Development of a serological based vaccine matching technique for Foot-and-Mouth Disease (FMD) SAT 2 viruses

by

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Supervisor: Dr PA Opperman
Co-supervisor: Dr LE Heath

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Declaration

I Ngwako Terrence Malesa, declare that the dissertation which I hereby submit for the degree MSc (Tropical animal health), in the Faculty of Veterinary Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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31 January 2024

Ethics statement

The author, whose name appears on the title page of this thesis, has obtained the required ethics approval/exemption for the research described in this work.

The author declares that they have observed the ethical standards required in terms of the University's Code of ethics for scholarly activities.

Abstract

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By

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For the degree MSc (**Tropical Animal disease**)

Foot-and-mouth disease (FMD) is a highly contagious viral disease that primarily affects domestic animals, including cattle, sheep, goats, and pigs. FMD virus is an RNA virus with seven serotypes, six of which are found in most of Africa. Africa relies on vaccination to control FMD, but antigenic variability among serotypes and subtypes limits cross-protection. Therefore, selection of vaccine strain that is antigenically close to the field virus is crucial for FMD control. Utilizing the relationship coefficient r_1-value, serological assays including virus neutralizing test (VNT), liquid phase blocking ELISA (LPBE), and solid phase competition ELISA (SPCE) have been crucial in vaccine matching. Since the year 2000, the antigenically diverse SAT 2 serotype, which has been responsible for the majority of FMD outbreaks, has not been effectively neutralized by trivalent FMD vaccines in Southern Africa, particularly in South Africa. Pentavalent FMD vaccines have been developed by the Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) in an effort to reduce and increase antigenic coverage against circulating and emerging SAT 2 strains. The study examined the FMD pentavalent vaccine's ability to protect against emerging and circulating SAT 2 strains using r_1-value antigenic matching. Firstly, SAT 2 virus prevalence in South Africa and

Zimbabwe was determined, yielding six strains (SAR/1/2001; SAR/1/2003; SAR/1/2013; SAR/15/2013, KNP/12/2008 and ZIM/2/2013). Secondly, the SPCE assay was optimised for vaccine matching due to the assay being robust in comparison to LPBE. Thirdly, involves assessing the cross-reactivity of SAT 2 viruses with sera (serum1-7) acquired from cattle. Serum sample 1-5 originated for cattle induced with pentavalent vaccine, while serum sample 6-7 where from not induced cattle. The serum sample 1-3 indicated high cross-reactivity resulting in high titre ranging $\log_{10}$ 2.8 and $\log_{10}$ 3.1 against the SAT 2 field viruses, while the serum sample 4-5 cross -reactivity were moderate with average titre ranging between $\log_{10}$ 1.4 and $\log_{10}$ 1.8 against the SAT 2 field viruses. Serum titre of serum 6-7 were between $\log_{10}$ 0.3 to $\log_{10}$ 0.8. and was expected. The titres resulted in r_1 -values; for SAR/1/2001 with the vaccine strain ranged between 0.65 to 1.19; SAR/1/2013 ranged between 0.54 to 1.17; ZIM/2/2013 ranged between 0.60 to 1.20; SAR/15/2013 ranged between 0.59 to 1.18; SAR/1/2003 range between 0.50 to 1.14; and KNP/12/2008 ranged between 0.54 to 1.15. R_1-values of the vaccine strain/homologous strain (SAR/03/2004) were 1.00 which was expected due to antigenic similarity existence between the strains. The r_1-values indicate a strong antigenic similarity between the SAT 2 field viruses and the pentavalent vaccine, suggesting that the pentavalent vaccine has the potential to provide protection against the selected SAT 2 field viruses. The optimised SPCE was instrumental in cross-reactivity of SAT 2 field viruses against sera. SPCE indicated the capability to determine the r_1-values which were significant in demonstrating antigenic similarity between SAT 2 field viruses and FMD pentavalent vaccine. Availability of such a SPCE assay as a vaccine matching tool will be valuable for the swift assessment of vaccine matching between a vaccine strain and circulating SAT 2 virus strains. This will provide immediate crucial information regarding the antigenic spectrum of the vaccine strain and whether the vaccine strain has the potential to provide for cross-protection or require optimization.

Dedication

This thesis is dedicated to almighty **God** and my late mother **Mrs. Moyahabo Victoria Malesa** who believed and invested in my education with hope that our circumstance will change for the better. May her soul rest in peace.

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List of Abbreviations

ARC	Agricultural Research Council
AEC	Animal Ethics Committee
BEI	binary ethylenimine
BD	Becton Dickson
BHK	baby hamster kidney cell line
BLAST	basic Local Alignment Search Tool
BSL-3	Bio-security level 3
˚C	degree Celsius
cDNA	complementary Deoxyribonucleic acid
cm	centi-meter
CPE	cytopathic effect
DE	diatomaceous earth
DNA	deoxyribonucleic acid
dpv	day post vaccinal
et al.	*et alia* (and others)
ELISA	enzyme linked Immunosorbent Assay
FMD	foot and mouth disease
FMDV	FMD virus
GMEM	Glasgow minimum essential medium
hpi	hour post infection
H_2SO_4	sulphuric acid
hrp	horse radish peroxidase.
IB-RS-2	Instituto Biologico-Rim Suino-2
Ig(A)	immune-globulin A
Ig(G)	immune-globulin G
Ig(M)	Immuno-globulin M
kbp	kilobase per
KNP	Kruger National Park
LPBE	liquid phase blocking ELISA

M	Molar
µg/ml	microgram per millilitre
ml	millilitre
MOI	Multiplicity of infection
mM	millimolar
NCBI	National Center for Biotechnology Information
No:	Number
nm	nanometre
OD	optical density
OIE	Office International des Epizooties (Known as WOAH)
OVR	Onderstepoort Veterinary Research
PCR	polymerase chain reaction
PEG	polyethylene glycol
PBS	phosphate buffer saline
PBS/T	1 x PBS containing 0.1 % (v/v) Tween 20
Pfu	plaque forming unit
%	Percentage
RGD	Arginine-Glycine-Aspartic
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
RPM	rotation per minute
SAT	South African Territories
SPCE	solid phase competition ELISA
TAE	tris-acetate-EDTA
TAD	transboundary Animal Disease
$TCID_{50}$	50 % tissue culture infective dose
TCC	Tissue cell culture
TPB	tryptose phosphate broth
™	trademark
VNT	virus neutralization test
v/v	volume per volume
VPg	viral genome linked protein
WOAH	World Organization for Animal Health

w/v weight per volume

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1. Literature View

1.1. Introduction of Foot and Mouth Disease

Foot and mouth disease (FMD) is a highly transmissible and economically important disease that affects a wide range of cloven-hoofed animals, such as cattle, sheep, pigs and goats (Coetzer et al., 1994, Hunter, 1998). In the late 19th century, the disease was widely spread throughout the world (Thomson et al., 2003) and it continues to be prevalent in developing countries, especially in Africa, South America, and Asia, where it is considered endemic (OIE., 2021, Santos et al., 2018). Seventy-five percent of the world's impoverished people live in developing countries, where they primarily rely on livestock farming for their nutrition (meat and milk), power for crop production, and animal trading (Perry and Rich, 2007, Rweyemamu et al., 2014). The prevalence of Foot-and-Mouth Disease (FMD) has a detrimental impact on the livelihoods of livestock farmers, especially in subsistence settings. This is primarily due to the negative effects it has on livestock productivity, leading to food insecurity and economic disadvantages due to trade restrictions for international markets (Thomson, 1995). Livestock farmers residing in countries endemic to FMD possess the capacity to enhance their livestock productivity and gain entry into the commercial export sector, thereby creating opportunities for additional revenue generation that may mitigate poverty and elevate their quality of life (Perry and Rich, 2007). The eradication and control of FMD continue to be shared objectives among countries with and without the disease (FAO/OIE, 2012, Thomson, 1995). The implementation of FMD vaccines has facilitated the eradication of FMD in certain regions of Asia, Western Europe, and North America (Diaz-San Segundo et al., 2017b, Thomson, 1995). However, the lack of effective FMD control measures such as animal movement control and inadequate support from various stakeholders results in increased disease outbreaks (Roberts and Fosgate, 2018). Furthermore, the absence of effectively coordinated vaccination strategies and the limited availability of appropriate FMD vaccines in regions where the disease is widespread continue to be barriers to the eradication of FMD entirely (Hunter, 1998, Rweyemamu et al., 2014, Diaz-San Segundo et al., 2017b).

Foot and mouth disease is highly infectious among cloven-hoofed animals, its causative agent is foot and mouth disease virus (FMDV) which is characterised by epithelial tissue infection causing the manifestation of vesicular lesions on the mouth, snout, feet, and mammary glands mainly in domestic livestock (Arzt et al., 2011b, Paton et al., 2018). It has been established that the severity of clinical manifestations in infected animals varies according to the species of

virus and host (Alexandersen et al., 2003). The acute infection phase of the infection in domestic livestock is characterized by a discernible severity that can result in myocarditis in young animals, leading to the mortality of a meagre proportion of the infected young (Belsham et al., 2020, Diaz-San Segundo et al., 2017b). On the contrary, morbidity observed in adult animals is generally diverse and persists for a period of two weeks, accompanied by negligible mortality (Santos et al., 2018). The infection significantly impairs the health of domestic livestock, rendering them vulnerable to secondary infections, causing weight loss, stunted growth, and diminished milk production in dairy cattle (Grubman and Baxt, 2004, Hunter, 1998, Belsham et al., 2020). Although the Office International des Epizooties (OIE: World Organization for Animal Health) has classified FMD as a list-A disease due to its ability to spread extensively and rapidly between regions and countries, the disease is not lethal (Vosloo and Thomson, 2017, Alexandersen et al., 2003).

Foot and mouth disease virus and equine rhinitis A are the prototype members of the *Aphthovirus* genus within the family of *Picornaviridae* (Fox et al., 1987, Mason et al., 2003). Morphology of FMDV virion has a non-enveloped icosahedral capsid structure of 30 nm that comprises of a long single-stranded positive sense ribonucleic acids (RNA) molecule of *ca.* 8500 nucleotides in length (Carroll et al., 1984, Fox et al., 1987, Galloway and Elford, 1931). FMDV RNA genome is flanked by dual untranslated regions (UTR), the 5'-terminus that is attached covalently to a small protein of 25-26 residues (VPg), and the 3' terminus linked to polyadenylated sequence, both UTR display a complex secondary structure (Carroll et al., 1984, Martinez-Salas and Belsham, 2017). Internal components of the RNA genome comprised of the poly (C) tract, the internal ribosome entry site (IRES) and an open reading frame (ORF) which encodes *ca*. 2332 amino acids that are cleaved into four structural proteins (VP1, VP2, VP3 and VP4) and several non-structural proteins that are involved in immune modulation and virus replication (Domingo et al., 2002, Forss et al., 1984).

