Molecular cloning and expression of the 3ABC non-structural protein-coding region from a SAT2 foot-and-mouth disease virus

by

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SUMMARY

Molecular cloning and expression of the 3ABC non-structural protein-coding region from a SAT2 foot-and-mouth disease virus

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Foot-and-mouth disease (FMD) virus causes a highly contagious, economically important disease of cloven-hoofed animals, including livestock animals such as cattle and swine. In South Africa, the disease is controlled primarily through prophylactic vaccination and strict animal movement control. To control effectively the spread of FMD, it has become increasingly more important to develop diagnostic tests that can differentiate FMDV-infected animals from those that have been vaccinated. Currently, the detection of antibodies to nonstructural proteins, especially the 3ABC nonstructural polypeptide, is considered to be the most reliable method to distinguish virus-infected from vaccinated animals. Towards the development of such a serological test, the primary aim of this investigation was to express the 3ABC nonstructural polypeptide of the SAT2 type FMD virus ZIM/7/83 in both a prokaryotic and eukaryotic expression system in order for the recombinant protein to be used as a diagnostic antigen.

The nucleotide sequence of the 3ABC-encoding region of SAT2/ZIM/7/83 was determined, the amino acid sequence deduced and subsequently compared to corresponding sequences of other virus isolates representing all seven FMDV serotypes. Phylogenetic analysis revealed that the 3ABC polypeptide of the SAT serotypes, which are mostly restricted to sub-Saharan

Africa, clustered separately from the euroasiatic FMDV serotypes (types A, O, C and Asia1). Amino acid sequence alignments also indicated considerable variation in the 3A, 3B and 3C proteins between the SAT and euroasiatic types located mainly in previously identified epitope-containing regions. These results suggest that the sensitivity and specificity of diagnostic tests based on the 3ABC nonstructural polypeptide of the European FMDV types may be compromised when applied to the African sub-continent. Therefore, a SAT-specific diagnostic assay is required to distinguish virus-infected from vaccinated animals.

The 3ABC-encoding region of SAT2/ZIM/7/83 was subsequently expressed in *Escherichia coli* as a glutathione *S*-transferase (GST) fusion protein using the bacterial expression vector pGEX-2T, and in *Spodoptera frugiperda* insect cells using the BAC-to-BAC™ baculovirus expression system. Although high-level expression of the recombinant GST-3ABC protein was obtained, the GST-3ABC protein was insoluble and could not be purified by glutathione affinity chromatography. Therefore, the recombinant GST-3ABC fusion protein was purified from reverse-stained SDS-polyacrylamide gels and shown to be immunoreactive in Western blot analysis using an FMDV-specific serum. Expression of the 3ABC polypeptide in insect cells infected with a recombinant bacmid yielded soluble recombinant protein, but the level of expression was lower compared to that obtained in *E. coli*. In addition, Western blot analysis of cell extracts prepared from recombinant bacmid-infected cells revealed the presence of three immunoreactive proteins of 47, 25 and 18 kDa. These correspond with the size of the FMDV proteins 3ABC, 3AB and 3A, respectively, suggesting that the 3C protease was responsible for proteolytic cleavage of the 3ABC polypeptide. Based on the results obtained, the bacmid expression system appears to be more suitable for the production of the 3ABC polypeptide.

LIST OF ABBREVIATIONS

$3C^{pro}$	3C protease
$3D^{pol}$	3D polymerase
aa	amino acid
BEI	binary ethyleneimine
BHK	baby hamster kidney
bp	base pair
BSA	bovine serum albumin
BT-ELISA	blocking trapping-ELISA
C	carboxy
ca.	approximately
cDNA	complementary DNA
C-ELISA	competitive-ELISA
CF	complement fixation
cm^2	cubic centimeter
cre	*cis*-acting replication element
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside-5'-triphosphate
DTT	dithiothreitol
DMSO	dimethylsulfoxide
e.g.	for example
EDTA	ethylenediaminetetra-acetic acid
eIF	eukaryotic initiation factor
EITB	immunoelectrotransfer blot
ELISA	enzyme-linked immunosorbent assay
EMCV	encephalomyocarditis virus
ER	endoplasmic reticulum
EtBr	ethidium bromide
FCS	fetal calf serum
Fig.	figure
FMD	foot-and-mouth disease
FMDV	foot-and-mouth disease virus
g	gram
GST	glutathione *S*-transferase
h	hour
H_2O_2	hydrogen peroxide
His	histidine
HSPG	heparin sulphate proteoglycan
IB-RS-2	Instituto Biologica Rim Suino (a pig kidney cell line)
I-ELISA	indirect-ELISA
IG	immunoglobulin
IL	interleukin
INF	interferon
IPTG	isopropyl-β-D-thiogalactoside
IRES	internal ribosome entry site
IT-ELISA	indirect trapping-ELISA
kb	kilobase pairs
kDa	kilodalton

L^{pro}	Leader protease
lacZ	β-galactosidase gene
LB	Lauria-Bertani
LPB-ELISA	liquid-phase blocking-ELISA
M	molar
mA	milliampere
mg	milligram
MHC	major histocompatability complex
min	minute
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
N	amino
NA	not available
NaOAc	sodium acetate
NJ	neighbour-joining
nm	nanometer
NSP	nonstructural protein
nt	nucleotide
°C	degrees Celsius
OD	optical density
OIE	Office International des Epizooties
ORF	open reading frame
PABP	poly(A)-binding protein
PBS	phosphate-buffered saline
PSB	protein sample buffer
PCBP	poly(C)-binding protein
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
PKs	pseudoknots
pmol	picomole
poly(A)	polyadenylated
poly(C)	polycytidylate
PTB	polypyrimidine tract-binding protein
RGD	Arg-Gly-Asp
RNA	ribonucleic acid
RNase	ribonuclease
RP	recombinant proteins
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
s	second
SAT	South African Territories
SDS	sodium dodecyl sulphate
***Sf* 9**	*Spodoptera frugiperda* 9 (insect cell line)
SFGM	serum-free Grace's medium
SNT	serum neutralization test
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TF	trigger factor
TNF	tumour necrosis factor

Tris	Tris-hydroxymethyl-aminomethane
U	units
UHQ	ultra-high quality
UTR	untranslated region
UV	ultraviolet
V	Volts
v/v	volume per volume
VIAA	virus infection-associated antigen
VNT	virus neutralization test
VP	virus protein
w/v	weight per volume
WT	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
λ	lambda
µg	microgram
µl	microliter
µM	micromolar

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CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Foot-and-mouth disease (FMD), of which foot-and-mouth disease virus (FMDV) is the aetiological agent, is a highly contagious, acute infection that affects all cloven-hoofed animals (Thomson, 1994). In addition to important livestock animals such as cattle, sheep, goats and swine; FMDV can also infect more than 30 species of wildlife (Thomson *et al.,* 2003). FMD is recognized as the most important constraint to the international trade of livestock and animal products and is categorized as a list A disease by the Office International des Epizooties (OIE) due to its highly contagious nature, its potential for rapid spread and for causing severe economic losses (OIE, 2004). In young animals infected with the disease the mortality rates can be high, resulting from a multifocal myocarditis. Although the mortality rate in adult animals is low (less than 5%), FMD has a major impact on the livestock industry and economic losses are incurred mainly as a result of the decline in meat and dairy production in infected animals and the loss of export markets during an outbreak (Thomson, 1994).

FMDV can be transmitted to susceptible animals through indirect or, more commonly, direct mechanisms. The most common route of infection is by direct contact between infected animals that excrete the virus and susceptible animals (Thomson, 1994). FMDV is present in most excretions and secretions of viraemic animals, such as milk, faeces, secretions of the nasal passages, urine, semen and saliva, which contains the highest concentrations of virus. Indirect transmission of the disease may occur by contact with virus-contaminated animal products (e.g. offal, milk and meat), but humans and vehicles have also been implicated in mechanical transmission of the disease (Thomson, 1994; Quinn and Markey, 2001). In addition, under favourable environmental conditions, FMDV in infective aerosols may be transmitted over long distances (Gloster *et al.,* 1982). In natural infections, the main route of virus entry is via the respiratory tract. Following viral multiplication in the pharynx epithelium, the virus enters the blood stream and lymphatic system and spreads rapidly to different organs and tissues (Sobrino *et al*., 2001). The incubation period ranges between two to eight days during which time significant amounts of virus may be shed and thus facilitates dissemination of the disease (Quinn and Markey, 2001). Whereas the earliest clinical signs of disease include fever, dullness, inappetence, cessation of rumination and reduction in milk production, it is shortly followed by signs more related to the development of lesions in the mouth and feet of infected animals such as lameness, disinclination to stand, salivation,

smacking of the lips and grinding of the teeth (Thomson, 1994). Following recovery from the acute stage of the disease, infectious virus disappears from all secretions and excretions. In approximately 50% of ruminants an asymptomatic, persistent infection can be established that may persist for a few weeks to several years (Thomson, 1994) during which virus can be isolated from the oesophageal-pharyngeal (OP) fluids (Van Bekkum *et al.,* 1959). Both naïve and vaccinated animals can become persistently infected following acute infection and could serve as the source of new FMD outbreaks (Salt, 1993; Alexandersen *et al*., 2003).

In FMD-free countries the disease is controlled through strict trade restrictions on the importation of livestock and animal products from countries where FMD occurs. In the event of an outbreak, a "stamping out" policy is enforced whereby all infected and susceptible in-contact animals are slaughtered (Barteling and Vresswijk, 1991). By contrast, in countries where FMD is endemic the disease is primarily controlled by prophylactic vaccination and strict zoosanitary measures (Hunter, 1998; Quinn and Markey, 2001). The first step in effective control is undoubtedly rapid and accurate diagnosis of the disease. Due to the highly infectious nature of the virus and its potential for rapid spread, handling of the virus and procedures related to diagnosis of the disease is restricted to high containment facilities (OIE, 2004), e.g. the Institute for Animal Health (Pirbright) and the Exotic Diseases Division, Onderstepoort Veterinary Institute. Laboratory diagnosis of the disease is based on identification of the virus and viral antigens during the early stages of infection, and serology in instances where the infection has lasted longer than two weeks (Thomson and Bastos, 2004; OIE, 2004). Although the prescribed diagnostic tests for identification of FMDV are the liquid-phase blocking ELISA (LPB-ELISA) and virus neutralization test (VNT) (OIE, 2004), reverse transcriptase-polymerase chain reaction (RT-PCR) procedures have been described that allow for rapid detection of FMDV genomic RNA in clinical specimens (Bastos, 1998; Reid *et al.,* 1999).

1.2 CLASSIFICATION AND GEOGRAPHICAL DISTRIBUTION OF FMDV

FMDV belongs to the family *Picornaviridae* and is the prototype member of the *Aphthovirus* genus (Rueckert, 1996; Stanway *et al.,* 2000). The Greek word "aphtho" refers to lesions in the mouth, which are characteristic of the acute phase of foot-and-mouth disease (Thomson and Bastos, 2004). The only other member of this genus is Equine rhinitis A virus, previously known as equine rhinovirus type 1, which has been grouped with FMDV based on

its genome sequence similarity with FMDV and its genomic organization resembling more closely that of FMDV compared to other picornaviruses (Li *et al.,* 1996).

Seven immunologically distinct FMDV serotypes have been identified (types A, O, C and Asia1, which are collectively known as the euroasiatic types, and the three South African Territories [SAT] types SAT1, SAT2 and SAT3) (Pereira, 1981; Brooksby, 1982). Within each serotype, multiple subtypes and topotypes can be identified by immunological assays, suggesting considerable genetic variability (Domingo *et al.,* 2002; Bastos *et al.,* 2003a;b). FMDV isolates are assigned to their respective serotypes and subtypes by serological criteria and phylogenetic analysis, particularly of the 1D-encoding region of the FMDV genome, which contains the major immunogenic determinant (Domingo *et al.,* 2003). The distribution of the seven FMDV serotypes around the world is indicated in Fig. 1.1. European serotypes O and A have the broadest distribution, occurring in many parts of Africa, southern Asia, South America and the Far East (not type A), and type C is mostly confined to the Indian sub-continent and to South America. Whereas, the Asia1 serotype occurs in southern Asia and in the Middle East, the SAT serotypes are normally confined to sub-Saharan Africa (Knowles and Samuel, 2003; Grubman and Baxt, 2004).

1.3 VIRAL RIBONUCLEIC ACID AND PROTEINS

The FMD virion is composed of a single-stranded RNA genome of approximately 8 500 nucleotides in length, enclosed within a non-enveloped spherical protein capsid of icosahedral symmetry (Sobrino *et al.,* 2001). The genome is a positive-sense infectious RNA molecule, which is polyadenylated at its 3' terminus and a small virus-encoded protein, VPg, is covalently attached to its 5' terminus (Putnak and Phillips, 1981; Sobrino *et al.,* 2001). The FMDV genome encodes a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs), which display complex secondary structure and do not encode viral proteins (Mason *et al.,* 2003). A diagrammatic representation of the FMDV genome is shown in Fig. 1.2.

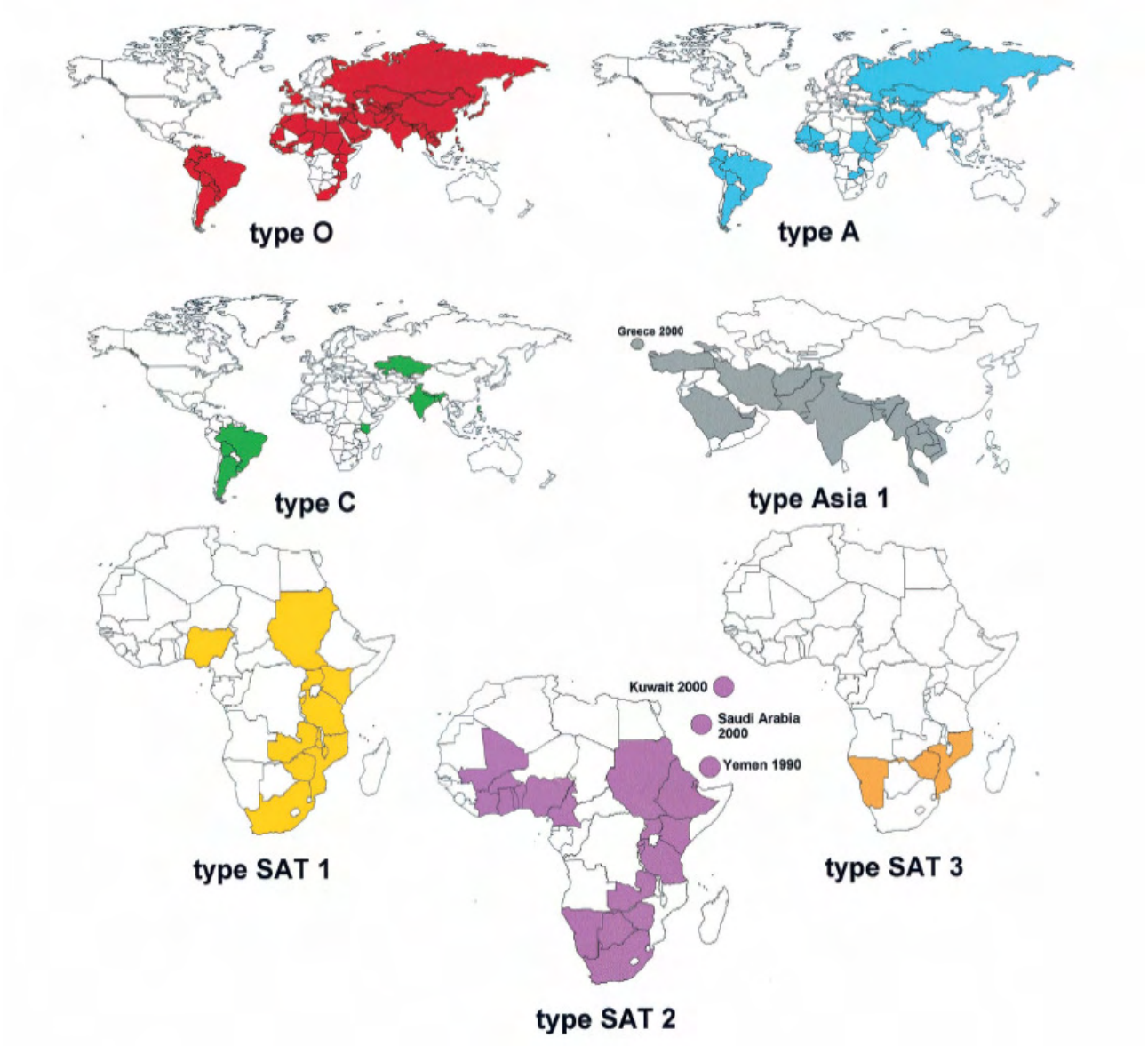


Fig. 1.1 World-wide distribution of the seven FMDV serotypes between 1990 and 2002. (Taken from Grubman and Baxt, 2004).

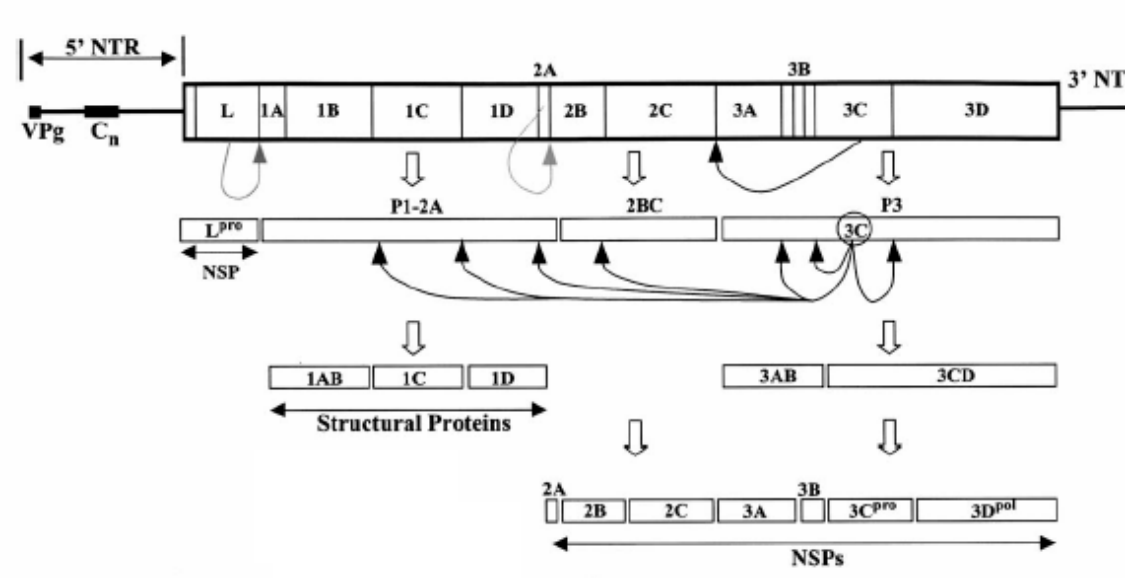
1.3.1 The 5' untranslated region (UTR)

The viral genome-linked protein (VPg), which is covalently linked to the 5' UTR of the picornaviral genome (Fig. 1.2; Lee *et al.,* 1977; Grubman, 1980), and encoded by the 3B genomic region, is involved in the initiation of RNA synthesis and encapsidation of viral RNA (Barclay *et al.,* 1998). In addition, *in vitro* studies have demonstrated that 3B (VPg) of poliovirus could be uridylylated by the virus-encoded RNA polymerase ($3D^{pol}$) and serves as a primer for RNA replication (Paul *et al*., 1998). The FMDV genome encodes three non-identical yet highly conserved 3B proteins, each of which contains Tyr-3, which is known to be involved in the phosphodiester linkage to the viral RNA (Forss and Schaller, 1982). The 3B-copy number is thought to relate to the pathogenic potential and host range determination of FMDV (Falk *et al.,* 1992; Pacheco *et al.,* 2003).

The 5' UTR of FMDV is approximately 1 300 bases in length (Forss *et al.,* 1984) and is composed of the S fragment, the poly(C) tract, the pseudoknots (PKs), the *cis*-acting replication element (*cre*), and the internal ribosome entry site (IRES). The first 360 bases of the 5' UTR, known as the S fragment, is capable of folding into a long stem-loop structure (Newton *et al*., 1985; Bunch *et al*., 1994). Although the exact function of the S fragment is not known, analogies with other picornaviral genomes suggest that the 5' cloverleaf structure may play a role in the binding of proteins involved in genome replication, as well as in regulating RNA stability and translation (Barton *et al*., 2001). In addition, it may prevent exonuclease digestion of the genome in infected cells, since the VPg protein is absent from cellular forms of the genome (Grubman and Bachrach, 1979).

In both aphthoviruses and cardioviruses, the S fragment is followed by a long (usually 100-250 residues) polycytidylate tract (poly(C) tract), composed mainly of cytosine (C) residues. Initially, it was thought that the length of the poly(C) tract may be an important determinant of virulence, since attenuated virus strains have been reported that possessed short poly(C) tracts (Harris and Brown, 1977; Black *et al.,* 1979). However, other studies, using isolates with varying poly(C) tract lengths and little variation elsewhere in the genome, have not been able to substantiate these findings (Costa Giomi *et al*., 1984; Rieder *et al.,* 1993). The poly(C) tract may be involved in genome circularization (Mason *et al.,* 2003). Studies with poliovirus have shown that the cellular poly(C)-binding protein (PCBP) can participate with other viral and cellular proteins in binding to the 5'-end of the genome and that the PCBP

(a)



(b)

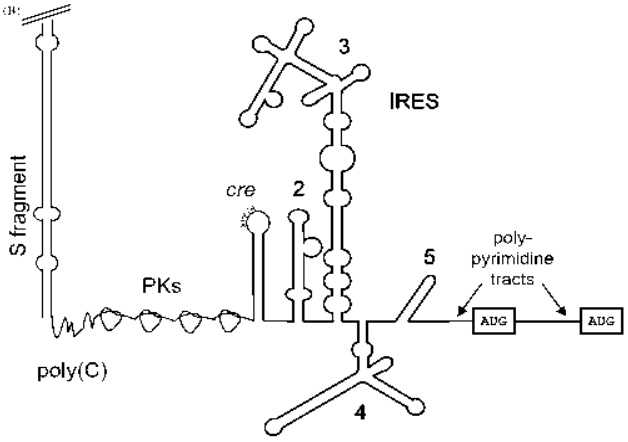


Fig. 1.2 (a) FMDV genome structure and expressed proteins. Black arrows indicate sites of cleavage by the viral proteinases. Structural proteins 1A, 1B, 1C and 1D form the viral capsid. Nonstructural proteins L^{pro}, 2A, 2B, 2C, 3A, 3B, 3C and 3D are involved in viral replication. The 5' untranslated region (UTR) is capped by a small protein VPg and the 3' UTR contains a poly(A) tail (Adapted from Clavijo *et al*., 2004a). (b) Expanded view of the 5' UTR, indicating the S fragment, poly(C), polycytidylate tract; PK, pseudoknot; *cre*, *cis*-acting replicative element; IRES, internal ribosome entry site (Adapted from Mason *et al.*, 2003).

could bring together the 5'- and 3'- ends of the poliovirus genome in a structure that could serve to regulate the switch from translation to genome replication (Barton *et al.,* 2001; Herold and Andino, 2001).

Following the poly(C) tract are several tandemly repeated pseudoknots (PKs) (Clarke *et al*., 1987) of which the function is not yet known, but has been proposed to influence viral virulence (Feng *et al*., 2004). The *cre*, a short hairpin element containing a highly conserved AAACA sequence in the loop region, is required for picornavirus genome replication (McNight and Lemon, 1998). By contrast to other picornaviruses in which the *cre* structure is located in protein-encoding regions of the viral genome, the *cre* of FMDV is located in the 5' UTR (Mason *et al*., 2003). The last functional motif of the 5' UTR is the highly structured IRES, which directs cap-independent translation of the viral RNA (Jackson *et al.,* 1990; Martinez-Salas, 1999).

1.3.2 The FMD virus polyprotein

The FMD viral genome encodes a single, long polyprotein that can be divided into four polyproteins, namely L protease, P1-P2A, P2 and P3 (Fig. 1.2a). Whereas the L protease cleaves at its own carboxy (C)-terminus, the P1-P2A polypeptide is the precursor of the viral capsid proteins and P2 and P3 are cleaved by viral proteases to yield different nonstructural proteins that are involved in functions essential to the virus replication cycle (Mason *et al.,* 2003).

1.3.2.1 Proteinases

The aphthovirus Leader proteinase (L^{pro}) is located in the amino (N)-terminal region of the FMDV-encoded polyprotein (Robertson *et al*., 1985). The L^{pro}-encoding region contains two in-frame AUG codons, which result in synthesis of two forms of the Leader proteinase, namely proteins L_b, which is the major protein synthesized *in vivo*, and L_{ab} (Clarke *et al*., 1985; Sanger *et al*., 1987). Both L_b and L_{ab} catalyse their proteolytic excision from the polyprotein at the L/1A junction at a Arg/Lys-Gly dipeptide (Strebel and Beck, 1986a; Palmenberg, 1990; van Rensburg *et al.,* 2002). The L^{pro} cleaves the host cell protein eukaryotic initiation factor 4G (eIF-4G), thus

inhibiting host cell translation of its own capped mRNAs. This results in the eventual shut-off of host cell protein synthesis in infected cells (Devaney *et al.*, 1988). Based on studies with a genetically engineered L^{pro}-deleted virus, it has been reported that the L^{pro} is an important determinant of FMDV virulence and is essential for pathogenesis (Brown *et al.*, 1996; Mason *et al.*, 1997).

The second proteinase contained in the FMDV genome is the 3C proteinase ($3C^{pro}$). The $3C^{pro}$ is responsible for catalyzing most of the proteolytic cleavages necessary for FMDV polyprotein processing (Vakharia *et al.,* 1987; Clarke and Sanger, 1988), the exceptions being the cleavage of L^{pro} from P1, 2A from P1-2A and the maturation cleavage of 1AB into 1A and 1B (Mason *et al.,* 2003). The FMDV $3C^{pro}$ cleavage sites display heterogeneity and cleavage occurs between several different dipeptides, including Gln-Gly, Glu-Gly, Gln-Leu and Glu-Ser (Robertson *et al.*, 1985; Palmenberg, 1990). Like the L^{pro} the $3C^{pro}$ also cleaves eIF-4G, but at different sites to L^{pro} and at a later stage in the infection cycle. The $3C^{pro}$ also cleaves the host cell protein eIF-4A, an RNA helicase and member of the cap-binding complex, thus contributing to inhibition of host cell translation (Belsham *et al.*, 2000). Moreover, $3C^{pro}$ also cleaves host cell protein histone H3 and may be involved in the shut-down of host cell transcription (Falk *et al.,* 1990; Tesar and Marquardt, 1990; Capozzo *et al.*, 2002).

The cleavage of the 2A-2B junction is mediated by the 2A peptide, a short 18-amino-acid "autoproteinase" (Ryan *et al.,* 1991; Donnelly *et al.*, 1997). Together with the N-terminal proline of the 2B protein, 2A represents an autonomous element capable of mediating cleavage at its own C-terminus, resulting in 2A and P1 being separated from 2BC-P3. The FMD 2A protein does not act as a substrate for either the L^{pro} or $3C^{pro}$ (Donnelly *et al.,* 1997). The 2A-2B cleavage is considered a single turn-over event either by providing a sequence substrate for a host cell proteinase, or by interrupting the elongation cycle during protein synthesis (Ryan and Drew, 1994; Ryan and Flint, 1997). Subsequent studies have led to the suggestion that the 2A-2B cleavage event is not a proteolytic event, but rather the result of the 2A sequence modifying the activity of the ribosome to promote hydrolysis of a peptidyl (2A)-$tRNA^{Gly}$ ester linkage. This may result in the release of the 2A protein from the ribosome in a manner that allows the synthesis of a downstream protein to proceed (Donnelly *et al.,* 2001 a;b).

1.3.2.2 Structural proteins

The P1 polyprotein is the precursor of the viral capsid proteins 1A, 1B, 1C and 1D. The $3C^{pro}$ cleaves the P1 polyprotein into 1AB, 1C and 1D proteins (Bablanian and Grubman, 1993). Upon encapsidation of the viral RNA, 1AB is autocatalytically cleaved into 1A and 1B (Harber *et al.,* 1991). The icosahedral viral capsid (Fig. 1.3) is composed of 60 heteromeric structural units, termed protomers. Each protomer consists of a single copy of each of the 1AB, 1C and 1D capsid proteins. Five protomers assemble into a pentamer and twelve pentamers assemble into a complete viral capsid that encloses the RNA genome (Sobrino *et al.,* 2001). Five molecules of the 1D protein are arranged around the icosahedral five-fold axis of symmetry, and proteins 1B and 1C alternate around the two- and three-fold axes (Acharya *et al.,* 1989). These capsid proteins are orientated to the surface of the virion, whereas the 1A protein, which has a myristylated N-terminus, is located internally (Chow *et al.,* 1987). The core of the surface capsid proteins (1B, 1C and 1D) form a wedge-shaped eight-stranded anti-parallel β-barrel with two flanking α-helices connected by surface-exposed loops (Acharya *et al.,* 1989). This three-dimensional arrangement of the structural proteins within the virion provides the antigenic sites that elicit responses to vaccination or infection, in addition to providing stability for the capsid (Mason *et al.,* 2003). The 1D protein contains a highly mobile loop, the G-H loop, that protrudes from the capsid surface and contains a highly conserved Arg-Gly-Asp (RGD) motif at its apex, which mediates cell attachment to susceptible host cells (Fox *et al.,* 1989; Mason *et al.,* 1994). The 1A protein is thought to assist in capsid assembly and to provide stability to the protein capsid (Chow *et al.,* 1987). It has been suggested that a histidine (His)-rich region at the pentamer interface (1B-1C) is responsible for acid-induced disassembly. Protonation of these residues at a pH below 6.5 may cause electrostatic repulsive forces across the pentamer interface, thus resulting in the capsid opening up (Acharya *et al.,* 1989; Curry *et al.,* 1995; Ellard *et al.,* 1999).

