INTRODUCTION

There are seven immunologically distinct serotypes of foot-and-mouth disease (FMD) virus with different geographical distributions. The South African Territories (SAT) serotypes of FMD virus are prevalent in sub-Saharan Africa where outbreaks attributed to them have been recorded in many countries in East, West and southern Africa (Vosloo, Bastos, Sangare, Hargreaves & Thomson 2002a). The SAT serotypes have been shown to be endemic to most African buffalo (*Syncerus caffer*) populations in southern Africa and, although not studied in such detail elsewhere, evidence suggests that buffaloes in East Africa are also persistently infected with SAT-1, SAT-2 and SAT-3 (Hedger, Forman & Woodford 1973; Thomson & Bastos 2004; Vosloo & Thomson 2004). Although these animals generally do not show clinical disease, they excrete virus throughout the acute phase of the disease (Gainaru, Thomson, Bengis, Esterhuysen, Bruce & Pini 1986) during which time they can infect other susceptible species. This is followed by a persistent infection where virus can only be found in the oro-pharyngeal region and, for buffaloes in particular, this period has been shown to be up to 5 years in a single animal (Condy, Hedger, Hamblin & Barnett 1985). Circumstantial as well as experimental evidence have pointed to persistently infected buffaloes precipitating disease outbreaks in cattle and other susceptible species (Gainaru, Thomson, Bengis, Esterhuysen, Bruce & Pini 1986).
when in close contact with other susceptible species (Dawe, Flanagan, Madekurozwa, Sorensen, Anderson, Foggin, Ferris & Knowles 1994a; Dawe, Sorenson, Ferris, Barnett, Armstrong & Knowles 1994b; Vosloo, Bastos, Kirkbride, Esterhuysen, Janse van Rensburg, Bengis, Keet & Thomson 1996; Bastos, Boshoff, Keet, Bengis & Thomson 2000; Vosloo, Bastos & Boshoff 2006) and new variants of virus being generated that could have severe implications on disease control reliant on the use of vaccines (Vosloo et al. 1996). Previous studies focusing mainly on buffalo isolates have indeed demonstrated large numbers of FMD virus variants present within specific geographic regions and in only a few cases could the transmission of virus from carrier buffaloes to susceptible livestock and wildlife be verified by phylogenetic analysis (Dawe et al. 1994a; Bastos et al. 2000; Vosloo, Boshoff, Dwarka & Bastos 2002b; Vosloo et al. 2006). Other wild ungulates do not become long term carriers of FMD virus, but can spread the disease during acute infection (Hedger, Condy & Golding 1972; Hedger 1981; Thomson, Bengis & Brown 2001; Thomson, Vosloo & Bastos 2003).

The causative virus, FMD virus, is one of two members of the *Aphthovirus* genus belonging to the *Picornaviridae* family. The single stranded RNA genome is 8 500 nucleotides in length and encodes a single open reading frame. The viral RNA dependent RNA polymerase lacks proof reading ability which leads to significant nucleotide changes during each round of viral replication (Sobrino, Dávila, Ortín & Domingo 1983). The rate of change for RNA viruses ranges between $10^{-1}$ and $10^{-4}$ substitutions per nucleotide per year (reviewed in Domingo, Baranowski, Escarmís & Sobrino 2002) while rates in excess of $10^{-2}$ substitutions per nucleotide per year within the VP1-VP3 coding region have been found during an outbreak of FMD virus (Sobrino, Palma, Beck, Dávila, De la Torre, Negro, Villanueva, Ortín & Domingo 1986; Villaverde, Martínez-Salas & Domingo 1988) with the estimation that clones from a single isolate differ in approximately 0.6–2 genomic positions, contemporary isolates in 2–20 positions and different isolates from a single outbreak differ in 50–100 genomic positions (reviewed in Domingo, Escarmís, Martínez, Martínez-Salas & Mateu 1992; Sobrino, Saiz, Jimenez-Clavero, Nunez, Rosas, Baranowski & Ley 2001).

