UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Investigating potential factors affecting foot-and-mouth disease virus internalisation

by

Melanie Chitray

Submitted in partial fulfilment of the requirements for the degree of

Master of Science

Faculty of Veterinary Science

Department of Veterinary Tropical Diseases

University of Pretoria

Onderstepoort

Supervisor: Dr. W. Vosloo

Co-supervisor: Dr. F. F. Maree

August 2008

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Investigating potential factors affecting foot-and-mouth disease virus internalisation

by

Melanie Chitray

Supervisor **Dr. W. Vosloo**
ARC-Onderstepoort Veterinary Institute,
Transboundary Animal Diseases programme;
Department of Veterinary Tropical diseases,
University of Pretoria;

Co-supervisor **Dr. F. F. Maree**
ARC-Onderstepoort Veterinary Institute,
Transboundary Animal Diseases programme

Degree **MSc**

ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious disease caused by the FMD virus (FMDV) belonging to the *Picornaviridae* family. The virus affects cloven-hoofed animals and occurs as seven immunologically distinct serotypes where six of the seven serotypes occur in Africa. This fact, as well as the role of wildlife in virus maintenance, makes eradication and control of FMDV in Africa difficult. Thus, it is imperative to attain more information regarding the genetic diversity of FMD viruses prevalent on the African continent to further our knowledge of the virus as well as to enable better control strategies and the development of improved vaccines. Sufficient genetic information regarding the Leader (L) and complete capsid-coding, P1, region of serotype A and O viruses prevalent on the African continent is lacking, although the SAT isolates have been extensively characterised in the past.

In this study the sequence of the L/P1-coding region was successfully determined using a genome-walking approach for a small number of A and O viruses recovered from outbreaks isolated from various species in East and West Africa over the last 33 years. Phylogenetic analysis of the P1 and capsid-coding regions 1A, 1B, 1C and 1D revealed that the African isolates grouped strictly according to serotype and geographic region which indicated the possibility of transboundary spread of the virus within East and West African countries respectively. In contrast, phylogenetic analysis of the non-structural, L^{pro}-coding region revealed a different tree topology compared to the capsid-coding regions for the A and O isolates with sub-grouping according to serotype and geographic regions was less apparent. The relatedness between the serotype A and O L region might be the result of genetic recombination.

The inter and intratypic nucleotide and amino acid variation of the A and O isolates revealed that the most variable capsid-coding region was the externally located VP1 whilst the internally located VP4 capsid protein was the most conserved. The observed variation is in agreement with other studies and reflects the selective pressures on these proteins which either allow or prevent the occurrence of genetic changes for structural constraints or immune escape. Surprisingly, the L protease-encoding region also displayed a high degree of variation. A detailed analysis of the L/P1 amino acid alignment of the A and O isolates revealed that although the extent of variation is high in these regions, the amino acids identified in previous studies as important for FMDV structure (for the capsid-coding regions) and function were found to be conserved, indicating that the virus has adapted itself to elude the host immune response without affecting its vital functional and structural abilities. Additionally, it was observed that the amino acid residues identified as being important for FMDV attaching to the host cell receptors e.g. the RGD amino acid motif of VP1 was highly conserved for all isolates.

To further investigate the FMDV-receptor interaction, RT-PCRs were developed to examine the mRNA expression level of the known FMDV receptors. The β integrins that facilitate FMDV cell entry *i.e.* β1, β3, β6, β8 and heparan sulphate proteoglycans (HSPG) were investigated in susceptible cell lines used for FMDV

vaccine production *i.e.* IB-RS-2 and BHK-21. The RT-PCRs were successfully developed and optimised. The results showed that the mRNA expression levels were variable for all receptor cDNAs tested across 36 passage levels of IB-RS-2 and BHK-21 cells. No distinct differences in virus susceptibility for three FMDV strains with continuous cell passage of IB-RS-2 and BHK-21 cells at passage levels 5, 21 and 36 could be found.

The information gained from this study regarding the viral L and P1 region genetic diversity, and phylogenetic analysis has indeed impacted on our understanding of FMDV African viruses. Additionally, the investigation of the FMDV receptor mRNA expression levels and virus susceptibility on two cell lines with continuous cell passage has proved a vital starting point to determine the possible receptors expressed on the surface of cells used by the vaccine production division at the ARC-OVI-TADP and forms the basis for further investigations of the FMDV receptors on the protein level and the development of a real-time RT-PCR for FMDV receptor expression.

ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to:

Drs. Wilna Vosloo and Francois Maree for allowing me this opportunity to work on this project, for your continued supervision, support, advice and encouragement throughout my project.

Dr. Rahana Mohan Dwarka for your friendship, for your support and always being ever so willing to assist me in time of need.

Ms. Belinda Blignaut for providing the receptor RT-PCR oligonucleotides and for her assistance with the virus susceptibility studies.

Dr. Livio Heath for his assistance with GelTrak.

Ms. Marie Smit at the ARC-Biometry Unit for her assistance with the statistical analysis.

The staff at ARC-OVI-TADP.

My husband, Enver, for the sacrifices you've had to make and for your invaluable support, love, encouragement and patience. You got me through it.

My parents, Anusha and Jayce Moonsamy, for always being there for me and making my dreams come true.

My brothers, Rodney and Vicky Moonsamy and their families who have supported me in so many ways throughout my studies.

I also wish to thank the following organisations for providing financial support:
The Department of Science and Technology (DST) and
The National Research Foundation (NRF)

"I CAN DO ALL THINGS THROUGH CHRIST WHO STRENGTHENS ME"

Philippians 4:13

CONTENTS

CHAPTER 2:

HETEROGENEITY IN THE LEADER (L) AND CAPSID - CODING (P1) REGION OF AFRICAN A AND O FOOT-AND-MOUTH DISEASE VIRUS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

aa	-	Amino acid
a.u.	-	Arbitrary units
ABI	-	Applied Biosystems
ARC	-	Agricultural research council
ATCC	-	American type culture collection
ATV	-	Active trypsin-versene
α	-	Alpha
β	-	Beta
bp	-	Base pairs
BHK-21	-	Baby hamster kidney cells
BTY	-	Bovine thyroid
ca.	-	Approximately
CAV-9	-	Coxsackievirus A9
CIV	-	Cóte d'Ivoire
cDNA	-	Complementary deoxyribonucleic acid
CHO	-	Chinese hamster ovary
CNI	-	Close-neighbour-interchange
CO_2	-	Carbon dioxide
CPE	-	Cytopathic effect
CS	-	Chondroitin sulphate
CV	-	Coefficient of variation
°C	-	Degrees celcius
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribonucleoside triphosphate
DS	-	Dermatan sulphate
EDTA	-	Ethylenediaminetetra-acetic acid
ERI	-	Eritrea
ETH	-	Ethiopia
e.g.	-	*Exempli gratia* (for example)

et al.	-	*et alia* (and others)
etc.	-	*et cetera* (and so forth)
EtBr.	-	Ethidium Bromide
eIF	-	Elongation factor
FACS	-	Fluorescence-activated cell sorting
FCS	-	Fetal calf serum
FMD	-	Foot-and-mouth disease
FMDV	-	Foot-and-mouth disease virus
GAG	-	Glycosaminoglycan
GuSCN	-	Guanidinium thiocyanate
h	-	Hour
HS	-	Heparan sulfate
HSPG	-	Heparan sulfate proteoglycans
HSV-1	-	Herpes simplex virus type 1
IB-RS-2	-	Instituto Biologico renal suino
IC	-	Interstitial cells
IRES	-	Internal ribosomal entry site
ITG	-	Integrin
KEN	-	Kenya
KS	-	Keratin sulphate
L	-	Leader
L^{pro}	-	Leader proteinase
LSD	-	Least significant difference
M	-	Molar
ME	-	Minimum evolution
ML	-	Maximum likelihood
MP	-	Maximum parsimony
mM	-	Millimolar
MAb	-	Monoclonal antibody
ml	-	Millilitre
min	-	Minute
mRNA	-	Messenger ribonucleic acid
MRC-5	-	Human embryonic lung fibroblasts
n/a	-	Not applicable

ng	-	Nanogram
NaOH	-	Sodium hydroxide
NIG	-	Nigeria
NJ	-	Neighbour joining
nt.	-	Nucleotide
OIE	-	Office International des Epizootis
ORF	-	Open reading frame
OVI	-	Onderstepoort veterinary institute
pfu	-	Plaque forming units
pmol	-	Picomole
PCR	-	Polymerase chain reaction
PGs	-	Proteoglycans
PK	-	Porcine kidney
PL	-	Passage level
PTBP	-	Polypyrimidine tract-binding protein
RGD	-	Arginine-glycine-aspartic acid
RNA	-	Ribonucleic acid
RT-PCR	-	Reverse transcription-polymerase chain reaction
s	-	Second
SAT	-	South African territories
SEM	-	Standard error of the means
SEN	-	Senegal
SOM	-	Somalia
SUD	-	Sudan
TAN	-	Tanzania
TADP	-	Transboundary animal diseases programme
TMJ	-	Temporomandibular joint
Tris	-	Tris(hydroxymethyl)-aminomethane
U	-	Units
µl	-	Microliter
µg	-	Microgram
UGA	-	Uganda
µM	-	Micromolar

UTR	-	Untranslated region
UV	-	Ultraviolet
V	-	Volt
v/v	-	Volume per volume
VP	-	Viral protein
w/v	-	Water for volume

CHAPTER 1
LITERATURE REVIEW

1.1. INTRODUCTION

Foot and mouth disease (FMD) is a highly infectious disease of livestock. All domestic and wild cloven-hoofed species such as the bovidae (cattle, zebus, domestic buffaloes, yaks), sheep, goats, swine, all wild ruminants and suidae are susceptible to FMD whereas camelidae (camels, dromedaries, llamas, vicunas) have low susceptibility to the disease (Thomson and Bastos, 2004). The damaging effects of FMD on livestock production make FMD an economically important, debilitating disease. Despite all the information accumulated over the years on the disease, including the availability of vaccines, FMD still occurs extensively around the world and is one of the most transmissible animal diseases (Kitching *et al*., 1998). Typically, when an animal becomes infected, most members of the herd or flock will also become infected and this has led the World Organisation for Animal Health (OIE) to include FMD on its list of highly infectious diseases. Outbreaks of the disease can result in high mortality of young animals due to myocarditis, as well as decreased production of milk and meat. The costs of eradication and control are enormous and trade restrictions could severely affect economies that are reliant on agricultural produce. Areas that are 'free' of FMD have been able to achieve freedom due to the maintenance of quarantine measures, vaccination and/or geographical isolation (Knowles and Samuel, 2003). Also, a non-vaccination, stamping out policy (slaughtering of infected and contact animals) as well as restricted movement of animals have resulted in maintaining areas free of FMD (Donaldson *et al*., 1992).

1.2. FOOT-AND-MOUTH DISEASE VIRUS

The virus that causes FMD is the foot-and-mouth disease virus (FMDV). Loeffler and Frosch in 1897 demonstrated that a filterable infectious agent smaller than bacteria caused the disease and this was the first description of a virus producing an animal disease. FMDV belongs to the family *Picornaviridae* and is one of two members of the *Aphthovirus* genus. Equine rhinitis A virus is the only non-foot-and-

mouth disease virus member of this genus (Pringle *et al*., 1999). Replication of FMDV is prone to error such that viral populations consist of mutant spectra (quasispecies) rather than a defined genomic sequence (Domingo *et al*., 1985; 2002). Most variation occurs within the capsid-coding region of the FMD genome resulting in antigenic variation (reviewed in Grubman and Baxt, 2004). FMDV has a high potential for genetic and antigenic variation; this has led to the virus classification of seven immunologically distinct serotypes. Serotypes O, A and C are the so-called European serotypes (which also occur in South America) where O and A were identified by Vallee and Carre in 1922, whilst type C was identified by Waldmann and Trautwein in 1926 (Brooksby, 1967). The other serotypes include the South African Territories (SAT) types 1, 2 and 3 and type Asia 1 (Brooksby, 1967; Galloway *et al*., 1948).

FMDV has a narrow pH stability range with the optimal pH between 7.2 and 7.6. At pH levels above 9 and below 6, the FMD virus capsid becomes unstable and the ribonucleoprotein is dissociated when FMDV is inactivated by acidification (v Vlijmen *et al.,* 1998). The acid-lability of FMDV is vital for efficient viral uncoating and endocytic entry into the host cell. The virus can survive in lymph nodes and bone marrow at neutral pH, but it is destroyed in muscle when the pH is <6.0 *i.e.* after *rigor mortis*. FMDV survives best in aerosols and microdroplets, where relative humidity exceeds 60% (Alexanderson *et al*., 2003).

1.3 THE VIRION

FMDV is a non-enveloped virus containing a single-stranded ribonucleic acid (RNA) genome of positive polarity. The viral RNA is infectious, thus the virus particle serves to protect the genome while it is outside of cells and also to aid the delivery of the RNA to the cytoplasm of the cell where infection can initiate. The surface of the virus particle is relatively smooth when compared to the other picornaviruses since it lacks peaks and depressions. The virus has a roughly spherical capsid, which results in icosahedral symmetry (figure 1.1). The diameter of the virus is 27 – 28nm and the virion consists of ~70% protein, 30% RNA and a small amount of lipid (Bachrach *et al.*, 1964). The total weight of an infectious virion is ~8.5 x 10^6Da. The viral protein coat consists of 60 copies of each of the four viral capsid proteins *i.e.*

VP1, VP2, VP3 and VP4 (Parry *et al*., 1989). VP1 to VP3 have molecular weights of ~30kDa which form the external surface of the icosahedral shell and constitute the bulk of the viral capsid (Vasquez *et al*., 1979). The small VP4, in conjunction with the amino termini of VP1 and VP2 form an interface between the capsid and the internal RNA genome. After cleavage of the structural precursor, P1, three polypeptides *i.e.* VP0 (precursor of VP4 and VP2 present in immature virions), VP1 and VP3 assemble into asymmetric units or protomers. VP0 is cleaved into VP4 and VP2 in the final stages of viral assembly (Pallansch *et al.*, 1984). This cleavage is considered autocatalytic and is observed during the encapsidation of RNA (Curry *et al*., 1996). Five copies of VP1 are clustered around the 5-fold symmetry whilst VP2 and VP3 are positioned at the two- and three-fold axes of symmetry (Archarya *et al*., 1990). Five protomers associate to form a pentamer and twelve pentamers enclose a newly synthesised RNA molecule to form a virus particle. Assembly of FMDV proceeds from 6S protomers of VP1, VP3 and VP0 via 14S pentamers of five 6S protomers, to form the mature virion (Rossman and Johnson, 1989).

1.3.1 FMD VIRAL STRUCTURAL PROTEINS

VP1 - 3 have similar structural patterns, which consist of an eight-stranded β-barrel composed of two four stranded β-sheets (figure 1.1). The loops that join these strands together with the C-termini of VP1 - 3 are exposed on the surface of the capsid. However, the N-termini are located facing its interior. The antigenicity of the viral particles is dependant on the amino acid residues that are exposed on the surface of the capsid (Mateu, 1995; Usherwood, 1995).

VP1 is comprised of ~213 residues in FMDV serotype O (Ferrer-Orta and Fita, 2004). A disordered protrusion is present on the particle surface, which is situated between the βG and βH-strands of the capsid protein VP1, also known as the G-H loop (at residues ~141-161 in serotype O), which is a prominent feature of the virion. Two VP1 protein regions show the highest antigenicity *i.e.* the G-H loop (the virus major antigenic site) and residues 200-213 of the C-terminal end (a minor viral antigenic site). Within the G-H loop there is a conserved amino acid sequence *i.e.* Arg-Gly-Asp (RGD) at residues ~145 to 147. The RGD sequence has a dual

function as it can recognize integrins that serve as cellular receptors for FMDV and in binding antibody (Domingo *et al.,* 2002; Mason *et al.,* 1994).

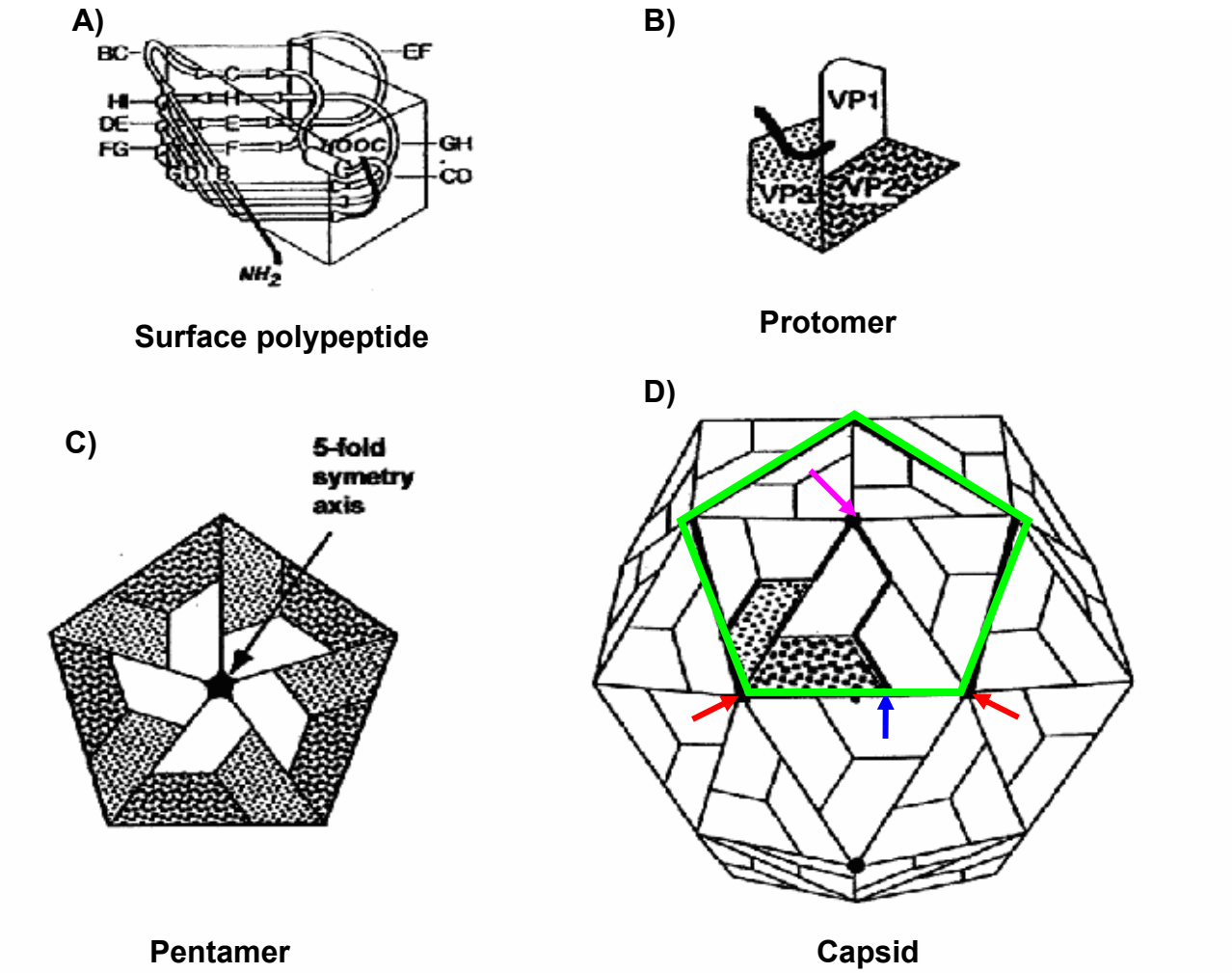


Figure 1.1: Schematic representation of the surface structure of FMDV capsid proteins, the subunits and the virus capsid. A) Schematic, tertiary structure of VP1 (1D), VP2 (1B) and VP3 (1C). The eight β-strands are shown *i.e.* B, I, D, G, C, H, E and F. B) Arrangement of VP1 – 3, the external capsid proteins, in a biological protomer. A black ribbon represents the location of the amino-terminal domain of VP1. C) The arrangement of five protomers making up a pentamer. D) Structure of the capsid, outlining the pentamer (in green) and the protomer (in black). The two-fold, three-fold and five-fold axes are indicated by the blue, red and pink arrows respectively. Taken from Saiz *et al*. (2002).

The flexibility of the loop and its loose connection to the capsid is due to the residues that lack secondary structure, which are found flanking the loop. A long 'arm' is formed with the 17 C-terminal residues of VP1, which is quite unique and is on the

surface of the capsid and extends at a constant radius to the 5-fold axis from the VP1 of one sub-unit over VP3 (Acharya *et al*., 1989).

In FMDV serotype O the VP2 protein is comprised of ~218 residues, whilst the VP3 protein is comprised of ~220 residues, the latter being the most conserved among the structural proteins of picornaviruses (Ferrer-Orta and Fita, 2004). The association of the N-termini VP3 molecules form a five-stranded β-barrel at the 5-fold axis, which 'knits' the protomers into pentamers (Jackson *et al*., 2003; Ferrer-Orta and Fita, 2004). The VP4 protein varies considerably in length when compared to the other capsid proteins and is comprised of approximately 85 residues in serotype O (Ferrer-Orta and Fita, 2004).

1.4 FMDV GENOME

The FMDV genome is a positive sense, single-stranded infectious RNA, where a single copy is contained within each virus particle (Belsham and Bostock, 1988). FMDV viral RNA is over 8, 500 nucleotides (nt) long. A small virus-encoded peptide termed VPg is covalently attached to the 5' terminus and there is a genetically encoded polyriboadenylic acid or poly (A) tail at the 3' end. At the 5' end an untranslated region (UTR) of ~1, 300nt is found followed by a large open reading frame (ORF) that is ~6, 996 nt and a short 3' UTR of ~90nt (figure 1.2).

The large ORF within the RNA encodes a single polypeptide, which is co- and post-translationally cleaved by viral proteases to give rise to the structural and non-structural proteins (Belsham, 1993). There are four distinct regions in the FMDV polyprotein *i.e.* the Leader (L), P1 – 2A, P2 and P3. The polyprotein is processed in a series of proteolytic cleavage steps to give rise to the virion capsid proteins as well as other non-capsid viral proteins. There are two different sites on the RNA at which the initiation of protein synthesis occurs, which are responsible for generating two forms of L^{pro} (see section 1.5).

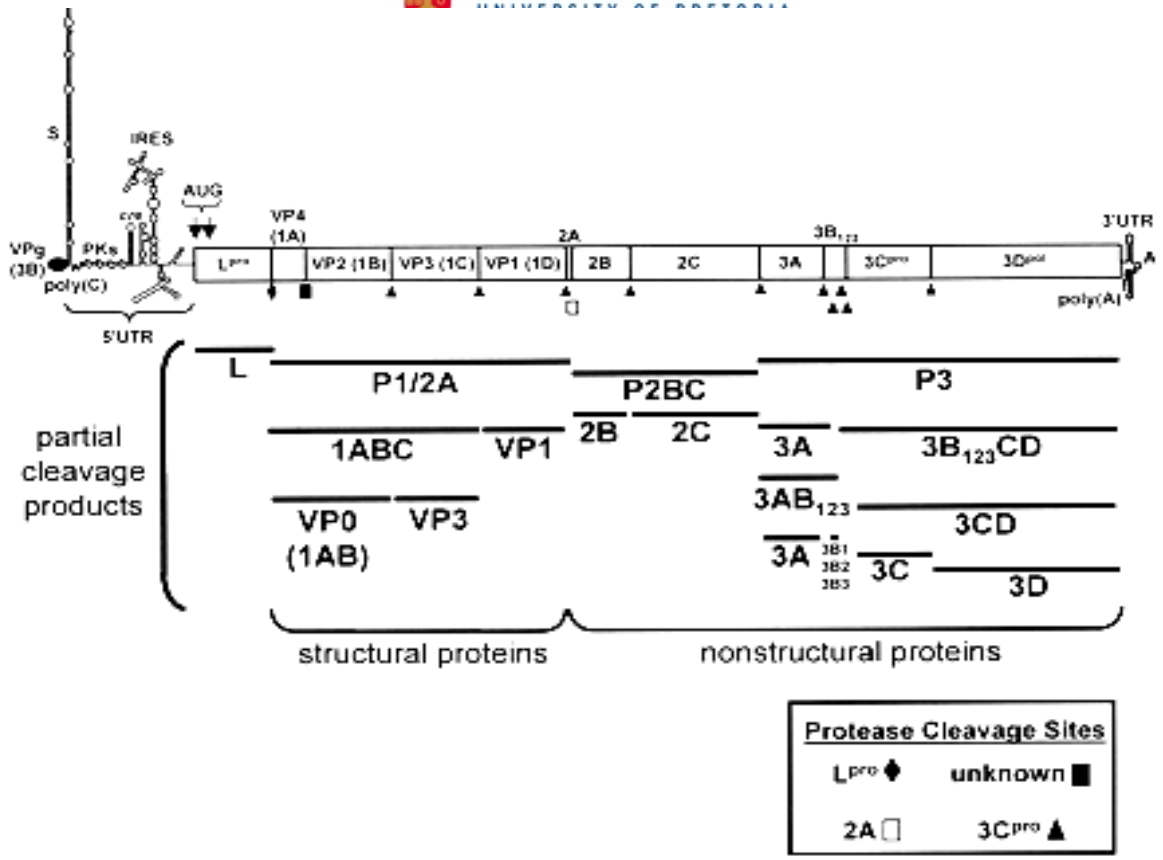


Figure 1.2: Map of the FMDV genome showing the functional elements of the genome as well as the protein cleavage products (taken from Grubman and Baxt, 2004).

The P1 region is the viral capsid precursor and consists of the proteins 1A (VP4), 1B (VP2), 1C (VP3), 1D (VP1) and 2A (short oligopeptide). The L, P2 and P3 are precursors for the non-structural proteins. The non-structural proteins play a role in protein processing and RNA replication (Mason *et al*., 2003a). An S fragment is present within the 5' UTR (~400nt long). The S-fragment is the name given to the smaller of the two RNA fragments, which are generated when viral RNA is treated with oligo(dG) and RNAseH (Rowlands *et al*., 1978). On the 3`side of the S-fragment there is a region containing cytidyl (C) residues (~50 – 250nt), which is referred to as the polyribocytidylic or poly C tract (Mason *et al*., 2003a). Downstream of the poly C tract, a region of ~720nt, consisting of inverted repeats and predicted to form pseudoknots, occurs. The internal ribosomal entry site (IRES) of ~435nt long is highly structured and located several hundred nucleotides away from the uncapped 5` end of the genome (Clarke *et al*., 1985; Domingo *et al*., 2002).

The 3C protease, which is a cysteine protease belonging to the family of chymotrypsin-like proteases and contains a serine protease fold (Seipelt *et al*., 1999;

Birtley *et al*., 2005; Curry *et al*., 2007), is the major FMDV protease, responsible for most of the cleavages within the polyprotein. FMDV 3C has been shown to modify certain cellular proteins. The histone, H3, has been shown to be cleaved by 3C as well as the translation initiation factors eIF4A and eIF4GI, within FMDV infected cells (Falk *et al*., 1990, Belsham *et al*., 2000). The 3D protein encodes for the RNA-dependant RNA polymerase and 3B encodes for three non-identical functional copies of VPg (Grubman and Baxt, 2004).

1.5 VIRUS REPLICATION AND TRANSLATION

FMDV RNA has all the information required for the virus to take control of the host cell machinery. The FMD virus enters the cell by attaching to viral receptors present on the host cell (figure 1.3). The virus is then internalised by endocytosis to form an endosome (Madshus *et al*., 1984a; b). Once the virus is inside an endosome, the low pH environment triggers uncoating of the virus and the viral RNA is translocated across the endosomal membrane into the cytosol (Fry *et al*., 1999; Jackson *et al*., 1997). The virus undergoes eclipse (initial part of infection, no complete virus particles present) and uncoating in a single step where the 146S virion degrades into 12S protein subunits and RNA (Baxt and Morgan, 1985). Thereafter viral replication and translation can occur in the cytoplasm via viral encoded polymerase and proteases. Before any viral processes can occur, the virus must inhibit certain host processes and redirect them into viral multiplication. Inhibition of host cell translation occurs by cleavage of p220 [one component of the cap-binding protein complex and is a 220 000 dalton polypeptide (Etchison *et al*., 1982)], which is induced by the L protein (Belsham, 1993).

Since the FMD RNA is a single-stranded plus sense RNA, it acts as a messenger RNA for the host cell translation machinery and as a template for RNA replication via the viral RNA polymerase so that both viral RNA and virus proteins are produced (Belsham, 1993). These viral proteins are necessary for the formation of the RNA replication complex as well as to produce the capsid proteins that are required to form new virus particles. The synthesis of viral RNA occurs by the virus encoded RNA-dependant RNA polymerase (3D pol). This process requires other viral proteins such as VPg (3B), which acts as the primer for RNA synthesis (Belsham

and Martinez-Salas, 2004). The IRES then forms a secondary structure, which is able to bind ribosomes and deliver them directly to the polyprotein initiation codon in a cap-independent manner. Translation initiation factors involved in cap-dependent mRNA translation, such as the eukaryotic translation initiation factor eIF-4B and the cellular polypyrimidine tract-binding protein (PTBP), have been reported to play a role in stimulation of the internal translation initiation at the picornaviral IRES (Meyer *et al*., 1995). Translation results in a large polyprotein, which is cleaved into the FMDV structural and non-structural proteins.

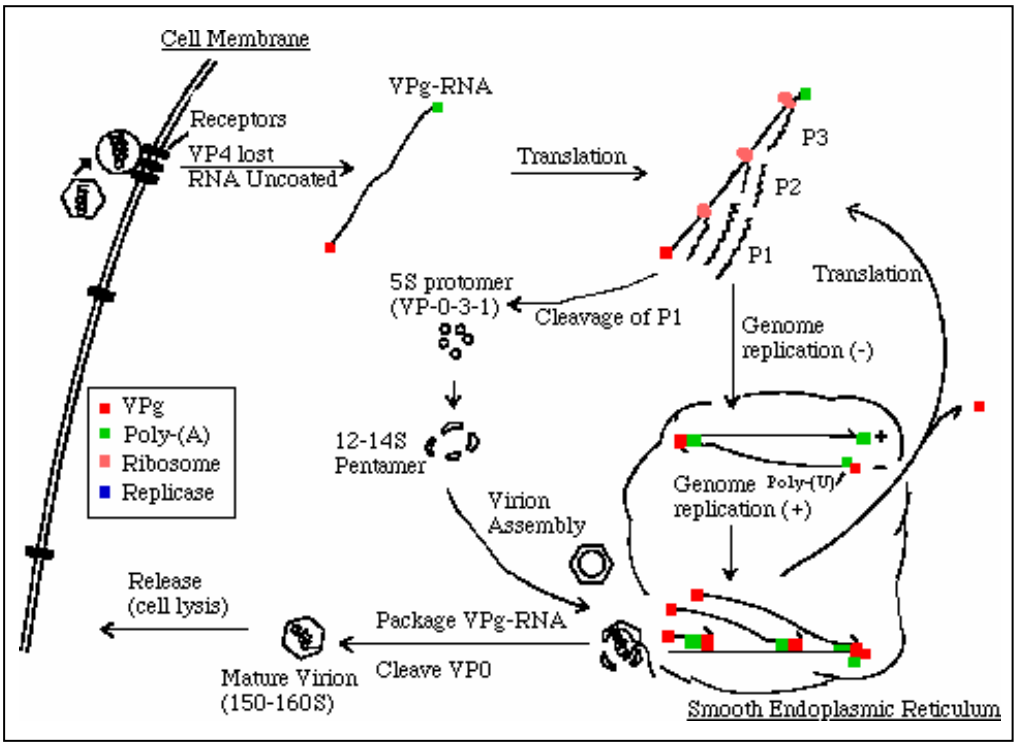


Figure 1.3: Diagram depicting FMDV replication and translation. Following entry of FMDV RNA into the cytoplasm of the cell, the RNA has to be translated to generate viral proteins required for viral RNA replication. Taken from http://www.microbiologybytes.com/virology/Picornaviruses.html

Co-translational cleavage of the L/P1 junction (by L proteinase) occurs. The leader protein is a papain-like cysteine proteinase (L^{pro}), which cleaves between its C terminus and the N terminus of VP4. There are two forms of the L proteinase *i.e.* Lb and the less abundant Lab, where Lb is the truncated version, which arises after the initiation of translation at the second AUG start codon (Belsham, 1993). Lab and Lb can cleave the L/P1 junction and ensure the proteolytic degradation of the cellular

cap-binding protein complex (eIF4G), which results in the shutoff of host translation (Devaney *et al.*, 1988). Thereafter, co-translational cleavage of the 2A/2B junction (by 2A) produces a P1-2A product. Post-translational cleavage of P1 via the 3C viral protease produces 1AB (VP0), 1C (VP3) and 1D (VP1). The assembly of the virus involves the formation of capsid protomers where 5 protomers assemble into pentamers. The packaging of the VPg-RNA to form provirions follows this (Belsham, 1993). The final proteolytic cleavage, which is the final step in virion maturation, occurs at VP0 but only upon encapsidation of the RNA and produces VP2 and VP4 (Harber *et al*., 1991). This is necessary to form infectious virus particles. The virions are then released from the host cell by lysis.

Due to the absence of proofreading by the viral replicase, there are high levels of variation of FMDV types that arise from viral RNA replication, which is error prone (Domingo *et al*., 2002).

1.6 THE CELLULAR RECEPTORS OF FMDV

For FMDV to initiate infection, it needs to attach to the host cell surface molecules on the plasma membrane (Neff *et al*., 1998). For FMDV, two families of cellular receptors have been identified *i.e.* integrins and heparan sulfate proteoglycans (HSPG).

1.6.1 INTEGRIN RECEPTORS:

Integrins can be described as cell surface adhesion receptors and are type I membrane proteins (Hynes, 1992). These cellular receptors contribute to a number of important processes in development and in adult organisms (Hynes, 1992). The integrins consist of α-β heterodimeric glycoproteins (figure 1.4) and there are at least 18α and 8β subunits, which associate to form over 20 different combinations of α-β (Jackson *et al*., 2000a). Each α subunit is noncovalently associated with a β subunit. A wide variety of cells express integrins and most cells express several integrins. Also, individual integrins can bind to more than one ligand and similarly, individual ligands are recognised by more than one integrin (Hynes, 1992). The first binding site to be defined as an integrin recognition site was the RGD sequence, which is

present in fibronectin, vitronectin and other adhesive proteins (Hynes, 1992). The RGD sequence is highly conserved in all FMDV serotypes and is involved in receptor binding (Fox *et al*., 1989; Baxt and Becker, 1990; Knowles and Samuel, 2003). To date, only eight integrins have been found to bind to their ligands by recognizing the RGD motif *viz.* $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_V\beta_8$, $\alpha_5\beta_1$, $\alpha_8\beta_1$ and $\alpha_{IIb}\beta_3$ (Jackson *et al*., 2000b). The specificity of the ligand binding of integrins is determined by the αβ combination while the sequences flanking the RGD motif and its hypervariable characteristics are involved in determining both the binding specificity and affinity of integrins for their ligands (Kunicki, *et al*., 1997; Pfaff, *et al*., 1994). The amino acid changes immediately flanking the G-H loop RGD of FMDV have been shown to influence the ability of several FMDV serotypes to bind and infect cells in culture (Lieppert *et al*., 1997; Mason *et al*., 1994; Mateu *et al*., 1996).

Both integrin subunits are transmembrane glycoproteins, each with a single hydrophobic transmembrane segment. Each subunit is composed of a large N-terminal extracellular domain, a smaller transmembrane region and in most cases a short cytoplasmic tail (50 amino acids or less) (Jackson *et al*., 2004). All β subunits have a four-fold repeat of a cysteine-rich segment (figure 1.4) (Calvete *et al*., 1991).

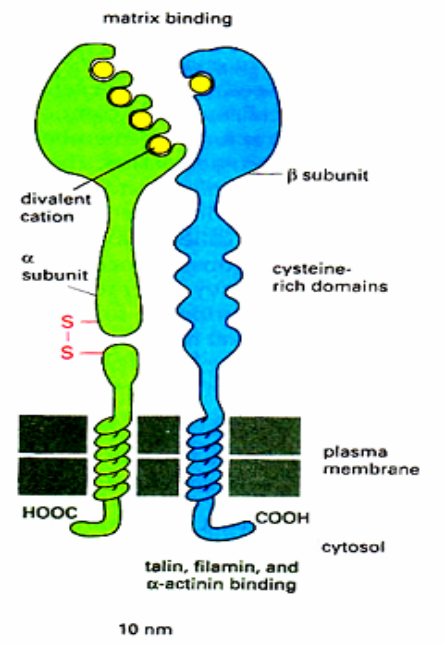


Figure 1.4: - Diagram depicting the integrin α and β subunits. The putative locations of the cysteine-rich repeats of the β subunit as well as the extracellular, transmembrane and the intracellular domains are indicated. Taken from http://personalpages.manchester.ac.uk/staff/j.gough/lectures/the_cell/cell_adhesion/page23.html

1.6.1.1 INTEGRIN ACTIVATION

Integrins exist in at least two conformations *i.e.* active, where it is able to bind the ligand and inactive, where it is unable to bind the ligand. Changing from the inactive to active state occurs via 'inside-out' signaling or integrin activation, which involves two different mechanisms (figure 1.5). The first mechanism is avidity modulation and this is mediated by the clustering of heterodimers at the cell surface. The second mechanism is affinity modulation and this is mediated via conformational changes in the integrin ectodomain (Jackson *et al*., 2002). Integrin activation is energy dependant and cell type specific, which requires both α and β subunit cytoplasmic domains (O'Toole *et al*., 1994). Both activation and inactivation of various integrins can be mediated from within cells. Although the exact mechanisms are not fully elucidated, presumptive evidence indicates that both of the integrin activation processes involve the cytoplasmic domains of the integrins, as well as cellular proteins (Hynes, 1992; O'Toole *et al*., 1994). Integrin inactivation is equally important so that cells do not attach at the wrong times and places e.g. without integrin inactivation, inappropriate adhesion of platelets leads to thrombosis and inappropriate adhesion of leukocytes leads to inflammation.

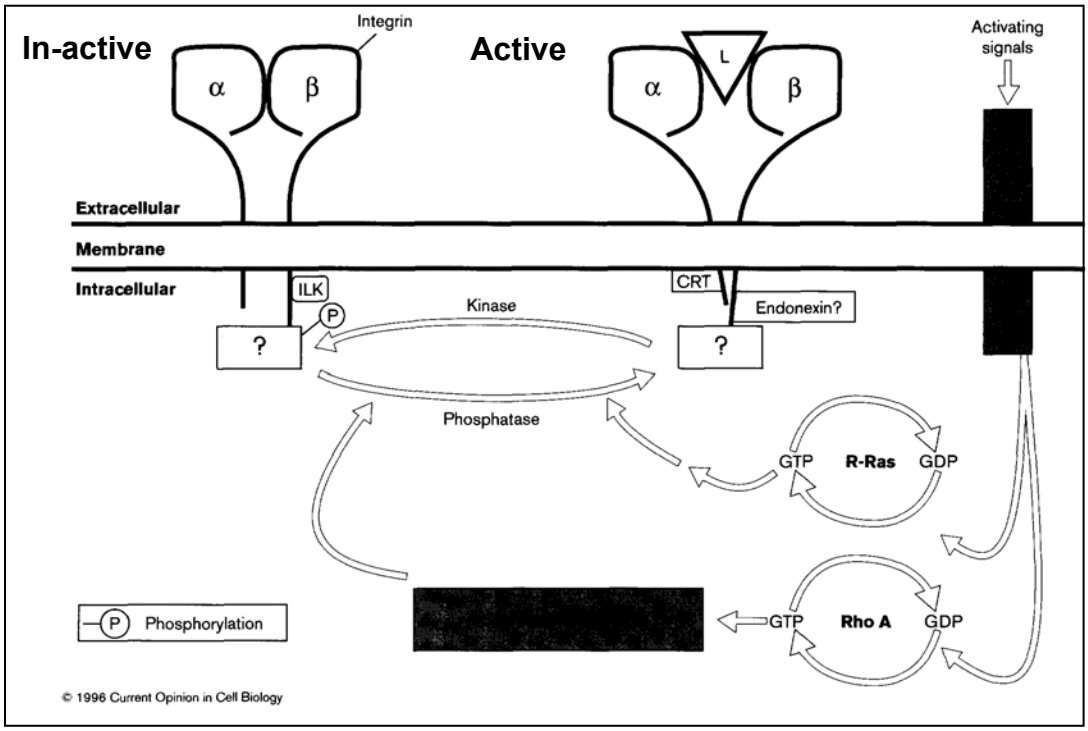


Figure 1.5: A schematic diagram representing currently postulated integrin-mediated 'inside-out' signaling. The activities of both protein (serine/threonine)

kinases and phosphotases are involved in the signaling regulation. Phosphorylation/dephosphorylation of integrin cytoplasmic domains (no evidence yet) or of associated regulatory molecules (? in diagram), may allow for the interaction of other proteins (e.g. endonexin) with the integrin cytoplasmic domains. Calreticulin (CRT) associates only with the 'active' integrin form of α subunits which are involved in the regulation of integrin-affinity states. Integrin-linked kinase (ILK) associates with the β integrin subunits and may play a role in regulating integrin-affinity states. The GTPases, R-ras and Rho A may be implicated in the regulation of integrin-affinity states (taken from Dedhar and Hannigan, 1996).

1.6.1.2 FMDV INTEGRIN BINDING

Baxt *et al*. (1984) were able to deduce that the VP1 protein of FMDV acts as a carrier of major antigenic determinants and also as the viral attachment protein, which reacts with the cellular receptor. Compared to the four viral proteins *i.e.* VP1 – 4, VP1 is the outermost. Treatment of FMDV with trypsin (proteolytic enzyme) results in the loss of FMD infectivity due to the cleavage Arg 144 of VP1, since the non-infectious virus particles are not able to bind to the cellular receptor (Fox *et al*., 1989). This led to the suggestion that Arg144 of the VP1 region (serotype O) must play a role in virus attachment to the cellular receptor. Arg144 of VP1 for serotype A, O and C was demonstrated to be the attachment site of the virus to the host cell receptor (Baxt and Becker, 1990). Due to virus evolution in cell culture, FMD viruses propagated in cell culture can utilise alternative pathways, independent of integrin-binding, to recognise and enter cells.

The RGD sequence is highly conserved but the sequences flanking the RGD are hypervariable, which does not affect receptor binding (Stanway, 1990). This introduces two important points: -

(i) The changes in the flanking RGD sequences result in mutations producing antigenic diversity without altering receptor binding.

(ii) The specificity of binding to different integrins may be affected due to the changes in the flanking RGD sequences.

1.6.1.3 INTEGRINS IDENTIFIED FOR FMDV ATTACHMENT

(i) $\alpha_V\beta_3$

The first to be identified as the primary receptor for FMDV was the integrin $\alpha_V\beta_3$, also referred to as the vitronectin receptor (Berinstein *et al*., 1995). Competition binding studies of coxsackievirus A9 (CAV-9) and FMDV in monkey kidney cells and BHK-21 (baby-hamster kidney) (Chang *et al*., 1989; Roivainen, *et al*., 1994) as well as antibodies blocking the $\alpha_V\beta_3$ integrin (Berinstein *et al*. 1995) suggested that FMDV could use $\alpha_V\beta_3$ as a receptor in certain cells. In addition, studies by Neff *et al*. (1998) concluded that FMDV type A12 replication was dependent on expression of the integrin $\alpha_V\beta_3$. However, in cattle and pigs the expression of $\alpha_V\beta_3$ is in the epithelium of many organs such as the small intestine, kidney, endometrium, etc. Considering that the initial infection sites of FMDV are the lung and pharyngeal areas, it was thought that other integrins must be receptors for FMDV.

(ii) $\alpha_V\beta_6$

The expression of $\alpha_V\beta_6$ has been observed in many sites such as epithelia of the uterus, bladder, respiratory tract and salivary gland (Breuss *et al*., 1995). This led Jackson *et al*. (2000a) to investigate if $\alpha_V\beta_6$ could act as a receptor for FMDV by transfecting the human colon carcinoma cell line to express $\alpha_V\beta_6$ at the cell surface and they found that these cells became susceptible to FMDV infection as a result of transfection with the β_6 subunit. In addition it was shown that virus binding to $\alpha_V\beta_6$ and infection of the β_6-transfected cells is RGD-dependant and established $\alpha_V\beta_6$ as a cellular receptor for FMDV.

Miller *et al*. (2001) demonstrated that the β_6 cytoplasmic domain is not required for virus binding; however, this domain is essential for infection, indicating a critical role in post-attachment events. Furthermore, it was shown that $\alpha_v\beta_6$-mediated infection is dependant on active endosomal acidification, which implies that infection, mediated by $\alpha_v\beta_6$ most likely proceeds through endosomes (Miller *et al*., 2001). Using immunofluorescence confocal microscopy and real-time RT-PCR, Monaghan *et al*. (2005), proved that in epithelia normally targeted by FMDV in cattle, integrin

$\alpha_v\beta_6$ and not $\alpha_v\beta_3$ serves as a major receptor that determines virus tropism and $\alpha_v\beta_6$ is expressed constitutively at levels sufficient to allow initiation of infection.

(iii) $\alpha_V\beta_1$

Jackson and coworkers (2002) investigated whether $\alpha_V\beta_1$ could serve as a receptor for FMDV by transfecting Chinese hamster ovary cells (CHOB2) cells to express two integrins *i.e.* $\alpha_V\beta_1$ and $\alpha_V\beta_5$. Since $\alpha_V\beta_5$ was previously shown to not mediate infection by FMDV, the results proved that FMDV infection occurred with the CHOB2 cells utilising the $\alpha_V\beta_1$ receptor. It was also shown that $\alpha_V\beta_1$ serves as a major receptor for FMDV attachment on CHOB2 cells, since virus binding was inhibited >98% by a function-blocking monoclonal antibody (MAb). Furthermore, RGD-containing peptides were shown to specifically inhibit virus attachment and infection mediated by $\alpha_V\beta_1$ (Jackson *et al*., 2002).

(iv) $\alpha_V\beta_8$

SW480 cells stably transfected with integrin β_8 cDNA resulted in the expression of $\alpha_v\beta_8$ at the cell surface and competition experiments demonstrated that virus binding and infection are inhibited by function-blocking MAbs specific for the $\alpha_v\beta_8$ heterodimer or the α_v chain confirming that the $\alpha_v\beta_8$ receptor was also used by FMD virus for infection (Jackson *et al*., 2004).

1.6.1.4 SUMMARY OF FMDV INTEGRIN RECEPTORS

The efficiency with which a receptor is used is dependant upon the FMD viral serotype and structural difference on the viral surface (Duque *et al*., 2004). Duque and Baxt (2003) showed that FMDV type O viruses used $\alpha_V\beta_6$ with the highest efficiency, following in descending order by $\alpha_V\beta_1$, $\alpha_V\beta_3$ and $\alpha_V\beta_5$. In addition, type A viruses used $\alpha_V\beta_3$ with a high efficiency and $\alpha_V\beta_6$ with either an equal or lesser efficiency. The FMDV type A_{24} Cru used $\alpha_V\beta_1$ with a low efficiency. Overall, it was concluded that all FMDV type A and O viruses used $\alpha_V\beta_5$ with low efficiency and that FMDV type O1 uses $\alpha_V\beta_6$ and $\alpha_V\beta_1$ as receptors *in vitro* (Duque and Baxt, 2003).

1.6.2 HEPARAN SULFATE (HS):

Proteoglycans (PGs) are sulfated macromolecules composed of glycosaminoglycan (GAG) chains covalently bound to a protein core (figure 1.6). PGs are widely distributed in animal tissues and appear to be synthesized by almost all cell types. The GAG components consist of hexosamine [D-glucosamine (GlcN) or D-galactosamine] and either hexuronic acid [D-glucuronic acid (GlcA)] or L-iduronic acid (IdoA), which carry random patterns of sulfation and are negatively charged (Jackson *et al*., 2003). The common GAGs include the galactosaminoglycans *i.e.* chondroitin sulphate (CS) and dermatan sulphate (DS), and the glucosaminoglycans *i.e.* heparan sulfate (HS), heparin and keratin sulphate (KS).

HS is another receptor implicated in FMDV cell adhesion. They are found distributed on cell surfaces as the carbohydrate component of proteoglycans (Fry *et al*., 1999). HS is able to distinguish between different cell types via specific protein-sugar interactions and it is able to do this due to the complex heterogeneous structure that it possesses (Fry *et al*., 1999).

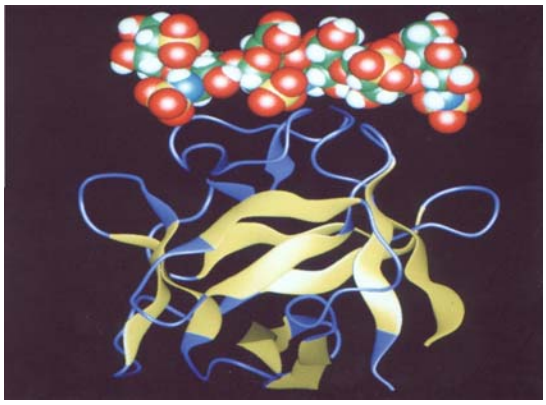


Figure 1.6: - Diagram depicting HSPGs. Taken from www.biosciences.bham.ac.uk

FMDV serotypes O, A, and C have been shown to bind HSPGs (Jackson *et al*., 1996, Baranowski *et al*., 1998). The FMDV type O virus O_1BFS was the first FMD virus identified as being able to use HSPG as cellular receptors (Jackson *et al*., 1996) while certain strains of FMDV O1 use HS as the predominant receptor. Adaptation of FMDV to cell culture can result in the selection of virus variants that have a high affinity for HS and a single amino acid change can alter receptor usage

(Jackson *et al*., 1996; Sa-Carvalho *et al*., 1997). This is true for FMDV type O viruses, which bind to HS in cell culture due to the substitution of an arginine for a histidine at position 56 of VP3 (Fry *et al.*, 1999). The additional positive charge on the surface of the virion could mediate electrostatic binding to HS, which is negatively charged (Sa-Carvalho *et al*., 1997). Sulphated heparan is thought to bind to a shallow depression of the FMDV protomer, making contact with all three major capsid proteins. The integrin recognition site is unaffected by heparan binding and the two receptor binding sites appear to be independent of each other (Baranowski *et al*., 2000).

1.7 PATHOGENESIS OF THE DISEASE

Infection with FMDV can occur in a number of ways e.g. via inhalation, ingestion, iatrogenic or via damaged epithelia and contact with contaminated persons or objects (Clavero *et al*., 2001). All secretions and excretions of the infected animal contain high levels of virus but decline 4 – 5 days after infection due to the presence of FMD-specific antibodies (Bartley *et al*., 2002). The primary route of infection by FMDV is via the upper respiratory tract where the virus shows a strong tropism for epithelial cells and the epithelial cells of the oropharynx & associated lymphoid tissues are the initial viral replication sites (Jackson *et al*., 2000a, Jackson *et al*., 2003). As the FMDV infection progresses in an animal, it disseminates throughout the body with secondary sites of replication in other epithelial tissues. Infection of an animal with FMDV results in a humoral response and the virus specific antibodies protect an animal against re-infection or infection of the same or related virus (Grubman and Baxt, 2004).

