AN EPIDEMIOLOGICAL STUDY ON THE GENETIC RELATIONSHIPS OF FOOT AND MOUTH DISEASE VIRUSES IN EAST AFRICA

by

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Dedicated to my parents Sahle Fursa and Desta Sheferaw

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ABSTRACT

Within East African countries many of the known infectious diseases of animals occur commonly and are poorly controlled. Foot-and-mouth disease (FMD) is one of the contagious viral diseases that has great impact on economic development both in terms of direct and indirect losses. The epidemiology of the disease is complex due to the presence of six of the seven serotypes and the presence of large numbers of both wild and domestic susceptible animals in the region. Decision-making to determine the importance of FMD control relative to the economic consequences and what FMD control strategies should be applied based on the epidemiological information is required. In this regard the first step is to investigate the genetic relationships/variability of East African isolates and their phylogeographic distribution. These can provide base-line information for

designing control strategies by vaccination as well as the determination of the sources of infection.

Sufficient genetic information on the FMD serotypes O, SAT-1 and SAT-2 are lacking and therefore the number of viral lineages and genotypes or topotypes from East African countries could not be determined. Published studies on the relative occurrence and genotype distribution of FMD are largely confined to the southern and western part of the continent. In this study, the genetic profile of the 3 most prevalent serotypes (O, SAT-2 and SAT-1) recovered from outbreaks in East Africa between 1957 and 2003 was addressed. Phylogenetic analysis of partial and complete sequences of the 1D gene revealed the presence of distinct lineages and genotypes for East Africa as well as historical relationships of some of the genotypes with isolates from other regions. A great variation in the occurrence and distribution of these serotypes were found.

All the African and the Middle East/South East Asian isolates of serotype O included in this study clustered into one lineage having 8 distinct topotypes. These results indicated that between countries as well as inter-regional (east and west Africa) spread of viruses occurred in the past. Inter-regional spread of the virus between eastern Africa and western Africa was also confirmed for SAT-1 viruses. The fact that phylogenetic links are found with both serotypes implies that the spread of viruses was possibly associated with unrestricted animal movement due to nomadic movement in Africa. The phylogenetic relationships of SAT-1 viruses are more diversified in Africa. Eight lineages and 11 genotypes were identified when the optimal nucleotide sequence differences of $\geq$ 23% for lineages and $\geq$ 16% for genotypes were used as a cut-off values.

It was observed that viruses from Uganda are evolving independently from viruses elsewhere on the continent and clustered into 3 discrete lineages. In contrast, viruses from countries neighbouring Uganda, Kenya and Tanzania, clustered into one lineage. Uganda also harboured 3 topotypes of SAT-2 virus isolates, one is distinct for Uganda and the other are shared with Kenya and Zaire (DRC). This study highlighted distinct lineages found in Uganda and needs further investigation.

Within SAT-2, 67 isolates from 22 African countries and Saudi Arabia clustered into 5 lineages which consisted of 15 genotypes. Clustering of viruses into distinct genotypes (topotypes) according to year of isolation and geographical origin was observed showing countries with common boundaries shared common epizootics in the past. These results also showed a link between eastern and southern African countries.

Attempts were also made to investigate the incidence of FMD in Ethiopia using sera collected from cattle, small ruminants and wildlife. The results obtained from the liquid phase blocking ELISA and the 3ABC ELISA indicated the presence of SAT-1 and SAT-2 in buffalo populations in the southern part of Ethiopia while results from small ruminants and other wildlife were not indicative of any significant role in the epidemiology of FMD. Serological results also indicated that SAT-1 is present in cattle, although this serotype has not been previously identified.

The cumulative molecular epidemiological results from this and previous studies indicated that genetic variability of FMD viruses can be independently maintained within country/countries or regions as well as inter-regions of Africa. The serological results from buffaloes in East Africa are also suggestive of a possible reservoir of the SAT types FMD in the region which has a great impact on the control of the disease. Furthermore, the numerous lineages and genotypes of FMD virus isolates in Africa having distinct or overlapping distributions as well as the genetic linkage between regions will complicate the epidemiology of the disease. Therefore, it is strategically important to consider a regional approach and the use of a vaccine which contains a cocktails of antigens of FMD virus strains.

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LIST OF ABBREVIATIONS

BHK	Baby Hamster Kidney cells
bp	base pair
CBPP	Contagious bovine pleuropneumonia
CCPP	Contagious caprine pleuropneumonia
CFT	Complement fixation test
cDNA	Complementary deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
ed.	editor
edt.	edition
e.g	for example
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethyl alcohol
Fig.	Figure
FMD	Foot and mouth disease
FMDV	Foot and mouth disease virus
g	Gram
x g	Unit of acceleration
GDP	Gross domestic production
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
IBRS-2	Instituto Biologico Rim Sunio
Lpb ELISA	Liquid phase blocking ELISA
kDa	Kilo dalton
masl	meter above sea level
mbsl	meter below see level
M	Molar
ML	Maximum likelihood
MM	Master mix
MOD	Maximum optical density
MOE	Ministry of Agriculture of Ethiopia
MP	Maximum parsimony
m/v	mass by volume
NaAc	Sodium acetate

NJ	Neighbour-joining
nt	nucleotide
OD	Optical density
OIE	Office International des Epizooties
OPD	o- phenelene diamine
PBS	Phosphate buffered saline
PBS-C	Phosphate buffer saline and casein
PBS-T	Phosphate buffer saline and twee-20
PK	Pig kidney
RT-PCR	Reverse transcriptase polymerase chain reaction
RGD	Arginine-Glycine-Asparic acid
Rnase	Human placental ribonuclease
S	Sedimentation coefficient
ssRNA	Single stranded ribonucleic acid
SAT	South African Territories
$TCID_{50}$	Tissue culture infective dose
UK	United Kingdom
UPGMA	Unweighted Pair-Group method using arithmetic average
VNT	Virus neutralization test
WRL	World Reference Laboratory

CHAPTER I

LITERATURE REVIEW

1.1 Introduction

Foot-and-mouth disease (FMD) is endemic to most of sub-Saharan Africa, except in a few countries in southern Africa, where the disease is controlled by the separation of infected wildlife from susceptible livestock as well as by vaccination. Largely due to the endemicity of the disease, and the fact that FMD does not normally cause high rates of mortality in adult animals, FMD outbreaks are not perceived as important and are not reported or investigated further to determine the causative serotypes. However, a number of countries now realise that FMD is one of the transboundary diseases that should be controlled to ensure economic stability and access to lucrative international export markets for animal and animal products. Furthermore, they recognise that a regional approach would be needed to succeed. Knowledge about the epidemiology of FMD can greatly assist in developing control policies for such a regional approach.

1.2 Historic and economic significance of foot-and-mouth disease

Foot and mouth disease was described for the first time during the XVI century (Bulloch, 1927). Bovines refused to eat as the internal parts of their mouths were covered with redness and small vesicles which then spread to the feet of the animals. Most of the individuals that were affected showed the same clinical signs. However, the disease spread to several provinces around Verona, Italy, and it was believed that the infection spread through air but other vectors such as water and swamps were not excluded. This description, carried out more than 400 years ago, is absolutely comparable to what we know today as FMD.

In 1898, Loeffler and Frosch proved the filterability of the pathogenic agent causing FMD through bacterial filters and started the notion of another living entity, *viz.*, viruses (Bos, 2000; Brown, 2003). This was the first evidence of a virus causing an animal disease. Hence forth, a significant aspect in the field of

FMD research was the discovery of the sensitivity of guinea pigs to FMD by Waldmann and Pape in 1920. During 1922 progress was made when Vallée and Carrée proved that there were different antigenic types of FMD, thus explaining the possibility of successive infections in the same animal. They discovered 2 serotypes and named them after the place of origin, O for Oise and A for Allemagne in France. In 1926, Waldmann and Trautwein discovered the third antigenic type which they called C. In the 1940s, 3 additional serotypes from Southern Africa were discovered at the Pirbright laboratory in England and named as South African Territories 1-3 (SAT-1, SAT-2, SAT-3) and the last serotype Asia I was discovered from Turkey in 1954 (Brown, 2003). At present 7 immunologically distinct serotypes of FMD viruses are known based on the fact that there is no cross protection between these serotypes (Brooksby, 1982). In addition, within each serotype a number of genetic and antigenic variants with different degrees of virulence exist (Vallée and Carrée, 1922; Pereira, 1977; Blood *et al.,* 1983; Chenug *et al.,* 1983; Kitching *et al.,* 1989). Vosloo *et al.* (2002) reviewed the genetic and geographical distribution of FMD viruses in Africa and showed that the SAT-2 viruses appear to be more diverse in topotypes and prevalent in sub-Saharan African countries. The prevalence of the other serotypes of FMD viruses in Africa was also reported from high to low prevalence as O → A → SAT-1 → SAT-3→C.

A break-through in the control of FMD was made when Vallée and co workers (1925) utilised formaldehyde-inactivated vesicular fluid from infected calves as a vaccine. In 1947, Frenkel started the large-scale production of virus on surviving bovine lingual epithelium to incorporate into the inactivated vaccine, which was subsequently adopted by a number of other laboratories (Fogedby, 1963). Since then various cell lines, *e.g.* Baby Hamster Kidney cells (Mowat and Chapman, 1962) have been investigated for virus propagation which opened a new era in vaccine production resulting in better control of the disease and fundamental studies on virus-cell interaction (cited in Bos, 2000; Brown, 2003).

Foot-and-mouth disease has considerable economic consequences. Losses can be attributed to both direct and indirect costs. The direct effects of the disease are loss of milk production, loss of draught power, retardation of growth, abortion in pregnant animals, death in calves and lambs while indirect losses can be

attributed to the disruption in trade of animals and derivative products. Its sequelae are found to be more important than the acute illness (Woodbury, 1995). A striking example is the recent outbreak of serotype O (the PanAsian strain) in Great Britain, a country which had been free of FMD since 1981. This devastating epidemic of 2001 spread to Ireland, France and the Netherlands where the United kingdom alone were forced to slaughter about 4 million infected and in contact animals. The cost of this epidemic in the UK was estimated to be more than US $29 billion (Samuel and Knowles, 2001a).

1.3 Taxonomy of Picornaviruses

The virus causing FMD was defined in 1963 by the International Committee of Taxonomy of viruses as belonging to the genus *Aphthovirus*, one of the genera of the family *Picornaviridae*. The name *Picornaviridae* is derived from the Latin word 'pico' (small) and 'rna' (RNA) which refers to the size and genome type while the genus name '*aphthovirus*' refers to the vesicular lesions produced in cloven hoofed animals. The *Picornaviridae* family consists of various virus species which cause diseases of medical and agricultural importance which are summarized as the various genera in Table 1.1.

Table 1.1 Summary of the genus composition of the family *Picornaviridae* (http://www.iah.bbsrc.ac.uk/virus/).

Genus	Virus name
1. *Aphtovirus*	Foot-and-mouth disease virus Equine rhinitis A virus
2. *Cardiovirus*	Encephalomyocarditis virus (mengovirus, mouse Elberfield virus, Columbia SK)
3. *Parechovirus*	Human parechovirus
4. *Enterovirus*	Human enteroviruses, human polioviruses bovine enteroviruses, porcine enteroviruses, simian enterovirus, human coxsackievirus A and human coxsackievirus B
5. *Hepatovirus*	Hepatitis A virus and semian hepatitis
6. *Rhinovirus*	Human common cold virus and bovine rhinoviruses
7. *Kobuvirus*	Aichi virus and bovine kobuvirus
8. *Teschovirus*	Consists of a single species, porcine teschovirus
9. *Erbovirus*	Equine rhinitis B virus

1.3.1 Types (serotypes) and subtypes

Serotype and subtype differentiation within viruses are based on the complete or partial lack of cross protection between FMD viruses. In addition, serological tests *e.g.* the complement fixation test (Forman, 1974), the serum neutralisation test (Rweyemamu *et al.,* 1977) or the enzyme linked immunosorbent assay (ELISA) (Ouldridge *et al.*, 1982) are used to assist in virus typing and subtyping. Initially over 60 different subtypes were identified by the World Reference Laboratory at Pirbright, UK. Later it became difficult to identify specific subtypes due to the continuous appearance of intratypic antigenic variants (Pereira, 1977). However, it is still imperative to test for the antigenic similarity or disparity between field isolates and vaccine strains. Most laboratories stock vaccine strain antisera and can therefore quickly do a one-way relationship between vaccine strain and outbreak isolates. A quantitative relationship 'r' between the viruses is interpreted as follows (Rweyemamu *et al.,* 1977).

r = heterologous neutralisation titre / homologous neutralisation titre.
r = > 0.4 (a suitable vaccine strain).
r = < 0.4 (indicate poor antigenic relationship between the outbreak and the vaccine strain).

At present, sequencing of a part of the 1D gene (Fig. 1.1) of FMD viruses is increasingly being used to establish intratypic variations of FMD viruses and classifying viruses into genotypes and lineages.

1.4 Foot-and-mouth disease virus

1.4.1 Physico-chemical properties of the FMD viruses

Picornaviruses are small RNA viruses that are enclosed with a non-enveloped protein shell (capsid). The capsid consists of polypeptides which are devoid of lipo-protein and hence is stable to lipid solvents like ether and chloroform (Cooper *et al.,* 1978). The most important difference between the physico-chemical properties of viruses of five of the genera of the *Picornaviridae* family is their pH stability (Pereira, 1981). *Entero-* and *cardioviruses* are stable at pH 4 while the

rhinoviruses loose activity lower than pH 5. The *hepatoviruses* are relatively stable at acid pH and high temperature.

Foot-and-mouth disease viruses can be inactivated by a number of chemical substances at the acidic and alkaline pH ranges, however, the virus is stable between pH 7 and 9 and at 4°C and -20°C (Mann and Sellers, 1990; Wilks, 1992). Two percent solution of NaOH or KOH and 4% Na_2CO_3 are effective disinfectants for FMDV contaminated objects, but the virus is resistant to alcohol and phenolic and quaternary ammonium disinfectants (Russell and Edington, 1985). However, the FMD virus is also sensitive to a range of other chemicals like trypsin which causes cleavage and denaturation of the vital capsid protein, VP1 (Wild *et al.,* 1969; Rowlands *et al.*, 1971). The size of droplet aerosol also plays a role in the survival or drying out of the virus, where a droplet aerosol size of 0.5 - 0.7 µm is optimal for longer survival of the virus in the air while smaller aerosols dry out. In dry conditions the virus also survives longer in proteins *e.g.* in epithelial fragments (Donaldson, 1983, 1987).

1.4.2 Virus morphology

The virus is composed of an icosahedral protein coat (capsid) and the RNA core has a diameter of 22 - 25 nm (Melnick *et al.,* 1975; Cooper *et al.,* 1978; Robert & Bruce, 1981). The sedimentation coefficient (S) of the intact virus particle is 146S (Barteling 2002). The capsid consists of 60 capsomers each consisting of four proteins (VP1-4). VP1 is the most antigenic protein, is involved in cell attachment and carries an immunological important G–H loop which is one of the most important neutralizing sites of the virus (Logan *et al.*, 1993).

1.4.3 Genome organization and protein processing

The genome consists of a positive sense single stranded RNA (ssRNA). The RNA genome is approximately 8500 bases long with a poly A tail at its 3' end and a viral genome protein (VPg) at its 5' end (Fig. 1.1) (Sanger, 1979; King *et al.,* 1980; Bittle *et al.,* 1982; Forss *et al.,* 1984; Chow *et al.,* 1987; Belsham, 1993). The RNA of serotype O, with molecular mass of 2.8 X 10^6 Da, consists of a single large open reading frame of 6996 nucleotides encoding a polyprotein of 2332

amino acids (Forss *et al.,* 1984). Four polyproteins (L, P1, P2 and P3) are translated and processed into the different structural and non-structural proteins by viral encoded proteases (L^{pro}, 2A oligopeptide and $3C^{pro}$) (Rueckert, 1996) (Fig. 1.1).

The L protein represents the leader protein, where 2 initiation sites (AUG codons) have been identified in FMD virus, namely *Lab* and *Lb* (Sangar *et al.,* 1988). The P1 gene product is the precursor of the capsid proteins 1D, 1B, 1C and 1A (Fig. 1.1). Firstly, the intermediate P1 precursor is processed with the help of viral protease $3C^{pro}$ to produce VP0, VP1 and VP3 where the products combine to form empty capsid particles. The mature virion is produced after the encapsidation of the virion RNA which is accompanied by the cleavage of VP0 to VP2 and VP4. VP1-3 are exposed on the capsid surface (Acharya *et al.*, 1989). The P2 (2A, 2B, 2C) and P3 (3A, 3B, 3C, 3D) regions encode for non-structural proteins that are involved in viral RNA replication and protein processing (Fig. 1.1) (Sangar, 1979; Forss *et al.,* 1984; Belsham, 1993).

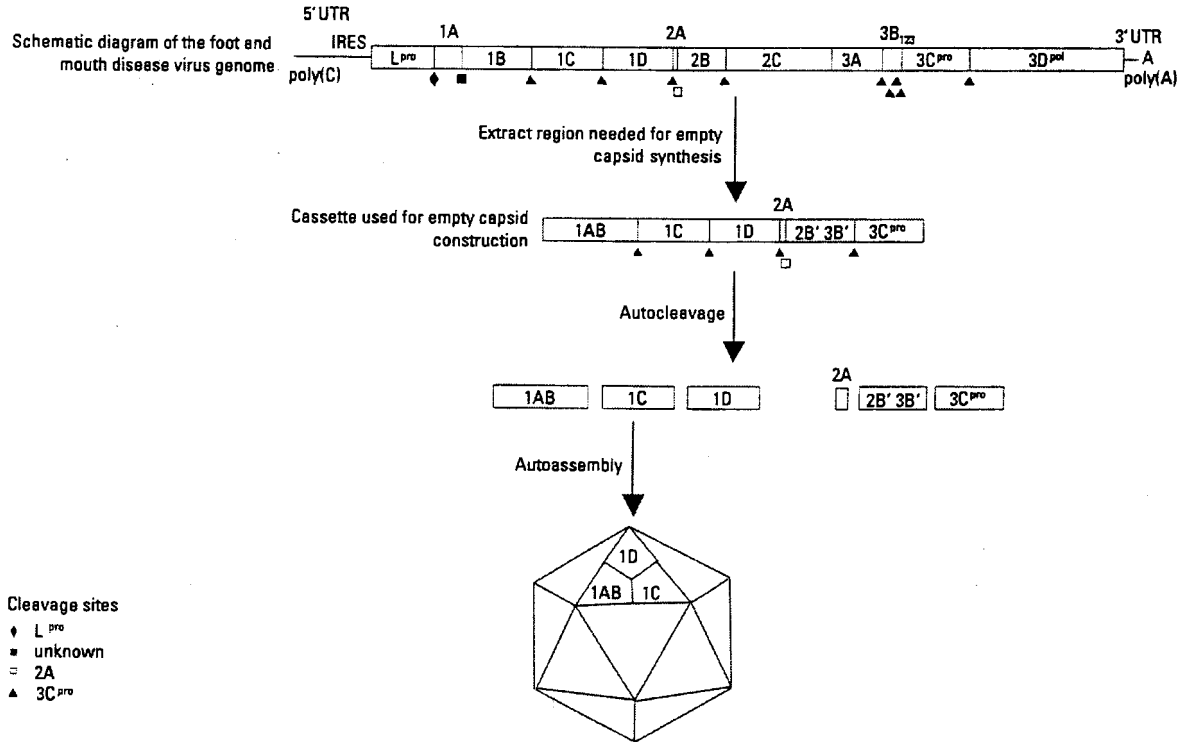


Fig. 1.1 Schematic representation of the FMD virus genome and empty capsid construction and auto-assembly. The untranslated regions of the genome are represented by lines and the protein-encoding regions are represented by boxes. The symbols below the protein-encoding regions identify the proteinases responsible for cleavage of the viral polyprotein. The 2A autoproteinase and $3C^{pro}$ regions are included in the empty capsid construct since processing of the capsid proteins to 1AB, 1C, and 1D by $3C^{pro}$ and removal of 1P-2A from the remainder of the viral polyprotein are required for autoassembly, and assembly is necessary for induction of neutralising and protective immunity (cited from Grubman and Mason, 2002).

1.4.4 Genetic variation of FMD virus genome

Mutations

Foot-and-mouth disease virus undergoes a high rate of mutation during replication. This is mainly due to a lack of replication error checking mechanisms. RNA viruses which exhibit such a deficiency mutate at the rate of one nucleotide base change per 10^3 bases per replication cycle (Holland *et al.*, 1982). Gebauer *et al.* (1988) reported a mutation rate of 10^{-2} substitutions per nucleotide site per

year (s/n/y) in serotype O, A and C. It is also estimated that a mutation rate of up to 10^{-8} – 10^{-9} nucleotide substitution per year during an epizootological cycle of FMD viruses can occur (Domingo *et al.,* 1990). Vosloo *et al.* (1996) estimated a mutation rate between 1.54 x 10^{-2} and 1.64 x 10^{-2} in African buffalo (*Syncerus caffer*) persistently infected with SAT type viruses. Therefore, new variants of FMD viruses are continuously arising after each replication cycle which constitute an intratypic population of FMD viruses with different degrees of genetic relationships, previously described as the quasispecies phenomena (Domingo *et al.,* 1985, 1990). Analysis of nucleotide substitutions indicated that synonymous mutations play a major role in FMD viruses evolution (De La Torre *et al.,* 1988; Sáiz *et al.,* 1993).

Modifications in the genome of viruses isolated from persistently infected cattle and buffalo (Dawe *et al.,* 1994; Vosloo *et al.,* 1996) and during virus replication in cell culture (Sobrino *et al.,* 1983) have been reported to occur. This may result in the generation of viral diversity. Changes in the nucleotide compositions of the capsid genes are responsible for the genetic or antigenic variability of the virus (Mateu *et al.,* 1989; Strohmaier *et al.,* 1982; Sobrino *et al.,* 1983; Carroll *et al.,* 1984; Beck and Strohmaier, 1987; Baxt *et al.,* 1989; Lews *et al.,* 1991; Meyer *et al.,* 1994). Thus, the generation of new variants is considered as one of the major problems in the control of FMD by vaccination.

Recombination

It has been shown that genetic recombination occurs between viruses of the same serotype (Pringle, 1965) as well as between serotypes, for example recombination has been demonstrated between isolates of serotype O and SAT-2 (McCahon *et al.,* 1985) and between serotypes O and C (Krebs and Marquardt, 1992). Mutations through recombination could result in the exchange of genetic material that could lead to the generation of new antigenic variants that may escape immune pressure (King *et al.,* 1982).

1.4.5 Antigenic variation

Among the capsid proteins, VP1 is the most antigenic protein and carries the domain mainly responsible for antigenic heterogeneity and cell-virus interaction

(Sanger, 1979; Bittle *et al.*, 1982; Robertson *et al.,* 1984; Morrell *et al.,* 1987; Dopazo *et al.,* 1988; Acharya *et al.,* 1989; Palmenberg, 1989; Rotbart and Kirkegaard, 1992; Logan *et al.,* 1993; Rueckert, 1996). Inoculation of this protein into pigs has produced high levels of neutralizing antibodies (Laporte *et al.,* 1973; Bittle *et al.,* 1982). The conserved Arginine-Glycine-Aspartic acid (RGD) site within the G-H loop spanning amino acid positions 140 –160 of the VP1 protein protrudes from the virion surface and is mobile (Acharya *et al.,* 1989) and constitutes the host cell binding motif in FMD viruses (Mateu *et al.*, 1996). This G-H loop often experiences a higher rate of non-synonymous substitution and exhibits greater genetic variability in the 1D gene (Haydon *et al.*, 1998).

The contribution of capsid proteins other than VP1 to the antigenicity of the virus was also demonstrated by many researchers (Barnett *et al.,* 1989; Baxt *et al.,* 1989; Kitson *et al.,* 1990; Lea *et al.,* 1994; Meyer *et al.,* 1997). These independent antigenic sites were identified on the VP2 and VP3 genes, *e.g.* the B-C loop (VP2) was found in serotypes A, O and Asia 1 (Sáiz *et al.,* 1991; Aktas and Samuel 2000; Marquardt *et al.,* 2000). Changes to the genes encoding capsid proteins can result in antigenic variation and evolvement of new subtypes (Haydon *et al.,* 2001). This may give rise to immunologically distinct variants that can re-infect individuals that have been previously infected by related viruses. The degree of cross protection among different subtypes of the same serotype thus varies (Grubman and Mason, 2002). Since there is continual antigenic drift in enzootic situation this is an important factor to consider when selecting vaccine strains.

1.5 Diagnosis of FMD

Diagnosis of FMD normally involves obtaining clinical information, examination of sick animals and sampling as well as laboratory tests. Due to the number of atypical cases as well as sub-clinical infections and apparently recovered animals that harbour the infection, clinical diagnosis can present many difficulties. Other viral infections of the mucous membrane, which produce similar clinical signs must first be eliminated *e.g.* vesicular stomatitis, rinderpest, malignant catarrhal fever, the bovine herpes 1 infections, exanthema of pigs and swine vesicular disease (Blood *et al.*, 1983). An appropriate sample can be collected and due to the

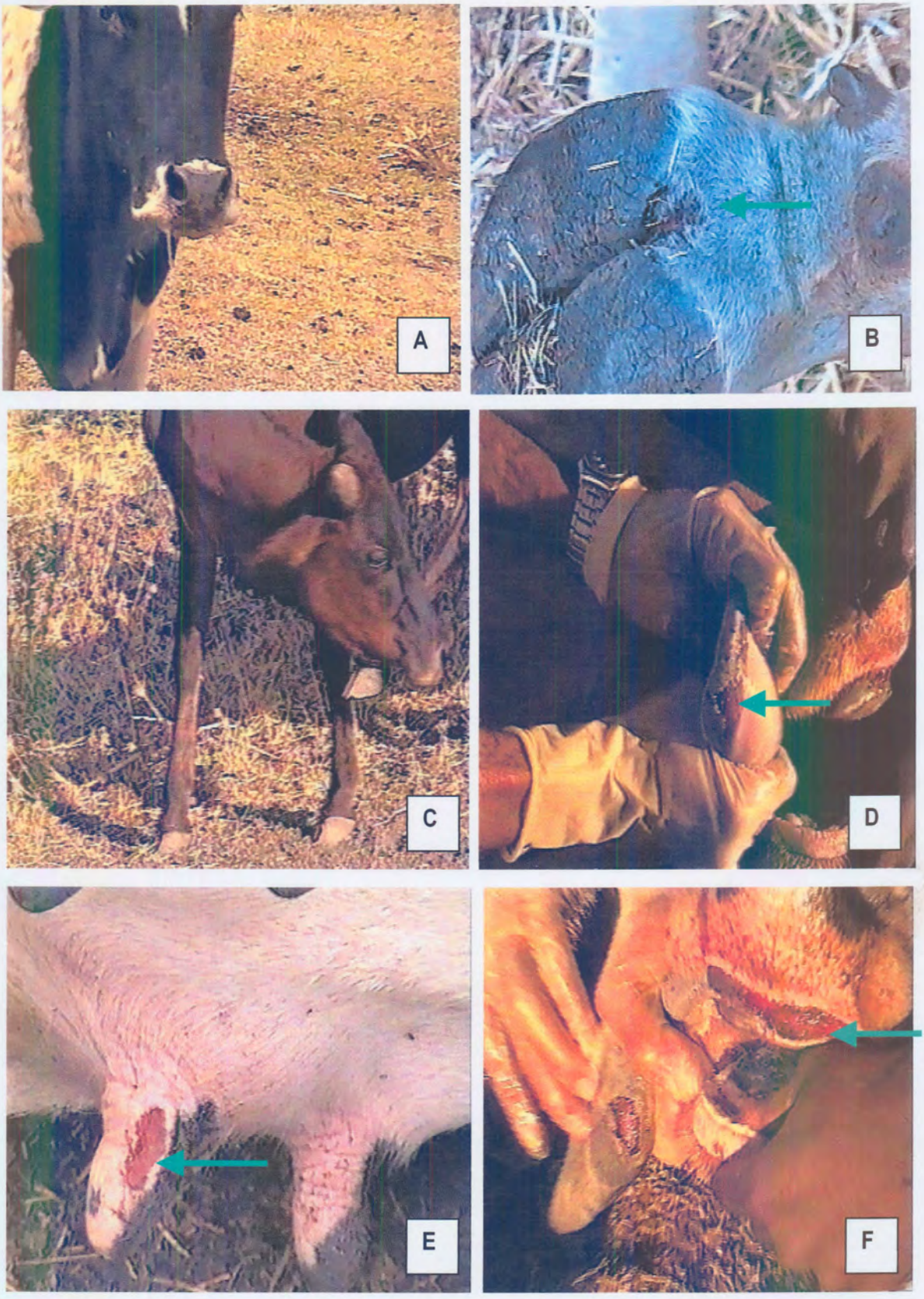
potentially devastating effect the disease can have on the economies of FMD free countries or zones, fast and reliable laboratory diagnosis is essential.

1.5.1 Clinical signs

The disease has an incubation period of 3-14 days and excretion of the virus from infected animals in all secretions and excretions usually begins before the appearance of visible clinical signs (Kitching, 2002a). Initial virus multiplication occurs mainly in the pre-pharyngeal area and the lungs (Van Bekkum *et al.,* 1960 Burrows, 1966, 1968; Sutmoller and McVicar, 1976; Burrows *et al.,* 1981).

The clinical manifestation of the disease may vary depending on the host species involved and the virus strain. Common clinical signs of the disease in cattle is presented in Fig. 1.2 from the picture taken during FMDV sample collection in Ethiopia (2001). The most severe disease occurs in cattle and pigs while in sheep and goats the disease may be sub-clinical. Lameness is usually the first indication of FMD in sheep and goats. Vesicles in the interdigital cleft are the most common findings in sheep while lesions at other sites are less regular (Hughes *et al.,* 2002).

Acutely infected cattle salivate profusely and develop a nasal discharge (mucoid and then mucopurulent). Following pyrexia (about 40°C), vesicles appear on the dorsum of the tongue, hard palate, dental pad, lips, gums, muzzle, coronary bands (Fig. 1.2 D, F, G and H) and interdigital space with consequent lameness. The vesicles usually rupture within 24-48 hours leaving shallow erosions and these lesions are susceptible to secondary bacterial infection (Fig. 1.2, I and J). At this stage animals are reluctant to eat and move. Other signs include licking of the feet or shifting weight from one leg to the other, holding one hoof off the ground, lagging behind the herd, lying down and reluctance to rise (Brooksby, 1982; Woodbury, 1995). Vesicles may also be seen on the teats of lactating animals. Morbidity is high and young calves may die before the appearance of clinical signs due to virus infection of the developing heart muscle and the production of a severe myocarditis (Woodbury, 1995). However, most animals recover within 2 weeks.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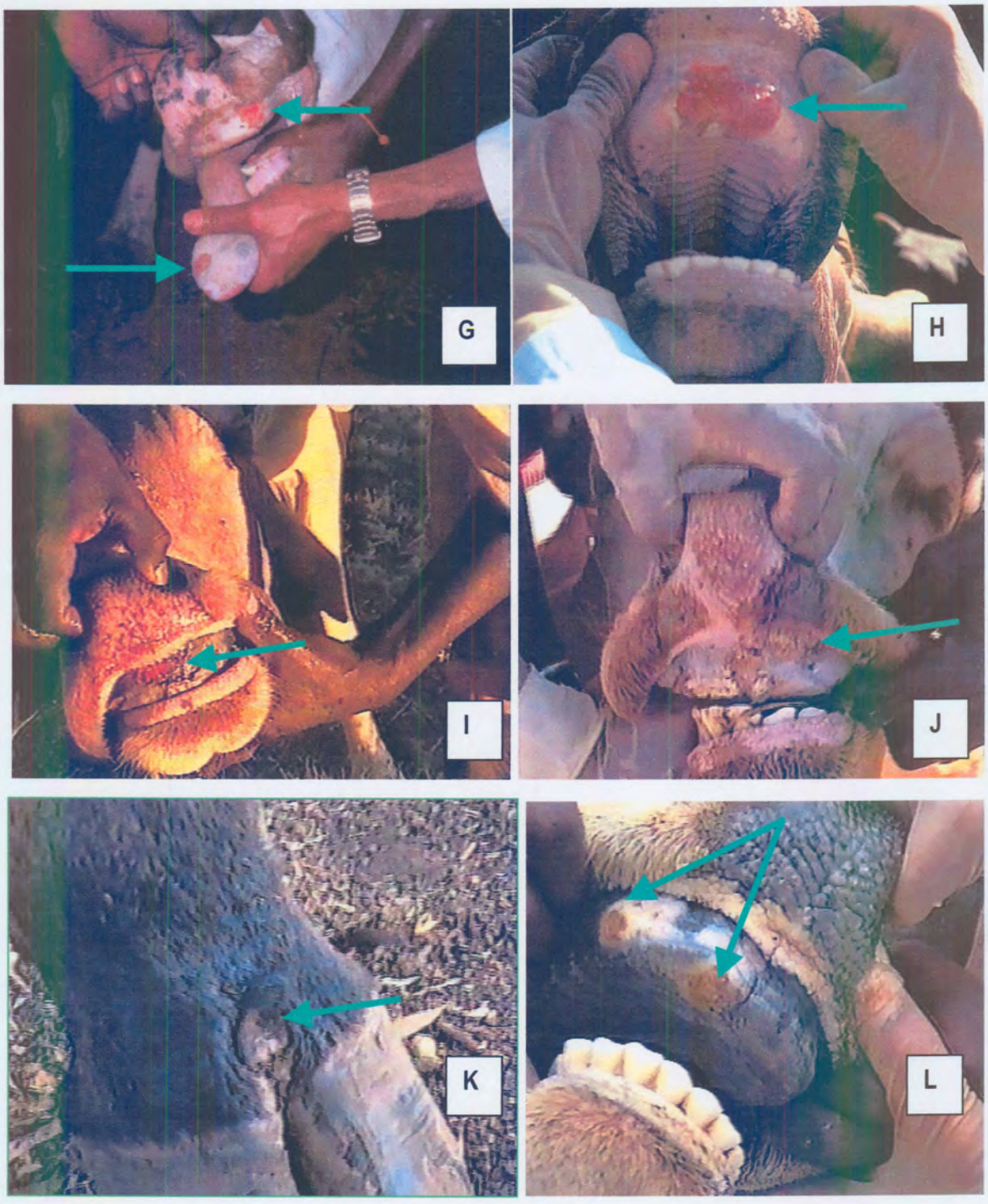

Fig. 1.2 Clinical signs of FMD in indigenous Ethiopian cattle. Pictures were taken during the outbreaks of FMD in 2001. Salivation (A), lesion at the coronary bands and interdigital space (B), lifting and licking of the feet (C), Vesicular lesion followed by ulceration and erosion (D-H), Bacterial complication (I-J), at the end the lesion heals (K-L).

1.5.2 Laboratory diagnosis

Several techniques are described in the OIE Manual of Diagnostic Standards for the confirmation of FMD infection. Virus can be isolated on cell cultures or the viral antigen detected using ELISAs, while the presence of viral genomic material can be detected using PCR assays. Alternatively, techniques that detect the presence of antibodies to the structural proteins in non-vaccinated animals, and those that detect antibodies to the non-structural proteins in both non-vaccinated and vaccinated animals can be used to determine infection. In most laboratories two approaches are used to determine the presence of FMDV infection: either cell culture and an antigen assays for the detection of the virus or assays for the detection of specific antibodies from sick or recovered animals. Virus isolation is a very sensitive method, but laborious and expensive and there is the risk of the dissemination of the virus in the environment (Kitching *et al.,* 1989). In most laboratories primary cell lines are used for virus isolation and IBRS-2 (Instituto Biologico Rim Suino, De Castro, 1964) and BHK cell lines are used for virus propagation. A number of other tests for the detection of either antigen or antibody may be used, *e.g.* the complement fixation test (CFT), virus neutralization test (VNT) and enzyme linked immunosorbent assay (ELISA) (Anonymous, 1996). Some of these tests require laboratory facilities of a high standard and the sensitivity and specificity of these conventional diagnostic tests are not always satisfactory (Have *et al.,* 1984; Hamblin *et al.,* 1986a, 1986b; Westbury *et al.,* 1988).

Some laboratories are using advanced techniques like polyacrylamide gel electrophoresis, isoelectric focusing, peptide mapping, polymerase chain reaction (PCR) and nucleotide sequencing for analysis of more detailed relationships of different isolates of the virus. Some of these tests are more sensitive and specific and can also indicate specific changes in the nucleotide sequence and polypeptide structures, which cannot be detected by conventional methods.