Seven distinct FMD serotypes have been recognised; type O, A, C, Asia 1 and Southern African Territory (SAT) 1, SAT 2, SAT 3 (Bachrach, 1968). Each virus serotype has a myriad of virus variants (subtypes) which are attributed to the absence of proof reading mechanism during the virus replication phase (Bachrach, 1968, Alexandersen et al., 2003). This has a detrimental impact on the effectiveness of vaccines. Usage of vaccine to provide immunity against one serotype or convalescence from a certain serotype infection does not assure cross-protection against other serotypes or subtypes within each serotype (Alexandersen et al., 2003, Domingo et al., 2002). The African continent consists of six serotypes, excluding Asia-1, and the sub-Saharan Africa region exclusively harbours SAT types (Thomson, 1995).

The inclusion of wildlife, particularly buffalo, in the disease's epidemiology adds complexity to understanding the pathogenesis, persistence, and antigenic properties of the SAT serotypes (Bastos et al., 2000, Capstick, 1978, Maree et al., 2011, Thomson, 1995). Although SAT serotypes have been distinguished primarily by frequency in relation to incidence (Maree et al., 2016), antigenic profiling has revealed a wide range of antigenic variation among them (Bastos et al., 2003). SAT serotypes are associated with African buffalo (Syncerus caffer) in the southern African region because it maintains SAT serotypes and is the source of the majority of FMD outbreaks. As a result, this affects the expansion of antigenicity within SAT serotypes (Paton et al., 2018, W.Vosloo et al., 2002). The antigenic diversity of the SAT type viruses has not been extensively studied, unlike the A, O, and C serotypes. (Bastos et al., 2003). Since the year 2000, the SAT 2 serotype has been consistently identified as the most prevalent and causative agent of numerous FMD outbreaks in both livestock and wildlife in the southern African region, in contrast to SAT 1 and SAT 2 serotypes (FMD-Bulletin, 2010, OIE., 2021, Bastos et al., 2003, Dyason, 2010, Hall et al., 2013). The SAT 2 serotype consists of 14 distinct evolutionary virus lineages that are geographically clustered and referred to as topotypes (Maree et al., 2011). The presence of antigenic variation within these topotypes contributes to a lack of vaccine coverage, which may explain the increase in SAT 2 outbreaks (Maree et al., 2011, Rweyemamu et al., 2008). Furthermore, extensive antigenic variation has serious implications for the selection of the appropriate vaccine strain and its efficacy in controlling FMD (Maree et al., 2011, FMD-Bulletin, 2010).

Various countries, including the United States of America (Bachrach, 1968) and the United Kingdom (Thompson et al., 2002) have implemented FMD control measures such as stamping out or culling, decontamination, and quarantine. These measures have enabled these countries to reclaim their FMD-free status after an outbreak, which is required for international trade. However, the use of these measures caused a large public outcry in terms of animal welfare, especially during the 2001 FMD outbreak in the United Kingdom, when approximately 10 million cattle, sheep, and pigs were slaughtered and scorched (Feng et al., 2017, Rowlands, 2017). This prompted discussions on the use of vaccination in these countries, which eventually resulted in the authorization of countries in the European Union to use emergency vaccines as a prevention and control measure against FMD rather than the unpalatable culling method (Scudamore and Harris, 2002). However, routine vaccination has been used in South America, Asia and Africa and is currently the recommended used method to control, eradicate and prevent FMD in these countries (Goris et al., 2007). Extensive vaccination has the capacity to hinder the dissemination of FMD by interrupting the transmission of the virus among

susceptible animals and decreasing the infection rate during initial outbreaks (Hunter, 1998). Although vaccination does not provide absolute immunity against infection, it does decrease the spread of the virus to other cattle and hinder the development of severe clinical symptoms (Hunter, 1998, Belsham et al., 2020). The efficacy of a vaccine depends on the quality and suitability of the strain integrated into the vaccine, taking into account the specific geographical area where the vaccine will be administered (Goris et al., 2007, Thompson, 1994). Vaccine strain suitability is determined by the antigenic correspondence between the circulating field virus strain and the vaccine strain that is capable of delivering adequate protection against the emerging field virus strain (Paton et al., 2005). The current recommended FMD vaccine involves the appropriate selection of virus strain that is propagated and cultivated in baby hamster kidney (BHK) cells, and the culture is inactivated chemically using a binary ethyleneimine (BEI) (Doel, 2003, Belsham et al., 2020), usually all the processes occur in fermenter vessels (Telling and Elsworth, 1965). Although vaccination is the suggested method for controlling FMD, the FMDV genome of the seven serotypes and their subtypes exhibits significant antigenic diversity, especially in the P1 region (Domingo et al., 1990) . The P1 region contains the antigenic properties used in selecting vaccine strains, therefore affecting vaccine effectiveness (Mateu, 2017).

1.2 Properties of FMDV

1.2.1. FMDV classification and its RNA genome structure

The systematic nomenclature of picornaviruses was adopted during the third meeting on picornavirus Molecular biology in Italy in 1983 by European Study Group, and Rueckert and Wimmer formalized the systematic nomenclature (Rueckert and Wimmer, 1984). A uniform nomenclature approach was adopted because various nomenclatures were used at that moment, which confused researchers and complicated picornavirus teachings (Rueckert and Wimmer, 1984). The adopted nomenclature of picornaviridae was based on the order of the gene segment i.e.; the Leader region, which is a polyprotein that precedes the capsid protein; the P1 region which encodes structural proteins; the P2 region or the "midpiece" polyprotein and the P3 region (Rueckert and Wimmer, 1984). Viruses that follows these gene segment order include poliovirus, enteroviruses human rhinovirus, mengovirus, encephalomyocarditis (EMC) virus and FMDV classified within the *Picornaviridae* family in different genus group Aphthovirus (Forss et al., 1984, Mateu, 2017, Rueckert and Wimmer, 1984).

The organization and nucleotide sequences of the FMDV virus were established by S.Forss, K.Strebel, and H.Schaller (Forss et al., 1984). The complete sequence of the viral genome,

which consists of a long single-stranded positive sense RNA of approximately 8,500 nucleotides encoding both structural and non-structural proteins, was elucidated by A.R. Carroll, D.J. Rowlands, and B.E. Clarke (Carroll et al., 1984). Additional investigation conducted by D. Stuart and colleagues revealed that the FMD virion possesses a crystal structure with a nearly spherical shape and icosahedral symmetry, lacking an envelope (Acharya et al., 1989). This characteristic facilitated extensive crystallographic analysis to determine and compare various serotypes, including O (Acharya et al., 1989), A (Curry et al., 1996), C (Lea et al., 1994), and SAT 1 (https://www.rcsb.org/structure/2wzr, unpublished).

The atomic details revealed matured FMD virion being a roughly spherical with a smooth surface of 30 nm diameter capsid icosahedral symmetry structure. (Acharya et al., 1989, Forss et al., 1984, Galloway and Elford, 1931). The capsid is encapsulated by twelve pentamers that enclose the compactly organized single copy of viral RNA (Acharya et al., 1989). Twelve pentamers form the capsid, and each has a pentagon structure formed by five protomers held together by extensive non-covalent interactions in a five-fold axis (Acharya et al., 1989, Arnold and Rossmann, 1990). A protomer is formed by assembling a single copy of each of the capsid structural proteins VP1, VP2, VP3, and VP4, which interacts with the genome (Acharya et al., 1989). Proteins VP1, VP2, and VP3 have an antiparallel β-barrel structure and contain antigenic determinant sites on their outer surface (Acharya et al., 1989, Fry et al., 1999). FMDV capsid outer-surface structure proteins stimulate neutralizing and non-neutralizing antibodies, with the primary immunogenic determinant in the VP1 polypeptide (Ouldridge et al., 1984, Parry et al., 1989).

1.2.1.1. The FMDV genome contains an ORF region that encodes structural and non-structural proteins.

FMDV open reading frame (ORF) polyprotein is ca. 7000 nucleotides in length, is generated from the translation of RNA and is cleaved post-translationally (Grubman and Baxt, 2004, Belsham, 2005a). The ORF is preceded by 5’ end, known as the 5' untranslated region (UTR) polypeptide region, has an excess of ca. 1300 nucleotides that is linked to the short viral protein (VPg /3B) of 24–25 residues by a phosphodiester bond (Forss et al., 1984, Rowlands, 2008). Five components have been identified within the 5’UTR which include, the small (S)-fragment, the poly-cytidylate poly (C) tract, the RNA pseudoknot, the 3B uridylylation renowned as cis-acting replication element or *cre* site, and the internal ribosome entry sites (IRES) (Grubman and Baxt, 2004, Martínez-Salas, 1999). The precise functions of the S-fragment, poly(C), and pseudoknot components have not been clearly elucidated (Escarmís

et al., 1992, Grubman and Baxt, 2004, Mason et al., 2002, Mason et al., 2003). The function of the cis-acting replication element (cre) is required for RNA genome replication (Mason et al., 2003, Mason et al., 2002). The internal ribosomal entry site forms a secondary and tertiary high-order structure and instructs cap-independent translation of the viral RNA (Martínez-Salas, 1999). It contains a conserved polypyrimidine tract that is preceded by two in-frame initiation AUG codons, detached by 84 nucleotides, which are involved in the initiation of protein synthesis encoded in the translation ORF polypeptide (Beales et al., 2003, Martínez-Salas, 1999, Forss et al., 1984).

The ORF consists of four basic components: the leader protein (L), the viral capsid proteins (P1-2A), and the non-structural proteins (P2, P3) (Syed and Graham, 2013). The leader proteinase (L^{pro}), which is located at the N-terminus of the ORF and is related to papain-like cysteine proteases, comprises two conserved in-frame start codons (AUG) responsible for encoding two constituents of the L protein, namely Lab and Lb (Beck et al., 1983, Sangar et al., 1987). These distinct bicistronic initiation sites, separated by 84 nucleotides, permit the expression of virus genes and are therefore essentially involved in the inhibition of cellular protein synthesis (Cao et al., 1995). Lab and Lb together catalyse their proteolytic cleavage from the developing polyprotein at its carboxy (C)-terminus (Beck et al., 1983, Strebel et al., 1986, Vakharia et al., 1987b), and commence the excision of the eukaryotic translation initiation factor eIF-4G multiprotein complex (Devaney et al., 1988, Medina et al., 1993). Meanwhile, eIF-4G is a constituent of the cap-binding complex that is needed for translation of the majority of cellular mRNAs (Meerovitch and Sonenberg, 1993). Excision of eIF-4G ensues in shutting off the host cap-dependent mRNA translation (Devaney et al., 1988, Mayer et al., 2008) and is detected approximately 30 minutes after infection due to the significant decline in host synthesis. (Rueckert, 1996). It is presumed that the cessation of host protein synthesis within infected cells inhibits the production of alpha/beta interferon that has the ability to induce various cell responses, such as activation of antiviral protein and dsRNA-activated protein kinases that impede host and viral protein synthesis (Chinsangaram et al., 1999, Kaufman, 2000).

