Improvement of a Liquid Phase Blocking ELISA for enhanced detection and measurement of antibodies against the SAT 3 serotype of FMDV

By

Iolanda Vieira Anahory

A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Science in the Department of Veterinary Tropical Disease, Faculty of Veterinary Science, University of Pretoria, South Africa.

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Declaration

I, Iolanda Vieira Anahory, do declare that this dissertation hereby submitted to the University of Pretoria for the degree of MSc in Veterinary Tropical Diseases is my own original work, and, to the best of my knowledge it has not been previously submitted by me or anyone for a degree at this or any other University. All materials contained therein has been duly cited.

Student signature

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(Iolanda Vieira Anahory)

Pretoria, ____/___/___

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Contents

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Abbreviations

µg - Microgram
ATV - Active tripsin versene
BEI - Binary ethyleneimine
BHK-21- Baby hamster kidney clone 21 cell line
BOT - Botswana
BTY - Bovine Thyroid
CPE - Cytopathic effect
EDD - Exotic Diseases Division
ELISA - Enzyme linked immunosorbent assay
et al - *et alia* (and others)
FBS - Foetal bovine serum
FMD - Foot and mouth disease
FMDV- Foot and mouth disease virus
GMEM - Glasgow minimal essential medium
Gp3 - Guinea- pig polyvalent antisera
h - Hour
IB-RS-2 - Instituto Biologico Rim Suino-2 cells
KNP – Kruger National Park
L - Litre
LPBE - Liquid phase blocking ELISA
ml - Millilitre
nm – Nanometre
MOI - Multiplicity of infection
º C - Degree Celsius
OD - Optical density
OVI - Onderstepoort Veterinary Institute
p.i. - Post infection
PBS - Phosphate buffered saline
pH - Logarithm of the reciprocal of the hydrogen ion concentration
RPMI 1640 - Roswell Park Memorial Institute - 1640 medium
Rb3 - Rabbit polyvalent antisera
SADC - Southern African Development Communities

SAR - South African Region

SAT - Southern African Territories

SDG - Sucrose density gradient

TADP - Transboundary Animal Diseases Program

$TCID_{50}$ - Fifty percent tissue culture infective dose

VNT - Virus neutralisation test

SUMMARY

Improvement of the Liquid Phase Blocking ELISA for Enhanced Detection and Measurement of Antibodies against the SAT3 Serotype of FMDV

by

Iolanda Anahory

Supervisor: Dr F. F. Maree
Transboundary Animal Diseases Programme
ARC - Onderstepoort Veterinary Institute

Co - supervisor: Dr R. M. Dwarka
Transboundary Animal Diseases Programme
ARC - Onderstepoort Veterinary Institute

Department: Department of Veterinary Tropical Diseases
Faculty of Veterinary Science

Degree MSc

Foot-and-mouth disease virus (FMDV) is the causative agent of one of the most serious infections of cloven-hoofed animals. An outbreak of FMD not only severely decreases livestock productivity, but also impacts on both the local and export trade of susceptible animals and their products. The Southern African Territories (SAT) types 1-3 are endemic to sub-Saharan Africa and display greater intratypic genomic and antigenic variation than the traditional “Euro-Asian” types. Southern Africa has an abundant wildlife, especially in National Parks and game reserves. Wildlife, particularly African buffalo (*Syncerus caffer*), is involved in virus maintenance and epidemiology of the disease. In communities within the proximity of National Parks and game reserves, the wildlife-livestock interface presents a challenge and poses difficulty to livestock disease eradication and control in Africa.

In this study, the influence of modifications to the reagents has on the specificity, sensitivity and repeatability of a LPBE, used for the detection of antibodies against FMD,

was determined. The sensitivity of the LPBE is dependent on the antigen used in the test and the ability of the sera to cross-react with the antigen. The purified and non-purified virus used as antigen and the capture and detector antibodies were prepared and standardized for this purpose. An attempt was made to reduce the subtype-specificity of the LBPE by including antigens from all the relevant SAT3 strains.

A total of 515 sera from FMDV exposed cattle in Mpumalanga during 2011-2012; 1398 sera from unexposed cattle obtained during an FMD survey conducted in the Northern Cape, and 286 sera from FMDV vaccinated cattle next to the Kruger National Park (KNP) were tested with the improved ELISA. A statistical analysis was conducted to compare the results obtained with the newly developed ELISA and the current in house ELISA. The new assay has higher sensitivity for detecting antibodies in vaccinated animals compared to the standard LPBE. The test is specific and suitable for detection of antibodies, and plays a key role toward the control of FMD, specific and suitable for identification and typing of all SAT3 serotype across the range of the genetic variations in the SAT3 serotype of FMDV.

Chapter 1

Literature review

1.1. Introduction

Foot and mouth disease (FMD) is a highly contagious vesicular disease affecting cloven-hoofed livestock (Rémond *et al.*, 2002; Hughes *et al*., 2002a; Li *et al.*, 2012) such as cattle, sheep, goats and pigs, (Grubman & Baxt, 2004) as well as wildlife such as water buffalo (*Bubalus bubalis*) and camelids (Samuel & Knowles, 2001a; Thomson *et al*., 2003). The African buffalo (*Syncerus caffer*) is the principal source of FMD virus (FMDV) infection in ungulates (Bastos *et al*., 2000; Maree *et al.*, 2014) due to their ability to maintain South African Territories (SAT) type viruses (Condy *et al.*, 1985; OIE, 2012; Maree *et al.*, 2014) and to transmit FMD (Thomson, 1996; Vosloo *et al*., 2007), posing a danger to livestock in sub-Saharan Africa (Maree *et al*., 2014).

The disease is easily transmissible, spreads rapidly to susceptible animal populations (Haydon *et al*., 2001) and can cause persistent infection and long-lasting effects on the productivity of the affected animals (Knowles & Samuel, 2003). An outbreak of FMD can have negative economic consequences (Hughes *et al.*, 2002a; Klein, 2009) which upset international trade of live animals, meat and animal products (Hamblin, 1986b; Leforban, 1999; Li *et al.*, 2012). It reduces productivity in adult animals and causes considerable mortality in young animals, especially if the disease spreads from rural to intensive farming practices (Samuel & Knowles, 2001b; Grubman & Baxt, 2004).

In Southern Africa, the disease places an embargo on the export of animal products and derivatives to developed countries (Vosloo *et al.*, 1992). The disease is widely distributed in the developing world, in particular Africa, Asia and South America. In these regions, livestock farming forms the backbone of rural economies that supports approximately 70 % of the world's poor. (Sobrino *et al*., 2001; Sutmoller *et al.*, 2003; Perry & Rich, 2007, Scoones, *et al.*, 2010; Miguel *et al*., 2013; Ferguson *et al*., 2013). In countries considered free of the disease, which do not vaccinate against the virus, tansboundary public and animal mobility is considered a substantial risk for the introduction of FMDV (Li *et al.*, 2012; Garcia & Romanowski, 2012). In event of an outbreak, these countries are subject to trade restrictions which carry a considerable economic penalty (Samuel & Knowles, 2001b). However, the occurrence of the disease not only affects international trade in livestock and animal products but also results in damaging consequences for the livelihoods of local

farmers due to impacts upon productivity, food security and losses of income. For these reasons identification of virus strains is essential, and continuous monitoring of field strains facilitates the selection of vaccine strains and the development of a proper disease control strategy (Hemadri *et al*., 2000).

1.2. The Foot-and-mouth disease virus

1.2.1. Classification and morphology of the virus

Friedrich Loeffer and Paul Frosch in 1897 reported that FMD is caused by an ultra-visible and ultra-filterable infectious agent smaller than any bacteria and this was the first indication of the virus (Sobrino *et al.*, 2001) causing morbidity in animals. FMDV is the prototype member of the *Aphtovirus* genus of the family *Picornaviridae* (Rodrigo & Dopazo 1995; Samuel & Knowles, 2001b; Domingo *et al*., 2002); it is distinguished from other picornaviruses by its smooth surface. Studies of electron micrographs revealed an icosahedral particle (Sangar, *et al*., 1987; Ellard *et al*., 1999), with a diameter of 22 to 25 nm in size (Crowther, 1986; Domingo *et al*. 2002).

1.2.2. Biochemistry of FMDV

The outer capsid of the virion is composed of 60 copies each of four capsid proteins VP4 (1A), VP2 (1B), VP3 (1C) and VP1 (1D) (Acharya *et al*., 1989; Logan *et al*., 1993; Fry *et al*., 1999; Grubman & Baxt, 2004). A single copy of each capsid protein assembles to produce a protomer, five protomers form a pentamer and twelve pentamers assemble into a complete icosahedral capsid that encloses the viral RNA genome (figure 1) (Acharya *et al*., 1989; Kitching *et al*., 1989; Sobrino *et al.*, 2001; Grubman, 2005). The proteins VP2, VP3 and VP1 are positioned externally (Knipe *et al.*, 1997; Haydon, *et al.*, 2001), while VP4 is localized internally in contact with the RNA molecule. The structural proteins VP1 to VP3 consist of eight-stranded anti-parallel β-barrel loops exposed to the outer surface of the virion. Nomenclature for the viral proteins was established by Rueckert and Wimmer (1984).

FMDV is acid-labile, the capsid is unstable below pH 6.8 (Acharya *et al*., 1989; Curry *et al*., 1995; Ellard *et al*., 1999). Under conditions of low pH, the capsid disassembles into 12S pentameric subunits releasing the RNA molecule. This instability or pH lability most likely has an important function in the infection of susceptible cells and organs in a host (Ellard *et al*., 1999; Grubman & Baxt, 2004). The sedimentation constant of the intact particle is 146S. Empty FMDV particles have a sedimentation constant of 75S and are structurally similar to the intact particle but contain no RNA, and the two proteins 1A and 1B remain

covalently attached as 1AB; subviral particles have a sedimentation constant of 12S and are pentamers of the proteins 1B, 1C and 1D (Crowther, 1986; Kitching *et al*., 1989; Acharya *et al*., 1989).

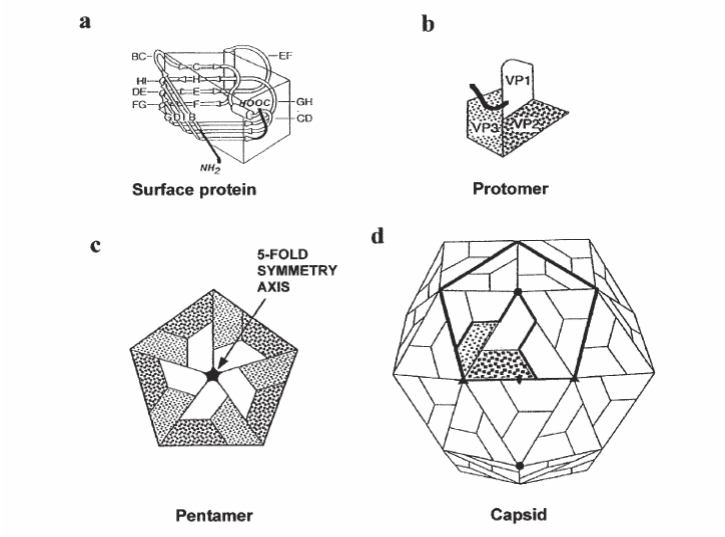


Figure 1: Schematic view of the foot and mouth disease virus capsid. (a) Schematic representation indicating the eight-stranded β-sandwich core of proteins VP2, VP3 and VP1. The strands are indicated as B, I, D, G, C, H, E and F and are joined through connecting loops. (b) The arrangement of the external capsid proteins (VP2, VP3 and VP1) in a biological protomer. The location of the C- and N-terminal domain of VP1 is indicated by a black ribbon. (c) Arrangement of five protomers into a pentamer. (d) Structure of the virion capsid, consisting of 60 protomers. Each protomer is composed of one copy of VP4, VP2, VP3 and VP1. The 2-fold, 3-fold and 5-fold axes are indicated, in blue by an ellipse (1), in red by a triangle (2) and in green by a circle (3), respectively. A pentamer is outlined in the capsid, and a protomer is indicated inside the pentamer. (Original taken from Sobrino and collaborators, 2001).

The G-H loop of VP1 is considered disordered, as revealed by crystallography studies (Logan *et al*., 1993; Verdaguer *et al*., 1995); and a significant "hot-spot" for genetic modification because of its unusual variability (Araujo *et al*., 2002). The G-H loop of VP1 contains a conserved Arg-Gly-Asp (RGD) motif which is involved in attachment to the cell surface and in virus infectivity (Domingo *et al*., 2002; Gulbahar *et al*., 2007). The RGD motif of the G-H loop interacts with the integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$, known as cellular receptors for FMDV, to facilitate cell entry (Berinstein *et al*., 1995; Jackson *et al*., 1997; Neff

et al., 1998; Neff *et al*., 2000; Jackson *et al.*, 2000; Jackson *et al.*, 2002; Jackson *et al.*, 2004; Duque & Baxt, 2003;). The VP1 G-H loop is also important for antibody binding and neutralization of the virus (Mateu, 1995; Domingo *et al.*, 2002; Grubman & Baxt, 2004). VP1 on its own can induce an immune response in animals (Araujo, *et al.*, 2002), eliciting antibodies which can bind and neutralise the virus and can be detected in a liquid-phase ELISA (Crowther, 1986).

1.2.3. Antigenic structure of the virus

FMDV displays a wide spectrum of genetic (Cottam *et al.*, 2006) and antigenic variability and the continuous emergence of new mutants from populations that escape the host immune response (Holland *et al*., 1982; Domingo & Holland 1997; Haydon *et al*., 2001). Most of the impacts of this variation derive from changes within the three major surface-exposed capsid proteins of the virus, i.e. VP1, VP2 and VP3. At least 30-50 % of the residues that constitute the capsid proteins are surface exposed, many of which encompass neutralising epitopes (Mateu *et al*., 1995; Usherwood & Nash 1995; Reeve *et al.*, 2010). This variation in antigenicity of FMDV gives rise to the seven serotypes and several subtypes (Forss *et al*., 1984; Haydon *et al*., 2001; Grubman & Baxt 2004). This complicates control of FMD by vaccination as vaccination against one serotype of FMDV does not provide protection against the other serotypes, or even other subtypes within the same serotype (Brooksby, 1982; Cartwright *et al*., 1982; Mattion *et al.*, 2004; Paton *et al*., 2005; Maree *et al.*, 2011). Therefore, there is currently no universal FMD vaccine available and the vaccines currently used in endemic countries normally contain more than one serotype of virus, depending on the epidemiological situation of the country (Parida, 2009).

The main antigenic site is positioned in the G-H loop of capsid protein VP1 (Domingo *et al*., 2003), which is common to the seven serotypes and of indispensable importance for the immune response (Mateu *et al*., 1995). This segment has been added in synthetic vaccine formulations against FMD (reviewed by Verdaguer *et al.*, 1995). It is important to recognise that the SAT1 and SAT2 viruses display greater antigenic variation compared to the Euro-Asian serotypes (O, A, C, Asia-1) (Reeve *et al*., 2010; Maree *et al*., 2011). The variation is not random, but tends to be concentrated at the surface-exposed β-barrel connecting loops. Therefore, knowledge of the amino acid residues that comprise the antigenic determinants of FMDV, and those that function as protective epitopes in particular, will greatly improve vaccine selection (Dunn *et al*., 1998; Juleff *et al*., 2009).

FMDV, like most RNA viruses, has high mutation rates due to the absence of proofreading enzymes during RNA replication (Holland *et al*., 1982; Cottam *et al.,* 2006). The high mutation rate results in different FMDV replicated genomes occurring together with the original parental genome, thus resulting in quasispecies (Eigen, 1971; Eigen & Schuster, 1979). The newly replicated variants can differ from their parental strands at an average of between 0.1 to 10 base positions (Haydon *et al*., 2001). The environment in which the virus replicates and adapts influences the mutations that will be selected and become fixated in the population. Immunological pressure and physical conditions, e.g. pH or temperature, can lead to changes such as thermal and acid lability of the FMDV particles, a change in plaque morphology, as well as variants with altered host range, antigenicity and virulence in vitro (Beard & Mason, 2000; Nunez *et al*., 2001). In addition to variation as a result of mutation, FMDV has also been shown to undergo RNA recombination in tissue culture. Recombination events were originally thought to occur mostly within the non-structural protein-coding regions. More recent studies have, however, indicated that RNA recombination can take place at the outside boundaries of the outer capsid-coding regions, thus contributing to the genetic diversity in FMDV field isolates (Tosh *et al*., 2002; Heath *et al*., 2006; Simmonds, 2006; Jackson *et al*., 2007).

The reduced effectiveness of the current vaccines against field strains necessitates the continuous development of new vaccines which includes several topotype-specific strains to provide suitable protection against a broad variety of types in the field (Maree *et al*., 2011). In order to affect successful control of the disease during outbreaks, rigorous selection of virus strains is necessary to accurately match the vaccine to the field strains in the region in which the vaccine is to be used (Rweyemamu, 1984). Serological tests have been used to determine antigenic correlation between FMDV isolates to identify the appropriate vaccine strain (Samuel & Knowles, 2001b).

1.2.4. Organization of the FMDV genome

The FMDV genome is a single strand, positive-sense RNA molecule (Grubman, 2005) with approximately 8.500 nucleotides (Forss, *et al*., 1984; Sangar, *et al.*, 1987) surrounded by the viral capsid (Sobrino *et al*., 2001; Domingo *et al.,* 2002; Grubman & Baxt, 2004; Garcia & Romanowski, 2012). The organization of the viral genome is presented in the Figure 2. The RNA is polyadenilated (poly A tail) at its 3’ terminus, and has a small viral protein 3B (VPg) covalently linked to the 5’ terminus of the molecule (Forss, *et al*., 1984;

Sangar, *et al*., 1987; Domingo *et al*., 2002). The viral genome is divided into two non-coding, regulatory region (NCR) at the 5' and 3' ends, and the protein-coding region is subdivided into L/P1, P2 and P3 (Domingo *et al.,* 2002; Grubman & Baxt, 2004). The RNA is translated as a unique open reading frame (ORF) into a polyprotein. Subsequently a number of enzymatic cleavages occur to create structural and non-structural viral proteins (Grubman *et al.*, 1984).