A reverse trancriptase polymerase chain reaction (RT-PCR) and nucleotide sequencing analysis have been developed for diagnostic purposes (Kitching *et al.,* 1988), but has also evolved into useful tools for molecular epidemiological studies. The PCR provides an extremely sensitive and rapid assay for the identification of

the viral genome. This technique allows the detection of genetic material even in the absence of infectivity for tissue culture or laboratory animals. This was demonstrated from samples of oesophageal-pharyngeal scrapings of carrier cattle taken at 180 and 560 days post infection (Laor *et al.,* 1992). The detection of genetic material by PCR was also demonstrated in aerosols (Suryanarayana *et al.,* 1999), clinical samples and cell culture isolates (Laor *et al.,* 1992; Meyer *et al.,* 1991; Amaral-doel *et al.,* 1993; Locher *et al.,* 1995; Vangrysperre and De Clercq, 1996; Niedbalski *et al.,* 1998; Zhu-CaiZhu *et al.,* 1998), nasal swabs of asymptomatic cattle (Marquardt *et al.,* 1995; Callens and De Clercq, 1997), oesophageal-pharyngeal samples (House and Meyer, 1993; Murphy *et al.,* 1994; Zhu-CaiZhu *et al.,* 1998), skin and tongue epithelium (Laor *et al.,* 1991) and blood (Höfner *et al.,* 1993; Locher *et al.,* 1995; Bastos, 1998). Serotype-specific PCRs have been developed using serotype-specific primers derived from sequences coding for the structural protein VP1 (Strohmaier *et al.,* 1982; Rodriquez et al., 1992; Suryanarayana *et al.,* 1999). The differences in VP1 sequences are the basis for developing RT-PCR assays to identify FMD viruses (Rodriguez *et al.,* 1992; Stram *et al.,* 1993).

1.6 Epidemiology and control of FMD

Foot-and-mouth disease is a highly contagious viral vesicular disease of cloven-hoofed domestic and wild animal species and is characterized by fever, salivation and vesicular eruptions on the feet and mouth (Brooksby, 1982; Blood *et al.,* 1983; Thomson, 1994). Morbidity is up to 100% in susceptible animal populations but mortality is low in adults. Infected animals show a spectrum of responses to FMD ranging from inapparent infection to severe disease and death. Cattle and pigs are more frequently affected (Dijkhuizen, 1989) while the clinical disease in sheep, goats, and wild ruminants is usually milder than in cattle and is characterized by foot lesions accompanied by lameness. In Africa, indigenous breeds of cattle generally do not develop such severe lesions as high producing exotic breeds. Recovered cattle produce neutralizing antibodies and can resist re-infection by the same subtype of virus for up to one to 3 years (Samina *et al.,* 1998).

1.6.1 Geographic distribution of FMD

Except for Greenland, New Zealand and the smaller islands of Oceania, many countries of the world have experienced outbreaks of FMD. The United States of America, Canada, Mexico, Australia, and Scandinavia haven't had the disease for many years (Samuel and Knowles, 2001b). At present, most developed countries have successfully controlled or eradicated the infection and have implemented strict control measures, especially regarding imports of animals and animal products to prevent re-introduction of the disease. According to the Office International des Epizooties (OIE) report of 2002, 50 of the 162 member countries of the OIE have obtained FMD disease free status.

The disease is prevalent in Asia, the Middle East, Africa and in some countries in South America with most outbreaks due to serotypes O and A (Anonymous 1998a; Kitching, 1998; Samuel and Knowles, 2001b Knowles and Samuel, 2003). A number of FMD outbreaks have been reported in Europe since the cessation of vaccination in 1991, *viz*. Bulgaria in 1991, Italy, Bulgaria and Russia in 1993, Greece during 1994, Turkish Thrace and Russia in 1995, Albania, the former Yugoslav Republic of Macedonia, Turkish Thrace, Bulgaria and Greece in 1996, the former Soviet Republics of Georgia and Armenia in 1997, Greece, Britain, France and The Netherlands in 2001 and Greece in 2001 (Leforban and Gerbier, 2002). The Pan-Asian serotype O virus was responsible for the outbreaks throughout the Middle East, South-East Asia and South Africa (Knowles *et al.,* 2000; Sangare *et al.,* 2001).

1.6.2 Serotype distribution of FMD in Africa

Foot-and-mouth disease is endemic in sub-Saharan African countries, except for Madagascar (Kitching, 1998, Vosloo *et al*., 2002). Six serotypes, namely O, A, C, SAT-1, SAT-2, SAT-3, are endemic in most sub-Saharan African countries with marked differences in the distribution and prevalence of serotypes (Pereira, 1981; Anderson, 1981; Abu Elzein, 1983; Abu Elzein *et al*., 1987; Kitching, 1998; Vosloo *et al.,* 2002) as indicated in Fig. 1.3. Serotypes A and O are wide-spread throughout sub-Saharan Africa while serotype C rarely occurs. The last outbreaks of serotype C were reported in Kenya in 1996 and 2000, a country where numerous

outbreaks due to the other serotypes, *viz.*, A, O, C, SAT-1 and 2 have also been reported. Type O is endemic in some countries of northern Africa such as Egypt and Libya, while outbreaks due to this serotype have also been reported in Algeria, Morocco and Tunisia. In Central Africa and West Africa serotypes O, A, SAT-1 and SAT-2 have been recorded since 1958 while most outbreaks were attributed to serotypes A and SAT-2 (Vosloo *et al.,* 2002). The three SAT types are also prevalent in southern and eastern Africa with SAT-3 demonstrating the most restricted distribution (Vosloo *et al.,* 2002).

Table 1.2 Topotypes of FMD serotypes O, A, C, and South African Territories types (SAT-1, -2 and -3) in Africa (cited from Vosloo *et al.*, 2002).

Sero-types	Topo-types	Representative country (ies)
SAT-1	I	South Africa, southern Zimbabwe, Mozambique
	II	Botswana, Namibia, western Zimbabwe
	III	Zambia, Malawi, Tanzania, northern Zimbabwe
	IV	Uganda
	V	Nigeria
	VI	Nigeria, Niger
SAT-2	I	South Africa, Mozambique, southern Zimbabwe
	II	Namibia, Botswana, northern and western Zimbabwe
	III	Botswana, Zambia
	IV	Burundi, Malawi, southern Kenya
	V	Nigeria, Senegal, Liberia, Ghana, Mali, Cote d'Ivoire
	VI	Gambia, Senegal
	VII	Eritrea
	VII	Rwanda
	IX	Kenya
	X	Democratic Republic of the Congo
	XI	Angola
SAT-3	I	South Africa, southern Zimbabwe
	II	Namibia, Botswana, western Zimbabwe
	III	Zambia
	IV	Northern Zimbabwe
	V	Uganda
O	I	South Africa
	II	Kenya, Uganda
	III	Algeria, Cote d'Ivoire, Guinea, Morocco, Niger, Ghana, Burkina Faso, Tunisia
	IV	Eritrea, Ethiopia, Tunisia, Egypt
	V	Angola
A	I	Mauritania, Mali, Cote d'Ivoire, Ghana, Niger, Nigeria, Cameroon, Chad, Senegal
	II	Angola, Algeria, Morocco, Libya, Tunisia, Malawi
	III	Tanzania, Burundi, Kenya, Somalia, Malawi
	IV	Ethiopia
	V	Sudan, Eritrea
	VI	Uganda, Kenya, Ethiopia
C	I	Kenya

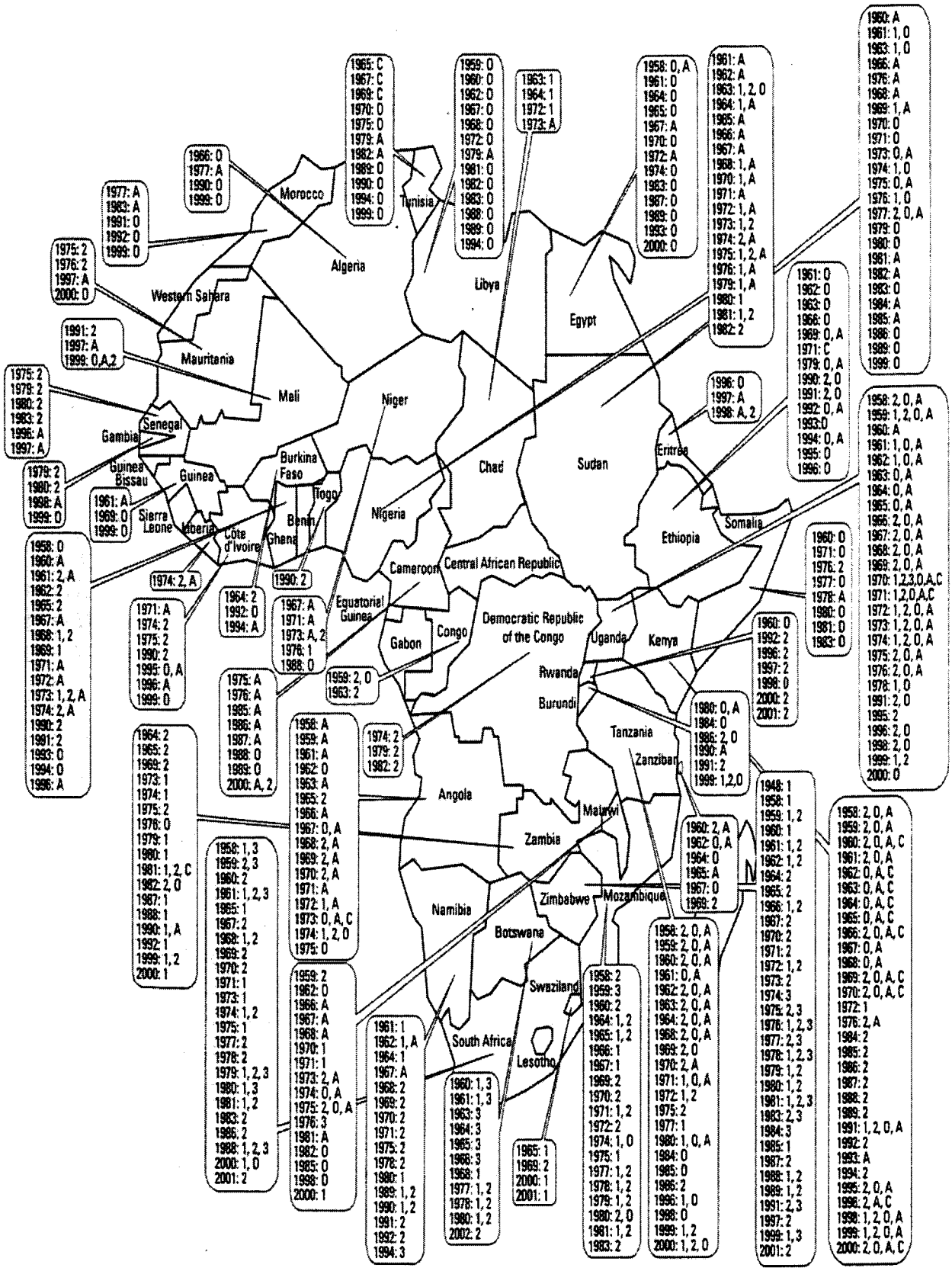

Fig. 1. 3 Map of Africa demonstrating the outbreaks of foot-and-mouth disease between 1948 - 2002 (cited from Vosloo *et al.*, 2002). The numbers 1, 2 and 3 represent the serotype SAT-1, SAT-2 and SAT-3, respectively.

1.6.3 Susceptible host range

Foot-and-mouth disease is highly contagious and affects over 70 domestic and wild *Artiodactyla* species (Hedger, 1981). It naturally infects and causes disease in cattle, pigs, sheep, goats and many wild ruminants and susceptibility of these animals can vary with breed of animal and strain of virus (Thomson, 1994; Kitching, 2002a; Kitching and Hughes, 2002; Kitching and Alexandersen, 2002). The disease is considerably less obvious or sub-clinical in breeds of cattle, sheep and goats indigenous to Africa and Asia, where FMD is endemic and these animals are believed to have been the source of infection for countries previously considered disease-free.

Foot-and-mouth disease has been reported in several species of African antelope (Anderson *et al.,* 1975; Ferris *et al.,* 1989; Hedger *et al.,* 1972; Thomson, 1996; Thomson *et al.*, 2003) and serological surveys showed that most species of animals in sub-Saharan Africa possessed antibodies to one or more serotype (Condy *et al.,* 1969; Hedger, 1976; Paling *et al.*, 1979; Anderson, 1981). Experimental infection of warthog (*Phacochoerus aethiopicus*) and bush pig (*Patomachoerus porcus*) with SAT-2 viruses showed severe clinical signs of infection and transmission to in-contact animals (Hedger *et al.,* 1972). However, these animals do not excrete virus to the levels of domestic pigs, and are not believed to play an important role in the epidemiology of FMD in Africa. Impala (*Aepyceros melampus*) is the most frequently infected species in South Africa (Thomson, 1996). Rare cases of FMD have also been reported in Indian elephants (*Elephas maximus*) and in the African elephant (*Loxodonta Africana*) (Howell *et al.*, 1973; Pyakural *et al.,* 1976). However, there is no evidence of the natural occurrence of the disease in the latter species in Africa (Howell *et al.,* 1973). Llamas (*Llama glama*) are susceptible to FMD infection but do not become FMD carriers (David *et al.,* 1993; Lubroth *et al.,* 1990). Foot-and-mouth disease is not a zoonosis, and only a few possible cases of infection of humans have been described (Bauer, 1997) but man is an important mechanical carrier of the virus.

1.6.4 The role of carriers in the epidemiology of the disease

FMDV may persist undetected in cattle and African buffalo and consequently may become the source of new infection to other susceptible animals (Rina and Martin, 1976). Persistence of infection and the presence of the virus in the pharyngeal region of cattle have been demonstrated after months or 1-2.5 years post infection (Van Bekkum *et al.,* 1960; Sutmoller and Gaggero, 1965; Hedger, 1968, 1970; Burrows *et al.,* 1971; Rossi *et al.,* 1988). Carrier state of animals depends on the ability of the virus to persist in the pharyngeal area of these ruminants which signifies a special virus-host relationship while the duration of the state of persistence differs from species to species. The carrier state in sheep varies between 1-5 months (Burrows, 1968). It is also reported that the African buffalo persistently infected with SAT type viruses can maintain the infection for up to 5 years (Condy *et al.,* 1985) and transmit the disease to other susceptible animals in close proximity (Dawe *et al.,* 1994; Vosloo *et al.,* 1996; Thomson, 1996; Bastos *et al.,* 2000). This may provide a mechanism for the maintenance of the virus in nature and the cause of acute episodes of disease and may also contribute to the emergence of new antigenically variant viruses (De La Torne *et al*, 1988; Donaldson and Kitching, 1989; Terpstra *et al.,* 1990; Domingo *et al.,* 1992; Malirat *et al.,* 1994). Cattle vaccinated against FMD and subsequently infected with FMD virus can also become persistently infected (Borrego *et al.,* 1993; Salt *et al.,* 1996). Antigenic variability and the possibility of a carrier state among infected animals complicate the diagnosis and control of FMD (Wittmann, 1990).

1.6.5 Transmission of FMD

Direct contact

Foot-and-mouth disease is highly contagious and transmission of the disease can occur by direct contact between infected and susceptible animals during the acute phase of the disease, by animal products (*e.g.* meat, milk, wool), by airborne route (Cooper *et al.,* 1978; Brooksby, 1982, Woodbury, 1995) and by contaminated animal handlers.

The released viruses can also survive in dry blood and defragmented epitelium in the environment for varying periods of time depending on the weather condition.

Immediate freezing of carcasses after dressing enhances preservation of live infectious virus and outbreaks across international borders have been ascribed to this manner through meat trading. The source of the 1967/68 outbreak of FMD on the British mainland was attributed to infected sheep meat imported from Argentina (Leforban and Gerbier, 2002). In addition vehicles and fomites have also been responsible for transmission of the disease (Hyslop, 1970, 1973; Sellers, 1971).

Aerosol transmission

Cattle, sheep and goats infected with FMD can excrete between 10^3 to 10^8 infectious virus units/day as an aerosol (Mann and Sellers, 1990), while aerosol production of infected pigs can be as high as 4×10^6 infectious units/day (Gibson and Donaldson, 1986; Donaldson, 1987; Donaldson *et al.,* 1987; Kitching, 1992). Cattle are very susceptible to infection by the respiratory route and a dose of 20 $TCID_{50}$ (tissue culture infective dose) of virus is sufficient to establish infection (Donaldson *et al.,* 1987). During the FMD outbreak that occurred in France and then in the UK (1981), virus spread from France to the UK over 250 km. Kitching (1992) also emphasized the possible spread of FMD up to 250 km across the sea and up to 60 km across land if conditions are suitable. At present there are computer models which can predict the most likely wind-borne spread of the virus from infected herds and allow the examination of a variety of control strategies (Dijkhuizen, 1989; Sanson *et al.,* 1991).

1.6.6 Control of FMD

Today, several countries have either eradicated FMD by compulsory slaughter of infected animals, or have greatly reduced its incidence or eradicated the disease by extensive vaccination programs. The control of FMD is dependent on the understanding of the disease epidemiology in specific areas or regions (Rweyemamu and Ouldridge, 1982; Sáiz *et al.,* 1993; Stram *et al.,* 1995).

1.6.6.1 Control by vaccination

In endemic areas the disease is generally controlled by vaccination and movement restriction of animals. Vaccination against FMD virus is achieved within activated vaccines that should induce protective immunity against each type of antigens incorporated in the vaccine. Intratypic variation of the field strains of FMD viruses must also be considered in the selection of seed virus for vaccine production. Immunity to one serotype provides protection against homologous viruses. In some cases, immunity to one subtype will not protect against other members of the same serotype. An inactivated bi-, tri-, or polyvalent vaccine, which contains the representative strains of the serotypes that are in circulation in the region, must be used (Gonzalez *et al.,* 1992). Therefore, active disease surveillance must be effective which needs a strong field service as well as proper laboratory facilities with efficient methods of detection and characterisation of the virus.

1.6.6.2 Control by stamping out

The United Kingdom, Ireland, countries of Scandinavia, Japan, Canada and the United States of America were able to control the disease by stamping-out. In Europe, FMD has been successfully controlled for several decades by extensive vaccination of the cattle population. Most of the European countries have agreed to a policy of non-vaccination and in the case of an outbreak, infected as well as in contact animals will be slaughtered (Locher *et al.,* 1995; Vangrysperre and De Clercq, 1996). The strategy used to combat the outbreak of FMD that occurred in the UK in 2001 has stimulated a larger debate on the policy of disease control by stamping out. This provokes the reassessment of the policy and re-consideration of vaccination as part of alternative and more flexible FMD control strategies (Rossides, 2002). The Nethlands adopted emergency vaccination to control outbreak in 2001.

1.6.6.3 Control of FMD in developing countries

Numerous natural as well as political and socio-economic problems complicate control and finally the eradication of FMD in developing countries where FMD is mostly endemic. Control strategies followed by many African countries include the

slaughter of infected animals and animals at risk, control of contact between infected wildlife and domestic animals by fencing and ring vaccination (southern African countries), control of cattle movement and the introduction of strict quarantine measures. Since FMD has become a great concern in many African countries, it also requires the co-operation of countries to share their experiences, information and technology in order to stop trans-boundary spread of the disease.

1.7 Molecular Phylogeny

Phylogenetic analysis of the VP1 region of FMD viruses has been used extensively to investigate the molecular epidemiology of the disease worldwide. These techniques have assisted in studies of the genetic relationships between different FMD virus isolates, geographical distribution of lineages and genotypes, the establishment of genetically and geographically linked topotypes and tracing the source of virus during outbreaks (Strohmaier *et al.,* 1982; Domingo *et al.,* 1985; Beck and Strohmaier, 1987; Dopazo *et al.,* 1988; Laor *et al.,* 1991; Meyer, *et al.,* 1991; Vosloo *et al.,* 1992; Sáiz *et al.,* 1993; Locher *et al.,* 1995; Marquardt *et al.,* 1995; Stram *et al.,* 1995; Yehuda *et al.,* 1995; Vangrysperre and De Clercq, 1996; Callens *et al.,* 1997; Bastos, 1998, 2001; Bastos *et al.*, 2001; Bastos *et al.*, 2003; Samuel and Knowles, 2001a, b, Knowles *et al.*, 2000; Knowles & Samuel, 2003; Sangare *et al.,* 2001, 2003). Sequence differences of 30 to 55% of the VP1 gene were obtained between 7 serotypes of FMD while different subgroups (genotypes, topotypes) were defined by differences of 15 to 20% (Knowles and Samuel, 2003). Since 1987, the analysis of the genetic distance and phylogenetic resolution of the sequence of VP1 encoding gene have provided crucial epidemiological information covering different degree of genetic relationships between field isolates (Beck and Strohmaier 1987; Vosloo *et al.*, 1992; Samuel *et al.*, 1997; Samuel *et al.*, 1999; Bastos *et al.*, 2001; Samuel and Knowles, 2001a,b; Knowles and Samuel, 2003) as follows:

Virus isolates from the same epizootic differ by $\leq$ 1%
Viruses belonging to the same epizootics (common origin) differ by < 7%
Viruses of the same genotype differ up to 15%
Viruses from different genetic lineages differ by ($\geq$ 20%)

The evolutionary changes of viruses are determined by comparing genomic material from more than one virus with each other. The basic process in the evolution of DNA/RNA sequence is the substitution of one nucleotide for another over evolutionary time. Changes in nucleotide sequences are used in molecular evolutionary studies both for estimating the rate of evolution and for re-constructing the evolutionary history of organisms (Graur and Wen-Hsiung, 2000). At present, DNA sequencing and phylogenetic trees are widely used to illustrate the genetic relationships between viruses. In order to construct evolutionary trees assumptions are made about the substitution process and these assumptions are stated in the form of a model.

Several assumptions exist regarding the probability of substitution of one nucleotide by another. For example the one parameter model of Juke and Cantor (1969) is based on the assumption that substitutions occur with equal probability among the four nucleotide types (Fig. 1.4) while Kimura's two-parameter model (Nei and Kumar, 2000) assumes transitions are generally more frequent than transversions. A simple measure of the extent of sequence divergence is the proportion (p) of nucleotide sites at which the two sequences are different.

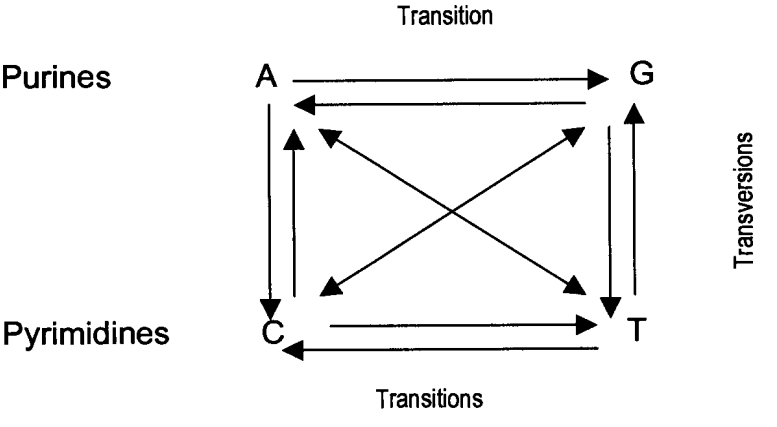

Fig. 1.4 The probability of substitution of one nucleotide by another between purines and/or pyrimidines.

Phylogenetic analysis

The evolutionary relationships among a group of organisms are illustrated by means of phylogenetic trees where the phylogeny is the branching history of route of inheritance of species populations or genes and is microevolutionary informative (Maddison *et al.*, 1992). Phylogeny is misleading unless it is based on a

reasonable alignment of the sequences used in the analysis and computer programs are available for obtaining optimal alignment of sequences.

Methods for constructing phylogenies.

There are a variety of methods available to construct trees from sequence data which use two primary approaches to tree construction: algorithmic and tree-searching. The algorithmic approach uses an algorithm to construct a tree from the data while the tree-searching method constructs many trees and then uses some criterion to decide which is the best tree or best of trees. Currently, three primary methods for constructing phylogenies from nucleic acid alignments *viz.*, Neighbour-joining (NJ), Maximum Parsimony (MP) and Maximum likelihood (ML) methods are in use (Nei and Kumar, 2000). The method of choice depends both on what you want to learn and on the size and complexity of the data set. It also depends on the speed of your computer and the ease of implementing the particular method. However, some criteria like efficiency, robustness, computational speed and discriminating ability are considered to select the best method for constructing evolutionary trees.

A. Distance methods

Distance methods convert the aligned sequences into a distance matrix of pair-wise differences (distances) between the sequences. The NJ and Unweighted Pair-Group Method (UPGMA) using arithmetic average methods are currently in use which are both algorithmic methods *i.e.*, they use a specific series of calculations to estimate a tree. The calculation involves manipulations of a distance matrix that is derived from multiple alignments. Starting with the multiple alignments, both programmes calculate for each pair of taxa the distance, or the fraction of differences, between the two sequences and write that distance to a matrix.

B. Character-based methods

Character-based methods include Parsimony, Maximum Likelihood, and Bayesian methods; all use the multiple alignments directly by comparing characters within each column (each site) in the alignment. Parsimony looks for the tree or trees with the minimum number of changes (Parsimony-informative sites). Maximum likelihood tries to infer an evolutionary tree, under some model of evolution, by

finding that tree that maximizes the probability of observing the data. The Bayesian analysis is a recent variant of Maximum Likelihood. This method, instead of seeking the tree that maximizes the likelihood of observing the data, seeks those trees with the greatest likelihoods given the data.

1.8 Summary and objective of the study

The improvement of diagnostic techniques will help overcome the existing diagnostic problems in many developing countries. Continuous research is needed to establish the geographic relatedness between isolates, the genetic variation, and molecular evolution of viruses in carriers. During outbreaks, it is also important to identify the origin of infection and its relationship to vaccines available for protection which will assist in planning a control programme in the country.

The molecular epidemiology of FMDV has been studied in some detail for southern and western Africa using nucleotide sequencing of the main antigenic determinant of the virus and phylogenetic analysis. However, sufficient genetic information of viruses from East Africa has not been available to determine the number of viral lineages and genotypes and to investigate whether certain patterns of spread between countries have occurred in the past. The role of wildlife and small stock in the maintenance of the disease also needs to be studied.

Objectives of study

1. To study the phylogenetic relationships of serotype O, SAT-1 and SAT-2 FMD virus isolates circulating in East Africa.
2. To determine the genetic variability of serotype O and SAT-2 FMD viruses in Ethiopia and their phylogenetic relationships in comparison to other East African isolates.
3. To identify trends with regards to the spread of FMD viruses within/across East African countries.
4. To assess the role of small ruminants and wildlife in the epidemiology of FMD in Ethiopia.
5. To provide Ethiopia and East African countries with a viable genetic database which will serve as a basis for future epidemiological studies.

CHAPTER II

MOLECULAR EPIDEMIOLOGY OF SEROTYPE O FOOT-AND-MOUTH DISEASE VIRUSES ISOLATED FROM CATTLE IN ETHIOPIA BETWEEN 1977 - 2001

2.1 Introduction

Ethiopia is one of the few countries in Africa with huge livestock resources that play a crucial role in the livelihoods of the majority of Ethiopians. Animal rearing is an integral part of the agricultural production in Ethiopia and animals represent the major draught power (95%) for crop production. The livestock population comprises of approximately 30 million cattle, 23 million sheep, 18 million goats, 7 million equines and 1.2 million camels while the agricultural sector constitutes about 45% of the gross domestic production (GDP), more than 90% of foreign exchange earnings, 85% of employment opportunities and most of the domestic food supply (Anonymous, 1998b; Anonymous, 2000).

However, due to various constraints such as poor genetic potential of animals, lack of proper livestock management and prevalent livestock diseases, the benefit from livestock production is very low. Animal diseases are currently widespread in all agro-ecological zones of the country and annual mortality rates due to diseases is estimated at 8 -10% for cattle herds and 15% and 12% for sheep and goat flocks, respectively. It is estimated that animal diseases reduce the production and productivity of livestock by 50 to 60% per year (Anonymous, 1998b).

At present FMD is one of the major livestock diseases of socio-economic importance. FMD occurred frequently in pastoral herds in the lowland areas of Ethiopia (Haile-Yesus, 1988) but in recent years the incidence of this disease has increased and became apparent in the highland areas where > 60% of the total livestock population occurs (Mengistu, 1997; Anonymous, 2000). According to the Animal Health Division of the Ministry of Agriculture of Ethiopia (2000), the incidence of FMD has increased between 1.3 to 1.5 times since 1990. The high incidence of the disease may be associated with extensive movement of livestock

and the high rate of contact between animals at marketing and common grazing places as well as at watering points (Mersie, *et al.*, 1992; Anonymous, 1998b). The role of wildlife in the epidemiology of FMD in Ethiopia has not been investigated, but it is accepted that the disease is maintained mostly in a domestic cycle.

Outbreaks caused by serotypes O and A are unusual in southern Africa (Vosloo, *et al.,* 2002), but frequently occur elsewhere on the subcontinent. During the period 1957-73, 62 isolates were typed as serotype O in Ethiopia, 24 as serotype C and 12 as serotype A. Based on comparisons with European isolates using virus neutralization and serum precipitation tests, serotype A isolates from Ethiopia appeared to be distinct from the European A types, while types C and O isolates differed little from the European C and O types respectively (Martel, 1974; Martel & Gallon, 1975). During the following years until 1992, outbreaks due to serotypes O and A were common while serotype C viruses seemed to disappear (Berson *et al.*, 1972; Prunet, 1972; Fikre, 1975; Anonymous, 1976; Haile-Yesus, 1988; Roeder *et al.*, 1994). Between 1990 and 1999, the World Reference Laboratory (WRL) at Pirbright typed outbreaks as serotype O (42), A (13) and SAT-2 (6). It is clear that serotype O remains dominant in outbreaks and has lead to a considerable economic crisis for the rural communities. However, it is important to note that only a small percentage of outbreaks in Ethiopia is reported and typed, therefore the above mentioned is a underestimation of the actual problem caused by FMD outbreaks.

Despite the wide distribution and economic impact of FMD in Ethiopia, few clinical and serological studies have been reported by the Regional Veterinary Laboratories and the National Animal Health Institute in Ethiopia (Anonymous, 1998b). The only attempt to date by the government to control the disease is by limited vaccination campaigns in dairy herds.

The epidemiology of the circulating viruses and their genetic relation compared to the vaccine strain is unknown. This study attempts to elucidate the genetic variation between Ethiopian type O isolates obtained from 1977 to 2001 and their relationship with other published type O FMD viruses as well as with the type O vaccine strain (ETH/19/77) currently in use in Ethiopia.

2.2 Materials and Methods

2.2.1 Viruses studied

Ethiopian serotype O outbreak strains of 2001 (ETH15/01, ETH16/01 and ETH 22/01) were isolated on primary pig kidney (PK) cells and then passaged on IB-RS-2 (Instituto Biologico Rim Sunio, De Castro, 1964) cell line. All other viruses from Ethiopia and Eritrea isolated between the years 1977 to 1996 were supplied by the WRL for FMD at the Institute for Animal Health, Pirbright (United Kingdom). These isolates were stored at –70°C for protracted periods of time and were therefore propagated on IB-RS-2 cells prior to further use. The laboratory designation, sampling date and geographical origin of all isolates are indicated in Table 2.1.

Table 2.1 Summary of serotype O FMD viruses included in this study.

Virus designations	Sampling year	Country of origin	Reference	Genbank Accession No.
*1. ETH/1/79	1979	Ethiopia	This study	AY283376
*2. ETH/3/79	1979	Ethiopia	This study	AY 283377
•3. ETH 19/77	1977	Ethiopia	This study	AY283378
*4. ETH/3/90	1990	Ethiopia	This study	AY283379
*5. ETH/8/90	1990	Ethiopia	This study	AY283380
*6. ETH/12/90	1990	Ethiopia	This study	AY283381
*7. ETH/9/92	1992	Ethiopia	This study	AY283382
*8. ETH/2/93	1993	Ethiopia	This study	AY283383
*9. ETH/8/94	1994	Ethiopia	This study	AY283384
*10. ETH/24/94	1994	Ethiopia	This study	AY283385
*11. ETH/30/94	1994	Ethiopia	This study	AY283386
*12. ETH/1/95	1995	Ethiopia	This study	AY283387
*13. ETH/5/95	1995	Ethiopia	This study	AY283388
*14. ETH/3/96	1996	Ethiopia	This study	AY283392
**15. ETH/15/01	2001	Ethiopia	This study	AY283393
**16. ETH/16/01	2001	Ethiopia	This study	AY283394
**17. ETH/22/01	2001	Ethiopia	This study	AY283395
*18. ERI/1/96	1996	Eritrea	This study	AY283390
*19. ERI/2/96	1996	Eritrea	This study	AY283391
20. KEN/77/78	1978	Kenya	Sangare *et al.* (2001)	AF300812

21. KEN/10/95	1995	Kenya	Samuel & Knowles, (2001b)	AJ303514
22. GHA/5/93	1993	Ghana	Sangare *et al.*, (2001)	AF300806
23. GHA/6/93	1993	Ghana	Sangare *et al.* (2001)	AF300807
24. GHA/9/93	1993	Ghana	Sangare *et al.* (2001)	AF300809
25. BFK/1/92	1992	Burkina Faso	Sangare *et al.* (2001)	AF300804
26. ALG/1/99	2000	Algeria	Sangare *et al.* (2001)	-
27. SAU/100/94	1994	Saudi Arabia	Samuel *et al.* (1997)	AJ004660
28. BAN/1/97	1997	Bangladesh	Freiberg *et al.* (1999)	-
29. IRQ/30/2000	2000	Iraq	Samuel & Knowles (2001b)	AJ303499
30. SAR/15/2000	2000	South Africa	Sangare *et al.* (2001)	AF306647
31. O5India	1962	India	Sangare *et al.* (2001)	AF274297
32. O1Manisa	1969	Turkey	Unpublished	AJ251477
33. Moscow/95	1995	Russia	Unpublished	AJ004662
34. Taiwan/97	1997	Taiwan	Tsai *et al.* (2000)	AF026168
35. Taiwan/98	1998	Taiwan	Tsai *et al.* (2000)	AF095877
36. O1 Yrigoyen	1982	Argentina	Sáiz *et al.* (1993)	Z21862

• Vaccine strain * viruses supplied by WRL ** viruses isolated from FMD outbreaks in Ethiopia

2.2.2 Nucleic acid isolation

Total RNA was extracted from tissue culture samples using a guanidium thiocyanate-silica method (Boom *et al.*, 1990). Lysis buffer (940µl, Appendix I) and 80 µl silica mix (Appendix I) were added to 200 µl of cell culture supernatant containing FMD virus in a 1.5 ml Eppendorf tube, vortexed and allowed to stand for 5 min at room temperature. After incubation, it was mixed using a vortex and centrifuged at 10000 x g for 15 sec to sediment the silica with the bound nucleic acid. The supernatant was removed (disposed into 10 M NaOH) and 900 µl of L2 wash buffer (Appendix I) was added to the pellet and vortexed until the silica was completely in suspension. This was centrifuged for 15 sec at 10 000 x g and all the supernatant was carefully removed. The pellet was resuspended in 800 µl of 70% ethyl alcohol (EtOH) (Sigma), mixed, centrifuged as above and the supernatant removed. The same process was repeated with 700 µl acetone. The silica pellet was allowed to dry by placing the open tubes in a heating block at 56°C for 15 min under flux laminar air flow. The pellet was suspended in 30 µl of 1 X TE (10mM Tris-HCl, pH 7.6; 1mM EDTA, Appendix I) containing 4U of RNasin (Promega) and incubated at 56°C for 2 min. The tubes were then centrifuged at 10000 x g for 1 min and the supernatant containing the eluted nucleic acid was

carefully removed and transferred into a new, clean, RNase free Eppendorf tube. The tube was labelled and the RNA was either used directly for cDNA synthesis or stored at -70°C until needed .

2.2.3 Complementary DNA synthesis (cDNA)

The RNA viral template was reverse transcribed using AMV reverse transcriptase (Promega) with the 2A/B junction antisense primer (P1) of Beck & Strohmaier (1987) (5'-GAAGGCCCAGGGTTGGACTC- 3') as described previously (Bastos, 1998). Each reaction tube contained an equal volume of RNA template and master mix (MM) composed of 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates (dNTPs) (Roche), 10 pmol/µl of primer P1, 200 pmol/µl of random hexanucleotides, 40 U/µl human placental ribonuclease (RNase) inhibitor (Promega). The mixture was centrifuged and incubated at 70°C for 3 min in a water bath and snap-frozen in liquid nitrogen. Tris-acetate buffer (40 mM Tris-acetate pH 7.4 and 1 mM EDTA) containing 4 U of RNasin and 10 U of AMV-reverse transcriptase was added to the mixture, the tube centrifuged at 13 000 g for 5 sec, and incubated at 42°C for 1 hour. After incubation, the enzyme was inactivated at 80°C in a water bath for 1 min followed by chilling and stored at -20°C until needed.