The P1 polyprotein, which is situated directly following the L protein, functions as the precursor for the immunogenic determinants for FMD, namely the viral capsid proteins VP1, VP2, VP3, and VP4 (Belsham, 1993, Domingo et al., 2002, Domingo et al., 1990, Grubman and Baxt, 1982, Sobrino et al., 2001). Structure proteins VP1, VP2, VP3, and VP4 are produced by processing the P1-2A precursor (Belsham, 2005a). The polyprotein is swiftly excised into proteins VP0, VP3 and VP1 by the virus-encoded 3C proteinase ($3C^{pro}$) (Bablanian and

Grubman, 1993b). Immediately following encapsidation of the RNA, VP0 is autocatalytically excised into proteins VP4 and VP2 (Harber et al., 1991, Lee et al., 1993). The FMDV structural proteins that have been processed self-assemble into an empty capsid (Abrams et al., 1995, Gullberg et al., 2013, Porta et al., 2013). This capsid is similar to that of a full virus, but it lacks infectious properties (Rowlands et al., 1975). A notable level of differentiation exists among these proteins, with the exception of VP4, which is an internally located and conserved protein. All picornaviruses possess the three surface-positioned proteins of the capsid, VP1-VP3, which each contain eight-stranded β-barrels (Logan et al., 1993). The identified antigenic sites have been associated with the most accessible outer-surface capsid proteins. (Fry et al., 2005, Logan et al., 1993). Using the neutralizing resistant mutant approach with monoclonal antibodies (mAbs), the locations of FMDV's neutralizing antigenic sites have been determined. This method has identified crucial amino acid residues (VP1, VP2, and VP3) of each capsid protein that are involved in antigenicity (Fry et al., 1999, Logan et al., 1993).

Serotypes O, A, and C have played a pivotal role in the identification of numerous immunologically significant sites located on VP1, VP2, and VP3 proteins (Crowther et al., 1993, Kitson et al., 1990, Mateu et al., 1995). VP1 proteins contain immunogenic and dominant antigenic sites (epitopes), with the carboxy terminus and βG-βH loop contributing to the antigenic sites (McCullough et al., 1985). VP1 protein, which is composed of amino acid residues with highly variable structures and is the most exposed surface of the virus particle, utilized the G-H loop (G-H loop), a protruding, highly mobile loop that is responsible for virus attachment and entry .(Acharya et al., 1989, Fry et al., 1999, Logan et al., 1993). The G-H loop, which is a flexible and disorderly external loop, exhibits significant variation in length and sequence across different serotypes of FMDV (Curry et al., 1996, Fox et al., 1989, Lea et al., 1994, Logan et al., 1993, Mason et al., 1994). Notably, situated at its apex is a sequence of three amino acids known as Arg-Gly-Asp (RGD), which is exceptionally conserved and plays a pivotal role in virus attachment and entry (Fox et al., 1989, Logan et al., 1993). The stability of these FMDV RGD triplets is maintained by the disulphide bonding of amino acid residues 145–146 in the β-sheet interaction (Logan et al., 1993). The VP1 surrounds the capsid's five-fold axes, while VP2 and VP3 alternate around its three-fold axes (Fry et al., 2005), and acid-induced disassembly has been attributed to the histidine-rich region at the VP2/VP3 interface (Ellard et al., 1999). Potential dissociation between pentamers may result from electrostatic repulsive forces across pentamers induced by acidic conditions in these residues to a pH below 6.5 (Acharya et al., 1989, Curry et al., 1995, Ellard et al., 1999). The myristate group, present at the amino N-terminus of the VP4 protein, an intracellular component, has been

suggested to have a substantial impact on the assembly and stability of the capsid (Acharya et al., 1989, Chow et al., 1987).

The 2A protein, the precursor linked to the P1 polyprotein after primary cleavage (Vakharia et al., 1987b), is short with 18 amino acids and lacks a protease motif (Belsham, 2005b, Donnelly et al., 1997, Robertson et al., 1985). The 2A protein seems to be proteolytically active itself (Ryan et al., 1991) and its 18 amino acids sequence catalysed the secondary primary cleavage at the 2A and 2BC junction using alternative mechanism of translation referred as ribosomal skipping instead of proteolytic cleavage (Rowlands, 2008). The 2BC protein components of the P2 polyprotein precursor are proteolytically processed by the 3C protease into the 2B and 2C protein products (Rueckert and Wimmer, 1984). Limited research has been done on the 2B and 2C proteins and their functions are not yet clearly elucidated in the FMDV, although in picornaviral, the 2B and 2C proteins have been associated in virus-induced cytopathic effects (CPE) (Grubman et al., 2008, Martinez-Salas and Belsham, 2017).

The FMDV P3 polypeptide precursor undergoes proteolytic cleavage by the 3C protease ($3C^{pro}$) to yield four mature proteins: 3A, 3B, $3C^{pro}$, and $3D^{pol}$ (Belsham, 2005b). The FMDV 3A protein contains a hydrophobic sequence and it's role has been implicated in viral replication and predicted to be a membrane anchor for the picornavirus replication complex (Xiang et al., 1997, Martinez-Salas and Belsham, 2017). It is linked to viral-induced membrane vesicles and supports CPE and suppression of protein secretion (Doedens and Kirkegaard, 1995, Wessels et al., 2006), including FMDV pathogenesis (Mason et al., 2003, Nunez et al., 2006). During RNA synthesis, the 3A protein is implicated in transporting the 3B gene to the membrane vesicles, which are the sites where RNA replication occurs, in order to act as a primer (Moffat et al., 2005). The 3B gene encode the three copies of the VPg protein, which are linked to the 5' terminus of RNA polypeptide and are considered to function as a primer for RNA synthesis and encapsidation of viral RNA (Barclay et al., 1998, Hogle et al., 1985, Xiang et al., 1997). The proteinase 3C ($3C^{pro}$) is a cysteine protease (Birtley et al., 2005) task for catalysing 10 of the 13 proteolytic excision occurrence that are essential for polyprotein processing (Vakharia et al., 1987a, Clarke and Sangar, 1988). Although the $3C^{pro}$ does not process the excision of L^{pro} from P1, 2A from P1-2A, and the maturation cleavage of VP0 into VP4 and VP2 (Bablanian and Grubman, 1993a), however is capable of modifying certain cellular protein (Belsham, 2005b). Protein $3D^{pol}$ is greatly maintained in both nucleotide and amino acid sequence between the different FMDV serotypes (George et al., 2001, Martínez-Salas et al., 1985), and is the RNA-dependent RNA polymerase (RDRP), responsible for RNA synthesis and VPg Uridylylation. (Belsham et al., 2000, Rowlands, 2008). The ORF is linked 3'untranslated region

polypeptide with poly (A) tract participates in genome circularization and other processes (Barton et al., 2001, Herold and Andino, 2001). Poly (A) has no role in FMDV antigenic activity and is thought to contain cis-acting sequences needed for replication (Grubman and Baxt, 2004, Mason et al., 2003).

1.3. FMD transmission and VP1 protein as virus entry site

FMDV is contagious in a wider range of cloven-hoofed animals, so disease transmission and pathogenesis have been studied more in cattle than pigs or small ruminants and wildlife (Arzt et al., 2011a). The general ways diseases spread among ruminants and from pigs to ruminants are through infected animals interacting with susceptible animals and through aerosol produced by infected animals (Donaldson and Alexandersen, 2002). Typical pathogenesis is characterised by high fever and vesicular lesions within the epithelial areas including mammary glands, feet, nasal and oral cavity. Variability of transmission have been stated between the cattle and pigs, where pigs have been characterised as airborne emitters of droplets-nuclei during exhaling excreting high amount of aerosol containing FMDV (Donaldson and Alexandersen, 2002, Sellers and Parker, 1969). The pigs are typically infected through direct or indirect routes, such as through skin cuts or by consuming FMD-contaminated food products, rather than through respiratory infection (Alexandersen et al., 2003). However, the cattle are susceptible to a significant degree of infection via the respiratory tract, specifically in the pharyngeal area (Alexandersen and Mowat, 2005). A substantial quantity of virus is released by vesicular lesions throughout the acute phase of infection (Alexandersen and Mowat, 2005, Donaldson and Kitching, 1989). A minimal amount of virus entering through the respiratory tract or damaged skin can trigger an infection, during which the virus quickly reproduces and disseminates throughout the body (Donaldson and Alexandersen, 2002, Pacheco et al., 2010). The virus during the acute phase of cattle infection can be identified in the bloodstream, organs, tissues, secreted semen, milk, saliva, as well as excretions such as faeces and urine (Bachrach, 1968).

The depth of elucidation of the natural mechanism of infection and dissemination of the virus between animals is still lacking. However, *in vitro* studies have shown that during infection, the FMD virus attaches to cell culture receptors through the mediation of the highly conserved RGD tripeptide (Arg-Gly-Asp) motif on the G-H loop of the VP1 capsid protein located on the surface of the virus particles (Rowlands, 2003, Mateu et al., 1994). All FMDV serotypes have a conserved RGD tripeptide motif and bind to cell culture as an integrin receptor (Acharya et al., 1989, Fry et al., 1999, Rowlands, 2003). The RGD tripeptide motif sequence was initially

identified by Geysen as occupying positions 145 -147 of VP1 (Geysen et al., 1985). Trypsin has been demonstrated to affect FMDV infectivity by hydrolysing the amino acids of the RGD sequence, which disrupts the ability of cell attachment (Strohmaier et al., 1982). Multiple cell lines, including epithelial, endothelial, fibroblast, Madin-Darby bovine kidney cells, primary lamb kidney cells, primary bovine cells (BTY), and the porcine kidney cell line Instituto Biologico Renal Suino-2 (IB-RS-2), have been shown to utilize the RGD tripeptide motif for cell attachment. Attachment is facilitated via a subset of immunoglobulins or integrins located on the cell surfaces (Fox et al., 1989, Berinstein et al., 1995, Burman et al., 2006). Integrins are heterodimer type 1 membrane glycoproteins that are constituted of two components (α and β), and FMDV integrins utilised for cell attachment are $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$, which interact noncovalently at the cell surface (Fry et al., 1999, Berinstein et al., 1995, Jackson et al., 2004). The virus RGD motif interacts with cell receptor integrins to stimulate clathrin-dependent endocytosis into early sorting and recycling endosomes, where low pH dissociates virus capsid and viral RNA, which is transferred into the cytosol by an unknown mechanism (Baxt, 1987, Berryman et al., 2005, O'Donnell et al., 2005). The antibody-complexed virus uses heparan sulfated (HS) and Fc receptors instead of integrins (Mason et al., 1993).