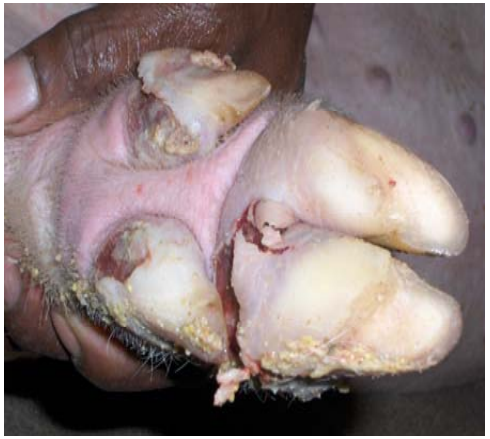
1.8 CLINICAL SIGNS OF FMDV

FMD is characterised by high fever, salivation as well as lameness due to vesicular lesions and erosions on the feet. Vesicular lesions also occur on the snout and teats (figure 1.7). The severity of the disease can vary depending on the infectious dose, the virus strain, the level of immunity and the species. FMDV infects various cloven-hoofed animals with a different degree of severity that can also vary between

animals within the same species. The deterioration of animal body condition can lower animal productivity by 25%.

The incubation period of the disease is ~3 – 14 days and virus excretion from infected animals begins before the development of clinical signs (Thomson, 1994). In cattle, between 2 – 3 days post infection, the virus causes pyrexia, anorexia, shivering and a reduction in milk. Thereafter, due to the vesicles (aphthae) on buccal and nasal mucous membranes between the claws and coronary band, the following occurs: smacking of lips, grinding of teeth, drooling, lameness and stamping or kicking of the feet. After 24 hours the vesicles rupture which result in erosions (Alexandersen *et al*., 2002).

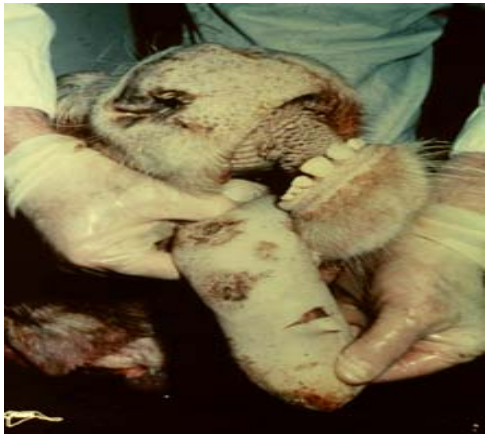
The lesions that occur in infected sheep and goats are not very pronounced. Lesions occur on the dental pad of the sheep, but feet lesions are more common. The initial pyrexic phase may not be noticeable (Saiz *et al*., 2002). Pigs tend to develop severe foot lesions especially if they are kept on hard floors. There is usually a high mortality rate in piglets. Pigs are less susceptible to aerosol infection but excrete more virus in aerosols than do cattle (Quan *et al.,* 2004).



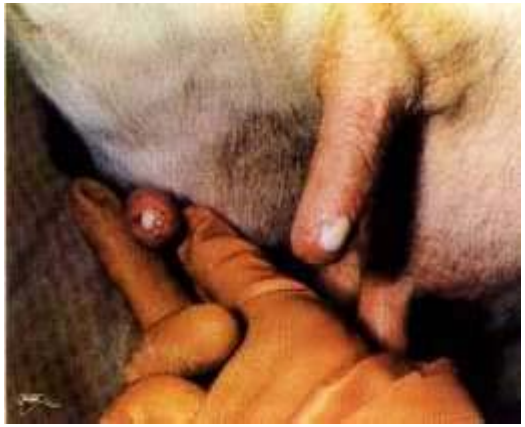
Rupture of a vesicle on the bulb of the heel of a pig

Ulcers on the gum, after rupture of the blister in cattle

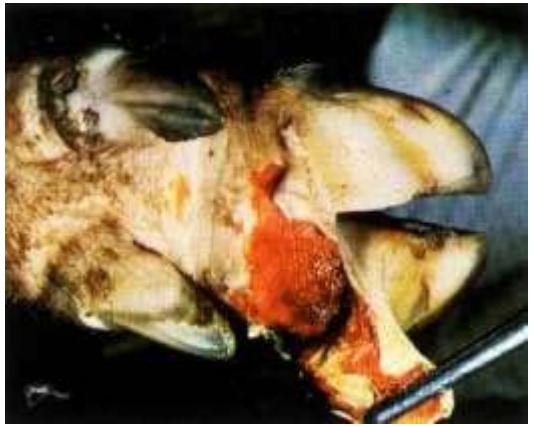
Salivation



Ulcers on the tongue, after rupture of the blister



Vesicles on the teats



Rupture of a vesicle on the bulb of the heel

Figure 1.7: Common clinical signs of FMDV

1.9 EPIDEMIOLOGY OF FMDV

1.9.1 DISTRIBUTION

To date, the FMD virus is still very active in all continents whilst areas that are FMD-free are investing enormous resources to maintain their status by implementing strict control measures especially regarding imports and exports of animals and animal products to prevent the occurrence of FMDV. The least developed countries seem to have a higher occurrence of FMDV than the developed countries where FMDV types A and O are prevalent in South America, Asia, Africa and the Middle East (Kitching, 1998; Samuel and Knowles, 2001b; Knowles and Samuel, 2003). The type C virus had a similar distribution but now seems to be eradicated and the three SAT types are restricted to sub-Saharan Africa (Ferris *et al*., 1992; Pereira, 1977). The Asia-1 serotype was confined to the Far and Middle East and the Balkans but in the year 2000, it occurred in Greece for the first time (Valarcher *et al*., 2005). The widest distribution and prevalence of all the FMDV serotypes is type O (Kitching, 1998; Kitching, 1999).

1.9.2 MOLECULAR EPIDEMIOLOGY

Molecular epidemiology of FMDV has become fundamental in FMD research. Determining the nucleotide sequence of the viral RNA has enabled the characterisation of genetic relationships between strains and provides accurate evidence for epidemiological studies (Beck and Strohmaier, 1987; Samuel *et al*., 1988). Epidemiological studies based on nucleotide sequence comparisons of FMDV show that if the number of differences in the sequences compared are large, then the virus strains are distantly related. The construction of phylogenetic trees results in visualising virus relationships. Using sequence data of the VP1 region, P1 region as well as the complete FMDV genome has become common to determine virus relationships. Molecular epidemiology based on nucleotide sequence determination has also been useful to successfully trace the origin of FMD outbreaks, inter-species transmissions as well as trans-continental introductions (Zadoks and Schukken, 2006).

The epidemiology of FMD in Africa is influenced by two different patterns *i.e.* a cycle involving wildlife and a cycle that is independent of wildlife but maintained within domestic animals (Vosloo *et al*., 2005). Based on nucleotide sequence analysis of a portion of viral genomes obtained from buffalo and domestic animals, in sub-Saharan Africa, the topotypes identified for SAT-1 are 8, which correspond to separate geographic regions whilst 14 and 6 topotypes have been identified for SAT-2 and SAT-3 respectively and for FMDV serotypes O, A and C; 8, 6 and 3 topotypes were identified (Vosloo *et al*., 2005).

1.9.3 FMDV CARRIERS

The control of FMDV can be problematic especially with the existence of FMDV 'carriers'. Carriers can be described as animals in which the virus persists in the pharyngeal region for more than four weeks after FMDV infection but do not show any clinical signs or symptoms of FMDV (persistently infected animals) (Salt, 1993). Additionally, unvaccinated infected animals can become carriers and also, the carrier state is not caused by vaccination but a vaccinated animal must be exposed to FMDV in order to become a carrier (Sutmoller *et al*., 2003). To date, the mechanism by which the carrier state is established and maintained is still unknown. Cattle, sheep and goats have been shown to be carriers for differing periods of time (Panina *et al*., 1989). Pigs were shown to be non-carriers until a recent study by Carrillo *et al*. (2007) who showed that after 26 days of contact with FMDV infected animals; two pigs had become infected with FMDV but did not show any clinical symptoms indicating that the virus had persisted for four weeks, resembling the FMDV carrier state. If successfully repeated this may be the first case described for pigs.

Africa is the only region where FMDV is maintained in wildlife *i.e.* the African buffaloes (*Syncerus caffer*), which can carry the virus for up to 5 years, act as maintenance hosts for all three of the FMDV SAT types (Condy *et al.*, 1985). This makes the eradication of FMDV in sub-Saharan Africa unattainable as these buffaloes have the ability to transmit the virus to other susceptible wild (e.g. impala) and domestic species that they may come into contact with in and around the Kruger National Park and other game reserves. In addition, the three FMDV SAT types

1.9.4 TRANSMISSION OF FMD

The transmission of FMDV usually occurs by direct contact between susceptible animals and animals excreting virus during transport, markets, shows, etc. Indirect transmission occurs via contact with contaminated objects or materials or animal products. Farmers, veterinarians, vehicles etc. can also be a source of indirect contact. Airborne transmission is favoured by humid and cold weather, where virus aerosols can be carried by the wind (Donaldson, 1972). Three factors determine FMD transmission *i.e.* the quantity, duration and means by which the virus is released into the environment, the virus's ability to survive outside the host and the amount of virus required to initiate infection at the primary infection sites of animals that are exposed.

A factor that plays a role in FMDV transmission is the stability of the virus under suitable conditions e.g. cool temperatures, neutral or alkaline conditions or organic material protecting it from the environment (Sellers *et al*., 1971). FMDV can survive outside the body of infected animals and can be preserved by refrigeration and freezing but at temperatures above 50°C it becomes inactivated.

1.9.5 CONTROL AND ERADICATION OF FMDV

Due to the economic importance of FMD, control and ultimate eradication of FMD is the aim of various regions of the world. Some areas have managed to eradicate the disease but in large areas of Asia, Africa and even South America, FMD persists. Countries that are free of FMD are under threat of disease due to the often illegal international movement of animals and animal products.

In endemically infected areas animals are usually vaccinated regularly with an inactivated vaccine to prevent outbreaks (Brown, 1999). FMDV that is incorporated into the vaccine is treated with an inactivant, which destroys viral infectivity but retains viral antigenicity (Brown, 1999). A vaccine can contain one or more strains of any of the FMD types depending on the area where it is used. Vaccines that are inactivated and have been at least partially purified cause the production of antibodies only against the viral structural proteins exposed on the virion surface *i.e.* VP1 – 3 and to a lesser extent to the polymerase. Vaccinated animals do not produce antibodies against the FMDV non-structural proteins whereas non-vaccinated, infected animals do. Thus this serves as a way to differentiate between a vaccinated and infected animal, which is useful to declare a country free from FMD infection (Sobrino *et al*., 2001, Barteling 2002).

In countries usually free of FMD, the first step to control FMDV during an outbreak is to stop movement of livestock in the infected area, which would then be followed by 'stamping-out', a process where all infected and susceptible animals are killed and their carcasses disposed of, followed by thorough cleaning and disinfection of the affected area. However, taking into account that rapid culling may not in all cases guarantee that an outbreak will not develop into an epidemic and to prevent unnecessary mass slaughter of animals, the European Union has put out a new directive to allow vaccination in future to control outbreak situations.

The control of FMDV in sub-Saharan Africa is difficult due to the occurrence of six of the seven FMDV serotypes. Also, the presence of the FMDV carrier, the African buffalo and the lack of controlled borders and animal movement in Africa contribute to the difficulty in achieving FMDV control in Africa. To control the transmission of

FMDV, the buffaloes need to be separated from other susceptible animals by wildlife fences. Also, there needs to be control of animal movement and vaccination of domestic animals adjacent to or in areas were infected buffalos occur (Vosloo *et al*., 2007).

The control and eradication of FMDV is difficult considering that there are seven FMDV serotypes where animals recovered from infection with one FMDV serotype are susceptible to re-infection with any of the other six serotypes (Thomson, 1994). FMDV eradication does not seem attainable in the near future and whilst the disease exists anywhere in the world, any country is at risk.

1.10 AIMS OF THE STUDY

FMDV possesses many advantageous characteristics for a pathogen e.g. it is highly contagious but mostly does not kill its host, infects a variety of cloven hoofed animals, has a wildlife maintenance host, consists of a large number of virus types and subtypes and is widely prevalent. These characteristics make eradication virtually impossible. However, well controlled regular vaccination of domestic animals, strict movement control of both animals and their products as well as limited slaughtering and good sanitation procedures have proven to reduce the occurrence of FMDV and in some countries have led to the eradication of the disease.

In order to develop better vaccines, more needs to be learnt of the different FMDV serotypes and the known FMDV receptors *i.e.* heparan sulfate and the various integrins. The SAT types of FMDV *i.e.* SAT1, SAT2 and SAT3 have been extensively studied in South Africa in the past as it is the most prevalent FMDV serotype in southern Africa. However, there is a dearth of knowledge regarding the A and O serotypes that occur in Africa.

This study will focus on the FMDV A and O serotypes where the capsid-coding, P1, region as well as the leader region of the FMDV A and O types will be sequenced, which will result in providing more information on hypervariable regions, receptor binding sites, possible antigenic sites, etc. Access to this information can be

beneficial in understanding FMD virus entry into the host cell and can also help in the choice of more relevant vaccine strains.

Primary and immortalised cell lines of FMDV natural bovine and porcine hosts play an important role in the diagnosis of live virus. In addition baby hamster kidney cells (BHK-21) are used during vaccine production. The adaptation process of the virus on these cell lines are hampered due to insufficient knowledge regarding the FMDV receptors, as well as the amino acid motifs on the virus capsid that bind to the receptors. Knowledge about the receptor preference of FMDV *in vitro* and the level of expression of these receptors in cultured cell lines that are used for the large-scale production of FMDV becomes more important to predict the outcome of antigen production. This led to the second objective to this study *i.e.* to investigate the level of expression of the known FMDV integrin receptors on BHK-21 and IB-RS-2 (Instituto Biologico renal suino, a pig kidney cell line) cells used in the vaccine production plant at the Agricultural Research Council (ARC), Onderstepoort Veterinary Institute (OVI), Transboundary Animal Diseases programme (TADP), Pretoria.

CHAPTER 2

HETEROGENEITY IN THE LEADER (L) AND CAPSID-CODING (P1) REGION OF AFRICAN A AND O FOOT-AND-MOUTH DISEASE VIRUS

2.1 INTRODUCTION

Despite the vast knowledge of FMDV and the availability of diagnostic tests and vaccines, FMD remains one of the most important diseases of livestock where control seems elusive. The devastation caused by the 2001 UK FMD outbreak is proof of the destruction the disease can cause politically, socially and especially economically (Knowles *et al*., 2001b). FMDV has the ability to infect numerous cloven-hoofed animals with different degrees of severity and this has resulted in new avenues of research into host and viral factors, which control the host range and virulence of individual isolates.

In the case of FMDV, its rapid rate of evolution, which is a result of a polymerase that lacks proof-reading activity (Domingo and Holland, 1997) and the ability of the genome to accommodate considerable amounts of mutations (Holland *et al*., 1982; Domingo and Holland, 1997), have made tracking outbreaks for this pathogen through comparisons of the nucleic acid sequences of the viral genome possible (Samuel & Knowles, 2001a). Also, the nucleotide changes that occur during FMD virus replication can alter the antigenic makeup of the virus, which in turn helps the virus to evade the host immune system (Kitching, 1998). Thus, generating FMDV sequence data becomes more important for a precise evaluation of the degree of relationships among viral strains than serological and physiochemical methods (Beck *et al*., 1987). In addition, it will provide the recognition of antigenic sites that correspond to those already identified and may also aid in the identification of potential antigenic epitopes, such as hypervariable regions.

The FMDV P1 region encodes four capsid proteins *i.e*. VP4, VP2, VP3, and VP1 (in this order) (Acharya *et al*., 1990). FMDV is a non-enveloped virus,

thus the capsid is exposed to the external environment of the virus and is the first part of the virus to attach to host cells. The VP1 protein has been shown to be important for FMDV integrin receptor binding, thus analysis of this region between different FMDV serotypes will enable the differentiation of amino acid residues vital for receptor binding. The capsid also serves to protect the viral RNA, which is in itself infectious, and is important for virus replication and as a result determines virus survival. It is for these reasons that the FMDV capsid proteins have been so extensively studied since some of the key elements to the FMDV survival must be due to some properties contained within the virus capsid proteins. Therefore, sequence analysis of the FMDV capsid-coding regions will enable the identification of possible sequence differences as well as conserved regions in the FMDV functional elements between serotypes which could account for FMDV diversity and may also improve our understanding of receptor-virus interactions.

The leader (L) region is encodes for the FMDV L proteinase (L^{pro}), which is important to cleave itself from the nascent protein as well as cleavage of the host cell translation factor eIF4G (Strebel and Beck, 1986; Gorbalenya *et al*., 1991; Kronovetr *et al*., 2002). As a result, gaining L sequences for the FMDV African A and O serotypes will allow the identification of conserved regions, which may be important for the functioning of the proteinase.

In light of these facts and to also expand the African sequence database of the FMD viruses at the Agricultural Research Council (ARC), Onderstepoort Veterinary Institute (OVI), Transboundary Animal Diseases Programme (TADP), this study aimed to sequence the L and complete P1 region of FMDV serotype A and O isolates that are prevalent on the African continent and to compare these sequences to a small number of FMDV non-African A and O virus L and P1 sequences available on GenBank. Although the P1 region of various SAT and 1D region of African A and O type viruses had been characterised extensively in the past, there is a lack of knowledge about the L and complete capsid coding, P1, region of A and O type viruses prevalent in Africa.

2.2 MATERIALS AND METHODS

2.2.1 Viruses

The viruses chosen for this project belong to different topotypes of FMDV serotypes A and O that had been identified on the African continent. For the FMDV O types, a total of 9 viruses from 4 topotypes, identified by Sahle (2004), were chosen while 8 viruses from 4 topotypes described by Knowles *et al*. (1998) were used for serotype A (table 2.1, viruses marked by *).

Table 2.1: Details of FMDV serotype A and O viruses included in this study

Virus strain	Country of origin	Reference	Genbank Accession No.	Passage History
O/ETH/3/96 *	Ethiopia	This study	EU919240	RS2
O/UGA/5/96 *	Uganda	This study	EU919247	RS2
O/KEN/10/95 *	Kenya	This study	EU919242	RS3
O/SUD/4/80 *	Sudan	This study	EU919239	RS2
O/UGA/17/98 *	Uganda	This study	EU919245	RS2
O/UGA/1/75 *	Uganda	This study	EU919244	RS2
O/UGA/6/76 *	Uganda	This study	EU919246	RS2
O/TAN/3/96 *	Tanzania	This study	EU919241	RS2
O/UGA/7/03 *	Uganda	This study	EU919243	PK1 RS1
O/UKG/35/2001 #	United Kingdom	Carrillo *et al*., 2005	AJ539141	-
OFRA/1/2001 #	France	Nobiron *et al*., 2005	AJ633821	-
O/SAR/19/2000 #	South Africa	Carrillo *et al*., 2005	AJ539140	-
O/TAW/2/99 #	Taiwan	Carrillo *et al*., 2005	AJ539137	-
O/TIBET/CHA/99 #	China	Carrillo *et al*., 2005	AJ539138	-
O/CHINA/1/99 #	China	Zhang *et al*., 2004	AF506822	-
O/SKR/2000 #	South Korea	Carrillo *et al*., 2005	AJ539139	-
O/O10PHIL76	Philippines	Carrillo *et al*., 2005	AY593812	-
O/O10PHIL54	Philippines	Carrillo *et al*., 2005	AY593811	-
O/O1MANISA87	Turkey	Carrillo *et al*., 2005	AY593823	-
O/AKESU/58	China	Li *et al*., 2007	AF511039	-
O/11INDONESIA52	Indonesia	Carillo *et al*., 2005	AY593813	-
O/O1BRUGGE79	Belgium	Carrillo *et al*., 2005	AY593817	-
O/O1ARGENTINA65	Argentina	Carrillo *et al*., 2005	AY593814	-
O/O1CAMPOS94	Argentina	Carrillo *et al*., 2005	AY593819	-

O/O1CAMPOS96	Brazil	Carrillo *et al*., 2005	AY593818	-
O/O1BFS46	United Kingdom	Carrillo *et al*., 2005	AY593816	-
O/O1BFS18	United Kingdom	Carrillo *et al*., 2005	AY593815	-
A/CIV/4/95 *	Ivory Coast	This study	EU919236	BTY1 RS2
A/ERI/3/98 *	Eritrea	This study	EU919238	BTY1 RS2
A/ETH/2/79 *	Ethiopia	This study	EU919233	BTY5 RS2
A/ETH/7/92 *	Ethipoia	This study	EU919235	BTY1 RS2
A/NIG/4/79 *	Nigeria	This study	EU919234	BTY2 BHK4 RS2
A/SEN/10/97 *	Senegal	This study	EU919237	BTY2 RS2
A/SOM/1/78 *	Somalia	This study	EU919231	BTY2 RS2
A/TAN/4/80 *	Tanzania	This study	EU919232	BTY2 RS2
A/A18ZULIA40	Venezuela	Carrillo *et al*., 2005	AY593758	-
A/A1BRAZIL75	Brazil	Carrillo *et al*., 2005	AY593753	-
A/A17AGUARULBOS83	Brazil	Carrillo *et al*., 2005	AY593757	-
A/APHILIPPINES50	Philippines	Carrillo *et al*., 2005	AY593793	-
A/A29PERU37	Peru	Carrillo *et al*., 2005	AY593773	-
A/BRAZIL67	Brazil	Carrillo *et al*., 2005	AY593788	-
A/A24CRUZEIRO71	Brazil	Carrillo *et al*., 2005	AY593768	-
A/A4SPAIN62	Spain	Carrillo *et al*., 2005	AY593778	-
A/A14SPAIN39	Spain	Carrillo *et al*., 2005	AY593754	-
A/A5WESTERWALD73	West Germany	Carrillo *et al*., 2005	AY593781	-
A/A5ALLIER45	France	Carrillo *et al*., 2005	AY593780	-
A/A12VALLE119/20	Great Britain	Carrillo *et al*., 2005	AY593752	-
A/A10HOLLAND82	Holland	Carrillo *et al*., 2005	AY593751	-
A/A1BAYERN41	Germany	Carrillo *et al*., 2005	AY593759	-
A/A3MECKLENBURG81	Germany	Carrillo *et al*., 2005	AY593776	-
A/A2SPAIN7	Spain	Carrillo *et al*., 2005	AY593774	-
A/A4WGERMANY72	West Germany	Carrillo *et al*., 2005	AY593779	-
A/A4WGERMANY42	West Germany	Carrillo *et al*., 2005	AY593777	-

BHK: baby hamster kidney cells; BTY: primary bovine thyroid cells; PK: primary porcine kidney cells; RS: IB-RS-2 porcine kidney cells. The number following the cell line indicates the number of times a virus was passaged in that particular cell line. #: FMDV O viruses belonging to the Pan-Asian lineage; *: African viruses

All the FMDV A & O type viruses were obtained from the Institute for Animal Health, Pirbright Laboratory, Pirbright, United Kingdom. The isolates were either used directly from the stock vials for RNA extraction or alternatively, propagated on IB-RS-2 cells to improve virus titres prior to processing. FMDV L and P1 sequences of A and O non-African isolates available on GenBank

were used for phylogenetic, nucleotide and amino acid analysis (table 2.1), which included viruses belonging to the Pan-Asian lineage (table 2.1, viruses marked by #).

2.2.1.1 Cell culture propagation of viruses

IB-RS-2 (Instituto Biologico renal suino cell line – a pig kidney cell line) cells were used for the propagation of all the African serotype A viruses (table 2.1*). The IB-RS-2 cells were passaged (as described by Freshney, 1994) and maintained in RPMI medium (Sigma, Appendix A1). Cell counts were performed for each cell sample using a haemocytometer as described by Freshney *et al*. (1987) to determine the amount of cells that was needed to seed new T25 flasks. Once the cells reached confluency (after 24hrs), the spent RPMI medium in the T25 flasks were replaced with 5ml RPMI virus growth medium (VGM) at room temperature {VGM = RPMI supplemented with 1% (v/v) FCS (Delta Bioproducts) and 5ml of 100x antibiotic mixture [Invitrogen, Appendix A1]}. Thereafter, 1ml of virus sample was added to the T25 flask and incubated for 1hr at 30°C with gentle agitation. VGM was added and the flask was incubated at 30°C for 48hrs or until CPE was observed. The supernatant was harvested and to this 20% sterile glycerol was added and mixed by gently swirling. One millilitre of this virus sample was transferred into sterile cryotubes and stored at -80°C for further use.

2.2.2 Nucleic acid extraction

Total viral RNA was extracted using a modified guanidinium thiocyanate (GuSCN) / silica method, as described by Boom *et al*. (1990). Briefly, 100µl of virus sample was lysed by addition of 900µl L6 buffer (Appendix A1) containing GuSCN and an aliquot of a 4% silica suspension (Appendix A1) while working in a fume hood. The mixture was vortexed and incubated for 5min at room temperature and the silica-bound nucleic acid was collected by centrifugation using a bench top centrifuge (13 200 x g, 15s). The supernatant was discarded into 10M NaOH and the pellet rinsed with 900µl L2

wash buffer (Appendix A1) followed by vortexing for 15s and centrifugation at 13 200 x g for 15s to pellet the silica. After discarding the supernatant into NaOH (the L2 and L6 buffers contain GuSCN, which is chaotropic and harmful, thus GuSCN-containing waste is discarded in a strong alkaline solution *i.e*. 10M NaOH to reduce its harmful nature), a wash step with 900µl of 70 % ethanol was performed and followed by 900µl of acetone as the final cleaning step, with vortexing and centrifugation as described above. After the acetone was discarded the silica was air-dried at 56ºC for 15min. To elute the nucleic acid from the silica matrix, 30µl 1 × TE buffer (10mMTris-HCl, 2mM EDTA; pH7.4) containing RNasin® ribonuclease inhibitor (40U/µl; Promega) was added followed by vortexing for 10s. The tube was incubated at 56°C for 2min, centrifuged at 13 200 x g for 3min and the supernatant containing the eluted nucleic acids was transferred to a new, sterile 1.5ml microtube and stored at -80ºC for further use.

2.2.3 Complementary DNA (cDNA) synthesis

The viral RNA was reverse-transcribed using a mixture of random hexanucleotides, together with the antisense primer WDA (5'-GAAGGGCCCAGGGTTGGACTC-3'), which anneals at the 2A/2B junction. The reaction mixture contained ~3µg RNA, 0.23µM primer WDA, 4.55µM of a random hexanucleotide mixture (Roche), 1 × AMV-RT buffer (50mM KCl, 10mM $MgCl_2$, 0.5mM spermidine, 10mM DTT), 0.34mM of each dNTP (Roche), 2% (v/v) DMSO and 20U RNasin® (40U/µl; Promega) in a final volume of 17µl. Following denaturation of the RNA by incubating at 70°C for 3min, 40U AMV-Reverse Transcriptase (10U/µl; Promega) was added and the RNA was reverse transcribed at 42ºC for 2h. After incubation, heating at 80ºC for 2min inactivated the enzyme and the resulting cDNA sample was stored at -20ºC.

2.2.4 Amplification of the complete L/P1-coding region using the polymerase chain reaction (PCR)

PCR was used to amplify the L/P1-coding region for sequencing. The cDNA was amplified using two primers for all the FMDV African serotype A and O samples *i.e*. the sense primer, NCR1 (10pmol) (5'-TACCAAGCGACACTCGGGATCT-3'), which anneals in the 5' non-coding region and the antisense primer, WDA (10pmol). The 50μl PCR reaction mixture contained 10μl cDNA, 0.5μM of each of the sense and antisense primers, 1 × long template PCR buffer (Roche; 50mM KCl, 10mM Tris-HCl [pH8.3], 1.5mM $MgCl_2$), 0.2mM of each dNTP (Roche) and 2.5U long template DNA polymerase (Roche). The PCR tube was placed in an ABI thermalcycler and underwent an initial denaturation step at 94°C for 2min, thereafter the reaction was subjected to 35 cycles using the following temperature profile: denaturation for 30s at 94°C, annealing for 30s at 55°C and elongation for 4 min at 68°C and finally a cycle of 7 min at 68°C to allow the synthesis of full-length products for the FMDV serotype A and O samples.

2.2.5 Agarose gel electrophoresis and PCR product purification

The PCR amplicons were separated by agarose gel electrophoresis. A 1% (w/v) agarose gel containing 1 × TAE electrophoresis buffer (40mM Tris-acetate, 2mM EDTA; pH8) was prepared as described by Ausubel *et al*. (1987). The gel contained 0.5μg/ml ethidium bromide (Promega; EtBr), to allow visualization using a UV transilluminator (Uvitec). During gel electrophoresis, the negatively charged DNA molecule migrates towards the positively charged cathode in the presence of an electrical current (100V). A marker DNA, Lambda (λ) DNA/HindIII, was loaded onto the gel.

The PCR fragment of interest was excised from the agarose gel. Thereafter, PCR product purification was performed using the Nucleospin® Extract kit (Macherey-Nagel) and the protocol was followed as per the manufacturer's instructions. Briefly, 200μl of Nucleospin® buffer NT (a chaotropic salt) was

added per 100mg agarose gel to the excised PCR fragment in a sterile 1.5ml microtube followed by 5-10min incubation at 50°C until the agarose gel-PCR fragment was completely dissolved. This mixture was loaded into a NucleoSpin® Extract II column placed in a 2ml NucleoSpin collecting tube followed by 1min centrifugation at 11 000 x g where the DNA binds to the silica membrane in the presence of a chaotropic salt. The silica membrane was washed by adding 600µl of an ethanolic buffer *i.e*. Nucleospin® buffer NT3 and centrifuged for 1min at 11 000 x g (allowing contaminants like salts and soluble macromolecular components to be removed) where after the flow-through was discarded. The silica membrane was dried by centrifuging for 2 min at 11 000 x g to remove buffer NT3 and the flow-through discarded. The NucleoSpin® Extract II column was placed into a sterile 1.5ml microtube and 30µl of prewarmed (70°C for 5 min) NucleoSpin® elution buffer (NE, 5mM Tris-Cl, pH 8.5) added and the tube incubated at room temperature for 1min to increase the yield. The sample was centrifuged for 1min at 11 000 x g. The resulting PCR purified products were stored at -20°C for further use. The purified product (3µl) was subjected to agarose gel electrophoresis (described above) to verify the presence of the expected size amplicon as well as to determine the estimated concentration of the product. In general, higher DNA quantities give higher PCR product signal intensities on the agarose gel.

2.2.6 Nucleotide sequencing of the L/P1-coding region

To sequence the entire L/P1-coding region a 'genome-walking' sequencing approach was followed. A summary of the primers are listed in Appendix A2. The amount of PCR product to use for nucleotide sequencing depends on the length of the PCR product. For a PCR product >2000bp in size, 40-100ng of PCR product was used for sequencing. The purified PCR product was subjected to cycle sequencing (ABI thermalcycler). The ABI PRISM™ BigDye® terminator cycle ready reaction kit v3.1 (Applied Biosystems) was utilised and followed as per the manufacturer's instructions. Briefly, a mixture of DNA template (3µl) at a concentration of ~100ng/µl, 2µl of 1.6pmol of the appropriate primer (Appendix A2), 2µl of 5x sequencing buffer (Applied

Biosystems), 4µl BigDye® sequencing reaction mix (Applied Biosystems) in a total of 20µl was used in the PCR cycle sequencing reaction. The cycle sequencing was performed using the following thermal profile: one cycle of 95°C for 2 min; 25 cycles of 96°C for 10s, 50°C for 5s, 60°C for 4min and one cycle of 60°C for 7min.

The DNA fragment was recovered with alcohol precipitation where 2µl of 3M NaAc (pH 7.4), 50µl 100% EtOH and 2µl 125mM EDTA were added to the completed sequencing reaction mix, briefly vortexed and left at room temperature for 15min. The mixture was centrifuged at 13 200 x g for 20min, the resulting supernatant was discarded and the pellet rinsed with 70µl of 70% EtOH. The sample was mixed and centrifuged at 13 200 x g for 10min and the supernatant discarded. The pellet was dried in a heating block (tube lid open but covered with foil due to sample being sensitive to light) at 90ºC for 1min and resuspended in 12µl of Hi-Di formamide (Applied Biosystems). The sample was vortexed for 5s, centrifuged at 2 000 x g and transferred into injection vials. Sequencing was undertaken at the ARC-TADP using the ABI Prism 310 Genetic Analyser and Inqaba Biotech.

2.2.7 Analysis of nucleotide sequences

The partial L/P1 sequences obtained from the chromatograms for each virus were edited and ambiguous nucleotide bases were resolved and assembled to form a contig using the SEQUENCHER™ 4.7 DNA sequence analysis software (Gene Codes Corporation, Ann Arbor, MI, USA). The nucleotide sequences were translated using BioEdit 5.0.9 DNA sequence analysis software (Hall, 1999) and the complete L/P1 sequences were aligned using ClustalX 1.8.1 (Thompson *et al*., 1997).

Neighbour-joining (NJ), minimum evolution (ME) and maximum parsimony (MP) methods included in the MEGA 4.0 program (Kumar *et al*., 1993) were used to construct the L, 1A, 1B, 1C, 1D gene trees as well as the P1 trees. The phylogeny included sequences of the FMDV African A and O viruses as

well as available/published sequences (table 2.1) of serotype A and O non-African isolates obtained from Genbank. Node reliability was estimated by 1000 bootstrap replications for NJ, ME and MP trees. For the NJ and ME trees, the nucleotide substitution model of Kimura 2-parameter was employed and for the MP and ME trees, close-neighbour-interchange (CNI) with search level 1 was in effect. Maximum likelihood (ML) trees were constructed using PAUP (Swofford, 1998). From Model Test (Posada, D. and Crandall, K.A., 1998), the criterion for the L, 1A, 1B, 1C, 1D and P1 ML trees were selected.

The MEGA 4.0 program (Kumar *et al*., 1993) was utilised to determine the amount of aa and nucleotide sequence variation for the FMDV A and O viruses. Entropy plots were constructed using BioEdit 5.0.9 (Hall, 1999) to determine sites of variation within the P1 alignments of the FMDV African A and O viruses.

2.3 RESULTS

2.3.1 Amplification of the L/P1 region of FMDV African serotypes A and O

The complete L/P1 region was successfully amplified via PCR for all the FMDV O and A viruses included in this study (figures 2.1 and 2.2) and the expected fragment size of *ca.* 2,820bp was obtained, excised, purified from the agarose gel and sequenced.

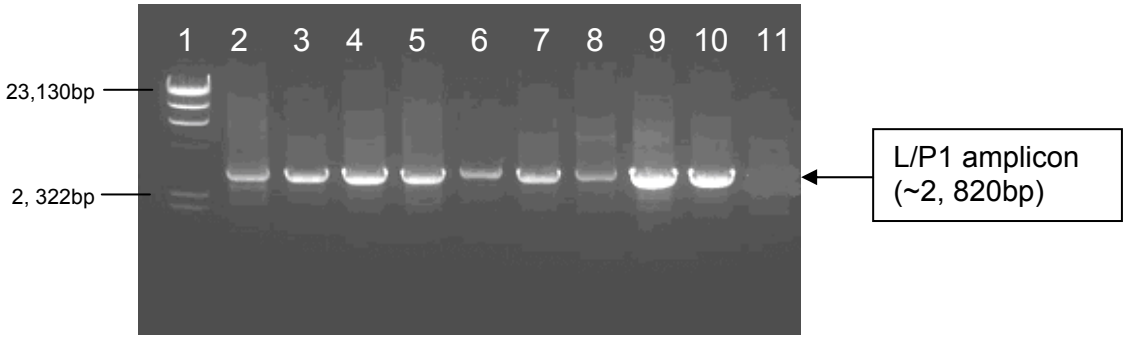


Figure 2.1: Agarose gel electrophoresis analysis of the amplicons obtained by RT-PCR amplification of the L/P1 region of all the FMDV O type virus samples. 1: λ DNA/*Hind*III molecular weight marker, 2: O/ETH3/96, 3: O/UGA/1/75, 4: O/UGA/5/96, 5: O/SUD/4/80, 6: O/TAN/3/96, 7: O/KEN/10/95, 8: O/UGA/6/76, 9: O/UGA/17/98, 10: O/UGA/7/03 and 11: negative control.

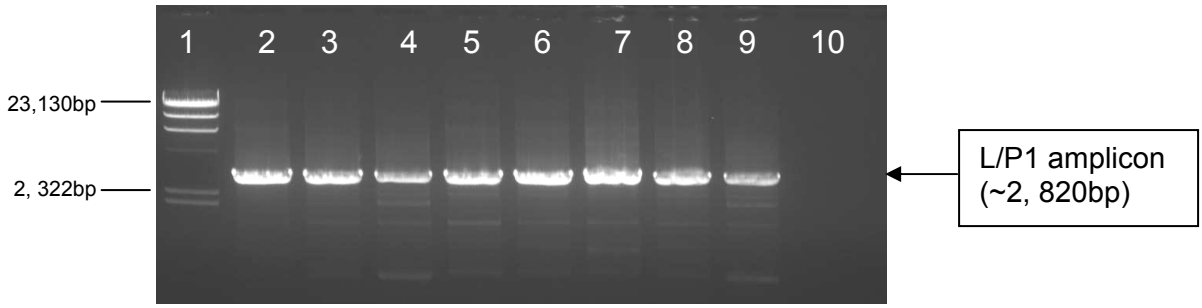


Figure 2.2: Agarose gel electrophoresis analysis of the amplicons obtained by RT-PCR amplification of the L/P1 region of all the FMDV A type virus samples. 1: λ DNA/*Hind*III molecular weight marker, 2: A/NIG/4/79, 3: A/ETH/7/92, 4: A/SOM/1/78, 5: A/TAN/4/80, 6: A/CIV/4/95, 7: A/ERI/3/98, 8: A/ETH/2/79, 9: A/SEN/10/97 and 10: negative control.

2.3.2 Nucleotide sequence and phylogenetic analysis of the L/P1-coding region of FMDV serotypes A and O

Genetic characterisation and variability of the L/P1-coding region was determined for isolates from different countries and regions in West and East Africa, isolated from various species over the last 33 years. The complete L/P1 sequences of serotype A and O viruses were determined following a genome walking approach (figure 2.3) and the primers designed and used are summarised in Appendix A2. At the end of 'genome-walking' sequencing, partial sequences were obtained, which overlap each other to form the complete L/P1 sequence. Sequences generated in this study were submitted to Genbank under the accession numbers indicated in table 2.1.

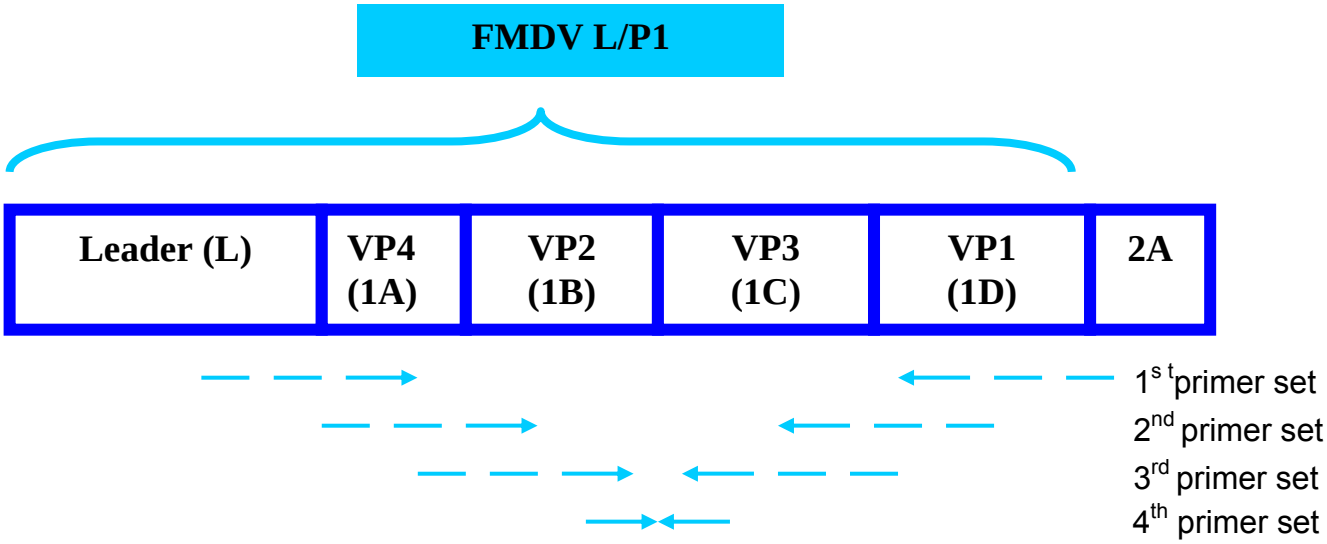


Figure 2.3: Diagram depicting the genome-walking sequencing of the FMDV L/P1 region. For genome walking sequencing, the L/P1 region was sequenced in sections by first utilising the WDA antisense primer annealing to the 2A/2B junction, and NCR1 sense primer annealing to the 5' UTR (Appendix A2). The resultant partial sequences were used to design a second set of sequencing primers (sense and antisense) and the process was repeated until the entire L/P1 region was sequenced.

The complete L/P1 sequences were successfully determined for the isolates chosen in this study and compared to cognate coding regions of other serotype A and O viruses, which included the Pan-Asian O isolates (table 2.1). The Pan-Asian O virus has been identified as spreading from India throughout southern Asia and the Middle East and during the year 2000, this

strain caused outbreaks in Korea, Japan, Russia, Mongolia and South Africa whereas in February 2001, it spread to the United Kingdom, Ireland, France and Netherlands (Mason *et al*., 2003b). The FMDV serotype O Pan-Asian virus isolates have been shown to be closely related (Knowles *et al*., 2001a; Samuel and Knowles, 2001b) thus some of these isolates were included in this study to analyse the relationship between the African and the Pan-Asian O isolates.

The criterion for the construction of all the ML trees are indicated in table 2.2.

Table 2.2: Criterion for the L, 1A, 1B, 1C, 1D and P1 ML trees selected under the Aikake Information Criterion

Phylogenetic Trees	Nucleotide substitution model	Base frequency for A	Base frequency for C	Base frequency for G	Base frequency for T	Proportion of invariable sites (I)	Gamma distribution shape parameter (G)
L	TVM+I+G	0.2180	0.3357	0.2626	0.1838	0.2983	0.9059
1D	GTR+I+G	0.2538	0.3207	0.2577	0.1678	0.3576	1.1849
1B	GTR+I+G	0.2220	0.3162	0.2676	0.1942	0.4977	1.2176
1C	GTR+I+G	0.2060	0.3266	0.2610	0.2064	0.4690	1.1373
1A	TVM+I+G	0.2570	0.3547	0.1949	0.1935	0.5991	1.4364
P1	GTR+I+G	0.2321	0.3268	0.2541	0.1870	0.4536	1.1966

2.3.2.1 Phylogenetic analysis of the FMDV O isolates: P1, 1B, 1C and 1D regions

The combined (including all the A and O isolates) P1, 1B, 1C and 1D NJ trees revealed groupings according to serotype *i.e*. the FMDV O and A viruses clustered separately (figure 2.4; Appendix A4, A5, A3). Additionally, the combined ME and MP trees showed similar groupings to the NJ trees for P1, 1B, 1C and 1D (results not shown). However, the combined P1 ML tree clusters were similar to the NJ except for one isolate, O/SUD/4/80, which did not group with any of the African or non-African A and O isolates but instead formed a separate branch, within the cluster of the non-African A isolates

(results not shown). In addition, the ML combined tree based on the 1D sequences did not provide an accurate reflection of the serotype divisions while the 1B and 1C ML tree groupings were similar to that described for the NJ trees (results not shown).

The phylogenetic analysis of the FMDV P1 (figure 2.4), 1B (Appendix A4), 1C (Appendix A5) and 1D (Appendix A3) regions for the serotype O isolates only, revealed similar groupings for all the NJ trees where separate clusters were observed for the Pan-Asian, the non-African and the African O viruses included in this study, which were supported by high bootstrap values. From the P1 NJ tree (figure 2.4), significant bootstrap support was observed for a group consisting of O/TAN/3/96 (East African) and O/UGA/7/03 (East African) (also observed for 1C, Appendix A5), a group with O/KEN/10/95 (East African) and O/UGA/5/96 (East African) (also observed for 1D, Appendix A3), and lastly for O/UGA/17/98 (East African) and O/UGA/6/76 (East African) (observed for 1B and 1C, Appendix A4 and A5 respectively). Furthermore, these groupings were observed when other phylogenetic methodologies were utilised.

Overall, for all the serotype O isolates for the P1, 1B, 1C, and 1D regions, it was observed that although the African O isolates formed a strong grouping; they did however fall in a strongly supported cluster with all the Pan-Asian isolates as well as one Manisa and two Philippines isolates (figure 2.4).

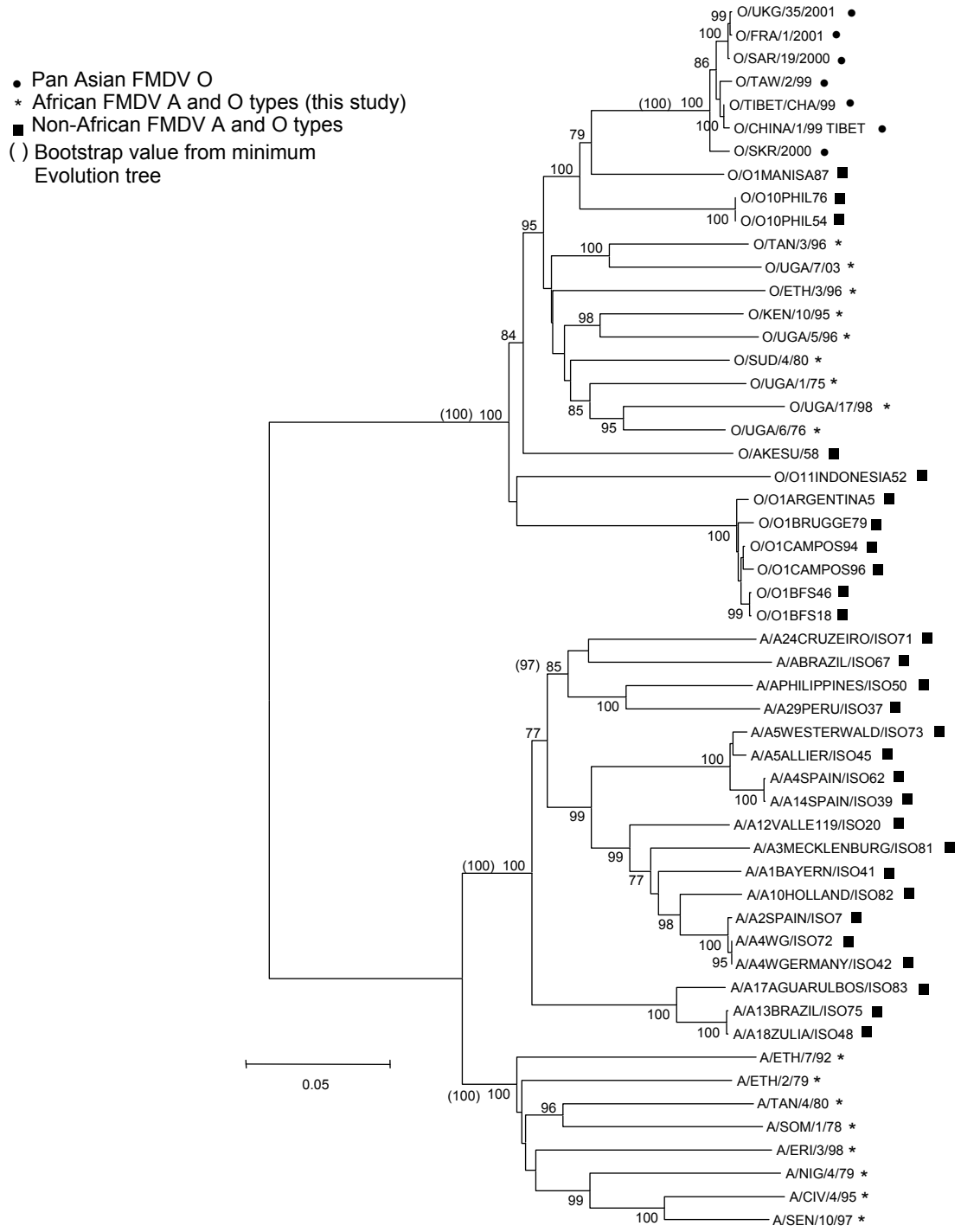


Figure 2.4: Neighbour-joining tree depicting gene relationships for the P1 region of FMDV A and O type viruses. Kimura-2 parameter and bootstrap (1000 replications) were applied.

2.3.2.2 Phylogenetic analysis of the FMDV A isolates: P1, 1B, 1C and 1D regions

The clustering of the African and non-African A viruses for the P1, 1B, 1C and 1D NJ (figure 2.4, Appendix A3 to A5), ME, MP and ML trees (results not shown) were all similar. Two separate clusters were observed for the non-African and African A isolates supported by 100% bootstrap values (figure 2.4, Appendix A3 to A5). Two East African isolates, A/TAN/4/80 and A/SOM/1/78 formed a well supported sub-group for the P1 and 1D NJ trees (figure 2.4, Appendix A3), which was consistently observed for the other phylogenetic methods applied. In addition, there was a consistently strong grouping for 3 west African isolates A/NIG/4/79, A/CIV/4/95 and A/SEN/1/97 for the P1, 1B, 1C and 1D NJ (figure 2.4, Appendix A3 to A5), ME, MP and ML trees (results not shown).

2.3.2.3 Phylogenetic analysis of the FMDV A and O viruses: 1A region

The 1A gene region resulted in phylogenetic groupings that differed from the P1, 1B, 1C and 1D analysis. It was observed for the combined 1A NJ tree that FMDV A and O isolates grouped according to serotype except for three FMDV non-African A strains, isolated from the South American countries Brazil and Venezuela *i.e.* A17/AGUARULBOS/ISO83, A18/ZULIA/ISO48 and A13/BRAZIL/ISO75 which formed a grouping, with significantly high bootstrap support within the cluster of the non-African O viruses (Appendix A6). This grouping was also observed using all other phylogenetic methodologies (results not shown).

