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SHORT REPORT
Molecular characterization of SAT 2 foot-and-mouth disease virus from post-outbreak slaughtered animals: implications for disease control in Uganda

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SUMMARY

In Uganda, limiting the extent of foot-and-mouth disease (FMD) spread during outbreaks involves short-term measures such as ring vaccination and restrictions of the movement of livestock and their products to and from the affected areas. In this study, the presence of FMD virus RNA was investigated in cattle samples 3 months after FMD quarantine measures had been lifted following an outbreak in 2004. Oropharyngeal tissue samples were obtained from 12 cattle slaughtered in a small town abattoir in Kiboga. FMD virus RNA was detected by diagnostic RT–PCR in nine of the 12 tissue samples. Part of the coding region for the capsid protein VP1 was amplified and sequenced. All samples were identified as belonging to the SAT 2 serotype. The implications for FMD control of both virus introduction into Uganda and the presence of carrier animals following outbreaks are discussed.

Key words: FMD control, SAT 2, sequence divergence.

Foot-and-mouth disease virus (FMDV) belongs to the genus *Aphthovirus* within the family Picornaviridae and is the causative agent of foot-and-mouth disease (FMD), a highly contagious infection of cloven-hoofed animals. Disease spread is mainly through direct and indirect contact, the former involving mechanical transfer of droplets from infected animals to other susceptible animals while the latter route is through contaminated personnel, vehicles and all classes of fomities [1]. Airborne transmission over long distances has been implicated under certain climatic and meteorological conditions particularly in respect of domestic pigs that exhale the highest quantities of airborne virus [2]. This is easily passed onto ruminants that are highly susceptible to infection by the respiratory route. In recent years, FMD has crossed international borders to areas that were previously considered FMD free, as was the case in Japan and UK in 2000 and 2001, respectively. The economic impact experienced by affected countries is usually in terms of reduction in animal production, costs of disease control and restrictions to trade at both local and international levels resulting in reduced GDP. Grave losses can be experienced especially by FMD-free countries as was the case in the UK where a total cost of over £3 billion [3] was incurred in 2001.

In Uganda, FMD control has mainly been through the use of ring vaccination, zoosanitary measures, restrictions on the movement of livestock and livestock

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products including the suspension of slaughtering within abattoirs in affected areas. Despite this, the level of success in containing the disease has been limited with the number of FMD outbreaks increasing; 25–38 reported per year between 2000 and 2006 in contrast to 1–15 between 1996 and 1999 [4]. The persistence and increase in FMD outbreaks could be, amongst other things, attributed to farming systems, e.g. communal grazing and pastoralism that are still practised by some communities. Wildlife, particularly the African buffalo (*Syncerus caffer*), have also been suggested to play a significant role in the epidemiology of the disease in Uganda [5]. Trans-boundary animal movements within the region may also contribute to the increased number of outbreaks that are reported. Last, current control measures may be inadequate, especially with respect to enforcement and, as such, could require further strengthening.

In this study, we have performed molecular characterization of recently circulating viruses and discuss the implications for FMD control of both virus introductions and the presence of carrier animals in Uganda.

Twelve clinically normal cattle from different farms that had been slaughtered in an abattoir were sampled and oropharyngeal tissues were collected in the Kiboga district around 3 months after quarantine measures, imposed during an outbreak, were lifted. These tissues were stored in RNAlater (Ambion, USA) at ambient temperature until they could be frozen at −80 °C in the laboratory. Subsequently, the 12 tissue samples (500 mg) were ground using sterile sand, re-suspended in phosphate-buffered saline (PBS, 500 μl) and clarified by centrifugation at 3000 rpm for 5 min. The resultant supernatant was frozen in aliquots.