Several studies have linked heparan sulfate to cellular receptor mediation, including the Chinese hamster ovary (CHO) cell line and Baby hamster kidney cell (BHK 21), which is preferred for FMDV vaccine production (Jackson et al., 1996, Rieder et al., 1994, Maree et al., 2010). Heparan sulfate is a glycosaminoglycan (GaGs) polysaccharide, which is classified as a proteoglycan and is composed of hydrogen-sulfated polymers of replicated disaccharides that are found in carbohydrates (Jackson et al., 1996). Heparan sulfate carries a negative charge (Fry et al., 1999, Kjellén and Lindahl, 1991) and binding involves electrostatic interactions among the positively charged amino acid residues lysine and arginine group on the FMDV capsid surface and negatively charged N and O-glycan sulfated group of GAG molecule (Byrnes and Griffin, 2000, Fromm et al., 1995). The process by which FMDV capsid amino acids acquire a positive charge involves serial passaging of the virus in tissue cultures (Fry et al., 1999, Maree et al., 2010, Sa-Carvalho et al., 1997). Upsurge of net positive charge on the virion surface revealed a pattern of mutation during the adaptation of SAT 1 and SAT 2 FMDV (Maree et al., 2010), which is believed to facilitate cell entry through heparan-sulfated proteoglycan (HSPG) molecules. The location of the heparan binding site is where VP1, VP2, and VP3 intersect; these three VP1–VP3 appear as a narrow depression on the surface of the virus. (Fry et al., 1999), and various studies have implicated amino acids residues flanking RGD motif in determining receptor specificity of FMDV (Burman et al., 2006). The arginine located

on residue 56 of VP3 is crucial for cell recognition for adapted virus (Fry et al., 1999), amino acids 147 and 152 of VP1 influence the binding of FMDV to BHK-21 cells (Rieder et al., 1994). In a study conducted by Maree et al., it was observed that SAT 1 utilized positively charged amino acids located at positions 110–112 of VP1 to gain cell entry for the BHK 21 cell via GAG (Maree et al., 2010). In contrast, SAT 2 viruses obtained positively charged residues at positions 83 and 85 of VP1, which are readily adaptable (Maree et al., 2010). The internalization of cells by FMDV field isolates is dependent on integrin receptors, whereas viruses that have been adapted to cell culture can utilize integrin receptors or heparan sulfate (Baranowski et al., 1998b, Neff et al., 1998, Sa-Carvalho et al., 1997). Integrin- and HS-independent pathways allow FMDV to infect susceptible cells (Baranowski et al., 2000, Baranowski et al., 1998b, Baxt and Mason, 1995, Mason et al., 1993, Mason et al., 1994, Zhao et al., 2003), suggesting FMDV possesses a third cell recognition mechanism. Nevertheless, the G-H loop, a mobile loop that is exposed and connects the G and H strands in VP1 across all FMDV, is critical for the binding of integrins to receptors and antigenic sites (Jackson et al., 2003, Baranowski et al., 1998a). The RGD motif sequence and some residues flanking the RGD motif sequence involve in cell entry form part of the antigenic site that interacts with antibodies neutralization (Núñez et al., 2007). Through selective pressure during cell entry, virus adaptation requires the mutation of one or two capsid amino acids via positive charge net gain (Sa-Carvalho et al., 1997, Maree et al., 2010, Mohapatra et al., 2015) has the potential to inadvertently influence virus antigenic sites (Tami et al., 2003). The cumulative impact of this antigenic site mutation has the potential to render currently administered vaccines ineffective, complicate the assessment of disease surveillance, and necessitate routine vaccine matching (Fry et al., 2005).

1.4. The surfaces of FMDV possess antigenic sites.

The revelation of the three-dimensional structure of FMDV has prompted research into the atomic characteristics of the virus, including the composition and arrangement of its capsid components, the locations of its antigenic sites, the interactions between the virus and antibodies, and the ability of antigenic variant viruses to evade the immune system through antigenic variation (Grazioli et al., 2013, Mateu, 2017). An in-depth knowledge of the antigenic sites of FMDV would be beneficial in developing accurate diagnostic assays, evaluating antigenic variability, and choosing appropriate vaccine strains (Grazioli et al., 2013, Opperman et al., 2012). The antigenic sites on the viral capsid, which is a sizable protein, were identified using the techniques of electron microscopy, X-ray crystallography, and nuclear magnetic resonance (Guthridge et al., 2001, Kleymann et al., 1995). The monoclonal antibodies

neutralizing resistant mutants (MAR-mutants)(Grazioli et al., 2013) and recombinant single chain variable fragments (scFvs) methods (Opperman et al., 2012) have supported FMDV antigenic site identification. These methods have enabled the identification of numerous amino acid residues that interact with neutralizing antibodies, revealing the VP1 loop as the most accessible capsid protein and the principal immunodominant region (Fry et al., 2005). Variability of VP1 loop causes antigenic diversity between FMDV serotypes and subtypes, making disease control difficult (Grazioli et al., 2013). Detection of amino acids and their positions at antigenic sites (epitopes) has been conducted for serotype A (Baxt et al., 1989), serotype O (Crowther et al., 1993, Kitson et al., 1990), serotype C (Mateu et al., 1990) and Asia 1 (Sanyal et al., 1997, Grazioli et al., 2013), with few studies being conducted for the SAT 1-3 serotypes (Grazioli et al., 2006, Opperman et al., 2012). Studies show that FMDV's antigenic sites are on the three capsid proteins (VP1, VP2, and VP3), and the amino acids in residues 140-160 of VP1 are the main antigenic determinants, with few conserved epitopes (Bittle et al., 1982, Strohmaier et al., 1982, Mateu et al., 1988).

The x-ray crystallographic structure and amino acid sequence determination using MAbs mutants mapped five antigenic sites of serotype O, the most common serotype worldwide (Crowther et al., 1993, Kitson et al., 1990). Antigenic site 1, which is linear, trypsin-sensitive, identified as the immunodominant region, and conserved across all seven FMDV serotypes, is situated on the VP1 G-H loop that encompasses the C-terminus (Acharya et al., 1989, Grazioli et al., 2006, Kitson et al., 1990). The essential locations of the identified amino acid residues are 144, 148, 154, and 208. The amino acid residues found at site 2 are 70-73, 75, 77, and 131. These residues are located within the βB-βC and βE-βF loops of VP2. Site 3 consists of amino acid residues 43 to 45 and 48 located in the βB-βC loop of the VP1 protein. Site 4 comprises residues 56 and 58 to 59 located within the βB "knob" region of VP3 (Kitson et al., 1990, Mateu et al., 1990). Site 5 is composed of a single residue, 149, of VP1 and is understood to be moulded by the specific orientation of the VP1 βG-βH loop, for instance, in the event that the loop is in a “down” position and is in proximity to other surface residues (Crowther et al., 1993, Parry et al., 1990). The antigenic site on the VP1 B-C loop has only been identified for serotypes O, A, and C, and its exact position varies slightly between the different serotypes (Grazioli et al., 2013). In addition, the VP2 B-C loop, VP3 B-B knob, and VP1 C-terminal segment are identified as non-conserved antigenic sites due to the presence of discontinuous epitopes in other serotypes (Mateu et al., 1994, Parry et al., 1989).

The mapping of neutralizing antigenic sites for the SAT 1-3 serotype, which is predominantly found in southern Africa and the African continent, has been relatively limited (Grazioli et al.,

2006). Four antigenic sites for SAT 1 have been identified: (i) the βG-βH-loop of VP1; (ii) VP3 residue positions 135, 71, or 76 and VP1 residue 179 or 181; (iii) conformation sites VP1 residue 181 and VP2 residue 72; and (iv) VP1 residue 111 (Grazioli et al., 2006). The neutralizing antigenic sites of Serotype SAT 2 are located in the following regions: (i) the βG-βH loop of VP1; (ii) VP1 residue 210; (iii) VP1 loop residues 84-86 and 109-111, as well as VP2 residues 71, 72, and 133-134; (iv) VP1 residue 159, and VP2 residues 71, 72, 133-134, and 148-151 (Crowther et al., 1993, Grazioli et al., 2006, Opperman et al., 2012, Opperman et al., 2014). Regarding SAT 3, there is currently no identified neutralizing antigenic site (Chitray et al., 2020, Maake et al., 2020). Additional characterization of antigenic epitopes for SAT serotypes is essential for the development of a vaccine, as all antigenic determinants must be considered in order to design an effective vaccine for southern Africa (Opperman et al., 2012). In southern Africa, SAT serotype outbreaks, with SAT 2 being the most frequently linked to livestock and wildlife incidents, followed by SAT 1 and SAT 3, suggest that the current vaccine is ineffective (FMD-Bulletin, 2010, Lazarus et al., 2018). Southern Africa's SAT serotype distribution involves livestock and wildlife, particularly the African buffalo, which is the virus reservoir and source of transmission, creating a unique ecosystem for disease control compared to other FMD-endemic regions (FMD-Bulletin, 2010, Thompson, 1994). Prevention of livestock-wildlife interaction via fencing, animal movement control, and livestock vaccination are subsequently the focal points of disease control in the region in an effort to avert or reduce the spread of disease (Hunter, 1998, Vosloo and Thomson, 2017, W.Vosloo et al., 2002).

1.5. Southern African FMDV distribution and vaccination effects

Southern African countries have well-established small and large commercial farms, rural pastoral livestock production, and wildlife conservation parks (W.Vosloo et al., 2002), some of which are adjacent to one another. Countries such as Namibia, Botswana, Lesotho and Eswatini in the southern African region are free or have FMD-free zones, except for South Africa, Zambia, Zimbabwe, Mozambique and Malawi (Rweyemamu et al., 2014, Roberts and Fosgate, 2018, Sikombe et al., 2015). Recently, in July 2021, Namibia has detected its first serotype O outbreak, which threatens its FMD-free status (Banda et al., 2022). In 2021, South Africa, previously recognized as free from Foot-and-Mouth Disease (FMD) without vaccination, had its status revoked due to multiple recurring outbreaks. These outbreaks led to a total of 191 incidents between 2021 and 2022 (OIE., 2021, Department of Agriculture, 2022). The occurrence of FMD in countries such as South Africa, Namibia, Botswana, and Eswatini with high-value commercial exports of livestock and livestock products remains a threat to their FMD-free status, which allows trade into international markets. Furthermore, the presence of

transboundary animal diseases, including Rift Valley fever, bovine spongiform encephalopathy and bovine brucellosis, which are endemic and indigenous to the southern Africa region, also contributes to barrier to trade in the developed countries markets (Cassidy et al., 2013, Thomson et al., 2013). Embargo of trade due to FMD causes great economic constraints to those commercialised farms subsequent to the requirement for geographic freedom from those diseases as prescribed by the World Organization for Animal Health (WOAH) formerly known as Office International des Epizooties(OIE) (Thomson et al., 2013). Therefore, countries must adopt and maintain strict control measures stipulated in the WOAH Terrestrial Animal Health Code (2021) to protect their market share in livestock exports, especially FMD, which is highly infectious (Bruckner et al., 2002, Vosloo et al., 1992, OIE., 2021, Rweyemamu et al., 2014).