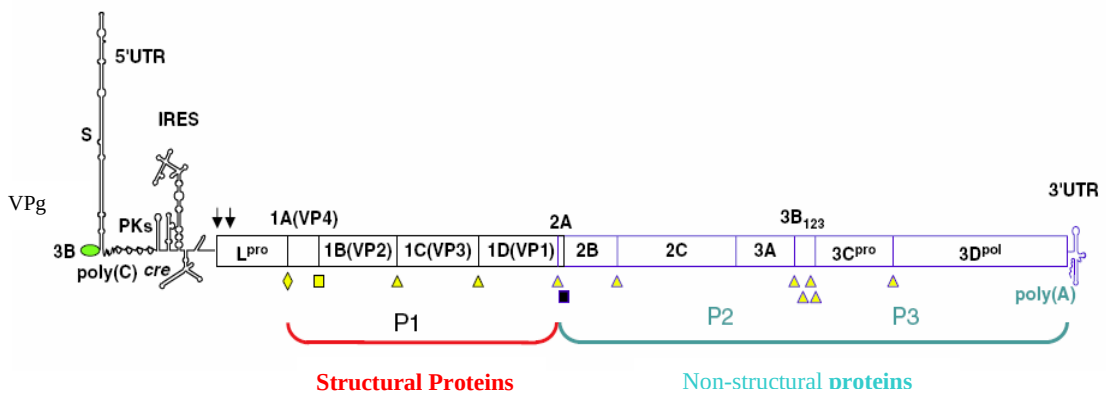


Figure 2: Scheme of the foot and mouth disease virus genome. Original taken from Grubman (2005).

Five distinct regions can be found at the 5' non-coding region, important for virus translation and RNA replication (Bunch *et al*., 1994). From 5' end there is an S fragment of about 370 residues (Domingo *et al*., 2002) essential in genome stability in host cells and may also be involved in the binding of proteins involved in genome replication (Barton *et al*., 2001). Following the S fragment, there is a variable length of poliribocytidylate (poly C) tract of about 100 - 400 residues; after the 3' end of the poly C tract there are pseudoknot structures. The cre (cis-acting replicative element), responsible for RNA genome replication, immediately precedes the internal ribosome entry site (IRES) of about 440 residues which allow the initiation of protein synthesis (Kuhn *et al*., 1990).

Two functional AUG codons separated by about 80 nucleotides indicate the start of the long open-reading frame and results in the synthesis of two forms of the Leader (L) protease (Lab and Lb) (Sangar, *et al.,* 1987; Kuhn, *et al.*, 1990; Domingo *et al.,* 2002). The four capsid proteins VP4 (1A), VP2 (1B), VP3 (1C) and VP1 (1D) are encoded by the P1-region (Rueckert & Wimmer, 1984) located following the L-region. The non-structural proteins 2A protease ($2A^{pro}$), 2B and 2C are encoded by the P2 region that follows the P1

region, and the P3 region encodes non-structural proteins 3A, three copies of 3B (VPg), 3C protease ($3C^{pro}$) and the $3D^{pol}$ (Vakharia, *et al.*, 1987).

According to studies by Cao and collaborators (1995), omission of the first AUG codon had no effect in viral replication although omission of the second AUG from infectious FMDV abolished viral replication. Also Piccone, and collaborators, (1995) produced a synthetic FMDV genome lacking the L-coding region to test the viability of the virus in the host cells and concluded that only polyprotein synthesised by the second AUG codon produced live virus.

The non-structural proteins are responsible for RNA replication, proteolytic cleavage of the viral polyprotein and packaging. The L^{pro} obstructs host protein synthesis, initiates RNA translation and is a viral virulence determinant (de Quinto & Martinez-Salas, 2000). The $3C^{pro}$ is a cysteine protease (Birtley *et al.*, 2005) responsible for catalysing 10 of the 13 proteolytic cleavage events necessary for polyprotein processing (Vakharia *et al.*, 1987; Clarke & Sangar, 1988). Protein 2B has been implicated in enhancing membrane permeability and blocking of protein secretory pathways, as well as virus-induced CPE (Doedens & Kirkegaard, 1995; van Kuppeveld *et al.*, 1997a; van Kuppeveld *et al.*, 1997b; Jecht *et al.*, 1998). The 2C is a conserved peptide with ATPase and RNA-binding activity and plays a role in viral RNA replication (Saunders *et al.*, 1985; Klein *et al.*, 2000; Sweeney *et al.*, 2010). The protein 3A is associated to viral-induced membrane vesicles and contributes to CPE and the inhibition of protein secretion (Doedens & Kirkegaard, 1995; Wessels *et al.*, 2006). The 3B encodes three copies of VPg and participates in encapsidation of the viral RNA (Hogle *et al.*, 1985; Xiang *et al.*, 1997; Barclay *et al.*, 1998). The $3D^{pol}$ is the viral RNA-dependent RNA polymerase (viral replicase) (Martinez-Salas *et al.*, 1985; George *et al.*, 2001). The 3' non-coding region contains a poly A tail consisting of about 90 residues and is the site of interaction of viral and host proteins for RNA replication (Domingo *et al.*, 2002).

Foot and mouth disease virus RNA is potentially infectious. It possesses a positive polarity and a polyadenylate 3' end that allow the RNA to act as a messenger (mRNA) (Forss, *et al.*, 1984; Grubman *et al.*, 1984) *in vivo* and *in vitro* (Domingo *et al.*, 2002); it has been characterized by full genome sequencing. Copies of cDNA obtained from FMDV RNA genome permit manipulation of specific segments of the FMDV genome to study the effects of mutations and others alterations of the genome. Studies using reverse genetics permitted the construction of chimeric FMD viruses to find determinants of viral replication, cell

recognition and virulence. This has improved development of treatment and vaccines (Domingo *et al*., 2002; Van Rensburg *et al*., 2004; Blignaut *et al*., 2011; Carrillo, 2005).

1.2.5. Infection cycle of FMDV

Foot and mouth disease virus has a tropism for epithelial cell in adults and myocardial cells in young animals (Salt, 1993; Kitching & Hughes 2002; Klein, 2009). The RNA virus replicates fast, leading to vast populations in a small period, a characteristic which facilitates the rapidly evolution of a huge number of diverse, but interrelated genomes (Manrubia *et al*., 2005). The FMDV replication cycle is initiated by the attachment of the virus to receptors exposed on the cell surface (Tamkun *et al*., 1986) via an RGD sequence found within the surface-exposed βG-βH loop of capsid protein VP1 (Fox *et al*., 1989; Mason *et al*., 1994). Although the highly conserved RGD tripeptide is characteristic of the ligands of several members of the integrin family (Hynes, 1992), tissue culture-adapted FMDV strains can utilize other receptors such as HSPG in an RGD-independent manner (Jackson *et al*., 1996; Sa-Carvalho *et al*., 1997).

Following binding of the virion to the cell surface receptor, the virus-receptor complex is invaginated and internalized by endocytosis to form a clathrin-coated vesicle (endosome) (Madshus *et al*., 1984a, b; Berryman *et al*., 2005; O'Donnell *et al*., 2005). Virus uptake via HSPG receptors also occurs by endocytosis, but is caveola-mediated (O'Donnell *et al*., 2005; O'Donnell *et al*., 2008). Acidification of the endosome leads to the release of 12 pentameric units and viral RNA (Cavanagh *et al*., 1978; Grubman & Baxt, 2004), as well as the unfolding of the hydrophobic regions buried inside the viral capsid (Curry *et al*., 1995). Fusion of the lipid bilayer with the hydrophobic regions of the exposed capsid proteins leads to the formation of a pore through which the viral RNA is transferred across the endosome membrane to the cytosol (Madshus *et al*., 1984a; b; Rueckert, 1996).

Once in the cytoplasm, induction of viral RNA translation and cessation of cellular RNA translation occurs simultaneously. The VPg protein is released from the 5'-UTR of the viral RNA (Lee *et al*., 1977; Ambros *et al*., 1978). Host translation is down-regulated by the L^{pro} and the IRES forms a secondary structure, which is able to bind ribosomes and deliver them directly to the polyprotein initiation codon in a cap-independent manner (Kuhn *et al*., 1990; Martinez-Salas *et al*., 1996), resulting in synthesis of a single polypeptide. Translation is initiated in the L-fragment of the viral genome by the two in-frame AUG codons (Beck *et al*., 1983; Sangar *et al*., 1987). The L^{pro}, which is the first protein to be synthesized, cleaves

itself from the rest of the growing polyprotein (Strebel & Beck, 1986) before cleaving eIF-4G. The RNA strand directs synthesis of the viral polyprotein, which is cleaved into individual proteins as synthesis progresses (Vakharia *et al*., 1987; Bablanian & Grubman, 1993; Rueckert, 1996; Belsham, 2005).

The single polyprotein encoded by the viral ORF is processed to produce the three polyprotein precursors P1-2A, P2 and P3 (Domingo *et al*., 1990; Belsham, 1993). The P1-2A polyprotein is obtained following the autocatalytic cleavage of L^{pro} from P1 (Strebel & Beck, 1986), and the 2A cleavage between P1-2A and 2B (Ryan *et al*., 1989). The P1-2A precursor is then cleaved by the $3C^{pro}$ to produce VP0, VP3 and VP1. Besides the cleavage of L^{pro} from P1, the cleavage of 2A and the maturation cleavage of VP0 to VP4 and VP2, all other cleavages are performed by 3Cpro and results in several mature structural and non-structural proteins (Vakharia *et al*., 1987; Clarke & Sangar, 1988; Bablanian & Grubman, 1993).

The RNA-dependent RNA polymerase 3Dpol, produced by the cleavage of P3, copies the positive-sense viral RNA to produce complementary negative-sense RNA. Progeny virus positive-sense strands are synthesized repeatedly from these negative-sense templates by a peeling-off mechanism (Joklik, 1980). The progeny positive-sense RNA strands are either translated or packaged into progeny virions (Joklik, 1980; Rueckert, 1996; Nayak *et al*., 2005). The RNA synthesis takes place in a membranous complex from Golgi and reticulum membranes, mediated by non-structural proteins encoded by the P2 (2B, 2BC and 2C) and P3 (3A, $3C^{pro}$ and $3D^{pro}$) regions. Protein 2B is involved in membrane permeability and inhibits protein secretion. Protein 2C is associated with FMDV RNA synthesis.

Virus assembly involves the formation of capsid protomers, five of which assemble into pentamers, followed by packaging of the positive-sense VPg-RNA to form provirions (uncleaved VP0) (Guttman & Baltimore, 1977; Belsham, 1993; Rueckert, 1996) or empty capsids (uncleaved VP0 lacking RNA) with a sedimentation rate of 75S and of unknown significance (Rueckert, 1996; Grubman & Baxt, 2004). The final step in virion maturation involves the autocatalytic cleavage of VP0 into VP4 and VP2, which not only completes the assembly process but is also required for the formation of infectious virus particles (Harber *et al*., 1991; Lee *et al*., 1993; Knipe *et al*., 1997). The mechanism of this maturation cleavage is unknown. Studies reported that an abnormal cleavage of VP0 in FMDV empty capsid can occur indicating that viral RNA is critical for the successful cleavage process (Curry *et al*., 1995). Maturation cleavage is important to produce infectious virus (Harber *et al*., 1991; Knipe *et al*., 1997), and VP0 cleavage is necessary to release RNA into the cytoplasm (Knipe

et al., 1997). The mature virions, with a sedimentation rate of 146S, are then released from the host cells by lysis (Belsham, 1993; Rueckert, 1996).

The replication is prone to errors; the mutation rate ranges between 0.1 and 10, and may take place each time the RNA genome is copied. As mentioned above, because of mutations, the genomes of FMDV populations are related but not identical; the high evolution and antigenic diversity of FMDV complicate the diagnosis, prevention and control of FMD.

1.3. Pathogenesis of foot and mouth disease

The efficiency and dissemination of FMD are influenced by various factors but are reliant on the amount of the virus, susceptibility of the host and the environment. The disease can be spread across borders by the movement of infected animals (inhalation or contact with vulnerable species), contaminated animal products (milk or unprocessed waste food) and mechanically by transport vehicles or hands of animal attendants (Alexandersen *et al.*, 2003). Sheep and goats are also involved in the spread of FMDV (OIE, 2012).

1.3.1. Disease transmission

The main sources of FMDV are infected animals. Virus can be found in all the secretion and excretion (Kitching *et al*., 2005), in expired air of acutely infected animals and in tissues before the clinical signs are noticeable. Transmission occurs generally by direct contact with infected animals (Maree *et al.*, 2014) or rarely, indirect exposure of susceptible animals to the excretion and secretion of acutely infected animals and by aerosol (Kitching & Hughes 2002; Grubman & Baxt, 2004) due to great quantities of infectious particles excreted by infected animals (Alexandersen *et al.*, 2003).

Infection can be established through abrasions on the skin or mucous membrane, or orally. Non-sterilised veterinary surgical instruments, hands and clothes of individuals dealing with infected animals can unconsciously carry virus between flocks or herds (Kitching *et al*., 2005). Contaminated animal products, movement of people and vehicles because of global trade, can carry infected materials between farms and across borders (Alexandersen *et al*., 2002a; Alexandersen *et al.*, 2003; Thomson *et al*., 2003), wild animals and birds can transport the pathogenic agents over long distances (reviewed by Samuel & Knowles 2001a). Animals vaccinated against FMDV as well animals recovering from infection can be infected if exposed to live virus (Kitching *et al*., 2005).

The three SAT serotypes, i.e. SAT1, SAT2 and SAT3, are maintained within the African buffalo populations (Hedger, 1972; Hedger *et al*., 1972; Condy *et al*., 1985; Bengis *et al*., 2002; Thomson *et al*., 2003). In the Kruger National Park, it has been demonstrated that buffalo is the source of infection for impala (*Aepycerus melampus*) and domestic livestock in surrounding areas of KNP and other game parks in southern Africa. Impala is the wildlife species other than buffalo that can transmit the disease during the acute phase of the infection in the KNP (Vosloo *et al*., 2006; Vosloo *et al*., 2007). In addition, it has been reported that experimentally-infected buffalo can infect susceptible cattle (Hedger & Condy, 1985; Vosloo, *et al*., 1996). These are the reasons that South Africa devote enormous efforts separating wild animals and domestic stock, with fences providing the basis to preserve a zone of "disease-free without vaccination'' in the country (Vosloo *et al*., 2007).

1.3.2. Incubation period

The incubation period is relatively brief in natural infection with FMDV the incubation period ranges between 3 to 8 days post-infection (Rémond *et al.*, 2002; Hughes *et al.*, 2002b; Grubman & Baxt, 2004). In experimental challenge, the incubation period can be less than 24 hours, depending on the susceptibility of the animal, the amount of virus and the route of administration (Kitching & Hughes, 2002). Recuperation usually occurs in 2 weeks but secondary infections can prolong recuperation.

1.3.3. Clinical signs of FMD

Clinical signs of the disease in susceptible animals may vary from mild or imperceptible infection in sheep and goats (Geering, 1967; Kitching & Hughes, 2002; Watson, 2004) to severe (Esterhuysen, 1994; OIE, 2012). The severity of the infection depends on the strain of the virus, breed and age of the animal (Kitching & Hughes 2002), environment (Geering, 1967; Garcia & Romanowski, 2012), dose (Hughes *et al.*, 2002b), host species and level of immunity (Vosloo *et al*., 2007; OIE, 2012), animal concentration, housing, diet and supervision (Watson, 2004).

The disease is characterized by fever, intense salivation, loss of appetite, vesicles and erosions in the buccal mucosa as well as tongue (figure 3A and 3B), dental pad, gums and lips from one to two days post infection. On the feet, erosions are prominent at the bulbs of the heel, skin of the interdigital space (figure 3C and 3D) and coronary bands, mammary glands in females, which leads to mastitis in dairy cattle (Gulbahar *et al*., 2007). Death can

occur mainly in newborn young cloven-hoofed livestock (Alexandersen *et al.*, 2003; Garcia & Romanowski, 2012) due to myocarditis (Kitching & Hughes 2002; Gulbahar *et al.*, 2007). Nevertheless, in adult cattle, the mortality rate is less than 5 % (reviewed by Samuel & Knowles 2001a). Sheep and goats are less affected than other cloven-hoofed animals.

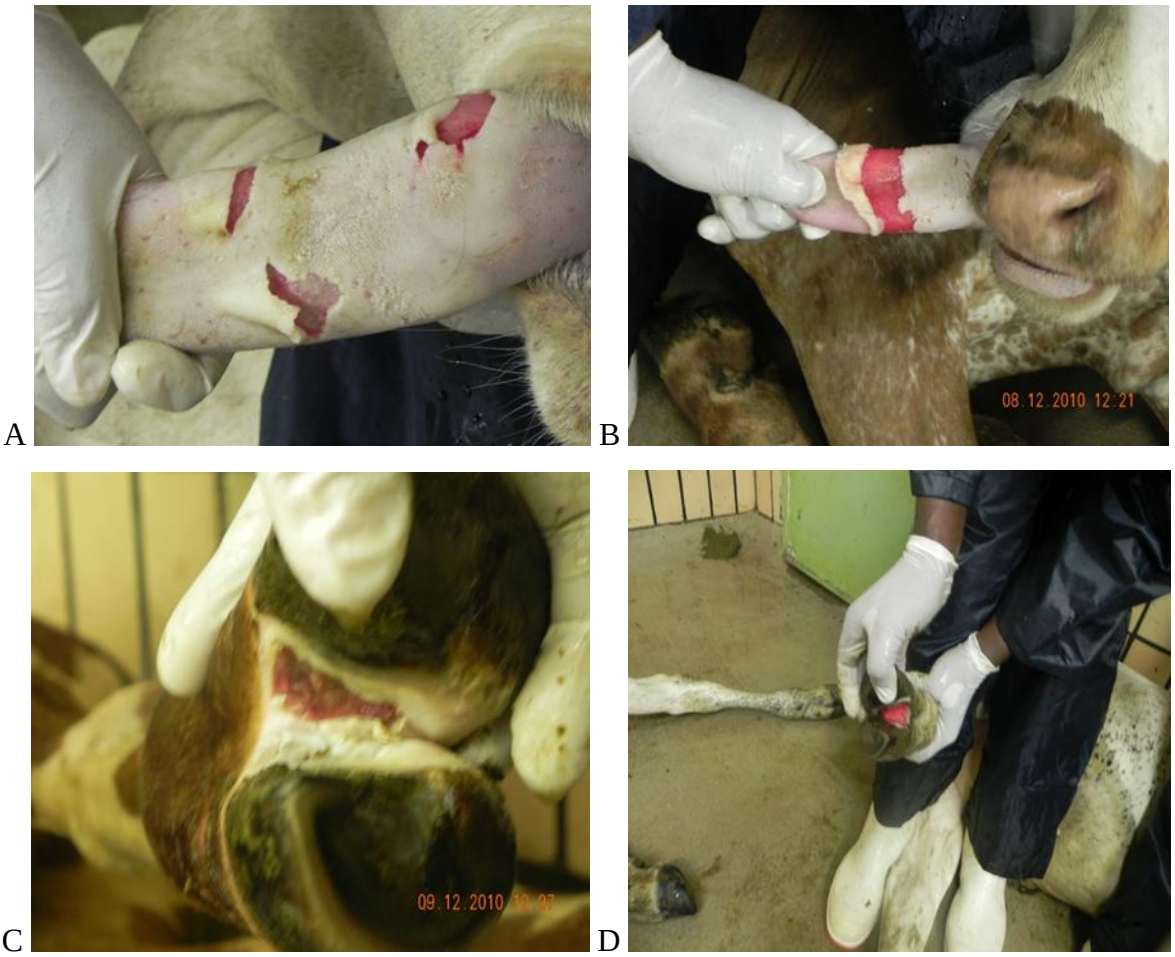


Figure 3: Foot and mouth disease clinical signs. A and B erosions in the tongue; C and D prominent erosions in the interdigital space. Pictures taken during experimental work at TADP of the ARC-OVI.

