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 2.8×10^6 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).

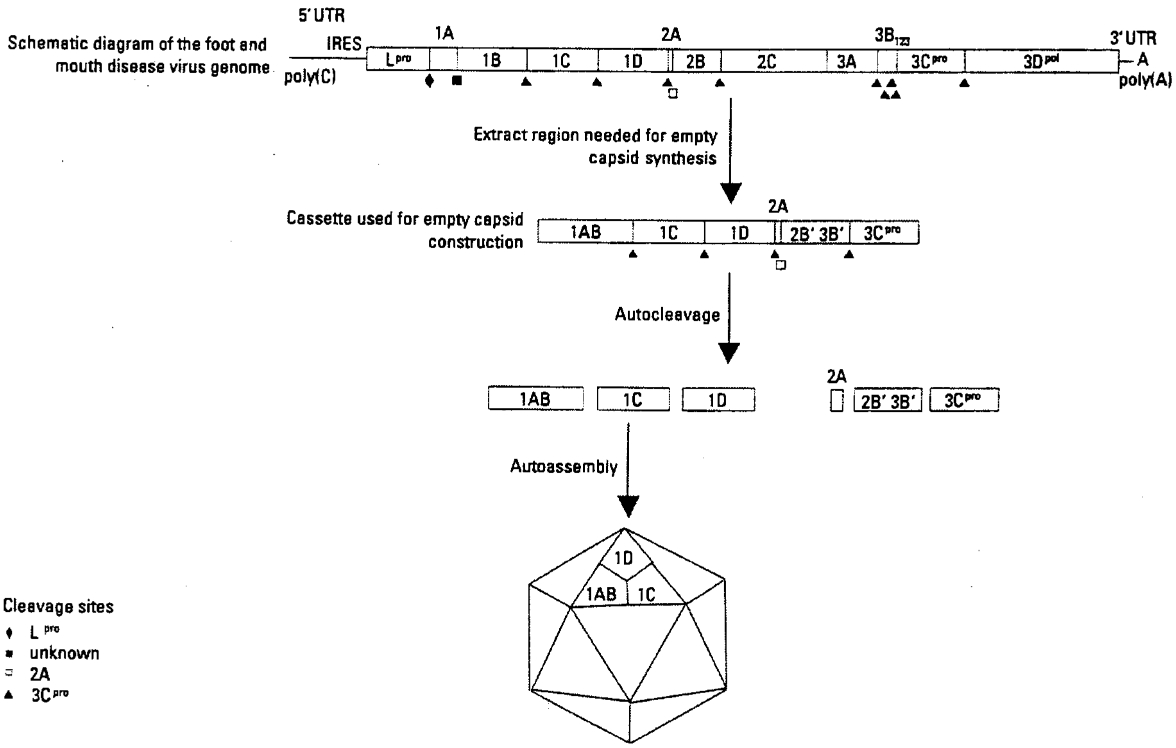


Fig. 1.1 Schematic representation of the FMD virus genome and empty capsid construction and auto-assembly. The untranslated regions of the genome are represented by lines and the protein-encoding regions are represented by boxes. The symbols below the protein-encoding regions identify the proteinases 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