The best method to date to differentiate between FMD virus isolates has been the determination of the RNA sequence encoding the VP1 protein which contains the major antigenic determinants of the virus (Beck & Strohmaier 1987; Samuel, Knowles & Kitching 1988). Despite the success in elucidating the epidemiology of the disease, sequence data do not predict with accuracy the influence on the antigenicity of the virus and therefore cannot at present be used in isolation to recommend vaccine strains. This is of particular interest in regions where vaccination is used to control and eradicate the disease, as it is imperative that vaccines be used that are antigenically closely related to viruses circulating in the field (Hunter 1998).

Of the SAT serotypes most outbreaks in domestic animals have been recorded for SAT-2 (Thomson & Bastos 2004) giving credence to the fact that SAT-2 may be most efficient in crossing species barriers (Bastos 2001). This serotype has also spread into the Middle East on at least two occasions (Ferris & Donaldson 1992; Bastos, Haydon, Sangare, Boshoff, Edrich & Thomson 2003b). In contrast, most buffaloes first become infected with SAT-1, followed by SAT-2 and lastly by SAT-3 as determined by serological responses in young buffaloes in the Kruger National Park (KNP), South Africa (Thomson & Bastos 2004). The genetic diversity of SAT-2 FMD isolates has been published previously for African countries with specific focus on southern Africa (Vosloo, Knowles & Thomson 1992; Vosloo, Kirkbride, Bengis, Keet & Thomson 1995; Bastos et al. 2003b) with limited data on isolates from East Africa. The present study was carried out to determine the genetic variability of the SAT-2 FMD isolates in East Africa and to elucidate their epidemiology on a sub-continental basis.

**MATERIALS AND METHODS**

**Viruses included in this study**

A total of 41 SAT-2 FMD virus isolates from Ethiopia, Sudan, Kenya, Uganda, Tanzania and Eritrea isolated between 1975 and 2000 were supplied by the World Reference Laboratory (WRL) for FMD at the Institute for Animal Health, Pirbright (United Kingdom). These isolates were propagated once on IBRS-2 cells before further processing.

**Nucleic acid isolation and RT-PCR amplification**

Total RNA was extracted from cell culture supernatant using a guanidium thiocyanate-silica method (Boom, Sol, Salimans, Jansen, Wertheim-van Dillen & Van der Noordaa 1990). The RNA viral template was reverse transcribed using AMV reverse transcrip-
tase (Promega) with antisense primer (P1) of Beck & Strohmaier (1987) and DNA amplification has been described previously (Bastos 1998). The P1 primer complementary to the conserved 2A/B junction site and the forward primer binding within 1C (VP3) termed VP3AB (5’-CACTGCTACCACTCRG AGTG-3’) (Bastos 1998), were used to amplify an approximately 880 bp fragment.

DNA purification and cycle sequencing

The PCR amplicon was excised from a 1.5% agarose gel and purified using the Qia Quick Gel Extraction Kit (Qiagen). Purified PCR products were sequenced using the Big Dye® version 3.0 Cycle Sequencing kit and the ABI Prism 310 Genetic Analyzer (Applied Biosystems). Two sequencing reactions were performed per isolate using identical upstream and downstream primers as in the PCR.

Phylogenetic analysis

The DAPSA program (Harley 2001) was used to align generated nucleotide sequences to data previously published for 26 isolates bringing the total number of isolates to 67 from 20 countries (Table 1). A homologous region of 648 bp corresponding to the complete VP1 encoding gene and 6 nucleotides of the 2A region was used for phylogenetic analysis. Phylogenetic reconstructions [neighbour joining (NJ) and minimum evolution (ME)] were carried out using methods of analysis included in MEGA version 2.0 (Kumar, Tamura, Jakobsen & Nei 2001), with p-distance, pair-wise deletions of gaps and confidence levels assessed by 1 000 bootstrap replications. A gamma shape parameter of 0.9059 as determined in Model Test (Posada & Crandall 1998) was used to construct the minimum evolution tree. Parsimony and UPGMA analysis were performed using MEGA version 2.0. Average pair-wise comparisons were conducted to estimate divergence within and between lineages and genotypes. A variability plot of sequences of the 1D gene of all virus isolates included in this study was determined using MEGA version 1.02 (Kumar, Tamura & Nei 1993) with numbers of variable sites in overlapping windows of 10 and >70% variation taken as an indication of hyper-variability.