RNA was extracted directly from 140 μl of the 12 thawed supernatant samples using the QIAamp® Viral RNA kit (Qiagen, Germany) according to the manufacturer's instructions. The cDNA synthesis was carried out using Ready-To-Go™ You-Prime First-Strand beads (GE Healthcare Life Sciences, Sweden), using random hexamer (pdN_6) primers. A standard PCR, targeting cDNA sequences corresponding to the 5′-untranslated region of the FMDV RNA, used two forward primers:

multi-II(F): 5′-CAC(T/C)T(T/C)AAG(G/A)TGACA-(T/C)TG(G/A)TACTGGTAC-3′,
multi-IISAT(F): 5′-CAC(T/C)T(T/C)AAG(G/A)-TAACA(T/C)TG(G/A)GACTGGTAC-3′

and a single reverse primer

multi-II(R-1): 5′-CAGAT(C/T)CC(G/A)AGTG(T/A)-C(I)TGTT-3′

in an 18 μl reaction volume to identify the presence or absence of FMDV cDNA in the samples [6]. Nine of the tissue samples were positive in this assay and produced a clear fragment of 96 bp (data not shown). To identify the virus serotype present within the positive samples, two types of PCR experiments using serotype-specific primers for O and SAT types were performed. The PCRs were performed using primers designed to amplify part of the VP1 capsid protein-coding sequence. Two primer sets, 8-A-PN 84 (TACTACACCCAGTACAGCG) with 8-A-PN-85 (GGAGCACCCGAAGCTGCA) and 8-A-PN 98 (GCATCCACTTACTACTTTGC) with 8-A-PN-64 (GGAGATGTGGAGTCCAACC) designed for serotype O yielded no product.

For the SAT types, the partial FMDV VP1-coding region was amplified using the forward primer SAT-1D209F (5′-CCACATACTACTTTTGTGACCTG-GA-3′) which binds within the VP1-coding region at nucleotide position 209–234, and a reverse primer P1 (5′-GAAGGGCCCAGGGTTGGACTC-3′) targeting the conserved 2A/2B junction [7]. These PCR reactions were performed in a final volume of 50 μl using 2–5 ng cDNA, 0·2 pmol of each primer and 2·5 U AmpliTaq gold DNA polymerase (Applied Biosystems, USA), 200 μM of each dNTP (dATP, dCTP, dGTP, dTTP) and 1·5 mM $MgCl_2$. Following the activation of AmpliTaq gold DNA polymerase at 95 °C for 5 min, reaction mixtures were heated to 95 °C for 15 s followed by 60 °C for 2 min to allow for primer annealing. For each cycle, a chain elongation step at 72 °C for 1 min 20 s was allowed. This process was repeated 30 times with a final extension at 72 °C for 5 min. The PCR products (expected size 496 bp) were analysed on 2% agarose gel electrophoresis using a molecular-weight marker ΦX174-RF DNA (Amersham Biosciences, UK). Purification of the PCR products to remove excess oligonucleotide primers, dNTPs and enzyme was performed using a QIAquick® PCR purification kit (Qiagen, Germany). Cycle sequencing was performed for both forward and reverse strands (using the same primers as employed in the PCRs) using the Big Dye Terminator version 3.1 kit (Applied Biosystems) and run on an automated DNA sequencer (ABI Prism® 3700) by Macrogen in Korea.

Sequencher software 4.8 (Gene Code Corporation, USA) was used to analyse the forward and reverse

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strands, resulting in sequence overlaps of 88%. Using BLAST (http://blast.ncbi.nlm.nih.gov), the partial VP1-coding sequences identified all nine FMDV samples as belonging to the SAT 2 serotype with relatedness values ranging between 84% and 90% to SAT 2 sequences present within the Genbank database [accession numbers are indicated by a dagger (†) in Table 1 at 86–87% query coverage].

The partial coding sequence of VP1 corresponded to amino-acid residues 101–215 (that resulted from 347 bp characterized out of the 496 bp) allows the direct evaluation of relatedness among virus strains [7]. Multiple alignments of these sequences, together with known sequences from GenBank, were carried out using MUSCLE by log-expectation comparison incorporated within Geneious 4.7.6 software [8]. Phylogenetic analyses involving the determination of models of evolution were performed using hierarchical likelihood-ratio tests of 24 models using PAUP* (v. 4.0 beta 10) and MrModeltest (v. 2.2). The SYM + I + G model was used and Bayesian inference analysis performed using MrBayes (v. 3.1.2) with the settings below incorporated: maximum-likelihood model was six substitution types (NST = 6), with base frequencies equally fixed; STATEFREQPR = fixed (equal). Rate variation across sites with a proportion of invariable sites was modelled using a gamma distribution (rates = INVGAMMA). The Markov Chain Monte Carlo search was run with four chains for 500 000 generations with trees sampled every 100 generations, the first 1250 were discarded as burn-in [9].