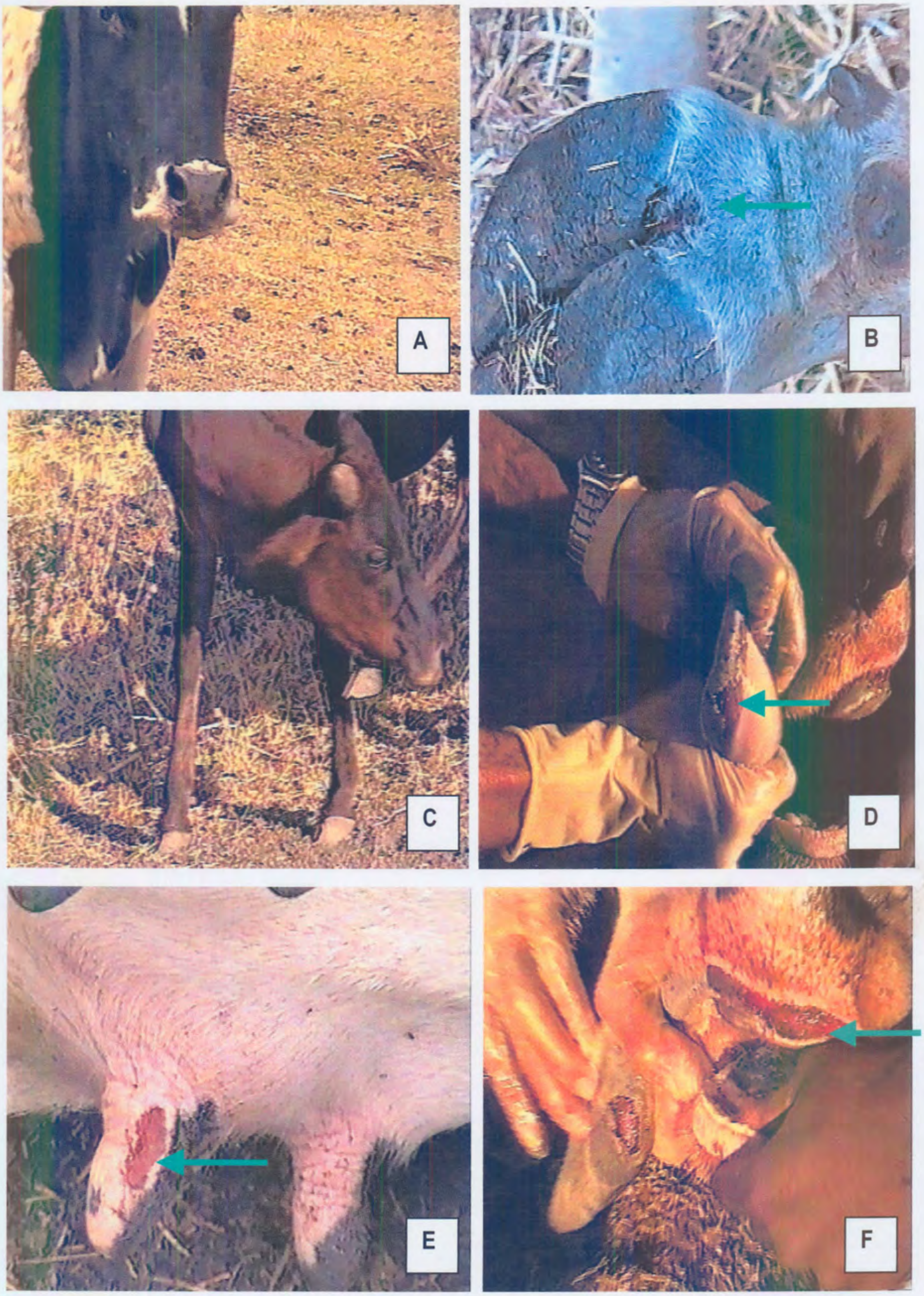
Diagnosis of FMD normally involves obtaining clinical information, examination of sick animals and sampling as well as laboratory tests. Due to the number of atypical cases as well as sub-clinical infections