RESULTS

Phylogenetic analysis

Complete 1D gene sequences (648 bp) were used to determine phylogenetic relationships between the 67 SAT-2 isolates from sub-Saharan Africa and one isolate that had caused outbreaks in Saudi Arabia during 2000 (Table 1). NJ, UPGMA, ME and parsimony methods produced trees with similar topology (results not shown) indicating that the recovered phylogeny is a good estimate and reliable. Only the ME tree is shown (Fig. 1). The ME tree revealed three lineages which are summarized below and were broadly geographically linked, with lineage I consisting of isolates from East Africa, Angola, the Democratic Republic of Congo (DRC; Zaire) and Saudi Arabia, lineage II containing isolates from West Africa, while lineage III encompassed southern and East Africa.

Lineage I


Lineage II

Genotype E (Ghana, Nigeria and Senegal 1975–1991) and genotype F (Gambia and Senegal 1979–1983)

Lineage III


Previously, 11 genotypes were described for sub-Saharan Africa based on genetic distance and bootstrap support (Bastos et al. 2003b) and in this study the genotypes are assigned the same alphabetical letters to facilitate comparison. The phylogeny corresponded well to what was previously described for SAT-2 in sub-Saharan Africa (Bastos et al. 2003b, Sangare, Bastos, Venter & Vosloo 2004). Three new genotypes were demonstrated in East Africa, viz. genotype L that contained isolates from Uganda (1975–1976), genotype M with two isolates obtained in Sudan during 1977 and genotype N with a single isolate from Ethiopia made during 1991. In this study, the number of isolates from East Africa was increased compared to earlier studies and the previously described genotype G was shown to contain two more isolates obtained in Eritrea during 1998, genotype H remained the same with a single isolate...
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All unmarked isolates were obtained from cattle

* Buffalo isolate

# Species of origin not known
FIG. 1 Minimum evolution tree based on the 1D gene depicting genetic relationships of SAT-2 FMD isolates from sub-Saharan Africa. Bootstrap values were estimated based on 1000 replications. I–III depict the major lineages, while A–N indicate genotypes.
Genetic heterogeneity of SAT-2 FMD virus in sub-Saharan Africa

**FIG. 2** Deduced amino acid alignment of VP1 of the newly sequenced isolates performed in this study (Table 1)
from Rwanda made during 2000 (RWA/1/00), while genotype I expanded with several more isolates from Kenya and Uganda in addition to demonstrating that this genotype had circulated for 41 years (1957–1998). Genotype J, that previously contained only one isolate from the DRC made during 1982 (ZAI/1/82), had two more isolates from Uganda 1998 (UGA/19/98 and UGA/28/98) and although these differed by 22% from the former, the cluster was supported by a bootstrap value of 95%. The within genotype variation based on pair-wise comparisons was up to 26% while between genotype differences varied from approximately 30–44%.

Closely related viruses, with sequence homology of >94%, caused a number of outbreaks between 1984 and 1998 in Kenya and Uganda, suggesting a direct epidemiological association between these outbreaks and possible long-term conservation (genotype I, lineage I; Fig. 1). A historical isolate made in 1957 in Kenya (KEN/3/57) clustered within this genotype with a high bootstrap support, but differed by 24% from the former cluster. In addition, three outbreaks caused by isolates from lineage III within genotype B had occurred in Kenya between 1976 and 1995 (KEN/2/76 and KEN/3/95), 1984–1987 (KEN/1/84, KEN1/86, KEN1/87, KEN2/87) and 1991–1999 (KEN/33/91, KEN/16/98, KEN/4/99, KEN/5/99, KEN/7/99, KEN/9/99). While the isolates obtained within an outbreak differed by less than 2% from each other, the distances between these outbreaks caused by isolates from lineage III within genotype B and seemed to be related to the ongoing outbreaks in the eastern African region based on a bootstrap support of 99% (Fig. 1).