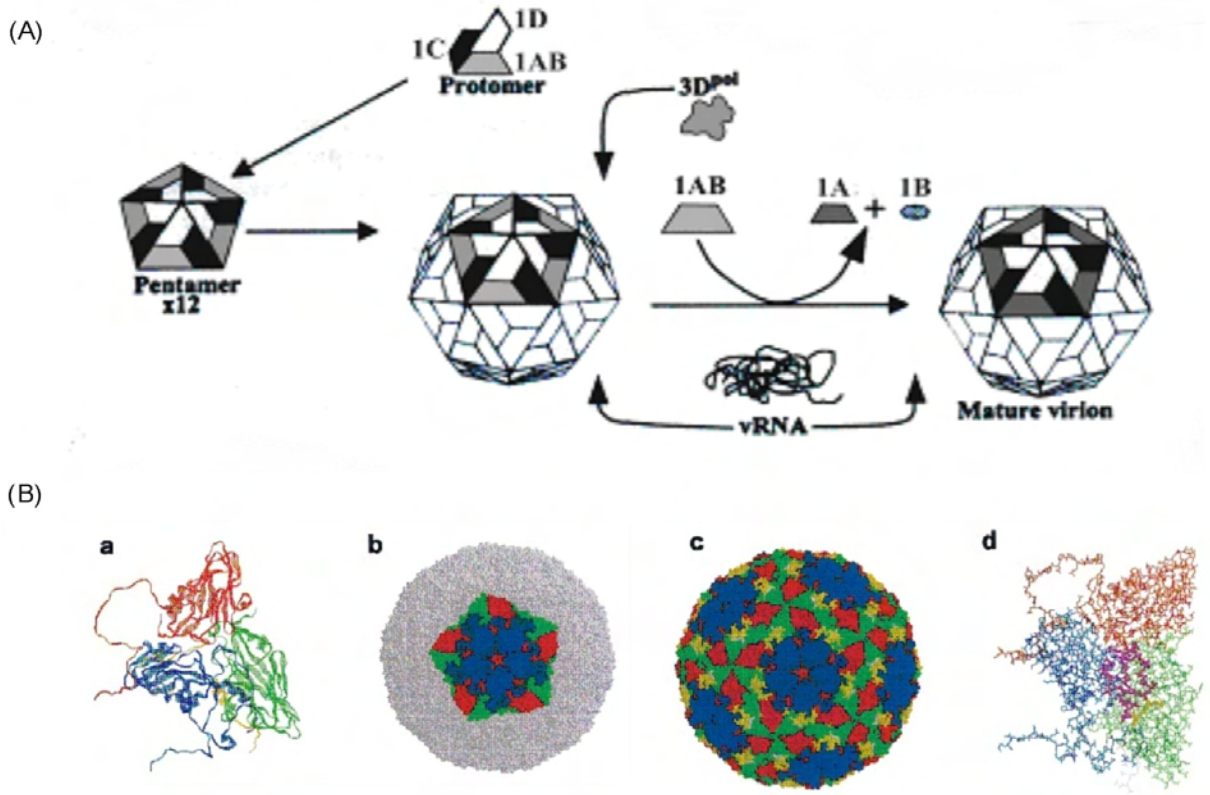


Fig. 1.3 (A) Assembly of the picornavirus capsid. Five protomers, which consist of one copy each of 1AB, 1C and 1D associate to form a pentamer. Twelve pentamers assemble into the provirion. With encapsidation of a single RNA molecule, 1AB is autocatalytically cleaved into 1A and 1B (Harber *et al.,* 1991) to form the mature viral capsid. (B) Structure of a FMD virion. (a) A viral protomer highlighting the β-barrel-and-loop organization of the viral proteins. (b) A pentamer positioned on the virion looking down the five-fold axis. (c) The organization of the entire virion, highlighting the G-H loop (yellow). (d) A protomer highlighting the positions of the G-H loop (purple) and RGD sequence (yellow). All structures are representative of the mature virion, and the viral proteins are colored blue (1D), green (1B), and red (1C). Protein 1A is buried within the particle and is visible only in panel a, where it is colored yellow. (Adapted from Clavijo *et al*., 2004a; Grubman and Baxt, 2004).

1.3.2.3 Nonstructural proteins

The P2 polyprotein precursor is proteolytically processed into three mature proteins, 2A, 2B and 2C (Rueckert and Wimmer, 1984). The 2A protein functions as an autoproteinase, as discussed previously. The FMDV 2B and 2BC proteins have been shown to associate with cellular membranes. The 2BC precursor blocks the delivery of proteins to the cell surface (Moffat *et al.,* 2005) and may induce cell membrane vesicle proliferation (Bienz *et al.,* 1990). It is well established that infection with related picornaviruses also results in the disruption of the host secretory pathway (Doedens and Kirkegaard, 1995; Van Kuppeveld *et al.,* 1997). However, it is apparent that the nonstructural proteins of different picornaviruses may differ in their ability to affect the secretory pathway, as the 3A protein and not the 2BC protein of poliovirus affects membrane trafficking (Van Kuppeveld *et al.,* 1997; Moffat *et al.,* 2005). The secretory pathway plays an essential role in the destruction of virus-infected cells by enabling the delivery of major histocompatability complex (MHC) class I peptide complexes to the surface of infected cells for presentation to cytotoxic T-cells (Heemels and Ploegh, 1995). Therefore, through disruption of the secretory pathway, it is possible that the FMD virus may gain an immunological advantage, but not only by affecting MHC class I antigen presentation, but also by interfering with the secretion of interferons, proinflammatory cytokines and by reducing expression of the tumour necrosis factor (TNF) receptor required for TNF-α-induced apoptosis, as has been suggested for poliovirus infections (Moffat *et al.,* 2005). It is this ability to evade the host's immune system that is though to contribute to the development of persistent infections in ruminants (Moffat *et al.,* 2005). In addition, mutations that confer resistance to guanidine hydrochloride, an inhibitor of viral RNA replication, are located in the 2C protein, thus suggesting a role for this protein in viral RNA synthesis (Saunders *et al.,* 1985).

The P3 polyprotein precursor is proteolytically processed into four mature proteins, 3A, 3B, 3C and 3D (Mason *et al.,* 2003). The FMDV 3A protein and its precursor 3AB has been shown to associate with intracellular membranes, in particular with the endoplasmic reticulum (ER), and has been proposed to be the membrane anchor for the picornaviral replication complex (Xiang *et al.,* 1997) and may also be involved in vesicle formation (Weber *et al.,* 1996). The 3A protein of FMDV has been shown to be associated with host range determination and pathogenicity.

Whereas a single amino acid substitution in 3A resulted in the adaptation of the virus to guinea pigs (Nũnez *et al.,* 2001), deletions in the C-terminus of the protein resulted in a serotype O outbreak strain being attenuated for cattle, but highly virulent in swine (Beard and Mason, 2000; Pacheco *et al.,* 2003). For poliovirus, the 3AB precursor can function as a co-factor of the viral replicase (Richards and Ehrenfeld, 1998). As discussed above, the 3C protein is a serine protease and is responsible for most of the proteolytic cleavages in the FMDV-encoded polyprotein (Ryan *et al.*, 1989). The FMDV 3D protein is the viral-encoded RNA-dependant RNA polymerase ($3D^{pol}$) (Newman *et al.*, 1979) and is responsible for the synthesis of positive- and negative-strand RNAs. The $3D^{pol}$ is highly conserved, both in nucleotide and amino acid sequence, among the different FMDV serotypes (Martinez-Salas *et al.*, 1985; George *et al.*, 2001; Carillo *et al.,* 2005). Since the presence of $3D^{pol}$ is indicative of FMDV replication, it was expected to serve as a reliable indicator of virus infection. Indeed, early studies detected antibodies to the virus infection-associated antigen (VIAA), of which the 3D protein is a major component, in serum from FMD convalescent animals (Cowan and Graves, 1966). It has since been reported that the $3D^{pol}$ is a component of FMDV particles, as these particles react with antibody against the *Escherichia coli*-expressed polymerase (Newman *et al.,* 1994). It appears that although the virus particles contain the enzyme, it is exposed in only 20-30% of the particles (Newman and Brown, 1997).

1.3.3 The 3' untranslated region (UTR)

The 3'-end of the FMDV genome contains two distinct elements, a 90-base untranslated region (UTR) and a genome-encoded polyadenylate tract (poly(A) tract) (Chatterjee *et al.*, 1976). Currently, the involvement of the 3' UTR and/or the poly(A) tract in translation is not known and the role of the 3' UTR in aphthovirus infection is poorly understood. The poly(A) tract terminal A residues may supply the template for hybridization of the 3B-pUpU primer, allowing synthesis of the viral negative-sense strands (Mason *et al.,* 2003). The picornavirus poly(A) tract may be involved in genome circularization, which could play a role in RNA replication (Barton *et al.*, 2001; Herold and Andino, 2001). The poly(A) tract probably binds to poly(A)-binding protein (PABP), forming a bridge to the 5'-end of the genome in the presence or absence of other host or viral proteins. The 3' UTR of FMDV may harbour *cis*-acting sequences required for

initiation of genome replication, since deletion of 74 bases of the 89 bases at the 3' UTR of FMDV type O_1 Kaufbeuren resulted in an inability to recover viable virus (Sáiz *et al*., 2001; López de Quinto *et al*., 2002). In addition, removal of the 3'-terminal sequences reduced the efficiency of viral protein translation (López de Quinto *et al*., 2002).

1.4 INFECTIOUS CYCLE OF FMDV

Replication and translation of FMDV RNA occurs in the cytoplasm of infected cells and these processes are associated with cell membranes (Bachrach, 1977). For comparative purposes the virus infection cycle of poliovirus is indicated Fig. 1.4. For FMDV field isolates, the virus infection cycle is initiated by attachment of the virus to any of four members of the α_v subgroup of the integrin family of cellular receptors ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$) (Berinstein *et al.,* 1995; Neff *et al.,* 1998; Duque *et al.,* 2004; Jackson *et al.,* 2002; 2004) via the highly conserved RGD sequence located within the surface-exposed G-H loop of the 1D capsid protein (Fox *et al.,* 1989; Mason *et al.,* 1994). By contrast, several cell culture-adapted viruses have been reported to utilise heparin sulphate proteoglycans (HSPG) for cell internalization (Jackson *et al.,* 1996; Sa-Carvalho *et al.,* 1997). Besides the RGD-dependent integrins or HSPG, an antibody-complexed virus has been shown to infect cells via F_C receptor-mediated adsorption (Mason *et al.,* 1993; Rieder *et al.,* 1996), suggesting that alternative pathways for virus entry may exist.

Following binding of the virus to the cell surface receptor, the virus-receptor complex is invaginated and internalized by endocytosis to form a clathrin-coated vesicle. Acidification of the vesicles leads to the release of protein 1A and unfolding of the hydrophobic regions of capsid proteins 1B, 1C and 1D. Fusion of the lipid bilayer with hydrophobic regions of the exposed capsid proteins leads to the formation of a pore through which the viral RNA can be transferred to the cytoplasm (O'Donnell *et al.,* 2005). In the cytoplasm, the 5' UTR-linked VPg protein is released from the positive-sense RNA genome by a cellular protease (Ambrose *et al.,* 1978) and polyprotein synthesis is initiated in a cap-independent manner at the internal ribosome entry site (IRES) (Lee *et al.,* 1977; Kühn *et al.*, 1990). In addition to viral proteins, the eukaryotic initiation factor eIF-4B (Meyer *et al.,* 1995) and the cellular polypyrimidine tract-binding protein (PTB) are believed to be involved in translation initiation at the picornaviral IRES (Niepmann *et*

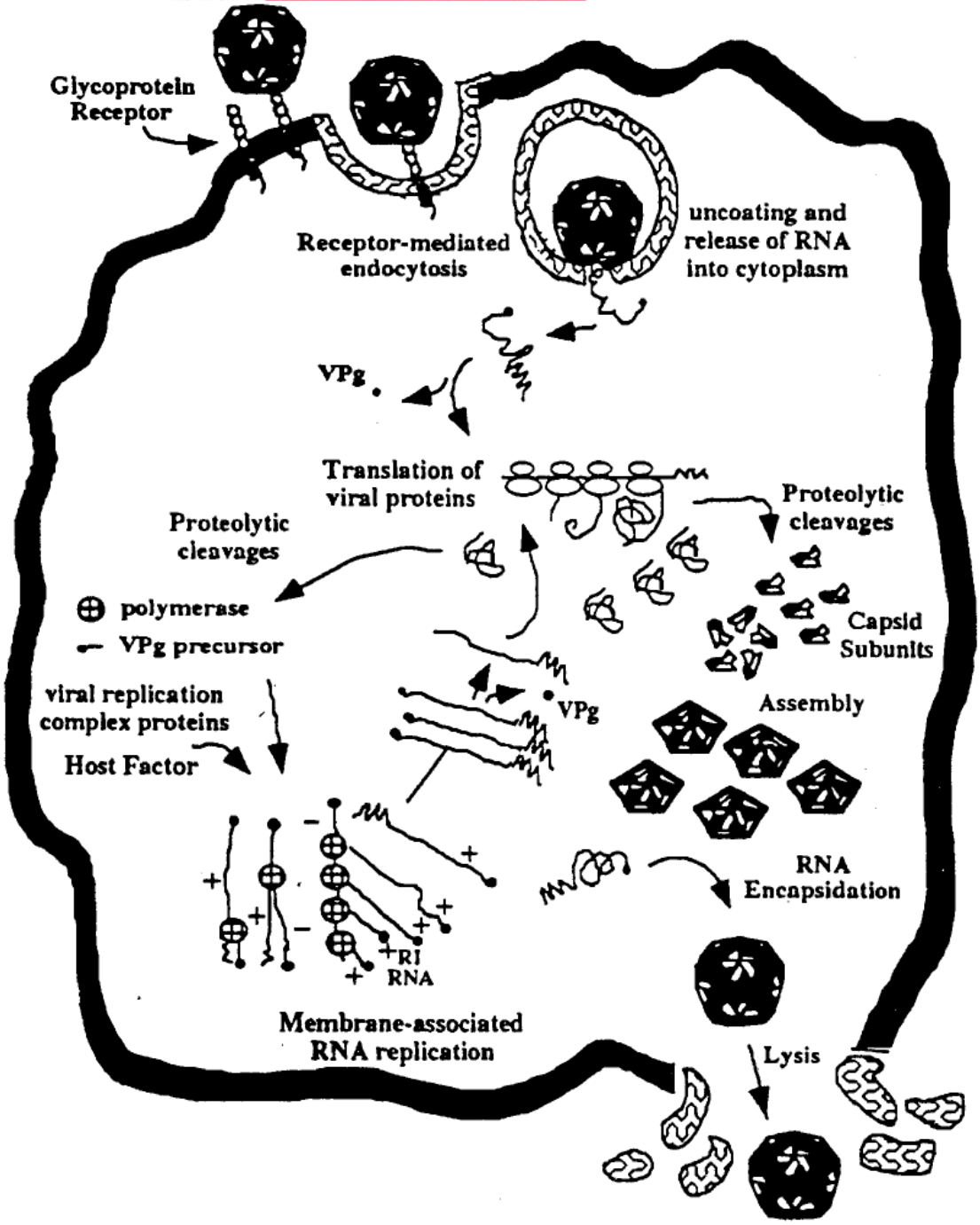


Fig. 1.4 Overview of the poliovirus infectious cycle. (Taken from Ansardi *et al*., 1996).

al., 1997). Translation is initiated at both in-frame AUG codons, 84 nt apart, in the L fragment of the viral genome (Beck *et al.,* 1983; Sanger *et al.,* 1987). The leader proteinase (L^{pro}) is the first protein to be synthesized and cleaves itself from the rest of the growing polypeptide at the L-P1 junction (Medina *et al.,* 1993). Both forms of the L^{pro}, L_{ab} and L_b, cleave eIF-4G, a member of the cellular cap-binding protein complex (Devaney *et al*., 1988). As a result, host cell cap-dependent mRNA translation is inhibited, whereas viral RNA synthesis and cap-independent protein synthesis of viral proteins proceed rapidly. The viral polyprotein is proteolytically processed by viral proteases into mature viral proteins to be used either in viral replication or for the formation of the viral capsid (Vakharia *et al.,* 1987; Mason *et al.,* 2003). Most of the polypeptide proteolytic processing events are performed by the 3C protease, which functions *in trans*. However, it is also responsible for cleavage of nuclear protein histone H3 and eukaryotic initiation factors eIF-4G and eIF-4A, resulting in inhibition of host cell transcription and translation, respectively (Falk *et al.,* 1990; Tesar and Marquardt, 1990; Meyer *et al.,* 1995; Belsham *et al.,* 2000; Capozzo *et al.,* 2002).

During the infection cycle, the positive-sense RNA viral genome is used as template for the synthesis of negative-sense RNA molecules. The viral RNA-dependent RNA polymerase catalyses the synthesis of new positive-sense RNA molecules from the negative-sense RNA template molecules, which are recruited as viral mRNA or packaged into virions (Rueckert, 1985). As synthesis of the positive-sense RNA continues and predominates, almost half of the RNA molecules are packaged into virions. Virion assembly involves the formation of capsid protomers, five of which assemble into pentamers and is then followed by assembly into either empty capsids (75S particles) or provirions (146S particles) that contain packaged positive-sense VPg-RNA (Guttman and Baltimore, 1977; Grubman *et al.,* 1985). The final step in virion maturation involves autocatalytic cleavage of 1AB into 1A and 1B, which is required for generation of infectious virus particles (Harber *et al.*, 1991). The mature infectious virions are subsequently released by infection-mediated disintegration of the cell (Rueckert, 1985).

1.5 DIAGNOSIS OF FMD

To implement effective control procedures and prevent the spread of FMD, early and accurate diagnosis of the disease is essential. Diagnosis may involve clinical and epidemiological assessment of the situation, followed swiftly by laboratory confirmation through identification of FMD viral nucleic acid, viral antigens or the detection of antibodies to infection (Rémond *et al.,* 2002; Thomson and Bastos, 2004). Furthermore, in order to administer emergency vaccination with the appropriate antigen and to trace the source of the outbreak, the determination of the specific serotype of the field virus and its molecular characterization are essential (Armstrong *et al.,* 1994; Bastos *et al.,* 2000; 2003a).

1.5.1 Clinical diagnosis

Clinical signs of FMD become evident following an incubation period of two to eight days. The disease is characterized by the appearance of lesions on the feet and are localized on the bulbs of the heel and along the interdigital cleft and coronary bands. Lesions also appear on the mucous membranes of the mouth, including the lips, tongue, dental pads and gums. Lesions may also appear on the mammary glands of infected females and on the snout of swine (Thomson, 1994; Rémond *et al.,* 2002; OIE, 2004). However, a definitive diagnosis cannot always be made based on clinical signs alone (Rémond *et al.,* 2002). At least two other viral diseases of livestock, namely swine vesicular disease (SVD) and vesicular stomatis (VS), produce lesions in cattle and swine that are indistinguishable from those of FMD (Grubman and Baxt, 2004). In addition, identification of FMD lesions may be obscured by ulceration, secondary infection or healing (Mann and Sellers, 1990). Also, the severity of clinical signs is dependent on virus strain and exposure dose, the age, breed and immune status of the animal (OIE, 2004). In sheep and goats the disease is often characterized by mild or subclinical symptoms. Therefore, clinical diagnosis requires laboratory confirmation through the recovery of virus from vesicular fluids and epithelial tissue associated with lesions, oesophageal secretions and blood (Rémond *et al.,* 2002).

1.5.2 Virological diagnosis

Complement fixation (CF) and serum neutralization (SN) tests, which are mostly based on the detection of the FMDV structural capsid proteins, are used for the routine detection of FMDV in clinical specimens (Buckley *et al.,* 1975; OIE, 2004). However, CF tests are relatively insensitive and is prone to difficult interpretation due to pro- and anti-complementary activity of the specimens (Rémond *et al.,* 2002). Consequently, CF tests have been largely replaced with an antigen capture ELISA (Ferris and Dawson, 1988) whereby FMDV isolates can be identified and typed. In general, these diagnostic tests make use of serotype-specific antisera and may require laborious and time-consuming propagation of the virus in cell culture if the titre is low or the results inconclusive (Thomson and Bastos, 2004). Primary cultures of calf thyroid cells have been shown to be as sensitive for virus detection as intradermal inoculation in cattle, but established cell lines such as baby hamster kidney cells (BHK-21) and pig kidney cells (IB-RS-2) exhibit considerable inconsistency (OIE, 2004).

Based on the above problems, RT-PCR assays have been developed for the specific detection and typing of FMDV genomic RNA in tissue culture fluid or in clinical specimens (Meyer *et al.*, 1991; Rodríguez *et al.*, 1992; Vangrysperre and De Clercq, 1996; Bastos, 1998; Reid *et al.*, 1999; Suryanarayana *et al*., 1999). In addition, RT-PCR, in combination with direct nucleotide sequencing, allows for the rapid characterization of field isolates and can provide important epidemiological information regarding the origin of an outbreak (Armstrong *et al.*, 1994; Bastos *et al.,* 2003b). For typing purposes, "universal" primer sets have been designed that target conserved sequences in the 1D gene of all seven FMDV serotypes (Callens and De Clercq, 1997). Although FMDV RT-PCR assays make for promising diagnostic assays, problems are experienced in the design of oligonucleotides and the efficiency of RNA extraction (Reid *et al.,* 1999). The performance of the RT-PCR on an extensive range of field samples has been compared to virus isolation from cell culture and antigen detection by ELISA. The results indicated that RT-PCR was the least sensitive and it was concluded that it may complement, but not yet replace the prescribed diagnostic assays (Reid *et al.,* 1998).

1.5.3 Serological diagnosis

Serological tests are particularly useful in identifying silent FMD infections in animals such as goats and sheep, which show little or no clinical symptoms (Blanco *et al.,* 2002; OIE, 2004). The prescribed FMD diagnostic assays, as stipulated in the Manual of Standards for Diagnostic Tests and Vaccines (OIE, 2004), are the virus neutralization test (VNT) (Golding *et al.,* 1976) and the ELISA (Mackay *et al.,* 2001; OIE, 2004). Both these tests measure antibodies to FMDV structural (capsid) proteins that can be acquired through infection or following vaccination. Although the VNT is serotype-specific it is laborious, requires cell culture facilities and may take two to three days to provide results (Rémond *et al.,* 2002; OIE, 2004). Consequently, a liquid-phase blocking ELISA (LPB-ELISA), of which the results correlate well with those obtained by VNTs (Rémond *et al.,* 2002), is used extensively for routine screening of a large number of sera (Hamblin *et al.,* 1986a;b; Mackay *et al.,* 1998). However, to overcome the variable stability of inactivated antigens and to increase the specificity of the LPB-ELISA, animal sera are currently tested in either a solid-phase blocking ELISA (Sørensen *et al.,* 1992; Chenard *et al*., 2003) or a solid-phase competition ELISA (Mackay *et al.,* 2001).

In addition to the structural proteins, the specific diagnosis of infection has also been performed using nonstructural proteins (mainly the $3C^{pro}$ and $3D^{pol}$) (Clavijo *et al.,* 2004a). These nonstructural proteins are expressed during the virus replication cycle and elicit an antibody response in infected animals, which theoretically is not present in animals vaccinated with purified viral particles. The first test developed was an agar gel immunodiffusion test to detect antibody to the virus infection-associated antigen (VIAA), now known as the viral replicase ($3D^{pol}$) (Cowan and Graves, 1966; McVicar and Sutmoller, 1970). However, it was soon realized that cattle vaccinated with conventional FMD vaccines may develop antibody to the VIAA antigen, especially after repeated vaccination (Pinto *et al.,* 1979; Mackay *et al.,* 1998). Therefore, other nonstructural proteins have been assayed as potential antigens to detect infected animals, as discussed in the following section.

1.6 DIFFERENTIATION OF INFECTION FROM VACCINATION IN FMD

Using traditional serological assays it is not possible to differentiate clearly FMDV-infected animals from vaccinated animals, as both groups of animals have neutralizing antibodies in their sera. The diagnostic challenge is therefore to distinguish FMDV-infected animals, either vaccinated or unvaccinated that have had contact with live virus, from those that have been only vaccinated against the disease (Clavijo *et al.,* 2004a). Such a diagnostic test would make it possible to monitor virus presence or circulation and therefore would be very useful for serological surveys to detect evidence of infection and to verify the naïve status of unvaccinated animals in FMD-free countries (Sutmoller *et al.,* 2003). Consequently, several diagnostic tests, which allow for differentiation of infected from vaccinated animals, have been developed and all of these are based on the detection of antibodies to nonstructural proteins (Rodríguez *et al.,* 1994; De Diego *et al.,* 1997; Silberstein *et al.,* 1997; Mackay *et al.,* 1998; Clavijo *et al.,* 2004b).

1.6.1 Immune response

Upon infection, FMD elicits rapid humoral and cellular immune responses that induce an efficient protection against re-infection with antigenically related or homologous viruses (McCullough *et al.*, 1992; Salt, 1993; Sobrino *et al.,* 2001). Neutralizing antibodies directed to B-cell epitopes located on the viral capsid can be detected soon after FMDV infection or vaccination. The first neutralizing antibodies elicited are IgMs and can be detected at 3 to 5 days post-infection or vaccination and reaches a peak in cattle between 5 to 10 days post-infection, and then declines (Doel, 1996). In both infected and vaccinated cattle, IgGs can be detected from 4 days post-infection and reach a peak at 10 to 14 days post-infection (Collen, 1994). IgGs are detected in swine between 4 to 7 days post-infection and become the major neutralizing antibodies by two weeks post-immunization (Francis and Black, 1983). By contrast, IgA antibodies peak at four to eight weeks post-infection, after the healing of vesicular lesions (Archetti *et al.,* 1995). The induction of high levels of neutralizing antibodies in serum coincides with resolution of lesions, progressive clearance of circulating virus and the reduction of virus excretion (Doel, 1996). Final clearance of the virus, however, requires the destruction of antibody-opsonized virus by macrophage phagocytosis (McCullough *et al.,* 1992; Brown, 1995).

Different studies have indicated that T-cell immune responses also contribute to the immunity to FMDV (Becker, 1993). In infected or vaccinated cattle and swine, B-cell activation and antibody production are associated with a lymphoproliferative response mediated mainly by T-cells ($CD4^+$). The T-helper cells recognize several viral epitopes located both in the capsid structural proteins (Collen *et al.*, 1991; Sáiz *et al.*, 1992; Mateu, 1995; García-Valcarcel *et al.*, 1996) and nonstructural proteins (Collen *et al.*, 1998; Shen *et al.*, 1999). Although T-cell responses mediated by $CD4^+$ cells appear to be required for protective immunity against FMDV, early events after FMDV infection result in a rapid reduction of MHC class 1 expression in the surface of FMDV-infected cells. Thus, presentation of viral peptides in FMDV-infected cells to the cytotoxic T-lymphocytes is impaired and may therefore facilitate virus escape from antiviral response by the host (Sanz-Parra *et al.,* 1998).

It has been suggested that initiation of FMDV persistence is correlated with the amount of interferon produced (Zhang *et al*., 2002). Studies have shown that interferon (IFN)-α, IFN-β, IFN-γ, as well as other cytokines, may play a role in host defence during the early stages of infection (Chinsangaram *et al.*, 1999; Chinsangaram *et al.*, 2001; Zhang *et al.*, 2002). Furthermore, cytokine-induced monocytic cell activity appears to play a role in protection from acute-phase disease. In swine that were either vaccinated only or was vaccinated and challenged, levels of interleukin (IL)-6, IL-8 and IL-12 in plasma increased after vaccination and/or challenge, suggesting monocyte/macrophage activation (Barnett *et al.,* 2002). Whereas IL-8 recruits the appropriate immune cells to the site of vaccination and infection, IL-6 is responsible for their enhanced immunological activity (Barnett *et al.,* 2002). Vaccinated swine, which were protected from contact challenge, showed the highest levels of IL-12. This interleukin is important for activation of innate host defences, including natural killer cells, and for the control of T-lymphocyte activity (Barnett *et al.,* 2002).

1.6.2 FMD vaccines and nonstructural proteins

The majority of FMD vaccines are produced in BHK-21 monolayer cells or in suspension cell cultures, and the live virus is inactivated using binary ethyleneimine (BEI). The inactivated antigen is then concentrated by polyethylene glycol (PEG) precipitation and ultrafiltration (Barteling and Vreeswijk, 1991). Precipitation of the antigen with PEG also concentrates tissue

culture-derived proteins and, therefore, a variable amount of nonstructural proteins, which are synthesized during the growth cycle of the virus in cell culture, may also be present in the final antigen preparation (Wagner *et al.,* 1970; Barteling and Vreeswijk, 1991; Doel, 1996). Finally, the antigen is diluted with buffers before blending it with an adjuvant, e.g. aluminium hydroxide/saponin or an oil adjuvant, which serves to stimulate and enhance the immune response to the FMD antigen (Barteling and Vreeswijk, 1991).

Since FMD vaccines may contain nonstructural proteins in addition to the inactivated virions, and depending on their concentration in the final vaccine preparation, they may induce a detectable immune response. For example, the VIAA (or $3D^{pol}$) is present in both the tissue culture from which vaccines are produced (O'Donnell *et al.,* 1997) and in the viral particle (Newman *et al.,* 1994). Antibodies to VIAA are therefore present in the sera of both infected and vaccinated animals, as well as in the sera of animals that have been given multiple vaccinations (Pinto and Garland, 1979; O'Donnell *et al.,* 1997; Mackay *et al.,* 1998). However, because of the low concentration of other nonstructural proteins in vaccines it is thought that detectable (positive) levels of antibodies to these nonstructural proteins may be of a shorter duration compared to those following infection with live virus (Clavijo *et al.,* 2004a).

1.6.3 Nonstructural proteins as indicators of infection

To overcome the above-mentioned problems, several research groups have attempted to identify antibodies to nonstructural proteins, other than the $3D^{pol}$, that could provide a reliable indicator of FMDV infection. In one of the first such experiments, Berger and co-workers analyzed bovine hyperimmune, convalescent sera for FMDV-specific antibodies and found that, unlike post-vaccination sera, the sera precipitated nonstructural proteins 3AB and/or 3C, 2C and occasionally 3A and 2B. It was therefore concluded that the presence of antibodies to these nonstructural proteins might provide a means to distinguish between animals that had recovered from FMDV infection and those vaccinated against FMD (Berger *et al.,* 1990). In a subsequent investigation, Lubroth and Brown reported that carrier convalescent cattle could be distinguished from vaccinated cattle based on the presence of antibodies to the 2C protein and to a lesser extent the 3ABC polyprotein (Lubroth and Brown, 1995). The absence of antibody to the 2C protein in

sera of vaccinated animals was thought to be due to the association of the 2C protein with cellular membranes, which are separated, along with the cellular debris, from the virus harvest prior to inactivation of the supernatant for vaccine production (Lubroth *et al.,* 1996; Meyer *et al.,* 1997). Similarly, it has been suggested that the absence of anti-3ABC and anti-3AB antibodies from post-vaccination sera is a likely consequence of the association of 3AB with cellular membranes (Silberstein *et al.,* 1997). Rodríguez and co-workers assessed the immunogenicity of the FMDV Leader, 2C, 3A, 3B, 3C, 3AB, 3ABC and 3D nonstructural proteins in swine and analyzed the specificity of the anti-FMDV antibodies against these nonstructural proteins in sera from infected or vaccinated swine. The results obtained indicated that the 3ABC polypeptide was the most immunogenic nonstructural protein and could be used to distinguish effectively between infected and vaccinated swine (Rodríguez *et al.,* 1994).

Mackay and co-workers compared the Leader, 2C, 3A, 3D proteins and the 3ABC polyprotein as markers of FMDV infection and reported that infected animals could be differentiated from vaccinated animals by the presence of antibodies to the nonstructural proteins 2C, 3A and the 3ABC polyprotein in the sera from infected animals. Of these, the 3ABC polyprotein was the most immunogenic and it was concluded that this protein is the most reliable single indicator of infection in both bovine and porcine sera (Mackay *et al.,* 1998). In addition, the immune response to 3ABC appeared 10 days post-infection and antibody to 3ABC could be detected for longer than antibodies to any of the other nonstructural proteins investigated (Mackay *et al.,* 1998). The majority of post-vaccination sera from cattle, which had received less than five vaccinations, were frequently positive for antibody to $3D^{pol}$, but were negative for antibody to the 3ABC polypeptide. However, cattle that had been vaccinated more than 10 times had nonstructural antibody profiles similar to those seen following infection, and therefore could not be differentiated using nonstructural protein-based serological assays. Nevertheless, it was concluded that such cattle would be rare and could be identified based on their age and vaccination history (Mackay *et al.,* 1998). In southern Africa where vaccination is used to control FMD, the vaccination history would aid in identifying such animals.