2.2.4 Polymerase chain reaction (PCR)

The P1 primer in combination with the serotype O specific sense primer VP_1O (5'-GATTTGTGAAGGTGACACC-3') (Rodriguez *et al.*, 1994) was used to amplify a 581 bp fragment of the 1D gene (VP1 encoding gene). The PCR was performed in a 50 µl volume Master Mix (Appendix I) in the presence of 3 µl of cDNA, 2,5 pmol of each primer (VP_1O and P1), 200 µl dNTPs, 1X Taq polymerase buffer containing 1.5 mM $MgCl_2$ and 2.5 U of Taq polymerase (Roche) using a thermal cycling profile of 39 cycles of denaturation at 96°C for 12s, annealing at 53°C for 20s and extension at 70°C for 40s.

2.2.5 Agarose gel electrophoresis of PCR products

A 1.5% (m/v) agarose gel (Appendix I) was used to confirm the amplification of the expected fragment (580 bp) by product size estimation against a DNA molecular weight marker (100 bp) (Promega). The band of the correct size was excised from the gel and purified using the Qia Quick Gel Extraction Kit (Qiagen) according to the guidelines of the manufacturer.

2.2.6 Nucleotide Sequencing

The ABI Prism 310 Genetic analyzer (Applied Biosystem, USA) was used for sequencing through the integration of fluorescent labelling, capillary electrophoresis and software for the collection and analysis of the fluorescent signal. To incorporate the fluorescent signal, the purified PCR product (DNA template) was subjected to cycle sequencing and alcohol precipitation. DNA template (at a concentration of approximately 100 ng/µl), 4 µl of sequencing primer (0.8 pmol/µl), 4 µl of the Big Dye® version 3.0 Cycle Sequencing kit, 2 µl sequencing buffer (Applied Biosystem) and ddH_2O were added to make the total volume 20 µl. The PCR reaction was performed using 25 cycles where each thermal cycle included 96°C for 2 min, 50°C for 10 seconds and 60°C for 4 min.

The extension product was precipitated with 2 µl 3 M NaAc pH 7.4, 52 µl 100% EtOH at room temperature for 15 min. The mixture was vortexed, centrifuged at 13 200 x g for 20 min and the supernatant discarded. The pellet was rinsed with 250 µl of 70% EtOH. After mixing the content, the tube was centrifuged at 13000 g for 5 min and the supernatant discarded. The pellet was dried by placing the tube, with the lid open, in a heating block at 96°C for 1 min after which the pellet was resuspended in 12 µl of Hi-Di formamide Dye (Applied Biosystems). The contents of the tube was vortexed, centrifuged at 2000 x g for 5 sec and then denatured at 95°C for 2 min and chilled on ice. The sample was transferred to a 0.5 ml sample tube (Applied Biosystem) and covered with a septa. The sample was loaded on the ABI Prism 310 Genetic Analyzer. Two independent sequencing reactions were performed per sample using the same sense and antisense primers utilized in the PCR. The procedure for the preparation of DNA and sequencing is detailed in the guideline manual supplied by the manufacturer.

2.2.7 Data Analysis

The generated nucleotide sequences were aligned using the DAPSA program (Harley, 1994). An homologous region of 495 bp corresponding to the C-terminus end of the VP1 gene was used for all phylogenetic analysis. Nucleotide sequences of serotype O isolates from West and South Africa were included to deduce the phylogeny of this serotype on the African continent as well as isolates from the Middle East, Asia and South America to ensure that all previously identified lineages and genotypes were represented (Sangare *et al.*, 2001). Phylogenetic trees were constructed using methods of analysis included in MEGA version 2.0 (Kumar *et al.*, 2001) and confidence levels were assessed by 1000 boot-strap replications. Lineages were distinguished on the basis of nucleotide sequence differences of > 20% and high boot-strap support (> 90%) while a divergence of > 13% distinguished genotypes. Average pair-wise comparisons (Distance, MEGA 2.0) were conducted to estimate divergence (Table 2.2) between genotypes. An amino acid variability plot (Kumar *et al.*, 1993) identified variable regions within the partial VP1 gene.

2.3 Results

2.3.1 Phylogenetic analysis of all serotype O isolates included in this study

Phylogenetic trees were constructed using the Unweighted Pair-Group Mean Average (UPGMA) and the pattern of topology was evaluated with Neighbour-joining and Maximum Parsimony methods. Identical genotypes and lineages with boot-strap support > 80% were consistently obtained irrespective of the method of analysis used indicating that the tree represented here reflects the correct phylogenetic tree.

The UPGMA tree demonstrated that the serotype O isolates clustered into 3 distinct lineages designated I, II and III (Fig. 2.1). The criteria used to distinguish lineages and genotypes were > 20% and > 13% nucleotide differences, respectively. The 3 lineages were confined to different geographical regions *viz.*, Lineage I: Africa-the Middle East/Asia which comprised 31/36 isolates; lineage II: Asia with 3/36 isolates and the South American strain (01Yirgoyen) together with

O1Kaufbeuren (European strain) which formed lineage III. The Africa-Middle East/Asian lineage (lineage I) contained 4 genotypes while lineage II consisted of 1 and lineage III of 2 genotypes (Fig. 2.1).

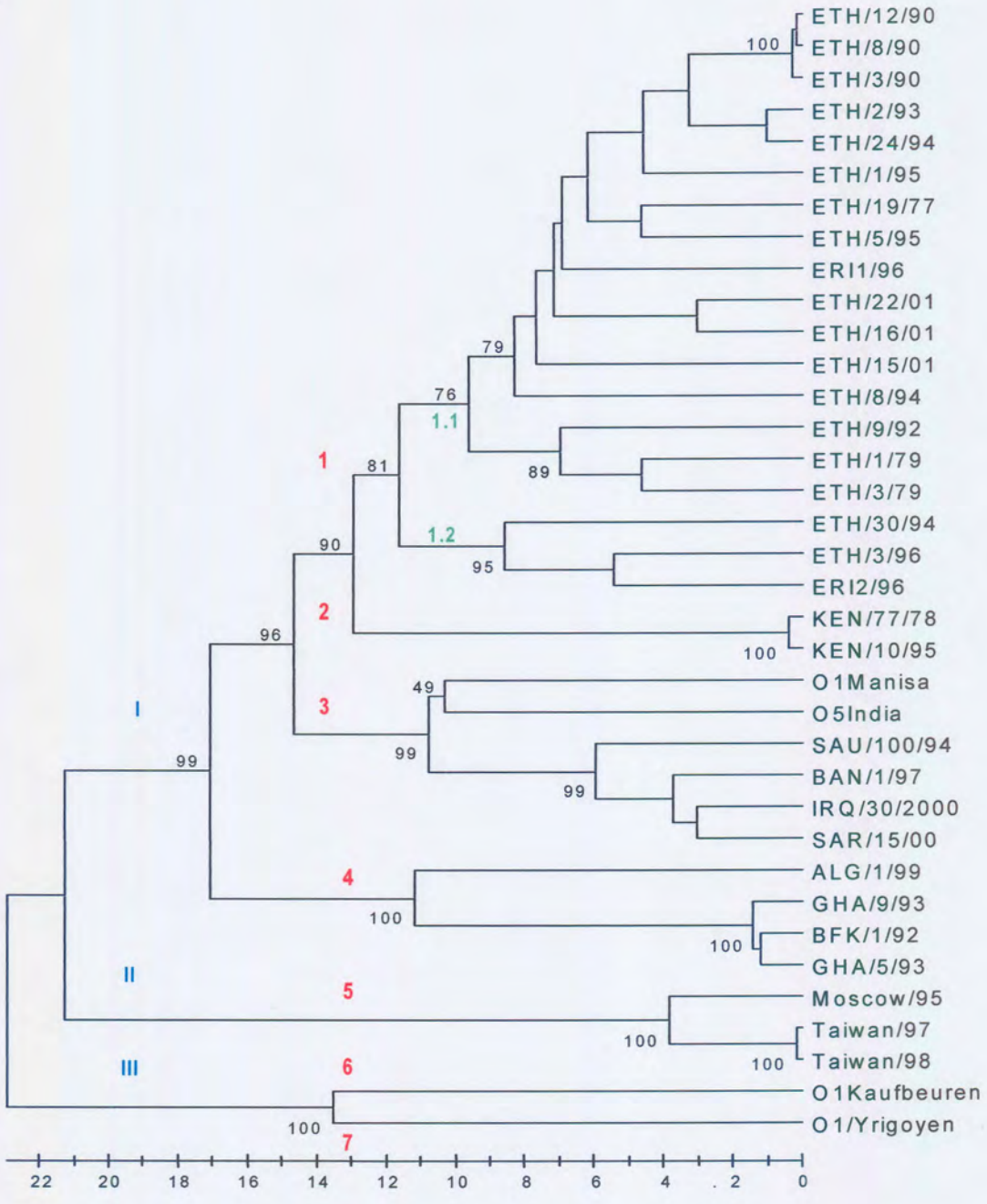

Fig. 2.1 UPGMA tree depicting genetic relationships of serotype O FMD viruses from Ethiopia, Africa (East, West & South), Africa-Middle East/Asia and Europe. I - III, 1 – 7 and 1.1 – 1.2 represented lineages, genotypes & clades, respectively.

Within lineage I genotype 1 was represented by 21 strains from Ethiopia and Eritrea which can be further divided into 2 main clusters (1.1 and 1.2) supported by statistically significant bootstrap values of 76 and 95%, respectively (Fig. 2.1). Within cluster 1.1 all isolates were obtained from Ethiopia and Eritrea during 1979 to 2001. Two sub-groupings within cluster 1.1 were also supported by boot-strap values of 79% and 89%, respectively. The first sub-grouping was represented by 13 isolates from Ethiopia and one Eritrean isolates and also contained the vaccine strain, ETH/19/77 that differed from ETH/5/95 by 4.6%. In the second sub-grouping 2 viruses obtained during 1979 and one virus during 1992 from Ethiopia differed by 5.5 – 8.5% from each other and demonstrated < 92% sequence similarities with the other viruses from Ethiopia and Eritrea within the same sub-grouping (1.1). This showed the heterogeneous nature of viruses between sub-groupings. The second cluster (1.2) consisted of isolates obtained from Ethiopia and Eritrea during 1994 and 1996 (Fig. 2.1). It is therefore possible that the outbreaks that occurred in both countries were linked due to uncontrolled cross border movement of animals. Likewise, virus isolates from Ethiopia, Eritrea and Kenya which demonstrated < 6% nucleotide differences and which had a possible common origin can be summarized as follows:

1. ETH/19/77, ETH/5/95
2. ETH/1/79, ETH/3/79
3. ETH/3/90, ETH/8/90, ETH/12/90, ETH/2/93, ETH/24/94
4. ETH/9/92, ETH/30/94
5. ETH/3/96, ERI/2/96
6. ETH/22/01, ETH/16/01
7. KEN/77/78, KEN/10/95

The isolates ETH/8/94, ETH/1/95, ERI/1/96 and ETH/15/01, each showed < 93% nucleotide sequence identity with the above isolates and they were part of different epidemics. The close genetic relationship of ETH/5/95 with vaccine strain ETH/19/77 could be due to incomplete inactivation of the vaccine of strain ETH/19/77.

Genotype 2 contained two isolates obtained from Kenya during 1978 and 1995. These two isolates demonstrated a surprisingly close genetic relationship although they were isolated 17 years apart (Fig. 2.1). Genotype 3 contained viruses from South Africa, the Middle East and Asia **that** displayed a similar clustering profile as previously described by Sangare *et al.*, (2001). Genotype 4 consisted of viruses

from North and West Africa (Fig. 2.1). The latter genotype and lineage II and III were consistent with previous findings by Sangare and co-workers (2001). The overall genetic distances between isolates and between lineages were estimated to be 4.6 – 25.7% and 21 – 25.7%, respectively.

2.3.2 Pair-wise comparison of partial VP1 gene sequences

Partial nucleotide sequences (495 bp) of the 1D gene obtained from all isolates (Table 2.1) were aligned and analysed for sequence homology and divergence. The average pair-wise distances between genotypes/groups of viruses from Ethiopia as well as strains from other countries are shown in Table 2.2.

Pair-wise distance comparisons revealed that the Ethiopian and Eritrean strains constituted two different clades (1.1 & 1.2, Fig. 2.1) within genotype 1 (divergence < 10. 8%). Genotype I, when compared with the remaining 5 different genotypes: Kenya, the Middle East and South Africa, West Africa, Asia and South America showed a high divergence percentage of up to 13.1, 15.6, 17.0, 24.0 and 21.9, respectively (Table 2.2). Thus viruses from Ethiopia and Eritrea (genotype 1) were clearly distinguishable from other isolates of genotypes 2 – 7 (divergence 13 – 24%) but comparatively, they showed less genetic distance (13%) to isolates from genotype 2 (Kenya) and therefore descended from a common ancestor (90% boot-strap support, Fig. 2.1).

Table 2.2 Average Pair-wise distance comparison between genotypes and/or clades (1.1 –1.2) of viruses studied. Distances of > 13% and < 11% were used to differentiate genotypes and clades (clusters), respectively.

Genotype and clades		**1.1**	**1.2**	**2**	**3**	**4**	**5**
1. East Africa	1.1 Ethiopia and Eritrea						
	1.2 Ethiopia and Eritrea	10.8					
2. Kenya		13.1	13.4				
3. South Africa -the Middle East /Asia		14.3	15.6	16.7			
4. West Africa		17.0	15.6	17.1	17.9		
5. Asia		24.0	23.7	24.4	23.3	25.5	
6. South America (outgroup)		21.3	21.9	21.8	18.8	24.0	25.7

2.3.3 Amino acid variability

Deduced amino acid sequences of the 36 viruses included in this study were aligned and investigated in an attempt to comment on the amino acid variation using the MEGA 2.0 program. In all isolates hypervariable regions were located at regions corresponding to positions 49 – 59, 83 – 85, 129 – 158, 194 – 209 (Fig. 2.2). The latter 2 regions correspond to the highly immunogenic sites, the G-H loop (133 – 158) and the C-terminus (194 – 213) of the VP1 gene. The RGD cell attachment site within the G–H loop of the gene was conserved in all isolates. All the amino acid positions mentioned above constitute more than 95% of the variation among the Ethiopian and Eritrea isolates.

A total of 24 different amino acid position changes were observed in the Ethiopian isolates in relation to the vaccine strain (ETH/19/77). At position 155 the majority of the isolates contained an A while the recent Ethiopian isolates ETH-15/01, 22/02, 8/94 and 30/94 had a V. At position 83 all isolates, except the ETH/15/01 and ETH/16/01, showed changes from K to E in relation to the vaccine strain. Isolates from a recent outbreak (ETH/ 15, 16/01, 22/01) had changes at residues $Y_{49} \rightarrow$ H and A, $D_{85} \rightarrow$ N, $M_{54} \rightarrow$ L, $Q_{55} \rightarrow$ L, $S_{140} \rightarrow$ P, $A_{154} \rightarrow$ V, $P_{158} \rightarrow$ A&T, $I_{205} \rightarrow$ M. This study therefore revealed that the type O FMD virus population circulating in Ethiopia, Eritrea and Kenya are heterogeneous in their amino acid composition and have numerous changes at the immunodominant-coding region of the VP1 gene in comparison with the vaccine strain.

2.4 Discussion

Foot-and-mouth disease is enzootic in Ethiopia as in most East African countries and very few reports on the molecular epidemiology of FMD viruses of this region are available. Nineteen isolates from Ethiopia and Eritrea as well as 17 viruses representing the 2 lineages and 4 genotypes previously identified in different countries (Sangare *et al.*, 2001) were compared. Phylogenetic analysis showed that the Ethiopian and Eritrean strains within genotype 1 clustered into two clades and shared a common ancestor with isolates in genotype 2 (Kenya) (Fig. 2.1) with significant boot-strap support (90%). These neighbouring genotypes again shared

a common ancestor with isolates from South Africa-the Middle East/Asia within genotype 3 (96% boot-strap support) where the South African outbreak strain was represented by the PanAsian strain (Sangare *et al.*, 2001). Stepwise the next genotype 4 (West Africa) (Fig. 2.1) shared common nodes with the previous 3 genotypes. This showed the historical genetic relation between the African and the Middle East/Asia isolates and clustering of the genotypes into defined geographical locations coincides with the FMD topotype concept. The least divergence value between genotypes was found within viruses isolated from countries with common boundaries. This is demonstrated by the genetic relationships of Ethiopian and Eritrean isolates in genotype 1 and descendant of these isolates with Kenyan isolates from a common ancestor. Despite having been sampled over a period of 23 years the Ethiopian, Kenyan and Eritrean isolates showed group mean divergence percentages between 13-14.2%.

The Ethiopian strains formed 2 distinct clusters (1.1 and 1.2, Fig. 2.1) within genotype 1. The first group (1.1), which also contained the currently used vaccine strain, showed divergence of 10.8% from cluster 1.2 (Table 2.2). Viruses isolated in 1994 and 1996 from Ethiopia and Eritrea were found in both groups. The isolates in cluster 1.2 differed from other Ethiopian and Eritrean isolates by 7-11% nucleotide sequence differences. This indicated that at least 2 separate groups of viruses were circulating in these countries.

Foot-and-mouth disease viruses that differ between 2-7% from each other are generally believed to come from the same epizootic (Samuel *et al.*, 1997; Bastos, 2001). Based on this assumption, the sequencing results of serotype O viruses showed the occurrence of at least 4 independent epizootics of disease between 1990 and 2001 in Ethiopia and Eritrea (section 2.3.1).

The lineage and genotype classifications are in concordance with the study carried out by Samuel and Knowles (2001b) and Sangare *et al.*, (2001) where the former authors identified 8 topotypes of serotype O across the world based on 15% sequence differences between the VP1 genes. According to their study the Ethiopian isolates (ETH/8/94) together with isolates from Tanzania (TAN/7/98) and Eritrea (ERI/1/96) fell in to the Middle East-South Asia (ME-SA) topotype. However, in this study, where more isolates from Ethiopia were included, the

Ethiopian isolates formed 2 sub-clusters (Fig 2.1) with significant boot-strap support (> 76%) within genotype 1 and were shown to differ by 14.3 - 15.6% uncorrected sequence divergence from genotype 3 (Table 2.2). Genotype 3 consisted of isolates previously identified as the ME-SA topotype and further studies are required to determine the genetic relationships of isolates within the ME-SA topotype with other East African FMD viruses.