1.3.4. Sub-clinical and persistent infections

Some animals can become infected and disseminate virus without evidence of clinical signs, in others the virus can persevere after recovering from FMD with clinical signs (Sutmoller & Casas, 2002). Unapparent persistent infections of FMDV or sub-clinically infected animals are considered potential disseminators of the virus and pose a great risk of the spread of infection to susceptible in-contact species (Salt, 1993; Gibbens *et al.*, 2001).

Animals in which the virus persists in the oropharynx for more than 28 days, after infection, are considered as persistently infected (Sutmoller *et al*., 1968) and commonly named carrier animals (Salt, 1993; OIE, 2012). Vaccinated animals (with low or high immunity) or naturally infected animal may become sub-clinically infected or carriers (Yadin *et al*., 2007; Alexandersen *et al.,* 2002b). These carrier animals may establish the disease in susceptible in-contact animals and if no efficient control policies are in place the disease may spread and persevere (Vosloo *et al.,* 2007). Natural and experimental transmissions have been verified from persistently infected buffalo to cattle (Dawe *et al*., 1994a; b; Vosloo *et al.,* 1996).

The duration of the carrier state depend on the species involved (Salt, 1993). Condy and co-workers (1985) in their investigation concluded that infections can persist for a minimum period of 5 years in individual buffalo, or more than two years (OIE, 2012). In cattle the maximum duration of the carrier state is 3.5 years; 6 months in sheep and 4 months in goats. However, FMDV persistence in pigs has not been confirmed (Alexandersen *et al*., 2002b).

1.3.5. Persistent infection in African buffalo

In Southern Africa, SAT-types are closely associated with African buffalo (Sutmoller & Casas, 2002). Persistent infection of buffalo with the SAT-types in the KNP was reported to be as high as 60 % (Vosloo *et al*., 1992; Vosloo *et al*., 2006), with more than one type of FMDV persistent in the pharyngeal region (Hedger, 1972; Vosloo *et al*., 1996; Sutmoller & Casas, 2002). During the persistent period the SAT viruses experience an elevated rate of mutation, leading to increased genetic and antigenic variations (Vosloo *et al*., 1996). So far, no indication of FMDV serotypes other than the SAT-types has been reported in buffalo (Vosloo *et al*., 1992).

1.3.6. Transmission from carrier animals

In the field, transmission from carrier to susceptible animals has been verified from African buffalo to impala (*Aepycerus melampus*) and cattle (Bastos *et al*., 2000; Vosloo *et al.,* 2006). In the endemic area of South Africa, the KNP, antelopes such as impala, play an important role in disease transmission (Thomson *et al*., 2003) as they act as intermediary host. They may cross the perimeter fence of the park and infect cattle on the outside (Sutmoller *at al*., 2000; Vosloo *et al*., 2006).

Transmission from carrier buffalo to cattle is significant, as is transmission from carrier cattle to in-contact cattle but carrier sheep are considered lower risk than carrier cattle (Sutmoller & Casas, 2002). Vaccination does not protect against the development of persistent infection in susceptible animals but in general decreases the prevalence of persistent animals in the field. A high coverage vaccination together with clinical surveillance of susceptible stock and laboratory test for antibodies to FMDV can guarantee that the incidence of carrier animals remains low (Alexandersen *et al.*, 2002b).

1.4. Global Distribution and Epidemiology of FMD

1.4.1. Geographical distribution of FMDV serotypes

The seven known serotypes of FMDV are irregularly disseminated over the world and they cluster into type-specific lineages (Knowles & Samuel 2003). The SAT3 serotype has a limited distribution and basically occurs only in southern Africa (reviewed by Bastos *et al*., 2003; Thomson *et al*., 2003; Klein, 2009). The serotypes A and O are widely dispersed and can be found in several regions of Africa, Asia, America and Europe (Samuel & Knowles, 2001b). The serotype Asia 1 is confined to Asia and the Middle East (Ansell, *et al*., 1994). Serotype C on the other hand has only been detected lately in Kenya in 2004 (Sangula *et al*., 2011).

Developed countries have eliminated FMD due to the negative economic effect of the disease. North America, a major part of Europe, Australia and New Zealand are countries free of the disease. Foot and mouth disease perseveres in African countries, the Middle East and some regions of south, central and south-east Asia (Thomson *et al.*, 2003). In South Africa, the KNP is the unique region where FMD has been reported frequently in wildlife (Thomson *et al*., 2003). Efforts have been focused to control rather than to eliminate the disease in Africa (Bastos *et al.*, 2001) due to the presence of free-living buffalo (*Syncerus caffer*) with high infection rates of SAT types. Buffalo is well known to be the maintenance host of FMDV (Condy *et al*., 1985) and potential source of FMDV transmission to other wildlife and domestic animals (Dawe *et al*., 1994a; Bastos *et al*., 2000). To date, no substantiation exists of buffalo that are infected with serotypes A and O virus in Africa (Thomson *et al*., 2003).

1.4.2. Epidemiology of FMD in southern Africa

In southern Africa, the epidemiology of FMD is unique in that the SAT1, SAT2 and SAT3 serotypes predominate. SAT2 is responsible for most of the FMD outbreaks (Condy *et al.*, 1969; Vosloo *et al.*, 2002), followed by SAT1 and then SAT3 (Thomson, 1994; Bastos *et al.*, 2001; Knowles & Samuel, 2003). These viruses are maintained in wildlife, particularly the African buffalo, which provide a potential source of infection for domestic livestock (Hedger 1972; Hedger *et al.*, 1972; Dawe *et al.*, 1994a; Condy *et al.*, 1985; Vosloo *et al.*, 2007; Bengis *et al.*, 2002; Thomson *et al.*, 2003). As a consequence of the Rinderpest panzootic of 1896-1905, which decimated the cattle population and the maintenance host of FMDV, the disease was absent from the southern African region for several decades. However, in March of 1931, FMD mysteriously re-appeared in south-eastern Zimbabwe. Since its re-emergence, regular outbreaks of FMD have occurred in Zimbabwe, Botswana and South Africa (Thomson, 1994). This prompted the Southern African Development Community (SADC) countries to implement improved disease control measures such as vaccination and fencing.

In the last decade, the numbers of FMD outbreaks in southern Africa have increased significantly. Outbreaks occurred in Mozambique (2001-2002, 2010), Zimbabwe (2000-2003, 2009-2010), Zambia (2004-2010), Botswana (2002 and 2005-2010), Namibia (2007-2010), Malawi (2008-2009) and Angola (2009) (Tekleghiorghis *et al.*, 2014; Brito *et al.*, 2015). South Africa experienced SAT1 outbreaks during 2000, 2002-2003, 2009-2011, SAT2 outbreaks during 2001, 2003- 2005, 2009, 2011-2012, and a SAT3 outbreak was reported in 2006 (Tekleghiorghis *et al.* 2014; Brito *et al.*, 2015). SAT3 virus was also recovered from buffalo in Kwazulu-Natal, immediately south of the Mozambique border, in 2011 (Tekleghiorghis *et al.*, 2014; Brito *et al.*, 2015). A serotype O outbreak occurred in 2001 that was thought to have started in East Asia and culminated in the 2001 United Kingdom outbreak (Knowles *et al.*, 2001). This was the first time that South Africa has experienced an FMD outbreak caused by a serotype other than the SAT serotypes (Sangare *et al.*, 2001).

1.5. Strategies for disease control

1.5.1. Biosecurity

According to the Office International des Epizooties (OIE), FMD is a notifiable disease of animals. In order to protect disease-free countries, control policy recommendations were established by the OIE for affected countries to achieve FMD free status and as a result participate in international trade.

In Southern Africa, the SAT serotypes of FMDV occur with high prevalence and are maintained by African buffalo (Thomson *et al*., 1992; Bastos *et al*., 2003). The major part of South Africa is recognised as free of FMD without vaccination by the OIE, only the KNP is an infected zone where infected buffalo exist, providing a potential source of sporadic infection to susceptible cloven hoofed species with which they come in to close contact (Bastos *et al*., 2000; Vosloo *et al*., 2007, Maree *et al.*, 2014). The existence of free-living, persistently infected animals, mainly buffalo is considered the source of infection for other animals, and complicates the control of the disease (Vosloo *et al*., 1992; Thomson *et al.*, 2003). In South Africa, the main strategy to control the disease is based on the use of fences to separate buffalo from susceptible livestock (Sutmoller *et al*., 2000), vaccination of domestic livestock in a restricted area along the border of KNP, and movement control (Thomson *et al*., 2003; Vosloo *et al*., 2007; Jori *et al.*, 2009). This situation is not only unique to South Africa and the close proximity of livestock and buffalo in southern Africa has necessitated that the SADC invest in regular vaccination programmes if they are to effectively manage FMD. In addition, post-vaccination monitoring is essential to determine the level of herd immunity (Hamblin *et al*., 1986b; 1987). The outcome of effective control is that a country can participate in international and regional trade in livestock and livestock products.

1.5.2. Vaccination

The existing vaccines against FMD consist of complete, chemically inactivated virions combined with an adjuvant (Doel, 2003). The current inactivated vaccines have proven effective in reducing clinical disease in FMD-endemic areas and have been critical to the success of FMD control programs in South America and Europe (Brown, 2003). Vaccines used in the control of FMD in endemic regions are mostly multivalent to provide protection against multiple serotypes (Rweyemamu *et al*., 2008). In Africa, the diversity of circulating field strains of FMDV makes the selection of sufficiently cross-protective FMD vaccines a challenge. Therefore, the success of any FMD control campaign ultimately depends on the abundant supply of vaccine of the appropriate strain composition and proven potency, adequate vaccine coverage, rapid vaccine development, overall planning and management by a well-resourced veterinary service and the involvement and cooperation of the livestock farmer (Rweyemamu & Garland, 2006). Formulation of efficient vaccine that induces a broad

antigenic response giving elevated values against a range of heterologous strains is an indispensable tool for the successful control of FMD by immunization (Perreira, 1978).

The two important determinants that will affect the efficacy of a vaccine and determine whether it will protect or not are (1) the ability of the vaccine strain to elicit antibodies that will cross-react and protect against the field or outbreak virus in question (defined as the vaccine or antigenic match), and (2) the potency of the vaccine to elicit a strong and long-lasting immune response. The quality and quantity of the antigen in the vaccine as well as the formulation of the vaccines and inclusion of immune-stimulating adjuvants are all factors that will influence and contribute to the overall potency of the vaccine (Paton *et al*., 2005). In addition to vaccine efficacy, the number of animals vaccinated in the target population during a vaccination campaign will determine effective protection at herd level and should be taken into consideration. The antigenic variation that exists within and among serotypes of the virus is a problem for the successful control of FMD (Esterhuysen, 1994). Knowledge about the antigenic differences existing between strains within serotypes was gained during the attempts to control FMD by immunization. Serological methods can be applied to assist in the selection of suitable vaccine strains for appropriate information on the control of FMD (Kitching *et al.*, 1989).

The implementation of control measures can be affected when laboratory test results are delayed. In the field, antigenic variation produces strains progressively different from the original virus strain. Specific serotyping of the field virus and molecular characterization must be performed in order to implement emergency vaccination with appropriate antigen and trace the source of an outbreak (Rémond *et al*., 2002). Serological assays are important tools in identifying FMDV by differentiating subtypes from other groups of strains to substantiate that failure in vaccination strategy was caused by the emergence of novel field strains and to support epidemiological studies aimed at generating appropriate vaccine strains and implementation of appropriate control strategies (Kitching *et al*., 1989).

1.6. Diagnosis of FMD

The accurate diagnosis of FMDV infection requires isolation and identification of live virus at the beginning of an outbreak. If the disease is disseminated, the diagnosis can be done by observation of clinical signs or information about infected herds (Kitching *et al*., 2005). Clinically, the disease cannot be differentiated from other vesicular infections (OIE, 2012). Lesions similar to FMD occur in diseases such as malignant catarrhal fever and

rinderpest in cattle; bluetongue, pest des petit ruminants and footrot in sheep, bovine stomatitis, bovine mucosal disease and bovine rhinotracheitis may also be mistaken for FMD (Alonso *et al*., 1991). Therefore, laboratory diagnosis for confirmation is indispensable in the presence of any suspected signs (Li *et al*., 2012) based on recovery of virus from vesicular fluids and epithelial tissue associated with lesions, oesopharyngeal secretion and blood serum for typing purposes. To prove clinical diagnosis of FMD, laboratory confirmation tests (Kitching & Hughes, 2002) includes either the detection of active virus, virus antigen or viral genome, or serological indication of virus occurrence (Kitching *et al*., 2005). Differential diagnosis with respect to other symptomatically related diseases requires precise and quick laboratory diagnosis (Alonso *et al*., 1991).

1.6.1. FMD samples for diagnostic testing

The ideal sample to diagnose FMD is epithelium from unruptured or fresh vesicles (OIE, 2012). In advanced disease or convalescent and carrier animals such as buffalo and cattle, oesophageal-pharyngeal (OP) sample obtained by probang cup, milk or blood are considered options to recover FMDV (Hedger, 1968).

1.6.2. FMD transport medium and sample transportation

To avoid loss or degradation of the virus, samples from the field must be placed in transport medium which contains 50 % phosphate/glycerol and antibiotics (OIE, 2012). The pH of the transport medium must be in between 7.4 - 7.6 (Kitching & Hughes, 2002). Samples must be transported in the gas-phase of liquid-nitrogen or on dry ice (solid carbon-dioxide) to the laboratory.

1.6.3. Laboratory diagnosis

Due to the highly contagious nature and economic importance of FMD, laboratory diagnosis and serotyping of the virus should be done in a high containment facility (Biosafety level 3), equipped to handle the diagnosis (OIE, 2012; Maree *et al*., 2014).

In the case of suspected disease, confirmation by laboratory diagnosis is a matter of urgency (Yadin *et al*., 2007; OIE, 2012; Maree *et al*., 2014). Diagnostic testing for FMD should be performed when clinical signs are present (Rémond *et al*., 2002). Two approaches must be taken in conjunction for detecting virus infection: to isolate the virus or its nucleic

acid and to identify specific antibodies to FMDV (Li *et al*., 2012). Enzyme-linked Immunosorbent Assay (ELISA) has been used for the detection of viral antigen and serotyping (OIE, 2012).

In addition to serological assays, reverse-transcriptase polymerase chain reaction (RT-PCR) (Rémond *et al*., 2002) is used to confirm the presence of FMDV in a sample. RT-PCR is highly sensitive, reproducible and reduces the time necessary for viral detection (Amaral-Doel *et al*., 1993; Marquardt *et al*., 1995). RT-PCR has valuable information and validation to virus isolation and serological data, and it is the first step to determine the nucleotide sequences. A combination of RT-PCR with nucleotide sequencing is an important tool for characterization of field virus isolates and to trace new outbreaks. Nucleotide sequence determination allows for the characterisation of FMDV below the level of serotype i.e. the identification of subtypes and strains. Sequencing for diagnostic and epidemiology purposes is mainly focused on the gene encoding capsid protein VP1. VP1-sequences are used to categorise field strains and for phylogenetic comparison (di Nardo *et al.,* 2011). This in turn allows for the determination of transboundary animal movements and provides a tool to support regional and country-wide FMD control programmes.

1.6.4. Serological Methods

Serological information is useful for assessment of the response to vaccination or whether animals have been infected with FMDV. Samples can be tested by serum neutralisation test, 3ABC ELISA and ELISA for the detection of antibodies. The liquid phase blocking ELISA (LPBE) and solid phase competition ELISA (SPCE) detect antibodies against structural proteins and the VNT are serotype-specific (OIE, 2012). The ELISA techniques are generally specific, sensitive and efficient, are not dependent on tissue culture systems, and not rely on the use of live virus; reducing the disease security risk (Mackay *et al*., 2001; Paiba *et al.,* 2004; King *et al*., 2012). Both ELISA's are relatively simple procedures and easily implementable in diagnostic laboratories in endemic regions. The results can be finalized within one day and the reagents can be prepared by the laboratory using viruses from that region. The VNT on the other hand, although a sensitive serotype-specific assay, requires technical skill to be performed accurately and is dependent on cell culture (Mackay *et al*., 1998a; Donaldson *et al*., 2000) and may not be practical for laboratories in endemic regions. The VNT quantify antibodies which neutralise the infection activity of the virus, whereas ELISA quantifies all types of antibodies reacting to the virus,

including those produced against incomplete and non-infectious virus (Hamblin *et al.*, 1987). The disadvantage of the VNT test is that it is laborious, expensive, time consuming, dependent on biological products with great variability (OIE, 2012) such as cell cultures (Hamblin *et al*., 1986a), and is prone to contamination and the analysis of the results may not be easy (Hamblin *et al*., 1986b). Consequently, it is rarely used and is performed only in reference laboratories. Therefore, the ELISA is the recommended method, for measuring antibodies in samples from convalescent and vaccinated animals, and for screening samples from animals proposed for export (Hamblin *et al*., 1986b; 1987).

The LPBE using a single dilution of serum for the detection and quantification of antibodies to FMDV SAT1, SAT2 and SAT3 was established (Sorensen *et al*., 1992). Ferris and colleagues (2009) used LPBE for screening large number of antibodies for their reactivity against strains of homologous and heterologous serotypes. The LPBE is an OIE recommended standard for the diagnosis of FMD, can detect antibody titres in early infection or vaccination, and plays a key role toward the control of FMD (Hamblin *et al*., 1986a; Hamblin *et al*., 1987). Furthermore, the LPBE is the preferred serological test for use in epidemiological studies; for screening animals before trade; to prove FMD positive animals; to confirm absence of infection; to reveal vaccine effectiveness and vaccine potency testing (Hamblin *et al.*, 1986b; OIE, 2012),

This method is serotype-specific and does not distinguish between infected and vaccinated animals (OIE, 2012). Since FMD vaccines contain inactivated virus, viral replication in vaccinated animals does not occur. These animals will not express antibodies to non-structural proteins (NSP). In infected animals, active viruses replicate and produce NSP's. A convalescent animal reveals both antibodies to structural proteins of the capsid and NSP of the virus. The NSP L, 2C, 3A, 3B, 3C and 3D are consistent indicators that the animal is recovering from infection (Kitching *et al*., 2005).