and apparently recovered animals that harbour the infection, clinical diagnosis can present many 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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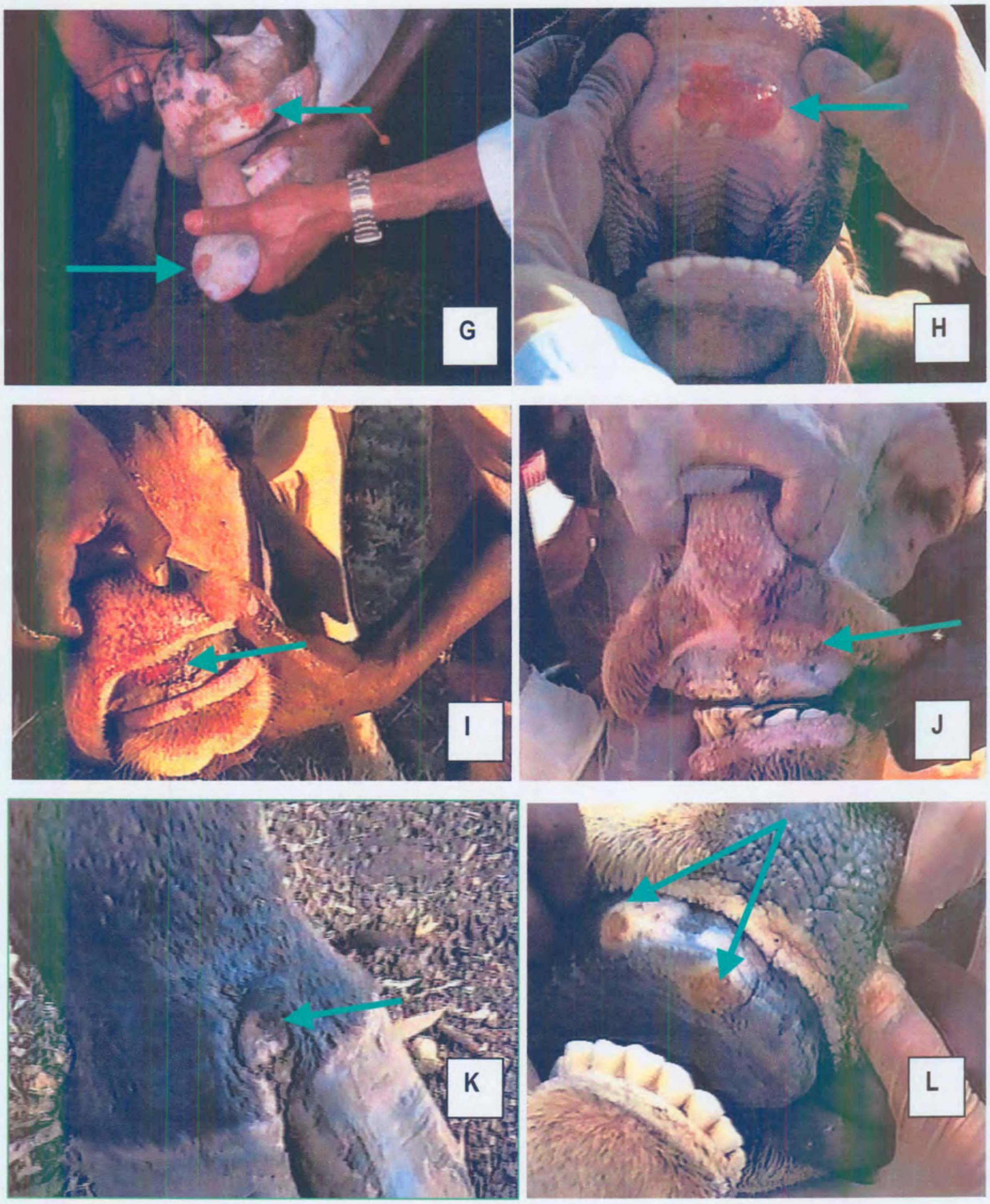