In addition, a separate cluster was observed for the Pan-Asian, non-African and African O isolates using all methodologies (Appendix A6, results not shown). For all 1A trees [NJ (Appendix A6), ME, MP and ML (results not shown)] the African A isolates clustered together except for one, A/SOM/1/78, which grouped with a non-African virus, A/BRAZIL/ISO67, but in each case, this grouping was not well supported.

2.3.2.4 Phylogenetic analysis of the FMDV A and O viruses: L region

The phylogenetic trees based on the L coding region had similar tree topologies for all the A and O isolates with all methods. The NJ tree of the L region showed that the viruses did not group strictly according to serotype (figure 2.5), thus it is evident that the trees representing the non-structural protein differ from that of the structural proteins. The non-African A and Pan-Asian O isolates formed separate sub-groupings in the L NJ tree (figure 2.5). Additionally, the African A and O viruses were combined within a separate cluster (figure 2.5). Most of the non-African O isolates formed a grouping except for five *i.e*. O/O10PHIL76, O/O10PHIL54, O/O1MANISA87, O/AKESU/58 and O/O11INDONESIA52, which grouped within the cluster of the African isolates (figure 2.5). Interestingly, for the NJ tree (figure 2.5) one West African, A/NIG/4/79, as well as five East African isolates, A/ETH/2/79, O/UGA/17/98, O/UGA/1/75, O/UGA/6/76 and A/ETH/7/92 formed a grouping with significantly high support. Also, a similarly strong grouping was observed for one East African, A/ERI/3/98 and two West African viruses, A/SEN/10/97 and A/CIV/4/95 using all phylogenetic methodologies. Two sub-groupings observed within the cluster including the African isolates were O/ETH/3/96 and A/SOM/1/78 as well as O/SUD/4/80 and A/TAN/4/80 (figure 2.5) and although these two groupings were not well supported, it is interesting to see that these viruses from different serotypes group together when all phylogenetic methods were applied (results not shown). The other A and O African viruses included in this study, which were the East African serotype O, grouped together for the NJ (figure 2.5), ME, MP and ML trees (results not shown).

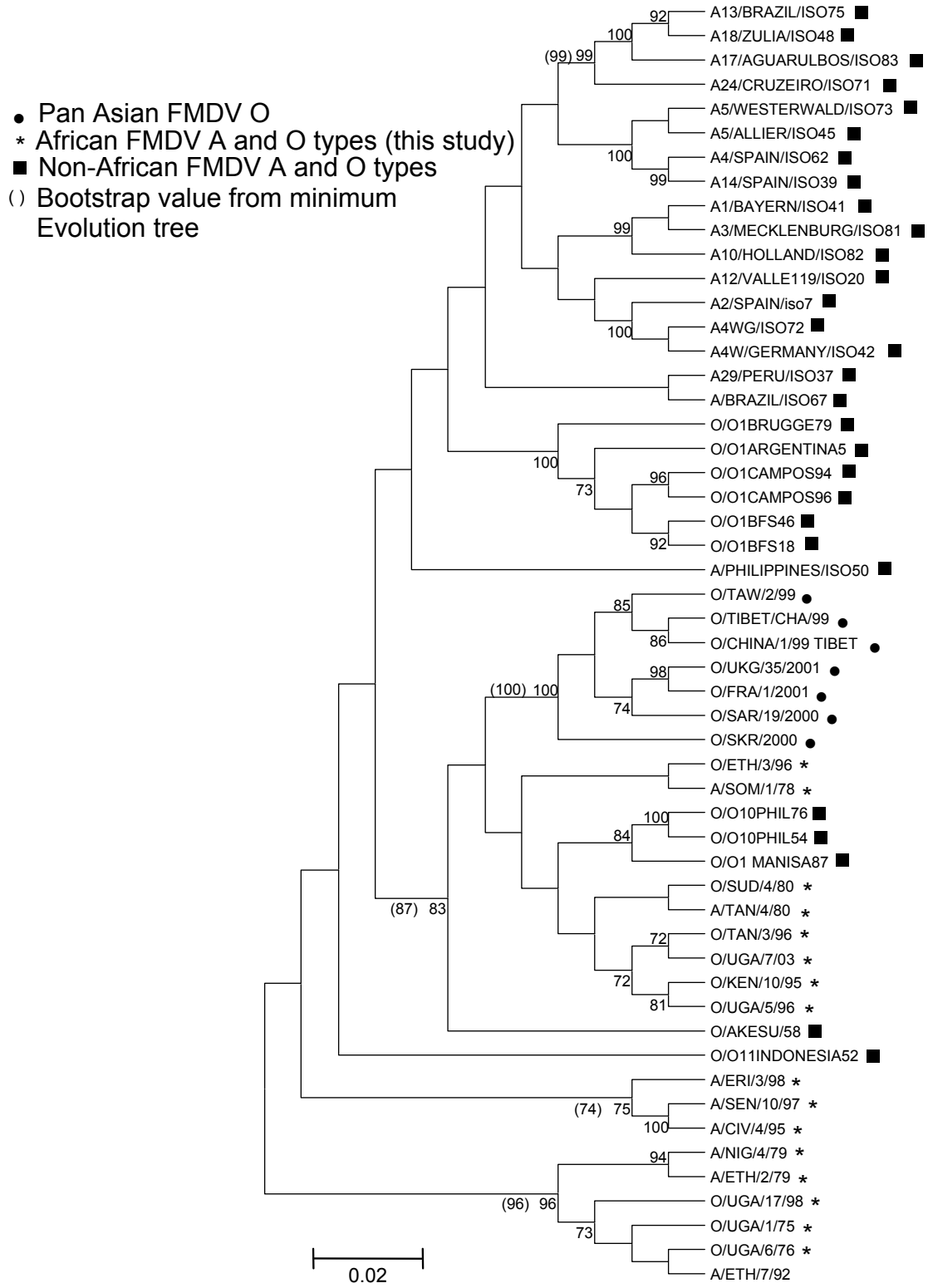


Figure 2.5: Neighbour-joining tree depicting gene relationships for the L region of FMDV A and O type viruses. Kimura-2 parameter and bootstrap (1000 replications) were applied.

2.3.2.5 Nucleotide variation for all the FMDV A and O isolates

For all the FMDV O isolates included in this study, the intratypic genetic variation of the L and P1 regions was calculated to be 43% and 41.7% respectively (table 2.3). The FMDV nucleotide variability for the individual FMDV capsid-coding regions showed that the 1A region (29.69% variability) was most conserved whilst the 1D region (43%) had the highest amount of variable nucleotides (table 2.3). Similar results were observed for the intratypic variation of all the FMDV A isolates where the L and P1 regions were variable (44.34% and 40.36% respectively) with 1D (45.64%) the most variable capsid region whilst 1A (36.7%) was the most conserved (table 2.4). Intratypically, for the O serotypes, 1C and 1D had the least average % transition/transversion (Ts/Tv) rate of 0.43% each (table 2.3) whilst for the A types 1D had the lowest (0.51%) (table 2.4).

Table 2.3: Intratypic variation: FMDV O type isolates nucleotide (nt.) sequence variability

Genome region	No. of nt. positions aligned	No. of variant nt.	% variant nt.	Av. % Ts/Tv rate	% Av pairwise id.	% Max pairwise id.
P1	2222	928	41.7	0.17	11.86	16.08
1A	256	76	29.69	2.05	11.76	27.45
1B	657	227	34.55	0.67	11.42	25.70
1C	667	275	41.23	0.43	11.26	24.53
1D	642	277	43	0.43	15.38	29.54
L	618	267	43	0.52	13.75	22.49

Table 2.4: Intratypic variation: FMDV A type isolates nucleotide (nt.) sequence variability

Genome region	No. of nt. positions aligned	No. of variant nt.	% variant nt.	Av. % Ts/Tv rate	% Av pairwise id.	% Max pairwise id.
P1	2222	897	40.36	1.68	13.19	18.15
1A	256	94	36.7	1.4	11.76	20.00
1B	657	253	38.5	0.57	13.55	18.72
1C	667	254	38.1	0.62	14.86	18.53
1D	642	293	45.64	0.51	13.85	20.46
L	618	274	44.34	0.51	13.75	21.52

The maximum percentage of nucleotide differences observed intratypically in any pairwise alignment comparing the capsid gene regions was highest for 1D

(29.54%) and lowest for 1C (24.53%) for the FMDV O types which was similarly observed for the FMDV A types *i.e*. 1D (20.46%) was the highest and 1C (18.53%) the lowest (tables 2.3 and 2.4).

As expected the intertypic variation (where the two different FMDV serotypes were compared) for all the A and O isolates included in this study was found to be much higher than the intratypic variation with the L and P1 regions demonstrating 51.50% and 52.29% variant nucleotide sequences respectively (table 2.5). The intertypic nucleotide variation for the four capsid-coding regions were also high and as seen with the intratypic variation, the 1A region (37.89% variability) was most conserved whilst 1D had the highest variability (58.70%) (table 2.5). Additionally, the intertypic maximum number of pairwise differences was higher than that observed for the intratypic variation where the L and P1 was 21.84% and 27.54% correspondingly whilst 1D (35.38%) > 1C (32.89%) > 1B (32.72%) > 1A (28.63%) (table 2.5). Comparing the capsid gene regions, the average %Ts/Tv rate was highest for the most conserved capsid region, 1A (1.045%) and lowest for the most variable capsid region, 1D (0.283%).

Table 2.5: Intertypic variation: FMDV non-African and African A and O types nt. sequence variability

Genome region	No. of nt. positions aligned	No. of variant nt.	% variant nt.	Av. %Ts/Tv rate	% Av pairwise id.	% Max pairwise id.
P1	2222	1162	52.29	0.104	19.43	27.54
1A	256	97	37.89	1.045	13.29	28.63
1B	657	309	47.03	0.314	20.06	32.72
1C	667	318	47.68	0.345	18.61	32.89
1D	642	377	58.70	0.283	22.05	35.38
L	618	318	51.50	0.470	14.21	21.84

2.3.3 Amino acid (aa) variation for the FMDV A and O isolates

The intratypic aa variation was higher for the FMDV A types (table 2.7) when compared to the FMDV O isolates (table 2.6) where it was observed that the VP4 region was the least variable and VP1 the most variable region in both cases *i.e*. for the FMDV O types: VP1 (29.90%) > VP3 (16.20%) > VP2

(11.47%) > VP4 (4.70%) whilst for the FMDV A types: VP1 (42.99%) > VP3 (28.40%) > VP2 (24.31%) > VP4 (15.30%) (tables 2.6 and 2.7). In addition, the L and P1 also displayed a high number of variant aa where for the FMDV O and A types whereas for the A types, it was higher (tables 2.6 and 2.7).

Table 2.6: Intratypic variation: FMDV O non-African and African aa sequence variability

Genome region	No. of aa positions aligned	No. of variant aa	% variant aa
P1	739	128	17.20
VP4	85	4	4.70
VP2	218	25	11.47
VP3	222	36	16.20
VP1	214	64	29.90
L	206	61	29.61

Table 2.7: Intratypic variation: FMDV A non-African and African aa sequence variability

Genome region	No. of aa positions aligned	No. of variant aa	% variant aa
P1	739	224	30.30
VP4	85	13	15.30
VP2	218	53	24.31
VP3	222	63	28.40
VP1	214	92	42.99
L	206	96	46.60

Similarly, as observed for the nucleotide intertypic variation, the aa intertypic variation was higher than the aa intratypic variation where VP4 (15.29%) remained the most conserved with VP1 (53.46%) the most variable FMDV capsid region whilst VP3 (39.91%) > VP2 (33.03%) (table 2.8). The intertypic variation of the L and complete P1 also displayed high aa variation (table 2.8).

Table 2.8: Intertypic variation: FMDV non-African and African A and O types aa sequence variability

Genome region	No. of aa positions aligned	No. of variant aa	% variant aa
P1	743	296	39.84
VP4	85	13	15.29
VP2	218	72	33.03
VP3	223	89	39.91
VP1	217	116	53.46
L	206	111	53.88

2.3.3.1 FMDV A and O serotype polyprotein cleavage site variability

The variability observed at the protease cleavage sites between L/VP4, VP4/VP2, VP2/VP3 and VP3/VP1 for the O and A viruses included in this study was investigated by alignment to O/O1MANISA87 (for the O isolates) and A/A24CRUZEIRO/ISO71 (for the A isolates) (table 2.9). The L proteinase (L^{pro}) is responsible for the L/VP4 cleavage (Strebel and Beck, 1986; Medina *et al*., 1993) whilst the 3C proteinase ($3C^{pro}$) cleaves at the VP2/VP3 and VP3/VP1 junctions (Ryan and Flint, 1997). In addition, the VP4/VP2 cleavage occurs autocatalytically, simultaneously with the FMDV viral RNA encapsidation (Harber *et al*., 1991). A comparison of the L/P1 cleavage sequence at the C-terminus of the L protein and N-terminus of the VP4 protein of the FMDV A and O types included in this study revealed a sequence of (K/R)(K/R)L(K/R)*GAGQ (* indicates cleaved peptide bond) for the FMDV O types and for the A types, the sequence was (Q/R/K/W)(K/R)LK*GAGQ (table 2.9). At the VP4/VP2 junction, the ALLA*DKKT sequence was conserved for all the A and O isolates (table 2.9). The most common sequence observed at the VP2/VP3 junction for all isolates was PSKE*GIFP with the exception of only one O isolate, O/AKESU/58 as well as 7 of the 8 African A viruses which exhibited the sequence PSKE*GIVP (table 2.9). The VP3/VP1 junction site sequences displayed a great deal of variation *i.e*. the O isolates had the sequence AR(T/V/A/R/S)(Q/E)*TTS(A/T/P/L) whilst for the A isolates, the sequence PR(Q/T/S/P/A)Q*TT(A/T/S)(T/V/A) was observed.

Table 2.9: Protein junction cleavage site alignment of the FMDV A and O isolates

FMDV-O types	L/VP4 junction	VP4/VP2 junction	VP2/VP3 junction	VP3/VP1 junction
O/O1MANISA87	KRLK*GAGQ	ALLA*DKKT	PSKE*GIFP	ARTQ*TTSA
O/CHINA/1/99_TIBET	...R*....	....*....	....*....	....*...T
O/TAW/2/99	...R*....	....*....	....*....	....*...T
O/UKG/35/2001	...R*....	....*....	....*....	....*....
O/SKR/2000	...R*....	....*....	....*....	....*...T
O/TIBET/CHA/99	...R*....	....*....	....*....	....*...T
O/SAR/19/2000	...R*....	....*....	....*....	....*...T
O/O10PHIL76	....*....	....*....	....*....	....*...T
O/O10PHIL54	....*....	....*....	....*....	....*...T
O/O11INDONESIA52	.K..*....	....*....	....*....	....*...T
O/O1BRUGGE79	RK..*....	....*....	....*....	..VE*....
O/AKESU/58	K...*....	....*....	....*..V.	....*...P
O/O1ARGENTINA5	RK..*....	....*....	....*....	..AE*....

O/O1BFS18	RK..*....	....*....	....*....	..AE*....
O/O1BFS46	RK..*....	....*....	....*....	..AE*....
O/O1CAMPOS94	RK..*....	....*....	....*....	..AE*....
O/O1CAMPOS96	RK..*....	....*....	....*....	..AE*....
O/FRA/1/2001	...R*....	....*....	....*....	....*....
O/SUD/4/80	....*....	....*....	....*....	..R.*...P
O/ETH/3/96	....*....	....*....	....*....	....*...P
O/TAN/3/96	R...*....	....*....	....*....	....*...P
O/KEN/10/95	....*....	....*....	....*....	..S.*...P
O/UGA/7/03	R...*....	....*....	....*....	....*...P
O/UGA/1/75	RK..*....	....*....	....*....	..R.*...P
O/UGA/17/98	RK..*....	....*....	....*....	..R.*...P
O/UGA/6/76	RK.R*....	....*....	....*....	..R.*...P
O/UGA/5/96	....*....	....*....	....*....	..S.*...L
FMDV-A types	**L/VP4 junction**	**VP4/VP2 junction**	**VP2/VP3 junction**	**VP3/VP1 junction**
A/A24CRUZEIRO/ISO71	QKLK*GAGQ	ALLA*DKKT	PSKE*GIFP	PRQQ*TTAT
A/A29PERU/ISO37	R...*....	....*....	....*....	....*....
A/ABRAZIL/ISO67	R...*....	....*....	....*....	..T.*....
A/A4WG/ISO72	R...*....	....*....	....*....	..T.*....
A/A2SPAIN/ISO7	R...*....	....*....	....*....	..T.*....
A/A4WGERMANY/ISO42	R...*....	....*....	....*....	..T.*....
A/A5WESTERWALD/ISO73	R...*....	....*....	....*....	....*...V
A/A14SPAIN/ISO39	R...*....	....*....	....*....	....*...V
A/A12VALLE119/ISO20	R...*....	....*....	....*....	..S.*....
A/A3MECKLENBURG/ISO81	R...*....	....*....	....*....	..P.*...A
A/A4SPAIN/ISO62	R...*....	....*....	....*....	....*...V
A/A5ALLIER/ISO45	R...*....	....*....	....*....	....*...V
A/A1BAYERN/ISO41	R...*....	....*....	....*....	..P.*..TA
A/A10HOLLAND/ISO82	R...*....	....*....	....*....	..P.*....
A/A17AGUARULBOS/ISO83	....*....	....*....	....*....	..S.*....
A/APHILIPPINES/ISO50	W...*....	....*....	....*....	....*....
A/A13BRAZIL/ISO75	....*....	....*....	....*....	..S.*..S.
A/A18ZULIA/ISO48	....*....	....*....	....*....	..S.*..S.
A/CIV/4/95	R...*....	....*....	....*..V.	..P.*..T.
A/ETH/7/92	R...*....	....*....	....*....	....*....
A/ERI/3/98	KR..*....	....*....	....*..V.	..A.*....
A/ETH/2/79	RR..*....	....*....	....*..V.	..T.*....
A/SOM/1/78	R...*....	....*....	....*..V.	..A.*....
A/SEN/10/97	K...*....	....*....	....*..V.	..P.*..T.
A/TAN/4/80	KR..*....	....*....	....*..V.	..A.*....
A/NIG/4/79	RR..*....	....*....	....*..V.	..A.*....

* indicates the protein cleavage point . indicates similarity with the reference sequence

2.3.3.2 Amino acid alignment of the L region

The L region displayed marked variation in both the Lab and Lb regions (Appendix A7) consistent with the high intertypic and intratypic amino acid variation observed in section 2.3.3. Despite this variation, the aa residues identified as being critical for the L function was highly conserved *i.e*. the residues C53, H153 and D168 required for L^{pro} catalytic activity; the E81 residue required for L^{pro} autocatalysis as well as two H residues (H114 and

H143) important for the translation initiation factor, eIF4G, cleavage (Appendix A7; aa numbered according to Appendix A7; Gorbalenya *et al*., 1991; Kronovetr *et al*., 2002; Piccone *et al*., 1995a; Piccone *et al*., 1995b).

2.3.3.3. FMDV P1 A and O entropy plots and antigenic variation

To have an indication of the regions of variation within the P1 region of the African FMDV A and O viruses, entropy plots were constructed for the complete P1 aa alignments (figure 2.6 a, b). Regions of variability are indicated by the vertical black bars on the entropy plots whilst regions that are conserved are indicated by the clear areas in the plots. In addition, entropy values of >1 were taken as regions that are prone to variation as greater entropy corresponds to greater variability at the aa residue position (Caspersen *et al*., 2002). Previously identified antigenic sites are indicated in figures 2.6 a and b in the orange blocks above the entropy plots.

For serotype O, the antigenic site 1 involves both the VP1 G-H loop, containing the receptor binding RGD sequence (site 1a), *ca*. aa 663 to 685 and the C-terminus of VP1 (site 1b), *ca*. aa 733 (aa residues numbered according to alignment in Appendix A8; Kitson *et al*., 1990). Site 2 of serotype O includes residues of the B-C and E-F loops of VP2 *i.e*. aa residues *ca*. 156-159, 161-163, 217, 220 whilst site 3 involves residues of the VP1 B-C loop *i.e. ca.* aa 569-571 and 574 (according to Appendix A8; Kitson *et al*., 1990). The B-B 'knob' of VP3 includes residues identified as site 4 for serotype O *i.e*. *ca.* aa 360, 362 (according to Appendix A8; Kitson *et al*., 1990). A fifth serotype O site corresponding to the VP1 G-H loop site 1a was identified by Crowther *et al*. (1993b) at *ca*. aa 675 (aa according to Appendix A8).

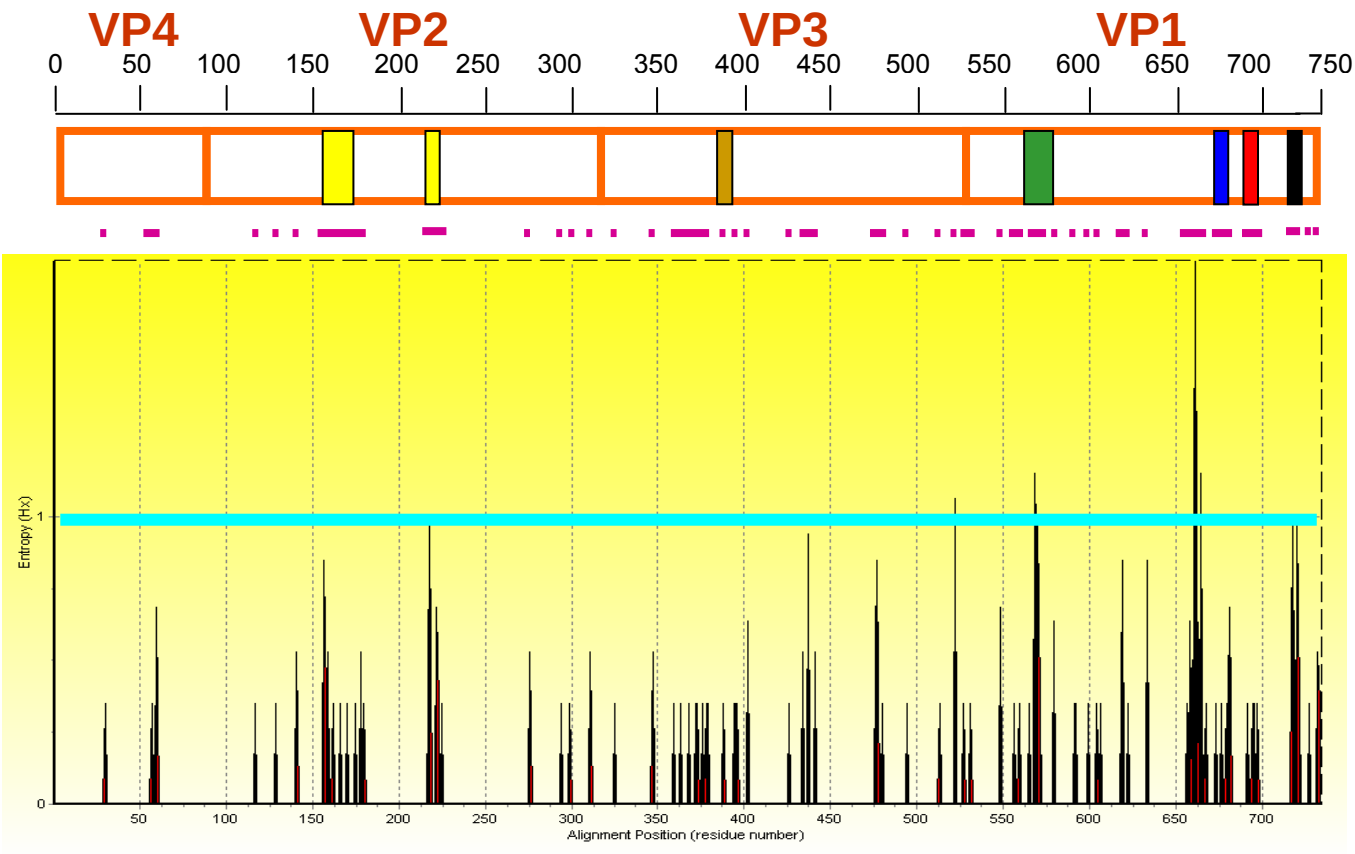
The results from the entropy plots for the African O isolates indicated that all these previously identified antigenic sites for the FMDV O serotypes corresponded to regions of entropy observed (figure 2.6 a). However, other regions of variability were also observed (indicated by the horizontal purple bars above the entropy plots, figure 2.6 a) and those regions of variation with

an entropy >1 are aa residues VP3: *ca*. 525 and VP1: *ca*. 660 for serotype O (figure 2.6 a).

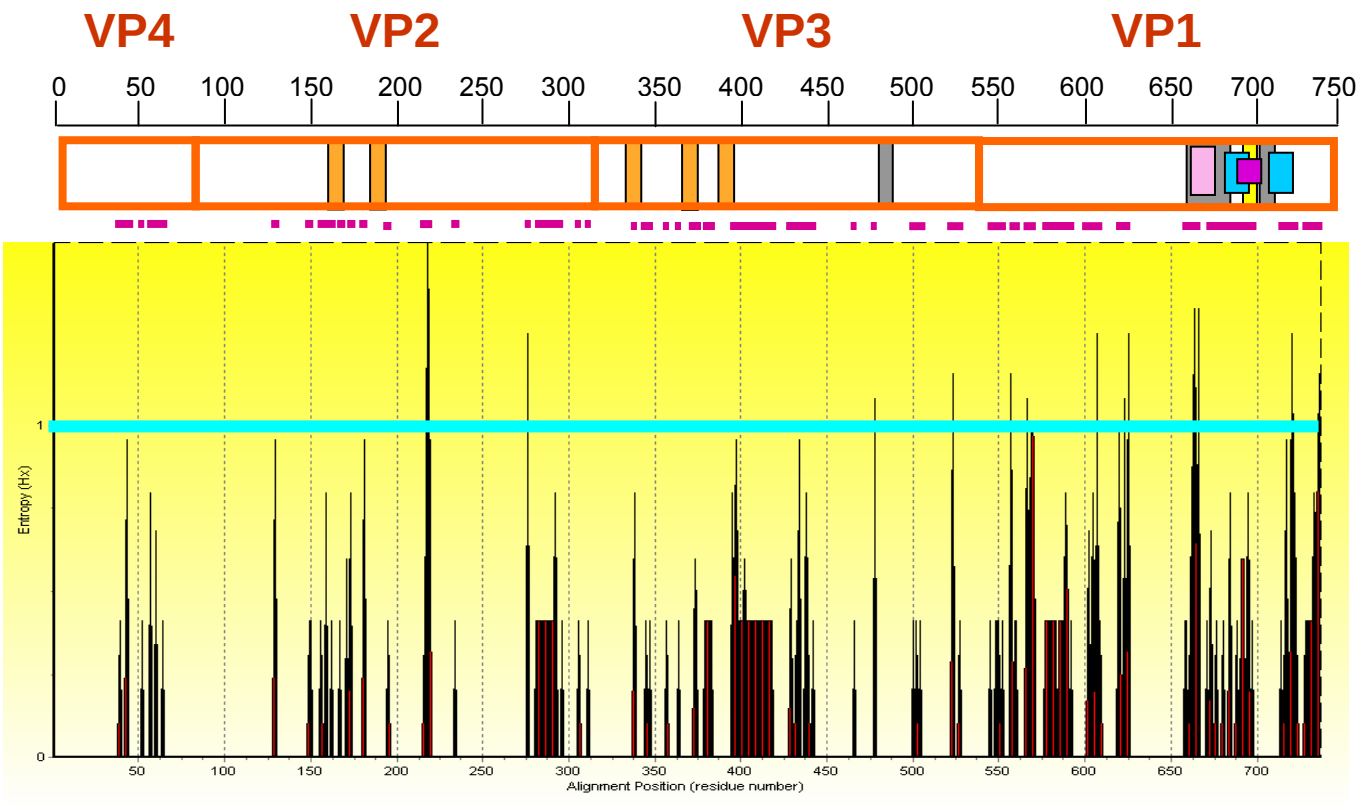
The serotype A site 1 involves the VP1 G-H loop, residues *ca*. aa 676, 678 as well as residues at the C-terminus, aa *ca.* 727, 734 plus the VP3 residues 479 and 482 (Appendix A8, Kitson *et al*., 1990). Other antigenic locations for serotype A include: site 2 (VP1 aa 699); group 1 (VP1 aa 666-678); group 2 (VP1 aa 681-682, 729); group 3 (VP2 aa 166 and 181, VP3 aa 339, 342, 361-364, 372-373 and 397) and group 4 (VP1 aa 694) (Kitson *et al*., 1990).

Similar to that observed for the African O isolates, all previously identified antigenic sites for serotype A corresponded to the regions of entropy for the African A isolates (figure 2.6 b). In addition to these antigenic sites, other regions of variability were identified, indicated by the horizontal purple bars above the entropy plots (figure 2.6 b) and those regions of variation with an entropy >1 are VP2 aa residues: *ca*. 225-227, 275; VP3 aa: *ca*. 475, 525; VP1 aa: *ca*. 560, 570, 610, 625. These results indicate that the FMDV African A viruses displayed more regions that are prone to variation/chaotropism than the FMDV African O isolates (entropy above 1; figure 2.6 a, b).

(a)



(b)



SITE 1 SITE 1b SITE 3 SITE 5 Group 1 Group 3
SITE 1a SITE 2 SITE 4 Regions of chaotropism Group 2 Group 4

Figure 2.6 Entropy plots indicating the variation expected at a specific aa residue position were determined using BioEdit for the African FMDV O viruses (a) and A viruses (b). Regions of chaotropism are indicated by a vertical black bar and the clear areas adjacent to the vertical black bars represent regions that are conserved. Black bars that are above an entropy of 1 (indicated by the blue line on the plot) are taken as regions which are prone to variation. Regions previously identified as antigenic sites are highlighted in the orange blocks, corresponding to the individual capsid coding regions, above the entropy plots (Crowther *et al*. 1993b and Kitson *et al*. 1990), which correspond to regions of variability on the entropy plot. The purple bars above the entropy plot indicate other regions of variability identified.

2.3.3.4. Important amino acids in the individual FMDV capsid protein regions

From the separate serotype A and O P1 aa alignments of all the FMDV viruses included in this study (results not shown), it was observed that most of the aa residues identified in previous studies as being critical for the function of the FMD virus was conserved (table 2.10). As expected, aa residues identified as being specific for the SAT types Q73, I76 and V80 of VP4 (Carrillo *et al*., 2005) were found to differ for the FMDV A and O viruses (table 2.10) that had S73, F76 and F80 respectively (aa numbered according to the Appendix A8 alignment). In addition, the N-terminal myristylation site and the swine and bovine T-cell epitope of VP4 aa 19-35 (Blanco *et al*., 2001) as well as the VP2 T-cell epitopes, aa 133-153, 198-215, 263-271 (Perez *et al*., 2000) were found to be reasonably conserved with only 1 or 2 amino acid changes for certain viruses (table 2.10, aa numbered as per Appendix A8). Furthermore, the P residue found to contribute to the VP4/VP2 cleavage pocket (Carrillo *et al*., 2005) at position 715 of VP1 (Appendix A8) was conserved for all viruses (table 2.10).

Also, a number of VP2 and VP3 residues previously shown as side chains involved in non-covalent interactions between the FMDV capsid pentamer subunits are pointed out in table 2.10 (Mateo *et al*., 2003) and were found to be conserved except for a few *i.e*. VP2: K173, K181, T195 as well as VP3: D372 and Q374 (table 2.10). Histidine residues located in the VP2/VP3 regions *i.e*. VP2 aa 106, 172, 230, 242, 259 as well as VP3 aa 388, 412, 446,

449 and 496 (aa numbered as per Appendix A8) which are proposed to mediate hydrogen bonding between these proteins (Acharya *et al*., 1989) were conserved for the African A and O serotypes (table 2.10). The VP3 H449 residue has been shown to play a significant role in FMDV capsid destabilisation (v. Vlijmen *et al*., 1998; Ellard *et al*., 1999) and was highly conserved for all the A and O serotypes (table 2.10).

In addition, FMDV O viruses have the potential to form a disulphide bond between residues C134 of VP1 and C130 of VP2 which links the base of the G-H loop (Logan *et al*. 1993). From the alignments, conserved C residues were found in all the African FMDV O viruses included in this study at both of these positions, however none of the African FMDV A viruses displayed a C residue at either of these positions [table 2.10; figure 2.7 b and d (in section 2.3.3.5 below); Appendix A8].

Table 2.10: A comparison of previously identified critical residues for FMDV to the FMDV A and O types from this study

aa Regions identified as important	Protein: aa residue/s *	A (African and non-African viruses): N (number of isolates aligned) = 26	A (African viruses): N=8	O (African and non-African viruses): N=27	O (African viruses): N=9
N-terminal myristylation site & swine and bovine T-cell epitope (Blanco *et al*., 2001)	**VP4**: 19 - 35	*c*	*c*	Y31H (1)	Y31H (1)
A Q residue distinguishes the SAT types from the Euro-Asiatic lineages (Carrillio *et al*., 2005).	**VP4**: 73	S (25) N(1)	S (8)	S (27)	S (9)
The I residue is potentially specific for the SAT2 and SAT3 viruses (Carrillo *et al*., 2005)	**VP4**: 76	F (26)	F (8)	F (27)	F (9)
A valine is potentially specific for SAT1 (Carrillo *et al*., 2005).	**VP4**: 80	F (26)	F (8)	F (27)	F (9)
Identified VP2 T-cell epitopes (Perez *et al*., 2000).	**VP2**: 133 – 153	I at position 141 (2) T at position 141 (1) Y at position 146 (3) R at position 149 (1) F at position 150 (17) H at position 150 (7) E at position 153 (3)	R at position 149 (1) H at position 150 (7)	A at position 141 (3)	A at position 141 (2)
	198 – 215	I at position 211 (1)	*c*	*c*	*c*
	263 - 271	I at position 265 (1) L at position 268 (1)	*c*	*c*	*c*
Side chains involved in non-covalent interactions between pentamer subunits in the FMDV capsid (Mateo *et al.*, 2003)	**VP2**: E96, I99, R103, H106, T108, T111, Q112, L136, T138, Q142, R145, K148, Y183, E193, V197,	*c*	*c*	*c*	*c*

	N199, Q200, F201, M239, K283, Y285, N287, E298, T107				
	K173	H (12), R (5), S (1), A (2), T (5)	A (2), T (5)	*c*	*c*
	K181	D (19), E (5), N (2)	D (5), E (1), N (2)	D (26), E (1)	D (9)
	T195	S(25)	S (7)	*c*	*c*
	VP3: K422, R424, M426, H446, H449, E451, D453, L456, N457, I494, T495, K498	*c*	*c*	*c*	*c*
	D372	G (2)	*c*	E (1)	E (1)
	Q374	T (16), N (3)	T (2)	D (25), F (1)	D (8), F (1)
H residues identified in the VP2/VP3 regions and are proposed to mediate VP2/VP3 hydrogen bonding (Acharya *et al*., 1989).	**VP2**: 106	*c*	*c*	*c*	*c*
	172	*c*	*c*	*c*	*c*
	230	*c*	*c*	*c*	*c*
	242	*c*	*c*	*c*	*c*
	259	*c*	*c*	*c*	*c*
	VP3: 388	*c*	*c*	T (1)	T (1)
	412	L (1)	L (1)	*c*	*c*
	446	*c*	*c*	*c*	*c*
	449	*c*	*c*	*c*	*c*
	496	*c*	*c*	D (1)	D (1)
C residues at these positions are important for the formation a disulphide bond at the base of	**VP2:** 215	K (26)	K (8)	C (27)	C (9)
	VP1: 660	S (15)	S (7)	S (1)	*c*

the G-H loop		N (9) D (2)	N (1)		
A R residue is important for HS binding (Fry *et al*., 1999).	**VP3**: 359	C (26)	C (8)	H (25) R (2)	H (8) R (1)
The H residue plays a significant role on FMDV capsid destabilization (v. Vlijmen *et al*., 1998; Ellard *et al*., 1999).	**VP3**: 449	*c*	*c*	*c*	*c*
The P residue contributes to the VP4/VP2 cleavage pocket (Carrillo *et al*., 2005).	**VP1**: 715	*c*	*c*	*c*	*c*
The RGD residue is important for receptor binding (Fox *et al.,* 1989; Mason *et al.,* 1994; Berinstein *et al.,* 1995; Jackson *et al*., 1997).	**VP1**: 671 - 673	*c*	*c*	SGD (1)	*c*
A L residue is most common and important for virus receptor ($\alpha v\beta 6$) recognition and plays a role in the stability of the virus/integrin complex (DiCara *et al*., 2008)	**VP1**: 674 677	M (6) S (2) T (3) H (2) P(2)	M (1) *c*	*c* *c*	*c* *c*

***** The aa numbering is according to the P1 alignment in Appendix A8. Note: Not all the sequences referred to in this table is included in Appendix A8. This table is based on the complete alignment of all the isolates included in this study (results not shown).

c Refers to the aa residues that are conserved in the P1 alignment.

The numbers in brackets ( ) indicate the number of isolates where there is a difference in aa and the letter next to the brackets refers to the aa change for those isolates.

N The number of isolates aligned

2.3.3.5. Residues important for FMDV receptor binding

As observed from the results thus far, the VP1 coding region of the African A and O types was found to have high variability. The RGD sequence of VP1 (aa 145 – 147, figure 2.7 c, e), which is essential for receptor binding (Fox *et al*., 1989; Baxt and Becker, 1990), was found to be conserved for all the FMDV A and O viruses although the adjoining sequences were variable (Appendix A8). The residues following the VP1 RGD motif are important for receptor recognition (Jackson *et al*., 2003) where the RGDLXXL (X represents any amino acid) sequence is most common for receptor recognition (Kraft *et al*., 1999). Alignment of all the FMDV O isolates included in this study revealed that the RGDLXXL sequences was highly conserved (table 2.10, alignment not shown). However, this sequence varied when compared to all the FMDV A isolates (table 2.10) at the RGD+1 position for 10 non-African and 1 African (A/SOM/1/78) isolate. Interestingly, 5 non-African and 1 African A viruses had a M at the RGD+1 position which was shown by Burman *et al*. (2007) to inhibit FMDV infection mediated by $\alpha_v\beta_6$ and $\alpha_v\beta_8$ receptors. In addition, at the RGD +4 position, most of the viruses had a L residue except for 4 non-African A viruses of which half had a H and the other two had a P residue (table 2.10). Most commonly, the RGD –1 position is a V residue and this was seen for 8 of the 9 African FMDV O sequences whilst O/UGA/1/75 had an R residue at this position (figure 2.7c). Additionally, it was observed that 14 of the 18 non-African O isolates displayed a V residue (results not shown) whilst for all of the FMDV A viruses included in this study there was a R residue at the RGD -1 position (figure 2.7 e).

Fry *et al*., 1999 showed that an R residue at position 359 of VP3 (according to the numbering of Appendix A8) is important for FMDV binding to the heparan sulphate receptor and from the alignments of all the A and O isolates in this study, it was found that only two viruses *i.e*. O/O1CAMPOS96 and O/KEN/10/95 had an R residue at this position whilst all the other FMDV O isolates had a H and all the FMDV A viruses had a C (table 2.10).

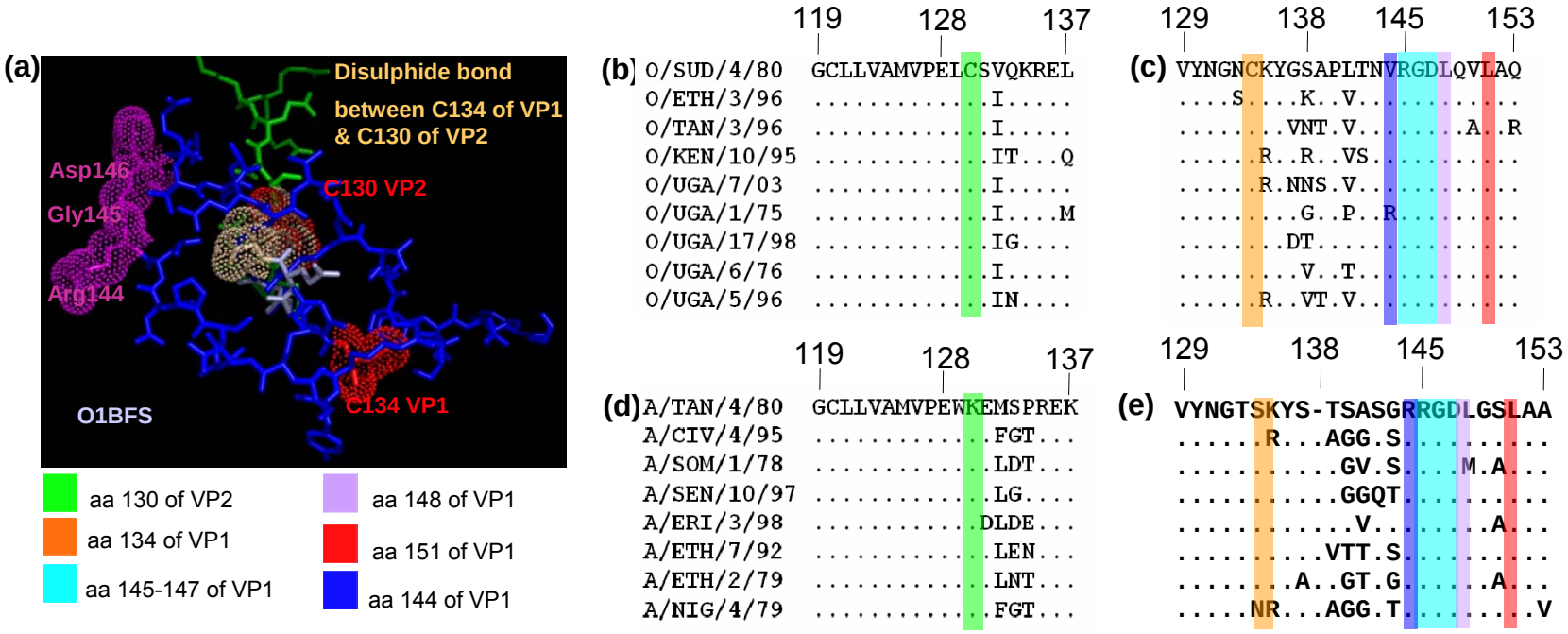


Figure 2.7: (a) Structural view of FMDV O1/BFS showing the disulphide bond at the base of the G-H loop. Amino acid sequence alignment showing amino acid (aa) residues 119 to 137 of the VP2 region of the African O viruses (b) and African A viruses (d). Part of the VP1 region showing residues 129 to 153 of the O viruses (c) and A viruses (e). The RGD sequence at aa position 145-147 on the βG-βH (G-H) loop of VP1 has been demonstrated to be the attachment site of the virus to the host cell receptor (Baxt and Becker, 1990). The RGD motif is highly conserved within all FMDV serotypes despite being located within the highly variable G-H loop. The sequences flanking the RGD motif are involved in determining both the binding specificity and affinity of integrins for their ligands. The aa position 144 of VP1 was found to have an R residue for all the FMDV A viruses. Positions 148 and 151 of VP1 for the O types and position 151 of the A types had a conserved leucine residue.

2.4 DISCUSSION

The first stage in a process of natural diversification of FMDV occurs during replication in infected animals and results in the rapid generation of mutants. Due to the high mutation rates and quasispecies dynamics of FMDV, it is important to continuously sequence FMD viruses to discover potentially important sequence differences that can contribute to the virus characteristics such as antigenicity and virus-receptor interactions. The widespread application of molecular and phylogenetic techniques has enabled tracing the origin of FMDV outbreaks and sequencing studies are required to investigate the possible genetic basis for various differences between strains. Due to rapid nucleotide sequencing techniques for the analysis of viral genomes, our understanding of RNA viruses has been greatly increased. Determining the P1 sequences of FMDV, and in particular the VP1 region, would give a better understanding of virus antigenicity where the important nucleotides are known for each serotype. In addition, antigenic sites have also been described for the VP2 and VP3 regions (Kitson e*t al*., 1990; Crowther *et al*., 1993) whilst the VP2 region plays a critical role in capsid stability and maturation (Curry *et al*., 1997). Previously, extensive studies were carried out to sequence the P1 regions of various SAT viruses even though FMDV A and O serotypes are also prevalent in Africa (van Rensburg and Nel, 1999). To this end, this study has successfully determined the complete L and P1 sequences of nine FMDV O and eight FMDV A viruses isolated in Africa. These sequences were compared to each other and with the reference strains listed in table 2.1.

The data from the analyses of the complete capsid-coding region, P1, as well as the individual capsid coding gene regions indicated that few incongruent tree topologies exist for the different genomic regions. In general, analyses of the entire structural protein encoding region improved bootstrap values relative to 1D analysis alone where the P1 trees gave the best resolution of virus distribution for the NJ, ME and MP phylogenetic trees. Consistent with previous findings (Knowles *et al*., 1998; Sahle *et al*., 2004) the NJ trees of the P1, 1B, 1C and 1D sequences resulted in the viruses grouping according to serotype. In addition, the A and O virus clusters could be further divided into

separate groupings of the African and non-African A and O isolates were observed for the P1, 1B, 1C and 1D NJ, ME and MP (Kumar *et al*., 1993) trees. The separate groupings of the African and non-African A viruses observed in this study for the P1, 1B, 1C and 1D trees support findings by Knowles and Samuel (2003) which stated that the 1D sequences of type A viruses could be grouped into three major restricted genotypes *i.e*. Euro-South America, Asia and Africa (this study only included FMDV A viruses from Euro-South America and Africa).

The ML (Swofford, 1998) trees for 1B and 1C also displayed similar groupings but there was a difference for the P1 ML tree where one African virus *i.e.* O/SUD/4/80 which did not group with any of the A or O isolates but instead formed an out-group. This could be due to the phylogenetic methodologies utilised when constructing the trees *i.e*. the ML and MP phylogenetic methods are character based where parsimony looks for trees with the minimum number of changes whilst ML tries to infer an evolutionary tree, under some model of evolution. In addition NJ and ME are distance methods. Thus, the difference observed for the P1 ML tree could be due to the difference in the way the ML tree is calculated. Moreover, taking into account that the O/SUD/4/80 isolate grouped with the African O viruses for the P1 NJ, ME and MP trees as well as the fact that the O/SUD/4/80 isolate is an African virus, the out-grouping of the O/SUD/4/80 virus observed in the P1 ML tree is possibly incorrect.

The clusters that were supported by high bootstrap values were observed using all phylogenetic methodologies for the P1 trees for O/TAN/3/96 and O/UGA/7/03 (including the 1C trees); O/KEN/10/95 and O/UGA/5/96 (including the 1D trees); A/TAN/4/80 and A/SOM/1/78 (including the 1D trees) as well as A/NIG/4/79, A/CIV/4/95 and A/SEN/10/97 (including the 1B, 1C, 1D trees) which is indicative of the transboundary spread of FMDV in Africa since the East African countries: Uganda, Kenya, Somalia and Tanzania are in close proximity to each other which is also true for the West African countries: Nigeria, Cote d'Ivoire and Senegal. These groupings also indicate that the East African and West African viruses fall into separate large groups. Another

well-supported grouping was observed for the P1, 1B and 1C trees (all methodologies) for O/UGA/1/75, O/UGA/6/76 and O/UGA/17/98 and this grouping signifies that the more recent outbreak strains could have evolved from older strains endemic in the area since the early 1970 *i.e*. from 1975 to 1998 (23 years).

There was a difference in the groupings for the 1A trees when compared to the P1 and other capsid-coding gene regions where three non-African A isolates clustered with the non-African O viruses (for all tree methodologies). This could be due to recombination events. Genetic diversification in FMDV can be attributed to recombination events (Pringle 1965; McCahon and Slade, 1981; King *et al*., 1982; 1985; McCahon *et al*., 1985; Giraudo *et al*., 1987; Wilson *et al*., 1988; Krebs and Marquardt, 1992) where Heath *et al*. (2006) showed that gene flow between serotypes can occur which may not affect viral fitness. Although there is scant evidence of recombination for the FMDV structural proteins (Krebs and Marquardt, 1992; Tosh *et al*., 2002), Tosh *et al*. (2002) demonstrated recombination in the P1 region of subtype A22 field isolates, proving that recombination in the P1 region of FMDV does exist that may provide selective advantage to the virus (an escape mechanism for the virus) as the pre-existing immunity towards either of the parental viruses may not afford complete protection.

The L gene encodes for a non-structural protein *i.e*. for a viral protease, L^{pro}. To date, studies to examine the genetic diversity in the FMDV genome at the ARC-TADP have focused primarily on the 1D or P1 regions because of their involvement in the formation of viral capsids and antibody-mediated virus neutralisation. In this study, in addition to the P1 and the individual capsid encoding regions, the L nucleotide sequence analysis of 9 African FMDV O and 8 FMDV A viruses were determined and compared to a few available/published A and O L sequences. It was evident from the results that the tree representing the region encoding the L protein differs from that of the structural proteins where sub-grouping according to serotype was much less apparent, which was consistent with previous findings of this region (van Rensburg *et al*., 2002; Tosh *et al*., 2004). Interestingly, certain A and O

African viruses clustered together and also did not separate into West and East African regions which were well supported in some instances (for all phylogenetic methods applied). These groupings suggest that the serotype A and O viruses from Africa are related and that recombination events might have occurred (Jackson *et al*., 2007; Heath *et al*., 2006). However, it is important to note that these groupings may change if more FMDV isolates and serotypes were included in this analysis. van Rensburg *et al*. (2002) showed that a L tree representing six of the seven FMDV serotypes displayed two clades where one clade included the European types (A, O and C) and the other clade included the SAT types.

It is generally accepted that due to the L functional features, it is more conserved within and between different FMDV serotypes as this region encoding the L^{pro} would not tolerate vast amounts of change. However the opposite was found to be true where both the intertypic and intratypic variations for the nucleotide as well as the amino acid sequence of the L region indicated high variation where Lab had a vast amount of variation. The L being a non-structural protein it is not under any direct selection pressure from the host immune system, and the only constraint to variation is possible mutations leading to a loss of proteolytic activity, thus the high degree of variability observed is difficult to explain. Probably the basic requirement of L^{pro} for FMDV biology is not as strict (Piccone *et al*., 1995a,b) as compared to other non-structural proteins and random mutations introduced in this gene in the course of viral replication would get fixed in the quasispecies as they might not lead to nonviable viruses (George *et al*., 2001). However, taking into account that both forms of the L protein are known to have all the processing functions of the proteinase (Medina *et al*., 1993), the variation of Lab and Lb might not interfere with the role of these proteins, where in this study all the important previously identified aa involved in the L^{pro} function was conserved.

From the phylogenetic analysis, using all methodologies, of the capsid coding regions as well as the P1 and L, the Pan-Asian FMDV O virus strains consistently formed a well-supported group within serotype O which

supporting previous results indicating their close relationship (Mason *et al*., 2003b). It is important to note that the results described in this study for the phylogenetic analysis included only a few FMDV A and O isolates, thus it is proposed that the tree topologies may change if more isolates were included for comparison.