1.6.4 Antibody responses to nonstructural proteins

From the literature, it appears that the antibody response to the nonstructural proteins is variable. Generally, antibodies to the nonstructural proteins (2C, 3A, 3D, 3ABC and 3AB) can be detected in the sera of infected animals between 8 to 11 days post-infection (Sørensen *et al.,* 1998; Mackay *et al.,* 1998). Antibody to the 3AB nonstructural protein was detected in the sera of experimentally infected cattle 7 days post-infection and could still be detected 560 days post-infection (Silberstein *et al.,* 1997). De Diego and co-workers reported that antibody to the 3ABC polyprotein could be detected between 8 to 10 days post-infection, and although anti-3ABC antibody declined gradually within 6 months, it could still be detected for up to 365 days post-infection in the sera of experimentally infected cattle (De Diego *et al.,* 1997). By contrast, Sørensen and co-workers did not observe declining anti-3AB or anti-3ABC antibodies levels and reported that the experimentally infected cattle remained strongly positive up to 395 days post-infection (Sørensen *et al.,* 1998). Malirat and co-workers reported that anti-3ABC antibody could be detected for longer, up to 742 days post-exposure to the virus (Malirat *et al.*, 1998). Antibodies to nonstructural proteins in sheep appear to develop much later in infection. Antibodies to the 3AB and 3ABC proteins were detected between 10 to 14 days post-infection, whilst antibody to the 3D protein in some sheep was only detectable at 22 days post-infection (Sørensen *et al.,* 1998). Similarly, Rodríguez and co-workers reported that antibody to the 3ABC polyprotein was detectable in the sera of experimentally infected swine at 14 days post-infection (Rodríguez *et al.,* 1994).

In general, antibodies to the structural (capsid) proteins can be detected earlier than those against the nonstructural proteins. In cattle, antibodies to the structural proteins can be detected as early as 3 to 4 days post-infection and are also detected for longer than antibodies to the nonstructural proteins (Thomson, 1994). Considering that antibody titres to the nonstructural proteins are highly variable, diagnostic tests relying on the detection of antibodies to the nonstructural proteins for evidence of infection in unvaccinated herds, would have a less relative sensitivity to detect infected (positive) animals early after infection than those that use structural proteins as antigen (Foster *et al.,* 1998). In addition, differentiation of vaccinated and infected animals is only possible on herd level as a consequence of the variability in the initiation, specificity and

duration of the immune response to the nonstructural proteins (Mackay *et al.,* 1998; Clavijo *et al.,* 2004a). Therefore, it has been proposed that the likelihood of detecting or confirming an infected animal may be greatly increased when multiple nonstructural proteins, excluding the $3D^{pol}$, are used as diagnostic antigens (Clavijo *et al.,* 2004a).

1.6.5 Diagnostic tests for the detection of antibodies to nonstructural proteins

To differentiate FMDV-infected animals, either vaccinated or not, from vaccinated animals, several research groups have developed diagnostic assays using recombinant nonstructural protein antigens. The nonstructural protein antigens have been produced either in *Escherichia coli* (Rodríguez *et al.,* 1994; De Diego *et al.,* 1997; Mackay *et al.,* 1998; Malirat *et al.,* 1998; Brocchi *et al.,* 1998) or in insect cells infected with recombinant baculoviruses (Sørensen *et al.,* 1998; Kweon *et al.,* 2003). One of the first tests developed was an enzyme-linked immunoelectrotransfer blot (EITB) using purified recombinant 3B, 2C, 3D and 3ABC antigens (Bergmann *et al.,* 1993). Although the test was highly specific and sensitive, it was not suited to screening large numbers of sera. Since then, several ELISAs have been developed (Table 1.1) to facilitate the screening of large numbers of sera.

A problem associated with the use of these recombinant nonstructural protein antigens is the presence of antibodies against host and expression vector antigens, which can cause non-specific reactions and complicate interpretation of the results (Clavijo *et al.,* 2004a). To address this problem, several strategies have been developed. Crude cell lysates of *E. coli* or insect cells have been used in ELISA to block non-specific reactions and were found to be largely successful in reducing the number of false positives (De Diego *et al.,* 1997; Kweon *et al.,* 2003). Many research groups have expressed the nonstructural proteins as a fusion protein in *E. coli* to facilitate its purification (Rodríguez *et al.,* 1994; De Diego *et al.,* 1997; Silberstein *et al.,* 1997; Mackay *et al.,* 1998; Malirat *et al.,* 1998). Indirect ELISAs, which use monoclonal antibodies to capture only the recombinant 3ABC polyprotein from partially purified cell extracts, have also been used (Brocchi *et al.,* 1998; Sørensen *et al.,* 1998; Chung *et al.,* 2002; Kweon *et al.,* 2003). In a recent study, Clavijo and co-workers used streptavidin-coated ELISA plates to capture biotinylated 3ABC protein from crude cell lysates, allowing for single-step purification of the

Table 1.1: Summary of nonstructural protein ELISA-based diagnostic tests. (Adapted from Clavijo *et al*. 2004a).

Test *	**System**	**Antigen(s)**	**Sensitivity**	**Specificity**	**Reference**	**Fusion proteins**
LPB-ELISA	RP(*E. coli*)	3D	90.9% (bovine)	100% (bovine)	O'Donnell *et al*. (1996)	GST
I-ELISA	RP(*E. coli*)	3ABC	NA	NA	Rodriguez *et al*. (1994)	MS2
I-ELISA	RP(*E. coli*)	Lb, 2C, 3A, 3D, 3ABC	94-100% for all antigens except Lb (64%)	95-100% for all antigens except 3D (64%)	Mackay *et al*. (1998)	GST
I-ELISA	RP(*E. coli*)	3ABC	100% (bovine)	99.2% (bovine)	Malirat *et al*. (1998)	MS2
IT ELISA	RP(*E. coli*)	3ABC	100% (bovine)	99.5% (bovine)	De Diego *et al*. (1997)	MS2
Mab-T-ELISA	RP(*E. coli*)	3ABC	100% (bovine)	99% (bovine)	Brocchi *et al*.(1998)	MS2
C-ELISA	RP (*E. coli*)	3ABC	NA	100% (bovine, ovine) 99.8% (porcine)	Clavijo *et al.* (2004)	Biotinylated
I-ELISA	RP(Baculovirus) +*E. coli*	$3AB_1$	NA	NA	Silberstein *et al*. (1997)	GST
IT-ELISA	RP(Baculovirus)	3ABC	NA	99% (bovine)	Kweon *et al*. (2003)	-
BT-ELISA	RP(Baculovirus)	3AB	95.8% (porcine)	100% (bovine)	Chung *et al*. (2002)	-
BT-ELISA	RP(Baculovirus)	3D 3AB 3ABC	98% (bovine) 84% (bovine) 92% (bovine)	99.8% (bovine) 99.8% (bovine) 100% (bovine)	Sorenson *et al*. (1998)	-
I-ELISA	Peptide	3B	98.2% (porcine) 100% (bovine)	99.3% (swine) 99.2% (bovine)	Shen *et al*. (1999)	-

*Abbreviations: LBP-ELISA, liquid phase blocking ELISA; I-ELISA, indirect ELISA; BT-ELISA, blocking trapping ELISA; IT-ELISA, indirect trapping ELISA; Mab-T-ELISA, monoclonal antibody trapping ELISA; C-ELISA, competitive ELISA; NA, not available; RP, recombinant proteins

antigen (Clavijo *et al.,* 2004b). Another strategy to circumvent the occurrence of false positive reactions could be to include a second confirmatory test such as an EITB, which can be used in combination with the 3ABC-ELISA (Bergmann *et al.,* 2000). Alternatively, instead of using recombinant antigens, synthetic peptides may rather be used for the identification of anti-nonstructural protein antibodies in an ELISA format (Shen *et al.,* 1999). Shen and co-workers have identified continuous antigenic determinants within the amino acid sequence of the 3ABC and 2C nonstructural proteins. By contrast to an ELISA based on the use of a 3A peptide, which reacted with some sera of vaccinated animals, an ELISA based on a 3B peptide was shown to differentiate vaccinated from infected animals and gave a positive reaction with the sera from cattle, swine and sheep (Shen *et al.,* 1999). Advantages associated with the use of peptides from the nonstructural proteins include their specificity, non-reactivity with antibodies against host-cell derived proteins and their ease of preparation.

1.7 PERSISTENT INFECTION, THE CARRIER PROBLEM

One of the most contentious issues in understanding the spread of FMD in the field, is the role of "carrier animals". The first report of the so-called "carrier status" was in 1959 when van Bekkum and co-workers demonstrated the recovery of infectious virus from the oesophageal-pharyngeal fluids of convalescent cattle (Van Bekkum *et al.,* 1959). Since then, carrier animals have been defined as animals from which live FMD virus can be isolated from the oesophagus and throat fluids at 28 days, or later, post-infection (Sutmoller *et al.,* 1968; Salt, 1993). The carrier state is characterized in ruminants by a long asymptomatic persistent infection, following the acute phase of infection (Salt, 1993). The situation is furthermore complicated by the fact that animals, which have been vaccinated, can also become carriers of FMDV following exposure to infectious virus (Salt, 1993; Alexandersen *et al.,* 2002; Sutmoller *et al.,* 2003). Whether or not carrier animals may serve as the source of new outbreaks of the disease remains unclear. However, there is epidemiological evidence to suggest that carrier animals may be the origin of outbreaks of acute disease when brought into contact with susceptible animals (Hedger and Condy, 1985; Dawe *et al.,* 1994a).

The duration of the carrier state has been documented for several animal species. In cattle, the carrier state can last up to 3.5 years (Thomson, 1994). In sheep and goats, the duration is shorter and lasts from 1 to 5 months (Burrows, 1968), but in some animals the carrier state may last as long as 12 months (McVicar and Sutmoller, 1968; Sharma, 1978). By contrast,

swine are rid of the infection within 3 to 4 weeks and therefore, as far as is known, do not become carriers of the virus (Alexandersen *et al.,* 2002). In addition to domestic livestock animals, wildlife have also been implicated as carriers of FMDV. For example, both fallow deer and white-tailed deer, which occur in the UK and in the USA, respectively, carried FMDV up to 5 weeks post-exposure to the virus (Forman *et al.,* 1974; McVicar *et al.,* 1974). In Africa, FMDV has been detected in kudu (*Tragelaphus strepsiceros*) for almost 5 months after artificial infection (Hedger, 1972). However, in impala (*Aepyceros melampus*), which is the antelope species most frequently affected by FMDV (Thomson, 1996), long-term viral persistence could not be demonstrated (Hedger, 1972; Anderson *et al.,* 1975). By contrast, in the African buffalo (*Syncerus caffer*) viral persistence can last for up to 5 years (Condy *et al.,* 1985) and individual buffalo may be persistently infected with multiple subtypes (Hedger, 1972; Anderson *et al.,* 1979). However, only the SAT type viruses and not the European types A, O and C become endemic in the buffalo population through the carrier status (Sutmoller *et al.,* 2003). In the African buffalo, the carrier state can be very high, ranging between 50 to 70% in the field (Condy *et al.,* 1985), but rates in cattle and sheep can vary widely, from 15 to 50% (Alexandersen *et al.,* 2002). The number of carrier animals in a population depends on several factors such as the species, the strain of the virus, incidence of infection and the immune state of the herd (vaccinated or not) (Alexandersen *et al.,* 2003).

As mentioned above, the role of carrier animals in the spread and transmission of FMDV is controversial. Although little direct evidence for carrier animals being the source of new infections or outbreaks has been provided, circumstantial evidence, together with the presence of live virus in oesophageal-pharyngeal fluids, suggests that they might indeed be responsible for outbreaks of FMD (Alexandersen *et al.,* 2003). The notion is supported by reports indicating that unexpected outbreaks occurred in countries free of FMD following the introduction of apparently healthy convalescent cattle. Subsequent vaccination led to a drastic reduction in the incidence of the virus circulating in the livestock population, thereby fuelling such suspicions (Sutmoller *et al.,* 2003). Dawe *et al.* (1994a) have provided evidence that persistently infected African buffalo were responsible for the transmission of FMDV to susceptible cattle during outbreaks of FMD in Zimbabwe, and Bastos *et al.* (1999) reported that such intra-species transmission might occur through sexual contact, as sightings of African buffalo mounting domestic cows has been recorded. However, there has been no evidence indicating that carrier cattle or sheep can transmit the virus to uninfected animals (Sutmoller *et al.,* 2003).

Although the mechanisms for the establishment and maintenance of the carrier state are not fully understood, two possible explanations for virus persistence in the pharynx of ruminants have been proposed. The first suggests that FMDV infects cells of the immune system such as macrophages, leading to evasion of the immune response, whereas, the second, more favoured suggestion, suggests that the virus exploits the host response to provide an intracellular milieu favourable for long-term persistence (Alexandersen *et al.,* 2002).

1.8 AIMS OF THIS STUDY

Foot-and-mouth disease (FMD) virus is a highly infectious viral agent, which causes an acute systemic disease of cloven-hoofed animals (Thomson, 1994). Since disease spreads rapidly among susceptible animals, rapid and sensitive diagnostic assays are required to accurately diagnose FMD in susceptible animals so as to allow prompt implementation of measures to control its spread. Current recommended diagnostic assays, the virus neutralization test (VNT) and the liquid-phase blocking ELISA (LPB-ELISA), which detect antibodies against primarily the capsid proteins, are not able to distinguish between vaccinated and infected animals (Quinn and Markey, 2001; OIE, 2004). In addition, since animals, irrespective of their vaccination state, may become persistently infected and display an asymptomatic infection, they may serve as sources of new FMD outbreaks (Salt, 1993; Sutmoller *et al.,* 2003).

Differentiation of FMDV-infected from vaccinated animals is possible by detecting antibodies against some of the nonstructural proteins. In particular, antibodies against the 3ABC polyprotein have been proposed to be the single most reliable indicator of infection (Mackay *et al.,* 1998). The majority of 3ABC-based serological assays are based on the use of recombinant proteins, produced either in *E. coli* or in insect cells by means of recombinant baculoviruses, as antigen in an ELISA format (Table 1.1). Although 3ABC-based diagnostic test kits are available commercially, they have been developed using the 3ABC polypeptide of European FMDV serotypes and is based on the assumption that the nonstructural protein-encoding regions are conserved across all FMDV serotypes (Mr. J. Esterhuysen, personal communication). Genetic characterization of SAT type 3A (Heath *et al.,* 2001) and 3C (Van Rensburg *et al.,* 2002) nonstructural proteins has indicated that these proteins are distinct from those of European types A, O and C. Thus, the sensitivity and specificity of

commercially available diagnostic 3ABC-ELISAs for detection of SAT type-specific infections may be compromised (Van Rensburg *et al.,* 2002).

Detection of SAT type-specific infections is of particular importance in sub-Saharan Africa, since 84% of identifiable FMD outbreaks during 1931 and 1990 were caused by SAT type viruses (Thomson, 1994). In southern Africa, SAT2 viruses are responsible for the majority of SAT type outbreaks (48%) and it is the most widely distributed FMDV serotype in sub-Saharan Africa (Ferris and Donaldson, 1992; Thomson, 1994). Between 1990 and 2001, the causative virus in outbreaks reported to the OIE from southern Africa, were almost equally divided between the respective SAT types (Thomson and Bastos, 2004). Towards the long-term aim of developing a SAT type-specific 3ABC-ELISA, capable of differentiating FMDV-infected from vaccinated animals, a first requirement would be to obtain sufficient amounts of a SAT type 3ABC polypeptide to allow for the development of such a diagnostic test.

Therefore, the objectives of this investigation were:

(i) To molecularly characterize the 3ABC-encoding genomic region of the current FMDV vaccine strain, a SAT2 type FMD virus, ZIM/7/83.

(ii) To express the 3ABC-encoding region of ZIM/7/83 in the gram-negative bacterium *Escherichia coli*.

(iii) To express the 3ABC-encoding region of ZIM/7/83 in the BAC-to-BAC™ baculovirus expression system.

CHAPTER 2

MOLECULAR CHARACTERIZATION AND ANALYSIS OF THE GENOMIC REGION ENCODING THE 3ABC NONSTRUCTURAL POLYPEPTIDE OF THE SAT2 TYPE FOOT-AND-MOUTH DISEASE VIRUS ZIM/7/83

2.1 INTRODUCTION

Foot-and-mouth disease virus (FMDV) is a highly infectious viral agent, causing an acute vesicular disease in both wild and domestic cloven-hoofed animal species. This disease is considered to be of great economic importance, since it results in a decline in meat and dairy production in infected animals and has led to the establishment of trade embargoes on the import and export of infected livestock and animal products (Thomson, 1994; Samuel and Knowles, 2001). Therefore, considerable importance has been placed on the control and eradication of foot-and-mouth disease (FMD) throughout the world.

In the African context, control of the disease is particularly complex due to several reasons. Firstly, seven immunologically distinct virus serotypes exist (euroasiatic types, A, O, C and Asia1 and the South African Territories [SAT] types, SAT1, SAT2 and SAT3) (Carrillo *et al.,* 2005), of which almost all (except Asia1) have caused outbreaks in livestock on the African continent (Vosloo *et al*., 2002). The FMDV types can be further divided into subtypes (Pereira, 1977) and geographically-localized topotypes (Vosloo *et al*., 1995; Bastos *et al*., 2001; Samuel and Knowles, 2001; Bastos *et al*., 2003a;b). Secondly, FMDV types are genetically (Vosloo *et al*., 1995; Van Rensburg and Nel, 1999; Van Rensburg *et al*., 2002) and antigenically (Esterhuysen, 1994) distinct from each other, so that animals infected with one serotype are not immune to re-infection with a virus from a different serotype or topotype (Thomson *et al*., 2003). Likewise, vaccines providing protection to one FMDV type are unlikely to provide full protection to re-infection with a different FMDV type (Cartwright *et al*., 1982). Furthermore, the SAT types have been shown through RNA hybridization assays (Robson *et al*., 1977), sequence comparisons of the capsid proteins (Carrillo *et al.,* 2005) and phylogenetic analysis of some nonstructural proteins (Heath *et al*., 2001; Van Rensburg *et al.,* 2002) to be distinct from the euroasiatic types. Finally, the SAT types are the only FMDV serotypes known to have a long-term wildlife reservoir host for continued viral persistence, making eradication of the disease in sub-Saharan Africa doubtful. This reservoir host, the African Buffalo (*Syncerus caffer*), is known to be an important source of FMD infection for cattle (Dawe *et al*., 1994a,b; Vosloo *et al*., 1996; Bastos *et al*., 1999). The SAT type viruses can persist for up to five years in an individual buffalo (Condy *et al*., 1985), during which time a variety of genetic and antigenic variants can be generated (Vosloo *et al*., 1996).

Consequently, control of FMD in sub-Saharan Africa is achieved with regular vaccination of susceptible livestock and by restriction of animal movement (Hunter, 1998). For effective control, it is important that vaccination programs are complemented with efficient routine diagnostic screening of livestock. The virus neutralisation test (VNT) and the liquid-phase blocking ELISA have been recommended for diagnosis of FMD in livestock (OIE, 2004). In addition, reverse-transcriptase polymerase chain reaction (RT-PCR) procedures for the detection of FMDV genomic RNA in tissue culture fluid and clinical specimens have been developed (Callens and De Clercq, 1997; Bastos, 1998; Reid *et al*., 1999). However, serological assays based on the detection of antibodies to the nonstructural viral proteins appear to be the most promising, because of its potential to differentiate between vaccinated and convalescent animals (Berger *et al*., 1990; Bergmann *et al*., 1993; Lubroth and Brown, 1995; Lubroth *et al*., 1996; De Diego *et al*., 1997). Of these, the 3ABC diagnostic ELISA is preferred, because the 3ABC antigen is considered to be the single most reliable indicator of viral infection (Mackay *et al*., 1998; Clavijo *et al*., 2004a).

Although 3ABC-ELISAs are commercially available, they have been developed for the detection of antibodies to the 3ABC polypeptide of the European types A, O and C (Mr J. Esterhuysen, personal communication). However, the SAT type viruses, which are generally restricted to sub-Saharan Africa, are responsible for the majority of identifiable FMD outbreaks in southern Africa (Thomson, 1994). Between 1850 and 1987, SAT2 accounted for 48% of all SAT type outbreaks, whereas, SAT1 and SAT3 accounted for 36% and 16%, respectively, of these outbreaks (Thomson, 1994). Occasionally SAT type viruses have spread beyond sub-Saharan Africa, causing outbreaks in the Middle East and Greece (SAT1) or in Saudi Arabia and Kuwait (SAT2) (Knowles and Samuel, 2003). The commercially available 3ABC-ELISAs have been developed based on the assumption that the 3ABC-encoding genomic region is conserved for all seven serotypes. However, the 3A- and 3C-encoding regions of the SAT type viruses have been reported to be distinct from the European types (Heath *et al*., 2001; Van Rensburg *et al*., 2002; Carrillo *et al.,* 2005) and may therefore influence the specificity and sensitivity of the available 3ABC-ELISAs.

The objectives for this part of the study were thus: (i) to determine the nucleotide and deduced amino acid sequence of the 3ABC-encoding region of the SAT2 type FMD virus ZIM/7/83 and (ii) to investigate the genetic variability of the 3ABC polypeptide between SAT type and euroasiatic type FMD viruses.

2.2 MATERIALS AND METHODS

2.2.1 DNA amplification

2.2.1.1 Oligonucleotides

A genome-length cDNA clone (pSAT2) of a SAT2 type FMD virus isolate, ZIM/7/83, has previously been constructed (Van Rensburg *et al.*, 2004) and was used as template DNA for PCR amplification of the 3ABC-encoding genomic region. Oligonucleotides containing unique restriction endonuclease recognition sites were designed using the nucleotide sequence of ZIM/7/83 (GenBank number: AF540910) (Van Rensburg *et al.,* 2004). Sense (3ABC-F; 5'-CGATT**GGATCC***ATG*ATTTCCATTC-3') and anti-sense (3ABC-R; 5'-CGC**GAATTC**CTACTCAGTG*TGA*GG-3') oligonucleotides, targeting the 3ABC coding sequence, were designed to include *Bam*HI and *Eco*RI restriction enzyme sites (bold) whereby cloning of the amplicon into bacterial (Chapter 3) and baculovirus (Chapter 4) vectors would be facilitated. For expression, an ATG codon (italics) in the 3ABC-F oligonucleotide and a TGA termination codon (italics) in the 3ABC-R oligonucleotide were included for translation initiation and termination, respectively.

2.2.1.2 Polymerase chain reaction (PCR) amplification

Per 50-µl PCR reaction, 25-50 ng of pSAT2 plasmid DNA, 50 pmol of each oligonucleotide, 1 × PCR buffer (50 mM KCl; 10 mM Tris-HCl [pH 9.0]; 0.1% [v/v] TritonX-100), 1.5 mM $MgCl_2$, 125 µM of each dNTP and 2.5 U *Taq* DNA polymerase (Promega) were added and placed in a Perkin-Elmer GeneAmp 2400 thermal cycler. In addition, a control reaction was prepared that contained all the above-mentioned reagents, except the template DNA. The thermocycling profile of the PCR reaction consisted of an initial denaturation step at 95ºC for 2 min, followed by 30 cycles of denaturation (94ºC for 1 min), annealing (60ºC for 1 min) and extension (72ºC for 2 min). A final extension step of 72ºC for 5 min was included to complete synthesis of all DNA strands. The reaction mixtures were analyzed on a 1% (w/v) agarose gel.

2.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis, as described by Sambrook *et al.* (1989), was used to visualize and size the DNA fragments. Molecular weight marker (lambda DNA digested with *Hind*III and *Eco*RI) and the DNA samples were mixed with loading buffer (40% [w/v] sucrose solution; 0.25% (w/v) bromophenol blue) and loaded into the wells of a 1% (w/v) agarose gel, containing 0.5 µg/ml ethidium bromide. Agarose gels were electrophoresed at 100 V in 1 × TAE buffer (40 mM Tris-HCl; 20 mM NaOAc; 1 mM EDTA; pH 8.5).

2.2.3 Purification of DNA fragments from agarose gels

Boyle and Lew (1995) developed a method for the purification of plasmid DNA from agarose gels. This method, as described by Van Sckalkwyk (2003), was used to obtain highly purified DNA fragments. A clean scalpel blade was used to excise the DNA fragment from the agarose gel, after which 3 volumes of 6 M NaI was added to the gel slice and incubated at 55ºC for 10 min. Ten µl of a silica suspension was added to the melted gel slice and vortexed for 10 s. The suspension was incubated on ice for 30 min to allow the DNA to bind to the silica and then pelleted for 30 s at 15 000 rpm. The pellet was washed five times with 0.5 ml chilled Wash buffer (50 mM NaCl; 10 mM Tris-HCl [pH 7.5]; 2.5 mM EDTA; 50% [v/v] ethanol) and the bound DNA was eluted at 55ºC for 10 min in 10 µl of UHQ water followed by a brief centrifugation step to pellet residual silica. The purity and concentration of the purified DNA was assessed by electrophoresis on a 1% (w/v) agarose gel.

2.2.4 Cloning of the 3ABC-encoding region into pGEM®-T Easy

2.2.4.1 Ligation of DNA fragments

The purified 3ABC amplicon was ligated into the pGEM®-T Easy (Promega) cloning vector. A 10-µl final reaction volume consisting of 50 ng of pGEM®-T Easy DNA, 150 ng of the purified 3ABC amplicon, 3 U of T4 DNA ligase (3 U/µl; Promega) and 5 µl of the supplied 2 × DNA ligation buffer (Promega) was prepared. The DNA fragments were ligated at 4ºC for 16 h.

2.2.4.2 Preparation of competent *E. coli* DH5α cells

Escherichia coli cells have been made competent for uptake of non-chromosomal and plasmid DNA by treatment with $CaCl_2$ (Cohen *et al.,* 1972; 1973). A variation of the method described by Cohen *et al.* (1972) was used to prepare competent *E. coli* DH5α cells for uptake of plasmid DNA (Sambrook *et al.,* 1989). Using aseptic techniques, an *E. coli* DH5α culture was streaked on Lauria-Bertani (LB) agar (1% [w/v] tryptone; 0.5% [w/v] yeast extract; 1% [w/v] NaCl; 1.2% [w/v] agar; pH 7.4) and incubated at 37ºC. A single colony was then inoculated into 5 ml of sterile LB broth (1% [w/v] tryptone; 0.5% [w/v] yeast extract; 1% [w/v] NaCl; pH 7.4). The culture was grown at 37ºC with shaking (200 rpm). After 16 h, 1 ml of the culture was inoculated into 100 ml sterile LB broth and grown at 37ºC until an optical density of 0.5 at 550 nm was reached. The cells were pelleted at 4 000 rpm for 5 min at 4ºC in chilled Corex tubes using a pre-cooled Sorvall SS-34 rotor. The pellet was suspended in 10 ml ice-cold 50 mM $CaCl_2$ and incubated on ice for 30 min, after which cells were pelleted as described above. The pellet was then resuspended in 1 ml of the 50 mM $CaCl_2$ solution. The competent cells were incubated on ice for 1 h prior to transformation with the plasmid DNA or, alternatively, were snap-frozen in 15% (v/v) sterile glycerol and stored at -70ºC until further use.

2.2.4.3 Transformation of competent cells

Plasmid DNA can be taken up by competent *E. coli* cells during a brief increase in incubation temperature, known as a "heat-pulse" or "heat-shock" step (Cohen *et al.,* 1972; Sambrook *et al.,* 1989). A heat-shock step was used to transform the competent *E. coli* DH5α cells (Sambrook *et al.,* 1989). For transformation, 5 µl of the ligation reaction mixture was added to 200 µl of competent *E. coli* DH5α cells and kept on ice for 30 min. A positive control (10 ng of pUC 18 plasmid DNA added to the competent cells) and a negative control (DNA was omitted) were included to determine the competency of the cells and as a test for contamination, respectively. The cells were subjected to a heat-shock at 42ºC for 90 s in a water bath (Branson) and returned to ice for 2 min. A volume of 800 µl of LB broth, heated to 37ºC, was added to the cells and then incubated at 37ºC for 1 h. The pGEM®-T Easy plasmid contains an ampicillin resistance gene and a T/A cloning site, which is located within the α-peptide coding region of the β-galactosidase enzyme (pGEM Manual; Promega). Therefore, 50 µl of the transformed cells were plated onto LB agar containing ampicillin (100

µg/ml) in the presence of 10 µl IPTG (100 mM stock solution) and 40 µl X-gal (2% [w/v] stock solution). The agar plates were incubated at 37ºC overnight until blue and white colonies were observed. Insertional inactivation of the marker gene with the cloned gene results in colonies with a white phenotype, which were selected as putative recombinants from the background of non-recombinant blue colonies.

2.2.5 Plasmid DNA isolation

Plasmid DNA was extracted from transformants using an alkaline lysis method (Sambrook *et al.,* 1989), as described previously (Van Sckalkwyk, 2003). Selected colonies were inoculated into 5 ml fresh LB broth containing ampicillin (100 µg/ml) and cultured overnight at 37ºC in a shaking incubator (200 rpm). The cells from 3 ml of the culture were pelleted at 15 000 rpm for 3 min. The pellet was suspended in 400 µl of a chilled isotonic solution (50 mM glucose; 10 mM EDTA; 10 mg/ml RNaseA; 25 mM Tris-HCl; pH 8.0) and incubated at room temperature for 10 min. A volume of 400 µl of freshly-prepared alkaline-SDS buffer (0.2 N NaOH; 1% [w/v] SDS) was added to the suspension, which was then kept on ice for 10 min. Following this lysis step, 300 µl chilled 7.5 M ammonium acetate (pH 7.6) was added and the suspension was kept on ice for a further 10 min. The plasmid-containing supernatant was collected after centrifugation at 15 000 rpm for 10 min and transferred to a microfuge tube containing 650 µl isopropanol to precipitate the plasmid DNA. After incubation at room temperature for 10 min, the plasmid DNA was collected by centrifugation at 15 000 rpm for 10 min. The pellet was resuspended in 100 µl 2 M ammonium acetate (pH 7.4) and incubated on ice for 10 min. Excess proteins were pelleted by centrifugation for 10 min at 15 000 rpm, and the plasmid-containing supernatant was transferred to a microfuge tube containing 110 µl isopropanol. The plasmid DNA was precipitated at room temperature for 10 min and pelleted by centrifugation at 15 000 for 10 min. The pellet was washed with 70% (v/v) ethanol, vacuum-dried for 10 min and resuspended in 30 µl 1 × TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8.0).

2.2.6 Restriction enzyme digestion

Restriction enzyme digestion, using *Bam*HI (Roche) and *Eco*RI (Roche) restriction endonucleases, was performed to confirm the presence of the cloned insert DNA. For restriction digestion, plasmid DNA, 5 U (per µg of plasmid DNA) of restriction enzyme, and

a 1× final concentration of compatible restriction buffer (supplied as a 10× buffer by the manufacturer) were added to a sterile microfuge tube. The final reaction volume was adjusted to 15 µl with UHQ water. For double-digests, where two enzymes were required to digest the plasmid DNA, a buffer compatible with both enzymes was used, or, alternatively, digests were performed sequentially in their recommended buffers. The reaction mixtures were incubated at 37ºC for 2 h for complete digestion. The DNA fragments were analyzed on a 0.8% (w/v) agarose gel in the presence of undigested plasmid DNA and a molecular weight marker.

