MOLECULAR EPIDEMIOLOGY OF FOOT-AND-MOUTH-DISEASE VIRUS IN WEST AFRICA

by

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submitted in partial fulfilment of the
requirements for the degree of

Doctor of Philosophy

in the
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria

June 2002

ACKNOWLEDGMENTS

The author wishes to thank the following persons:

Profs. J.A.W. Coetzer, and P.G. Howell for support and directing me into FMDV research

Dr. Gavin R. Thomson with assistance in securing sponsorship from Intervet International, The Netherlands

Prof. Estelle H. Venter and Drs Wilna Vosloo & Armanda D.S Bastos for their guidance

A special thanks to Dr. Armanda D.S. Bastos for her interest and invaluable contribution with data analysis

Dr. Nigel Ferris for providing viruses in this study

Drs Boubacar Diallo & Yaghouba Kane, centre National d'Elevage et de Recherches Veterinaires (C.N.E.R.V.), Nouakchott, Mauritania to make available the serum bank from their laboratory for this study

Drs A. Bado & Mamadou Sidibe, Laboratoire National d'Elevage, Ouagadougou, Burkina Faso for serum from Burkina Faso for this study

Drs Jacky Grimbeek & Van der Linde, Department of Statistics at the University of Pretoria for assistance in the sampling design

The personnel of Block G, Exotic Diseases Division, Onderstepoort Veterinary Institute and Molecular Biology section at the department of Zoology & Entomology, University of Pretoria, for their help in various ways during this study

My family, friends and colleagues for their encouragement, interest and understanding in the completion of this study

The author also wishes to express her sincere gratitude to:

Winrock International, Arkansas, USA for the PhD scholarship

Intervet International, The Netherlands for sponsoring this project
The University of Pretoria for financial support

The Government of Mali and the Laboratoire Central Veterinaire for giving me the opportunity to pursue my studies

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ABSTRACT

The economy of West African countries is dependent mainly on agriculture. Livestock production is a vital source of providing dietary protein for the rapidly growing human population and it is therefore important to define strategies for controlling infectious diseases that are undermining the livestock industry. Although the foot-and-mouth disease (FMD) virus causes one of the most devastating economical diseases, it has been mainly ignored in West Africa due to low mortality rates in the face of other diseases that cause significant mortalities. This may explain the lack of interest for studying FMD infections in the region. However, the eradication of other diseases such as Rinderpest together with an increase in the number of outbreaks of FMD in recent years has caused a renewed interest in understanding the epidemiology of the disease.

Foot-and-mouth disease is a highly contagious disease of cloven-hoofed animals. The causative agent, FMD virus, has a high rate of genetic variation in its single-stranded RNA genome. The genetic characterization of the surface capsid protein gene, VP1, is the most informative technique for studying the molecular epidemiology of FMD. The genetic profile of different serotypes of FMDV isolated across West Africa was investigated in this study using manual and automated nucleotide sequencing. A total number of 21 type O isolates from Ghana, Burkina Faso and South Africa (1992-2000), 23 SAT-1 viruses from Niger and Nigeria (1975-1981) and 30 SAT-2 viruses from Mali, Ivory Coast, Ghana, Nigeria, Liberia, Senegal and Gambia (1974-1991) were investigated. The sequence data was used to establish the phylogenetic relationships between the west African strains and those previously characterized from East, central and southern Africa as well as other regions of the world in the case of serotype O.

Viruses from West Africa formed a single genotype while the isolates from South Africa clustered with the Pan-Asian topotype (Bangladesh 1997 & Japan 2000). Sequence identity of 99 % and 95 % were found between Ghana-Burkina Faso and South Africa-Bangladesh type O viruses, respectively.

Within SAT-2, the viruses characterized were isolated over 27 years from seven countries in West Africa and two indigenous topotypes (> 97 % sequence identity in the cluster) were identified . Of interest was the clustering of Nigerian (1982) and Eritrea (1998), which has provided the first evidence of virus transmission between West and East Africa.

For SAT-1, two distinct lineages (I-II) were identified. Lineage I consisted of viruses isolated between 1975-1976 from neighboring countries Niger and Nigeria, while lineage II was composed of viruses recovered from outbreaks between 1979-1981 in Nigeria. Furthermore, viruses from the latter lineage shared > 98 % sequence identity across the VP1 gene providing a clear indication of a long circulation of virus in the field in West Africa.

For the serotypes investigated in this study viz. serotypes O, SAT-2 and SAT-1, indicated that the year of isolation is more important in the epidemiology of FMD in West Africa rather than country of origin. The phylogenetic analysis demonstrated that viruses from each serotype grouped according to year of isolation rather than their geographical origin. This is in contrast of what was reported previously for FMDV strains in southern Africa. Results further revealed that FMD viruses from West Africa are evolving independently from viruses elsewhere on the continent and clustered in discreet genotype. The genetic distinctiveness of west African FMD isolates is likely to be reflected antigenically and has implications in the selection of regionally appropriate field strains for the use in vaccines for use in vaccines to assist in the control of the disease.

SCIENTIFIC CONTRIBUTIONS EMANATING FROM THIS THESIS

Published Articles in International Scientific Journals

Sangare, O., Bastos, A.D.S., Marquardt, O., Venter, E.H., Vosloo, W., and Thomson, G.R. 2001. Molecular epidemiology of serotype o foot-and-mouth disease virus with emphasis on West and South Africa. Virus Genes, 22 (3): 345-351.

Vosloo, W., Bastos, A.D.S., **Sangare, O.**, Hargreaves, S., Thomson, G.R. 2001.Review of status and approaches to control and eradication of foot-and-mouth-disease in Sub-Sahara Africa. (OIE Scientific and Technical Review, 2002, In press).

Manuscripts presently under review

Sangare, O., Bastos, A.D.S., Venter, E.H., Vosloo, W. 2002. A first molecular epidemiological study of SAT-2 type foot-and-mouth disease viruses in West Africa. (Epidemiology & Infection).

Sangare, O., Bastos, A.D.S., Venter, E.H., Vosloo, W. 2002. Retrospective genetic analysis of SAT-1 type foot-and-mouth disease outbreaks in West Africa (1975-1981). (Veterinary Microbiology).

Published Conference Proceedings

Sangare, O., Bastos, A.D.S., Vosloo, W., and Venter, E.H. 2002. Molecular characterization of foot-and-mouth disease virus serotypes O, SAT-1 and SAT-2 in West Africa. Proceedings of the Southern African Society for Veterinary Epidemiology and Preventive Medicine, 22-23 July 2002, Onderstepoort, South Africa: 116-121 (Talk).

Bastos, A.D.S., & **Sangare, O**. 2001. Geographic distribution of SAT-2 type foot-and-mouth disease virus genotypes in Africa. Proceedings of the southern African Society for Veterinary Epidemiology and Preventive Medicine, 10-11 May 2001, Onderstepoort, South Africa: 20-26.

International Congresses

Sangare, O., Bastos, A.D.S., Venter, E.H., and Vosloo, W. Genetic Relationships of Type O Foot-and-mouth disease viruses recovered from outbreaks in West and South Africa. 2001. In Virus Evolution and Genomics. Sixth International Symposium on Positive Strand RNA Viruses, May 28 - 2 June, Institut Pasteur, Paris, France: P2-112 (Poster).

National Congresses

Sangare, O., Bastos, A.D.S., Vosloo, W., and Venter, E.H. 2001. Genetic Relationships of SAT-2 Type Foot-and-mouth disease virus isolates from outbreaks in West Africa, 1974-1991. Proceedings of the southern African Society for Veterinary Epidemiology and Preventive Medicine, 10-11 May, Onderstepoort, South Africa: 56 (Poster).

Sangare, O., Bastos, A.D.S., Venter, E.H., and Vosloo, W. Molecular epidemiology of serotype O foot-and-mouth disease (FMD) virus in West Africa (1988-1993). 2000. Sixteenth FACULTY DAY, Faculty of Veterinary Science, University of Pretoria, September 29, Onderstepoort, South Africa: 24 (Poster).

Sangare, O., Bastos, A.D.S., Vosloo, W., and Venter, E.H. Phylogenetic relationships of Foot-and-mouth disease virus Type SAT-2 in West Africa. 2001. Seventeenth Academic DAY of the Faculty of Veterinary Science, September 20, University of Pretoria, Onderstepoort, South Africa: 37 (Poster).

Sangare, O., Bastos, A.D.S., Vosloo, W., and Venter, E.H. Characterization of Foot-and-mouth disease viruses responsible for the disease in West Africa. 2001. Seventeenth Academic DAY of the Faculty of Veterinary Science, University of Pretoria, September 20, Onderstepoort, South Africa: 15 (Talk).

Sangare, O., and Bastos, D.S. Molecular Epidemiology of Foot-and-Mouth Disease virus in West Africa. 2000. Southern and Eastern African Division of C.L. Davis DVM Foundation, 4th Veterinary Pathology Symposium on African Epizootic Animal Diseases. National Faculty of Veterinary Science, Pretoria, South Africa, 9-13 October (Talk).

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LIST OF ABBREVIATIONS

(A/V)EET	(alanine/valine) glutamic acid-glutamic acid-threonine
A (Q/R)	Alanine (glutamine/arginine)
ALG	Algeria
ANG	Angola
BFK	Burkina Faso
BOT	Botswana
bp	base pair
cDNA	complementary DNA
CPE	Cytopathic effect
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
ddATP	dideoxyadenosine triphosphate
ddH_2O	deionized distilled water
dDTTP	deoxythymidine triphosphate
dGTP	deoxyguanidine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside-5'-triphosphate
EDD	Exotic Diseases Division
EMCV	encephalomyocarditis virus
ERI	Eritrea
EtOH	ethanol
e.g.	for example
FAO	world food and agricultural organization
Fig.	figure
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
g	gram
GAM	Gambia
GHA	Ghana
IVY	Ivory coast

KEN	Kenya
KQ/LC	lysine-glycine/leucine-cysteine
LBR	Liberia
LCV	Laboratoire Central Veterinaire
M	molar
MAI	Mali
MAL	Malawi
$MgCl_2$	magnesium chloride
min	minute
ml	millilitre
mM	millimolar
NaAc	sodium acetate
NCR	non-coding region
NEDT	arparagine-glutamic acid-aspartic acid-threonine
NGR	Niger
NIG	Nigeria
nt	nucleotide
^oC	degrees Celsius
OIE	Organisation International des Epizooties
OVI	Onderstepoort Veterinary Institute
P (I/T)	proline (isoleucine/threonine)
PCR	polymerase chain reaction
pmol	picomole
poly(A)	poly-adenosine
poly(U)	poly-uridine
PVE (N/S)	proline-valine-glutamic acid (asparagine/serine)
RE/QS	arginine glutamic acid/ glutamine serine
RGD	arginine-glycine-aspartic acid
RI	replicative intermediate
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute

RQ/TC	arginine glutamine/ threonine cysteine
SAR	South Africa Republic
SAT	southern African Territories
SDS	sodium dodecyl sulphate
sec	second
SEN	Senegal
TN	threonine-asparagine
Tris	Tris-hydroxymethyl-aminomethane
TTLV	threonine-threonine-leucine-valine
U	unit
UPGMA	unweighted pair-group method using arithmetic average
UV	ultra-violet
VP	virus protein
VSLI	valine-serine-leucine-isoleucine
WRL	world reference laboratory
ZAI	Democratic Republic of Congo
µg	microgram
µl	microlitre
µM	micromolar

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Foot-and-mouth disease virus (FMDV) was identified by Loeffler and Frosch in 1898 as the first filtrable viral agent to cause animal disease. It is a highly contagious virus affecting over 60 species of cloven-hoofed domestic and wild animals (Hedger, 1981). The disease has a high morbidity and mortality is rare in adult animals, however myocarditis may occur in young animals resulting in death. The recovered animals remain in poor physical condition over long periods of time leading to sustained economic losses for the livestock industry.

An understanding of the epidemiology of the disease is critical for the implementation of good control programmes and the eradication of the disease. An important part of combating foot-and-mouth disease (FMD) is virus characterization, where the relationships between field isolates using reference and historical viruses are used to investigate the possible origins of the disease. Unlike the southern region and part of central and East Africa, little is known about the FMD situation in West Africa. In addition, epidemiological studies in southern Africa were focused on the southern African Territories (SAT) types and little attention was paid to the European FMD virus serotypes on the continent (Vosloo *et al.,* 1992, 1995 &1996; Bastos *et al.,* 1998, 1999, 2000 & 2001; Esterhuysen, 1994 & 1985; Van Rensburg & Nel, 1999).

The lack of interest in FMD virus (FMDV) within west African countries was due to the prevalence of other major animal diseases such as rinderpest (Mukhopadhyay *et al.*, 1999). The recent FMD outbreaks in the United Kingdom and the evidence of trans-continental transmission of the virus (Samuel & Knowles, 2001) have highlighted the need for worldwide collaborative programmes to control the spread of the virus.

1. 2 Picornaviridae family

Viruses which are insensitive to ether, chloroform, non-ionic detergents and which have viral ribonucleic acid (RNA) functioning as messenger RNA or as template for progeny RNA are classified as members of the *picornavirus* family (Melnick *et al.,* 1975). Viruses within this family cause diseases of medical and

agricultural importance, e.g. poliovirus, common cold virus, human hepatitis A virus and FMDV.

The family consists of 20 virus species divided into nine genera and is summarized in Table 1.1 (Pringle, 1999):

Cardiovirus: encephalomyocarditis virus (Columbia SK virus, mengovirus, mouse Elberfield virus), *Theiler's murine*: encephalomyelitis virus (murine poliovirus).

Enterovirus (human polyoviruses, human enteroviruses, bovine enteroviruses, porcine enteroviruses, simian enterovirus and human coxsackieviruses (A1 to A22, A24, and B1 to B6).

Hepatovirus: hepatitis A virus and simian hepatitis A virus. The viruses are very stable at acid pH and high temperature (60^{o}C).

Rhinovirus: major cause of human common cold virus (100 human serotypes) and other upper and lower respiratory tract infections, and bovine rhinoviruses. The viruses are inactivated below pH 5.

Kobuvirus: (Aichi virus)

Teschovirus: (e.g. teschen disease virus of pigs),

Erbovirus, *parechovirus*.

Aphthovirus: foot-and-mouth disease virus is the prototype of the genus Aphthovirus. The aphthoviruses are named for the vesicular lesions that they produce in cloven-hoofed animals (Melnick, 1983). Foot-and-mouth-disease viruses are unstable below pH 7. Equine rhinovirus is also an aphthovirus.

1.3 Properties of foot-and-mouth disease virus

1.3.1 Morphology & biophysical characteristics of foot-and-mouth disease virus

The name picornavirus comes from the word "pico" which means very small and "rna" for the genome type. They are among the smallest known RNA viruses and have a naked, ether-resistant, icosahedral protein shell with a symmetry of 22-30 nm in diameter (Melnick *et al.,* 1975; Cooper *et al.*, 1978).

In acidic conditions the FMDV particles are disrupted into pentameric subunits composed of five copies each of the virus structural capsid proteins (VP1-3) with the liberation of the internal capsid protein (VP4) and the RNA. FMDV is also unstable at pH > 11 and when treated by heat or by gamma radiation loose infectivity for susceptible cells (Newman *et al.,* 1973; Acharya *et al.,* 1990).

1.3.2 Genome structure

The genome of FMDV consists of a positive sense single stranded RNA molecule of about 8500 nucleotides in length. The 5' non-coding region (NCR) is exceptionally long (about 1300 nt) and has a

virus encoded protein, 3B, called virus protein genome (VPg) attached to the 5' end (Fig. 1.1a & b) (Sangar *et al.*, 1977). The protein 3B occurs in three different forms, 3B1, 3B2 and 3B3 (Sangar *et al.*, 1977; Belsham, 1993). The aphthoviruses are unique in having these three similar, but not identical, VPg-encoding genes in tandem (Palmenberg, 1987). The 5' non-coding region consists of three segments: the S fragment (400 bases in length) is capable of forming a large hairpin structure (Newton *et al., 1986*), the cytidyl (poly[C]) tract (50-100 nt) followed by a non-translated segment (about 720 bases) which is predicted to form pseudo-knots (Clarke *et al.,* 1987). It has a poly [A] tail of about 50-100 nt at the 3' end (Bachrach, 1977). At the 3' end, there is also a short 3' NCR of 89 bases of which the function is not known.

The main portion of the virus genome is a single very large open reading frame of 6996 nucleotides encoding a polyprotein of 2332 amino acids (for serotype O) (Forss *et al.*, 1984). The polyprotein can be divided into four elements: L, P1-2A, P2 and P3 (Fig. 1.1b). Two initiation sites have been identified (AUG codons) resulting in two forms of the leader protein (Lab & Lb) in FMD virus (Sangar *et al.,* 1988).

The structural protein precursor (P1) is preceded by a short leader protein (L) which cleaves itself first from the P1 precursor followed by subsequent cleavages achieved by 3C which is a virus proteolytic enzyme (Doel *et al.,* 1978; Brown, 1981; Strebel & Beck, 1986). It is more likely that self-cleavage occur between 1A/1B. The 1A/1B cleavage occurs at a late stage in virus morphogenesis and is concomitant with encapsidation of the virion RNA. A second cleavage takes place at the junction between 2A/2B and results in the P1-2A polypeptide (Fig. 1.1b).

The poly[A] tract (named An) has been associated with infectivity of poliovirus, but the role of poly [C] remains unclear in FMD virus (Brown, 1976). However, studies have indicated that the truncation of the poly [C] tract reduce virulence in the natural hosts of mengovirus and poliovirus (Iizuka *et al.,* 1989; Duke *et al.*, 1990).

Table 1.1 The family of *Picornaviridae*

Genus	Number of serotypes	Members/Species	pH stability
Aphthovirus	7	Foot-and-mouth disease virus	Labile < 7
	1	Equine rhinovirus previously known as equine rhinovirus type 1	
Rhinovirus	102	Human rhinoviruses	Labile < 5
	3	Bovine rhinoviruses	
Enterovirus	3	Human polioviruses	Stable 3-9
	23	Human Coxsackieviruses A	
	6	Human Coxsackieviurses B	
	30	Human echoviruses	
	4	Human enteroviruses	
	1	Vilyuisk virus	
	18	Simian enteroviruses	
	2	Bovine enteroviruses	
	7	Porcine enteroviruses	
Cardiovirus	1	Encephalomyocarditis (EMC)	Stable 3-9
	1	Theiler's murine encepholomyelitis (TME) virus	
Parechovirus	1	Human parechovirus type 1 described previously as Human echovirus type 22	
Hepatovirus	1	Human hepatitis virus A	Stable < 5
Kobuvirus:	1	Aichi virus	
Teschovirus:	1	Porcine teschovirus or Teschen disease virus of pigs known previously as Porcine enterovirus 1	
Erbovirus	1	Equine rhinovirus 2 described previously as Equine rhinitis B virus	

From Pringle, (1999).

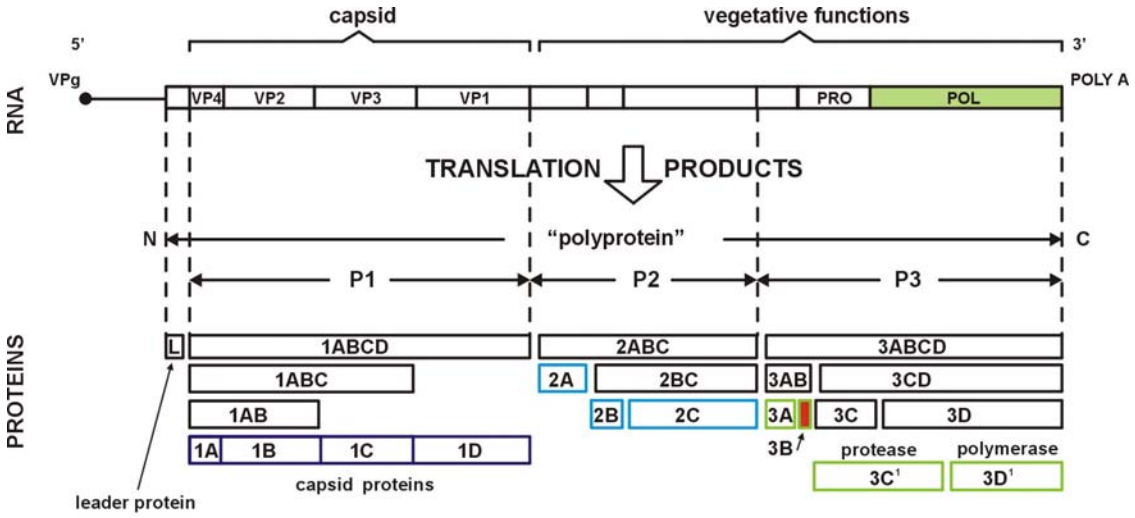


Figure 1.1a: Diagram of general structure of picornavirus genome with cleavage sites of the polyprotein adapted from Rueckert (1985).

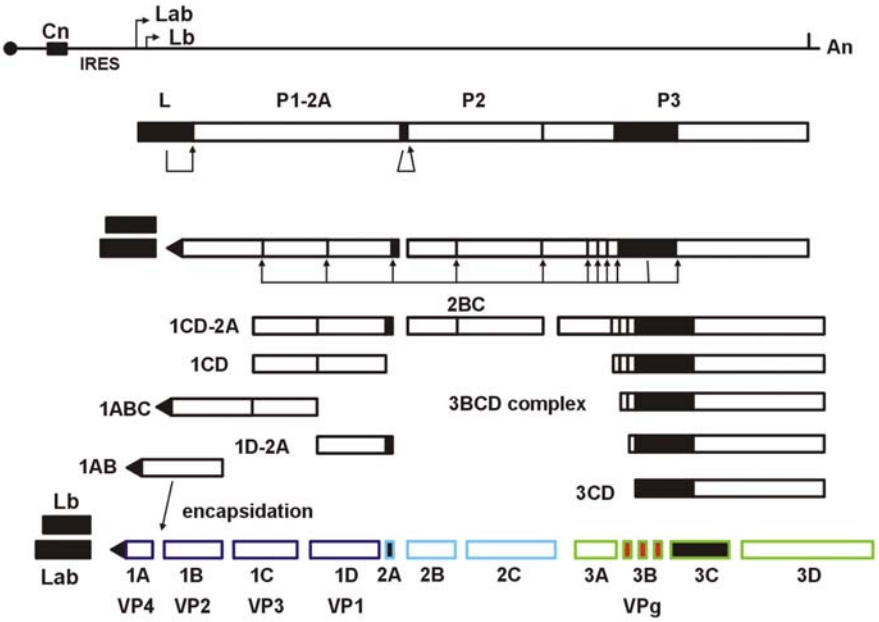


Figure 1.1b: The genome organization of FMD with cleavage sites of the polyprotein adapted from Belsham (1993)

1.3.3 Foot and mouth disease virus protein

Structural proteins

The P1-2A gene product is the precursor of the capsid proteins 1A, 1B, 1C, and 1D named viral proteins 1-4 (VP4, VP2, VP3, and VP1), while P2 and P3 are precursors to non-structural proteins (Fig. 1.1b) (Brown, 1976; Ryan *et al.,* 1989; Belsham, 1993). The genome segment P2 encodes for proteins 2B, 2C and 2A. While, the P3 region is a precursor of 3A, 3B, 3C and 3D. The protein 3C is the major protease and 3D is the RNA-dependent RNA polymerase or RNA replicase gene (Caliguiri, 1974).

The capsid is composed of the four major structural capsid proteins (VP1, VP2, VP3 & VP4) and contains 60 copies of each (Cooper *et al.,* 1978). Five copies of VP1 are clustered around the fivefold axis of symmetry; while VP2 and VP3 are positioned at the two and three-fold axis of symmetry (Acharya *et al.,* 1990) (Fig 1.2). These proteins are elements of identical four-segmented protein subunits called protomers which are defined as the smallest identical subunit of an oligomeric protein (Monod *et al.*, 1965).

To establish the icosahedral symmetry structure in the virus capsid the structural proteins are usually assembled as 12 pentamers (Fig. 1.2). The basic building block of the icosahedral capsid is a pentamer made up of five copies of VP1 to VP4. The pentamers are stable through the interactions involving the N and C terminus of VP1 and VP3 along with VP4. These three proteins together with VP2 form the protomeric subunit and adjacent pentamers are held together by hydrogen bonds between parts of VP2 and VP3. It has been reported that the relative weakness of these interactions may facilitate the uncoating of the viruses during replication (Stanway, 1990).

FMD viruses have a high concentration of histidine residues lining the pentamer interfaces that also play a major role in virus instability at acidic pH (Stanway, 1990). Heat or acid treatment of the virus disrupts the interactions between VP2 and VP3 and the pentameric interfaces resulting in pentamer dissociation with release of the internal capsid protein VP4 and the RNA genome.

VP1, VP2 and VP3 are situated at the surface of the FMD virus. The smallest capsid protein, VP4, is internal and can be thought of as an N-terminal extension of VP2 which is cleaved from the VP2/VP4 precursor at the final stage of maturation of the virus particle. The VP4 protein interacts with the viral RNA (Strohmaier & Adam, 1974; Chow *et al.,* 1987; Acharya *et al.,* 1989). VP1 is the most important protein for epidemiological studies of FMD viruses and will be discussed in section 1.4 in more detail. VP3 is the most conserved surface exposed structural protein among different FMD viruses (Acharya *et*

al., 1990).

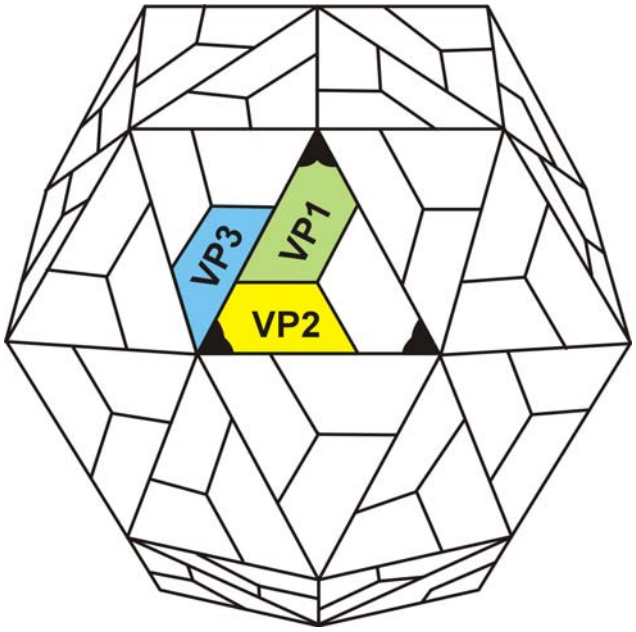


Fig. 1.2 Arrangement of surface proteins VP1, VP2 and VP3 in the picornaviral shell. Fields Virology (1996)

Non-structural proteins

The P2 and P3 precursors are processed into non-structural proteins which are involved in virus RNA replication and protein processing (Fig.1.1b) (Sanger, 1979; Forss *et al.*, 1984; Acharya *et al.*, 1989; Belsham, 1993). The genome segments P2 and P3 encodes the non-structural proteins 2A, 2B, 2C and 3A, 3B, 3C, 3D, respectively (Ryan *et al.*, 1989). The protein 2A is a protease and cleaves itself liberating the precursor of the capsid proteins whilst 3C carries out the majority of the processing of the polyprotein (Strebel & Beck, 1986; Stanway, 1990). The protein 3D is the RNA-dependent RNA polymerase and is required for the replicative intermediate (RI) stage described under the virus replication section (1.6). The role of the proteins 2B, 2C, and 3A still remains unclear.

1.4 Properties and functions of the VP1 protein

1.4.1 Importance of VP1 protein in the antigenicity of FMD virus

The antigenic diversity of FMD viruses has made the diagnosis and control of these viruses very difficult

in countries where the disease is endemic. Several studies have shown that VP1 (encoded by 1D) is the most important protein in FMD virus both for its antigenic properties and as the virus-cell attachment site.

Two regions of VP1, amino acids 140-160 and 200-213, have been shown to induce antibodies involved in neutralization of viral infectivity (Bittle *et al.,* 1982; Pfaff *et al.,* 1982; Strohmaier *et al.*, 1982). The electron density map has also revealed that the immunogenic site (140-154) is exposed on the surface of the virus particle and located at a highly disordered region (GH-loop) of the capsid (Acharya *et al.,* 1989), while the C- terminus antigenic residues (200-213) are highly ordered. Several subsequent studies have shown that purified VP1 alone, can elicit neutralizing and protective antibodies in mice, guinea pigs, cattle and pigs although the titre was low when compared when the whole virus particle was used (Laporte *et al.*, 1973; Bachrach *et al.*, 1975; Bittle *et al.,* 1982; Strohmaier *et al.,* 1982; Acharya *et al.*, 1989). The 201-213 sequence at the C-terminus also elicited neutralizing antibody but the levels were lower than those obtained with the 141-160 (G-H loop) sequence.

Treatment of the intact virus particle with proteolytic enzymes, such as trypsin leads to the loss of infectivity and immunogenic activities (Wild *et al.*, 1969, Rowlands *et al.*, 1971). Since VP1 is the only protein cleaved by trypsin it is believed to be responsible for both immunological and infectivity functions (Laporte *et al.,* 1973; Bachrach *et al.,* 1975; Bittle *et al.,* 1982).

Evidence of an antigenic site outside the 1D region was demonstrated in FMD virus mutants that were not neutralized by antibodies directed against the VP1 protein (Thomas *et al.*, 1988). Several studies have shown that mutations at the critical amino acid residues in 1D (VP1) can result in antigenic variations in FMD viruses. It is well documented that some substitutions, e.g. Leu□Ile at 154 or 208 of VP1 greatly reduce the antibody binding activity to FMD virus whereas a change at position 148 results in a complete resistance to neutralization using a monoclonal antibodies (Mab) (Geysen *et al.*, 1985; Sobrino *et al.*, 1986; Xie *et al.*, 1987; Domingo *et al.*, 1992).

Studies of type O1 Caseros and O1 Kaufbeuren using Mab have allowed the identification of four non-overlapping neutralization sites and a fifth conformational site in FMD virus (Crowther et al., 1993; Aktas & Samuel, 2000). Antigenic site 1 is located in the middle of the VP1 gene at amino acid position 140-160 (O1 Kaufbeuren). Antigenic site 2 involves the C-terminal segment of VP1 and VP2 and antigenic site 3 resides around the fivefold axis of the O1 Kaufbeuren type. This site is dependent on conformation.

Antigenic site four is located close to the threefold axis of FMD virus and involves VP2 and VP3. The fifth antigenic site is formed due to the interaction of the VP1 loop region with VP2 and VP3 capsid proteins (Kitson *et al.*, 1990; Crowther *et al.*, 1993; Lea *et al.*, 1994). The main immunogenic site 1 of FMD viruses is located on the 1D region which makes the VP1 region the gene of choice to study variation and relationships between isolates.

1.4.2 Importance of VP1 protein in virus-cell attachment

Several studies have reported that the RGD sequence within the G-H-loop of the VP1 is involved in attachment of the virus to susceptible cell receptors (Leippert *et al.*, 1997; Liebermann *et al.*, 1991). The cleavage of VP1 by trypsin highly reduces or abolishes the ability of the FMD virus to bind and infect susceptible cell cultures (Putnak *et al.*, 198l; Baxt *et al.,* 1989). Similar work conducted by Liebermann and co-workers (1991) on Type O1 Kaufbeuren reported that the highly conserved triplet, Arginine-Glycine-Aspartic acid (RGD) within the G-H-loop is responsible for binding of FMD virus to pig kidney cell receptors. FMD virus infection of susceptible cells is successfully blocked following the binding of antibodies directed against the RGD region as well as peptides representing part of its sequence. In addition, many studies have reported that the RGD binds to a large family of integrin receptors and many extracellular substrate proteins (Ruoslahti & Pierschbacher, 1987). Although, the RGD has been reported to play the main role in cell-virus attachment, it is not the sole element in the binding process since removal of the C-terminus of VP1 in the absence of cleavage within the FMD virus loop also affects the attachment (Fox *et al.*, 1989). Baranowski and co-workers (2000) also reported that on type C at least three different mechanisms for cell recognition by FMDV and suggest a potential for this virus to use multiple alternative receptors for entry even into the same cell type. These authors indicated that FMDV with RGG variants involves a mechanism of cell entry which does not implicate integrin $\alpha_v\beta_3$ or cell surface heparin.

Surprisingly, the highly conserved triplet, Arg-Gly-Asp at positions 145-147 amongst FMD viruses is flanked by the loop sequence, which is a hypervariable region both in composition and length between different FMD viruses (Sobrino *et al.*, 1986; Dopazo *et al.*, 1988; Plaff *et al.*, 1988). The sequence variation around the virus binding site is responsible for the antigenic diversity of the virus (Fry *et al.*, 1990; Logan *et al.*,1993). There is a hypothesis that this disordered region may affect the binding affinity to different integrin molecules on the cell surface. Therefore, it is believed that there is more than one host cell receptor for FMD virus partially amongst the integrin family since complete or incomplete

competition for cell receptors occurs in homologous situations as well as between viruses of different serotypes (Sekiguchi *et al.*, 1982). The FMD virus binding site to cell receptors is located within the most exposed site on the virus particle compared to other picornaviruses where it is found in a deep pocket on the virus surface, hidden from immune surveillance (Rossmann *et al.*, 1985).

1.5 Antigenic variation

The concept of antigenic variation came from the observation of Vallée & Carré in 1922 that an animal that has recovered from FMD virus infection can be re-infected and develops clinical signs. Subsequently, cross challenge experiments in cattle have allowed the classification of FMD virus into seven immunologically distinguishable serotypes: O, A which exist Asia, South America and Africa, and Asia-1 and the SAT types (South African Territories SAT1, SAT2 & SAT3) which occur in Asia and sub-Saharan Africa, respectively (Brooksby, 1982). Type C currently occurs only in East Africa (Thesis: Bastos, 2001).

Serological studies and the observation in the degree of virulence of the virus in recovered animals have shown that there are significant differences between strains within each serotype (subtypes) (Waldmann & Trautwein, 1926; Traub & Mohlmann, 1946; Brooksby, 1982). The study on field isolates of type C using a panel of monoclonal antibodies against virus C_1 and C_3 Indaial, identified several antigenic groups showing variation in FMD virus epitopes (Mateu *et al.*, 1987). Progress made in the understanding of the genetic differences underlying observed antigenic variation, has played a major role in the epidemiology of the disease. Today, nucleotide sequencing is routinely used to identify the genetic relationships between different isolates and historical strains. In this way the origin of a virus can be traced in outbreak conditions.

1.5.1 Mutations

Antigenic variation can be caused by nucleotide mutations or recombinations in the RNA viral genome. Studies revealed that the rates of mutations of the European serotype FMDV RNA genome can reach 10^{-2} substitutions per nucleotide site per year (s/n/y) (Gebauer *et al.*, 1988). Similar studies conducted on SAT1 and SAT2 FMD viruses have estimated nucleotide changes of 1,64 % and 1,54 %, respectively per year for the VP1 gene (Vosloo *et al.*, 1996). This rapid mutation rate is a million times greater than the rates in their natural hosts (10^{-8} to 10^{-9} nucleotide substitution per year) (Domingo *et al.*, 1990). These mutations may give rise to variant viruses that can be a source of new outbreaks (De La Torre *et al.*, 1985 & 1988; Domingo *et al.*, 1992; Vosloo *et al.*, 1992 & 1996).