Figure 1 shows the inferred phylogenetic relationships between these Kiboga isolates and other SAT 2 virus sequences previously deposited in Genbank (Table 1). The Kiboga 2004 samples are very similar to each other with an overall sequence divergence of only 4·6%. These strains were also closely related to viruses obtained previously in Kenya (1984, 1999), Tanzania (1975 and 1986), Ethiopia (1990 and 1991) and Malawi (1975) with a sequence divergence of 9·2%. However, these viruses are quite distinct from some other SAT 2 isolates from Uganda previously isolated in the years 1975, 1995, 1998 and 2002. These belong to a different lineage with the greatest similarity to viruses obtained from Zaire (1982), Eritrea (1998), Rwanda (2002) and Sudan (1977) with an average group sequence divergence of 23·6%. Furthermore, the mean sequence divergence between these earlier Ugandan strains and the recent strains from Kiboga (2004) was determined as 26·7%.

Figure 2 shows the sequence alignment of the variable nucleotide positions and corresponding amino acids within this partial VP1 capsid protein-coding region. The alignment is comprised of sequences from samples from the Kiboga district only. It should be noted that all of the recent Ugandan samples have been sequenced directly from the RNA extracted from animal tissues without any cell culture propagation of the viruses. Out of the 347 nucleotide bases characterized for each sample, a total of 44 nucleotide substitutions across all the Kiboga genotypes were identified. Of these changes, ten, four and 30 were at first, second and third codon positions, respectively (Fig. 2). Substitutions at 14 codon positions resulted in amino-acid changes with three of these positions (residues 112, 175 and 202) undergoing more than one change (Fig. 2). The amino-acid variations did not affect the integrin receptor-binding site motif 'RGDRAVL' within the VP1 GH loop. SAT 2 viruses are characterized by the presence of a RGDR motif within this loop including a positively charged arginine (R) residue in contrast to the RGDL with a non-polar leucine (L) residue in most other serotypes [10]. With the exceptions of amino acids at positions 139 and 140 (Fig. 2), that were found to be either glutamate (E) or aspartate (D), and glutamine (Q) or arginine (R), respectively, among the isolates, the entire GH loop region (amino-acid positions 135–161) was relatively conserved. The carboxy-terminal region (amino-acid positions 193–215) of VP1 exhibited rather more sequence variation in these isolates (see Fig. 2).

Phylogenetic analysis showed that the Kiboga 2004 isolates belong to a single lineage with an average sequence divergence of 4·6%, which falls within the range of 2–5%, consistent with origin from within the same epizootic [11]. Substitutions in nucleotide bases were reflected in changes in amino-acid sequence, some of which resulted in variations within the GH loop and carboxy-terminus region of VP1, these are important surface-exposed regions of this capsid protein which contribute to the antigenic characteristics of the virus. The sequence of the UGA/05/2004 sample predicted the loss of an amino-acid residue from within the C-terminal region of VP1 (see Fig. 2). Some earlier studies have shown that differences in genetic sequences within the same serotype do not necessarily show antigenic variation [12]. However, even limited genetic variation within regions corresponding to antigenic sites can alter the antigenic properties of FMD viruses [13]. The Kiboga 2004 viruses differ from the earlier SAT 2 viruses that have