Uganda suffered from unrelated outbreaks between 1975–1976 (genotype L; UGA/51/75, UGA/3/76, UGA/8/76) and 1998 (genotype J) bringing the total number of unrelated outbreaks to three, all from different genotypes. The introduction into Rwanda during 2000 was unrelated to any of these previous outbreaks in neighbouring countries. During 1990–1991 Ethiopia had at least two separate introductions, with one isolate clustering within genotype N (ETH/2/91; lineage I) and ETH1/90, ETH/2/90 and ETH/1/91 clustering within genotype B, lineage III. A single isolate from Burundi, made in 1991 also grouped in genotype B, indicated that this genotype had been circulating in East and southern Africa for 24 years.

Genetic variability and distribution of mutations

The invariable sites over the 1D gene for all the isolates included in the study were 236/638 (37%), with 56% (358/638) parsimoniously informative sites and 54 singletons. For the deduced amino acid sequences, the invariable sites were 42% (91/216), 43% (93/216) parsimoniously informative sites and 32 singletons occurred. Amino acid variability was plotted to determine whether mutations were randomly distributed or localized to specific regions of the VP1 gene. The result of the amino acid hyper-variability plots of 67 isolates from Africa indicated the hyper-variable regions were located at amino acid positions 45–50, 107–111, 135–141 and 148–160 (within the G-H loop) as well as 198–202, the C-terminal part of the protein. A putative hyper-variable site was also identified at positions 21–28, which corresponds with a T-cell epitope identified on O Kaufbeuren (Collen, Dimarchi & Doel 1991) and was also recognised as hyper-variable for SAT-1 (Sahle, Dwarka, Venter & Vosloo 2007).

When comparing only the newly generated deduced amino acid sequences of the 48 East African isolates, the RGD cell attachment site of the virus at amino acid positions 144–146 within the G-H loop was completely conserved across all isolates (Fig. 2). The C at the base of the 1D loop (position 134) was maintained as well as the R at position +1, the I at position –1 and the L at position +4. Of the previously described neutralisation sites identified by monoclonal antibodies at positions +2, +3 and +10 and +12 (Crowther, Rowe & Butcher 1993; Bastos et al. 2003b), only +3 was moderately conserved with three (V/A/L) options, while at positions +2 and +10, five different amino acids occurred and at position +12 seven differences were found (Fig. 2). Previously Bastos et al. (2003b) found that for representative isolates from sub-Saharan Africa, but with few of them being from East Africa, the +2 and +3 sites showed moderate levels of variation, while the +10 and +12 sites showed high levels. For the East African isolates, only the +3 site showed moderate variation. The VP1/2A cleavage site contained predominantly amino acid sequences VP1(K/R)Q/2A(L/T)VS/1D with the Q at the cleavage site conserved over all isolates.

DISCUSSION

Phylogenetic analysis has been of great benefit in determining possible origins of FMD outbreaks, interspecies transmission, tracing spread of virus over vast distances and ultimately to better understand
Genetic heterogeneity of SAT-2 FMD virus in sub-Saharan Africa