The immunogenic character of the 140-160 and 193-203 amino acid regions of VP1 has been discussed previously (see 1.4.1). The results of studies on the sequence of naturally occurring antigenic variants of the A12 virus, have also revealed that the amino acid residues at positions 148 and 153 were altered in the variant viruses, suggesting that these residues were important in the antigenicity of FMD virus (Rowlands *et al.*, 1983). Mutations, that lead to conformational changes, produce a population of neutralizing escape variants (Stave *et al.*, 1988; Domingo *et al.*, 1992; Sanyal *et al.*, 1997).

1.5.2 Recombination

Mutations through recombination were first reported in picornaviruses following the replication of a mixture of mutants in the same cell monolayer (Hirst, 1962). Later, Pringle (1965) presented evidence of genetic recombination between immunologically distinct strains of FMD virus SAT2 (Kenya-3 and Rho-1) multiplying in the same tissue.

The mechanism of recombination in FMD virus has been described as the modification of the surface proteins due to segment crossing over during co-infection of the animal cells by more than one FMD virus serotype (King *et al.*, 1982; Krebs & Marquardt, 1992). Methods such as electrofocusing using radioactive labelled structural polypeptides or SDS-polyacrylamide gel electrophoresis with molecular markers were also used by King and co-workers (1985) to determine recombination between different subtype strains in the unsegmented genome of FMD virus.

Crossing over or reciprocal recombination involves the even exchange of homologous sequences. Non-reciprocal recombination, on the other hand, involves the uneven replacement of a sequence which results in the loss of one of the variant sequences involved in the recombination event. This procedure has been reported to be responsible for severe types of mutational change that may affect the susceptibility of the natural hosts (King *et al.*, 1980 & 1982).

1.5.3 Quasi-species concept

The quasi-species concept was proposed by Eigen in 1971. RNA viruses have genomes that replicate in the absence of repair mechanisms, they evolve very rapidly with a mutation frequency per nucleotide site of 10^{-3} to 10^{-5} substitutions per year (Van Regenmortel *et al.*, 1997). The high rate of error during RNA replication in the picornaviruses gives rise to a range of multiple co-circulating viral genomes within a

host. A dominant virus genome sequence called a master or consensus sequence in regard to the specific environment emerges and competes with the others in the virus population. This is defined as the quasi-species principle of RNA viruses (Eigen, 1971; Holland *et al.*, 1982; Sobrino *et al.*, 1986; Domingo *et al.*, 1992; Van Regenmortel *et al.*, 1997). At a certain time a shift or change in the equilibrium will alter the consensus sequence and cause one of the variants to dominate. FMDV populations exist as mixtures of related but non-identical genomes that can have a competitive potential, and generate a dominant variant in a viral population (Domingo *et al.*, 1992).

Continual modification of the genome of FMD viruses isolated from persistently infected cattle and buffalo have been reported *(*Malirat *et al.*, 1994; Dawe *et al.*, 1994a; Vosloo *et al.*, 1996). Antigenic changes occur following replication of a virulent FMD virus in partially immune populations of cattle (Hyslop & Fagg, 1965; Hedger, 1968) and a large number of genetic and phenotypic variants have also been generated after limited replication in cell cultures (Sobrino *et al.*, 1983).

1.6 Virus replication

In the initial event of the replication process, FMD virus uses among others picornaviruses its highly conserved triplet sequence (Arg-Gly-Asp) motif on the G-H loop to attach to specific receptors on the cell membrane (Fox *et al.*, 1989; Mateu *et al.*, 1996). These receptors mediate the release of the viral genome from the protein shell into the cytoplasm. The incoming RNA uses the host cell protein-synthesizing machinery causing shut down of host cell replication. Complementary negative (-) RNA strand synthesis of the positive (+) RNA strand is initiated by a virally-encoded RNA polymerase. Further synthesis of (+) RNA strands leads to the formation of multi-stranded replicative intermediates (RI) with a 3' poly [A] which are transcribed from the poly [U] tract in the RI. The RI generates a pool of (+) RNA for translation and some for synthesis of additional (-) RNA. As the protein level increases, some (+) RNA are packaged into virions (Rueckert, 1985).

Proteolytic cleavages occur during shell assembly in a polyprotein precursor of structural and non-structural proteins. The precursor coat polyprotein is cleaved into three proteins (VP0, VP3 & VP1) by a viral coded protease. Protein VP0 is later cleaved to VP4 and VP2 in the maturation process. Complete virus particles are released by cell lysis (Rueckert, 1985;Belsham, 1993). The cycle is completed in 5 to 10 hours.

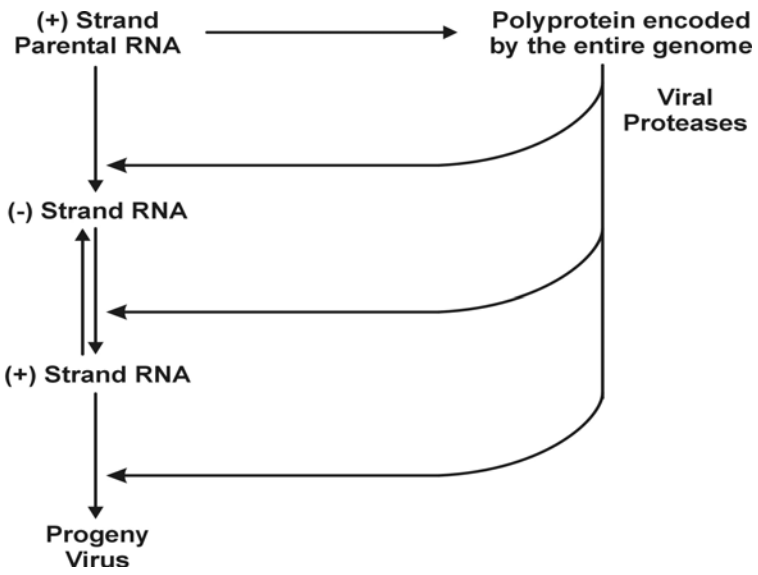


Fig. 1.3 Flow of events during the replication of positive strand RNA viruses that code for a single genome-sized(+) RNA. (Adapted from Fields Virology, 1996).

1.7 Epidemiolgy

1.7.1 Distribution

FMD has occurred in most areas of the world except Greenland, New Zealand, and the smaller islands of Oceania. Australia has not experienced an outbreak since 1870; the United States of America since 1929; Canada since 1952 and Mexico since 1954 (Samuel & Knowles, 2001). Europe has reported a number of sporadic outbreaks since the cessation of vaccination in 1990-1991. These outbreaks occurred in Bulgaria (1991, 1993 and 1996), Italy (1993), Greece (1994, 1996 and 2000), Turkish Thrace (1995 and 1996), Albania (1996), the former Yugoslav Republic of Macedonia (1996), and the Former Soviet Republics of Georgia and Armenia (1997) (Kitching, 1998; Samuel & Knowles, 2001). Europe lost its status of disease free continent following the type O outbreaks (2001) in England, Ireland, The Netherlands, and France. However, all these countries have regained their OIE disease free status again.

Type O currently poses the greatest threat for livestock production worldwide (Kitching, 1998; Samuel & Knowles, 2001a & b) due to its predominance in outbreaks. In the Middle East and Indian subcontinent, type O is endemic (Samuel & Knowles, 2001). Type O has also been reported to be the most prevalent

serotype in Abu Dhabi (United Arab Emirates) (Moustafa & Gadir, 1993). Although Israel is less successful in the eradication of the disease because of the nomadic customs in the region, they succeeded in controlling the disease with only a few isolated outbreaks in 1992, 1993 and 1994 caused by type O being reported (Stram *et al.*, 1995). On the African continent, the disease-free status from domestic animals in the Republic of South Africa has been hampered by the recent type O outbreak in the Kwa Zulu Natal province (Sangare *et al.*, 2001). In East Africa, type O is endemic in Egypt and was also involved in the majority of outbreaks in Ethiopia in 1992-1993 (Roeder *et al.*, 1994). In North Africa, type O outbreaks were reported in 1989 for the first time in Tunisia and spread into Algeria and Morocco. The disease is also endemic in Lybia (Samuel & Knowles, 2001). In West Africa, type O was involved in outbreaks in Burkina Faso in 1992, in Ghana in 1993 and in Mali in 1998 (Reports of the FAO/OIE; Sangare *et al.*, 2001).

The guarantee of freedom from FMD in Europe has been threatened by type A outbreaks in 1995 in Turkey, in 1996 in Albania and Macedonia (Kitching, 1998). In South America, type A outbreaks in 1997 were reported in Bolivia, Colombia, Ecuador and Brazil and in the Middle East in Saudi Arabia in 1991, 1992, 1993 and 1995, in Malaysia in 1995 (Kitching, 1998). Type A, was probably introduced to the southern African region (Thomson, 1994). This belief is supported by studies on the molecular comparison of isolates where recent isolates from Africa namely Algeria, Angola, Morocco and Malawi grouped with historical viruses from Europe and South America (Knowles *et al.*, 1998). Serotype A has now been reported in several countries (Eritrea and Gambia in 1998, Senegal, Mauritania and Mali in 1997, Kenya and Ghana in 1996, Cote d'Ivoire in 1995) across the African continent (Knowles *et al.*, 1998).

Serotype Asia-1 is confined to Asia and has also been isolated in Saudi Arabia in the middle East. The presence of these exotic serotypes in Saudi Arabia is certainly due to the fact that the country imports annually about 6.5 million live animals, mainly sheep and goats, from Africa, Asia and Australasia (Kitching, 1998).

SAT-1 occurs mainly in southern, East and Central Africa (Bastos *et al.*, 2001). The SAT-1 type has been reported in only two west African countries namely Nigeria and Niger between 1975-1981(FAO/OIE, WRL, Chapter 4, this thesis). SAT-1, is also the first SAT type to have made incursions into the Middle East (Pereira, 1981).

Prior to 1992, SAT-2 was the serotype most frequently involved in outbreaks in west African countries (Ghana and Ivory Coast in 1990, Ghana and Mali in 1991) (OIE/FAO, WRL; Chapter 3). It has also been reported regularly in outbreak conditions in southern and eastern Africa (Thomson, 1994). The first introduction of SAT-2 into Saudi Arabia and Kuwait was reported in 2000 (http://www.oie.int).

Although, SAT-3 exists in southern Africa, it has not been reported in West Africa. Studies have indicated that out of the ten southern African countries, SAT-3 occurs in Zimbabwe, Namibia, Zambia, Botswana and Malawi and type C has been reported only in Mozambique, northern Namibia and Malawi (Bastos, Thesis 2001).

Serotype C has not been identified in West Africa. Currently, this serotype is confined to three countries on the African continent namely Angola, Ethiopia and Kenya. Previous study revealed a probable link between Kenyan type C isolates in 1996 and the Middle East specific topotype (Reid *et al.*, 2001). Reporting of FMD from many countries in Africa is occasional and may therefore not reflect the true distribution of FMD in Africa. Further genetic characterization is thus needed to assist in clarifying the epidemiology of FMD on the continent.

Foot-and-mouth disease is endemic in most countries in sub-Sahara Africa such as Tanzania, Malawi, Zaire and Angola. The virus is confined primarily to wildlife in Zimbabwe, Botswana, Namibia and the Republic of South Africa. In East Africa, five serotypes, A, O, C, SAT1 and SAT2 have been reported in Kenya alone (Roeder *et al.*, 1994). In West Africa at least two or more serotypes exist in each country where FMD virus was isolated (Fig. 1.4). Madagascar is free of FMD. Due to the lack of serological data, the total number of countries infected by the virus is probably higher than presently verified.

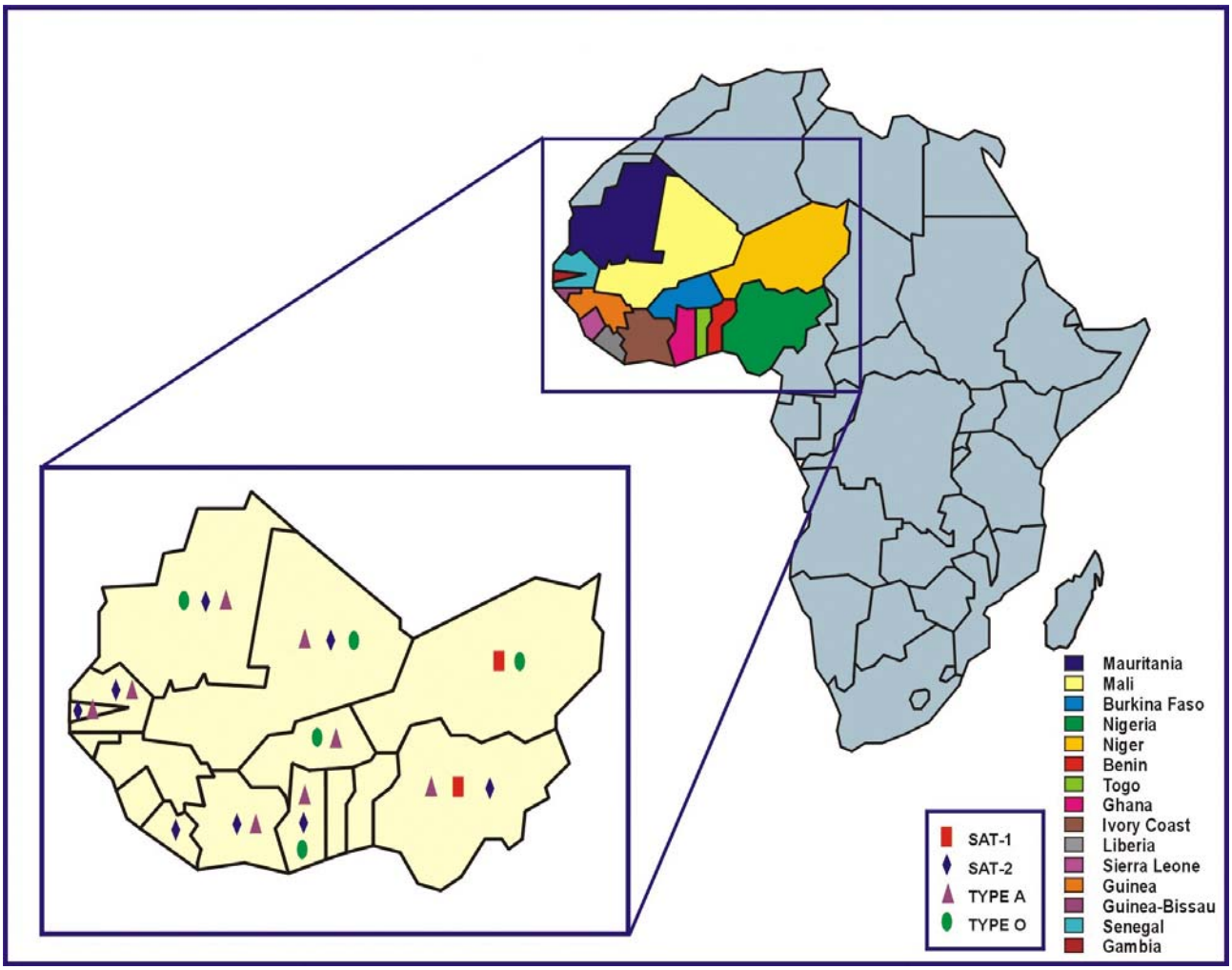


Fig 1.4 FMD distribution in West Africa

1.8 Foot and mouth disease

1.8.1 Clinical signs of the disease

The disease is highly contagious and known as one of the most economically devastating diseases of livestock (Brooksby, 1982). The incubation period in natural infections is usually between 2 and 3 days and could be as long as 14 days in cattle and lasts for 4 to 8 days in sows (Burrows *et al.*, 1981; Donaldson *et al.*, 1984). The incubation time may be even shorter in experimental infections. A rise in temperature (41^0 C - 42^0 C) occurs a day before the first appearance of mouth and feet lesions and viraemia in cattle lasts for 3-5 days (Cottral & Bachrach, 1968). The lesions develop 2-14 days post infection. The common clinical signs of the disease are vesicle formation on the mucous membranes of the tongue, lips,

interdigital spaces and on the coronary bands which make animals reluctant to eat or move (Geering, 1967; Brooksby, 1 982; Woodbury, 1995). However, mouth lesions are less common and less pronounced in other species such as sheep and pigs. Commonly in sheep and other small ruminants lesions occur on the dental pad, where they may be difficult to detect (Thomson, 1994). Mortality in adult animals is rare, while in young animals, death can occur due to myocarditis and mortality may exceed 50% in calves (Bachrach, 1977; Donaldson *et al.*, 1984; Mckercher *et al.*, 1985; Woodbury, 1995). Morbidity can approach 100% (Woodbury, 1995; Salt *et al.,* 1996). A chronic panting syndrome characterized by dyspnoea, anaemia, hair overgrowth, and lack of heat tolerance has been reported as a sequela in cattle (Geering, 1967).

1.8.2 Pathology

Foot-and-mouth disease virus may be detected in oesophageal/pharyngeal fluids and lymph nodes which constitute the site of primary replication after infection by the respiratory tract through inhalation (Burrows *et al.*, 1981). The lesions on the dental pad and tongue appear as reddened areas and progress within a few hours into vesicles. The vesicles are easily ruptured within 24 hours leaving a raw surface and healing occurs within one to two weeks of rupture. Lesions at interdigital areas occur and animals can loose their hooves in severe cases (Geering, 1967; Donaldson *et al.*, 1984). There has also been supportive evidence that FMD virus replicates in the bovine mammary gland and mastitis may occur due to secondary bacterial infection. Moreover, histological studies have revealed the presence of clumps of necrotic secretory epithelial cells in the mammary gland alveolar tissue. A week after the onset of the disease in cattle, an increase in the number of alveoli containing necrotic cells, and luminal exocytosis of all alveoli occurs with concomitant increase in non-secretory areas (Blackwell *et al.*, 1981 & 1983).

1.8.3 Susceptible host range

Aphthoviruses infect at least 200 species of mammals (Murphy *et al.*, 1995). The clinical and pathological expression of FMD varies among different species ranging from sub-clinical or mild to acute disease. FMD viruses infect cattle, swine, sheep, goats and most other cloven-hoofed animals (Cooper *et al.*, 1978). The susceptible hosts of FMD viruses are cattle and swine. Infection in sheep and goats in endemic areas is usually silent or sub-clinical, making sheep and goats potential reservoirs for spreading the virus (McVicar & Sutmoller, 1968; Falconer, 1972). However, severe clinical cases may also occur in sheep where the variation in clinical expression is due to several factors such as the strain of virus, the breed of sheep and environmental and stress conditions (Geering, 1967). FMD also affects wild animals such as

buffalo (*Syncerus caffer*), eland (*Taurotragus oryx*), and impala (*Aepyceros melampus*) (Hedger *et al.*, 1980; Bastos *et al.*, 1999; Vosloo *et al.*, 1996). Clinical disease in African elephants (*Loxodona africana*) is extremely rare (Howell *et al.*, 1973). It has been reported more frequently in the Indian elephant. Amongst wildlife, the disease can be severe or subclinical in impala making the impala a possible transmission route of FMD virus from buffalo to cattle (Bastos *et al.*, 2000).

1.8.4 Carrier animals

A carrier status is applied to an animal from which infectious FMD virus could be recovered 28 days after infection (Dawe *et al.,* 1994a & 1994b; Woodbury, 1995). Cattle were first identified as carriers by Van Bekkum *et al.*, (1959) while Hedger, (1968), later reported that cattle can carry FMD virus in the oropharyngeal areas for up to 2 and half years. The development of the carrier state in cattle occurs in both clinical and sub-clinical infections with FMD virus (Salt *et al.,* 1996). Studies have shown that there is a high incidence of sub-clinical infection in cattle in endemic areas (Malirat *et al.,* 1994). The carrier state has been attributed to a defective immune response in the animal or incomplete virus replication due to poor enzyme production. Cross species infection is also believed to play a role in carrier status (Rina & Martin, 1976; De La Torre *et al.,* 1988; Salt, 1993; Woodbury, 1995). Among livestock, cattle and pigs are regarded as the primary source for FMD virus transmission to other susceptible animals, followed by sheep in which a carrier status of up to 9 months has been recorded (McVicar & Sutmoller, 1968).

African buffalo can carry FMD virus without disease up to 5 years (Sutmoller & Gaggero, 1965) and experimentally infected buffalo developed disease with or without clinical lesions (Young *et al.*, 1972; Anderson *et al.*, 1979; Gainaru *et al.*, 1986). This contributes to the buffalo being important amplifiers and a big threat in spreading the virus to domestic livestock populations (Sutmoller & Gaggero, 1965; Gainaru *et al.*, 1986; Hedger, 1972; Vosloo *et al.,* 1996). Studies have reported the natural transmission of FMD virus between buffalo and impala *(Aepyceros melampus)* (Bastos *et al.*, 2000) and have proposed that impala can act as intermediaries in disease transmission between buffalo and cattle (Hargreaves *et al.*, 2001).

Antibodies to FMD virus have also been detected in other wildlife such as waterbuck (*Kobus ellipsiprymnus*), kudu (*Tragelaphus stresiceros*), sable (*Hippotragus niger*), and eland (*Taurotragus oryx*) (Anderson, 1993), with only the kudu being shown to have the carrier status (Hedger, 1972).

Besides living animals, FMD virus can also establish a persistent infection in cell cultures which undergo morphological and physiological changes (Rina & Martin, 1976; De La Torre *et al.*, 1989).

1.8.5 Transmission

The virus is transmitted by direct contact, from contaminated secretions, body fluids, contaminated fomites, animal products (e.g. meat, milk, wool), people, vehicles and inhalation of airborne virus (Cooper *et al.*, 1978; Brooksby, 1982, Woodbury, 1995).

1.8.6 Socio-economical impact of the disease

Although mortality is rare in adult cattle, FMD virus causes an economically devastating disease of cloven-hoofed animals. Studies have estimated 25% loss of productivity of animals following infection e.g. reduction or loss of milk production in lactating animals and the deterioration in body conditions (Bachrach, 1968).

The loss in animal production and international trade restrictions imposed following an outbreak make FMD of major concern for livestock owners. The control of an outbreak (slaughter of infected and in contact animals, disposal of carcasses in disease free zones) and the loss due to the ban on livestock exports cost several million US dollars for a single outbreak (Sellers & Daggupaty, 1990). The cost of the UK epidemic in 2001 was estimated to be more than US $29 billion (Samuel & Knowles, 2001).

1.9 Control of foot-and-mouth disease

Since 1909 research institutes have been established to develop new strategies to control FMD (Brooksby, 1982). The knowledge of the virus replication on cell culture, the identification of the main immunogenic sites of the virus and the fact that RGD is a critical receptor binding site have contributed to better understand and develop strategies to control the disease (Stanway, 1990; Brown, 1999). Amongst these strategies are the selection of appropriate vaccine strains, vaccination and re-vaccination policies, improved techniques in vaccine production and identification of FMD virus infected zones, ongoing serological surveillance, and improved information technologies (Garland, 1999). However, recent FMD pandemics in Asia, the Middle East, Africa and outbreaks in Europe have again emphasized the difficulties of controlling FMD because of the emergence of new strains due to the high rate of mutation of the virus (Kitching, 1998).

1.9.1 Vaccine and vaccination

The discovery of a Baby Hamster Kidney (BHK) cell line which was found susceptible to FMDV opened new areas in vaccine production resulting in better control of the disease (MacPherson & Stocker, 1962). Vaccination is essential for controlling FMD virus in endemic areas. If vaccination is to be used to control the disease, the vaccine and the outbreak strains must be antigenically similar. The outbreak strain is compared directly with the existing vaccine strains using serum from bovines 21 days after vaccination and antibodies are compared in this way. In addition, the vaccine must contain all the serotypes that are circulating in the field and should induce protective immunity against each type of the vaccine components. This is very important since studies have reported that the immune response to some serotypes such as SAT2, when injected in combination with other FMD vaccine types was poor compared to the response when injected on its own (Dawe & Pinto, 1978).

Vaccination against FMD virus is achieved with inactivated vaccines. These vaccines are proven to be effective however there is danger of incomplete inactivation (Beck & Strohmaier, 1987; Knowles *et al.*, 1988). In addition, FMD virus vaccines require appropriate handling because they are thermally unstable.

Several encouraging studies for the production of chimeric (antigen called antigenic chimeras contains one or more antigenic sites from one virus substituted by a sequence corresponding to an antigenic site from another virus) vaccine have been reported, however, none of the bio-engineered FMD vaccines has produced full protection in cattle against challenge compared to inactivated vaccines. Many problems related to inactivated vaccines would be overcome by the use of chimeric vaccines but effective chimeric vaccines are presently not available.

1.9.2 Control strategy in endemic and disease free zones

Vaccination against FMD virus has been used successfully to help in the eradication of the disease in some parts of the world. However, FMD is still endemic in many countries in Africa and Asia. In 1996, alone, Kamer et al., (1997) have reported about 2000 type A and O outbreaks from 44 countries (cited by Freiberg et al., 1999). The first aim of disease control is the local reduction of incidence of the infection by vaccination, movement restriction of animals and regular inspection of cloven-hoofed animals (Brown, 1981; Woodbury, 1995). In endemic areas, vaccination is very costly because the duration of immunity induced by the FMD vaccine is short and two to three vaccinations are required annually to maintain high levels of neutralizing antibodies in susceptible animals (Garland, 1999).

In countries free from FMD, stamping-out of infected and in contact animals is favoured in the case of outbreaks. Severe restriction on the import of animals and animal products from countries where FMD virus infections occur are also implemented (Brown, 1981; Woodbury, 1995). To contain the disease in outbreak conditions in countries from the disease with regard to the recent UK conditions, free ring vaccination around infected areas is required and precautions should also be taken to destroy the vaccinated animals (Samuel & Knowles, 2001). Unlike parts of Europe and South America, eradication of the disease in Africa is presently impossible because domestic and wild animals are sharing the grazing areas in many African countries (Anderson *et al.*, 1979; Thomson, 1995).

1.9.3 Immune response

The protection of a susceptible host against FMD virus correlates with the neutralizing antibody level (Brooksby, 1982). Infection with one serotype produces complete protection against homologous virus, but little or no protection against heterologous viruses (Samina *et al.*, 1998). Serotype-specific immunity is based on the presence of neutralizing antibodies to one of the viral capsid proteins, VP1. However, recent studies on FMD virus type O have reported that none of the three main antigenic sites (G-H loop and carboxy terminus of VP1 contribute to site 1, amino acids at positions 31, 70-73, 75 and 77 of VP2 form site 2, and site 3 consists of residues 43 and 44 of the G-H loop of VP1) can be considered immunodominant following vaccination with FMDV type O in cattle or sheep (Aggrawal & Barnett, 2002) since they showed a similar response to all three sites in their anti-FMDV polyclonal response.

Serum-specific neutralizing antibodies develop after exposure to virus and peak at 7 to 21 days p.i. At the early stage of infection early antibodies can cross-react with heterologous viruses but antibodies become more specific from 14-21 days post infection. Samina *et al.* (1998) reported that inoculation of a multivalent vaccine induces a cross reaction to at least one heterologous virus that was not present in the vaccine. Generally, the immunoglobulin M (IgM) is most prevalent in the early convalescent serum and is less specific to the different serotypes than Immunoglobulin G (IgG). IgG is produced in the later stage during the FMD virus infection and the reaction between the serotype and the homologous antibodies is highly specific. An increase in IgG1 & IgG2 subclasses is observed in the serum of naturally infected animals as well as animals vaccinated with peptides. It has been reported that the healing of lesions and clinical recovery in infected animals would not occur until a few days after the IgG1 antibodies have developed (Mulcahy *et al.*, 1990).

The localized antibody response occurred in FMD virus infection in the mucosal secretions of the oropharyngeal area of the respiratory tract, the primary site of virus replication (Burrows *et al.*, 1981). Francis and co-workers (1983) have reported the production of specific anti-FMD IgM and IgA antibodies in the pharyngeal fluid of cattle 7 days after exposure to the virus, while IgG activity reached a peak in serum only 14-21 days after infection.

The age of individuals has also been shown to influence the antibody response against FMD virus. Nicholls *et al*., (1985) reported that calves deprived of maternal antibodies from one week to six months responded as well as, or better than 18 months old cattle to initial vaccination against FMD. In young pigs, the immune system is less mature and can only respond effectively to FMD vaccination 2 weeks after birth (Francis & Black, 1986). Some studies indicated failure of aqueous FMD vaccines to produce antibody in neonates with colostrally derived FMD virus antibodies. Späth *et al.*, (1995) have reported that calves as young as three weeks of age, with high maternal antibody levels responded to vaccination with both aqueous and oil adjuvant vaccine. However, there was no significant difference in the antibody level required for protection against the disease for young calves compared to adult animals (Nicholls *et al.*, 1985). The breed or other non-identified factors may also be involved in the antibody response to FMD virus infection. Pay & Parker (1977) reported that cattle in southern and Central Africa responded less to FMD vaccination than those in England (UK).

The influence of the route of virus entry on vaccination has been investigated. Mulcahy *et al.*, (1990) have reported that animals immunized with O1K peptide in incomplete oil adjuvant by subcutaneous route had a strong antibody response at 21 days p.i. In contrast, animals vaccinated intramuscularly responded more favourably than those injected by the intradermal route, but strong IgG1 responses were obtained in both cases (Mulcahy *et al.*, 1990).

The antigens used also influence the antibody responses e.g. the use of synthetic peptides from VP1 and inactivated viral vaccines have comparable capacity to neutralize the virus in *in vitro* experiments, but differ in their capability to confer protection to virus challenge *in vivo* (DiMarchi *et al.*, 1986).

Although serum antibody levels play an important role in host protection against FMD virus infection, the cellular response to FMD virus has also been investigated. Sanz-Parra *et al.*, (1998) have cloned the FMD

virus genes encoding the structural proteins in recombinant vaccinia virus and evaluated the immune response in guinea-pigs. They reported that FMDV polypeptides expressed by vaccinia virus recombinants allows the presentation of viral peptides to the major histocompatibility complex (MHC) molecules on the surface of infected cells. These peptides are recognized by T-cells and induce both humoral-and cellular-specific immune responses to FMD virus in guinea pigs (Bartels *et al.*, 1994; Sanz-Parra *et al.*, 1998). They also reported that these T-cells can recognize naive FMD virus and the viral epitopes in FMD virus infected cells, suggesting that T-helper and T-cytotoxic cells play a role in the immune response to FMD virus infection. However, FMD virus neutralizing antibodies were not detected in the sera of immunized animals, indicating that the humoral response to vaccinia virus recombinants differed from that reported for conventional vaccines.

1.10 Diagnosis of foot-and-mouth disease

Beside clinical diagnosis based on lesion identification, in the early stage of infection, FMD virus or viral antigen can be detected using several diagnostic techniques. However, different serological methods are used to detect antibody against FMD virus and is the main indication that infection has taken place.

1.10.1 Agent detection tests

Specimens for antigen detection

Vesicular fluid usually contains the highest quantity of virus. Epithelium from early vesicles and from recently ruptured vesicles are tissues of choice for virus isolation. Epithelial samples are placed in transport medium composed of an equal quantity of glycerol and 0.0 4 M phosphate buffer pH 7.2-7.6. A suspension is prepared by grinding the sample with sterile sand, using a sterile pestle and mortar with a small volume of tissue culture media and antibiotics. Media is added so that the final volume is ten times that of the epithelial sample, producing a 10% suspension (OIE/FAO, 2000). The suspension is centrifuged at 13000 rpm for 10 minutes and the supernatant is used for virus isolation.

When epithelium tissue is not available from ruminant animals e.g. in advanced or convalescent cases and infection is suspected in the absence of clinical signs, samples of oesophageal-pharyngeal (OP) fluid is collected by means of a probang and used for virus isolation (OIE/FAO, 1992).

Virus isolation

The isolation and characterization of the virus is the "golden rule" for the diagnosis of a viral disease. The

suspensions obtained from the specimens are inoculated onto susceptible cells (e.g. primary pig kidney cells) incubated at 37° C and examined for cytopathic effect (CPE) 24 to 48 hours post infection (p.i.). CPE can be seen as early as 12 hours p.i. as cell detachment and destruction with high virus concentration. If no CPE is observed the cells are frozen and thawed followed by 2 blind passages on cell culture (Kahrs, 1981; Westbury *et al.,* 1988).

Complement fixation test

Since its was first described by Traub and Mohlmann in 1946 the complement fixation (CF) test was commonly used in the diagnosis of FMD. It has been an important tool in the early studies for the comparison of the antigenic variation of two viruses. The CF test is based on the principle that complement (a series of serum proteins) serves as a mediator of many antigen-antibody reactions in which it is fixed in the formation of immune complexes. The presence of complement (usually provided by addition of guinea-pig serum) is revealed by its ability to mediate lysis of sensitized sheep red blood cells. Currently, the test is time consuming, less sensitive and no longer used to diagnose FMD.

Enzyme-linked immunosorbent assays (ELISA)

Because the complement fixation (CF) test lacks sensitivity and cell culture isolation takes up to 2-7 days, a more sensitive, rapid and practical alternative to traditional assays was needed for an efficient diagnosis of FMD. The enzyme-linked immunosorbent assay (ELISA) was developed with a number of applications which include the detection of antigen, and antibody (Crowther & Abu-Elzein, 1979; Hamblin *et al.*, 1984). Later, the technique was improved by making of high-titer serotype-specific antisera, by the use of inactivated 146S virus antigens which give less cross-reaction and a higher sensitivity compared to the infective virus, and the establishment of a positive/negative threshold (Roeder & Le Blanc Smith 1986; Hamblin *et al.*, 1986). Currently, two procedures are used, the sandwich ELISA for antigen detection (Roeder & Le Blanc Smith, 1986) and the liquid phase blocking ELISA for antibody detection (1.10.2) (Hamblin *et al.,* 1986, Esterhuysen *et al.*, 1985); both tests are routinely used in the diagnosis of FMD.

Sandwich ELISA

In the sandwich ELISA, the plates are coated with rabbit antisera (capture sera) to each of the seven serotypes of FMD virus (Crowther & Abu Elzein, 1979). Test samples as well as controls are added to the plates. Guinea-pig antisera to each of the serotypes of FMD virus are added to the corresponding wells followed by rabbit anti-guinea-pig serum conjugated to an enzyme. Plates are washed between each step to

remove unbound reagents. A color development after the addition of a substrate indicates a positive reaction (OIE/FAO, 2000).

The sandwich ELISA is used to study antigenic relationships of field isolates and the selection of vaccine strains. Cross-reactions between serotypes occur in the ELISA and can be overcome by the optimization of all reagents. Unlike the monoclonal antibodies, the use of polyclonal antibodies can detect smaller antigen concentrations and makes the technique more effective for detecting and typing of FMD virus (Alonso *et al.*, 1992).