Table 1. *Summary of SAT 2 FMD virus sequences used in the study*

Virus name	Country of origin	Year of sampling	GenBank accession no.
TAN/1/1975	Tanzania	1975	AY343970†
MAL/3/1975	Malawi	1975	AF367099†
UGA/51/1975	Uganda	1975	AY343963
BOT/3/1977	Botswana	1977	AY254712
SUD/6/1977	Sudan	1977	AY343939
SUD/9/1977	Sudan	1977	AY442014
ZAI/1/1982	Zaire	1982	AF367100
K52/1984	Kenya	1984	Sangula *et al.*, unpublished
TAN/1/1986	Tanzania	1986	AY343971†
ZIM/1/1988	Zimbabwe	1988	AF367108
KNP/7/1988	Kruger National Park, SA	1988	AF367103
SWA/4/1989	Swaziland	1989	AY254717
ZIM/7/1989	Zimbabwe	1989	AY254718
KNP/2/1989	Kruger National Park, SA	1989	AF367109
ZIM/14/1990	Zimbabwe	1990	AY254719
ETH/1/1990	Ethiopia	1990	AY343935†
NAM/1/1991	Namibia	1991	AY254720
ZIM/16/1991	Zimbabwe	1991	AY254722
BUN/1/1991	Burundi	1991	AF367111†
KNP/160/1991	Kruger National Park, SA	1991	AF137011
ETH/1/1991	Ethiopia	1991	AY343937†
KNP/32/1992	Kruger National Park, SA	1992	AF137013
KNP/9/1993	Kruger National Park, SA	1993	AF137014
ZIM/8/1994	Zimbabwe	1994	AY254725
ZIM/7/1995	Zimbabwe	1995	AY254727
KNP/31/1995	Kruger National Park, SA	1995	AF137017
KNP/46/1995	Kruger National Park, SA	1995	AY254726
UGA/9/1995	Uganda	1995	AY343967
ZAM/8/1996	Zambia	1996	AY254728
KNP/24/1996	Kruger National Park, SA	1996	AF137019
KNP/6/1996	Kruger National Park, SA	1996	AF137018
ZIM/1/1997	Zimbabwe	1997	AF136981
ZIM/4/1997	Zimbabwe	1997	AF136982
UGA/19/1998	Uganda	1998	AY343969
ERI/12/1998	Eritrea	1998	AF367126
UGA/28/1998	Uganda	1998	AY343968
KEN/6/1999	Kenya	1999	AY254729
KEN/8/1999	Kenya	1999	AY254730
RWA/1/2000	Rwanda	2000	AF367134
UGA/02/2002	Uganda	2002	DQ009731
UGA/01/2004	Uganda	2004	Present study
UGA/03/2004	Uganda	2004	Present study
UGA/02/2004	Uganda	2004	Present study
UGA/08/2004	Uganda	2004	Present study
UGA/11/2004	Uganda	2004	Present study
UGA/12/2004	Uganda	2004	Present study
UGA/07/2004	Uganda	2004	Present study
UGA/09/2004	Uganda	2004	Present study
UGA/05/2004	Uganda	2004	Present study

SA, South Africa.

† GenBank sequences with 84–90% similarity to Kiboga (2004) viruses at 86–87% query coverage.