2.2.7 Nucleotide sequencing

2.2.7.1 Sequencing reactions

DNA sequencing was performed in an automated DNA sequencer, using the ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (v.3.0; Perkin Elmer Biosystems). In addition to the pUC/M13 forward (5'-GTAAAACGACGGCCAGTG-3') and reverse (5'-CAGGAAACAGCTATGAC-3') oligonucleotides, an internal sense oligonucleotide (5'-GGAMAACTTYGAGATYGTTGC-3') was used to determine the nucleotide sequence of the cloned 3ABC-encoding region of SAT2/ZIM/7/83. Cycle sequencing and precipitation reactions were performed according to ABI protocols, with the following modifications. Each 10-µl sequencing reaction consisted of 200-500 ng of plasmid DNA, 1.6 pmol of sequencing oligonucleotide and 2 µl Terminator Ready Reaction Mix. Cycle sequences were performed in a Perkin-Elmer GeneAmp 2400 thermal cycler with 25 cycles of denaturation at 96ºC for 15 s, annealing at 50ºC for 5 s, and extension at 60ºC for 4 min. The volume of the reaction mixture was adjusted to 20 µl with UHQ water and the extension products were precipitated at room temperature in the dark for 20 min with a precipitation mix (50 µl of 96% ethanol; 2 µl of 3 M NaOAc, pH 4.6). After centrifugation at 15 000 rpm for 30 min, the pellet was washed twice with 70% ethanol and vacuum-dried for 15 min. The sequencing reactions were then sent to the sequencing facility at the University of Pretoria where they were suspended in 3 µl sequencing loading buffer, denatured at 95ºC for 2 min and placed on ice. A 1.5-µl aliquot of each sample was loaded on a 4% denaturing polyacrylamide gel and electrophoresed at 1.6 kV for 7 h. Sequencing was performed with an automated Perkin-Elmer ABI 377 cycle sequencer.

2.2.7.2 Sequence analyses

Sequences were analyzed using the ABI PRISM sequence analysis™ and ABI PRISM Navigator™ software programs. The amino acid sequence of the 3A, 3B, 3C, 1D and the 3ABC polypeptide of SAT2/ZIM/7/83, as well as several other FMDV isolates, were deduced using DNAMAN (v.4.0 Lynnon Biosoft, Copyright©1994-1997) and aligned using ClustalX v.1.83 (Higgins and Sharp, 1988). The selected sequences, obtained from GenBank (http://www.ncbi.nlm.nih.gov), contain representatives of all seven FMDV serotypes and are summarized, together with their corresponding accession numbers, in Table 2.1. Phylogenetic analysis was carried out using the neighbour-joining (NJ) algorithm implemented in PAUP (v.4.0b10, Sinauer Associates). Statistical support for the trees was obtained by bootstrap analysis with 1000 replicates. The corresponding sequence of Equine Rhinitis A virus (GenBank accession number X96870) was selected as out-group. Hydrophobic regions and transmembrane domains were identified using Antheprot 2000 v.5.2 (Deleage, 2001), using the Kyte and Doolittle (1982) and Pearson/Olsen predictions, respectively.

Table 2.1: Foot-and-mouth disease viruses used in this study

Viruses	Serotype	Country of origin	Year of isolation	GenBank acc. no. (3ABC/1D)
A_{12}	A	United Kingdom	1932	M10975
A_{22}	A	USSR	1965	X74812
A_{23}Kenya	A	Kenya	1965	AY593766
A_3Mecklenburg	A	Germany	1968	AY593776
Argentina/01	A	Argentina	2001	AY593783
A_{16}Belem	A	Brazil	1959	AY593756
SAR/19/2000	O	South Africa	2000	AJ539140
O_1Campos	O	Brazil	1958	AJ320488
O_1Kaufbeuren	O	Germany	1966	X00871
O_1BFS	O	United Kingdom	1967	AY593815
C Waldman Str.149	C	Great Britain	1970	AY593810
C_5Argentina	C	Argentina	1969	AY593809
C_3Resende	C	Brazil	1955	AY593807
C_1Oberbayern	C	Germany	1960	AY593805
IND321/01	Asia1	India	2001	AY687333
IND491/97	Asia1	India: West Bengal	1985	AY687334
IND63/72	Asia1	India	1972	AY304994
Rhod5/66	SAT1	Zimbabwe	1966	AY593846
BOT1/68	SAT1	Botswana	1968	AY593845
6 SWA40/61	SAT1	Namibia	1940	AY593843
7 ISRL/4/62	SAT1	Israel	1962	AY593844
5 SA/61	SAT1	South Africa	1961	AY593842
4 SR2/58	SAT1	Zimbabwe	1968	AY593841
3 SWA1/49	SAT1	Namibia	1949	AY593840
1 BEC	SAT1	Botswana	1970	AY593838
RHO/1/48	SAT2	Zimbabwe	1948	AY593847
ZIM/7/83	SAT2	Zimbabwe	1983	AF540910
KEN/3/57	SAT2	Kenya	1957	AJ251473
4 BEC1/65	SAT3	Botswana	1965	AY593853
2 SA57/59	SAT3	South Africa	1957	AY593850
3 Kenya11/60	SAT3	Kenya	1960	AY593852
KNP/10/90	SAT3	South Africa	1990	Unpublished sequence data, kindly supplied by Dr. F. Maree, OVI-EDD

2.3 RESULTS

2.3.1 Construction of a recombinant pGEM®-T Easy vector containing the 3ABC-encoding region of SAT2/ZIM/7/83

Plasmid pSAT2, containing a cloned cDNA copy of the ZIM/7/83 FMD viral genome (Van Rensburg *et al.,* 2004), was obtained from Dr H.G. van Rensburg. The 3ABC-encoding region was PCR-amplified using pSAT2 as DNA template, as described under Materials and Methods (Section 2.2.1). Agarose gel electrophoresis of the reaction mixture revealed the presence of a single 1.3-kb DNA fragment, whereas no amplification product was observed in the control reaction mixture from which template DNA had been omitted (Fig. 2.1). The amplicon was purified from the agarose gel, ligated into the pGEM®-T Easy vector and the ligation reaction mixture was transformed into competent *E. coli* DH5α cells. Recombinant transformants were selected by blue/white colour selection, cultured overnight and the extracted plasmid DNA was analyzed by agarose gel electrophoresis. Plasmid DNA migrating slower than the parental pGEM®-T Easy vector DNA on agarose gels were selected (Fig. 2.2, lanes 2 and 3). These were investigated for the presence of a cloned insert DNA by restriction endonuclease digestion with both *Bam*HI and *Eco*RI, the recognition sequences of which were incorporated in the oligonucleotides used to PCR-amplify the 3ABC-encoding region. Following agarose gel electrophoresis, restriction fragments of 3.0-kb and 1.3-kb were observed, which is in agreement with expected sizes of the pGEM®-T Easy vector and insert DNA, respectively (Fig. 2.2, lane 5). The selected recombinant was designated pGEM-3ABC and used in all subsequent DNA manipulations.

2.3.2 Genetic characterization of the 3ABC-encoding region

The nucleotide sequence of the 3ABC-encoding region of SAT2/ZIM/7/83 was determined by sequencing the cloned insert DNA using pUC/M13 forward and reverse oligonucleotides, as well as an internal 3ABC-specific oligonucleotide. Additional nucleotide sequences, included in the sequence comparisons, were retrieved from GenBank (Table 2.1). Of the 32 FMDV isolates, 14 were European types, three were Asia1 types and 15 were SAT types. All nucleotide sequences were translated and the deduced amino acid sequences of the 3A, 3B and 3C proteins were aligned to determine amino acid sequence variation between the SAT and euroasiatic types. The nucleotide and deduced 3ABC amino acid sequence of SAT2/ZIM/7/83 is provided in the Appendix to this dissertation.

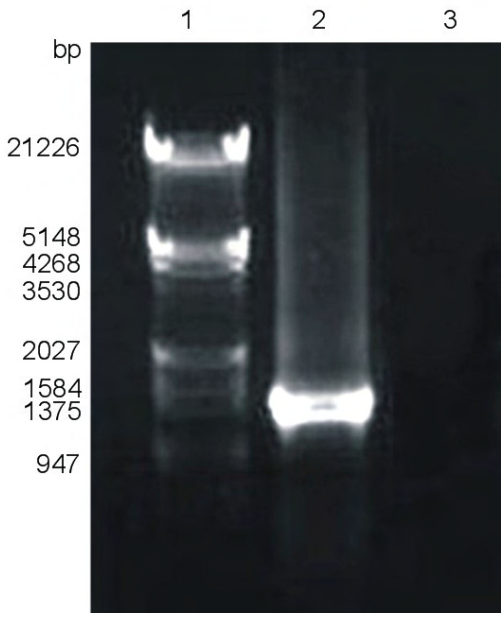


Fig. 2.1 Agarose gel electrophoresis of the amplicon obtained by PCR amplification using plasmid pSAT2 as template DNA. Lane 1, DNA molecular weight marker (λ DNA digested with *Hind*III and *Eco*RI); lane 2, sample of the reaction mixture following PCR; lane 3, sample of the negative control PCR reaction mixture, lacking template DNA.

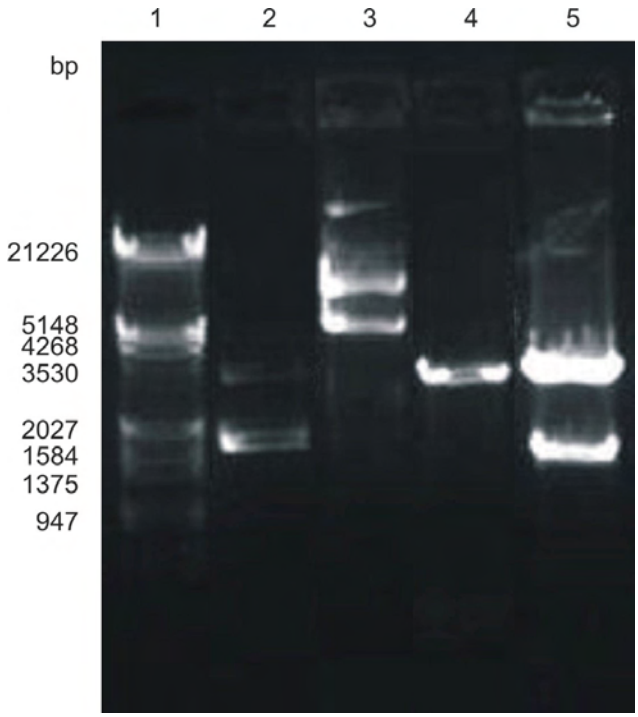


Fig. 2.2 Agarose gel electrophoresis of the recombinant pGEM-3ABC plasmid and non-recombinant pGEM®-T Easy vector DNA, following restriction enzyme digestion. Lane 1, Molecular weight marker (λ DNA digested with *Eco*RI and *Hind*III); lane 2, uncut pGEM®-T Easy plasmid DNA; lane 3, uncut recombinant pGEM-3ABC plasmid DNA; lane 4, pGEM®-T Easy vector DNA digested with *Eco*RI; lane 5, pGEM-3ABC plasmid DNA digested with *Eco*RI and *Bam*HI.

2.3.2.1 Comparative analysis of the 3A protein

Analysis of the deduced amino acid sequence of protein 3A led to the identification of a highly hydrophobic domain spanning amino acid residues 61 to 76, which contained a predicted transmembrane domain from residues 65 to 71 (Fig. 2.3). This is in agreement with results reported previously by Beard and Mason (2000). Sequence comparison of the transmembrane domain, which was present in all FMDV isolates included in this analysis, revealed a three-amino-acid difference (residues 65 to 67) of CLT in the euroasiatic types to VVV in the SAT types, except in SAT1/7ISRL/4/62 (VLA), SAT2/KEN/3/57 (CLA), and SAT2/RHO/1/48 (IVV) (Fig. 2.3).

Two major continuous epitope regions have been mapped in the 3A protein of O/HKN/14/82 (Sun *et al.,* 2004), A_{12} (Shen *et al.,* 1999) and O_1Kaufbeuren (Hölich *et al.,* 2003); one spanning amino acid residues 75 to 90 and a second general epitope region spanning amino acid residues 110 to 153 (Shen *et al.,* 1999; Hölich *et al*., 2003; Sun *et al*., 2004). Whereas the epitope region from amino acids 75 to 90 was relatively conserved, the second general epitope region, located in the C-terminal region of the 3A protein, displayed considerable variability between the SAT type viruses and the euroasiatic types (Fig. 2.3). The SAT type viruses, except for SAT2/KEN/3/57 (LETTGAS), SAT1/7ISRL/4/62 (LETTGTS), SAT3/4 BEC1/65 (QEAVDKP), SAT1/3 SWA1/49 (QEVIDKP) and SAT3/KNP/10/90 (QEVVGQT), contained the conserved amino acid sequence QEVVDKP (residues 114-120). Similarly, residues 134-137 (TDDE) were conserved for all SAT types, except in SAT2/KEN/3/57 (VSDD), SAT1/ISRL/4/62 (VDND), SAT3/3Kenya11/60 (TGNE) and SAT1/Rhod5/66 (TDND). A variable region spanning amino acids 143-149 in the C-terminal region of the 3A protein was identified. This region in the SAT type viruses (except SAT1/7ISRL/4/62) contained a single amino acid deletion corresponding to residue 148 of the euroasiatic types. Consequently, the 3A protein of most SAT types was 152 amino acids in length, whilst the 3A protein of the euroasiatic types was 153 amino acids in length (Fig. 2.3).

When all FMDV isolates used in this analysis were considered, an amino acid sequence identity of 50.3% for the 3A protein was obtained. However, when the euroasiatic types only were considered, the amino acid identity of protein 3A amounted to 73.2%. For the SAT types only, the amino acid sequence identity of protein 3A was 60.5%, but increased to 73.7% upon exclusion of SAT2/KEN/3/57 and SAT1/7ISRL/4/62 from the analysis.

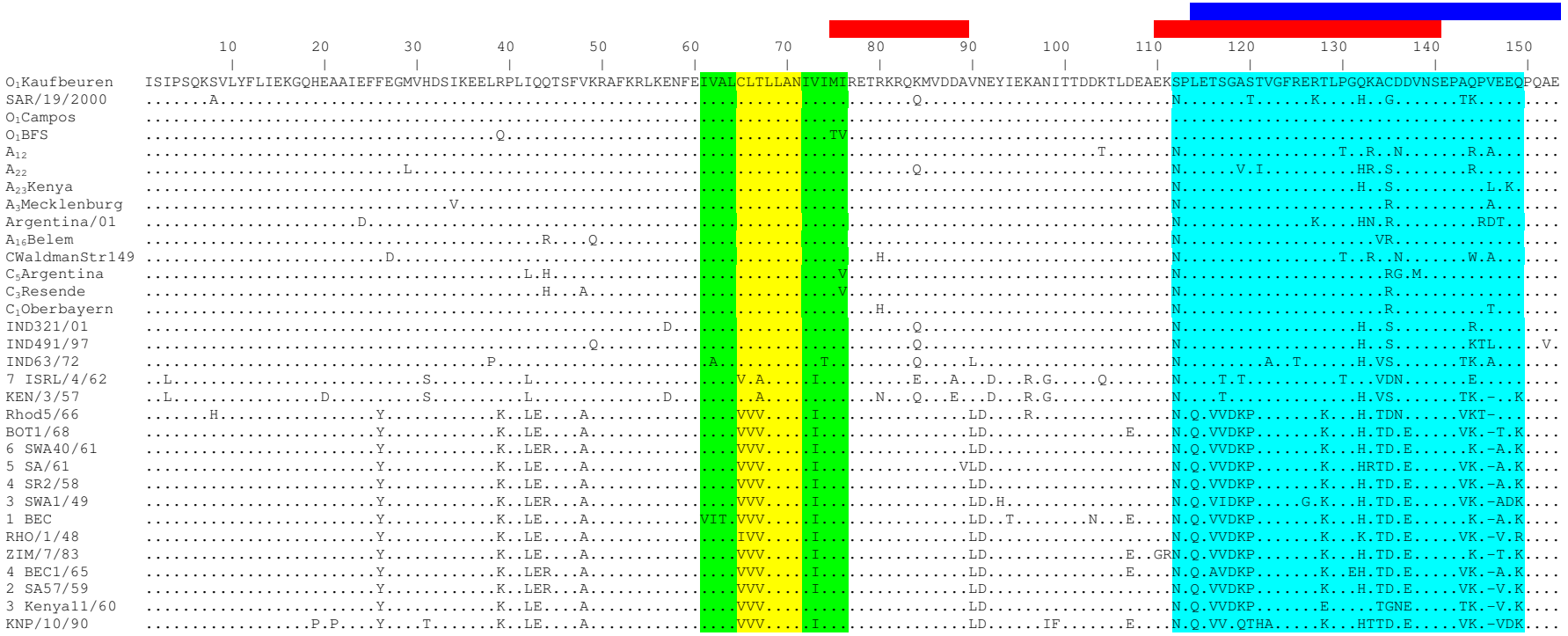


Fig. 2.3 Amino acid multiple sequence alignment of the 3A protein. Dots (.) indicate conserved amino acid residues. Dashes (-) indicate amino acid deletions. The highly hydrophobic domain is highlighted in green and the transmembrane domain is highlighted in yellow. Continuous epitopes on the 3A protein (Shen *et al.,* 1999) are indicated by red bars and a general epitope region (Shen *et al.,* 1999; Hölich *et al.,* 2003; Sun *et al.,* 2004) is indicated by a blue bar above the multiple sequence alignment. A region corresponding to a previously mapped general epitope region (amino acid residues 112 to 149) displays considerable hypervariability to the euroasiatic and SAT types (highlighted in light blue).

2.3.2.2 Comparative analysis of the 3B protein

All FMD viruses included in analysis contain three, non-identical tandem copies of the 3B gene, namely $3B_1$ (23 amino acids), $3B_2$ (24 amino acids) and $3B_3$ (24 amino acids). These tandem copies are illustrated in Fig. 2.4, together with their cleavage sites, as identified by Palmenberg (1990) and Robertson *et al*. (1985). The most variation was observed in the $3B_1$ and $3B_2$ isoforms, which comprised a general epitope region identified previously in O/HKN/14/82, A_{12} and O_1Kaufbeuren (Shen *et al*., 1999; Hölich *et al*., 2003; Sun *et al*., 2004) (Fig. 2.4). The amino acid sequence identity of the 3B protein of the SAT types was 69%, but increased to 81.7% upon exclusion of SAT2/KEN/3/57 and SAT1/7ISRL/4/62 from the analysis. The amino acid identity of protein 3B of the euroasiatic serotypes amounted to 83.1%. However, the overall amino acid identity of protein 3B amongst the SAT and euroasiatic type viruses was only 62%.

2.3.2.3 Comparative analysis of the 3C protein

Three epitope regions have been mapped in the 3C protein of FMDV isolate A_{12} and these span amino acid residues 1-18, 100-118 and 130-148 (Shen *et al.,* 1999). Although the overall 3C amino acid sequence was conserved between the SAT and euroasiatic FMDV isolates, the second epitope region (residues 100-118) displayed extensive variability. This region of the 3C protein contained a conserved motif (MKLSKGS) in the SAT type virus isolates (residues 107 to 113), except in SAT2/KEN/3/57 and SAT1/7ISRL/4/62, which contained an amino acid substitution at position 110 (R) (Fig. 2.5). Variability was also observed in the other two epitope regions, but not to the same extent as observed above. A transmembrane region spanning amino acids residues 27 to 39 was identified in the 3C protein of all FMDV isolates included in this analysis. All residues in the predicted transmembrane domain were invariant, except for a conservative amino acid substitution from I to L at position 30 in the SAT type viruses. The active site residues of the 3C protein (His_{46}-Asp_{84}-Cys_{163}) (Grubman *et al.,* 1995; Birtley *et al.,* 2005) were conserved in all FMDV isolates included in the analysis. The 3C protein displayed an overall amino acid sequence identity of 78.4%. Whereas the amino acid identity of the 3C protein was 83.1% for SAT types (96.2% if SAT2/KEN/3/57 and SAT1/7ISRL/4/62 was excluded from the analysis), the amino acid sequence identity of the 3C protein for euroasiatic types was 91.6%.

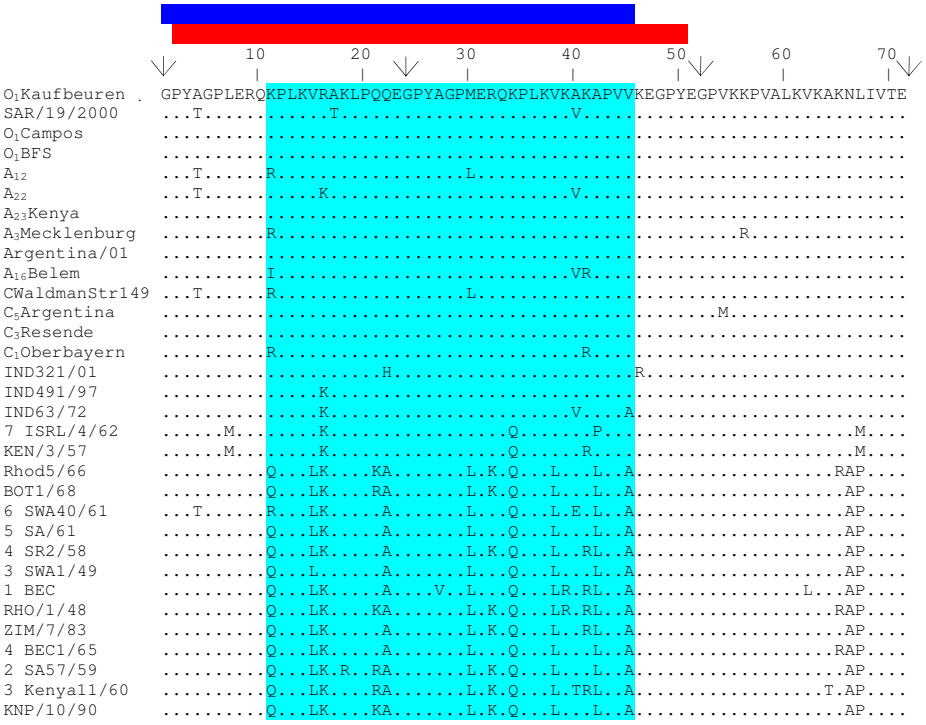
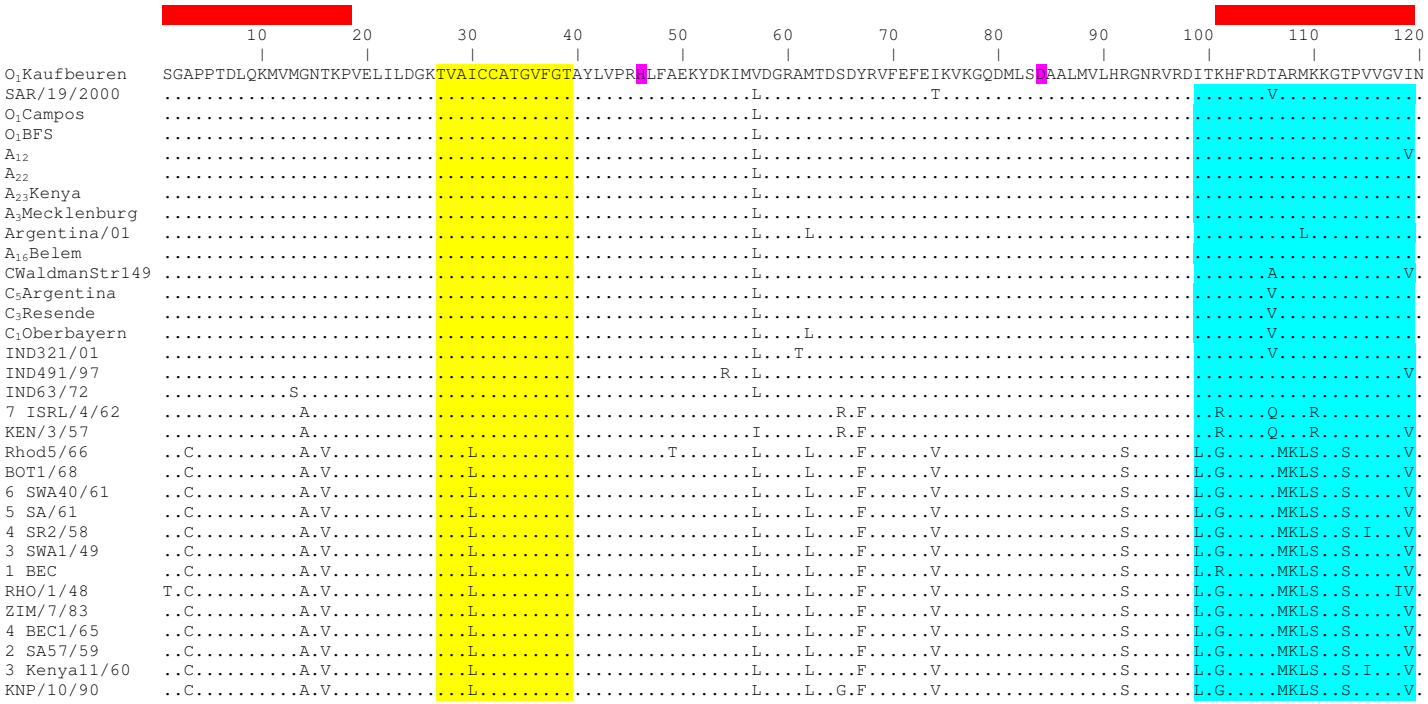


Fig. 2.4 Amino acid multiple sequence alignment of the 3B protein. Dots (.) indicate conserved amino acid residues. Cleavage sites between the 3B isoforms are indicated with arrows (Palmenberg, 1990). A continuous epitope on the 3B protein (Shen *et al.* 1999) is indicated by a red bar and a general epitope region (Shen *et al*., 1999; Hölich *et al*., 2003; Sun *et al*., 2004) is indicated by a blue bar above the multiple sequence alignment. A region from amino acid residues 11 to 45 (highlighted in light blue) displays considerable hypervariability between the SAT and euroasiatic types.


10 20 30 40 50 60 70 80 90 100 110 120
O1Kaufbeuren SGAPPTDLQKMVMGNTKPVELILDGKTVAICCATGVFGTAYLVPRHLFAEKYDKIMVDGRAMTDSDYRVFEFEIKVKGQDMLSDAALMVLHRGNRVRDITKHFRDTARMKKGTPVVGVIN
SAR/19/2000
O1Campos
O1BFS
A12
A22
A23Kenya
A3Mecklenburg
Argentina/01
A16Belem
CWaldmanStr149
C5Argentina
C3Resende
C1Oberbayern
IND321/01
IND491/97
IND63/72
7 ISRL/4/62
KEN/3/57
Rhod5/66
BOT1/68
6 SWA40/61
5 SA/61
4 SR2/58
3 SWA1/49
1 BEC
RHO/1/48
ZIM/7/83
4 BEC1/65
2 SA57/59
3 Kenya11/60
KNP/10/90

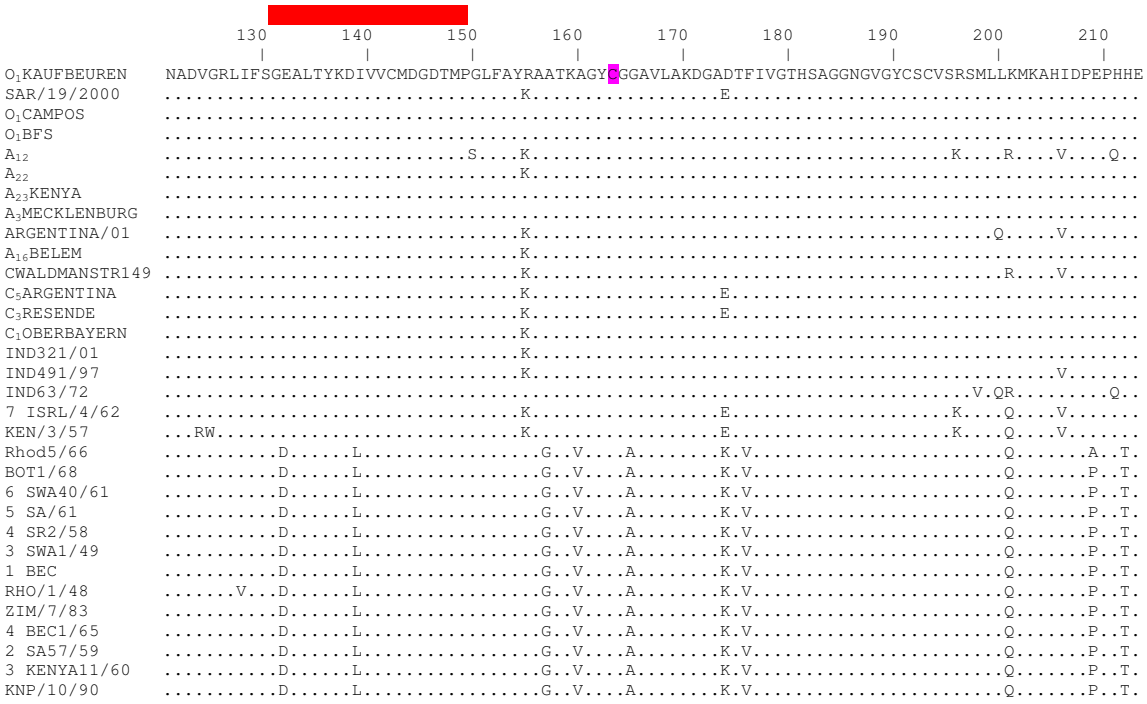


Fig. 2.5 Amino acid multiple sequence alignment of the 3C protein. Dots (.) indicate conserved amino acid residues. The transmembrane domain is highlighted in yellow. Continuous epitopes on the 3C protein, as identified by Shen *et al.* (1999), are indicated by red bars above the multiple sequence alignment. The conservation of the catalytic residues (His_{46}-Asp_{84}-Cys_{174}) in the isolates investigated is highlighted in pink (Birtley *et al.,* 2005). A region from amino acid residues 98 to 119 displays considerable variability and is highlighted in light blue.

2.3.2.4 Analysis of the complete 3ABC polypeptide of FMDV isolates

Euroasiatic and SAT type virus isolates were compared to determine their phylogenetic relationships with respect to the 3ABC polypeptide. A neighbour-joining tree was constructed using Equine Rhinitis A virus as the out-group (Fig. 2.6). The analysis revealed the presence of two distinct clades, each of which was supported by bootstrap values of 100%. The majority of the SAT type viruses grouped in clade A, whereas the euroasiatic viruses grouped together with two SAT types, SAT2/KEN/3/57 and SAT1/7ISRL/4/62, to form clade B (Fig. 2.6A). Grouping of the 3ABC polypeptide of the SAT2/KEN/3/57 and SAT1/7ISRL/4/62 viruses with euroasiatic type viruses is consistent with results presented by Heath *et al.* (2001) and by Van Rensburg *et al.* (2002) concerning the 3A and 3C proteins, respectively, of SAT2/KEN/3/57. In contrast, phylogenetic analysis based on the outer capsid 1D protein, which contains the major antigenic determinant, indicated grouping of the viruses strictly according to serotype (Fig. 2.6B), a result that has been described previously with 1D sequences (Palmenberg, 1989; Vosloo *et al.,* 1995; Bastos, 1998; Van Rensburg *et al.,* 2002; Carrillo *et al.,* 2005). Although 7ISRL/4/62 and KEN/3/57 grouped with SAT1 and with SAT2 type viruses, respectively, these isolates did not appear to be closely related to virus isolates within the respective serogroups. The amino acid sequence variation for the 3ABC polypeptide of euroasiatic types was 16.3% and that for SAT type viruses was 27.1%. However, when SAT2/KEN/3/57 and SAT1/7ISRL/4/62 were excluded from the analysis the amino acid sequence variation of the 3ABC polypeptide was 14.2%. When all FMDV isolates were considered the 3ABC amino acid variation was considerably higher at 34.1%.