The structural proteins of FMDV are involved in capsid assembly and stability, virus binding to target cells and antigenic specificity, which influences significant aspects of virus infection and immunity (Jackson *et al*., 2003). Due to the high mutation rates of FMDV, it is likely that even brief epidemics might result in the generation of substantial antigenic variability (Haydon *et al*., 2001). However, the adaptive significance of this variation remains unclear (Haydon *et al*., 1998). The antigenicity of FMDV is contributed to the amino acid residues that are well exposed on the surface of the capsid (Mateu *et al*., 1995). Typically it is known that the VP1 region is the most variant of the individual capsid-encoding regions which was found to be true in this study for all isolates for both the nucleotide and aa intertypic and intratypic variations whereas the variation for the VP2 and VP3 regions were also high. Thus, the variability observed in the external capsid proteins in this study likely reflects the selective pressures on them which allows for genetic changes in these proteins (Carrillo *et al*., 2005). In contrast, the VP4 capsid protein is the only capsid protein that is internally located in the capsid, thus the environmental and host immunological pressures on this region of the virus is different to that for VP1 – VP3 (Burroughs *et al*., 1971; Chow *et al*., 1987; Carrillo *et al*., 2005). Taking this into account, the nucleotide and amino acid diversity would be less for VP4, which is consistent with the nucleotide and amino acid variation observed in this study for all viruses, for both the intertypic and intratypic analysis where the VP4 region was found to be the most conserved capsid region. Overall the variation observed for the FMDV A and O viruses included in this study (both nucleotide and aa sequence) shows that the A viruses exhibited more variation, possibly indicating that the A viruses evolves rapidly which supports studies by Bachrach (1968) and Brooksby (1982).

The most important immunogenic determinant, the VP1 GH loop (Bachrach *et al*., 1982) exhibited a high degree of variation for the A and O isolates included in this study. Consequently nucleotide changes in this region are most likely involved in the appearance of new antigenic sites. In addition, Baxt and Bachrach (1982) and Baxt *et al*., (1984) have shown that this region of the VP1 protein is involved in virus attachment. Thus, this site may need to change for the virus to successfully bind to and proliferate in a new cell type with a different receptor, for example during transmission from one animal species to another (Weddell *et al*., 1985; Leippert *et al*., 1997). Such a mechanism would explain the ability of FMDV to rapidly adapt under different selective pressures and to grow in a wide range of hosts which would also account for the fact that antigenic variants arise among field isolates, especially during transmission from one species to another (Brooksby 1982; Ouldridge *et al*., 1982). Analyses of antigenic sites of picornaviruses have been carried out using neutralising monoclonal antibodies (Mabs) to select and screen Mab-resistant mutants. Sequence analysis of these mutants has resulted in the identification of 5 antigenic sites of serotype O virus *i.e*. O_1Kaufbeuren (Kitson *et al*., 1990, Crowther *et al*., 1993) and 6 sites for the FMDV A viruses (Kitson *et al*., 1990). Alignments of the aa sequences for the African A and O viruses indicated that regions of variability identified in this study correspond to the FMDV known antigenic sites which points to the fact that the antigenic sites are conserved for the African A and O viruses included in this study. In addition to these sites, from the entropy plots, other regions of variability were identified for both the FMDV O and A African isolates with 2 and 8 regions prone to variation (entropy >1) respectively. These regions identified for the African viruses could potentially be antigenic epitopes but this would need to be further investigated.

The VP4/VP2 cleavage is autocatalytic and the aa residues at the VP4/VP2 cleavage site was highly conserved for the A and O types, whilst VP2/VP3 and VP3/VP1 cleavage sites where $3C^{pro}$ is involved, displayed a small degree of variation in the protein junction site sequences. A study by Birtley *et al*. (2005) established from alanine-scanning mutagenesis studies that the $3C^{pro}$ substrate recognition site spans four aa residues on either side of the

protease cleavage site *i.e*. P4 to P4'. Additionally, at positions P4, P2, P1 and P4'; the residues P, K, Q and F respectively were found to be important for specific recognition by $3C^{pro}$ (Birtley *et al*., 2005). In particular, the K and F aa residues at the P2 and P4' $3C^{pro}$ substrate recognition site respectively was shown to contribute substantially to $3C^{pro}$ cleavage, however these residues were not conserved at the protease cleavage junction (Birtley *et al*., 2005). Most picornavirus 3C proteases are specific for either a E or Q aa residue at the P1 position (Long *et al*., 1989; Jewell *et al*., 1992). In this study, only the P4 P and P2 K aa residues were conserved for all isolates at the VP2/VP3 junction whilst at the VP3/VP1 junction, the P4 P aa was conserved for all FMDV A isolates and the P1 Q aa residue was conserved for all the A isolates as well as for 21 of the 27 FMDV O isolates.

The L^{pro}/VP4 cleavage sequence at the C-terminus of the L protein and N-terminus of the VP4 protein should be (K/R)KLK*GAGQ (Skern *et al.*, 1998). A detailed analysis of the L^{pro}/VP4 junction resulted in a similar cleavage motif than was previously described for serotypes A, O and C (Seipelt *et al*., 1999) where the K(R)R(K)LK(R) at the L^{pro} C terminus and the GAGQ residues at the VP4 N terminus was observed. These results may indicate that for all the A and O types included in this study, the conserved sequence XXLK(R)*GAGQ (where X is either K/R) is sufficient for L/P1 cleavage by the L^{pro}.

As indicated in the results, various aa residues that were previously identified as important for playing a role in various functions for FMDV were found to be conserved for the A and O isolates. To point out a few: (i) Several T-cell epitopes (important for eliciting the host immune response) have been identified for FMDV located on the capsid proteins and these were found to be fairly conserved for the A and O viruses in this study. (ii) The presence of the C residue for all the African FMDV O viruses which links the base of the GH loop at position 134 of VP1 and 130 of VP2 (figure 2.7) indicates that in theory, there could potentially be a disulphide bond between these residues (Parry *et al*., 1990; Logan *et al*., 1993). However none of the African FMDV A viruses displayed a C residue at these positions, indicating that this trait might

be present only for the FMDV O types. In addition, this difference, suggested by Duque and Baxt (2003), might propose that there are differences in the nature of the GH loop between type A and O viruses. (iii) The H residues at the interface between VP2 and VP3, which were highly conserved for the A and O isolates, play a major role for FMDV capsid stability as when these residues become protonated at low pH, the capsid weakens through electrostatic repulsion (Curry *et al*., 1995; Ellard *et al*., 1999). (iv) The stability of the FMDV capsid depend on the establishment of multiple non-covalent interactions between pentameric subunits that function as assembly or disassembly intermediates (Mateo *et al*., 2003) and the amino acids identified for this role were found to be highly conserved for most the A and O viruses (this study). On the whole despite the variability observed for the FMD virus capsids, those regions that are important for capsid stability and FMDV function were highly conserved. In addition, this was found to be true for the L^{pro} where the functional elements such as the catalytic and cleavage residues were conserved.

A number of aa residues have been identified as playing a role for FMDV receptor recognition and were mainly conserved for all the A and O types in this study. Unlike most picornaviruses, FMDV has a smooth capsid surface with a main protruding element *i.e*. the G-H loop in VP1. This loop contains the highly conserved RGD triplet, a universal cell recognition site, which interacts with cell-surface integrin receptors. The exposed, flexible G-H loop of VP1 of the African FMDV A and O types from this study contain a strictly conserved RGD motif which was flanked by hypervariable sequences. The residues that follow after the RGD motif are important for receptor recognition (Jackson *et al*. 2000a) and the RGD is flanked on both sides by hypervariable sequences which delivers a domain that is capable of adopting different conformations and presumably this situation ensures access to cells by the virus and at the same time, enables it to elude the immune response of the host (Rossman and Palmberg, 1988; Rossman and Rueckert, 1987). Additionally, the L residues at the RGD +1 and +4 positions have been shown to be important for virus receptor ($\alpha_v\beta_6$) recognition where the RGD +1 makes

a more significant contribution to the stability of the virus/integrin complex (DiCara *et al*., 2008). DiCara *et al*. (2008) showed by using resistance to EDTA-induced dissociation as an indicator of highly stable binding that the stabilisation of FMDV O and C peptide-integrin ($\alpha_v\beta_6$) complexes was dependant on the RGD +1 and +4 L residues. Additionally, the amino acids flanking the RGD, especially at positions RGD –1, +1 and +4 are known to affect integrin binding affinity and specificity although the relative importance of each of these flanking sites may vary between types (Mateu *et al*., 1996). A unique R residue was observed for 25 of the 26 FMDV A types at the RGD –1 position and it is proposed that this R residue could play an important role in receptor recognition for the FMDV A viruses. The RGD +1 and +4 positions was highly conserved for all viruses included in this study, confirming its importance for FMD virus-receptor interactions.

Sa-Carvalho *et al*. (1997) and Fry *et al*. (1999) confirmed the importance of the R 56 residue of VP3 for HS binding. From the alignments of all the African FMDV A and O viruses from this study only one African virus *i.e*. O/KEN/10/95, displayed an R at this position, which may be an indication that this virus utilises HS as its receptor. In addition, Neff *et al*. (1998) showed that a variant of the type O1 virus containing a R at residue 56 of VP3 required only HS to replicate in CHO cells but that another variant with a H residue at this position required the integrin to replicate in cell culture. In this study 25 of the 27 O isolates in this study had a H residue at VP3 56 which proposes that these viruses may use the integrin receptors for host cell entry.

It is clear from the outbreaks of FMD during the last two decades that there is a continuing threat to the livestock industry. In addition, there are seven FMDV serotypes, which have the ability to persist, to mutate and to spread amongst livestock. Thus, genome sequencing can provide a vital tool to increase our knowledge with regard to FMDV phylogenetic relationships, receptor recognition sites, virus antigenicity, etc. about the various FMD viruses. Six of the seven FMDV serotypes exist in Africa, which makes control and possible future eradication difficult. This study has been valuable

in increasing our understanding with regards to the FMDV African A and O viruses that are prevalent in Africa as various valuable information has been achieved regarding these viruses on the L and complete capsid-coding, P1, region through comparisons with a small number of non-African isolates .

CHAPTER 3

INVESTIGATING THE mRNA EXPRESSION LEVEL OF FOOT-AND-MOUTH DISEASE VIRUS RECEPTORS IN TWO DIFFERENT CELL LINES

3.1 INTRODUCTION

FMDV possesses many advantageous characteristics for a pathogen *viz*. it has a wide host range, rapid replication cycle, infection can be initiated by small amounts of virus and infected animals excrete high levels of virus, which make eradication and control of the disease difficult in many countries. Although FMDV research has been ongoing for many years, there are still gaps in our knowledge that impede obtaining FMD-free regions.

A considerable amount of effort has gone into the identification of those structures that mediate cell recognition by viruses and the transfer of their genetic material into cells. Structural and functional studies of FMDV over the past years have established the G-H loop of capsid protein VP1 and in particular, the RGD motif as being involved in virus interaction with the cellular integrins $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_6$ and $\alpha_V\beta_8$ in cell culture and cattle (Berinstein *et al*., 1995; Jackson *et al*., 2000b; Jackson *et al*., 2002; Jackson *et al*., 2004). However, viruses propagated in cell culture can also utilise alternative receptor pathways, which is independent of integrins, to enter cells (Jackson *et al*., 1996; Baranowski *et al*., 1998; Fry *et al*., 1999). One alternative receptor for FMDV adapted to cell culture is heparan sulphate proteoglycans (HSPG) (Sa-Carvalho *et al*. 1997; Fry *et al*., 1999; Baranowski *et al*., 2000).

The development of multiple receptor pathways for FMDV entry, even into the same cell type, confers the potential to regulate receptor usage in response to selective constraints. It was shown that when the interaction of the parental virus with integrins was blocked by RGD-containing peptides; the infectivity of cell culture adapted FMDV isolates was not affected by these peptides (Baranowski *et al*., 2000; Baranowski *et al*., 2001). This indicates a transition from an RGD dependant to an RGD independent mechanism of cell

recognition by FMDV. In addition, FMDV cell culture adapted isolates are able to interact with integrins (Baranowski *et al*., 2000). Furthermore, when HSPG binding was inhibited, the RGD-dependant pathway for FMDV entry was utilised (Berinstein *et al*., 1995; Jackson *et al*., 2000b). These facts indicate that the FMD virus has developed characteristics to ensure its infectivity and survival. Thus, it is of utmost importance to further investigate FMDV receptor usage so that the virus-receptor interactions can be better understood.

In order to develop improved vaccines and to understand the role of FMDV receptors, more needs to be learnt about the FMDV HSPG and integrin receptors. There is a dearth of knowledge regarding the level of expression of FMDV receptors in cultured cell lines which are used for the large-scale production of FMDV antigen for vaccines. This information could be used to predict the outcome of adaptation to cell cultures and possibly virus yields. As a starting point, this study investigated the levels of messenger RNA (mRNA) expression for the β_1, β_3, β_6, β_8 and HSPG cDNA regions on baby hamster kidney (BHK-21) and IB-RS-2 (Instituto Biologico renal suino, a pig kidney cell line) cells using newly developed reverse-transcription polymerase chain reactions (RT-PCRs). The results were normalised using a RT-PCR to the β-actin mRNA. Investigating the FMDV receptor mRNA expression levels in cell lines used at the vaccine production section at the Agricultural Research Council (ARC), Onderstepoort Veterinary Institute (OVI), Transboundary Animal Diseases Programme (TADP) can provide an indication of the possible receptors that are being expressed on the cells and could be utilised to facilitate faster adaptation of viruses to cell lines.

3.2. MATERIALS AND METHODS

3.2.1. Cell Cultures

Cell samples of BHK-21 *i.e*. clone 31 (BHK-31), strain 2/04 (BHK2/04), strain 6/06 (BHK6/06), strain 4/98 (BHK4/98) and BHK-21 from the American type culture collection [BHK-21(ATCC)] as well as IB-RS-2 were obtained from the tissue culture section at the ARC-TADP and 300µl of the sample was used directly to optimise the RT-PCR for the detection of β1, β3, β6 and β8 mRNA in both cell lines. In addition, one BHK-21 and one IB-RS-2 cell sample were obtained from frozen stocks at the ARC-TADP and grown for 36 sequential passages. At each passage level (PL), cells were passaged in a microbiological safety cabinet, which was wiped down with 70% ethanol. The cells were passaged every second day. The BHK-21 cells were maintained in Eagle's Basal Medium (Sigma; Appendix A1) whilst the IB-RS-2 cells were maintained in RPMI medium (Sigma; Appendix A1). For details of cell passaging, see section 2.2.1.1. At each PL 1ml of the cell sample was removed and adjusted to a final count of *ca*. 1 x 10^6 cells/ml for further use. From this 1ml cell sample at each PL, two samples (duplicates) with a volume of 300µl each were used for the optimised receptor RT-PCR (section 3.3.2).

3.2.2 Viruses and infection of cells

Plaque titration assays were performed at PLs 5, 21 and 36 for both cell lines using three pre-titrated FMD virus strains (table 3.1) to investigate the virus susceptibility with the increase in cell passage (kindly assisted by Ms. Belinda Blignaut, ARC-TADP). These isolates were chosen randomly.

Table 3.1: FMD virus strains used for the plaque titration assays.

Virus strain	Passage History	Initial Titres
vKNP/SAT2 (chimera strain)	PK_1 IB-RS-2_2 BHK_5	1.53 x 10^8 pfu/ml
SAT1/KNP/196/91 (vaccine strain)	PK_1 IB-RS-2_2 BHK_5	7.5 x 10^8 pfu/ml
O/UGA/5/96	IB-RS-2_2	3.30 x 10^8 pfu/ml

PK: primary porcine kidney cells; IB-RS-2: porcine kidney cells; BHK: baby hamster kidney cells. The number following the cell line indicates the number of times a virus was passaged in that particular cell line.

Briefly, cell monolayers of IB-RS-2 and BHK-21 cells were seeded in 35mm diameter wells to achieve 100% confluency following incubation overnight at 37°C in the presence of 5% CO_2. Log_{10} dilutions from 10^{-4} to 10^{-6} (performed in triplicate for each dilution) of the vKNP/SAT2, SAT1/KNP/196/91 and O/UGA/5/96 viruses were prepared in BME medium, containing 1% FCS, antibiotics (Appendix A1) and 25mM HEPES buffer (Invitrogen). Following aspiration of the medium, 200µl of each dilution was added to the cell monolayers and incubated for 1h at 37ºC in a CO_2 incubator with gentle shaking after which a 2ml Tragacanth overlay (Appendix A1) at room temperature was added to each well. Incubation was continued for 24h in a CO_2 incubator without shaking. The cell monolayers were stained overnight with 2ml of a 1% (w/v) methylene blue stain (Appendix A1) to visualise plaques.

3.2.3 RNA Extraction

RNA was extracted from cell samples using the Trizol method (Chomczynski *et al*., 1987). For each 1ml cell sample, RNA extraction was performed in duplicate, which served as verification of the results. Briefly, 300µl of the cell sample, previously adjusted to 1 x 10^6 cells/ml was added to 700µl of Trizol (Invitrogen). The Trizol reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Following 15s of vortexing and 5min incubation at room temperature, 200µl chloroform was added, followed by brief vortexing and 10min incubation at room temperature. Chloroform separates the solution into an aqueous phase containing the RNA and an organic phase. The aqueous phase was transferred into a sterile microfuge tube and 500µl isopropanol added followed by 10min incubation at room temperature and 10min centrifugation at 13 000 x g to recover the RNA. The supernatant was removed and discarded and 500ul 70% ethanol added where after the sample was centrifuged at 13 000 x g for 10 min. After removal of the supernatant, the pellet was dried at 56°C for 10min and re-suspended in a final volume of 30µl 1 × TE buffer (10mM Tris-HCl, 2mM

EDTA; pH 7.4) containing RNasin® ribonuclease inhibitor (40 U/μl; Promega) and stored at -70ºC.

3.2.3.1 RNA quantification and precipitation

The cellular RNA was quantified using the Nanodrop (ND-1000 Spectrophotometer, Inqaba Biotech) and adjusted to 100ng/μl. For RNA samples that exceeded 100ng/μl, RNA concentrations were adjusted by diluting the RNA in a mixture of 1 × TE buffer (10mM Tris-HCl, 2mM EDTA; pH 7.4) containing RNasin® ribonuclease inhibitor (40 U/μl; Promega). However, samples that had concentrations <100ng/μl were subjected to ethanol precipitation. Briefly, 30μl of RNA, 58μl of distilled water, 8μl 5M NaCl, 1μl 1M Tris (pH8), 2μl 0.5M EDTA, 2μl oyster glycogen (Sigma) and 300μl 100% ethanol was mixed in a 1.5ml microtube by gently tapping the tube and precipitated for 2hrs at -20°C. The RNA was pelleted by centrifuging for 18min at 13 000 x g and the resultant supernatant discarded. To the pellet, 200μl 70% ethanol was added and gently mixed by tapping the tube and centrifuged for 10min at 13 000 x g. The supernatant was removed and the pellet dried at 56°C and resuspended in a final volume of 7μl DEPC water (Invitrogen) and 1 × TE buffer (10mM Tris-HCl, 2mM EDTA; pH 7.4) containing RNasin® ribonuclease inhibitor (40 U/μl; Promega). For a few samples, the RNA concentrations were too low and could not be concentrated to 100ng/μl and were thus further used as is.

3.2.4 cDNA synthesis

The SuperScript III First-Strand synthesis system for RT-PCR kit (Invitrogen) was used to synthesize cDNA from the cellular RNA. The kit was followed as per the manufacturer's specifications. cDNA synthesis was carried out for each RNA sample and it's corresponding duplicate. Briefly a RNA/primer mixture was prepared consisting of 1μM of Oligo$(dT)_{20}$ primer, 1μl 10mM dNTP mix, 3μl DEPC-treated water and 5μl cellular RNA in a sterile microfuge

tube and incubated for 5min at 65°C followed by 1 minute on ice. A cDNA synthesis mix was prepared consisting of 2μl 10x RT buffer, 4μl 25mM $MgCl_2$, 2μl 0.1M DTT, 1μl RNaseOUT™ (40U/μl) and 1μl SuperScript™ III RT (200U/μl). Ten microliter of the cDNA synthesis mix was added to the RNA/primer mixture, which was vortexed briefly and centrifuged at 13 000 x g for 5s. This was incubated for 110min at 50°C to allow the synthesis of cDNA. The reaction was terminated by incubating for 5min at 85°C and the cDNA sample was stored at -20°C for future use.

3.2.5 Primers

Two different primer sets were utilized in order to develop an optimized RT-PCR to amplify the integrin cDNA, *i.e*. those called the hamster-specific primers, which amplified the β1, β3 and β6 cDNA (Appendix A9) and those called the integrin (ITG) primers (Appendix A10), which amplified the β1, β3, β6 and β8 cDNA. The differences between these primer sets are described in section 3.3.1 (kindly developed by Ms. Belinda Blignaut, ARC-TADP). Primers for PCR had to be designed to amplify the HSPG cDNA (see section 3.3.1, Appendix A11).

PCR amplification of a housekeeping gene, β-actin, was utilised as an internal control. The β-actin primer (obtained from the ARC-TADP primer stocks) sense and antisense sequences are shown in Appendix A9 and A10.

3.2.6 PCR amplification

PCR was performed for each cDNA sample as well as its corresponding duplicate. Each PCR reaction mixture (10μl) contained 1μl cDNA, 0.25μM of each of the appropriate sense and antisense primers (Appendix A9, A10, A11), 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$), 0.9μl of 200μM dNTP mix (Roche), 0.5μl 25mM $MgCl_2$, 4.3μl distilled H_2O and 0.3μl *Taq* DNA polymerase (5 U/μl; Roche). No additional $MgCl_2$ was

included for the β-actin PCR mix and thus 4.8μl of distilled H_2O was added. The tubes were placed in an ABI thermal cycler and after initial denaturation at 94°C for 2min, the reactions were subjected to 35 cycles using the following temperature profile: denaturation for 30s at 94°C, annealing for 30s at 53°C (for β6 and β8 primers, IB-RS-2 cells), 55°C (for β-actin and Heparan sulphate primers, BHK and IB-RS-2 cells), 57°C (for β1 and β6 primers, BHK cells) and 58°C (for β3 and β8 primers, BHK cells and β1 and β3 primers, IB-RS cells), elongation for 60s at 72°C and 5min at 72°C to allow the synthesis of full-length products. The details of the PCR reaction for each primer set are outlined in Appendix A12 and A13.

3.2.7 Agarose gel electrophoresis

The PCR amplicons were separated by agarose gel electrophoresis. A 1.5% (w/v) agarose gel in 1 × TAE buffer (40mM Tris-acetate, 2mM EDTA; pH 8) was used. The gel contained 0.5μg/ml ethidium bromide [EtBr (10mg/ml); Promega], which allowed visualization of the DNA banding pattern by UV fluorescence. A 100bp DNA marker (Promega) was used as an indication of the correct PCR product sizes. The electrophoresis tank was set at 100V for 30 min.

3.2.8 Analysis of the PCR product intensity

The mRNA expression level for each receptor for each cell sample, including the duplicate sample, was determined by the intensity of the PCR product on the agarose gel. To measure this intensity, the GelTrak virtual densitometer computer program [Dennis Maeder (c) 1995, 1996, 2004 Department of Biochemistry, University of Cape Town COMB, U. Maryland Biotechnology Institute] was used. The β1, β3, β6, β8, βactin and HS PCR products for each cell sample (including the duplicate PCR products) were loaded onto the same agarose gel and agarose gel electrophoresis was carried out as described in section 3.2.7. The agarose gel containing the separated PCR products was visualised using the Alpha imager®HP (Innotech) and saved as

a bitmap image. This bitmap image was loaded onto the GelTrak program. In GelTrak, parameters were set to include specific lanes and PCR products that needed analysing. The intensity value obtained from GelTrak for each PCR product was normalized to that of the β-actin PCR (described in section 3.3.3.1, figure 3.12).

3.2.9 Statistical analysis of plaque titration results

The average titer for each virus strain (table 3.1) and for each cell line (BHK-21 and IB-RS-2) at PLs 5, 21 and 36 were analysed statistically using the program GenStat (2003) by Ms Marie Smit at the ARC-Biometry unit to determine whether the differences observed in the titres (section 3.3.3.2) were statistically significant. The average titres from two cell lines (BHK-21 and IB-RS-2) and three PLs (5, 21 & 36) per virus strain were analysed as data from a completely random design (CRD). Analysis of variance (ANOVA) was used to determine differences between cell line (C) and PL (P), as well as the cell line-by-PL interaction. Typically, the ANOVA test is is used to test for differences among two or more independent groups. It is a general technique that can be used to test the hypothesis that the means among two or more groups are equal, under the assumption that the sampled populations are normally distributed. The data from this study was log-normally distributed with heterogeneous treatment variances. Therefore the log (base 10) transformation was applied to the data and estimated means were separated using Fisher's protected t-test least significant difference (LSD) at the 1% level of significance (Snedecor and Cochran, 1980), as some variances were still heterogeneous after transforming the data. The Fisher's LSD test is a t-test for all pairwise comparisons in an ANOVA, but using the variance estimate from the ANOVA.

3.3 RESULTS

3.3.1 Primers

A project was initiated by Ms. Belinda Blignaut (ARC-TADP) where the expression levels and genetic characterisation of the known FMDV receptors for cell entry in BHK-21, IB-RS-2 and CHO-K1 (Chinese hamster ovary) cells were examined. Through this investigation, nucleotide sequences from GenBank of β1, β3, β6 and β8 were attained from a range of different species such as rat, mouse and human, which were applied to design primers for the detection of these genes in BHK cells. The primers designed were called the integrin (ITG) primers (Appendix A10). Using the ITG primers Ms. Blignaut amplified the β1, β3 and β6 cDNA (via RT-PCR) of BHK-21 cells and the resulting PCR products were sequenced and these sequences were used to design BHK specific primers (Appendix A9). The expected sizes of the fragments of interest for the hamster-specific primers (Appendix A9) were ~150bp, ~155bp and ~200bp for β1, β3, and β6 primers and for the ITG primers (Appendix A10) were ~370bp, ~510bp, ~420bp and ~580bp for the ITGβ1, ITGβ3, ITGβ6 and ITGβ8 primers. The expected fragment size for the β-actin primers was *ca*. 250bp.

HSPG sequences were attained from GenBank (table 3.2) and aligned using the BioEdit sequence analysis software (Hall, 1999). From these alignments, primers were designed leading to a PCR product of ~300bp (Appendix A11).

Table 3.2: Details of HSPG sequences attained from GenBank to design primers for the amplification of the HSPG cDNA

NAME OF SEQUENCE	ACCESS NUMBER	SOURCE
Homo sapiens syndecan (SDC) 1	NM_001006946	GenBank
Rattus norvegicus syndecan (SDC) 1	NM_013026	GenBank
Syrian golden hamster (M.auratus) HSPG	M29967	GenBank
Mus musculus SDC 1	NM_011519	GenBank

3.3.2 Optimisation of the receptor PCR

3.3.2.1 BHK-21 Cells

To optimise the β-actin PCR, BHK-21 cells were used and annealing temperatures (Ta's) of 57°C, 58°C and 60°C were tested but resulted in PCR fragments of the incorrect size (results not shown). Annealing temperatures of 55°C and 56°C were tested using cell samples BHK31, BHK2/04 and BHK6/06. In addition $MgCl_2$ concentrations were varied *i.e.* the PCRs were carried out for each cell sample with and without the addition of 0.5µl 25mM $MgCl_2$. The results indicated that an annealing temperature of 55°C and no addition of $MgCl_2$ were the optimum conditions for the β-actin PCR (lanes 3, 5, and 7 of figure 3.1).

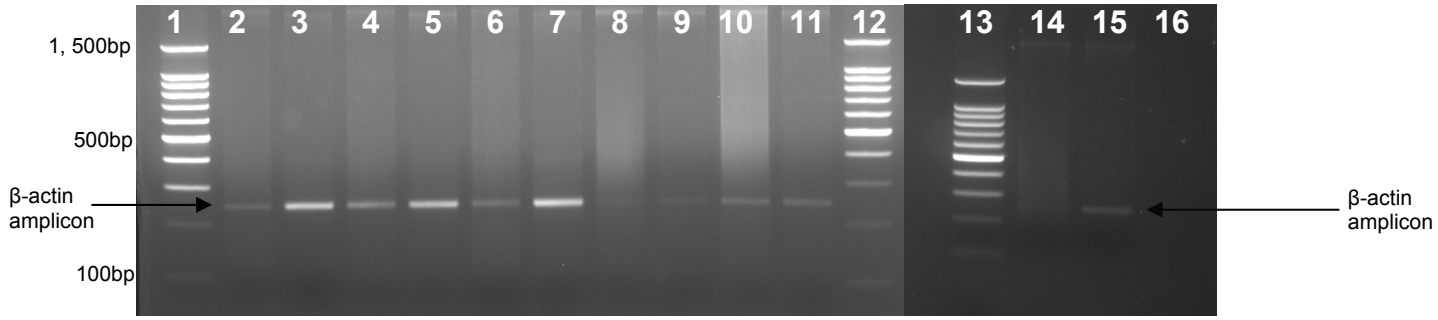


Figure 3.1: Agarose gel showing the PCR results for the β-actin PCR optimisations. The arrows indicate the amplicon size of interest. 1: 100bp marker; 2: BHK31, Ta: 55°C, 0.5µl $MgCl_2$; 3: BHK31, Ta: 55°C, no $MgCl_2$; 4: BHK2/04, Ta: 55°C, 0.5µl MgCl2; 5: BHK2/04, Ta: 55°C, no MgCl2; 6: BHK6/06, Ta: 55°C, 0.5µl $MgCl_2$; 7: BHK6/06, Ta: 55°C, no $MgCl_2$; 8: BHK31, Ta: 56°C, 0.5µl $MgCl_2$; 9: BHK31, Ta: 56°C, no $MgCl_2$; 10: BHK2/04, Ta: 56°C, 0.5µl MgCl2; 11: BHK2/04, Ta: 56°C, no MgCl2; 12: 100bp marker; 13: 100bp marker; 14: BHK6/06, Ta: 56°C, 0.5µl $MgCl_2$; 15: BHK6/06, Ta: 56°C, no $MgCl_2$; 16: negative control.

The hamster-specific β1, β3 and β6 primers (B. Blignaut, Appendix A9) were initially used to optimise the receptor PCR. Since these primers were designed specifically for this species, it was expected that the PCR would be easily optimised using BHK-21 cells. However, no PCR products were initially obtained when varying Ta's (58°C, 60°C) were tested (results not shown) and lowering the annealing temperature to 53°C resulted in non-specific amplification (figure 3.2).

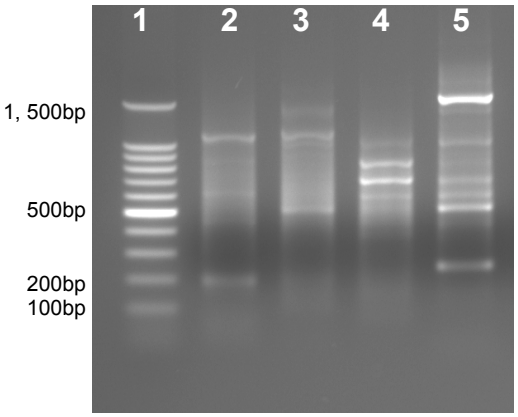


Figure 3.2: Agarose gel showing the PCR results where BHK-21 (ATCC) cellular cDNA and the hamster-specific BHK β1, β3, and β6 primers were utilised. 1: 100bp marker, 2: BHK-21 β1, 3: BHK-21 β3, 4: BHK-21 β6, 5: BHK-21 β-actin.

It was assumed that the non-specific products were due to the hexamers that were added in the cDNA synthesis steps and cDNA synthesis was repeated which excluded the hexamers, followed by PCR (utilising the hamster-specific BHK primers) at a Ta of 53°C (figure 3.3).

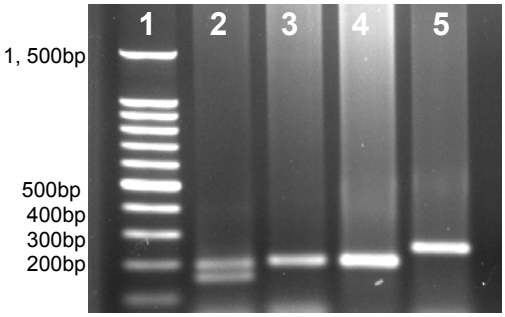


Figure 3.3: Agarose gel showing the PCR results of BHK-21 (ATCC) cellular cDNA using the hamster-specific BHK β1, β3, and β6 primers. 1: 100bp marker, 2: BHK-21 β1, 3: BHK-21 β3, 4: BHK-21 β6, 5: BHK-21 β-actin.

The correct sized fragments were obtained for all primers. However, there was a double fragment for the β1 integrin primer *i.e. ca*. 200bp and *ca*. 150bp respectively where the *ca*. 150bp PCR fragment was the fragment expected (figure 3.3, lane 2). This double fragment posed a problem for the interpretation of results as during the PCR reaction, it is possible that there would be competition between the two PCR products which would result in the incorrect mRNA intensity value for β1. Also, during agarose gel electrophoresis, the two fragments may not separate evenly which would make it difficult to distinguish between the two products. Additionally, despite

the success when using these primer sets during optimisation, when the PCRs were tested on various BHK-21 cell samples received from the cell fermenters from the tissue culture section at ARC-TADP, the results were inconsistent and in most cases unsuccessful, even though the β-actin PCR product control was present (results not shown). It was decided to test alternative primers.

The ITG primers (B. Blignaut, Appendix A10) were used to investigate the mRNA expression levels for the β1, β3, β6 and β8 cDNA. An experiment was performed where both the hamster-specific and the ITG integrin primers were tested on the same cell sample, BHK-21 (ATCC) with better results using the latter primers (figure 3.4).

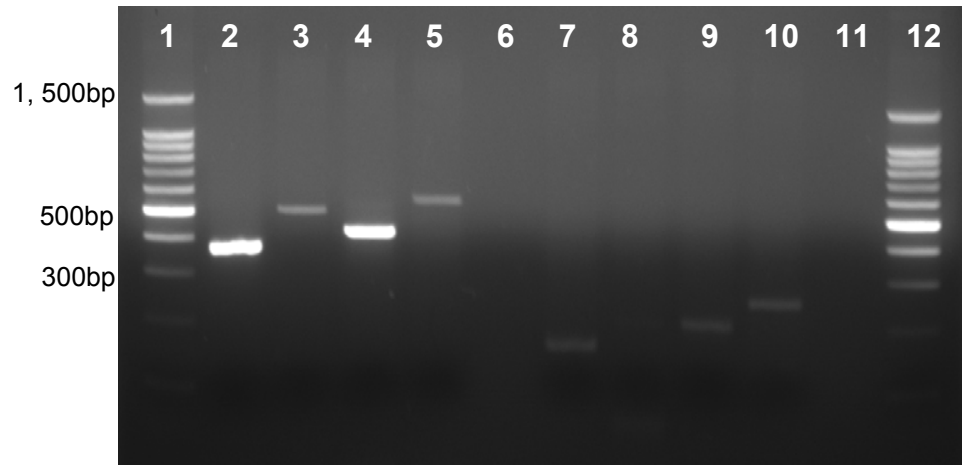


Figure 3.4: Agarose gel showing the PCR results of BHK-21 (ATCC) cellular cDNA showing the ITG primers (2 to 5) compared to the hamster-specific primers (7 to 9). 1: 100bp marker, 2: BHK-21 ITGβ1, 3: BHK-21 ITGβ3, 4: BHK-21 ITGβ6, 5: ITGβ8, 6: negative control, 7: BHK-21 β1, 8: BHK-21 β3, 9: BHK-21 β6, 10: BHK-21 β-actin, 11: negative control and 12: 100bp marker.

PCRs using the ITG primers were optimised and it was found that Ta's of 55°C and 56°C were unsuccessful using the β1, β3, β6 and β8 ITG primers (results not shown). However the Ta of 57°C for β1 and β6 and 58°C for β3 and β8 primer sets were found to be optimum (figure 3.5; lanes 8, 9, 10 and 11).

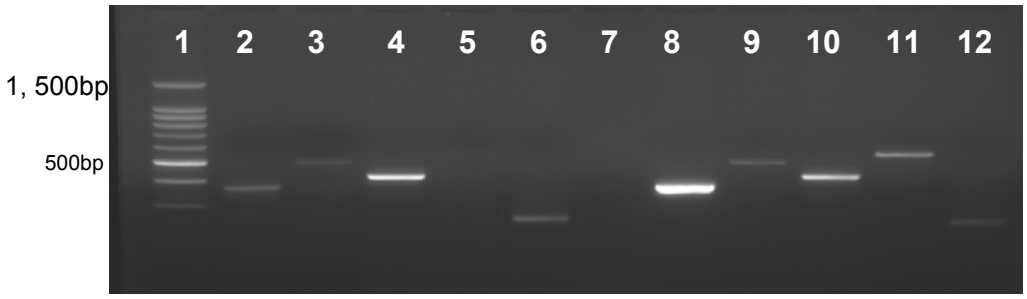


Figure 3.5: Agarose gel showing the PCR results of the ITG primers on BHK-21 (ATCC) cellular cDNA were Ta's of 57°C and 58°C were tested. 1: 100bp marker, 2: ITGβ1 Ta 58°C, 3: ITGβ3 Ta 57°C, 4: ITGβ6 Ta 58°C, 5: ITGβ8 Ta 57°C, 6: β-actin Ta 55°C, 7: negative control, 8: ITGβ1 Ta 57°C, 9: ITGβ3 Ta 58°C, 10: ITGβ6 Ta 57°C, 11: ITGβ8 Ta 58°C, 12: β-actin Ta 55°C.

To optimise the PCR utilising the HSPG PCR primers (Appendix A11), various Ta's were tested *i.e.* 55ºC, 57ºC and 58ºC (figure 3.6) using BHK4/98 and BHK31 cell samples and an annealing temp of 55ºC was optimum (lane 3).

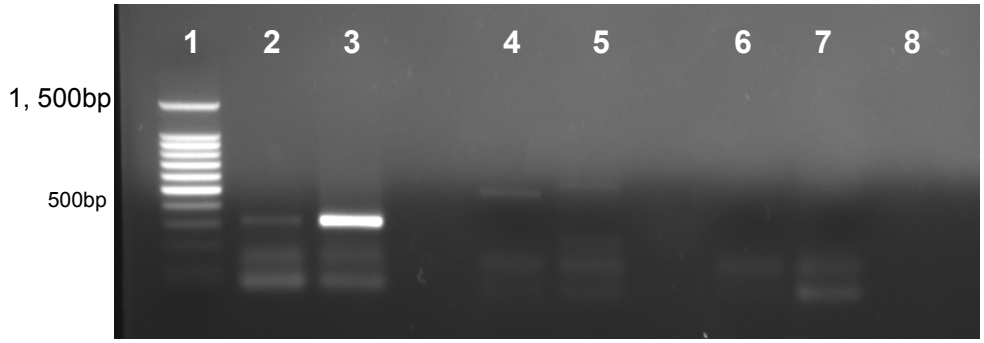


Figure 3.6:- Agarose gel showing the results of the HSPG RT-PCR when various annealing temperatures were tested. 1: 100bp marker, 2: BHK4/98: Ta = 55ºC, 3: BHK31: Ta = 55ºC, 4: BHK4/98: Ta = 57ºC, 5: BHK31: Ta = 57ºC, 6: BHK4/98: Ta = 58ºC, 7: BHK31: Ta = 58ºC, 8: Negative control.

3.3.2.2 IB-RS-2 cells

To develop an optimised PCR to detect the mRNA expression levels of HSPG, β1, β3, β6 and β8 cDNA in IB-RS-2 cells, the ITG primers (Appendix A10) and HSPG primers (Appendix A11) were used. For the PCR optimisation, varying annealing temperatures were tested (Appendix A14) and it was concluded that the optimised Ta for the IB-RS-2 mRNA PCR for β1 & β3 was 58°C (figure 3.7, lane 3 and lane 6 respectively), β6 & β8 was 53°C (figure 3.9, lane 2 and 5 respectively) and βactin was 55°C (figure 3.8, lane 7).

The expected fragment sizes were β1: ~370bp, β3: ~510bp, β6: ~420bp, β8: ~580bp and βactin: ~250bp.

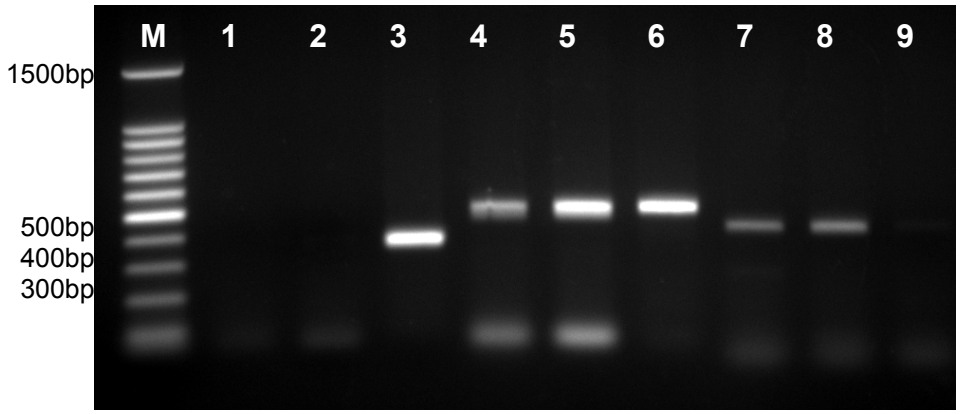


Figure 3.7: Agarose gel showing the IB-RS-2 β1 (1-3), β3 (4-6) and β6 (7-9) PCR at a Ta of 55°C (1, 4, 7), 57°C (2, 5, 8) and 58°C (3, 6, 9). The 100bp molecular weight maker is indicated by M.

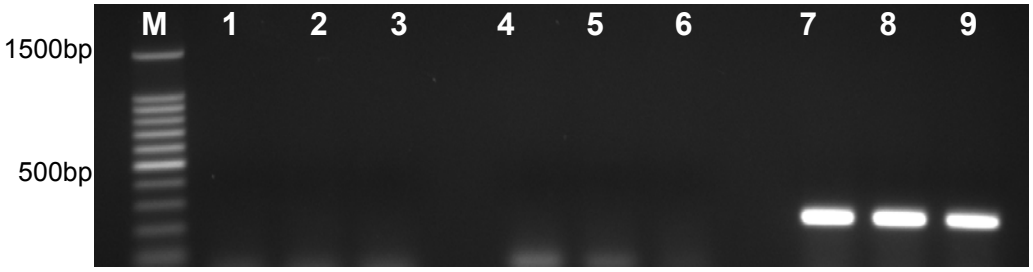


Figure 3.8: Agarose gel showing the IB-RS-2 β8 (1-3), HSPG (4-6) and βact (7-9) PCR at a Ta of 55°C (1, 4, 7), 57°C (2, 5, 8) and 58°C (3, 6, 9). The 100bp molecular weight maker is indicated by M.

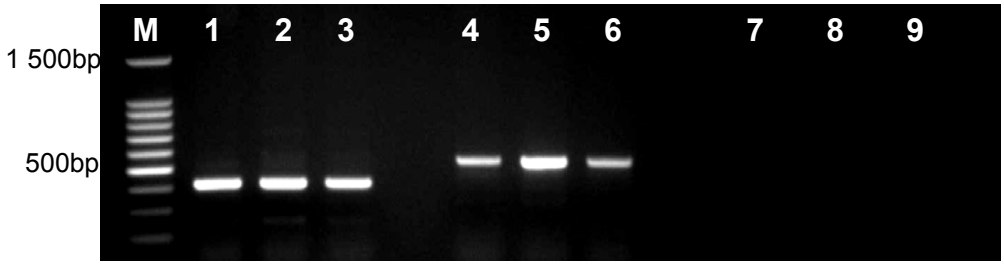


Figure 3.9: Agarose gel showing the IB-RS-2 β6 (1-3), β8 (4-6) and HSPG (7-9) PCR at a Ta of 52°C (1, 4, 7), 53°C (2, 5, 8) and 54°C (3, 6, 9). The 100bp molecular weight maker is indicated by M.

The HSPG PCR was continuously negative for IB-RS-2 cell samples despite various efforts to optimise the PCR (*i.e.* testing various Ta's and $MgCl_2$ concentrations) (figure 3.8 and 3.9) and was therefore excluded for this cell

line. All the integrin receptor RT-PCRs was successfully optimised for IB-RS-2 cells.

3.3.3 Investigating the mRNA expression levels on BHK-21 and IB-RS-2 cells

3.3.3.1 *Investigating the β1, β3, β6, β8 and HSPG mRNA expression levels with the increase in cell passage level (PL) for BHK-21 and IB-RS-2 cells*

The optimised BHK-21 and IB-RS-2 receptor RT-PCR was applied to investigate the intensity of the mRNA expression levels of the integrin subunits β1, β3, β6, β8 and HSPG cDNA on BHK-21 and IB-RS-2 cells during continuous cell passage. These results would indicate the effect, if any, that cell passaging has on the expression of the integrin and HSPG receptor mRNA. The intensity of the PCR products on the agarose gel was taken as an indication of the mRNA expression level of that particular cDNA and for each cell sample, RNA extraction; cDNA synthesis and PCR were performed in duplicate. It was decided to investigate the mRNA expression of β1, β3, β6, β8 and HSPG for BHK-21 and β1, β3, β6, β8 for IB-RS-2 cells from passage levels 2-26 and from 34-36. Due to time constraints PLs 27 to 33 were excluded but PLs 34 to 36 was included as we wanted to compare the mRNA expression level at early and late cell passage.

The intensity for each receptor PCR product for most cell samples varied significantly when compared to their corresponding duplicates. An example of this is shown in figures 3.10 and 3.11 for the BHK-21 and IB-RS-2 β1 PCRs respectively. From these figures, it can be seen that the PCR outlined in section 3.2.6 for the β1 cDNA, amplicon size of *ca*. 370bp, was successful. In some instances, when the PCR products were subjected to gel electrophoresis, it was observed that a second amplicon was present on the agarose gel (figures 3.10 and 3.11). This second fragment was probably due to the formation of primer-dimers which is defined as a small amplicon that results from the extension of self-annealed primers.

Due to steps taken to ensure that the cell count and RNA concentration for each sample were similar as well as using the β-actin PCR results as control and normaliser, similar expression levels were expected for a sample and it's duplicate. However, it is important to note that the RNA concentration for certain samples could not be adjusted to ~100ng/μl, even after ethanol precipitation, due to the initial concentrations being too low and thus could have had an effect on the results.

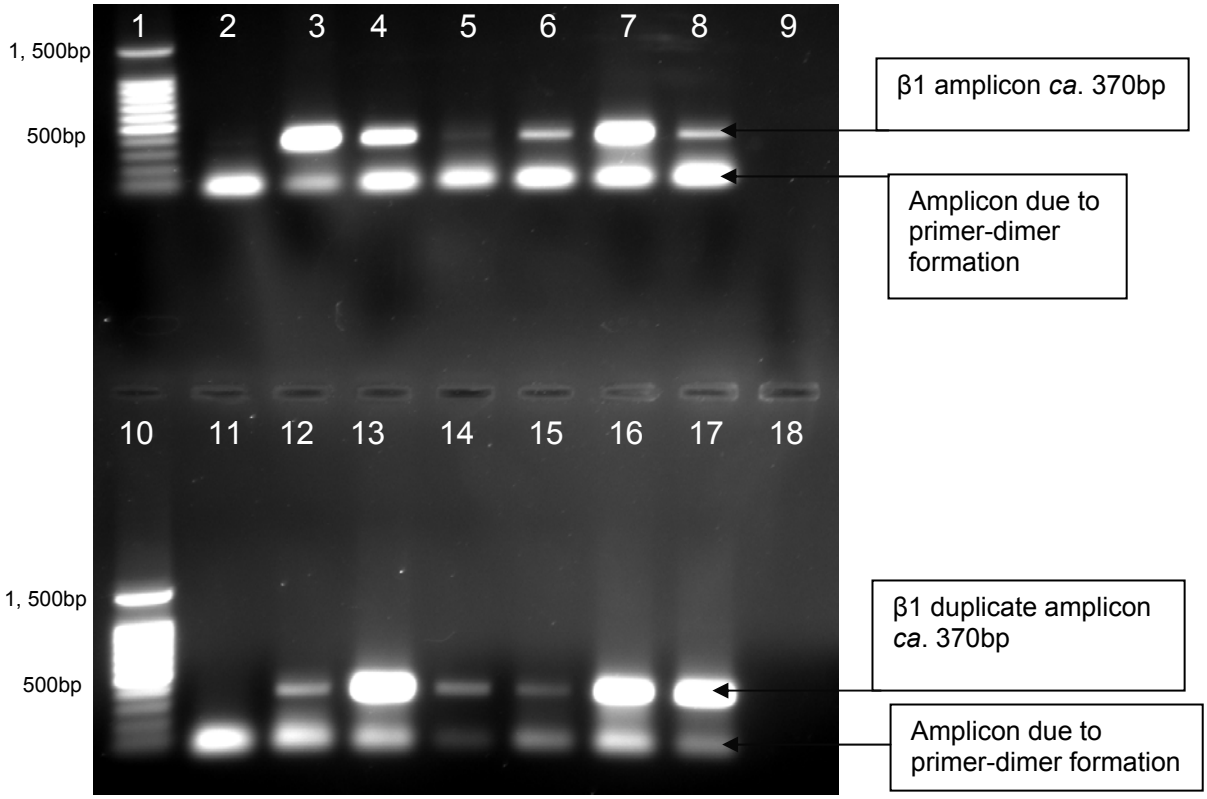


Figure 3.10: Agarose gel showing the BHK-21 β1 RT-PCR results with continuous cell passage from PL 20 to 26 as well as the corresponding duplicate PCR. The presence and intensity of the PCR product represent the mRNA intensity level for each sample. PCR of the cell samples at PL 20 (2), 21 (3), 22 (4), 23 (5), 24 (6), 25 (7), 26 (8) and each respective duplicate PCR result from lane 11 to 17 is shown. The negative control is indicated by 9 and 18 and the 100bp molecular weight marker by 1 and 10.