Polymerase chain reaction (PCR)

Due to the rapid spread of FMD virus and the devastating economic consequences of the disease, it is essential to have a diagnostic test that is sensitive, accurate, rapid and easy to use. Reverse transcriptase PCR (RT-PCR) is used as a diagnostic tool to transcribe the RNA genome and make the first cDNA strand. Pairs of oligonucleotide primers are chosen to flank the DNA region of interest that is amplified by a Taq DNA polymerase enzyme. Following cycles of, DNA denaturation by heat, primer annealing by cooling, and strand extension with a thermostabile enzyme such as Taq polymerase, DNA is synthesized from a very small amount of template (Belák & Ballagi-Pordány, 1993). Subsequent improvements in the PCR have reduced the time required for viral detection and for the accurate characterization of FMD viruses from diagnostic samples (Locher *et al.,* 1995; Bastos, 1998).

Polyacryalamide gel electrophoresis (PAGE)

The electrophoretic mobility of the proteins is dependent on both their size and charge. The technique developed by Laemmli (1970) was used to separate and study the variation in the pattern of the structural polypeptides of many human and animal viruses such as human adenoviruses (Wadell, 1979), FMDV (Robson *et al.*, 1979) and poliovirus (Nottay *et al.*, 1981; Romanova *et al.*, 1981). It was also demonstrated that PAGE of the structural polypeptides of swine vesicular disease virus (SVDV) could be used to distinguish virus strains exhibiting very small serological differences (Harris *et al.*, 1979).

Knowles & Hedger (1985) studied the antigenic variation of FMD virus type A serotype using PAGE and evaluated the technique as an aid to existing serological techniques. Using the PAGE technique, the majority of the subtypes studied had different polypeptide patterns, but some variation was observed between strains within a subtype. The migration pattern of VP2 and VP3 was often conserved in different

strains within a subtype and between geographically related subtypes whereas changes in the electrophoretic mobility of VP1 protein were observed within a subtype (Knowles & Hedger, 1985).

1.10.2 Antibody detection tests

Virus neutralization test (VNT)

The virus neutralization test is based on the principle that serum specific neutralizing antibodies bind to the virus and then block the virus from entering cells in cell cultures. Virus neutralization has been used to study the homologous and heterologous responses against FMD virus (Vallée & Carrée, 1922; Hyslop & Fagg, 1965; Hedger, 1968). The test identifies specific neutralizing antibodies whereas blocking ELISA detects FMD antibodies across all the serotypes. Serial dilution is performed on test serum after inactivation. The virus suspension (100 $TCID_{50}$) is mixed with a serum sample incubated and inoculated onto the susceptible cell cultures. The growth of virus in cell cultures is manifested by cell destruction or cytopathic effect (CPE). No CPE confirms the presence of FMDV antibodies in the serum samples.

Liquid phase blocking ELISA

The blocking ELISA detects and quantifies specific FMD antibodies in both infected and vaccinated animals (Hamblin *et al.*, 1986; McCullough *et al.*, 1992). The test is carried out in 96 well microplates. Rabbit antiserum to the 146S antigen of one of the seven types of FMD virus is used as the trapping antibody at predetermined concentration in bicarbonate buffer. Serial dilutions of test serum are mixed with a constant virus dilution and incubated overnight, then the mixture is transferred to a coated plate with trapping antiserum (rabbit FMDV antisera). Positive serum will block the antigen and prevent it from binding to the trapping antibody and no blocking will occur with negative serum. Guinea-pig antisera homologous to the viral antigen used in the previous step is added to each well, incubated and followed by rabbit anti-guinea-pig immunoglobulin conjugated to an enzyme (horseradish peroxidase). Following incubation, the substrate solution (orthophenylene diamine) containing 0.05 % H2O2 is added to each well. The reaction is stopped after 15 min by addition of 1.25 M sulphuric acid. Plates are washed between each step to removed unbound reagents. The plates are read at 492 nm on a spectrophometer linked to a microcomputer (OIE/FAO, 2000).

Results from the blocking ELISA are determined as follows: For each test serum, a test optical density (Test OD) is determined by subtracting the OD obtained with the serum in the presence of negative antigen from that obtained from the serum with positive antigen (Esterhuysen *et al.*, 1995). The maximum

optical density (Max OD) for each plate is determined by subtracting the OD value of the negative antigen from the OD value of the positive antigen. The OD of each serum sample is calculated by subtracting the OD obtained with the serum in the presence of negative antigen from the value obtained with serum with positive antigen. The degree of inhibition for each serum dilution is calculated using the formula:
% Inhibition = [(Max OD - Test OD)/(Max OD)] X 100. Any sera dilutions where 50% of the Max OD reading is inhibited are considered positive.

Antibody titres are expressed as the 50 % end-point titre., i.e. the dilution at which 50 % of the wells show greater than 50 % inhibition of the median OD of the reaction (antigen) control wells. Serum with titres > 1/40 or close to 1/40 are considered positive or suspect respectively. The latter must be re-tested using the virus neutralization test (OIE/FAO, 2000).

3ABC ELISA

The detection of antibody to the polyprotein 3ABC (see Fig.1.1b) is a useful indicator of FMD virus infection regardless of the serotype involved (Haas, 1997; Mackay *et al.*, 1998). Antibody to the 3ABC is only found in virus-infected animals but not in vaccinated animals (Diego *et al.*, 1997). It was also reported that the presence of antibody to one or more of the other NS proteins (2C, 2A, 3D) is an indication of previous infection (Diego *et al.*, 1997; Mackay *et al.*, 1998).

Briefly, the test is carried out as follows: plates are coated overnight at 4^{o}C with 3ABC antigen. Then test sera are added including a set of negative and positive references. Following 60 min incubation at 37^{o}C an enzyme conjugated to anti-species antibody that is added to the plate and incubated for another 30 min. The reaction is terminated after 15 min by adding a stop solution. The plates are washed between each step. An ELISA reader is used and the results are expressed as an index derived by dividing the absorbance value of the serum tested by that of the cut-off control.

Compared to the blocking ELISA, the advantage of the 3ABC ELISA is the differentiation between infected and vaccinated animals in the diagnosis of FMD exposure. The 3ABC ELISA is also a rapid test for screening of large number of sera. In areas where more than one serotype exist, the test is also cheap compared to the conventional liquid phase blocking ELISA, which has the disadvantage that each serum sample must be tested against all existing serotypes.

1.10.3 Nucleotide sequencing

Unlike many living organisms where the hereditary information is encoded within a DNA genome, FMD virus has a RNA genome that can be sequenced directly, but RNA is unstable and it usually first transcribed into cDNA prior of performing the nucleotide sequence. Reverse transcription (RT) when combined with PCR provides a rapid and powerful technique for studying diverse RNA genomes. In epidemiological studies of FMD virus, nucleotide sequencing of the VP1 gene, has been used extensively to determine the relationships between the field isolates. The technique is also routinely used to investigate genetic variation, molecular evolution in carrier animals, and to identify the source of an infection in outbreak conditions (Domingo *et al.,* 1985; Beck & Strohmaier, 1987; Dopazo *et al.,* 1988; Vosloo *et al.,* 1992; Saiz *et al.,* 1993; Bastos, 1998). The first genetic relationships of FMD virus type A, O, and C were constructed using this approach (Beck & Strohmaier, 1987).

The nucleotide sequence of the major immunogenic protein, VP1 was also used to subtype the European FMD viruses type A and O recovered from different outbreaks (Beck & Strohmaier, 1987). They reported that the use of nucleotide sequences is not only a rapid and accurate technique for subtyping FMD virus but also differentiates variants of a given subtype. They also demonstrated that a single nucleotide change could be detected in the nucleotide sequencing of the isolate from Germany in 1984 (O Zusmarshausen) and strain O1 Kaufbeuren. Subsequent studies using this approach have provided crucial epidemiological insights which include among others, the use of nucleotide sequences for the identification of virus variants arising from laboratory cell passage (Sáiz *et al.*, 1993), the identification of trans-boundary virus transmission (Sáiz *et al.*, 1993; Samuel *et al.*, 1999), and evidence of prolonged persistence of a particular virus type in the field (Freiberg, *et al.*, 1999; Samuel *et al.*, 1999). Sequence data has also been instrumental in identifying outbreaks resulting from inadequately inactivated vaccines (Beck & Strohmaier, 1987; Krebs & Marquardt, 1992) and for refuting vaccine involvement in outbreaks (Suryanarayana *et al.*, 1998).

Manual sequencing

There are two DNA sequencing techniques: the chemical degradation method and chain termination sequencing. The basic difference between these lies in the way in which the A, C, G and T families of molecules are generated.

The chemical degradation method described in 1977 by Maxam and Gilbert involves base-specific (A, C, G and T) cleavage of a 5' end-labeled DNA molecule by different chemicals to give a series of fragments of varying lengths of either radioactive adenine, thymine, cytosine or guanine, which can be analyzed by gel electrophoresis. Double stranded (dsDNA) or single stranded DNA (ssDNA) are used in this method. The nucleotides are separated and identified based on their electrophoretic mobility. To visualize the radioactively labelled nucleotides the sequencing gel is exposed to X-ray film and is read in a manner very similar to chain termination sequencing.

The chain termination sequencing described by Sanger *et al.*, (1977) is similar to the Maxam-Gilbert sequencing in that it generates a set of fragments with a common 5' origin and base-specific 3' termini. This method requires strand synthesis by enzymatic activity, and is more complex than simple chemical degradation. The technique is based on the principle that a specific primer is annealed to a ssDNA molecule. Primer extension is achieved in four individual reaction containing the deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, or dTTP) in the presence of a DNA polymerase enzyme. Each sequencing reaction mixture also contains a specific dideoxynucleotide (e.g. the "A" reaction has ddATP). The incorporation of ddATP into the growing chain stops the elongation of the DNA molecule because the dideoxyadenosine lacks the hydroxyl group at the 3' end that is required to form the phosphodiester bonds link with the 5'-hydroxyl group of the next nucleotide. This reaction generates polynucleotide chains of different lengths, all with a ddATP. The nucleotides are separated using gel electrophoresis and visualized by autoradiography.

Both sequencing reactions have some advantages and disadvantages. An unambiguous sequence is one of the main advantages of the chemical degradation method. The interpretation of the autoradiograph is easier and quicker than in the chain termination method. In addition, there is no enzyme used which can affect the quality of the sequencing results. However, there are some disadvantages such as the modification and cleavage reactions which take longer to carry out, involve more steps, and are more hazardous than the strand synthesis reactions required for chain termination sequencing. The length of the sequences is also shorter with the chemical degradation method than in the chain termination method.

When compared to chemical degradation, the disadvantages of the chain termination method are the presence of ambiguous sites which make the interpretation of the banding pattern on an autoradiograph difficult (e.g. hairpin loops may lead to band compression in one or more lanes). The reading of the

nucleotides on the X-ray film is also time consuming. Other problems such as poor quality enzyme, inappropriate dNTP:ddNTP ratio arise during the chain termination method affecting the quality of the sequencing results. There are however some advantages such as that the nucleotide sequences obtained are longer than those from the chemical reaction and it is also safer compared to the chemical degradation method.

Automated sequencing

Automated or cycle sequencing is a chain termination sequencing method incorporating PCR methodology that has revolutionized nucleotide sequencing. A small amount of sequencing template is needed which provides longer sequences which are read and analyzed using computer software. It is very quick with sequencing results being available within a day. The sequencing reaction contains either the fluorescent labeled sequencing primer or fluorescently labeled dideoxy nucleotides. The technique relies on repeated rounds of denaturation, primer annealing and extension/termination. After each round, the elongation product is dissociated from the template by heat denaturation, giving way to another round of DNA synthesis.

1.10.4 Data analysis

The identification of the origin of the disease or the transmission route is essential in the control of the disease. There is no doubt that DNA sequencing and phylogenetic tree building are the best ways to illustrate the evolution of relationships between viruses. Numerous computer programs are available to analyze the nucleotide or amino acid sequences. The results are reproducible, objective, and easy to interpret. A large number of isolates can be processed and their phylogenetic relationships determined in a very short time using the different methods.

A huge database of published and unpublished sequences is available for FMD viruses and can be accessed to determine the genetic relatedness of a current isolate to previously characterized viruses. A short overview of the most common methods used for phylogenetic inference are discussed below.

Sequence alignment

The first step in analyzing nucleotide data is to align the sequences under study with one another (Doolittle, 1990). Many procedures have been developed to align nucleotide or amino acid sequences. The manual approach to align sequences is very tedious and the results more subjective. A classical

technique involves positioning the two sequences in single letter code and with length m and n such that one is vertical along column (m) and the other horizontal along a row (n). Then, in matrix fashion, scores are inserted at each grid point (m x n) corresponding to every pairwise comparison of the amino acids in each sequence. This classic procedure has some drawbacks when alignments result in amino acid identity at 35% or less of the matched positions. Numerous computer programmes are available for obtaining optimal alignment of sequences.

Methods for phylogenetic inference

A phylogeny is the branching history of routes of inheritance of species, populations or genes and is microevolutionary informative (Maddison & Maddison, 1992). The relationship of a set of related nucleotide or protein sequences can be expressed quantitatively in terms of a phylogenetic tree. There are two types of trees e.g. rooted and un-rooted trees (Fig. 1.5) and the branching pattern on the tree is named a topology (Nei & Kumar, 2000). The accuracy of the tree depends on the alignment of the sequences. The order in which the sequences are aligned is important. In this regard, an approximate phylogenetic order of the sequences is first determined by a series of pairwise alignments.

Distance methods

When comparing two or more sequences, the first question that comes to mind is how much evolutionary difference has occurred between these sequences? Answering this question has resulted in the development of numerous methods for measuring sequence differences. There are many distance methods or distance matrix methods and a brief summary of some methods is given below.

Distance Methods using clustering algorithms

Neighbor-joining method

It is a clustering method which does not examine all possible topologies and uses the minimum evolution principle at each stage of taxon clustering (Saitou & Nei, 1987). It is a widely used tree building method. In neighbor-joining, each taxon is compared individually to all the other taxa in the data set. The concept is based on the principle that the taxa or gene sequences that are closely related are neighbors on the tree. The comparison of different topologies is built into the algorithm, and the final tree with both topology and branch lengths is automatically produced (Saitou & Nei, 1987).

UPGMA (unweighted pair-group method using arithmetic average)

The method is efficient for constructing gene trees when the rate of evolution is constant, which is equivalent to assuming a molecular clock (Nei *et al.*, 1983; Li *et al.*, 1987). UPGMA method is fast and all taxa on the tree are equidistant from the root of the tree. However, UPGMA can often generate an incorrect tree when the rate of gene substitution is not constant or when the number of nucleotide sequences is small (Nei & Kumar, 2000).

Discrete character method using an optimality criterion

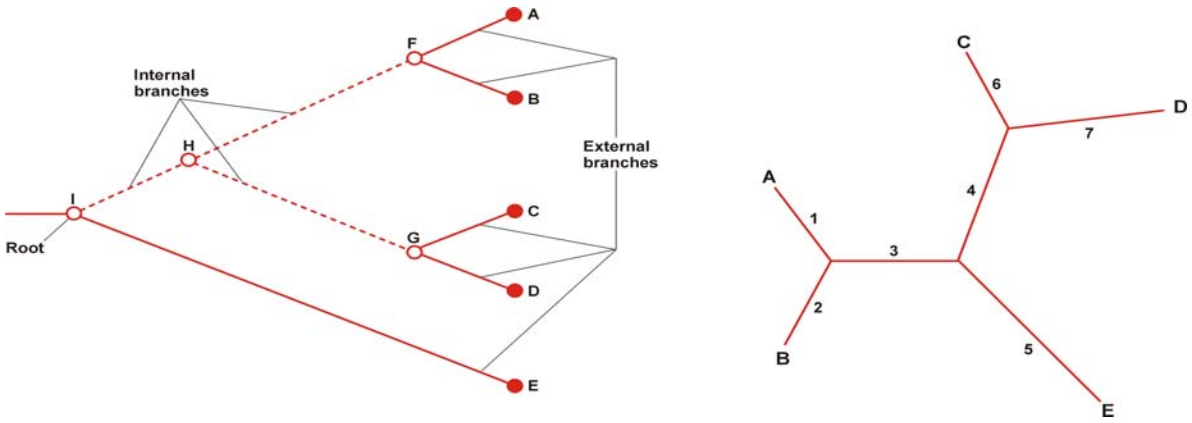
Unlike, the distance methods that convert aligned sequences into a pairwise distance matrix, then convert that matrix into a tree building method, the discrete methods consider individual nucleotide sites directly. A maximum parsimony which is a discrete method that chooses the tree that require the fewest evolutionary changes is discussed below.

A maximum parsimony method

A maximum parsimony is used both in judging the phylogenetic tree and in reconstructing the history of character change. Parsimony methods select the tree that requires the smallest number of evolutionary changes (e.g., change from one character state to another) caused by nucleotide substitutions, insertions or deletions (Hillis & Bull, 1993) to explain the observed differences among the operational taxonomic units (OTUs) under study. In all parsimony methods, change of character state in either direction is assumed to be equally probable, and character state may transform from one state to another and back again (Swofford *et al.*, 1996).

The correct evolutionary tree is a rooted tree that graphically depicts the cladistic relationships which exist among the OTUs. As described by Page & Holmes (1998) a tree consists of nodes connected by lines, and a rooted tree starts from the root, which is the ancestral node to all other nodes. Each ancestral node gives rise to two descendant nodes and terminal nodes corresponding to OTUs (Fig. 1.5).

Fig. 1.5 A rooted tree is depicted on the left, whilst an unrooted tree for the same OTUs (A-E) is shown on the right.



Adapted from Page & Holmes (2000).

Maximum likelihood method

The method gives the smallest variance of a parameter estimated when sample size is large. However, the method presents two major problems e.g. when the rate of nucleotide substitution varies extensively from branch to branch, incorrect topologies may be chosen more often than the true topology even with large size of samples. The reconstruction of topology is a second problem because there are no parameters for topologies in the likelihood function (Felsenstein, 1981).

Consensus trees

Consensus trees are used to compare trees obtained from different sequences or from the same sequences using different methods (Page & Holmes, 2000). They define trees resulting from combined data sets and produce trees that have common characteristics across the different methods of analysis or across different gene regions.

The phylogenetic reconstruction methods can be differentiated from each other on the basis of the treatment of the data, e.g. distances versus those that use discrete characters, and according to the tree building method, e.g. clustering algorithm versus optimality criterion (Page & Holmes, 2000) as follows:

Table1.2: Some common phylogenetic methods classified by the method used to build the tree, and by the type of data used.

	Type of data	
Tree building method	Distances	Nucleotide sites
Clustering algorithm	UPGMA Neighbor joining	
Optimality criterion	Minimum evolution	Maximum parsimony Maximum likelihood

However, the efficiency or program running speed, the volume of the data requires or power, the consistency, the robustness and the falsifiability must be taken into account when selecting a tree building method (Penny *et al.*, 1992).

Bootstrap method

The bootstrap method is a method for testing the reliability of the topology by re-sampling the data. This method was introduced into phylogenetic studies by Felsenstein (1985). It is not a tree making method but a statistical method that can be used to evaluate the reliability of each interior branch of a tree. The tree is reliable if the computing results give a positive value for every interior branch length of a tree. The bootstrap values are obtained by computing a quantity equivalent to the probability of confidence (1-Type 1 error) rather than the significance level. If this value is higher than 95%, the branch is statistically significant (Felsenstein, 1985).

However, it has been shown that the bootstrap method underestimates confidence at levels between 70 % - 90 % and overestimates confidence at levels lower than 60 % (Hillis & Bull, 1993). Thus bootstrap values ≥70 % are considered statistically significant.

1.11 Aims of this study

To understand the epidemiology of FMD is crucial for the implementation of good control measures. The identification of the possible origin of the virus is one of the major factors contributing to control of the disease. In recent years the nucleotide sequences of viruses generated much attention in this regard and sequence data has been instrumental to identify the origin of an outbreak (Beck & Strohmaier, 1987; Krebs & Marquardt, 1992). To facilitate studies on the genetic relationship of isolates in outbreaks conditions, the World Reference Laboratory for FMD virus has established a database of FMD virus sequences from isolates collected worldwide (Samuel *et al.*, 2001).

However, the literature review has shown that there is a paucity of information on FMD epidemiology in West Africa. A study was therefore undertaken in which the genetic characterization and comparison of historical and recent field isolates from several west African countries was conducted with the aim of addressing the lack of information on FMD in this region of Africa.

The main objectives of this study were:

- To evaluate the genetic diversity of serotypes circulating in West Africa by characterization of the VP1 gene of these isolates.
- To determine the regional topotypes of the different serotypes of West Africa.
- To identify the phylogenetic relationships between west African viruses and those previously isolated in East, Central, southern Africa and other continents.
- To establish a genetic database of FMD viruses for West Africa as a reference for:

  - Tracing the transmission route between historical and recent outbreaks.

  - Determining the origin of the virus in future outbreak conditions in the region.

CHAPTER 2

MOLECULAR EPIDEMIOLOGY OF TYPE O FOOT-AND-MOUTH DISEASE VIRUS IN WEST AFRICA

2.1 Summary

Genetic relationships of serotype O foot-and-mouth disease (FMD) viruses recovered from outbreaks of the disease in the west African countries of Niger, Burkina Faso and Ghana (1988-1993) and those from South Africa (2000) were determined by partial VP1 gene characterization. A 581 bp fragment, corresponding to the C-terminus end of the 1D (VP1 gene) region was amplified and sequenced. A homologous region of 495 nucleotides was ultimately used to determine genetic relationships of serotype O viruses from the Middle East, Europe, South America, North Africa, East Africa, southern Africa and Asia.

Seven independently evolving type O genotypes were identified by phylogenetic reconstruction, consisting of viruses from the following geographical regions: Genotype A: Asia, Middle East and South Africa, Genotype B: East Africa, Genotype C: West and North Africa, Genotype D: Taiwan and Russia, Genotype E: Angola and Venezuela, Genotype F: Western Europe and Genotype G: Europe and South America. The differentiation of the same genotypes was based on at least 86 % sequence identity in the C-terminus region of the VP1 gene.

The seven genotypes constitute three different evolutionary lineages (I-III), which correspond to three discrete continental regions, some of which display inter-continental distributions due to introductions. Results further indicate that the outbreaks in Burkina Faso (1992) and Ghana (1993) are part of the same epizootic and that the strain involved in recent outbreaks of the disease in South Africa is most closely related (97 % and 99 % sequence identity) to the 1997 Bangladesh and the 2000 Japan strains, respectively. Inter-continental virus transmission was also observed for lineage (III), in which viruses from Angola and those from South America (Venezuela) were closely related.

2.2 Introduction

The serotype O of FMD virus is of particular importance due to the recent and regular involvement of this

virus type in different outbreaks (Kitching, 1998). This includes outbreaks in North Africa (Samuel *et al.*, 1999), the Middle East (Stram *et al.*, 1995; Samuel *et al.*, 1997), Europe (Marquardt & Haas, 1998) and Asia (Pattnaik *et al.*, 1998; Freiberg *et al.*, 1999; Tsai *et al.*, 2000).

Molecular epidemiological studies of serotype O have primarily focused on outbreaks affecting Europe, South America and Asia, with limited data available for type O viruses of African origin (Samuel *et al.*, 1999). In part of Europe, Italy, Greece, Bulgaria, Moscow, Turkey, and Lebanon, type O outbreaks have been reported regularly between 1990-1996 (Kitching, 1998). The genetic relationships between these viruses were established by nucleotide sequencing and phylogenetic analysis. For example viruses involved in outbreaks in Bulgaria in 1991, were closely related to a serotype O virus circulating in Turkey. Similarly the type O virus causing an outbreak in Greece in 1994 was closely related to strains circulating in the Middle East in 1990 and Turkey in 1991 but distant from the strain responsible for an outbreak in Italy in 1993 (Kitching, 1998).

The first recorded outbreak of a type O virus in South Africa in 2000 has raised the profile of this virus type on the continent and stresses the importance of establishing a continental reference database suitable for tracing the origin and spread of viruses occurring here (Sangare *et al.*, 2001). It is with this in mind that the genetic characterization of viruses from West, North, South and East Africa has been undertaken. The aim was to determine the number of type O genotypes (regional variation) represented in West Africa and to assess their relationships with others on the continent.

2.3 Materials and methods

2.3.1 Viruses

Genetic characterization of viruses causing FMD in pigs (SAR/11/00 and SAR/15/00), goats (SAR/12/00) and cattle (SAR/13/00B, SAR/18/00, SAR/19/00, SAR/25/00, SAR/26/00) in South Africa was performed directly on clinical specimens. Cell culture isolates from West (Niger, Burkina Faso and Ghana), North (Algeria), East (Kenya) and southern Africa (Angola) were selected in order to assess the genetic variation of type O viruses on the African continent. Virus isolates were supplied by the Institute for Animal Health, Pirbright, UK and their geographical origin, sampling date and laboratory designation are indicated in Table 2.1.

2.3.2 Cell cultures

IB-RS-2 (Insituto Biologico Rim Suino, De Castro, 1964) a pig kidney cell line was grown in 25 cm^2 tissue culture flasks (Corning). GI-1640 medium (Sigma) supplemented with 10% fetal calf serum and 1x antibiotics (100 units/ml) was used. The cells were incubated at 37^oC until a confluent monolayer was formed.

2.3.3 Virus stocks and replication

Each initial cell culture isolate received from the Pirbright laboratory was passaged at least twice on IB-RS-2 cells. The cell monolayers were washed with 10 ml pre-warmed phosphate buffered saline (PBS) and inoculated with 0.5 ml of the stock virus sample. GI-1640 medium (2 ml) without fetal calf serum (maintenance medium) was added and the flasks were incubated at 37^oC for 30 min on a shaker. The supernatant was removed at the end of the incubation and the monolayers were rinsed very gently for 5 sec with phosphate buffer saline and the fluid discarded. Maintenance media (5 ml) was added to the cells and the flasks were incubated at 37^oC. After 2 days incubation, the cells without CPE, were blind passaged and considered negative after two blind passages. Monolayers which showed CPE were harvested when 90-100 % of CPE was observed. The virus suspensions were centrifuged at 15 000 g for 10 min to remove cell debris and the suspensions (10ml/flask) were collected by syringe aspiration. Glycerol was added to the virus suspensions so that a final concentration of 20% glycerol was obtained. The suspensions were aliquoted in 2 ml cryotubes and kept at -80^oC until further use.

2.3.4 Nucleic acid extraction

Total RNA was extracted from the cell culture isolates by a modified guanidium-thiocyanate-silica method based on that of Boom and co-workers (1990). Briefly, 900 µl lysis buffer and 40 µl silica was added to a 100 µl virus suspension. The mixture was vortexed and incubated at room temperature for 5 min. The sample was mixed intermittently during the incubation period. The suspension was centrifuged at 15 000 g for 15 sec using a benchtop centrifuge and the supernatant was discarded. The pellet was washed and re-suspended in 900 µl of a L2 wash buffer (60 g of Guanidine Thiocyanate in 50 ml ddH_2O of 0.1 Tris- HCI at 50^oC, pH 6.4) and centrifuged at 15 000 g for 15 sec. The supernatant was discarded and 900 µl of 70% ethanol (EtOH) was added to the pellet and centrifuged as before. The supernatant was discarded and 100% acetone was added to the pellet, mixed and centrifuged at 15 000 g for 15 sec and the supernatant discarded. The pellet was dried by incubating the tube in a heating block at 56^oC for 10-20 min under flux laminar air flow. The pellet was suspended in 30 µl of 1 X TE (10 mM Tris-HCI, pH 7.6; 1 mM EDTA)

containing 4 U of RNasin (Promega). The tubes were incubated at 56°C for 2 min, centrifuged and the supernatant containing the eluted nucleic acid was carefully removed and transferred into a new, clean, RNase free eppendorph tube. The RNA was used for cDNA synthesis or stored at -80°C until needed.

2.3.5 Complementary DNA synthesis (cDNA)

The first strand cDNA synthesis was carried out for 1 hour at 42°C in 10 µl reaction volume as described by Bastos (1998). Each reaction tube contained an equal volume of RNA and reaction mix composed of 50 mM Tris-HCL (pH 8.3), 50 mM KCL, 10 mM MgCL, 2.5 mM dNTPs, 10 pmol/µl of a antisense primer (P1) (5'GAA GGG CCC AGG GTT GGA CTC 3') published by Beck & Strohmaier (1987), 200 pmol/µl of random hexanucleotides ((Boehringer Mannhein), 40 U/µl RNase inhibitor (Promega), 100 U of AMV-RT (Promega). The tubes were centrifuged and first incubated at 70°C for 3 min using a dry block or water bath and snap-frozen in liquid nitrogen, then 1 X TE containing 4 U of Rnasin and 10 U AMV-reverse transcriptase (Promega) were added to the tube, centrifuged at 15 000 g for 5 sec and incubated as mentioned above, followed by heating for 1 min at 80°C and stored at 4°C until needed.

2.3.6 Polymerase chain reaction (PCR)

The PCR was performed as described by Bastos (1998). A total volume of 50 µl was used which contained the following: cDNA (2.5 µl), 5 µl of 10 X buffer (DynaZyme), 4 µl of 2.5 mM dNTPs, 2 µl of 10 pmol of each primer, a serotype O-specific sense strand primer (5' GAT TTG TGA AGG TGA CACC 3') (Rodriguez al., 1994) and antisense primer (5' GAA GGG CCC AGG GTT GGA CTC 3') (Beck & Strohmaier, 1987), 35 µl of double distilled water (ddH_20), and 1U/µl of Taq polymerase (Roche). The amplification of a 581 bp DNA fragment was performed using the following program: 96°C for 1 min., followed by 35 cycles of denaturation at 96°C for 12 sec, annealing at 55°C for 20 sec and extension at 70°C for 30 sec.

2.3.7 Gel electrophoresis and purification of amplified DNA

The PCR products were analyzed on a 1.5 % agarose gel containing 0.5 µg/ml of ethidium bromide. The DNA bands were visualized by UV illumination and the expected sizes were identified against a DNA molecular weight marker (100 bp) (Promega). The correct DNA bands were cut from the gel and purified using the cleanmix purification kit (Talent Kit) according to the instructions of the manufacturer.

2.3.8 Nucleotide sequencing

The purified DNA templates were sequenced manually with a radionucleotide as previously described

(Bastos, 1998) or alternatively using thermocycler sequencing. Two independent amplification and sequencing reactions were performed per sample with each of the primers using one or both sequencing methods.

Manual sequencing was performed using the T7-Sequence kit version 2.0 (Amersham). The termination mix (ddATP, ddCTP, ddGTP, ddTTP) supplied by the manufacturer were added to a 96-well microplate (2.5 µl/well). The purified DNA was resuspended in 12 µl ddH_2O and 6 µl was added to 4 µl of a upstream primer (5' GAT TTG TGA AGG TGA CACC 3'), and the same volume of DNA was added to 4 µl of a downstream primer (5' GAA GGG CCC AGG GTT GGA CTA 3') in a separate tube. The reaction mixes (DNA-primer) were boiled for 4 min and quickly frozen using liquid nitrogen. The samples were thawed and 4 µl of a 60 X labeling cocktail was added. Tubes were centrifuged at 15 000 g for 15 sec and incubated at room temperature for 5 min. The reaction mixes (3.2 µl) were added to the appropriate termination mixes in the pre-warmed microplate wells on a hotplate at 37^{o}C for 1-2 min. After a 5 min incubation, 4.2 µl of a stop solution was added to each well. The microplates were heated at 80^{o}C for 1 min in a water bath or 3 min on the heating block to stop the reaction and then put on ice. The DNA mixture (2.5 µl or 3.5 µl per well for single or double runs, respectively) was loaded on a sequencing gel and run for 2-3 hrs at 1950 Volts.

Automatic sequencing was performed using fluorescent dye deoxy-terminator cycle sequencing (Perkin Elmer) on an ABI Prism 377 DNA sequencer (Applied Biosystems). DNA template (5 µl) at a concentration of 100 ng/µl, 1 µl of primer (3.2 pmol/µl) and 4 µl of fluorescent dye deoxy-terminator were used in the PCR cycle sequencing reaction. A hot start PCR was performed using 25 cycles of the following thermal profile:
96^{o}C for 10 min, 50^{o}C for 5 seconds and 60^{o}C for 4 min. The DNA was precipitated in 50 µl of ice cold 100 % ethanol (EtoH) with 2 µl 3M NaAc and 10 µl of dH_2O. The mixture was vortexed and centrifuged at 13 500 g for 30 min. The supernatant was added to 100 µl of 70 % cold EtOH, centrifuged at 13 500 g for 15 min and the pellet dried on a heat block at 70-90^{o} C for 2 min. The DNA pellet was resuspended in 3 µl loading buffer (deionized formamide, 25 mM EDTA, Ph = 8.0 containing 50 mg/ml blue dextran in a ratio of 5:1 formamide to EDTA/blue dextran). The reactions were heated to 95^{o}C for 2 min prior to loading on the ABI Prism 377 DNA sequencer (Applied Biosystems). The software AM V3.01 (Pharmacia Biotech) was used to process the data which was then exported as an ASCII text file.

2.3.9 Data analysis

Sequences generated in this study were submitted to Genbank under the accession numbers indicated in Table 2.1. An homologous region of 495 nt, corresponding to amino acid positions 49-213 of the VP1 gene was identified and aligned using the DAPSA program (Harley, 1994). Neighbor-joining and UPGMA methods included in the MEGA program (Kumar *et al.*, 1993) were used to construct gene trees. UPGMA is considered a heuristic method (all tips are equidistant from the root of the tree, which is equivalent to assuming a molecular clock) for finding the best least squares ultrametric tree. Neighbor joining is a method for clustering and estimating the minimum evolution tree. Confidence limits were placed on the tree branches using the bootstrap resampling method with reliability being assessed by a 1000 replications (Felsenstein, 1985).

2.4 Results

2.4.1 VP1 gene relationships

In order to put our results within the context of earlier molecular epidemiological studies, nucleotide sequences of a suitable length for phylogenetic analysis (Martin *et al.*, 1995) of serotype O were obtained from the Genbank database. A total of 44 viruses were subsequently included in the analyses (Table 2.1) of which 21 were characterized in this study. Trees of identical topology were consistently obtained irrespective of the method of analysis used, indicating that the phylogenetic results are reliable (Kim, 1993). In the neighbor joining tree presented in Fig.2.2 three major evolutionary lineages, I-III are distinguished on the basis of nucleotide sequence differences > 20 % and statistically significant bootstrap support (95 %). These three discrete evolutionary lineages correspond to different geographical regions as follows: Lineage I: Africa-Asia; Lineage II: Asia-Europe; and Lineage III: Europe-South America-Africa. Within each of these lineages, further clusters or genotypes were similarly identified and labelled A-G. Viruses of the same genotype are defined here as those that differ from each other by no more than 14 % on nucleotide level across the 495 nt region characterized in this study (Fig. 2.3).