1.7. Objective of the study

Foot and mouth disease is a huge threat to the health and economic value of livestock species all over the world (reviewed by Grubman & Baxt 2004; Patch *et al*., 2011). Livestock farming forms the backbone of rural economies for most of the SADC member countries. More than 75 % of livestock is raised under the communal smallholder systems and sustains livelihoods of vulnerable groups such as women and children. (Scoones *et al*., 2010; Ferguson *et al.*, 2013; Miguel *et al.*, 2013). Southern Africa has been distinctively endowed

with an abundance of wildlife which has been well protected within national parks and game reserves (Chardonnet *et al.*, 2002). Inevitably, in communities within the proximity of these parks and game reserves, wildlife-livestock interface presents unique challenges to livestock disease control (Bruckner *et al*., 2002; de Garine-Wichatitsky *et al.*, 2013). Of particular note is the presence of the three SAT-type FMDV which are maintained within buffalo (*Syncerus caffer*) populations.

In southern Africa, the SAT serotypes of FMDV occur with high prevalence and have been largely studied in South Africa in the past. However, FMDV exists as distinct genetic and antigenic variants within the seven serotypes through southern Africa. Infection with one serotype does not confer immunity against another. In case of a suspected outbreak of the disease, a confirmed laboratory diagnosis is a matter of urgency. Delay in laboratory tests may hinder the effectiveness of control measures. Thus, fast, sensitive and reliable diagnostic measures are necessary (Oem, *et al.*, 2007). The disease needs to be controlled and prevented through vaccination. Post-vaccination monitoring is essential to determine the level of herd immunity.

The LPBE is used to detect antibodies raised against FMDV, and is an OIE recommended standard for the diagnosis of FMD. This assay is a safe, cost-effective, easy to perform (de Diego, *et al*., 1997), specific, sensitive, fast, reliable and plays a key role toward the control of FMD. Large numbers of sera can be tested in a short time, and evaluate the efficiency of the disease control measures adopted in response to an outbreak. Consequently, areas of poor herd immunity can be identified and corrective vaccination strategies applied. This method is a useful tool for hugely serological surveys (de Diego, *et al*., 1997; Lu *et al.*, 2007) and to evaluate the immune status, in large cattle populations (Smitsaart *et al.*, 1998). Precise diagnosis of FMDV is of huge importance for the control and eradication campaigns in FMD endemic areas and for supportive measure to the ‘stamping out’ policy in FMD free areas.

The LPBE uses inactivated antigens, allowing FMD laboratories to extend the range of their tests to exotic strains of FMD (Ferris *et al.*, 1990), and avoiding the need to handle live virus (Mackay *et al*., 1998b), making the test simple, safe and easy to standardise. The method is reproducible and can be used in laboratories with limited facilities for FMD diagnosis. The Agricultural Research Council (ARC), TADP has existing LPBEs for the detection of SAT1, SAT2 and SAT3 FMDV serotypes. These assays are well established and provide reproducible results. The sensitivity of the LPBE is dependent on the antigen used in the test and the ability of the sera from infected animals to cross-react with the antigen.

Therefore an ELISA test based on one antigen may not adequately detect antibodies raised against the various antigenic subtypes of FMDV field strains. This is substantiated by *in vitro* virus neutralization (VN) studies where sera raised against existing vaccine viruses does not sufficiently cross-react with emerging viruses within the same serotype (Hamblin *et al*., 1986a). Therefore, because of antigenic variants existing within each of the SAT-types, a possibility to increase the sensitivity of the LPBE was considered by incorporating the appropriate strains in the ELISA. The SAT3 serotype is restricted to seven African countries and has high levels of intra-typic variation. Researchers focus on other serotypes due to sporadic involvement of SAT3 outbreaks leading to poor information about antigenic variability of SAT3 virus (Bastos *et al.*, 2003).

The purpose of the present study was to apply certain modifications to the reagents and then experimentally determine if such changes would improve the test in any specific manner, and also, to improve the current LPBE by incorporating additional SAT3 antigens representative of the antigenic variants that occur within southern Africa. This would result in a diagnostic assay which is antigenically more diverse than the current LPBE, thus increasing the sensitivity of the assay. Such an improved assay would greatly benefit the diagnosis and control of FMDV in the SADC.

1.7.1. Specific project objectives

- To incorporate appropriate SAT3 antigens in the current LPBE, to represent antigenic variants representative of topotypes I and II prevalent in southern Africa.
- Preparation of reagents generated (FMDV antigens and antisera) to improve the current SAT3 LPBE.
- Optimisation of the LPBE with the new reagents.
- Validation and application of the new LPBE by determining the assay performance characteristics.

Chapter 2

Materials and Methods

2.1. Virus selection

Viruses used as reference strains in diagnostic tests were selected on the basis of serotype, topotype, ability to propagate in cell culture and immunological characteristics, which were determined by cross-reactivity in VNT. The viruses used in this study were obtained from the TADP of the Agricultural Research council (ARC) at Onderstepoort Veterinary Institute (OVI). The SAT3 viruses were selected based on the phylogeny (courtesy of Dr Dwarka, TADP) to represent topotype I and II (Table 1). All three viruses replicate well in cultured cells as described in the following sections.

Table 1: FMDV used in this study to improve the LPBE

Type	Strain	Topotype	Animal specie	Sample origin	Country of origin
SAT3	SAR/1/06	I	Cattle	Sibasa Matine diptank	South Africa
SAT3	KNP/10/90	I	Buffalo	Kruger National Park	South Africa
SAT3	BOT/6/98	II	Buffalo	Botswana	Botswana

The SAT3 viruses were originally isolated on primary pig kidney (PPK) cells at the TADP laboratory. The stock was stored at -80ºC; a blind passage of each virus was performed and the titres were calculated using $TCID_{50}$ by the Karber method (1931).

2.2. Cell lines

2.2.1. Mammalian cells

The baby hamster kidney-21 clone 13 (BHK-21, ATCC CCL-10) is a cell line derived from baby hamster kidney (MacPherson & Stocker, 1962). The BHK-21 cells were used because of their ability to grow as a monolayer culture or in suspension (Capstick *et al.*, 1962) and their susceptibility to FMDV (Mowat & Chapman, 1962). The cells were grown as monolayer cultures in 850 cm^3 roller bottles for FMDV adaptation and propagation and vaccine formulation (Amadori *et al.*, 1994). The cell lines used to adapt and propagate the selected SAT3 virus strains are presented (Table 2).

Instituto Biologico Renal Suino-2 (IB-RS-2) cell is a porcine Kidney (PK) cell line. The IB-RS-2 cells were grown as monolayer cultures in T165 (165 cm^3 growth area; 2.1 x 10^7 cells) flasks, in Roswell Park Memorial Institute (RPMI-1640) medium (Sigma-Aldrich), for FMDV adaptation and propagation to improve virus titres, because they are more favourable (produce higher virus titres) and susceptibility of the virus.

Table 2: Application of the different cell type used in this study obtained at OVI, TADP laboratory, BHK-21 and IB-RS-2 cells were supplied by the cell culture section of the TADP, at ARC-OVI, as a monolayer cells.

Cell Type	Purpose
IB-RS-2	Virus adaptation and propagation
BHK-21 clone 13	Virus adaptation, propagation and vaccine production

2.2.2. Media for cell cultures

Glasgow Minimum Essential Medium (GMEM, Sigma-Aldrich), supplemented with 10 % (v/v) of foetal bovine serum (FBS, Invitrogen), 10 % (v/v) tryptose phosphate broth (TPB, Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich), was used as growth medium to cultivate BHK-21 cells. Antibiotics 1x (100 U/ml penicillin and 100 U/ml streptomycin) were added to the medium prior to use. The propagation of virus on BHK-21 cells were performed in the presence of GMEM (Sigma-Aldrich) viral growth medium containing 1 % (v/v) FBS (Invitrogen), antibiotics 1x (100 U/ml penicillin and 100 U/ml streptomycin) and 25 mM HEPES (Invitrogen).

To cultivate porcine kidney cells (IB-RS-2), RPMI-1640 (Sigma-Aldrich) medium was used, supplemented with 10 % (v/v) FBS (Invitrogen) and 1ml each of 100 U/ml penicillin and 100 U/ml gentamycin (appendix 1.2). The propagation of virus on IB-RS-2 cells was done in the presence of RPMI-1640 (Sigma-Aldrich), maintenance medium containing 1 % (v/v) FBS (Invitrogen), antibiotics 1x (100 U/ml penicillin and 100 U/ml streptomycin) and 25 mM HEPES (Invitrogen).

2.2.3. Culturing of mammalian cells in monolayer

Baby hamster kidney monolayer cells were prepared by seeding 3.0 x 10^7 cells in GMEM (Sigma-Aldrich) containing 10 % (v/v) FBS (Invitrogen) and 1x (100 U/ml penicillin and 100 U/ml streptomycin) into 850 cm^3 cell culture roller bottles. Subsequently, the bottles were incubated in the roller apparatus at 37ºC for 72 h at 0.25 rpm, until 100 % confluence had been achieved. The cells were subcultured by adding 3 ml 0.25 % active trypsin versene (ATV, appendix 1.1) and allowing the cells to detach from the surface, then resuspended the cells in 20 ml of GMEM (Sigma-Aldrich) supplemented with 10 % (v/v) FBS and 1x (100 U/ml penicillin and 100 U/ml streptomycin) and cells counted (appendix 1.4). The resulting amount of cells to seed was dispensed in 200 ml of GMEM (Sigma-Aldrich) growth medium which had been added per roller bottle. The bottles were incubated at 37°C for almost 48 h at 0.25 rpm to obtain 90 - 100 % confluence. After 48 h, the medium was discarded and 60 ml of GMEM (Sigma-Aldrich) maintenance medium was added prior to virus infection.

The IB-RS-2 cell line was used to propagate virus previously isolated on primary pig kidney cells. Cell counts were performed for each cell suspension sample, using haemocytometer to determine the amount of cells necessary to seed new T165 (2.1 x 10^7 cells; 165 cm^3 growth area) flasks. When the cells achieved 100 % confluence (24 hours after), the RPMI-1640 medium (Sigma-Aldrich) in the T165 flasks were discharged and replaced with 25 ml of RPMI-1640 virus growth medium (VGM) supplemented with 1 % (v/v) FBS (Invitrogen) and 1x (100 U/ml penicillin and 100 U/ml streptomycin) prior to virus infection.

The cells were subcultured similar to BHK-21 cells, except that maintenance medium consisting of RPMI-1640 (Sigma-Aldrich), with 10 % (v/v) FBS (Invitrogen) and 1x (100 U/ml penicillin and 100 U/ml streptomycin) were supplemented.

2.3. Virus propagation

2.3.1. Propagation of viruses in IB-RS-2 cells

For virus adaptation on IB-RS-2 monolayer cells in 165 cm^3 cell culture flasks, the medium was discarded and the cells washed with RPMI-1640 (Sigma-Aldrich) medium containing 1x (100 U/ml penicillin and 100 U/ml streptomycin). Thereafter, 1 ml of original virus stock was added to the 165 cm^3 cell culture flask (90 - 100 % confluence), and incubated for 30 minutes at 37°C with gentle agitation to allow adsorption of the virus. Then 25 ml of RPMI-1640 (Sigma-Aldrich) maintenance medium was added to the inoculum and

the flask was incubated for two days at 37°C or until cytopathic effect (CPE) was advanced. The resulting suspension was frozen at -70ºC, thawed, harvested and clarified by centrifugation at 8000 rpm for 15 minutes. This process was then repeated for a further four blind serial passages. Four passages were considered minimum for growing viruses, to avoid accumulation of changes that may alter the virus antigenicity.

2.3.2. Adaptation and propagation of the viruses in BHK-21 cells

The SAT3 types were adapted and propagated in monolayers of BHK-21 cells to allow the production of a vaccine batch. Confluent BHK-21 cells monolayer 850 cm^3 roller bottles were washed twice with 25 ml of GMEM (Sigma-Aldrich) medium containing 1x (100 U/ml penicillin and 100 U/ml streptomycin), prior to infection with 5 ml of the viruses previously passaged on IB-RS-2 monolayer cells. These were incubated at 37ºC for 30 minutes to allow adsorption, then 55 ml of GMEM (Sigma-Aldrich) maintenance medium was added to each bottle and incubated at 37ºC in the roller machine until CPE was evident or up to 48 h post-infection. A multiplicity of infection of 1:1 (appendix 1.7) for SAT3/BOT/6/98 and SAT3/SAR/1/06 were determined to infect each BHK-21 monolayer 850 cm^3 cell culture roller bottle. After harvesting the supernatant, freezing, and thawing, cultures were centrifuged at 8000 rpm for 30 minutes to eliminate cell debris, and kept at -70ºC for virus propagation and further inactivation and purification.

Thus, four passages were performed on IB-RS-2 monolayer 165 cm^3 culture flask, harvested, clarified at low speed centrifugation, followed by another four passages on BHK-21 monolayer 850 cm^3 cell culture roller bottles. Table 3 summarise the passage history of the viruses used in this study.

Table 3: Passage history of the viruses used in this study

Virus stock	Passage history
SAT3/SAR/1/06	$BTY_1RS_4RS_4BHK_5$
SAT3/BOT/6/98	$BTY_1RS_4RS_4BHK_5$
SAT3/KNP/10/90	$BTY_1RS_4RS_4BHK_5$

2.4. Virus titration

The virus titres were determined by serially diluting virus in virus growth medium and dispensing into a 96-well flat-bottomed microtitre plate (appendix 1.6). Subsequently,

BHK-21 cells suspension was prepared, the number of cells was calculated (2.63 x 10^6 cell/ml), added to the microplate and incubated in 5 % CO_2 at 37ºC for 72 h. Plates were stained with 1 % (w/v) methylene blue (Merck, appendix 1.5), washed, dried and examined visually with an inverted microscope (10x amplification). Virus titres were determined according to the Karber (1931) method (appendix 1.6). Then the viruses were seeded into BHK-21 monolayer 850 cm^3 cell cultures roller bottle for four further serial blind passages, harvested, clarified and virus titres calculated (appendix 1.6). Subsequently, the viruses were stored at -70°C for further propagation into BHK-21 monolayer 850 cm^3 culture roller bottles for virus purification and vaccine production.

2.5. Vaccine preparation

2.5.1. FMD antigen inactivation

Fifty Percent Tissue Culture Infective Dose ($TCID_{50}$) is the measure of infectious virus titer. This method, determine the amount of virus necessary to destroy 50% of infected cells or to produce a cytopathic effect in 50% of inoculated tissue culture cells. The lethal dose of virus was determined. Host cells were plated and serial dilutions of the virus were added. After incubation, the percentage of infected cells was observed and recorded for each virus dilution, and results were used to mathematically calculate a $TCID_{50}$ result. Firstly, virus inoculum was determined ($10^{5.5}$ $TCID_{50}$) to infect BHK-21 850 cm^3 roller bottles. After incubation, 1/100 volume of 10 % Nonidet P40 (w/v) (Roche) was added to the BHK-21 850 cm^3 roller bottles containing the viruses and shaken vigorously to disrupt cell membranes. Then, 20 mM EDTA (containing 1M Tris pH 7.6 and 0.5 M EDTA pH 8.0; Merck) freshly prepared was added to stop protease activity and maintain the pH, and the cells were clarified at 8000 rpm, at 4ºC for 30 minutes to eliminate cellular debris. Afterward, the antigens were inactivated with 0.05 % (w/v) binaryethyleneimine (BEI) consisting of 2-bromethyl-ammoniumbromide (BEA, Merck) and sodium hydroxide pH 12.5 (Merck) at 28ºC for 26 h, stirring in water bath. Following inactivation, 0.2 % volume of sodium thiosulphate solution (Sigma-Aldrich) was added to neutralise the BEI, for further inactivating virus.

2.5.2. Safety testing

Inactivated FMDV suspension was tested for the presence of remnant live virus by titration on BHK-21 monolayer cells using a 96 wells flat-bottomed microtitre plate. Cells suspension (BHK-21) was prepared, the number of cells was calculated (2.63 x 10^6 cell/ml)

and added to the microplate and incubated for 72 h in the presence of 5 % carbon dioxide (CO_2) to confirm that there is no live virus present in the suspension. The plate was stained with 1 % (w/v) methylene blue (Merck, appendix 1.5), dried and examined visually and microscopically with an inverted microscope (10x amplification). No CPE was observed and it was concluded that the virus was completely inactivated.

2.5.3. Purification of 146S antigen

The BEI-inactivated antigens were concentrated by precipitation with 8 % volume of polyethyleneglycol (PEG, 8000 MW, Sigma-Aldrich) followed by centrifugation at 8000 rpm, at 4°C for 30 minutes. Complete 146S particles concentrated with PEG 8000 MW (Sigma-Aldrich), were resuspended with cold NET buffer [150 mM NaCl (Merck); 2 mM EDTA (Merck) and 50 mM Tris (Roche)], at pH 7.6. This was followed by a second clarification step in the presence of a 1/30 volume of cold NET buffer [150 Mm NaCl (Merck); 2 mM EDTA (Merck) and 50 mM Tris (Roche)] at pH 7.6 to dissolve and the particles remove unwanted cellular debris at low speed centrifugation. Then, 1/30 volume of 10 % (w/v) Sodium sarcosyl (Sigma-Aldrich) and 2 mM EDTA (Merck) at pH 7.6 was added and clarified by centrifugation at 8000 rpm, 4°C for 30 minutes.

Subsequently, the SAT3 inactivated antigen were layered on top of a continuous 10 – 50 % sucrose (Calbiochem, 99.9 % purity) density gradient (prepared in NET buffer consisting of 150 Mm NaCl (Merck), 2 mM EDTA (Merck) and 50 mM Tris (Roche)] at pH 7.6) and centrifuged at 16000 rpm for 17 h, at 4ºC. Peak fractions which contained the intact 146S particles were collected and pooled, and the concentration of the antigen was measured by Nanodrop spectrophotometer at λ_1 = 259 nm and λ_2 = 280 nm. The concentration of the antigen measured spectrophotometrically was 321.28 µg equivalents to 160 µg/ml. This was used to prepare a batch of vaccine.

2.5.4. Vaccine formulation

Vaccines for rabbits and guinea-pigs were prepared by emulsifying 1.2 ml of SAT3 inactivated and purified 146S antigen in 3.8 ml of phosphate buffered saline (PBS, appendix 1.8) and 5 ml ISA 206B double oil adjuvant and homogenised, equivalent to100 µg/ml. This was used immediately for animal inoculation.

2.6. Rabbit inoculation

Two groups of rabbits were inoculated with SAT3/BOT/6/98 and SAT3/SAR/1/06 146S antigens. For each strain of FMD SAT3 146S antigen, 2 rabbits were inoculated intramuscularly with 1 ml of vaccine. Each rabbit received vaccine equivalent to 100 µg of antigen. The animals were boosted 28 days post-inoculation with 50 µg in 0.5 ml of 146S antigen without adjuvant. Ten days later, the rabbits were sedated and bled for antisera preparation.