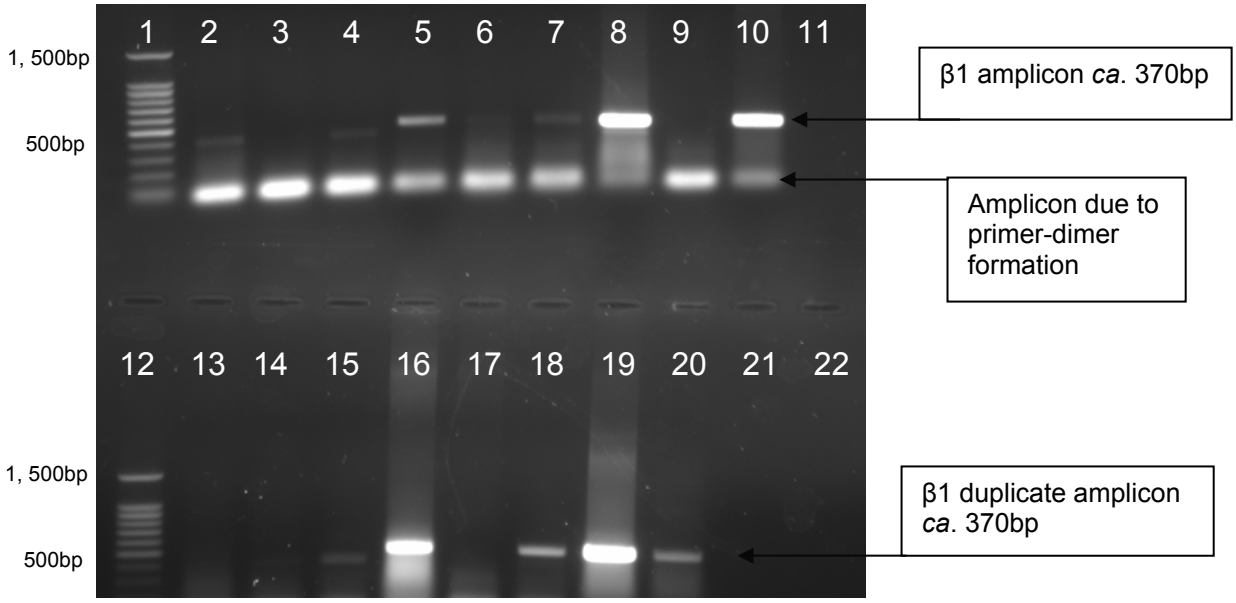
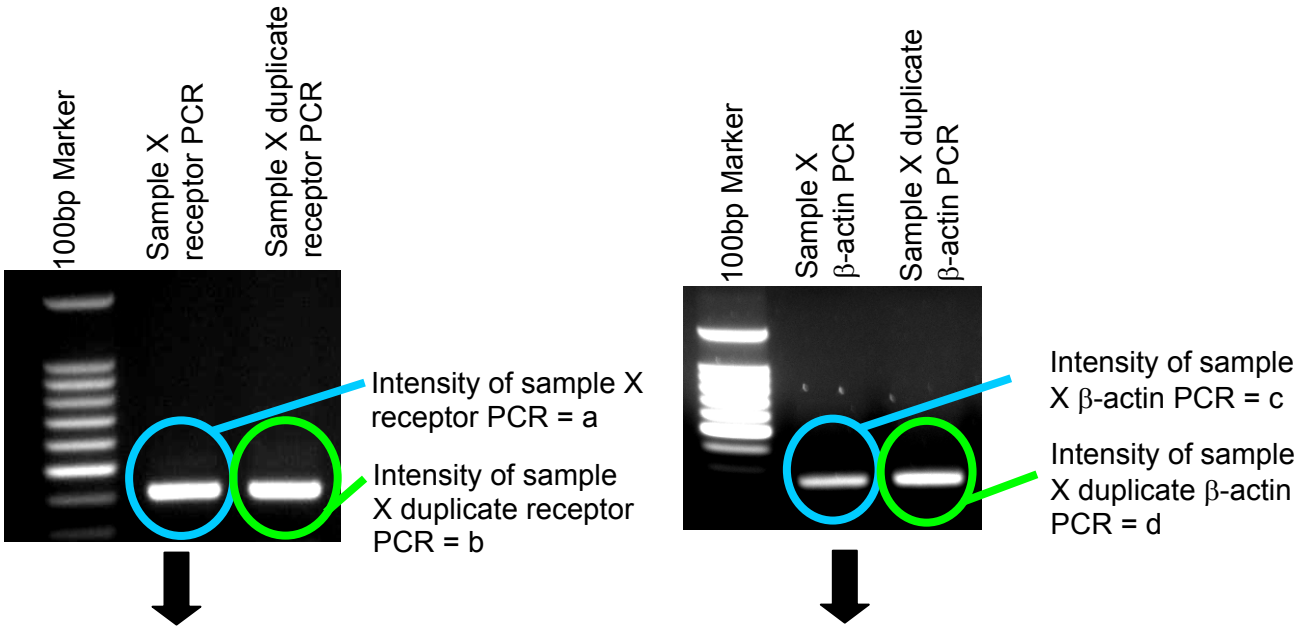


Figure 3.11 Agarose gel showing the IB-RS-2 β1 RT-PCR results from PL 11 to 19 as well as the corresponding duplicate PCR. The presence and intensity of the PCR product represents the mRNA intensity level for each sample. PCR of the cell samples at PL 11 (2), 12 (3), 13 (4), 14 (5), 15 (6), 16 (7), 17 (8), 18 (9), 19 (10) and each respective duplicate PCR result from lane 13 to 21 is shown. The negative control is indicated by 11 and 22 and the 100bp molecular weight marker by 1 and 12.

The mRNA expression level for each cell sample was calculated by taking the average of a sample and its corresponding duplicate intensity value. This calculation is illustrated in figure 3.12.



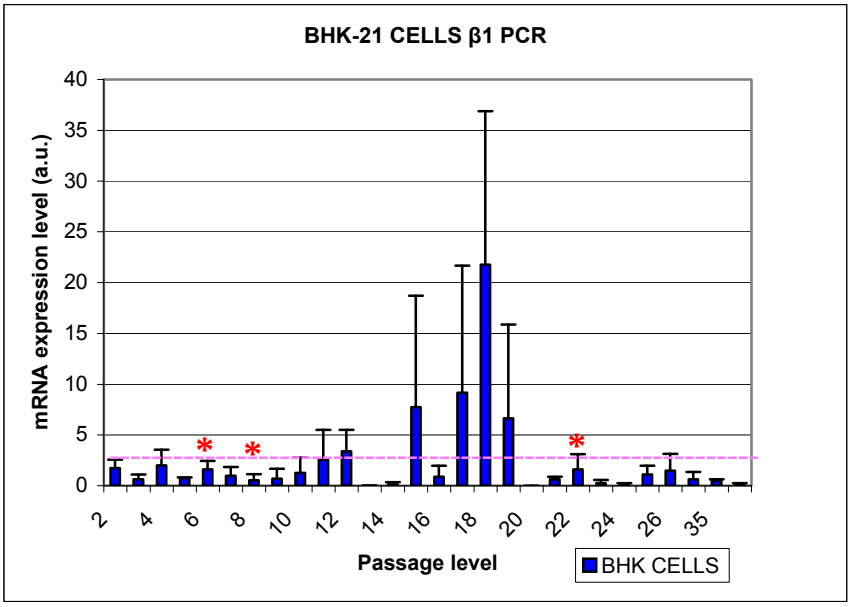
∴ **The mRNA expression level per sample = [(a / c) + (b / d)] / 2**

Figure 3.12: The mRNA expression level for each sample and its duplicate was determined by measuring the intensity of each PCR product on an

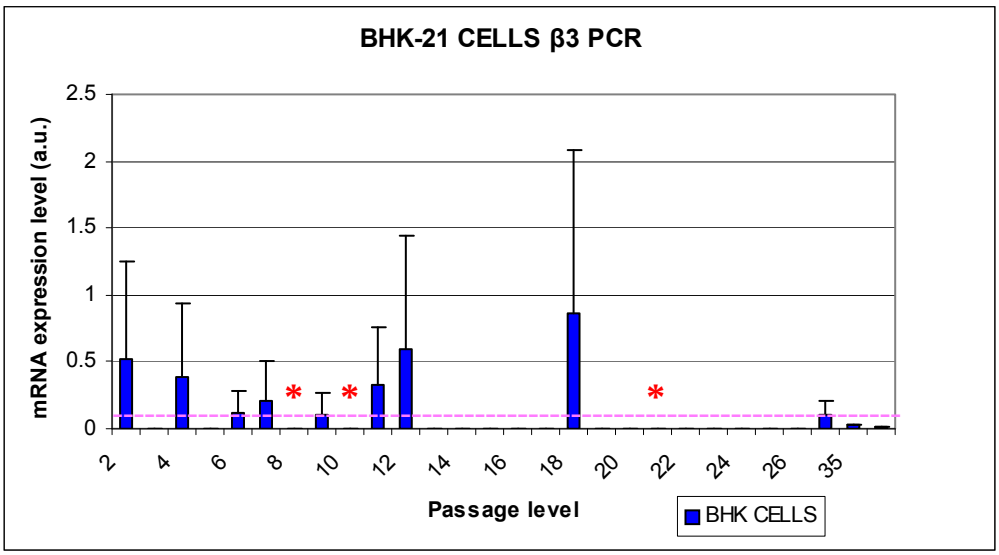
agarose gel using the GelTrak virtual densitometer computer program resulting in the values as arbitrary units (a.u.). These results were normalised to that of the β-actin PCR for the sample and its duplicate and thereafter the mRNA expression level calculated as per the above formula.

The averaged intensity value in arbitrary units (a.u.) for each cell sample and each receptor with continuous cell passage was plotted onto graphs from PLs 2 to 26 and 34 to 36 for BHK-21 cells (figure 3.13) and IB-RS-2 cells (figure 3.14).

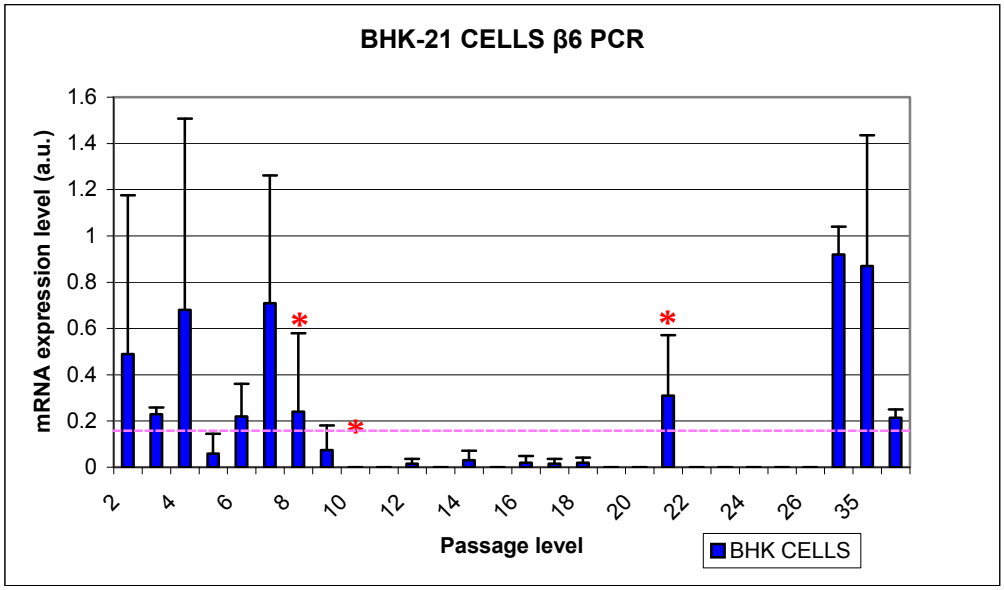
(a)



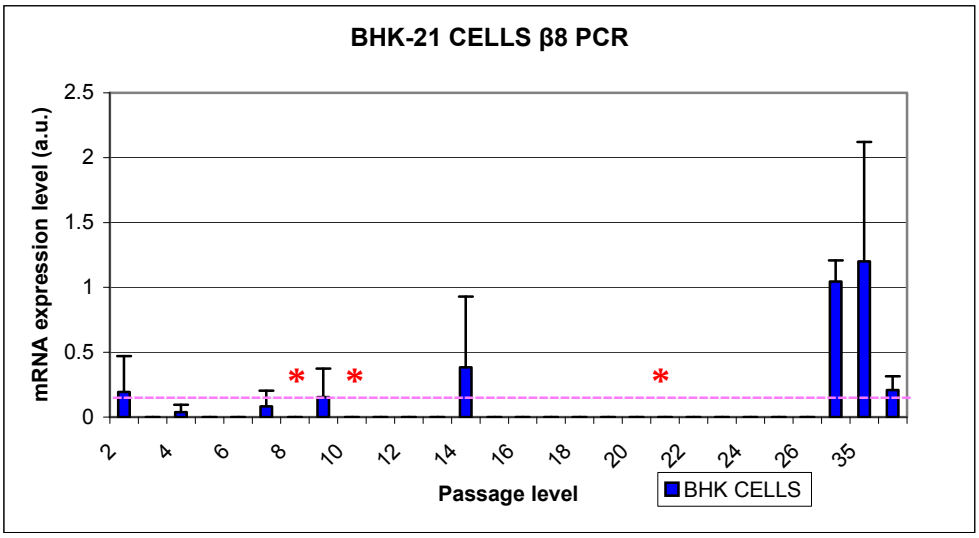
(b)



(c)


BHK-21 CELLS β6 PCR
mRNA expression level (a.u.)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2
4
6
8
10
12
14
16
18
20
22
24
26
35
Passage level
BHK CELLS

(d)


BHK-21 CELLS β8 PCR
mRNA expression level (a.u.)
2.5
2
1.5
1
0.5
0
2
4
6
8
10
12
14
16
18
20
22
24
26
35
Passage level
BHK CELLS

(e)

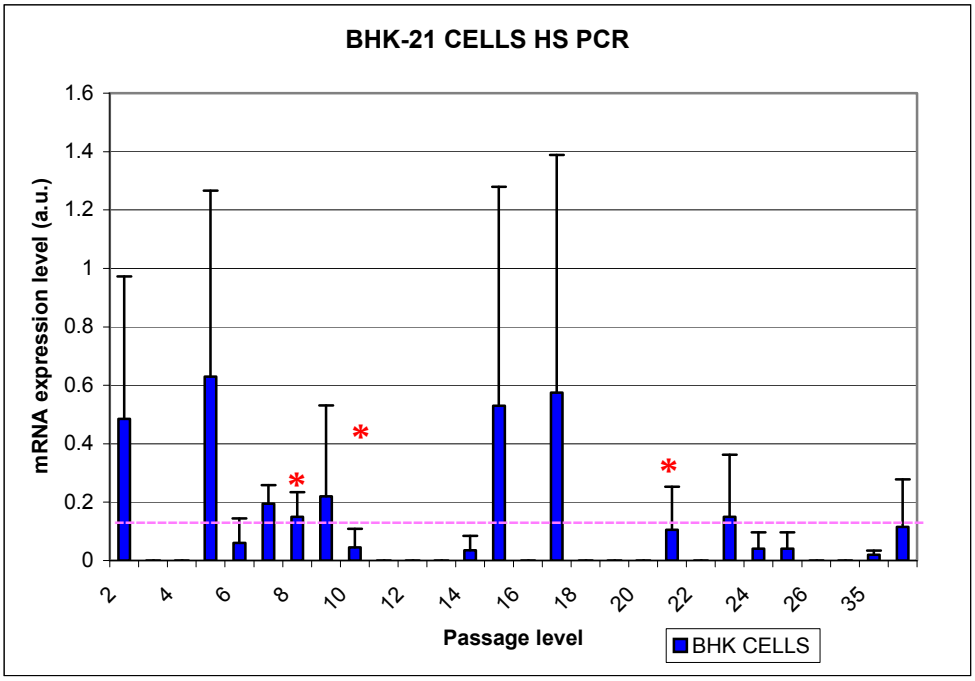


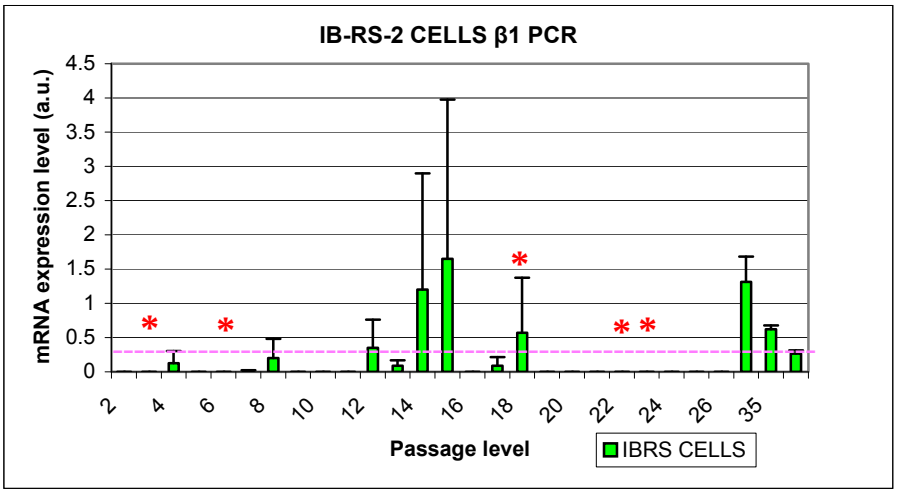
Figure 3.13: The β1 (a), β3 (b), β6 (c), β8 (d) and HSPG (e) mRNA expression levels from PLs 2 to 26 and 34 to 36 for BHK-21 cells as measured by the normalised intensity value of the RT-PCR product. The error bar indicates the standard deviation of the two values obtained for each sample. *: Represents samples where either both of the duplicate samples or one of the duplicates had low RNA concentrations *i.e.* below 100ng/ul. The --- line represents the average mRNA expression level for each receptor RT-PCR over 36 PLs.

The integrin and HSPG mRNA expression levels were normalised by comparison with the house-keeping gene, β-actin. The β-actin mRNA intensity value was thus 1a.u. An average of the mRNA expression levels were calculated across 36 passage levels for β1, β3, β6, β8 and HSPG for IB-RS-2 (figure 3.14) and BHK-21 (figure 3.13) cells which were found to be 2.47a.u., 0.12a.u., 0.18a.u., 0.12a.u. and 0.13a.u. for BHK-21 β1, β3, β6, β8 and HSPG whilst it was 0.23a.u., 0.52a.u., 0.11a.u. and 0.03a.u. for IB-RS-2 β1, β3, β6 and β8. These averages are indicated by the horizontal line (----) in figures 3.13 and 3.14.

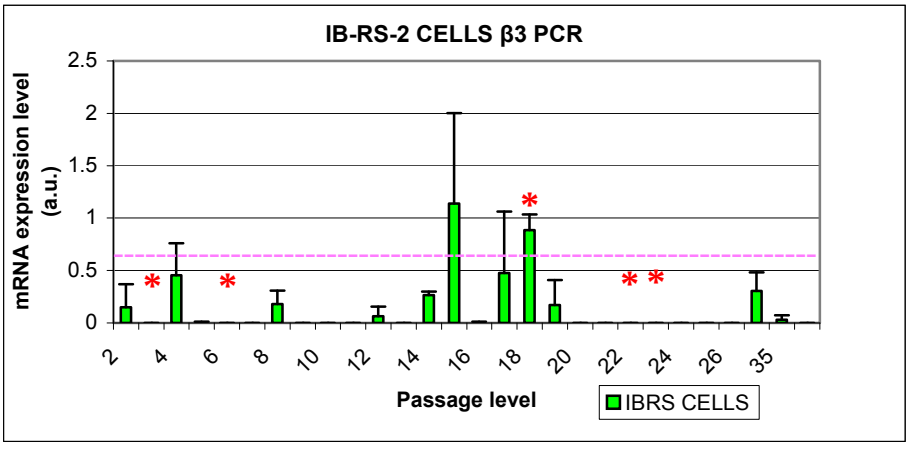
Comparing the β1 mRNA expression level for the BHK-21 cells to β-actin shows that the overall average expression levels observed was more than twice (2.47a.u.) that of β-actin (1a.u.) (figure 3.13a). For the 36 PLs, the β1

mRNA expression level was similar to the average expression (2.47a.u.) except for three PLs *i.e*. 15 and 17-19 where there was a substantial increase in the expression ranging from 7 – 22 a.u (figure 3.13a). Markedly lower than the average expression level for β1 was observed at only 6 PLs *i.e*. 13-14, 20, 23-24 and 36 (figure 3.13a). The average expression levels observed for BHK-21 β3 was lower *i.e*. 0.12a.u. than β-actin (figure 3.13b). An increase in β3 expression was observed at PLs 2, 4, 11-12 and 18 but the highest expression level was still less than β-actin *i.e*. ~0.8a.u. at PL 18 (figure 3.13b). The BHK-21 β6, β8 and HSPG average expression levels were also less than that of β-actin *i.e*. 0.18a.u., 0.12a.u. and 0.13a.u. where the highest expression levels observed for β6, β8 and HSPG were *ca*. 0.9a.u. (PL34), *ca*. 1.25a.u. (PL35) and *ca*. 0.62a.u. (PL5), which was almost equal to the β-actin expression (1a.u.) (figure 3.13c, d, e).

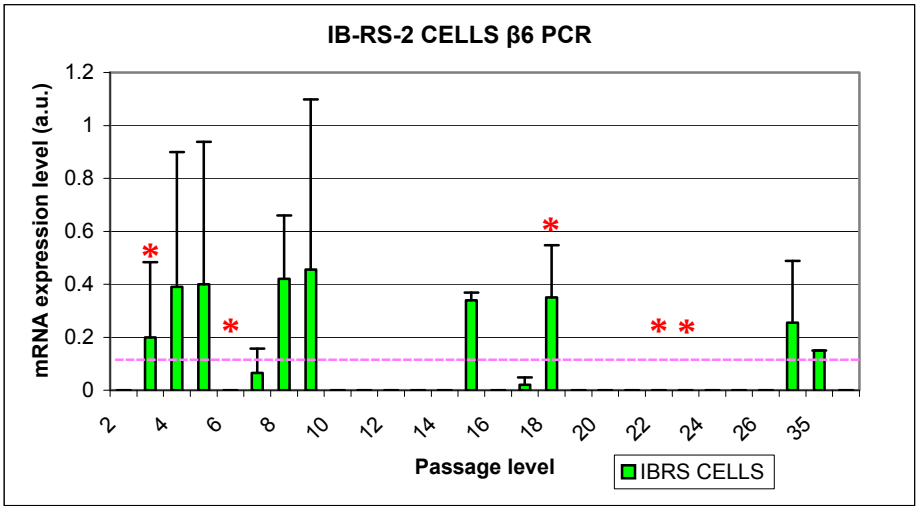
(a)



(b)



(c)



(d)

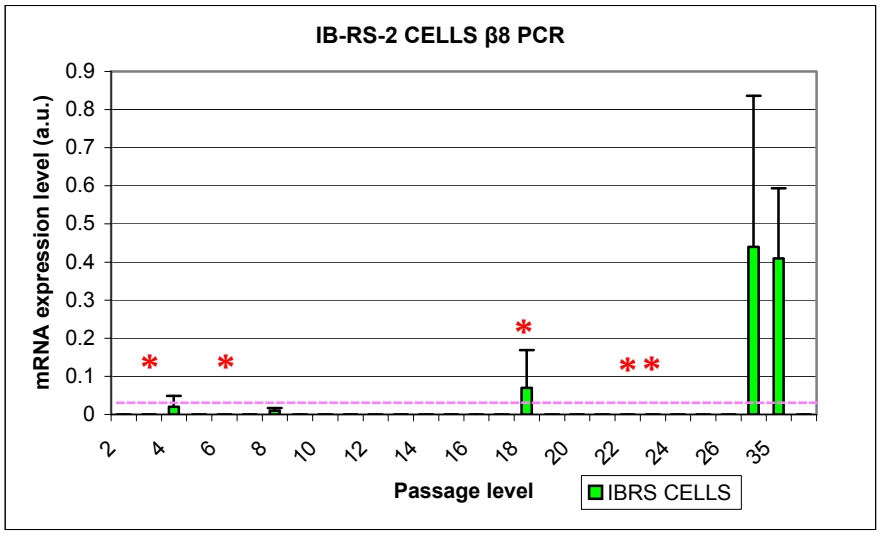


Figure 3.14: The β1 (a), β3 (b), β6 (c) and β8 (d) mRNA expression levels from PLs 2 to 26 and from 34 to 36 for IB-RS-2 cells. The error bar indicates the standard deviation of the two values obtained for each sample. *: Represents samples where either both of the duplicate samples or one of the duplicates had low RNA concentrations *i.e.* below 100ng/ul. The ---- line represents the average mRNA expression level for each receptor RT-PCR over 36 PLs.

The IB-RS-2 β integrin and HSPG mRNA expression levels observed were lower than that observed for the BHK-21 cells. An mRNA expression level of 0a.u. or near to 0a.u. was mostly observed for IB-RS-2 β1, β3, β6 and β8 over 36 PLs (figure 3.14). The average IB-RS-2 β1 expression was 0.23a.u., approximately 1/5th x that of β-actin, however an increase in expression level,

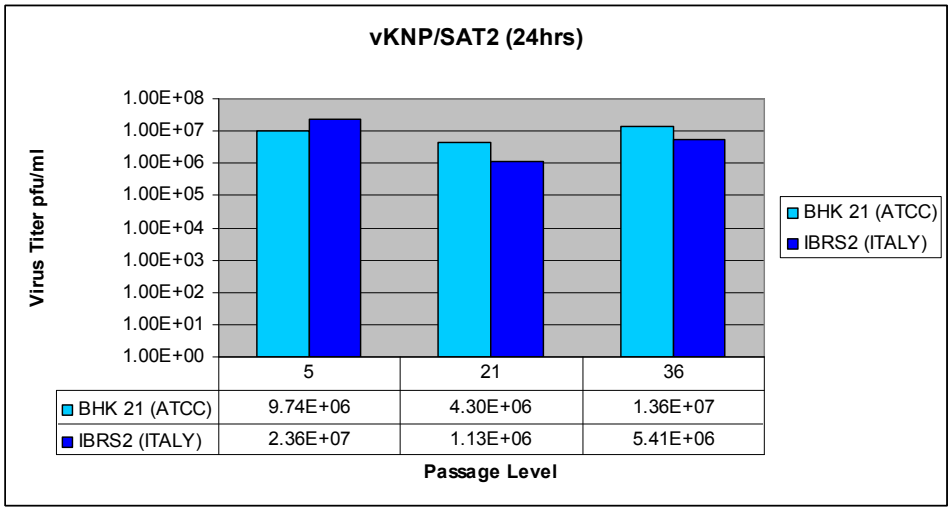
slightly higher to the β-actin expression level was observed at 3 PLs *i.e*. 14 (*ca*. 1.25a.u.), 15 (*ca.* 1.75a.u.) and 34 (*ca*. 1.48a.u.) (figure 3.14a). For β3 and β8, an evident increase in mRNA expression was observed during two PLs *i.e*. PLs 15 (*ca*. 1.25a.u.) and 18 (*ca*. 0.9a.u.) for β3 and PLs 34 (*ca*. 0.45a.u.) and 35 (*ca*. 0.4a.u.) for β8. The average expression observed for β6 was *ca*. 1/10th (0.11a.u.) that of β-actin where an increase in mRNA expression levels was at PLs 4 (*ca.* 0.4a.u.), 5 (*ca.* 0.4a.u.), 8 (*ca.* 0.42a.u.), 9 (*ca.* 0.45a.u.), 15 (*ca.* 0.38a.u.), 18 (*ca.* 0.39a.u.) and 34 (*ca.* 0.25a.u.). These results indicate that for all the β integrin subunits for IB-RS-2 cells, the mRNA expression levels were either noticeably lower than or similar to the β-actin expression of 1a.u. even during PLs were an increase was observed.

Overall, the mRNA expression levels were variable for the β integrins and HSPG with the increase in both IB-RS-2 and BHK-21 cell PL. In addition, at several PLs (figures 3.13 and 3.14) the RNA concentrations for these samples were below 100ng/μl, even after attempts to concentrate the RNA. However, due to the variability observed when the concentrations were optimal, it is assumed that the low RNA concentration probably did not influence the results too severely.

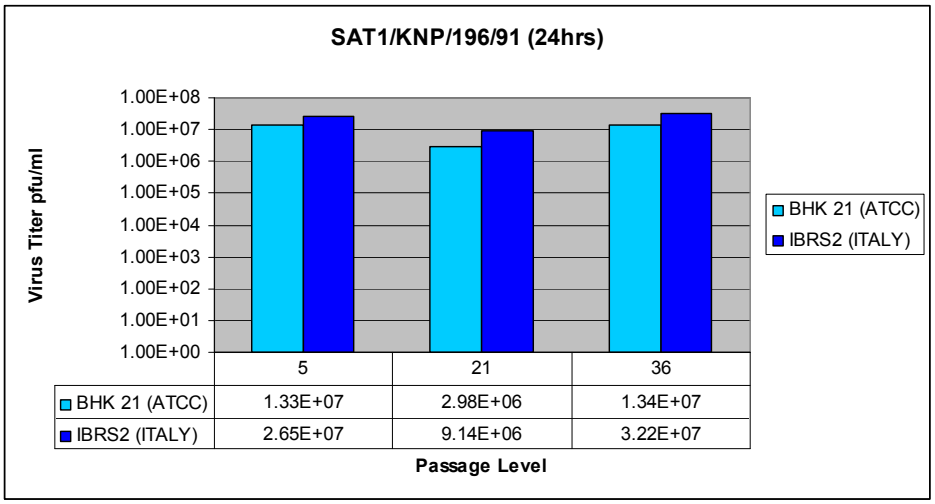
3.3.3.2 Plaque titration assays to measure the FMD virus susceptibility of the cells during increasing PLs

The susceptibility of BHK-21 and IB-RS-2 cells to infection by three FMDV strains with increasing cell PLs was determined by performing plaque titration assays (figure 3.15). It was also important to ascertain whether there was any correlation between the cell receptor mRNA expression levels and virus titre.

(a)



(b)



(c)

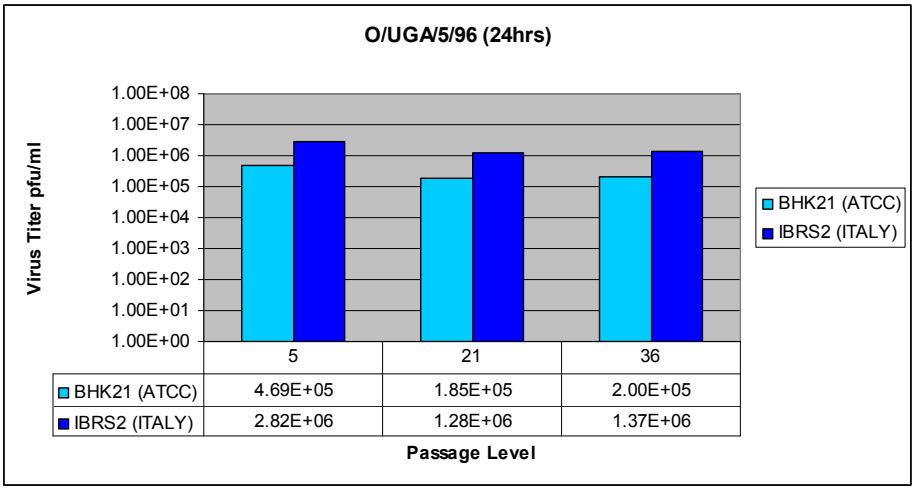


Figure 3.15: Plaque titration results for virus strains (a) vKNP/SAT2, (b) SAT1/KNP/196/91 and (c) O/UGA/5/96. The plaque titrations were performed for each virus strain in BHK-21 and IB-RS-2 cells at PLs 5, 21 and 36. The

titre at each PL represents an average of three plaque titration repeats and is indicated in the data table within the figures. In addition the standard deviation values in plaque forming units per millilitre (pfu/ml) are also shown in the data tables.

Virus titres were stable from PLs 5, 21 and 36 for all three virus strains. At the corresponding PLs for BHK-21 and IB-RS-2 cells, the β1, β3, β6, β8 and HSPG mRNA expression levels were low in all cases (figures 3.13 and 3.14). Thus, from this study, the susceptibility of BHK-21 and IB-RS-2 cells for FMDV infection after 36 sequential cell passaging could not be related to the receptor mRNA expression levels. Overall, the average titres for the IB-RS-2 cells were higher for all three virus strains at passage levels 5, 21 and 36 except for vKNP/SAT2 at PLs 21 and 36 only (figure 3.15) which may be an indication that the IB-RS-2 cells are more favourable (produce higher virus titres) for the FMDV type O, SAT1 and SAT2 infection. In addition, it was observed that there was a slight decrease in titre for all virus strains from PL 5 to 21 but an increase was observed from PL 21 to 36 for both BHK-21 and IB-RS-2 cells (figure 3.15). This variation may be due to the biological nature of the experiment such as variation within the cells and cell counts. The significance of this decrease in titre, if any, was investigated further through statistical analysis.

3.3.3.3 Statistical analysis of plaque titration assays

Statistical analysis was performed to determine if the variation in titre from PL 5 to 21 and from 21 to 36 was significant. The titres obtained for each Log_{10} dilutions from 10^{-4} to 10^{-6}, which were performed in triplicate for each dilution of the vKNP/SAT2, SAT1/KNP/196/91 and O/UGA/5/96 strains were used in the ANOVA test using the program GenStat (2003) (Ms Marie Smit, ARC-Biometry unit) to determine the differences between cell line and PLs as well as cell line by PL interaction (table 3.3). It is important to keep in mind for the statistical results (table 3.3) that if there is a significant difference in the F probability value, then this will have an affect on the Fisher's protected t-test

least significant difference (LSD) at the 1% level and thus there will be a value for the LSD.

Table 3.3:- Statistical results for three FMD virus strains with the increase in PLs for cell lines BHK-21 and IB-RS-2.

Cell Line	PL	Virus strain vKNP/SAT2	Virus strain SAT1/KNP196/91	Virus strain O/UGA/5/96
1. Statistical testing of differences between cell lines:				
BHK-21		7.00	6.961	3.08
IB-RS-2		6.06	7.403	5.69
SEM		0.503	0.0821	0.901
F probability		0.214	0.003	0.064
LSD(1%)		n/a	0.3678	n/a
2. Statistical testing of differences between PLs:				
n/a	5	7.12	7.358	5.23
n/a	21	5.39	6.712	3.88
n/a	36	7.08	7.476	4.04
SEM		0.616	0.1005	1.104
F probability		0.119	<0.001	0.648
LSD(1%)		n/a	0.4505	n/a
3. Statistical testing for BHK-21 and IB-RS-2 cell lines at PL 5, 21 and 36 for the three virus strains.				
BHK-21	5	7.09	7.126	3.85
	21	6.59	6.472	3.58
	36	7.30	7.284	1.83
IB-RS-2	5	7.15	7.589	6.62
	21	4.20	6.951	4.19
	36	6.85	7.667	6.25
SEM		0.871	0.1421	1.561
F probability		0.364	0.937	0.494
LSD(1%)		n/a	0.6371	n/a
CV%		23.1	3.4	61.7

SEM: Standard error of the means
LSD: Fisher's protected t-test least significant difference at the 1% level
CV%: Percentage coefficient of variation.
n/a: Not applicable

The statistical analysis revealed the following: -

1. Statistical testing of differences between cell lines: there was statistically a significant difference in susceptibility for virus strain SAT1/KNP196/91 between the two cell lines, BHK-21 and IB-RS-2, which is indicated by the low F probability value of 0.003 and the LSD of 0.3678 (table 3.3). No significant

difference was noted for vKNP/SAT2 and O/UGA/5/96 as the F probability was higher *i.e*. 0.214 and 0.064 respectively and there was no LSD value.

2. Statistical testing of differences between passage levels: the virus titres did not significantly change from passage level 5 to 21 to 36 for virus strains vKNP/SAT2 and O/UGA/5/96 (no LSD value therefore no significant difference found for the F probability, table 3.3). However, for SAT1/KNP196/91, there was a statistically significant difference between passage levels which was supported by a very low F probability *i.e*. <0.001 and a LSD of 0.4505 (table 3.3).

3. Statistical testing for BHK-21 and IB-RS-2 cell lines at passage level 5, 21 and 36 for the three virus strains: there was no significant difference between the cell line and the increase in passage levels for virus strains vKNP/SAT2 and O/UGA/5/96 (no LSD, table 3.3). However, for SAT1/KNP196/91 there was a significant difference for both cell lines where this difference was supported by a LSD of 0.6371 and a low CV% of 3.4% when compared to vKNP/SAT2 and O/UGA/5/96 (table 3.3).

The statistical analysis as previously said was performed to determine whether the decrease in titre observed for all three virus strains (SAT1/KNP196/91, vKNP/SAT2 and O/UGA/5/96) at PL 21 was significant. Overall the results indicate that this difference in titre observed from PLs 5 to 21 to 36 was statistically significant for only one virus strain *i.e*. SAT1/KNP196/91. However, the minor difference in values between PLs is expected and may be attributed to biological variation when performing the plaque titration assays.

3.4 DISCUSSION

From all the possible host factors involved in picornaviral pathogenesis, the viral receptor plays a major role in both host and tissue tropism (Evans and Almond, 1998; Rieder and Wimmer, 2002; Schneider-Schaulies, 2000). Thus, much research has been directed towards understanding the interactions of the virus with its host cell receptors. This study was a small step into improving our understanding of the expression of the β integrins and HSPG receptors at the mRNA level in BHK-21 and IB-RS-2 cells used at the FMDV vaccine production plant at ARC-TADP.

RT-PCRs were successfully developed and optimised to detect the presence and mRNA expression levels of β1, β3, β6, β8 and HSPG in BHK-21 and β1, β3, β6 and β8 in IB-RS-2 cell lines. The HSPG RT-PCR could not be established for IB-RS-2 cells, which was most likely due to sequence differences between the primers and the target. This was not surprising taking into account that the IB-RS-2 cells are a porcine kidney cell line and the HSPG sequences obtained from GenBank to design the HSPG primers were from different species (table 3.2). No HSPG porcine sequences could be obtained to design primers specifically for the IB-RS-2 cells.

The successfully optimised RT-PCRs were applied to determine if continuous cell passaging of a single cell sample (IB-RS-2 or BHK-21 cells) had an affect on the level of mRNA expression of the beta integrins and HSPG. The results showed that the mRNA expression levels were highly variable with continuous cell passage even though measures were taken to prevent inconsistent results. This variation in mRNA expression levels is normal as previous studies have been shown that integrin levels are often influenced by the differentiation state of the cell (Burger *et al*., 1992; Song *et al*., 1992; Ferreira *et al*., 1991) and can be modulated by soluble regulatory factors (Defilippi *et al*., 1991; Santala and Heino, 1991; Ignotz *et al*., 1989). Overall for the BHK-21 cells, the average mRNA expression levels across 36 PLs were highest for β1 followed by: β6 > HSPG > β3 = β8. However, the expression levels were

generally lower for the β integrins in IB-RS-2 cells than for the BHK-21 cells where the highest to lowest average expression across 36 PLs for the β integrins were β3 > β1 > β6 > β8. Thus, the results indicate that the β1 and β3 mRNA levels for BHK-21 and IB-RS-2 cells respectively were the highest but whether these mRNA expression levels correspond to receptor expression at the cell surface and to FMDV receptor usage is not well defined. However, Monaghan and co-workers (2005) showed that $\alpha_v\beta_6$ and not $\alpha_v\beta_3$ serves as a major receptor that determines the tropism of FMDV for the epithelia normally targeted by this virus in cattle. Additionally, the high expression of the αvβ6 protein at these sites correlated with the abundance of β6 mRNA, which proposes that the integrin mRNA expression levels may be indicative of the integrin protein expression levels on the cell surface *in vivo* but it is important to note that this might differ for in vitro studies.

Another study done by Duque and Baxt (2003) where the efficiencies of infection mediated by $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, and $\alpha_V\beta_6$ among three strains of FMDV serotype A and two strains of serotype O were compared using cells expressing these integins and the study showed that FMDV type O viruses used $\alpha_V\beta_6$ and $\alpha_V\beta_1$ with higher efficiency than $\alpha_V\beta_3$. In contrast, all the type A viruses used $\alpha_V\beta_3$ and $\alpha_V\beta_6$ with relatively high efficiency except for one type A virus which utilised $\alpha_V\beta_1$ with moderate efficiency, which indicated that FMDV serotypes can utilise any of its identified integrin receptors but with differences among different strains *in vitro*. Thus Duque and Baxt (2003) was able to show the efficiency of FMDV receptor use but whether this can be linked to the protein or mRNA receptor expression levels, still needs to be investigated for FMDV.

From the results obtained in this study, it can be concluded that 36 sequential cell passaging of either BHK-21 or IB-RS-2 cells does not have an effect on virus susceptibility as the change in virus susceptibility from PLs 5, 21 and 36 was not significant for three virus strains tested. Jensen and Norrild (2000) investigated the significance of cell passage on human embryonic lung fibroblasts (MRC-5) at various passage levels infected with herpes simplex

virus type 1 (HSV-1). Cells at high PLs contained significantly fewer infectious virus particles, very likely of lower virulence, and their expression of HSV-1 glycoproteins gC-1 and gD-1 were reduced, indicating that high PLs can have an effect on virus susceptibility. Thus the results obtained in this study do not rule out the possibility that FMDV susceptibility may have differed if the cells were passaged further than 36 PLs.

Unexpectedly the mRNA expression level for each sample and its corresponding duplicate differed despite the actions taken to equalize the outcome. The variability observed could be attributed to the fact that the RT-PCRs of the cell samples were performed at different times *i.e.* due to the high number of samples it was not possible to execute all the steps for all samples in one day. Thus, the variability that is observed could be due to variation in experimental conditions when the tests were performed on different days. The experimental techniques *i.e.* RNA extraction, cDNA synthesis and PCR were performed in duplicate for each cell sample, however, the results could have been more conclusive if the experimental techniques were carried out in triplicate or quadruplicate which was not possible due to time constraints. The RNA concentration could not be improved for certain samples due to the initial low RNA concentration; however the results indicated that this most probably did not increase the variability. Additionally, the cDNA was not normalised which may have impacted on the results.

The differences in mRNA expression levels observed may also be due to the mRNA half-life of the various receptors. Messenger RNA is transcribed as a complementary copy of DNA that will eventually be translated into an amino acid chain. In the cell, mRNA travels from the nucleus to the cytoplasm for translation and because mRNA is constantly degraded in the cytoplasm, it is synthesized at a much higher rate than necessary for maintenance of a steady amount (Wang *et al*., 2002; Jacobson and Peltz, 1999). Measuring the half-life of mRNA is difficult to determine experimentally because it is so short lived (Leclerc *et al*., 2002). However, knowledge of the half-life of mRNA can provide information about the stability of different types of mRNA, which in-

turn influences gene expression. No information could be gained about the mRNA half-life of the β1, β3, β6, β8 and HSPG in BHK-21 or IB-RS-2 cells and it is important to note that many factors affect mRNA half-lives. Ross (1995) indicated that environmental factors such as hormones, growth factors, ions etc. have an effect on mRNA stability, thus the mRNA half-life of a particular gene for one cell line may not necessarily be the same for another. It has been previously suggested that signals from extra-cellular matrix molecules and growth factors can modulate the mRNA decay rate of specific integrins (Xu and Clark, 1996). In addition, Feng *et al*. (1999) showed that the β3 mRNA stability could be increased by cell interaction with fibrin but not with collagen. It is therefore possible that various factors could have influenced the results in this study.

Integrin expression can be regulated by a variety of agents *i.e.* proinflammatory cytokines, growth factors, hormones, etc. (Delcommenne and Streuli, 1995; Kim and Yamda, 1997). For example the integrin $\alpha_v\beta_3$ has been shown to be up-regulated by transforming growth factor-β1 and platelet-derive growth factor-BB (Janat *et al*., 1992). Mechanisms regulating integrin expression include regulation of protein levels by transcriptional or post-transcriptional events or alternative splicing of RNA, which could in turn have an effect on mRNA expression (Kim and Yamda, 1997) and can explain why there were increases and decreases observed for continuous cell passage with the expression level of all the integrins and HSPG mRNA investigated in this study. Continuous cell passaging can also play a role in gene expression as indicated by a study where temporomandibular joint (TMJ) disc gene expression levels over multiple PLs using qRT-PCR were investigated for TMJ disc cells (Allen and Athanasiou, 2007). The results showed that gene expression levels of aggrecan, collagen type I and collagen type II dropped 20%, 23% and 73% per passage, respectively for 9 continuous cell passage levels (Allen and Athanasiou, 2007). Also, a study by O'Driscoll *et al*. (2006) showed that high passage or long-term culture of MIN-6 cells (a highly differentiated and glucose-responsive murine β-cell line) had significant affects on the expression of many mRNAs.

Retta *et al*. (2001) have investigated the novel mechanism of integrin cross talk between β1 and αv integrins and the affect of β1 on β3 stability. It was concluded that the expression of β1 integrins in GD25 cells (β1-null fibroblastic cell line) regulates the cell surface levels of $\alpha_v\beta_3$ where the decreased expression of integrin $\alpha_v\beta_3$ in these cells involves an increased decay rate of β3 mRNA, another factor that may have influenced the results of this study at particular PLs.

The choice of housekeeping gene *i.e.* β-actin could also have influenced the results. A housekeeping gene can be described as a gene that is constitutively expressed and is involved in basic functions needed for the sustenance of the cell (Synnergren *et al*., 2007). Yperman *et al*. (2004) evaluated β-actin as a housekeeping gene for interstitial cells (IC) of ovine heart valve tissue from PL 0 up to 5. A four-fold lower mRNA level was observed for freshly isolated IC than in cultured IC indicating variability of β-actin mRNA expression levels (Yperman *et al*., 2004). In addition, another study investigated the β-actin mRNA half-life for Nalm-6 and CCRF-CEM cells (Leclerc *et al*., 2002), which showed a two-fold difference for the β-actin mRNA half-life between the two cell lines. The explanation for this difference was attributed to the expression of the β-actin gene being regulated as a function of the cell cycle with transcription in the G_1 phase and mRNA decay in the G_2 phase. These studies show that there can be variation for the house-keeping gene, β-actin, which could explain why the β-actin mRNA expression levels in this study were found to be variable for each cell sample tested (results not shown) but the variation is also dependant on the type of study that is carried out.

It is important to note that the results from this study reflect the mRNA expression levels only and not the actual expression of the receptors on the cell surface. Translational processes may cause a difference in expression of the beta integrins and HSPG from the mRNA level compared to the expression at the protein level (Celis *et al*., 2000). In order to gain conclusive

results with regards to the mRNA expression levels of the beta integrins and HSPG on BHK-21 and IB-RS-2 cells, it is suggested that other experimental techniques be used such as real-time quantitative PCR, which is a highly sensitive, accurate and fast technique that offers high-throughput and the ability to quantify mRNA copy number. Alternatively investigating actual receptor expression in cells using immunofluorescence and confocal microscopy or fluorescence-activated cell sorting (FACS) is suggested.

This study has resulted in the development of optimised RT-PCRs for the detection of β1, β3, β6 and β8 cDNA on IB-RS-2 and BHK-21 cells and HSPG on BHK-21 cells, which could be a useful tool for the FMDV vaccine production section at ARC-TADP to provide an indication of the FMDV receptor mRNA expressed by the cells and hence success in virus propagation. Evidence to date indicates that the interaction of the FMD virus with host cell receptors mediates the foot-and-mouth disease infection process, however, it is not yet a well-understood process and there are many factors regarding FMDV infection that still remain unknown. This study was able to show that the mRNA expression levels of the FMDV receptors are most probably not stable with continuous cell passage, which could be due to many influencing factors. Information regarding the association of the mRNA expression levels, the expressed protein levels of the receptors and their presence on the cell surface would be more useful to determine the effect on virus susceptibility. However, whether the level of expression of the receptors on the cell surface has an influence on the FMDV receptor usage, still needs to be determined. This study was just a small step forward to understanding the FMDV receptor expression in cell lines used for FMDV vaccine production.

CHAPTER 4

CONCLUDING REMARKS AND FUTURE PROSPECTS

Globally FMDV poses a major problem as the adverse affects of the occurrence of the disease, outlined in chapter 1, show how the disease can directly affect a country's economical situation. The disease poses a major problem in Africa as it is highly contagious and endemic in most countries with six of the seven serotypes known to occur in Africa. In addition, the occurrence of FMDV in wildlife, where the African buffalo is the only wildlife species unequivocally shown to be capable of independently maintaining FMDV for long periods of time (Condy *et al*., 1985; Thomson, 1994, Thomson *et al*., 2003), makes the control and eradication of FMDV in Africa difficult. These facts emphasise the need to further our knowledge of the different FMDV serotypes by determining conserved, variable and functional differences in the FMDV genome sequences between and within the various FMDV serotypes in Africa and to further understand virus and host cell receptor interactions, which will advance our knowledge about the initiation of the FMD virus infection.

There is potentially a wide range of positive outcomes to be gained through FMDV sequencing. The Leader and P1 regions of 8 FMDV A and 9 FMDV O viruses from various regions in West and East Africa isolated over the last 33 years were successfully determined by genome-walking sequencing (chapter 2). These sequences allowed for various analyses to be undertaken such as the investigation of phylogenetic relationships between the African A and O viruses compared with GenBank available sequences of non-African A and O isolates for the L, P1, 1A, 1B, 1C and 1D coding regions. Additionally, nucleotide and amino acid sequence variation between and within the FMDV A and O serotypes as well as comparisons of the non-African and African A and O isolates with regards to previously identified antigenic sites were investigated.

Phylogenetic analysis revealed that the complete P1 region presented the best resolution of the relationships of the viruses included in this study with the isolates forming clusters according to serotype. The gene regions 1B, 1C and 1D encoding for the FMDV external structural proteins resulted in tree topologies similar to the P1 NJ tree but with inferior bootstrap support. For all these trees, the separate A and O serotype clusters were further divided into groups which contained either the African or non-African A and O isolates respectively. These groups had sub-clusters which were in some cases supported by high bootstrap values. It was generally observed that the viruses from West Africa and those from East Africa formed separate, strongly supported groupings, which indicated the distribution of the isolates to defined localities and point towards the existence of transboundary, but regionally spread of the African isolates. These results support previous studies where virus transmission between different countries has shown to be responsible for many outbreaks (Knowles *et al*., 2000).

The tree topology for the 1A gene region, which encodes for the internal capsid protein, only demonstrated one difference in that three non-African A isolates formed a strong grouping within the FMDV non-African O cluster, which could be due to recombination and although it is rare in the capsid coding region, it has been shown for FMDV (Pringle 1965; McCahon and Slade, 1981; King *et al*., 1982; 1985; McCahon *et al*., 1985; Giraudo *et al*., 1987; Wilson *et al*., 1988; Krebs and Marquardt, 1992).

The L region encodes for the non-structural protein *i.e*. the L proteinase and as expected the L NJ tree topology differed from those of the 1A, 1B, 1C, 1D and the complete P1. It was observed that the A and O isolates did not group strictly according to serotype or geographic region but instead there was a cluster which included the West and East African A and O viruses. This suggests that the African viruses share similarities or are closely related when comparing the L sequences, probably due to the occurrence of recombination events (King *et al*., 1982; 1985; Giraudo *et al*., 1987, van Rensburg *et al*., 2002; Jackson *et al*., 2007). The amount of variation observed for the L region was surprising as it is expected that a region encoding for the

proteinase would not tolerate vast genetic changes. However, it was observed that the regions within the L, found to be important for it's functioning e.g. its catalytic activity, were conserved for all isolates.