Viruses from Africa group within four distinct genotypes, A - C and E. Genotypes C (North and West Africa) and genotype B (East Africa) form strict localized groupings, whilst the South African (Genotype A) and Angolan viruses (Genotype E) are linked to viruses of Asian and South American origin, respectively. Within genotype E high levels of sequence identity (99 %) are noted for these viruses which were sampled one year apart (1974 and 1975). These results indicate that they are part of the same epizootic. Similarly, viruses from Burkina Faso 1992 and Ghana 1993, although derived from outbreaks

occurring in different years and in different countries, have 98 % sequence identity, again indicating a common origin. In contrast, only 89 % nucleotide sequence identity occurs between Burkina Faso and Niger isolates indicating that these viruses are derived from unrelated epizootics. The virus cluster within genotype A consists of strains from South Africa, Bangladesh, Japan, Sri Lanka, Taiwan and Saudi Arabia, has high bootstrap support (> 100 %) and shows a high degree of sequence identity (> 95 % sequence identity), providing strong evidence for a link between viruses from these two continents.

2.4.2 Sequence variation and determination of the geographical origin of type O outbreaks (1988-1993) in West Africa.

Conservation of 49.3 % was observed for nucleotide sequences with the alignment of the 44 virus sequences (Fig.2.2). From the deduced amino acid sequence alignment, it was shown that only 59.4 % were completely conserved across all protein sequences. Most mutations were clustered within two distinct regions corresponding to antigenic site 1 (Kitson *et al.*, 1990), comprising of the G-H loop (amino acid positions 133-158) and the C-terminus region (amino acid positions 194-213). The 'RGD' cell attachment site of the virus (amino acid positions 145-147) is completely conserved.

The comparison of viruses based on the partial sequence (495 nucleotides) data of the VP1 gene from different countries in West Africa namely three isolates from outbreaks in Niger (1988), three viruses recovered from outbreaks in Burkina Faso (1992) and four viruses isolated from Ghana (1993) display only 12 variable sites over the 495 nucleotides characterized here. Viruses from two neighboring countries shared > 97 % identity at the nucleotide sequence level (495 nt). This result demonstrated clearly that these viruses are closely related (< 3 % nucleotide difference) and probably were from the same outbreak.

Interestingly, viruses isolated in Niger in 1988 were genetically homogenous (> 98 % sequence identity) indicating that they share a common ancestor but were distinct from other outbreaks . A single virus from Algeria (1999) was included in the analysis. Although, this north African isolate clustered with the viruses recovered from outbreaks in West Africa between 1992 to 1993, it differed by >10 % from these viruses.

TABLE 2.1 Summary of type O FMD viruses used in this study

Virus name	Sampling year	Country of origin	Reference	Genbank Accession No
O1 Lombardy	1946	Italy	Krebs et al. 1991a	M58601
O2 Brescia	1947	Italy	Krebs et al. 1991b	M55287
O3 Venezuela	1951	Venezuela	Leister et al. 1993	AJ004645
O1 Campos	1958	Brazil	Jensen et al. 1993	M95781
* O5 India	1962	India	This study	AF274297
O1 Kaufbeuren	1966	Germany	Forss et al. 1984	X00871
O1 BFS	1967	United Kingdom	Makoff et al. 1982	J02185
O1 Manisa	1969	Turkey	§	AJ251477
*ANG/10/74	1974	Angola	This study	AF300810
*ANG/1/75	1975	Angola	This study	AF300811
*KEN/77/78	1978	Kenya	This study	AF300812
O1 Caseros	1982	Argentina	§	U82271
O1 Yrigoyen	1982	Argentina	Sáiz et al. 1993	Z21862
*NGR/1/88	1988	Niger	This study	AF300801
*NGR/2/88	1988	Niger	This study	AF300802
*NGR/3/88	1988	Niger	This study	AF300803
*KEN/1/91	1991	Kenya	Bastos 1998	AF023527
*BFK/1/92	1992	Burkina Faso	This study	AF300804
*BFK/2/92	1992	Burkina Faso	This study	AF274296
*BFK/3/92	1992	Burkina Faso	This study	AF300805
GHA/5/93P	1993	Ghana	Samuel & Knowles, 2001	AJ303488
*GHA/5/93	1993	Ghana	This study	AF300806
*GHA/6/93	1993	Ghana	This study	AF300807
*GHA/7/93	1993	Ghana	This study	AF300808
*GHA/9/93	1993	Ghana	This study	AF300809
SAU/100/94	1994	Saudi Arabia	Samuel et al. 1997	AJ004660
SAU/2/95	1995	Saudi Arabia	Samuel et al. 1997	AJ004662
Moscow/95	1995	Russia	§	AJ004680
BAN/1/97	1997	Bangladesh	Freiberg et al. 1999	NA
Taiwan/97	1997	Taiwan	Tsai et al. 2000	AF026168
Taiwan/98	1998	Taiwan	Tsai et al. 2000	AF095877
CIV/8/99	1999	Cote d'Ivoire	Samuel & Knowles, 2001	AJ303485
Taiw/2/99	1999	Taiwan	Knowles et al., 2001	AJ294927
ALG/1/99	1999	Algeria	This study	NA
SAR/11/00P	2000	South Africa	This study	AF306646
SAR/12/00G	2000	South Africa	This study	AY009087
SAR/13/00B	2000	South Africa	This study	AY009088
SAR/15/00P	2000	South Africa	This study	AF306647
SAR/18/00	2000	South Africa	This study	NA
SAR/19/00	2000	South Africa	This study	AY009088
SAR/25/00	2000	South Africa	This study	NA
SAR/26/00	2000	South Africa	This study	NA
SKR/1/00	2000	Sri Lanka	Knowles et al., 2000	NA
JPN/2000	2000	Japan	Kanno et al., 2000	AB050978

*Virus isolates provided by the Institute for Animal Health Pirbright, UK. § Unpublished Genbank sequence submissions. The virus name followed by P, G, or B indicated pig, goat or bovine origin expect GHA/5/93P where P indicated published sequence. NA = Not available

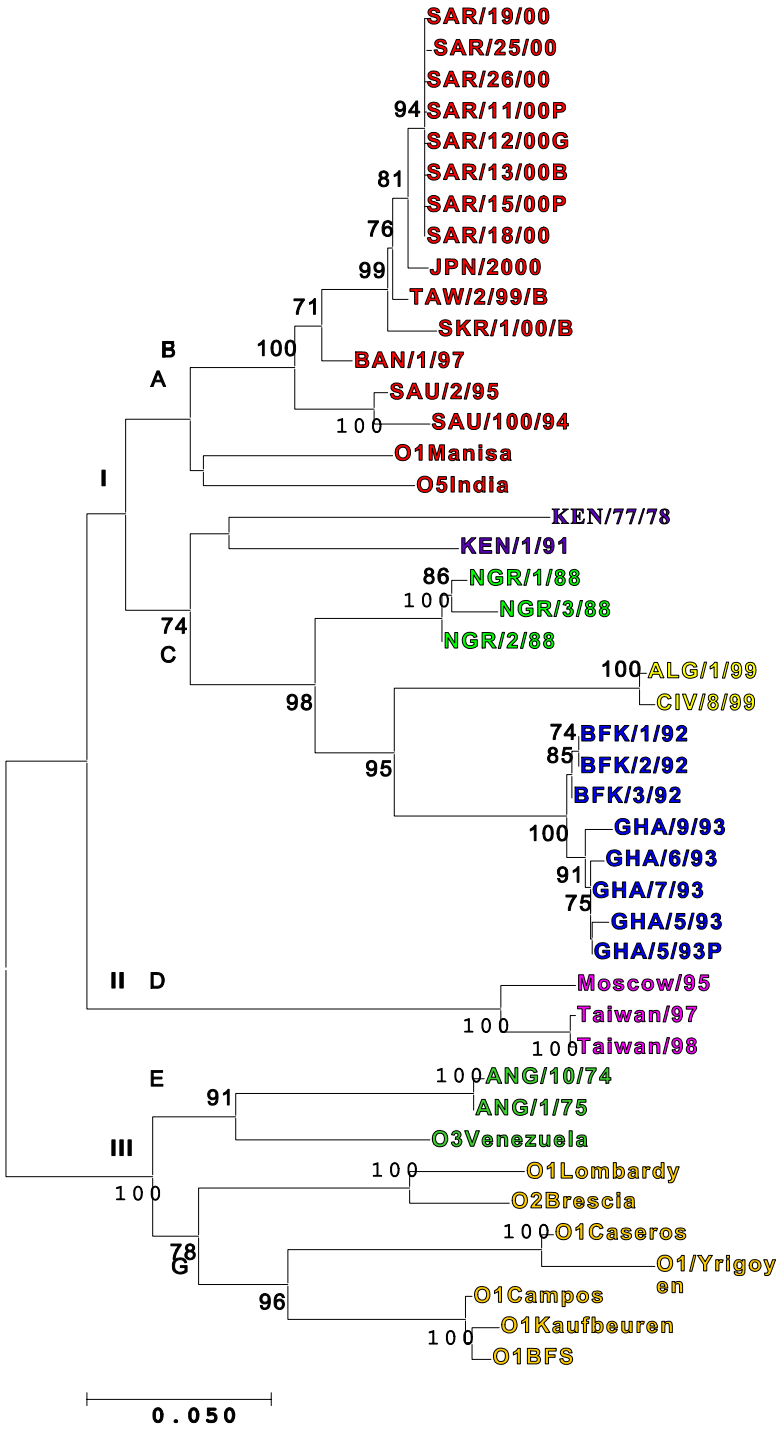


Fig. 2.1 Neighbor joining tree depicting VP1 gene relationships of serotype O viruses, based on 495 nt, corresponding to amino acid positions 49-213. Bootstrap values are indicated in the figure and the scale provides a measure of 5 % sequence difference. I-III indicates the major evolutionary lineages, whilst the

seven genotypes are indicated by the letters A-G. The virus names followed by P, G, or B indicated pig, goat or bovine origin expect GHA/5/93P where P means published sequence.

	58	68	78	88	98	108	118	128
O1Campos	NILDLMQIPS	HTLVGALLRA	STYYFSDLEI	AVKHEGDLTW	VPNGAPEKAL	DNTTNPTAYH	KAPLTRLALP	YTAPHRVLAT
O1Kaufbeuren	....	....	....	....	....	....	....	....
O1BFS	.......V..	....	....	.....R....	....	....	....	....
O1Caseros	.T........	....	....	V.........	......VQ..	G........Y	.K........	....
O1Lombardy	.V........	Y.........	....	......Y...	.......S..	E.........	..?.......	....
O2Brescia	.V.N......	....	....	....	.......?..	E.........	....	....
O1/Yrigoyen	.T........	....	....	V.........	......V...	G........Y	.K........	....Y.....
O3Venezuela	.V.......G	....	....	....	.......Q..	....	....	....
ANG/10/74	.V.......G	....	V	....K.....	.......Q..	....	....	....
ANG/1/75	.V.......G	....	V	....	.......Q..	....	....	....
O1Manisa	.V.....T.A	.........T	A....A...V	......N...	.......A..	....	....	....
BAN/1/97	.V.....T.A	.........T	A....A...V	......N...	.......T..	....	....	....
O5India	.V.....T.A	.........T	A....A...V	......N...	.......T..	....	....	....
SAU/2/95	TV.....T.A	?........T	A....A...V	......N...	.......T..	.........?	.......E..	....
SAU/100/94	TV?...KT.A	.........T	A....A...V	......N...	.......T..	.........R	.......?..	.R........
KEN/77/78	.V.....T.A	.........T	A....A.I.V	......N...	.......S..	....	....	....
KEN/1/91	.V.....T.A	.........T	A....A...V	..T...N...	.......T..	....	....	....
Moscow/95	.V.......A	.........T	A........L	....	.......T..	....	.E........	....
Taiwan/97	.V.......A	.........T	A........L	....	.......T..	....	.E........	....
Taiwan/98	.V.......A	.........T	A........L	....	.......T..	....	.E........	....
NGR/1/88	.V.....T.A	.........T	A....A...V	....	.......T..	....	....	....
NGR/2/88	.V.....T.A	.........T	A....A...V	....	.......T..	....	....	....
NGR/3/88	.V.....T.A	.........T	A....A...V	....	.......T..	....	....	....
BFK/1/92	.V.....A.A	.........T	A....A...V	....	.......S..	....	....	....
BFK/2/92	.V.....A.A	.........T	A....A...V	....	.......S..	....	....	....
BFK/3/92	.V.....A.A	.........T	A....A...V	....	.......S..	....	....	....
GHA/5/93	.V.....A.A	.........T	A....A...V	....	......KS..	....	....	....
GHA/5/93P	.V.....A.A	.........T	A....A...V.	....	......S...	....	....	....
GHA/6/93	.V.....A.A	.........T	A....A...V.	....	......S...	....	....	....
GHA/7/93	.V.....A.A	.........T	A....A...V.	....	......S...	....	....	....
GHA/9/93	.V.....A.A	.........T	A....A...V.	....	......S...	....	....	....
ALG/1/99	.V.....T.A	.........T	A....A...V.	....	......T...	....	....	?.........
CIV/8/99	.V.....T.A	.........T	A....A...V.	....	......S...	....	....	....
TAW/2/99/B	.V...V.T.A	.........T	A....A...V.	.....N....	......T...	....	....	....
SKR/1/00/B	.A.....T.A	.........T	A....A...V.	....	......T...	....	..T.......	....
SAR/11/00P	.V.....T.A	.........T	A....A...V.	.....N....	......T...	....	....	....
SAR/12/00G	.V.....T.A	.........T	A....A...V	......N...	.......T..	....	....	....
SAR/13/00B	.V.....T.A	.........T	A....A...V	......N...	.......T..	....	....	....
SAR/15/00P	.V.....T.A	.........T	A....A...V	......N...	.......T..	....	....	....
SAR/18/00	.V.....T.A	.........T	A....A...V	......N...	.......T..	....	....	....
SAR/19/00	.V.....T.A	.........T	A....A...V	......N...	.......T..	....	....	....
SAR/25/00	.V.....T.A	.........T	A....A...V	......N...	.......T..	....	....	....
SAR/26/00	.V.....T.A	.........T	A....A...V	......N...	.......T..	....	....	....
JPN/2000	.V.....T.A	.........T	A....A...V	......N...	.......T..	....	....	....

	138	148	158	168	178	188	198	↓213	
O1Campos	VYNGECRYSR	NAVPN**RGD**LQ	VLAQKVART	LPTSFNYGAI	KATRVTELLY	RMKRAETYCP	RPLLAIHPTE	ARHKQKIVAPVKQ	TL
O1Kaufbeuren	.....N.	.....L....	.....	.....	.....	.....	.....	.....	..
O1BFS	.....	.....L....	.....	.....	.....	.....	.....	.....	..
O1Caseros	.....K..S	K.....	N..E..A...	.....	.....	.....	.....D	.....	..
O1Lombardy	....GS....	.T.....	...T..A...	.....	R.....	.....	.....	.....	..
O2Brescia	.....S....	...?.....	.A....A...	.....	R.....	.....	.....	.....A..	..
O1/Yrigoyen	.....T..S	.....P	NL.E..A..M	..AY.....	.....	.....	.....D	....R.....R	..
O3Venezuela	....R.....	DV.T..I...	.A..H.A..S	.....	.....	.....	.....	.....	..
ANG/10/74	....R...GA	D.....	.....A..S	.....	....I.....	.....	.....	.....	S.
ANG/1/75	....R...GA	D.....	.....A..S	.....	....I.....	.....	.....	.....	S.
O1Manisa	....N.K.GD	GT.A.....	.....A..A	.....	.....	.....	.....DQ	.....	L.
BAN/1/97	....N.K.GE	SP.T.....	.....AT..	.....	.....	.....	.....S.	.....	L.
O5India	....N.K.AD	GP.A.....	.....A..A	.....	.....	.....	.....S.	.....	L.
SAU/2/95	....N.K.GE	SS.T.....	.....A...	.....	.....	.....	.....S.	.....	L.
SAU/100/94	....N.K.GE	SC.T.....	.....A...	.....	.....	.....	.....S.	.....	L.
KEN/77/78	....N...G.	AP.T.....	.....A...	.....	.....I....	.....	.....T..S.	.....R....A..	L.
KEN/1/91	....N.K.GG	TPTT.....	.....A...	.....	.....	.....	.....SQ	.....	L.
Moscow/95	....SSK.GD	TSTN.....	.....AE..	.....F...	.....	.....	.....Q.SD	.....?A..	L.
Taiwan/97	....SSK.GD	TSTN.....	.....AE..	.....F...	.....	.....	.....Q.SD	.....R....A..	L.
Taiwan/98	....SSK.GD	TSTN.....	.....AE..	.....F...	.....	.....	.....Q.SD	.....R....A..	L.
NGR/1/88	....S.K..G	VS.....	.....RA...	.....F...	.....	.....	.....S.	S..E.....	L.
NGR/2/88	....S.K..G	VS.....	.....RA...	.....F...	.....	.....	.....S.	S.....	L.
NGR/3/88	....S.K..G	VS.....	...R.RA...	.....F...	.....	.....	.....S.	S.....	L.
BFK/1/92	....S.K...	VE..K.....	.....RA...	.....F...	.....	.....	.....V..S.	T.....M..	L.
BFK/2/92	....S.K...	VE..K.....	.....RA...	.....F...	.....	.....	.....V..S.	T.....M..	L.
BFK/3/92	....S.K...	VE..K.....	.....RA...	.....F...	.....	.....	.....V..S.	T.....M..	L.
GHA/5/93	....S.K...	VE..K.....	....RRA...	.....F...	.....	.....	.....V..S.	T.R.....M..	L.
GHA/5/93P	....S.K...	VE..K.....	....RRA...	.....F...	.....	.....	.....V..S.	T.....M..	L.
GHA/6/93	....S.K...	VE..K.....	....RRA...	.....F...	.....	.....	.....V..S.	T.....M..	L.
GHA/7/93	....S.K...	VE..K.....	....RRA...	.....F...	.....	.....	.....V..S.	T.....M..	L.
GHA/9/93	....S.K...	VE..KL....	....RRA...	.....F...	.....	.....	.....V..S.	T.....M..	L.
ALG/1/99	....S....G	AVT.....	....RRA.PM	.....F...	.....	.....	.....F..S.	.....	L.
CIV/8/99	....S....G	AVT.....	....RRA.PM	.....F...	.....	.....	.....F..S.	.....A..	L.
TAW/2/99/B	....N.K.GE	SP.T.....	.....A...	.....	.....	.....	.....S.	.....	L.
SKR/1/00/B	....N.K.GE	SP.T.....	.....A...	.....	.....	.....	.....S.	.....	F.
SAR/11/00P	....N.K.GE	SP.T.....	.....A...	.....	.....	.....	.....S.	.....	L.
SAR/12/00G	....N.K.GE	SP.T.....	.....A...	.....	.....	.....	.....S.	.....	L.
SAR/13/00B	....N.K.GE	SP.T.....	.....A...	.....	.....	.....	.....S.	.....	L.
SAR/15/00P	....N.K.GE	SP.T.....	.....A...	.....	.....	.....	.....S.	.....	L.
SAR/18/00	....N.K.GE	SP.T.....	.....A...	.....	.....	.....	.....S.	.....	L.
SAR/19/00	....N.K.GE	SP.T.....	.....A...	.....	.....	.....	.....S.	.....	L.
SAR/25/00	....N.K.GE	SP.T.....	.....A..K	.....	.....	.....	.....S.	.....	L.
SAR/26/00	....N.K.GE	SP.T.....	.....A...	.....	.....	.....	.....S.	.....	L.
JPN/2000	....N.K.GE	SP.T.....	.....A...	.....	.....	.....	.....S.	.....	L.

Fig. 2.2 Deduced amino acid sequences of the 495 nt region corresponding to the C-terminus half of VP1 gene. Number of sequences = 44; Number of sites = 165; - identical to consensus sequence; ? = ambiguous sites; RGD is indicated in bold and is underlined; the cleavage site VP1/2A (QTL)is indicated by an arrow.

2.5 Discussion

The use of a partial nucleotide sequences of the VP1 gene of viruses from North, West, East and southern Africa has allowed assessment of the regional variation and structuring within serotype O viruses. Of the seven distinct genotypes (A-G) identified in this study, four of the genotypes (A, D, F and G) consist of isolates that have been characterized in earlier studies (Krebs & Marquardt, 1992; Sáiz *et al.*, 1993; Samuel *et al.*, 1997; Knowles, 1997; Freiberg *et al.*, 1999; Tsai *et al.*, 2000), two comprise viruses of African origin (B and C), whilst the remaining genotype (E), provides the first evidence of type O virus introduction into southern Africa from a different continent. Evidence for inter-continental transmission is similarly provided by the close genetic relationship of the south African type O strain and viruses constituting the 'PanAsian' lineage, as defined by Knowles and co-workers (2000).

These results were in agreement with studies on other FMD serotypes indicating that trans-continental transmission does occur (Samuel & Knowles, 1999). These authors also reported a single virus lineage spread from the Middle East that caused an epidemic in western North Africa in a FMD-free region. Similarly, the viruses isolated from Bangladesh in 1997 were found to be related to the genotype previously identified from India, Iran, and Saudi Arabia (Freiberg *et al.*, 1999). The viruses that caused outbreaks in Israel and other Middle-Eastern countries in 1994 were also linked to isolates from Jordan and Lebanon (Stram *et al.*, 1995).

The genotypes defined here constitute three discrete evolutionary lineages (I-III) which correspond to different continental regions. Lineage I is made up of viruses of Asian and African origin, with the latter being exclusively represented by countries north of the equator, Lineage II is represented by viruses recently introduced into Taiwan (1997-1998) and Russia (Tsai *et al.*, 2000), whilst lineage III comprises viruses from Europe and South America. Type O viruses recovered from outbreaks in South Africa and the Angola cluster fall within two different evolutionary lineages (I and III) and are caused by introductions from other continents. These results indicated that type O may be an exotic FMD serotype to the southern African region. However, this is the first evidence of the trans-continental introduction of type O to the sub-equator zone of the African continent (link between Venezuela one isolate in 1951 & Angola outbreak viruses isolated in 1974-1975; Fig 2.2). Similar transmission of the same serotypes from the Middle East to North Africa has been reported (Samuel *et al.*, 1999). Additional genetic characterization of southern African viruses would assist in the clarification of the type O status in this region.

Within Genotype C, which comprises viruses exclusively of African origin, there are four distinct sub-clusters. Viruses within one of these sub-clusters are from neighboring countries in West Africa (Burkina Faso and Ghana) and show high levels of sequence identity which indicates that they were from the same origin. However, it would only be possible to identify the virus origin if samples from more neighboring countries were included in the study. It has also been reported that closely related viruses could either be from the same outbreak or from viruses temporally closely related (Samuel et al., 1999). In contrast, although Algeria and Niger share borders, the viruses from these countries differ from each other by more than 10 % and make up distinct sub-clusters. It is important to mention that there were six years difference in the isolation date between viruses from West and North Africa. The mutations that occur during the replication process over 6 years may explain the high nucleotide difference between the Algeria and Niger virus isolates.

The second distinct African genotype, is the Kenyan virus lineage (genotype B) which although only represented by two viruses, shows the highest level of variation within genotype sequences. These viruses differ from each other by 13 % on nucleotide level. Previous studies also reported a homogenous clustering among Kenyan type O viruses, while isolates from other East African countries (Tanzania, Eritrea, and Ethiopia) were grouping with those from the Middle East and South Asia (Samuel & Knowles, 2001). The sample size in this case was also small. It is likely that further characterization of viruses from East Africa will identify additional virus types or genotypes and should be investigated further.

The presence of southern African viruses within a distinct European-South American lineage (III) is strongly indicative of an introduction from one of these mentioned continents, as all other viruses from Africa, cluster within the Afro-Asian lineage (I). It is important to note that O3 Venezuela and the Angolan isolates although temporally unrelated (23 years between isolations), form the same genotype E. Data analysis with a shorter genomic region, further indicates that there is statistically significant support for the Angola-Venezuela-Argentina genotype (results not shown). Genotype E, therefore provides the first evidence of virus introduction from another continent into southern Africa (Fig.2.1).

Genotype A which includes viruses from South Africa (2000) is primarily composed of viruses from the Middle East and Asia, that pre-date the South African outbreak. This together with the inability to demonstrate a link to any distinctly African genotypes (B and C) supports the believe that the type O virus was most likely imported into South Africa from one of the Asian countries most afflicted by FMD in 2000 (Knowles *et al.*, 2000). This is supported by the high level of sequence identity between viruses isolated previously from Japan and those recovered from the recent outbreak in South Africa. Further evidence includes the consistent recovery of the South Africa-Japan-Saudi Arabia-Bangladesh cluster, statistically significant bootstrap support (> 99 %) for this cluster, and anecdotal evidence of swine becoming infected after being fed ship swill obtained from Durban harbour. The sequence conservation between these viruses was 92.1 % at the nucleotide level and 95.8 % at the amino acid level. The complete sequence conservation of the eight South African viruses examined points to a single introduction of the virus that caused the outbreak. Retrospective studies indicate that the Pan-Asia strain which is dominant in the field was present in India in 1990, from where it appears to have spread westwards to the Middle East and eastwards to south-east Asia. Outbreaks originated from a single transmission of the virus had been reported previously (Tsai *et al.*, 2000). Tsai and co-workers (2000) investigated 49 clinical samples by direct amplification and cycle sequencing and a divergence of 0.2-0.9 % was observed at the nucleotide level indicating a single common source.

The results presented here support the genetic grouping of serotype O viruses on the basis of geographical origin (Sáiz *et al.*, 1993), in accordance with the topotype concept (Samuel & Knowles, 2001). The results here corresponds with prior molecular studies and confirm the prolonged circulation of viruses in the field. This study has also been useful in identifying the trans-boundary and inter-continental transmission of viruses in West and southern Africa. The cross border transmission of the viruses reported here has shown the need to implement a collaborative control programme between neighboring West African countries to combat the disease. The phylogenetic resolution of viruses of African origin has assisted in the identification of distinct virus groups, and forms a valuable basis for identifying the origin of future outbreaks across the continent.

CHAPTER 3

MOLECULAR EPIDEMIOLOGY STUDY OF SAT-2 TYPE FOOT-AND-MOUTH DISEASE VIRUSES RECOVERED FROM OUTBREAKS IN WEST AFRICA (1974-1991)

3.1 Summary

Thirty viruses from major outbreaks in seven West African countries and four isolates from East and Central Africa were genetically characterized in this study. Phylogenetic analysis of an homologous region of 480 nucleotides corresponding to the C-terminus end of the VP1 gene revealed four evolutionary lineages (I-IV). Lineage (I) comprised two West African genotypes with viruses clustering according to year of isolation rather than geographical origin. Lineage II was represented solely by viruses isolated between 1979 to 1983 in two neighbouring west African countries, Senegal and Gambia, whilst viruses from East and West Africa constituted lineage (III). Lineage (IV) was composed of isolates from Central and East Africa.

This study revealed that several epizootics caused by SAT-2 viruses in different West African countries shared a same origin possibly due to the fact that animal movement is not restricted in the region. This is a major factor in disease dissemination and complicates the epidemiology of FMD in West Africa. The study also showed a link between a virus from Nigeria (NIG2/82) with isolates from East Africa. This has provided the first evidence of transmission of viruses between East and West Africa indicating that there are no barriers to trans-regional virus infection.

3.2 Introduction

Amongst the four FMD virus serotypes identified in West Africa, SAT-2 was the most prevalent FMDV type recovered from outbreaks between 1974-1991 in the region. Of the 12 West African countries, SAT-2 has been recorded in eight, namely Mali, Ivory Coast, Ghana, Nigeria, Liberia, Senegal, Gambia and Mauritania (Records of the OIE/WRL) but it is possible that some outbreaks have not been reported due to the poor communication network in many rural areas in the region. Since the eradication of rinderpest, attention has shifted to other infectious diseases mainly FMD due to a marked increase of outbreaks in West Africa (Chapter 2).

SAT-2 is now endemic in many west African countries. Besides type O, cattle with severe clinical signs due to SAT-2 have been reported particularly in Mali (Report/LCV). This situation has raised concern because the disease is normally mild in the local breeds. During the last few years, a variation in the virulence of outbreaks of FMD in cattle in the region is widely observed across the region. In Mali, animals reluctant to move and to eat followed by death were reported in young animals (Report/LCV). It is important to note that cattle recovered from the disease are not removed from the herd. Since carrier stage has been reported in cattle, therefore this latter factor probably plays a role in the maintenance of the viruses in the field in the region (Gebauer *et al.*, 1988). Therefore, this predominance of SAT-2 and an increase in the virulence should be investigated to determine the origin of the virus and to control the disease.

Currently, the growing population's high demand for animal protein and the need to compete in international markets have raised the profile of the FMD in West Africa. Despite its importance and the regular involvement of SAT-2 type viruses in outbreaks in this region, little is known of the epidemiology of the disease. This study represents a first attempt to address this short-coming by focusing on elucidating the regional genetic relationships of SAT-2 viruses recovered from outbreaks between 1974 and 1991 in West Africa.

3.3 Materials and methods

3.3.1 Viruses used in this study

Tissue culture isolates were supplied by the World Reference Laboratory (WRL, UK). The study was conducted on six 1974 isolates from Ghana, Nigeria, Ivory Coast and Liberia, five 1975 viruses from Nigeria and Senegal, four 1979 viruses from Senegal and Gambia, one 1982 virus from Nigeria, four 1983 viruses from Senegal, three 1990 viruses from Ivory Coast and Ghana, and seven 1991 viruses from Mali and Ghana. Viruses which were previously characterized from Kenya, one 1982 virus from Zaire, one 1998 virus from Eritrea and one 2000 virus from Rwanda were also included. The summary of the viruses used in this study is indicated in Table 3.1.

3.3.2 Cell cultures, RNA extraction and cDNA synthesis

Viruses were grown in IB-RS-2 cells in 25 cm^2 tissue culture flasks (Corning). The medium and methods used for virus stocks and replication were the same as described in section 2.3.3. RNA was

extracted and cDNA synthesized as previously described in sections 2.3.4 & 2.3.5.

3.3.3 PCR amplification and DNA purification

A DNA band of 518 bp corresponding to the VP1 gene was amplified by RT-PCR following methodology outlined in section in 2.3.6. The universal antisense primer was combined with an upstream primer (VP1Ub) 5' CCA CGT ACT ACT TYT CTG ACC TGG A 3'. A low amplification rate in the PCR reaction was obtained with this primer combination for west African SAT-2 and SAT-1 isolates (Fig 3.1 & 3.2), although they were shown to successfully amplify >98 % of SAT-types viruses from southern Africa (Bastos, 1998). The DNA bands were faint and non-specific amplifications were observed (Fig. 3.1, 3.2). Amplification of SAT-2 type viruses from West Africa was then attempted with a primer binding in VP3 (Bastos et al., 2002); and amplifying a product of 880 bp. SAT-2 viruses which amplified successfully with this primer combination were sequenced and on the basis of these sequences a new West African-specific SAT-2 primer, termed SAT2U-OS was designed. This primer, 5' CCA CNT TCG AGG TCA ACT TGA T 3' binds at nucleotide positions 139-160 in the VP1 gene (Fig 4.1). All PCR reactions were performed in a total volume of 50 □l using both primers (SAT2U-OS and P1) with annealing temperature at 57^{o}C for 30 sec. A PCR product of 518 bp was obtained and processed as described in section 2.3.8 (Fig 3.3).

3.3.4 Sequencing and data analysis

Both manual and automated sequencing were conducted as described in 2.3.8 & 2.4. An homologous region of 480 nt, corresponding to the C-terminus region was determined and aligned using the same method as in 2.4. The variability plot was also constructed to identify high variable regions using the same computer program as described in 2.4.

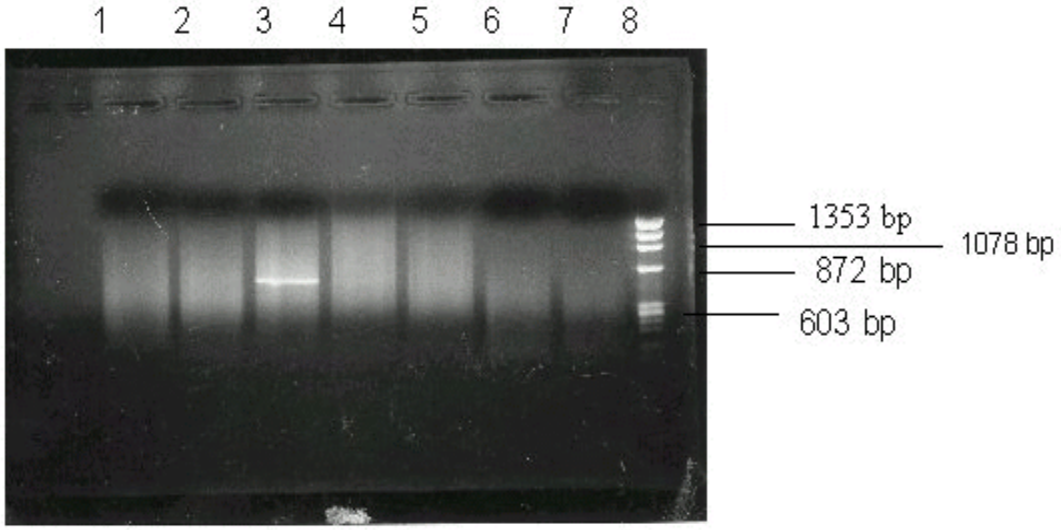


Fig. 3.1 Agarose gel showing amplification results using W-DA & VP1Ub primers. cDNA of the following SAT-2 was used (lanes 1-7): GAM8/79, SEN2/79, LIB1/74, MAI2/91, MAI6/91, MAU2/75 and IVY7/90. DNA size was determined using the □X174 (Hae III) molecular weight marker (Promega) in lane 8. The PCR product in lane 3 is approximately 500 bp. Figures 3.1 & 3.2 summarizes results obtained with the published primers W-DA & VP1Ub which were shown to successfully amplify >98 % of SAT-type viruses from southern Africa (Bastos, 1998).

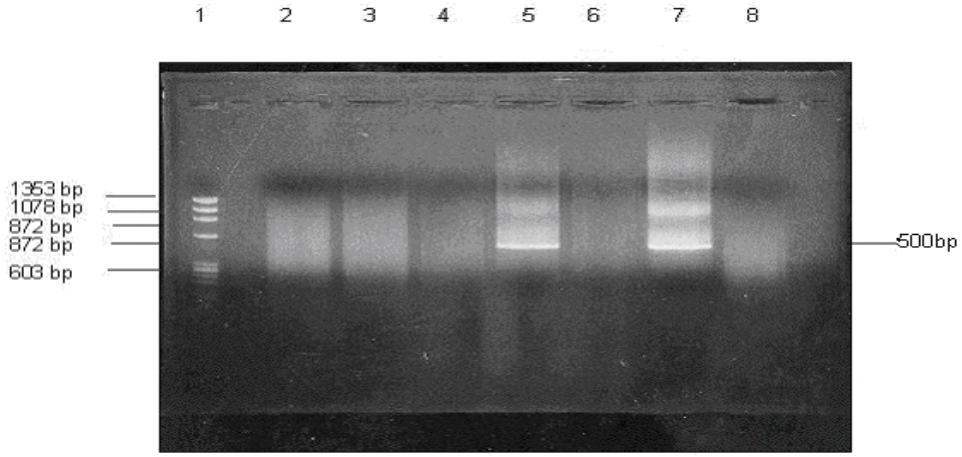


Fig. 3.2 Agarose gel showing amplification results using W-DA & VP1Ub primers. cDNA of the following SAT-1 was used (lanes 2-8): NGR5/76, NIG5/81, NIG11/81, NIG14/76, NIG1/76 and NIG5/76. The PCR product size was estimated using the □X 174 (Hae III) molecular weight marker (Promega) in lane 1. The PCR products in lanes 5 & 7 are approximately 500 bp.