2.7. Guinea-pigs inoculation

Two groups of guinea-pigs were inoculated with SAT3/BOT/6/98 and SAT3/SAR/1/06 146S antigens. For each strain of FMD SAT3 146S antigen, 10 guinea-pigs were inoculated intramuscularly with 0.5 ml of vaccine. Each guinea-pig received vaccine equivalent to 50 µg of vaccine antigen. The animals were boosted 28 days post-inoculation with 25 µg in 0.25 ml of 146S antigen without adjuvant. Ten days later, the guinea-pigs were sedated and bled for antisera preparation.

2.8. Preparation of antisera

Rabbit and Guinea-pig monovalent antisera were prepared against SAT3/SAR/1/06 and SAT3/BOT/6/98 strains obtained from OVI, TADP virus collection. The SAT3/KNP/10/90 strain used to prepare the pooled (rabbit and guinea-pig) antisera, antigen and positive control reference serum, was also obtained at OVI, TADP laboratory.

2.8.1. Rabbit antiserum stock preparation

Specific trapping antibodies to each FMD SAT3 virus are whole serum raised in rabbits for adsorption onto the microplates. A quantity of 60 - 80 ml/rabbit blood were collected in sterile containers during the terminal bleeding and left for at least 2 h at room temperature to allow clotting and serum separation. The blood was kept at 4ºC overnight then centrifuged at 8000 rpm for 30 minutes for serum separation. Aliquots of 5 ml were stored at -20ºC.

2.8.2. Guinea-pig antiserum stock preparation

The detecting antibodies are guinea-pig antiserum (whole serum) of the same specificity as the trapping antibody. A quantity of 20 ml/guinea-pig of blood were collected in sterile containers during the terminal bleeding and left for at least 2 h at room temperature to allow clotting and serum separation. The blood was kept at 4ºC overnight, then centrifuged at 5000 rpm for 30 minutes for serum separation. Subsequently the antiserum was pre-blocked with normal bovine serum (NBS) as follows: for each 1 ml of guinea-pig serum, 1 ml of NBS and 8 ml of PBS-casein (Sigma-Aldrich, appendix 1.10) were added to make 1:10 guinea-pig antibody stock. Aliquots of 5 ml were stored at -20ºC.

2.8.3. Cattle antiserum preparation

Specific anti-FMDV SAT3 cattle serum (whole serum) was prepared by infecting groups of 5 animals each with the selected viruses (SAT3/BOT/6/98, SAT3/SAR/1/06) for use as positive control in ELISA. The FMDV type-specific bovine reference sera were collected through venepuncture (jugular or caudal vein) from each animal at day zero prior to challenge to certify that the animals were healthy. The skin at the site of venepuncture was swabbed with 70 % alcohol and allowed to dry. Subsequently an amount of 1 ml of live FMDV physiological suspension at a $10^{4.75}$ $TCID_{50}$ of SAT3/BOT/6/98 and $10^{4.17}$ $TCID_{50}$ of SAT3/SAR/1/06 viruses were inoculated intradermolingually at two sites. The animals were observed daily from day 1 for clinical signs of FMD. Most of the animals had shown lesions in the tongue at day 2 p.i. At 7 and 14 days post experimental infection, the blood was collected from each animal in a vacuette serum separator tube for ELISA test. At day 21 after experimental infection, each animal was sedated and at least 1L of blood per animal was collected in sterile containers for sera preparation. Aliquots of 5 ml were stored at -70ºC.

2.9. Standardisation of reagents

The optimal concentration and working dilutions for rabbit and guinea-pig antisera were determined by chequerboard titration sandwich ELISA.

2.9.1. Titration of rabbit antiserum

In order to determine the optimum concentration of trapping antibodies to improve the current LPBE, a pool of the selected SAT3/BOT/6/98 and SAT3/SAR/1/06 rabbit antisera

including SAT3/KNP/10/90 antiserum were made (polyvalent rabbit antisera or Rb3), by mixing equal volume of monovalent antiserum. Microtitre plates (NUNC-Immunoplate F96 Maxisorp) flat bottom were coated with a two-fold dilutions series, from 1:100 to 1:204800 of the antiserum. A sandwich ELISA was performed to determine the optimum concentration of polyvalent antisera. Homologous virus preparation (SAT3/KNP/10/90) was used for the titration. The concentration of trapping antibodies was measured at optical density of 492 nm.

2.9.2. Titration of guinea-pig antiserum

In order to determine the optimum concentration of the detecting antibodies (secondary antibody or guinea pig antisera), to improve the current LPBE, a pool of the 1:10 stock of the SAT3/BOT/6/98, SAT3/SAR/1/06 and SAT3/KNP/10/90 guinea-pig antisera were prepared (guinea-pig polyvalent antisera or Gp3), by mixing equal volume of each monovalent antiserum stock. Two-fold dilutions ranging from 1:500 to 1:16000 of this serum were prepared. A cross-titration using sandwich ELISA was performed, where the coating sera (polyvalent antiserum or Rb3) and homologous antigen (SAT3/KNP/10/90) were used at predetermined optimum dilution to titrate Gp3. This detecting antibody was added after the addition of the antigen trapped by the detecting antibody (rabbit antisera), with the same specificity as the rabbit coating antisera. Then, Goat anti-guinea pig immunoglobulin G (IgG) conjugated to horseradish peroxidise (HRP), was added to the deteting antibody.

2.9.3. Titration of positive sera (control sera)

Serum obtained from cattle used as positive controls was titrated using sandwich ELISA. Table 4 illustrate the reagents dilutions used for titration of the positive sera (cattle sera) controls.

Table 4: Optimum dilutions for coating, typing and control sera used in the LPBE

Reagent	Strain (SAT3)	Optimum dilution	Function
Polyvalent rabbit antisera (Rb3)	BOT/6/98 SAR/1/06 KNP/10/90	1:2500	Trapping antibody
Polyvalent guinea-pig antisera (Gp3)	BOT/6/98 SAR/1/06 KNP/10/90	1:4000	Detecting antibody
FMDV antigen (Ag)	KNP/10/90	01:20	Antigen
Positive control reference serum	KNP/10/90	01:20	Positive control
Negative control reference serum	NBS	01:20	Negative control
Goat anti-guinea pig immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP)	-	1:80	Detecting bound IgG antibodies

2.10. Liquid phase blocking ELISA

This study describes the improvement of an existing liquid phase blocking ELISA (LPBE) for the detection of antibodies against SAT3 viruses in infected or vaccinated animals. An ELISA incorporating more than one SAT3 antigen was used to detect antibodies raised against the various antigenic subtypes of SAT3 field strains. Constant pre-titrated antigen was incubated in the LPBE with serial dilutions of test sera, and the results were considered valid by evaluating the OD values of the control antigens (Hamblin *et al*., 1986a). Results obtained by the new LPBE and the current LPBE in use at TADP laboratory were compared.

2.10.1. Principle of LPBE

The test is based upon specific blocking of liquid phase FMD antigen by antibodies (figure 4) in the sample to be tested (Hamblin *et al.*, 1986a; b). Rabbit antiserum raised to specific FMDV serotypes is passively adsorbed to polystyrene microwells to coat with the specific FMD antigen. A serial dilution of serum is made and specific FMD antigen is added and incubated in liquid phase and allowed to react. Subsequently, the serum/antigen mixture is transferred to an ELISA plate coated with FMDV serotype-specific trapping antibodies. If

antibodies to FMDV are present in the serum sample, they will bind to the trapping antibodies adsorbed into the polystyrene plate and immune complex will form. Following incubation period, plate is washed and a detecting antibody (pre-blocked with non-immune bovine serum of the same specificities as the trapping antibody is added which will react. Subsequently, specie-specific horseradish peroxidase conjugate is added after incubation period. Colour develops after the addition of substrate/chromogen solution when compared to controls containing free antigen only. If antibodies are present in the serum sample, they will block the antigen preventing it from binding to the coating antibody, resulting in no colour development. If there are no specific antibodies in the sera then the antigen will be available to be trapped onto the plates, this will be detected by development of colour indicating negative results. Suitable washing procedures to remove unbound reagents are essential at each step in the solid phase.

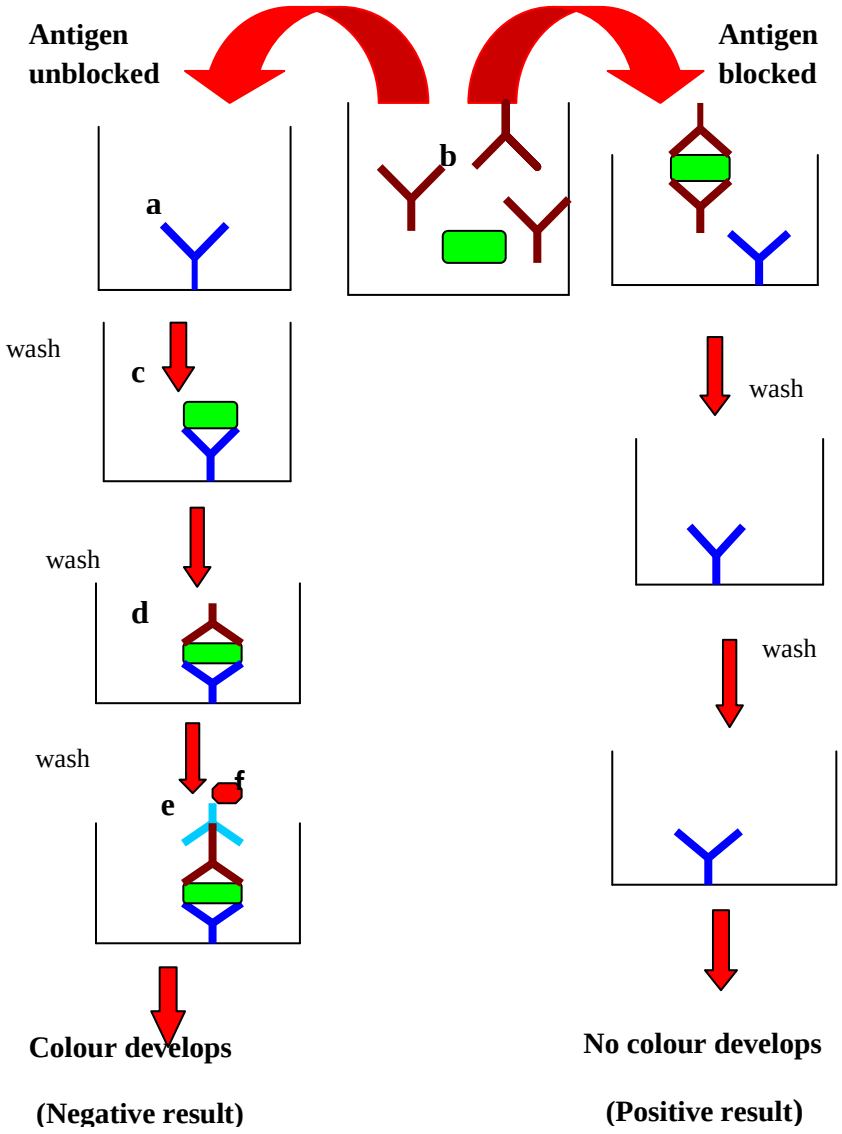


Figure 4: Flow diagram of the liquid phase blocking ELISA. Trapping Antibody (a); Serum sample antibody (b); Test and control serum incubation (liquid phase antigen) (c); Detector antibody (d); Conjugate (e); Substrate chromogenic (f)

2.10.2. LPBE Procedures

2.10.2.1. Coating of ELISA microplates

Rabbit antiserum to the 146S antigen of the selected SAT3 serotypes of FMDV was used as trapping antibody at a predetermined dilution. The stocks of the 1/50 diluted coating

rabbit sera were thawed just before use. All coating serum was used at a final dilution of 1:2500. Polystyrene microplates (Nunc Immunoplate - Maxisorp flat bottomed ELISA plate) were coated with 100 µl of the predetermined dilution of the mixed three SAT3 rabbit antisera (SAR/1/06; BOT/6/98 and KNP/10/90) in carbonate/bicarbonate buffer at pH 9.6 (appendix 1.9) (OIE, 2012), and left at room temperature for 24 h on the bench (figure 4a). The plates were washed for three cycles in an automated microplate washer with 1x PBS with 0.1 % (v/v) Tween (1x PBS-Tween; wash buffer) (appendix 1.11), dried and stored at -20ºC until required. At this temperature, plates can be stored for long periods of time.

2.10.2.2. Test and control serum incubation

Half titrations of control and test sera were prepared in microplates to determine the immune status of the samples. The optimal concentrations of all produced reagents were determined before performing the test. Afterward, different samples were tested per plate.

Reference control sera and test sera were added to the wells of polypropylene U-bottom microplates at a dilution of 1/20 (figure 4b). At least 100 µl of diluted serum and controls were dispensed in duplicate. Subsequently, 50 µl volumes of 2 % (w/v) casein in 1x PBS were dispensed. Control and test sera were titrated in duplicate wells against a reference antigen in a polypropylene U-bottom plate starting with initial dilution of 1:20 to 1:160. Twofold dilution series were made by transferring 50 µl from row to row consecutively, and discarding 50 µl from the last dilution.

2.10.2.3. Addition of FMDV antigen

Binary ethyleneimine-inactivated antigen was diluted (table 4) in 2 % (w/v) casein in 1x PBS (appendix 1.10) and 50 µl of the working dilution added to all 96 wells of the polypropylene U-bottom microplate, followed by incubation for 1 h at 37ºC on an orbital shaker (figure 4c).

2.10.2.4. Transference of the serum/antigen mixture to the ELISA plate

Fifty microliters of serum/antigen mixture was transferred from propylene plate to the corresponding wells of the coated ELISA plates (NUNC Maxisorp), and incubated overnight

at 4ºC. The microplates were washed for four cycles in an automated microplate washer with PBS-Tween (appendix 1.11).

2.10.2.5. Addition of detecting antibody

Guinea-pig antisera prepared by inoculating guinea-pigs with 146S antigen of the selected SAT3 viruses and preblocked with normal bovine serum (NBS) were used as detecting antibody (figure 4d). The detecting guinea-pig sera (designated Gp3) used was of the same specificity as the rabbit coating serum on the microplate. In order to avoid background reactions, these sera were treated with NBS to make 1:10 treated working stock and stored at -20ºC. Then, 50 µl volumes of guinea-pig serum at a pre-titrated optimal dilution (table 4) were dispensed in all wells of the plate and incubated for 1 h at 37ºC on an orbital shaker, and washed in an automated microplate washer with PBS-Tween (appendix 1.11) for four cycles.

2.10.2.6. Addition of conjugate

The anti-species conjugate is a Goat anti-guinea-pig immunoglobulin G (IgG) conjugated to horseradish peroxidease (Chemicon-Millipore AQ108P). To 1ml of commercial conjugate, 90 ml 0.2 % casein in PBS, (appendix 1.8) and 10 ml of NBS were added and stored in aliquots of 1 ml (stock) at -80ºC. Prior to use one ampoule was thawed and 50 µl volumes of 1:80 diluted in PBS-casein (0.2 % casein in PBS, appendix 1.10) were dispensed in all wells (figure 4e). The plates were incubated at 37ºC for 1 h on an orbital shaker, then washed in the automate microplate washer with PBS-Tween for four cycles.

2.10.2.7. Addition of substrate and stop solutions

Freshly prepared substrate [30 % (m/v) Hydrogen peroxide (Merck), blue substrate buffer: Citric acid monohydrate (Merck); Tri-Potassium citrate (Merck)] and Chromogen solution consisting of: N,N- Dimethylacetamide (Merck); Tetrabutylammonium borohydride (Sigma-Aldrich) and 3,3’5,5’-Tetramethylbenzidine (Sigma-Aldrich)] (appendix 1.15) was prepared just prior to use in the ELISA. A volume of 100 µl was added to each wells of the microplate (figure 4f). The reaction was allowed to proceed for 10 minutes at room temperature, and then stopped with the addition of 50 µl of 1.25 M Sulphuric acid (98 %;

Merck) solution (appendix 1.16). Absorbances were read in a Thermo Multiskan EX microplate reader with an interference filter of 450 nm.

2.11. Validation of liquid phase blocking ELISA

The goal to test validation is to estimate sensitivity and specificity with regards to clinical diagnosis, surveillance and risk assessment (Paweska *et al*., 2005a). The internationally accepted tests for the detection of antibodies to the structural proteins of FMDV, as prescribed by the OIE, are VNT and LPBE. While the VNT is considered the Gold Standard test, the LPBE is generally used for the routine screening of large numbers of field samples. To develop and validate the new SAT3 LPBE it was essential to optimize and standardise all the reagents necessary for antibody detection (OIE, 2012).

2.11.1. Foot-and-mouth disease free cattle

A total of 1398 serum samples from FMDV free cattle collected in FMD-free area of Northern Cape Province in South Africa were tested by the new LPBE for FMDV antibody and the specificity (proportion of cattle that tested negative by the assay) was estimated. The confidence interval was calculated using mid-P exact methods.

2.11.2. Foot-and-mouth disease virus infected cattle

A total of 515 sera from exposed cattle collected in Mpumalanga province during 2011-2012, stored at -20°C, were tested by the new LPBE for FMDV antibody. The repeatability of the new assay was estimated by testing two aliquots and calculating the coefficient of variation. The relative sensitivity and specificity of the current SAT3 LPBE was estimated. Agreement between the new assay and the current SAT3 LPBE was estimated using the kappa statistic. The strength of agreement was determined based on the following Kappa values: $\leq$ 0.20 poor agreement, 0.21-0.40 fair agreement, 0.41 - 0.60 moderate agreement, 0.61 - 0.80 good agreement, and 0.81-1.00 very good agreement (Altman, 1991). The antibody titre distribution of the new and the current SAT3 LPBE were compared using the Wilcoxon signed-rank test.

2.11.3. Foot-and-mouth disease virus vaccinated cattle

Relative sensitivity and specificity to the current SAT3 LPBE was estimated by testing 286 cattle sera samples, collected in three rounds, in FMDV vaccinated cattle from Mnisi, neighbouring area of Kruger National Park. Agreement between new assay and the current LPBE was estimated using the kappa statistic. The titre distributions of the new assay and the current LPBE were compared using Wilcoxon signed-rank test. Figure 5 presents study region in which diptanks (red dots) where chosen to collect sera used in this study.