the epidemiology of the disease in sub-Saharan Africa (Vosloo et al. 1992; Dawe et al. 1994a; Vosloo et al. 1995; Bastos et al. 2000; Bastos 2001; Bastos, Haydon, Forsberg, Knowles, Anderson, Bengis, Nel & Thomson, 2001; Sangare, Bastos, Marquardt, Venter, Vosloo & Thomson 2001; Bastos, Anderson, Bengis, Keet, Winterbach & Thomson 2003a; Bastos et al. 2003b; Sangare, Bastos, Venter & Vosloo 2003; Sahle, Venter, Dwarka & Vosloo 2004; Sangare et al. 2004; Vosloo & Thomson 2004; Vosloo et al. 2006). SAT-2 isolates from East Africa have not been studied in detail and compared to those obtained from other regions to better understand and assess the molecular epidemiology of SAT-2 in sub-Saharan Africa. The phylogeny has expanded with three new genotypes identified in East Africa, bringing the total number to eight belonging to two different lineages. The previously identified lineages (Bastos et al. 2003b; Sangare et al. 2004) could not be followed in this study, as the inclusion of more isolates has altered the structure of the phylogeny at that level, albeit not on genotype level. Only three lineages were assigned in this study that covered East Africa and south-western Africa, one consisting solely of West African isolates and the third from East and southern Africa.

These linkages between different geographical regions of the subcontinent demonstrate clearly the potential for FMD virus to disperse over considerable distances and emphasize the need to investigate the main factors which play a role in exchange of subtypes of the virus between countries and its spread within and between regions. Transboundary transmission of the disease due to animal movement is possible as a number of countries share common boundaries and animal trading across borders is common practice (Ndiritu 1984). Added to this, the population of susceptible hosts for FMD in East African countries is high [the cattle and sheep population were estimated to be 57.6 and 98.9 million, respectively (McDermott & Arimi 2002)], and can easily maintain cycles of FMD epizootics. The livestock and the livestock production systems, illegal trading of animal and animal products as well as the presence of cloven-hoofed wild animals in the region favour the transmission of disease between neighbouring countries and could lead to endemic cycles. A study performed in West Africa indicated clearly that the role of sheep and goats in the epidemiology of FMD is not fully understood either due to a real low prevalence of disease or, more likely, because clinical disease is not apparent and the importance of these species is overlooked (Bronsvoort, Tanya, Kitching, Nfon, Haman & Morgan 2003). In Sudan it was shown that sheep and goats play an important role in the epidemiology based on serological studies following natural infection (Abu Elzein, Newman, Crowther, Barnett & McGrane 1987). Cross-border transmission was aptly demonstrated where an outbreak in Saudi Arabia was shown to cluster with three isolates previously obtained from Eritrea which was possibly due to spread of virus to Saudi Arabia arising from trade in livestock between the two countries (Bastos et al. 2003b). Similarly rare incursions of SAT-1 into the Middle East have been recorded (Knowles & Samuel 2003). Within a geographical region such as East Africa, cross-border movement most probably leads to dissemination of disease between various countries sharing borders.

More surprising was the demonstration that isolates from Angola and DRC clustered with the East African lineage I which is supported by a high bootstrap value. However, due to the low numbers of isolates available from central and south-western Africa, it is not possible to determine whether these were accidental introductions over large distances, or whether there are indeed similar isolates circulating within this geographical region.

A total of 14 genotypes were identified in sub-Saharan Africa. Of these, six may be extinct (E, F, K, L, M and N) as no isolates similar to those included in this study have been found since 1996. However, in endemic areas the disease is often not reported nor material submitted for further investigation, implying that the exact distribution and occurrence of serotypes is not known. Bronsvoort et al. (2003) found by using questionnaires that the prevalence may be up to 58% in specific regions of Mali but outbreaks are not reported to veterinary services. Investigations into more recent isolates may prove that these and new genotypes are currently circulating within sub-Saharan Africa.