The variation observed for the capsid-coding regions were highest for VP1 and the nucleotide and amino acid variation observed in the outer capsid coding regions *i.e.* VP1, VP2 and VP3 is probably an indication of external immune pressures. Despite this marked variation, those regions previously identified as important for the virion stability was conserved (chapter 2). The high rate of genetic changes leading to antigenic differences of the FMD virus enables it to evade the host immune response; however the regions vital for its stability and functioning are conserved. In particular, the known integrin binding site, the RGD, was completely conserved for all viruses, which is vital for FMDV infection. Fox *et al*. (1989) demonstrated that mutations within the RGD or in the flanking leucine residue (RGDL) lead to a decrease of 60-73% in virus attachment depending on the serotype. Leippert *et al*. (1997) showed that mutations at and bordering the RGD affect the binding of FMD virus to susceptible cells. In this study, only one virus, A/SOM/1/78 out of all the African viruses had a L→M substitution at the RGD +1 position, which indicates the importance of the RGDL sequence. Furthermore, the RGDM sequence has been shown to play a role in inhibition of FMDV infection mediated by both $\alpha v\beta 6$ and $\alpha v\beta 8$ (Burman *et al*., 2007), which proposes that A/SOM/1/78 may not use these receptors for infection. The internally located VP4 protein was found to be the most conserved, which is probably due to its structural constraints and the fact that it faces few external pressures, resulting in hardly any genetic changes.

The attachment of FMDV to the host cell initiates the FMDV infection process. It is to this end that the FMDV cellular receptors have been investigated so extensively in the past (Berinstein *et al*., 1995; Sa-Carvalho *et al*., 1997 ; Fry *et al*., 1999; Jackson *et al*. 2000a; 2002; 2004). From chapter 2, it was shown that the previously identified important amino acids involved in both integrin and heparan sulphate receptor binding were conserved for all isolates.

However, there is still a lack of knowledge regarding FMD virus interaction with its identified cell receptors, which is the reason that in this study, a starting point was to investigate the mRNA expression of the known FMDV receptors for the β integrins (β1, β3, β6, β8) and HSPG on BHK-21 and IB-RS-2 cells (chapter 3). In addition, the mRNA expression levels with regards to continuous cell passage on these cell lines as well as virus susceptibility of three FMD virus strains at early and late passage levels were investigated. RT-PCRs were successfully developed and optimised and it was observed that the mRNA expression levels were highly variable with the increase in cell passage for all the β integrins and HSPG on both cell lines. As indicated from this study, the mRNA expression variation could be due to many factors (chapter 3). The investigation of the virus susceptibility for three FMD virus strains with continuous cell passage revealed that there was slight variation over 36 cell passages. This result, however, does not rule out the possibility that FMDV susceptibility may have differed if the cells were passaged further than 36 PLs. Studies have shown that cells at high passage levels can have an effect on virus susceptibility and result in fewer infectious virus particles (Jensen and Norrild, 2000). In addition, due to the variable levels of mRNA, the susceptibility of the BHK-21 and IB-RS-2 cells for FMDV infection could not be related to the receptor mRNA expression levels

This study has contributed to the better understanding of FMDV phylogenetic relationships of a small number of African isolates, the P1 and L sequence variation, important conserved functional regions as well as the mRNA expression of the receptors identified for FMDV. Additionally, this study forms the platform for FMDV complete genome sequencing where only the 5' and 3' untranslated regions as well as the P2 and P3 regions need to be sequenced in the future to obtain the complete genome of the FMD African viruses included in this study. This information would be useful for FMDV phylogenetic and molecular epidemiological studies as well as for comparative genomics.

The development and successful optimisation of the FMDV receptor mRNA RT-PCRs can now be taken further and adapted for real-time RT-PCR, which would make analysis of the mRNA expression for the β integrins and HSPG on BHK-21 and IB-RS-2 cells rapid and the results more reliable. Future investigations could involve determining the receptor protein expression levels on the cell surface and to determine whether the receptor mRNA expression levels correlate to the receptor protein levels expressed on the cell surface. Thereafter, whether the protein levels expressed on the cell surface influence FMDV receptor usage, could potentially be examined in future investigations.

CONFERENCE CONTRIBUTIONS

Chitray, M., Blignaut, B., Maree, F.F., Vosloo, W. Investigating the role of cellular receptors in foot-and-mouth disease virus adaptation to in vitro growth. Faculty Day 2007, University of Pretoria, Onderstepoort, South Africa, September 2007 (Poster).

Chitray, M., Blignaut, B., Maree, F.F., Vosloo, W. Heterogeneity in the outer capsid-coding region of foot-and-mouth disease virus A and O serotypes in Africa. World Association of Veterinary Laboratory Diagnosticians (WAVLD) 2007, Melbourne, Australia, 11 – 14 November 2007 (Poster).

Chitray, M., Blignaut, B., Maree, F.F., Vosloo, W. Investigating the mRNA expression level of foot-and-mouth disease virus receptors in production cells. Bio 08, Grahamstown, South Africa, 21 – 25 January 2008 (Poster).

REFERENCES

Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D.J. and Brown, F. (1989). The three-dimensional structure of foot-and-mouth disease virus at 2.9A resolution. *Nature* **337:** 709–716.

Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D.J. and Brown, F. (1990). The structure of foot-and-mouth disease virus: implications for its physical and biological properties. *Vet. Micro.* **23:** 21-34.

Alexandersen, S., Zhang, Z. and Donaldson, A.I. (2002). Aspects of the persistence of foot and mouth disease virus in animals – The carrier problem. *Microbes & Infect.* **4:** 1099-1110.

Alexandersen, S., Zhang, Z., Donaldson, A.I. and Garland, A.J.M. (2003). The pathogenesis and diagnosis of foot-and-mouth disease. *J. Comp. Path.* **129:** 1-36.

Allen, K.D. and Athanasiou, A. (2007). Effect of passage and topography on gene expression of temporomandibular joint disc cells. *Tissue Eng.* **13:** 101-110.

Bachrach, H.L., Trautman, R. and Breese, S.S. (1964). Chemical physical properties of virtually pure foot-and-mouth disease virus. *Am. J. Vet. Res.* **25:** 333-342.

Bachrach, H.L. (1968). Foot-and-mouth disease. *Annu. Rev. Microbiol*. **22:** 201-244.

Bachrach. H.L., Morgan, D.O., McKercher, P.D., Moore, D.M. and Robertson, B.H. (1982). Foot-and-mouth disease virus: immunogenicity and structure of fragments derived from capsid protein VP and of virus containing cleaved VP. *Vet. Microbiol.* **7:** 85-96.

Baranowski, E., Sevilla, N., Verdaguer, N., Ruiz-Jarabo, C.M., Beck, E. and Domingo, E. (1998). Multiple virulence determinants of foot-and-mouth disease virus in cell culture. *J. Virol.* **72:** 6362-6372.

Baranowski, E., Jarabo, R.C.M., Sevilla, N., Andreu, D., Beck, E. and Domingo, E. (2000). Cell recognition by foot and mouth disease virus that lacks the RGD integrin-binding motif: Flexibility in aphthovirus receptor usage. *J. Virol.* **74:** 1641-1647.

Baranowski, E., Ruiz-Jarabo, C.M. and Domingo, E. (2001). Evolution of cell recognition by viruses. *Science* **292:** 1102–1105.

Barteling, S.J. (2002). Development and performance of inactivated vaccines against foot-and-mouth disease. *Rev. Sci. Tech. Off. Int. Epiz.* **21:** 577-588.

Bartley, L.M., Donnelly, C.A. and Anderson, R.M. (2002). Review of foot-and-mouth disease virus survival in animal excretions and on formites. *Vet. Rec.* **151:** 667-669.

Baxt, B. and Bachrach, H.L. (1982). The Adsorption and degradation of foot-and-mouth disease virus by isolated BHK-21 cell plasma membranes. *Virol.* **116:** 391-405.

Baxt, B., Morgan, D.O., Robertson, B.H. and Timpone, C.A. (1984). Epitopes on foot-and-mouth disease virus outer capsid protein VP1 involved in neutralization and cell attachment. *J. Virol.* **51:** 298-305.

Baxt, B. and Morgan, D.O. (1985). Nature of the interaction between foot-and-mouth disease virus and cultured cells. In *Virus Attachment and Entry into Cells*, *Proceedings of an ASM Conference.* Edited by Cowell, R.L., Holm, K.L. ASM Press.

Baxt, B. and Becker, Y. (1990). The effect of peptides containing the arginine-glycine-aspartic acid sequence on the adsorption of foot-and-mouth disease virus to tissue culture cells. *Virus Genes* **4:** 73-83.

Beck, E. and Strohmaier, K. (1987). Subtyping of European foot-and-mouth disease virus strains by nucleotide sequence determination. *J. of Virol.* **61:** 1621-1629.

Belsham, G.J. and Bostock, C.J. (1988). Studies on the infectivity of foot-and-mouth disease virus RNA using microinjection. *J. Gen. Virol.* **69:** 265–274.

Belsham, G.J. (1993). Distinctive features of foot-and-mouth disease virus, a member of the picornavirus family; aspects of virus protein synthesis, protein processing and structure. *Biophys. Molec. Biol.* **60:** 241-260.

Belsham, G.J., McInerney, G.M. and Ross-Smith, N. (2000). Foot-and-mouth disease virus 3C protease induces cleavage of translation initiation factors eIF4A and eIF4G within infected cells. *J. Virol*. **74:** 272-280.

Belsham, G. J. and Martínez-Salas, E. (2004). Genome organisation, translation and replication of foot-and-mouth disease virus RNA. In *Foot and Mouth Disease: Current Perspectives*, pp. 19–52. Edited by Domingo, E., Sobrino, F. Norfolk, UK. Horizon Bioscience.

Berinstein, A., Rovainen, M., Hovi, T., Mason, P.W. and Baxt, B. (1995). Antibodies to the vitronectin receptor (integrin αvβ3) inhibit binding and the

infection of foot-and-mouth disease virus to cultured cells. *J. of Virol.* **69:** 2664-2666.

Birtley, J.R., Knox, S.R., Jaulent, A.M., Brick, P., Leatherbarrow, R.J. and Curry, S. (2005). Crystal structure of foot-and-mouth disease virus 3C protease: New insights into catalytic mechanism and cleavage specificity. *J. Biol. Chem.* **280:** 11520–11527.

Blanco, E., Garcia-Briones, M., Sanz-Parra, A., Gomes, P., De Oliveira, E., Valero, M.L., Andreu, D., Ley, V. and Sobrino, F. (2001). Identification of T-Cell epitopes in nonstructural proteins of foot-and-mouth disease Virus. *J. Virol.* **75:** 3164-3174.

Boom, R., Sol, C.J., Salimans, M.M.M., Jansen, C.L., Wertheim-van Dillen, P.M.E. and van Der Noordaa, J. (1990). Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28:** 495-503.

Breuss, J.M., Gallo, J. DeLisser, H.M., Klimanskaya, I.V., Folkesson, H.G., Pittet, J.F., Nishimura, S.L., Aldape, K., Landers, D.V., Carpenter, W., Gillett, N., Sheppard, D., Matthay, M.A., Albelda, S.M., Kramer, R.H. and Pytela, R. (1995). Expression of the β6 integrin subunit in development, neoplasia and tissue repair suggests a role in epithelial remodeling. *J. of Cell Science* **108:** 2241-2251.

Brooksby, J.B. (1967). Foot-and-mouth diseases – A world problem. *Nature* **231:** 120-122.

Brooksby, J.B. (1982). Portraits of viruses: foot-and-mouth disease virus. *Intervirology* **18:** 1–23.

Brown, F. (1999). Foot and mouth disease and beyond: Vaccine design, past, present and future. *Arch. Virol.* **15:** 179-188.

Burger, S.R., Zutter, M.M., Sturgill, K.S. and Santoro, S.A. (1992). Induced cell surface expression of functional alpha 2 beta 1 integrin during megakaryocytic differentiation of K562 leukemic cells. *Exp. Cell. Res*. **202:** 28–35.

Burman, A., Clark, S., Abrescia, N.G.A., Fry, E.E., Stuart, D.I. and Jackson, T. (2007). Specificity of the VP1 GH loop of foot-and-mouth disease virus for αv integrins. *J. Virol.* **80:** 9798-9810.

Burroughs, J.N., Rowlands, D.J., Sangar, D.V., Talbot, P. and Brown, F. (1971). Further evidence for multiple proteins in the foot-and-mouth disease virus particle. *J Gen Virol* **13:** 73-84.

Burroughs, J.N., Sangar, D.V., Clarke, B.E., Rowlands, D.J., Billiau, A. and Collen, D. (1984). Multiple proteases in foot-and-mouth disease virus replication. *J. Virol.* **50:** 878-883.

Calvete, J.J., Henschen, A. and González-Rodriguez, J. (1991). Assignment of disulphide bonds in human platelet GPIIIa. A disulphide pattern for the β-subunits of the integrin family. *Biochem. J.* **274:** 63–71.

Carrillo, C., Tulman, E.R., Delhon, G., Lu, Z., Carreno, A., Vagnozzi, A., Kutish, G.F. and Rock, D.L. (2005). Comparative genomics of Foot and mouth disease virus. *J. Virol.* **79:** 6487-6504.

Carrillo, C., Lu, Z., Borca, M.V., Vagnozzi, A., Kutish, G.F. and Rock, D.L. (2007). Genetic and phenotypic variation of FMDV during serial passages in a natural host. *J. Virol.* **81:** 11341-11351.

Caspersen, K., Park, J., Patil, S. and Dumler, J.S. (2002). Genetic variability and stability of *Anaplasma phagocytophila msp2* (p44). *Infection and Immunity* **70:** 1230-1234.

Celis, J.E., Kruhoffer, M., Gromova, I., Frederiksen, C., Ostergaard, M., Thykjaer, T., Gromov, P., Yu, J., Palsdottir, H., Magnusson, N. and Orntoft, T.F. (2000). Gene expression profiling: monitoring transcription and translation products sing DNA mircoarrays and proteomics. *FEBS Letters* **480:** 2-16.

Cheung, A., De Lamarter, J., Weiss, S. and Kupper, H. (1983). Comparison of the major antigenic determinants of different serotypes of foot-and-mouth disease virus. *J. Virol*. **48:** 451-459

Chomczynski, P. and Sacchi, N. (1987). Single Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction. *Analytical Biochemistry* **162:** 156.

Chow, M., Newman, J.F.E., Filman, D., Hogle, J.M., Rowlands, D.J. and Brown, F. (1987). Myristylation of picornavirus capsid protein VP4 and its structural significance. *Nature* **327:** 482-486.

Clarke, B.E., Sangar, D.V., Burroughs, J.N., Newton, S.E., Carroll, A.R. and Rowlands, D.J. (1985). Two initiation sites for foot-and-mouth disease virus polyprotein *in vivo. J. Gen. Virol.* **66:** 2615-2626.

Clavero, M.A.J., Nunez, J.I., Rosas, M.F., Baranowski, E. and Ley, V. (2001). Foot-and-mouth disease virus: A long known virus, but a current threat. *Vet. Res.* **32:** 1-30.

Condy, J.B. and Hedger, R.S. (1974). The survival of foot-and-mouth disease virus in African buffalo with non-transference of infection to domestic cattle. *Res. Vet. Sci*. **16:** 182-185.

Condy, J.B., Hedger, R.S., Hamblin, C. and Barnett, I.T.R. (1985). The duration of the foot-and-mouth disease virus carrier state in african buffalo (i) in the individual animal and (ii) in a free-living herd. *Comp. Immun. Microbiol. Infect. Dis.* **8:** 259–265

Crowther, J.R., Farias, S., Carpenter, W.C. and Samuel, A.R. (1993). Identification of a fifth neutralisable site on type O foot-and-mouth disease virus following characterization of single and quintuple monoclonal antibody escape mutants. *J. Gen. Virol.* **74:** 1547–1553.

Curry, S., Abrams, C.C., Fry, E., Crowther, J.C., Belsham, G.J., Stuart, D.I. and King, A.M.Q. (1995). Viral RNA modulates the acid sensitivity of foot-and-mouth disease virus capsids. *J. Virol.* **69:** 430-438.

Curry, S., Fry, E., Blakemore, W., Abu-Ghazaleh, R., Jackson, T., King, A., Lea, S., Newman, J., Rowlands, D. and Stuart, D. (1996). Perturbations in the surface structure of A22 Iraq foot-and-mouth disease virus accompanying coupled changes in host cell specificity and antigenicity. *Structure* **4:** 135-145.

Curry, S., Fry, E., Blakemore, W., Abu-Ghazaleh, R., Jackson, T., King, A., Lea, S., Newman, J. and Stuart, D. (1997). Dissecting the roles of VP0 cleavage and RNA packaging in Picornavirus capsid stabilization: the structure of empty capsids of foot-and-mouth disease virus. *J. Virol.* **71:** 9743–9752.

Curry, S., Roqué-Rosell, N., Zunszain, P.A. and Leatherbarrow, R.J. (2007). Foot-and-mouth disease virus 3C protease: recent structural and functional insights into an antiviral target. *Int J Biochem Cell Biol*. **39:** 1-6.

Dedhar, S. and Hannigan, G.E. (1996). Integrin cytoplasmic interactions and bi-directional transmembrane signaling. *Curr. Opin. Cell. Bio.* **8:** 657–669.

Defilippi, P., Truffa, G., Stefanuto, G., Altruda, F., Silengo, L. and Tarone, G. (1991). Tumor necrosis factor alpha and interferon gamma modulate the expression of the vitronectin receptor (integrin beta 3) in human endothelial cells. *J. Biol. Chem*. **266:** 7638-7645.

Delcommenne, M. and Streuli, C.H. (1995). Control of integrin expression of extracellular matrix. *J. Biol. Chem.* **270:** 26794-26801.

Devaney, M.A., Vakharia, V.N., Lloyd, R.E., Ehrenfeld, E. and Grubman, M.J. (1988). Leader protein of foot-and-mouth disease virus is required for cleavage of the p220 component of the cap-binding protein complex. *J. Virol.* **62:** 4407-4409.

DiCara, D., Burman, A., Clark, S., Berryman, S., Howard, M.J., Hart, I.R., Marshall, J.F. and Jackson, T. (2008). Foot-and-mouth disease virus forms a highly stable EDTA-resistant complex with its principle receptor, integrin αvβ6: implications for infectiousness. *J. Virol*. **82:** 1537-1546.

Domingo, E., Martinez-Salas, E., Sobrino, F., de la torre, C.J., Portela, A., Ortin, J., Lopez-Galindez, C., Perez-Brena, P., Villanueva, N., Najera, R., VandePol, S., Steinhauer, D., DePolo, N. and Holland, J. (1985). The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance – a review. *Gene* **40:** 1-8.

Domingo, E. and Holland, J.J. (1997). RNA virus mutations and fitness for survival. *Annu. Rev. Microbiol*. **51:** 51–178.

Domingo, E., Baranowski, E., Escarmis, C. and Sobrino, F. (2002). Foot-and-mouth disease virus. *Comp. Imm. Micro. & Infect. Dis.* **25:** 297-308.

Domingo, E., Escarmis, C., Baranowski, E., Ruiz-Jarabo, C.M., Carrillo, E., Nunez, J.I. and Sobrino, F. (2003). Evolution of foot-and-mouth disease virus. *Virus Res.* **91:** 47-63.

Donaldson, A.I. (1972). The influence of relative humidity on the aerosol stability of different strains of foot-and-mouth disease virus suspended in saliva. *J. Gen. Virol.* **15:** 25–33.

Donaldson, A.I., Doel, T.R. (1992). Foot-and-mouth disease: the risk for Great Britain after 1992. *Vet. Rec.* **131:** 114–120.

Donaldson, A.I. and Kitching, R.P. (1989). Transmission of foot-and-mouth disease by vaccinated cattle following natural challenge. *Research in Vet. Sci.* **46:** 9–14.

Duque, H. and Baxt, B. (2003). Foot-and-mouth disease virus receptors: Comparison of bovine αv integrin utilization by type A & O viruses. *J. Virol.* **77:** 2500-2511.

Duque. H., LaRocco, M., Golde, W.T. and Baxt, B. (2004). Interactions of foot-and-mouth disease virus with soluble bovine αvβ3 and αvβ6 integrins. *J. Virol.* **78:** 9773-9781.

Ellard, F.M., Drew, J., Blakemore, W.E., Stuart, D.I. and King, A.M. (1999). Evidence for the role of His-142 of protein 1C in the acid-induced disassembly of foot-and-mouth disease virus capsids. *J. Gen. Virol*. **80:** 1911-1918.

Etchison, D., Milburn, S.C., Edery, I., Sonenberg, N. and Hershey, J.W.B. (1982). Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. *J. Biol. Chem*. **257:** 14806-14810.

Evans, D.J. and Almond, J.W. (1998). Cell receptors for picornaviruses as determinants of cell tropism and pathogenesis. *Trends in Micro.* **6:** 198-202.

Falk, M.M., Grigera, P.R., Bergmann, I.E., Zibert, A., Multhaup, G. and Beck, E., (1990). FMDV protease 3C induces specific proteolytic cleavage of host cell histone H3. *J. Virol*. **64:** 748- 756.

Feng, X., Clark, R.A., Galanakis, D. and Tonnesen, M.G. (1999). Fibrin and collagen differentially regulate human dermal microvascular endothelial cell integrins: stabilization of αv/β3 mRNA by fibrin1. *J. Invest. Dermatol.* **113:** 913-919.

Ferreira, O.C.J., Valinsky, J.E., Sheridan, K., Wayner, E.A., Bianco, C. and Garcia-Pardo, A. (1991). Phorbol ester-induced differentiation of U937 cells enhances attachment to fibronectin and distinctly modulates the alpha 5 beta 1 and alpha 4 beta 1 fibronectin receptors. *Exp. Cell. Res*. **193:** 20-26.

Ferrer-Orta, C. and Fita, I. (2004). Structure of foot-and-mouth disease virus particles. In *Foot-and-mouth disease: Current perspectives*, pp 77-92. Edited by Sobrino, F., Domingo, E. Horizon Biosciences.

Ferris, N.P. and Donaldson, A.I. (1992). The World Reference Laboratory for foot-and-mouth disease: a review of 33 years of activity (1958-1991). *Rev. Sci. Tech. Off. Int. Epiz.* **11:** 657-684.

Fox, G., Parry, N.R., Barnett, P.V., Mcginn, B., Rowlands, D.J. and Brown, F. (1989). The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). *J. Gen. Virol.* **70:** 625–637.

Freshney, R. (1987). Culture of Animal Cells: In *A Manual of Basic Technique*, pp 117. Edited by Alan, R. Wiley-Liss, Inc., New York.

Freshney, R.I. (1994). Culture of animal cells. In *A manual of basic technique*, 3rd edition. Edited by Alan, R. Wiley-Liss, Inc., New York, USA.

Fry, E., Lea, S.M., Jackson, T., Newman, J.W.I., Ellard, F.M., Blakemore, W.E., Ghazaleh, R.A., Samuel, A., King, A.M.Q. and Stuart, D.I. (1999). The structure and function of a foot and mouth disease virus-oligosaccharide receptor complex. *The EMBO Journal.* **18:** 543-554.

Galloway, I.A., Henderson, W.M. and Brooksby, J.B. (1948). Strains of the virus of foot-and-mouth disease recovered from outbreaks in Mexico. *Virology* **67:** 57–84.

George, M., Venkatatamanan, R., Gurumurthy, C.B. and Hemadri, D. (2001). The non-structural Leader protein of foot-and-mouth disease virus is highly variable between serotypes. *Virus Genes* **22:** 271-278.

Giraudo, A.T., Sagedahl, A., Bergmann, I.E., La Torre, J.L. and Scodeller, E.A. (1987). Isolation and characterization of recombinants between attenuated and virulent aphthovirus strains. *J. Virol*. **61:** 419-425.

Gorbalenya, A.E., Koonin, E.V. and Lai, M.M. (1991). Putative papain-related thiol proteases of positive strand RNA viruses. Identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi-, alpha- and coronaviruses. *FEBS Lett.* **288:** 201-205.

Grubman, M.J. and Baxt, B. (2004). Foot-and-mouth disease. *Clin. Micro. Rev.* **17:** 465-493.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser*. **41:** 95-98.

Harber, J.J., Bradley, J., Anderson, C.W. and Wimmer, E. (1991). Catalysis of poliovirus VP0 maturation cleavage is not mediated by serine 10 of VP2*. J. Virol.* **65:** 326-334.

Haydon, D., Lea, S., Fry, L., Knowles, N., Samuel, A.R., Stuart, D. and Woolhouse, .E.J. (1998). Characterising sequence variation in the VP1 capsid proteins of foot-and-mouth disease virus (serotype O) with the respect to virion structure. *J. Mol. Evol.* **46:** 465–475.

Haydon, D.T., Bastos, A.D., Knowles, N.J. and Samuel, A.R. (2001). Evidence for positive selection in foot-and-mouth disease virus capsid genes from field isolates. *Genetics* **157:** 7-15.

Heath, L., van der Walt, E., Varsani, A. and Martin, D.P. (2006). Recombination patterns in Aphthoviruses mirror those found in other Picornaviruses. *J. Virol.* **80:** 11827-11832.

Holland, J. J., Spindler, K., Horodyski, F., Garabau, E., Nichols, S. and Vandepol, S. (1982). Rapid evolution of RNA genomes. *Science* **215:**1577–1585.

Hynes, R.O. (1992). Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* **69:** 11-25.

Ignotz, R.A., Heino, J. and Massague, J. (1989). Regulation of cell adhesion receptors by transforming growth factor-beta. Regulation of vitronectin receptor and LFA-1. *J. Biol. Chem*. **264:** 389-392.

Jackson, T., Ellard, F.M., Ghazaleh, R.B., Brookes, S.M., Blakemore, W.E., Corteyn, A.H., Stuart, D.I., Newman, J.W.I. and King, A.M. (1996). Efficient infection of cells in culture by type O foot-and-mouth disease virus requires binding to cell surface heparan sulfate. *J. Virol.* **70:** 5282-5287.

Jackson, T., Sharma, A., Ghazaleh, R.A., Blakemore, W.E., Ellard, F.M., Simmons, D.L., Newman, J.W.I., Stuart, D.I. and King, A.M.Q. (1997). Arginine-glycine-aspartic acid-specific binding by foot-and-mouth disease viruses to the purified integrin αvβ3 *in vitro*. *J. Virol.* **71:** 8357-8361.

Jackson, T., Sheppard, D., Denyer, M., Blakemore, W. and King, A.M.Q. (2000a). The epithelial integrin αvβ6 is a receptor for foot-and-mouth disease virus. *J. Virol.* **74:** 4949-4956.

Jackson, T., Blakemore, W., Newman, J.W.I., Knowles, N.J., Mould, A.P., Humphries, M.J. and King, A.M.Q. (2000b). Foot-and-mouth disease virus is a ligand for the high-affinity binding conformation of integrin α5β1: influence of the leucine residue within the RGDL motif on selectivity of integrin binding. *J. Gen. Virol* **81:** 1383–1391.

Jackson, T., Mould, A.P., Sheppard, D., King, A.M.Q. (2002). Integrin αvβ1 is a receptor for foot-and-mouth disease virus. *J. Virol.* **76:** 935-941.

Jackson, T., King, A.M.Q., Stuart, D.I. and Fry, E. (2003). Structure and receptor binding. *Virus Res.* **91:** 33-46.

Jackson, T., Clark, S., Berryman, S., Burman, A., Cambier, S., Mu, D., Nishimura, S. and King, A.M.Q. (2004). Integrin αvβ8 functions as a receptor of foot-and-mouth disease virus: Role of the β-chain cytodomain in integrin-mediated infection. *J. Virol.* **78:** 4533-4540.

Jackson, A.L., O'Neill, H., Maree, F.F., Blignaut, B., Carrillo, C., Rodriguez, L. and Haydon, D.T. (2007). Mosaic structure of foot-and-mouth disease virus genomes. *J. Gen. Virol.* **88:** 487-492.

Jacobson, A. and Peltz, S.W. (1999). Tools for turnover: Methods for analysis of mRNA stability in eukaryotic cells. *Methods* **17:** 1-2.

Janat, M.F., Argraves, W.S. and Liau, G. (1992). Regulation of vascular smooth muscle cell integrin expression by transforming growth factor β1 and by platelet-derived growth factor-BB. *J. Cell. Physiol.* **151:** 588-595.

Jensen, H.L. and Norrild, B. (2000). The effects of cell passages on the cell morphology and the outcome of herpes simplex virus type 1 infection. *J. Virol. Methods* **84:** 139-152.

Jewell, D.A., Swietnicki, W., Dunn, B.M. and Malcolm, B.A. (1992). Hepatitis A virus 3C proteinase substrate specificity. *Biochemistry* **31:** 7862-7869.

Kim, L.T. and Yamada, K.M. (1997). The regulation of expression of integrin receptors. *Proc. Soc. Exp. Biol. Med.* **214:** 123-131.

King, A.M.Q., McCahon, D., Slade, W.R. and Newman, J.W.I. (1982). Recombination in RNA. *Cell* **29:** 921-928.

King, A.M.Q., McCahon, D., Saunders, K., Newman, J.W.I. and 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. *J. Comp. Path.* **118:** 89–108.

Kitching, R.P. (1999). Foot and mouth disease: current world situation. *Vaccine* **17:** 1772-1774.

Kitson, J.D.A., McCahon, D. and 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 surface exposed capsid proteins in four antigenic sites. *Virology* **179:** 26–34.

Knowles, N.J., Ansell, D.M. and Samuel, A.R. (1998). Molecular comparison of recent foot-and-mouth disease type A viruses from West Africa with historical and reference virus strains. *Session of the research group of the standing technical committee of the European Commission for the control of FMD,* Pirbright.

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. and Xie, Q.-G. (2000). Emergence of a pandemic strain of foot-and-mouth disease virus serotype O. *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, 2000 Sep 5-8. Rome: Food and Agriculture Organization; Appendix 1. p. 20-31.

Knowles, N.J., Davies, P.R., Henry, T., O'Donnell, V., Pacheco, J.M. and Mason, P.W. (2001a). Emergence in Asia of foot-and-mouth disease viruses with altered host range: characterisation of alterations in the 3A protein. *J. Virol.* **75:** 1551-1556.

Knowles, N.J., Samuel, A.R., Davies, P.R., Kitching, R.P. and Donaldson, A.I. (2001b). Outbreak of foot-and-mouth disease virus serotype O in the UK caused by a pandemic strain. *Vet. Rec.* **148:** 258-259.

Knowles, N.J. and Samuel, A.R. (2003). Molecular epidemiology of foot-and-mouth disease virus. *Virus Res.* **91:** 65-80.

Kraft, S., Diefenbach, B., Mehta, R., Jonezyk, A., Luckenbach, A. and Goodman, S.L. (1999). Definition of an unexpected ligand recognition motif for $\alpha_v\beta_6$ integrin. *J. Biol. Chem.* **274:** 1979-1985.

Krebs, O. and Marquardt, O. (1992). Identification and characterization of foot and mouth disease virus O1 Burgwedel/1987 as an intertypic recombinant. *J. Gen. Virol*. **73:** 613-619.

Kronovetr, J. and Skern, T. (2002). Foot-and-mouth disease virus leader proteinase: a papain-like enzyme requiring and acidic environment in the active site. *FEBS Lett.* **528:** 58-62.

Kumar, S., Tamura, K. and Nei, M. (1993). MEGA. Molecular evolutionary genetics analysis version 3.0. Pennsylvania State University.

Kunicki, T.J., Kritzik, M., Annis**,** D.S. and Nugent, D.J. (1997). Hereditary variation in platelet integrin $\alpha_2\beta_1$ density is associated with two silent polymorphisms in the α_2 gene coding sequence. *Blood* **89:** 1939-1943.

Leclerc, G.J., Leclerc, G.M. and Barredo, J.C. (2002). Real-time RT-PCR analysis of mRNA decay: half-life of beta-actin mRNA in human leukemia CCRF-CEM and Nalm-6 cell lines. *Cancer Cell Int.* **2:** 1-5.

Leippert, M., Beck, E., Weiland, F. and Pfaff, E. (1997). Point mutations within the βG-βH loop of foot-and-mouth disease virus O_1K affect virus attachment to target cells. *J. of Virol.* **71:** 104-1051.

Li,D., Liu,Z.X., Bao,H.F., Lu,Z.J., Guo,J.H., Cao,Y.M., Li,P.H., Bai,X.W., Chen,Y.L., Xie,B.X., Cai,X.P. and Xie,Q.G. (2007). The complete genome sequence of foot-and-mouth disease virus O/Akesu/58 strain and its some molecular characteristics. *Arch. Viro*l. **152:** 2079-2085.

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. and Fry, E. (1993). Structure of a major immunogenic site on foot-and-mouth disease virus. *Nature* **362:** 566–568.

Long, A.C., Orr, D.C., Cameron, J.M., Dunn, B.M. and Kay, J. (1989). A consensus sequence for sunstrate hydrolysis by rhinovirus 3C proteinase. *FEBS lett* **258:** 75-78.

Madshus, I.H., Olsnes, S. and Sandvig, K. (1984a). Different pH requirements for entry of the two picornaviruses, human rhinovirus 2 and murine encephalomyocarditis virus. *Virology* **139:** 346-357.

Madshus, I.H., Olsnes, S. and Sandvig, K. (1984b). Mechanism of entry into the cytosol of poliovirus type 1: requirement for low pH. *J. Cell Biol.* **98:** 1194-1200.

Mason, P.W., Rieder, E. and Baxt, B. (1994). RGD sequence of foot-and-mouth disease virus is essential for infecting cells via the natural receptor but can be bypassed by an antibody-dependant enhancement pathway. *Proc. Natl. Acad. Sci.* **91:** 1932-1936.

Mason, P.W., Grubman, M.J. and Baxt, B. (2003a). Molecular basis of pathogenesis of FMDV. *Virus Res.* **91:** 9-32.

Mason, P.W., Pacheco, J.M., Zhao, Q.Z. and Knowles, N.J. (2003b). Comparisons of the complete genomes of Asian, African and European isolates of a recent foot-and-mouth disease virus type O pandemic strain (PanAsia). *J. Gen. Virol.* **84:** 1583-1593.

Mateo, R., Díaz, A., Baranowski, E. and Mateu, M.G. (2003). Complete alanine scanning of intersubunit interfaces in a foot-and-mouth disease virus capsid reveals critical contributions of many side chains to particle stability and viral function. *J. Biol. Chem*. **278:** 41019-41027.

Mateu, M.G., Camarero, J.A., Giralt, E., Andreu, D. and Domingo, E. (1995). Direct evaluation of the immunodominance of a major antigenic site of foot-and-mouth disease virus in a natural host. *Virology* **206:** 298 – 306.

Mateu, M.G., Valero, M.L., Andreu, D. and Domingo, E. (1996). Systemic replacement of amino acid residues within an Arg-Gly-Asp-containg loop of foot-and-mouth disease virus and effect on cell recognition. *J. Biol. Chem.* **271:** 12814–12819.

McCahon, D. and Slade, W.R. (1981). A sensitive method for the detection and isolation of recombinants of foot-and-mouth disease virus. *J. Virol*. **53:** 333-342.

McCahon, D., King, A.M.Q., Roe, D.S., Slade, W.R., Newman, J.W.I. and Cleary, A.M. (1985). Isolation and biochemical characterization of intertypic recombinants of foot-and-mouth disease virus. *Virus Res*. **3:** 87-100.

Medina, M., Domingo, E., Brangwyn, J.K. and Belsham, G.J. (1993). The two species of the foot-and-mouth disease virus leader protein, expressed individually, exhibit the same activities. *Virol.* **194:** 355-359.

Meyer, K., Petersen, A., Niepmann, M. and Beck, E. (1995). Interaction of eukaryotic initiation factor eIF-4B with a picornavirus internal translation initiation site. *J. Virol.* **69:** 2819-2824

Miller, L.C., Blakermore, W., Sheppard, D., Atakilit, A., King, A.M.Q. and Jackson, T. (2001). Role of the cytoplasmic domain of the β-subunit of integrin αvβ6 in infection by foot-and-mouth disease virus. *J. of Virol.* **75:** 4158-4164.

Monaghan, P., Gold, S., Simpson, J., Zhang, Z., Weinreb, P.H., Violette, S.M., Alexandersen, S. and Jackson, T. (2005). The αvβ6 integrin receptor for foot-and-mouth disease vius is expressed constitutively on the epithelial cells targeted in cattle. *J. Gen. Virol.* **86:** 2769-2780.

Neff, S., Sa-Carvalho, D., Rieder, E., Mason, P.W., Blystone, S.D., Brown, E.J. and Baxt, B. (1998). Foot-and-mouth disease virus virulent for cattle utilizes the integrin αvβ3 as its receptor. *J. Virol.* **72:** 3587-3594.

Nobiron,I., Remond,M., Kaiser,C., Lebreton,F., Zientara,S. and Delmas,B. (2005). The nucleotide sequence of foot-and-mouth disease virus O/FRA/1/2001 and comparison with its British parental strain O/UKG/35/2001. *Virus Res*. **108:** 225-229.

O'Driscoll, L., Gammell, P., McKiernan, E., Ryan, E., Jeppesen, P.B., Rani, S. and Clynes, M. (2006). Phenotypic and global gene expression profile changes between low passage and high passage MIN-6 cells. *J. Endocrinology* **191:** 665-676.

O'Toole, T.E., Katagiri, Y., Faull, R.J., Peter, K., Tamura, R., Quaranta, V., Loftus, J.C., Shattil, S.J. and Ginsberg, M.H. (1994). Integrin cytoplasmic domains mediate inside-out signal transduction. *J. Cell Biol.* **124:** 1047–1059.

Ouldridge, E.J., Francis, M.J. and Black, L. (1982). Antibody response of pigs to foot-and-mouth disease oil emulsion vaccine: the antibody classes involved. *Res Vet Sci.* **32:** 327-331.

Pallansch, M.A., Kew, O.M., Semler, B.L., Omilianowski, D.R., Anderson, C.W.., Wimmer, E. and Rueckert, R.R. (1984). Protein processing map of poliovirus. *J. Virol.* **49:** 873–880.

Panina, G.F., Civardi, A., Massirio, I., Scatozza, F., Baldini, P. and Palmia, F. (1989). Survival of foot-and-mouth disease virus in sausage meat products (Italian salami). *Int. J. Food Micro.* **8:** 141–148.

Parry, N.R., Barnett, P.V., Ouldridge, E.J., Rowlands, D.J. and Brown, F. (1989). Neutralizing epitopes of type O foot-and-mouth disease virus. II. Mapping three conformational sites with synthetic peptide reagents. *J. Gen. Virol.* **70:** 1493-1503.

Parry, N., Fox, G., Rowlands, D., Brown, F., Fry, E., Acharya, R., Logan, D. and Stuart, D. (1990). Structural and serological evidence for a novel mechanism of antigenic variation in foot-and-mouth disease virus. *Nature* **347:** 569 – 572.

Pereira, H. G. (1977). Subtyping of foot-and-mouth disease virus. *Developments in Biological Standardization* **35:** 167-174.

Perez, F.M., Wigdorovitz A., Romera, A., Zamorano, P., Borca, M.V. and Sadir, A.M. (2002). Detection and Characterization of Functional T-Cell Epitopes on the Structural Proteins VP2, VP3, and VP4 of Foot and Mouth Disease Virus O1 Campos. *Virology* **271:** 234-239.

Pfaff, M., Tangemann, K., Muller, B., Gurrath, M., Muller, G., Kessler, H., Timpl, R. and Engel, J. (1994). Selective recognition of cyclic RGD peptides of NMR defined conformation by alpha IIb beta 3, alpha V beta 3 and alpha 5 beta 1 integrins. *J. Biol. Chem.* **269:** 20233-20238.

Piccone, M.E., Rieder, E., Mason, P.W. and Grubman, M.J. (1995a). The foot-and-mouth disease virus leader proteinase gene is not required for viral replication. *J. Virol.* **69:** 5376-5382.

Piccone, M.E., Zellner, M., Kumosinski, T.F., Mason, P.W. and Grubman, M.J. (1995b). Identification of the active-site residues of the L-proteinase of foot-and-mouth disease virus. *J. Virol.* **69:** 4950-4956.

Posada, D. and Crandall, K.A. (1998). MODELTEST: testing the model of DNA substitution. *BioInformatics* **14:** 817–818.

Pringle, C.R. (1965). Evidence of genetic recombination in foot-and-mouth disease virus. *Virol*. **25:** 48-54.

Pringle, C. R. (1999). Virus Taxonomy at the XIth International Congress of Virology, Sydney, Australia, 1999. *Archives of Virology* **144:** 2065-2070.

Quan, M., Murphy, C.M., Zhang, Z. and Alexandersen, S. (2004). Determinants of early foot-and-mouth disease virus dynamics in pigs. *J. Comp. Path.* **131:** 294-307.

Retta, S.F., Cassara, G., D'Amato, M., Alessandro, R., Pellegrino, M., Degani, S., de Leo, G., Silengo, L. and Tarone, G. (2001). Cross talk between β1 and αv integrins: β1 affects β3 mRNA stability. *Mol. Biol. Of the Cell*. **12:** 3126-3138.

Rieder, E. and Wimmer, E. (2002). Cellular receptors of picarnoviruses: an overview, pg 61-70. In *Molecular biology of picarnoviruses.* Edited by Semler, B.L., Wimmer, E. ASM Press, Washington D.C.

Ross, J. (1995). mRNA stability in mammalian cells. *Microbiol. Rev.* **59:** 423-250.

Rossman, M.G. and Rueckert, R.R. (1987). What does the molecular structure of viruses tell us about viral functions? *Microbiological sciences* **4:** 206-214.

Rossman, M.G. and Palmenberg, A.A. (1988). Conservation of the putative receptor attachment site in picornaviruses. *Virology* **164:** 373-382.

Rossman, M.G. and Johnson, J. E. (1989). Icosahedral RNA virus structure. *Annual Review of Biochemistry* **58:** 533-573.

Rowlands, D.J., Harris, T.J.R. and Brown, F. (1978). More precise location of the polycytidylic acid tract in foot-and-mouth disease virus RNA. *J. Virol*. **26:** 335–343.

Ryan, M.D. and Flint, M. (1997). Virus-encoded proteases of the picornavirus super-group. *J. Gen. Virol*. **78:** 699–723.

Sa-Carvalho, D., Reider, E., axt, B., Rodarte, R., Tanuri, A. and Mason, P.W. (1997). Tissue culture adaptation of foot-and-mouth disease virus selects viruses that bind to heparin and are attenuated in cattle. *J. of Virol.* **71:** 5115-5123.

Saiz, M., Nunez, J.I., Clavero, M.A.J., Baranowski, E. and Sobrino, F. (2002). Foot-and-mouth disease virus: biology and prospects for disease control. *Microbes & Infect.* **4:** 1183-1192.

Sahle, M., Venter, E.H., Dwarka, R.M. and Vosloo, W. (2004). Molecular epidemiology of serotype O foot-and-mouth disease virus isolated from cattle in Ethiopia between 1979-2001. *Onderstepoort J. Vet. Res.* **71:** 129-138.

Salt, J.S. (1993). The carrier state in foot-and-mouth disease – an immunological review. *Br. Vet. J.* **149:** 207-223.

Samuel A.R., Knowles, N.J. and Kitching, R.P. (1988). Serological and biochemical analysis of some recent type A foot-and-mouth disease virus isolates from the Middle East. *Epidem. Inf.* **101:** 577–590.

Samuel, A.R. and Knowles, N.J. (2001a). Foot-and-mouth disease virus: cause of the recent crisis for the UK livestock industry. *TRENDS in Gen.* **17:** 421-424.

Samuel, A.R. and Knowles, N.J. (2001b). Foot-and-mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). *J. Gen. Virol.* **82:** 609-621.

Santala, P. and Heino, J. (1991). Regulation of integrin-type cell adhesion receptors by cytokines. *J. Biol. Chem*. **266:** 23505-23509.

Schneider-Schaulies, J. (2000). Cellular receptors for viruses: links to tropism and pathogenesis. *J. Gen. Virol.* **81:** 1413-1429.

Seipelt, J., Guarne´, A., Bergmann, E., James, M., Sommergruber, W., Fita, I. and Skern, T. (1999). The structures of picornaviral proteinases. *Virus Res*. **62:** 159–168.

Sellers, R.F., Herniman, K.A.J. and Donaldson, A.I. (1971). The effects of killing or removal of animals affected with foot-and-mouth disease on the amounts of airborne virus present in looseboxes. *Br. vet. J.* **127:** 358 – 364.

Skern, T., Fita, I. and Guarne, A. (1998). A structural model of picornavirus leader proteinases based on papain and bleomycin hydrolase. *J. Gen. Virol.* **79:** 301-307.

Sobrino, F., Saiz, M., Clavero, M.A.J., Nunez, J.I., Rosas, M.F., Baranowski, E. and Ley, V. (2001). Foot-and-mouth disease virus: a long known virus, but a current threat. *Vet. Res.* **32:** 1–30.

Song, W.K., Wang, W., Foster, R.F., Bielser, D.A. and Kaufman, S.J. (1992). H36-alpha7 is a novel integrin alpha chain that is developmentally regulated during skeletal myogenesis. *J. Cell Biol*. **117:** 643-649.

Snedecor, G.W. and Cochran, W.G. (1980). Statistical methods, 7th edition, pp 507. Ames: Iowa State University Press.

Stanway, G. (1990). Structure, function and evolution of picornaviruses. *J. Gen. Virol.* **71:** 2483-2501.

Strebel, K. and Beck, E. (1986). A second protease of foot-and-mouth disease virus. *J. Virol*. **58:** 893-899.

Sutmoller, P., Barteling, S.S., Olascoaga, R.C. and Sumption, K.J. (2003). Control and eradication of foot-and-mouth disease. *Virus Res* **91:** 101–144.

Swofford, D.L. (1998). PAUP: phylogenetic analysis using parsimony (and other methods). Sinauer Associates, Sunderland, MA.

Synnergren, J.E., Giesler, T.L., Adak, S., Tandon, R., Noaksson, K., Lindahl, A., Nilsson, P., Nelson, D., Olsson, B., Englund, M.C.O., Abbot, S. and Sartipy, P. (2007). Differentiating Human Embryonic Stem Cells Express a Unique Housekeeping Gene Signature. *Stem Cells* **25:** 473-480.

Thomson, G.R., Vosloo, W., Esterhuysen, J.J. and Bengis, R.G. (1992). Maintenance of foot-and-mouth disease viruses in buffalo (*Syncerus caffer Sparrman*, 1779) in southern Africa*. Rev Sci Tech.* **11:** 1097-107.

Thomson, G. R. (1994). Foot-and-mouth disease. In *Infectious diseases of livestock with special reference to Southern Africa*, pp 825-852. Edited by Coetzer, J.A.W., Thomson G.R., Tustin, R.C., Kriek, N.P.J. Oxford University Press, Cape Town.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24:** 4876-4882.

Thomson, G.R., Vosloo, W. and Bastos, A.D.S. (2003). Foot and mouth disease in wildlife. *Virus Res.* **91:** 145-161.

Thomson, G.R. and Bastos, A.D.S. (2004). Foot-and-mouth disease. In *Infect. Diseases of Livestock*, Volume 2, 2nd edition, pp 1324-1365. Edited by Coetzer, J.A.W., Tustin, R.C. Oxford University press.

Tosh, C., Hemadri, D. and Sanyal, A. (2002). Evidence for recombination in the capsid-coding region of type A foot-and-mouth disease virus. *J. Gen. Virol*. **83:** 853-869.

Tosh, C., Mittal, M., Sanyal, A., Hemadri, D. and Bandyopadhyay, S.K. (2004). Molecular phylogeny of leader proteinase gene of type A of foot-and-mouth disease virus from India. *Arch. Virol*. **149:** 523-536.

Usherwood, E.J., Johnston, I.C.D., Lovelidge, L.J., Tonks, P. and Nash, A.A. (1995). Lymphocyte recognition elements on the VP1 protein of Theiler's virus. *Immunology* **85:** 190– 97.

van Vlijmen, H. W., Curry, S., Schaefer, M. and Karplus, M. (1998). Titration calculations of foot-and-mouth disease virus capsids and their stabilities as a function of pH. *J. Mol. Biol.* **275:** 295 – 308.

Valarcher, J.F., Knowles, N.J., Ferris, N.P., Paton, D.J., Zakharov, V., Sherbakov, A., Shang, Y., Zai-xin, L., Xiang-tao, L., Sanyal, A., Hemadri, D., Tosh, C. and Rasool, T.J. (2005). Recent spread of FMD virus serotype Asia 1. *Vet Rec*. **157:** 30.

van Rensburg, H.G. and Nel, L.H. (1999). Characterisation of the structural-protein-coding region of SAT 2 type foot-and-mouth disease virus. *Virus Genes* **19:** 229-233.

van Rensburg, H., Haydon, D., Joubert, F., Bastos, A., Heath, L. and Nel, L. (2002). Genetic heterogeneity in the foot-and-mouth disease virus Leader and 3C proteinase. *Gene* **289:** 19-29.

Vasquez, C., Denoya, C.D., La Torre, J.L. and Palma, E.L. (1979). Structure of the foot-and-mouth disease virus capsid. *Virology* **97:** 195–200.

Vosloo, W., Bastos, A.D., Kirkbride, E., Esterhuysen, J.J., van Rensburg, D.J., Bengis, R.G., Keet, D.W. and Thomson, G.R. (1996). Persistent infection of African buffalo (Syncerus caffer) with SAT-type foot-and-mouth disease viruses: rate of fixation of mutations, antigenic change and interspecies transmission. *J. Gen. Virol.* **77:** 1457-1467.

Vosloo, W., Bastos, A.D.S., Sahle, M., Sangare, O. and Dwarka, R.M. (2005). Chapter 10: Virus topotypes and the role of wildlife in foot and mouth disease in Africa. In: *Conservation and development interventions at the wildlife/livestock interface: Implications for wildlife, livestock and human health*. IUCN, Gland, Switzerland and Cambridge, UK.

Vosloo, W., de Klerk, L.M., Boshoff, C.I., Botha, B., Dwarka, R., Keet, D. and Haydon, D.T. (2007). Characterisation of a SAT-1 outbreak of foot-and-mouth disease in captive African Buffalo (*Syncerus caffer)*: Clinical symptoms, genetica characterization and phylogenetic comparison of outbreak isolates. *Vet. Micro.* **120:** 226-240.

Wang, Y., Liu, C.L., Storey, J.D., Tibshirani, R.J., Herschlag, D. and Brown, P.O. (2002). Precision and functional specificity in mRNA decay. *PNAS* **99:** 5860-5865.