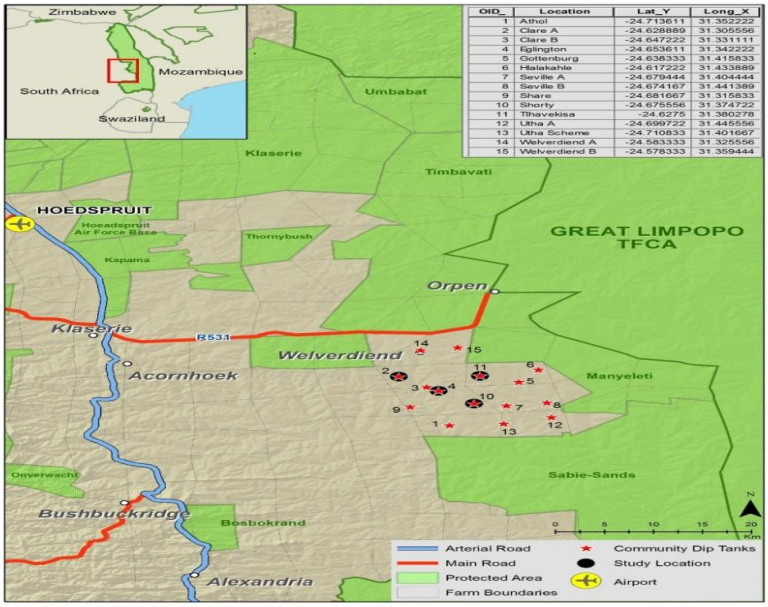


Figure 5: Mnisi region orientation map. The map presents study region in which diptanks (red dots) where chosen to collect sera samples used in this study

Chapter 3
Results

3.1. Introduction

Paweska and co-workers (2005b) in a study for ELISA validation reported that ELISA is safe, robust and highly accurate and can be used in early diagnosis of infection, disease surveillance and for monitoring of immune response in vaccine. Dekker and collaborators (1995), in their paper, on validation of LPBE, collected sera from two farms where an outbreak had occurred, evaluated the specificity and sensitivity of two distinct ELISAs and the results tests were compared with the results obtained by VNT.

Procedures for the validation of a serological assay for the diagnosis of infectious disease are complex. The main consideration of assay validation is the capacity of the test result to predict accurately the infection or exposure status of the animal or population of animals as positive or negative. Assay performance characteristics should be accurate and resultant from testing samples from reference animals of known infection status relative to the disease or infection and relevant to the region in which the test is to be used (OIE, 2012).

3.2. Phylogenetic analysis of SAT3 serotype

Phylogenetic trees show the relationship between viruses strains (Saiz *et al*., 1993; Hemadri *et al*., 2000; Knowles & Samuel, 2003), and are useful to study the source of an outbreak (Samuel & Knowles, 2001a; Domingo *et al*., 2003). Phylogeny based on the outer-capsid protein, VP1, shows that SAT3 viruses in southern Africa exist as four genetic groups, I to IV, with high levels of bootstrap support. The groupings based on genetic analysis corresponds to geographically distinct regions (figure 6), in accordance to the FMD topotype concept as it applies to European and SAT virus types (Bastos *et al.*, 2001; Samuel and Knowles, 2001b). These four topotypes are distributed in southern Africa as follows: (1) topotype I in South Africa and southern Zimbabwe; (2) topotype II in Namibia, Botswana and western Zimbabwe; (3) topotype III in Malawi and northern Zimbabwe, (4) topotype IV in Zambia (Bastos *et al*., 2003; Knowles and Samuel, 2003).

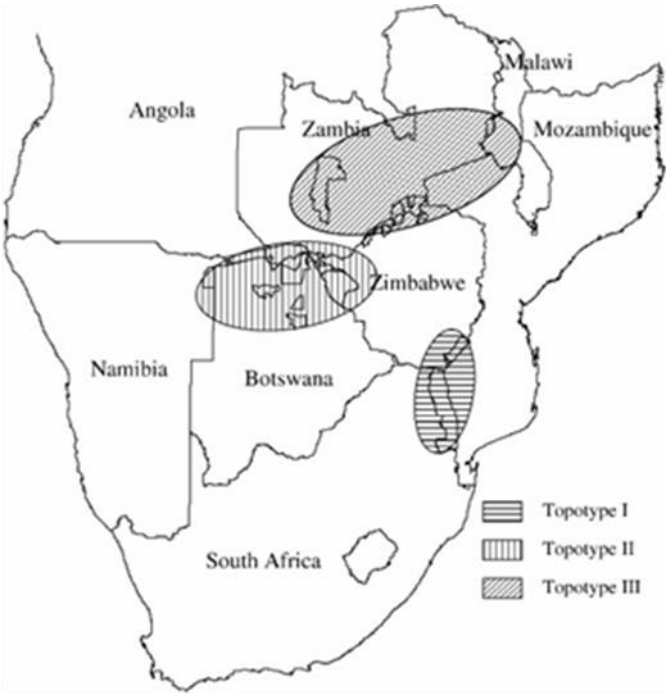


Figure 6: Map of southern African game parks indicating the geographical distribution of topotypes in distinct regions. Original taken from Bastos *et al.*, 2001

A dendogram derived from nucleotide sequence of the VP1 gene of FMDV serotype SAT3, illustrating genetic relationship between SAT3 viruses selected in this study is presented in the figure 7.

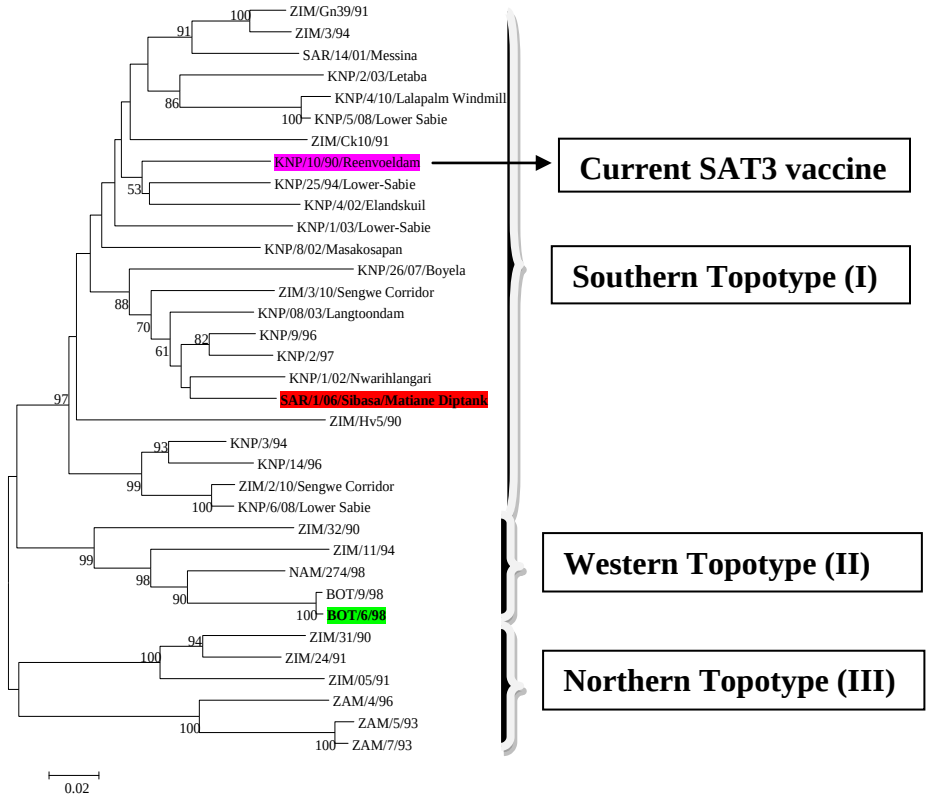


Figure 7: Dendogram derived from nucleotide sequence of the VP1 gene of the FMDV serotype SAT3. SAT3/SAR/1/06 strains from Sibasa Matine diptank (topotype I); SAT3/BOT/6/98 strain from Botswana (topotype II) and SAT3/KNP/10/90/Reevoeldam (topotype I), the current vaccine.

From the phylogenetic tree, three isolates were selected for the development of an improved VP3 LPBE, based on (i) the most recent emergence in the field and (ii) representatives of topotypes most relevant to South Africa. These include SAT3/SAR/1/06, a strain from an outbreak at Sibasa Matine diptank (topotype I); SAT3/BOT/6/98 from Botswana (topotype II) and SAT3/KNP/10/90/Reenvoeldam (topotype I), the current vaccine strain in use in the southern Africa region. These strains were selected to include representatives of the topotypes existing in the southern Africa region, to be included in the ELISA.

Determination of the mean p-distance between the SAT3 topotypes indicates a minimum sequence divergence of about 18 % on nucleotide level. Within the four southern African topotypes, multiple genotypes were identified with viruses of the same genotype generally sharing 90 % or more sequence identity.

The viruses selected as representatives of the three relevant topotypes were passaged in monolayer cell cultures and titrated. According to the Karber (1931) method (appendix 1.6), the SAT3/BOT/6/98 and SAT3/SAR/1/06 viruses titres calculated were $10^{5.1}$ $TCID_{50}$ and $10^{5.19}$ $TCID_{50}$ respectively. These titres were used to seed BHK-21 monolayer 850cm^3 cell cultures roller bottle.

3.3. Antigen preparation and concentration

Foot and mouth disease virus (SAT3/BOT/6/98, SAT3/SAR/1/06 and SAT3/KNP/10/90) for production of a vaccine batch were acquired at the TADP laboratory strain collection of the ARC-OVI. The SAT3 viruses were selected based on the phylogeny to represent topotype I and II. All three viruses replicated well in cultured cells.

Antigen preparation was performed in monolayers of BHK-21 (ATCC) cells for the SAT3/BOT/6/98 and SAT3/SAR/1/06 viruses. Initially both viruses were harvested and purified, without inactivation, to determine the effectiveness of live virus recovery. This allowed us to facilitate troubleshooting. Between the initial harvest and sucrose gradient purification there was no significant loss of virus. The SAT3/BOT/6/98, SAT3/SAR/1/06 and SAT3/KNP/10/90 146S particle purifications were repeatedly successful and a summary of the data is given in Table 5. The purified 146S antigens were used as positive controls in the new LPBE assay. Therefore, the SAT3/BOT/6/98 and SAT3/SAR/1/06 146S antigen provided low optical density as a result, compared to the results obtained when used SAT3/KNP/10/90 146S particles as positive control for the new LPBE. Thus, the SAT3/KNP/10/90 146S particles were used as positive control for the improved LPBE.

Table 5: Indicating summary of the titers of SAT3/BOT/6/98, SAT3/SAR/1/06, NKP/10/90 positive controls and FBS as negative control

Reagent	ID	Titer
SAT3/BOT/6/98	1084	2.5
SAT3/SAR/1/06	1070	2.4
SAT3/KNP/10/90	Positive control	> 3.1
FBS	Negative control	< 1.3

The same purification protocol was applied in the preparation of inactivated antigen. Table 6 is a summary of the data obtained in separate inactivation experiments. Inactivation

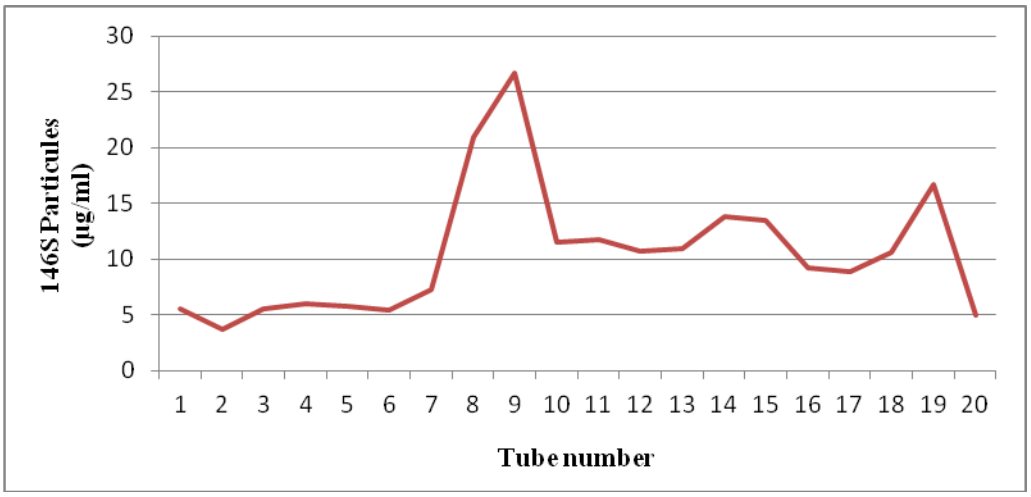
was performed with 5 mM BEI for 28 hours at 28°C, and from Table 6 it is clear that the yield of 146S particles (determined from OD_{259nm} readings of sucrose fractions), was taken at the peak fraction (tube number 9), considered the fraction with high antigen concentration (purified antigen), without proteins, suitable for vaccine preparation. Those fractions that form a peak consistent with the 146S component were pooled, to determine the amount of the antigen.

Table 6: Summary of the data obtained in separate inactivation experiments. SAT3/BOT/6/98 and SAT3/SAR/1/06 146S particle purifications

SAT3/BOT6/98			**SAT3/SAR1/06**		
Tube n.	λ1 259 ηm	λ1 280 ηm	Tube n.	λ1 259ηm	λ1 280 ηm
1	0.029	0.036	1	0,023	0.036
2	0.019	0.032	2	0.019	0.031
3	0.029	0.039	3	0.022	0.030
4	0.031	0.040	4	0.026	0.039
5	0.030	0.041	5	0.029	0.044
6	0.028	0.036	6	0.027	0.061
7	0.038	0.044	7	0.048	0.610
8	0.109	0.090	8	0.060	0.065
9	**0.139**	**0.105**	9	**0.162**	**0.121**
10	0.060	0.062	10	0.073	0.076
11	0.061	0.066	11	0.058	0.068
12	0.056	0.066	12	0.061	0.067
13	0.057	0.062	13	0.061	0.070
14	0.072	0.073	14	0.067	0.072
15	0.070	0.072	15	0.078	0.084
16	0.048	0.059	16	0.061	0.075
17	0.046	0.063	17	0.049	0.069
18	0.055	0.079	18	0.063	0.091
19	0.087	0.123	19	0.083	0.119
20	0.126	0.160	20	0.012	0.164

The amount of 146S particles, was determined (figure 8), from OD_{259nm} readings of sucrose fractions. The peak fraction (tube number 9), considered the fraction with high antigen concentration, without proteins, was selected for vaccine preparation.

A



B

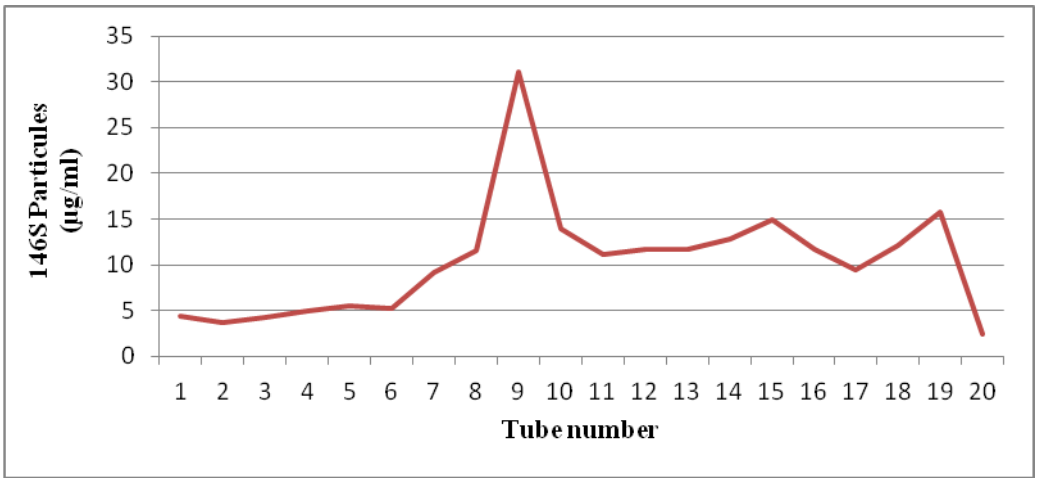


Figure 8: Graphs ilustrating the concentrarion of the purified 146S particles (purified antigen). A: Concentration of SAT3/ Bot6/98; and B: SAT3/SAR1/06

A vaccine batch of SAT3/BOT/6/98 and SAT3/SAR/1/06 was subsequently prepared in *ca.* 1.5 x 10^8 BHK-21 cells/roller, infected at a MOI of 1:10 for 17 hours. Following treatment with 5 mM BEI at 28°C for 28 h the viruses was successfully inactivated. During inactivation of the viruses, samples were taken at 24h and 26h for the purpose of monitoring inactivation process. To confirm inactivation (absence or freedom from infectious virus), the samples were inoculated in BHK-21 monolayer cell cultures and incubated at 37ºC for 72 h in the presence of CO_2. Then examined by inverted microscope and no CPE was observed. It was concluded that the virus was completely inactivated. At the end of the inactivation period, 0.2 % of 50 % sodium thiosulphate solution was added to neutralize the inactivant (BEI) for further inactivating the antigen.

The antigen was concentrated by 8 % PEG precipitation and purified by sucrose density (10 - 50 %) gradient (SDG) centrifugation. The gradient was fractionated and the fractions were read on a spectrophotometer and verified by SDS-PAGE analysis. A fraction with high antigen concentration, which contains no background proteins (table 6, tube number 9), was used to determine the 146S value for further processing. The reading at 259 nm was multiplied by 127 (extinction coefficient E_{259nm} = 79.9; Doel & Mowat, 1985) to determine the antigen concentration in µg/ml (SDG fraction 8: 0.79 x 127= 100 µg/ml). The integrity of the antigen was verified by ELISA and indicated that a high concentration of immunologically relevant antigen was purified.

Vaccine batches for SAT3/BOT/6/98 and SAT3/SAR/1/06 was prepared to inoculate groups of two rabbits and ten guinea pigs. The ratio of the aqueous antigen to the oil adjuvant was 50:50. A volume of 1:1 of chemically inactivated antigen in Montanide ISA 206B oil adjuvant (consisting of 5 ml of 146S antigen diluted in PBS and mixed in to 5 ml of Montanide ISA 206B oil adjuvant) and emulsified. This is equivalent to 100 µg/ml, to stimulate antibody production and increase the duration of immunity of FMD vaccine.

3.4. Preparation and titration of rabbit antisera

In order to produce trapping antibody coated onto the microtitre plates used in the improved LPBE, rabbit monovalent antisera were prepared by inoculating two rabbits with 100 µg antigen of each of SAT3/SAR/1/06, SAT3/BOT/6/98 and SAT3/KNP/10/90 vaccines. The animals were boosted 28 days post-inoculation with 50 µg in 0.5 ml of 146S antigen without adjuvant. Ten days later, the rabbits were sedated and bled and the serum collected. The rabbit antisera were used as trapping antibodies in the LPBE.

To determine the optimum concentration of trapping antibodies in the LPBE, a pool of the SAT3/BOT/6/98, SAT3/SAR/1/06 and SAT3/KNP/10/90 antisera were prepared by mixing equal volume of monovalent antiserum. A sandwich ELISA was performed to determine the optimum concentration of polyvalent antisera. The optimal dilution to be used was 1:3200. Since this dilution gave low readings, it was necessary to raise the concentration of the rabbit antiserum by adjusting to 1:2500 (figure 9). The latter was found to offer higher readings. The guinea-pig antiserum gave higher readings when used at final dilution of 1:4000 (table 4).