2.4 DISCUSSION

The nucleotide sequence of the 3ABC-encoding region of a SAT2 type virus isolate, ZIM/7/83, was determined by automated sequencing and the amino acid sequence was deduced. The 3A, 3B and 3C amino acid sequence of SAT2/ZIM/7/83 and that of several other SAT virus isolates were compared to the corresponding sequences of several European and Asian type viruses. Although Asia1 outbreaks in livestock have not occurred on the African continent, three Asia1 isolates were nevertheless included in the analysis for comparative purposes. The 3A protein of the SAT type viruses, except that of SAT1/7ISRL/4/62, contained a single amino acid deletion in the C-terminus of the protein. Therefore, the 3A protein of SAT type viruses is 152 amino acids in length compared to the

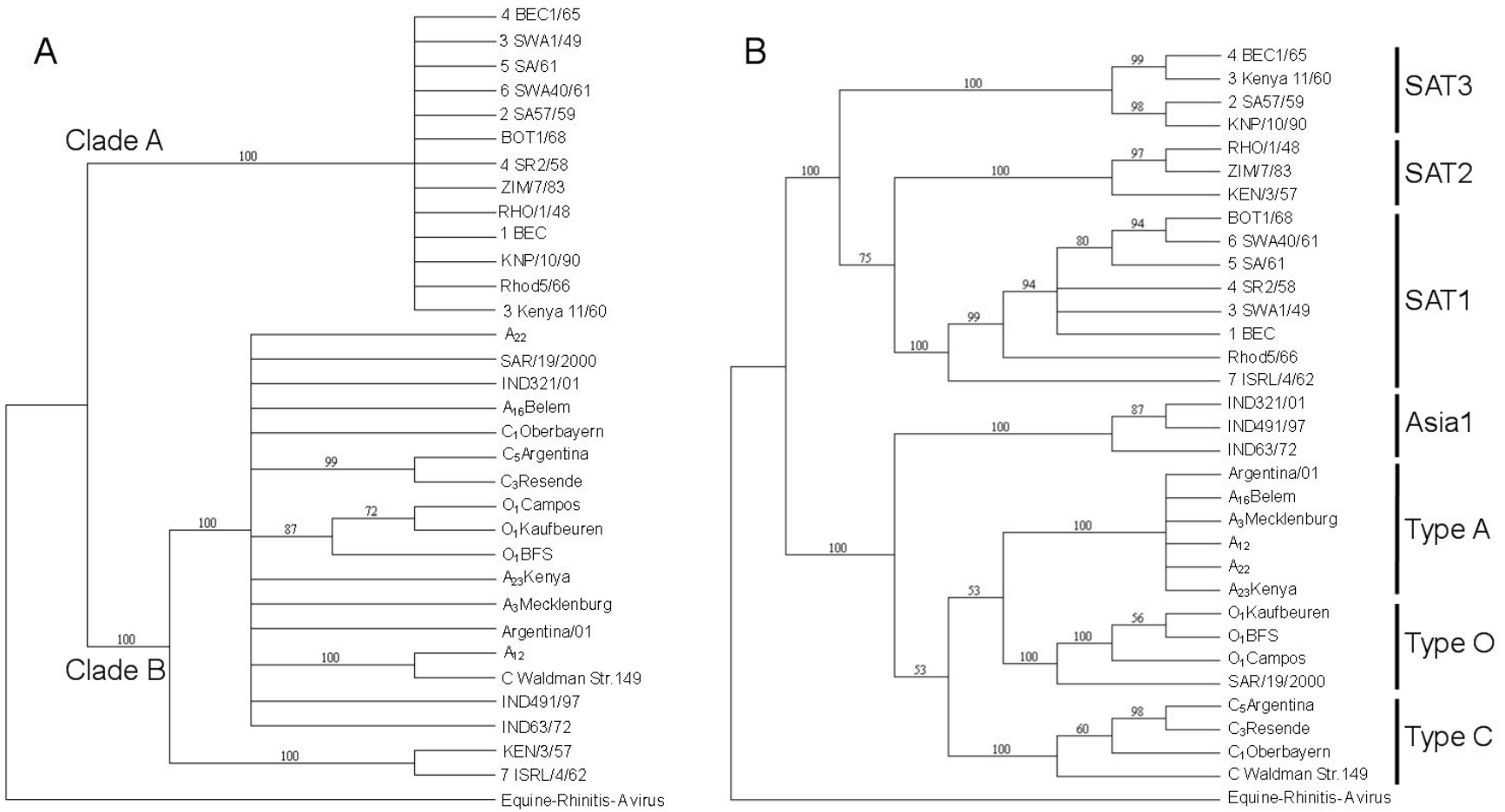


Fig. 2.6 Distance neighbour-joining tree constructed using the 3ABC amino acid sequence (A) and the 1D amino acid sequence (B) of different FMDV isolates, with Equine rhinitis A as the out-group. Bootstrap values are indicated on the branches of the respective phylogenetic trees. In (A) the clades are indicated. In (B) the different serotypes are indicated.

3A protein of euroasiatic type viruses that is 153 amino acids in length. These results are in agreement with those reported by Heath *et al.* (2001). Deletions in the 3A region have been implicated in host range specificity (Knowles *et al.,* 2001). However, the observed deletion has been identified in various other SAT type viruses isolated from not only domestic livestock species such as cattle, but also from African wildlife species such as buffalo and impala. These results would therefore suggest that the deletion does not play a role in host range specificity (Heath *et al.,* 2001).

The amino acid sequence alignments revealed considerable variability between the FMDV 3A, 3B and 3C amino acid sequences. The 3A protein was found to be the most variable (49.7% amino acid variation) and the 3C protein was the most conserved (21.6% amino acid variation). The results obtained in this part of the study correlates well with those reported previously, supporting the distinctiveness of the SAT type 3A and 3B proteins compared to the euroasiatic types (Heath *et al.,* 2001; Van Rensburg *et al.,* 2002; Carrillo *et al.,* 2005). The amino acid sequence variation of the 3ABC polypeptide amongst euroasiatic type viruses was 16.3% and 27.1% amongst the SAT type viruses. However, by excluding two SAT type viruses, SAT2/KEN/3/57 and SAT1/7IRSL/4/62, which upon phylogenetic analysis grouped more closely with the euroasiatic types, the amino acid sequence variation amongst the SAT type viruses decreased to 14.2%. For all FMDV isolates included in the analysis, amino acid sequence variation of the 3ABC polypeptide was 34.1%. The two SAT isolates, SAT2/KEN/3/57 and SAT1/7ISRL/4/62, could be, as suggested by Heath *et al.* (2001), the products of recombination events. Although previous reports regarding FMD viral recombination suggest that this is a ubiquitous event, occurring as a result of the high number of available recombination sites in the FMDV RNA genome (King *et al.,* 1985), only occasionally have intertypic (Krebs and Marquardt, 1992) and inter-regional recombination (Bastos *et al.,* 2003a) been documented. Therefore, it is possible that these virus isolates have evolved independently in different geographical regions.

The high level of amino acid sequence conservation of the 3C protein is a likely consequence of the structural constraints and conserved functionality of the 3C protease (Birtley *et al.,* 2005), which is responsible for most of the viral polyprotein proteolytic cleavage events and for cleavage of biochemically important host cell factors such as eIF-4G, eIF-4A (Belsham *et al.*, 2000) and histone protein H3 (Falk *et al.*, 1990). Despite the amino acid sequence conservation of the 3C protein, a variable region was identified that was located in a

previously identified epitope region. Interestingly, an epitope region on the 3C protein of serotype A_{12} was absent from the 3C protein of virus types O_1 Kaufbeuren and O/HKN/14/82 (Hölich *et al.*, 2003; Sun *et al.*, 2004). Furthermore, Rodríguez *et al.* (1994) found that FMDV convalescent sera of swine mostly recognized 3ABC, 3AB, 3A and 3B polypeptides. These results suggest that continuous epitopes on the 3C protein may therefore not be ideal candidates for diagnostic purposes (Sun *et al.,* 2004). A highly variable region spanning amino acids 114 of 3A to amino acid 45 of 3B was identified and is also localized in a previously identified epitope region (Shen *et al.*, 1999; Hölich *et al.*, 2003; Sun *et al.*, 2004). The epitope, located at positions 75-90 in 3A, occurs in a region that is deleted from several porcinophilic type O virus isolates (Knowles *et al.*, 2001; Hölich *et al.*, 2003). Note should be taken that the distribution patterns of epitopes on the 3ABC polypeptide among different virus strains may be similar but not identical (Shen *et al.*, 1999; Hölich *et al.*, 2003; Sun *et al.*, 2004). Therefore, the observed variability in these previously identified epitope regions may influence the sensitivity and specificity of diagnostic 3ABC-ELISAs to detect SAT type viruses.

In conclusion, the results presented in this Chapter indicate that the 3ABC polypeptide of the SAT FMDV types is distinct from the euroasiatic types and therefore validate the need for a SAT type-specific 3ABC ELISA assay. Variable regions are located especially in putative epitope-containing regions, which may considerably influence the probability of detecting infected animals using serological assays based on detection of antibodies to the 3ABC of European types O, A and C. Towards the development of such a diagnostic assay, sufficient amounts of a SAT type-specific 3ABC antigen would need to be produced. The use of two different expression systems for the expression of recombinant SAT2/ZIM/7/83 3ABC was subsequently investigated.

CHAPTER 3

EXPRESSION OF THE 3ABC NONSTRUCTURAL POLYPEPTIDE OF ZIM/7/83, A SAT2 TYPE FOOT-AND-MOUTH DISEASE VIRUS, IN *Escherichia coli*

3.1 INTRODUCTION

Detection of antibodies to the 3ABC polypeptide is considered to be the most reliable marker of FMDV-infection in vaccinated populations (Rodríguez *et al.,* 1994; De Diego *et al.,* 1997; Mackay *et al.,* 1998). Although commercially available 3ABC-ELISA kits have been developed for detection of antibodies to the 3ABC polypeptide of European (A, O and C) types, their sensitivity and specificity for the detection of antibodies to the 3ABC of SAT type FMD viruses is compromised (Mr J. Esterhuysen, personal communication). This may be due to the considerable amino acid sequence variation between SAT type and European type 3ABC polypeptides (Chapter 2). Therefore, the development of a SAT type-specific 3ABC ELISA is required for use in sub-Saharan Africa, where the SAT types prevail. Towards the development of such an assay, large quantities of a SAT type 3ABC antigen would be required. The availability of various different biological expression systems and molecular cloning techniques has made it possible to produce large quantities of proteins in heterologous hosts. In particular, prokaryotic expression systems have been used for the production of viral nonstructural proteins (Bergmann *et al*., 1993; Laviada *et al*., 1995; Malirat *et al*., 1998) and are less costly than their eukaryotic counterparts. Ideally, heterologous gene expression should result in high-level production of the recombinant protein in a soluble form that can be easily recovered and purified (Weickert *et al.,* 1996; Sørensen and Mortensen, 2005). Therefore, the expression host and choice of expression vector, in combination with the most suitable cellular site for heterologous protein production, should be considered carefully (Baneyx, 1999).

Secretion of heterologous proteins into the extracellular medium greatly simplifies protein purification, since there are comparatively fewer cellular proteins (Stander and Silhavy, 1990; Cornelis, 2000). Furthermore, the secreted proteins are more likely to be properly folded and soluble (Khushoo *et al.,* 2004). *Bacillus subtilis*, a gram-positive bacterium, has been used frequently for the production of heterologous proteins, because of its existing secretory pathways that allow export of recombinant proteins across a single cell membrane into the extracellular culture medium (Simonen and Palva, 1993; van Wely *et al.,* 2001). However, there are two main drawbacks with the *B. subtilis* expression system, namely plasmid instability (Haima *et al.,* 1987; Westers *et al.,* 2004) and low production yields of recombinant protein. The latter results from degradation of the recombinant protein by secreted host cell proteases (Simonen and Palva, 1993; Bolhuis *et al.,* 1999).

The gram-negative bacterium, *Escherichia coli,* is highly favoured for expression of heterologous proteins, because of the rapid generation of biomass as a result of high cell growth rates and its utilization of inexpensive carbon sources (Baneyx, 1999). For *E. coli*, the extracellular environment is relatively free of contaminating proteins and proteases, since *E. coli* secretes only a limited number of endogenous proteins into the extracellular environment (Khushoo *et al.,* 2004). However, secretion in *E. coli* is more complicated than in *Bacillus* spp., as translocation to the extracellular medium requires the passage of proteins through both the inner and the outer cell membranes (Choi and Lee, 2004). This is generally ineffective, unless the secreted protein is a naturally secreted bacterial exoprotein (Stander and Silhavy, 1990). Even when recombinant proteins are co-expressed with permeabilizing proteins, which require controlled low-level expression of the co-expressing gene, problems such as cell lysis, lethality and non-specific secretion may arise (Makrides, 1996; Jonasson *et al.,* 2002).

Alternatively, recombinant proteins can be targeted to the periplasmic space by fusing appropriate signal sequences to their N-termini (Kareem *et al.,* 1992; Holt and Raju, 2000). The oxidizing environment of the periplasm allows for accurate disulphide bond formation and the authentic N-terminus of the protein can be acquired, following removal of the signal sequence by signal peptidases (Jonasson *et al.,* 2002; Baneyx and Mujacic, 2004). Furthermore, recombinant proteins targeted to the periplasm can be purified with relative ease, since there are comparatively fewer cellular proteins in the periplasm than in the cytoplasm of *E. coli* (Jonasson *et al.,* 2002; Hanning and Makrides, 1998). However, problems encountered when targeting heterologous proteins to the periplasm often include inefficient export, overloading of the transport machinery and competition for processing of signal sequences (Makrides, 1996). These problems can result in degradation or aggregation of the pre-proteins in the cytoplasm or, alternatively, the pre-proteins can become lodged in the inner membrane (Baneyx, 1999). Consequently, heterologous proteins are typically expressed in the cytoplasm of *E. coli*. This is despite observations that over-expression of heterologous proteins in this cellular compartment often coincides with the target protein failing to adopt its native conformation and accumulating as insoluble aggregates, termed inclusion bodies (Villaverde and Carrió, 2003; Baneyx and Mujacic, 2004). In addition, there are many cytoplasmic proteases that may degrade the recombinant proteins (Swamy and Goldberg, 1981; 1982; Gottesman, 1996).

Besides selecting the most appropriate cellular location for production of the recombinant protein, the high-level synthesis of heterologous protein is also dependent on a number of factors. These factors include, the plasmid copy number (Nordstrộm and Uhlin, 1992), the growth conditions, the choice of antibiotic resistance that the plasmid confers (Makrides, 1996), the stability of the mRNA (Hayashi and Hayashi, 1985) and the secondary structure of the mRNA that can affect accessibility to the ribosome binding site (De Smit and van Duin, 1990). Transcription terminators are also important, since they prevent read-through transcription into vector sequences (Stueber and Bujard, 1982) and enhance mRNA stability, thereby increasing the level of protein synthesis (Hayashi and Hayashi, 1985). Furthermore, the choice of promoter that drives transcription of the heterologous gene is based on criteria such as strength, regulation, cost of the inducer and the conditions under which the promoter is to be used (Yansura and Henner, 1990; Makrides, 1996).

Based on the above, the pGEX expression system (Smith and Johnson, 1988) was selected for expression of the 3ABC-encoding region of ZIM/7/83, a SAT2 type FMDV. These vectors contain strong IPTG-inducible *tac* promoters for high level cytoplasmic synthesis of recombinant proteins, which typically accumulate up to 30% of the total cellular protein (de Boer *et al.,* 1983). Furthermore, the pGEX vectors contain the *lacI*^{q} allele and therefore expression of the GST-fusion proteins from the *tac* promoter is strongly repressed until IPTG induction (Smith and Johnson, 1988). Because the recombinant proteins are expressed as fusions with the C-terminus of the *Schistosoma japonicum* 27-kDa glutathione *S*-transferase (GST) protein, they can be easily purified from the bacterial crude cell lysates by glutathione affinity chromatography under non-denaturing conditions (Smith and Johnson, 1988).

Therefore, the aims of this part of the study were (i) to express the 3ABC-encoding region of ZIM/7/83 in *E. coli* as a recombinant GST-fusion protein and (ii) to purify the recombinant GST-3ABC protein to high homogeneity.

3.2 MATERIALS AND METHODS

3.2.1 Construction of a recombinant pGEX-2T bacterial expression vector

For the construction of a recombinant pGEX plasmid, the same molecular techniques, as described in Chapter 2, were used. The 3ABC-coding sequence was recovered from the pGEM-3ABC construct after digestion with *Bam*HI and *Eco*RI. The pGEX-2T vector (Pharmacia Amersham) was simultaneously digested with the same restriction endonucleases. The 1.3-kb 3ABC-encoding region and the digested pGEX-2T vector were purified from a 0.8% (w/v) agarose gel, using the modified method of Boyle and Lew (1995), and ligated for 16 h, using the same procedures as described in section 2.2.4.1, in a vector-to-insert ratio of 1:6. The ligation reactions were then transformed into competent *E. coli* DH5α cells and the transformation mixtures were plated onto LB agar containing ampicillin (100 µg/ml). The transformants were characterized, following plasmid extraction, by restriction endonuclease digestion. The nucleotide sequence of the cloned 3ABC insert DNA in recombinant plasmid pGEX-3ABC was determined using an ABI PRISM™ BigDye™ Terminator Sequencing Ready Reaction kit (PE Applied Biosystems). Oligonucleotides pGEX-F (5'-CCAGCAAGTATATAGCATGG-3') and pGEX-R (5'-CCGGGAGCTGCATGTGTCAGAGG-3') were used as sequencing oligonucleotides and the extension products were resolved on a model 377 automated sequencer (Perkin-Elmer).

3.2.2 Expression of the recombinant 3ABC protein in *E. coli*

The method of Smith and Johnson (1988), as described by Theron (1996), was used to induce expression of the GST fusion proteins. Overnight cultures of *E. coli* DH5α, containing the pGEX-3ABC and pGEX-2T plasmids, were prepared by inoculating single colonies into sterile test tubes containing 1 ml of ampicillin (100 µg/ml)-supplemented LB broth and incubated for 16 h at 37ºC with shaking (250 rpm). The cultures were diluted 1:10 in 1 ml of fresh medium and incubated at 37ºC for 90 min. Expression of the fusion proteins was then induced with the addition of IPTG to a final concentration of 0.5 mM, followed by incubation at 37ºC with shaking. Cells were harvested 3-4 h post-induction at 15 000 rpm for 1 min. The cell pellets were suspended in 200 µl 2 × Protein Sample Buffer (PSB: 125 mM Tris [pH 6.8]; 4% [w/v] SDS; 20% [v/v] glycerol; 10% [v/v] 2-mercaptoethanol; 0.002% [w/v] bromo-phenol blue), heated to 95ºC for 5 min, vortexed for 2 min and sonicated for 15 min. The whole-cell lysates were analyzed by SDS-PAGE analysis.

3.2.3 Purification of the recombinant GST-3ABC protein by glutathione affinity chromatography

Expression of the GST-3ABC fusion protein was induced, as described above, except that culture volumes were increased to 10 ml. Cells from the 10-ml cultures were pelleted by centrifugation at 8 000 rpm for 5 min and lysed at room temperature for 15 min in 1 ml lysis buffer (20% [w/v] sucrose; 100 mM Tris-HCl [pH 8.0]; 10 mM EDTA) containing 8.4 µl lysozyme (5 mg/ml). The lysates were placed on ice for 30 min, after which DTT was added to 10 mM, TritonX-100 and Tween-20 to 1% (v/v) and PBS to a final concentration of 1× (13.7 mM NaCl; 0.27 mM KCl; 0.43 mM $Na_2HPO_4.7H_2O$; 0.14 mM KH_2PO_4; pH 7.3). Incubation on ice was continued for an additional 90 min (Theron, 1994). Alternatively, DTT was substituted with 1.5% (v/v) *N*-lauryl sarcosine (Sigma-Aldrich) dissolved in 0.15 M STE (150 mM NaCl; 10 mM Tris-HCl [pH 7.4]; 1 mM EDTA) (Burgess, 1996; Clavijo *et al.*, 2004b). Soluble cytoplasmic (supernatant) and insoluble particulate (pellet) fractions were obtained following centrifugation at 15 000 rpm for 15 min and stored at -20°C until needed.

Purification of the recombinant GST-3ABC protein from the soluble fraction was performed by glutathione agarose (Sigma-Aldrich) affinity chromatography (Smith and Johnson, 1988). For the insoluble fraction, pellets were extracted sequentially with 550 µl 1 M urea and 550 µl 7 M urea, each for 30 min at 37ºC (Strebel *et al.*, 1986b; Mackay *et al.*, 1998). The 7-M urea fraction was further purified by glutathione affinity chromatography, as described below. The glutathione beads were pre-swollen in MTPBS buffer (150 mM NaCl; 16 mM Na_2HPO_4; 4mM NaH_2PO_4; pH 7.3), washed twice and stored in MTPBS at 4°C as a 50% (v/v) slurry. Fusion proteins were allowed to adsorb to the glutathione agarose beads by mixing the cleared supernatant or urea-treated fractions (500 µl) with 125 µl of glutathione agarose beads and incubating at room temperature for 45 min with gentle agitation. Glutathione agarose beads were pelleted by centrifugation at 2 000 rpm for 1 min and washed three times each with 1 ml MTPBS. Bound fusion proteins were eluted from the glutathione agarose beads with 500 µl of freshly prepared reduced glutathione solution (50 mM Tris-HCl [pH 8.0]; 5 mM reduced glutathione; pH 7.5). Following centrifugation (2 000 rpm, 1 min), an aliquot of the fusion protein-containing supernatant was analyzed by SDS-PAGE.

3.2.4 Purification of the recombinant GST-3ABC protein by elution from reverse-stained SDS-polyacrylamide gels

3.2.4.1 Preparation and electrophoresis of protein samples

The cells from induced cultures were pelleted at 2 000 rpm for 10 min and suspended in 5 ml TBS buffer (50 mM Tris-HCl [pH 7.5]; 100 mM NaCl; 0.1% [v/v] TritonX-100). The cells were lysed by a freeze-thaw process, homogenized by 30 strokes with a Dounce homogenizer (Wheaton) and sonicated for 15 min. The lysates were centrifuged at 10 000 rpm for 20 min, and the resulting pellets suspended in 500 µl of 2 × PSB and boiled for 5 min. Sample volumes of 200 µl were loaded onto 8% SDS-polyacrylamide gels and electrophoresed in a Hoefer Sturdier™ electrophoresis unit at 90 V for 16 h. To visualize the proteins, the SDS-polyacrylamide gels were reverse-stained according to the method of Fernandez-Patron *et al.* (1995). The gels were rinsed with dH_2O for 1 min, soaked in 0.2 M imidazole containing 0.1% (w/v) SDS for 15 min and then immersed in 100 ml 0.2 M ZnS until the gel background turned white, leaving unstained transparent protein bands. Staining was stopped by rinsing the gel in dH_2O and the GST-3ABC protein band was excised from the stained gel using a clean scalpel blade.

3.2.4.2 Elution of the recombinant GST-3ABC protein

Elution of the GST-3ABC protein from the reverse-stained SDS-polyacrylamide gel slice was performed according to the method of Szewczyk and Summers (1988). First, the gel slice was soaked in protein mobilization buffer (25 mM Tris-HCl [pH 8.3]; 192 mM glycine) until the band became translucent and then macerated using an ULTRA-TURRAX T25 homogenizer (Janke and Kunkel IKA-Labortechnik). Then, 0.5 ml elution buffer (50 mM Tris [pH 9.0]; 1% [v/v] TritonX-100; 2% [w/v] SDS) per cm^2 of the gel was added and the proteins were allowed to elute at 37°C for 2 h with shaking. The polyacrylamide gel pieces were removed from the suspension by brief centrifugation at 12 000 rpm. The protein-containing supernatant was retained and the proteins precipitated at -70ºC for 1 h with 4 volumes of ice-cold acetone (Sambrook *et al.,* 1989). Following precipitation, the proteins were collected by centrifugation at 15 000 rpm for 20 min at 4ºC and the acetone discarded. The proteins were briefly air-dried and suspended in 2 × PSB for SDS-PAGE and Western blot analysis.

3.2.4.3 Protein concentration determination

The concentration of the purified protein was quantified by the Bradford method using a commercial kit (Pierce Coomassie Plus) and bovine serum albumin (BSA) was used to generate a standard curve (Bradford, 1976). Recombinant GST-3ABC protein, eluted from six SDS-polyacrylamide gels, was dissolved in 1 ml of 1 × PBS at a concentration of at least 3 µg/µl.

3.2.5 SDS-PAGE analysis

SDS-PAGE was performed according to the discontinuous buffer system of Laemmli (1970) using a 5% stacking gel and a 10% resolving gel. The 5% stacking gel (5% [w/v] acrylamide; 0.17% [w/v] bis-acrylamide; 125 mM Tris-HCl; 0.1% [w/v] SDS; pH 6.8) and the 10% resolving gel (10% [w/v] acrylamide; 0.34% [w/v] bis-acrylamide; 0.375 mM Tris-HCl; 0.1% [w/v] SDS; pH 8.8) were polymerized with 0.08% (w/v) ammonium persulfate and 10 µl TEMED. Protein samples were loaded onto the SDS-polyacrylamide gel and electrophoresed in a Hoefer Mighty Small™ electrophoresis unit at 120 V for 2.5 h in 1 × TGS (0.025 M Tris [pH 8.3]; 0.192 M glycine; 0.1% [w/v] SDS). The gels were immersed in Coomassie Staining Solution (0.125% [w/v] Coomassie Brilliant Blue; 50% [v/v] methanol; 10% [v/v] glacial acetic acid) for 20 min at room temperature and then soaked in a 5% (v/v) acetic acid and 5% (v/v) methanol solution, until the proteins were visible. The sizes of the resolved proteins were estimated by comparison to reference marker proteins (BioRad).

3.2.6 Western blot analysis

For Western blot analysis, proteins resolved by SDS-PAGE were electrotransferred from the unstained SDS-polyacrylamide gel onto a Hybond™-C$^+$ nitrocellulose membrane (Amersham Bioscience) of equal size (Winston *et al.,* 1988). This was performed by pre-soaking both the SDS-polyacrylamide gel and the membrane for 30 min in transfer buffer (120 mM Tris-HCl; 40 mM glycine; pH 8.3), followed by assembly of the blotting apparatus and electrotransfer at 100 mA and 24 V for 2 h in a Mighty Small™ Transphor Electrophoresis unit (Hoefer) in the presence of transfer buffer. After electrotransfer, the membrane was rinsed for 5 min in 1 × PBS. Non-specific binding of antibodies to the membrane was blocked by incubating the membrane in blocking buffer (1 × PBS containing 1% [w/v] fat-free milk powder) for 30 min at room temperature or at 4ºC overnight. The membrane was transferred to blocking buffer

containing the diluted antibodies. These comprised a polyclonal anti-GST antibody (Santa Cruz Biotechnology, Inc.) diluted 1:1000, or a FMDV-specific antiserum (SAT3/KNP/10/90; kindly provided by Mr J. Esterhuysen, OVI) diluted 1:5. The membrane was incubated in the primary antibody solution for 2 h at room temperature with gentle shaking and then washed three times for 5 min each in wash buffer (1 × PBS containing 0.05% [v/v] Tween-20). The membrane was transferred to the secondary antibody solution, namely protein A conjugated to horseradish peroxidase diluted 1:1000 in fresh blocking solution, and incubated with gentle shaking at room temperature for 1 h. Following incubation, membranes were washed three times for 5 min each in wash buffer and once for 5 min in 1 × PBS. The membrane was then placed in an enzyme substrate solution (60 mg of 4-chloro-1-naphtol dissolved in 20 ml ice-cold methanol, mixed just prior to use with 100 ml of 1 × PBS and 60 µl H_2O_2) to visualize the immunoreactive proteins. Thereafter, the membrane was rinsed with water and air-dried.

3.3 RESULTS

3.3.1 Construction of recombinant plasmid pGEX-3ABC containing the 3ABC-encoding region of SAT2/ZIM/7/83

To obtain high amounts of a SAT type 3ABC polypeptide that could be purified from crude *E. coli* cell lysates, the 3ABC-encoding sequence of the SAT2/ZIM/7/83 virus isolate was cloned into a pGEX expression vector. The pGEX expression system consists of three expression vectors to allow for correct in-frame synthesis of a recombinant protein as a GST fusion protein in one of the three reading frames (Smith and Johnson, 1988). For translation of the 3ABC polypeptide in the correct reading frame, the pGEX-2T expression vector was selected. To construct the recombinant pGEX-3ABC plasmid (Fig. 3.1a), the pGEM-3ABC construct (Chapter 2) and the pGEX-2T vector DNA were both digested with *Eco*RI and *Bam*HI to allow for directional cloning. The 1.3-kb DNA fragment obtained from digestion of the pGEM-3ABC plasmid and the 4.9-kb digested pGEX-2T vector DNA were purified from the agarose gel, ligated and transformed into competent *E. coli* DH5α cells. Plasmid DNA extracted from ampicillin-resistant transformants were subsequently characterized by agarose gel electrophoresis and by restriction enzyme digestion.

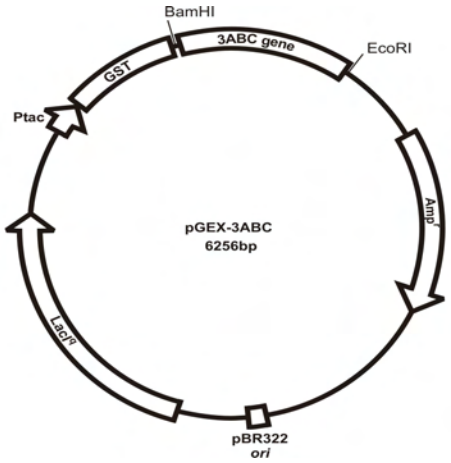


Fig. 3.1a Recombinant pGEX-2T prokaryotic expression vector containing the cloned 3ABC-encoding region of SAT2/ZIM/7/83.