Weddell, G.N., Yansura, D.G., Dowbenko, D.J., Hoatlin, M.E., Grubman, M.J., Moore, D.M. and Kleid, D. (1985). Sequence variation in the gene for the immunogenic protein VP1 of foot-and-mouth disease virus type A. *Proc. Natl. Acad. Sci. USA* **82:** 2618-2622.

Wilson, V., Taylor, P. and Desselberger, U. (1988). Crossover regions in foot-and-mouth disease virus (FMDV) recombinants correspond to regions of high local secondary structure. *Arch. Virol*. **102:** 131-139.

Xu, J. and Clark, R.A. (1996). Extracellular matrix alters PDGF regulation of fibroblast integrins. *J. Cell. Biol.* **132:** 239-249.

Yperman, J., de Visscher, G., Holvoet, P. and Flameng, P. (2004). Beta-actin cannot be used as a control for gene expression in ovine interstitial cells derived from heart valves. *J. Heart Valve Dis.* **13:** 848-853.

Zadoks, R.N. and Schukken, Y.H. (2006). Use of molecular epidemiology in veterinary practice. *Vet. Clin. Food Anim*. **22:** 229-261.

Zhang,X., Liu,Z., Zhao,Q., Chang,H. and Xie,Q. (2004). Sequencing and analysis for the full-length genome RNA of foot-and-mouth disease virus China/99. *Sci. China, C, Life Sci.* **47:** 74–81.

APPENDIX

A1: Buffers and solutions

- L6 Buffer:
  8.3 M GuSCN, 80 mM Tris-HCl (pH 6.4), 36 mM EDTA, 33 mM TritonX-100

- L2 Wash Buffer:
  10.2 M GuSCN, 0.1 M Tris-HCl (pH 6.4)

- Methylene Blue Stain:
  1% (w/v) methylene blue dissolved in absolute EtOH to an equal volume of formaldehyde prepared with phosphate-buffered saline; pH 7.4

- Tragacanth overlay Media:
  2 × MEM, 1.2% gum Tragacanth, 1% FCS

- Silica suspension:
  6g of silica dioxide suspended in 50ml sterile distilled H_2O in a 50ml-measuring cylinder sedimented for 24 hours at room temperature. The supernatant (43ml) was removed by suction with pipette-aid. The sedimented silica was re-suspended to a final volume of 50ml. A second sedimentation was allowed for the duration of 5 hours at room temperature. The supernatant (44ml) was removed and the pH adjusted to 2 by adding 60µl of HCl (32% w/v).

- Antibiotic-Antimycotic mixture (Invitrogen):
  10,000 units of penicillin G (sodium salt), 10,000 µg of streptomycin sulphate and 25 µg of amphotericin B/ml as Fungizone® Antimycotic in 0.85% saline

- Preparation of Eagle's Basal Medium for BHK cells:
  500ml of medium was supplemented with 10% (v/v) fetal calf serum (FCS; Delta Bioproducts), 5ml of 100x antibiotic-antimycotic mixture **(**Invitrogen,

see above), 1 mM L-glutamine (Invitrogen) and 10% (v/v) tryptose phosphate broth (TPB; Sigma)

- Preparation of RPMI medium for IBRS cells:
  500ml of RPMI medium was supplemented with 5% (v/v) FCS and 5ml of 100x antibiotic-antimycotic mixture (see above).

- Trypan blue stain (0.5%):
  1L dH_2O, 5g Trypan blue (Merck), 8.5g NaCl (Merck)

A2: List of oligonucletides used for the L/P1sequencing of the FMDV A and O types

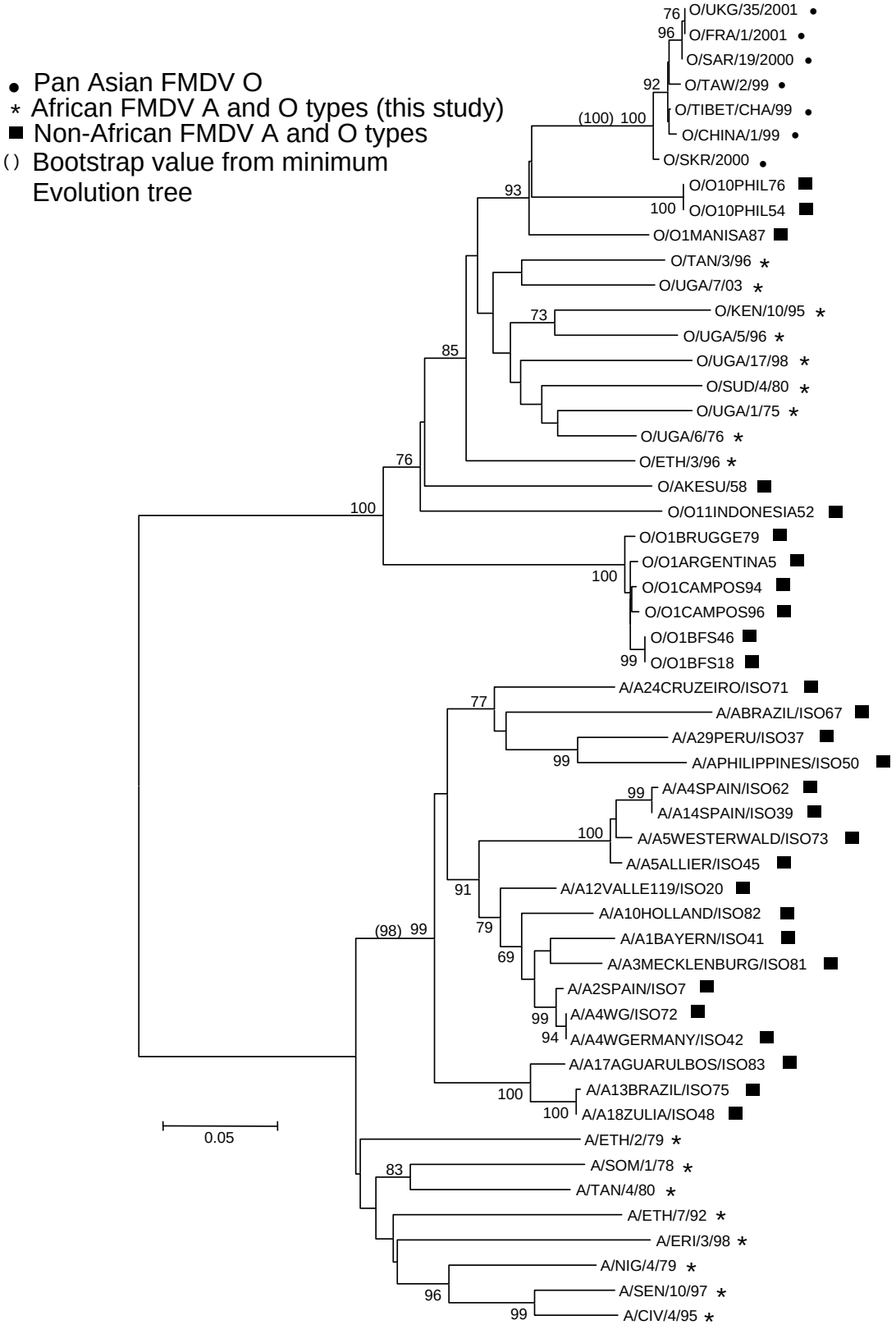
Oligonucleotide	❖ Sequence	Sense or antisense	FMDV A Oligonucleotide	FMDV O Oligonucleotide	Reference sequence Genbank no.
O–TAN-VP1	5’-AGCAGGGGTCTGCATCAGGTCCAA-3’	antisense	-	✓	EU919241
O–UGA-VP2	5’-GCTGGAATCTTCCTAAAAGGACAG-3’	sense	-	✓	EU919243
O–UGA-VP1	5’-TGCATATGAGTCGGTCAGGCTGCC-3’	antisense	-	✓	EU919243
A–ETH-VP4	5’-AYTAYATGCAGCAGTACCARAA-3’	sense	✓	-	EU919233
A–ETH-VP3	5’-ATATGAGAAATGGCTGGGA-3’	sense	✓	-	EU919233
A–ETH-VP1	5’-TCCGCCGCAGACATGTACGGGAT-3’	antisense	✓	-	EU919233
A–NIG-VP2/3	5’-ACGGTACGGACATGTGTTTGGCT-3’	antisense	✓	-	EU919234
A–NIG-VP1/3	5’-AGGTCAGAGAAGTAGTACGTAGCT-3’	sense	✓	-	EU919234
A–NIG–4/79-VP/VP3-F	5’-ACGGTACGGACATGTGTTTGGCT-3’	sense	✓	-	EU919234
A–NIG–4/79-VP/VP3-R	5’-AGGTCAGAGAAGTAGTACGTAGCT-3’	antisense	✓	-	EU919234
A–CIV–4/95-VP/VP3-F	5’-TAGTTACATGCARCAGTACC-3’	sense	✓	-	EU919236
A–CIV–4/95-VP/VP3-R	5’-TGGTGTGTCTGCATGAGGTCTAA-3’	antisense	✓	-	EU919236
A–SOM–1/78-VP3-F	5’-TAYGACCARTACAARAAGCACA-3’	sense	✓	-	EU919231
A–SOM–1/78-VP3-R	5’-TGGGTCTGCATGAAGTCTATGA-3’	antisense	✓	-	EU919231
A-NIG-4/79-VP1-F1	5'-GTCACRTACGGGTACTCCAC-3'	sense	✓	-	EU919234
A–NIG–4/79-VP1-F2	5'-CTGTTCGGCGCACTGCTCGCCGAC-3'	sense	✓	-	EU919234
A–NIG–4/79-VP3-R1	5'-ACATAGGCCACCATGTAGCGGG-3'	antisense	✓	-	EU919234
A–NIG–4/79-VP3-R2	5'-GCATAGTCCGCGGCAGACATGTAC-3'	antisense	✓	-	EU919234

Oligonucleotide	❖ Sequence	Sense or antisense	FMDV A Oligonucleotide	FMDV O Oligonucleotide	Reference sequence Genbank no.
NCR1	5’-TACCAAGCGACACTCGGGATCT-3’	sense	✓	✓	N/A
WDA	5’-GAAGGGCCCAGGGTTGGACTC-3’	antisense	✓	✓	N/A
O-Lin-F	5’-CAARCAYYTGCTCCACACCGG-3’	sense	-	✓	EU919242
O-Lin-R	5’-TCRTCRATCGCGTACCACCC-3’	antisense	-	✓	EU919242
VP1OA–R	5’-GGTGTYACTTTCACAAATCTGTCC-3’	antisense	-	✓	EU919243
VP1OB–R	5’-GGTGTAACTTTCACAAATCTATCC-3’	antisense	-	✓	EU919243
O-VP2–F	5’-CCACCTGTTCGACTGGGTCAC-3’	sense	-	✓	EU919240
O–VP3–R	5’-GGTTGATGGTGCCRCTGTACTG-3’	antisense	-	✓	EU919240
A–Lint–F	5’-ACCCAGYGAGGTGTGYATGG-3’	sense	✓	-	EU919237
A–1D–R	5’-TTTGTGGTAGGCYGTGGGGTTGC-3’	antisense	✓	-	EU919234
A-1D4-R	5'-TGTGGTAGGCCGTGGGGTTGCTC-3'	antisense	✓	-	EU919234
A-VP4INT-F	5'-CGCAACGGRCACACCACCTCGAC-3'	sense	✓	-	EU919238
A-VP4INT2-F	5'-CAGTACCAGAACTCCATGGACAC-3'	sense	✓	-	EU919238
A-LINT2-F	5'-CGATGAAYCGTTCTTCGACTGGGTC-3'	sense	✓	-	EU919231
A-1D2-R	5'-TGTGGTAGGCTGTGGGGTTGCTC-3'	antisense	✓	-	EU919233
A-1D3-R	5'-TGTGGTAGGCGGTGGGGTTGCTC-3'	antisense	✓	-	EU919233
O–TAN-VP2	5’-GTGACGTTCCGTACGTTACCACG-3’	sense	-	✓	EU919241
O–TAN-VP3	5’-ACTACTACATGCAGCAGTACCA-3’	sense	-	✓	EU919241

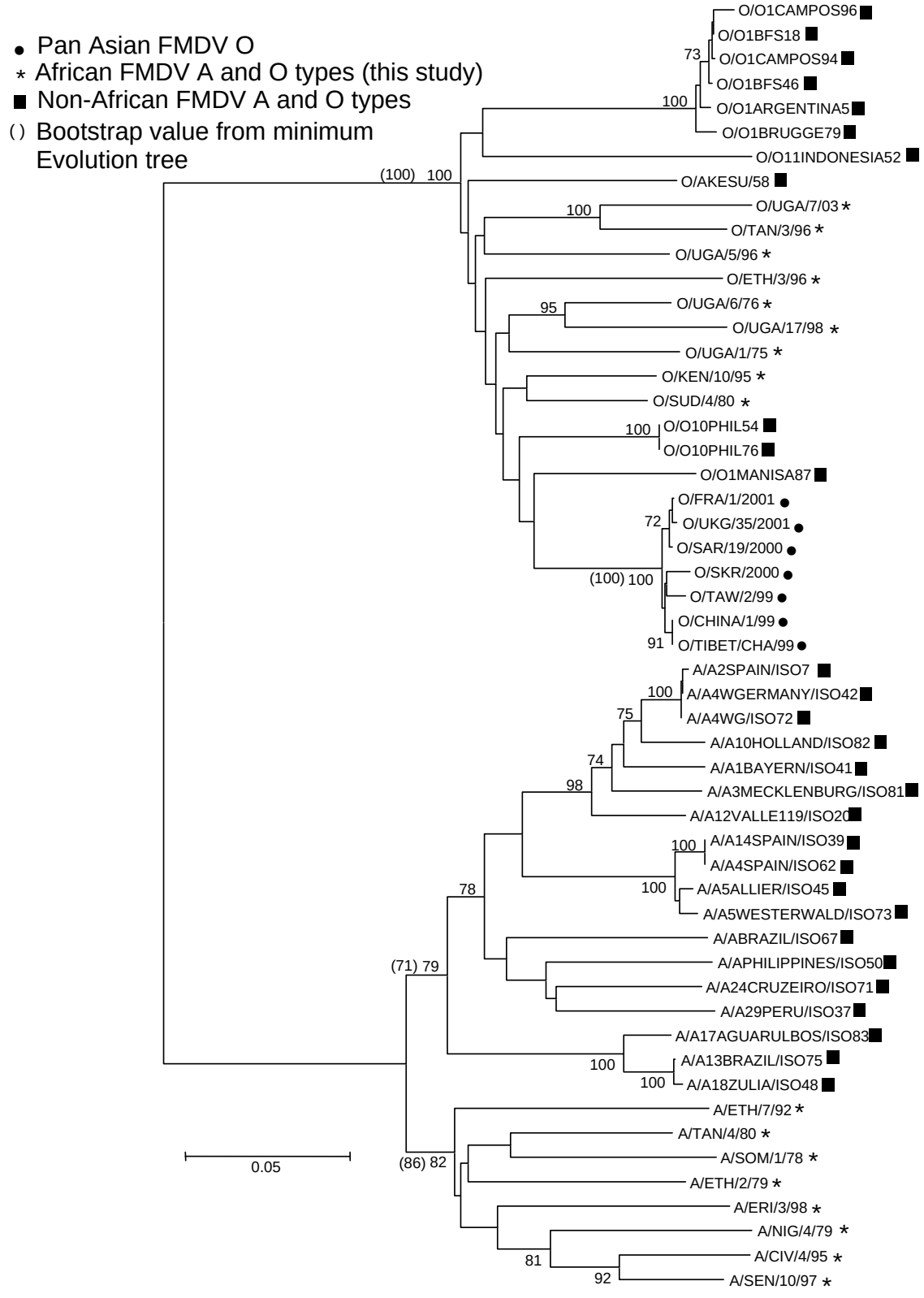
Oligonucleotide	❖ Sequence	Sense or antisense	FMDV A Oligonucleotide	FMDV O Oligonucleotide	Reference sequence Genbank no.
A–CIV-4/95-F/seq	5'-GTGGTGCAGGCAGAAAGGTTC-3'	sense	✓	-	EU919236
A–ETH-2/79-F/seq	5'-ACGGCGGTCTGGTGACAACAG-3'	sense	✓	-	EU919233
A–ETH-7/92-F/seq	5'-AAAGGACAGGAACACGCTGTG-3'	sense	✓	-	EU919235
A–ETH-7/92-R/seq	5'-TTGCCAGCACTTACTGACACG-3'	antisense	✓	-	EU919235
A–SEN-10/97-F/seq	5'-TCGCCGCTTACAGTCAGCGCG-3'	sense	✓	-	EU919237
A–TAN-4/80-F/seq	5'-ATCGTCCCGGTCGCTTGTTC-3'	sense	✓	-	EU919232
O–ETH-3/96-F/seq	5'-CGTGCTGGCTAGCGCTGGCAAG-3'	sense	-	✓	EU919240
O–KEN-10/95-F/seq	5'-AGTTGCGTCTACCAGTKGACGC-3'	sense	-	✓	EU919242
O–UGA-1/75-F/seq	5'-CAAACCTTGGACCCTTGTGGTC-3'	sense	-	✓	EU919244
O–UGA-5/96-F/seq1B	5'-CAACTACTACATGCAGCARTACC-3'	sense	-	✓	EU919247
O–UGA-5/96-F/seq1C	5'-GACAACCAATGTGCAGGGATGG-3'	sense	-	✓	EU919247
O–UGA-6/76-F/seq1A	5'-CCTCGCGACCCAGCGAGGTGTG-3'	sense	-	✓	EU919246

❖ Abbreviations representing ambiguities are Y = C/T; R = A/G; K = T/G and N/A = not applicable

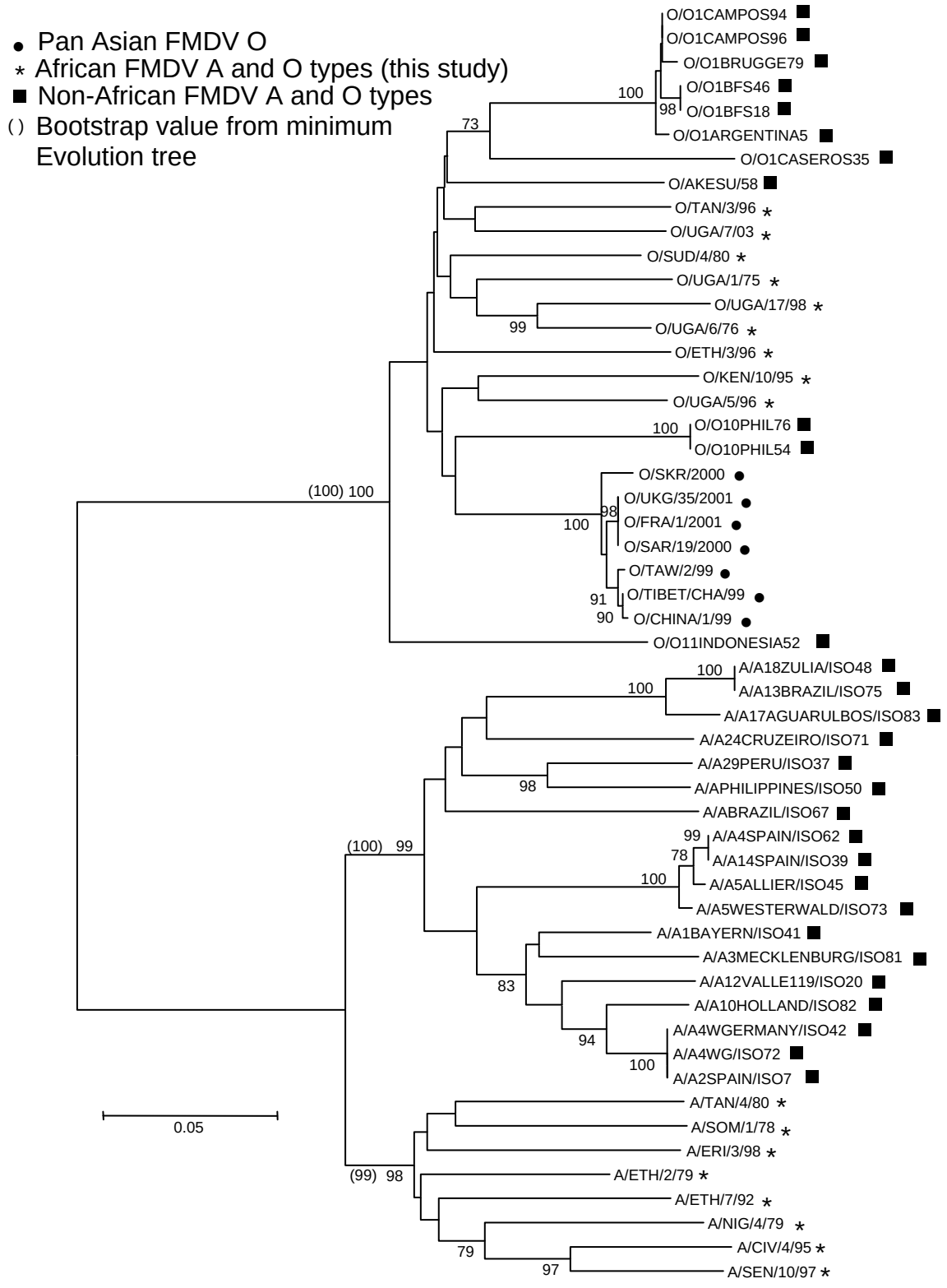
A3: Neighbour-joining tree depicting gene relationships for the 1D region of A and O type viruses. Kimura-2 parameter and bootstrap (1000 replications) were applied.



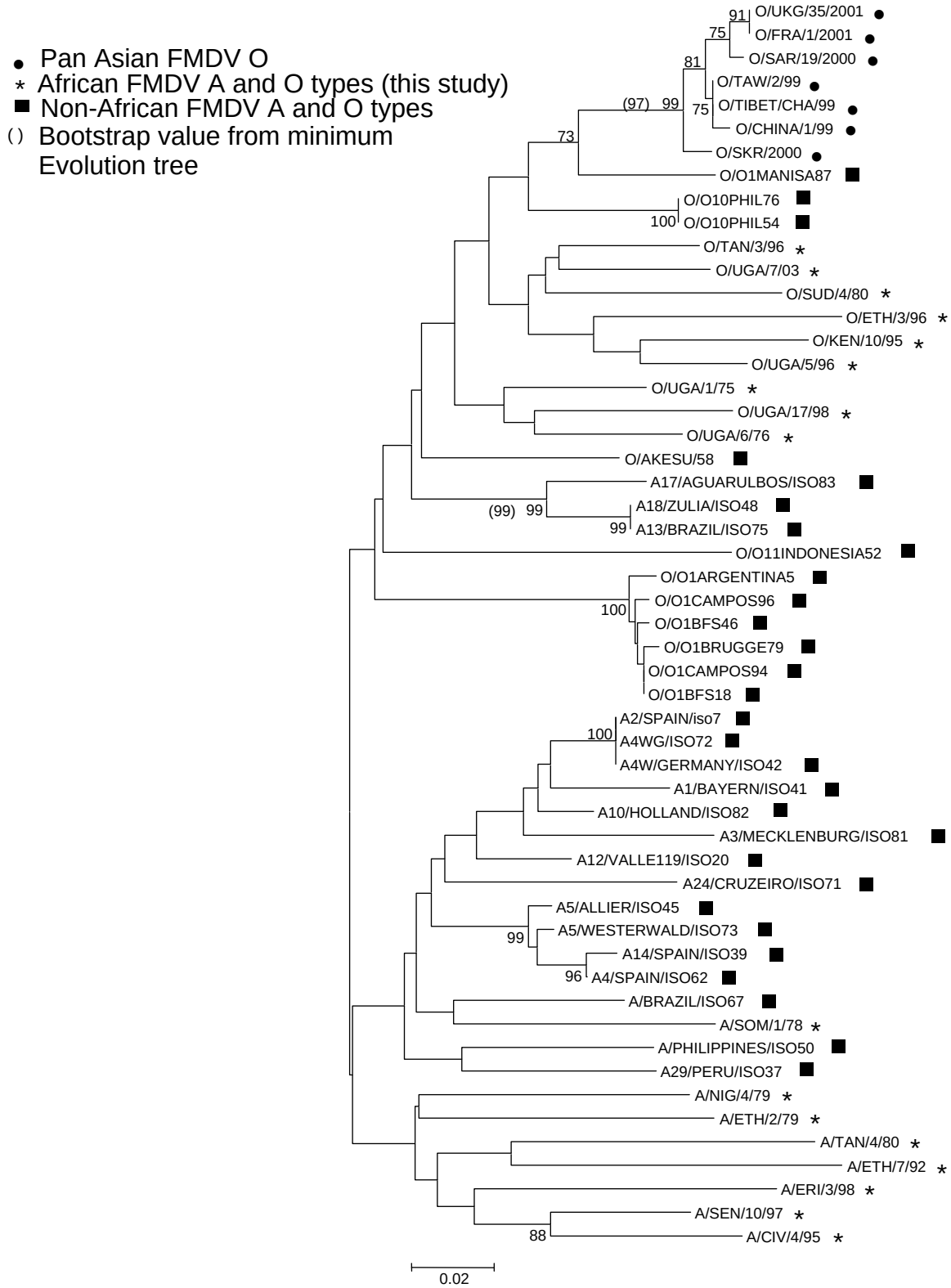
A4: Neighbour-joining tree depicting gene relationships for the 1B region of A and O type viruses. Kimura-2 parameter and bootstrap (1000 replications) were applied.



A5: Neighbour-joining tree depicting gene relationships for the 1C region of A and O type viruses. Kimura-2 parameter and bootstrap (1000 replications) were applied.

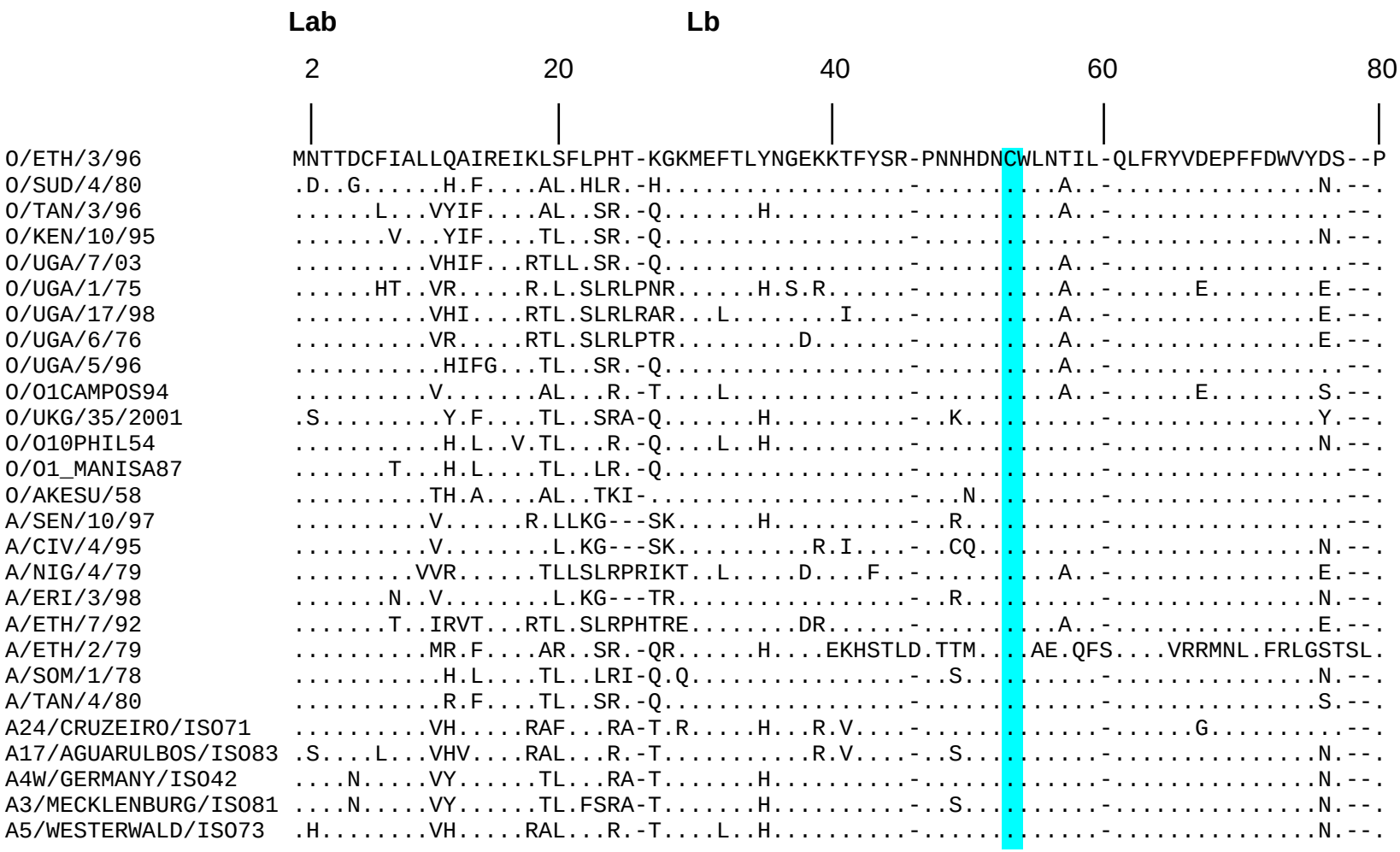


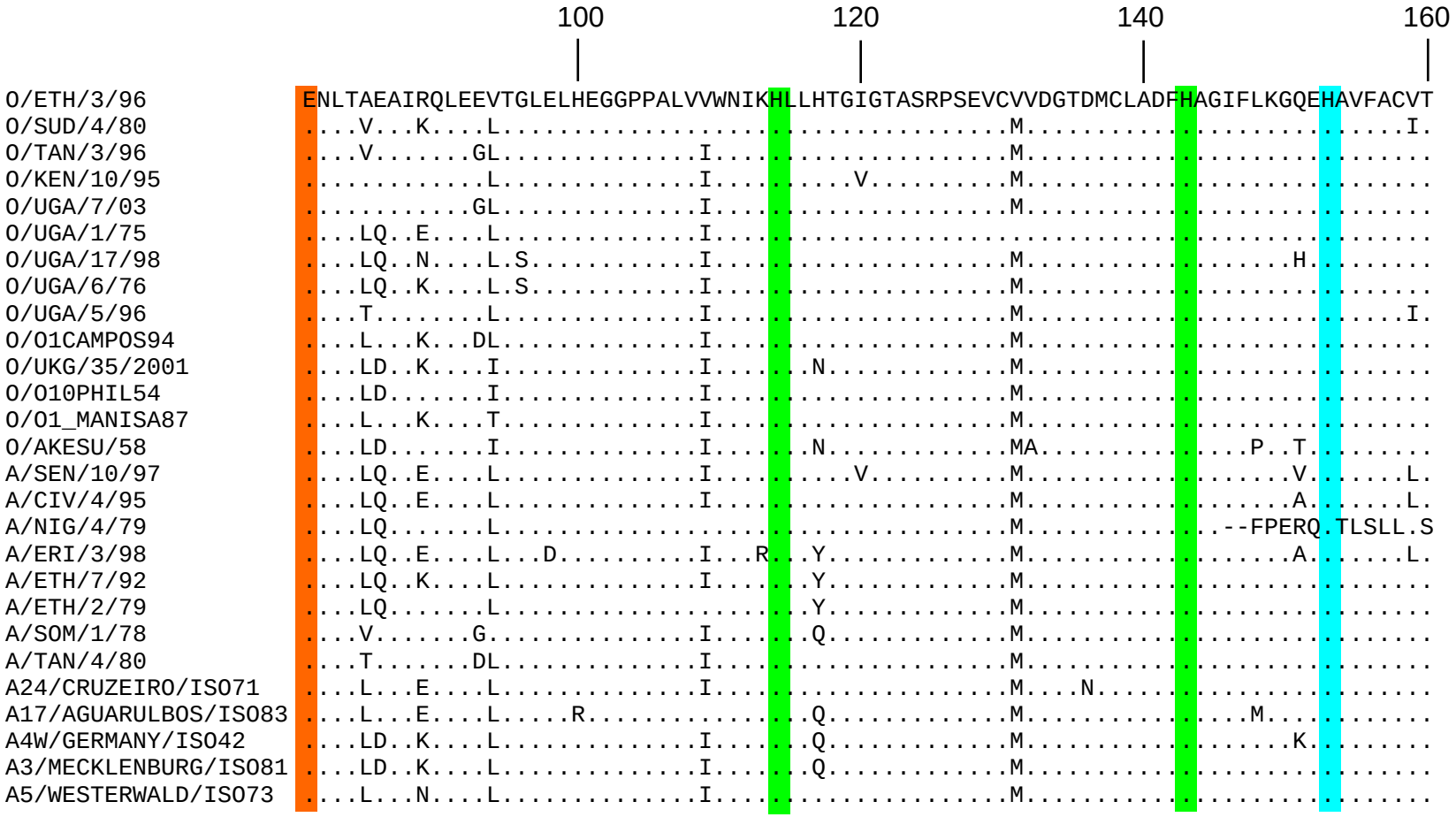
A6: Neighbour-joining tree depicting gene relationships for the 1A region of A and O type viruses. Kimura-2 parameter and bootstrap (1000 replications) were applied.

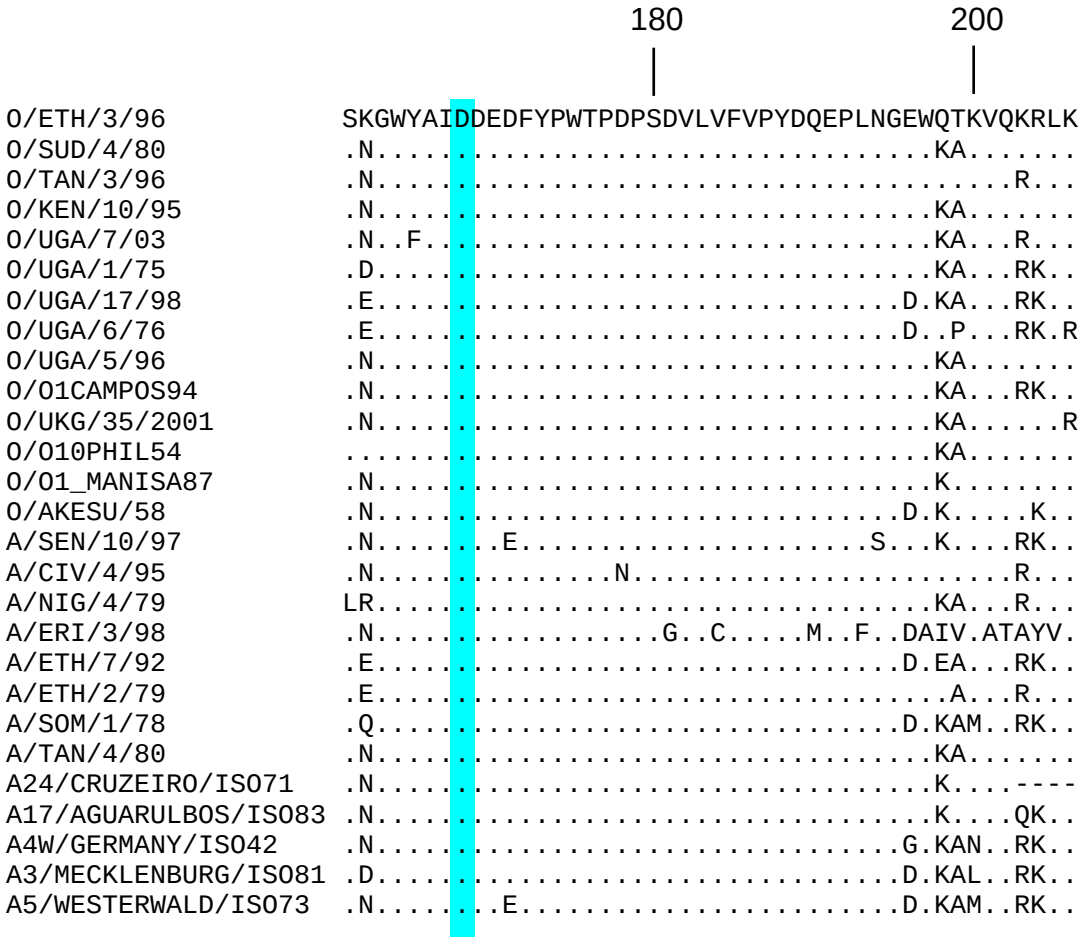


A7: Amino acid alignment of FMDV African A and O serotypes Leader region. Both the Lab^{pro} and Lb^{pro} are indicated.

(A small number of non-African A and O isolates are also included in the alignment)

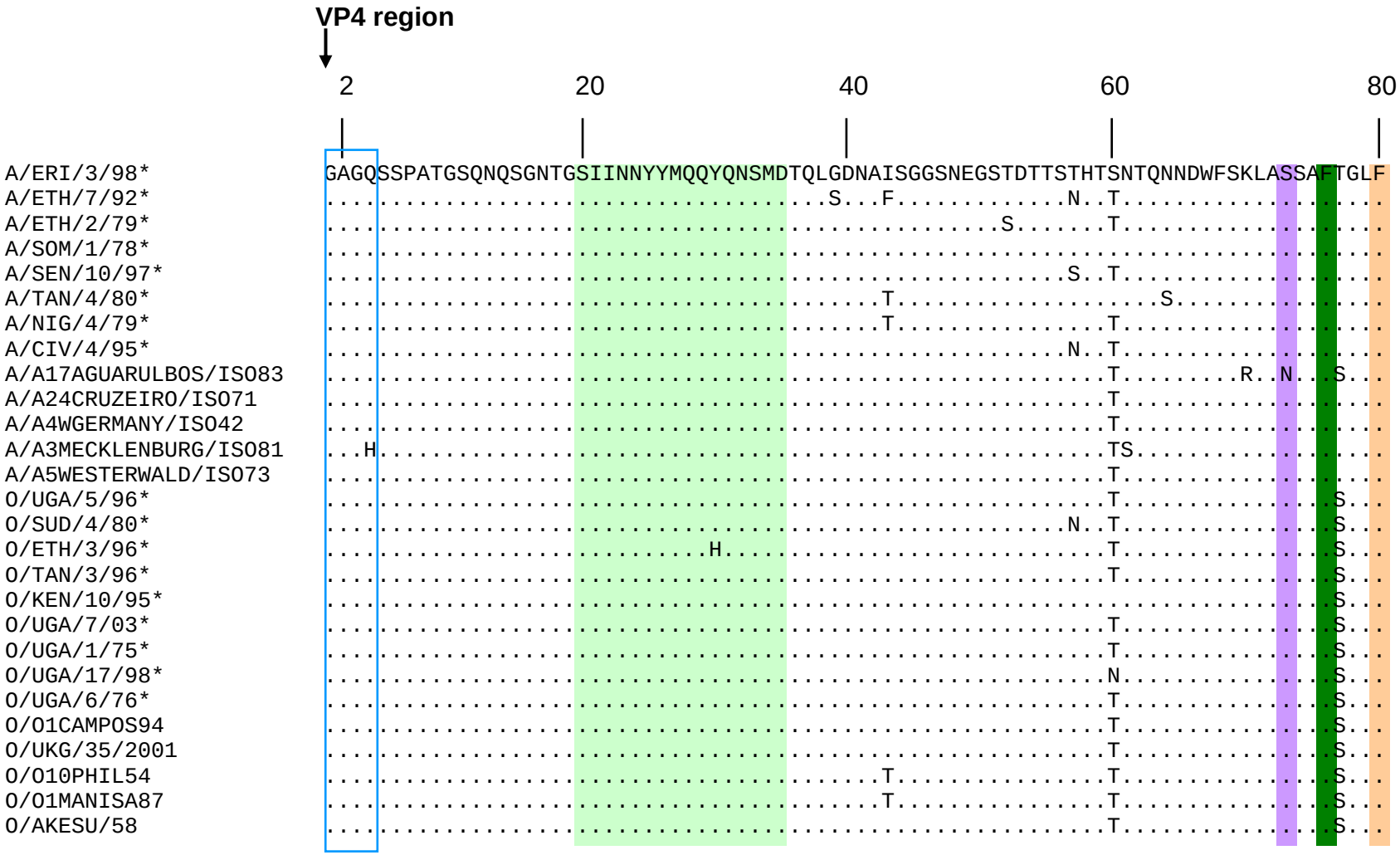


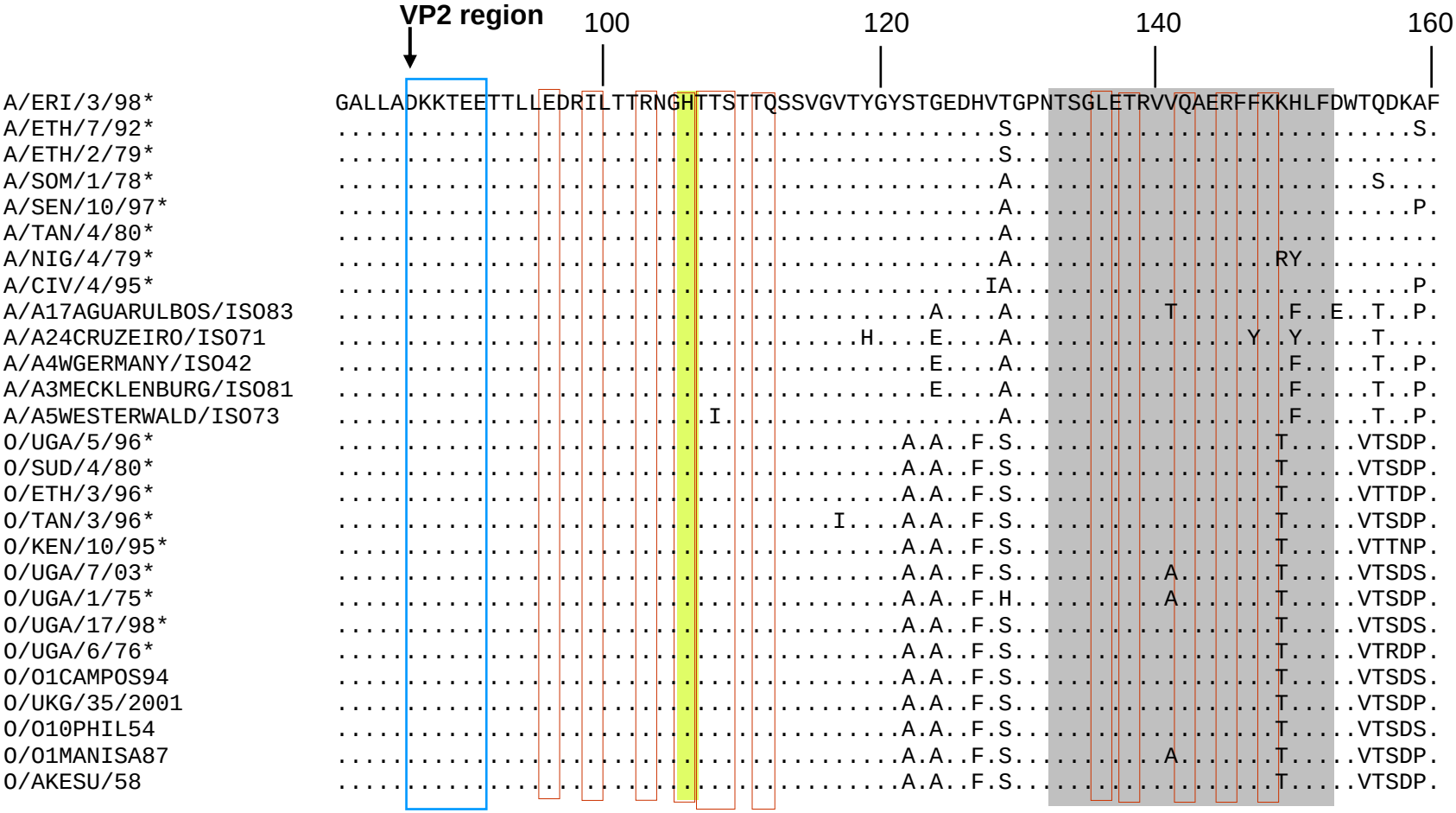

100
120
140
160
O/ETH/3/96 ENLTAEAIRQLEEVTGLELHEGGPPALVVWNIKHLLHTGIGTASRPSEVCVVDGTDMCLADFHAGIFLKGQEHAVFACVT
O/SUD/4/80 ...V...K....L.....M.....I.
O/TAN/3/96 ...V.....GL.....I.....M.....
O/KEN/10/95 .....L.....I.....V.....M.....
O/UGA/7/03 .....GL.....I.....M.....
O/UGA/1/75 ...LQ..E....L.....I.....
O/UGA/17/98 ...LQ..N....L.S.....I.....M.....H.....
O/UGA/6/76 ...LQ..K....L.S.....I.....M.....
O/UGA/5/96 ...T.....L.....I.....M.....I.
O/O1CAMPOS94 ...L...K...DL.....I.....M.....
O/UKG/35/2001 ...LD..K....I.....I.....N.....M.....
O/O10PHIL54 ...LD.....I.....I.....M.....
O/O1_MANISA87 ...L...K....T.....I.....M.....
O/AKESU/58 ...LD.....I.....I.....N.....MA.....P..T.....
A/SEN/10/97 ...LQ..E....L.....I.....V.....M.....V.....L.
A/CIV/4/95 ...LQ..E....L.....I.....M.....A.....L.
A/NIG/4/79 ...LQ.....L.....M.....--FPERQ.TLSLL.S
A/ERI/3/98 ...LQ..E....L...D.....I...R..Y.....M.....A.....L.
A/ETH/7/92 ...LQ..K....L.....I.....Y.....M.....
A/ETH/2/79 ...LQ.....L.....Y.....M.....
A/SOM/1/78 ...V.....G.....I.....Q.....M.....
A/TAN/4/80 ...T.....DL.....I.....M.....
A24/CRUZEIRO/ISO71 ...L...E....L.....I.....M....N.....
A17/AGUARULBOS/ISO83 ...L...E....L.....R.....Q.....M.....M.....
A4W/GERMANY/ISO42 ...LD..K....L.....I.....Q.....M.....K.....
A3/MECKLENBURG/ISO81 ...LD..K....L.....I.....Q.....M.....
A5/WESTERWALD/ISO73 ...L...N....L.....I.....M.....

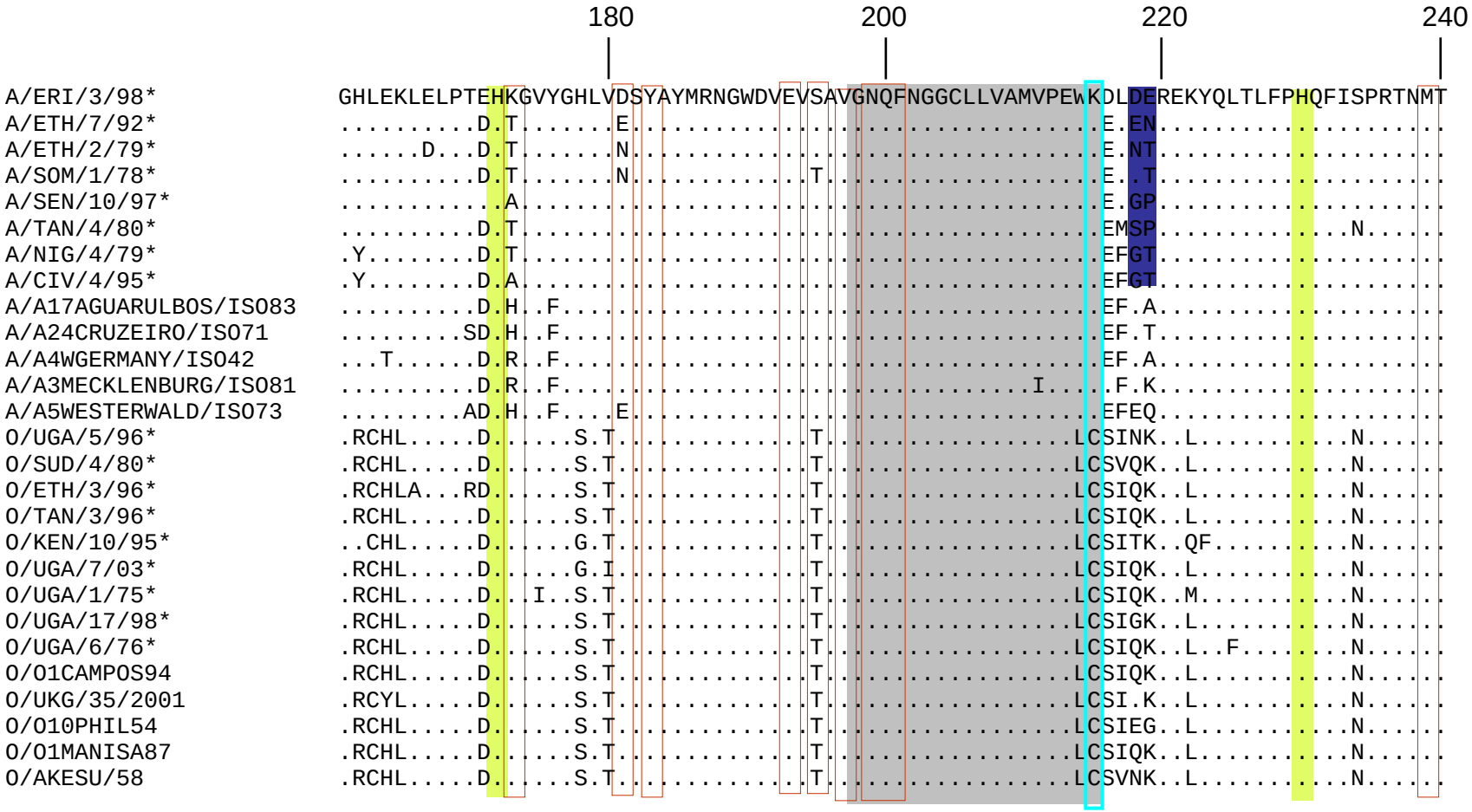


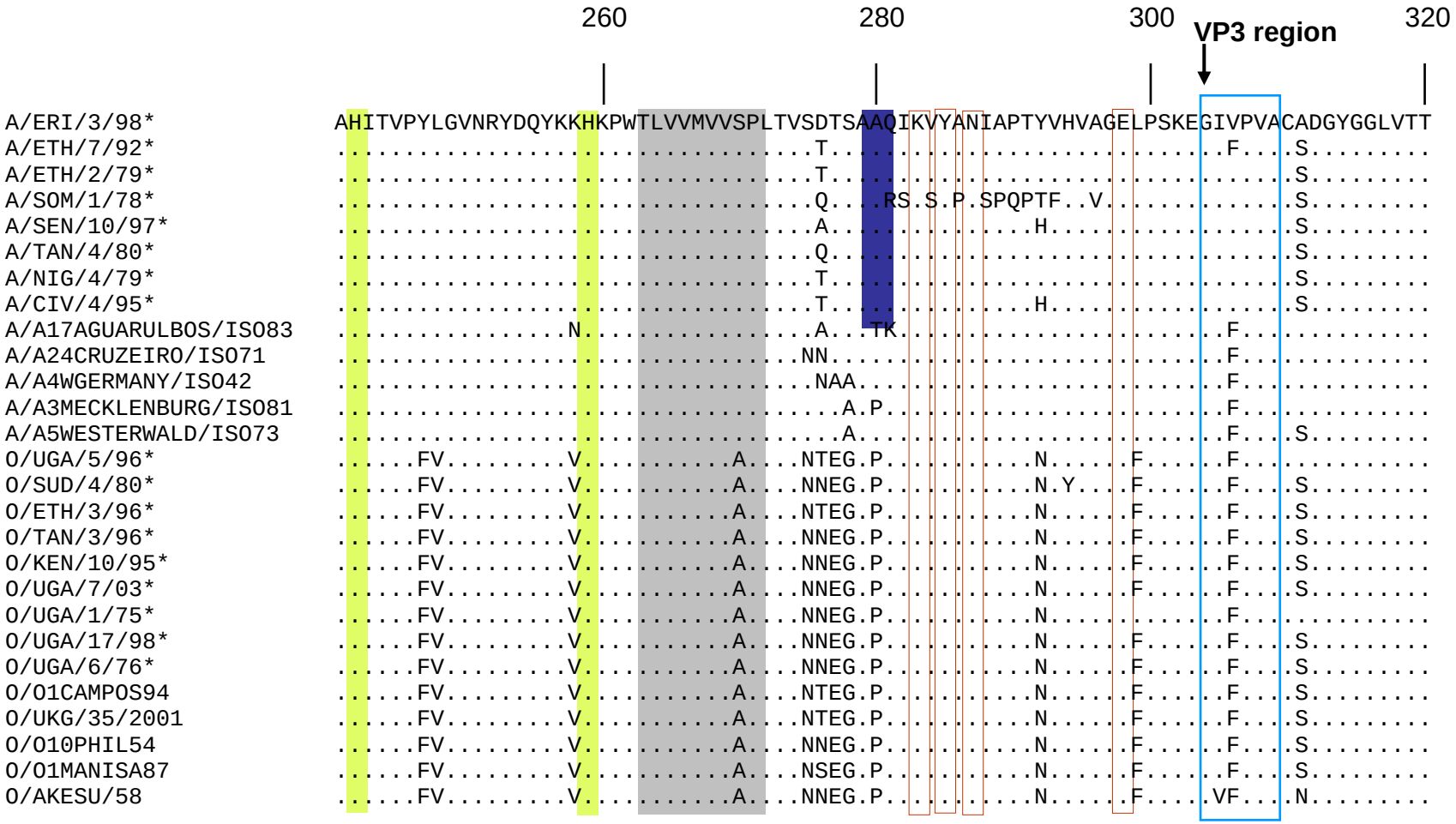
Residues required for L^{pro} catalytic activity (Gorbalenya *et al*., 1991; Kronovetr *et al*., 2002)
Residue involved in L^{pro} autocatalysis (Piccone *et al*., 1995a)
Residues important for eIF4G cleavage (Piccone *et al*., 1995b)
Dot (.) indicates identity with O/ETH/3/96, aa differences are indicated by single letter code.