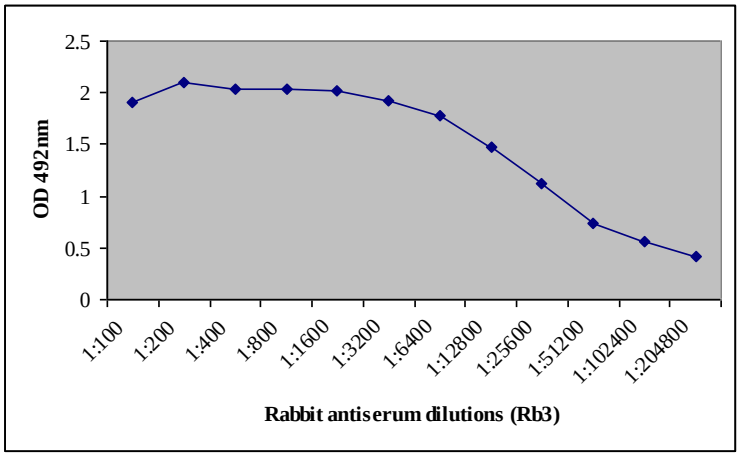


Figure 9: Titration of the polyvalent rabbit coating antisera (Rb3), composed of SAT3/BOT/6/98, SAT3/SAR/1/06 and SAT3/KNP/10/90 in a sandwich ELISA

A sigmoid shaped curve was obtained. The plateau area of the curve represents the area where the highest binding capacity of the capture antibody was exceeded and the optimum amount of antigen was trapped by the coating plate. The linear area of the curve represents the diminishing amount of trapping antibody that was no longer sufficient to trap the antigen optimally. The optimum concentration of rabbit antiserum for coating plates was regarded as the highest dilution where the maximum amount of antigen could be trapped. The dilution used to coat the plates as trapping antibodies in the LPBE was 1:2500 (table 4).

3.5. Preparation and titration of guinea-pig antisera

To produce detecting antibody used in the improved LPBE, guinea pig monovalent antisera were prepared by inoculating ten guinea pigs with 100 µg antigen of each of SAT3/SAR/1/06 and SAT3/BOT/6/98 vaccines. The animals were boosted 28 days post-inoculation with 50 µg in 0.5 ml of 146S antigen without adjuvant. Ten days later, the guinea pigs were sedated and bled and the serum collected. The guinea pig antisera were used as detecting antibodies in the LPBE.

To determine the optimum concentration of detecting antibodies in the LPBE, a pool of the SAT3/BOT/6/98, SAT3/SAR/1/06 and SAT3/KNP/10/90 antisera were prepared by mixing equal volume of monovalent antiserum. A sandwich ELISA was performed to determine the optimum concentration of polyvalent antisera. The optimum dilution (figure 10) at which the guinea pig antisera should be used was regarded as that dilution where the highest possible value is obtained in the ELISA. The optimum concentration at which guinea-pig antisera were diluted was 1:4000 (table 4).

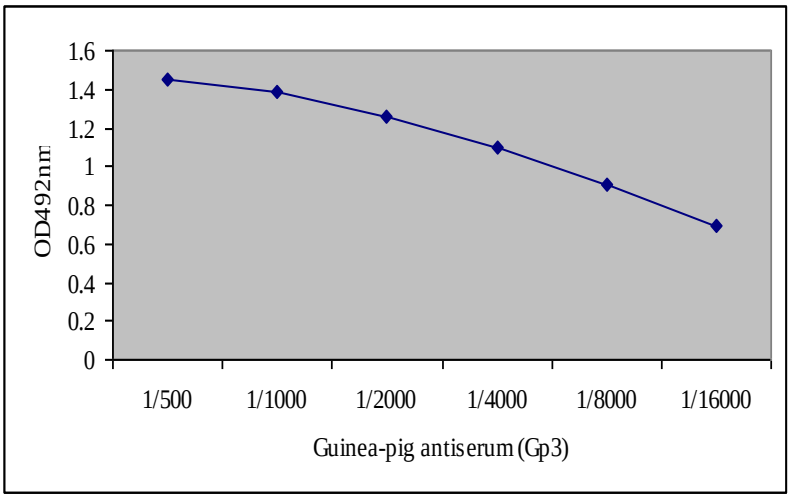


Figure 10: Titration of the pooled SAT3/BOT/6/98; SAT3/SAR/1/06 and SAT3/KNP/10/90 guinea-pig (Gp3) in a sandwich ELISA.

3.6. Statistical analysis

The purpose of the present study was to improve the current LPBE, by incorporating suitable SAT3 antigens (SAT3/BOT/6/98, SAT3/SAR/1/06 and SAT3/KNP/10/90) in the assay, to represent antigenic variants representative of topotypes I and II prevalent in southern Africa and to increase the sensitivity of the assay. To evaluate the sensitivity and the specificity of the improved LPBE, the results were compared with those of the current LPBE.

Diagnostic sensitivity and specificity were calculated relative to the current SAT3 LPBE. Relative specificity and sensitivity to the current SAT3 LPBE was estimated, agreement between new assay and the current LPBE was estimated, and the titre distributions of the new and the current SAT3 LPBE were compared. Sera were tested for antibodies against FMDV at a screening dilution of 1:20 in LPBE. Known sera from FMDV infected cattle from Mpumalanga province; sera from FMD free cattle from Northern Cape region available at TADP laboratory sera bank, and sera from FMDV vaccinated cattle collected in Mnisi area were used to validate the proposed improved LPBE.

3.6.1. Foot-and-mouth disease free cattle

Seventeen out of 1386 FMD free cattle sera from Northern Cape Province (figure 11) had titres equal or greater than 1.6 cut-off. Therefore, the specificity of the new LPBE was 98.8 % (98.1, 99.3) considering a 95 % confidence interval.

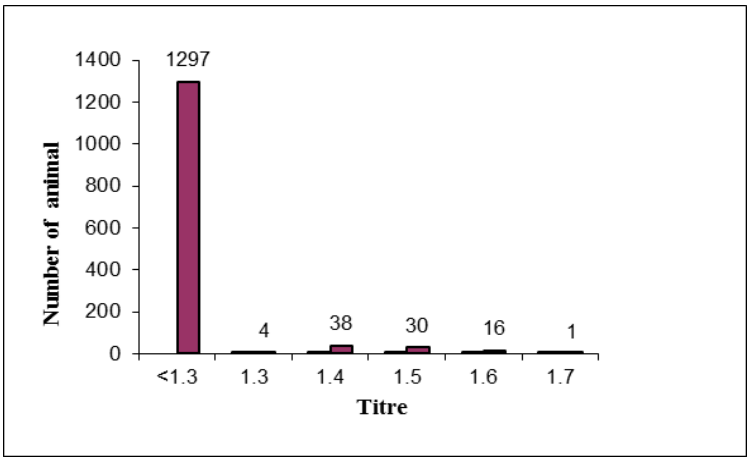


Figure 11: Histogram illustrating the distribution of the values, obtained from FMD free cattle in the Northern Cape Province.

3.6.2. Foot-and-mouth disease infected cattle

Regarding the repeatability of the assay, the estimated coefficient of variation (figure 12) range from 0 - 26.2 % and the mean was 1.4 %. Comparison to the current SAT3 LPBE as the reference test, 303 sera were positive based on cut-off ≥ 1.6, and 212 sera were negatives based on cut-off < 1.6; the relative sensitivity estimated was 75.2 % [70.2 %, 79.9 %] and the relative specificity estimated was 80.7 % [74.9 %, 85.6 %]. The agreement between the new assay and the current SAT3 LPBE was 77.5 %, considering cut-off of 1.6; Kappa = 0.546 (0.460 - 0.632). Considering P < 0.001, the distribution of values of the new assay and the current SAT3 LPBE are not the same.

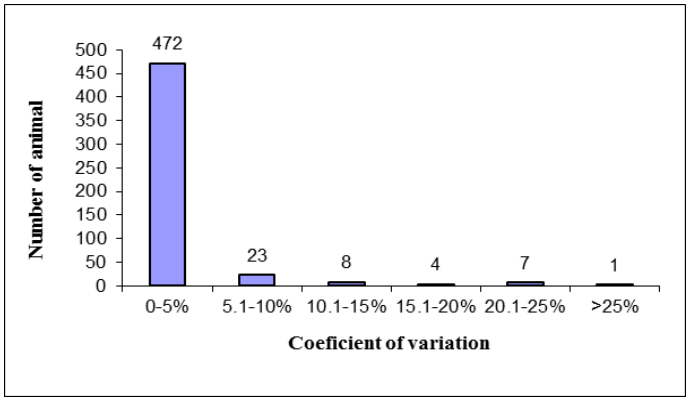


Figure 12: Histogram presenting the covariance obtained from FMDV infected cattle in Mpumalanga province. Comparison to the current SAT3 LPBE as the reference test

The scatter plots (figure 13) are the comparison of results from both tests. Each point represents the values from both assays. Each animal had 2 titers (new LPBE and the current LPBE). The titers were plotted on the graph. For a single point, to the y-axis, that is the value on new LPBE assay. From the point down, to the x-axis, that is the titer on the current LPBE on that same sample. The difference in the location along the axes is the difference in the results between the 2 assays. From the graph, it is clear that the distributions of values are not the same, meaning that the new LPBE assay is tending to have much higher values. This could be related to a greater good sensitivity rather than simply a poor specificity.

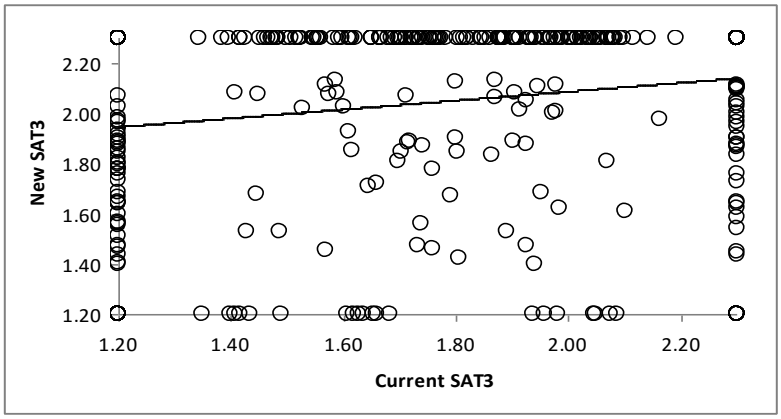


Figure 13: Scatter plot comparing the new SAT3 LPBE results to the current assay using FMDV infected cattle sera from Mpumalanga province

3.6.3. Foot-and-mouth disease vaccinated cattle sera

Comparison to the current SAT3 as the reference test (figure 14), 68 sera were positive based on cut-off ≥ 1.6 and 218 sera were negative based on cut-off < 1.6. The relative sensitivity estimated was 86.8 % [77.1 %, 93.3 %] and relative specificity estimated was 15.1 % [10.8 %, 20.4 %]. The agreement between the new assay and the current LPBE was 32.2 % based on cut-off of 1.6, Kappa = 0.010 [- 0.041, 0.061], P = 0.349.

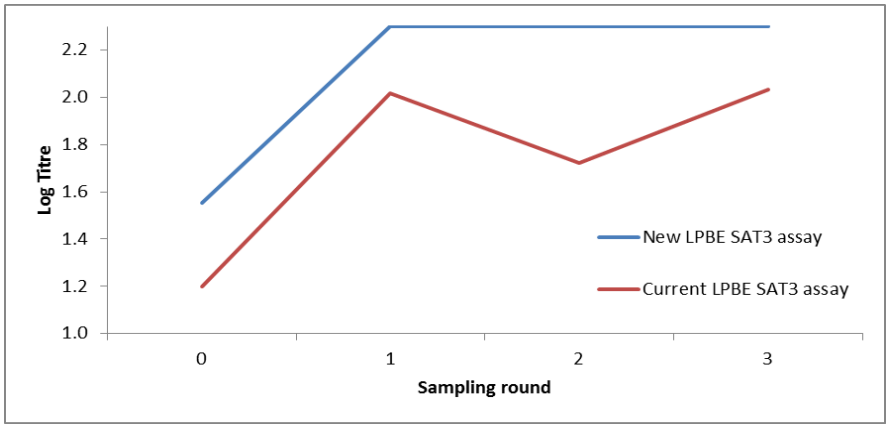


Figure 14: Graph showing the sera titres obtained from vaccinated cattle in Mnisi area. The same sera were tested by the current and the new LPBE assay

Assuming that all animals 2 weeks after vaccination had a titre ≥ 2.0, then the sensitivity and statistical comparison is as follows for the 109 cattle tested at round 1: Current SAT3 LPBE: Sensitivity (titre 1.6) = 58/109 = 53.2 % [43.8 %, 62.4 %] and the new SAT3 LPBE: Sensitivity (titre 1.6) = 84/109 = 77.1 % [68.5 %, 84.2 %]. These are statistically different using $P < 0.001$.

Chapter 4
Discussion

Because FMD in ruminants cannot be distinguished from others vesicular diseases based on clinical signs alone, laboratory identification of the agent involved is essential for diagnosis. The improvement of techniques in this field is of great importance for prevention, control and eradication programs of FMD. The current in-use LPBE performed in the laboratories has solved problems associated with VNT for the diagnosis of FMD by using antisera adjusted to an optimum dilution against homologous antigen. In addition, the ELISA detects smaller amounts of antibodies and is more sensitive than VNT tests.

The Liquid phase blocking ELISA is one of the simplest and fastest among different immunoassay techniques used for the detection of antibodies, but it can be difficult to validate due to signal amplification of both specific and non-specific components. The major problem hindering the diagnostic application of a LPBE for FMD has been the extensive spectrum of genetic and antigenic variability of the FMDV and the continuous appearance of new mutants from populations that escape the host immune response (Domingo *et al*., 1997; Haydon *et al*., 2001). Production of viral antigen stocks for a LPBE requires propagation of live virus and chemical inactivation by BEI, as the source of antigen. To overcome the problem of low sensitivity, an ELISA that utilises an antibody system to trap the antigens, it was necessary to select the appropriate SAT3 viruses based on the phylogeny which represents topotype I and II and create a broad spectrum of the SAT3 antigen detection in the new assay.

Outbreaks of FMD in domestic animals in southern Africa is associated with the presence of African buffaloes (*Sincerus caffer*) (Dawe *et al*., 1994b; Bastos *et al.*, 1999), which are efficient maintenance host (Vosloo *et al.*, 1996; Bastos *at al.*, 2003), and they have been demonstrated to be persistently infected with SAT-type viruses (reviewed by Thomson *et al.*, 2003), and play a key role in the epidemiology of the disease due to their capability to maintain and spread FMDV (Vosloo *et al*., 1996; Bastos *et al*., 2003; Maree, 2014). A liquid phase blocking ELISA for the detection of antibodies to FMDV is the OIE recommended standard for the serological diagnosis of FMDV (OIE, 2012). This assay plays a key role towards the control of FMD and is valuable in establishing FMD free status in animals destined for export from South Africa or import into the region.

It is known that high genetic and antigenic variants occur within each SAT-type (Bastos *et al.*, 2001, 2003; reviewed by Maree *et al*., 2011, 2014). Samuel and Knowles

(2001b), based on phylogenetic analysis of the outer capsid-coding genes have showed that the FMDV serotypes have distinct lineages which correlate with the serotypes. Looking at the cross-protection provided by a FMD vaccine against emerging field strains, it was therefore decided to investigate the sensitivity of the LPBE using homologous antigens. The sensitivity of the LPBE is dependent on the antigen used in the test and the ability of the sera from infected animals to cross-react with the antigen. Therefore, an ELISA test based on one antigen may not adequately detect antibodies raised against the various antigenic subtypes of FMDV field strains.

Considering the extensive antigenic variability that occurs between SAT-types of FMDV, it was important to use similar SAT3 antigens. As the SAT3/KNP/10/90 is the current vaccine strain, and used as reference antigen in LPBE at TADP, it was used as antigen against rabbit antibody (trapping antibody) in the improved LPBE. In accordance with the FMDV, geographically and genetically different groups are known as topotypes (Knowles and Samuel, 2001b). By using three antigenically different strains for SAT3 serotype viruses it was possible to improve the current LPBE to detect accurately antibodies to any SAT3 isolate from southern Africa. Three SAT3 virus isolates (SAT3/BOT6/98, SAT3/SAR/1/06 and SAT3/KNP/10/90) were selected to represent topotypes I and II. These strains were adapted on IB-RS-2 monolayer cells and propagated on BHK-21 C13 monolayer cells.

The three viruses used in this study were chemically inactivated with BEI and purified 146S particles were obtained. Vaccine was formulated and rabbits and guinea-pigs were vaccinated. Antisera raised from these animals were titrated to determine the optimal antibody concentration to be used in the LPBE. A cocktail of SAT3/BOT6/98; SAT3/SAR/1/06 and SAT3/KNP/10/90 antisera from rabbits was made. A sandwich ELISA was used to determine the optimal dilution of all reagents.

To validate the assay, 515 known bovine sera from FMDV infected cattle from the Mpumalanga province, 1398 known bovine sera from FMDV uninfected cattle from the Northern Cape and 286 sera from FMDV vaccinated cattle were tested by the new LPBE against homologous antigen, and the results of the test compared with the results obtained by the current LPBE. The analytical specificity of the multiple SAT3 antigens ELISA was confirmed in this study. The ELISA was effective to recognize antibodies present in the sera, produced by multiple homologous antigens used in this study.

The diagnostic accuracy of the multiple SAT3 antigens ELISA was also confirmed in this study. Validation of the test is necessary to determine reliable estimates of diagnostic

sensitivity and specificity (diagnostic accuracy) with respect to clinical diagnosis, risk assessment and risk-factor studies (Jacobson, 1998). The current international recommendations for the validation of serological assays for the diagnosis of infectious diseases are complex and subject to many limitations, including availability of standards and representative reference sera (OIE, 2012). The ultimate goal of assay validation is to provide test results that identify objects as positive or negative, and by inference accurately predicts the infection status of individuals with a predetermined degree of statistical certainty. The determination of the performance characteristics of a diagnostic assay should be based on testing samples from individuals of known infection status relative to the disease of interest. A number of standards can be applied to verify the infection or exposure status of animals (Jacobson, 1998; OIE, 2012).

In this study, the existing mono-antigen based LPBE was used as relative standard of comparison to distinguish animals according to their FMD infection status. The test is serotype-specific and detects antibodies elicited by vaccination and infection (Hamblin *et al*., 1986; 1987). This test is serotype-specific, on condition that the virus or antigen used in the test is closely matched to the strain circulating in the field. Low titre false-positive reactions can be expected in a small proportion of the sera in the assay (OIE, 2012).