In southern Africa, FMD-free zones are maintained by restricting cloven-hoofed animal movement, erecting game-proof fences to separate wildlife and livestock, frequently vaccinating livestock around game parks, and vigorous FMD surveillance (Rweyemamu et al., 2014, Roberts and Fosgate, 2018). Wildlife animals play an essential role in socio-economic advancement in southern Africa, particularly in rural areas (Thomson et al., 2013). A significant threat to the control of FMD is posed by the fact that these wildlife animals, specifically African buffaloes, are abode in game reserves and established trans-frontier conservation areas (TFCAs), including the Kavango-Zambezi TFCA and Great Limpopo TFCA (Jori and Etter, 2016, Roberts and Fosgate, 2018, Thomson et al., 2013). Disease control in these established TFCA's becomes complex because it enhances the opportunity for the interaction of livestock and wildlife animals which is the ideal ecosystem for disease dissemination as has been the supposition in the Kruger National Park (KNP) in South Africa (Bastos et al., 2000, Bruckner et al., 2002, Jori et al., 2016a, Vosloo and Thomson, 2017). South Africa was FMD-free until 2021, with Kruger National Park (KNP) being the endemic site for FMD-SAT serotypes (SAT 1; 2; and 3) maintained by African buffalo (Bruckner et al., 2002, Condy and Hedger, 1974, Lazarus et al., 2017, Bastos et al., 2000, FMD-Bulletin, 2010, Department of Agriculture, 2022, OIE., 2021). Adjacent areas to the KNP are known as the protection zone (control zone), which are considered FMD high risk areas due to livestock in this vicinity surrounding the KNP (Bruckner et al., 2002). In order to mitigate and manage FMD, cattle are vaccinated biannually with trivalent inactivated vaccine strains (SAT 1; 2; and 3) and inspected at dip tanks every two weeks for FMD clinical signs by para-veterinarians working under the supervision of state veterinarians (Bruckner et al., 2002, Lazarus et al., 2018). Cattles on the western border of the protection zone and along the South African geographical borderline of Namibia,

Botswana, Zimbabwe, and Eswatini are monitored for FMD clinical signs and tested by the state veterinarian and animal health technicians in surveillance zones (Bruckner et al., 2002, Roberts and Fosgate, 2018). Although the key primary methods adopted by the South African government to prevent and control FMD, such as erecting of fences within the game reserves; movement control of cloven-hoofed animals; clinical surveillance and bi-annual prophylactic vaccination; has not deterred the occurrence of the disease in the FMD free zone hence the repeated recent outbreaks (Department of Agriculture, 2022). Compared to SAT 1 and SAT 3 outbreaks, FMD SAT 2 serotype outbreaks in cattle have increased since 2000, and vaccine has not fully prevented this recrudescence (FMD-Bulletin, 2010, Lazarus et al., 2018, OIE., 2021). This phenomenon may be attributed to the considerable antigenic diversity exhibited by the SAT 2 serotype, which impedes the effectiveness of vaccines (Maree et al., 2015, Rweyemamu et al., 2014, Tekleghiorghis et al., 2016, Vosloo and Thomson, 2017). Recent outbreaks in South Africa, which have been attributed to serotypes SAT 2 and SAT 3, render the currently employed vaccine ineffective. (Department of Agriculture, 2022, Lazarus et al., 2018). It is worth noting that cattle residing within the control zone are vaccinated every two years (Lazarus et al., 2017). Observation of trivalent vaccine (SAT 1; 2 and 3) used during a vaccination programme in the protection zone elicited adequate humoral antibody titres; however, the antibody response was transient, which is a challenge that requires repetitive immunization (Lazarus et al., 2018). The persistence and recurrence of SAT 2 outbreaks in vaccinated populations demonstrates that the current vaccine being used lacks adequate antigenic coverage against the recent SAT 2 outbreak isolates, and vaccine matching must be carried out to broaden the vaccine protection spectrum and increase the vaccine effectiveness against SAT2 isolates.

1.6. Various control measures in FMD endemic and non-endemic countries and how vaccine function

1.6.1. Control measure of FMD applied

FMD outbreaks have short- and long-term social and economic impacts and disrupt the productivity of livestock and country's trade of livestock and its products (Kitching et al., 2007). Although the impact of FMD differs throughout the world, the poorest countries are severely affected due to being directly dependent on livestock, and the presence of FMD influences their level of economic development to some degree (Kitching et al., 2007, Knight-Jones et al., 2016). However, FMD being low mortality disease, the control of FMD is an

essential task because of its transboundary and contagious capabilities to affect larger number of livestock, which can cause colossal global impact (Knight-Jones and Rushton, 2013). Most countries have devised various effective control measures in place to avert FMD incursion or dissemination depending on the country's FMD endemic or non-endemic status. Countries or regions with FMD non-endemic status have control measures in place mainly to restrict trade of animal products from countries affected with FMD, with a purpose to prevent introduction of FMD into their countries. These countries maintain well-coordinated veterinary services and rapid diagnostic capabilities; during an outbreak, infected animals are culled along with in-contact animals; and animals within close proximity are quarantined in attempt to suppress the disease (Parida Satya, 2009, Schley et al., 2009, Lyons et al., 2015, Santos et al., 2018). In some cases, emergency vaccination under the vaccine-to-live policy is used to limit disease spread when culling animals is not possible. (Ryan, 2001). To regain FMD-free status, the country have to demonstrate the absence of virus circulation six months after the last reported case (Parida Satya, 2009, OIE, 2021).

In 2001 the outbreak cost the United Kingdom economy was eight billion dollars (Thompson et al., 2002) and the disease extended to the Netherland were the country resorted to cull the vaccinated animals as part of control programme to rapidly regain the FMD status and re-establish trading position (Pluimers et al., 2002). The endemic countries, primarily developing nations, employ routine vaccination and restrictions on animal movement as primary control measures, despite the fact that the latter imposes socio-economic difficulties by limiting local livestock trade (Santos et al., 2018, Knight-Jones and Rushton, 2013). Vaccination has been the prevailing method of disease control in both endemic and non-endemic nations, and developed countries have embraced this approach since the 19th century, when it proved instrumental in eradicating the disease.(Bachrach, 1968, Rodriguez and Gay, 2011, Santos et al., 2018). In South America, vaccination has played a pivotal role in the reduction or eradication of disease in the majority of nations, including Paraguay, Brazil, Argentina, and Colombia (Naranjo and Cosivi, 2013). Vaccination in southern Africa began in 1970 and has reduced FMD severity and spread (Hunter, 1998, Vosloo and Thomson, 2017). Nonetheless, a number of factors, including inadequate vaccine compatibility with field strains, non-compliance with vaccine manufacturer recommendations, and inadequate cold chain maintenance that aids in vaccine stability, have impeded the effectiveness of vaccine use (Rweyemamu et al., 2014, Santos et al., 2018). The efficacy of the inactivated FMD vaccine utilized in the countries of the Southern African Development Community (SADC) has shown a notable decline due to the persistence of infection and the frequent rise of FMD outbreaks

(FMD-Bulletin, 2010, Lazarus et al., 2018, Vosloo and Thomson, 2017). The majority of studies have demonstrated that adequate protection against the outbreak virus can be achieved through the use of suitable vaccines that are closely related to the incursion virus (Brehm et al., 2008, Orsel and Bouma, 2009, Pereira, 1978), and repeated vaccination in endemic settings is recommended to maintain protective levels (Paton et al., 2019).

1.6.2. Function of vaccine

The immune response to FMDV infection and vaccination involves humoral immunity (innate and adaptive), which varies qualitatively and quantitatively depending on the effective antigen dose, animal species, nutrition, and health (Doel, 2005, Di Giacomo et al., 2015, Paton et al., 2019, Lazarus et al., 2018). Immune response against FMDV infection or exposure and vaccination is still being studied, but the humoral immune system elicits anti-FMDV antibodies, a robust immune response (Black et al., 1984, McCullough et al., 1986, McCullough et al., 1992a, Ostrowski et al., 2007, Pay and Hingley, 1992). Infection or vaccination elicits serotype-specific protective antibodies directed to outer capsid structural protein epitopes. High levels of neutralizing antibodies are associated with good protective immunity (Grubman and Baxt, 2004, McCullough et al., 1992a). Various studies have linked high neutralizing antibodies to protection based on the strong association of induced FMD virus-specific antibody levels with clinical symptom reduction and FMD prevention (McCullough et al., 1992a, McCullough et al., 1988, McCullough et al., 1992c, Sutmoller and Vieira, 1980, Van Bekkum, 1969). Furthermore, the neutralising immunoglobulin (Ig) M (IgM) have been detected within 3-4 day of infection or vaccination in cattle which decline after 14 days (Parida Satya, 2009). The IgG_1 and IgG_2 immunoglobulins are also recruited within 4-7 days, however IgG_1 is produced in high quantity in contrast to IgG_2 and within 14 days both become the main neutralizing antibodies during vaccination (Parida Satya, 2009, Habiela Mohammed et al., 2014, Salt, 1993, Salt et al., 1996).

The immunoglobulin A (IgA) has been found early during infection in upper respiratory and nasal oropharyngeal secretions, and its presence post-28 days has been linked to carrier animals, making the oropharynx a replication site for carrier animals (Parida Satya, 2009, Burrows, 1966, Prato Murphy et al., 1994). The Immunoglobulin G (IgG) and IgM activity have been observed after vaccination and during revaccination the neutralising antibody in oropharynx region was primarily associated with IgG (Francis et al., 1983). Frequent cattle vaccinations maintain high levels of neutralizing antibodies and specific IgG, which can reduce FMD virus in the oropharynx and shedding (Francis et al., 1983, Pinto and Garland, 1979).