East Africa demonstrates the most variation of all regions in sub-Saharan Africa with at least eight genotypes in two lineages consisting almost exclusively of cattle isolates, compared to southern Africa where three genotypes have been described (Bastos et al. 2003b), the latter consisting mostly of buffalo isolates. Previously, Bastos et al. (2003b) found that the highest rates of nucleotide substitution for SAT-2 groups were those that were recovered from cattle, while the lowest rates were recovered from wildlife. They speculated that these different rates could have been due to a higher number of cases during cattle outbreaks resulting in more virus replication and more opportunity for divergence although
their study could not support this assumption statistically. From the data included in this study, it is clear that within East Africa at least, more variation is observed, giving credence to the fact that the disease is most probably maintained by livestock. Other factors cannot be excluded in generating this diversity. The role of buffaloes in these regions is largely unknown, and more studies into the presence of SAT serotype viruses in buffaloes in the area could provide an explanation. In addition, the role of other wildlife species is also not clear. In southern Africa and in the KNP in particular, it has been shown that impalas can play an important role in transmitting disease to other species (Vosloo et al. 2006). In addition to transboundary movement of livestock which could spread disease, introduction of strains due to vaccine cannot be excluded. The latter could explain the long term conservation of genotypes observed over extended time periods.

A number of East African countries have had separate incursions of disease belonging to different lineages and genotypes such as Kenya, Ethiopia and Uganda. These genetic differences lead to antigenic differences (Vosloo, Dwarka, Bastos, Esterhuysen, Sahle & Sangare 2004) and have an important bearing on the use of vaccination to control the disease. Cross neutralisation studies have shown that even within a genotype of SAT-2, the antigenic relationships are sufficiently poor to warrant specific vaccines strains and there will probably be no protection between genotypes (Vosloo et al. 2004). Countries will have to consider the strains and genotypes included in vaccines to ensure that vaccination will be effective. These differences could also have a negative impact on diagnostic tests relying on antigenic relationships between viruses and should be considered when diagnoses are required. In contrast Tanzania, Rwanda and Eritrea had outbreaks caused by single genotypes, but this could be due to under representation, rather than a true reflection of the current status.

The seven serotypes of FMD virus cluster into lineages that differ by approximately 30–50% over the 1D gene (Knowles & Samuel 2003). In this study it was observed that lineages differ by up to 44% from each other, nearly as much as was found for serotypes, indicating the high level of mutation found in SAT-2. A similar level of variation was observed for SAT-1 when isolates from over sub-Saharan Africa were investigated (Sahle et al. 2007). Knowles & Samuel (2003) suggested that variation of up to 20% could be used for inclusion into a SAT topotype. However, it was found in this study that certain genotypes that corresponded to geographical locations (ie toptypes) had up to 26% within group variation (genotype D) when pairwise comparisons were performed and it seems plausible that these cut-off levels should be redefined, especially for the SAT types.

The hyper-variable regions of the East African isolates compared to those identified for SAT-1 and SAT-2 with 135–141 and 148–160 corresponding to the βG-βH loop (Bastos et al. 2001; Bastos et al. 2003b; Vosloo et al. 2006; Sahle et al. 2007). Sites 107–111 correspond to the βF-βG loop shown to be hyper-variable for SAT-1 and SAT-3 (Bastos 2001; Bastos et al. 2003a, b), while 45–50 correspond to the βB-βC loop identified on O1 BFS (Acharaya, Fry, Stuart, Fox, Rowlands & Brown 1989) and shown to be hyper-variable for SAT-1 (Vosloo et al. 2006; Sahle et al. 2007). This high level of variation around the immunologically important GH loop which also plays a role in cell receptor recognition (Logan, Abu-Ghazaleh, Blakemore, Curry, Jackson, King, Lea, Lewis, Newman & Parry 1993) could have a severe impact on the efficacy of vaccines. As was demonstrated in previous studies investigating SAT-2, the arginine at position 148 was conserved in all isolates investigated. The role of this change from leucine (as in most other serotypes) to arginine is not clear as the leucine has been shown to stabilize alpha helix formation (France, Platti, Newman, Toth, Gibbons & Brown 1994).

This study adds to our understanding of the molecular epidemiology of SAT-2 FMD isolates in sub-Saharan Africa and demonstrates clearly that control of this disease should be seen as a regional priority due to the virus’ ability to spread over vast distances. It also indicates that our understanding of the factors that lead to the generation of variants, disappearance and re-emergence of strains and regional patterns is inadequate and that more research is needed to ensure better prediction of disease emergence and effective control policies.

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