Fig. 1.2 Clinical signs of FMD in indigenous Ethiopian cattle. Pictures were taken during the outbreaks of FMD in 2001. Salivation (A), lesion at the coronary bands and interdigital space (B), lifting and licking of the feet (C), Vesicular lesion followed by ulceration and erosion (D-H), Bacterial complication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

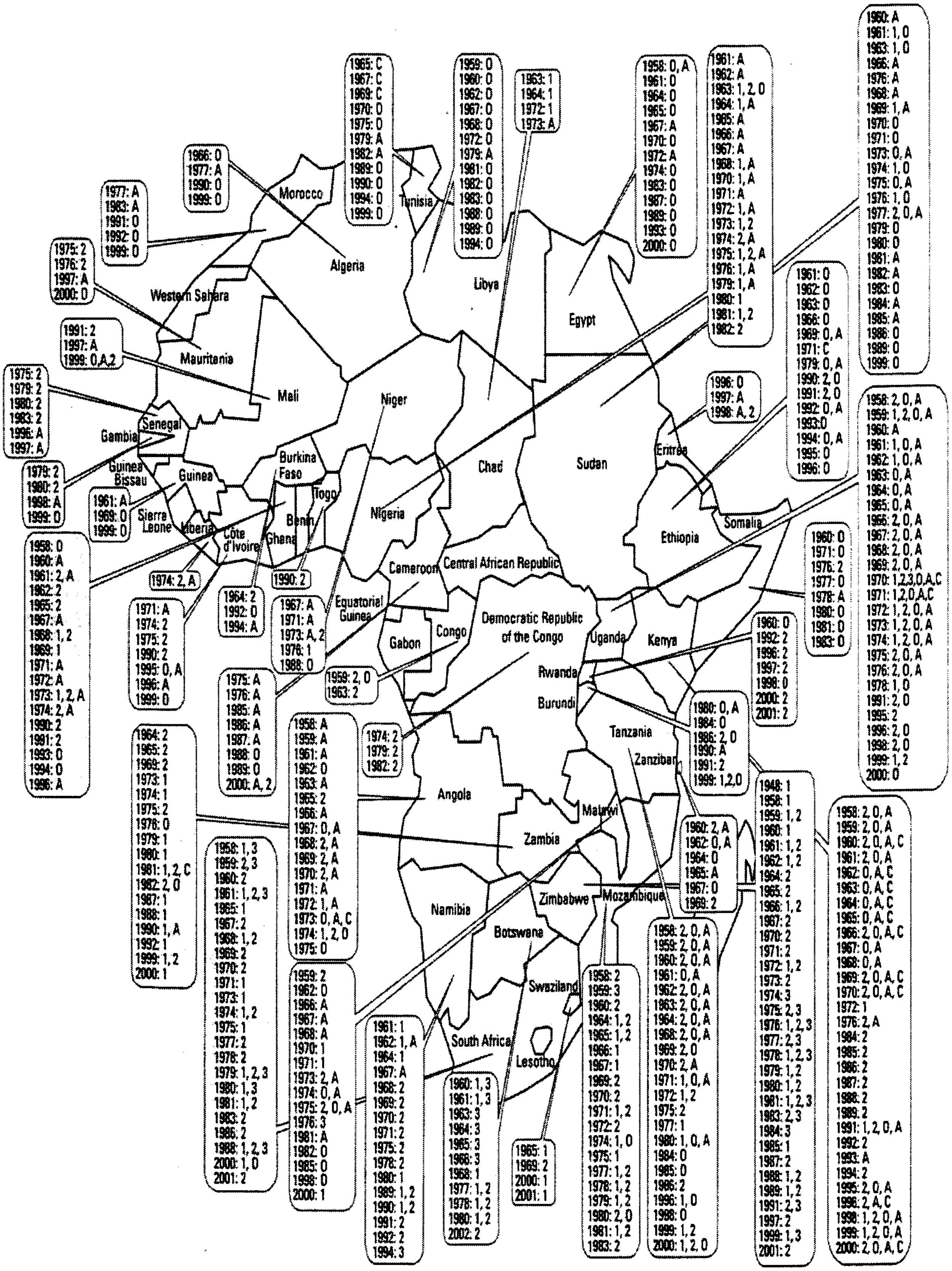

Fig. 1. 3 Map of Africa demonstrating the outbreaks of foot-and-mouth disease between 1948 - 2002 (cited from Vosloo *et al.,* 2002). The numbers 1, 2 and 3 represent the serotype SAT-1, SAT-2 and SAT-3, respectively.

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 x 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 ≤ 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 (≥ 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.

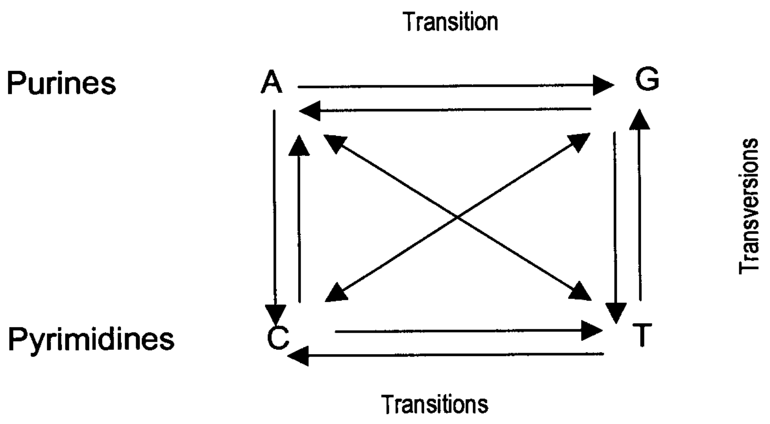

Fig. 1.4 The probability of substitution of one nucleotide by another between purines and/or pyrimidines.