Cellular immunity against FMDV has been less studied than humoral immunity, but oil-adjuvant FMD vaccines have been shown to stimulate specific and non-specific cell-mediated immunity (Grubman and Baxt, 2004, Salt et al., 1998). Use of vaccine has been linked with the increase of macrophages which have the ability to eliminate the FMD virus through phagocytosis and has been a crucial effector against the virus (Leslie, 1985, McCullough et al., 1992a, Ostrowski et al., 2007). Macrophages (mononuclear phagocytes) are crucial in facilitating the effector of innate immune response and its ability to clear FMDV depend on antibody-antigen complex which enhance the phagocytosis (McCullough et al., 1988, McCullough et al., 1992b). Phagocytosis is considered as mechanism for virus clearance and had been deemed as important protective immune response against FMDV (McCullough et al., 1992a).The natural killer (NK) cells are known to play a critical role in protection against numerous viral infections and are capable of eliminating the marked Immunoglobulins G through antibody-dependent cytotoxicity, (Chung et al., 2006, Toka et al., 2009). However, during the acute FMDV infection in porcine, the NK cells tend to dysfunction presumed due to pyrexia, virus proliferation and lymphopenia, although the role of NK cells in cattle and porcine is not fully elucidated (Toka et al., 2009, Salt et al., 1998). Dendritic (Dc) cells are involved in the immune response of viral infection and stimulate T-dependent antibody production, proliferation, and differentiation of T- and B-cells during viral infection, however, its function against FMDV still obscure (Inaba and Steinman, 1985, Inaba et al., 1983, Steinman, 2003, Steinman and Witmer, 1978). Cellular-mediated immunity may have a role to play during FMDV eradication in carrier animals, and their function is still not understood; however, the use of vaccination offers some protection in neutralising the virus and localizing the infection site (Amadori et al., 1992, McCullough et al., 1992c). The hallmark of a good or effective vaccine is to mimic the adaptive immune response and induce a protective immune response that is durable, as well as provide cross-protection against multiple serotypes (McCullough et al., 2017).

1.7. Contemporary FMD vaccine and potential future vaccines

Routine vaccination in endemic countries is an effective method for FMD prevention and control, while FMD-free countries use vaccine as an integrated control measure in addition to practice eradication. Emergency vaccine is used in the event of an FMD outbreak (Barnett et al., 2002b, Elnekave et al., 2013, Alexandersen et al., 2002). FMD-free countries use emergency vaccine, which is considered more potent, to induce protective immunity, but conventional vaccine also induces protective immunity after a few days (Sellers and Herniman, 1974). The potency is indirectly linked to protective immunity and broader vaccine coverage

(Salt et al., 1998), which can be inferred as the vaccine's ability to induce immunity needed for protection (Doel, 2003). High-potency vaccines are ≥6 PD_{50} which are recommended for emergency vaccination of naïve animal populations due to their high antigen payload, broad coverage, and prompt protective immunity (Barnett and Carabin, 2002). Conventional vaccines, which are recommended for routine vaccination campaigns, are considered to have standard potency. They are formulated with sufficient antigen and adjuvant to meet the minimum potency requirement of 3 PD_{50} (50 % protective dose) (Elnekave et al., 2013). In vaccine formulations, the PD_{50} represents the factor of dilution at which 50% of immunized animals continue to receive protection (Barnett et al., 2003). Despite previous efforts to develop vaccines with a ≥10 PD_{50} or higher (Barnett and Carabin, 2002), the limited understanding of how the immune system reacts to FMDV has prompted investigations into the creation of different vaccine formulations in an attempt to identify a vaccine that can induce a protective immune response against multiple serotypes. Presently, inactivated FMDV is the recommended vaccine; however, care must be taken when selecting strains that are in circulation or accountable for outbreaks, as the virus mutation rate generates quasi-species that have a direct impact on the effectiveness of vaccines (Brown, 2003, Mahapatra and Parida, 2018).

1.7.1. Inactivated whole virus vaccine

The development of inactivated FMD vaccines made it possible for FMD-endemic countries to adopt palatable methods to control FMD outbreaks. The reluctance of FMD-free countries to adopt vaccination as a priority measure rather than slaughtering is due to carrier state of animal that can develop after virus exposure despite animals being vaccinated (Doel et al., 1994). However, some studies have shown the potential of inactivated vaccine to reduce carrier state in vaccinated animals compared to non-vaccinated animals (Anderson et al., 1974). The development and advancement of inactivated vaccine, was initially explored study by Vallée using the vesicular fluid from infected calves which was inactivated using formalin, and the product was used to immunise cattle (Vallee et al., 1925). Currently, the Baby hamster kidney 21 cell line adapted to suspension are used in fermenters for virus cultivation (Doel, 2003, Smitsaart and Bergmann, 2017). Utilization of large-scale fermenters was adapted by Telling and Elsworth, which is the technology that is capable of producing large quantities of virus (Telling and Elsworth, 1965) and is currently being used widely to produce hundreds millions of vaccine doses in high containment facilities (Doel, 2003, Tom Willems et al., 2020). In this developed inactivated vaccine, the virus is chemically inactivated with Binary ethyleneimine (BEI), which alkylates the viral RNA genome and disrupts the RNA-protein

interaction (Bahnemann, 1975). Inactivant BEI is capable of protecting the integrity of the non-infectious complete capsid structure (146S particle) that comprises the antigenic properties capable of triggering the immune response (Bahnemann, 1990, Brown, 2003, Lombard et al., 2007, Barteling and Vreeswijk, 1991). Ultrafiltration is utilized to remove impurities and remnants of non-structural proteins from inactivated virus, particularly the 3ABC proteins, which are diagnostic core proteins used to distinguish between vaccinated and infected animals (Diaz-San Segundo et al., 2017a, Doel, 2003, Barnett and Carabin, 2002). Purified antigen can be stored in liquid nitrogen and made into single or multivalent vaccine doses. The antigen is combined with adjuvants like saponin and aluminum hydroxide gel to boost vaccine potency (OIE, 2021, Grubman and Baxt, 2004, Rowlands, 2008). Although inactivated vaccines do not prevent infection, they provide serotype-specific protection in ruminants for six months against antigenically similar strains of the same serotype (Kitching et al., 2007). Parida et al. found that the high-potency sheep vaccine fully protected against clinical symptoms and reduced FMDV virus in saliva and nasal secretions after challenge, limiting shedding and disease transmission (Parida Satya, 2009). Comparable results were observed when evaluating the efficacy of a high-potency vaccine in swine, which reduced virus excretion by 90–98% compared to unvaccinated pigs and provided early protection against direct and indirect contact infection (Barnett et al., 2002a). When cattle were challenged with the virus 10 days after receiving a high-potency vaccine, Cox et al. discovered that clinical symptoms and virus excretion decreased significantly (Cox et al., 2007). For cattle, the high-potency vaccine induces protective antibodies 3–4 days after vaccination, while livestock vaccination induces antibodies that peak 14–28 days later (Doel et al., 1994). Certain drawbacks have been associated with inactivated vaccines. These include restricted immunogenicity, which necessitates the incorporation of newly circulating virus strains during the vaccine formation process, the need for re-vaccination to sustain high levels of protective antibodies, the inability to prevent carrier status, and the expensive implementation of high containment measures for vaccine preparation (Beck and Strohmaier, 1987, Blanco et al., 2016, van Bekkum, 1978b, King et al., 1981).

1.7.2. Live attenuated vaccine

Live attenuated vaccines (LAV) and inactivated vaccines are both considered "classical FMD vaccines" (Blanco et al., 2017). LAV can imitate viruses to induce rapid and effective immunity, but an attenuated FMDV vaccine has yet to be developed despite extensive research (Diaz-San Segundo et al., 2016). Advancements in molecular biology have enabled the development of novel attenuated vaccines via modifications such as generation of a leaderless virus

(Chinsangaram et al., 1998, Mason et al., 1997), elimination of the conserved SAP domain from the L-protease (Diaz-San Segundo et al., 2016), codon pair deoptimization (Diaz-San Segundo et al., 2016), chimeric viruses (Uddowla et al., 2013), and modification of replication fidelity (Rai et al., 2017); have been possible through the advancement of molecular biology that have made the virus more stable (Blanco et al., 2017, Kamel et al., 2019). The majority of research on live attenuated vaccines has focused on the L-protease (non-structural protein), which is responsible for the development of leaderless viruses, because of its virulence determinants that impede the induction of beta interferon mRNA and innate immunity (de Los Santos et al., 2006). Leaderless virus technique may elicit protective neutralizing antibodies, but the antibodies produced are insufficient to protect cattle and pigs (Chinsangaram et al., 1998, Mason et al., 1997). Furthermore, other research has shown that chimeric FMDV fused with bovine rhinitis B virus L-protease can induce protective immunity in pigs (Uddowla et al., 2013). Mice, swine, and cattle showed some protection from this attenuated vaccine (Hollister et al., 2008, Uddowla et al., 2013), however more research is still required to be conducted.

Further attenuated vaccine attempts were made with the discovery of the conserved SAP motif (SAF-A/B ; Acinus; and PIAS) within the L protease region, where two amino acids that signify virulence factor were replaced within the SAP domain (Díaz-San Segundo et al., 2012). In swine, the SAP mutant of L protease conferred some prompt protection two days after vaccination; nevertheless, the substitution of these two amino acid residues posed a risk that the virus would revert to its virulence phase in response to immune selective pressure (Diaz-San Segundo et al., 2016). Deoptimization of synonymous codon bias usage is the contemporary approach for the development of attenuated vaccines (Diaz-San Segundo et al., 2016), and deoptimization has been instrumental in genetic engineering of attenuated poliovirus (Mueller et al., 2006). The deoptimization of codon usage bias is based on the approach of modifying the synonymous codon pairs that are either overrepresented or underrepresented using the assistance of synthetic attenuated virus engineering (SAVE) technology (Coleman et al., 2008, Diaz-San Segundo et al., 2016). The study by Diaz-San Segundo et al. of codon pair bias deoptimization in the FMD strain A12-P1 virus P1 structural region, the results showed that mice had strong protective immunity and that swine had constant high levels of antibodies when challenged with wild-type virus (Diaz-San Segundo et al., 2016) . Even at a marginal dose, which is considered safe, no clinical signs or virus shedding were observed. Additional studies of polymerase engineering approach have been explored, where a tryptophan residue located within motif A at position 273, has been replaced with phenylalanine on the $3D^{pol}$ RNA dependent RNA polymerase that is responsible for viral

replication in FMDV (Rai et al., 2017). During replication, the replacement of tryptophan by phenylalanine increases the polymerase fidelity (proofreading), while the replacement of isoleucine or leucine reduces the polymerase fidelity (Rai et al., 2017). The increase of high fidelity has been pursued to develop more stable attenuated FMDV vaccines with less mutations, although the research has been limited to cell culture (Rai et al., 2017).