The proportion of known infected reference animals that provide positive results in the assay is the diagnostic sensitivity; infected animals that offer negative results are considered to produce false negative results. The proportion of uninfected reference animals that produce negative results in the assay diagnostic specificity is the diagnostic specificity; uninfected reference animals that give positive results are considered to produce false positive results. The number and origin of reference samples used to originate diagnostic sensitivity and diagnostic specificity are of greatest importance for proper assay validation (Jacobson, 1998). A high sensitivity and type specificity can be achieved in a diagnostic test since the appropriate reagents and test formulation are selected (Roeder & Le Blanc, 1987). The percentage recommended for the repeatability of the assay should be < 20 %, and, the repeatability for the new LPBE assay was less than 20 %. The value of 77.5 % determined was considered absolute agreement. Kappa = 0.546 (0.460 - 0.632), is the amount of agreement expected by chance alone.

Comparison to the current SAT3 LPBE suggests that the new assay is tending to have much higher values in vaccinated population (figure 12). This could be related to a greater true sensitivity (86.8 % [77.1 %, 93.3 %]) rather than simply a poor specificity (15.1 % [10.8 %, 20.4 %]). The agreement between current and new SAT3 LPBE of 32.2 % based on cut-

off of 1.6 was considered absolute agreement. Using $P < 0.001$ the distributions of the values are not the same.

Chapter 5
Conclusions

Foot-and-mouth disease (FMD) virus occurs as a multiple serotype causing a highly contagious disease and great economic loss in susceptible cloven-hoofed animals. Livestock farming forms the backbone of rural economies for most of the SADC member countries. More than 75% of livestock is raised under the communal smallholder systems and sustains livelihoods of vulnerable groups such as women and children. Southern Africa has been distinctively endowed with an abundance of wildlife which has been well protected within national parks and game reserves. Inevitably, in communities within the proximity of these parks and game reserves, wildlife-livestock interface presents unique challenges to livestock disease control. Of particular note is the presence of the three SAT-type FMD viruses (FMDV) which are maintained within buffalo (*Syncerus caffer*) populations. This unique situation has necessitated that SADC countries invest in regular vaccination programmes if they are to effectively manage FMD and consequently participate in international and regional trade in livestock and livestock products.

FMDV exists as distinct genetic and antigenic variants within the seven serotypes. Infection with one serotype does not confer immunity against another. An indispensable component of the disease control consists of diagnostic assay to rapidly confirm the early clinical determination of infection. It is of particular importance since other vesicular diseases (such as swine vesicular disease, vesicular stomatitis and vesicular exanthema of swine) cause vesicular lesions in swine and cattle that cannot be distinguished from those caused by FMD (Bachrach, 1968; Ma *et al*., 2011). Also, FMDV infection of sheep and goat can be difficult to identify clinically (Geering, 1967). The improvement of techniques in this field is of great importance for prevention, control and eradication programs of FMD. Sensitive diagnostic assay are necessary to distinguish vaccinated from infected or convalescent animals, so that trade market can be possible to countries that may have used vaccination as part of their disease control strategy and to detect carrier animals. Besides, this assay can be used for epidemiological surveillance to confirm the naïve status in field situations.

The liquid phase blocking ELISA for detection of antibodies to FMD virus is OIE recommended standard for the diagnosis of FMDV. Liquid-phase-blocking ELISA is the current conducted serological test for FMD. The test is highly sensitive and adaptable to sera

samples of susceptible species, and appropriate for testing herd samples (Dekker *et al*., 1995). This assay is fast and reliable and plays a key role toward the control of FMD. The sensitivity of the LPBE is dependent on the antigen used in the test and the ability of the sera to cross-react with the antigen. Therefore an ELISA test based on one antigen may not adequately detect antibodies raised against the various antigenic subtypes of FMDV field strains. This is substantiated by in vitro virus neutralization (VN) studies where sera raised against existing vaccine viruses does not sufficiently cross-react with emerging viruses within the same serotype. Challenge with simultaneous infection and vaccination, and accurate assessment to susceptible animal in an extensive range is urgent for the decision of the following control methods but also difficult due to lack of effective investigation approach. These obstacles make the search for stable and safe test become a dynamic subject of investigation (Ma *et al*., 2011).

In southern Africa, the SAT serotypes of FMDV occur with high prevalence. The disease needs to be controlled and prevented through vaccination. Post-vaccination monitoring is essential to determine the level of herd immunity. Improvement of the current SAT3 liquid phase blocking ELISA to enhance the control of FMD in SADC was the main objective in this study. The LPBE is used to detect antibodies raised against FMDV. The current SAT3 LPBE only uses one strain to detect antibodies to the various antigenic subtypes of SAT3.

Because of antigenic variants existing within each of the SAT-types, the sensitivity of the current LPBE for the detection of specific antibodies was improved by incorporating additional SAT3 isolates (homologous antigens), to represent topotypes I and II prevalent in southern Africa. Virus strains were selected from phylogenetic analysis database existing at TADP for SAT3 serotype in order to provide a broader coverage in this study, using two SAT3 topotypes (I-II) that occur in southern Africa. The FMD SAT3 viruses were adapted and propagated on monolayer of IB-RS-2 cells, and then on BHK-21 cells until 100% CPE was observed. The supernatant was harvested, clarified and inactivated with binary ethyleneimine. Inactivated antigen was precipitated with polyethyleneglycol and then purified by sucrose density gradient centrifugation and vaccines formulated to inoculate animals.

Reagents (positive sera, antisera) to strains of FMD SAT3 virus were raised in rabbits and guinea pigs by immunization with inactivated purified 146S antigen. Then optimal dilutions (working concentrations) of all reagents: capture antibody; detector antibody and virus antigen were determined. Known positive and negative sera from the Mnisi region were used to validate the proposed newly developed LPBE by comparison to the current LPBE at

TADP. The newly developed LPBE was validated using three groups of sera: FMD free cattle sera; FMD infected cattle sera and FMD vaccinated cattle sera. The assay performance characteristic (specificity and sensitivity) of the LPBE were determined. To determine the distribution of values of the assay, Wilcoxon signed-rank test was used, and the correlation of the assay was determined based on Spearman's rho.

The new SAT3 LPBE assay is tending to have much larger values in vaccinated population (higher sensitivity for detecting vaccinated animals). This could be related to a greater true sensitivity rather than simply a poor specificity. The new assay has higher sensitivity for detecting vaccinated animals. The results confirm the greater sensitivity of the new LPBE, 77.1 % [68.5 %, 84.2 %] positive in comparison to the current LPBE 53.2 % [43.8 %, 62.4 %] positive, performed at TADP laboratory. The inclusion of additional antigens in the new LPBE resulted in a diagnostic assay which is antigenically more diverse than the current LPBE, thus increasing the sensitivity of the assay across the range of genetic and antigenic variability in SAT3 serotype. Thus, the new LPBE would increase the success rate of antibody detection from serum sample. This would greatly benefit the diagnosis, screening antibodies in animal sera before export, epidemiological studies and vaccine potency testing and monitoring the efficacy of vaccination. This assay is suitable for large-scale evaluation of the effectiveness of the disease control measures adopted in response to an outbreak.

In conclusion, the improved LPBE reported in this study, taking into account the antigenic diversity within the SAT serotypes, the sensitivity of the SAT specific LPBE was improved by incorporating the appropriate SAT3 strains, representative of the antigenic variants that occur within southern Africa. This makes the diagnostic assay antigenically more diverse than the current LPBE, by increasing the sensitivity of the assay across the range of genetic and antigenic variability in SAT3 serotype. The new assay is considered a reliable method to evaluate protective antibodies induced by FMD vaccines, hence can be suggested for use either for assessment of vaccines or for monitoring the effectiveness and extent of a field vaccination program. In summary, this would benefit the control of FMD in the SADC.

For the next future, the newly developed LPBE and skills, which show great promise but is still in the early stages of development need to be confirmed by assay validity during routine use and enhance assay validation criteria, by monitoring and maintenance of the assay performance (precision and accuracy) and proficiency testing.

Limitations

One of the difficulties confronted during this work, was the adaptability of the selected SAT3 viruses to the tissue cultures, which required propagation of live virus and chemical inactivation by BEI, as the source of antigen. To overcome the problem of low sensitivity, an ELISA that utilises an antibody system to trap the antigens was necessary to amplify the selected antigens by tissue culture passage and testing the supernatant once a CPE has developed. Thus, to adapt the antigens, four blind serial passages were performed on IB-RS-2 monolayer cells culture, harvested, clarified at low speed centrifugation. Followed by another four passages on BHK-21 monolayer cell culture roller bottles to amplify the antigens. When 100% CPE was observed, the supernatant was harvested, frozen and thawed, and centrifuged at 8000 rpm for 30 minutes to eliminate cell debris, and kept at -70ºC for further inactivation.

Another constraint found was the high background which resulted in false readings leading to repeats of the test in some samples. The sera used as reagents (detecting and traping antisera) contain considerable quantities of antibodies that reacts against bovine IgG and others bovine serum components, due to the fact that the 146S particles inoculated in guinea-pigs were purified on sucrose gradients. This could be avoided by mixing of equal volumes of normal bovine serum with the guinea-pig antiserum and conjugate antiserum which are very important procedure for the quality of the ELISA (Roeder & Le Blanc, 1987).

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Appendix 1

1.1. Active Trypsin Versene (ATV)

Composition:

NaCl (Merck)	160.0 gr
KCl (Merck..	8.0 gr
NaHCO3 (Merck)	11.6 gr
Trypsin (1:250) (Sigma)	10.0 gr
D-Glucose (Merck)	20.0 gr
EDTA (BDH) .	5.0 gr
Phenol red 0.5 % solution	8.0 ml
Neomycin	10.0 ml
Streptomycin	10.0 ml
Penicillin	10.0 ml

Add deionised distilled water to a final volume of 2.0 litres.

After mixing, adjusted the pH to 7.1-7.2 with CO_2 or air.

Sterile filter used 0.2 µm.

Aliquots of 5 ml in sterile McCartney bottles and stored at -20°C.

1.2. Antibiotics preparation

Penicillin (Sterile)

Penicillin (Benzylpenicillin) (Sigma)	30.0 gr
Distilled water (OIED)	1.0 L
Sterile filter used	0.2 µm

Aliquots of 5 ml in sterile McCartney bottles and stored at -2

Gentamycin (sterile)

Gentamycin	2.0 gr
Distilled water (OIED)	1.0 L
Sterile filter used	0.2 µm

Aliquots of 5ml in sterile McCartney bottles and stored at -20°C

1.3. Cells counting

To determine the percentage of viability within population cells trypan blue 0.25 % w/v in 1x PBS (Merck) solution was used for cell counting. The cells suspension were mixed with vital dye and observed under an inverted microscope (Olympus). Vital dye stains dead cells and exclude live cell.

A 1:10 dilution (100 µl cells suspension and 900 µl dye) were made and the cells counted on a haemocytometer.

1.4. Calculation of number of cells/ml

[(Total number of viable cells counted) / (Number of squares counted)] 10^6 cells/ml x dilution factor.

= (Total) 10^6 cells/ml to seed per flask

= (Total)10^6 cells/ml to seed per roller bottle / Number of days required to achieve confluence.

1.5. Methylene blue stain:

Methylene blue stain (1 % w/v; Merck)	1.0 gr
Ethanol (98 %)	100.0 ml
Formaldehyde (37 %; Merck) solution	100.0 ml
PBS	800.0 ml

Mixture well and store at room temperature.

1.6. Example of virus titre determination

$TCID_{50}$/ml = 50 per cent tissue culture infectious dose per ml

Calculation of a $TCID_{50}$ end point (Karber, 1931)

Example:

Virus dilution	Infected well ratio	Infected %
10^{-1}	8/8	100
10^{-2}	8/8	100
10^{-3}	8/8	100
10^{-4}	6/8	75
10^{-5}	3/8	37.5

[(Sum % infected wells at each dilution- 0.5) x (log dilution)] ÷ 100

= -1.0 – [(100 + 100 + 100 + 75 + 37.5)/100] - 0.5 x (log 10)]

= -1- [(4.1-0.5) x 1.0]

= -1 – 3.6

= -3.6

$TCID_{50} = 10^{-3.6}$

1.7. Determination of multiplicity of infection (moi)

(Number of roller bottles x cell count /bottle x 10^6 x moi)/virus titre (log_{10}/ml)

= Volume virus to seed/roller.

or

(Total volume x cell/ml x moi)/Virus titre

= Volume virus to seed /roller.

1.8. Phosphate buffer Saline (PBS)

Solution A:

Potassium chloride (Kcl; Merk)	8.0 gr
Sodium chloride (Nacl; Merck)	320.0 gr
Sodium hydrogen orthophosphate (Na_2HPO_4 H_2O; Merck)	115.0 gr
Potassium dihydrogenorthophosphate (KH_2PO_4; Analar)	8.0 gr
Phenol red (1 % solution)	40.0 ml
Distilled water	2.0 L

Aliquots of 50 ml stock were sterilised for 30 minutes, and kept at room temperature.

Solution B:

Calcium chloride ($Cacl_2$ $6H_2O$; BHD Prolabo)	2.6 gr
Distilled water	1.0 L

Aliquots of 25 ml stock were sterilised for 30 minutes, and kept at room temperature.

Solution C:

Magnesium chloride ($Mgcl_2$ $6H_2O$; Merck)	4.0 gr
Distilled water	1.0 L

Aliquots of 25 ml stock were sterilised for 30 minutes, and kept at room temperature.

Mixture solutions B, C and A in 900 ml distilled water. The pH automatically must be 7.3-7.5.This is ready to use.

1.9. Coating buffer (Carbonate/bicarbonate buffer 0.05 M)

Preparation of 10x concentrated stock:

1) Sodium hydrogen Carbonate ($NaHCO_3$ 0.5 M; Merck)	42.0 gr
Distilled water	1.0 L
2) Sodium carbonate anhydrous (Na_2CO_3 0.5M; Merck)	53.0 gr
Distilled water	1.0 L

Store at 4ºC.

Preparation just before to use:

Prepare 1/10 of both the 10x concentrates of $NaHCO_3$ and Na_2CO_3 by adding 100ml to 900ml distilled water to obtain 0.05M concentrations.

Mixture quantities of 0.05M Na_2CO_3 and 0.05M $NaHCO_3$ until pH 9.6.

1.10. PBS-Casein

To prepare 2% casein stock solution:

Casein (Sigma-Aldrich)/ (from bovine milk)	40 gr
Solution A	100.0 ml
Solution B	25.0 ml
Solution C	25.0 ml

Method:

Mixture distilled water with solutions B, C and A (2 litres of PBS). Heat the PBS to 60ºC. Add magnet stirrer and while stirring the PBS start adding the casein. As the casein dissolves, the pH must be set to neutral (red colour) by adding drops of 0.5M NaOH. The casein must be completely dissolved. Set the pH to 7.2 - 7.4. Aliquots of 80ml (stock) must be stored at -20°C.

To prepare 0.5 % casein for use:

Thaw 2% casein stock solution. Diluted each 80ml casein stock into 240ml prepared PBS. This is ready to use.

1.11. Washing buffer (PBS-Tween)

Potassium chloride (Kcl; Merck)	40.0 gr
Sodium chloride (Nacl; Merck)	1600.0 gr
Potassium dihydrogen orthophosphate (KH_2PO_4; Merck)	40.0 gr
Disodium hydrogen phosphate dodecahidrate (Na_2HPO_4 .$12H_2O$; Merck)	574.48gr
Distilled water (up to)	6.0 L
Tween 20 (0.05%)	100 ml

Set the pH to 7.4 - 7.6 (with Hcl/NaOH)

Aliquot in 175ml (stock) and kept at - 20°C until needed.

Dilute 175ml in 5L of sterile water. This is ready to use.

1.12. Blue substrate buffer

Solution A:

Citric acid monohydrate (Merck)	21.0 gr
Distilled water	1.0 L

Solution B:

Tri-Potassium citrate (Merck)	32.4 gr
Distilled water	1.0 L

Mixed solutions A and B to obtain pH 4.0 and stored at 4ºC.

1.13. Chromogen solution

N,N- Dimethylacetamide (Merck)	100.0 ml
Tetrabutylammonium borohydride (TBABH, Sigma-Aldrich)	0.21 gr
3,3'5,5'-Tetramethylbenzidine (Sigma-Aldrich)	1.0 gr

Stored in the dark at 2 - 8ºC.

1.14. Substrate

H_2O_2 30 % m/v (Merck).

1.15. Substrate/chromogen

Fresh preparation:

Chromogen solution	1.0 ml

Blue substrate buffer	100.0 ml
H_2O_2	50 µl

1.16. Stopping solution

1.25M H_2O_2 (98 %; Merck)	67.0 ml
Water with crushed ice	933.0 ml

Appendix 2

Plate layout for LPBE (screening test)

	Pos Ag	Neg.	Pos Ag	Neg.	Pos Ag	Neg.	Pos Ag	Neg.	Pos Ag	Neg.	Pos Ag	Neg.	Serum dilution
	1	2	3	4	5	6	7	8	9	10	11	12	
A	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	C-	C-	1:20
B	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	C-	C-	1:40
C	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	C-	C-	1:80
D	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	C-	C-	1:160
E	S6	S6	S7	S7	S8	S8	S9	S9	S10	S10	C+	C+	1:20
F	S6	S6	S7	S7	S8	S8	S9	S9	S10	S10	C+	C+	1:40
G	S6	S6	S7	S7	S8	S8	S9	S9	S10	S10	C+	C+	1:80
H	S6	S6	S7	S7	S8	S8	S9	S9	S10	S10	C+	C+	1:160

1-12	Column numbers
A-H	Rows
S1-10	Test serum
1:20 to 1:160	Serum dilution
C-	Control negative (NBS)
C+	Control positive serum (derived from cattle inoculated with SAT3/KNP/10/90)
Pos Ag	Positive antigen column (SAT3/KNP/10/90)
Neg.	Negative antigen column

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
ANIMAL USE AND CARE COMMITTEE
Private Bag X04
0110 Onderstepoort

Tel +27 12 529 8434 / Fax +27 12 529 8300
e-mail: aucc@up.ac.za

Ref: V032-12

1 August 2012

Dr F Maree
ARC-OVI (TADP)
Old Soutpan Road
Onderstepoort Veterinary Institute
(MareeF@arc.agric.za)

Dear Dr Maree

V032-12 : Improvement of the surrent SA-3 liquid phase blocking ELISA (LPBE) to enhance the control of FMD in SADC *(IV Anahory)*

The application for ethical approval, dated 30 March 2012 was discussed and approved by the Animal Use and Committee at its meeting held on 30 July 2012.

The following concerns were raised:

- The rabbits and guinea pigs even though they are separated, should not be kept in the same room. Cross contamination may occur if they are kept together. Additional stress is also caused having different animal species together.

It is suggested that either the experiments on the two species are separated in time or that arrangements be made to separate them by partitioning.

Kind regards

E Mostert

Elmarie Mostert

AUCC Coordinator

Copy Dr IV Anahory