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).

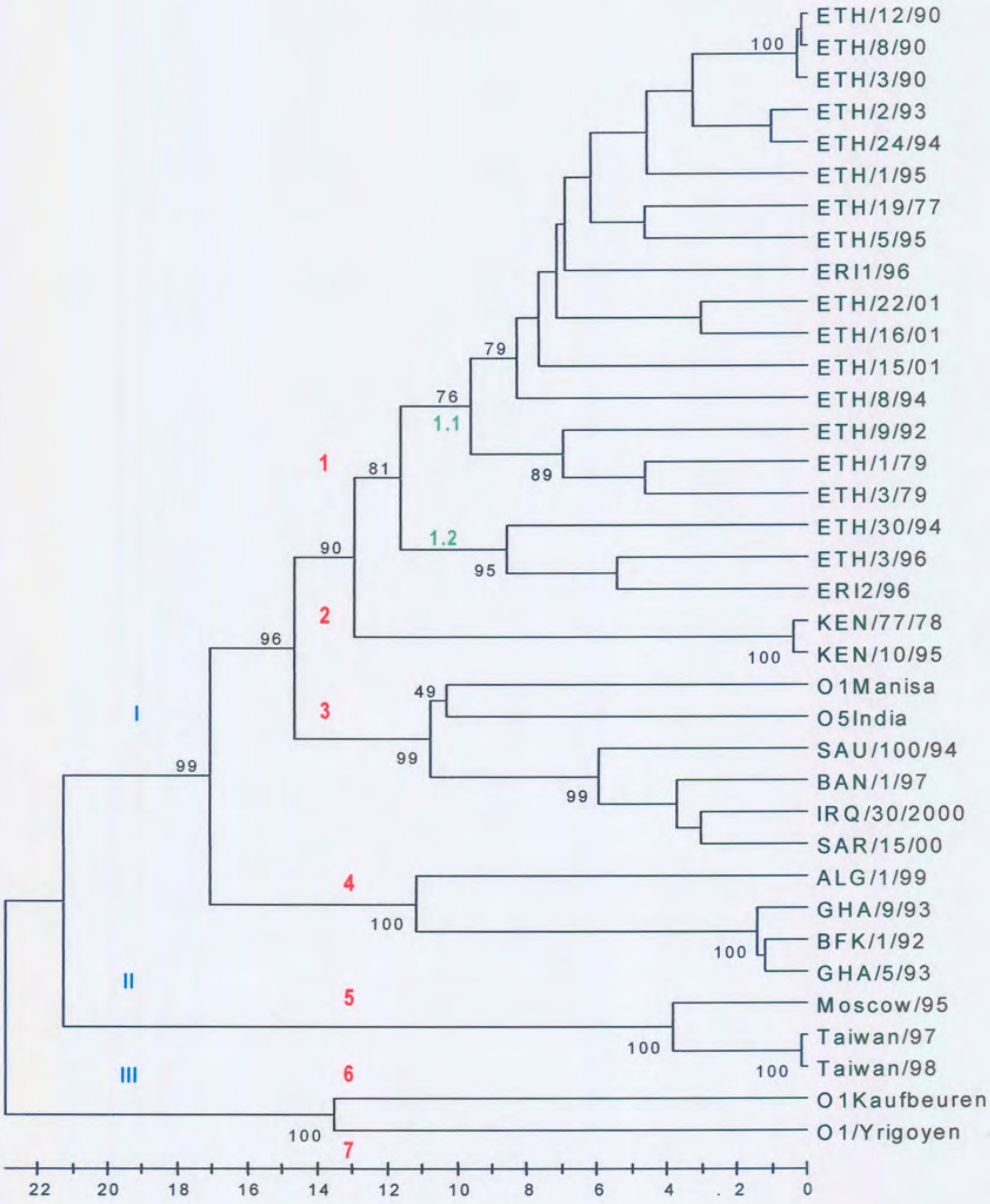

Fig. 2.1 UPGMA tree depicting genetic relationships of serotype O FMD viruses from Ethiopia, Africa (East, West & South), Africa-Middle East/Asia and Europe. I - III, 1 – 7 and 1.1 – 1.2 represented lineages, genotypes & clades, respectively.