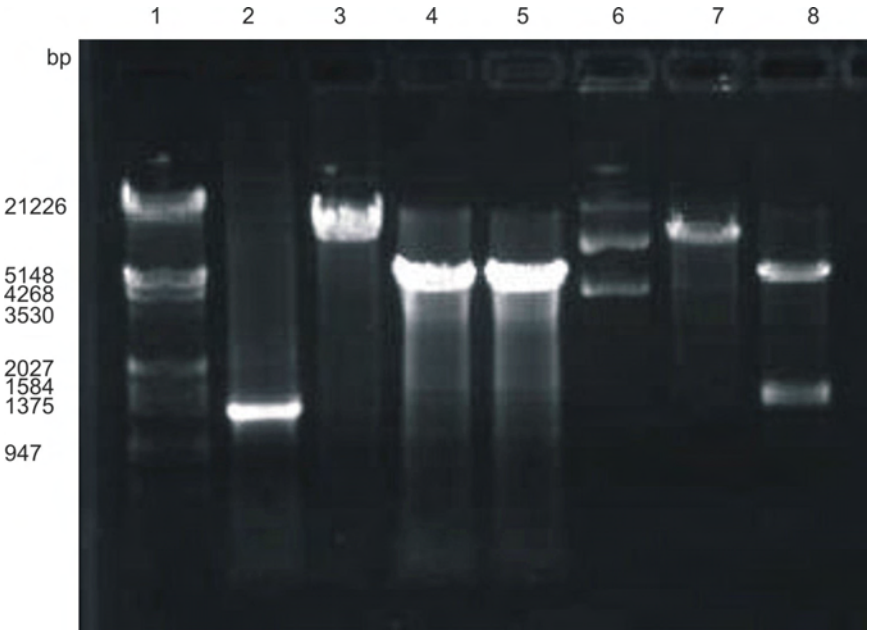


Fig. 3.1b Agarose gel electrophoresis of the recombinant pGEX-3ABC plasmid and the pGEX-2T parental vector DNA, following restriction enzyme digestion. Lane 1, Molecular weight marker (λ DNA digested with *Eco*RI and *Hind*III); lane 2, 1.3-kb 3ABC amplicon; lane 3, uncut parental pGEX-2T vector DNA; lane 4, pGEX-2T vector DNA digested with *Eco*RI; lane 5, pGEX-2T vector DNA digested with *Eco*RI and *Bam*HI; lane 6, uncut recombinant pGEX-3ABC plasmid DNA; lane 7, recombinant pGEX-3ABC plasmid DNA digested with *Eco*RI; lane 8, recombinant plasmid pGEX-3ABC digested with *Eco*RI and *Bam*HI.

Plasmid DNA migrating slower than the parental pGEX-2T vector DNA on agarose gels were selected (Fig. 3.1b, lanes 3 and 6) and investigated for the presence of a cloned insert DNA by restriction enzyme digestion with *Eco*RI. Agarose gel electrophoresis revealed that digestion of the non-recombinant plasmid DNA with *Eco*RI yielded a DNA fragment of 4.9 kb (Fig. 3.1b, lane 4), but digestion of the recombinant plasmid DNA yielded a 6.2-kb DNA fragment (Fig. 3.1b, lane 7). This corresponds to the size of the pGEX-2T vector (4.9 kb) together with the 3ABC-encoding region (1.3 kb). To further verify the presence of the cloned 3ABC-encoding region, the recombinant and parental pGEX-2T plasmids were digested with both *Eco*RI and *Bam*HI, which flank the cloned DNA insert in the multiple cloning site of the pGEX-2T vector. When parental pGEX-2T plasmid DNA was digested, a DNA fragment of 4.9 kb was obtained (Fig. 3.1b, lane 5), whereas digestion of the recombinant plasmid DNA yielded not only the vector fragment, but also a 1.3-kb DNA fragment (Fig. 3.1b, lane 8). A recombinant clone was selected (pGEX-3ABC) and the cloned 3ABC insert DNA sequenced using pGEX-specific sequencing oligonucleotides. Sequence analysis revealed that no mutations had been introduced into the 3ABC sequence when compared to the corresponding sequence obtained previously in Chapter 2 (results not shown).

3.3.2 Expression of the recombinant GST-3ABC fusion protein

For expression of the GST fusion proteins, overnight cultures of *E. coli* DH5α cells harbouring the non-recombinant pGEX-2T or the recombinant pGEX-3ABC plasmid were diluted in fresh medium and grown in the presence of IPTG to induce high-level protein expression. The cells were harvested and cell lysates were then prepared and analyzed by SDS-PAGE (Fig. 3.2). Analysis of the Coomassie blue-stained SDS-polyacrylamide gel indicated the presence of a 27-kDa protein in the lysate prepared from cells containing the non-recombinant pGEX-2T plasmid. In contrast to the cell lysates prepared from non-recombinant and untransformed cells, a unique over-expressed 74-kDa protein was detected in the cell lysate prepared from cells containing the recombinant pGEX-3ABC plasmid. The size of the 74-kDa protein is in agreement with that calculated for the GST-3ABC fusion protein (47 kDa for the 3ABC polypeptide and 27 kDa for the GST protein).

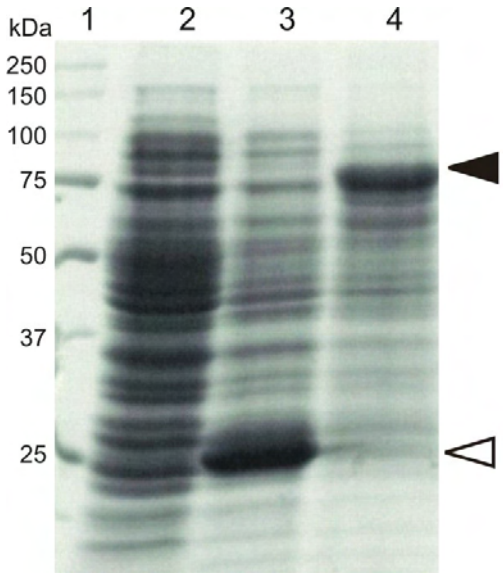


Fig. 3.2 SDS-PAGE analysis, of *E. coli* DH5α cell lysates prepared from cells expressing the recombinant GST-3ABC fusion protein (lane 4) and GST protein (lane 3). As a control, IPTG-induced *E. coli* DH5α cells (lane 2) were included. The recombinant GST-3ABC fusion protein is indicated with a solid arrow, whereas the GST protein is indicated with an open arrow. The sizes of the protein molecular weight markers (lane 1) are indicated (in kDa) to the left.

To determine if the recombinant GST-3ABC protein was present in a soluble form, an attempt was made to purify the GST-3ABC protein from the crude cell lysates by glutathione affinity chromatography. The soluble GST protein was used as a control. The purity and yield of the purified fusion proteins were assessed by SDS-PAGE (Fig. 3.3). Analysis of the Coomassie blue-stained gel indicated that the GST-3ABC and GST proteins were expressed to high levels in the bacterial cells (Fig. 3.3, lanes 2 and 6, respectively). However, although the GST protein was purified to near homogeneity (Fig. 3.3, lane 8), the GST-3ABC protein could not be purified with glutathione affinity chromatography under the conditions used. Furthermore, the GST-3ABC protein was located in the particulate fraction of the fractionated cell lysates, which may suggest that the protein accumulated in an insoluble form within the *E. coli* host (Fig. 3.3, lane 5).

3.3.3 Attempts to obtain soluble recombinant GST-3ABC protein

High-level expression of heterologous proteins in the *E. coli* cytoplasm often results in the accumulation of large quantities of incorrectly folded insoluble heterologous proteins, which tend to aggregate in what is termed inclusion bodies (Krueger *et al.*, 1989; Villaverde and Carrió, 2003). However, several approaches have been reported that focus on reducing the rate of protein synthesis, thus enabling more accurate folding of the heterologous proteins *in vivo* and consequently, improving the yield of soluble heterologous protein. These approaches include, amongst other, growth of the cultures at a reduced temperature (Schirano and Shibata, 1990; Vasina and Baneyx, 1997), altering the pH of the culture medium (Sugimoto *et al.*, 1991) and reducing the concentration of the chemical inducer (Bentley and Kompala, 1990). The afore-mentioned approaches were therefore used in an attempt to obtain soluble GST-3ABC recombinant protein *in vivo*. In this study, the incubation temperature was lowered from 37^{o}C to 20^{o}C, 15 min prior to addition of IPTG and then maintained at 20^{o}C for the duration of culturing; the pH of the culture medium was adjusted from pH 7.4 to pH 9.0 by addition of NaOH; and the concentration of the chemical inducer, IPTG, was varied (100 µM to 0.5 mM). None of these approaches or any combination thereof yielded soluble GST-3ABC (results not shown).

As an alternative to obtaining soluble protein *in vivo*, it was attempted to purify the GST-3ABC protein from the insoluble inclusion bodies, since they can be readily purified and the proteins can then be solubilized *in vitro* (Rudolph and Lilie, 1996). Insoluble inclusion bodies

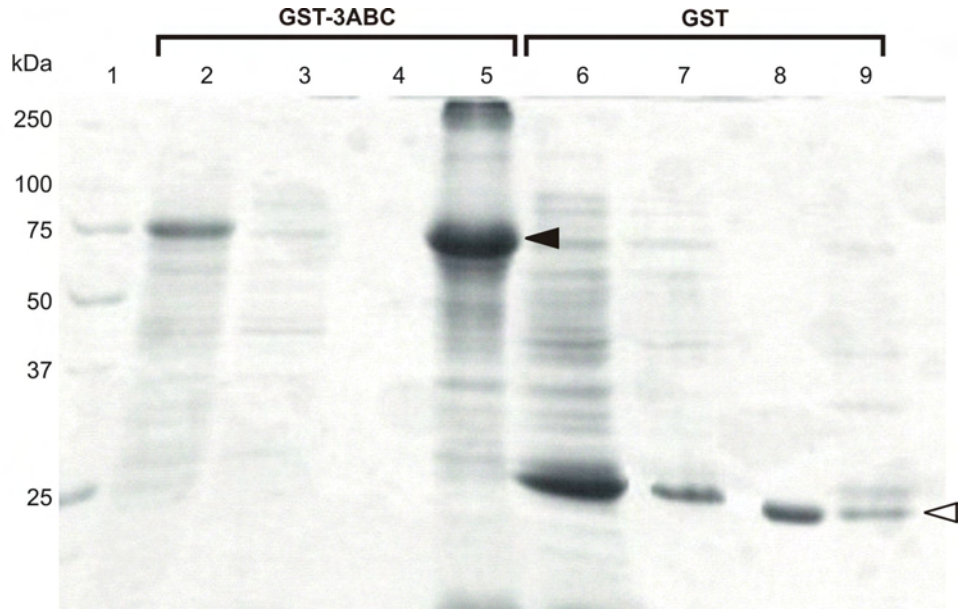


Fig. 3.3 SDS-PAGE analysis following expression and purification of the GST-3ABC and GST proteins. Proteins in the different fractions obtained during the glutathione affinity chromatography purification procedure were resolved on a 10% SDS-polyacrylamide gel. Lane 1, protein molecular weight marker (sizes indicated in kDa); lanes 2 and 6, cell lysates of the IPTG-induced *E. coli* DH5α cultures; lanes 3 and 7, cytoplasmic (supernatant) fractions obtained following cell lysis and centrifugation to pellet cellular debris; lanes 4 and 8, purified proteins obtained following elution from the glutathione agarose; lanes 5 and 9, particulate (pellet) fraction of cell extracts from induced cultures. The GST-3ABC fusion proteins are indicated with a closed arrow and the GST protein with an open arrow.

are frequently solubilized with strong detergents such as guanidium hydrochloride and urea or with organic solvents, alkaline pH or with *N*-lauryl sarcosine (Fischer *et al.,* 1992; Umetsu *et al.,* 2004; Wang *et al.,* 2005). Consequently, to solubilize the over-expressed GST-3ABC protein the usefulness of urea and *N*-lauryl sarcosine was investigated, since soluble FMDV O_1Campos (Clavijo *et al.,* 2004b) and O_1Kaufbeuren (De Diego *et al.,* 1997) 3ABC proteins have been obtained previously using these solubilizing reagents. The results that were obtained indicated that the recombinant GST-3ABC protein could not be solubilized in 1 M urea (Fig. 3.4, lane 4), but it could be solubilized in 7 M urea (Fig. 3.4, lane 5). However, the solubilized recombinant GST-3ABC protein could not be purified using glutathione agarose affinity chromatography (Fig. 3.4, lane 6). This may be as a result of the urea interfering with binding of the GST fusion partner to the glutathione agarose matrix. This was confirmed in a control experiment in which GST, also solubilized in 7 M urea, was not able to bind to the glutathione agarose matrix (results not shown).

3.3.4 Purification of the recombinant GST-3ABC protein by elution from reverse-stained SDS-polyacrylamide gels

Since glutathione agarose affinity chromatography was ineffective for purification of the recombinant GST-3ABC protein, a different strategy was subsequently investigated. To obtain highly purified GST-3ABC, the recombinant protein was purified directly from reverse-stained SDS-polyacrylamide gels. Reverse staining immobilizes the proteins in the gel by their association to zinc ions and the addition of imidazole causes a reaction with the unbound Zn^{2+}, resulting in a white precipitate on the gel background but leaving the resolved protein bands unstained (Fernandez-Patron *et al.*, 1992; Castellanos-Serra *et al.*, 1996). Using this technique, proteins can be renatured in the gel if the recovery of biologically active proteins is required (Hardy *et al.*, 1996).

The GST-3ABC protein was eluted from SDS-polyacrylamide gel slices and concentrated by acetone precipitation prior to analysis by SDS-PAGE (Fig. 3.5). Analysis of the Coomassie-stained gel indicated that no proteolytic degradation of the recombinant GST-3ABC protein had occurred, as was evidenced by the detection of a single protein band. The size of the protein was in agreement with that expected for the GST-3ABC fusion protein (74 kDa). The identity of the purified GST-3ABC protein was confirmed by Western blot analysis. The proteins from unstained SDS-polyacrylamide gels were electroblotted onto nitrocellulose membranes and the GST-3ABC recombinant protein was detected using a polyclonal anti-

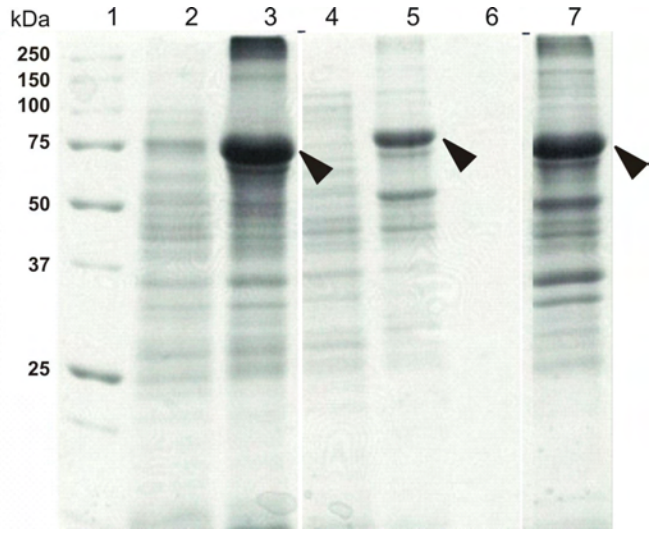


Fig. 3.4 SDS-PAGE of glutathione affinity chromatography-purified recombinant GST-3ABC fusion protein, following solubilization of the recombinant protein with urea. Lane 1, protein molecular weight marker (sizes indicated in kDa); lane 2, *E. coli* DH5α whole-cell lysate; lane 3, whole-cell lysate of induced cells that contained the pGEX-3ABC plasmid; lane 4, 1 M urea eluate; lane 5, 7 M urea eluate; lane 6, protein eluted following glutathione affinity chromatography from a 7 M urea eluate; lane 7, unbound protein fraction following glutathione agarose purification of the urea-solubilized GST-3ABC fusion protein. The recombinant GST-3ABC fusion protein is indicated with a closed arrow.

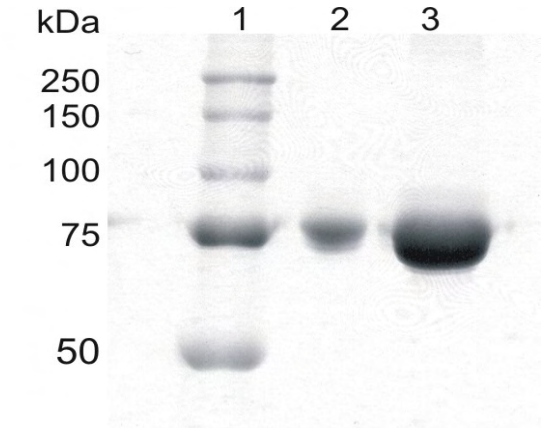


Fig. 3.5 SDS-PAGE analysis of the recombinant GST-3ABC fusion protein eluted from reverse-stained SDS-polyacrylamide gels. Lane 1, protein molecular weight marker (sizes indicated in kDa); lane 2, 1 µl of purified recombinant protein (representing 3 µg); lane 3, 5 µl of purified recombinant protein (representing 15 µg). The concentration of the purified GST-3ABC protein was determined using the Bradford assay, as described under Materials and Methods.

GST antibody and FMDV-specific antiserum. Western blot analysis performed using the anti-GST antibody (Fig. 3.6a) indicated that the antibody reacted with a 74-kDa protein in the cell lysate of IPTG-induced *E. coli* DH5α cells containing the recombinant pGEX-3ABC plasmid and with the 74-kDa protein eluted from reverse-stained gels, as well as with the 27-kDa GST protein. Western blot analysis performed using the FMDV-specific serum (Fig. 3.6b) indicated that the serum reacted only with the 74-kDa GST-3ABC protein and not with the 27-kDa GST protein. Taken together, these results therefore confirmed that the 3ABC polypeptide of ZIM/7/83 was expressed in *E. coli* and that recombinant GST-3ABC can be purified to near homogeneity from reverse-stained SDS-polyacrylamide gels.

3.4 DISCUSSION

Through advances in biotechnology the production of large quantities of desired protein products in biological systems has been made possible. Although there are a multitude of expression hosts to choose from, *E. coli* has remained one of the most popular and cost-effective expression hosts to date (Baneyx, 1999). Different FMDV nonstructural proteins, namely 2C, 3AB, 3ABC and 3D, have previously been expressed successfully in *E. coli* (Bergmann *et al.*, 1993; Rodríguez *et al.*, 1994; O'Donnel *et al.,* 1996; De Diego *et al.*, 1997; Mackay *et al.*, 1998; Clavijo *et al.*, 2004b). Consequently, *E. coli* was used as expression host for production of the 3ABC polypeptide of a SAT type FMD virus, SAT2/ZIM/7/83. However, previous reports have indicated that antibodies to *E. coli*, which are present in animal serum, may cause false positive reactions in an ELISA (De Diego *et al.,* 1997; Meyer *et al.,* 1997). Since it would therefore be necessary to purify the recombinant 3ABC polypeptide from the bacterial cell lysates, the 3ABC polypeptide was expressed as a fusion protein with GST using the pGEX expression system (Smith and Johnson, 1988). This system allows recombinant fusion proteins to be purified from crude cell lysates to homogeneity by affinity chromatography under non-denaturing conditions (Smith and Johnson, 1988; Theron *et al.*, 1996).

The 3ABC-encoding region of SAT2/ZIM/7/83 was cloned into the pGEX-2T expression vector and crude cell lysates of IPTG-induced recombinant *E. coli* DH5α cells were analyzed by SDS-PAGE. The results indicated that a protein of 74 kDa was expressed to a high level and its identity was verified by Western blot analysis using an anti-GST antibody and FMDV-specific antiserum. Since no other immunoreactive proteins were detected, it would suggest

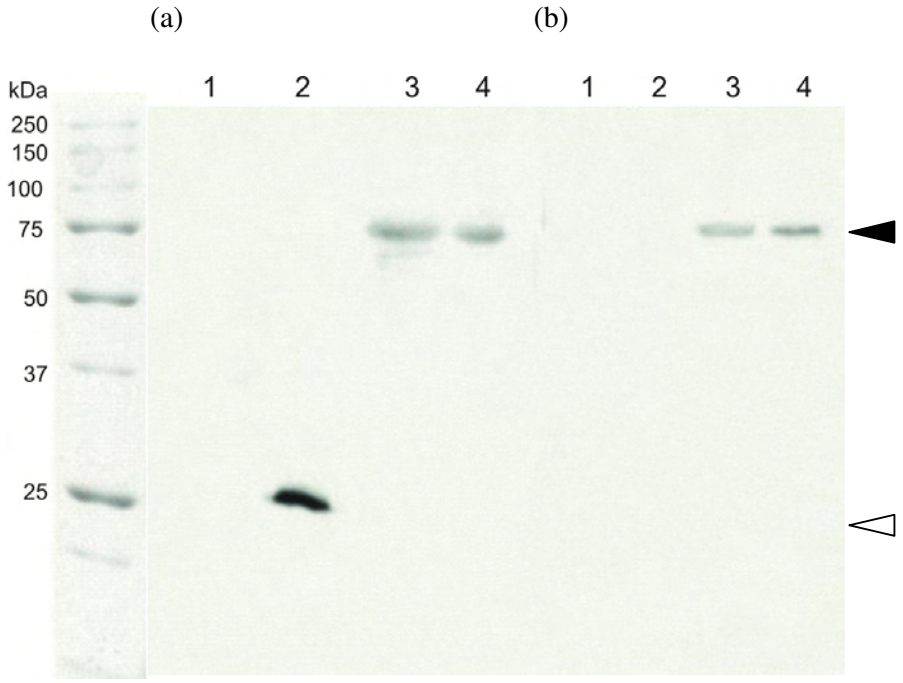


Fig. 3.6 Western blot analysis of the GST-3ABC fusion protein using (a) polyclonal anti-GST antibody or (b) anti-FMDV serum as primary antibody. Protein samples identical to those used for Fig. 3.2 and the gel-purified GST-3ABC fusion protein were resolved on 10% SDS-polyacrylamide gels. The unstained proteins were transferred to a nitrocellulose membrane for Western blot analysis to verify the identity of the over-expressed 74-kDa protein. Lanes 1, IPTG-induced *E. coli* DH5α culture; lanes 2, lysate of IPTG-induced cells that contained the pGEX-2T plasmid; lanes 3, lysate of IPTG-induced cells that contained the pGEX-3ABC plasmid; lanes 4, purified GST-3ABC protein obtained by elution from reverse-stained SDS-polyacrylamide gels. The GST-3ABC fusion protein and GST protein are indicated with closed and open arrows, respectively.

that the GST-3ABC fusion protein was neither degraded by host cell proteases nor processed by the 3C protease. The GST-3ABC fusion protein was found to be insoluble and could not be purified under non-denaturing conditions by glutathione affinity chromatography. Similarly, the 3ABC polypeptide of FMDV type O_1Kaufbeuren was insoluble when expressed in *E. coli* (Strebel *et al*., 1986b; Rodríguez *et al*., 1994; De Diego *et al*., 1997; Mackay *et al*., 1998). In contrast, Cappozzo *et al*. (2002) obtained functionally active but low yields of soluble 3ABC of FMDV type O_1 Campos using the pET expression vector.

A common problem associated with the over-production of heterologous proteins in *E. coli* is the inability of the host cell molecular chaperones to effectively fold the rapidly synthesized heterologous protein into its native, biologically active conformation (Baneyx and Mujacic, 2004). This leads to an increased local concentration of the partly-folded or misfolded recombinant proteins, which are thought to associate by exposed hydrophobic regions and interchain hydrogen bonds, thereby leading to the formation of dense protein aggregates, known as inclusion bodies (Villaverde and Carrió, 2003). These inclusion bodies are therefore insoluble, protease-resistant aggregates of mainly misfolded, biologically inactive recombinant protein (Rudolph and Lilie, 1996). Inclusion body formation may simplify recombinant protein purification procedures, but, in most cases, extensive optimization of refolding procedures is required and only low yields of soluble protein are obtained (Tsumoto *et al.,* 2003; Singh and Panda, 2005). Therefore, instead of refolding the heterologous protein it is often more advantageous to increase synthesis of soluble recombinant protein *in vivo*.

One way of increasing the yield of soluble recombinant proteins *in vivo* is to reduce the rate of protein synthesis through alteration of the cultivation conditions (Makrides, 1996). A reduction in cultivation temperature not only reduces the rate of transcription and translation, but also increases the probability of obtaining more accurately folded protein by minimising the hydrophobic interactions that contribute to protein aggregation (Baneyx and Mujacic, 2004). Furthermore, at lower cultivation temperatures there is a reduction in the activity of heat-shock proteases whose expression is induced under over-expression conditions (Chesshyre and Hipkiss, 1989). However, the recombinant GST-3ABC protein was insoluble when cultures were grown at 20ºC before and after induction with IPTG. Alternatively, lowering the concentration of the chemical inducer (Bentley and Kompala, 1990) can reduce the rate of protein synthesis and thus increase the amount of properly folded recombinant protein. However, in this study, reducing the concentration of IPTG to as low as 100 µM did

not result in soluble recombinant GST-3ABC protein. The addition of certain co-factors to the cultivation medium or a change in pH can also substantially improve the yield of soluble recombinant proteins (Sugimoto *et al*., 1991; Weickert *et al.,* 1999; Yang *et al.,* 2003). In this study, only the influence of pH on obtaining soluble GST-3ABC *in vivo* was investigated. However, no soluble GST-3ABC was obtained when the pH of the culture medium was changed from pH 7.4 to pH 9.0.

It has been reported that partitioning of a given target protein into the soluble or insoluble fraction can be strongly influenced by the nature of the lysis buffer (Brennan and Lin, 1996). Proteins containing hydrophobic or membrane-associated domains may partition into the insoluble fraction, but may not actually be present in inclusion bodies. Furthermore, proteins in the insoluble fraction due to association with bacterial lipids or membranes may often be converted to the soluble fraction by adding low concentrations of non-ionic or Zwitterionic detergents to the lysis buffer (Rabilloud, 1996; Shaw and Riederer, 2003). The lysis buffer used in this study contained the reducing reagent DTT, to break all disulphide bonds and to maintain all proteins in their reduced state, and TritonX-100, to prevent aggregation of the proteins through hydrophobic interactions (Rabilloud, 1996; Middleberg, 2002). In addition, these reagents do not disrupt binding of GST fusion proteins to glutathione agarose beads (Smith and Johnson, 1988). Despite the composition of the lysis buffer used in this study, no soluble GST-3ABC polypeptide was recovered by affinity chromatography purification, but instead could only be detected in the insoluble protein fraction.

Insoluble misfolded proteins can be denatured and then renatured to their native, biologically active conformation, albeit that the yield of soluble protein is low (Rudolph and Lilie, 1996). Often, strong chaotrophic agents such as urea at concentrations of 6-8 M can be used to disrupt hydrogen bonds and hydrophobic interactions (Herskovits *et al.,* 1970), thus denaturing the insoluble recombinant protein completely. The denatured protein is then refolded *in vitro* by reducing the denaturant concentration either through dilution or dialysis, and by supplying an oxidative environment for correct disulphide bonds to form (Rudolph and Lilie, 1996). However, refolding is dependent on the nature of the particular recombinant protein and is hampered by re-aggregation of folding-intermediates (Singh and Panda, 2005). Nevertheless, urea concentrations of 6-8 M have been used to solubilize *E. coli*-expressed FMDV 3ABC polypeptides (Strebel *et al*., 1986b; De Diego *et al*., 1997; Bergmann *et al.,* 2000). Although the recombinant GST-3ABC protein could be solubilized in 7 M urea, it

could not be purified by glutathione affinity chromatography. This may be due to the urea influencing binding of the fusion protein to the glutathione matrix in it's denatured form. Therefore, an alternative means of obtaining purified GST-3ABC was investigated. Previously, proteins used as antigens have been purified from SDS-polyacrylamide gels (Uitenweerde *et al.,* 1995). Using a similar approach, the recombinant GST-3ABC protein was eluted from reverse-stained gels and yielded recombinant protein purified to near homogeneity. However, as a procedure for the large-scale purification of the recombinant protein, the assay is not ideal as it is laborious and expensive.

Based on the results obtained, it can be concluded that although the 3ABC polypeptide of SAT2/ZIM/7/83 was expressed to high levels in *E. coli* the recombinant protein was insoluble and could not be purified by affinity chromatography. Although different approaches were used to improve the *in vivo* production of soluble recombinant GST-3ABC, no soluble protein was obtained. Furthermore, it is possible to solubilize insoluble recombinant proteins by treatment with high concentrations of denaturants, followed by removal of the denaturing agent by dialysis or dilution, but these procedures require extensive optimization on a case-by-case basis. Therefore, as an alternative, the recombinant GST-3ABC protein was purified from reverse-stained SDS-polyacrylamide gels and resulted in high concentrations of highly purified recombinant protein. However, because the recombinant proteins were purified under denaturing conditions only linear epitopes on the 3ABC polypeptide would be available for antibody recognition. Therefore, it is likely that the sensitivity of the diagnostic 3ABC-ELISA may be compromised when using the *E. coli*-expressed 3ABC polypeptide as diagnostic antigen.

CHAPTER 4

EXPRESSION OF THE 3ABC NONSTRUCTURAL POLYPEPTIDE OF ZIM/7/83, A SAT2 TYPE FOOT-AND-MOUTH DISEASE VIRUS, IN INSECT CELLS USING THE BAC-TO-BAC™ BACULOVIRUS EXPRESSION SYSTEM

4.1 INTRODUCTION

The early and accurate diagnosis of foot-and-mouth disease (FMD) virus-infected animals from animals vaccinated against the disease is particularly important in controlling the spread of FMD, since infected animals may become carriers of the virus and the source of new FMD outbreaks (Salt, 1993; Alexandersen *et al*., 2003). The 3ABC-ELISA is considered to be the most appropriate assay to distinguish between FMDV-infected and vaccinated animals, and is based on the detection of antibodies to the 3ABC nonstructural polypeptide (Rodríguez *et al.,* 1994; Mackay *et al*., 1998; Clavijo *et al*., 2004b). To develop a 3ABC-diagnostic ELISA for use in southern Africa, large quantities of the nonstructural 3ABC polypeptide of a SAT type virus is required as antigen. Towards this aim, the 3ABC-encoding region of SAT2/ZIM/7/83 was expressed previously in *E. coli*. Although a high level of the recombinant protein was synthesized, it accumulated in an insoluble form (Chapter 3). One of the disadvantages of using *E. coli* as an expression host is its inability to perform post-translational modifications on the expressed heterologous proteins (Villaverde and Carrió, 2003). As a result, the recombinant protein often accumulates as insoluble aggregates (Georgiou and Valax, 1996). Therefore, eukaryotic expression systems are often used as an alternative for production of heterologous proteins.

Some of the more commonly used eukaryotic expression systems are yeast and baculovirus expression systems. The methylotroph *Pichia pastoris* is often used for recombinant protein production (Sudbery, 1996; Cereghino and Cregg, 2000), since it allows for high levels of recombinant proteins to be produced either intracellularly or to be secreted into the extracellular environment. The latter facilitates recombinant protein purification, since only a few endogenous proteins are secreted (Romanos, 1995; Sreekrishna *et al*., 1997; Cereghino and Cregg, 2000). However, expression in *P. pastoris* is often met with inefficient secretion of complex heterologous proteins (Scorer *et al.,* 1993) or proteolysis of the secreted proteins (Cregg *et al.,* 1993). In addition, the secretion of some proteins is highly dependent upon the intrinsic properties of the protein (Shuster, 1991). Furthermore, some proteins are produced in low amounts or are barely detectable, because the sequence of some heterologous genes may give rise to fortuitous transcription termination signals that result in truncated mRNA (Scorer *et al.,* 1993; Romanos, 1995). Heterologous proteins are also expressed in insect cells using recombinant baculoviruses. This approach frequently results in high-level production of correctly folded post-translationally modified soluble foreign proteins (Luckow and

Summers, 1988; O'Reilly *et al.,* 1992). Two dispensable baculovirus genes, the polyhedrin and p10 genes, have strong promoters and their encoded gene products accumulate to high levels in the late stages of the virus infection cycle (Possee, 1997; Vlak *et al.,* 1988; Weyer and Possee, 1991). Since these genes are dispensable, they can be substituted by heterologous genes and the expressed proteins can represent up to 50% of the total cellular protein (Matsuura *et al*., 1987).