Differences in the genetic content of viruses of the same serotype do not necessarily reflect differences in antigenicity (Esterhuysen, 1994). On the other hand, it has also been shown that very limited genetic variation in the immunodominant regions can alter the antigenic specificity of FMD virus isolates (Mateu *et al.*, 1990; Mateu *et al.*, 1995; Vosloo *et al.*, 1996). The nucleotide and deduced amino acid variation found in the VP1 encoding-gene of the field isolates and the vaccine strain currently in use in Ethiopia confirmed the genetic heterogeneity of the viral population. Such differences through accumulation of mutation can lead to vaccine failure. The choice of strains of FMD viruses to use in the vaccine is important (Cartwright *et al.*, 1982) and should be protective against a wide spectrum of different strains within the same serotype. The genetic variation at the nucleotide level of the ETH/19/77 vaccine strain was not high when compared with the recent outbreaks strains (7.1 – 10.8%), but substitutions of amino acids took place in the immunodominant region of the VP1 region. This shows the need to evaluate the immunogenic ability of the current vaccine against different field isolates in Ethiopia. As the National Veterinary Institute in Ethiopia becomes more aware of the existence of different strains within the same serotype they should implement the appropriate disease control measures related to the production of potent vaccines.

```
                          58         68         78         88         98         108        118        128
1.  ETH/19/77     YVLDLMQTPA HTLVGALLRT ATYYFADLEV AVKHKGDLTW VPNGAPESAL DNTTNPTAYH KAPLTRLALP YTAPHRVLAT
2.  ETH/8/90      .......... Q......... .......... ....E.N... .......... .......... .......... ..........
3.  ETH/3/90      .......... .......... .......... ....E.N... .......... .......... .......... ..........
4.  ETH/5/95      .......... .......... .......... ....E.N... .......... .......... .......... ..........
5.  ETH/12/90     .......... .......... .......... ....E.N... .......... .......... .......... ..........
6.  ETH/2/93      ?.....L... Q......... .......... ....E.N... .......... .......... .......... ..........
7.  ETH/1/95      .......... .......... .......... ....E.N... .......... .......... .......... ..........
8.  ETH/24/94     H......... .......... .......... ....E.N... .......... .......... .......... ..........
9.  ERI1/96       .......... .......... .......... ....E.N... .......... .......... .......... ..........
10. ETH/22/01     H.....L... .......... .......... ....E.N... .......... .......... .......... ..........
11. ETH/16/01     A....L.... .......... .......... ......N... .......... .......... .......... ..........
12. ETH/15/01     H......... .......... .......... ......N... .......... .......... .......... ..........
13. ETH/8/94      .......... .......... .......... ....E.N... .......... .......... .......... ..........
14. ETH/1/79      N......... .......... .......... ....E.N... .......... .......... .......... ..........
15. ETH/3/79      .......... .........A .......... ....E.N... .......... .......... .......... ..........
16. ETH/9/92      .......... Y......... .......... ....E.N... .......... .......... .......... ..........
17. ETH/30/94     .......... .......... .......... ....E.N... .......T.. .......... .......... ..........
18. ETH/3/96      .......... .......... .......... ....E.N... .......TV. N......... .......... ..........
19. ERI/2/96      H......... .......... .......... ....E.N... .......A.. .......... .......... ..........
20. KEN/77/78     N......... .......... .......I.. ....E.N... .......... .......... .......... ..........
21. KEN/10/95     .......... .......... .......I.. ....E.N... .......... .......... .......... ..........
22. O1Manisa      N......... .......... .......... ....E.N... .......A.. .......... .......... ..........
23. O5India       N......... .......... .......... ....E.N... .......T.. .......... .......... ..........
24. BAN/1/97      N......... .......... .......... ....E.N... .......T.. .......... .......... ..........
25. IRQ/30/2000   N......... .......... .......... ....E.N... .......T.. .......... .......... ..........
26. SAR/15/00     N......... .......... .......... ....E.N... .......T.. .......... .......... ..........
27. ALG/1/99      N......... .......... .......... ....E..... .......T.. .......... .......... ?.........
28. GHA/9/93      N......A.. .......... .......... ....E..... .......... .......... .......... ..........
29. GHA/6/93      N......A.. .......... .......... ....E..... ......K... .......... .......... ..........
30. GHA/5/93      N......A.. .......... .......... ....E..... ......K... .......... .......... ..........
31. BFK/1/92      N......A.. .......... .......... ....E..... .......... .......... .......... ..........
32. SAU/100/94    T.?...K... .......... .......... ....E.N... .......T.. .........R .......?.. .R........
33. Moscow/95     N......I.. .......... .....S...L ....E..... .......T.. .......... .E........ ..........
34. Taiwan/97     N......I.. .......... .....S...L ....E..... .......T.. .......... .E........ ..........
35. Taiwan/98     N......I.. .......... .....S...L ....E..... .......T.. .......... .E........ ..........
36. O1/Yrigoyen   NT.....I.S .........A S....S...I V...E..... ......VK.. G........Y .K........ ....Y.....
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		138	148	158	168	178	188	198	213
1.	ETH/19/77	TYNGNCKYGE	TSVTNVRGDL	QVLAQKAVRP	LPTSFNYGAI	KATRVTELLY	RMKRAETYCP	RPLLAIHPSE	ARHKQKIVAP	VKQLL
2.	ETH/8/90	V.....	.....	.....A.A	.....	.....	.....	.....	.....	.....
3.	ETH/3/90	V.....	.....	.....A.A	.....	.....	.....	.....	.....	.....
4.	ETH/5/95	V.....	.....	.....A..	.....	.....	.....	.....	.....	.....
5.	ETH/12/90	V.....	.....	.....A.A	.....	.....	.....	.....	.....	.....
6.	ETH/2/93	V.....	.....	.....A.A	.....	.....	.....	.....	.....	.....
7.	ETH/1/95	V.....	.....	.....A.A	.....	.....	.....	.....	.....	.....
8.	ETH/24/94	V.....	.....	.....A.A	.....	.....	.....	.....	.....	.....
9.	ERI1/96	V.....R...	.P.....	.....VA.T	.....	.....	.....	.....	.....	.....
10.	ETH/22/01	V.....	.P.....	.....A.A	.....	.....	.....	.....	.....	.....
11.	ETH/16/01	V.....	.P.....	.....A	.....	.....	.....	.....	.....M...	?....
12.	ETH/15/01	V.....	.P.....	.....VA.T	.....	.....	.....	.....	.....	.....
13.	ETH/8/94	V.....	.P.....	.....VA.T	.....	.....	.....	.....K	.....	.....
14.	ETH/1/79	V...D....K	AP.....	.....T	.....	.....	.....	.....	.....	.....
15.	ETH/3/79	V.....R...	AP.....	.....A.T	.....	.....	.....	.....	.....	.....
16.	ETH/9/92	V...K.R...	AP.....	.....A.A	.....	.....	.....	.....Q...	.....	.....
17.	ETH/30/94	V.....R...	AP.....	.....VA.T	.....	.....	.....	.....T.	.....	.....
18.	ETH/3/96	V...S....K	AP.....	.....AWT	.....	.....	.....	.....K.	.....	.....
19.	ERI/2/96	V.....R...	AP.....	.....A.T	.....	.....	.....	.....T.	....T.....	.....
20.	KEN/77/78	V.....R..R	AP.....	.....A.T	.....	.....I....	.....	.....T....	.....R....	A....
21.	KEN/10/95	V.....R..R	AP.....	.....A.T	.....	.....I....	.....	.....T....	.....R.I..	A....
22.	O1Manisa	V.....D	GT.A.....	.....A.A	.....	.....	.....	.....DQ	.....	.....
23.	O5India	V.....AD	GP.A.....	.....A.A	.....	.....	.....	.....	.....	.....
24.	BAN/1/97	V.....	SP.....	.....T.T	.....	.....	.....	.....	.....	.....
25.	IRQ/30/2000	V.....	SP.....	.....A.T	.....	.....	.....	.....	.....	.....
26.	SAR/15/00	V.....	SP.....	.....A.T	.....	.....	.....	.....	.....	.....
27.	ALG/1/99	V...S.R.SG	AVTP.....	....RR.APM	......F...	.....	.....	.....F....	.....	.....
28.	GHA/9/93	V...S...SR	VE.PKL....	....RR.A.T	......F...	.....	.....	.....V....	T.....	.....
29.	GHA/6/93	V...S...SR	VE.PK.....	....RR.A.T	......F...	.....	.....	.....V....	T.R.R.....	M....
30.	GHA/5/93	V...S...SR	VE.PK.....	....RR.A.T	......F...	.....	.....	.....V....	T.R.....	M....
31.	BFK/1/92	V...S...SR	VE.PK.....	.....R.A.T	......F...	.....	.....	.....V....	T.....	M....
32.	SAU/100/94	V.....	SC.....	.....A.T	.....	.....	.....	.....	.....	.....
33.	Moscow/95	V...SS...D	..TN.....	.....E.T	......F...	.....	.....	......Q..D	.....?	A....
34.	Taiwan/97	V...SS...D	..TN.....	.....E.T	......F...	.....	.....	......Q..D	.....R....	A....
35.	Taiwan/98	V...SS...D	..TN.....	.....E.T	......F...	.....	.....	......Q..D	.....R....	A....
36.	O1/Yrigoyen	V...E.T.SS	NA.P.....P	NL.E...A.M	..AY.....	.....	.....	.....TD	....R.....	..RT.

Fig. 2.2 Amino acid alignment of 165 amino acids of the C terminus of the 1D gene of 36 type O FMD viruses. The cell attachment site of the viruses (RGD) in the GH-loop is highlighted at positions 145-147. Dots (.) indicates amino acids identical to the vaccine strain ETH/19/77. '?' indicates amino acids that could not be determined due to unresolved nucleotide sequences.

CHAPTER III

MOLECULAR EPIDEMIOLOGY OF SEROTYPE O FOOT-AND-MOUTH DISEASE VIRUSES FROM EAST AFRICAN COUNTRIES IN RELATION TO THE REST OF THE WORLD

3.1 Introduction

Foot-and-mouth disease is the most common viral infection in cattle in East African countries (Ethiopia, Somalia, Kenya, Sudan, Tanzania & Uganda) with serotype O having the widest geographical distribution (Kitching, 1998; Vosloo *et al.*, 2002). Despite the widespread distribution and economic impact of FMD, only a few studies have been conducted on the epidemiology of the circulating type O FMD viruses in East Africa. These molecular studies have also provided the means to characterize individual strains of viruses which is vital in the understanding of the epidemiology of FMD (Kitching, 1992).

To determine the number of viral lineages and genotypes and their distribution between East African countries sufficient genetic information of serotype O viruses is lacking. However, in this study an attempt was made to update and to supplement the limited molecular epidemiological information described above. Comparisons were also made to elucidate the genetic relationships between East African serotype O isolates from 1974 to 2003 and previously reported sequences of the same serotype from Genbank.

3.2 Materials and Methods

3.2.1 Viruses studied

A total of 52 isolates from East Africa were obtained from the WRL for FMD while 12 viruses were isolated from FMD outbreaks in Ethiopia (2001) and Uganda (2002, 2003) which were labelled with an * in Table 3.1. The sequences of 21 viruses, which represented different lineages and genotypes, were obtained from

GenBank and included to assess the genetic relatedness of East African isolates with the rest of the world. The viruses from the WRL were grown on a IBRS-2 cell line prior to further processing. The viruses that were collected from field outbreaks of FMD in Ethiopia and Uganda were first isolated on primary pig kidney cells and then passaged on IBRS-2 cells.

Table 3.1 Summary of serotype O FMD viruses included in this study

Isolate name	Sampling year	Country of origin	Reference	Genbank Accession No.
ALG/1/99	1999	Algeria	Sangare *et al.* (2001)	NA
ANG/1074	1974	Angola	Sangare *et al.* (2001)	AF300810
ANG/1/75	1975	Angola	Sangare *et al.* (2001)	AF300811
BAN/1/97	1997	Bangladesh	Freiberg *et al.* (1999)	NA
BEK/1/92	1992	Burkina Faso	Sangare *et al.* (2001)	AF300804
ERQ/30/2000	2000	Iraq	Samuel & Knowles (2001b)	AJ303499
ERI/1/96	1996	Eritrea	Sahle *et al.* 2004 (in press)	AY283390
ERI/2/96	1996	Eritrea	Sahle *et al.* 2004 (in press)	AY283391
ETH/1/79	1979	Ethiopia	Sahle *et al.* 2004 (in press)	AY283376
ETH/3/79	1979	Ethiopia	Sahle *et al.* 2004 (in press)	AY283377
ETH/19/77	1977	Ethiopia	Sahle *et al.* 2004 (in press)	AY283378
ETH/3/90	1990	Ethiopia	Sahle *et al.* 2004 (in press)	AY283379
ETH/8/90	1990	Ethiopia	Sahle *et al.* 2004 (in press)	AY283380
ETH/12/90	1990	Ethiopia	Sahle *et al.* 2004 (in press)	AY283381
ETH/9/92	1992	Ethiopia	Sahle *et al.* 2004 (in press)	AY283382
ETH/2/93	1993	Ethiopia	Sahle *et al.* 2004 (in press)	AY283383
ETH/24/94	1994	Ethiopia	Sahle *et al.* 2004 (in press)	AY283385
ETH/30/94	1994	Ethiopia	Sahle *et al.* 2004 (in press)	AY283386
ETH/8/94	1994	Ethiopia	Sahle *et al.* 2004 (in press)	AY283384
ETH/1/95	1995	Ethiopia	Sahle *et al.* 2004 (in press)	AY283387
ETH/5/95	1995	Ethiopia	Sahle *et al.* 2004 (in press)	AY283387
ETH/3/96	1996	Ethiopia	Sahle *et al.* 2004 (in press)	AY283392
ETH/15/01 *	2001	Ethiopia	Sahle *et al.* 2004 (in press)	AY283393
ETH/16/01 *	2001	Ethiopia	Sahle *et al.* 2004 (in press)	AY283394
ETH/22/01 *	2001	Ethiopia	Sahle *et al.* 2004 (in press)	AY283395
GHA/9/93	1993	Ghana	Sangare *et al.* (2001)	AF300809
GHA/6/93	1993	Ghana	Sangare *et al.* (2001)	AF300807
KEN/77/78	1978	Kenya	Sangare *et al.* (2001)	AF300812
KEN/10/95	1995	Kenya	Samuel & Knowles (2001b)	AJ303514
KEN/17/98	1998	Kenya	This study	AY344594
KEN/19/98	1998	Kenya	This study	AY344593
KEN/3/98	1998	Kenya	This study	AY344592
KEN/6/99	1999	Kenya	This study	AY344591
Moscow/95	1995	Russia	Unpublished	AJ004680
O5India	1962	India	Sangare *et al.* (2001)	AF274297
O1Kaufbeuren	1966	Germany	Forss *et al.* (1984)	X00871
O1BFS	1967	United Kingdom	Makoff *et al.* (1982)	J02185
O1Manisa	1969	Turkey	Unpublished	AJ251477
O1Yirigoyen	1982	Argentina	Sáiz *et al.* (1993)	Z21862
SAU/100/94	1994	Saudi Arabia	Samuel *et al.* (1997)	AJ004660
SAR/15/00	2000	South Africa	Sangare *et al.* (2001)	AF306647
SOM1/77	1977	Somalia	This study	AY344597

SOM/1/80	1980	Somalia	This study	AY344595
SOM/1/81	1981	Somalia	This study	AY344598
SOM/1/83	1983	Somalia	This study	AY344596
SUD/10/74	1974	Sudan	This study	AY344600
SUD/5/74	1974	Sudan	This study	AY344602
SUD/1/76	1976	Sudan	This study	AY344599
SUD/1/77	1977	Sudan	This study	AY344605
SUD/1/80	1980	Sudan	This study	AY344604
SUD/2/80	1980	Sudan	This study	AY344608
SUD4/80	1980	Sudan	This study	AY344610
SUD/2/83	1983	Sudan	This study	AY344607
SUD/3/83	1983	Sudan	This study	AY344606
SUD/2/86	1986	Sudan	This study	AY344601
SUD/6/89	1989	Sudan	This study	AY344603
SUD5/99	1999	Sudan	This study	AY344609
Taiwan/97	1997	Taiwan	Tsai *et al.* (2000)	AF026168
Taiwan/98	1998	Taiwan	Tsai *et al.* (2000)	AF095877
TAN/1/80	1980	Tanzania	This study	AY344615
TAN/1/85	1985	Tanzania	This study	AY344616
TAN/3/96	1996	Tanzania	This study	AY344611
UGA/1A/74	1974	Uganda	This study	AY344626
UGA/2C/74	1974	Uganda	This study	AY344627
UGA/9/74	1974	Uganda	This study	AY344630
UGA/1/75	1975	Uganda	This study	AY344623
UGA/28/75	1975	Uganda	This study	AY344625
UGA/42/75	1975	Uganda	This study	AY344628
UGA/12/76	1976	Uganda	This study	AY344621
UGA/6/76	1976	Uganda	This study	AY344622
UGA/9/78	1978	Uganda	This study	AY344631
UGA/1/96	1996	Uganda	This study	AY344617
UGA/29/96	1996	Uganda	This study	AY344618
UGA/5/96	1996	Uganda	This study	AY344619
UGA/17/98	1998	Uganda	This study	AY344624
UGA/9/98	1998	Uganda	This study	AY344629
UGA/05/02 *	2002	Uganda	This study	NA
UGA/11/02 *	2002	Uganda	This study	AY349950
UGA03/02 *	2002	Uganda	This study	NA
UGA/1/03 *	2003	Uganda	This study	AY349951
UGA/2/03 *	2003	Uganda	This study	AY349952
UGA/3/03 *	2003	Uganda	This study	AY349953
UGA/4/03 *	2003	Uganda	This study	AY349954
UGA/5/03 *	2003	Uganda	This study	AY349955
UGA/7/03 *	2003	Uganda	This study	NA

NA - not available. * - viruses isolated by the authors from FMD outbreaks and all other viruses isolates used in this study were supplied by WRL for FMD

3.2.2 RT-PCR amplification of the 1D gene and nucleotide sequencing

The methods used for the isolation of viral RNA from cell culture as well as RT/PCR have been described previously (Boom *et al.*, 1990; Bastos, 1998) and the methodologies are outlined in section 2.2.2 and 2.2.4. Viral RNA was reverse

transcribed using AMV-RT (Promega) and the oligonucleotide primer P1 5'-GAAGGGCCCAGGGTTGGACTC-3' (Beck & Strohmair, 1987). The antisense primer P1 and serotype O specific sense primer VP1 O (5'-GATTTGTGAAGGTGACACC-3') (Rodriguez *et al.*, 1994) were used to amplify a 581 bp fragment of the 1D gene. The amplified VP1 fragment was electophoresed on a 1.5% low melting-point agarose gel (Roche) containing 0.5 µg/ml ethidium bromide and the amplified PCR product was visualized using a transilluminator. The PCR product was excised from the gel, purified using a QIA quick Gel Extraction Kit (Qiagen, USA) and was sequenced using the Big Dye® version 3.0 Cycle Sequencing kit and the ABI Prism 310 Genetic Analyzer (Applied Biosystems) (section 2.2.6). All the nucleotide sequences determined in this study have been submitted to GenBank and the accession numbers are indicated in Table 3.1.

3.2.3 Sequence data analysis

Nucleotide sequences alignment and analysis were carried out according to methods described in section 2.2.7 and the phylogenetic tree was constructed according to the sequence relatedness of 495 bp using the Neighbour-joining method and fitted models to include p-distance and transition-transversion bias. Confidence limits were placed on the tree branches using the boot-strap resampling method (1000 replicates). The value of > 13% nucleotide sequence difference of the VP1 encoding gene was used to differentiate genotypes in concordance with genotype concept as described previously (Vosloo, *et al.*, 1992; Samuel and Knowles, 2001b; Sangare *et al.*, 2001). Nucleotide sequence differences of < 7% were considered as closely related viruses (Bastos 1998; Bastos et al., 2001, 2002; Samuel *et al.*, 1997; Samuel *et al.*, 1999). Lineages were defined as clusters demonstrating $\geq$ 20% nucleotide differences. Nucleotide sequence divergence percentages were also determined using the distance method provided in the Mega 2.1 program.

3.3 Results

3.3.1 Genetic relationships of serotype O FMD viruses

Phylogenetic analysis of serotype O FMD viruses included in this study revealed the presence of three distinct lineages. Lineage I comprised of 8 genotypes that

comprised of isolates from East and West Africa as well as isolates from the Middle East/Asia and South Africa (Fig. 3.1). Lineage II consisted of one genotype from Asia while lineage III included two genotypes, one contains isolates from Angola, and the other with isolates from South America and Europe. These genotypes corresponded to geographically distinct regions in concordance with the FMD topotype concept as it is applied to European and SAT type viruses (Bastos, 2001; Samuel and Knowles, 2001b). Within each lineage the genotypes corresponded to different geographical locations and therefore they were designated into topotypes (Fig. 3.1 and Table 3.2). Defining Uganda and Kenya as central within the East African country's the distribution of topotypes can be summarized as follows:

Lineage 1:

- Topotype A: North Eastern Africa: isolates from Ethiopia, Eritrea, Somalia, Sudan and Kenya
- Topotype B: Sudan-West Africa: isolates from Sudan, Algeria, Ghana and Burkina Faso
- Topotype C: Central East Africa: isolates from Uganda and Kenya
- Topotype D: Sudanese: isolates from Sudan
- Topotype E: Ugandan I: isolates from Uganda
- Topotype F: Ugandan II: isolates from Uganda
- Topotype G: South Eastern Africa: isolates from Uganda and Tanzania
- Topotype H: Middle-East, South Asia and South Africa: isolates from India, Turkey, Saudi Arabia, Bangladesh, Iraq and South Africa

Lineage 2:

- Topotype I (Cathay): isolates from Taiwan and Russia

Lineage 3:

- Topotype J Angolan: isolates from Angola
- Topotype K (Euro-S. American): isolates from Argentina, UK and Germany

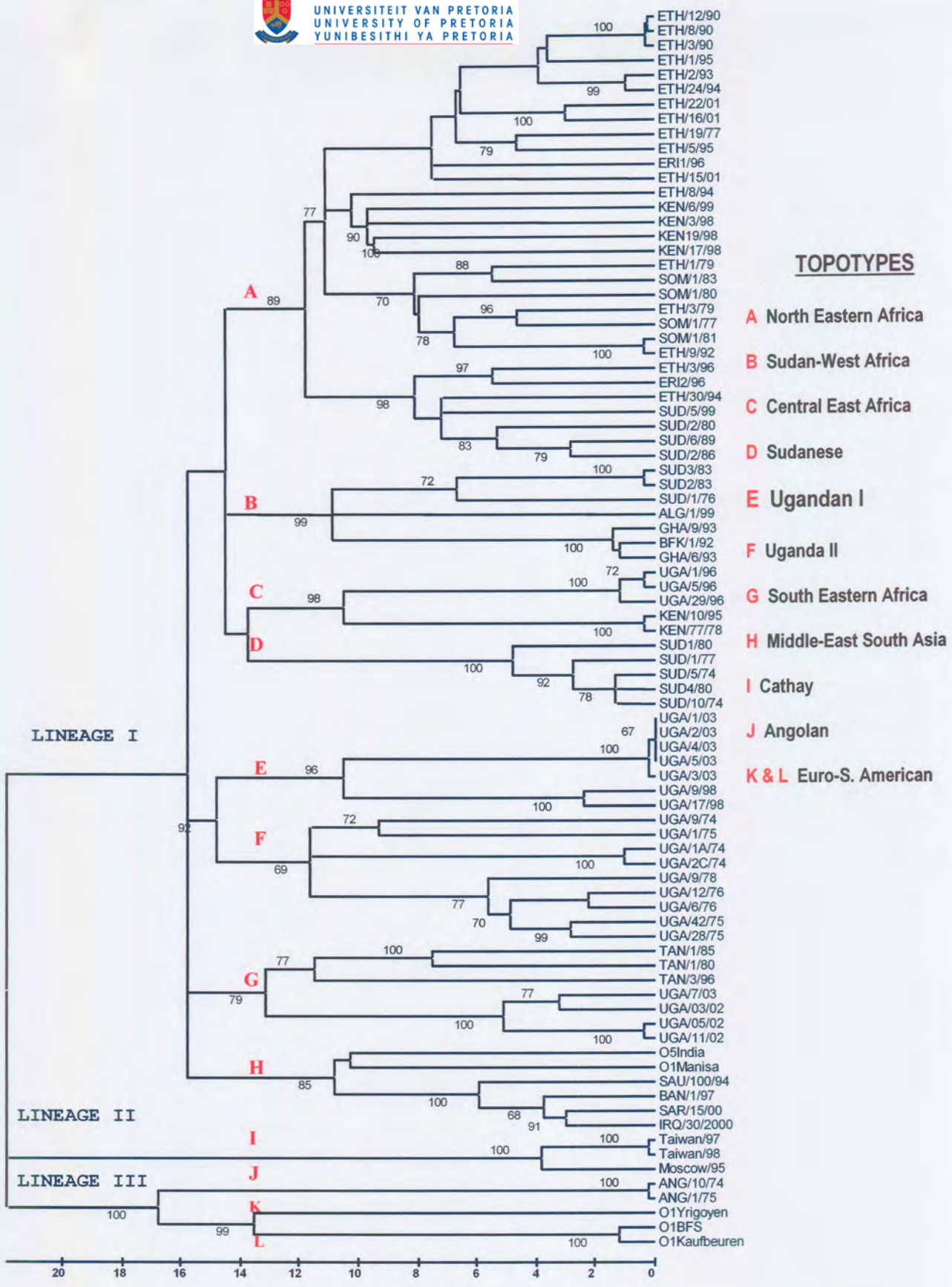

Fig. 3.1 Neighbour-joining tree depicting genetic relationships of serotype O FMD viruses from Africa (East, West & South), the Middle East, Europe, South America and Asia. lineages (I-III) were identified. Genotypes are indicated based on > 13% nucleotide differences among clusters and are indicated by A-L. The scale provides a measure of percentage of sequence differences along the branches.

The viruses in lineage I, isolated between 1974 – 2003 from East African countries, revealed the presence of 6 distinct topotypes for this region as well as one topotype with isolates from the Middle East, South Asia and South Africa. The Sudan-West Africa topotype included isolates from Sudan and West Africa (see summary above). Uganda harboured two distinct topotypes belonging to that country alone and another 2 shared topotypes, one with Kenya and one with Tanzania, bringing the total number of topotypes present in Uganda to four (Fig. 3.1). Between the two distinct Ugandan topotypes a divergence of 17.4% was observed while these topotypes differed from the other African topotypes by percentage of less than 17% (Table 3.2). The North Eastern topotype has the widest distribution and involved 5 countries of the region (Ethiopia, Eritrea, Kenya, Somalia and Sudan), which may indicate that spread of FMD between these countries had occurred in the past. This was further demonstrated by the surprisingly close genetic relationship (2% nucleotide differences) between SOM/1/81 and ETH/9/92 (Table 3.3).

Table 3.2 Average Pair-wise distance comparison between genotypes 1 – 11 of serotype O FMD viruses identified in this study.

Genotypes	1	2	3	4	5	6	7	8	9	10
1. North- Eastern										
2. Sudan-Western	13.0									
3. Central	15.9	16.2								
4. Sudanese	14.4	14.0	14.4							
5. Ugandan I	15.4	15.7	16.0	14.3						
6. Ugandan II	15.7	16.3	17.8	15.2	17.5					
7. South Eastern	14.9	16.9	16.5	15.0	15.7	16.6				
8. Middle East-South Asia	16.6	17.2	14.9	15.6	17.2	17.4	17.1			
9. Cathay	18.2	19.9	18.4	20.0	18.8	19.0	20.0	19.0		
10. Angolan	22.4	24.0	23.8	23.2	22.4	23.2	21.3	22.0	20.3	
11. Euro-S. American	20.5	22.0	22.8	21.1	20.6	21.3	20.1	21.0	20.1	16.8

Sequence similarities between East African isolates also indicated the occurrences of outbreaks from genetically closely related viruses (≥ 94% sequence similarities) and related epizootics and are summarized in Table 3.3. According to Table 3.3 and previous chapter (section 2.3.1) Ethiopia has had at least 6 separate epizootics between 1977 and 2001, while Uganda has had 8 epizootics between 1974 and 2003. Six of the Ugandan epizootics were illustrated in Table 3.3 while UGA/9/74 and UGA/1/75 (Ugandan II topotype) were part of two more epizootics (< 91% nucleotide sequence similarity). The ETH/9/92 and the SOM/1/81 viruses were isolated in two different countries (Ethiopia and Somalia) that were sampled

over a 10 year period and shared 98% sequence similarities. The Sudanese isolates sampled over a 6 year period (1974 – 1980, Table 3.3) exhibited ≥ 96% sequence similarity. Obviously these viruses were not from the same outbreak but closely related viruses were circulating during these years.

Table 3.3 Summary of genetically closely related viruses which demonstrated < 6% nucleotide differences and possibly originated from common sources.

Countries	Isolates	Seq. similarities (%)
Ethiopia (ETH)	6 epizootics (section 2.3.1)	>94
Sudan	1. SUD/2/86 & SUD/6/89	96
	2. SUD/2/83 & SUD 3/83	99
	3. SUD/5/74, SUD/10/74 SUD/1/77, SUD/1/80, SUD/4/80	>94
Uganda	1. UGA/1/96, UGA/5/96 & UGA/29/96	>97
	2. UGA/1-5/03 (5 isolates)	>98
	3. UGA/9/98 & UGA/17/98	97
	4. UGA/1A/74 &UGA/2C/74	98
	5. UGA/42/75, UGA/2875, UGA/6/76 & UGA/12/76	96
	6. UGA/7/03 & UGA/03/02, UGA/05/02 & UGA/11/02	94
ETH & Somalia	1. SOM/1/81 & ETH/9/92	98

The average pair-wise distance comparisons in Table 3.2 indicated that each African topotype differed by between 13 and 17% from each other, compatible with previous definitions for genotypes within serotype O (Samuel and Knowles, 2001b; Sangare *et al.*, 2001). The highest divergence percentage was observed between the Angolan and Sudan-Western topotypes (24%) while the former showed less divergence with the Euro-S. American (16.8%) topotype than the African topotypes (>16.8%). The branch linking the Angolan and Euro-S. American genotypes in Fig. 3.1 is well supported (100%) which indicated that member of these genotypes may have a common ancestor. The three lineages differed from each other by more than 21%.

3.4 DISCUSSION

This study demonstrated the presence of 7 distinct genotypes of serotype O in East Africa which is in contrast with previous studies where a limited number of isolates from the region were included and only one topotype from East Africa was indicated (Samuel and Knowles, 2001b; Sangare *et al.*, 2001). It illustrates the importance of performing comprehensive studies for molecular epidemiology and to include representative samples from all regions in the analysis to reach correct

conclusions. However, even this study may not be a true reflection of the number of topotypes present in East Africa, as it is a well known fact that outbreaks are not reported or investigated due to the endemic nature of the disease in this part of the continent.

Genotype A (North Eastern topotype) represents isolates from 5 countries bordering each other (Eritrea, Sudan, Kenya, Somalia and Ethiopia) that were sampled over a 29 year period (1974 – 2003) and showed a sequence identity > 85% as well as providing evidence for links between outbreaks. This was clearly demonstrated by isolates from different countries grouping into two sub-clusters in genotype A whilst significant boot-strap support (77 & 98%) indicated common ancestry (Fig.1). Interestingly isolates from Ethiopia were found in each sub-cluster indicating that transboundary movement of viruses between Ethiopia and neighbouring countries has occurred from different directions in the past. Similarly, isolates from East African countries comprised distinct clusters (topotypes B, C, G), indicating that the outbreaks due to these isolates were most probably spread by uncontrolled transborder animal movements.

Two topotypes consisted of isolates obtained solely from Uganda, *viz*., Ugandan I (E) and Ugandan II (F). These topotypes differ by more than 17.5% from each other and it indicates that serotype O strains have been evolving in Uganda over time and possibly did not transcend the Ugandan border. Uganda II is made up of viruses isolated between 1974 –1978 and this topotype seems to be extinct. However, Uganda also shares topotypes with Kenya (C) and Tanzania (G). Similarly, the Sudanese topotype constituted of serotype O viruses solely of Sudanese origin (D) which spans a 7 year period (1974 - 1980) where isolates demonstrated a > 94% sequence identity and most probably this topotype is also extinct. Sudanese isolates also cluster as part of the Sudan-West African topotype (B). This study highlighted distinct topotypes found in Uganda and Sudan need further investigation.

Sequence divergence between topotypes (Table 3.2) and phylogenetic tree topology (Fig. 3.1) confirmed the genetic distinctiveness of type O FMD viruses from East Africa from the rest of the isolates included in this study. The Sudan-West Africa topotype is exceptional in that it links West and East African isolates.

However, the East, West, South Africa, the Middle East/Asia isolates form part of a major lineage (based on ≤ 20% sequence differences). The molecular epidemiology of this serotype has been investigated and reviewed by Samuel and Knowles, (2001b) and Knowles and Samuel, (2003) who demonstrated the existence of 8 topotypes within samples collected around the world based on the comparison of sequence data of the VP1 gene. Among these topotypes two were found in Africa, one in East Africa and one in West Africa. The genetic diversity of type O viruses from West Africa has also been determined by Sangare *et al.* (2001) who identified four genotypes from the African isolates. Isolates from West and North Africa, East Africa, South Africa and Angola constituted genotypes 1 to 4, respectively (Sangare et al., 2001). However, with the exception of 12 isolates (KEN77/78, KEN/83/79, KEN/2/91, KEN/4/91, KEN/2/95, KEN/4/95, KEN/5/95, KEN/10/95 UGA/5/96, ETH/8/94, ERI/1/96 and TAN/7/98) which were included in the study by Sangare *et al.*, (2001) and Samuel and Knowles (2001b), the molecular epidemiology of serotype O FMD viruses from East African countries has not been intensively studied.

Present study results indicated that similar strains of viruses can be confined to a certain country and evolved within that country over time while other strains can transcend country boundaries. One could speculate that the sharing of genotypes between countries or the confinement of certain genotypes to a specific country could be largely influenced by the social, economic, climatic and political situation in that specific area in any given point in time.

This molecular epidemiological study for type O FMD viruses of East African origin has provided valuable information with respect to the epidemiology of the disease in this region. These results demonstrated a distinct geographical grouping of serotype O in East Africa and indicated that control of FMD will only be effective if the region cooperates to prevent transboundary spread of the disease. It is also recommendable that vaccines should be custom-made for the East African region, as the viruses showed genetic heterogeneity and differed significantly from other viruses on the continent.

CHAPTER IV

GENETIC HETEROGENEITY OF SAT-2 FOOT-AND-MOUTH DISEASE VIRUSES IN EAST AFRICA

4.1 Introduction

The SAT serotypes of FMD are prevalent in southern and eastern Africa and are endemic to most African buffalo populations in sub-Saharan Africa (Hedger *et al.*, 1973; Anderson, 1981; Vosloo *et al.*, 2002). The SAT-2 serotype has been recorded among East African countries *viz.,* Eritrea, Ethiopia, Sudan, Kenya, Uganda and Tanzania. However, there is no official record of this serotype in Somalia as very few samples from FMD outbreaks were submitted to the World Reference Laboratory (WRL) for FMD between 1960 and 1983.

The genetic diversity of SAT-2 FMD viruses has been investigated previously for eastern, western, central and southern African countries by Sangare *et al.* (2002) and Bastos *et al.* (2003). A total of V lineages of this serotype were identified in Africa where isolates from East Africa constituted two separate lineages. However, with the exception of a few isolates from Kenya and Eritrea previously described and included in the above studies, the molecular epidemiology of other SAT-2 FMD viruses from Ethiopia, Somalia, Sudan, Tanzania and Uganda has not been intensively studied.

The present study was carried out to determine the genetic variability of SAT-2 FMD viruses in East Africa and to compare them to previously reported VP1 gene sequences of SAT-2 viruses (Table 4.1).

4.2 Materials and Methods

4.2.1 Viruses studied

A total of 41 SAT-2 FMD viruses from Ethiopia, Sudan, Kenya, Uganda, Tanzania and Eritrea isolated between 1975 and 2000 were supplied by the WRL for FMD at the Institute for Animal Health, Pirbright (United Kingdom). These isolates were managed as described previously. To complement the study sequences of 26

isolates from 16 other countries in Africa and Saudi Arabia were selected from Genbank. The laboratory virus designations, country of origin, year of sampling and Genbank accession number of all isolates are indicated in Table 4.1.

Table 4.1 Summary of SAT-2 FMD viruses included in this study

Virus designations	Country of origin	Year of sampling	Reference	Genbank accession No.
ANG/4/74	Angola	1974	Bastos *et al.* (2003)	AF479417
BOT/1/98	Botswana	1998	Bastos *et al.* (2003)	AF367122
BOT/31/98	Botswana	1998	Bastos *et al.* (2003)	AF367125
BUN/1/91	Burundi	1991	Bastos *et al.* (2003)	AF367111
ERI/1/98	Eritrea	1998	This study	AY343933
ERI/12/98	Eritrea	1998	Bastos *et al.* (2003)	AF367126
ERI/4/98	Eritrea	1998	This study	AY343934
ETH/1/90	Ethiopia	1990	This study	AY343935
ETH/2/90	Ethiopia	1990	This study	AY343936
ETH/1/91	Ethiopia	1991	This study	AY343937
ETH/2/91	Ethiopia	1991	This study	AY343938
GAM/9/79	Gambia	1979	Sangare (2002)	AF426078
GAM/8/79	Gambia	1979	Sangare (2002)	AF426093
GHA/2/90	Ghana	1990	Sangare (2002)	AF426081
GHA/8/91	Ghana	1991	Sangare (2002)	AF426083
KEN/3/57	Kenya	1957	Unpublished	AJ251473
KEN/2/76	Kenya	1976	This study	AY343940
KEN/1/84	Kenya	1984	This study	AY344505
KEN/2/84	Kenya	1984	This study	AY343941
KEN/1/85	Kenya	1985	This study	AY343942
KEN/1/86	Kenya	1986	This study	AY343943
KEN/1/87	Kenya	1987	This study	AY343944
KEN/2/87	Kenya	1987	This study	AY343945
KEN/2/88	Kenya	1988	This study	AY343946
KEN/1/89	Kenya	1989	This study	AY343947
KEN/28/91	Kenya	1991	This study	AY343948
KEN/33/91	Kenya	1991	This study	AY343950
KEN/8/91	Kenya	1991	This study	AY343949
KEN/1/92	Kenya	1992	This study	AY343953
KEN/3/92	Kenya	1992	This study	AY343951
KEN/6/92	Kenya	1992	This study	AY343952
KEN/1/94	Kenya	1994	This study	AY343954
KEN/2/94	Kenya	1994	This study	AY343955
KEN/3/95	Kenya	1995	This study	AY343957
KEN/7/95	Kenya	1995	This study	AY343956
KEN/1/96	Kenya	1996	This study	AY343960
KEN/11/96	Kenya	1996	This study	AY343958
KEN/7/96	Kenya	1996	This study	AY343959
KEN/16/98	Kenya	1998	This study	AY343962
KEN/7A/98	Kenya	1998	This study	AY343961
KEN/5/99	Kenya	1999	Bastos *et al.* (2003)	AF367131
KEN/7/99	Kenya	1999	Bastos *et al.* (2003)	AF367132
KEN/9/99	Kenya	1999	Bastos *et al.* (2003)	AF367135

KNP/31/95	South Africa	1995	Bastos *et al.* (2003)	AF367119
MAL/3/75	Malawi	1975	Bastos *et al.* (2003)	AF367099
NIG/2/75	Nigeria	1975	Sangare *et al.* (2002)	AF367139
PAL/5/83	South Africa	1983	Bastos *et al.* (2003)	AF367102
RWA/1/00	Rwanda	2000	Bastos *et al.* (2003)	AF367134
SAU/6/00	Saudi Arabia	2000	Bastos *et al.* (2003)	AF367132
SEN/5/75	Sénégal	1975	Bastos *et al.* (2003)	AF367099
SEN/2/75	Sénégal	1975	Sangare (2002)	AF431732
SUD/6/77	Sudan	1977	This study	AY343939
SUD/9/77	Sudan	1977	This study	AY442014
TAN/1/75	Tanzania	1975	This study	AY343970
TAN/1/86	Tanzania	1986	This study	AY343971
UGA/51/75	Uganda	1975	This study	AY343963
UGA/3/76	Uganda	1976	This study	AY343964
UGA/8/76	Uganda	1976	This study	AY343965
UGA/3/91	Uganda	1991	This study	AY343966
UGA/9/95	Uganda	1995	This study	AY343967
UGA/19/98	Uganda	1998	This study	AY343969
UGA/28/98	Uganda	1998	This study	AY343968
ZAI/1/82	Zaire	1982	Bastos *et al.* (2003)	AF367100
ZAM/10/96	Zambia	1996	Bastos *et al.* (2003)	AF367121
ZIM/7/83	Zimbabwe	1983	van Rensburg & Nel (1999)	AF136607
ZIM/1/00	Zimbabwe	2000	Bastos *et al.* (2003)	AF367136
ZIM/267/98	Zimbabwe	1998	Bastos *et al.* (2003)	AF367130

4.2.2 Nucleic acid isolation and RT-PCR amplification

All procedures for RNA extraction, cDNA synthesis and DNA amplification were described previously sections 2.2.2, 2.2.3 and 2.2.4 with the exception of the downstream primer. The P1 primer and the upstream primer termed VP 3AB, binding to the VP3 encoding gene (5'-CACTGCTACCACTCRGAGTG-3') (Bastos *et al.*, 1998), were used to amplify an approximately 880 bp fragment.

4.2.3 DNA purification and Cycle Sequencing

The 880 bp 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) as previously described in section 2.2.6. Two independent sequencing reactions were performed per sample using the P1 and upstream primers as in the PCR. All data were submitted to Genbank under the accession numbers provided in Table 4.1.

4.2.4 Phylogenetic Analysis

The generated nucleotide sequences were aligned to published reference strains (Table 4.1) using the DAPSA program (Harley, 2001) and trimmed so that a homologous region of 648 bp corresponding to the complete VP1 encoding gene was used for phylogenetic analysis. Phylogenetic reconstruction was carried out using methods of analysis included in MEGA version 2.0 (Kumar *et al.*, 2001), with p-distance, pair-wise deletions of gaps and confidence levels assessed by 1000 boot-strap replications. Average pair-wise comparisons (distance, MEGA 2.0) were conducted to estimate divergence (Table 4.2) between lineages. Evolutionary lineages were distinguished on the basis of nucleotide sequence differences of $\geq$ 26% and boot-strap support > 80% while a divergence of $\geq$ 20% and < 26% distinguished genotypes. A variability plot of sequences of the 1D gene of 48 SAT-2 viruses from East Africa was represented using MEGA V1.02 (Kumar *et al.*, 1993) with the numbers of variable sites in overlapping windows of 10.

4.3 RESULTS

4.3.1 Phylogenetic analysis

The complete 1D gene sequences (648 bp) were used to compare phylogenetic differences between the SAT-2 viruses studied (Table 4.1). The Neighbour-joining (NJ) tree revealed 5 lineages and 15 genotypes from 67 virus isolates from Africa of which 41 East African isolates were characterized in this study. Identical genotypes were consistently obtained irrespective of the method of analysis used. Neighbour-joining (NJ), UPGMA and Parsimony methods produced trees with identical clusters (results not shown) indicating that the recovered phylogeny is a good estimate and reliable (Kim, 1993). It was observed that the NJ method gave rise to 3 distinct regionally associated grouping of lineages *viz.*, East African (lineages I & II), East-southern African (lineage V) and West African (III and IV) (indicated by bold arrow in Fig. 4.1). However, with all phylogenetic reconstruction methods used all the African isolates clustered into five lineages as this classification was further supported by the result of genetic distance comparison of the lineages (Table 4.2). The identified lineages (I – V) and genotypes (A – O) are illustrated in the phylogenetic tree (Fig. 4.1) and phylogeographic distribution of

genotypes (supported by 78% to 100% boot-strap support) can be summarized as follows:

East Africa

Lineage I: isolates from Eritrea, Saudi Arabia, Ethiopia, Sudan, Uganda, Zaire (DRC), Rwanda and Kenya.

- Genotype A: Kenya and Uganda
- Genotype B: Uganda
- Genotype C: Uganda and Zaire (DRC)
- Genotype D: Sudan
- Genotype E: Ethiopia
- Genotype F: Eritrea and Saudi Arabia
- Genotype G: Rwanda

Angola

Lineage II: isolate from Angola (Genotype H).

West Africa

Lineage III: isolates from Ghana, Niger and Senegal (Genotype I).

Lineage IV: isolates from Gambia and Senegal (Genotype J)

East-Southern Africa

Lineage V: isolates from Kenya, Malawi, Tanzania, Ethiopia, Burundi, South Africa, Zambia, Botswana and Zimbabwe.

- Genotype K: Zimbabwe and Botswana
- Genotype L: Zimbabwe
- Genotype M: Botswana and Zambia
- Genotype N: South Africa
- Genotype O: Kenya, Malawi, Tanzania, Ethiopia, Burundi

Most of the isolates within these lineages clustered into geographical localities in accordance with the FMD topotype concept of Knowles and Samuel (2003).

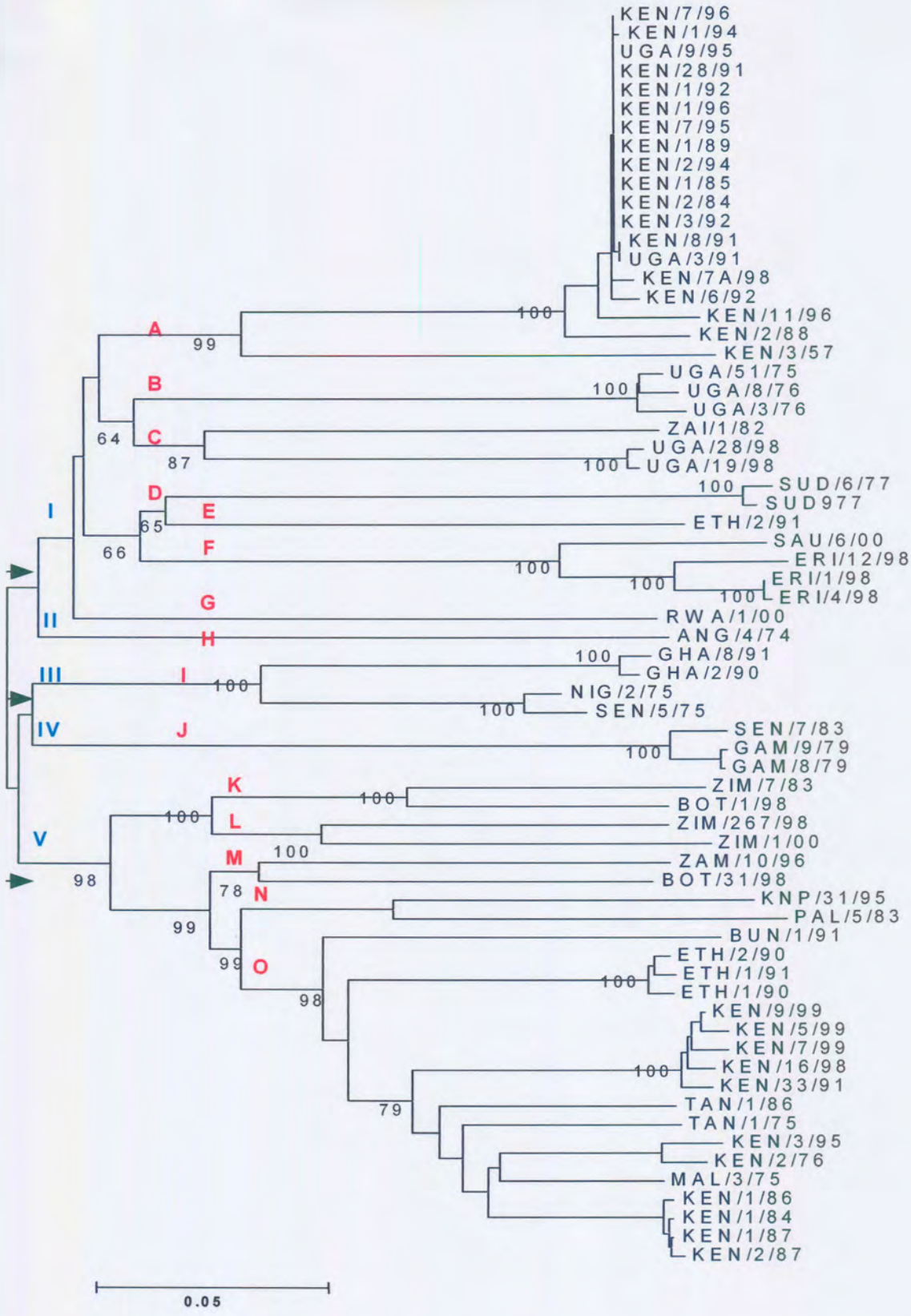

Fig. 4.1 Neighbour-joining tree depicting genetic relationships of serotype SAT-2 FMD viruses from East, West and southern Africa and Saudi Arabia. Boot-strap values were estimated based on 1000 replications. I - V and A – O correspond to lineages and genotypes, respectively. ► Indicates 3 distinct regionally associated grouping of lineages. The scale represents 5% differences at the nucleotide level.

Isolates from lineages I to IV were distributed into geographically defined areas, *viz*., East Africa, Angola and 2 in West Africa which corresponded to lineages I, II and III and IV, respectively. Isolates within lineage V were distributed throughout the southern African region and extended into central and East Africa, thus these isolates were not restricted to one geographically defined region. However, within this lineages 26 isolates from 9 countries clustered into 5 genotypes (K-O) and at the genotype level showed defined areas of distribution. Genotypes A to N appeared to cluster into geographically defined countries with common boundaries which can be termed topotypes. Eighteen isolates from 5 countries clustered in genotype O which showed a wider geographical distribution and this topotype should be further investigated by including more isolates from these countries.

The East African isolates in particular, constituted distinct regional associations where 7 topotypes (genotype A – F, O) were assigned. The only exception being genotype F where isolates from Eritrea and Saudi Arabia clustered together with sequence similarity of between 90 - 99%, thus providing evidence for links between outbreaks from these countries.

The five lineages demonstrated a higher level of genetic distance (divergence) ranging from 27 – 31% (Table 4.2). The sequence homology within each lineage varied between 80 - 99% and between lineages sequence similarities were found to be < 80%.

Table 4.2 Average pair-wise distance comparisons between lineages (I – V) of SAT-2 FMD viruses included in this study.

Lineages	**I**	**II**	**III**	**IV**
I East Africa				
II Angola	27.7			
III West Africa	27.1	28.0		
IV West Africa	29.3	30.4	27.3	
V East and Southern Africa	27.6	29.1	27.9	29.9

Description: No. of Taxa : 67, No. of Groups : 5, Gaps/Missing data : Pair-wise Deletion, Codon Positions : Noncoding, Distance method : Nucleotide: p-distance [Between group average], No. of Sites : 648, distance : estimate

4.3.2 Sequence variations and distribution of mutations

Nucleotide sequence similarities of isolates within genotypes were found to vary from genotype to genotype (Table 4.3). The lowest sequence variation (< 5%) was observed in genotype A between 16 Kenyan isolates (1984 – 1996) and 2 Ugandan isolates (1991, 1995) which revealed that the same virus was circulating over a period of 13 years between the two countries. Similarly, genotype O, spanning 23 years constituted of 18 isolates from Burundi (1), Ethiopia (3), Kenya (11), Tanzania (2) and Malawi (1) and demonstrated > 87% sequence identity.

Amino acid variability was plotted to determine whether mutations were randomly distributed or localized to specific regions of the VP1 gene. The nucleotide sequences of the full-length VP1 encoding gene of 48 isolates of East African origin showed 43.4% (171/648) of the sites were invariant across all isolates while at the amino acid level this translated into 52.7% (114/216) (underlined amino acids, Fig. 4.2). The hypervariable regions were located at amino acid positions 46 – 59, 76 – 90, 135 – 142, 148 – 160 (Fig. 4.2, 4.3). The latter 2 regions of variations were found within the immunogenic G-H loop of the gene (Pfaff *et al.*, 1988; Parry *et al.*, 1990) and amino acid positions 76-99 incorporates the D-E loop. The 'RGD' cell attachment site of the virus at amino acid positions 144-146 within the G-H loop is completely conserved across all isolates. Complete conservation was also observed at amino acids position between 175 – 191 in all 48 viruses while the amino acids position 192 – 210 exhibited variation between 20 - 40%. The VP1/2A cleavage site contained predominantly amino acid sequences KQ/LL, KQ/LC and RQTL.

Table 4.3. Pair-wise sequence divergence using the p-distance model. 0 indicate distance < 1%

Virus designation	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
1.KEN/1/85																																																										
2. KEN/1/94	0																																																										
3. KEN/3/92	0	0																																																							
4. KEN/7/96	0	0	0																																																					
5. KEN/7/95	0	0	0	0																																																				
6.KEN/28/91	0	0	0	0	0																																																				
7. KEN/2/84	0	0	0	0	0	0																																																			
8. KEN/1/89	0	0	0	0	0	0	0																																																	
9. UGA/9/95	0	0	0	0	0	0	0	0																																																
10. KEN/1/96	0	0	0	0	0	0	0	0	0																																															
11. KEN/2/94	0	0	0	0	0	0	0	0	0	0																																														
12. KEN/1/92	0	0	0	0	0	0	0	0	0	0	0																																													
13. KEN/8/91	0	0	0	0	0	0	0	0	0	0	0	0																																											
14. UGA/3/91	0	0	0	0	0	0	0	0	0	0	0	0	0																																									
15. KEN/7A/98	0	1	0	0	0	0	0	0	0	0	0	0	1	1																																							
16. KEN/6/92	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1																																						
17. KEN/11/96	2	3	2	2	2	2	2	2	2	2	2	2	3	3	3	3																																						
18. KEN/2/88	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	6																																						
19. KEN/3/57	18	18	18	18	18	18	18	18	18	18	18	18	18	18	19	19	20	20																																						
20. UGA/28/98	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	25	24	27																																						
21. UGA/19/98	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	25	24	27	1																																				
22. UGA/3/76	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	26	26	25	22	22																																				
23. UGA/51/75	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	24	25	25	25	22	22	2																																				
24. UGA/8/76	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	26	26	26	22	21	2	1																																				
25. ETH/2/91	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	25	26	26	27	26	26	26	26	26																																				
26. SUD/6/77	26	27	26	26	26	26	26	26	26	26	26	27	27	27	27	28	27	30	26	26	26	26	26	24																																				
27. SAU/6/00	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	27	28	27	26	27	27	27	27	27	26	27																																		
28. ERI/12/98	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27	29	27	28	29	28	27	27	27	25	26	9																																
29. ERI/1/98	26	27	26	26	26	26	26	26	26	26	26	26	27	27	27	27	28	27	27	28	27	27	27	27	26	26	9	4																																
30. ERI/4/98	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27	28	27	28	28	28	27	27	27	26	27	9	4	0																																
31. GHA/8/91	27	27	27	27	27	27	27	27	27	27	27	27	27	27	28	28	29	28	29	29	28	25	25	25	28	29	30	30	31	31																																
32. GHA/2/90	27	27	27	27	27	27	27	27	27	27	27	27	27	27	28	28	29	27	29	29	29	25	25	25	28	28	30	30	30	30	1																																
33. ZIM/7/83	29	29	29	29	29	29	29	29	29	29	29	29	28	28	28	29	30	27	32	31	31	29	28	28	29	32	30	31	30	30	27	27																																
34. BOT/1/98	27	27	27	27	27	27	27	27	27	27	27	27	27	27	28	28	29	27	31	30	30	29	28	28	28	32	31	31	30	30	28	28	12																																
35. ZIM/267/98	27	27	27	27	27	27	27	27	27	27	27	27	28	28	29	27	30	27	27	28	27	27	29	30	30	30	29	29	27	27	21	19																																
36. ZIM/1/00	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	30	28	32	29	29	30	29	29	31	29	30	30	29	29	27	27	21	21	16																																
37. ZAM/10/96	27	27	27	27	27	27	27	27	27	27	27	27	27	27	28	29	26	31	31	31	29	28	29	28	29	32	30	31	30	31	27	27	24	24	26	24																																
38. BOT/31/98	27	27	27	27	27	27	27	27	27	27	27	27	27	27	28	28	29	25	30	30	30	29	28	29	28	32	30	30	30	30	28	27	25	24	26	25	17																																
39. KNP/31/95	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	31	32	30	32	29	29	29	28	28	25	32	28	29	28	29	28	27	26	26	27	26	21	21																																
40. PAL/5/83	31	32	31	31	31	31	31	31	31	31	31	31	32	32	32	32	33	31	31	29	29	29	29	29	27	31	28	27	27	27	28	28	26	26	27	27	22	22	16																																
41. ETH/2/90	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	29	26	31	29	29	29	28	29	28	30	29	29	28	29	29	29	25	24	24	25	20	21	20	23																																
42. ETH/1/91	27	27	27	27	27	27	27	27	27	27	27	27	27	28	28	29	25	31	29	29	29	28	29	27	30	28	28	28	28	29	29	25	24	24	26	20	22	20	23	1																																
43. ETH/1/90	27	28	27	27	27	27	27	27	27	27	27	28	28	28	28	29	26	31	29	29	29	28	28	31	29	29	29	29	28	28	25	24	24	25	20	22	20	23	1	1																																
44. TAN/1/86	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	29	26	30	28	28	29	28	29	27	30	28	29	28	29	29	28	23	24	24	25	20	22	21	23	14	15	15																																
45. KEN/9/99	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	30	27	31	30	30	28	27	28	27	30	30	30	29	29	29	28	25	25	26	27	21	22	20	23	15	15	15	13																																
46. KEN/5/99	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	30	31	27	32	30	30	28	27	28	28	31	30	31	30	30	29	29	26	26	26	28	21	22	21	24	15	16	15	13	1																																
47. KEN/7/99	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	30	31	27	32	30	30	28	28	28	29	27	31	30	30	30	30	29	29	26	25	26	28	21	22	20	24	15	16	15	13	1	2																																
48. KEN/16/98	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	30	27	32	30	30	28	28	28	27	30	30	30	29	29	29	29	26	25	26	27	21	22	20	23	15	15	15	13	1	1	2																																
49. KEN/4/99	28	29	28	28	28	28	28	28	28	28	28	28	29	29	29	29	30	27	31	30	30	28	27	28	28	30	29	30	29	29	29	28	26	25	26	27	21	22	21	23	14	15	14	13	1	2	2	1																																
50. KEN/33/91	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	29	30	26	31	30	30	28	28	28	27	31	29	30	29	29	29	29	26	26	26	27	21	23	21	24	15	15	15	14	1	2	2	1	2																														
51. TAN/1/75	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	29	30	26	30	31	31	29	28	29	26	30	28	29	28	28	28	28	25	24	24	25	21	22	19	22	13	13	13	10	10	11	11	10	10	11																														
52. KEN/3/95	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	31	27	32	29	29	29	29	29	29	31	29	30	30	30	27	27	25	24	24	24	20	22	21	24	14	14	14	12	11	12	12	12	12	12	10																														
53. KEN/2/76	29	30	29	29	29	29	29	29	29	29	29	29	30	30	30	30	31	27	31	29	29	29	29	29	28	30	29	30	29	29	27	26	24	23	24	23	19	21	20	23	13	14	14	10	11	12	11	12	12	12	8	2																														
54. KEN/1/86	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	29	25	29	29	29	29	28	29	28	30	29	29	28	28	27	27	23	24	23	25	19	22	21	22	12	13	12	11	11	12	11	11	11	12	10	8	9																														
55. KEN/1/84	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	29	24	29	29	29	29	29	29	28	30	29	29	28	28	27	27	23	25	24	25	19	22	22	22	13	13	13	11	11	12	11	11	11	12	10	8	9	0																														
56. KEN/1/87	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	29	25	29	29	29	29	28	29	28	30	29	29	28	28	27	27	23	24	23	25	19	21	21	22	12	13	13	11	11	11	11	11	11	12	10	8	9	0	0																														
57. KEN/2/87	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28	29	30	25	29	29	29	29	29	29	28	31	30	29	28	28	27	27	24	25	24	25	19	22	22	22	13	13	13	11	11	12	12	12	12	12	10	9	9	1	0	0																														

