity (99.4%) with Togolese human strain MRC-DPRU1689 (G12P[6]) and Zambian human strain MRC-DPRU3507 (G12P[6]). On phylogenetic analysis, strains KDH633 and KDH684 were found to be closely related with these human G12P[6] strains from Africa in a common branch with Brazilian porcine-like human strains NB150 (G1G4P[6]) (Mascarenhas et al., 2007a; Maestri et al., 2012) and COD064 (G4P[6]) (Mascarenhas et al., 2007b) within the porcine-like E1 subcluster (Fig. 3j). In contrast, the NSP4 gene of strain KDH651 exhibited the highest nucleotide sequence identity (99.4%) with American human strain USA2007719739 (G1P[8]) (Table 1). Phylogenetically, strain KDH651 was found to cluster with strain USA2007719739 and several human G1 strains within the human-like E1 subcluster (Fig. 3j).

The NSP5 genes of strains KDH633 and KDH684 exhibited complete nucleotide sequence identity (100%) (Supplementary Table S2), and the highest nucleotide sequence similarity (99.8%) with that of American human strain USA2007719825 (G1P[8]) (Table 1). On phylogenetic analysis, strains KDH633 and KDH684 were found to be clustered near strain USA2007719825, and several human G1 and G12 strains (Fig. 3k). On the other hand, the NSP5 gene of strain KDH651 showed the maximum nucleotide sequence similarity (99.6%) with Australian human strain CK20043 (G1P[8])

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