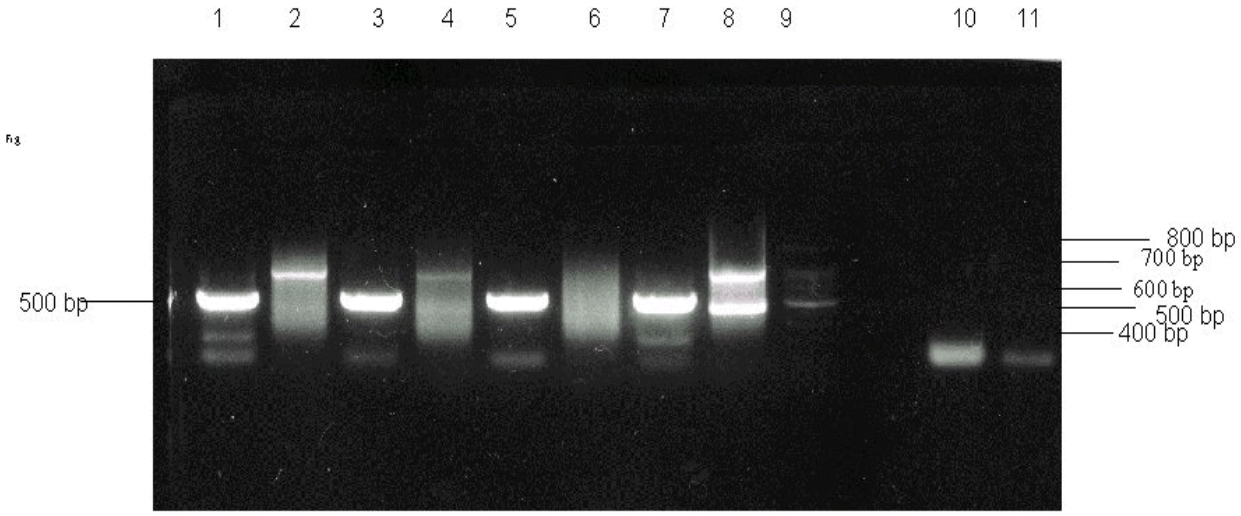


Fig. 3.3 This figure compares W-DA & VP3-AB (lanes 2, 4, 6 & 8) with those obtained with the west African specific primer SAT2-OS & W-DA (lanes 1, 3, 5, & 7). The cDNA templates used for the PCR reactions were the following: GHA2/90 (lanes 1 & 2), NIG1/75 (lanes 3 & 4), NIG2/75 (lanes 5 & 6), GAM9/79 (lanes 7 & 8) and a negative control (lanes 10 & 11). Size of amplicons was determined against the 100 bp ladder molecular weight marker in lane 9.

3.4 Results

3.4.1 VP1 sequence analysis

For comparison of the genetic relationships of SAT-2 FMD viruses north of the African equator, 34 viruses from different geographical locations were selected for this study. Nucleotide sequences were determined for 30 SAT-2 viruses involved in outbreaks in West Africa between 1974-1991 and four viruses previously sequenced from East and Central Africa recovered between 1957 and 2000. An homologous region of 480 nt was ultimately used for phylogenetic analysis which meets the general concept that the longer the sequence analyzed the more accurate the FMD phylogeny that is inferred (Martin *et al.*, 1995). Eight major genotypes were identified by phylogenetic reconstruction (Fig. 3.4) based on high bootstrap support of > 70 % and the cut-off criterion that picornaviruses with more than 85% sequence identity belong to the same genotype (Rico-Hesse *et al.*, 1987). These genotypes (labeled A-H) constitute four major evolutionary lineages (I - IV) that are associated with geographically distinct regions (Fig. 3.4). Lineages (I) and (II) were made up of viruses of West African origin, whilst lineages III and IV include viruses from West-East Africa and Central-East Africa, respectively. Of interest is the presence of a single west African virus (NIG/2/82) in the East African lineage (III).

Eight major SAT-2 genotypes were consistently recovered with neighbor joining, UPGMA and parsimony methods indicating that the trees were a robust estimate of the true viral relationships (Kim, 1993). The geographical distribution of these genotypes is indicated in Fig. 3.6 and constitute the following major viral lineages:

Lineage I	-	Genotype A:	Ghana, Liberia, Ivory Coast, Nigeria and Senegal (1974 - 1975)
		Genotype B:	Ghana, Ivory Coast and Mali (1990 -1991).
Lineage II	-	Genotype C:	Gambia and Senegal (1979 - 1983)
Lineage III	-	Genotype D:	Nigeria (1982)
		Genotype E:	Eritrea (1998)
Lineage IV	-	Genotype F:	Democratic Republic of the Congo (1982)
		Genotype G:	Kenya (1957)
		Genotype H:	Rwanda (2000)

Viruses from West Africa grouped according to the year of isolation rather than outbreak locality. In the case of genotypes A and B, viruses were recovered over a one year period. Extensive circulation of a single virus type in the field was however indicated by genotype C which included viruses recovered over a four year period (1979-1983). In all three genotypes (A-C), sequence identity values were consistently . 97 %. Together these genotypes (A, B & C) constitute a distinct west African assemblage of viruses (lineages I-II). The viruses representative of the remaining five genotypes displayed a geographical rather than temporal association. This was particularly true for lineage IV which comprised viruses from three neighboring East and Central African countries despite these viruses being sampled over a 43 year period (1957-2000).

3.4.2 Amino acid variation

In order to determine whether the patterns of variation were random or specific to some regions of the gene, hypervariable regions were identified in which > 40 % of the sites were shown to vary (Fig. 3.7). These hypervariable regions were located at amino acid positions 80-89, 137-161, and 199-214 (Fig. 3.7). The latter two regions of variation corresponded to the highly immunogenic G-H loop and C-

terminus parts of the gene, respectively (Xie *et al.*, 1987, Pfaff *et al.*, 1988, Parry *et al.*, 1990) whilst amino acid positions 80-89 incorporate the D-E loop. The cell attachment site, Arg-Gly-Asp (RGD) at amino acid residues 145-147, within the G-H loop was completely conserved across all viruses included in this study. The arginine residue at position 148 was present in all but two viruses from West Africa (SEN1/75 and SEN2/75 had methionine at position 148). It has been reported previously that mutations within amino acids bordering the RGD sequence influence the binding of FMD virus to cells (Bittle *et al.*, 1982). The amino acid position 148 is located in the middle of the hydrophobic arc of the loop and leucine at that position has been reported to enhance and stabilize the -helix formation (France *et al.*, 1994). A cysteine residue at the base of the G-H loop (position 135) was conserved among all the isolates (Fig 3.5). Studies on type O virus have indicated that a cysteine at this position is associated with disulphide bond formation with a corresponding cysteine in VP3 that appears to enhance the disordered structure of the G-H loop and promotes conformational epitope formation (Parry *et al.*, 1990, Logan *et al.*, 1993). The cleavage site VP1/2A was not conserved in SAT-2 viruses of west African origin. Two distinct cleavage site sequences were observed namely, KQ/LC (lysine-glycine/leucine-cystein) and RQ/TC (arginine glutamine/ threonine cysteine) (Fig. 3.3). The KQ variant was always associated with an LC in 2A, whilst a change from KQ to RQ in VP1 was always coupled with a change from LC to TC in 2A. Variation in sequences on the 2A side of the cleavage have been reported previously (Van Rensburg *et al.*, 2001).

TABLE 3.1: Summary of SAT-2 isolates used in this study

Virus designation	Year isolation	Country	Reference	Genbank No
GHA/10/74	1974	Ghana	This study	AF426068
GHA/14/74	1974	Ghana	This study	AF426069
IVY/9/74	1974	Ivory Coast	This study	AF426070
LBR/1/74B	1974	Liberia	This study	AF426071
NIG/1/75	1975	Nigeria	This study	AF426074
NIG/2/75	1975	Nigeria	This study	AF367139
SEN/1/75	1975	Senegal	This study	AF426076
SEN/5/75	1975	Senegal	This study	AF367140
GAM/9/79	1979	Gambia	This study	AF426078
SEN/1/83	1983	Senegal	This study	AF426079

SEN/3/83	1983	Senegal	This study	AF426080
GHA/2/90	1990	Ghana	This study	AF426081
IVY/2/90	1990	Ivory Coast	This study	AF426082
GHA/8/91	1991	Ghana	This study	AF426083
MAI/1/91	1991	Mali	This study	AF426084
MAI/2/91	1991	Mali	This study	AF426085
MAI/3/91	1991	Mali	This study	AF426086
MAI/5/91	1991	Mali	This study	AF426087
MAI/6/91	1991	Mali	This study	AF426088
MAI/7/91	1991	Mali	This study	AF426089
NIG/1/74	1974	Nigeria	This study	AF426091
NIG/35/74	1974	Nigeria	This study	AF426092
GAM/8/79	1979	Gambia	This study	AF426093
SEN/7/79	1979	Senegal	This study	AF426094
SEN/8/79	1979	Senegal	This study	AF426095
NIG/2/82	1982	Nigeria	This study	AF426096
SEN/5/83	1983	Senegal	This study	AF426097
SEN/7/83	1983	Senegal	This study	AF426098
IVY/7/90	1990	Ivory Coast	This study	AF426099
SEN/2/75	1975	Senegal	This study	AF431732
ERI/12/98	1998	Eritrea	Ref. 31	AF367126
KEN/3/57	1957	Kenya	Unpublished	AJ251473
ZAI/1/82	1982	D.R.C.	Ref. 31	AF367100
RWA/1/00	2000	Rwanda	Ref. 31	AF367134

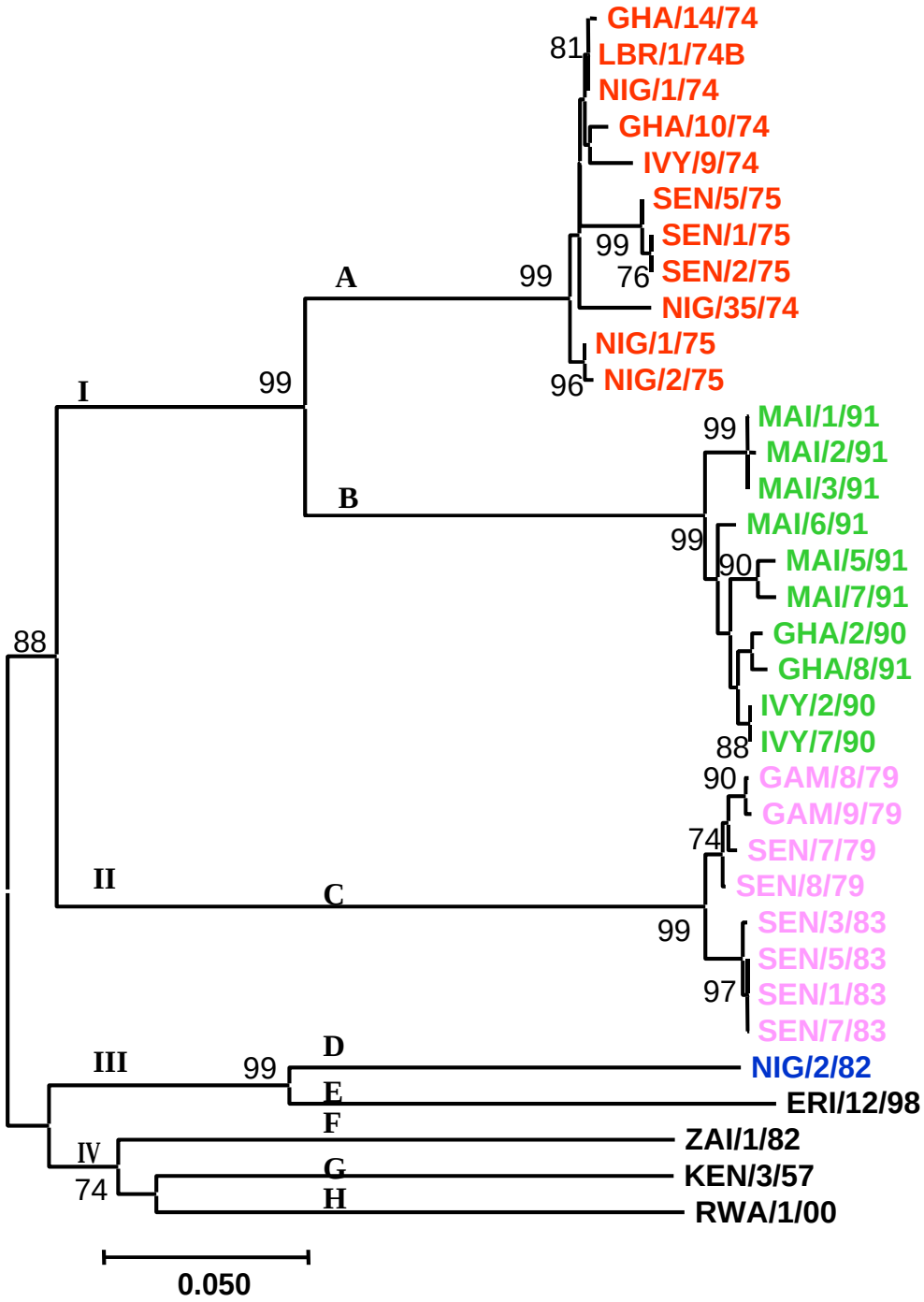


Fig. 3.4 Neighbor-joining tree depicting VP1 gene relationships of SAT-2 type FMD viruses from West Africa (1974-1991) and Central and East Africa (1957-2000). Bootstrap values >70 based on 10000 replications are indicated as are the SAT-2 genotypes (labelled A-H) and the genetic lineages (labelled I-IV).

	66	**76**	**86**	**96**	**106**	**116**	**126**	**136**
KEN/3/57	KEKALVGAIL	RSATYYFCDL	E**VACVGKHKR**			**VF**WQPNGAPR	TTQLGDNPMV	FSHNNVTRFA
							IPFTAPHRLL	STVYNGEC**EY**
GHA/10/74	.K.....V.	.A.....	.I....E...	.....	.....	....K.....	.....	.....N.
GHA/14/74	.....V.	.A.....	.I....E...	.....	.....	....K.....	.....	.....N.
IVY/9/74	.K.....V.	.A.....	.I....E...	.....	.....	....K.....	.....	.....N.
LBR/1/74B	.....V.	.A.....	.I....E...	.....	.....	....K.....	.....	.....N.
NIG/1/74	?.....V.	.A.....	.I....E...	.....	.....	....K.....	.....	.....N.
NIG/35/74	.....V.	.A.....	.I....E...	.....	A.....	....K.....	.....	.....N.
NIG/1/75	.....V.	.A.....	.I....E...	.....	.....	....K.....	.....	.....N.
NIG/2/75	.....V.	.A.....	.I....E...	.....	.....	...KK.....	.....	.....N.
SEN/1/75	.....V.	.A.....	.I....D...	.....	A.....	....K.....	.....	.....N.
SEN/2/75	.....V.	.A.....	.I....D...	.....	A.....	....K.....	.....	.....N.
SEN/5/75	.....V.	.A.....	.I....D...	.....	A.....	....K.....	.....	.....N.
GAM/8/79	.D.T....L.	.AT....A..	.I....D...		.Y.....	.....	....R.....	..Y.....	.....V.
GAM/9/79	.D.T....L.	.AT....A..	.I....D...		.Y.....	.....	....R.....	..Y.....	.....V.
SEN/7/79	.D.T....L.	.AT....A..	.I....D...		.Y.....	.....	....R.....	..Y.....	.....V.
SEN/8/79	.D.T....L.	.AT....A..	.I....D...		.Y.....	.....	....R.....	..Y.....	.....V.
NIG/2/82	.....	.AS.....	.I....E...	.Y.....	.....	.AK.G.....	.....	.....S.
SEN/1/83	.D.T....L.	.AT....A..	.I....D...		.Y.....	.....	....R.....	..Y.....	.....V.
SEN/3/83	.D.T....L.	.AT....A..	.I....D...		.Y.....	.....	....R.....	..Y.....	.....V.
SEN/5/83	.D.T....L.	.AT....A..	.I....D...		.Y.....	.....	....R.....	..Y.....	.....V.
SEN/7/83	.D.T....L.	.AT....A..	.I....D...		.Y.....	.....	....R.....	..Y.....	.....V.
GHA/2/90	.....V.	.A.....	.IT...E.A.		.....	.....	.....	..Y.....	.....K.
IVY/2/90	.....V.	.A.....	.I....E.A.	.....	.....	.....	..Y.....	.....K.
IVY/7/90	.....V.	.A.....	.I....E.A.	.....	.....	.....	..Y.....	.....K.
GHA/8/91	.....V.	.A.....	.IT...E.A.		.....	.....	.....	..Y.....	.....K.
MAI/1/91	.....V.	.A.....	.I....E.T.	.....	.....	.....	..Y.....	.....K.
MAI/2/91	.....V.	?A.....	.I....E.T.		.....	.....	.....	..Y.....	.....K.
MAI/3/91	.....V.	.A.....	.I....E.T.	.....	.....	.....	..Y.....	.....K.
MAI/5/91	.....V.	.A.....	.IT...E.A.		.....	.....	..R.....	..Y.....	.....K.
MAI/6/91	.....V.	.A.....	.I....E.A.	.....	.....	.....	..Y.....	.....K.
MAI/7/91	.....V.	.A.....	.IT...E.A.		.....	.....	..R.....	..Y.....	.....K.

ZAI/1/82 .... .I....D.T. ....K..... YA..H..... ....?.....
ERI/12/98 .G..... .AS..... .I....D.T. .... YAKGG..... ....T.
RWA/1/00 .....V. ....D.A. .... .A..K..... ..Y..... .....

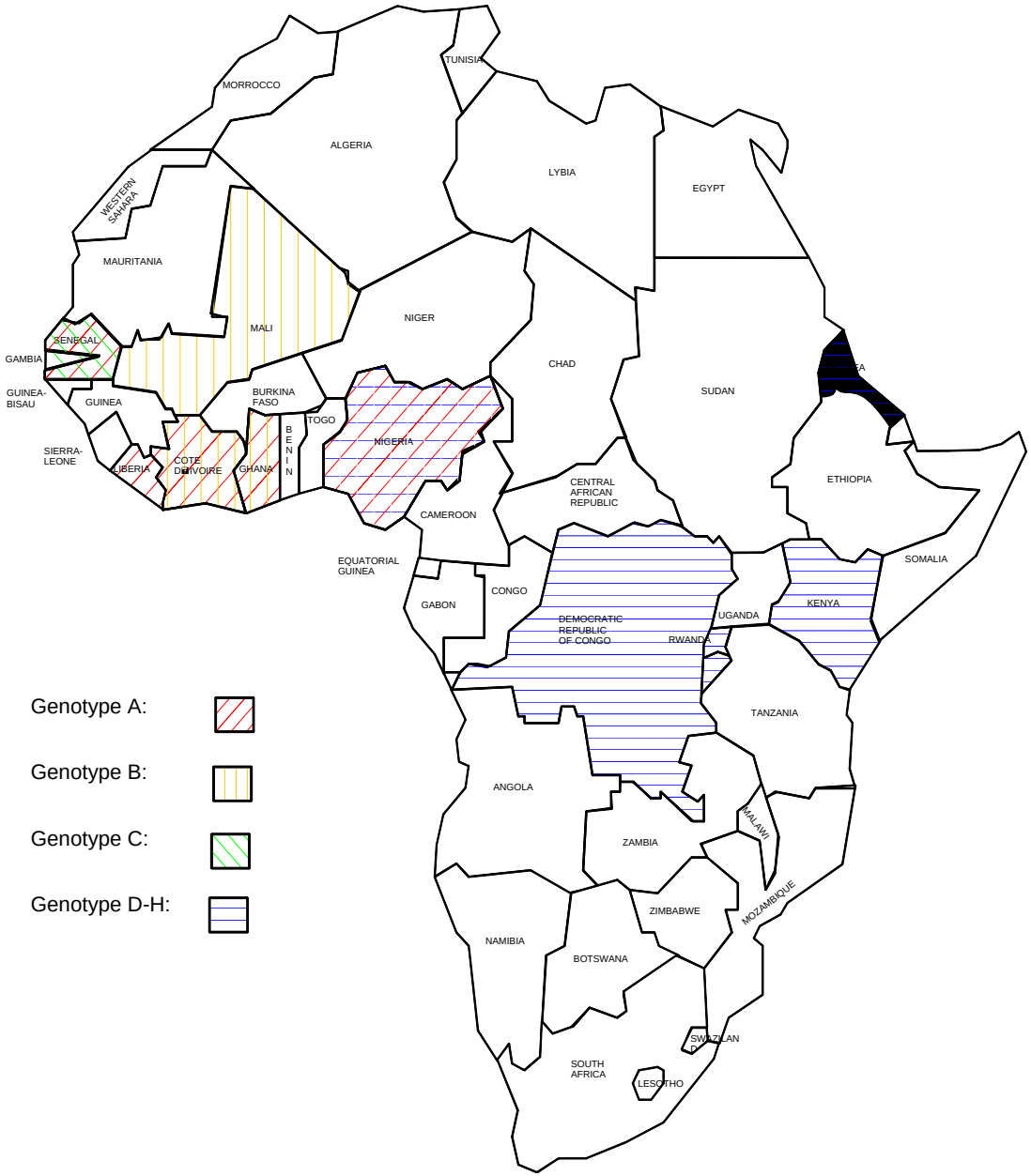
146 156 166 176 186 196 206 214 ↓216

KEN/3/57 **TKTVTAI<u>RGD</u>** REVLAQKYSS AKHSLPSTFN FGFVTADKPV DVYYRMKRAE LYCPRALLPA YT**HAGGDRFD A**PIGVAKQ LL
GHA/10/74 STS..P.... .A...A..AN T..T..... ..Y....A.. .....S. .....P.... .D.QSR.... .....E.. .C
GHA/14/74 STS..P.... .A...A..AN T..T..... ..Y....A.. .....S. .....P.... .D.QSR.... .....E.. .C
IVY/9/74 STS..P.... .A...A..AN T..T..... ..Y....A.. .....S. .....P.... .D.QSRH.V. .....E.. .?
LBR/1/74B STS..P.... .A...A..AN T..T..... ..Y....A.. .....S. .....P.... .D.QSR.... .....E.. .C
NIG/1/74 STS..P.... .A...A..AN T..T..... ..Y....A.. .....S. .....P.... .D.QSR.... .....E.. .C
NIG/35/74 STS..PV... .A...A..AN T..T..... ..Y....A.. .....S. .....P.... .D.QSR.... .....E.. .C
NIG/1/75 STS..P.... .A...A..AN T..T..... ..Y....A.. .....S. .....P.... .D.QSR.... .....E.. .C
NIG/2/75 STS..P.... .A...A..AN T..T..... ..Y....A.. .....S. .....P.... .D.QSR.... .....E.. .C
SEN/1/75 STS..P.... MA...A..A. T..T..... ..Y....A.. .....S. .....P.... .D.QSH.... .....E.. .C
SEN/2/75 STS..P.... MA...A..A. T..T..... ..Y....A.. .....S. .....P.... .D.QSH.... .....E.. .C
SEN/5/75 STS..P.... .A...A..A. T..T..... ..Y....A.. .....S. .....P.... .D.QSH.... .....E.. .C
GAM/8/79 AD...P.... .Q...A...N R..Q..... Y.Y....E.. ..... F..... .D.HSR.... S....E.. .C
GAM/9/79 AD...P.... .Q...A...N R..Q..... Y.Y....E.. ..... F..... .D.HSR.... S....E.. .C
SEN/7/79 AD...P.... .Q...A...N R..Q..... Y.Y....E.. ..... F..... .D.HSR.... S....E.. .C
SEN/8/79 AD...P.... .Q...A...N R..Q..... Y.Y....E.. ..... F..... .D.HSR.... S....E.. .C
NIG/2/82 K.ET..... .A...A..A. T..T..... .....A.. .....T. .....P.... .D...R.... .....ER. T.
SEN/1/83 AD..AP.... .Q...A...N R..Q..... Y.Y....E.. ..... ?..... .D.HSR.... S....E.. .C
SEN/3/83 AD..AP.... .Q...A...N R..Q..... Y.Y....E.. ..... F..... .D.HSR.... S....E.. .C
SEN/5/83 AD..AP.... .Q...A...N R..Q..... Y.Y....E.. ..... F..... .D.HSR.... S....E.. .C
SEN/7/83 AD..AP.... .Q...A...N R..Q..... Y.Y....E.. ..... F..... .D.HSR.... S....E.. .C
GHA/2/90 NT..QP.... .A...N..AN R..T..... ..Y..... ....P.... .D.QSR.... .....E.. .C
IVY/2/90 NT..QP.... .A...N..AN R..T..... ..Y..... ....P.... .D.QSH.... .....E.. .C
IVY/7/90 NT..QP.... .A...N..AN R..T..... ..Y..... ....P.... .D.QSH.... .....E.. .C
GHA/8/91 DT..QP.... .A...N..AN R..T..... ..Y..... ....P.... .D.QSR.... .....E.. .C
MAI/1/91 NT..QP.... .A...N..AH RE.T..... ..Y..... ....P.... .D.QSR.... .....E.. .C

MAI/2/91
..Y.....
MAI/3/91
.Y.....
MAI/5/91
.....
MAI/6/91

MAI/7/91
ZAI/1/82
.....

.D..SR....



Genotype A:

Genotype B:

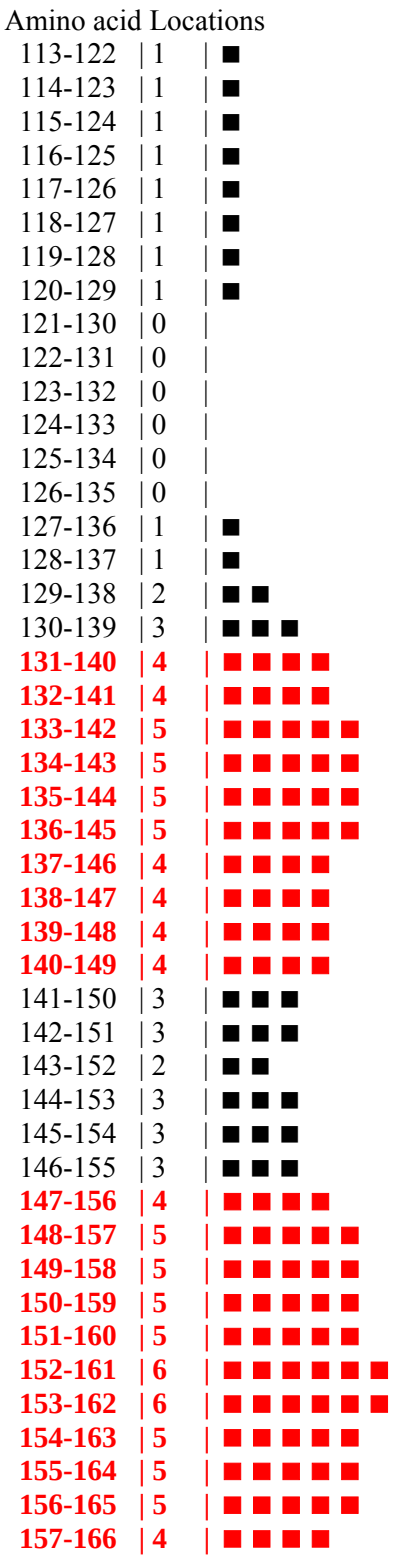
Genotype C:

Genotype D-H:

NT..QP.... .A...N..AH RE.T.....
....?P.... .D.QSR..V. .....E.. .C
NT..QP.... .A...N..AH RE.T.....
....P.... .D.QSR.... .....E.. .C
NT..QP.... .A...S..AN R..T..... .Y.....
.....P.... .D.QSR.... .....E.. .C
DT..QP.... .A...N..AN R..T.....
.Y....R... ....P.... .D.QSR....
.....E.. .C
NT..QP.... .A...N..AN R..T..... .Y..... ....
RTN..... .Q.....A. ..T..A.... .....T....
.....P.... .D.QNR.... .....E.. .C
ERI/12/98 A..A..... .AA..A..AA
..SV.T..Q. .....V.... ....P....
.....ER. T.
RWA/1/00 ....S..... .A...A..A. ..G..T....
..... N..... .....PF... .D..SR....
.....E.. .A

Fig. 3.5: Deduced amino acid sequences of the C-terminal half of the VP1 genes of SAT-2 strains from West, East and Central Africa. Dots indicate sequence identity to the master sequence, KEN/3/57. ? indicates undefined amino acids due to sequence ambiguities. The RGD hypervariable regions are embolded and VP1/2A is underlined. The cleavage site of vertical (KQ/LC or RQ/TL) is indicated by a arrow.

Fig. 3.6: Genotype distribution of SAT-2 type FMDV in West, East and Central Africa



158-167 | 3 | ■ ■ ■
159-168 | 3 | ■ ■ ■
160-169 | 2 | ■ ■
161-170 | 2 | ■ ■
162-171 | 1 | ■
163-172 | 1 | ■
164-173 | 1 | ■
165-174 | 1 | ■

166-175 | 2 | ■ ■
167-176 | 2 | ■ ■
168-177 | 2 | ■ ■
169-178 | 1 | ■
170-179 | 1 | ■
171-180 | 1 | ■
172-181 | 1 | ■
173-182 | 1 | ■
174-183 | 1 | ■
175-184 | 1 | ■
176-185 | 0 |
177-186 | 1 | ■
178-187 | 1 | ■
179-188 | 2 | ■ ■
180-189 | 2 | ■ ■
181-190 | 2 | ■ ■
182-191 | 2 | ■ ■
183-192 | 2 | ■ ■
184-193 | 3 | ■ ■ ■
185-194 | 3 | ■ ■ ■
186-195 | 3 | ■ ■ ■
187-196 | 2 | ■ ■
188-197 | 2 | ■ ■
189-198 | 1 | ■ ■
190-199 | 1 | ■
191-200 | 1 | ■
192-201 | 2 | ■ ■
193-202 | 2 | ■ ■
194-203 | 2 | ■ ■
195-204 | 3 | ■ ■ ■
196-205 | 3 | ■ ■ ■
197-206 | 4 | ■ ■ ■ ■
198-207 | 4 | ■ ■ ■ ■
199-208 | 5 | ■ ■ ■ ■ ■
200-209 | 5 | ■ ■ ■ ■ ■
201-210 | 5 | ■ ■ ■ ■ ■
202-211 | 4 | ■ ■ ■ ■
203-212 | 4 | ■ ■ ■ ■
204-213 | 3 | ■ ■ ■
205-214 | 2 | ■ ■

206-215 | 2 | ■ ■
207-216 | 1 | ■
208-217 | 1 | ■

Fig. 3.7: Variability plot: Location of SAT-2 from West Africa amino acid variable sites in overlapping windows of size = 10
Number = amino acid position
Red = high variable region (40 - 60 %)
Black = conserved region

3.5 Discussion

Four distinct evolutionary lineages (Fig. 3.4) were identified by sequencing the VP1 gene of 34 SAT-2 viruses consisting of 30 isolates from the west African countries of Mali, Ghana, Ivory Coast, Liberia, Nigeria, Senegal and Gambia. Four viruses from Central and East African countries of Eritrea, Kenya, Zaire and Rwanda were also included. Lineages I and II consisted of viruses from West Africa exclusively. A unique west African lineage has also been reported for serotype A FMD viruses, where all west African viruses were shown to constitute a single large evolutionary lineage covering a period of 26 years (Knowles *et al.,* 1998). Thus a west African topotype is distinguishable, however, viruses within this topotype region did not display the same locality-specific grouping of viruses found for the southern African topotype viruses (Bastos *et al.*, 2001; Bastos & Sangare, 2001). Like type O, the SAT-2 viruses from different west African countries group according to year of isolation rather than geographical origin indicating a link between the epizootics.

The sequencing results of SAT-2 viruses indicate that three unrelated epizootics of viruses from three different genotypes (A-C) occurred in West Africa between 1974 and 1991 (Fig. 3.4 & 3.6). The first epizootic between 1974 and 1975 affected numerous neighboring west African countries, including Ghana, Ivory Coast, Liberia, Nigeria and Senegal. The second (1979-1983) involving Gambia and Senegal, was caused by a virus with an extended field presence. The third occurred over a two year period (1990-1991) and affected Mali, Ivory Coast and Ghana. For each of these epizootics it was shown that the viruses differed from each other by no more than 3 % across the nucleotide region characterized in this study. Furthermore, bootstrap values, based on 10 000 replications, were 100 % for each of the epizootics / genotypes identified here.

These genotypes constitute two distinct evolutionary lineages (I-II), which may be discerned on the basis of high levels of bootstrap support and nucleotide sequence differences in excess of 20 %, as indicated in other molecular epidemiological studies of FMD viruses (Bastos *et al.*, 2001). The grouping of viruses from different countries from West Africa, although isolated in different years, is

in agreement with type O epidemiology in the region where viruses recovered from Burkina Faso (1992) and Ghana (1993) shared 98 % sequence identity over their VP1 gene nucleotide sequences (Chapter 2).

Whilst lineages I and II are distinct for West Africa, the inclusion of other viruses from African localities north of the equator indicated that a further two major lineages are present in this continental region. Lineages III and IV comprised viruses primarily from Central (Democratic Republic of Congo-DRC & Rwanda) and eastern Africa (Eritrea & Kenya). One exception was NIG/2/82, which was the only virus from a west African country to occur within these viral lineages. The presence of this virus within east and central African topotypes is a strong indication of an introduction of virus from one of these former regions into Nigeria in 1982. There is not only extensive movement of virus within the west African region, but introduction from other African regions also occurs, leading to extensive genetic heterogeneity in the field. This heterogeneity is likely to be reflected antigenically (Esterhuysen, 1994) and will influence a vaccination strategy including updating the vaccine strains. The identification of virus movement between West and East Africa showed the need for a continent approach to control the disease.

In contrast to southern Africa where the geographical origin of an outbreak can be traced with great accuracy (Bastos *et al.*, 2000; Bastos *et al.*, 2001) the outbreaks in West Africa appear to have multiple foci in different countries, making it difficult to determine the original source of the infection. This grouping of viruses from West Africa according to year of isolation rather than sampling locality indicates that unrestricted animal movement occurs. In order to adequately control the disease it is therefore clear that restriction of animal movement will be a key factor in achieving this. In addition, the phylogenetic trees have shown that viruses from West Africa are genetically distinct from those occurring elsewhere. Therefore, the control of FMD in the west African region would not only require regulation at the border areas but also the development and administration of custom-made vaccines if study on the antigenic characterization will correlate with the genetic divergence found in this study.