1.7.3. DNA vaccines

The research into the use of DNA vaccine as potential vaccine to confer protection against diseases, including FMD, has been broadly undertaken (Cedillo-Barrón et al., 2001, Wong et al., 2000). The investigation of different plasmid DNA plasmid-borne FMD gene insertions, including capsid gene P1-2A, 3C, and 3D 3D proteases, has demonstrated their capacity to induce humoral and cellular responses (Beard et al., 1999, Cedillo-Barrón et al., 2001, Nobiron et al., 2001, Wong et al., 2000). This insertion of FMDV sequence has been an alternative route to classical FMD vaccines and has been shown to elicit an immune response, particularly in mice, but protein expression is insufficient to trigger an immune response in cattle, pigs, and sheep (Niborski et al., 2006, Ward et al., 1997). When plasmid DNA encoded with a modified full-length FMD genome was expressed in cattle and swine, partial and full protective immunity were observed, but multiple vaccine doses with more adjuvants and cytokines like GM-CSF and IL-2 were needed (Cedillo-Barrón et al., 2001, Ward et al., 1997, Wong et al., 2002) DNA vaccines have the following advantages over inactivated vaccines: they are stable and do not necessitate cold chain storage; they do not require a high containment facility for production; and they stimulate B and T cells (Kamel et al., 2019) (Niborski et al., 2006). In contrast to inactivated vaccines, the protective neutralizing antibodies produced by DNA vaccines are transient, albeit they do prevent the onset of clinical symptoms (Chaplin et al., 1999, Niborski et al., 2006).

1.7.4. Peptide Vaccines

Molecular biology techniques have made significant progress in identifying the structural protein, with VP1 being a major antigenic determinant. Isolating VP1 has been shown to elicit neutralizing antibodies in cattle and swine, which confers protective immunity (Diaz-San Segundo et al., 2017b, DiMarchi et al., 1986, Bachrach et al., 1975). This achievement led to the development of the subunit peptide vaccine, which had advantages over classical vaccines (inactivated and live attenuated), some of which have been commercialized (Blanco et al., 2017). The peptide vaccine contains a purified VP1 single linear peptide encoding structural proteins or B- and T-cell epitopes. (Wang et al., 2002, Bachrach, 1977). Inoculating cattle and swine with purified E.coli VP1 provided partial protection against homologous FMD virus

(Bachrach et al., 1975). The most antigenic site on VP1, the G-H loop, was synthesized, and repeated vaccination has conferred a degree of partial protection (Taboga et al., 1997). The combination of the G-H loop and T-cells peptides in a vaccine showed some promising protection in swine; however, an inadequate protective immune response was observed in cattle (Rodriguez et al., 2003). The inadequate immune response and protection are believed to be caused by the hypervariability of the G-H loop domain in VP1 (Szczepanek et al., 2012). The incorporation of a multiple-epitope recombinant vaccine has been demonstrated to elicit an immune response that generates substantial quantities of antibodies that confer complete immunity. Moreover, this vaccine has provided some protection in swine when exposed to homologous strain serotype O (Shao et al., 2011). The approach showed that the vaccinated pigs developed minimal clinical signs and did not disseminate disease to other animals in the experiment (Cubillos et al., 2008). Due to its low costs in large-scale production, use of non-infectious material, and stability without a cold chain, this approach is preferred over inactivated vaccines.(Rodriguez et al., 2003). Given the potential for peptide vaccines to serve as the preferred vaccine for the management of FMD, this stimulated further investigation into its development.

1.7.5. Virus like particles

Effectiveness in producing antigens that elicit an immune response has been demonstrated through the use of virus-like particles (VLP), which are generated by introducing recombinant genes into diverse prokaryotic and eukaryotic expression systems (DiMarchi et al., 1986). A virus-like particle can be used as a vaccine because it can express viral proteins encoded in the recombinant genome and spontaneously self-assemble into icosahedral or rod-like shapes (Bachmann and Jennings, 2010). Due to the absence of nucleic acids and replicases that encode viral proteins, VLPs have the advantage of preventing particle replication (Bachmann and Jennings, 2010). The VLPs fall within the size range of 20–200 nm, which is optimal for antigen-presenting cell (APC) uptake efficiency and lymphatic vessel drainage to lymphatic organs for follicular B cell interaction, which induces an effective antibody response within several hours (Oussoren et al., 1997, Swartz, 2001). The VP1 structural protein of the FMD virus, with the inclusion of non-structural proteins 2A, 2C, and 3C proteinases, has been expressed in transgenic plants such as *Arabidopsis* and alfalfa (Carrillo et al., 1998, Wigdorovitz et al., 1999a) , and the production of antigen was quite minimal in yield, which became a challenge to implement the technology (Wigdorovitz et al., 1999b). Utilization of mammalian cells expression systems, e.g the *baculovirus* and *E.coli* , to express VLP demonstrated protection in pigs however did not prevent viral replication (Grubman et al.,

1993, Rodriguez and Gay, 2011). This promising results of using VLP was observed when larvae lysate was used to derive VLP and vaccinated cattle showed protection (Li et al., 2011). Research on the use of *E.coli* expressions system was explored, which showed promising results after providing protective immunity in cattle and swine (Guo et al., 2013, Lee et al., 2009, Xiao et al., 2016). The VLP provides a more cost-effective and secure alternative to inactivated and attenuated vaccines; consequently, its performance in large-scale animals and large-scale expression have imposed limitations on its application. Consequently, further investigation is required to determine the full potential of this technology.

1.7.6. Live viral vector

A live viral vector (LVV) vaccine is technology that involves the use of various virus vectors, such as the vaccinia virus (Sanz-Parra et al., 1999), fowl-pox virus (Ma et al., 2008), herpesvirus (pseudorabies) (Zhang et al., 2011), where virus-vectors infected into the host are used to convey genetic material in *in vivo* with the objective of expressing virus-like particles rather than delivering protein synthesised outside the host (Diaz-San Segundo et al., 2017b, Santos et al., 2018). Expression of virus-like particles can induce humoral and cell-mediated immunity (de los Santos et al., 2018). An extensive research has been conducted on the use of live viral vectors, with the implementation of recombinant replicative human defective adenovirus type 5 (Ad5) serving as an example of a successful live viral vector (Moraes et al., 2003). The Ad5 construct targets FMD subunits like capsid VP1 region, 2A and 3C protease, making the vaccine an FMD subunit vaccine (Mayr et al., 1999, Mayr et al., 2001). As a vaccine, the Ad5-FMD construct distinguishes between vaccinated and infected animals by utilizing diagnostic techniques to detect the 3B and 3D FMD. (Rodriguez and Gay, 2011, Grubman et al., 2012). The advantage of the Ad5-FMD vaccine is that it does not require the use of a high containment facility; no adaptation of a field strain is needed nor is the production of vaccine in cells as the expression of virus-like particles is directly within the host (Pacheco et al., 2005). The Ad5-FMD vaccine may be an alternative to inactivated and attenuated vaccines, and the United States of America (USA) has endorsed it as an emergency vaccine (Santos et al., 2018). The inability of Ad5-FMD to induce rapid protection under 7 days, despite its potential to induce protective immunity, prompted the design of an Ad5 virus containing antiviral porcine Interferon-alpha (Ad5-pINFα) to inducer rapid protection (Moraes et al., 2003). Expression of porcine interferon alpha in adenovirus (Ad5-pIFNα) and FMDV subunit vaccine induced robust neutralising antibody response that conferred protection in swine and prevented viral replication (Moraes et al., 2003). The efficacy and viability of combining antivirals with vaccines have shown promising results, but additional research is required to assess its reliability and

practicality in larger animal populations. Vaccines under development have the potential to be successful vaccines to replace inactivated vaccines; however, while the research is still progressing, it is still important to improve the vaccine in use by improving the vaccine strains that are added during vaccine formulation. Improvement of the inactivated vaccine require some of the method that are used to in FMD diagnosis.

1.8. FMD diagnostic methods are instrumental in vaccine matching

FMD diagnostic methods are essential for determining and distinguishing the disease from similar-looking diseases like vesicular exanthema, stomatitis, and swine vesicular disease (Bachrach, 1968). Diagnostic assays FMDV serotyping and characterization of the viral genome utilised in antigenic profiling and vaccine matching are imperative in outbreak confirmation (Ludi et al., 2017, Ma et al., 2011). FMDV diagnostic methods include virological tests that detect the virus or antigens and serological tests that detect antibodies in blood or serum samples (Ludi et al., 2017).

The serological assays, which recognize FMD antibodies via the formation of virus-antibodies complexes, consist of the virus neutralizing test (VNT), the enzyme-linked immunosorbent assay (ELISA), and the complement fixation test (CFT) (Hamblin et al., 1986). The VNT and ELISA methodologies are extensively employed in the field to ascertain the immune status of animals, distinguish infected from vaccinated animals (DIVA) via non-structural target proteins (3B and 3D), and match antigens from field isolates to vaccine strains (Brehm et al., 2008, Ludi et al., 2017, Rodriguez and Gay, 2011). In endemic regions, antigenic matching, also known as vaccine matching, is a crucial method for determining the efficacy of inactivated vaccines used to control FMD (Paton et al., 2005).

1.8.1. Vaccine matching techniques

Vaccine matching techniques aim to achieve the highest degree of similarity between the vaccine strain and circulating field strains in order to elicit a robust and all-encompassing neutralizing immune response (Brehm et al., 2008, Rweyemamu et al., 2014). The emergence of new FMD variants and the antigenic disparity of field isolate and vaccine strains leads to ineffective vaccines that affect the control of FMD (Mahapatra and Parida, 2018). Africa has six FMDV serotypes, and new variants with diverse antigenic and immunogenic properties make choosing an FMDV vaccine strain difficult (Domingo and Holland, 1997, Haydon et al., 2001, Maree et al., 2011, Rweyemamu et al., 2014). In contrast to the widely dispersed O and A serotypes, the SAT 1 and SAT 2 serotypes demonstrate significant antigenic variation across the African continent. This antigenic variation is determined by the variation in the three major

capsid-displayed proteins, namely VP1, VP2, and VP3 (Esterhuysen et al., 1988, Maree et al., 2011, Reeve et al., 2010, Rweyemamu et al., 2014, Paton et al., 2019). The presence of antigenic variability among serotypes leads to the development of an ineffective vaccine as a consequence of differences in antigenicity (Bastos et al., 2003, Lazarus et al., 2018, Maree et al., 2011, Maree et al., 2015) , therefore vaccine matching is important in this regard.