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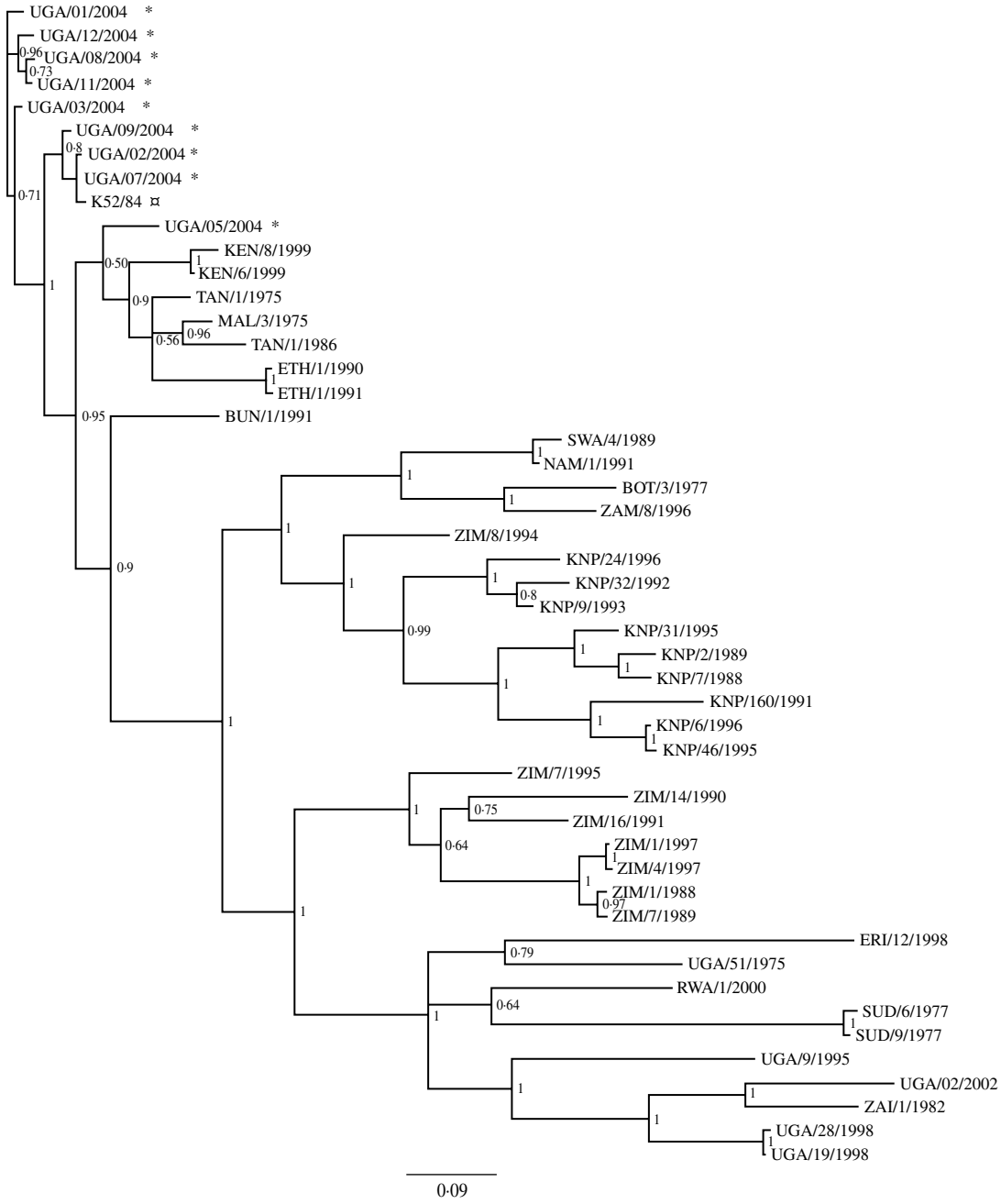

Fig. 1. Phylogenetic relationship of 47 partial VP1 sequences in the study. Genetic relationships of SAT 2-type FMD viruses from East and Southern Africa were derived from partial VP1-coding sequences (347 nt). The tree was constructed using Bayesian inference analysis (MrBayes) with the SYM + I + G model of nucleotide substitution, rate variation across sites and a proportion of invariable sites. Asterisks (*) indicate recent SAT 2 (2004) viruses from Kiboga.

been characterized from within Uganda with a sequence divergence of 26·7%, a value greater than the 20% level which indicates that they belong to different lineages that have evolved independently [14]. The recent SAT 2 viruses from Uganda and those from the neighbouring countries of Kenya, Tanzania, Ethiopia and Malawi belong to a single lineage with an average sequence divergence of 9·2%, a value $<20\%$. This suggests close similarity and provides evidence consistent with trans-boundary movement of the disease in the region. Indeed, uncontrolled animal movement even at border points is probably responsible for a significant proportion of outbreaks known to have occurred in Uganda.