Unlike southern Africa, the role of wildlife in the transmission of FMDV in West Africa is unclear. Wildlife populations in West African countries are comparatively small and their role in the epidemiology is therefore likely to be of minor importance due to the lower incidence of interaction between livestock and wildlife. A factor which may well be significant for virus spread in this region is the massive trans-boundary movement of livestock between neighboring countries (Bizimana,

1994). This is due to seasonal droughts which drive nomadic ranching and is exacerbated by a low standard of husbandry and a lack of fencing and defined grazing areas. One major problem for the extensive livestock system in West Africa is that the number of cattle is used to evaluate a family's wealth status.

Serological surveys against FMDV in various species of Sudanese livestock following natural infection has indicated that 53 % of cattle, 2 % of sheep and 4 % of goat populations were positive to antibodies against FMD virus (Abu Elzein *et al.*, 1987). A similar survey in Saudi Arabia where over a million sheep and goats are imported annually, reported that 20 % of sheep, 9 % of goats, 16 % of cattle and 5 % of other ruminant species had sero-converted to FMD virus (Hafez *et al.*, 1994). Although several million small ruminants occur in West Africa (OIE website: http://www.oie.int/), their role in the epidemiology is presently not known. Therefore, a large serological survey will help to understand the role of this species in West Africa.

In summary, this first study of the molecular epidemiology study of FMD virus SAT-2 in West Africa has provided valuable insights into the epidemiology of the disease in this region. Unrestricted animal movement is a major factor in disease transmission, resulting in the rapid spread of infection which in turn leads to the inability to discern the source of the outbreak. The epidemiology of FMD in West Africa is exacerbated by the presence of four of the seven known serotypes, and by intratypic complexities as illustrated here for the SAT-2 serotype. Of importance is the threat of the introduction of new strains from neighboring countries and from different continental regions. The latter significantly affects the antigenic and genetic diversity of viruses in the west African region and has implications for control of the disease through vaccination.

CHAPTER 4

RETROSPECTIVE GENETIC ANALYSIS OF SAT-1 TYPE FOOT-AND-MOUTH DISEASE OUTBREAKS IN WEST AFRICA (1975-1981)

4.1 Summary

Twenty-three SAT-1 viruses causing foot-and-mouth disease outbreaks in West Africa between 1975 and 1981 were obtained from the World Reference Laboratory for FMD to conduct a retrospective study of this serotype in the region. The complete 1D genome region encoding the immunogenically important protein (VP1) was amplified by RT-PCR and genetically characterized. Phylogenetic reconstruction of viral relationships using an homologous 666 nucleotide sequence indicated that two independent outbreaks occurred, the first involved Niger and Nigeria, whilst the second affected Nigeria alone. In the former epizootic, virus circulation spanned over a period of two years, whilst in the latter virus was recovered from the field over a three years period.

The comparison of the west African SAT-1 viruses with those from other regions in Africa revealed six different lineages of which two are composed exclusively of isolates from West Africa. The two west African lineages identified in this study displayed a highly distinct and homogenous regional grouping. Furthermore, variation in VP1 gene length was identified in SAT-1 viruses for the first time, further emphasizing the uniqueness of these pathogens in West Africa.

This retrospective analysis in which the molecular epidemiology of SAT-1 viruses in West Africa is reported for the first time provides useful data of the regional variation of SAT-1 viruses and is an essential first step in the establishment of a regional sequence database that will be used for future outbreaks.

4.2 Introduction

In Africa, the epizootical situation of FMD varies according to geographical location. The complexity of the disease on the continent has been discussed previously (see 1.7.1). Among the FMD viruses that occur in West Africa , SAT-1 has only been identified in Niger and Nigeria between 1975-1981. It is also important to note that some of the isolates in this study have been identified over a two decade period

(Table 4.1). Despite SAT-1 circulating in West Africa over 6 years and the irregular movement of animals across borders, SAT-1 has been confined only to Niger and Nigeria whereas studies of type A, SAT-2 and O epizootics have shown that these outbreaks occur across country borders in West Africa (Knowles *et al.*, 1998; Chapter 2 & 3). SAT-1 is widely distributed and exists in eight countries in southern Africa (Thesis, Bastos, 2001, Bastos *et al.*, 2001). Therefore, the reason for SAT-1 being confined to these two countries only is not known or probably because of the fact that the disease is under-reported. Until 2000, SAT-1 was the only SAT-type reported outside the African continent for the past two decades (Pereira, 1981; WRL, OIE/FAO).

The epidemiology of SAT-1 is of particular interest because of its restriction to Niger and Nigeria. Viruses isolated in 1975 and 1976 from Niger and Nigeria and those isolated only from Nigeria in 1979 and 1981 were investigated in this study. These were the only SAT-1 viruses available from West Africa since the WRL started receiving samples. Historical isolates from East and southern Africa were included for comparative purposes. Nucleotide sequencing of several SAT-1 viruses of the two countries was performed and the data used to construct the gene tree to determine the heterogeneity and genetic relationships of SAT-1 in sub-Saharan-Africa.

In previous studies, genetic characterization of SAT-type viruses have reported the geographical clustering of the SAT-1 isolates and a higher level of intratypic variation than that reported for European serotypes (Vosloo *et al.*, 1995; Bastos *et al.* 2001). This chapter describes an attempt to assess the regional variation of SAT-1 isolates from West Africa and to establish a regional reference VP1 gene sequence database. However, it not known if the SAT-1 type in West Africa has been extinct or exist in silent form in the region.

4.3 Materials and methods

4.3.1 Viruses used in this study

The viruses were supplied by the World Reference Laboratory (WRL) for FMD at the Institute for Animal Health, Pirbright (United Kingdom). The geographical origin and isolation dates of nineteen viruses from Nigeria (1975-1981), and four isolates from Niger (1976) are indicated in Table 4.1. A 1968 SAT-1 strain isolated previously from Botswana with full length VP1 gene sequence available was included as a reference strain. Since most of the isolates were stored at –70^{o}C for protracted periods of time, it was

necessary to propagate all isolates on IB-RS-2 cells to reach sufficient virus titres for future use. Passage levels are indicated in Table 4.1.

4.3.2 Reverse transcriptase, PCR amplification, nucleotide sequencing and data analysis

Total RNA was extracted and PCRs were performed as described previously for serotypes O and SAT-2 with a few modifications. Initially, genomic amplification of the cDNA was performed with published primers, the antisense universal oligonucleotide P1 (Beck & Strohmaier, 1987) combined with one of the following sense primers W-US (Vosloo *et al.*, 1996), VP1Ub (Bastos, 1998) or VP3AB (Fig. 4.1). PCR with SAT-1 cDNA samples using the sense primers W-US or VP1Ub were unsuccessful or gave faint amplicons with many non-target amplification and high background. A limited numbers of successful amplification were obtained using the primer set P1 and VP3AB (Fig. 3.3). Because of the low success rate of amplification with the available existing primers, a new primer was designed specifically for this study. The primer design was based on a multiple alignment of the nucleotide sequences obtained from the PCR products using P1 and VP3AB primers. From this alignment, a totally conserved region of 20 nucleotides was identified on the VP3 neighboring gene of the VP1 among all the SAT-1 isolates from West Africa. A SAT-1 specific primer termed SAT1U-OS (5' GTG TAC CAG ATC ACT GAC AC 3') was synthesized (Life Technologies). This primer binds 105 nucleotides upstream of the VP1 gene within the VP3 (1C) genome region (Table 4.2). A product of 750 bp was ultimately obtained (Fig. 4.1). PCR products were electrophoresed on 2 % agarose gels stained with ethidium bromide (10 mg/ml) at 55-100 V for 30 min in TAE buffer. A 100 bp ladder DNA molecular weight marker (Promega) was included to identify the size of 750 bp PCR products using UV light. The same standard procedures used in 2.3.8; 2.3.10 and 2.4 were applied for nucleotide sequencing and analysis of the DNA samples with confidence levels being assessed by 10 000 bootstrap replications. An isolate obtained from Botswana in 1968 was included as a reference strain (Table 4.1).

4.4 Results

Complete VP1 genome sequences of 23 SAT-1 type FMD isolates were generated in order to determine the genetic relationships of viruses recovered from several outbreaks in Niger and Nigeria between 1975 and 1981. Viruses representative of the four major topotypes in south-eastern Africa (Bastos *et al.*, 2001) were included in the phylogenetic analyses (Table 4.1). All methods of analysis (UPGMA, NJ and parsimony) produced trees of similar topology (results not shown) indicating that the recovered phylogeny was reliable (Kim, 1993).

The gene trees revealed six major evolutionary lineages (I-VI) supported by high bootstrap values (Fig. 4.2). Two separate lineages (I and II) were observed in West Africa while the remaining lineages (III-VI) represented previously identified viral topotypes from East and southern Africa (Bastos *et al.* 2001). Lineage I comprised viruses involved in outbreaks in Nigeria (1975-1976) and those responsible for the disease during 1976 in Niger, indicating that the outbreak possibly originated in Nigeria and subsequently spread to Niger. These viruses shared a high degree of sequence identity across the full-length VP1 sequences with between 0.6 % to 2.2 % nucleotide differences observed with pairwise comparisons.

The viruses from Nigeria that formed part of Lineage II (Fig. 4.2) differed from those in Lineage I by more than 25 % across the full-length VP1 gene sequences, indicating that they were evolutionary distinct lineages. Within Lineage II all viruses were of Nigerian origin and were recovered from outbreaks occurring between 1979 and 1981. These viruses differed by between 0.4 %-3.5 % from each other indicating that they were part of the same epizootic which lasted at least 3 years.

The deduced amino acid sequences of the full-length VP1 of 19 isolates from Nigeria and 4 from Niger are shown in Fig. 4.3. The viruses belonging to Lineage I have a codon deletion corresponding to amino acid position 81 when compared with the reference strain BOT/1/68 (Botswana, 1968) and a single codon deletion at position 148, two amino acid positions downstream of the RGD sequence (Fig. 4.3). Viruses from Lineage II (Nigeria 1979-1981) also have the deletion at position 81, but not at position 148 where the amino acid Asparagine (ACC), is similar to that occurring in BOT/1/68.

The isolates from Lineage I also differed from those in Lineage II at several amino acid positions. Around the main antigenic region containing the RGD cell adhesion sequence (positions 150-152), viruses from Lineage I had A(Q/R)PVE(N/S) [Alanine (glutamine/arginine) proline-valine-glutamic acid (asparagine/serine)] at positions 140-145, while isolates from Lineage II had P(I/T)NEDT. Furthermore, the Lineage II isolates had TN at position 147-148, while Lineage I isolates had E and a deletion (Fig. 4.3). More differences were observed at position 155 where Lineage I isolates had V compared to T in Lineage II. Furthermore, at positions 161-164 Lineage I isolates had (A/V)EET and Lineage II isolates had REQS. Several differences were observed between viruses belonging to the two lineages around position 200-214. At positions 212-215 Lineage I isolates had TTLV while Lineage II had VSLI.

Table 4.1: List of viruses used in this study

Virus Name	Year of isolate	Origin	Passage history	Reference	GenBank access N_0
NIG/14/75	1975	Nigeria	RS1BTY1RS2	This study	AF431709
NIG/15/75	1975	Nigeria	RS1BTY1RS3	This study	AF431710
NIG/11/75	1975	Nigeria	RS3	This study	AF431711
NIG/17/75	1975	Nigeria	BTY2RS2	This study	AF431712
NIG/20/75	1975	Nigeria	RS3	This study	AF431713
NIG/24/75	1975	Nigeria	BTY2RS1	This study	AF431714
NIG/25/75	1975	Nigeria	BTY1RS2	This study	AF431715
NIG/6/76	1976	Nigeria	BTY1RS2	This study	AF431716
NGR/1/76	1976	Niger	BTY2RS2	This study	AF431717
NGR/2/76	1976	Niger	BTY2RS1	This study	AF431718
NGR/4/76	1976	Niger	BTY1RS1	This study	AF431719
NGR/5/76	1976	Niger	BTY2RS1	This study	AF431720
NIG/1/76	1976	Nigeria	BTY1RS3	This study	AF431721
NIG/2/76	1976	Nigeria	BTY2RS2	This study	AF431722
NIG/5/76	1976	Nigeria	BTY1RS2	This study	AF431723
NIG/8/76	1976	Nigeria	BTY1RS1	This study	AF431724
NIG/14/76	1976	Nigeria	BTY2RS1	This study	AF431725
NIG/7/76	1976	Nigeria	BTY1RS2	This study	AF431726
NIG/20/76	1976	Nigeria	BTY2RS2	This study	AF431727
NIG/2/79	1979	Nigeria	RS4	This study	AF431728
NIG/3/80	1980	Nigeria	BTY3RS1	This study	AF431729
NIG/5/81	1981	Nigeria	BTY2RS2	This study	AF431730
NIG/10/81	1981	Nigeria	RS1BTY1RS1	This study	AF431731
BOT/1/68	1968	Satau, Bostwana	BHK3B1	Unpublished	NA
UGA/1/97	1997	Uganda	PK1	Bastos et al., 2001	AF056513
ZIM/3/88	1988	Zimbabwe	CFK1RS1	Bastos et al., 2001	AF056520
SAR/09/81P	1981	South Africa	B1BHK4RS2	Bastos et al., 2001	AF056511
ZAM/2/93	1993	Zambia	PK1RS3	Bastos et al., 2001	AF056514
MOZ/3/77	1977	Mozambique	BHK1B1BHK4	Bastos et al., 2001	AFO56510
MAL/0/185	1985	Malawi	CFK2BTY1BHK5	Bastos et al., 2001	AF056509

BHK = baby hamster kidney: B = bovine; CFK = calf fetal kidney; BTY = bovine thyroid; RS = pig kidney, NA= Not available

Table 4.2: Summary of published and designed primers used in this study.

Name	Sequence	Length	Position	Orientation	Reference/Consensus sequence	Tm	Ta	Expected product size combined with P1
P1	GAA GGG CCC AGG GTT GGA CTC	21 mer	2A/2B - 33 - 53 nt downstream of VP1	Antisense	Beck & Strohmaier 1987	65°C	61°C	----
W-US	CC ACG TAT TAC TTT TGT GAC	20 mer	primes at position 206 - 225 nt in VP1	Sense	Vosloo et al., 1996	53°C	49°C	500 bp
VP1Ub	CC ACG TAC TAC TTY TCT GAC CTG GA	25 mer	primes at position 206 - 230 in VP1 gene	Sense	Bastos, 1998	64°C	60°C	500 bp
VP3AB	CAC TGC TAC CAC TCR GAG TG	20 mer	primes at position 241 - 260 upstream of VP1	Sense	Bastos et al., 2002	59°C	55°C	850 bp
VP1U-O	GAT TTG TGA AGG TGA CACC	19 mer	primes at position 113 - 131 nt in VP1 gene	Sense	Rodriguez et al., 1994	54°C	50°C	581 bp
SAT1-OS	GTG TAC CAG ATC ACT GAC AC	20 mer	VP3-105 nt upstream of VP1	Sense	Sangare et al., 2002	59°C	55°C	750 bp
SAT2-OS	CCA CNT TCG AGG TCA ACT TGA T	22 mer	139-160 nt in VP1	Sense	Sangare et al., 2002	61°C	57°C	518 bp

The melting temperature (Tm) of each oligonucleotide was calculated following the formula: Tm = [69.3°C + 0.41 (% GC)] - 650/primer length (MWG-Biotech GmbH, Ebersberg, Germany). The PCR annealing temperature (Ta) was calculated using the formula: Ta = Tm - 4°C.

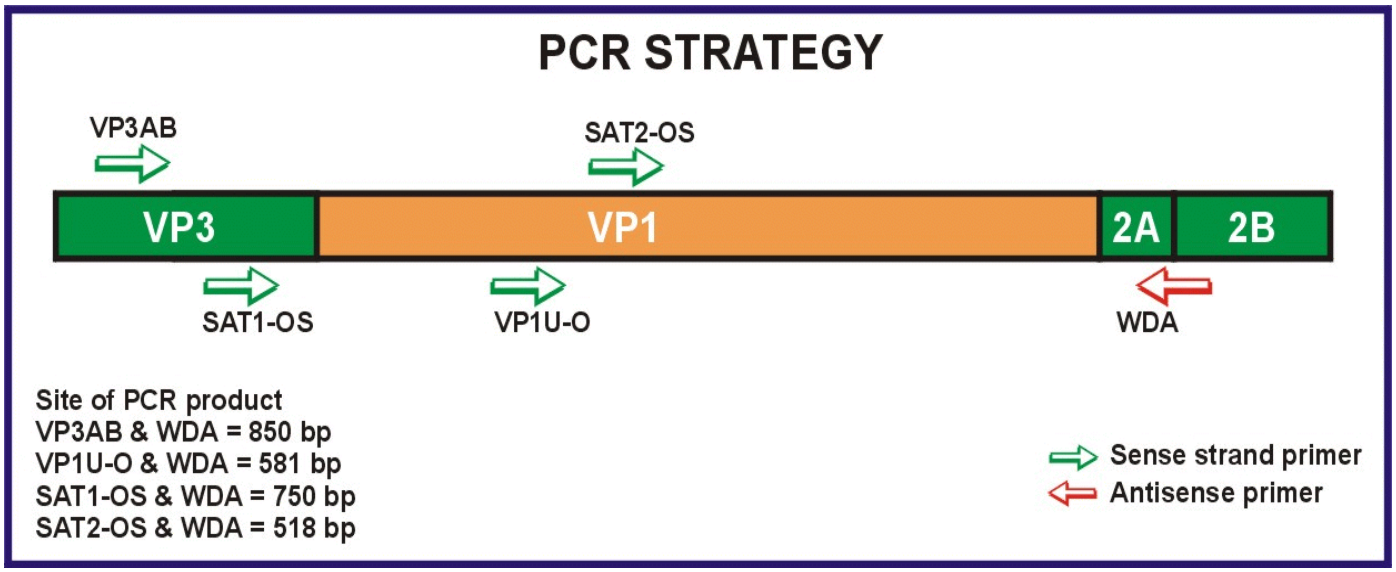


Fig. 4.1: Polymerase chain reaction strategy. Primers binding sites are indicated by the arrows.

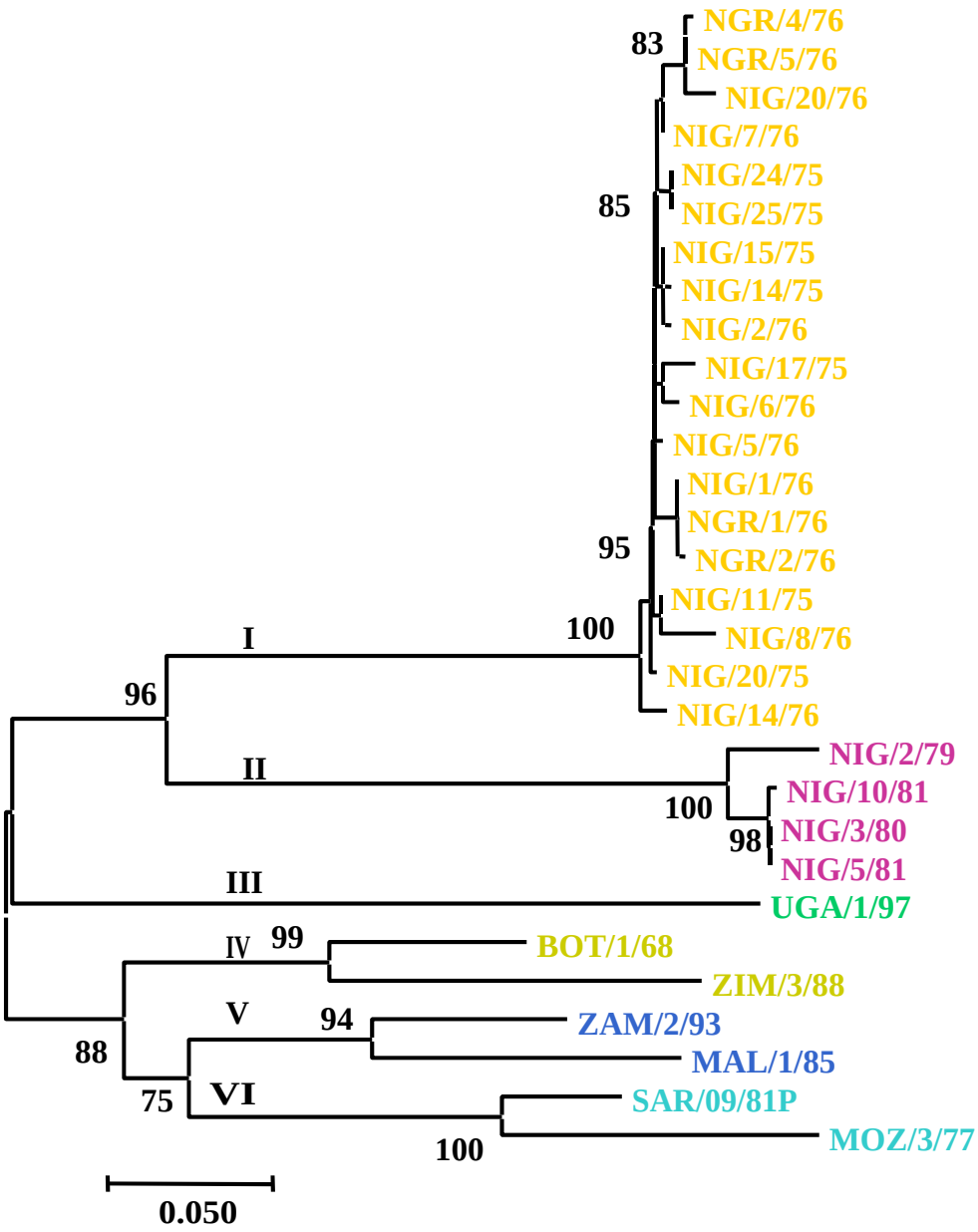


Fig.4.2 Neighbor-joining tree depicting VP1 gene relationships of SAT-1 type FMD viruses from West, East and southern Africa. Six major lineages labelled I-VI were identified. Bootstrap values ≥70 are indicated and are based on 10 000 replications

```
                    10          20          30          40          50          60
       70          80
BOT/1/68  TTSAGEGADP VTTDASAHGG NTRTTSRAHT DVTFLLDRFT LVGKTNDKKL VFDLLSTKEK
SLVGALLRAS TYYFSDLEVA
NIG/11/75 .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....I..SA ..........
NIG/14/75 .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....V..SA ..........
NIG/15/75 .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....V..SA ..........
NIG/17/75 .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....I..SA ..........
NIG/20/75 .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K..?.
A....I..SA ..........
NIG/24/75 .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....I..SA ..........
NIG/25/75 .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....I..SA ..........
NIG/6/76  .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....V..SA ..........
NGR/1/76  .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....I..SA ..........
NGR/2/76  .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....I..SA ..........
NGR/4/76  .........V ?......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....V..SA ..........
NGR/5/76  .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....V..SA ..........
NIG/1/76  .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....I..SA ..........
NIG/2/76  .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....I..SA ..........
NIG/5/76  .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....T..SA ..........
NIG/8/76  .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....V..SA ..........
NIG/14/76 .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....I..SA ..........
NIG/7/76  .........V .......... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....V..SA ..........
NIG/20/76 .........V ..?....... ...P.R.V.. ..A....... .....V.N.M .L...K....
A....V..SA ..........
NIG/2/79  .........V ..V.TA.... .Q.R.R.V.. ..A....... .....Q.N.M .L.M.K....
A....I..SA ....A.....
NIG/3/80  .........V ..V.TA.... .Q.R.R.V.. ..A....... .....R.N.M .L.M.K....
A....I..SA ....A.....
NIG/5/81  .........V ..V.TD?... ??.R.R.V.. ..A....... .....R.N.M .L.M.K....
A....I?.SA .?..A.....
NIG/10/81 .........V ..V.TA.... .Q.R.R.V.. ..A....... .....R.N.M .L.M.K....
A....I..SA ....A.....
```

```
                  90        100       110       120       130       140       150       160
BOT/1/68  DCVGTNAWVG WTPNGSPVLT EVGDNPVVFS RRGTTRFALP YTAPHRVLAT VYNGDCKYKP TGTAPRENIR GDLATLAARI
NIG/11/75 -.....K... .V...A..PK .......... HN........ .......... T........A RPVEN..-.. ....V....V
NIG/14/75 -.....K... .V...A..PK .......... HN........ D......... T........A QPVEN..-.. ..M.V....V
NIG/15/75 -.....K... .V...A..PK .......... HN........ .......... T........A QPVEN..-.. ..M.V....V
NIG/17/75 -.....K... .V...A..PK .......... HN........ .......... T........A QPVEN..-.. ....V....V
NIG/20/75 -.....K... .V...A..PK .......... HN........ .......... T........A QPVEN..-.. ....V....V
NIG/24/75 -.....K... .V...A..PK .......... HN........ .......... T........A QPVEN..-.. ....V....V
NIG/25/75 -.....K... .V...A..PK .......... HN........ .......... T........A QPVEN..-.. ....V....V
NGR/1/76  -.....K... .V...A..PK .......... HN........ .......... T........A QSVES..-.. ....V....V
NGR/2/76  -.....K... .V...A..PK .......... HN........ .......... T....Y...A QSVES..-.. ....V....V
NGR/4/76  -.....K... .V...A..PK .......... HN........ .......... T........A QPVEN..-.. ....V..V.V
NGR/5/76  -.....K... .V...A..PK .......... HN........ .......... T........A QPVEN..-.. ....V..V.V
NIG/1/76  -.....K... .V...A..PK .......... HN........ .......... T........A QSVES..-.. ....V....V
NIG/2/76  -.....K... .V...A..PK .......... HN........ .......... T........A QPVEN..-.. ..M.V....V
NIG/5/76  -.....K... .V...A..PK .......... HN........ .......... T........A QPVEN..-.. ....V....V
NIG/6/76  -.....K... .V...A..PK .......... HN........ .......... T........A QPVEN..-.. ....V....V
NIG/7/76  -.....K... .V...A..PK .......... HN........ .......... T........A QPVEN..-.. ....V....V
NIG/8/76  -.....K... .V...A..PK G......... HN........ .......... T........A RPVEN..-.. ....V....V
NIG/14/76 -.....K... .V...A..PK .......... HN.A...... .......... T........A QPVEN..-.. ....VV...V
NIG/20/76 -.....K... .V...A..PK .......I.. HN........ .......... T........A QPVEN..-.. ....V..V.V
NIG/2/79  -.....K... .L...A..PR .......... .N........ F......... .....Y.... INEDT.T... .........V
NIG/3/80  -.....K... .L...A..PR .......... HN........ F......... .......... .NEDT.T... .........V
NIG/5/81  -.....K... .L...A..PR .......... HN........ F......... .......... .NEDT.T... .........V
NIG/10/81 -.....K... .L...A..PR .......... HN........ F......... .......... .NEDT.T... ..........
```

							VP1 ↓ 2A
	170	180	190	200	210	220	222
BOT/1/68	ASETHIPTTF	NYGMIYTKAE	VDVYLRMKRA	ELYCPRPVLT	HYDHNGRDRY	KTTLVKPAKQ	LS
NIG/11/75	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NIG/14/75	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NIG/15/75	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NIG/17/75	.E.....	.....L.ES.	....V.....	......FL..	T.....A..H	.....A.E..	.A
NIG/20/75	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NIG/24/75	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NIG/25/75	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NGR/1/76	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NGR/2/76	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NGR/4/76	.E.....	.....L.ES.	....V.....	......FL..	T...D.A...	.....A.E..	.A
NGR/5/76	.E.....	.....L.ES.	....V.....	......FL..	T...D.A...	.....A.E..	.A
NIG/1/76	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NIG/2/76	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NIG/5/76	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NIG/6/76	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NIG/7/76	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NIG/8/76	.E.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NIG/14/76	VE.....	.....L.ES.	....V.....	......FL..	T.....A...	.....A.E..	.A
NIG/20/76	.E.....	.....L.ES.	....V.....	......FL..	T...D.A...	.....A.E..	.A
NIG/2/79	REQS.....	...I.L.E..	....V.....	....	T...ASA...	.VS.IA.E..	MA
NIG/3/80	REQS.....	...I.L.E..	....V.....	....	T...ALA...	.VS.IA.E..	MA
NIG/5/81	REQS.....	...I.L.E..	....V.....	....	T...ALA...	.VS.IA.E..	MA
NIG/10/81	REQS.....	...I.L.E..	....V.....	....	T...ALA...	.VS.IA.E..	MA

Fig. 4.3 Deduced amino acid sequence of the complete VP1 gene of SAT-1 isolates from West Africa and BOT/1/68 (reference strain). Dots indicate sequence identity with BOT/1/68. ? indicates undefined amino acids due to sequence ambiguities The RGD is bold and is underlined. Gaps included for alignment purposes are denoted by a (-).

4.5 Discussion

Nineteen historical SAT-1 viruses from Nigeria and four from Niger were characterized genetically by nucleotide sequencing of the VP1 gene. Although it is possible that SAT-1 outbreaks have occurred in West Africa subsequent to the 1981 outbreak, these may have gone unnoticed due to the endemic nature of the disease and the comparatively minor effects of the disease in this region. However, a low incidence of SAT-1 outbreaks in livestock has also been reported in southern Africa (Thomson, 1995).

Thomson, (1995) reported that since 1931 of the 350 epizootics in cattle in southern Africa, only 25 % were caused by SAT-1 despite the high prevalence of serum antibodies against this specific SAT type in carrier buffalo, the main source of virus transmission to livestock. Outbreaks due to SAT-2 and SAT-3 were 41 % and 17 %, respectively. A similar observation has been reported by Bastos *et al.*, (2000) that between 1985 to 1995 the FMD outbreaks in Impala were caused mainly by the SAT-2 type despite the higher recovery of SAT-1 type virus from probang specimens of buffalo in the same geographical location and within the same time period. The reason for the differences in the prevalence of SAT-1 types in the maintenance host and in cattle are not known. However, Bastos *et al.*, (2001) have speculated that the differences in the incidence of SAT-types in cattle may be due to the differential abilities of the different SAT- types to cross species barriers. Therefore, survey sampling in a large scale in Niger, Nigeria and if possible all their neighboring countries can assist with the clarification of the epidemiology of SAT-1 in West Africa.

The two SAT-1 type west African epizootics identified here were shown to differ to such an extent (25 %) from each other at nucleotide sequence level, that two evolutionary distinct lineages could be assigned. Isolates from one epizootic (1975 - 1976) that occurred over a period of 2 years differed by between 0.6-2.2 % whilst isolates from another epizootic (1979 - 1981) that lasted 3 years, differed between 0.4-3.5 %. These values fall within the expected ranges of genetic change for viruses recovered from a single epizootic over a number of years.

The nucleotide sequence identity which was in excess of 96 % confirms that isolates from Nigeria collected over a 3 year period (1979-1981), are clearly part of the same epizootic. SAT-2 isolates from West Africa had similarly long circulation periods of up to 4 years (Chapter 3). Based on lineage cut-off values previously described for this serotype and a bootstrap support, six distinct SAT-1 lineages were identified in this study (Fig. 4.2). Four lineages corresponded to known southern and east African lineages (Bastos *et al.* 2001), with the remaining two being representative of West Africa. The genetic distinctiveness of the west African SAT-1 viruses reported here has similarly been observed for serotype O (Chapter 2), serotype A (Knowles *et al.* 1998) and SAT-2 (Chapter 3). Although, SAT-1 type has not been reported in West Africa since 1981, the information obtained from this study can be used to investigate new SAT-1 outbreaks, should they occur in the region.

Amino acid deletions were observed in SAT-1 viruses obtained from West Africa (Fig. 4.3). These occurred at two positions (residues 81 and 148) for viruses isolated in 1975 and 1976. Interestingly, the viruses isolated from 1979 to 1981 had only one deletion at position 148. The amino acid position 148 is located within the GH-loop sequence (140-160) that contains the main immunogenic site of FMD virus and it is likely to affect the antigenicity of the virus as changes to this residue result in complete resistance to neutralization in serotypes O viruses (Xie *et al.* 1987; Acharya *et al.*, 1989). A codon deletion unique to west African serotype A and SAT-2 viruses has also been reported (Knowles *et al.* 1998; Bastos et al. 2002). Thus amino acid length variation within different FMD serotypes seems to be a special feature of strains from West Africa. The precise antigenic implication of the intratypic length variability still needs to be determined.

The virus binding site to cells, amino acid sequence Arg-Gly-Asp (RGD) (Leippert *et al.,* 1997) was highly conserved in the G-H loop amongst all the SAT-1 isolates in this study. It is important to indicate that 86.9 % of the viruses had leucine flanking aspartic acid (RGDL). The remaining had methionine (RGDM) (Fig. 4.3). Fox and co-workers (1989) have reported that mutations in the RGD or the flanking amino acid leucine, RGDL, decrease virus attachment to susceptible cells by up to 60-73 % depending on the serotype. The predominance of leucine flanking the RGD motif in SAT1 type from West Africa was absent from SAT-2 type viruses in this region (Chapter 3).

The results presented here show a distinct geographical grouping of SAT-1 isolates from West Africa. Although, SAT-1 type viruses have been investigated in southern Africa (Bastos *et al.*, 2001), the genetic diversity remains obscure for other regions regularly afflicted by SAT-1 outbreaks. This first molecular epidemiology of SAT-1 type from West Africa thus provides a valuable contribution for assessing SAT-1 type variation at the continental level. Most importantly however are the benefits derived from the understanding of the genetic relationships of FMD viruses in this part of the African continent, where a regional approach to disease control should be advocated for disease eradication.

CHAPTER 5

5.1 Summary and conclusions

The objective of this study was to characterize FMD viruses that have caused outbreaks in West Africa by comparing partial and full length VP1 gene sequences in order to assess the regional genetic heterogeneity. The information was used to assist in the clarification of the epidemiology of FMD in this region and in the establishment of a regional sequence database. Before this study little was known about the epidemiology of FMD virus in West Africa with the exception of serotype A (Knowles *et al.*, 1998). This dearth of knowledge is emphasized by a general absence of published data about the viruses circulating in this part of the African continent.

Foot-and-mouth disease is a highly contagious disease that requires rapid and accurate diagnosis for effective disease control. Valuable contributions in investigating the epidemiology of the disease were made possible by using the nucleotide sequencing of the VP1 gene of FMD virus (Beck & Strohmaier, 1987; Marquardt & Haas, 1998; Samuel *et al.*, 1999). One of the main objectives of this study was to use this molecular technology to characterize the VP1 genes of FMD virus serotypes O, SAT-1 and SAT-2 from countries in West and South Africa and thus to address the lack of knowledge regarding the epidemiology of the disease.