```
                          10        20        30        40        50        60        70        80
                 1. KEN/3/57  TTSAGEGAEV VTTDPTTHGG KVTTPRRVHT DVAFLLDRST HVHTNTTAFV VDLMDTKEKA LVGAILRSAT YYFCDLEVAC
                 2. ERI/1/98  ........D. .....S.... N.QEG..K.. E......... .....K.S.. .......K.. .......AS. .......I..
                 3. ERI/4/98  ........D. .....S.... N.QEG..K.. E......... .....K.S.. .......K.. .......AS. .......I..
                 4. ETH/2/90  ........D. .....S.... N.LEK.?M.. ....V...F. ....SK.T.N .......QH. ....L..AS. .......I..
                 5. ETH/1/91  ........D. .....S.... N.LEK..M.. ....V...F. ....SK.T.N .......QH. ....L..AS. .......I..
                 6. ETH/2/91  ........D. ..I.....  S..PA..I.. .......... .....K.T.N I......... .......... .......I..
                 7. SUD/6/77  ....S...D. I..G.A....  TEG.A..I.. .......... .....K...A .......R.. .......... ..........
                 8. SUD/9/77  ....S...D. I..G.A....  TEG.A..I.. .......... .....K...A .......R.. .......... ..........
                 9. ETH/1/90  ........D. .....S.... N.LEK..M.. ....V...F. ....SK.T.N .......QH. ....L..AS. .......IT.
                10. KEN/1/85  ........D. .......... T..AA..... .......... .....K.T.A .......... .......... .......I..
                11. KEN/11/96 ........D. .......... T..AA..... .......... .....NL.D. KGK...N... .......... .......I..
                12. KEN/2/84  ........D. .......... T..AA..... .......... .....K.T.A .......... .......... .......I..
                13. KEN/1/87  ........D. .....S.... S.VEK..M.. ....V...F. .....K.T.N .......QQ. ....L..AS. .......I..
                14. KEN/1/89  ........D. .......... T..AA..... .......... .....K.T.A .......... .......... .......I..
                15. KEN/1/92  ........D. .......... T..AA..... .......... .....K.T.A .......... .......... .......I..
                16. KEN/3/95  ........D. .....S.... S.VEK..M.. ....V...F. .....K.T.K ....N..QQ. ....L..AS. .......I..
                17. KEN/7/96  ........D. .......... T..AA..... .......... .....K.T.A .......... .......... .......I..
                18. KEN/16/98 ........D. .....SS... S.VEK..M.. ....V...F. ....SK.T.N ...L...QH. ....L..AS. .......I..
                19. KEN/4/99  ........D. .....SS... S.VEK..M.. ....V...F. ....SK.T.N ...L...QH. ....L..AS. .......I..
                20. KEN/2/76  ........D. .....S.... S.VEK..M.. ....V...F. .....K.T.N .......QQ. ....L..AS. .......I..
                21. KEN/1/94  ........D. .......... T..AA..... .......... .....K.T.A .......... ...T...... .......I..
                22. KEN/1/84  ........D. .....S.... S.VEK..M.. ....V...F. .....K.T.N .......QQ. ....L..AS. .......I..
                23. KEN/1/96  ........D. .......... T..AA..... .......... .....K.T.A .......... .......... .......I..
                24. KEN/1/86  ........D. .....S.... S.VEK..M.. ....V...F. .....K.T.N .......QQ. ....L..AS. .......I..
                25. KEN/28/91 ........D. .......... T..AA..... .......... .....K.T.A .......... .......... .......I..
                26. KEN/8/91  ........D. .......... T..AA..... .......... .....R.T.A .......... .......... .......I..
                27. KEN/2/87  ........D. .....S.... S.VEK..M.. ....V...F. .....K.T.N .......QQ. ....L..AS. .......I..
                28. KEN/2/94  ........D. .......... T..AA..... .......... .....K.T.A .......... .......... .......I..
                29. KEN/7/95  ........D. .......... T..AA..... .......... .....K.T.A .......... .......... .......I..
                30. KEN/6/92  ........D. .......... T..AA..... .......... .....K.T.A .......... .......... .......I..
                31. KEN/2/88  ........D. .....S.... T.MAA..... ........F. .....K.T.A ......N.Q. ....L...T. .......I..
                32. KEN/33/91 ........D. .....SS... S.VEK..M.. ....V...F. ....SK.T.N ...L...QH. ....L..AS. .......I..
                33. KEN/7A/98 ........D. .......... T..AA..... .......... .....K.T.A .......... .......... .......I..
                34. KEN/3/92  ........D. .......... T..AA..... .......... .....K.T.A .......... .......... .......I..
                35. UGA/9/95  ........D. .......... T..AA..... .......... .....K.T.A .......... .......... .......I..
                36. UGA/3/91  ........D. .......... T..AA..... .......... .....R.T.A .......... .......... .......I..
                37. UGA/51/75 .......... .......... S.GA...... .......... ....QK.S.A ...L...... .......... .....MDIT.
                38. TAN/1/75  ........D. .....S.... S.VEK..M.. ....V...F. .....K.T.S .......QQ. ....L..AS. .......I..
                39. TAN/1/86  ........D. .....S.... S.EEK..M.. ....V...F. .....KAT.N .......QQT ....L..AS. .......I..
                40. UGA/28/98 ........D. .......... S.RN...I.. ..T....... .....K.S.A .......... .......... H......I..
                41. UGA/3/76  .......... .......... S.GA...... .......... ....QK.S.A ...L...... .......... .....M.IT.
                42. UGA/8/76  .......... .......... S.G....... .......... ....QK.S.A ...L...... .......... .....M.IT.
                43. UGA/19/98 ........D. .......... S.RN...I.. ..T....... .....K.S.A .......... .......... H......I..
                44. ERI/12/98 ........D. .....S.... N.QEG..K.. E......... .....K.S.. .......G.. .......AS. .......I..
                45. SAU/6/00  ......S.D. .....S.... N.QEG..K.. E......... .....K.S.. .......... .......AS. .......I..
                46. KEN/9/99  ........D. .....SS... S.VEK..M.. ....V...F. ....SK.T.N ...L...QH. ....L..AS. .......I..
                47. KEN/7/99  ........D. .....SS... S.VEK..M.. ....V...F. ....SK.T.N ...L...QH. .I..L..AS. .......I..
                48. KEN/5/99  ........D. .....SS... S.VEK..M.. ....V...F. ....SK.T.N ...L...QH. ....L..AS. .......I..
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                          90        100       110       120       130       140       150       160
1.  KEN/3/57   ?GQTQARVLQ PNGAPRTTQL GDNPMVLSRN NVTRFAIPFT APHRLLSTVY NGECEYTKTV TAIRGDREVL AQKYSSAKHS
2.  ERI/1/98   V.DHTRVFW. .......... ......YAKG G......... .......... ....T....A .......AA. .A..ADNV.T
3.  ERI/4/98   V.DHTRVFW. .......... ......YAKG G......... .......... ....T....A .......AA. .A..ADNVYT
4.  ETH/2/90   V.EHTRVYW. .......... ......FAH. G.....V.Y. ....V.A.R. ....K..DR. SP.....A.. .A..ADSR..
5.  ETH/1/91   V.EHTRVYW. .......... ......FAH. G.....V.Y. ....V.A.R. ....K..DR. SP.....A.. .A..ADSR..
6.  ETH/2/91   V.EHKRVFW. ........T. ......Y.H. G.......Y. .......... ....D.K.AS .......A.. .A..ANT..T
7.  SUD/6/77   V.EHKRVFW. ........V. ......FAH. G.......Y. .......... ......N..S NP.....A.. .A.HKDVA..
8.  SUD/9/77   V.EHKRVFW. ........V. ......FAH. G.......Y. .......... ......N..S NP.....A.. .A..KDVA..
9.  ETH/1/90   V.EHSRVYW. .......... ......FAH. G.....V.Y. ....V.A.R. ....K..DR. SP.....A.. .A..ADSR..
10. KEN/1/85   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
11. KEN/11/96  V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
12. KEN/2/84   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
13. KEN/1/87   V.KHTRVFW. ........T. ......FAH. R.......Y. ......A.R. ....K..DR. S......A.. .A..ADSR.T
14. KEN/1/89   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
15. KEN/1/92   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
16. KEN/3/95   V.THTRVYW. ......A.A. ......FAH. G.....V.Y. ......A.R. ....N.KDR. S......A.. .A..A.SR.A
17. KEN/7/96   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
18. KEN/16/98  V.THKRVYWH ........T. ......FAH. G.......Y. ......A... ....K..DR. S......A.. .A..AESR.T
19. KEN/4/99   V.THKRVYW. ........T. ......FAH. G.......Y. ......A... ....K..DR. S......A.. .A..AESR.T
20. KEN/2/76   V.THTRVYW. ......A.A. ......FAH. G.....V.Y. ......A.R. ....K..DR. .......A.. .A..A.SR.A
21. KEN/1/94   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
22. KEN/1/84   V.KHTRVFW. ........T. ......FAH. R.......Y. ......A.R. ....K..DR. S......A.. .A..ADSR.T
23. KEN/1/96   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
24. KEN/1/86   V.KHTRVFW. ........T. ......FAH. R.......Y. ......A.R. ....K..DR. S......A.. .A..ADSR.T
25. KEN/28/91  V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
26. KEN/8/91   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
27. KEN/2/87   V.KHTRVFW. ........T. ......FAH. R.......Y. ......A.R. ....K..DR. S......A.. .T..ADSR.T
28. KEN/2/94   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
29. KEN/7/95   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
30. KEN/6/92   V.EHKRVFW. ....S.S... .V....F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
31. KEN/2/88   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
32. KEN/33/91  V.THKRVYW. .......MT. ......FAH. G.......Y. ......A... ....K..DR. S......A.. .A..AESR.T
33. KEN/7A/98  V.EHKRVFW. .......PKF ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
34. KEN/3/92   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
35. UGA/9/95   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
36. UGA/3/91   V.EHKRVFW. .......... ......F.HK K......... .......... ....K..EKT I......A.. ....A.T..A
37. UGA/51/75  V.EHARVFW. .......NE. .E....F.H. K.......Y. .......... ....A.S.P. S......QA. .A..A.GR.T
38. TAN/1/75   V.THTRVYW. ........P. ......FAH. G.......Y. ......A.M. ....K..DR. S......A.. .A..ADSR.T
39. TAN/1/86   V.THKRVYW. ........V. ......FAH. G.......Y. ......A... ....R..DK. .......A.. .A..ADSR.A
40. UGA/28/98  V.DHKRVFW. .......V.. ......F.H. G.P..... .......... ....D.NSK. .......Q.. Q...A.TR.A
41. UGA/3/76   V.EHARVFW. .......NE. .....IF.H. K.......Y. .......... .K.A.S.P. S......QA. .A..A.GG.T
42. UGA/8/76   V.EHARVFW. .......NE. ......F.H. K.....V.Y. .......... ....A.S.P. S......QA. .A..A.GR.T
43. UGA/19/98  V.DHKCVFW. .......V.. ......F.H. G......... .......... ....D.NSK. .......Q.. Q...A.TR.A
44. ERI/12/98  V.DHTRVFW. .......... ......YAKG G......... .......... ....T.A..A .......AA. .A..AASV.T
45. SAU/6/00   V.D?TRAFW. .......... ......FAKG G......... .......... ....V.K..P .......AA. .A..ADST.T
46. KEN/9/99   V.THKRVYW. ........T. ......FAH. G.......Y. ......A... ....K..DR. S......A.. .A..AESR.T
47. KEN/7/99   V.THKSVYW. ........T. ......FAH. G.......Y. ......A... ....K..DR. S......T.. .A..AESR.T
48. KEN/5/99   V.THKRVYW. ........T. ......FAH. G.......Y. ......A... ....K..DR. S......LL. .A..AESR.T
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                          170        180        190        200        214↓216
1.  KEN/3/57   LPSTFNFGFV TADKPVDVYY RMKRAELYCP RALLPAYTHA GGDRFDAPIGVAKQ LL
2.  ERI/1/98   ..Q.......  .V........ .......... .P.....D.. SR.........ER. T.
3.  ERI/4/98   ..Q.......  .V........ .......... .P.....D.. SR.........ER. T.
4.  ETH/2/90   ........H.  ...R...... .......... .P.....Q.N NR.........E.. .C
5.  ETH/1/91   ........H.  ...R...... .......... .P.....Q.N NR.........E.. ..
6.  ETH/2/91   ........H.  ...A...... .......... .......D.V .R.........ER. ..
7.  SUD/6/77   .......... .......... .......... .P.....S.N .R.........E.. .C
8.  SUD/9/77   .......... .......... .......... .P.....S.N .R.........E.. .C
9.  ETH/1/90   ........H.  ...R...... .......... .P.....Q.N NR.........E.. .C
10. KEN/1/85   .......... .......... .......... .........Q DR.........E.. ..
11. KEN/11/96  .......... .......... .......... .........Q DR.........E.. ..
12. KEN/2/84   .......... .......... .......... .........Q DR.........E.. ..
13. KEN/1/87   ........H.  ...Q...... .......... .P.....Q.G NR.........E.. .C
14. KEN/1/89   .......... .......... .......... .........Q DR.........E.. ..
15. KEN/1/92   .......... .......... .......... .........Q DR.........E.. ..
16. KEN/3/95   ........Y.  ...Q...... .......... .P.....Q.N NR.........K.. VC
17. KEN/7/96   .......... .......... .......... .........Q DR.........E.. ..
18. KEN/16/98  ........H.  ...Q...... .......... .P.....Q.G IR.........E.. .C
19. KEN/4/99   ........H.  ...Q...... .......... .P.....Q.G IR.........E.. .C
20. KEN/2/76   ........H.  ...Q...... .......... .P.....Q.N DR.........E.. .C
21. KEN/1/94   .......... .......... .......... .........Q DR.........E.. ..
22. KEN/1/84   ........H.  ...Q...... .......... .P.....Q.G NR.........E.. .C
23. KEN/1/96   .......... .......... .......... .........Q DR.........E.. ..
24. KEN/1/86   ........H.  ...N...... .......... .P.....Q.G NR.........E.. .C
25. KEN/28/91  .......... .......... .......... .........Q DR.........E.. ..
26. KEN/8/91   .......... .......... .......... .........Q DR.........E.. ..
27. KEN/2/87   ...R....H.  ...Q...... .......... .P.....Q.G NR.........E.. .C
28. KEN/2/94   .......... .......... .......... .........Q DR.........E.. ..
29. KEN/7/95   .......... .......... .......... .........Q DR.........E.. ..
30. KEN/6/92   .......... .......... .......... .........Q DR.........E.. ..
31. KEN/2/88   .......... .......... .......... .........Q DR.........E.. ..
32. KEN/33/91  ........H.  ...Q...... .......... .P.....Q.G IR.........E.. ..
33. KEN/7A/98  .......... .......... .......... .........Q DR.........E.. ..
34. KEN/3/92   .......... .......... .......... .........Q DR.........E.. ..
35. UGA/9/95   .......... .......... .......... .........Q DR.........E.. ..
36. UGA/3/91   .......... .......... .......... .........Q DR.........E.. ..
37. UGA/51/75  .......... .......... .......... .......D.. NR.........E.. .H
38. TAN/1/75   ........H.  ...Q...... .......... .P.....D.S DR.........E.. ..
39. TAN/1/86   ........H.  ...Q...... .......... .P.....Q.G DR.........E.. .C
40. UGA/28/98  .......... .......... .......... .P.....D.K NR.........ER. .S
41. UGA/3/76   .......... .......... .......... .......D.. NR.........E.. .H
42. UGA/8/76   .......... .......... .......... .......D.. NR.........E.. .H
43. UGA/19/98  .......... .......... .......... .P.....D.K NR.........ER. .Y
44. ERI/12/98  ..Q.......  .V........ .......... .P.....D.. SR.........ER. T.
45. SAU/6/00   ..........  .V........ .......... .P.....E.T ..........ER. T.
46. KEN/9/99   ........H.  ...Q...... .......... .P.....Q.G NR.........E.. .C
47. KEN/7/99   ........H.  ...Q...... .......... .P.....Q.G NR.........E.. .C
48. KEN/5/99   ........H.  ...Q...... .......... .P.....Q.G NR.........E.. .C
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Fig. 4.2 Amino acid sequence alignment of the 1D gene of 48 SAT-2 FMD viruses from East African countries. '.' Indicates an amino acid site identical to the sequence of KEN/3/57 and '?' indicates ambiguous sites. The cell attachment site the 'RGD' is in bold and the conserved amino acids are underlined.

Amino acid in windows of 10	variable sites	Variablity plot
1-10	3	ÞÞÞ
2-11	4	ÞÞÞÞ
3-12	4	ÞÞÞÞ
4-13	5	ÞÞÞÞÞ
5-14	6	ÞÞÞÞÞÞ
6-15	5	ÞÞÞÞÞ
7-16	6	ÞÞÞÞÞÞ
8-17	6	ÞÞÞÞÞÞ
9-18	6	ÞÞÞÞÞÞ
10-19	5	ÞÞÞÞÞ
11-20	5	ÞÞÞÞÞ
12-21	5	ÞÞÞÞÞ
13-22	6	ÞÞÞÞÞÞ
14-23	6	ÞÞÞÞÞÞ
15-24	6	ÞÞÞÞÞÞ
16-25	7	ÞÞÞÞÞÞÞ
17-26	6	ÞÞÞÞÞÞ
18-27	5	ÞÞÞÞÞ
19-28	6	ÞÞÞÞÞÞ
20-29	6	ÞÞÞÞÞÞ
21-30	6	ÞÞÞÞÞÞ
22-31	6	ÞÞÞÞÞÞ
23-32	5	ÞÞÞÞÞ
24-33	5	ÞÞÞÞÞ
25-34	4	ÞÞÞÞ
26-35	4	ÞÞÞÞ
27-36	4	ÞÞÞÞ
28-37	4	ÞÞÞÞ
29-38	3	ÞÞÞ
30-39	4	ÞÞÞÞ
31-40	4	ÞÞÞÞ
32-41	3	ÞÞÞ
33-42	3	ÞÞÞ
34-43	2	ÞÞ
35-44	2	ÞÞ
36-45	2	ÞÞ
37-46	3	ÞÞÞ
38-47	4	ÞÞÞÞ
39-48	5	ÞÞÞÞÞ
40-49	5	ÞÞÞÞÞ
41-50	6	ÞÞÞÞÞÞ
42-51	7	ÞÞÞÞÞÞÞ
43-52	8	ÞÞÞÞÞÞÞÞ
44-53	9	ÞÞÞÞÞÞÞÞÞ
45-54	10	ÞÞÞÞÞÞÞÞÞÞ
46-55	10	ÞÞÞÞÞÞÞÞÞÞ
47-56	9	ÞÞÞÞÞÞÞÞÞ
48-57	9	ÞÞÞÞÞÞÞÞÞ
49-58	9	ÞÞÞÞÞÞÞÞÞ
50-59	9	ÞÞÞÞÞÞÞÞÞ
51-60	9	ÞÞÞÞÞÞÞÞÞ
52-61	8	ÞÞÞÞÞÞÞÞ
53-62	8	ÞÞÞÞÞÞÞÞ
54-63	7	ÞÞÞÞÞÞÞ
55-64	7	ÞÞÞÞÞÞÞ
56-65	7	ÞÞÞÞÞÞÞ
57-66	7	ÞÞÞÞÞÞÞ
58-67	6	ÞÞÞÞÞÞ
59-68	6	ÞÞÞÞÞÞ
60-69	6	ÞÞÞÞÞÞ
61-70	5	ÞÞÞÞÞ
62-71	6	ÞÞÞÞÞÞ
63-72	5	ÞÞÞÞÞ
64-73	5	ÞÞÞÞÞ
65-74	4	ÞÞÞÞ
66-75	3	ÞÞÞ
67-76	4	ÞÞÞÞ
68-77	5	ÞÞÞÞÞ
69-78	5	ÞÞÞÞÞ
70-79	5	ÞÞÞÞÞ
71-80	5	ÞÞÞÞÞ
72-81	4	ÞÞÞÞ
73-82	4	ÞÞÞÞ
74-83	5	ÞÞÞÞÞ
75-84	6	ÞÞÞÞÞÞ
76-85	7	ÞÞÞÞÞÞÞ
77-86	7	ÞÞÞÞÞÞÞ
78-87	7	ÞÞÞÞÞÞÞ
79-88	7	ÞÞÞÞÞÞÞ
80-89	7	ÞÞÞÞÞÞÞ
81-90	8	ÞÞÞÞÞÞÞÞ
82-91	8	ÞÞÞÞÞÞÞÞ
83-92	8	ÞÞÞÞÞÞÞÞ
84-93	7	ÞÞÞÞÞÞÞ
85-94	6	ÞÞÞÞÞÞ
86-95	6	ÞÞÞÞÞÞ
87-96	5	ÞÞÞÞÞ
88-97	5	ÞÞÞÞÞ
89-98	5	ÞÞÞÞÞ
90-99	5	ÞÞÞÞÞ
91-100	5	ÞÞÞÞÞ
92-101	5	ÞÞÞÞÞ
93-102	6	ÞÞÞÞÞÞ
94-103	6	ÞÞÞÞÞÞ
95-104	6	ÞÞÞÞÞÞ
96-105	5	ÞÞÞÞÞ
97-106	6	ÞÞÞÞÞÞ
98-107	6	ÞÞÞÞÞÞ
99-108	6	ÞÞÞÞÞÞ
100-109	6	ÞÞÞÞÞÞ
101-110	6	ÞÞÞÞÞÞ
102-111	7	ÞÞÞÞÞÞÞ
103-112	6	ÞÞÞÞÞÞ
104-113	7	ÞÞÞÞÞÞÞ
105-114	7	ÞÞÞÞÞÞÞ
106-115	7	ÞÞÞÞÞÞÞ
107-116	6	ÞÞÞÞÞÞ
108-117	6	ÞÞÞÞÞÞ
109-118	5	ÞÞÞÞÞ
110-119	5	ÞÞÞÞÞ
111-120	4	ÞÞÞÞ
112-121	3	ÞÞÞ
113-122	3	ÞÞÞ
114-123	2	ÞÞ
115-124	2	ÞÞ
116-125	3	ÞÞÞ
117-126	3	ÞÞÞ
118-127	3	ÞÞÞ
119-128	3	ÞÞÞ
120-129	3	ÞÞÞ
121-130	3	ÞÞÞ
122-131	3	ÞÞÞ
123-132	3	ÞÞÞ
124-133	4	ÞÞÞÞ
125-134	4	ÞÞÞÞ
126-135	4	ÞÞÞÞ

127-136	4	þþþþ
128-137	4	þþþþ
129-138	5	þþþþþ
130-139	5	þþþþþ
131-140	6	þþþþþþ
132-141	7	þþþþþþþ
133-142	8	þþþþþþþþ
134-143	7	þþþþþþþ
135-144	7	þþþþþþþ
136-145	6	þþþþþþ
137-146	6	þþþþþþ
138-147	5	þþþþþ
139-148	5	þþþþþ
140-149	5	þþþþþ
141-150	4	þþþþ
142-151	4	þþþþ
143-152	4	þþþþ
144-153	4	þþþþ
145-154	5	þþþþþ
146-155	6	þþþþþþ
147-156	7	þþþþþþþ
148-157	8	þþþþþþþþ
149-158	8	þþþþþþþþ
150-159	8	þþþþþþþþ
151-160	9	þþþþþþþþþ
152-161	8	þþþþþþþþ
153-162	7	þþþþþþþ
154-163	8	þþþþþþþþ
155-164	8	þþþþþþþþ
156-165	7	þþþþþþþ
157-166	6	þþþþþþ
158-167	5	þþþþþ
159-168	4	þþþþ
160-169	4	þþþþ
161-170	3	þþþ
162-171	3	þþþ
163-172	4	þþþþ
164-173	3	þþþ
165-174	3	þþþ
166-175	3	þþþ
167-176	3	þþþ
168-177	3	þþþ
169-178	3	þþþ
170-179	2	þþ
171-180	2	þþ
172-181	2	þþ
173-182	1	þ
174-183	1	þ
175-184	**0**	
176-185	**0**	
177-186	**0**	
178-187	**0**	
179-188	**0**	
180-189	**0**	
181-190	**0**	
182-191	**0**	
183-192	1	þ
184-193	1	þ
185-194	1	þ
186-195	1	þ
187-196	1	þ
188-197	1	þ
189-198	2	þþ
190-199	2	þþ
191-200	3	þþþ
192-201	4	þþþþ
193-202	4	þþþþ
194-203	4	þþþþ
195-204	4	þþþþ
196-205	4	þþþþ
197-206	4	þþþþ
198-207	4	þþþþ
199-208	3	þþþ
200-209	3	þþþ
201-210	2	þþ
202-211	1	þ
203-212	1	þ
204-213	2	þþ
205-214	2	þþ
206-215	3	þþþ
207-216	4	þþþþ

Fig. 4.3 Variability plot of sequences of 1D gene of SAT-2 viruses from East Africa. The numbers of variable sites in overlapping windows of size = 10 > 4 and < 7 = variable region 7-10 (Red)= Hyper variable region (>70%)
Black = conserved region

4.4 Discussion

Two lineages (I and V) with divergence of > 26% were identified for East Africa from isolates characterized in this study based on the phylogenetic analysis of the complete 1D gene sequences of SAT-2 viruses from 5 East African countries (Eritrea, Ethiopia, Kenya, Uganda and Tanzania). These lineages comprised of 12 genotypes including the 4 (4/7) new genotypes (A, B, D, E) identified for East Africa. The 3 other genotypes (C, F & G) which consisted of isolates from East Africa as identified by Bastos *et al.* (2003) clustered into two separate lineages whilst in this study the 3 genotypes clustered into one lineage (lineage I). The remaining genotypes within lineages I and V and the 3 other lineages (II – IV) consisted of isolates that have been characterized in earlier studies (Table 4.1; van Rensburg & Nel, 1999; Sangare *et al.*, 2002; Bastos *et al.*, 2003).

Twelve independently evolving genotypes were identified within lineages I and V. Genotype O within lineage V shared a genetic relationship with isolates from southern African countries and interestingly shared a common ancestor with buffalo viruses from South Africa (PAL/5/83, KNP/31/95; boot-strap support of 98%). This genotype (O) comprised of isolates from Ethiopia, Kenya, Malawi, Burundi and Tanzania collected over 20 years with a sequence similarity > 80% indicating a higher level of mutations compared to isolates in genotype A from lineage I (84 - 99%). Clustering of isolates from East Africa within lineage V with buffalo viruses from South Africa might indicate that the ancient migration of buffaloes caused the spread of the disease between East and southern African countries as this species is known to harbour viruses for up to 5 years (Condy *et al.*, 1985) and transmitting disease to other susceptible animals (Dawe *et al.*, 1994; Vosloo *et al.*, 1996; Bastos *et al.*, 2000).

The virus isolates comprising of genotypes A-G within lineage I were generally found to originate either from countries with common boundaries or from specific East African countries (Fig. 4.1) and were genetically heterogeneous. The Ugandan isolates of genotypes B and C were genetically sufficiently diverged to be assigned into two distinct genotypes and differed from other SAT-2 East African isolates. This indicated that similar strains of viruses can be confined to certain

localities and evolve within that locality over time while other strains can transcend country boundaries. This emphasizes the need to investigate the main factors which play a role in exchange of viruses between countries to minimize complication of the epidemiology of the disease in the regions. Restriction of animal movement in this region must be the first step in the control of the disease as well as development of polyvalent vaccines that contains a region specific cocktail of strains.

Genotype F within lineage I consisted of three isolates from Eritrea and Saudi Arabia, the latter being a country where SAT-2 does not normally occur. This result is consistent with the findings of Bastos *et al.* (2003) and the outbreak in Saudi Arabia was most probably caused by trade in livestock between the two countries.

Genotype A, within lineage I, which has a sequence identity between 96 – 99.5% (Table 4.3), is shared between 17 Kenyan and 2 Ugandan viruses and spans a 13 year period. This high level of sequence identity over such a long period is surprising and should be investigated further to determine whether the spread of these outbreaks were not due to incompletely inactivated vaccines. Of interest was that the isolate KEN/3/57 from the same genotype showed > 17% sequence dissimilarities with Kenyan isolates where this difference is very close to cut-off value for genotype, whilst high boot-strap support (99%) points to common ancestry.

Trans-boundary transmission of the disease due to animal movement is possible within lineages as the countries included in these lineages share common boundaries and animal trading across borders is a common practice (Ndiritu, 1984). 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 and 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 favours the transmission of the disease between neighbouring countries and maintenance of the endemicity of FMD in the region.

It was also observed that the genetic diversity between topotypes (A-O) was more than 20% at nucleotide level. This genetic diversity could also be the reflection of the existence of extensive antigenic variation within SAT-2 viruses as was shown to be the case in SAT-1 and serotype A viruses (Vosloo *et al.*, 1996; Bastos *et al.*, 2003, the references therein). The result of the amino acid hypervariability plots of 48 isolates from Africa indicated that the GH-loop region had a continuous hypervariable region upstream and downstream of the 'RGD' which probably indicates the importance of this region for the virus to escape immune pressure. In contrast to other SAT type viruses the region within the C-terminus were not shown to be hypervariable (< 20%) whilst the amino acid positions 46-69 and 76-99 of the 1D gene accommodated hypervariable regions (> 70%).

This molecular epidemiological study of SAT-2 FMD viruses obtained from East Africa has provided valuable information with respect to the epidemiology of the disease in this region. These results demonstrated a distinct geographical grouping of SAT-2 in East Africa and stressed the genetic diversity within the serotype that reflects on the control of the disease by vaccination. Therefore, control of FMD will only be effective if the region co-operates to prevent transboundary spread of the disease which necessitates the need for regional or Pan-African management programme.

CHAPTER V

MOLECULAR EPIDEMIOLOGY OF SAT-1 FOOT-AND-MOUTH DISEASE VIRUSES IN EAST AFRICA ISOLATED BETWEEN 1971 AND 2000

5.1 Introduction

Over the past 30 years data from the WRL indicated that SAT-1 FMD viruses maintained endemicity in four East African countries, namely Uganda, Tanzania, Sudan and Kenya. This serotype has not been reported in Ethiopia, Eritrea and Somalia. Due to poor reporting from East African countries this figure is unlikely to provide a true distribution of SAT-1 in the region. Movement of animals across borders of countries is a common practice due to nomads and illegal animal trading. This favours the spread of the disease between countries as has been shown to occur in other African countries (Bastos and Sangare, 2001; Sangare *et al.*, 2001). The livestock population of 5 East African countries (Ethiopia, Kenya, Somalia, Tanzania and Uganda) is estimated to be over 57 million cattle and 98 million goats and sheep (McDermott and Arimi, 2002). There are also large numbers of African buffalos (*Syncerus caffer*) in East Africa, capable of maintaining and transmitting the SAT type FMD viruses (Thomson, 1996) and three independently evolving viral SAT-1 lineages were previously identified from persistently infected African buffalo populations within southern Africa (Bastos *et al.*, 2001).