The large size of the baculovirus genome (approximately 135 kb) requires that the first step in generating a recombinant baculovirus is the construction of a recombinant transfer vector, where the gene of interest is cloned downstream of a viral promoter and is flanked by baculovirus sequences (Luckow *et al.,* 1993). Following co-transfection of the recombinant transfer vector and infectious baculovirus DNA into insect cells, homologous recombination occurs and the promoter and target gene sequences are incorporated into the viral genome (Kitts *et al.,* 1990; O'Reilly *et al*., 1992). However, the percentage of recombinant viruses obtained by this method is only 0.1 to 1% of the progeny viruses (Kitts *et al.,* 1990) and multiple rounds of plaque purification is required to obtain pure stocks of the recombinant virus (O'Reilly *et al*., 1992). Consequently, the BAC-to-BAC™ baculovirus expression system (Invitrogen) has been developed. With this system, recombinant baculovirus shuttle vectors (bacmids) are constructed in *E. coli* by site-specific transposition of an expression cassette from a "donor" plasmid, which contains the gene of interest and a baculovirus promoter, into the bacmid DNA (Luckow *et al*., 1993). The mini-Tn7 element of the donor plasmid transposes to the mini-*att*Tn7 attachment site on the bacmid DNA when the Tn7 transposition functions are provided in *trans* by a helper plasmid (Luckow *et al*., 1993). With the BAC-to-BAC™ expression system, there is no need to perform multiple rounds of plaque purification, since the recombinant bacmids are selected for in *E. coli*. As a result, recombinant viral DNA is transfected into the insect cells, allowing for stocks of the recombinant virus to be obtained within seven to ten days (Luckow *et al*., 1993).

Based on the above, the BAC-TO-BAC™ baculovirus expression system may provide a useful means whereby the 3ABC polypeptide of SAT2/ZIM/7/83 can be produced. Furthermore, the 3ABC and 3AB polypeptides of European type FMDV isolates have been expressed successfully using recombinant baculoviruses (Silberstein *et al.,* 1997; Sørensen *et al.,* 1998; Chung *et al.,* 2002, Kweon *et al.,* 2003). Therefore, the aim of this part of the study

was to use of the BAC-TO-BAC™ expression system to produce large amounts of the 3ABC polypeptide of SAT2/ZIM/7/83 in insect cells.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture

Spodoptera frugiperda (*Sf* 9) insect cells were maintained at 27ºC as adherent cultures in 75-cm^2 cell culture flasks (Starsted) in Grace's insect medium (Highveld Biological) supplemented with 10% (v/v) fetal calf serum (FCS) and antibiotics (0.12 mg/ml penicillin G; 0.12 mg/ml streptomycin sulphate; 0.0325 µg/ml Fungizone; Highveld Biological), or as suspension cultures in identical medium supplemented with 10% (v/v) Pleuronic-F68 (Sigma-Aldrich). *Sf* 9 cells were counted on a haemocytometer under 10× magnification immediately after staining with an equal volume of 0.4% trypan blue solution (in 1 × PBS) (Summers and Smith, 1987). Wild-type and recombinant baculoviruses were propagated in the *Sf* 9 cells, as described by O'Reilly *et al*. (1992).

4.2.2 Construction of a recombinant pFastBac1 transfer vector

The molecular techniques, as described in Chapter 2, were used in this part of the study. For construction of the recombinant baculovirus transfer vector, the 3ABC-encoding region of SAT2/ZIM/7/83 was recovered from the recombinant pGEM-3ABC plasmid DNA (Chapter 2) by digestion with *Eco*RI and *Bam*HI. Likewise, the pFastBac1 vector was digested with the same restriction enzymes. Following agarose gel electrophoresis of the digested plasmids, the 1.3-kb 3ABC-encoding region and the pFastBac1 vector DNA fragments were excised from the 0.8% (w/v) agarose gel, purified using a silica suspension and ligated overnight at 16ºC. The ligated products were transformed into competent *E. coli* DH5α cells, following which the transformed cells were plated onto LB agar containing 100 µg/ml ampicillin. Transformants were cultured overnight and the plasmid DNA was extracted and characterized by restriction enzyme digestion. A recombinant clone was selected and designated pFastBac-3ABC.

4.2.3 Construction and characterization of the recombinant bacmid

4.2.3.1 Preparation of competent *E. coli* DH10Bac™ cells

E. coli DH10Bac™ cells (Invitrogen), containing the bacmid genome and helper plasmid, were made competent for the uptake of the transfer vector DNA according to the method of Chung and Miller (1988). One ml of an *E. coli* DH10Bac™ cell culture grown overnight at 37ºC in LB broth containing 10 µg/ml tetracycline and 50 µg/ml kanamycin was inoculated into 100 ml of the same medium and grown at 37ºC with shaking until an OD_{600} of 0.3-0.6 was reached. Cells from 30 ml of the culture were harvested by centrifugation at 4 000 rpm for 5 min at 4ºC, and the pellet suspended in 3 ml of chilled TSB (LB broth; 10% [w/v] PEG-3350; 5% [v/v] DMSO; 10 mM $MgCl_2$, 10 mM $MgSO_4$). The cells were then aliquoted (100 µl) into sterile microfuge tubes and incubated on ice for at least 20 min.

4.2.3.2 Transformation of competent *E. coli* DH10Bac™ cells

For transformation, 100 ng of the pFastBac-3ABC donor plasmid DNA was added to 100 µl of the competent *E. coli* DH10Bac™ cells and incubated on ice for 30 min. Since a heat-shock step was not required to transform the competent DH10Bac™ cells, 900 µl of heated (37ºC) TSBG broth (TSB with 20 mM glucose) was added directly to the cells and incubated at 37ºC for 4 h with shaking to allow for transposition to occur and to permit expression of the antibiotic resistance genes. The transformation mixtures (100 µl) were plated onto LB agar containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal and 40 µg/ml IPTG. The agar plates were incubated at 37ºC up to 48 h to allow for the appearance of blue and white colonies. White colonies were selected as possible recombinants and re-streaked on the same agar medium, as above, to confirm the white colony-phenotype.

4.2.3.3 Extraction of recombinant bacmid DNA

Selected colonies were inoculated into 2 ml LB medium containing antibiotics (50 µg/ml kanamycin; 7 µg/ml gentamicin; 10 µg/ml tetracycline) and grown at 37ºC with shaking (300 rpm). High-molecular-weight recombinant bacmid DNA was isolated by the rapid alkaline lysis method modified for the isolation of large plasmid DNA (Invitrogen). Essentially, cells from 3 ml of the overnight culture were harvested by centrifugation at 15 000 rpm for 1 min.

The cell pellet was suspended in 300 µl Solution I (15 mM Tris-HCl [pH 8.0]; 10 mM EDTA; 10 mg/ml RNase A), following which 300 µl of Solution II (0.2 N NaOH; 1% SDS) was added. The suspensions were inverted several times and incubated at room temperature for 5 min. Subsequently, 300 µl of 3 M KOAc (pH 5.5) was added drop-wise, followed by incubation on ice for 10 min. The proteins and *E. coli* genomic DNA were pelleted by centrifugation at 15 000 rpm for 10 min. The resulting supernatant was added to 800 µl isopropanol and then left on ice for 10 min to precipitate the bacmid DNA. The sample was centrifuged at 15 000 rpm for 15 min at room temperature and the DNA pellet washed with 500 µl of 70% ethanol. The DNA pellet was air-dried for 10 min at room temperature and resuspended in 20 µl of 1 × TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8.0). An aliquot of the DNA was electrophoresed on a 0.5% (w/v) agarose gel at 70 V for 45 min in 1 × TAE buffer (40 mM Tris-HCl; 20 mM NaOAc; 1 mM EDTA; pH 8.5).

4.2.3.4 Analyses of recombinant bacmid DNA

Successful transposition of the 3ABC-encoding region of SAT2/ZIM/7/83 into the bacmid DNA was verified by PCR analyses using pUC/M13 oligonucleotides, which anneal at sites flanking the mini-*att*Tn7 site. The 50-µl PCR reaction mixtures contained 50 pmol of the pUC/M13 forward (5'-GTTTTCCCAGTCACGAC-3') and the 50 pmol of pUC/M13 reverse (5'-CAGGAAACAGCTATGAC-3') oligonucleotides, 30 ng of bacmid DNA, 1 × PCR buffer (50 mM KCl; 10 mM Tris-HCl [pH 9.0]; 0.1% [v/v] TritonX-100), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 5% (v/v) DMSO and 2.5 U of *Taq* DNA polymerase (Promega). To test for contaminating DNA, a negative control was included from which template DNA was omitted. PCR amplification was performed in a Perkin-Elmer GeneAmp 2400 thermal cycler with an initial denaturation step at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 5 min. A final extension step at 72°C for 7 min was included to complete synthesis of all DNA strands. The reaction mixtures were analyzed on a 1% (w/v) agarose gel in the presence of DNA molecular weight marker.

4.2.4 Transfection of *S. frugiperda* cells

The extracted recombinant bacmid DNA was transfected into *Sf* 9 insect cells using a lipid reagent, Cellfectin™ (Invitrogen) according to the manufacturer's instructions. For each transfection, *Sf* 9 cells (9×10^5 cells per 35-mm-diameter well) were seeded in 2 ml Grace's

medium containing antibiotics and allowed to attach for at least 1 h at 27ºC. The lipid reagent (6 µl) and the recombinant bacmid DNA (5 ng) were diluted separately into 100 µl of serum-free Grace's medium (SFGM) without antibiotics, then mixed gently to form lipid-DNA complexes. The lipid-DNA complexes were incubated at room temperature for 45 min, while the medium from the attached cells was removed and the cells washed three times with 2 ml SFGM. Thereafter, 800 µl of SFGM was added to the lipid-DNA complexes, which was then added slowly in a drop-wise manner to the washed cells. Following incubation for 5 h at 27ºC, the transfection mixtures were removed and replaced with 2 ml Grace's medium containing 10% (w/v) FCS and antibiotics. The cells were incubated at 27ºC for 72 h. The supernatants containing the recombinant baculoviruses were subsequently collected by pelleting the cells and cellular debris by centrifugation at 2 000 rpm for 5 min. The supernatants were stored at 4ºC until required. For control purposes, mock-transfected and cells transfected with wild-type bacmid DNA were included.

4.2.5 Preparation and titration of viral stocks

For the preparation of virus stocks, 100 µl of the virus-containing supernatants obtained by transfection was used to infect *Sf* 9 cell monolayers in 75-cm^2 cell culture flasks (1×10^7 cells/flask). Following incubation at 27ºC for 48 h, the supernatants were harvested by centrifugation at 2 000 rpm for 5 min, filter-sterilized and stored at 4ºC. To determine the virus titre of the stocks, plaque assays were performed (Brown and Faulkner, 1977) by seeding 6-well tissue culture plates with *Sf* 9 cells at 1×10^6 cells/well. The cells were allowed to attach for 1 h at 27ºC and the virus stocks were serially diluted to 10^{-9} in 900 µl Grace's medium. The medium was removed from the cells and replaced with the virus dilutions (10^{-4} to 10^{-9}). Following incubation for 1 h at 27ºC, the virus dilutions were removed and replaced with an agarose overlay (sterile 3% [w/v] BacPlaque^{TM} agarose [Novagen] diluted to 1.5% [w/v] in Grace's medium containing 10% [v/v] FCS and antibiotics). The tissue culture plates were incubated at 27ºC for 5 days in a humidified environment and the cells were then stained by addition of 0.5 ml Neutral Red (0.1% [w/v] in sterile UHQ water). Excess staining solution was removed and the plates were incubated at 27ºC for approximately 2 h or until plaques were visible.

4.2.6 Fractionation of bacmid-infected *S. frugiperda* cells

The recombinant baculovirus was used to infect *Sf* 9 monolayers cultures (1×10^7 cells/75-cm^2 flask) at a multiplicity of infection (MOI) of 5 pfu/cell. The infected cultures were incubated at 27ºC for 72 h before being harvested and pelleted by centrifugation at 2 000 rpm for 10 min. The cell pellets were washed three times with 1 × PBS (13.7 mM NaCl; 0.27 mM KCl; 0.43 mM $Na_2HPO_4.7H_2O$; 0.14 mM KH_2PO_4; pH 7.3), resuspended in 1 ml of 0.01 M STE (10 mM NaCl; 10 mM Tris-HCl [pH 7.4]; 1 mM EDTA) containing 0.5% (v/v) TritonX-100 and placed on ice for 10 min. Thereafter, the method described by Huismans *et al.* (1987) was used to prepare sub-cellular fractions of the proteins. The cells were disrupted with 15 strokes of a Dounce homogenizer and nuclei were pelleted by centrifugation at 2 000 rpm for 5 min. The supernatant or cytoplasmic fraction (S10) was recovered and loaded onto a 1-ml 40% (w/v) sucrose cushion (prepared in 0.1 M STE) and centrifuged at 40 000 rpm for 1 h in a Beckman SW50.1 rotor to obtain the supernatant (S100) and pellet (P100) fractions. The P100 fraction was suspended in 0.01 M STE buffer in $1/10^{th}$ of the original S10 fraction volume. The fractions were analyzed by SDS-PAGE, as described below.

4.2.7 SDS-PAGE analysis of recombinant bacmid-infected *S. frugiperda* cells

To prepare cell lysates, *Sf* 9 cells (1×10^6) were infected with recombinant and wild-type bacmids at a MOI of 0.5-5 pfu/cell in 35-mm-diameter wells. For control purposes, *Sf* 9 cells were mock-infected. The cells were incubated at 27°C for 96 h. The cells were suspended in the medium by scraping and pelleted at 2 000 rpm for 5 min. The pelleted cells were washed three times in 1 × PBS and suspended in 30 µl of 1 × PBS. For SDS-PAGE, an equal volume of 2 × PSB (125 mM Tris [pH 6.8]; 4% [w/v] SDS; 20% [v/v] glycerol; 10% [v/v] 2-mercaptoethanol; 0.002% [w/v] bromophenol blue) was added and the samples were heated to 100ºC for 5 min, followed by sonication for 15 min. The cell lysates were resolved on a 10% SDS-polyacrylamide gel, as described previously (Section 3.2.5). After electrophoresis, the gels were stained with 0.125% (w/v) Coomassie Brilliant Blue and destained with a solution containing 5% (v/v) glacial acetic acid and 5% (v/v) ethanol.

4.2.8 Western blot analysis

Proteins resolved by SDS-PAGE were electroblotted from an unstained SDS-polyacrylamide gel onto an equally-sized Hybond^{TM}-C^+ nitrocellulose membrane (Amersham Biosciences),

following soaking of the membrane and gel in dH_2O for 5 min and then in transfer buffer (24 mM Tris; 190 mM glycine; 20% methanol) for 15 min. Proteins were transferred at 30 mA for 1.5 h using a Model TE70 SemiPhor™ Semi-Dry Transfer unit (Amersham Bioscience). The membrane was then immersed for 1 h in TBS-T buffer (200 mM NaCl; 50 mM Tris-HCl; 0.2% [v/v] Tween-20; pH 7.6) containing 5% (w/v) blocking reagent (Amersham Bioscience). The membrane was transferred to TBS-T containing 5% (w/v) blocking reagent and the primary antibody, an FMDV-specific antiserum (SAT3/KNP/10/90; kindly provided by Mr J. Esterhuysen, OVI). Following incubation at room temperature for 1 h with gentle shaking, the membrane was washed three times for 5 min each in TBS-T buffer. The membrane was then transferred to the secondary antibody, horseradish peroxidase-conjugated goat anti-bovine IgG (0.2 µg/µl in fresh blocking solution), and incubated at room temperature for 1 h with gentle shaking. The membrane was then washed three times, as described above. The bound antibody was detected using the ECL Western blotting detection and analysis system (Amersham Bioscience) according to manufacturer's instructions.

4.3 RESULTS

4.3.1 Construction of a recombinant pFastBac1 donor plasmid containing the 3ABC-encoding region

A recombinant bacmid donor plasmid was constructed by ligating the 1.3-kb 3ABC-encoding region of SAT2/ZIM/7/83, which had been recovered from plasmid pGEM-3ABC (Chapter 2) by digestion with *Bam*HI and *Eco*RI, into identically prepared pFastBac1 vector DNA to allow for directional cloning. Following transformation of competent *E. coli* DH5α cells with the ligation reaction, ampicillin-resistant transformants were cultured overnight and the extracted plasmid DNA was characterized by agarose gel electrophoresis and by restriction enzyme digestion.

Plasmid DNA migrating slower than the parental pFastBac1 vector DNA on agarose gels were analyzed for the presence of the cloned 3ABC insert DNA. Digestion of the non-recombinant plasmid DNA with *Eco*RI yielded a DNA fragment of 4.7 kb (Fig. 4.1b, lane 4), whereas digestion of the recombinant plasmid DNA (Fig. 4.1a) yielded a 6-kb DNA fragment (Fig. 4.1b, lane 7). Furthermore, when non-recombinant plasmid DNA was digested with both *Bam*HI and *Eco*RI, a DNA fragment of 4.7 kb was obtained (Fig 4.1b, lane 5), whereas digestion of the recombinant plasmid yielded DNA fragments of 4.7 kb and 1.3 kb (Fig. 4.1b,

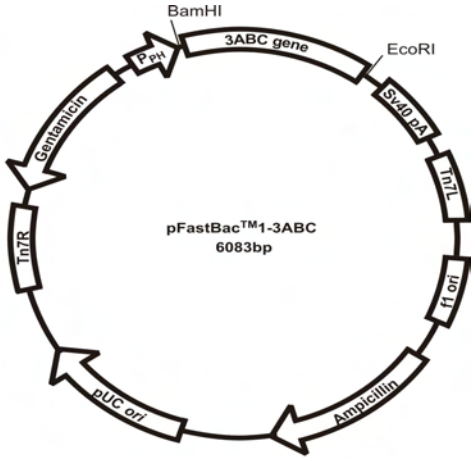


Fig. 4.1a Recombinant pFastBac-3ABC donor plasmid containing the cloned 3ABC-encoding region of SAT2/ZIM/7/83.

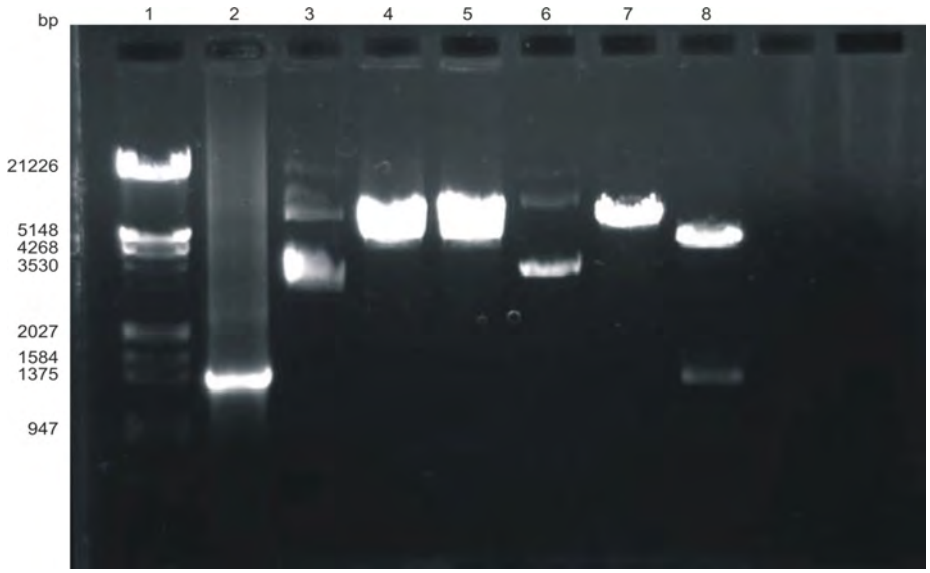


Fig. 4.1b Agarose gel electrophoresis of the recombinant pFastBac-3ABC plasmid and pFastbac1 parental vector DNA, following restriction enzyme digestion. Lane 1, Molecular weight marker (λ DNA digested with *Eco*RI and *Hind*III); lane 2, amplicon of the 3ABC-encoding region (1.3 kb); lane 3, uncut pFastBac1 vector DNA; lane 4, pFastBac1 vector DNA digested with *Eco*RI; lane 5, pFastBac1 vector DNA digested with *Eco*RI and *Bam*HI; lane 6, uncut recombinant pFastBac-3ABC plasmid DNA; lane 7, recombinant pFastBac-3ABC plasmid DNA digested with *Eco*RI; lane 8, recombinant pFastBac-3ABC plasmid DNA digested with *Eco*RI and *Bam*HI.

lane 8). The sizes of these DNA fragments are in agreement with that expected for the pFastBac1 donor plasmid and 3ABC-encoding region of SAT2/ZIM/7/83, respectively. These results therefore confirmed that the 3ABC-encoding region was successfully cloned into the pFastBac1 donor plasmid. The recombinant clone was designated pFastBac-3ABC and used in subsequent experiments.

4.3.2 Engineering of a recombinant bacmid

To engineer the recombinant bacmid, the pFastBac-3ABC donor plasmid was transformed into *E. coli* DH10Bac^{TM} cells, which contain the bacmid DNA and helper plasmid. During site-specific transposition of the recombinant donor plasmid, the mini-Tn7 cassette is inserted from the donor plasmid into the mini-*att*Tn7 attachment site on the bacmid DNA, thereby disrupting expression of the LacZα peptide from the bacmid genome. The transposase required for this process is encoded by the helper plasmid (Luckow *et al.,* 1993). Following transformation, recombinant bacmid DNA was selected by plating the transformed cells onto a selective medium (Section 4.2.3.2) and colonies containing the recombinant bacmid displayed a white colony-phenotype that could be readily distinguished from blue colonies that harboured the unaltered bacmid DNA. The high-molecular-weight recombinant bacmid DNA was extracted from selected transformants and used as template DNA in PCR assays to confirm successful transposition of the 3ABC-encoding region into the bacmid DNA. For this purpose, the universal pUC/M13 oligonucleotides, which anneal to sequences flanking the mini-*att*Tn7 attachment site within the *lac*Zα gene of the bacmid DNA (Fig. 4.2a), were used.

By making use of recombinant bacmid DNA transposed with the pFastBac-3ABC donor plasmid as template for the PCR, a single 3.6-kb band was obtained (Fig. 4.2b, lane 2). The size of the amplicon corresponds with the combined size of the mini-Tn7 cassette (*ca*. 2 kb), the 3ABC-encoding region (*ca.* 1.3 kb) and bacmid DNA flanking the mini-*att*Tn7 site (*ca.* 300 bp) (Fig. 4.2a). In contrast, when either bacmid DNA transposed with the parental pFastBac1 donor plasmid (Fig. 4.2b, lane 3) or wild-type bacmid DNA (Fig. 4.2, lanes 4 and 5) was used as template in the PCR reactions, amplicons of approximately 2.3 kb and 300 bp were observed, respectively. No amplicons were observed in the control reaction from which template DNA was omitted (Fig. 4.2b, lane 6). The recombinant bacmid was designated Bac-3ABC and subsequently used in the transfection of *Sf* 9 cells.

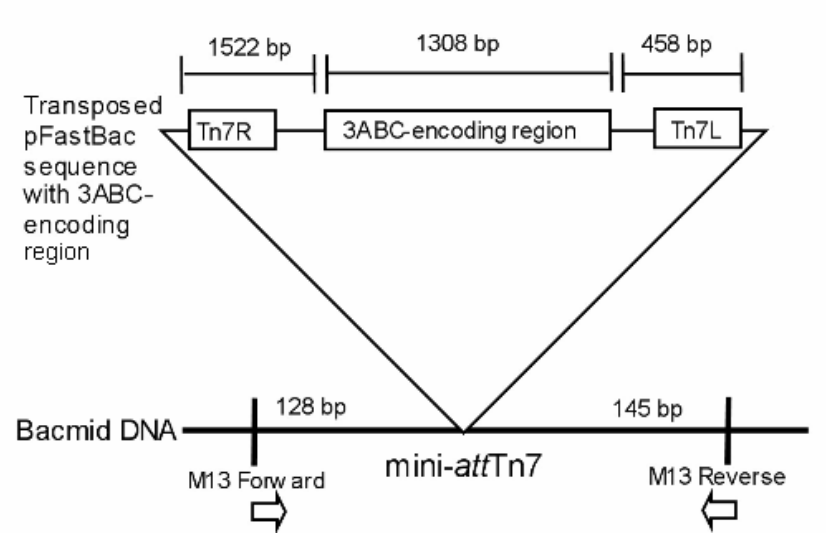


Fig. 4.2a Diagrammatic representation of the transposed region indicating insertion of the expression cassette containing the SAT2/ZIM/7/83 3ABC-encoding region into the bacmid DNA. The annealing sites of the universal pUC/M13 forward and reverse oligonucleotides are indicated by arrows.

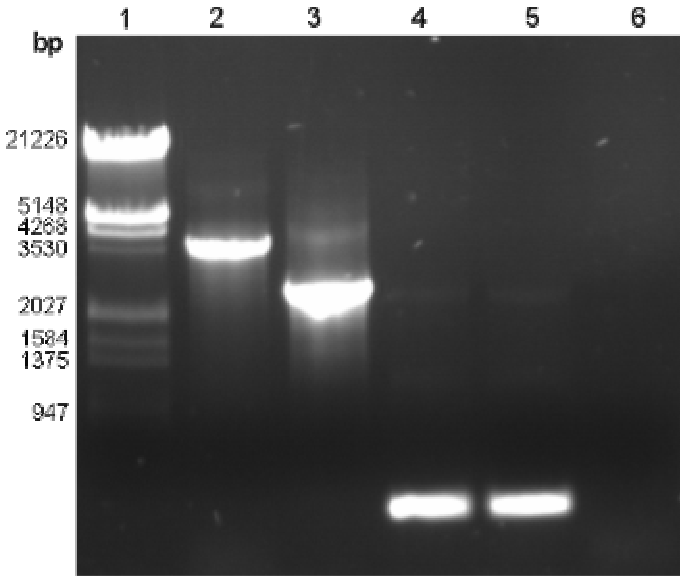


Fig. 4.2b Agarose gel electrophoresis of amplicons obtained, following PCR analyses using the universal pUC/M13 forward and reverse oligonucleotides to verify the presence of the 3ABC-encoding region of SAT2/ZIM/7/83 in recombinant bacmid DNA. Lane 1, DNA molecular weight marker (λ DNA digested with *Hind*III and *Eco*RI); lane 2, amplicon obtained using recombinant bacmid DNA as template; lane 3, amplicon obtained using bacmid DNA transposed with non-recombinant pFastBac1 vector as template; lanes 4-5, amplicons obtained using wild-type bacmid DNA as template; lane 6, control PCR reaction mixture in which template DNA was omitted.

4.3.3 Recombinant protein expression in *S. frugiperda* cells

The recombinant Bac-3ABC bacmid DNA was isolated from *E. coli* DH10Bac^{TM} cultures, transfected into *Sf* 9 insect cells and the recombinant virus was then subjected to three rounds of amplification in *Sf* 9 monolayer cultures. The virus titre was determined by plaque assays to be 3.8×10^6 pfu/ml. To determine whether the 3ABC polypeptide of SAT2/ZIM/7/83 was expressed in recombinant bacmid-infected insect cells, *Sf* 9 cell monolayers were infected with the recombinant or wild-type virus or were mock-infected. Following incubation at 27ºC for 72 h, whole-cell lysates were prepared and analyzed by SDS-PAGE and Western blot analysis using an FMDV-specific antiserum. Analysis of the Coomassie-stained gel indicated the presence of three faint but unique proteins in the cell lysate prepared from BAC-3ABC-infected cells. These had estimated sizes of 47, 30 and 18 kDa, respectively, and were not observed in the cell lysates prepared from either mock-infected or wild-type bacmid-infected cells (Fig. 4.3a). Subsequent Western blot analysis of the respective cell lysates indicated that these three unique proteins observed in the cell lysate from cells infected with Bac-3ABC was recognized by the FMDV-specific serum. No proteins of similar size were detected in either the wild-type bacmid-infected or mock-infected cell lysates, despite the presence of cross-reacting proteins in the different cell lysates (Fig. 4.3b).

Analysis of the Coomassie-stained SDS-polyacrylamide gel indicated that the 3ABC polypeptide was not expressed to high levels in the recombinant bacmid-infected *Sf* 9 cells. Consequently, sub-cellular fractions were prepared to obtain fractions enriched with the recombinant 3ABC polypeptide. *Sf* 9 cell monolayers were therefore infected with the recombinant bacmid and the cells were fractionated as described under Materials and Methods (Section 4.2.6). Following SDS-PAGE of the respective fractions, the results indicated that not only was the 3ABC polypeptide mostly present in the P100 (particulate) fraction, but the 3ABC polypeptide was also purified away from the cellular proteins (results not shown). The 3ABC polypeptide may require further purification or, alternatively, the partially-purified 3ABC polypeptide could be used directly as antigen for the development of a SAT type-specific ELISA.

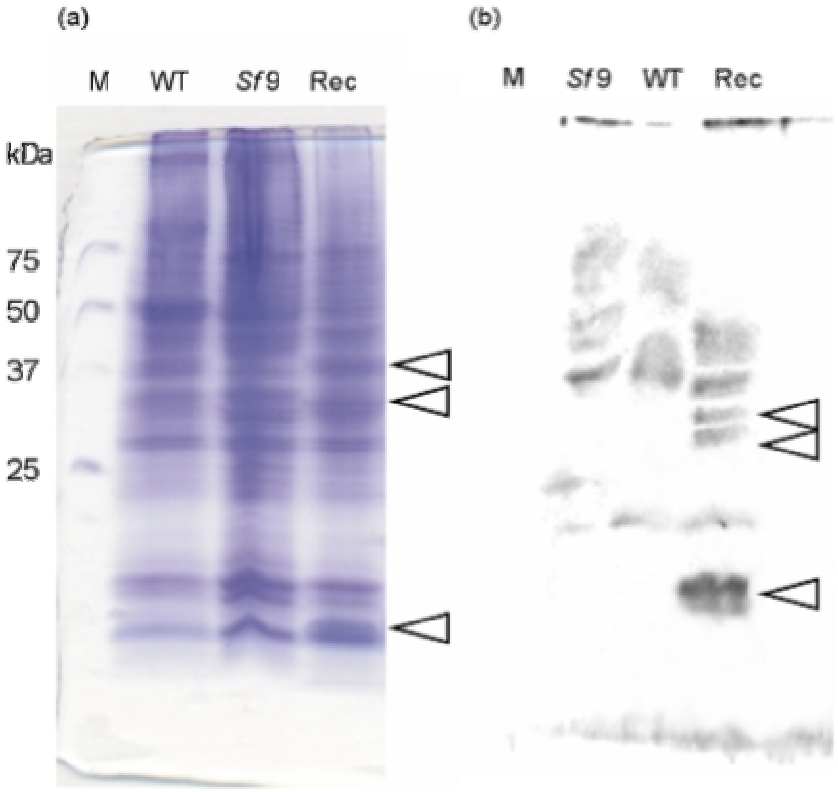


Fig. 4.3 SDS-PAGE analysis (a) and Western blot analysis (b), using an FMDV-specific antiserum, of *S. frugiperda* cells harvested at 72 h post-infection. The *Sf* 9 cells were mock-infected (*Sf* 9), infected with wild-type bacmid (WT) or with the recombinant Bac-3ABC bacmid (Rec). Proteins recognized specifically by the FMDV antiserum and the corresponding proteins on the SDS-polyacrylamide gel are indicated with arrows. The sizes of the protein molecular weight marker (M) are indicated to the left of the figure.