Type O was first characterized due to its involvement in a large number of outbreaks in recent years and high mortality in young cattle particularly in Mali. In total 19 new viruses including those from Angola (1974-1975), Niger (1988), Kenya (1978 and 1991), Burkina Faso (1992), Ghana (1993) and South Africa (2000) were characterized in this study. Other sequences were obtained from Genbank for comparative purposes. The phylogenetic results based on 44 viruses indicated three evolutionary lineages (I-III) irrespective of the method of analysis (Fig. 2.1). Lineage I consisted of three genotypes (A-C). The viruses from West Africa (genotype C) formed a regionally distinct genotype as did those from East Africa (genotype B), whilst, South African isolates clustered within the "Pan-Asia" lineage (Knowles *et al.*, 2000) in genotype A. Viruses from two neighboring countries displayed 98 % nucleotide sequence identity over a period of a year (Burkina Faso in 1992 & Ghana in 1993) showing that they shared common ancestry. However, viruses from the 1988 outbreak in Niger formed a sub-cluster in the west African genotype and showed a difference of up to 11 % across the VP1 region sequenced when compared to those from Burkina Faso (1992) and Ghana (1993). This result is in agreement with previous

studies (Knowles *et al.*, 2000; Islam *et al.*, 2001) where virus transmission between different countries have shown to be responsible for many outbreaks. This was exemplified by the close relationships of type O isolates from Bangladesh in 1987-1997, India in 1990 and Iran in 1997 (Freiberg *et al.*, 1999). Similarly isolates obtained from Hong Kong in 1994 and Taiwan in 1997 were also closely related (Tsai *et al.*, 2000) indicating that they shared a common origin.

RNA viruses lack error correction mechanisms in their replication process (Holland *et al.*, 1982) and in previous studies of FMDV, the rates of fixation mutations were estimated at 0.5×10^{-2} - 1.5×10^{-2} per nucleotide site variation per year in the field (Holland *et al.*, 1982). Therefore, the difference of 2 % obtained over a year period between Burkina Faso (1992) and Ghana (1993) serotype O isolates concur with the rates of mutation which were evaluated around 0.5 - 1.5 % in field FMDV viruses and from infected animals sampled over a long time period epidemics (Holland *et al.*, 1982; Drake & Holland, 1999). Therefore, the difference of 11 % between Niger (1988) and Burkina Faso -Ghana (1992-1993) viruses over 4 years indicated clearly that these isolates were unrelated and had different sources of infection. The extent of difference is such that it could not be due to persistence in the field over the time span investigated.

A difference in excess of 20 % was observed in the nucleotide sequences between the serotype O viruses isolated from West Africa included in this study and those isolated from other regions on the continent and elsewhere in the world. The clustering of type O viruses from West Africa is in agreement with a previous study where genetic analysis using the north African type O strains indicated that all the isolates from the 1989-1992 epidemic in this region formed a cluster differing by no more than 6 % from each other (Samuel *et al.*, 1999).

The historical type O characterized from Venezuela in 1951 clustered with the Angolan isolates of 1974-1975 and the viruses from Angola differed by only 1 % at the nucleotide sequences level indicating that the Angolan outbreaks were closely related and part of the same epizootic. It is important to realize that trans-continental transmission occurred in Africa and that there are indigenous and exotic type O strains for West and southern Africa, respectively. When the isolates causing the first type O outbreak in South Africa were sequenced, the data indicated that these viruses shared 99 % nucleotide identity across the VP1 gene with those responsible for outbreaks of the disease in Bangladesh in 1997, Taiwan in 1999 and Japan in 2000 (Sangare *et al.*, 2001). The transmission of type O between continents revealed in this study

is of major importance as countries are under constant threat of importing exotic viruses. This is in agreement with previous studies such as Freiberg *et al.*, (1999) where viruses recovered from outbreaks in Bangladesh in 1987-1997, Greece and Bulgaria in 1996 were found likely to have had a common origin. Similarly a link between Campos Brazil/58 and European O1 has been reported (Sáiz *et al.*, 1993) whilst the type O from Hong Kong (1994), Moscow (1995) and Taiwan in 1997 were shown to have common ancestry (Tsai *et al.*, 2000). The isolates from the recent epidemic in the UK were also of Asian origin and the viruses involved in the UK outbreak shared up to 95 % sequence identity within the gene encoding the immunogenic capsid protein VP1 with those from Asia in 2000 (Samuel & Knowles, 2001). The conclusion drawn from this study that the type O outbreak in South Africa was due to an introduction of virus of Asian origin was supported by Samuel & Knowles (2001) who found the isolates of South Africa in 2000, Japan in 2000 and UK in 2001 to be genetically related, pointing to a common source.

Nucleotide sequencing is one of the best methods to clarify the source of epizootics occurring simultaneously by the same serotype within a single country. Stram *et al.*, (1995) reported that two different viruses that differed by more than 6 % in their nucleotide sequence were involved in the Israeli type O outbreaks of 1994 in South Lebanon and the Golan Heights pointing to two separate foci caused by different viruses. Viruses isolated from Lebanon (1992-1993) and Egypt (1993) and those of 1994 from Jordan and the Golan Heights were closely related and differences in the nucleotide sequences of viruses within each group did not exceed 2 %. These outbreaks possibly had a common origin. Tracing the route of transmission is greatly facilitated by the availability of a global nucleotide databases worldwide.

The sequence information generated in this study for west African viruses adds valuable new information to these databases and will assist in the identification of the source of infection in the west African region. This is critical for animal health personnel for implementing adequate measures to limit the spread and control of the disease. Because of the endemic situation of FMD in West Africa and the distinctiveness of the isolates from the region, vaccination is strongly advised to control the disease. Since the vaccines should include viruses that match the field isolates, the results reported in this study will contribute in investigating the antigenic relationships between the west African viruses and those used in the current vaccine on the market.

The second serotype of interest was SAT-2 because of its wide distribution in Africa and the fact that it was the most prevalent FMD virus type recovered from outbreaks between 1974-1991 in West Africa.

Like other serotypes in the region, little is known about the epidemiology of this type despite its importance and regular involvement in outbreaks. Two major lineages and three genotypes were identified by phylogenetic reconstruction of viruses from Mali, Liberia, Nigeria, Ghana, Ivory Coast, Senegal, and Gambia. Three main clusters composed of isolates from 1974-1975; 1979-1983 and 1990-1991 were distinguished in West Africa regardless to the country of origin of the isolates showing that the year of isolation is much more important than the geographical origin in the epidemiology of FMD virus in this region. A total of four major lineages and eight genotypes were identified when viruses from East and Central Africa were included. Comparison of the amino acid sequences indicated a difference of up to 20 % between the 1974-1975 and 1979-1983 isolates and 18 % between the 1979-1983 and 1990-1991 isolates. However, there was a high degree of homology between all VP1 sequences within the viruses of the same year from West Africa with less than 3 % amino acid differences being noted. Sequence identity of up to 99 % was observed between 1979 isolates and also between 1983 viruses from Senegal and Gambia. Unlike West Africa, the viruses from East Africa formed a homogenous cluster according to geographical location despite being isolated over a 40 year period (Fig. 3.4). This could be due to the low number of isolates from East Africa included in this study and may change once more data have been generated.

Of interest was that all SAT-2 viruses from West Africa clustered within regionally distinct groups with the exception of NIG/2/82 which was most closely related to ERI/12/98. These two viruses were recovered from the East and West of the continent and separated in time by 16 years. They had 89 % sequence identity across the VP1 gene region characterized in this study. The 11 % difference is very close to the 10 % genotype cut-off (Bastos, 2001), whilst high bootstrap support (99 %) points to common ancestry. These results are significant as it indicates that East-west movement of virus on the African continent, north of the equator has occurred in the past and that the threat of importing viruses from unrelated lineages is possible. This has important implications as vaccine stocks against all major lineages north of the equator should be readily available in case of an outbreak caused by a genetically “exotic” virus.

SAT-1 viruses recovered from outbreaks in Niger and Nigeria between 1975 and 1981 were also characterized. The gene trees of SAT-1 revealed two major evolutionary lineages (I-II) for isolates from West Africa supported by high bootstrap values and lineage cut-off values previously described for this serotype (Bastos, 2001) (Fig. 4.2). A total of six lineages were identified when viruses isolated and previously characterized from East, Central and southern Africa were included. Results showed that the

outbreaks probably originated in Nigeria in 1975 - 1976 and subsequently spread to Niger in 1976. These viruses shared a high degree of sequence identity across the full-length VP1 sequences with between 0.6 %-2.2 % nucleotide differences observed in pairwise comparisons. Within Lineage II all viruses were of Nigerian origin and were recovered from outbreaks occurring between 1979 and 1981. Nucleotide sequence differences between 0.4 % and 3.5 % over the 3 year circulation period were observed. These values fall within the expected range of genetic change (0.5 - 1.5 % per year).

The deduced amino acid sequences of the full-length VP1 gene of 19 SAT-1 isolates from Nigeria and 4 from Niger are shown in Fig. 4.3. The viruses belonging to Lineage I have a codon deletion corresponding to amino acid position 81 of the reference strain Bot/1/68 (Botswana, 1968) and another single codon deletion at position 148, two amino acid positions upstream of the RGD. Viruses from Lineage II (Nigeria 1979-1981) also had a deletion at position 81, but not at position 148. A codon insertion at 29 has also been reported for SAT-2 viruses from West Africa (Bastos & Sangare, 2001) whilst serotype A has a deletion at amino acid 196 of VP1 (Knowles *et al.*, 1998). In addition to these deletions there were many differences amongst the amino acid residues between Lineages I and II around the main antigenic region containing the RGD cell adhesion sequence (positions 150-152). Changes to residue 148 observed in SAT-1 are likely to affect antigenicity of the virus as complete and partial resistance to neutralization in serotype O viruses were associated with mutations at sites 148 and 144, respectively (Xie *et al.*, 1987). Escarmis and co-workers (1995) have speculated that deletion events in the genes encoding capsid proteins will affect the folding patterns of these proteins and the stability of the virus particle. It has also been reported that some mutations during the replicating process in cell cultures may contribute to an increase in virulence (Sevilla & Domingo 1996) or to an expanded host range and therefore to an increased probability of virus survival (Baranowski *et al.*, 1998). Although deletion events in the genes of the capsid proteins are not common, they do occur at the amino acid level of non-structural proteins such as deletions on the 3A gene for type C and O (Giraudo *et al.*, 1990; Beard & Mason, 2000). Deletions in the 3A gene were associated with attenuation of FMD virus type O and C in cattle (Giraudo *et al.*, 1990) and resulted in an increase in virulence for type O in swine (Beard & Mason, 2000). Although the actual implications of the codon deletions found in SAT-type isolates from West Africa are not known, it can be assumed that the missing codons in the VP1 gene are likely to be reflected antigenically. Bastos (Thesis, 2002) reported that the diversity in SAT-types is higher compared to the European serotypes. However, the later studies were conducted on viruses isolated from sub-sahara Africa. The results in this study will assist to understand the continental genetic diversity of FMDV in Africa.

Viruses of all three serotopes characterized in this study showed several amino acid differences clustering at known antigenic sites on the GH-loop around RGD sequence residues 144-146 (serotype O and SAT-2), 150-152 (SAT-1). Differences were also observed at the C-terminus region. The virus binding site to cells, the RGD motif was completely conserved in the G-H loop amongst all the serotypes investigated in this study. Fox *et al.*, (1989) reported that mutations within the RGD or in the flanking amino acid leucine (RGDL) leads to a decrease of 60-73 % in virus attachment depending on the serotype. A similar study in 1997 by Leippert and co-workers revealed that variation at the RGD motif and other amino acids in the bordering region affects the binding of FMD virus to susceptible cells. However, Sevilla *et al.*, (1996) indicated that a substitution in the RGD flanking Leucine, L → V of clone C-S8c1 (clone of serotype C derived from persistently infected cell culture) does not seem to induce significant conformational rearrangements in the peptide backbone, and it is compatible with maintaining the helical conformation to the carboxy side of the RGD as well as of the intrapeptide hydrogen bonds found in the wild-type structure. The RGD motif in 93 % of SAT-2 isolates from West Africa was flanked by arginine (R). Although a mutation in the RGD flanking leucine to valine has minor effects, it has been reported from the same study that substitution of the RGD flanking arginine by proline (R → P) required some chain rearrangements to relax steric hindrances imposed by the bulkier P residue (Sevilla *et al.*, 1996). Clearly, further studies are required to elucidate the implication of a total absence of leucine bordering the RGD motif from SAT-2 in the G-H loop. For SAT-1 in this study 86.9 % of the viruses had leucine flanking aspartic acid while the remaining isolates had methionine. The leucine flanking the RGD motif was conserved in type O and predominant in SAT-1 type viruses from West Africa but was absent from SAT-2 types that had either arginine or methionine (Fig. 2.3; 3.5 & 4.3).

During 1975-1976 and 1979-1981 serotype A and SAT-1 outbreaks were simultaneously reported in Nigeria (Records of the WRL) indicating that it is not uncommon to have outbreaks caused by viruses from different serotypes and genotypes within an overlapping time period. This emphasizes the importance of molecular epidemiological studies as a means of accurately differentiating the viruses involved in simultaneously occurring epizootics.

It is also important to investigate the manner in which FMD virus is maintained in the field in West Africa as there are no known wildlife maintenance hosts for FMD virus in the region. This is in contrast with southern Africa where it is well documented that carrier African buffalo (*Syncerus caffer*) play an

important role in the maintenance of the disease and act as a source of virus for other susceptible species (Dawe *et al.*, 1994a; Dawe *et al.*, 1994b; Thomson, 1994; Vosloo *et al.*, 1996; Bastos *et al.*, 2000; Bastos *et al.* 2001). In West Africa where wildlife is scarce, it is not clear how the virus is maintained in inter-epidemic periods and this is one of the reasons why the epidemiology remains obscure. A long period of virus circulation of up to 4 years in the field was observed for SAT-1 and SAT-2. Therefore, it is possible that the disease is maintained by livestock and facilitated by the nomadic movement of animals and people. Due to under reporting of the disease it is possible that minor outbreaks are constantly occurring in livestock which go unnoticed. The disease may also be maintained by sheep and goats where outbreaks are usually difficult to detect. In order to further clarify the epidemiological situation, an inter-dependent, multi-pronged approach is envisaged. An extensive serological survey of cloven-hoofed animals needs to be conducted and the possible role of small ruminants in virus maintenance during inter-epidemic periods needs to be addressed.

A first attempt to investigate the antibody status against FMDV in cattle in Mali, Burkina Faso and Mauritania was made by testing serum samples from the rinderpest campaign for antibodies to the structural and non-structural proteins (3ABC). Since animals are not vaccinated in West Africa, a positive result on the liquid phase blocking ELISA (lpbELISA) and to the non-structural proteins (3ABC ELISA) will be an indication of infection. Antibodies to this non-structural protein last up to 1 year after infection (Mackay *et al.*, 1998). Three hundred sera (100 from Mali, 100 from Mauritania and 100 from Burkina Faso) were used in a preliminary survey to test for antibodies to the non-structural. All positive and few negative to 3ABC were tested to the structural proteins. Due to the high discrepancies found between the results, all positive as well as negative sera to the 3ABC were tested against 6 of the 7 FMD serotypes (except Asia-1) using a liquid phase blocking ELISA according to the standard procedure of the OVI-EDD (Onderstepoort Veterinary Institute-Exotic Diseases Division).

The preliminary results showed that up to 87 % of samples positive to the non-structural proteins were negative when tested with the liquid phase blocking ELISA. The high discrepancy between the results of the 3ABC and blocking ELISA may be due to the fact that the former identifies antibodies against non-structural protein whereas the latter detects antibodies against serotype-specific antigen. The blocking ELISA used in this study was developed against the southern Africa viruses and the serum samples were from West Africa. It is well documented that antibody molecules will only bind to epitopes whose shape exactly matches the conformation of its own binding site (Avrameas *et al.*, 1983). The degree of antigenic

variation between isolates from West and southern Africa is not known, but the nucleotide and amino acid differences observed in this study would most probably cause antigenic differences. Conclusions about the immune status and prevalence of the different serotypes could not be reached in the serological study. Therefore, the need to develop an ELISA capable of accurately identifying the FMDV topotypes in West Africa is crucial to determine the prevalence of viruses and to control the disease.

Although SAT- type viruses have been investigated in southern Africa (Vosloo *et al.*, 1992, 1995 & 1996; Bastos *et al.*, 1998, 1999, 2000 & 2001), the genetic diversity of viruses in West and East Africa where regular outbreaks of FMDV occur is not known. The results presented here show distinct geographical groupings of type O, SAT-1 and SAT-2 isolates from West Africa. This first report on the molecular epidemiology of west African type O, SAT-1 and SAT-2 viruses provides a valuable contribution for assessing the variation of these specific serotypes at the continental level. Perhaps most importantly are the benefits derived from the increased understanding of the genetic relationships of FMD viruses in this part of the African continent, where a regional approach to disease control is advocated for disease eradication.

REFERENCES

ABU ELZEIN, E.M.E., NEWMAN, B.J., CROWTHER, J.R., BARNETT, I.T.R. & McGRANE, J.J. 1987. The prevalence of antibodies against foot-and-mouth disease in various species of Sudanese livestock following natural infection. Review d'Elevage et de Medecine Veterinaire des Pays Tropicaux, 40 (1): 7-12.

ACHARYA, R., FRY, E., STUART, D., FOX, G., ROWLANDS, D. & BROWN, F. 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9 Å resolution. Nature, 337: 709-716.

ACHARYA, R., FRY, E., STUART, D., FOX, G., ROWLANDS, D. & BROWN, F. 1990. The structure of foot-and-mouth-disease virus: implications for its physical and biological properties. Veterinary Microbiology,
23: 21-34.

AGGARWAL, N. & BARNETT, P.V. 2002. Antigenic sites of foot-and-mouth-disease virus (FMDV): an analysis of the specificities of anti-FMDV antibodies after vaccination of naturally susceptible host species. Journal of General Virology, 83: 775-782.

AKTAS, S. & SAMUEL, A.R. 2000. Identification of antigenic epitopes on the foot-and-mouth-disease virus isolate O1/Manisa/Turkey/69 using monoclonal antibodies. O.I.E. Scientific and Technical Review, 19: 744-753.

ALONSO, A., MARTINS MAURICIO, A., GOMES PENDA MARIA, D.A.D., ALLENDE, R. & SONDAHL MAGNUS, S. 1992. Foot-and-mouth-disease virus typing by complement fixation and enzyme linked immunosorbent assay using monovalent and polyvalent antisera. Journal of Veterinary Diagnostic Investigation, 4: 249-253.

ANDERSON, E.C., DOUGHTY, W.J., ANDERSON, J. & PALING, R. 1979. The pathogenesis of foot-and-mouth-disease in the African buffalo (*Syncerus caffer*) and the role of this species in the epidemiology of the disease in Kenya. Journal of Comparative Pathology, 89: 541-549.

AVRAMEAS, S., DRUET, P. & MASSEUEFF, R. 1983. Immunoenzymatic techniques. Proceedings of the second international symposium on immunoenzymatic techniques, Elsevier Science Publishers, New York, N.Y., 410.

BACHRACH, H.L. 1968. Foot-and-mouth disease. Annual Revue of Microbiology, 22: 201-244.

BACHRACH, H.L., MOORE, D.M., MCKERCHER, P.D. & POLATNICK, J. 1975. Immune and antibody responses to an isolated capsid protein of foot-and-mouth-disease virus. Journal of Immunology, 115: 1636-1641.

BACHRACH, H.L. 1977. Foot-and-mouth-disease virus: properties, molecular biology and immunogenicity. Beltsville Symposia in Agricultural Research I. Virology in Agriculture. Edited by J. A. Romberger, Allanheld, Osmun and Co. Montclair.

BARANOWSKI, E., SEVILLA, N., VERDAGUER, N., CARMEN, M., RUIZ-JARABO, C.M., BECK, E. & DOMINGO, E. 1998. Multiple virulence determinants of foot-and-mouth disease virus in cell culture. Journal of Virology, 72(8): 6362-6372.

BARANOWSKI, E., RUIZ-JARABO, C.M., SEVILLA, N., ANDREU, D., BECK, E. & DOMINGO, E. 2000. Cell recognition by foot-and-mouth disease virus that lacks the RGD integrin-binding motif: flexibility in aphthovirus receptor usage. Journal of Virology, 74 (4): 1641-1647.

BARTELS, T., SCHÄFER, H., LIEBERMANN, H., BURGER, R. & BEYER, J. 1994. T-lymphocyte responses in guinea pigs vaccinated with foot-and-mouth-disease virus. Veterinary Immunology And Immunopathology, 40: 213-223.

BASTOS, A.D.S. 1998. Detection and characterization of foot-and-mouth disease virus in sub-Saharan Africa. Onderstepoort Journal of Veterinary Research, 65: 37-47.

BASTOS, A.D.S., BERTSCHINGER, H.J., CORDEL, C.,DE W VAN VUUREN, C., KEET, D., BENGIS, R.G., GROBLER, D.G. & THOMSON, G.R. 1999. Possibility of sexual transmission of foot-and-mouth disease from African buffalo to cattle. Veterinary Record, 145: 77-79.

BASTOS, AD.S., BOSHOFF, C.I., KEET, D.F., BENGIS, R.G. & THOMSON, G.R. 2000. Natural transmission of foot-and-mouth-disease virus between African buffalo (*Syncerus caffer*) and impala (*Aepyceros melampus*) in the Kruger National Park, South Africa. Epidemiology & Infection, 124: 591-598.

BASTOS, A.D.S., HAYDON, D.T., FORSBERG, R., KNOWLES, N.J., ANDERSON, E.C. BENGIS, R.G., NEL, L.H. & THOMSON, G.R. 2001. Genetic heterogeneity of SAT-1 type foot-and-mouth disease viruses in southern Africa. Archives of Virology, 146: 1537-1551.

BASTOS, A.D.S. & SANGARE, O. 2001. Geographic distribution of SAT-2 type foot-and-mouth-disease virus genotypes in Africa. Proceedings of the southern African Society for Veterinary Epidemiology and Preventive Medicine, Onderstepoort, South Africa, 2001; 10-11 May, pp. 20-26.

BASTOS, A.D.S. 2001. Molecular epidemiology and diagnosis of SAT-Type foot-and-mouth disease in southern Africa. Thesis, PhD.

BAXT, B., VAKHARIA, V., MOORE, D.M., FRANKE, A.J. & MORGAN, D.O. 1989. Analysis of neutralizing antigenic sites on the surface of type A12 foot-and-mouth-disease Virus. Journal of Virology, 63 (5): 2143-2151.

BECK, E. & STROHMAIER, K. 1987. Subtyping of European FMDV outbreak strains by nucleotide sequence determination. Journal of Virology, 61: 1621-1629.

BELÁK, S. & BALLAGI-PORDÁNY, A. 1993. Application of the polymerase chain reaction (PCR) in veterinary diagnostic virology. Veterinary Research Communication, 17: 55-72.

BELSHAM, G.J. 1993. Distinctive features of foot-and-mouth disease virus, a number of the picornavirus family; aspects of virus protein synthesis, protein processing and structure. Progress in Biophys Molecular Biology, 60: 241-260.

BEARD, C.W. & MASON, P.W. 2000. Genetic determinants of altered virulence of Tiawanese foot-and-mouth disease virus. Journal of Virology, 74: 987-991.

BITTLE, J.L., HOUGHTEN, R.A., ALEXANDER, H., SHINNICK, T.M., SUTCLIFFE, J.G. & LERNER, R.A. 1982. Protection against foot-and-mouth-disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. Nature, 298: 30-33.

BIZIMANA, N. 1994. Epidemiology, surveillance and control of the principal infectious animal diseases in Africa. Review Scientifiques et Techniques des Offices International des Epizooties, 13 (2): 397-416.

BLACKWELL, J.H., MCKERCHER, P.D., KOSIKOWSKI, F.V., CARMICHAEL, L.E. & GOREWIT, R.C. 1983. Histological and histochemical characterisation of mammary gland tissue of cows infected with foot-and-mouth-disease by contact exposure. Research in Veterinary Sciences, 35: 106-113.

BOOM, R., SOL, C.J., SALIMANS, M.M.M., JANSEN, C.L., WERTHEIM-VAN DILLEN, P.M.E. & VAN DER NOORDAA, J. 1990. Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology, 28: 495-503.

BROOKSBY, J.B. 1982. Portraits of Viruses: foot-and-mouth-disease Virus. Intervirology, 18: 1-23.

BROWN, F. 1976. Replication of Picornaviruses. Chemotherapy, Volume 6, Edited by Williams, J.D & Geddes, A.M. Plenum Publishing Corporation New York, N.Y. 10011.

BROWN, F. 1981. Foot-and-mouth-disease virus. Trends in Biochemical Sciences, 6 (12): 325- 327.

BROWN, F. 1999. Foot-and-mouth disease and beyond: vaccine design, past, present and future. Archives of Virology, 15: 179-188.

BURROWS, R., MANN, J.A., GREIG, A., CHAPMAN, W.C. & GOODRIDGE, D. 1981. The pathogenesis of natural and simulated natural foot-and-mouth-disease infection in cattle. Journal of Comparative Pathology, 91: 599-609.

CALIGUIRI, L.A. 1974. Analysis of RNA associated with the poliovirus RNA replication complexes. Virology, 58: 526-535.

CHOW, M., NEWMAN, J.F.E., FILMAN, D., HOGLE, J.M., ROWLANDS, D.J. & BROWN, F. 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature, 327: 482-486.

CLARKE, B.E., BROWN, A.L., CURREY, K.M., NEWTON, S.E., ROWLANDS, D.E. & CARROL, A.R. 1987. Potential secondary and tertiary structure in the genomic RNA of foot-and-mouth disease virus. Nucleic Acids Research, 15: 7067-7079.

COTTRAL, G.E. & BACHRACH, H.L. 1968. Foot-and-mouth-disease viraemis Proceedings. 72nd Annual meeting. U.S. Animal Health Association, p: 383-399.

.,

COOPER, P.D., AGOL, V.I., BACHRACH, H.L., BROWN, F., GHENDON, Y., GIBBS, A.J., GILLESPIE, J.H., LONBERGHOLM, K., MANDEL, B., MELNICK, J.L., MOHANTY, S.B., POVEY, R.C., RUECKERT, R.R., SCHAFFER, F.C. & TYRRELL, D.A.J. 1978. Picornaviridae: Second report. Intervirology, 10: 165-180.

CROWTHER, J.R. & ABU-ELZEIN, E.M.E. 1979. Application of the enzyme-linked-immunosorbent assay to the detection and identification of foot-and-mouth-disease viruses. Journal of Hygiene, 83: 513-519.

CROWTHER, J.R., FARIAS, S., CARPENTER, W.C. & SAMUEL, A.R. 1993. Identification of a fifth neutralizable site on type O foot-and-mouth-disease virus following characterization of single and quintuple monoclonal antibody escape mutants. Journal of General Virology, 74: 1547-1553.

DAWE, P.S. & PINTO, A.A. 1978. Antibody responses to type-specific and virus-infection-associated antigens in cattle vaccinated with inactivated polyvalent foot-and-mouth-disease virus in North Malawi. British Veterinary Journal, 134: 504-511.

DAWE, P.S., SORENSON, K.J., FERRIS, N.P., BARNETT, I.T.R., ARMSTRONG, R.M. & KNOWLES, N.J. 1994a. Experimental transmission of foot-and-mouth disease virus from carrier African

buffalo (Syncerus caffer) to cattle in Zimbabwe. Veterinary Record, 134: 211-215.

DAWE, P.S., FLANAGAN, F.O., MADEKUROZWA, R.L., SORENSON, K.J., ANDERSON, E.C., FOGGIN, C.M., FERRIS, N.P. & KNOWLES, N.J. 1994b. Natural transmission of foot-and-mouth disease from African buffalo (*Syncerus caffer*) to cattle in a wildlife area of Zimbabwe. Veterinary Record, 134: 230-232.

DE LA TORRE, J.C., DÁVILA, M., SOBRINA, G., ORTÍN, J. & DOMINGO E. 1985. Establishment of cell lines persistently infected with foot-and-mouth disease virus. Virology, 145: 24-35.

DE LA TORRE, J.C., MARTÍNEZ-SALAS, E., DIEZ, J., VILLVERDE, A., GEBAUER, F., ROCHA, E., DÁVILA, M. & DOMINGO, E. 1988. Coevolution of cells and viruses in a persistent infection of foot-and-mouth-disease virus in cell culture. Journal of Virology, 62: 2050-2058.

DE LA TORRE, J.C., MARTÍNEZ-SALAS, E., DIEZ, J. & DOMINGO, E. 1989. Extensive cell heterogeneity during persistent infection with foot-and-mouth disease virus. Journal of Virology, 63: 59-63.

DIEGO, A. DE, BROCCHI, E., MACKAY, D. & SIMONE, F. 1997. The use of the non-structural polyprotein 3ABC of FMD virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. Archives of Virology, 142: 2021-2033.

DIMARCHI, R., BROOKE, G., GALE, C., CRACKNELL, V., DOEL, T. & MOWAT, N. 1986. Protection of cattle against foot-and-mouth disease by a synthetic peptide. Science, 232: 639-641.

DOEL, T.R., SANGAR, D.V., ROWLANDS, D.J. & BROWN, F. 1978. A re-appraisal of the biochemical map of foot-and-mouth-disease virus RNA. Journal of General Virology, 41: 395-404.

DOMINGO, E., MARTINEZ-SALAS, E., SOBRINO, F., DE LA TORRE, J.C., PORTELA, A., ORTÍN, J., LÓPEZ-GALINDEZ, C., PÉREZ-BRE, A.P., VILLANUEVA, N., NÁJERA, R., VANDEPOL, S., STEINHAUER, D., DEPOLO, N. & JOHN HOLLAND. 1985. The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance- Review. Gene, 40: 1-8.

DOMINGO, E., MATEU, M.G., MARTINEZ, M.A., DOPAZO, J., MOYA, A. & SOBRINO, F. 1990. Genetic variability and antigenic diversity of foot-and-mouth-disease virus. Applied Virology Research, 2: 233-266.

DOMINGO, E., ESCARMIS, M.A., MARTINEZ-SALAS, E. & MATEU, M.G. 1992. Foot-and-mouth-disease virus populations are quasispecies. Current topics in Microbiology and immunology, 176: 33-47.

DONALDSON, A.I., FERRIS, N.P. & WELLS, G.A.H. 1984. Experimental foot-and-mouth-disease in fattening pigs, sows and piglets in relation to outbreaks in the field. Veterinary Record, 115: 509-512.

DOOLITTLE, R.F. 1990. Searching through sequence databases. In: Molecular Evolution: computer analysis of protein and nucleic acid sequences. Methods in enzymology. Edited by Doolittle, R.F. Academic press, New York, p: 99-110.

DOPAZO, J., SOBRINO, F., PALMA, E.L., DOMINGO, E. & MOYA, A. 1988. Gene encoding capsid protein VP1 of foot-and-mouth-disease virus: a quasispecies model of molecular evolution. Proceedings of the National Academy of Sciences of the USA, 85: 6811-6815.

DRAKE, J.W. & HOLLAND, J.J. 1999. Mutation rates among RNA viruses. Proceedings of the National Academy of Sciences of the USA, 96: 3910-3913.

DUKE, G.M., OSORIO, J.E. & PALMENBERG, A.C. 1990. Attenuation of Mengo virus through genetic engineering of the 5' non-coding poly(C) tract. Nature, 343: 474-476.

HOWEL, P.G., YOUNG, E. & HEDGER, R.S. 1973. Foot-and-mouth disease in the African elephan (*Loxodonta Africana*). Onderstepoort Journal for Veterinary Research, 40: 41-52

EIGEN, M. 1971. Self-organization of matter and the evolution of biological macromolecules. Naturwissenschaften, 58: 465-523.

ESCARMIS, C., DOPAZO, J., DáVILA, M., PALMA, E.L. & DOMINGO, E. 1995. Large deletion in the 5'-untranslated region of foot-and-mouth-disease virus of serotype C. Virus Research, 35: 155-167.

ESTERHUYSEN, J.J., THOMSON, G.R., FLAMMAND, J.R.B. & BENGIS, R.G. 1985. Buffalo in the northern Natal Game Parks show no serological evidence of infection with foot-and-mouth-disease virus. Onderstepoort Journal of Veterinary Research, 52: 63-66.

ESTERHUYSEN, J.J. 1994. The antigenic variation of foot-and-mouth-disease viruses and its significance in the epidemiology of the disease in southern Africa. Msc. dissertation, University of Pretoria.

ESTERHUYSEN, J.J., PREHAUD, C. & THOMSON, G.R. 1995. A liquid-phase blocking ELISA for the detection of antibodies to rabies virus. Journal of Virological Methods, 51: 31-42.

FALCONER, J. 1972. The epizootiology and control of foot-and-mouth disease in Botswana. The Veterinary Record, 91: 354-359.

FELSENSTEIN, J. 1981. Evolutionary trees from DNA sequences: A maximum likelihood approach. Journal of Molecular Evolution, 17: 368-376.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 39: 783-791.

FORSS, S., STREBEL, K., BECK, E. & SCHALLER, H. 1984. Nucleotide sequence and genome organization of foot-and-mouth-disease virus. Nucleic Acids Research, 12: 6587-6601.

FOX, G., PARRY, N.R., BARNETT, P.V., McGINN, B., ROWLANDS, D.J. & BROWN, F. 1989. The cell attachment site on foot-and-mouth-disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). Journal of General Virology, 70: 625-637.

FRANCIS, M.J., OULDRIDGE, E.J. & BLACK, L. 1983. Antibody response in bovine pharyngeal fluid following foot-and-mouth-disease vaccination and/or exposure to live virus. Research in Veterinary Sciences, 35: 206-210.

FRANCIS, M.J. & BLACK, L. 1986. Response to young pigs to FMD oil emulsion vaccination in the presence and absence of maternally derived neutralizing antibodies. Research in Veterinary Science, 41: 33-39.

FREIBERG, B., RAHMAN, M.M. & MARQUARDT, O. 1999. Genetical and immunological analysis of recent Asian type A and O foot-and-mouth-disease virus isolates. Virus Genes, 19 (3): 167-182.

FRY, E., LOGAN, D., ACHARYA, R., FOX, G., ROWLANDS, D., BROWN, F. & STUART, D.I. 1990. Architecture and topography of an Aphtovirus. Virology, 1: 439-451.

GAINARU, M.D., THOMSON, G.R., BENGIS, R.G., ESTERHUYSEN, J.J., BRUCE, W. & PINI, A. 1986. Foot-and-mouth-disease and the African buffalo (Syncerus caffer). II. Virus excretion and transmission during acute infection. Onderstepoort Journal of Veterinary Research, 53: 75-85.

GARLAND, A.J.M. 1999. Vital elements for the successful control of foot-and-mouth-disease by vaccination. Vaccine, 17: 1760-17.

GEBAUER, F., DE LA TORRE, J.C., GOMES, I., MATEU, M.G., BARAHONA, H., TIRABOSCHI, B., BERGMANN, I., AUGÉ DE MELLO, P. & DOMINGO, E. 1988. Rapid selection of genetic and antigenic variants of foot-and-mouth-disease virus during persistence in cattle. Journal of Virology, 62: 2041-2049.