The SAT 2 vaccine strain, K52/84, one of the vaccines used in Uganda to combat the disease, mainly

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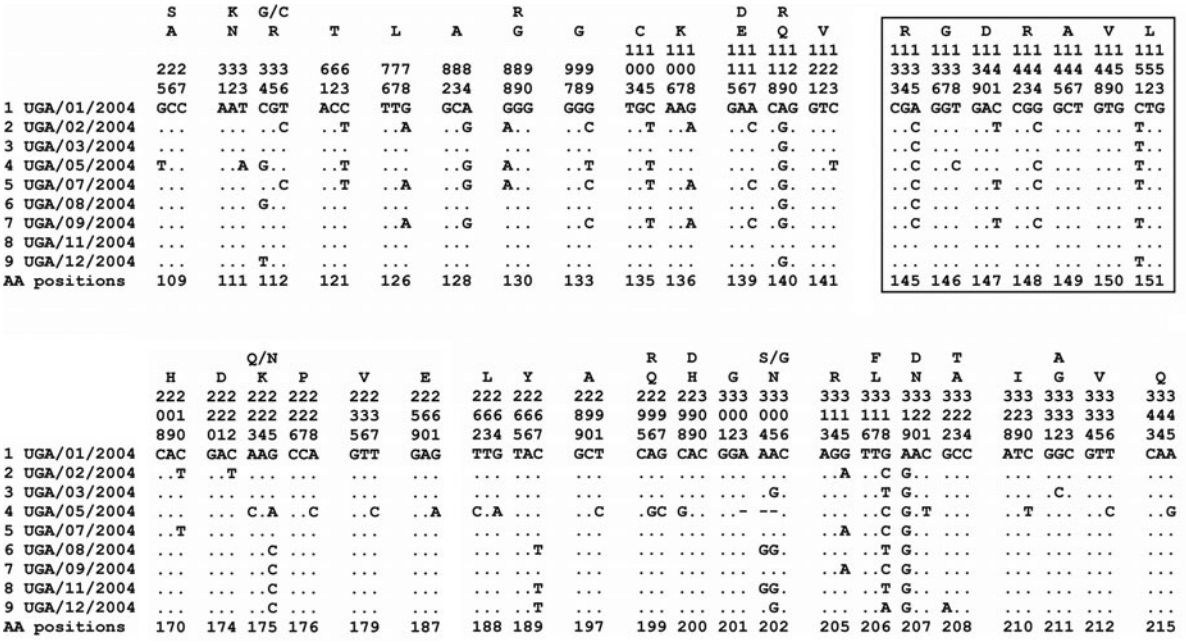

	S	K	G/C				R				D	R		R	G	D	R	A	V	L
	A	N	R	T	L	A	G	G	C	K	E	Q	V	R	G	D	R	A	V	L
									111	111	111	111	111	111	111	111	111	111	111	111
	222	333	333	666	777	888	889	999	000	000	111	112	222	333	333	344	444	444	445	555
	567	123	456	123	678	234	890	789	345	678	567	890	123	345	678	901	234	567	890	123
1 UGA/01/2004	GCC	AAT	CGT	ACC	TTG	GCA	GGG	GGG	TGC	AAG	GAA	CAG	GTC	CGA	GGT	GAC	CGG	GCT	GTG	CTG
2 UGA/02/2004	...	...	..C	..T	..A	..G	A..	..C	..T	..A	..C	.G.	...	..C	...	..T	..C	...	...	T..
3 UGA/03/2004	...	...	...	...	...	...	...	...	...	...	...	.G.	...	..C	...	...	...	...	...	T..
4 UGA/05/2004	T..	..A	G..	..T	...	..G	A..	..T	..T	...	...	.G.	..T	..C	..C	...	..C	...	...	T..
5 UGA/07/2004	...	...	..C	..T	..A	..G	A..	..C	..T	..A	..C	.G.	...	..C	...	..T	..C	...	...	T..
6 UGA/08/2004	...	...	G..	...	...	...	...	...	...	...	...	.G.	...	..C	...	...	...	...	...	...
7 UGA/09/2004	...	...	...	...	..A	..G	...	..C	..T	..A	..C	.G.	...	..C	...	..T	..C	...	...	T..
8 UGA/11/2004	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
9 UGA/12/2004	...	...	T..	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...	...	T..
AA positions	109	111	112	121	126	128	130	133	135	136	139	140	141	145	146	147	148	149	150	151