There is little data available on the nucleotide sequence database of SAT-1 viruses of East African origin. With the exception of a number of isolates from Tanzania and Uganda (Bastos *et al.*, (2001) the phylogenetic relationships of SAT-1 viruses in East Africa has not been studied in any detail. In the present study, sequences from East African isolates and from previous studies were used to address the phylogeographical distribution of SAT-1 type viruses in Africa.

5.2 Materials and Methods

5.2.1 Viruses used in this study

This study included 23 new East African and 30 West, South and Central African virus isolates from previous studies. The WRL for FMD at the Institute for Animal Health, Pirbright (UK) supplied twenty (20/23) tissue culture virus isolates from East Africa and these viruses were propagated once on IB-RS-2 cells and stored at –70°C prior to further use. Sequences of all other isolates from Africa were obtained from Genbank. The laboratory designation, sampling date and geographical origin of all isolates are indicated in Table 5.1.

Table 5.1 Summary of SAT-1 FMD viruses used in this study.

Virus designations	Sampling year	Country of origin	Reference	Genbank Accession No
BOT/1/68	1968	Botswana	Knowles, 1998 (direct submission)	Z98203
BOT/24/77	1977	Botswana	Bastos *et al.* (2001)	AF301423
BOT/2/98	1998	Botswana	Bastos *et al.* (2001)	AF137402
BOT/37/98	1998	Botswana	Bastos *et al.* (2001)	AF137405
BOT/8/98	1998	Botswana	Bastos *et al.* (2001)	AF301432
KEN/11/91	1991	Kenya	This study	AY441994
KEN/9/91	1991	Kenya	This study	AY441995
KEN/4/98	1998	Kenya	This study	AY441993
KNP/96/81	1981	South Africa	This study	
SAR/9/81	1981	South Africa	Bastos *et al.* (2001)	AF056511
KNP/196/91	1991	South Africa	Van Rensburg *et al.* (2002)	AF283429
MAL/1/85	1985	Malawi	Bastos *et al.* (2001)	AF056509
MOZ/3/77	1977	Mozambique	Bastos *et al.* (2001)	AF056510
NAM/307/98	1998	Namibia	Van Rensburg (unpublished data)	
NGR/2/76	1976	Niger	Sangare *et al.* (2003)	AF431718
NGR/4/76	1976	Niger	Sangare *et al.* (2003)	AF431719
NIG/17/75	1975	Nigeria	Sangare *et al.* (2003)	AF431712
NIG/25/75	1975	Nigeria	Sangare *et al.* (2003)	AF431715
NIG/14/76	1976	Nigeria	Sangare *et al.* (2003)	AF431719
NIG/1/76	1976	Nigeria	Sangare *et al.* (2003)	AF431721
NIG/20/76	1976	Nigeria	Sangare *et al.* (2003)	AF431727
NIG/6/76	1976	Nigeria	Sangare *et al.* (2003)	AF431716
NIG/8/76	1976	Nigeria	Sangare *et al.* (2003)	AF431724
NIG/2/79	1979	Nigeria	Sangare *et al.* (2003)	AF431728
NIG/3/80	1980	Nigeria	Sangare *et al.* (2003)	AF431729
NIG/10/81	1981	Nigeria	Sangare *et al.* (2003)	AF431731
NIG/5/81	1981	Nigeria	Sangare *et al.* (2003)	AF431730
SUD/13/74	1974	Sudan	This study	AY442000
SUD/8/74	1974	Sudan	This study	AY441998
SUD/9/74	1974	Sudan	This study	AY441999
SUD/3/76	1976	Sudan	This study	AY441996
SUD/4/76	1976	Sudan	This study	AY441997
TAN/2/77	1977	Tanzania	This study	AY442008
TAN/3/80	1980	Tanzania	This study	AY442006
TAN/1/96	1996	Tanzania	This study	AY442003
TAN/19/96	1996	Tanzania	This study	AY442013
TAN/2/96	1996	Tanzania	This study	AY442001
TAN/5/96	1996	Tanzania	This study	AY442007
TAN/1/99	1999	Tanzania	Bastos *et al.* (2001)	AF301434

TAN/2/99	1999	Tanzania	Bastos *et al.* (2001)	AF301435
TAN/37/99	1999	Tanzania	This study	AY442005
TAN/51/99	1999	Tanzania	This study	AY442004
TAN/60/99	1999	Tanzania	This study	AY442002
UGA/13/74	1974	Uganda	This study	AY442010
UGA/1/97	1997	Uganda	Van Rensburg *et al.* (2002)	AF283439
UGA/3/99	1999	Uganda	This study	AY442009
UGA/7/99	1999	Uganda	This study	AY442011
ZAM/2/93	1993	Zambia	Bastos *et al.* (2001)	AF056514
ZAM/1/99	1999	Zambia	Bastos *et al.* (2001)	AF301436
ZIM/14/98	1998	Zimbabwe	Bastos *et al.* (2001)	AF137411
ZIM/3/88	1988	Zimbabwe	Bastos *et al.* (2001)	AF056520
ZIM/5/99	1999	Zimbabwe	Bastos *et al.* (2001)	AF301437
ZIM/7/99	1999	Zimbabwe	Bastos *et al.* (2001)	AF301439

5.2.2 Nucleic acid isolation and RT-PCR amplification

Viral RNA isolation, cDNA synthesis and RT-PCR amplification was carried out following the procedure described in section 2.2.2 - 2.2.4. The P1 primer and a primer VP 3AB binding to the VP3 encoding gene (5' CACTGCTACCACTCRGAGTG 3') (Bastos *et al.*, 2003) were used to amplify the complete 1D gene (880 bp) of SAT-1 viruses.

5.2.3 DNA purification and Cycle Sequencing

The 880 bp PCR amplicon was purified and sequenced as described in sections 2.2.6 and 4.3.4 respectively. Two independent sequencing reactions were performed per sample using the P1 and upstream primers VP 3AB (Bastos *et al.*, 2003). All data were submitted to Genbank under the accession numbers provided in Table 5.1.

5.2.4 Phylogenetic analysis

Two sets of sequences were used in this study and sequence alignments and phylogenetic analysis were carried out as described in previous chapters. The 880 bp sequence fragment was trimmed resulting in homologous regions of 396 and 663 base pairs corresponding to the partial and complete 1D encoding gene respectively and these were used as two sets of data for phylogenetic analysis. Lineages were distinguished on the basis of nucleotide sequence differences of > 23% and boot-strap support $\geq$ 80% while a divergence of $\geq$16% distinguished genotypes.

5.3 Results

5.3.1 Phylogenetic analysis

In order to assess the phylogenetic relationships of SAT-1 type FMD viruses from East Africa and other African countries, two sets of sequence data were analyzed based on the availability of sequences of viruses from Genbank. An homologous region of 396 nt corresponding to amino acid positions 90 – 221 C-terminus of the 1D gene and the complete sequence (663 nt) of the 1D/2A protein were used for phylogenetic analysis.

In order to place our results within the context of earlier molecular epidemiological studies and to have a better representation of viruses from different regions of the continent, a total of 53 viruses with sequence length of 396 nt were analysed. The NJ method using the p-distance model revealed 8 major lineages labelled I - VIII and 11 distinct genotypes labelled A – K (Fig. 5.1) from the partial sequences of the 1D gene of 39 isolates.

Figure 5.2 represents analysis of the complete 1D gene sequences of 21 isolates from East Africa and 18 isolates from other African countries. The NJ method of analysis recovered the same clustering of isolates *ie.*, 8 lineages as revealed with the partial sequence analysis of the 1D gene. The recovered genotypes in Fig. 5.2 were also found to coincide with genotypes as in the partial sequence analysis (Fig. 5.1).

Analysis of the two sets of data with the UPGMA and Maximum Parsimony methods recovered the same clustering of viruses as distinguished by NJ tree. The average pair-wise distance comparisons between lineages (I – VIII) revealed uncorrected sequence divergence in excess of 23% (Table 5.2).

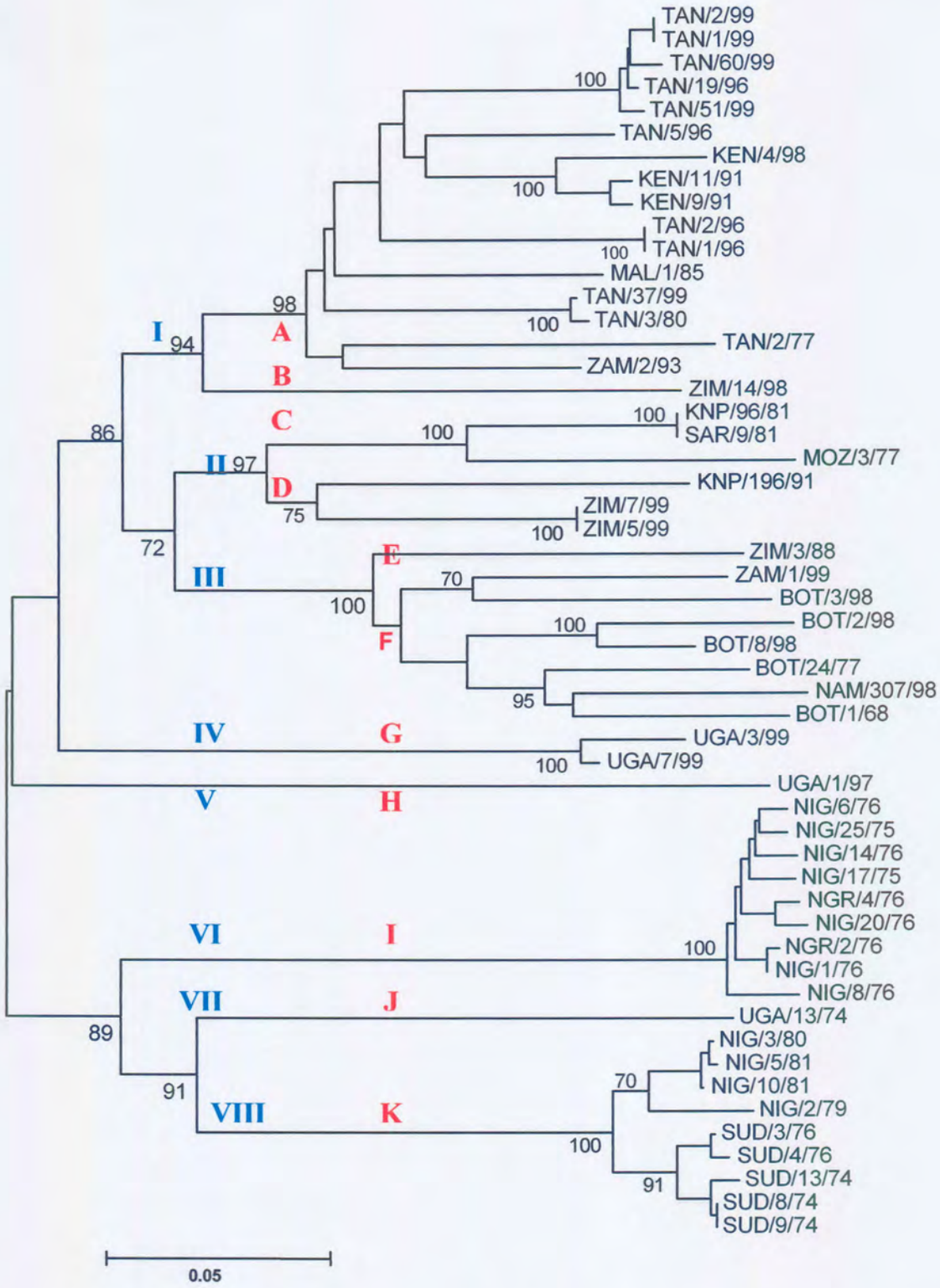


Fig. 5.1 Neighbour-joining tree based on 396 nt of the 1D gene (amino acid position 90 - 221) depicting genetic relationships of 53 SAT-1 FMD viruses from Africa. lineages (I-VIII) and genotypes (A-K) were identified. P-distance was used to estimate genetic distances and boot-strap values of ≥ 70% are indicated and are based on 1000 replications. The scale represents 5% differences at the nucleotide level.

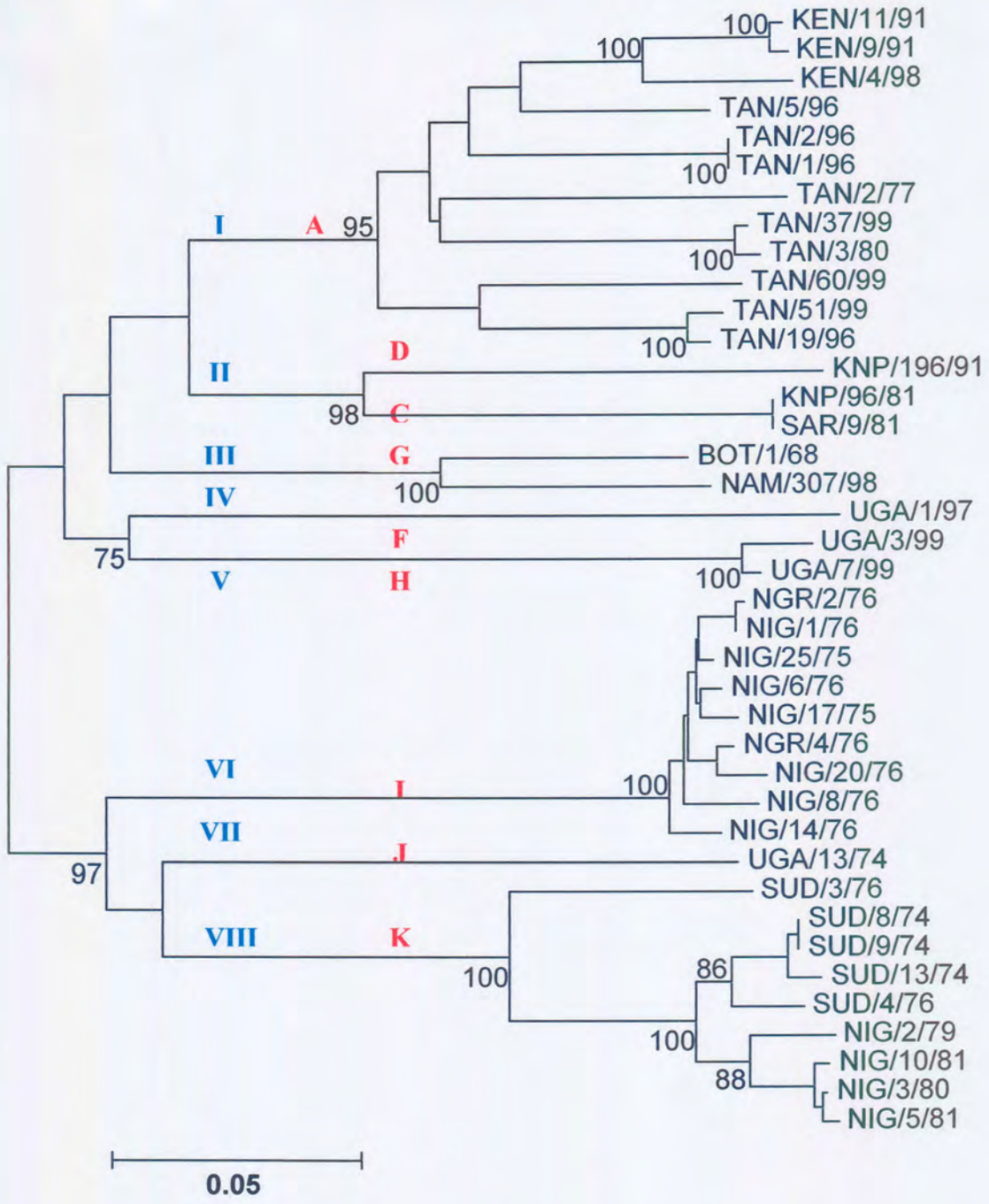


Fig. 5.2 Neighbour-joining tree based on 663 nt of 1D/2A gene (6 nt from 2A) depicting phylogenetic relationships of 39 SAT-1 FMD virus isolates from Africa. Eight major lineages labelled I -VIII were identified based on uncorrected sequence divergence in excess of 23% and genotypes labelled A – K coincided with Fig. 5.1 (genotype B & E in Fig.5.1 were not presented here as their complete 1D gene sequence were not available). The scale represents 5% differences at the nucleotide level.

Lineage I (East-southern Africa)

Genotype A: Tanzania, Kenya, Zambia and Malawi

Genotype B: Zimbabwe

Lineage II (Southern Africa)

Genotype C: South Africa and Mozambique

Genotype D: Zimbabwe

Lineage III (Southern Africa)

Genotype E: Zimbabwe

Genotype F: Zambia, Botswana and Namibia

Lineage IV (East Africa)

Genotype G: Uganda

Lineage V (East Africa)

Genotype H: Uganda

Lineage VI (West Africa)

Genotype I: Nigeria and Niger

Lineage VII (East Africa)

Genotype J: Uganda

Lineage VIII (East-west Africa)

Genotype K: Nigeria and Sudan

Isolates from East Africa were distributed into 5 lineages (Fig. 5.1) where the 3 lineages IV, V and VII were found to be distinct to the Uganda. The other 2 lineages (I and VIII) comprised of viruses from East Africa clustering with viruses from other regions in Africa. Viruses from Zambia, Malawi, Zimbabwe and East Africa (Tanzania and Kenya) clustered in lineage I (Fig. 5.1) while lineage VIII showed historical relationships between Sudan and West African (Nigerian) isolates. The latter lineage represented one of the two lineages identified by Sangare *et al.* (2003).

The complete 1D gene sequences of 3 isolates KNP/196/91, UGA/1/97 (van Rensburg *et al.*, 2002) and NAM/307/98 (Van Rensburg, unpublished) included in this study were found to be heterogeneous and clustered in lineages II, IV and III, respectively (Fig. 5.2). These three lineages showed high divergent values (> 30%) (Table 5.2) and represented previously identified lineages by Bastos and co-workers (2001).

Table 5.2 Average pair-wise distance comparisons between lineages (I – VIII) of SAT-1 FMD viruses included in this study.

Lineages	I	II	III	IV	V	VI	VII
I (East Africa & Zimbabwe)							
II (Southern Africa)	23.5						
III (Southern Africa)	25.8	23.8					
IV (Uganda)	26.1	28.0	27.5				
V (Uganda)	31.2	30.8	30.8	32.3			
VI (West Africa)	32.3	31.9	31.2	31.1	32.5		
VII (Uganda)	29.9	35.8	31.7	31.4	34.8	29.3	
VIII (West and East Africa)	29.2	34.4	32.8	33.8	35.0	27.6	23.7

Description: No. of Taxa : 53, No. of Groups: 8, Gaps/Missing data : Pair-wise Deletion, Codon Positions : Noncoding, Distance method : Nucleotide: p-distance [Between group average], No. of Sites : 396, Distance (d) : estimate .

5.3.2 Sequence variations

Sequencing results showed that 53% of the amino acids across the complete 1D gene of the 36 SAT-1 viruses compared in this study were conserved (Fig. 5.3). In the remaining part of the 1D gene dispersed variability (for overlapping windows of 10 amino acids) was observed and hypervariable regions were located at amino acid positions 135 – 163 (135 – 147, 152 –163) and 196 – 220 in which 70% or more of the sites varied. These regions coincided with the immunogenic G-H loop and the C-terminus, respectively. The RGD motive at positions 149 – 151 were conserved in all 36 isolates and two amino acids at the +1 (L) or –1 (I) positions of the motive were also conserved in 32 isolates. Conserved sites (amino acids) were also found at amino acid positions 131-133 (YNG), 137 (Y), 156 (A) and 158 (R) within the immunogenic G-H loop of the 1D gene of all isolates which possibly plays a crucial role in virus cell interaction.

The variability plot revealed that distinct amino acids were only shared between the Sudanese and the Nigerian isolates at deduced amino acid positions 141-144 (NEDT), 160 (R), 161 – 163 (RQS) and 220 (M). Variable regions were also observed at amino acid positions 13 – 35, 44 –56, 91 – 112 and 172 - 179 (Fig. 5.4) in which 40 - 70% of the sites varied. The average proportion of nucleotide differences across the complete 1D sequence between all isolates was estimated to be 24%.

	10	20	30	40	50	60	70	80
BOT/1/68	TTSAGEGADP	VTTDASAHGG	DTRTTRRAHT	DVTFLLDRFT	LVGKTNDNKL	VLDLLSTKEK	SLVGALLRAA	TYYFSDLEVA
KEN/4/98	....	.....TQ...	GR..A..H..	..S.......	.....QN.R.	T....Q....	A....I....	....
KEN/11/91	........E.	.....TQ...	GR..AY.H..	..S.I.....	...R.QN.R.	T....Q....	A....I....	....
KEN/9/91	........E.	.....TQ...	GR..AY.H..	..S.I.....	...R.QN.R.	T....Q....	A....I....	....
SUD/3/76	........E.	......Q...	GR.AA..Q..	..S.......	.....VN...	T....K....	A....I..S.	....A.....
SUD/4/76	V	..V..A....	NQ.R...V..	..A.......	.....Q...M	...M.K....	A....I..S.	....A.P...
SUD/8/74	V	..V..A....	NQ.R...V..	..A.......	.....QN.RM	...M.K....	A....I..S.	....A.....
SUD/9/74	V	..V..A....	NQ.R...V..	..A.......	.....QN.RM	...M.K....	A....I..S.	....A.....
SUD/13/74	V	..V.TA....	NQ.R...V..	..A.......	.....QN.RM	...M.K....	A....I..S.	....A.....
TAN/2/96	........E.	......Q...	GR.A.H.H..	..S.......	.....VA...	T....Q....	A....I....	....
TAN/60/99	V	..V..A....	NQ.R...V..	..A.......	.....Q...M	...M.K....	A....I....	....
TAN/1/96	........E.	......Q...	GR.A.H.H..	..S.......	.....VA...	T....Q....	A....I....	....
TAN/51/99	........E.	......Q...	GR.AA..Q..	..S.......	.....VN...	T....Q....	A....I....	....
TAN/37/99	........E.	......Q...	GR.VA..H..	..S.I.....	.....VN.R.	T....Q....	A....I....	....
TAN/3/80	........E.	......Q...	GR.V...H..	..S.I.....	.....VK.R.	T....Q....	A....I....	....
TAN/5/96	........E.	......Q...	GR..A..H..	..S.I.....	.....V....	T....Q....	A....I....	....
TAN/2/77	........E.	......Q...	GR..A..H..	..S.I.....	.....V....	T....Q....	A....I..V.	....
UGA/3/99	........E.	......Q...	TS.RV..Q..	..S.......	.I...H....	.....Q....	A....I..S.	....
UGA/13/74	V	..V..T....	TQ.A...V..	..A.......	.....V...M	...M.Q..K.	A....I..S.	....A.....
UGA/7/99	........E.	......Q...	TS.RV..Q..	..S.......	.I...HN...	.....Q....	A....I..S.	....
UGA/1/97	........E.	......Q...	NA.P...Y..	N.E.......	.I...HN..M	...M.R.EK.	A....I..S.	....
TAN/19/96	........E.	......Q...	GR.AA..Q..	..S.......	.....VN...	T....Q....	A....I....	....
KNP/96/81	........E.	..V...Q...	NS.GVH.Q..	..S.......	.....QN..M	T....Q....	A....I....	....
NIG/10/81	V	..V.TA....	NQ.R...V..	..A.......	.....R...M	...M.K....	A....I..S.	....A.....
NIG/2/79	V	..V.TA....	NQ.R...V..	..A.......	.....Q...M	...M.K....	A....I..S.	....A.....
NIG/3/80	V	..V.TA....	NQ.R...V..	..A.......	.....R...M	...M.K....	A....I..S.	....A.....
NIG/5/81	V	..V.TD?...	??.R...V..	..A.......	.....R...M	...M.K....	A....I?.S.	.?..A.....
NGR/2/76	V	....	N..P...V..	..A.......	.....V...M	.....K....	A....I..S.	....
NGR/4/76	V	?.........	N..P...V..	..A.......	.....V...M	.....K....	A....V..S.	....
NIG/1/76	V	....	N..P...V..	..A.......	.....V...M	.....K....	A....I..S.	....
NIG/6/76	V	....	N..P...V..	..A.......	.....V...M	.....K....	A....V..S.	....
NIG/8/76	V	....	N..P...V..	..A.......	.....V...M	.....K....	A....V..S.	....
NIG/17/75	V	....	N..P...V..	..A.......	.....V...M	.....K....	A....I..S.	....
NIG/25/75	V	....	N..P...V..	..A.......	.....V...M	.....K....	A....I..S.	....
NIG/14/76	V	....	N..P...V..	..A.......	.....V...M	.....K....	A....I..S.	....
NIG/20/76	V	..?.......	N..P...V..	..A.......	.....V...M	.....K....	A....V..S.	....

	90	100	110	120	130	140	150	160
BOT/1/68	CVGTNAWVGW	TPNGSPVLTE	VGDNQVVFSR	RGTTRFALPY	TAPHRVLATV	YNGDCKYKPT	GTAPRENIRG	DLATLAARIA
KEN/4/98	...E.K....	....A.E.S.	....P....H	N.........	.....C...A	.........N	AE...TH...	...V..E...
KEN/11/91	...V.K..R.	....A.E.S.	....P....H	N.........	.....C...A	.........N	TE...TH...	......E...
KEN/9/91	...V.K..R.	....A.E.S.	....P....H	N.........	.....C...A	.........N	TE...TH...	......E...
SUD/3/76	.....R....	L...A..PQ.	....P....H	N........F	....	I	NEDT.T....	.........VR
SUD/4/76	.....R....	L...A..PQ.	....P....H	N........F	....	I	NEDT.T....	.........VR
SUD/8/74	.....K....	L...A..PQ.	....P....H	N........F	....	I	NEDT.T....	.........R
SUD/9/74	.....K....	L...A..PQ.	....P....H	N........F	....	I	NEDT.T....	.........R
SUD/13/74	.....K....	L...A..PQ.	....P....H	N.A......F	....	I	NEDT.T....	.........R
TAN/2/96	.....K....	....A.E.A.	....P....H	N.........	.....C...A	....S..RSH	NEE..AH...	....
TAN/60/99	.....K....	....A.E.N.	....P....H	NE........	.....C...A	.........N	SE...TH...	.FT...E...
TAN/1/96	.....K....	....A.E.A.	....P....H	N.........	.....C...A	....S..RSH	NEE..AH...	....
TAN/51/99	.....K....	....A.E.N.	....P....H	NE........	.....C...A	.........N	SE...THF..	......E...
TAN/37/99	...V.G....	....A.E.S.	....P....H	N.........	.....C...T	....	.E...TH...	......E...
TAN/3/80	...V.G....	....A.E.S.	....P....H	N.........	.....C...T	....	.E...TH...	......E...
TAN/5/96	.....K....	....A.E.S.	....P.....	N.........	.....C...A	.........N	HET..TH...	......T...
TAN/2/77	...E.K....	....A.E.S.	....P....H	N.........	.....C....	.....R....	TES..TH...	......E...
UGA/3/99	.....K....	....A.Q...	....PSH..H	N.........	.....L....	....	EQP..T....	...V..Q...
UGA/13/74	.....K....	L...A..PS.	....P....H	N.V.......	....	...N.R...N	TE...PT...	....
UGA/7/99	.....K....	....A.Q...	....P.I..H	N.........	.....L....	....	EQP..T....	...V..Q...
UGA/1/97	.....E....	V...T.DVS.	....P.....	D.A......F	......V..S	...N...QDQ	S.T.LTHV..	......E...
TAN/19/96	.....K....	....A.E.N.	....P....H	NE........	.....C...A	.........N	SE...TH...	.F....E...
KNP/96/81	.L.E.K....	....A.E.E.	....P....N	..A......F	.....C...T	.........A	.....D....	...V..Q...
NIG/10/81	.....K....	L...A..PR.	....P....H	N........F	....	....	NEDT.T....	.........R
NIG/2/79	.....K....	L...A..PR.	....P.....	N........F	....	....Y....I	NEDT.T....	.........VR
NIG/3/80	.....K....	L...A..PR.	....P....H	N........F	....	....	NEDT.T....	.........VR
NIG/5/81	.....K....	L...A..PR.	....P....H	N........F	....	....	NEDT.T....	.........VR
NGR/2/76	.....K....	V...A..PK.	....P....H	N.........	.........T	....Y...AQ	SVES..-...	...V....V.
NGR/4/76	.....K....	V...A..PK.	....P....H	N.........	.........T	.........AQ	PVEN..-...	...V..V.V.
NIG/1/76	.....K....	V...A..PK.	....P....H	N.........	.........T	.........AQ	SVES..-...	...V....V.
NIG/6/76	.....K....	V...A..PK.	....P....H	N.........	.........T	.........AQ	PVEN..-...	...V....V.
NIG/8/76	.....K....	V...A..PKG	....P....H	N.........	.........T	.........AR	PVEN..-...	...V....V.
NIG/17/75	.....K....	V...A..PK.	....P....H	N.........	.........T	.........AQ	PVEN..-...	...V....V.
NIG/25/75	.....K....	V...A..PK.	....P....H	N.........	.........T	.........AQ	PVEN..-...	...V....V.
NIG/14/76	.....K....	V...A..PK.	....P....H	N.A.......	.........T	.........AQ	PVEN..-...	...VV...VV
NIG/20/76	.....K....	V...A..PK.	....P.I..H	N.........	.........T	.........AQ	PVEN..-...	...V..V.V.

						VP1 ↓ 2A
	170	180	190	200	210	221
BOT/1/68	SETHIPTTFN	YGMIYTQAEV	DVYLRMKRAE	LYCPRPVLTH	YDHNGRDRYK	TTLVKPAKQ LS
KEN/4/98	....	..R...E.D.	...V......	....	...K......	VA.T..... .C
KEN/11/91	....	..RL..E.D.	...V......	....	...K.N....	VA.T..... .C
KEN/9/91	....	..RL..E.D.	...V......	....	...K.N....	VA.T..... .C
SUD/3/76	EQS.....	..I.L.E...	...V......	.........T	...ASA....	VP..A.E.. MA
SUD/4/76	EQS.....	..I.L.E...	...V......	.........T	...ASA....	VP..A.E.. MA
SUD/8/74	EQS.....	..I.L.E...	...V......	.........T	...ASA....	VS..A.E.. MA
SUD/9/74	EQS.....	..I.L.E...	...V......	.........T	...ASA....	VS..A.E.. MA
SUD/13/74	EQS.....	..I.L.E...	...V......	.........T	...ASA....	VS..A.E.. MA
TAN/2/96	....	..R...E...	...V......	....	...Q.K....	VA.T..... .C
TAN/60/99	....	..R...E...	...V......	....	...Q......	VA.T..... .C
TAN/1/96	....	..R...E...	...V......	....	...Q.K....	VA.T..... .C
TAN/51/99	....	..R...E...	...V......	....	...Q.K....	VA.T..... .C
TAN/37/99	....	..R.......	...V......	....	...Q......	VA.T..... .C
TAN/3/80	....	..R.......	...V......	....	...Q......	VA.T..... .C
TAN/5/96	....	..R...E...	...V......	....	.E.Q.K....	VA.T..... .C
TAN/2/77	....	..R...E...	...V......	V.........	.N.Q.K....	.A.T..... .C
UGA/3/99	.K.....S..	..Q...ES..	...V......	....	...GDK....	IE..... .E
UGA/13/74	EQA.....	..I.L.EG..	...V......	.....F...M	...A.Q..R.	IR..A.... VC
UGA/7/99	.......S..	..Q...ES..	...V......	....	...GDK....	IE..... .C
UGA/1/97	QNA....S..	..Q...E...	...V......	T.....L...	...A.KN...	.K..A.... .C
TAN/19/96	....	..R...E...	...V......	....	...Q.K....	VA.T..... .C
KNP/96/81	G.........	..R...E...	...V......	......L...	.....K....	.AIT..... .G
NIG/10/81	EQS.....	..I.L.E...	...V......	.........T	...ALA....	VS.IA.E.. MA
NIG/2/79	EQS.....	..I.L.E...	...V......	.........T	...ASA....	VS.IA.E.. MA
NIG/3/80	EQS.....	..I.L.E...	...V......	.........T	...ALA....	VS.IA.E.. MA
NIG/5/81	EQS.....	..I.L.E...	...V......	.........T	...ALA....	VS.IA.E.. MA
NGR/2/76	E.........	....L.ES..	...V......	.....FL..T	.....A....	....A.E.. .A
NGR/4/76	E.........	....L.ES..	...V......	.....FL..T	...D.A....	....A.E.. .A
NIG/1/76	E.........	....L.ES..	...V......	.....FL..T	.....A....	....A.E.. .A
NIG/6/76	E.........	....L.ES..	...V......	.....FL..T	.....A....	....A.E.. .A
NIG/8/76	E.........	....L.ES..	...V......	.....FL..T	.....A....	....A.E.. .A
NIG/17/75	E.........	....L.ES..	...V......	.....FL..T	.....A..H.	....A.E.. .A
NIG/25/75	E.........	....L.ES..	...V......	.....FL..T	.....A....	....A.E.. .A
NIG/14/76	E.........	....L.ES..	...V......	.....FL..T	.....A....	....A.E.. .A
NIG/20/76	E.........	....L.ES..	...V......	.....FL..T	...D.A....	....A.E.. .A

Fig. 5.3 Sequence alignment of 221 amino acids of the 1D gene of 36 SAT-1 FMD viruses. The cell attachment site of the viruses (RGD) in the GH-loop is highlighted at positions 149-51. Dots (.) indicate amino acids identical to BOT/1/68. '?' indicates amino acids that could not be determined. '-' indicates viruses that have a codon deletion corresponding to amino acid position 147.