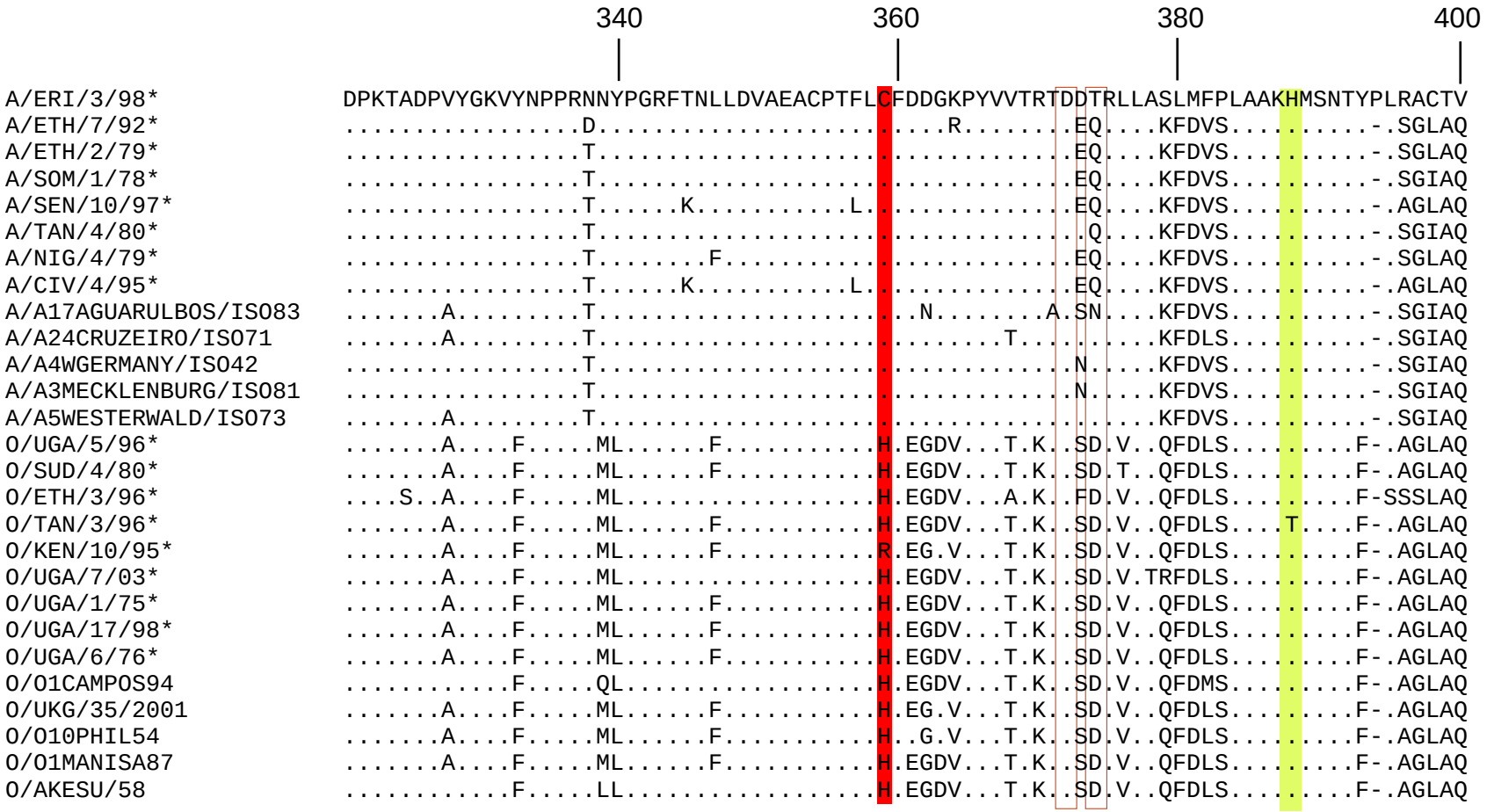
A8: P1 amino acid alignment of FMDV A and O serotypes

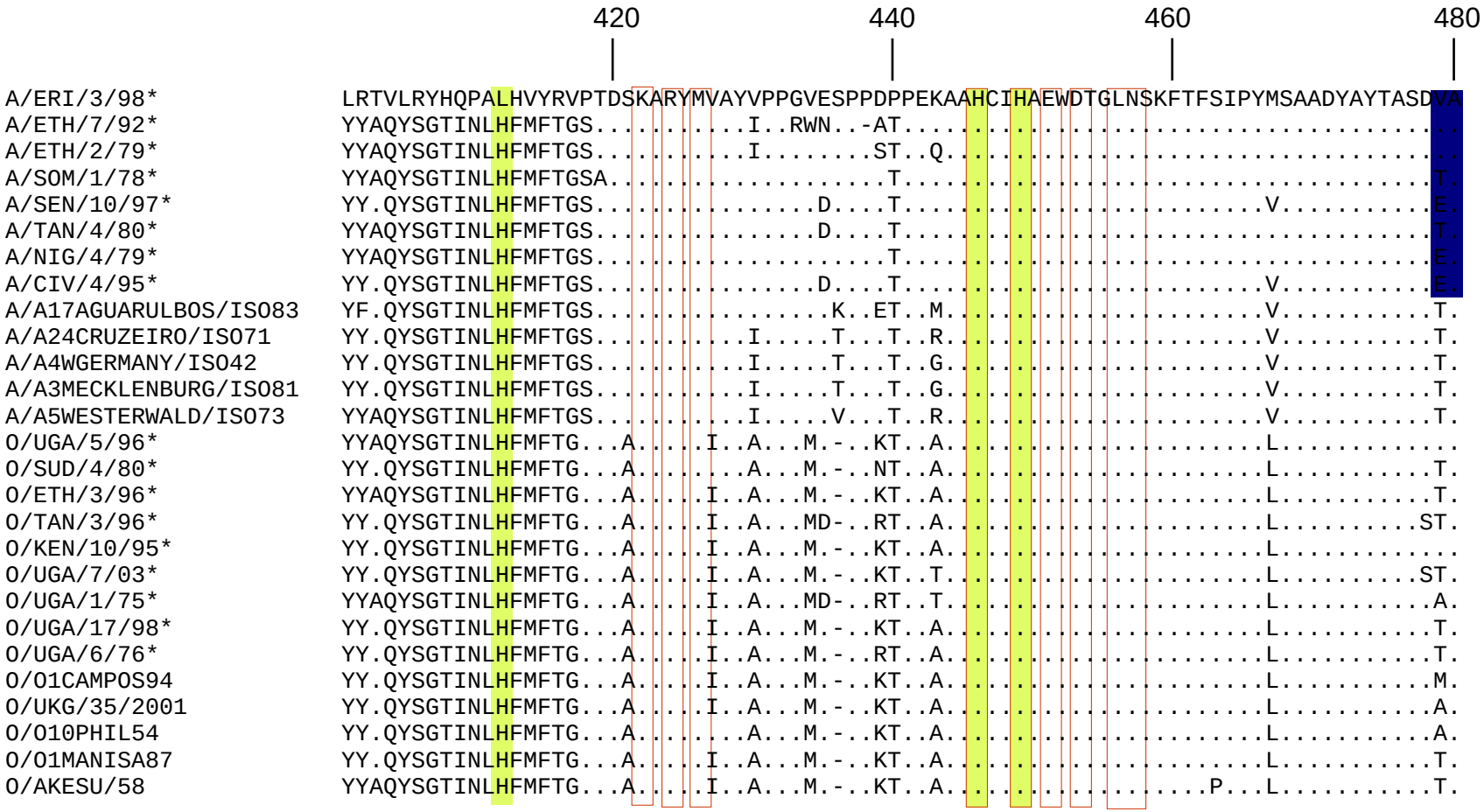


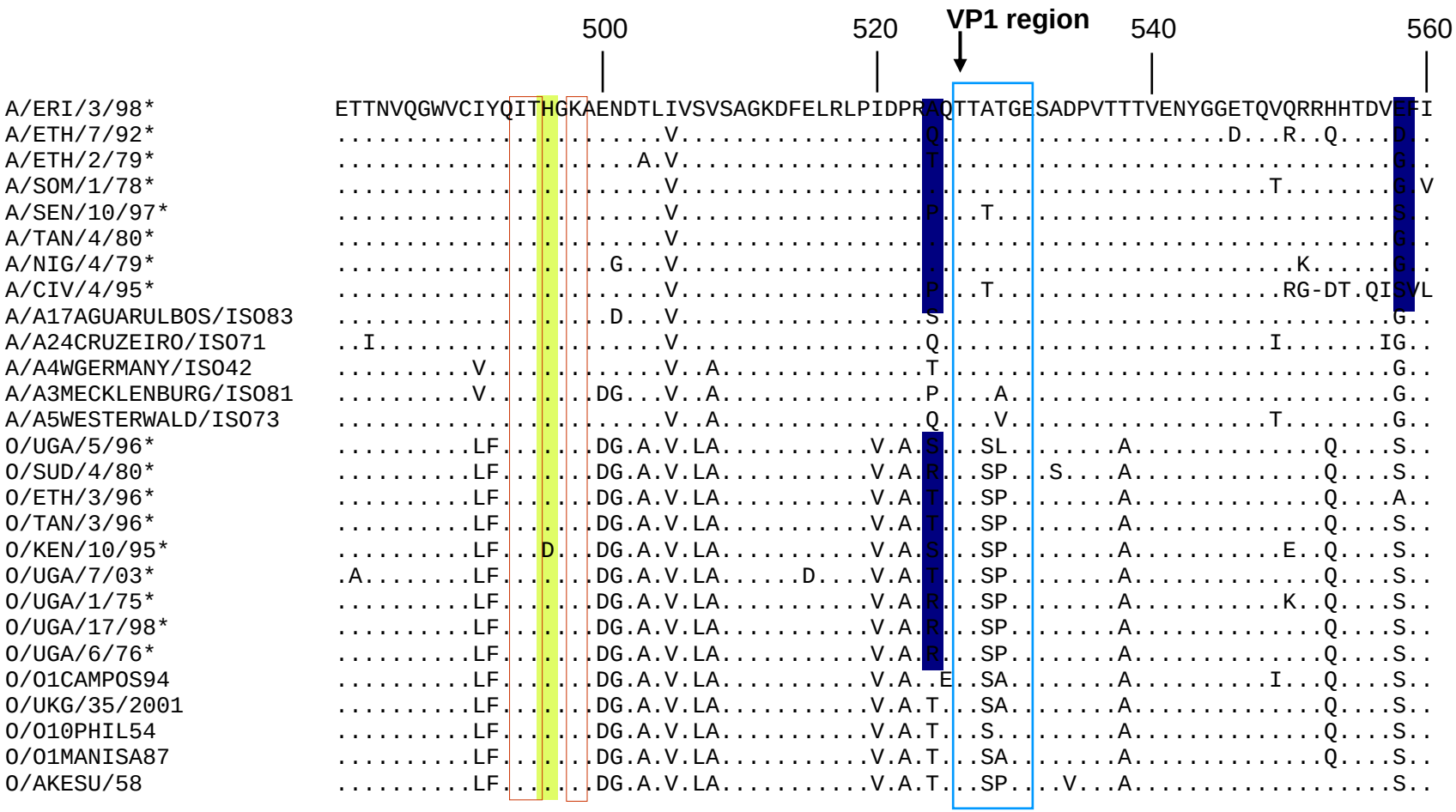

VP2 region
100
120
140
160
A/ERI/3/98* GALLADKKTEETTLLEDRILTTRNGHTTSTTQSSVGVTYGYSTGEDHVTGPNTSGLETRVVQAERFFKKHLFDWTQDKAF
A/ETH/7/92* .....S.....S.
A/ETH/2/79* .....S.....
A/SOM/1/78* .....A.....S....
A/SEN/10/97* .....A.....P.
A/TAN/4/80* .....A.....
A/NIG/4/79* .....A.....RY.....
A/CIV/4/95* .....IA.....P.
A/A17AGUARULBOS/ISO83 .....A....A.....T.....F..E..T..P.
A/A24CRUZEIRO/ISO71 .....H....E....A.....Y..Y.....T....
A/A4WGERMANY/ISO42 .....E....A.....F.....T..P.
A/A3MECKLENBURG/ISO81 .....E....A.....F.....T..P.
A/A5WESTERWALD/ISO73 .....I.....A.....F.....T..P.
O/UGA/5/96* .....A.A..F.S.....T.....VTSDP.
O/SUD/4/80* .....A.A..F.S.....T.....VTSDP.
O/ETH/3/96* .....A.A..F.S.....T.....VTTDP.
O/TAN/3/96* .....I....A.A..F.S.....T.....VTSDP.
O/KEN/10/95* .....A.A..F.S.....T.....VTTNP.
O/UGA/7/03* .....A.A..F.S.....A.....T.....VTSDS.
O/UGA/1/75* .....A.A..F.H.....A.....T.....VTSDP.
O/UGA/17/98* .....A.A..F.S.....T.....VTSDS.
O/UGA/6/76* .....A.A..F.S.....T.....VTRDP.
O/O1CAMPOS94 .....A.A..F.S.....T.....VTSDS.
O/UKG/35/2001 .....A.A..F.S.....T.....VTSDP.
O/O10PHIL54 .....A.A..F.S.....T.....VTSDS.
O/O1MANISA87 .....A.A..F.S.....A.....T.....VTSDP.
O/AKESU/58 .....A.A..F.S.....T.....VTSDP.

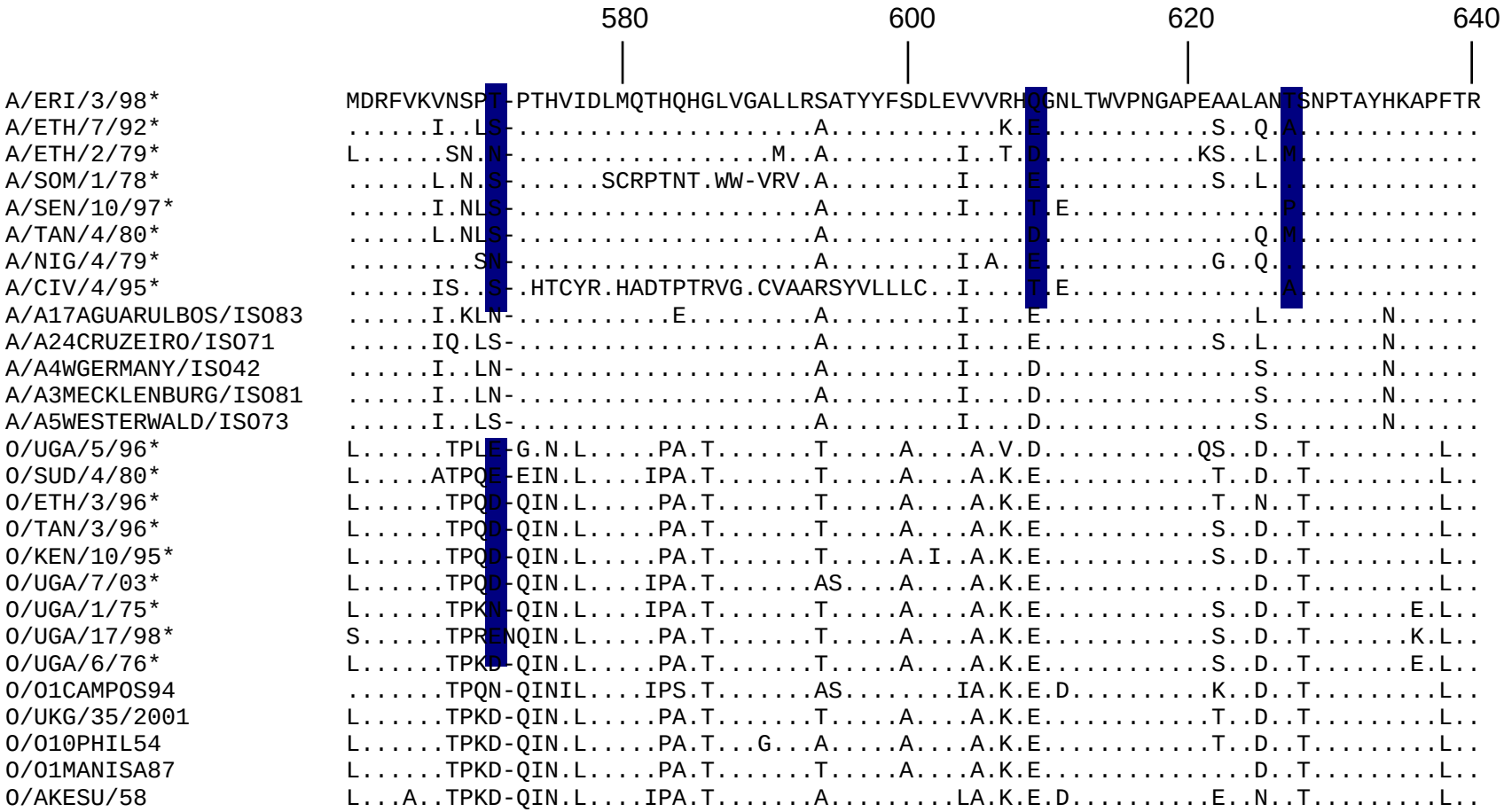

180
200
220
240
A/ERI/3/98* GHLEKLELPTEHKGVYGHLVDSYAYMRNGWDVEVSAVGNQFNGGCLLVAMVPEWKDLDEREKYQLTLFPHQFISPRTNMT
A/ETH/7/92* .....D.T.....E.....E.EN.....
A/ETH/2/79* .....D...D.T.....N.....E.NT.....
A/SOM/1/78* .....D.T.....N.....T.....E..T.....
A/SEN/10/97* .....A.....E.GP.....
A/TAN/4/80* .....D.T.....EMSP.....N.....
A/NIG/4/79* .Y.....D.T.....EFGT.....
A/CIV/4/95* .Y.....D.A.....EFGT.....
A/A17AGUARULBOS/ISO83 .....D.H..F.....EF.A.....
A/A24CRUZEIRO/ISO71 .....SD.H..F.....EF.T.....
A/A4WGERMANY/ISO42 ...T.....D.R..F.....EF.A.....
A/A3MECKLENBURG/ISO81 .....D.R..F.....I.....F.K.....
A/A5WESTERWALD/ISO73 .....AD.H..F....E.....EFEQ.....
O/UGA/5/96* .RCHL.....D.....S.T.....T.....LCSINK..L.....N.....
O/SUD/4/80* .RCHL.....D.....S.T.....T.....LCSVQK..L.....N.....
O/ETH/3/96* .RCHLA...RD.....S.T.....T.....LCSIQK..L.....N.....
O/TAN/3/96* .RCHL.....D.....S.T.....T.....LCSIQK..L.....N.....
O/KEN/10/95* ..CHL.....D.....G.T.....T.....LCSITK..QF.....N.....
O/UGA/7/03* .RCHL.....D.....G.I.....T.....LCSIQK..L.....N.....
O/UGA/1/75* .RCHL.....D...I..S.T.....T.....LCSIQK..M.....N.....
O/UGA/17/98* .RCHL.....D.....S.T.....T.....LCSIGK..L.....N.....
O/UGA/6/76* .RCHL.....D.....S.T.....T.....LCSIQK..L..F.....N.....
O/O1CAMPOS94 .RCHL.....D.....S.T.....T.....LCSIQK..L.....N.....
O/UKG/35/2001 .RCYL.....D.....S.T.....T.....LCSI.K..L.....N.....
O/O10PHIL54 .RCHL.....D.....S.T.....T.....LCSIEG..L.....N.....
O/O1MANISA87 .RCHL.....D.....S.T.....T.....LCSIQK..L.....N.....
O/AKESU/58 .RCHL.....D.....S.T.....T.....LCSVNK..L.....N.....

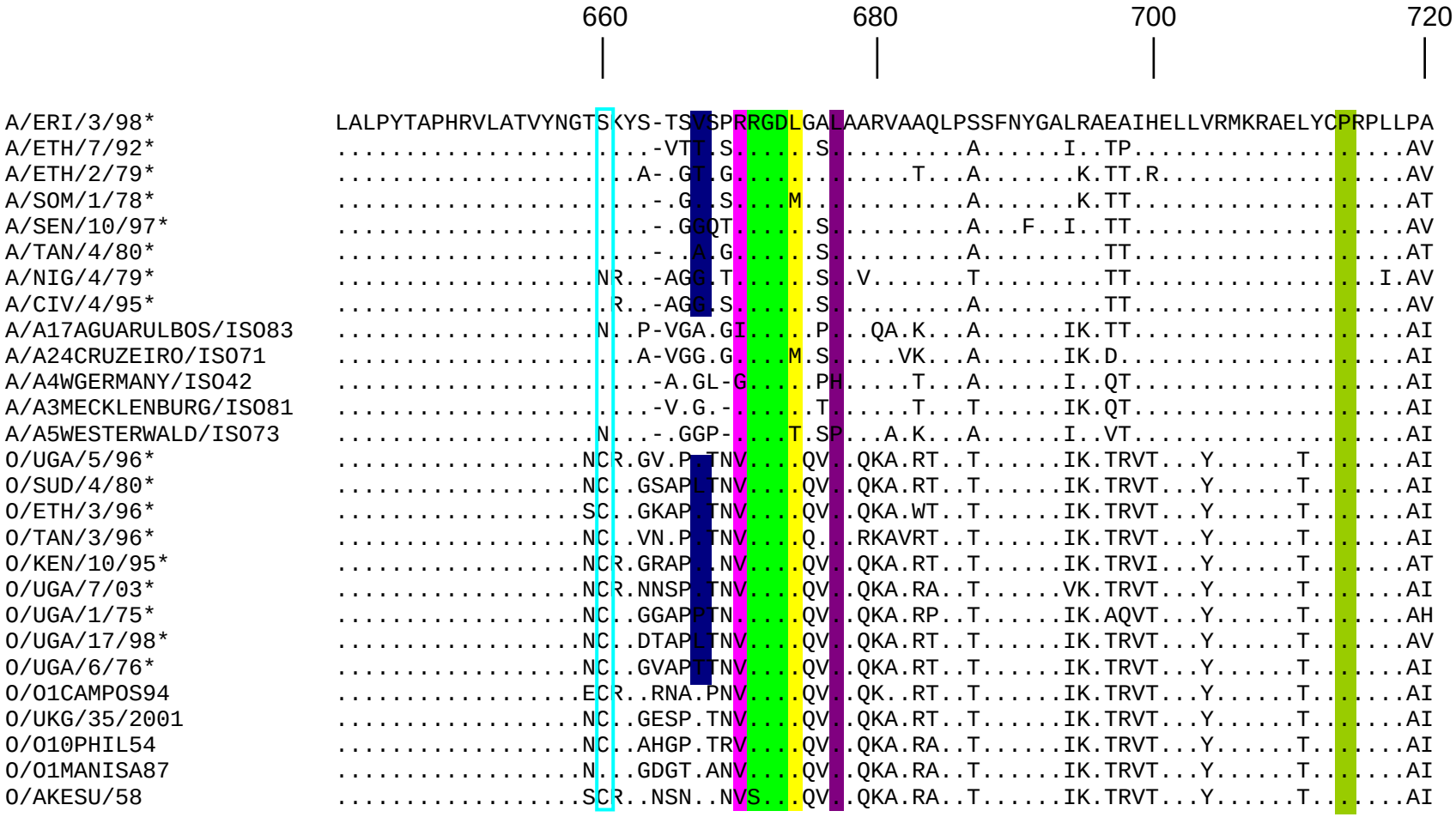

260
280
300
VP3 region
320
A/ERI/3/98* AHITVPYLGVNRYDQYKKHKPWTLVVMVVSPLTVSDTSAAQIKVYANIAPTYVHVAGELPSKEGIVPVACADGYGGLVTT
A/ETH/7/92* .....T.....F...S.....
A/ETH/2/79* .....T.....S.....
A/SOM/1/78* .....Q...RS.S.P.SPQPTF..V.....S.....
A/SEN/10/97* .....A.....H.....S.....
A/TAN/4/80* .....Q.....S.....
A/NIG/4/79* .....T.....S.....
A/CIV/4/95* .....T.....H.....S.....
A/A17AGUARULBOS/ISO83 .....N.....A...TK.....F.....
A/A24CRUZEIRO/ISO71 .....NN.....F.....
A/A4WGERMANY/ISO42 .....NAA.....F.....
A/A3MECKLENBURG/ISO81 .....A.P.....F.....
A/A5WESTERWALD/ISO73 .....A.....F....S.....
O/UGA/5/96* .....FV.....V.....A....NTEG.P.....N.....F.....F.....
O/SUD/4/80* .....FV.....V.....A....NNEG.P.....N.Y...F.....F....S.....
O/ETH/3/96* .....FV.....V.....A....NTEG.P.....N.....F.....F....S.....
O/TAN/3/96* .....FV.....V.....A....NNEG.P.....N.....F.....F....S.....
O/KEN/10/95* .....FV.....V.....A....NNEG.P.....N.....F.....F....S.....
O/UGA/7/03* .....FV.....V.....A....NNEG.P.....N.....F.....F....S.....
O/UGA/1/75* .....FV.....V.....A....NNEG.P.....N.....F.....
O/UGA/17/98* .....FV.....V.....A....NNEG.P.....N.....F.....F....S.....
O/UGA/6/76* .....FV.....V.....A....NNEG.P.....N.....F.....F....S.....
O/O1CAMPOS94 .....FV.....V.....A....NTEG.P.....N.....F.....F....S.....
O/UKG/35/2001 .....FV.....V.....A....NTEG.P.....N.....F.....F....S.....
O/O10PHIL54 .....FV.....V.....A....NNEG.P.....N.....F.....F....S.....
O/O1MANISA87 .....FV.....V.....A....NSEG.P.....N.....F.....F....S.....
O/AKESU/58 .....FV.....V.....A....NNEG.P.....N.....F.....VF....N.....

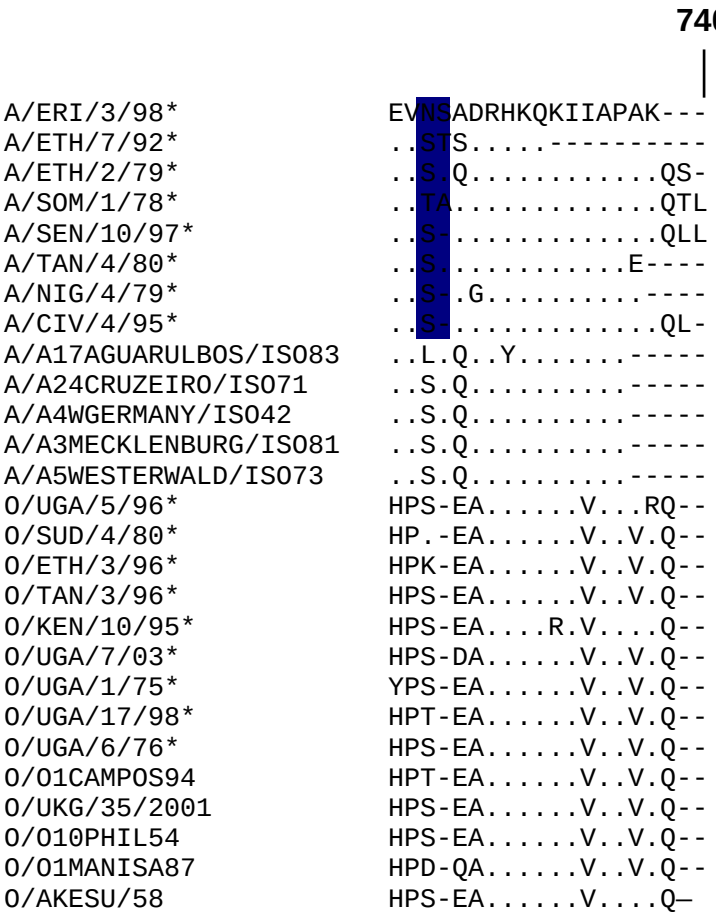

340
360
380
400
A/ERI/3/98* DPKTADPVYGKVYNPPRNNYPGRFTNLLDVAEACPTFLCFDDGKPYVVTRTDDTRLLASLMFPLAAKHMSNTYPLRACTV
A/ETH/7/92* .....D.....R.....EQ....KFDVS.....-.SGLAQ
A/ETH/2/79* .....T.....EQ....KFDVS.....-.SGLAQ
A/SOM/1/78* .....T.....EQ....KFDVS.....-.SGIAQ
A/SEN/10/97* .....T.....K.....L.....EQ....KFDVS.....-.AGLAQ
A/TAN/4/80* .....T.....Q....KFDVS.....-.SGIAQ
A/NIG/4/79* .....T.....F.....EQ....KFDVS.....-.SGLAQ
A/CIV/4/95* .....T.....K.....L.....EQ....KFDVS.....-.AGLAQ
A/A17AGUARULBOS/ISO83 .....A.....T.....N.....A.SN....KFDVS.....-.SGIAQ
A/A24CRUZEIRO/ISO71 .....A.....T.....T.....KFDLS.....-.SGIAQ
A/A4WGERMANY/ISO42 .....T.....N.....KFDVS.....-.SGIAQ
A/A3MECKLENBURG/ISO81 .....T.....N.....KFDVS.....-.SGIAQ
A/A5WESTERWALD/ISO73 .....A.....T.....KFDVS.....-.SGIAQ
O/UGA/5/96* .....A....F.....ML.....F.....H.EGDV...T.K..SD.V..QFDLS.....F-.AGLAQ
O/SUD/4/80* .....A....F.....ML.....F.....H.EGDV...T.K..SD.T..QFDLS.....F-.AGLAQ
O/ETH/3/96* ....S..A....F.....ML.....H.EGDV...A.K..FD.V..QFDLS.....F-SSSLAQ
O/TAN/3/96* .....A....F.....ML.....F.....H.EGDV...T.K..SD.V..QFDLS....T....F-.AGLAQ
O/KEN/10/95* .....A....F.....ML.....F.....R.EG.V...T.K..SD.V..QFDLS.....F-.AGLAQ
O/UGA/7/03* .....A....F.....ML.....H.EGDV...T.K..SD.V.TRFDLS.....F-.AGLAQ
O/UGA/1/75* .....A....F.....ML.....F.....H.EGDV...T.K..SD.V..QFDLS.....F-.AGLAQ
O/UGA/17/98* .....A....F.....ML.....F.....H.EGDV...T.K..SD.V..QFDLS.....F-.AGLAQ
O/UGA/6/76* .....A....F.....ML.....F.....H.EGDV...T.K..SD.V..QFDLS.....F-.AGLAQ
O/O1CAMPOS94 .....F.....QL.....H.EGDV...T.K..SD.V..QFDMS.....F-.AGLAQ
O/UKG/35/2001 .....A....F.....ML.....F.....H.EG.V...T.K..SD.V..QFDLS.....F-.AGLAQ
O/O10PHIL54 .....A....F.....ML.....F.....H..G.V...T.K..SD.V..QFDLS.....F-.AGLAQ
O/O1MANISA87 .....A....F.....ML.....F.....H.EGDV...T.K..SD.V..QFDLS.....F-.AGLAQ
O/AKESU/58 .....F.....LL.....H.EGDV...T.K..SD.V..QFDLS.....F-.AGLAQ


420
440
460
480
A/ERI/3/98*
A/ETH/7/92*
A/ETH/2/79*
A/SOM/1/78*
A/SEN/10/97*
A/TAN/4/80*
A/NIG/4/79*
A/CIV/4/95*
A/A17AGUARULBOS/ISO83
A/A24CRUZEIRO/ISO71
A/A4WGERMANY/ISO42
A/A3MECKLENBURG/ISO81
A/A5WESTERWALD/ISO73
O/UGA/5/96*
O/SUD/4/80*
O/ETH/3/96*
O/TAN/3/96*
O/KEN/10/95*
O/UGA/7/03*
O/UGA/1/75*
O/UGA/17/98*
O/UGA/6/76*
O/O1CAMPOS94
O/UKG/35/2001
O/O10PHIL54
O/O1MANISA87
O/AKESU/58


500
520
VP1 region
540
560
A/ERI/3/98* ETTNVQGWVCIYQITHGKAENDTLIVSVSAGKDFELRLPIDPRAQTTATGESADPVTTTVENYGGETQVQRRHHTDVEFI
A/ETH/7/92* .....V.....Q.....D...R..Q....D..
A/ETH/2/79* .....A.V.....T.....G..
A/SOM/1/78* .....V.....T.....G.V
A/SEN/10/97* .....V.....P...T.....S..
A/TAN/4/80* .....V.....G..
A/NIG/4/79* .....G...V.....K.....G..
A/CIV/4/95* .....V.....P...T.....RG-DT.QISVL
A/A17AGUARULBOS/ISO83 .....D...V.....S.....G..
A/A24CRUZEIRO/ISO71 ..I.....V.....Q.....I.....IG..
A/A4WGERMANY/ISO42 .....V.....V..A.....T.....G..
A/A3MECKLENBURG/ISO81 .....V.....DG...V..A.....P...A.....G..
A/A5WESTERWALD/ISO73 .....V..A.....Q...V.....T.....G..
O/UGA/5/96* .....LF.....DG.A.V.LA.....V.A.S...SL.....A.....Q....S..
O/SUD/4/80* .....LF.....DG.A.V.LA.....V.A.R...SP...S....A.....Q....S..
O/ETH/3/96* .....LF.....DG.A.V.LA.....V.A.T...SP.....A.....Q....A..
O/TAN/3/96* .....LF.....DG.A.V.LA.....V.A.T...SP.....A.....Q....S..
O/KEN/10/95* .....LF...D...DG.A.V.LA.....V.A.S...SP.....A.....E..Q....S..
O/UGA/7/03* .A.....LF.....DG.A.V.LA.....D....V.A.T...SP.....A.....Q....S..
O/UGA/1/75* .....LF.....DG.A.V.LA.....V.A.R...SP.....A.....K..Q....S..
O/UGA/17/98* .....LF.....DG.A.V.LA.....V.A.R...SP.....A.....Q....S..
O/UGA/6/76* .....LF.....DG.A.V.LA.....V.A.R...SP.....A.....Q....S..
O/O1CAMPOS94 .....LF.....DG.A.V.LA.....V.A..E..SA.....A.....I...Q....S..
O/UKG/35/2001 .....LF.....DG.A.V.LA.....V.A.T...SA.....A.....Q....S..
O/O10PHIL54 .....LF.....DG.A.V.LA.....V.A.T...S.....A.....Q....S..
O/O1MANISA87 .....LF.....DG.A.V.LA.....V.A.T...SA.....A.....Q....S..
O/AKESU/58 .....LF.....DG.A.V.LA.....V.A.T...SP....V...A.....S..


580
600
620
640
A/ERI/3/98* MDRFVKVNSPT-PTHVIDLMQTHQHGLVGALLRSATYYFSDLEVVVRHQGNLTWVPNGAPEAALANTSNPTAYHKAPFTR
A/ETH/7/92* ......I..LS-.....A.....K.E.....S..Q.A.....
A/ETH/2/79* L......SN.N-.....M..A.....I..T.D.....KS..L.M.....
A/SOM/1/78* ......L.N.S-......SCRPTNT.WW-VRV.A.....I....E.....S..L.....
A/SEN/10/97* ......I.NLS-.....A.....I....T.E.....P.....
A/TAN/4/80* ......L.NLS-.....A.....D.....Q.M.....
A/NIG/4/79* .........SN-.....A.....I.A..E.....G..Q.....
A/CIV/4/95* ......IS..S-.HTCYR.HADTPTRVG.CVAARSYVLLLC..I....T.E.....A.....
A/A17AGUARULBOS/ISO83 ......I.KLN-.....E.....A.....I....E.....L.....N.....
A/A24CRUZEIRO/ISO71 ......IQ.LS-.....A.....I....E.....S..L.....N.....
A/A4WGERMANY/ISO42 ......I..LN-.....A.....I....D.....S.....N.....
A/A3MECKLENBURG/ISO81 ......I..LN-.....A.....I....D.....S.....N.....
A/A5WESTERWALD/ISO73 ......I..LS-.....A.....I....D.....S.....N.....
O/UGA/5/96* L......TPLE-G.N.L.....PA.T.....T.....A....A.V.D.....QS..D..T.....L..
O/SUD/4/80* L.....ATPQE-EIN.L....IPA.T.....T.....A....A.K.E.....T..D..T.....L..
O/ETH/3/96* L......TPQD-QIN.L.....PA.T.....T.....A....A.K.E.....T..N..T.....L..
O/TAN/3/96* L......TPQD-QIN.L.....PA.T.....T.....A....A.K.E.....S..D..T.....L..
O/KEN/10/95* L......TPQD-QIN.L.....PA.T.....T.....A.I..A.K.E.....S..D..T.....L..
O/UGA/7/03* L......TPQD-QIN.L....IPA.T.....AS....A....A.K.E.....D..T.....L..
O/UGA/1/75* L......TPKN-QIN.L....IPA.T.....T.....A....A.K.E.....S..D..T.....E.L..
O/UGA/17/98* S......TPRENQIN.L.....PA.T.....T.....A....A.K.E.....S..D..T.....K.L..
O/UGA/6/76* L......TPKD-QIN.L.....PA.T.....T.....A....A.K.E.....S..D..T.....E.L..
O/O1CAMPOS94 .....TPQN-QINIL....IPS.T.....AS.....IA.K.E.D.....K..D..T.....L..
O/UKG/35/2001 L......TPKD-QIN.L.....PA.T.....T.....A....A.K.E.....T..D..T.....L..
O/O10PHIL54 L......TPKD-QIN.L.....PA.T...G...A.....A....A.K.E.....T..D..T.....L..
O/O1MANISA87 L......TPKD-QIN.L.....PA.T.....T.....A....A.K.E.....D..T.....L..
O/AKESU/58 L...A..TPKD-QIN.L....IPA.T.....A.....LA.K.E.D.....E..N..T.....L..


660
680
700
720
A/ERI/3/98* LALPYTAPHRVLATVYNGTSKYS-TSVSPRRGDLGALAARVAAQLPSSFNYGALRAEAIHELLVRMKRAELYCPRPLLPA
A/ETH/7/92* .....-VTT.S.....S.........A......I..TP.....AV
A/ETH/2/79* .....A-.GT.G.....T...A.......K.TT.R.....AV
A/SOM/1/78* .....-.G..S....M.....A.......K.TT.....AT
A/SEN/10/97* .....-.GGQT.....S.....A...F..I..TT.....AV
A/TAN/4/80* .....-..A.G.....S.....A.....TT.....AT
A/NIG/4/79* .....NR..-AGG.T.....S..V.....T.....TT.....I.AV
A/CIV/4/95* .....R..-AGG.S.....S.....A.....TT.....AV
A/A17AGUARULBOS/ISO83 .....N..P-VGA.GI.....P...QA.K...A.....IK.TT.....AI
A/A24CRUZEIRO/ISO71 .....A-VGG.G....M.S....VK...A.....IK.D.....AI
A/A4WGERMANY/ISO42 .....-A.GL-G....PH.....T...A.....I..QT.....AI
A/A3MECKLENBURG/ISO81 .....-V.G.-.....T.....T...T.....IK.QT.....AI
A/A5WESTERWALD/ISO73 .....N...-.GGP-....T.SP...A.K...A.....I..VT.....AI
O/UGA/5/96* .....NCR.GV.P.TNV....QV..QKA.RT..T.....IK.TRVT...Y.....T.....AI
O/SUD/4/80* .....NC..GSAPLTNV....QV..QKA.RT..T.....IK.TRVT...Y.....T.....AI
O/ETH/3/96* .....SC..GKAP.TNV....QV..QKA.WT..T.....IK.TRVT...Y.....T.....AI
O/TAN/3/96* .....NC..VN.P.TNV....Q...RKAVRT..T.....IK.TRVT...Y.....T.....AI
O/KEN/10/95* .....NCR.GRAP..NV....QV..QKA.RT..T.....IK.TRVI...Y.....T.....AT
O/UGA/7/03* .....NCR.NNSP.TNV....QV..QKA.RA..T.....VK.TRVT...Y.....T.....AI
O/UGA/1/75* .....NC..GGAPPTN.....QV..QKA.RP..T.....IK.AQVT...Y.....T.....AH
O/UGA/17/98* .....NC..DTAPLTNV....QV..QKA.RT..T.....IK.TRVT...Y.....T.....AV
O/UGA/6/76* .....NC..GVAPTTNV....QV..QKA.RT..T.....IK.TRVT...Y.....T.....AI
O/O1CAMPOS94 .....ECR..RNA.PNV....QV..QK..RT..T.....IK.TRVT...Y.....T.....AI
O/UKG/35/2001 .....NC..GESP.TNV....QV..QKA.RT..T.....IK.TRVT...Y.....T.....AI
O/O10PHIL54 .....NC..AHGP.TRV....QV..QKA.RA..T.....IK.TRVT...Y.....T.....AI
O/O1MANISA87 .....N...GDGT.ANV....QV..QKA.RA..T.....IK.TRVT...Y.....T.....AI
O/AKESU/58 .....SCR..NSN..NVS...QV..QKA.RA..T.....IK.TRVT...Y.....T.....AI



N-terminal myristylation site and previously identified swine and bovine T-cell epitope (Blanco *et al*., 2001)

This position is a conserved Q residue for the SAT types, which distinguishes them from the Euroasiatic lineages. (Carrillio *et al*., 2005).

At this position, the I residue is potentially specific for the SAT2 and SAT3 viruses (Carrillo *et al*., 2005)

A valine at this position is potentially specific for SAT1 (Carrillo *et al*., 2005).

The P residue contributes to the VP4/VP2 cleavage pocket (Carrillo *et al*., 2005).

A R at this position (only observed for O/KEN/10/95 in this study) was identified by Fry *et al.* (1999) as being important for HS binding.

The RGD residue (important for receptor binding) was conserved for all viruses in the alignment except for O/Akesu/58.

A unique R residue for all the FMDV A serotypes except for A17AGUARULBOS/ISO83 and A/A4WGERMANY/ISO42 was observed.

At this position the L residue is most common and was observed for all viruses except for A/SOM/1/78 and A/A24CRUZEIRO/ISO71, which had a M and A/A5WESTERWALD/ISO73 had a T at this position.

The L is also comonly found at this position, which was observed for all viruses except A/A4WGERMANY/ISO42 and A/A5WESTERWALD/ISO73, which had a H and P residue at this position respectively.

Identified VP2 T-cell epitopes (Perez *et al*., 2000).

H residues have been identified in the VP2/VP3 regions and is proposed to mediate VP2/VP3 hydrogen bonding (Acharya *et al*., 1989; Curry *et al*, 1999).

Regions prone to variation for the FMDV A and O African viruses (from the entropy plots, values above an entropy of 1.

Side chains involved in non-covalent interactions between pentamer subunits in the FMDV C-S8c1 capsid.

C residues at these positions are important for the formation of a disulphide bond, linking the base of the GH loop

Amino acid sequence alignment of the complete capsid-coding (P1) region of the African FMDV A and O types (included in this study) and European A and O serotypes. The start of each region *i.e*. VP4, VP2, VP3 and VP1 are indicated by the blue blocks. In addition the cut sites are indicated by the black arrows. Dot (.) indicates identity with A/ERI/3/98, aa differences are indicated by single letter code. * indicates the FMDV A and O African isolates.

A9: Hamster specific oligonucleotides specific for BHK cells

Oligonucleotide	Sequence	Expected fragment size
BHKβ1-F	5'–TTGCGATCAGGAGAACCACAG–3'	~150bp
BHKβ1-R	5'-CCTCATTTCATTCATCAGATCTG–3'	
BHKβ3-F	5'-TTGGCAGTGACAACCATTACTC–3'	~155bp
BHKβ3-R	5'-AGGACATTGCTTGAGTCATCAG–3'	
BHKβ6-F	5'-GTGATGCCGATTCTCATTTTGG–3'	~200bp
BHKβ6-R	5'-GGACTTGTTCTTGGGTTACAGC–3'	
β-actin -F	5'-TCGTCACCAACTGGGACGAC–3'	~250bp
β-actin -R	5'-CGGAGTCCATCACGATGCC–3'	

A10: List of integrin (ITG) primers

Oligonucleotide	Sequence	Expected fragment size
ITGβ1–F	5'–GGAATGCCTACTTCTGCACGATG–3'	~370bp
ITGβ1–R	5'-GAAGTAATCCTCCTCATTTCATTC–3'	
ITGβ3–F	5'-GCTGGAGGAATGATGCATCCC–3'	~510bp
ITGβ3–R	5'-ATGCTGAAGCTCACCGTGTCTCC–3'	
ITGβ6–F	5'-ACACCCGAAGGTGGATTTGATGC–3'	~420bp
ITGβ6–R	5'-CCACCTCAGACCGCAGTTCTT–3'	
ITGβ8–F	5'–AAGATTGCTGCTGGTGATGACAG–3'	~580bp
ITGβ8–R	5'–GTGGTTTCATTGAAACCAATAGG–3'	
β-actin -F	5'-TCGTCACCAACTGGGACGAC–3'	~250bp
β-actin -R	5'-CGGAGTCCATCACGATGCC–3'	

A11: Details of HS oligonucleotide sequences

Oligonucleotide	**Sequence**	**Expected fragment size**
HSPG-F	5'–GTCGAGAGCTGACTCCAGCCG–3'	~300bp
HSPG-R	5'–GTTGTCAGAGTCATCCCCAGAGC–3'	

A12: Volume of each reagent used in the PCR mix for the different primer sets

	HS	ITGβ1	ITGβ3	ITGβ6	ITGβ8	β-ACTIN
REAGENTS (FINAL CONCENTRATION)						
DNA template	1µl	1µl	1µl	1µl	1µl	1µl
dH_2O	4.3µl	4.3µl	4.3µl	4.3µl	4.3µl	4.8µl
PCR Buffer (1X)	1µl	1µl	1µl	1µl	1µl	1µl
DNTPs (200µM)	0.9µl	0.9µl	0.9µl	0.9µl	0.9µl	0.9µl
$MgCl_2$ (2.75mM)	0.5µl	0.5µl	0.5µl	0.5µl	0.5µl	0µl
Forward Primer (10pmol)	1µl	1µl	1µl	1µl	1µl	1µl
Reverse Primer (10pmol)	1µl	1µl	1µl	1µl	1µl	1µl
Taq Polymerase (2.5U)	0.3µl	0.3µl	0.3µl	0.3µl	0.3µl	0.3µl
Total volume	**10µl**	**10µl**	**10µl**	**10µl**	**10µl**	**10µl**

A13: PCR conditions for the different primer sets

	ITGβ1	ITGβ3	ITGβ6	ITGβ8	βACTIN
94°C2min	✓	✓	✓	✓	✓
94°C30s	✓	✓	✓	✓	✓
55°C30s					✓
57°C30s	✓		✓		
58°C30s		✓		✓	
72°C60s	✓	✓	✓	✓	✓
72°C5min	✓	✓	✓	✓	✓

A14: PCR conditions tested (using various annealing temperatures) for IB-RS-2 optimisation

	ITGβ1	ITGβ3	ITGβ6	ITGβ8	HS	βACTIN
94°C2min	✓	✓	✓	✓	✓	✓
94°C30s	✓	✓	✓	✓	✓	✓
52°C30s			✓	✓	✓	
53°C30s			✓	✓	✓	
54°C30s			✓	✓	✓	
55°C30s	✓	✓	✓	✓	✓	✓
57°C30s	✓	✓	✓	✓	✓	✓
58°C30s	✓	✓	✓	✓	✓	✓
72°C60s	✓	✓	✓	✓	✓	✓
72°C5min	✓	✓	✓	✓	✓	✓