	H	D	Q/N K	P	V	E	L	Y	A	R Q	D H	G	S/G N	R	F L	D N	T A	I	A G	V	Q
	222	222	222	222	222	222	222	222	222	222	223	333	333	333	333	333	333	333	333	333	333
	001	222	222	222	333	566	666	666	899	999	990	000	000	111	111	122	222	223	333	333	444
	890	012	345	678	567	901	234	567	901	567	890	123	456	345	678	901	234	890	123	456	345
1 UGA/01/2004	CAC	GAC	AAG	CCA	GTT	GAG	TTG	TAC	GCT	CAG	CAC	GGA	AAC	AGG	TTG	AAC	GCC	ATC	GGC	GTT	CAA
2 UGA/02/2004	..T	..T	...	...	...	...	...	...	...	...	...	...	...	..A	..C	G..	...	...	...	...	...
3 UGA/03/2004	...	...	...	...	...	...	...	...	...	...	...	...	.G.	...	..T	G..	...	...	.C.	...	...
4 UGA/05/2004	...	...	C.A	..C	..C	..A	C.A	...	..C	.GC	G..	..-	--.	...	..C	G.T	...	..T	...	..C	..G
5 UGA/07/2004	..T	...	...	...	...	...	...	...	...	...	...	...	...	..A	..C	G..	...	...	...	...	...
6 UGA/08/2004	...	...	..C	...	...	...	...	..T	...	...	...	...	GG.	...	..T	G..	...	...	...	...	...
7 UGA/09/2004	...	...	..C	...	...	...	...	...	...	...	...	...	...	..A	..C	G..	...	...	...	...	...
8 UGA/11/2004	...	...	..C	...	...	...	...	..T	...	...	...	...	GG.	...	..T	G..	...	...	...	...	...
9 UGA/12/2004	...	...	..C	...	...	...	...	..T	...	...	...	...	.G.	...	..A	G..	A..	...	...	...	...
AA positions	170	174	175	176	179	187	188	189	197	199	200	201	202	205	206	207	208	210	211	212	215

Fig. 2. Summary of nucleotide substitutions and amino-acid changes in the Kiboga SAT 2 FMD virus. The nucleotide positions in the SAT 2 sequences obtained from Kiboga in 2004 which vary and the corresponding amino acids of the partial VP1-coding region of SAT 2 viruses from Kiboga (Uganda) are shown. The loss of the codon in the UGA/05/2004 sequence is indicated by three dashes (–). The highly conserved integrin receptor-binding site (RGDRXXL) is highlighted by a rectangle.

among cattle, is purchased as a trivalent preparation comprising serotypes O, SAT 1 and SAT 2 from Embakasi (Nairobi). The vaccine virus is closely related to the SAT 2 viruses from Kiboga (2004) within the VP1 region (Fig. 1). It can be argued that this suggests that the vaccine against SAT 2 currently in use in Uganda may be responsible for these outbreaks, presumably due to incomplete virus inactivation. However, more than one source of FMDV SAT 2 vaccine was used in this district during 2004. Thus, the precise origin of these virus strains is uncertain. Clearly, the presence of two highly divergent SAT 2 lineages in Uganda coupled with evidence for movement of the disease in the region suggested in this study, implies that constant molecular characterization of FMDV strains circulating within Uganda is required in order to determine the nature of the circulating strains as well as the relevance and probable efficacy of vaccines.

A large proportion of animals (75%) slaughtered in the Kiboga abattoir on 3 August 2004 had FMDV RNA in their oropharyngeal tissue some 3 months after quarantine measures (following the outbreak of FMD in the area) had been lifted. Unfortunately virus isolation from these animals was not attempted. However, it is most unlikely that FMDV RNA could persist for any significant time within an animal in the absence of virus replication and/or enclosure within intact virus particles. Thus, the presence of SAT 2 FMDV RNA detected at this stage, after the apparent end of an outbreak, indicates that the cattle may have developed so-called 'carrier' status, defined as the maintenance of virus, particularly in the oropharyngeal region, more than 28 days after infection [15, 16]. This may have important implications for disease control. Although there is currently no experimental evidence for spread of disease from carrier cattle, there is evidence indicating that carrier buffalo can spread the disease to cattle [17–19]. The RNA detected in these animals may be an indicator of a potential risk factor, further complicating the epidemiology of this disease in Uganda.

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DECLARATION OF INTEREST

None.

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