4.4 DISCUSSION

Due to problems encountered with the insolubility and purification of the 3ABC polypeptide of SAT2/ZIM/7/83 expressed in *E. coli* (Chapter 3), an alternative expression system, the BAC-TO-BAC™ baculovirus expression system (Invitrogen), was investigated as a means whereby the 3ABC polypeptide could be produced. This system frequently allows for high-level expression of heterologous proteins that are conformationally more closely related to native proteins than those produced in a prokaryotic system (Luckow and Summers, 1988; O'Reilly *et al.,* 1992). Furthermore, purification of the recombinant bacmid-expressed 3ABC polypeptide to homogeneity prior to its use as a diagnostic antigen may not be required, since the host range of baculoviruses is restricted to specific insect species or cell lines (Blissard and Rohrmann, 1990; Hu, 2005). It is therefore unlikely that animal sera will contain anti-baculovirus or anti-insect cell antibodies (Silberstein *et al.,* 1997; Sørensen *et al.,* 1998).

A recombinant bacmid was generated harbouring the 3ABC-encoding region of SAT2/ZIM/7/83, under the transcriptional control of the strong polyhedrin promoter. SDS-PAGE analysis of cell lysates prepared from the recombinant bacmid-infected cells indicated the presence of three unique proteins of which the level of expression was much lower compared to that of the 3ABC polypeptide expressed previously in *E. coli*. The identity of these proteins was confirmed by Western blot analysis using an FMDV-specific antiserum that reacted specifically with proteins of 47 kDa, 30 kDa and 18 kDa. No proteins of similar size were detected in either mock-infected or wild-type bacmid-infected insect cells. Therefore, the lower molecular weight proteins could represent truncated versions of the full-length 3ABC polypeptide that resulted from proteolytic degradation by host cell proteases, or, more likely, these proteins may have resulted from proteolytic processing of the 3ABC polypeptide by the 3C protease.

The 3C protease catalyses most of the proteolytic cleavage events required for FMDV polyprotein processing, including processing of the P3 polypeptide precursor into different cleavage intermediates and into the mature viral proteins 3A, 3B, 3C and 3D (Mason *et al.,* 2003). Since the 3ABC polypeptide of SAT2/ZIM/7/83 has a predicted molecular mass of 47.7 kDa, cleavage of the recombinant bacmid-expressed 3ABC polypeptide by the 3C protease would yield mature viral proteins 3A (17.4 kDa), $3B_{123}$ (7.6 kDa) and 3C (22.7 kDa). Incomplete proteolytic processing of the 3ABC polypeptide would yield several cleavage

intermediates, namely $3AB_1$ (20 kDa), $3AB_{12}$ (22 kDa) and $3AB_{123}$ (25 kDa), in addition to the above-mentioned viral proteins. It was therefore concluded that the immunoreactive 47-kDa protein corresponded to the 3ABC polypeptide, whilst the immunoreactive 30- and 18-kDa proteins corresponded to the $3AB_{123}$ and 3A proteins, respectively. The accumulation of uncleaved 3AB is in agreement with studies using synthetic peptides, which showed that the 3AB linkage is a relatively poor substrate for the 3C protease (Pallai *et al.,* 1989; Birtley *et al.,* 2005). The identity of these immunoreactive bands may in future be confirmed by immunoprecipitation studies using 3A, 3B and 3C monoclonal antibodies. The inability to detect the 3C protein by Western blot analysis is in agreement with previous reports regarding detection of FMDV and other picornavirus 3C proteases. The 3C protease of FMDV was reported to be barely detectable in immunoprecipitates of virus-infected cell extracts (Strebel *et al.,* 1986b) or in the protein profile of *in vitro* translation products of FMDV RNA (Ryan *et al.,* 1989). The 3C protein of human rhinovirus 1A is a minor component of virus-infected HeLa cells (Aschauer *et al.,* 1991), whereas the 3C protease of poliovirus was reported to be unstable in virus-infected HeLa cells (Thomas *et al.,* 1983). Furthermore, *in vitro* translated 3C protease of encephalomyocarditis virus (EMCV) was reported to be rapidly degraded after its *in vitro* synthesis (Oberst *et al.,* 1993).

SDS-PAGE and Western blot analysis of the recombinant bacmid-infected cell lysate revealed that the 3ABC polypeptide and its cleavage products differed in size from their calculated sizes. Similar discrepancies between the observed and calculated sizes of FMDV proteins have been reported previously, especially for the 3AB protein of FMDV (Strebel *et al.,* 1986b). These differences may be the result of post-translational modifications of one or more of the 3A, 3B and 3C proteins. The amino acid sequence of the 3ABC proteins of SAT2/ZIM/7/83 was scanned using the ProScan function of the EXPASY Molecular biology server (available at http://www.expasy.ch). The results indicated that the 3A and 3C proteins contain potential phosphorylation sites for two different kinases, whereas the 3A protein also contains a potential *N*-glycosylation site. These post-translational modifications may account for the differences between the observed and calculated protein sizes; however, further investigations would be required to ascertain whether the proteins are indeed glycosylated or phosphorylated.

Baculovirus expression of the 3ABC polypeptide resulted in low expression levels of the 3ABC polypeptide. This may be attributed to the apparent cytotoxic nature of the 3C

protease. Notably, several reports regarding the expression of protease-processed empty viral capsids have reported low yields of expression, apparently as a result of the cytotoxic *Aphthovirus* protease (Lewis *et al.*, 1991; Belsham *et al.*, 1990; Roosien *et al.*, 1990). In addition, toxicity of the picornaviral 3C protease has been linked to its ability to cleave host cell proteins involved in cellular transcription or in chromatin structure (Falk *et al.,* 1990; Clark *et al.,* 1991; Capazzo *et al.,* 2002; Tesar and Marquardt, 1990). Furthermore, the FMDV protease may exert a negative effect on cap-dependent translation (Martínez-Salas and Domingo, 1995), since it cleaves cellular translation initiation factors, namely eukaryotic initiation factors eIF-4G and eIF-4A (Belsham *et al.,* 2000).

In conclusion, the 3ABC polypeptide of SAT2/ZIM/7/83 was expressed successfully in insect cells using a recombinant bacmid. Although the 3ABC polypeptide was expressed to a low level and appeared to be proteolytically cleaved by the 3C protease, a fraction of the 3ABC polypeptide was nevertheless expressed in a soluble form. Therefore, the BAC-TO-BAC™ baculovirus expression system could be applicable for the mass production of the 3ABC polypeptide for use in the development of a SAT type-specific diagnostic serological assay.

CHAPTER 5

CONCLUDING REMARKS

Foot-and-mouth disease (FMD) is a highly contagious livestock disease and presents a threat to livestock industries worldwide (Sobrino *et al.,* 2001). Economic losses as a result of a single outbreak can amount to millions in currency as a consequence of livestock losses, loss of export markets, and the cost of control procedures (Hunter, 1998). As a result, countries with FMD-free status are wary of importing the disease, hence trade barriers on the importation of animals and animal products from countries that do not have FMD-free status have been implemented (Samuel and Knowles, 2001). In southern Africa, the African buffalo is the long-time reservoir host for the SAT type viruses and therefore the prospect of completely eliminating the disease from southern African countries is unlikely to transpire (Thomson *et al.,* 2003). Consequently, in southern Africa great emphasis has been placed on effective control and preventative measures. In particular, animal movement restrictions, vaccination of domestic animals adjacent to wildlife reserves and disease surveillance through serological testing have been the key focal points of control (Thomson and Bastos, 2004).

In FMD-free countries, serological diagnosis is based on the detection of antibodies to the structural proteins (OIE, 2004). However, detection of FMDV infection in countries where vaccination is used, is not based on the detection of antibodies to the structural proteins since antibodies to these proteins are induced upon infection and vaccination. Rather antibodies to the nonstructural proteins are detected, which are induced during replication of the virus and therefore is indicative of FMDV infection (Mackay *et al.,* 1998; Sørensen *et al.,* 1998). Several diagnostic assays have been developed based on the detection of antibodies to the 2C, 3A, 3B, 3AB and 3ABC nonstructural proteins (Berger *et al.*, 1990; Bergmann *et al.*, 1993; Lubroth and Brown, 1995; Lubroth *et al.*, 1996; Silberstein *et al.*, 1997; Kweon *et al.*, 2003). However, most of the current assays are based on detection of antibodies to the 3ABC polypeptide, as this serological marker has proven to be most reliable for the detection of FMDV infection in vaccinated populations (Bergmann *et al.*, 1993; Rodríguez *et al.*, 1994; De Diego *et al,.* 1997; Mackay *et al.,* 1998). Current 3ABC-based serological assays have been developed using 3ABC polypeptide of the European serotypes of FMDV. However, the assays are not optimal for the detection of SAT type FMD virus infections (Mr. J. Esterhuysen, personal communication). It was thus suggested that a SAT type-specific assay needs to be developed for detection of antibodies to the 3ABC polypeptide of SAT serotypes (Mr. J. Esterhuysen, personal communication).

The nucleotide sequence of the 3ABC-encoding region of SAT2/ZIM/7/83 was determined and the deduced amino acid sequence was compared to the corresponding region of other SAT, European and Asia1 FMDV isolates (Chapter 2). The results obtained indicated that the 3A, 3B and the 3C amino acid sequences of the SAT type viruses varied significantly from that of the euroasiatic isolates, and that the amino acid sequence variation was located mostly in previously predicted epitope containing regions. These results lend further support to the necessity of developing a SAT type-specific 3ABC diagnostic ELISA for use in southern Africa. When two SAT type strains (SAT2/KEN/3/57 and SAT1/7ISRL/4/62), which grouped more closely with the European type isolates were included in the 3ABC polyprotein sequence analysis, a high level of amino acid sequence variation (27.1%) was observed. Although, this might influence the ability of the SAT specific diagnostic ELISA to detect such SAT type infections, all negative results could be confirmed with the use of a European type diagnostic test.

Towards the development a SAT type-specific 3ABC diagnostic ELISA, large quantities of a SAT type 3ABC polypeptide would be required. Two expression systems, known for high-level gene expression, were thus selected for production of the 3ABC polypeptide of SAT2/ZIM/7/83. The first expression system to be investigated was the well-characterized and cost-effective *E. coli* expression system (Chapter 3). The 3ABC polypeptide of SAT2/ZIM/7/83 was expressed in *E. coli* as a fusion with a GST protein to allow for subsequent purification of the recombinant protein by glutathione affinity chromatography (Chapter 3). Although the recombinant protein was expressed to high levels, it accumulated in an insoluble form and could not be purified by affinity chromatography. Different approaches aimed at reducing the rate of protein synthesis, thus allowing for the formation of correctly folded and soluble recombinant protein, were unsuccessful. However, the recombinant GST-3ABC protein could be solubilized by treatment with high concentrations of urea (7 M), but it could not be purified by glutathione affinity chromatography. It is possible that the high concentration of urea used influenced binding of the GST-3ABC fusion protein to the glutathione agarose beads. The recombinant protein was subsequently purified directly from reverse-stained SDS-polyacrylamide gels and shown to be immunoreactive in Western blot analysis using an FMDV-specific serum. However, although high concentrations of the purified product were obtained, the extensive time required to purify the protein and the costs and labour involved made it unsuitable for mass production of the 3ABC polypeptide.

Other approaches may in future be utilized to improve the yield of soluble GST-3ABC. For instance, since the recombinant GST-3ABC protein could be solubilized using high concentrations of urea, the purification procedure could be improved by incorporating a renaturation step prior to glutathione affinity chromatography. The recombinant protein may be dialysed or diluted into a non-denaturing buffer to allow refolding to occur (De Diego *et al.,* 1997; Mackay *et al.,* 1998). However, protein folding must be optimized on a case-by-case basis and the yield of accurately refolded protein may be low, which would subsequently result in lower yields of purified protein (Schein, 1989; Rudolph and Lilie, 1996; Sørensen and Mortensen, 2005). Alternatively, a different expression vector can be used. Capozzo *et al.* (2002) have demonstrated that expression of the 3ABC polypeptide of FMDV type O_1 Campos in the pET expression vector resulted in expression of the soluble recombinant protein. Since the pET vector allows regulated expression of the foreign gene by bacteriophage T7 RNA polymerase, this result suggests that careful consideration should be given to the choice of promoter. Instead of using the strong inducible *tac* promoter (de Boer *et al.,* 1983) for high-level expression of the recombinant GST-3ABC protein, it may be more advantageous to use a weaker promoter or the protein may be expressed constitutively at a low level (Martínez-Salas and Domingo, 1995). A different approach may involve the use of promoters such as the *csp*A cold-shock promoter (Goldstein *et al.,* 1990). The promoter is efficiently repressed at 37ºC, but rapidly induced upon a temperature-downshift to 15-30ºC (Vasina and Baneyx, 1996; 1997; Mujacic *et al.,* 1999). This is well-suited to expression of heterologous proteins that tend to aggregate when expressed at 37ºC, because at lower cultivation temperatures, rates of transcription and translation and the strength of hydrophobic interactions are reduced, thus favouring correct protein folding and increasing the likelihood of obtaining soluble recombinant protein (Graig and Kumar, 1996; Vasina and Baneyx, 1997; Baneyx and Mujacic, 2004).

Another strategy to prevent accumulation of the recombinant GST-3ABC protein as insoluble aggregates could involve co-over-expression of molecular chaperones. Chaperones are involved in protein folding and the prevention of protein aggregation (Hartl and Hayer-Hartl, 2002). The chaperone systems are cooperative and often successful expression of recombinant proteins requires co-expression of combinations of the chaperones (Sørensen and Mortensen, 2005). Often, the GroEL-GroES and DnaK-DnaJ-GrpE molecular chaperone systems are used (Sørensen and Mortensen, 2005). DnaK and its co-chaperone DnaJ bind to

exposed hydrophobic regions of the unfolded polypeptide, thereby preventing aggregation of the polypeptide (Thomas and Baneyx, 1996; Hartl and Hayer-Hartl, 2002). Dissociation of DnaK and DnaJ from the polypeptide is subsequently facilitated by protein GrpE (Hartl and Hayer-Hartl, 2002; Young *et al.,* 2004). The GroES-GroEL chaperonin system mediates solubilization of proteins by binding to misfolded polypeptides and allows them to refold when released (Hartl and Hayer-Hartl, 2002). However, there is no guarantee that this approach will ensure expression of soluble protein, as the "correct" substrate-chaperone combination is required (Lilie *et al.*, 1998). In addition, the molecular chaperones may be co-precipitated and co-purified with the target protein (Chen *et al.,* 2003). These *E. coli* proteins could therefore react with sera from animals in serological assays and cause false positive reactions.

Another means of obtaining properly folded soluble recombinant protein, yet maintaining high levels of recombinant protein expression, is to select an alternative expression host. In this study, *Spodoptera frugiperda* (*Sf* 9) insect cells and the BAC-to-BAC™ baculovirus expression vector system (Invitrogen) were used for the expression of the 3ABC polypeptide of SAT2/ZIM/7/83 (Chapter 4). The results obtained indicated that the 3ABC polypeptide of ZIM/7/83 was successfully expressed in the *Sf* 9 insect cells, but that the yield was significantly lower than that obtained in *E. coli*. Upon Western blot analysis, using an FMDV specific antiserum, three immunoreactive proteins corresponding to the 3ABC (47 kDa), 3AB (30 kDa) and 3A (18 kDa) proteins were detected. This finding suggests that the 3ABC polypeptide was cleaved by the 3C protease, following its own cleavage from the recombinant protein. Furthermore, a fraction of the 3ABC polypeptide was detected in the cytoplasmic fraction of the recombinant bacmid-infected infected cell extracts, indicating the presence of soluble 3ABC polypeptide.

Several research groups have commented on the apparent toxicity of the 3C protease to prokaryotic and eukaryotic cells (Roosien *et al.,* 1990; Lewis *et al.,* 1991; Grubman *et al.,* 1993; Abrams *et al.,* 1995). The 3C protease of FMDV is not only responsible for extensive proteolytic processing of the viral polyprotein (Vakharia *et al.*, 1987; Clarke and Sanger, 1988), but also for degradation of eukaryotic initiation factors eIF4G and eIF4A, which influences host translation (Belsham *et al.,* 2000), as well as histone H3, which leads to inhibition of host cell transcription (Falk *et al.,* 1990). Furthermore, the 3A protein is associated with the cellular membranes where virus replication occurs. Due to the membrane

association of the 3A protein, and therefore possibly also 3ABC, it can be hypothesized that this association alters membrane permeability, thereby leading to osmotic disregulation and eventual cell death (Prof J. Theron, personal communication). These properties of the 3A and 3C proteins may be directly or indirectly responsible for the low expression levels observed for the 3ABC polypeptide in the bacmid/insect cell expression system. Previous reports have also shown that when certain membrane or membrane-associated proteins are expressed in the baculovirus expression system, low yields of the respective recombinant proteins are obtained (Schmaljohn *et al.*, 1989; Takehara *et al.*, 1990; Van Staden *et al.*, 1995).

Since the low levels of 3ABC polypeptide expression in *Sf* 9 cells are a likely consequence of the cytotoxicity associated with the 3C protease, expression of either the 3AB protein alone and/or the use of alternative insect cells may circumvent this problem. For example, the use of High-Five insect cells, derived from *Trichoplusia ni*, has been reported to result in higher yields of baculovirus-expressed FMDV 3ABC and 3AB proteins when compared to those obtained in *Sf* 9 cells (Sørensen *et al.,* 1998). Similarly, the yield of FMDV 3D protein expressed in High-Five insect cells was three to four times that obtained in *Sf* 9 cells (Meyer *et al.,* 1997). Furthermore, by expressing the 3AB protein alone, the yield of soluble 3AB was approximately 10-20 times higher than that of 3ABC (Sørensen *et al.,* 1998). Nevertheless, it should be noted that inclusion of the 3C protein in the 3ABC polypeptide has been reported to increase the reliability of the antigen as a marker of FMDV infection (Mackay *et al.,* 1998). However, considering that 3C protease is highly conserved amongst FMDV serotypes and that epitopes on 3C are not ideal candidates for diagnostic purposes (Sun *et al.,* 2004), expression of the 3AB protein alone may be more suitable to the development of a FMDV diagnostic assay. Alternatively, instead of making use of a different culture cell line, live insect larvae, such as *Rachiplusia nu* larvae (López *et al.,* 2005), could be used for production of the recombinant 3ABC polypeptide. There are clear advantages to using live insect larvae, such as rapid and high-level expression, as well as cost benefits. However, infection of insect larvae with recombinant baculoviruses is hampered by the difficulty in obtaining consistent infection (Hu, 2005), the need to tend living insects and the greater difficulty of recombinant protein purification (O'Reilly *et al.*, 1992).

A different approach whereby the yield of recombinant 3ABC polyprotein could be increased relies on mutating the 3C protease cleavage sites at the 3A/3B and 3B/3C junctions by site-directed mutagenesis. It is important to note that cleavage sites in the 3ABC polypeptide are

heterogeneous since the 3C protease is capable of cleaving the dipeptides Gln-Gly; Glu-Gly, Glu-Thr, Gln-Leu and Glu-Ser (Robertson *et al.*, 1985; Palmenberg, 1990). Therefore, care should be taken when mutating the 3C protease cleavage site in the 3ABC polypeptide so as to prevent cleavage of the mutated 3ABC polypeptide. Alternatively, the catalytic triad domain (His_{46}-Asp_{84}-Cys_{163}) of the 3C protease could be inactivated through site-directed mutagenesis (Grubman *et al.,* 1995). It has previously been reported that substitution of Cys_{163} of the catalytic triad with Gly inactivates the 3C proteolytic activity (Grubman *et al.,* 1995; Mayr *et al.,* 1999).

In conclusion, the 3ABC polypeptide of the SAT2 virus isolate, ZIM/7/83, was successfully expressed in *E. coli* and in insect cells with the use of the BAC-TO-BAC™ baculovirus expression system. The results obtained during this investigation indicate that the bacmid/insect cells expression system is suitable for the production of the 3ABC polypeptide as a diagnostic antigen. Although the 3ABC polypeptide was expressed in insect cells to lower yields than in the bacterial *E. coli* host, its accumulation in the soluble form is likely to facilitate purification of the recombinant bacmid-expressed 3ABC polypeptide. The yield of soluble protein may be increased with alterations of specific amino acids in the 3ABC sequence or alternatively by optimizing expression of the 3ABC polypeptide. It should be noted that purification of the recombinant bacmid-expressed 3ABC polypeptide may not necessarily be required, as animal sera are unlikely to contain anti-insect and anti-baculovirus antibodies. In addition, the recombinant bacmid-expressed 3ABC antigen may be used in a trapping ELISA format (Silberstein *et al.*, 1997), since it allows the use of a semi-purified preparation and thus avoids purification procedures.

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CONGRESS CONTRIBUTIONS

Sorrill, M.J., Van Rensburg, H.G. and Theron, J. Development of a diagnostic assay for the differentiation of foot-and-mouth disease infected animals from vaccinated and naïve animals for use in sub-Saharan Africa. 18th Congress of the South African Society of Biochemistry and Molecular Biology, Pretoria, South Africa, 6-9 July 2003. (Poster).

Sorrill, M.J., Theron, J., Böhmer, B., Lekoana, T., Maree, F., Esterhuysen, J., Phiri, O.C., Vosloo, W. and Van Rensburg, H.G. Application of recombinant SAT2 foot-and-mouth disease virus nonstructural 3ABC polyprotein, in a diagnostic ELISA. South African Society for Biochemistry and Molecular Biology XIXth Conference. 16-20 January 2005, Stellenbosch, South Africa. (Poster).

Böhmer, B., Sorrill, M.J., Maree, S., Theron, J., Maree, F. F., Esterhuysen, J., Lekoana, T., Phiri, O.C., Vosloo, W. and Van Rensburg, H.G. Recombinant SAT2 foot-and-mouth disease virus nonstructural 3ABC polyprotein, in a diagnostic ELISA. 2005 EUROpic: XIIIth Meeting, 23-29 May 2005, Lunteren, The Netherlands. (Poster).

APPENDIX I

3A

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atttccattccttcccaaaagtccgtgctctacttcctcatcgagaaaggacagcacgaagcagcaattgaattttacgagggaatggtgcacgacagcattaaggaagaacttaagc
 I  S  I  P  S  Q  K  S  V  L  Y  F  L  I  E  K  G  Q  H  E  A  A  I  E  F  Y  E  G  M  V  H  D  S  I  K  E  E  L  K

ccttgttggagcaaaccagcttcgccaagcgtgcttttaaacgcctcaaggaaaacttcgagatcgttgctctcgttgttgtgctgttggcaaacatcatcatcatgatccgcgagac
P  L  L  E  Q  T  S  F  A  K  R  A  F  K  R  L  K  E  N  F  E  I  V  A  L  V  V  V  L  L  A  N  I  I  I  M  I  R  E  T

tcgcaagcgccagaagatggtggacgatgctctcgatgagtacattgagaaggcaaacatcaccaccgacgacaaaacgcttgaagaggcgggaagaaaccctcaagaggttgtcgac
  R  K  R  Q  K  M  V  D  D  A  L  D  E  Y  I  E  K  A  N  I  T  T  D  D  K  T  L  E  E  A  G  R  N  P  Q  E  V  V  D
                                                                                                          3B1
aaacccactgtcggcttccgcgagagaaaactccctgggcataaaactgacgatgaagtgaactctgagccagccaaacccacggagaaaccacaagctgaa↓ggaccctacgctggcc
 K  P  T  V  G  F  R  E  R  K  L  P  G  H  K  T  D  D  E  V  N  S  E  P  A  K  P  T  E  K  P  Q  A  E  G  P  Y  A  G
                                                3B2
ccctcgagcgacagcagccgctaaagctcaaggccaagctccctcaggcagag↓gggccttacgccgggccgctagagaaacaacaaccactgaaactgaaagcgagactgcctgtggc
P  L  E  R  Q  Q  P  L  K  L  K  A  K  L  P  Q  A  E  G  P  Y  A  G  P  L  E  K  Q  Q  P  L  K  L  K  A  R  L  P  V  A
      3B3                                                                    3C
caaggaa↓gggccatatgaaggaccagtgaagaaacctgtcgctttgaaagtgaaagcaaaagccccgattgtcactgaa↓agcggatgcccaccgaccgacttgcaaaagatggtcatg
  K  E  G  P  Y  E  G  P  V  K  K  P  V  A  L  K  V  K  A  K  A  P  I  V  T  E  S  G  C  P  P  T  D  L  Q  K  M  V  M

gcaaacgtgaagcccgttgagctcatcctcgacgggaagacagttgcgctctgctgcgcgactggagtgttcgggacggcttacctcgtgcctcgtcatcttttcgcagagaagtatg
 A  N  V  K  P  V  E  L  I  L  D  G  K  T  V  A  L  C  C  A  T  G  V  F  G  T  A  Y  L  V  P  R  H  L  F  A  E  K  Y
                                                                                                    *
acaagatcatgctggacggccgcgccctgacagacagtgacttcagagtgtttgagttcgaggtgaaagtgaaaggacaggacatgctttcagatgccgcgctgatggttctccactc
D  K  I  M  L  D  G  R  A  L  T  D  S  D  F  R  V  F  E  F  E  V  K  V  K  G  Q  D  M  L  S  D  A  A  L  M  V  L  H  S
                                                                                                 *
tggaaaccgagtgcgtgatctcacggggcacttccgtgacaccatgaaactgtcgaaaggcagccccgtcgttggcgtggtcaacaacgccgacgtcggaagactcatcttctcagga
  G  N  R  V  R  D  L  T  G  H  F  R  D  T  M  K  L  S  K  G  S  P  V  V  G  V  V  N  N  A  D  V  G  R  L  I  F  S  G

gacgctctaacctacaaagacctagtcgtttgtatggacggtgacaccatgcctggactcttcgcgtaccgcgctgggaccaaggttggatactgtggagccgctgttctcgcaaagg
 D  A  L  T  Y  K  D  L  V  V  C  M  D  G  D  T  M  P  G  L  F  A  Y  R  A  G  T  K  V  G  Y  C  G  A  A  V  L  A  K
                                                                                              *
acggcgccaaaacagtgatcgtcggcacccactctgccggaggcaacggagtaggctactgctcctgcgtctcacgatccatgctcctgcagatgaaggcccacatcgaccctccccc
D  G  A  K  T  V  I  V  G  T  H  S  A  G  G  N  G  V  G  Y  C  S  C  V  S  R  S  M  L  L  Q  M  K  A  H  I  D  P  P  P

tcacactgag
  H  T  E
```

Nucleotide and deduced amino acid sequence of the 3ABC-encoding region of SAT2/ZIM/7/83 determined in this study. The 3C protease cleavage sites (Birtley *et al.*, 2005) are indicated by arrows (red). Catalytic residues in the 3C sequence are indicated in bold with a green asterisk (*) (Grubman *et al.*, 1995; Birtley et al., 2005). The conserved GPYXGP motif at the N terminus of each of the three 3B proteins is depicted in bold (blue).

APPENDIX II

LIST OF BUFFERS

0.01 M STE: 10 mM NaCl; 10 mM Tris-HCl (pH 7.4); 1 mM EDTA.

0.15 M STE: 150 mM NaCl; 10 mM Tris-HCl (pH 7.4); 1 mM EDTA.

1 × PCR buffer: 50 mM KCl; 10 mM Tris-HCl (pH 9.0); 0.1% (v/v) TritonX-100.

1 × TAE buffer: 40 mM Tris-HCl; 20 mM NaOAc; 1 mM EDTA; pH 8.5.

1 × TE buffer: 10 mM Tris-HCl; 1 mM EDTA; pH 8.0.

1 × PBS: 13.7 mM NaCl; 0.27 mM KCl; 0.43 mM $Na_2HPO_4.7H_2O$; 0.14 mM KH_2PO_4; pH 7.3.

1 × TGS: 250 M Tris (pH 8.3); 0.192 M glycine; 0.1% (w/v) SDS.

2 × PSB: 125 mM Tris (pH 6.8); 4% (w/v) SDS; 20% (v/v) glycerol; 10% (v/v) 2-mercaptoethanol; 0.002% (w/v) bromophenol blue.

10% resolving gel: 10% (w/v) acrylamide; 0.34% (w/v) bis-acrylamide; 0.375 mM Tris-HCl; 0.1% (w/v) SDS; pH 8.8.

5% stacking gel: % (w/v) acrylamide; 0.17% (w/v) bis-acrylamide; 125 mM Tris-HCl; 0.1% (w/v) SDS; pH 6.8.

Agarose overlay: sterile 3% (w/v) BacPlaque™ agarose (Novagen) diluted to 1.5% (w/v) in Grace's medium containing 10% (v/v) FCS and antibiotics.

Alkaline-SDS buffer: 0.2 N NaOH; 1% (w/v) SDS.

Blocking buffer: 1 × PBS containing 1% (w/v) fat-free milk powder.

Coomassie Staining Solution: 0.125% (w/v) Coomassie Brilliant Blue; 50% (v/v) methanol; 10% (v/v) glacial acetic acid.

Elution buffer: 50 mM Tris (pH 9.0); 1% (v/v) TritonX-100; 2% (w/v) SDS.

Enzyme substrate solution: 60 mg of 4-chloro-1-naphtol dissolved in 20 ml ice-cold methanol, mixed just prior to use with 100 ml of 1 × PBS and 60 µl H_2O_2.

LB agar: 1% (w/v) tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl; 1.2% (w/v) agar; pH 7.4.

LB broth: 1% (w/v) tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl; pH 7.4.

Loading buffer: 40% (w/v) sucrose solution; 0.25% (w/v) bromophenol blue.

Lysis buffer: 20% (w/v) sucrose; 100 mM Tris-HCl (pH 8.0); 10 mM EDTA.

MTPBS buffer: 150 mM NaCl; 16 mM Na_2HPO_4; 4mM NaH_2PO_4; pH 7.3.

Neutral Red: 0.1% (w/v) Neutral Red in sterile UHQ water.

Plasmid DNA isolation isotonic solution: 50 mM glucose; 10 mM EDTA; 10 mg/ml RNaseA; 25 mM Tris-HCl; pH 8.0.

Precipitation mix: 50 µl of 96% ethanol; 2 µl of 3 M NaOAc; pH 4.6.

Protein mobilization buffer: 25 mM Tris-HCl (pH 8.3); 192 mM glycine.

Reduced glutathione solution: 50 mM Tris-HCl (pH 8.0); 5 mM reduced glutathione; pH 7.5.

Solution I: 15 mM Tris-HCl (pH 8.0); 10 mM EDTA; 10 mg/ml RNase A.

Solution II: 0.2 N NaOH; 1% SDS.

TBS buffer: 50 mM Tris-HCl (pH 7.5); 100 mM NaCl; 0.1% (v/v) TritonX-100.

TBS-T buffer: 200 mM NaCl; 50 mM Tris-HCl; 0.2% (v/v) Tween-20; pH 7.6.

Western blot transfer buffer: 120 mM Tris-HCl; 40 mM glycine; pH 8.3.

ECL Western blot Transfer buffer: 24 mM Tris; 190 mM glycine; 20% methanol.

TSB: LB broth; 10% (w/v) PEG-3350; 5% (v/v) DMSO; 10 mM $MgCl_2$; 10 mM $MgSO_4$.

TSBG broth: TSB with 20 mM glucose.

Wash buffer (Western blot analysis): 1 × PBS containing 0.05% (v/v) Tween-20.

Wash buffer (purification of DNA from agarose gels): 50 mM NaCl; 10 mM Tris-HCl (pH 7.5); 2.5 mM EDTA; 50% (v/v) ethanol.

Silica suspension: 10 g silica (Sigma); 100 ml PBS. The silica was allowed to settle for 2 h after which the supernatant was removed and the procedure repeated. The silica was pelleted at 5 000 rpm, 2 min and then suspended in 3 M NaI at 100 mg/ml.