A	B	C
1-10	2	þþ
2-11	2	þþ
3-12	2	þþ
4-13	3	þþþ
5-14	3	þþþ
6-15	4	þþþþ
7-16	5	þþþþþ
8-17	6	þþþþþþ
9-18	6	þþþþþþ
10-19	5	þþþþþ
11-20	4	þþþþ
12-21	5	þþþþþ
13-22	6	þþþþþþ
14-23	5	þþþþþ
15-24	6	þþþþþþ
16-25	6	þþþþþþ
17-26	6	þþþþþþ
18-27	5	þþþþþ
19-28	6	þþþþþþ
20-29	6	þþþþþþ
21-30	6	þþþþþþ
22-31	6	þþþþþþ
23-32	5	þþþþþ
24-33	6	þþþþþþ
25-34	5	þþþþþ
26-35	5	þþþþþ
27-36	4	þþþþ
28-37	4	þþþþ
29-38	3	þþþ
30-39	3	þþþ
31-40	3	þþþ
32-41	2	þþ
33-42	3	þþþ
34-43	2	þþ
35-44	3	þþþ
36-45	2	þþ
37-46	3	þþþ
38-47	4	þþþþ
39-48	4	þþþþ
40-49	5	þþþþþ
41-50	6	þþþþþþ
42-51	7	þþþþþþþ
43-52	6	þþþþþþ
44-53	6	þþþþþþ
45-54	6	þþþþþþ
46-55	6	þþþþþþ
47-56	6	þþþþþþ
48-57	5	þþþþþ
49-58	6	þþþþþþ
50-59	6	þþþþþþ
51-60	5	þþþþþ
52-61	5	þþþþþ
53-62	5	þþþþþ
54-63	5	þþþþþ
55-64	4	þþþþ
56-65	4	þþþþ
57-66	4	þþþþ
58-67	4	þþþþ
59-68	3	þþþ
60-69	3	þþþ
61-70	3	þþþ
62-71	2	þþ
63-72	2	þþ
64-73	2	þþ
65-74	2	þþ
66-75	3	þþþ
67-76	2	þþ
68-77	3	þþþ
69-78	3	þþþ
70-79	2	þþ
71-80	2	þþ
72-81	2	þþ
73-82	3	þþþ
74-83	3	þþþ
75-84	4	þþþþ
76-85	3	þþþ
77-86	4	þþþþ
78-87	3	þþþ
79-88	3	þþþ
80-89	4	þþþþ
81-90	4	þþþþ
82-91	5	þþþþþ
83-92	4	þþþþ
84-93	4	þþþþ
85-94	3	þþþ
86-95	4	þþþþ
87-96	3	þþþ
88-97	4	þþþþ
89-98	5	þþþþþ
90-99	5	þþþþþ
91-100	6	þþþþþþ
92-101	5	þþþþþ
93-102	5	þþþþþ
94-103	5	þþþþþ
95-104	5	þþþþþ
96-105	5	þþþþþ
97-106	6	þþþþþþ
98-107	6	þþþþþþ
99-108	5	þþþþþ
100-109	4	þþþþ
101-110	4	þþþþ
102-111	5	þþþþþ
103-112	6	þþþþþþ
104-113	7	þþþþþþþ
105-114	7	þþþþþþþ
106-115	6	þþþþþþ
107-116	5	þþþþþ
108-117	4	þþþþ
109-118	4	þþþþ
110-119	4	þþþþ
111-120	4	þþþþ
112-121	3	þþþ
113-122	2	þþ
114-123	1	þ
115-124	1	þ
116-125	1	þ
117-126	2	þþ
118-127	3	þþþ
119-128	3	þþþ
120-129	3	þþþ
121-130	3	þþþ
122-131	3	þþþ
123-132	3	þþþ
124-133	3	þþþ
125-134	4	þþþþ
126-135	5	þþþþþ
127-136	5	þþþþþ
128-137	4	þþþþ
129-138	5	þþþþþ

130-139	6	þþþþþþ
131-140	6	þþþþþþ
132-141	**7**	**þþþþþþþ**
133-142	**8**	**þþþþþþþþ**
134-143	**9**	**þþþþþþþþþ**
135-144	**9**	**þþþþþþþþþ**
136-145	**9**	**þþþþþþþþþ**
137-146	**9**	**þþþþþþþþþ**
138-147	**10**	**þþþþþþþþþþ**
139-148	**10**	**þþþþþþþþþþ**
140-149	**9**	**þþþþþþþþþ**
141-150	**8**	**þþþþþþþþ**
142-151	**7**	**þþþþþþþ**
143-152	**7**	**þþþþþþþ**
144-153	**7**	**þþþþþþþ**
145-154	**7**	**þþþþþþþ**
146-155	**7**	**þþþþþþþ**
147-156	6	þþþþþþ
148-157	6	þþþþþþ
149-158	5	þþþþþ
150-159	6	þþþþþþ
151-160	**7**	**þþþþþþþ**
152-161	**8**	**þþþþþþþþ**
153-162	**8**	**þþþþþþþþ**
154-163	**8**	**þþþþþþþþ**
155-164	**7**	**þþþþþþþ**
156-165	6	þþþþþþ
157-166	6	þþþþþþ
158-167	5	þþþþþ
159-168	6	þþþþþþ
160-169	5	þþþþþ
161-170	4	þþþþ
162-171	3	þþþ
163-172	2	þþ
164-173	2	þþ
165-174	3	þþþ
166-175	4	þþþþ
167-176	4	þþþþ
168-177	5	þþþþþ
169-178	5	þþþþþ
170-179	6	þþþþþþ
171-180	6	þþþþþþ
172-181	6	þþþþþþ
82	6	þþþþþþ
174-183	5	þþþþþ
175-184	5	þþþþþ
176-185	4	þþþþ
177-186	4	þþþþ
178-187	3	þþþ
179-188	2	þþ
180-189	1	þ
181-190	1	þ
182-191	2	þþ
183-192	2	þþ
184-193	2	þþ
185-194	1	þ
186-195	1	þ
187-196	2	þþ
188-197	3	þþþ
189-198	3	þþþ
190-199	3	þþþ
191-200	4	þþþþ
192-201	3	þþþ
193-202	4	þþþþ
194-203	4	þþþþ
195-204	5	þþþþþ
196-205	6	þþþþþþ
197-206	6	þþþþþþ
198-207	6	þþþþþþ
199-208	6	þþþþþþ
200-209	**7**	**þþþþþþþ**
201-210	**7**	**þþþþþþþ**
202-211	**8**	**þþþþþþþþ**
203-212	**8**	**þþþþþþþþ**
204-213	**9**	**þþþþþþþþþ**
205-214	**9**	**þþþþþþþþþ**
206-215	**9**	**þþþþþþþþþ**
207-216	**8**	**þþþþþþþþ**
208-217	**8**	**þþþþþþþþ**
209-218	**8**	**þþþþþþþþ**
210-219	**7**	**þþþþþþþ**
211-220	**7**	**þþþþþþþ**
212-221	7	þþþþþþþ

Fig. 5.4 Variability plot of complete sequences of the 1D gene of SAT-1 viruses from Africa. Under **A** amino acids in a window of 10 were indicated while **B** and **C** indicated the number of variable sites and variability per amino acid, respectively.

Key: Between 4-7 = variable region. 7-10 = Hyper variable region (≥7)

5.4 Discussion

Genetic characterization of SAT type viruses is well documented for the southern African region with the emphasis on buffalo isolates (Vosloo *et al.*, 1992; Esterhysen, 1994, Vosloo *et al.*, 1995; Van Rensburg and Nei, 1999; Bastos, 2001; Bastos *et al.*, 2001; Van Rensburg *et al.*, 2002; Bastos *et al.*, 2003) as well as for west Africa (Sangare, 2002; Sangare *et al.*, 2003). A total of 23 (23/53) virus isolates from East Africa as well as 30 geographically representative isolates from previous studies were included in this study to determine the genetic relationships of African SAT-1 isolates. In the present analysis of nucleotide sequences of the 1D gene, divergence between lineages and genotypes varied from 23.5 to 35% and 16.9 to 23.5%, respectively. Thus, the SAT-1 virus types are genetically heterogeneous and distantly related at both lineage and genotype level. This shows an independent evolution of lineages and genotypes and a high rate of mutation of FMD viruses (Sobrino *et al.*, 1983) and a higher level of intratypic variation for SAT type viruses (Vosloo *et al.*, 1995; Bastos *et al.*, 2001; Bastos *et al.*, 2003).

A divergence of > 20% in nucleotide sequences distinguishes topotypes for the SAT type FMD viruses (Bastos *et al.*, 2003; Knowles and Samuel, 2003). The present divergence in nucleotide sequence was > 23% for lineages and > 16% for genotypes. Since viruses within genotypes were distributed into distinct geographical localities, topotypes could be assigned. The three Ugandan lineages (IV, V and VII) represented isolates from 1974, 1997 and 1999 and are distinct for Uganda (Fig. 5.2).

The historical evolutionary relationships of SAT-1 type viruses in Africa can clearly be seen in Fig. 5.1 and Fig. 5.2. These phylogenetic trees revealed three major genetically related combinations of lineages. The first major cluster composed of lineages I – III which included virus isolates from East and southern Africa while the second major cluster (lineages IV and V) consisted of isolates from Uganda and is distinct for East Africa. The last major cluster (lineage VI - VIII) constituted virus isolates from West Africa (Nigeria and Niger) and East Africa (Uganda and Sudan). This showed that East African isolates are historically related with both

West and southern African isolates. The phylogenetic link between the West and the East African isolates was further supported by the observation that distinct amino acids located within the GH-loop (NEDT, R, RQS) and C-terminus (M) which were not found in the other African isolates (Fig. 5.3).

Despite this progress there are still some very basic questions that need to be addressed. It is interesting to note that Uganda has 3 distinct lineages within its border. There is also very little movement control within Uganda and between bordering countries. Why should one country then have such different viruses. As mentioned above the question of the heterogeneity of the Ugandan isolates which clustered into three distinct lineages needs to be addressed. Analysis of the complete 1D gene sequence indicated that these lineages differed by more than 30% to each other and > 26% to other lineages found in Africa (Table 5.2). The high divergence between lineages (> 23%) can be explained by multiple cycles of epizootics and independent evolution of variants. The overlapping distribution of lineages of different serotypes can also lead to intra- and intertypic recombination of viruses in endemic countries like Uganda. Study based on the complete genome sequences of viruses may be very useful in determining the prevalence and the frequency of genetic recombination in the field (Knowles and Samuel, 2003).

Different isolates of SAT-1 viruses from Tanzania included in this study clustered into genotype A. The 10 Tanzanian isolates obtained from 1977 to 1999 (Fig. 5.1 and Fig. 5.2) constituted of 5 groups of unrelated viruses (base on < 90% sequence similarities). Within each of the first (TAN/2/99, 1/99, 60/99, 19/96, 51/99) and second groups (TAN/1/96 and TAN2/96) a high percentage of sequence similarity (> 97%) were found which on the other hand indicated that closely related viruses in each group were responsible for the outbreaks. However, the third (TAN/5/96), fourth (TAN/3799, TAN/3/80) and fifth groups (TAN/2/77) were found to differ from each other and from the first 2 groups by > 10%. Isolates of the same year *ie*., from 1996 were distributed into more than 3 groups which showed a minimum of 3 variant of viruses that were responsible for outbreaks in Tanzania. Phylogenetic analysis also showed that the Kenyan (1991 and 1998), Malawian (1985), Zambian (1993) and the Tanzanian isolates

clustered as genotype A whereas one Tanzanian isolate from 1977 clustered with a Zambian isolate of 1993 within genotype B (Fig. 5.1).

In the phylogenetic analysis by Bastos and co-workers (2001) two 1999 isolates from Tanzania clustered with Zambian isolates while one Ugandan isolate (UGA/1/97) differed from southern African isolates and appeared as a distinct lineage. The other isolates from southern Africa included in the same study showed a geographical clustering and 3 topotypes were assigned. Sangare *et al.* (2003) have also identified two independently evolving viral lineages of this serotype in West Africa. The present study showed that the SAT-1 type FMD virus populations circulating in East Africa are genetically heterogeneous and phylogenetically clustered into 3 distinct Ugandan lineages and 2 shared lineages with West and southern Africa. These results also complemented the previous studies by Bastos *et al.* (2001), Sangare *et al.* (2003) and Knowles and Samuel, (2003) and assisted in complying a comprehensive SAT-1 sequence database for East African countries.

CHAPTER VI

SEROLOGICAL SURVEY TO DETERMINE THE ROLE OF SMALL RUMINANTS AND WILDLIFE IN THE EPIDEMIOLOGY OF FOOT AND MOUTH DISEASE IN ETHIOPIA AND EVALUATION OF SEROLOGICAL TESTS

6.1 INTRODUCTION

Ethiopia is characterized by its central highlands, rising up to 4600 metres above sea level (masl) and peripheral lowlands descending down to 100 metres below sea level (mbsl). Three major agro-climatic zones occur in Ethiopia *viz.*, dry land (north-east and the rest of low-lying areas in the peripheries), tropical rainy climate (west mainly, also eastern regions) and moderate climate (central plateau) (Anonymous, 1998b).

Animal diseases are widespread in all agro-ecological zones of Ethiopia. At present the three major diseases of socio-economic importance are FMD, contagious bovine pleuropneumonia (CBPP) and trypanosomosis. However, diseases such as contagious caprine pleuropneumonia (CCPP), peste des petit ruminants, lumpy skin disease, African horse sickness, Newcastle disease, cowdriosis, fasciolasis, haemonchosis, etc. are still rampant (Anonymous 1998b; Anonymous, 2000). The conventional methods of controlling these diseases either through immunization or therapy are in most instances not completely effective. Almost all vaccines for the major animal diseases are produced in the country while the vaccines for CCPP, rabies and FMD are imported on occasion. In Ethiopia, rinderpest is the only animal disease investigated by sero-surveillance at national level and the country has been declared free of the disease (Abraham *et al.*, 1998).

The current situation of FMD in Ethiopia is alarming. There is no national control strategy, no legislation exists for making FMD notifiable to the veterinary authorities nor for animal movement restrictions to be imposed. Therefore, livestock are at risk from endemic strains as well as from antigenic variants prevailing in neighbouring countries. The official data may not exhibit the reality of the disease, due to the

insidious nature of the disease, the unreported cases by farmers, as well as the few samples submitted to Pirbright for identification. However, records from the National Animal Health Research Center and the National Veterinary Institute of Ethiopia indicated that serotypes O, A, C and SAT-2 were responsible for FMD outbreaks during 1974 – 2003. However, a national survey on the incidence and distribution of the disease has not yet been conducted. The role of small and wild ruminants in the epidemiology of FMD is therefore not known.

Good quality data on the impact of FMD on animal production in Ethiopia are lacking. The prevalence of the disease, the existing circulating viral isolates, variation in the severity of outbreaks and the role of small ruminants and cloven-hoofed wild animals in the country need to be investigated. This chapter describes an initial attempt of using sero-surveillance of FMD through the detection of antibodies in serum samples collected from cattle, small ruminants and cloven-hoofed wild animals to address this need.

6.2 Materials and Methods

6.2.1 Study area

Ethiopia is divided into 8 administrative regions (Fig. 6.1). Within each region there are zones which are further divided into districts. There are 3 major agro-ecological zones in Ethiopia *viz*., the dry climate, the tropical rainy climate and the temperate climate. Classification of the country into agro-ecological zones or administrative regions is of no value to study FMD. However, classification according to farming systems is more beneficial as the type of farming system may influence the transmission and spread of the disease. The two major farming systems in Ethiopia are indicated in Fig. 6.2:

1. The communal (pastoral) farming system is occupied mainly by nomads and it represents the dry climate and low land (green-shaded).
2. The semi-intensive and intensive farming system covers the tropical rainy climate and temperate climate which are mostly high land and mid altitude areas (yellow-shaded).

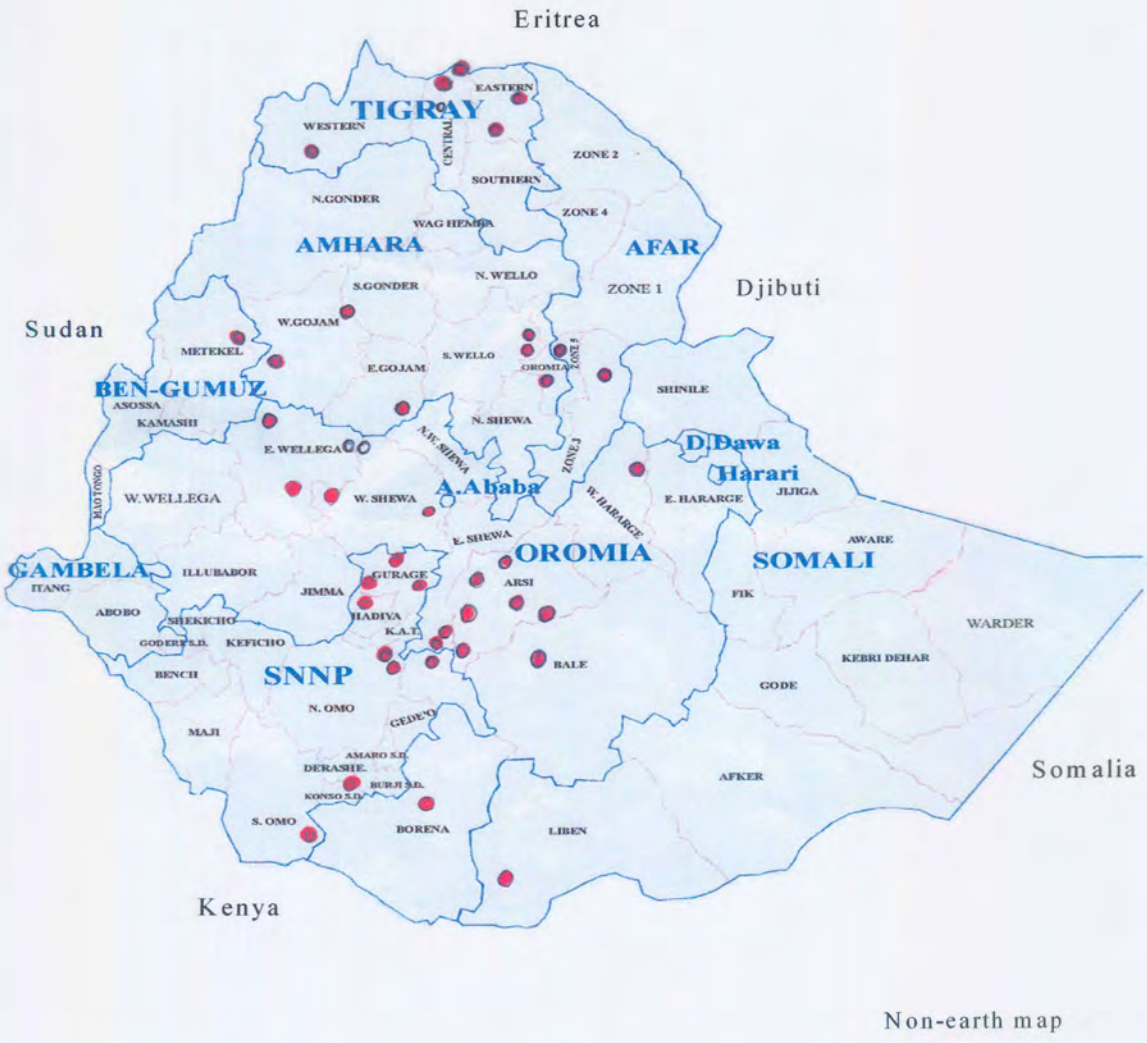


Fig. 6.1 Administrative map of the Federal Republic of Ethiopia, depicting regions (blue print) and zones (black). ● indicates distribution of reported foot-and-mouth disease outbreaks during serum sampling in 2001 (January to March)

6.2.2 Serum samples

Serum samples from cattle collected over a 5 year period for sero-surveillance of rinderpest and sera from sheep, goats and wild ruminants were collected. Field sera were sampled from sheep and goats above one year of age and cattle ranging between 1 and 3 years of age. All serum samples collected represent sera from non-vaccinated animals. The species of wildlife sampled are indicated in Table 6.3a.

Fig. 6.2 Map of Ethiopia showing the different farming systems (source: Ministry of Agriculture of Ethiopia, 1999).

Serum samples collected from each farming system (Fig. 6.2) and stored in the serum bank were selected by applying simple random or systematic random sampling. The same protocol was designed for the control of rinderpest to detect a sero-prevalence of 5%, with a precision level of 5% of the true level 95% of the time. This allowed the detection of one antibody positive case to rinderpest in the sampling unit (herd).

Serum sample size determination: The districts within each farming system as well as the size of the livestock population were considered in the preparation of

the sample framework so that all the study populations had an equal chance of being selected.

It was assumed that 50% of the cattle and small ruminants (sheep and goats) had antibodies to FMDV. This estimate was assumed to be within 5% (allowable error) for cattle and small ruminants of the true level, 95% of the time. It was also estimated that 20% of wildlife (buffalo, warthog, hart beest, lesser kudu, eland, menlik's bush buck and mountain nyala) sampled would be sero-positive with a desired absolute precision of 10% of the true level, 95% of the time.

$n = 1.96^2 P\hat{O} / L^2$ (where $\hat{O} = 1 - P$)

n = required sample size

P = estimate of prevalence (%)

L = desired absolute precision (%)

Cattle (P = 50%, L = 5%)

$n = 1.96^2 \times 0.5(1-0.5) / (0.05)^2 = 384.16$ ... (400)

Small ruminants (P = 50%, L = 5%)

$n = 1.96^2 \times 0.5(1-0.5) / (0.05)^2 = 384.16$ ... (400)

Wild animals (P=20%, L = 10%)

$n = 1.96^2 \times 0.2(1-0.2) / (0.1)^2 = 61.46$ ... (62)

To compensate for the error due to multistage sampling the number of samples was doubled. Therefore, the total number of serum samples for both cattle and small ruminants (sheep and goats) were 800 each (Table 6.1). Since the population of wild animals is very small compared to cattle and small ruminants, the sample size remained the same.

Criteria of sampling percentage: It has been established that 40% of the animals are farmed in the communal farming system, while 60% of them are farmed in the intensive and semi-intensive farming system (Anonymous 1998b). Thus, the number of serum samples collected is a reflection of the representative percentage livestock population per farming system (Table 6.1) and the sample size within each category is summarized in Table 6.2. Sampling units within administrative regions were randomly selected representing the 2 different farming

systems. The number of serum samples tested per administrative region is a reflection of the percentages of livestock in relation to the total livestock population in the 2 farming systems.

Table 6.1 Total number of serum samples collected per farming system.

Farming system	**Serum samples**		
	Cattle	Sheep & goats	Wild ruminants
Communal farming	320 (40%)	320 (40%)	77
Intensive & semi-intensive	480 (60%)	480 (60%)	
Total	800	800	77

Table 6.2 Number of serum samples collected from randomly selected districts within the administrative regions, representing the 40 and 60% animal distribution in the (A) communal and (B) intensive/semi-intensive farming systems, respectively.

Adminstrative region	**Cattle**		**Small ruminants**		**Cattle**	**Small ruminant**
	A	B	A	B	Total	Total
Tigray	7	54	50	46	61	96
Afar	101		80		101	80
Amhara		124		139	124	139
Oromia	42	214	54	219	256	273
Somali	100		80		100	80
Benshangule	30		24		30	24
S.N.N.P.R.	20	88	14	76	108	90
Gambela	20		18		20	18
Total	320	480	320	480	800	800

6.2.3 Antibody detection tests

6.2.3.1 Liquid phase blocking ELISA

An adaptation of the method described by Hamblin *et al.* (1986a & 1986b) and applied by Esterhuysen *et al.* (1994) was used.

Serum samples: - Serum samples were stored at -20°C. Once thawed, samples were kept at 4°C until used. Phosphate buffered saline (PBS) containing 0.05% casein (PBS-C) was used as diluent for sera and conjugate while PBS containing Tween 20 (PBS-T) was used as washing buffer to remove unbound reagents. Sera were initially diluted (1:10) in PBS-C and two fold dilutions of these sera (1:20 – 1:80) in duplicates were tested for serotyping against 6 serotypes of FMD virus where the antigen (inactivated virus from cell culture supernatant) and antisera used in the liquid phase blocking ELISA (Lpb ELISA) was prepared from the following isolates (Esterhuysen, personal communication).

SAT-1: SAR/9/81 and BOT/1/68	A: A_5 Allier
SAT-2: ZIM/7/83 and KNP/19/89/2	O: SAR/11/00
SAT-3: KNP/10/90/3	C: C_1 Noville

Antibody blocking titres were expressed at the 50% level of inhibition compared with the virus control. Any sera dilution where 50% of the maximum optical density (MOD) reading was inhibited were considered reactive (positive) where the MOD for each plate was determined by subtracting the OD value of the negative antigen from the OD value of the positive antigen. Sera with titres $\geq$ 1/40 were considered positive.

6.2.3.2 The UBI FMDV non-structural enzyme-linked immunosorbent assay (3ABC ELISA)

The United Biomedical Inc. FMD virus enzyme-linked immunosorbent assay (UBI FMDV NS EIA) is a useful test for identifying infected animals (Mackay, 1998 and references therein). All the reagents, buffers, microplates, control reactive and non-reactive sera were supplied by the manufacturer (UBI, USA). They were stored at +4°C and always pre-warmed to room temperature before used. Briefly, the test sera as well as a set of negative and positive references were added to ELISA plates pre-coated with 3ABC antigen and incubated for 60 min at 37°C. After incubation, the plates were washed (at least 300 µl/well/wash) with diluted wash buffer (PBS) using an automatic microplate washer. Excess liquid was removed from the reaction microplate by inverting and tapping on absorbent paper until no further moisture appeared on the paper. An enzyme conjugated to anti-species antibody was added to the plate and incubated for another 30 min. After incubation the plates were washed as described above. One hundred µl of TMB substrate solution was added to each well. After incubation of the microplate in

the dark for 15 min at 37°C, 100 µl of stop solution (1M H_2SO_4) was added to each well. The absorbance of each well was read at 450 nm. The positive and negative results of test sera for each plate were established using the formula provided by the manufacturer where the results were expressed as an index derived by dividing the absorbance value of the serum tested by that of the cut-off control.

6.2.4 Data analysis

The sample size was determined in accordance with the estimation of the prevalence of FMD in Ethiopia (Section 6.2.2). All data collected were edited and evaluated from the sample sheet. Descriptive statistical methods were applied to estimate the percentage of agreement between the two different ELISAs in detecting antibody to FMD virus. The data comparing the Lpb ELISA and the 3ABC ELISA were assessed by cross tabulating and the percentage of agreement was estimated. The results were analysed in three groups representing serum samples from three species of animals (wildlife, small ruminants and cattle).

6.3 Results

The Lpb ELISA and 3ABC ELISA were used to test sera for the presence of antibodies mounted against structural and non-structural (3ABC) proteins of FMDV respectively. Heterogeneity of FMDV due to changes in the structural proteins of the virus may have an effect on the sensitivity of the Lpb ELISA and the antigen used in this test may not be recognized by some of the antibodies in the test sera. Therefore, the 3ABC ELISA was used since it employs synthetic peptides to the more conserved non-structural proteins compared with the solid-phase immunosorbent assay for detection of antibodies to infectious FMDV and may be more sensitive to score positive sera (Mackay, 1998).

In this study the origin of the reactive and non-reactive sera was used to evaluate the potential role of the farming systems on the incidence of FMD as well as the role of wildlife and small ruminants in the epidemiology FMD. The serotype identification by Lpb ELISA and the reactive and non-reactive sera detected by the 3ABC ELISA (per row) in each table represents the same animals i.e. if the serum scored positive in Lpb ELISA and positive in 3ABC ELISA in the same row, it

represents the same animal. All reactive (positive) sera are denoted by R (+) and all non-reactive sera by NR (-).

6.3.1 Analysis of sera from wildlife

Cross tabulation representing the number of positive and negative serum samples from wildlife are presented in Table 6.3a. The different serotypes identified are presented in Table 6.3b. Sera reactive to both tests were detected in buffalo, eland and mountain nyala while sera from other wild species included in this study were found to be non-reactive (Table 6.3a). One serum from eland and mountain nyala each tested with the Lpb ELISA, was positive for serotype C of FMD (Table 6.3b) but had low titres (1.7, 1/52) and was probably due to false positive reactions. These reactions were not confirmed on serum neutralization test and tested negative on the 3ABC ELISA (Table 6.3a). Serotype A, SAT-1 and SAT-2 were identified in 21 (n = 25) sera from buffaloes where only 16/21 (76.19%) of the same sera were found to be positive with the 3ABC ELISA (Table 6.3b). There were therefore 7 sera positive with the Lpb ELISA that didn't react with 3ABC ELISA. In contrast, 2 sera were detected as reactors in the 3ABC ELISA and not in the Lpb ELISA. Five doubtful reactive sera to SAT-3 by the Lpb ELISA were considered as negative as low values for the same sera were detected by the 3ABC ELISA (Appendix II). A total of 52/77 sera were detected as non-reactors in both tests (Table 6.6).

Table 6.3a Summary of reactivity of serum samples tested by the Lpb ELISA and 3ABC ELISA. The numbers in each row represent the same serum samples.

Origin of serum	Lpb ELISA		3ABC ELISA	
	R (+)	NR (-)	R	N R
Buffalo	21		14	7
		4	2	2
Eland (Tautotragus oryx)	1	4		5
Mountain Nyala (Tragelaphus buxtfoni)	1	6		7
Tiang (Taurotragus oryx)		11		11
Lelwel H. beest (Alcelaphus buselaphus lelwel)		5		5
Swayne's H. beest (A. budelaphus swaynei)		11		11
White earned kob (Kobus kob cencotis)		4		4
Lesser kudu (Tragelaphus imberbis)		3		3
Menlik's bush buck (T. scriptus meniliki)		2		2
Warthog (Phacocherus aethiopicus)		4		4
Total	23	54	16	61

R(+) = Reactive serum NR(–) = Non-reactive serum

Table 6.3b: Foot-and-mouth disease virus serotypes identified from reactive sera of wildlife.

Origin of serum	Serotype identified				3ABC ELISA
	SAT-1	SAT-2	A	C	
Buffalo (Omo National Park)	+13				+8, –5
	+2				+1, –1
	+3				+3
		+1			+1
		+2			+1, –1
	– 4				(+2, -2)
Eland (Omo National Park)				+1	–1
Mountain Nyala (Bale Mountain Park)				+1	–1

6.3.2 Analysis of sera from small ruminants

Eight hundred serum samples from sheep and goats (400 + 400) were tested using the Lpb ELISA and the 3ABC ELISA (Table 6.4). Antibodies to serotypes A (25), O (43) and to both A & O (19) were identified. There were no serum samples reactive to serotype C and the 3 SAT types of FMD virus. Both tests detected the same 692 sera as non-reactive and the 87 sera mentioned above as reactive. An additional 21 sera were positive on the 3ABC ELISA while not reacting on the Lpb ELISA (692 + 21 = 713). This indicated that 14.9% (21/108) of the sera were not recognized as reactors by the Lpb ELISA. Of interest is that all the reactive sera to 3ABC ELISA were collected from the areas where there were FMD outbreaks (Fig. 6.2) due to serotypes A and O. All the serum samples taken from the low-land (Somalia, Gambela and Benshanule) were found to be non-reactive in both assays (Table 6.4).

Table 6.4 Summary of results of Lpb ELISA and 3ABC ELISA using sera from small ruminants.

Regions	Lpb. ELISA			3ABC ELISA		Total
	RS and serotype identified		NR			
	A	O		R	NR	
Tigray		4	92	4	92	96
Afar	5 (A+O)	11 (O)	64	20	60	80
Amhara	4 (A+O) 2 (A)	20 (O)	113	29	110	139
Oromia	10 (A+O) 12 (A)	8 (O)	243	33	240	273
Somali			80		80	80
Benshangule			24	4	20	24
S.N.N.P.R.	11 (A)		79	16	74	90
Gambela			18	2	16	18
Total	87		713	108	692	800

6.3.3 Analysis of sera from cattle

The number of reactive and non-reactive sera as well as the serotypes identified in sera from cattle in each administrative region are presented in Table 6.5. Serotypes O, A, SAT-1 and SAT-2 were identified in bovine serum samples but there were no sera reactive to serotype C and SAT-3. Serotype O (150/210) was most prevalent and had a wide distribution throughout Ethiopia while serotype A was limited to 3 administrative regions with common boundaries (Table 6.5). The positive sera due to serotypes A and O were detected in both farming systems in the administrative regions of Afar, Amhara and Oromia where more than 80% of the livestock population of Ethiopia occur. Serotype O was also detected from serum collected from the low-land area of Tigray. The distribution of serotypes A and O was not confined to a particular farming system as it covered the low-land area in the south (Oromia), central high lands and low land areas of the North East and Western part of Ethiopia. It seems that the size of susceptible cattle population plays a role in the maintenance of FMD rather than the farming system.

Table 6.5 Summary of test results of Lpb ELISA and 3ABC ELISA using sera from cattle.

Regions	Lpb ELISA					3ABC-ELISA		Total
	RS & serotype of FMDV identified				NR	R	NR	
	A	O	SAT-1	SAT-2				
Tigray		12 (O)			49	14	47	61
Afar	12(A+O) 11(A)	20 (O)			58	40	61	101
Amhara	7 (A)	23 (O)			94	34	90	124
Oromia	12(A+O) 8 (A)	39 (O)	7 (SAT-1&2) 11 (SAT-1)	16 (SAT-2)	163	95	161	256
Somali					100		100	100
Benshangule					30		30	30
S.N.N.P.R.		32 (O)			76	31	77	108
Gambela					20		20	20
Total	210 (26.5%)				590	214	586	800

The distribution of all reactive sera to SAT types (34) was limited to the southern part of the country (Borena and Southern Shoe zones). Antibodies to serotype SAT-1 and SAT-2 (24) were detected from cattle in the low land area of Borena zone adjacent to the Kenyan border and Omo National Park which is a rich habitat for wild ruminants. Nearly equal numbers of non-reactive sera (307) (52.1%) were collected from the low-land areas in the region of Tigray, Oromia, Somali, Benshangule and Gambela while 283 (47.9%) non reactive sera were from intensive and semi-intensive farming systems (Fig. 6.2).

Summary of cross tabulation comparisons of test results of serum samples from the three groups of animals detected by blocking ELISA and 3ABC-ELISA is presented in Table 6.6. The results indicated that both assays identified the same 306/1677 and 1327/1677 sera as reactive and non-reactive, respectively. Disagreement between the two tests was found on test results of 44/1677 sera. The 3ABC ELISA identified 13/44 (29.5%) as reactive (negative on Lpb ELISA) and the remaining 31/44 (70.4%) as non-reactive (positive on Lpb ELISA).

Table 6.6 Cross tabulation comparisons of reactive and non-reactive sera from bovine, small ruminants and buffaloes detected by blocking ELISA and 3ABC ELISA.

Wildlife		3ABC ELISA		Total
		R	NR	
Lpb ELISA	R	14	2	16
	NR	9	52	61
Total		23	54	77
Small ruminants		3ABC ELISA		Total
		R	NR	
Lpb ELISA	R	87	21	108
	NR	0	692	692
Total		87	713	800
Cattle		3ABC ELISA		Total
		R	NR	
Lpb ELISA	R	205	8	213
	NR	4	583	587
Total		209	591	800

6.4 Discussion

In a country such as Ethiopia where FMD is endemic, and where large numbers of susceptible domestic and wild ruminants exist with limited vaccination on some dairy farms, serological surveys are a pre-requisite to understand the epidemiology of FMD. To delineate the epidemiological profiles of the endemic occurrence of FMD in Ethiopia, 1677 sera from different species were investigated using serological tests. Of the animal species included in this study, cattle, sheep, goats and buffalo are more susceptible to FMD (FAO, 1984). In addition the few species of cloven-hoofed wildlife from Ethiopia investigated in this study may become infected with FMD but their role in the epidemiology of FMD is unclear. In Ethiopia the highest sero-prevalence was detected in buffalo 84% (21/25) where antibodies to serotypes A, SAT-1 and SAT-2 were identified for the first time in this species. Antibodies to the same serotypes of FMD viruses were also identified in cattle serum collected in the vicinity of the buffalo populations (Omo National Park). Both tests confirmed the presence of antibodies to FMDV in clinically normal buffalos, indicating a possible carrier state as has been recognized previously (Hedger, 1968,1970; Burrows *et al.,* 1971; Rossi *et al.,* 1988). These animals could represent a potential risk of FMD maintenance and a potential

source of infection for other susceptible cloven-hoofed animals in Ethiopia. This is likely to be the same scenario in the neighbouring country Kenya, as the presence of antibodies against 5 serotypes of FMD in buffalo was previously reported in Kenya by Anderson (1981, 1985).

Serological results indicated that the two ELISAs were able to detected 306 (18%) and 1327 (79%) sera as seropositive and seronegative, respectively. However, thirty one sera were reactive with the Lpb ELISA while they were non-reactive in the 3ABC ELISA (Table 6.6). This may be explained by differences in the life span of antibodies to structural and non-structural (NS) proteins (3ABC) in infected animals or false positive reaction on the Lpb ELISA, but also false negative reaction on the 3ABC ELISA. Antibodies to the non-structural proteins can be detected in infected cattle and pigs for up to one year after infection (reviewed by Mackay, 1998) while antibodies to the structural proteins can last 1-2 years (McCullough *et al.*, 1992) and even longer. On the other hand another 13 sera that were positive with the 3ABC ELISA appeared as negative in the Lpb ELISA which could be possible due to heterogeneity of the viruses included in the latter assay and prevalent viruses in Ethiopia. For example, the ZIM/7/83 (SAT-2) that was used for Lpb ELISA reagent preparation and the ETH/2/91 Ethiopian field isolate clustered into 2 different lineages (divergence 27.6%) (Fig. 4.1). Thus the Lpb ELISA used in this study may lack sensitivity to some genetic variants of FMDV (previous chapters) as the immuno-reagents for this test were developed against southern African isolates (Section 6.2.3.1). The results indicate that the Lpb ELISA should either be customized for each region in Africa to ensure sensitivity or that viruses from different regions should be included as a mixture in the assay. It would seem that ELISA kits will provide better results if designed for specific geographic locations, incorporating immuno-reagents that can recognize homologous and heterologous population of circulating variants.

Sample size was determined on the basis of the assumption that 50% of cattle and small ruminants and 20% of wild ruminants had antibodies to FMD. In this study from the results indicated that 26.25% (210/800) cattle, 10.8% (87/800) small ruminants and 29.8% (23/77) of wild ruminants have been exposed. This showed that more serum samples must be tested to estimate the actual sero-prevalence of the disease. In conclusion therefore, the detection of antibody to the NS protein

3ABC in conjunction with specific antibody detection by the Lpb ELISA provided conclusive evidence of previous infection with FMD. In addition, the distribution of reactive sera (Table 6.4, 6.5) and non-reactive sera did not show the importance of farming systems in the occurrence of the disease. Although the number of sera analysed in this study is small, the serology results indicated that buffaloes are the only wildlife species that play an important role in the epidemiology of FMD in Ethiopia. Although, small ruminants become infected, the low percentage of positive animals indicated that they are probably not important in the epidemiology of the disease in Ethiopia. It has also been reported that clinically healthy sheep was shown to be seropositive to infection with FMDV in Kenya and Morocco (Ndarathi, 1991; Blanco *et al.*, 2002). Therefore more epidemiological studies including serosurveillance and virus isolation is required to confirm the role of small ruminants and wildlife in Ethiopia.