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.
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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 ≥ 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

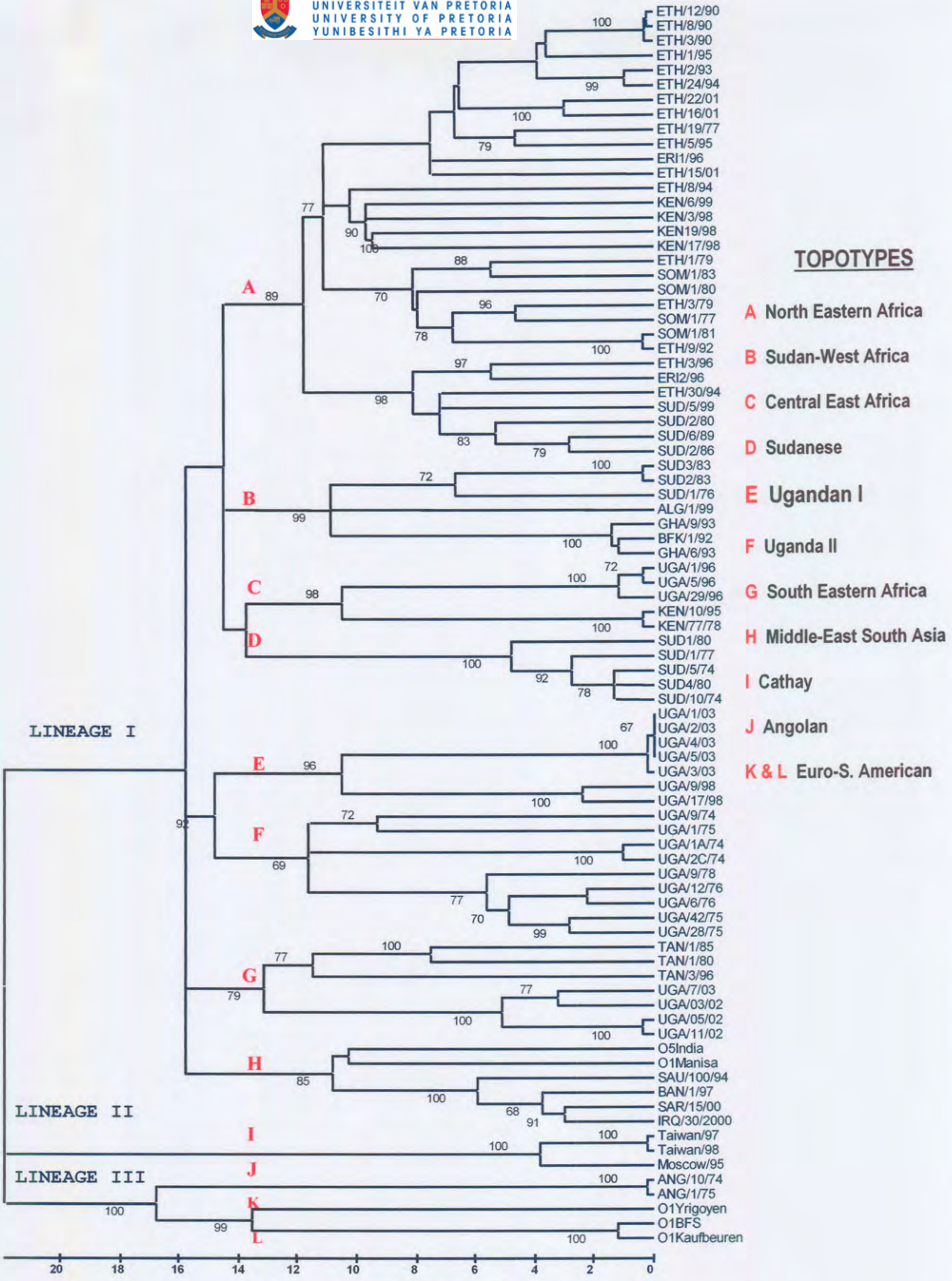

Fig. 3.1 Neighbour-joining tree depicting genetic relationships of serotype O FMD viruses from Africa (East, West & South), the Middle East, Europe, South America and Asia. lineages (I-III) were identified. Genotypes are indicated based on > 13% nucleotide differences among clusters and are indicated 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 ($\geq$ 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.