GEERING, W.A. 1967. Foot-and-mouth-disease in Sheep. Australian Veterinary Journal, 43: 485-489.

GEYSEN, H.M., BARTELING, S.J. & MELOEN, R.H. 1985. Small peptides induce antibodies with a sequence and structural requirement for binding antigen comparable to antibodies raised against the native protein. Proceedings of the National Academy of Sciences, USA, 82: 178-182.

GIRAUDO, A.T., BECK, E., STREBEL, K., AUGE DE MELLO, P., LA TORRE, J.L., SCHODELLER, E.D. & BERGMAN, I.E 1990. Identification of a nucleotide deletion in parts of polypeptide 3A in two independent attenuated aphthovirus strains. Virology, 177: 780-788.

HAAS, B. 1997. Possible role of 3ABC-ELISA in screening of cattle after an outbreak. Session of the research group of the standing technical committee of the European Commission for the control of foot-and-mouth-disease, Brasov, 23-27 September 1997, 52-54.

HAFEZ, S.M., FARAG, M.A., MAZLOUM, K.S. & AL-BOKMY, A.M. 1994. Serological survey of foot-and-mouth disease in Saudi Arabia. Review Scientific & Technique des Offices International des Epizooties, 13 (3): 711-719.

HAMBLIN, C., ARMSTRONG, R.M. & HEDGER, R.S. 1984. A rapid enzyme-linked-immunosorbent assay for the detection of foot-and-mouth-disease virus in epithelial tissues. Veterinary Microbiology, 9: 435-443.

HAMBLIN, C., BARNETT, I.T.R. & CROWTHER, J.R. 1986. A new enzyme-linked-immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth-disease virus. I. Development and method of ELISA. Journal of Immunological Methods, 93: 115-122.

HARLEY, E.H. 1994. DAPSA. DNA and protein sequence analysis, version 2.9. University of Cape Town: Department of Chemical Pathology, 1994.

HARRIS, T.J.R., UNDERWOOD, B.O., KNOWLES, N.J., CROWTHER, J.R. & BROWN, F. 1979. Molecular approach to the epidemiology of swine vesicular disease: correlation of variation in the virus structural polypeptides with serological properties. Infection & Immunology, 24: 593-599.

HAVE, P., LEI, J.C. & SCHJERNING-THIESEN, K. 1983. An enzyme-linked-immunosorbent assay (ELISA) for the primary diagnosis of foot-and-mouth-disease: characterization and comparison with complement fixation. In: Report of the session of the research group of the standing technical committee of the European Commission for the control of foot-and-mouth-disease, Lelystad, Netherlands, 20-22 September, 1983. FAO, Rome, p: 34-42.

HEDGER, R.S. 1968. The isolation and characterization of foot-and-mouth-disease virus from clinically normal herds of cattle in Botswana. Journal of Hygiene Cambridge, 66: 27-36.

HEDGER, R.S., CONDY, J.B. & GOLDING, S.M. 1972. Infection of some species of African wildlife with foot-and-mouth-disease virus. Journal of Comparative Pathology, 82: 455-461.

HEDGER, R.S., CONDY, J.B. & GRADWELL, D.V. 1980. The response of some African wildlife species to foot-and-mouth-disease vaccination. Journal of Wildlife Diseases, 16 (3): 431-438.

HEDGER, R.S. 1981. Foot-and-mouth disease. In Infectious diseases of wild animals. Edited by J.W. DAVIS, L.H. KARSTAD & D.O. TRAINER. 2nd Edition, Ames, Iowa State University Press, p: 87-96.

HILLIS, D.M. & BULL, J.J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Systematic Biology, 42: 182-192.

HIRST, G.K. 1962. Genetic recombination with newcastle disease virus, polioviruses and influenza. Cold Spring Harbor Symposium of Quantitative Biology, 27: 303-308.

HOLLAND, J.J., SPINDLER, K., HORODYSKI, GRABEU, E., NICHOL, S. & VANDE POL, S.1982. Rapid evolution of RNA genomes. Science, 215: 1577-1585.

HOWELL, P.G., YOUNG, E. & HEDGER, R.S. 1973. Foot-and-mouth disease in the African elephant (*Loxodonta Africana*). Onderstepoort Journal for Veterinary Research, 40: 41-52.

HYSLOP, N.St.G. & FAGG, R.H. 1965. Isolation of variants during passage of a strain of foot-and-mouth-disease virus in partly immunized cattle. Journal of Hygiene, 63: 357.

IIZUKA, N., KOHARA, M., HAGINO-YAMAGISHI, K., ABE, S., KOMATSU, T., TAGO, K., ARITA, M. & NOMOTO, A. 1989. Construction of less neurovirulent polioviruses by introducing deletions into the 5' non-coding sequence of the genome. Journal of Virology, 63: 5354-5363.

ISLAM, M.A., RAHMAN, M.M., ADAM, K.H. & MARQUARDT, O. 2001. Epidemiological implications of the molecular characterization of foot-and-mouth-disease virus isolated between 1996 and 2000 in Bangladesh. Virus Genes, 23 (2): 203-210.

KAHRS, R.F. 1981. Foot-and-mouth-disease In: Viral diseases of cattle. Printed by Iowa State University Press, Ames, Iowa 50010, p: 255-262.

KIM, J. 1993. Improving the accuracy of phylogenetic estimation by combining different methods. Systematic Biology, 42: 331-340.

KING, A.M.Q., SANGAR, D.V., HARRIS, T.J.R. & BROWN, F. 1980. Heterogeneity of the genome-linked protein of foot-and-mouth-disease virus. Journal of Virology, 34: 627-634.

KING, A.M.Q., McCAHON, D., SLADE, W.R. & NEWMAN, J.W.I. 1982. Biochemical evidence of recombination within the unsegmented RNA genome of Aphtovirus. Journal of Virology, 66-77.

KING, A.M.Q., McCAHON, D., SAUNDERS, K., NEWMAN, J.W.I. & SLADE, W.R. 1985. Multiple sites of recombination within the RNA genome of foot-and-mouth-disease virus. Virus Research, 3: 373-384.

KITCHING, R.P. 1998. A recent history of foot-and-mouth-disease. Journal of Comparative Pathology, 118: 89-108.

KITSON, K.D.A., McCAHON, D. & BELSHAM, G.J. 1990. Sequence analysis of monoclonal antibody resistant mutants of type O foot-and-mouth-disease virus: evidence for the involvement of the three exposed capsid proteins in four antigenic sites. Virology, 179: 26-34.

KNOWLES, N.J. & HEDGER, R.S. 1985. A study of antigenic variants of foot-and-mouth-disease virus by polyacrylamide gel electrophoresis of their structural polypeptides. Veterinary Microbiology, 10: 347-357.

KNOWLES, N.J., MARQUARDT, O. & SAMUEL, A.R. 1988. Antigenic and molecular characterization of virus isolates from recent outbreaks of foot-and-mouth-disease in the Federal Republic of Germany. Research group of European commission control of FMD, Prague, Czechoslovakia, Rome: FAO, p: 145-55.

KNOWLES, N.J. 1997. In: Proceedings of an International Symposium on diagnosis and control of livestock diseases using nuclear and related techniques (IAEA-SM-348/16), Vienna, 1-22 April, 1997, p: 185-198.

KNOWLES, N.J., ANSELL, D.M. & SAMUEL, A.R. 1998. Molecular comparison of recent foot-and-mouth-disease type A viruses from West Africa with historical and reference virus strains. Paper presented to the session of the research group of the standing technical committee of the European commission for control of foot-and-mouth-disease, Pirbright, U.K., 14-18th September, 1998.

KNOWLES, N.J., SAMUEL, A.R., DAVIES, P.R., KITCHING, R.P., VENKATARAMANAN, R., KANNO, T., SCHERBAKOV, A.V., DRYGIN, V.V., ZHAO, Q-Z. & XIE, Q.G. 2000. Emergence of a pandemic strain of foot-and-mouth-disease virus serotype O. In: Report of the session of the research group of the standing technical committee of the European commission for the control of foot-and-mouth-disease. Borovets, Bulgaria, 5-8 September, 2000.

KREBS, O. & MARQUARDT, O. 1992. Identification and characterization of foot-and-mouth-disease virus O1. Burgwedel/1987 as an intertypic recombinant. Journal of General Virology, 73: 613-619.

KUMAR, S., TAMURA, K. & NEI, M. 1993. MEGA. Molecular evolutionary genetics analysis, version 1.0. Pennsylvania State University.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

LAPORTE, J., GROSCLAUDE, J., WANTYGHEM, J., BERNARD, S. & ROUZE, P. 1973. Neutralisation en culture cellulaire du pouvoir infectieux du virus de la fièvre aphteuse par des sérums provenant de porcs immunisés à l'aide d'une protéine virale purifiée. Comptes rendus hebdomadaires des séances de l'Academie des sciences serie D, Paris 276, 3399-34-2.

LEA, S., HERNÁNDEZ, J., BLAKEMORE, W., BROCCHI, E., CURRY, S., DOMINGO, E., FRY, E., ABU-GHAZALEH, R., KING, A., NEWMAN, J., STUART, D. & MATEU, M.G. 1994. The structure

and antigenicity of a type C foot-and-mouth-disease virus. Structure, 2: 123-139.

LEIPPERT, M., BECK, E., WEILAND, F. & PFAFF, E. 1997. Point mutations within the βG-βH Loop of foot-and-mouth-disease virus O_1 K affect virus attachment to target cells. Journal of Virology, 71(2): 1046-1051.

LI, W.-H., TANIMURA, M. & SHARP, P.M. 1987. An evaluation of the molecular clock hypothesis using mammalian DNA sequences. Journal of Molecular Evolution, 25: 330-342.

LIEBERMANN, H.T., DÖLLING, R., SCHMIDT, D. & THALMANN, G. 1991. RGD containing peptides of VP1 of foot-and-mouth-disease virus (FMDV) prevent virus infection in vitro. Acta Virology, 35: 90-93.

LOCHER, F., SURYANARAYANA, V.V. & TRATSCHIN, J.D. 1995. Rapid detection and characterization of foot-and-mouth disease virus by restriction enzyme and nucleotide sequence analysis of PCR products. Journal of Clinical Microbiology, 33: 440-444.

LOEFFLER, F. & FROSCH, P. 1898. Berichte der Kommission zur Erforschung der Maul-und Klauenseuche bei dem lnstitut für lnfektrionskrankheiten in Berlin. Zbl Bakter Abt I Orig., 23: 371-391.

LOGAN, D., ABU-GHAZALEH, R., BLAKEMORE, W., CURRY, S., JACKSON, T., KING, A., LEA, S., LEWIS, R., NEWMAN, J., PARRY, N., ROWLANDS, D., STUART, D. & FRY, E. 1993. Structure of a major immunogenic site on foot-and-mouth-disease virus. Nature, 362: 566-568.

MacPHERSON, I.A. & STOKER, M.G.P. 1962. Polyoma transformation of hamster cell clones - an investigation of genetic factors affecting cell competence. Virology, 16: 147-151.

MACKAY, D.K.J., FORSYTH, M.A., DAVIES, P.R., & SALT. J.S. 1998. Antibody to the nonstructural proteins of foot-and-mouth-disease virus in vaccinated animals exposed to infection. The Veterinary Quarterly, 20(2): S9-S11.

MADDISON, W.P. & MADDISON, D.R. 1992. MacClade: Analysis of phylogeny and character evolution. Publishers, Sinauer Associates, Inc. Sunderland, Massachusetts, U.S.A. University of Arizona.

MALIRAT VIVIAN, PAULO AUGÉ DE MELLO, BEATRIZ TIRABOSHI, EWALD BECK, LVO GOMES, & INGRID E. BERGMANN. 1994. Genetic variation of foot-and-mouth-disease virus during persistent infection in cattle. Virus Research, 34: 31-34.

MARQUARDT, O., & HAAS, B. 1998. VP1 coding sequences of recent isolates of foot-and-mouth-disease Types A, O, Asia1. Virus Genes, 16: 185-193.

MARTIN, M.J., GONZÁLEZ-CANDELAS, SOBRINO, F. & DOPAZO, J. 1995. A method for determining the position and size of optimal sequence regions for phylogenetic analysis. Journal of Molecular Evolution, 41:1128-1138.

MATEU, M.G., ROCHA, E., VICENTE, O., VAYREDA, F., NAVALPOTRO, C., ADREU, D., PEDROSO, E., GIRALT, E., ENJUANES, L. & DOMINGO, E. 1987. Reactivity with monoclonal antibodies of viruses from an episode of foot-and-mouth-disease. Virus Research, 8: 261-274.

MATEU, M.G., VALERO, M.L., ADREU, D. & DOMINGO, E. 1996. Systematic replacement of amino acid residues within an Arg-Gly-Asp containing loop of foot-and-mouth-disease virus: effect on cell recognition. Journal of Biological Chemistry, 271: 12814-12819.

MAXAM, A.M. & GILBERT, W. 1977. A new method for sequencing DNA. Proceedings in National Academy of Science, U.S.A., p: 560-564.

McCULLOUGH, K.C., BRUCKNER, L., SCHAFFNER, R., FRAEFEL, W., MÜLLER H.K. & KIHM, U. 1992. Relationship between the anti-FMD virus antibody reaction as measured by different assays, and protection in vivo against challenge infection. Veterinary Microbiology, 30: 99-112.

McKERCHER, P.D., MOORE, D.M., MORGAN, D.O., ROBERTSON, H., CALLIS, J.J., KLEID, D.G., SHIRE, S.J., YANSURA, D.J., DOWBENKO, D. & SMALL, B. 1985. Dose response evaluation of a

genetically engineered foot-and-mouth-disease virus polypeptide immunogen in cattle. American Journal of Veterinary Reseach, 46: 587

McVICAR, J.W. & SUTMOLLER, P. 1968. Sheep and goats as foot-and-mouth-disease virus carriers. Proceeding of Meetings of the United States Livestock and Sanitary Association, 72: 400-406.

MELNICK, J.L., AGOL, V.I., BACHRACH, H.L., BROWN, F., COOPER, P.D., FIFRS, W., GARD, S., GEAR, J.H., GHENDON, Y., KASZA, L., LAPLAGA, MANDEI, B., McGREGOR, S., MOHANTY, S.B., PLUMMER, G., RUECKERT, R.R., SCHAFFER, F.L., TAGAYA, I., TYRRELL, D.A.J., VOROSHILOVA, M. & WENNER, H.A. 1975. Picornaviridae. Intervirology, 4: 303-316.

MOUSTAFA, T. & ABD EL-GADIR, F. 1993. Studies on foot-and-mouth-disease in the eastern region of Abu Dhabi, United Arab Emirates. Review Scientifiques et techniques des Office International des Epizooties, 12 (3): 831-837.

MUKHOPADHYAY, A.K., TAYLOR, W.P. & ROEDER, P.L. 1999. Rinderpest: a case study of animal health emergency management. Review Scientifiques et techniques des Office International des Epizootie, 18(1): 164-178.

MULCAHY, G., GALE, C., ROBERTSON, P., IYISAN, S., DIMARCHI, R.D. & DOEL, T.R. 1990. Isotype responses of infected, virus-vaccinated and peptide-vaccinated cattle to foot-and-mouth-disease virus. Vaccine, 8: 249-256.

MURPHY, F.A., FAUQUET, C.M., BISHOP, D.H.L., GHABRIAL, S.A., JARVIS, A.W., MARTELLI, G.P., MAYO, M.A. & SUMMER, M.D. 1995. Virus taxonomy: Classification and nomenclature of Viruses. Sixth report of the International Committee on Taxonomy of Viruses. Springer-Verlag, Wen.

NEI, M. & KUMAR, S. 2000. Molecular evolution and phylogenetics. Oxford press, U.S.A, p: 87-113.

NEWMAN, J.F.E., ROWLANDS, D.J. & BROWN, F. 1973. A physico-chemical sub-grouping of the mammalian picornaviruses. Journal of General Virology, 18: 171-180.

NICHOLLS, N.J., BLACK, L., RWEYEMAMU, M.M. & GRADWELL, D.V. 1985. Effect of age on response of cattle to vaccination against foot-and-mouth-disease. British Veterinary Journal, 141(1): 17-26.

MONOD, J., WYMAN, J. & CHANGEUX, J. 1965. On the nature of allosteric transitions. A plausible model. Journal of Molecular Biology, 12: 88-118.

NEI, M., TAJIMA, F. & TATENO, Y. 1983. Accuracy of estimated phylogenetic trees from molecular data. II. Gene frequency data. Journal of Molecular Evolution, 19: 153-170.

NOTTAY, B.K., KEW, O.M., HATCH, M.H., HEYWARD, J.T. & OBIJESKI, J.F. 1981. Molecular variation of type 1 vaccine-related and wild poliovirus during replication in humans. Virology, 108: 405-423.

OFFICE INTERNATIONAL DES EPIZOOTIES. 1992. Manual of standards for diagnostic tests and vaccines. 4th Edition, 1992, p: 77-92.

OFFICE INTERNATIONAL DES EPIZOOTIES. 2000. Manual of standards for diagnostic tests and vaccines. Second Edition, 2000, p: 1-12.

PAGE, R.D.M. & HOLMES, E.C. 1998. Trees. In Molecular Evolution: A phylogenetic approach. Edited by Blakwell Science Ltd. Osney Mead, Oxford, U.K., p: 11-36

PALMENBERG, A.C. 1987. Comparative organization and genome structure in picornaviruses. In Positive Strand RNA Viruses, UCLA Symposium on molecular and cellular biology, New Series, 54: 25-34. Edited by M.A. Brinton & R. Rueckert, New York, Alan, R. Liss.

PATTNAIK, P., VENKATARAMANAN, R., TOSH, C., SNYAL, A., HEMADRI, D., SAMUEL, A.R., KNOWLES, N.J. & KITCHING, R.P. 1998. Genetic heterogeneity of Indian field isolates of foot-and-mouth-disease virus serotype O as revealed by partial sequencing of 1D gene. Virus Research, 55: 115-127.

PAY, T.W.F. & PARKER, M.J. 1977. Some statistical and experimental design problems in the assessment of foot-and-mouth-disease vaccine potency. Developments of Biological Standardization, 35: 369-383.

PENNY, D., HENRY, M.D. & STEEL, M.A. 1992. Progess with methods for constructing evolutionary trees. Trends in Ecology and Evolution, 7: 73-79.

PEREIRA, H.G. 1981. Foot-and-mouth-disease. In: Virus diseases of food animals. Vol. II. Disease Monographs, Edited by E.P.J. Gibbs: London, New York. Academic Press Inc.

PFAFF, E., MUSGAY, M., BOHM, H.O., SCTHULZ, G.E. & SCHALLER, H. 1982. Antibodies against a preselected peptide recognize and neutralize foot-and-mouth-disease virus. EMBO Journal, 1: 869-874.

PFAFF, E., THIEL, H.J., BECK, E., STROHMAIER, K. & SCHALLER, H. 1988. Analysis of neutralizing epitopes on foot-and-mouth-disease virus. Journal of Virology, 62: 2033-2040.

PRINGLE, C.R. 1965. Evidence of genetic recombination in foot-and-mouth-disease virus. Virology, 25: 48-54.

PRINGLE, C.R. 1999. Virus taxonomy at the XIth international congress of virology, Sydney, Australia. Archives of Virology, 144 (10): 2065-2070.

REID, S.M., FERRIS, N.P., HUTCHINGS, G.H., DE CLERQ, K., NEWMAN, B.J., KNOWLES, N.J. & SAMUEL, A.R. 2001. Diagnosis of foot-and-mouth disease by RT-PCR: use of phylogenetic data to evaluate primers for the typing of viral RNA in clinical samples. Archives of Virology, 146: 2421-2434.

RICO-HESSE, R., PALLANSCH, M.A., NOTTAY, B.K. & KEW, O.M. 1987. Geographic distribution of wild poliovirus type 1 genotypes. Virology, 160: 311-322.

RINA, B.K. & MARTIN, S.J. 1976. Persistent infection of tissue culture cells by RNA viruses. Medecine of Microbiological Immunology, 162: 89-118.

ROBSON, K.J.H., CROWTHER, J.R., KING, A.M.Q. & BROWN, F. 1979. Comparative biochemical and serological analysis of five isolates of a single serotype of foot-and-mouth-disease virus. Journal of General Virology, 45: 579-590.

RODRIGUEZ, A., NUNEZ, J.I., NOLASCO, G., PONZ, F., SOBRINO, F. & DE BLAS, C. 1994. Direct PCR detection of foot-and-mouth disease virus. Journal of Virological Methods, 47: 345-349.

ROEDER, P.L. & LE BLANC SMITH, P.M. 1987. Detection and typing of foot-and-mouth-disease virus by enzyme-linked-immunosorbent assay: a sensitive, rapid and reliable technique for primary diagnosis. Research in Veterinary Science, 43: 225-232.

ROEDER, P.L., ABRAHAM, G., MEBRATU, G.Y. & KITCHING, R.P. 1994. Foot-and-mouth-disease in Ethiopia from 1988 to 1991. Tropical Animal Health Production, 26: 163-167.

ROMANOVA, L.I., TOLSKAYA, E.A. & AGOL, V.I. 1981. Antigenic and structural relatedness among non-capsid and capsid polypeptides of polioviruses belonging to different serotypes. Journal of General Virology, 52: 279-289.

ROSSMAN, M.G., ARNOLD, E., ERICKSON, J.W., FRANKENBERGER, E.A., GRIFFITH, J.P., HECHT, H.J., JOHNSON, J.R., KAMER, G., LUO, M., MOSSER, A.G., RUECKERT, R.R., SHERRY, B. & VRIEND, G. 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. Nature, 317: 145-153.

ROWLANDS, D.J., SANGAR, D.V. & BROWN, F. 1971. Relationships of the antigenic structure of foot-and-mouth-disease virus to the process of infection. Journal of General Virology, 13: 85-93.

RUECKERT, R.R. 1985. Picornaviruses and their replication. In: Virology, second edition, edited by Fields, B.N., Knipe, D.M., Chanock, R.M., Melnick, J.L., Roizman, B., Shope, R.E.,. Raven Press, New York, p: 705-738.

RUOSLAHTI, E. & PIERSCHBACHER, M.D. 1987. New perspectives in cell adhesion: RGD and integrins. Science, 238: 491-497.

RYAN, M.D., BELSHAM, G.J. & KING, A.M.Q. 1989. Specificity of enzyme-substrate interactions in foot-and-mouth-disease virus polyprotein processing. Virology, 173: 35-45.

RWEYEMANY, M.M., PAY, T.W.F. & PARKER, M.J. 1977. Serological differentiation of foot-and-mouth-disease virus strains in relation to the selection of suitable vaccine viruses. Development of biological standard, 35: 205-214.

SAITOU, N. & NEI, M. 1987. The neighbor-joining method: a new method for reconstructing phyologenetic trees. Molecular Biology & Evolution, 4: 406-425.

SÁIZ, J.C., SOBRINO, F. & DOPAZA, J. 1993. Molecular epidemiology of foot-and-mouth-disease virus type O. Journal of General Virology, 74: 2281-2285.

SALT J S, 1993. The carrier state in foot-and-mouth disease-an immunological review. British Veterinary Journal, 149: 207-223.

SALT, J.S., SAMUEL, A.R. & KITCHING, R.P. 1996. Antigenic analysis of type O foot-and-mouth disease virus in the persistently infected bovine. Archives of Virology, 141: 1407-1421.

SAMINA ITZCHAK, ZICHRIA ZAKAY-RONES & BEN-AMI, 1998. Homologous and heterologous antibody response of cattle and sheep after vaccination with foot-and-mouth-disease and influenza viruses. Vaccine, 16: 551-557.

SAMUEL, A.R., KNOWLES, N.J., KITCHING, R.P. & HAFEZ, S.M. 1997. Molecular analysis of type O foot-and-mouth-disease viruses isolated in Saudi Arabia between 1983 and 1995. Epidemiology & Infection, 119: 381-389.

SAMUEL, A.R., KNOWLES, N.J. & MACKAY, D.K.J. 1999. Genetic analysis of type O viruses responsible for epidemics of foot-and-mouth-disease in North Africa. Epidemiology & Infection, 122: 529-538.

SAMUEL, A.R. & KNOWLES, N.J. 2001a. Foot-and-mouth-disease virus: cause of the recent crisis for the UK livestock industry. Trends Genetics, 17 (8): 421-424.

SAMUEL, A.R. & KNOWLES, N.J. 2001b. Foot-and-mouth-disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). Journal of General Virology, 82: 609-621.

SANGAR, D.V., ROWLAND, D.J., HARRIS, T.J.R. & BROWN, F. 1977. A protein covalently linked to foot-and-mouth-disease virus RNA. Nature, 268: 648-650.

SANGAR, D.V. 1979. The replication of picornaviruses. Journal of General Virology, 45: 1-13.

SANGAR, D.V., CLARK., R.P., CARROLL, A.R., ROWLANDS, D.J. & CLARKE, B.E. 1988. Modification of the leader protein (Lb) of foot-and-mouth-disease virus. Journal of General Virology, 69: 2327-2333.

SANGARE, O., BASTOS, A.D.S., MARQUARDT, O., VENTER, E.H., VOSLOO, W. & THOMSON, G.R. 2001. Molecular epidemiology of serotype O foot-and-mouth-disease virus with emphasis on West and South Africa. Virus Genes, 22(3): 345-351.

SANZ-PARRA, A., SOBRINO, F. & LEY, V. 1998. Infection with foot-and-mouth-disease virus results in a rapid reduction of MHC class I surface expression. Journal of General Virology, 79: 433-436.

SANYAL, A., VENKATATARAMANAN, R. & PATTNAIK, B. 1997. Antigenic features of foot-and-mouth-disease virus serotype ASIA1 as revealed by monoclonal antibodies and neutralization escape mutants. Virus Research, 50: 107-117.

SEKIGUCHI, K., FRANKE, A.J. & BAXT, B. 1982. Competition for cellular receptor sites among selected aphthoviruses. Archives of Virology, 74: 53-64.

SELLERS, R.F. & DAGGUPATY, S.M. 1990. The epidemic of foot-and-mouth-disease in Saskatchewan, Canada, 1951-1952, Canadian Journal of Veterinary Reseach, 54: 457-464.

SEVILLA, N. & DOMINGO, E. 1996. Evolution of a persistent aphthovirus in cytolytic infections: partial reversion of phenotypic traits accompanied by genetic diversification. Journal of Virology, 70: 6617-6624.

SEVILLA, N., VERDAGUER, N. & DOMINGO, E. 1996. Antigenically profound amino acid substitutions occur during large population passages of foot-and-mouth disease virus. Virology, 225: 400-405.

SOBRINO, F., DÁVILA, M., ORTÍN, J. & DOMINGO, E. 1983. Multiple genetic variants arise in the course of replication of foot-and-mouth-disease virus in cell culture. Virology, 128: 310-318.

SOBRINO, F., PALMA, E.L., BECK, E., DÁVILA, M., DE LA TORRE, J.C., NEGRO, P., VILLANUEVA, N., ORTIN, J. & DOMINGO, E. 1986. Fixation of mutations in the viral genome during an outbreak of foot-and-mouth-disease: heterogeneity and rate variations. Gene, 50:149-159.

SPÄTH, E.J.A., SMITSAART, E., CASARO, A.P.E., FONDEVILA, N., FERNÁNDEZ, F., LEUNDA, M.R., COMPAIRED, D., BUFFARINI, M. & PESSI, H. 1995. Immune response of calves to foot-and-mouth-disease virus vaccine emulsified with oil adjuvant. Strategies of vaccination. Vaccine, 13 (10): 909-914.

STANWAY, G. 1990. Review Article: Structure, function and evolution of picornaviruses. Journal of Virology, 71: 2483-2501.

STAVE, J.W., CARD, J.L., MORGAN, D.O. & VAKHARIA, V.N. 1988. Neutralization sites of type O1 foot-and-mouth-disease virus defined by monoclonal antibodies and neutralization-escape virus variants. Virology, 162: 21-29.

STRAM Y., CHAI D., FAWZY H.E.D., MOLAD T., MEIRI N., VAN-HAM M., EL-KILANI S., FAHAMY F., MOUSSA A.A.M. & YADIN H. 1995. Molecular epidemiology of foot-and-mouth-disease (FMD) in Israel in 1994 and in other Middle-Eastern countries in years 1992-1994. Archives of Virology, 140: 1791-1797.

STREBEL, K. & BECK, E. 1986. A second protease of foot-and-mouth-disease virus. Journal of Virology, 58 (3): 893-899.

STROHMAIER, K. & ADAM, K.H. 1974. Comparative electrophoretic studies of foot-and-mouth-disease virus proteins. Journal of General Virology, 22: 105-114.

STROHMAIER, K., FRANZE, R. & ADAM, K.H. 1982. Location and characterization of the antigenic portion of the FMDV immunizing protein. Journal of General Virology, 59: 295-306.

SUTMOLLER, P. & GAGGERO, A. 1965. Foot-and-mouth-disease carriers. Veterinary Record, 77: 968-969.

SURYANARAYANA, V., TULASIRAM, P., PRABHUDAS, K.S., MISRA, L.D. & NATATAJAN, C. 1998. The foot-and-mouth-disease type O outbreak of 1992 is not related to vaccine strains (O/R2/75). Virus Genes, 16: 167-172.

SWOFFORD, D.L., OLSEN, G.L., WADDELL, P.J. & HILIS, D.M. Phylogenetic inference. In: Molecular systematics, Edited by Hillis, D.M, Moritz, C. & Mable, B.K. Publishers, Sunderland, Massachusetts, U.S.A., 1996, p: 415.

THOMAS, A.A.M., WOORTMEIJER, R.J., BARTELING, S.J. & MELOEN, R.H. 1988. Antigenic sites on foot-and-mouth-disease virus type A10. Journal of Virology, 62: 2782-2789.

THOMSON G R, 1994. Foot-and-mouth disease. In: Infectious diseases of livestock with special reference to Southern Africa. Edited by Coetzer, J. A. W., Thomson, G.R., & Tustin, R.C. Cape Town, London, New York: Oxfort University Press*: 825-952.*

THOMSON, G.R. 1995. Overview of foot-and-mouth-disease in southern Africa. Review Scientifiques et techniques des Offices International des Epizoties, 14(3): 503-520.

THOMSON GR. 1996. The role of carrier animals in the transmission of foot-and-mouth-disease. O.I.E. Comprehensive Reports on Technical Items Presented to the International Committee or to Regional Commissions, p: 87-103.

THOMSON, G. R., VOSLOO, W., ESTERHUYSEN, J. J. & BENGIS, R. G. 1992. Maintenance of foot-and-mouth-disease viruses in buffalo in Southern Africa. Review Scientifiques & Techniques de l' Office International des Epizooties, 11: 1097-107.

TRAUB, E. & MOHLMANN, H. 1946. Untersuchungen überim monologische Varianten der Typen A und B des Maul- und Klauenseuche- Virus. Berl. Münch, tierärztl. Wschr. 62:1

TSAI, C.P., PAN, C.H., LIU, M.Y., LIN, Y.L., CHEN, C.M., HUANG, T.S., CHENG, I.C., JONG, M.H. & YANG, P.C. 2000. Molecular epidemiological studies on foot-and-mouth-disease type O Taiwan viruses from the 1997 epidemic. Veterinary Microbiology, 74: 207-216.

VALLÉE, H. & CARRÉE, H. 1922. Sur la pluralité du virus aphteux. C.r. hebd. Séanc. Academy in Sciences, Paris 174:1498.

VAN BEKKUM, J.G., FRENKEL, H.S., FREDERIKS, H.H.J. & FRENKEL, S. 1959. Observation on the carrier state of cattle exposed to foot-and-mouth disease virus. Tijdschrift voor Diergeneeskunde 84: 1159-1164.

VAN REGENMORTEL, M.H.V., BISHOP, D.H.L., FAUQUET, C.M., MAYO, M.A., MANILOFF, J. & CALISHER, C.H. 1997. Guidelines to the demarcation of virus species. Archives of Virology, 142 (7): 1505-1518.

VAN RENSBURG, H. G. & NEL, L. H. 1999. Characterization of the structural-protein-coding region of SAT2 type foot-and-mouth disease virus. Virus Genes 19 (3): 229-233.

VAN RENSBURG, H.G., HAYDON, D., JOUBERT, F., BASTOS, A., HEATH, L. & NEL, L. 2001. Genetic heterogeneity in the foot-and-mouth-disease virus leader and 3C proteinases. (In press, Genes).

VOSLOO, W., KNOWLES, N.J. & THOMSON, G.R. 1992. Genetic relationships between southern Africa SAT-2 isolates of foot-and-mouth-disease virus. Epidemiology & Infection, 109: 547-558.

VOSLOO, W., KIRKBRIDE, E., BENGIS, R.G., KEET, D.F. & THOMSON, G.R. 1995. Genome variation in the SAT types of foot-and-mouth disease viruses prevalent in buffalo (Syncerus caffer) in the Kruger National Park and other regions of southern Africa, 1986-1993. Epidemiology & Infection, 114: 203-218.

VOSLOO, W., BASTOS, A.D., KIRKBRIDE, E., ESTERHUYSEN, J.J., JANSE VAN RENSBURG, D., BENGIS, R.G., KEET, D.F. & THOMSON, G.R. 1996. Persistant infection of African buffalo (Syncerus caffer) with SAT- type foot-and-mouth disease viruses: rate of fixation of mutations, antigenic change and interspecies transmission. Journal of General Virology, 77: 1457-1467.

WADELL, G. 1979. Classification of human adenoviruses by SDS-polyacrylamide gel electrophoresis of structural polypeptides. Intervirology, 11: 47-57.

WALDMANN, O. & TRAUTWEIN, K. 1926. Experimentelle Untersuchungen über die Pluralität des Maul- und Klauenseuche- Virus. Berl. Tierärztl. Wschr., 42: 569.

WESTBURY, H.A., DOUGHTY, W.J., FORMAN, A.J., TANGCHAITRONG SUCHINTA & KONGTHON, A.B. 1988. A comparison of enzyme-linked-immunosorbent assay, complement fixation and virus isolation for foot-and-mouth disease diagnosis. Veterinary Microbiology, 17: 21-28.

WILD, T.F., BURROUGHS, J.N. & BROWN, F. 1969. Surface structures of foot-and-mouth-disease virus. Journal of General Virology, 4: 313-320.

WOODBURY, E.L, 1995. A review of the possible mechanisms for the persistence of foot-and-mouth disease virus. Epidemiology & Infection, 114: 1-13.

XIE, Q.C., McCAHON, D., CROWTHER, J.R., BELSHAM, G.J. & McCULLOUGH, K.C. 1987. Neutralization of foot-and-mouth-disease virus can be mediated through any of at least three separate antigenic sites. Journal of General Virology, 68:1637-1647.

YOUNG, E., HEDGER, R.S. & HOWELL, P.G. 1972. Clinical foot-and-mouth-disease in the African buffalo (*Syncerus caffer*). Onderstepoort Journal of Veterinary Research, 39:181-184.