CHAPTER VII

GENERAL DISCUSSION AND RECOMMENDATIONS

Complete and partial nucleotide sequence data of the 1D gene were used to study the genetic variation and relationships of isolates within serotype O, SAT-1 and SAT-2 FMD from East Africa. These three serotypes as well as serotype A have been reported to be most prevalent in sub-Saharan African countries (Vosloo *et al.*, in 2002). Previous studies on the molecular epidemiology of FMD virus focused mainly on southern and West African isolates (section 5.4, the references therein). Knowles *et al.* (1998) studied serotype A in some detail and reported the presence of six regionally and genetically distinct virus lineages. Serotype C is rare and the last outbreak was recorded in Kenya in 2000 (Kitching, 2002a). Serotype SAT-3 rarely occurred in East Africa and has been reported only in Uganda (Vosloo *et al.*, 2002). Therefore, serotypes A, C and SAT-3 were not included in this study.

A summary of lineages and genotypes (topotypes) identified in this and other studies are presented in Table 7.1 and Fig 7.1A-C. In this study distinct geographical localization of virus isolates were observed at genotype level. The distribution of isolates to defined localities can also be seen at lineage level (Table 7.1). In some cases 2 to 3 genotypes were found to be overlapping (*e.g.* Ugandan isolates).

The interpretation of the genetic relationships of FMD virus isolates in terms of lineage and genotype was based on uncorrected sequence divergence values between clusters in the phylogenetic tree with significant boot-strap support and agreements between different methods of phylogenetic reconstruction applied. The optimum cut-off values for clearer definition of genotypes and lineages are summarized in Table 7.2. Differences of approximately > 15% in nucleotide sequence to distinguish genotype (Rico-Hesse *et al.*, 1987) and cut-off values in excess of 15 - 20% nucleotide differences (VP1 gene) to distinguish between subgroups/topotypes have previously been used (Vosloo *et al.*, 1992, Bastos *et al.*, 2001, 2003: Sangare *et al.*, 2001, 2003; Samuel and Knowles 2001a,b; Knowles and Samuel 2003).

Table 7.1 Summary of phylogenetic analysis of FMD virus isolates from East Africa (EA) and elsewhere. References are indicated for previously established topotypes.

Lineages	Topotypes	Country
O		
I	A (North-Eastern EA)	Ethiopia, Eritrea, Kenya, Somalia, Sudan
	B (Sudan-West Africa)	Sudan, Algeria, Ghana, Burkina Faso
	C (Central EA)	Kenya, Uganda
	D (Sudanese)	Sudan
	E (Uganda I)	Uganda
	F (Uganda II)	Uganda
	G (South-Eastern EA)	Tanzania, Uganda
	H (The Middle East-South Asia)	India, Turkey, Saudi Arabia, Bangladesh, Iraq, South Africa (Sangare *et al.*, 2001; Samuel & Knowles, 2001b)
II	I (Cathay)	Taiwan, Russia (Samuel & Knowles, 2001b)
III	J (Angolan)	Angola (Sangare *et al.*, 2001)
	K (Euro-S. America)	United Kingdom, Argentina (Samuel & Knowles, 2001b)
SAT-1		
I	A	Tanzania, Kenya, Zambia, Malawi
	B	Zimbabwe (Bastos *et al.*, 2001)
II	C	South Africa, Mozambique (Bastos *et al.*, 2001)
	D	Zimbabwe (Bastos *et al.*, 2001)
III	E	Zimbabwe (Bastos *et al.*, 2001)
	F	Zambia, Botswana, Namibia (Bastos *et al.*, 2001)
IV	G	Uganda
V	H	Uganda
VI	I	Nigeria, Niger (Sangare *et al.*, 2003)
VII	J	Uganda
VIII	K	Nigeria, Sudan (Sangare *et al.*, 2003 and this study)
SAT-2		
I (East Africa)	A	Kenya, Uganda
	B	Uganda
	C	Uganda, Zaire (DRC)
	D	Sudan
	E	Ethiopia
	F	Eritrea, Saudi Arabia (Bastos *et al.*, 2003 and this study)
	G	Rwanda (Bastos *et al.*, 2003)
II (Angola)	H	Angola (Bastos *et al.*, 2003)
III (West Africa)	I	Ghana, Niger, Senegal (Sangare *et al.*, 2003)
IV (WA)	J	Gambia, Senegal (Sangare *et al.*, 2003)
V (East-Southern Africa)	K	Zimbabwe and Botswana (Bastos *et al.*, 2003)
	L	Zimbabwe (Bastos *et al.*, 2003)
	M	Botswana and Zambia (Bastos *et al.*, 2003)
	N	South Africa (Bastos *et al.*, 2003)
	O	Kenya, Malawi, Tanzania, Ethiopia, Burundi

In this study, the cut-off values determined for genotypes varied from 13 to 20% and lineages from 20 to 26% (Table 7.2). The differences in cut-off values for each serotype are most probably associated with the differences in the level of intratypic

variation between serotypes. Higher cut-off values were observed for SAT type virus isolates than for serotype O. Similarly, higher levels of intratypic variations were observed for SAT type virus isolates than for serotypes O, A, C (Vosloo *et al.*, 1995; Bastos, 1998, Bastos *et al.*, 2001, 2003; Van Rensburg and Nel 1999). The nucleotide sequence divergence between genotypes within each serotype was found to be higher in the SAT-1 and SAT-2 virus isolates compared to O viruses (Table 7.2). The variation level between some lineages within SAT-1 (30-35%) is almost borderline for variation between serotypes, as was indicated between serotypes O and C (Domingo *et al.*, 1990).

Table 7.2 Summary of nucleotide sequence differences used as cut-off values to define genotypes and lineages as well as divergence between genotypes within each serotype.

Serotypes	Genotypes (%)	Lineages (%)	Divergence between genotypes (%)
O	> 13	> 20	13 – 20
SAT-1	> 16	> 23	16 - 24
SAT-2	> 20	> 26	20 - 27

For serotype O all 66 isolates from Africa as well as 6 isolates from the Middle East/Asia and South Africa clustered into one evolutionary lineage containing 8 distinct genotypes (Table 7.1). Isolates from East Africa clustered into 7 distinct topotypes (A - G), except one topotype (B) which included isolates from Sudan and West Africa and this study has revealed the historical relationship between East and North-western African isolates. Furthermore, the phylogenetic tree linked all 7 topotypes as well as one topotype (H) which included isolates from the Middle East and South-East Asia to the same node with significant boot-strap support of 92%, suggesting the possibility of the same ancestor. This lineage also diverged (20-24%) from the previously determined 2 lineages included in this study (Samuel and Knowles, 2001b; Knowles and Samuel, 2003; Sangare *et al.*, 2001) where one lineage included the Cathay topotype and the other the Angolan, and Euro-South American topotypes (Fig. 3.1).

The phylogeographical distribution of serotype O topotypes in East Africa can be divided into 2 major areas. Three topotypes (Table 7.1, A, B & D) were found to

be distributed in the northern part of East Africa (Kenya and north of Kenya) while the other 4 topotypes (C, E, F & G) were distributed in Kenya and countries to the south. The North Eastern African topotype (A) has a wide distribution across 5 countries with common boundaries namely Ethiopia, Eritrea, Somalia, Kenya and Sudan. One of these countries, Sudan harboured another 2 topotypes, the Sudanese (D) as well as the Sudan-West Africa topotypes (B). From countries south of Kenya, Uganda was extremely diverse in its topotype distribution with one topotype being shared with Kenya (C) and the other (G) with Tanzania as well as having two distinct topotypes (D & E) exclusive to Uganda. The overlapping topotype distribution in East Africa provides the basis for a proposal of FMD control. Countries north and south of Kenya have to consider the genetic diversity of isolates within topotypes of their respective sub-regions for the implementation of effective control strategies. Kenya, being centrally situated within East Africa has to consider all topotype distributions since the country harboured both groups of topotypes. Kenya and Ethiopia are producing vaccines against this serotype but detailed studies on the level of protection against all circulating field strains within these countries should be determined.

Comparison of amino acids at the immunodominant sites between the vaccine strain (ETH19/77) and other Ethiopian outbreak isolates revealed genetic variation. In all 36 compared isolates (Fig. 2.2) the hypervariable region was located at the highly immunogenic site of the 1D gene, the G-H loop. These results also indicated that further research is needed to assess whether the vaccine strain will provide adequate protection against field variants of type O FMD viruses currently circulating in Ethiopia. The distribution of East African topotypes revealed in this study, is of major importance as each country in this region is under constant threat of importing new variants. Therefore, it is strategically important to consider a regional approach for effective disease control.

In Ethiopia, FMD is a very common disease in cattle. The clinical signs of the disease in this species of animals are well recognized by herds men. The majority of the farmers interviewed during sample collection expressed the exact clinical signs of the disease in cattle. However, the majority of the same farmers didn't know the occurrence of the disease in small ruminants. The pastoral community in the southern part of the country (Borena) observed 2-3 outbreaks of FMD per year

and this was also confirmed by animal health assistance personnel in the region. In this study, serological survey results also indicated the involvement of 2 to 3 serotypes in outbreaks within the same herds of cattle and small ruminants (Table 6.4, 6.5). Alternatively, serotypes may persist in a region and rarely appear clinically, producing only mild infection where it can be only detected serologically. Generally the disease is clinically less obvious in indigenous breeds to the country where FMD is endemic (Kitching, 2002a).

Serological evaluation was used to determine the FMD incidence in Ethiopia using a liquid phase blocking ELISA and 3ABC ELISA from sera of non-vaccinated cattle, small ruminants and wild ruminants. The results obtained from these tests identified SAT-1 and SAT-2 FMD viruses circulating in buffalo and cattle populations in the southern part of the country. Since 1992, SAT-2 viruses have been isolated from FMD outbreaks while to date no SAT-1 virus has been isolated in the country. However, it is clear that SAT-1 viruses are present, but remain unrecorded as the surveillance system is inadequate. Furthermore, the highest sero-prevalence was detected in buffalo (84%) where antibodies to SAT-1 and SAT-2 were identified for the first time in these species in Ethiopia while both serotypes were also identified in cattle serum collected in the vicinity of buffalo populations (Omo National Park). Although antibodies to the 2 SAT types as well as serotype A and O have been identified from cattle and small ruminants sera, outbreaks due to O and A are predominant, with SAT-2 having the lowest incidence in Ethiopia and SAT-1 being the least frequently recovered serotype from outbreaks in East Africa. Antibodies to serotype A and O were also identified from the sera of small ruminants but for all of these reactive sera the distribution was limited to the areas where FMD outbreaks were reported during the period of sample collection. The present study highlighted the importance of buffalo in the epidemiology of FMD in Ethiopia and showed that other wild and domestic small ruminants are insignificant in the epidemiology of the disease in Ethiopia. The susceptibility of small ruminants to FMD can vary with strains of virus and the clinical signs may be indistinguishable from those produced by a variety of other causes and for this reason most cases of FMD in sheep and goats are misdiagnosed. However, this species have played a significant role in the transmission of the disease to susceptible hosts in other parts of the world

(Kitching and Hughes, 2002). Furthermore, small ruminants (sheep and goats) may become carriers of FMDV for variable periods of time (Callis, 1996).

Research addressing the role of small ruminants and other wildlife in the epidemiology of FMD in Ethiopia and other East African countries has been very limited in comparison to southern African countries. In order to further clarify the possible role of small ruminants and wildlife in virus maintenance and virus transmission during inter-epizootic periods and to gather more information on epidemiological data which can assist in the control the disease, extensive epidemiological studies involving both serological surveys and virus isolations need to be conducted.

The molecular epidemiology of SAT-2 was based on the phylogenetic analysis of the complete VP1 nucleotide sequences of 41 East African and 26 isolates from other African countries. Phylogenetic analysis identified 5 lineages and 15 genotypes from 13 African countries and Saudi Arabia (Table 7.1). Lineage I comprised 7 genotypes which consisted of viruses from East Africa, Zaire (DRC), Rwanda and Saudi Arabia while lineage V consisted of viruses occurring throughout southern African countries and extending into East Africa. Previously identified lineages (III and IV) from West Africa and one lineage (II) from Angola (Sangare *et al.*, 2003) included in this study were found to represent distinct isolates for their respective regions. These results indicated that the SAT-2 viruses were extremely diverse, but according to genotype distribution could be divided into 15 topotypes. Some of the East African topotypes showed links between countries within the region as well as with the southern and central African countries.

The East African isolates were distributed within lineages I and V (Table 7.1). Lineage I consisted of the majority of East African isolates as well as individual isolates from Saudi Arabia and Rwanda and 7 topotypes were assigned. The distribution of each topotype was limited to one or 2 countries. These genetically independent entities corresponded to geographically defined areas. Alternatively, the other topotype (O) within lineage V showed wide distribution which covered 5 countries (Burundi, Malawi, Tanzania, Kenya and Ethiopia). The geographic distribution of topotype O stretches from south to north across these countries.

Interestingly this topotype shared a common ancestor with buffalo virus isolates from South Africa in topotype N within the same lineage which indicated historical relationships between East African and South African isolates. It is possible that isolates within this topotype have a wider range of susceptible hosts.

The presence of 3 topotypes within Uganda (Table 7.1) showed the higher level of intratypic variation of SAT-2 virus isolates from this country. In contrast, the majority of virus isolates in genotype A, from Kenya and Uganda, were less diverged (< 6%) even though these isolates span a period of 13 years (Table 4.3). This genetic conservation over 13 years needs further investigation. Similarly, a close genetic relationship was observed between the vaccine strain ETH/19/77 and the field strain ETH/5/95 in Ethiopia. One speculation can be the re-introduction of the same virus from a common source such as a poorly inactivated vaccine.

SAT-1 maintains endemicity in many sub-Saharan African countries with marked regional differences in distribution. The Neighbour-joining method was used to construct phylogenetic trees based on partial nucleotide sequencing of the 1D gene (396 bp) from 52 SAT-1 virus isolates from Africa. This analysis identified 8 lineages and 11 genotypes (Table 7.1) when nucleotide differences of > 23% for lineages and > 16% for genotypes were used as cut-off values. The same pattern of phylogenetic relationships was confirmed using the complete 1D gene sequence of 39 selected isolates of SAT-1 viruses from Africa.

High intratypic variation (divergence 23.5 – 35%) was observed across lineages, except for the lineages distinct to Uganda, the other lineages were distributed between two or more countries. It seems reasonable to assume that viruses in each genotype evolved independently over years. A historical relationship between Sudan and West African (Nigerian) isolates were also observed in one of the genotypes. The genetic relationships of this serotype as well as serotype O was indicative of some exchange of viruses taking place in the past which can be related to the nomadic movement of animals and links between East and West Africa. These preliminary results showed the importance of molecular studies to assist in a better understanding of the genetic relationships of FMD viruses in Africa.

Three distinct lineages for Uganda were identified with high divergent values between each other (> 30%) and are by far the most heterogeneous. The Ugandan isolates characterized in this study including the above two serotypes revealed the presence of 8 genotype in this country. As this study highlighted, there is no explanation why are some strains are restricted to certain areas, while others are not. The precise mechanism that enable the virus to achieve this in not clear. It can however be predicted that within each serotype there is a spectrum of strains with their own antigenic and epidemiological characteristics (Kitching, 2002) which are likely to be important in survival mechanism of the viruses.

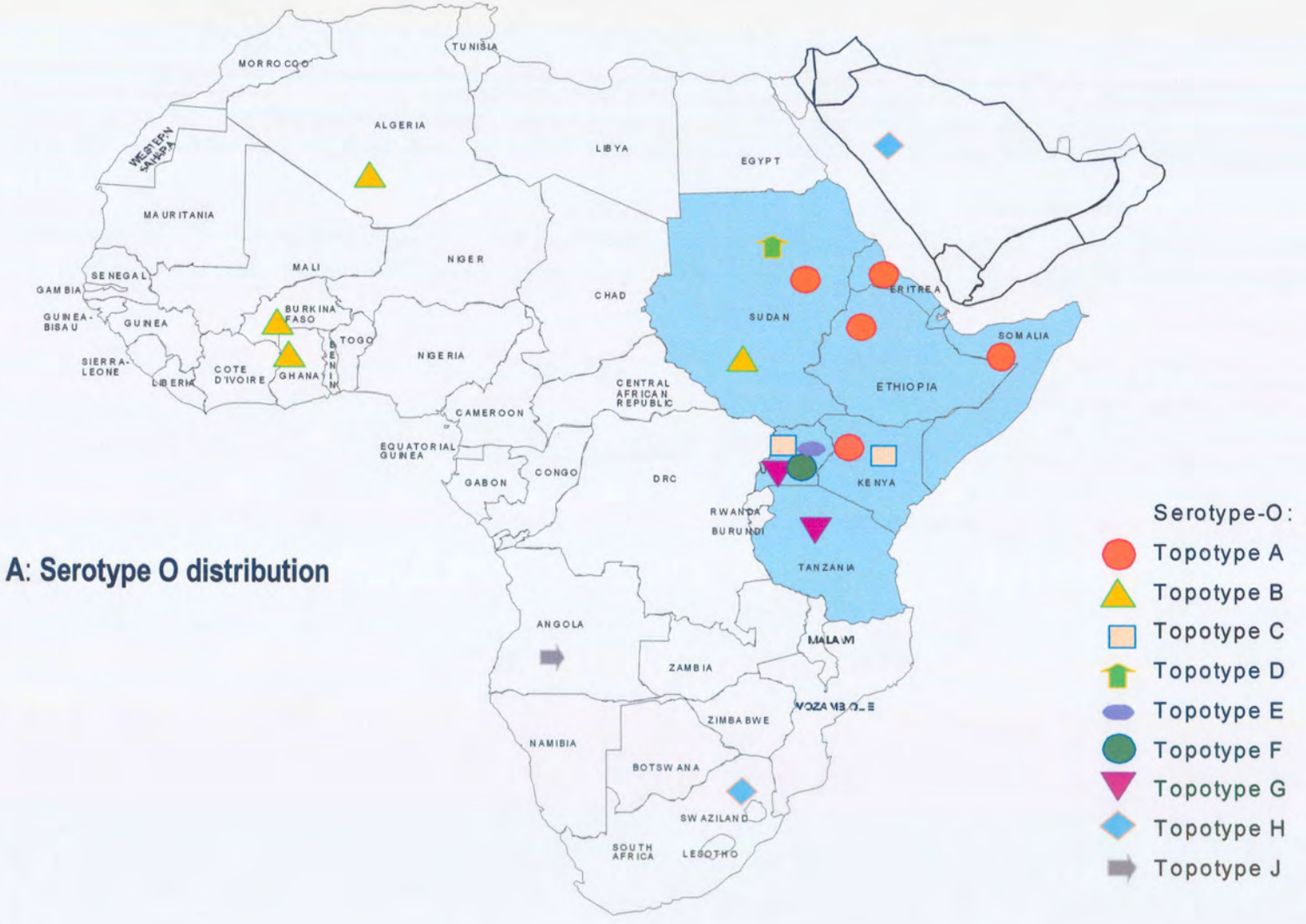
A variability plot based on sequence alignment of 221 amino acids of the 1D gene of 52 SAT-1 viruses correspond with immunogenic sites the G-H loop and the C-terminus regions (Fig. 5.4) which coincided mostly with the previous finding from southern and western African isolates (Bastos *et al.*, 2001; Sangare *et al.*, 2001). SAT 2 in contrast with SAT-1, were not hypervariable at amino acid positions 192 – 208 (the C-terminus) of the 1D gene. This was found with 48 SAT-2 viruses (Fig. 4.2) which exhibited a variation between 20 – 40% in this region. In addition within SAT-2 viruses two regions at amino acid positions 46 – 59 and 76 – 90 were shown to be hypervariable which were not observed in SAT-1 viruses.

The identification of numerous lineages and genotypes of FMD virus in East Africa having distinct or overlapping distribution and the links between East Africa and West Africa as well as southern Africa indicate that exchange of viruses between these regions has occurred in the past and the highest interatypic variation was observed in SAT-1 and SAT-2 viruses. The intratypic genetic variation in excess of 20% on the nucleotide sequence level were shown to be antigenically poorly related. This may lead to difficulties in the effective control of FMD by vaccination (Bastos *et al.*, 2003). Therefore, it is vital that the correct vaccine strains are selected that induce antibodies capable of full protection against field variants circulating within a country or region.

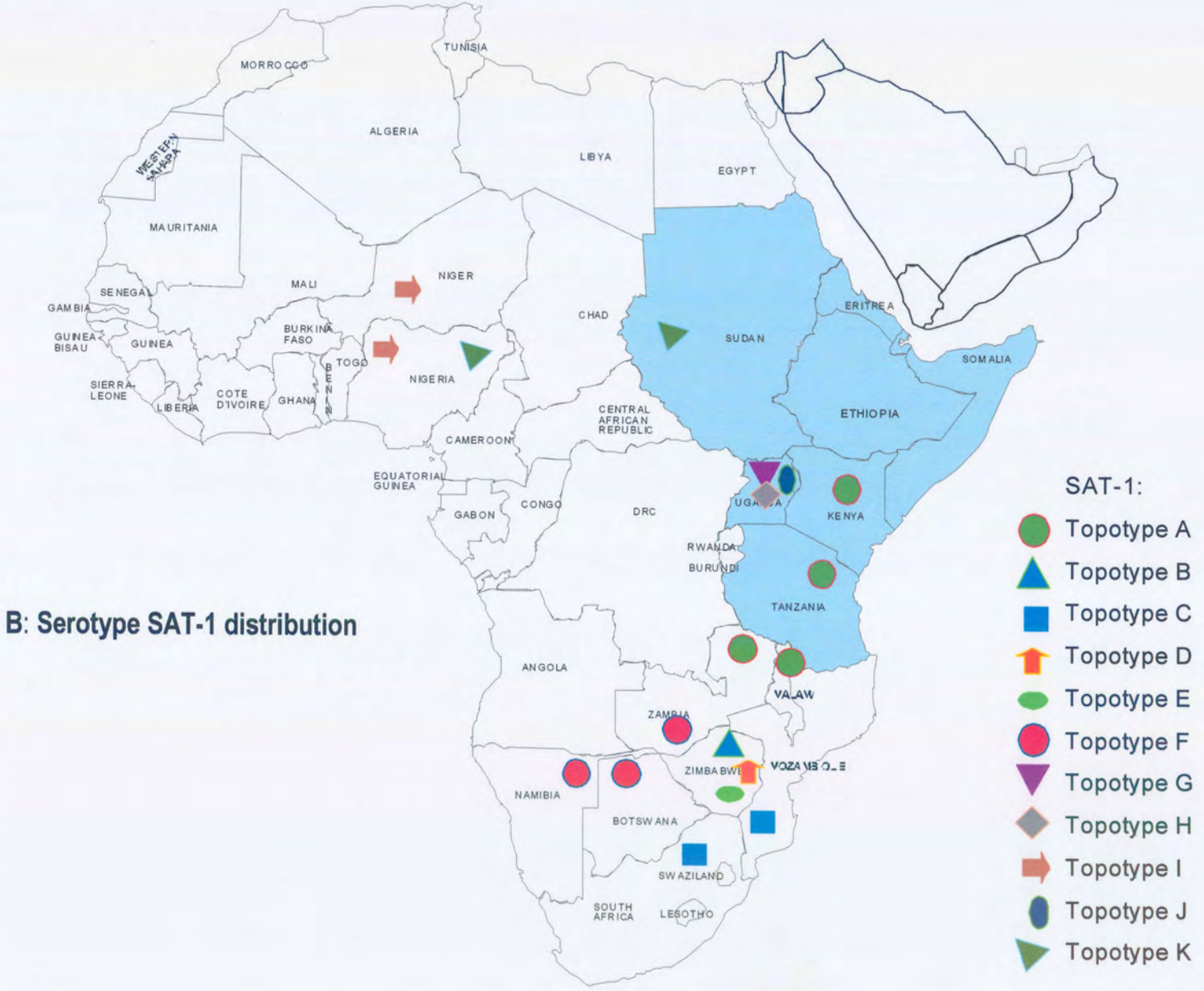
Understanding of the distribution of genotypes and lineages in Africa is vital and important in the development of future disease control strategies. It is likely that the genetic diversity on the nucleotide sequence level of the VP1 gene observed in this study is reflected antigenically. It can be seen that the one strategy by

vaccination only is complex partly because of the complex epidemiology involved. Our result indicated that some topotypes have a wider geographical distribution when others restricted within a country. This might be explained by the differences in survival mechanism of FMD viruses which might be linked to host rang and their ability to transmit rapidly and cause infection. It was reported that the PanAsian strain has been isolated from cattle, sheep, goats, pigs, water buffalo, deer, gazelles, antelopes and camels (Samuel and Knowles, 2001a) while other virus of serotype O was found to be a pig-specific strain in South-East Asia (Kitching, 2002b).

This study as well as previous studies based on the major immunogenic regions of the VP1 gene of SAT types (Bastos *et al.*, 2001, 2003; Vosloo *et al.*, 2002; Sangare *et al.*, 2003; Knowles and Samuel 2003) illustrated the distribution of distinct topotypes in Africa and a higher level of intratypic variation within each SAT type. Thus, a further detailed study on the evaluation of available vaccines and the development of multivalent vaccines for most developing countries of Africa on the basis of strict geographical clustering of independently evolving virus populations should be encouraged. Furthermore, restrictions on animal movement must be enforced to minimize further transboundary transmission of the disease. The virus characterization approach also provides a base-line database for tracing the origin of outbreaks. Effective control of FMD also requires a regional approach where the governments must be accountable for the planning and co-ordination of disease control programmes.



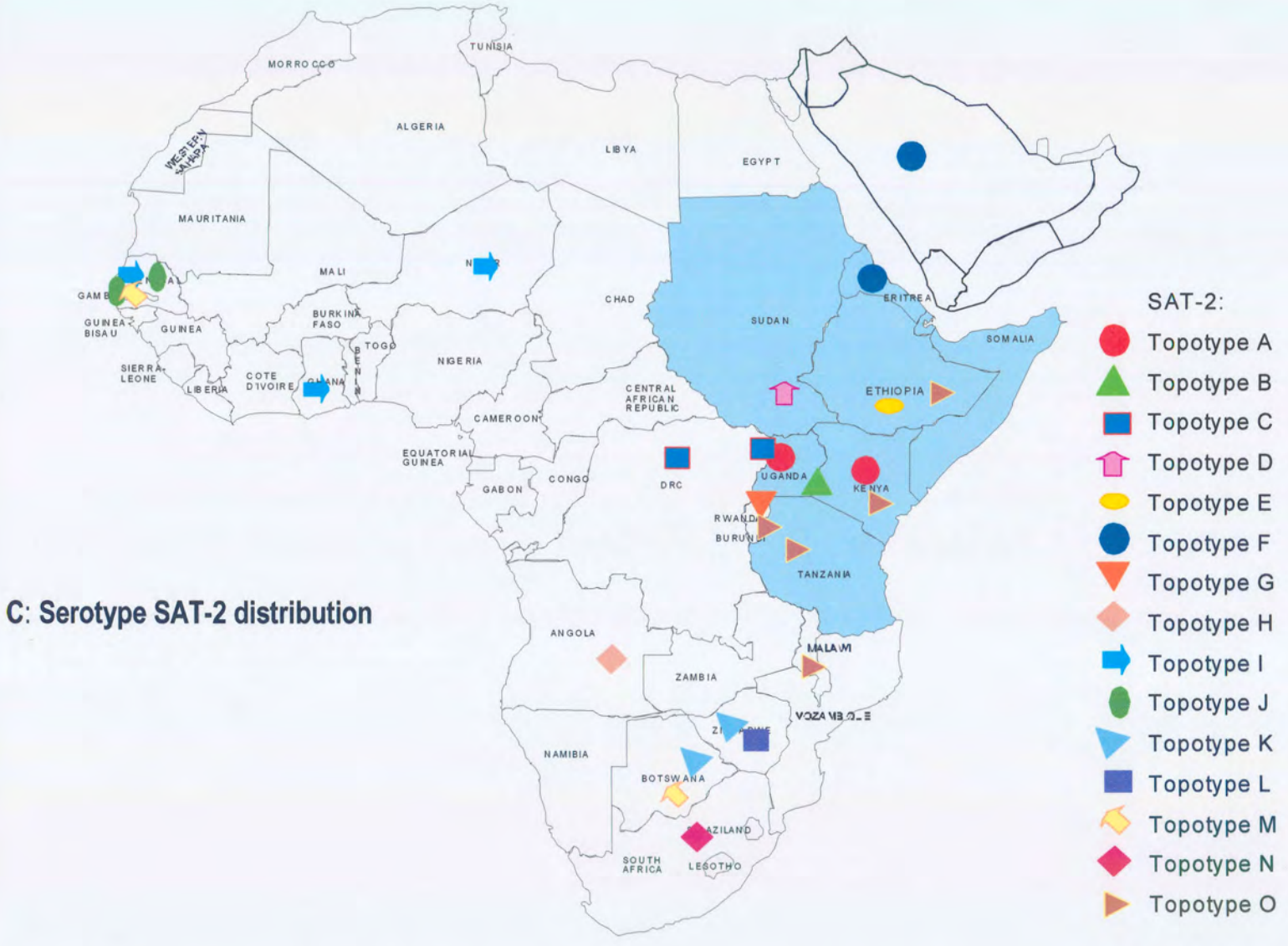
A: Serotype O distribution



B: Serotype SAT-1 distribution

SAT-1:

- Topotype A
- Topotype B
- Topotype C
- Topotype D
- Topotype E
- Topotype F
- Topotype G
- Topotype H
- Topotype I
- Topotype J
- Topotype K



C: Serotype SAT-2 distribution

Fig. 7.1 Distribution of various Topotypes of FMD virus isolates identified in Africa (A: type O, B:SAT-1, C: SAT-2).

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APPENDIX I

Commonly used reagents, buffers and other preparations for molecular work.

Molecular biology grade chemicals were used for making reagents and solutions for molecular biology. Double glass distilled deionised water (ddH_2O) was used for all molecular tests.

Preparation of size fractionated silica

Six grams of silicon dioxide (99% Sigma) was suspended in 50 ml of sterile ddH_2O in a glass bottle and allowed to sediment for 24 hours at room temperature. The supernatant (43 ml) was removed and the sedimented silica was re-suspended to a final volume of 50 ml. A second sedimentation was allowed for the duration of 5 hours at room temperature. The supernatant (44 ml) was removed and then the pH of silica was adjusted to 2 by adding 60 µl of HCl (32% W/V). Aliquots of 240, 280, 360 and 400 µl were prepared in 1.5 eppendorf tubes and stored in dark. Fourty µl of this suspension was used per tissue cultured supernatant of the virus samples.

EDTA (1M)

An amount of 372.2 g of disodium ethylenediaminetetra-acetate·$2H_2O$ (EDTA) was added to 800 ml of water, vigorously stirred on a magnet stirrer, and the pH adjusted to 8.0 with NaOH (~20 g of NaOH pellets). The solution was dispensed into aliquots and sterilised by autoclaving.

Preparation of L6 lysis buffer

Sixty grams of Guanidine Thiocyanate (GuSCN, Promega) was dissolved in 50 ml of 0.1M Tris-HCl (pH 6.4). 11 ml of 0.2M EDTA and 1.3 g of Triton X-100 were added and kept at 50°C in water bath until they completely dissolved. The bottle was wrapped in aluminium foil and stored at room temperature.

Preparation of L2 wash buffer

L2 wash buffer was prepared in the same way as L6 lysis buffer preparation but without the addition of EDTA and Triton X-100.

Tris (1 M) preparation

Tris base (121.1 g) was dissolved in 800 ml of dH_2O, and the solution was allowed to cool to room temperature before final adjustment of pH to 7.4 with concentrated HCl. After adjusting the volume of the solution to 1 litre with dH_2O, it was dispensed into aliquots and sterilised by autoclaving.

DNA loading buffer (6x)

DNA loading buffer was made by dissolving 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose in sterile distilled water (Sambrook *et al.*, 1989). The buffer was stored at – 4°C in 1 ml aliquots.

Tris acetate buffer

Tris-acetatte (TAE) was used for electrophoresis of the RT-PCR products in agarose gels. It consisted of 40 mM Tris-acetate and 1 mM EDTA. Generally a 50X solution was made by dissolving 242 g of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) in deionised distilled water to a final volume of 1l. The buffer was autoclaved, and diluted 1:50 in deionised distilled water before use.

Ethidium bromide (10 mg/ml)

One gram of ethidium bromide was dissolved in 100 ml of dH_2O. The solution was stirred on a magnetic stirrer for several hours to ensure that the dye was completely dissolved. The container was wrapped in aluminium foil and stored at – 4°C. This dye was used to track PCR products in agarose gel electrophoresis. Because of the powerful mutagen and moderately toxic property of ethidium bromide, all the material that has been in contact with the reagent when working with solutions had been disposed in hazard bags, and necessary precautions were taken to ensure maximum safety and avoid skin or any other contact with the reagent.

Deoxyribonucleoside triphosphates (dNTPs)

Free dNTPs are required for DNA synthesis. Equimolar amounts of the four dNTPs (dATP, dGTP dCTP dTTP) were used in the reaction mixture to minimize misincorporation errors. All DNA polymerase enzymes add deoxynucleotides (dNTP) in a 5' to 3' direction to a primer. From stock solutions of each dNTP commercially available (Roch), dilutions were made to obtain final concentrations of

10pmol/µl of working solutions and each were stored separately at minus 20°C in small aliquots.

PCR Master Mix per reaction

The PCR was performed in a 50 µl volume in the presence of 3 µl of cDNA were constituted as follows:

ddH_2O	X µl
Sense primer (Inqba Biotech)	2.5 µl (stock = 10 pmol, final conc = 25 pmol)
Antisense primer (Inqba Biotech)	2.5 µl (stock = 10 pmol, final conc = 25 pmol)
dNTPs (Roche)	1 µl (stock = 10 mM, final conc = 200 µM)
Taq buffer (Promega)	1 µl (stock = 10X, final conc = 1X)
Taq (Roche)	0.5 µl (stock = 5U, final conc = 2,5 U)
cDNA template	3 µl
Total	50 µl

Detail information of the sense and Antisense primers used for PCR against each serotype of FMDV are described in their respective chapter.

Summary of reactivity of serum samples from buffalo tested by the Lpb ELISA and 3ABC ELISA

Serum samples ident. No.	SAT-			A	O	C	3ABC ELISA
	1	2	3				
152	2.003	1.687	-	-	-	-	0.838
153	2.005	2.055	1.559	-	-	-	-
154	-	-	-	-	-	-	0.705
155	-	-	-	-	-	-	0.678
156	1.742	-	-	-	-	-	0.995
157	-	2.086	-	-	-	-	-
158	1.617		-	-	-	-	0.671
159	2.238	2.108	1.600	-	-	-	-
160	1.680	-	-	-	-	-	1.256
161	2.005	1.735	-	-	-	-	0.911
162	2.355	2.239	1.626	-	-	-	0.863
163	1.886	1.874	-	1.702	-	-	-
164	2.355	2.355	1.800	-	-	-	-
165	2.172	2.255	-	-	-	-	1.124
166	2.199	2.164	1.600	-	-	-	0.782
167	2.055	2.267	-	1.772	-	-	0.958
168	1.982	1.953	-	-	-	-	-
169	2.013	1.942	-	-	-	-	1.063
170	1.927	1.681	-	-	-	-	1.079
171	2.089	2.104	-	-	-	-	-
172	2.165	2.102	-	-	-	-	1.055
173	-	2.055	-	1.910	-	-	0.661
178	-	1.760	-	-	-	-	0.880
179	-	-	-	-	-	-	-
180	-	-	-	-	-	-	-

Total serum samples from Buffalo = 25 (152 – 180) (Origin Omo national park).
- indicates non-reactive sera

Lpb ELISA: Sera with titres > 1/40 that showed an OD of > 1.6 were considered as reactive sera (non-reactive sera ≤ 1.600) (Section 6.2.3.1). Results indicated under column SAT-3 were considered as negative.

3ABC ELISA: an index values of > 0.5 accepted as reactive sera and those results below this value considered as non reactive sera (Section 6.2.3.2).