In vivo potency tests are a direct and accurate way to assess a vaccine's protection against circulating field isolate (Brehm et al., 2008). *In vivo* potency test, live vaccinated animals are challenged with a homologous live virus strain to determine if they are protected (Brehm et al., 2008, Paton et al., 2019, Rweyemamu et al., 2014). *In vivo* potency test has the advantage of having a defined standard approach, although it does not simulate the usual animal infection route such as inhalation and ingestion (Paton et al., 2005). Drawbacks of the *In vivo* potency test are: variability when using the heterologous strain, resulting in the test being unreliable; operation cost; and not being ideal to use during FMD outbreaks because the result turnaround time exceeds 30 days and the decision to select the appropriate vaccine strain to be used in vaccine formulation is required within days (Brehm et al., 2008, Paton et al., 2019, Goris et al., 2007, Lavoria et al., 2012). The implementation of an in vivo potency test entails operational costs such as the need for a specialized bio-containment facility and a larger group of animals to ensure accurate results; this could give rise to significant animal welfare concerns (Rweyemamu et al., 2014). FMD serological test assays, including the virus neutralizing test (VNT), liquid phase blocking ELISA (LPBE), and solid phase competition ELISA (SPCE), are in vitro test assays that have emerged as alternative options for vaccine matching. These assays are characterized by their rapidity, reliability, robustness, cost-effectiveness, and ease of use (Brehm et al., 2008, Paton et al., 2019, Chénard et al., 2003, Mackay et al., 2001). These *in vitro* test assays measure the antibody titre of sera samples from vaccinated animals against both the vaccine strain and field isolate to indirectly determine antigenic match (Brehm et al., 2008, Paton et al., 2005). This indirect antigenic measure is denoted by the r1-value, which indirectly establishes the field isolate and vaccine strain serological antigenic relationship (Mahapatra and Parida, 2018).

1.8.1.1. r-value as antigenic relationship determinant for vaccine matching

Determination of antigenic relatedness using the relationship coefficient r_1-value through the use of serological methods have been utilised by FMD reference laboratories and vaccine manufactures to assess the appropriateness of vaccine strain candidate or sera cross-reactivity against the excursion virus (OIE, 2021, Paton et al., 2005). R_1-value is one-way antigenic relationship which is determined as; ratio of the titre acquired from sera against

heterologous strain (field isolate) and titre acquired from sera against the homologous strain (vaccine strain) (Brehm et al., 2008, Mattion et al., 2009). This concept is based on studies that shown a strong correlation between high antibody titre and protective immunity (Hingley and Pay, 1987, Maradei et al., 2008, Van Maanen and Terpstra, 1989). Serology tests VNT and ELISA (LPBE and SPCE) measure total cross-reactivity between sera antibodies and field isolate, using sera titres to establish antigenic relationship r_1-value and estimate vaccine matching between vaccine strain and field isolate (Mattion et al., 2009, Tekleghiorghis et al., 2016).

Estimated r_1-values and interpretation depend on the type of serological assay used between VNT or ELISA tests. Low values below 0.3 for VNT and 0.4 for ELISA indicate distant antigenic relatedness, while high values above 0.3 to 1.0 for VNT and 0.4 to 1.0 for ELISA indicate close antigenic relatedness (Barnett et al., 2001, Ferris and Donaldson, 1992, OIE., 2021, Rweyemamu, 1984). To choose a vaccine strain, consistence antibody titres must be determined using VNT, LPBE, and SPCE, and the estimated r_1-value must be comparable to the gold standard *in vivo* test for protective immunity. (Mattion et al., 2009, Paton et al., 2019, Tom Willems et al., 2020). It is recommended that the test be repeated and the sera in use, typically a bovine vaccinal sera from vaccinated cattle, be standardized by means of sera pools collected from a minimum of five vaccinated animals within 28 days post-vaccination and exclusion of sera with low antibody titres (Mattion et al., 2009, Paton et al., 2005, Rweyemamu, 1984, Rweyemamu and Hingley, 1984). The use of low serum antibody titre to estimate the r_1-value leads to inaccurate predictions, whereas the approach of pooled sera for VNT, LPBE and SPCE tests decreases the inter-animal and inter-assay variation (Mattion et al., 2009). In the investigations conducted by Brito et al., Mattion et al., and Brehm et al., the utilization of FMDV serotype A against heterologous strains had an impact on the precision of the r_1-value in correlation with cross-protection to *in vivo* potency testing. (Brehm et al., 2008, Brito et al., 2014, Mattion et al., 2009). The determined r_1-values in the studies were below 0.3 and 0.4 for VNT and liquid blocking ELISA, respectively, indicating low degree of antigenic relatedness between vaccine strain and challenge virus despite cross-protection conferred on the animals (Brehm et al., 2008, Brito et al., 2014, Mattion et al., 2009). The inaccuracy of r_1-values is suggested to be contributed by low antigenic relatedness of the vaccine used to vaccinate the cattle, which did not induce sufficient neutralising antibody that confer cross-protection (Brehm et al., 2008, Mattion et al., 2009). Additionally, the broader antigenic diversity exhibited by the serotype A could be another rationale for inaccuracy of r_1-value in correlation to cross-protection with *in vivo* potency test (Arrowsmith, 1975, Paton et al., 2019). The degree of

antigenic relationship and high potency of the vaccine are interrelated, and this two factors influence cross-protection in heterologous virus challenge (Brehm et al., 2008, Paton et al., 2005).

The application of a high-potency vaccine resulted in substantial protection against heterologous strains, despite the estimated r_1-value being relatively low. This can be attributed to the high-potency vaccine's ability to generate robust homologous protective immunity, which effectively cross-protects against heterologous strains (Brehm et al., 2008, Paton et al., 2019). The challenge with r_1-value concept is non-inclusion of antibody dependent phagocyte and cytotoxicity, which are involve in protective immunity *in vivo* (Mattion et al., 2009, McCullough et al., 1992a, Ostrowski et al., 2007), hence in some instances the low r_1-value is capable of conferring protective immunity. The study by Cao et. al., showed a strong induced protective immunity when the closest antigenic vaccine strain was used against heterologous serotype O when the boost vaccine was administered (Cao et al., 2021). The determined antibody titres in VNT and ELISA tests were consistent, which showed a comparability between VNT and ELISA titres, and the established ELISA titres were correlated with protection *in vivo* (Cao et al., 2021). In the study of Sirdar et al. the concept of r_1-value to determine antigenic relatedness between vaccine strain and field isolate was undertaken, where vaccine matching was determining between trivalent inactivated vaccine (consisting of SAT 1, SAT 2, and SAT 3 antigen) against SAT 1 and SAT 2 FMDV viruses that were responsible for outbreak during the 1991 – 2015 period in South Africa (Sirdar et al., 2019). Overall concept of r_1-value indicates potential of r_1-value to become an alternative vaccine matching predicting tool. The limited investigation into this notion may be attributed to the correlation between cross-protection using heterologous strains and in vivo potency tests, which have only been observed to function with serotype A (Brehm et al., 2008, Brito et al., 2014). Therefore, extensive research on the r_1-value concept will aid in its validation and determine the feasibility and reliability of the r_1-value (Brehm et al., 2008, Sirdar et al., 2019). For vaccine matching, r1-value must be accurate and precise in correlating cross-protection with in vivo, which requires serological tests like VNT, LPBE, and SPCE to determine consistent and comparable antibody titres (Paton et al., 2019, Paton et al., 2005).

1.8.1.2. Virus neutralising test (VNT)

The World Organization for Animal Health (WOAH) has designated the virus neutralizing test as the global benchmark for identifying FMDV-specific antibodies in animals. Its principal function is to validate the positive antibody detection in the enzyme-linked immunosorbent

assay (ELISA) (Chénard et al., 2003, Paton et al., 2005). Due to its capacity to quantify the cross-reactivity between antibodies in the sera elicited from vaccinated animals and a field isolate in cell culture, VNT has been implemented as a vaccine matching method (Maree et al., 2011, Syed and Graham, 2013, Reeve et al., 2010). Sera antibodies' ability to neutralize field viruses before they infect cell cultures is the basis of the VNT and indirectly indicates the antigenic match between a vaccine strain and field isolate. The standardised method for estimating the antigenic match between vaccinal sera and field isolates is to determine the r1-value using the heterologous and homologous titre (Rweyemamu et al., 2014). Cross-reactivity antibody titres with homologous strain (vaccine strain) and heterologous strain (field isolate) are determined, which are then used to establish antigenic relatedness using r_1-value (Jamal and Belsham, 2013, OIE, 2021). A r_1-value equal to or above 0.3–1.0 signifies a strong antigenic similarity between the vaccine strain and the field strain, suggesting that the vaccine is likely to provide effective cross-protection (Rweyemamu, 1984, OIE, 2021, Barnett et al., 2001). When the r_1-value is less than 0.3, it signifies that the vaccine strain and the field strain have a distant antigenic relationship, and the vaccine will not provide sufficient cross-protection (OIE, 2021, Rweyemamu, 1984). The VNT is one of the standard cross-reactivity tests and is sensitive and specific, making it essential for determining if the vaccine will provide enough protection (OIE, 2021). Antibody titre acquired from vaccinated animal have been generally acknowledged to provide a good correlation for protection against virus challenge (Maradei et al., 2008, Pay, 1983, Pay and Hingley, 1992, Van Maanen and Terpstra, 1989) and studies has shown the ELISA test lacking in terms of reliability in correlation despite being more producible than VNT (Amadori et al., 1991, Goris et al., 2008, Maradei et al., 2008, Periolo et al., 1993, Van Maanen and Terpstra, 1989). A limitation of this method is that it is not reproducible; it requires biocontainment for the use of live viruses and requires large quantities of serum; sensitivity can vary among cell cultures, which makes them susceptible to contamination (Syed and Graham, 2013). In addition, the procedure requires considerable effort and time, with a turnaround time of 2–3 days for the results (Rweyemamu et al., 2014).

1.8.1.3. Liquid phase blocking elisa (LPBE)

Liquid phase blocking ELISA is an *in vitro* serological method that has been adopted by various laboratories for the detection and quantification of FMD antibodies in infected and/or vaccinated animals (Hamblin et al., 1986). The LPBE, which measures the total cross-reactivity of the sera antibody titre elicited from vaccinated animals against the field isolate (heterologous strain) and vaccine strain (homologous strain) via ELISA, has been implemented as an in vitro serological vaccine matching technique (Kitching et al., 1988, Hamblin et al., 1986). In contrast

to VNTs, the LPBE does not necessitate the utilization of cell culture. Nevertheless, to ensure adequate antigen-antibody interaction, bovine vaccinal serum is incubated with the field-isolated antigen in suspension for a specified duration. The quantification of antibodies follows a sequence of procedures (McCullough et al., 1985, Hamblin et al., 1986). The LPBE plate is coated with serotype-specific rabbit antiserum and test serum (bovine vaccinal serum) is mixed with virus isolate in question (homologous strain or heterologous strain) in a separate carrier container and incubated for defined period to allow cross-reactivity. Following incubation, the mixture will be added to plate and incubate. Afterwards, series of washes will follow and addition of guinea-pig antiserum detected antigen trapped by coated rabbit antisera. This would be followed by binding of rabbit anti-guinea pig immunoglobulin conjugate to horse radish peroxidase. Addition of substrate will react with conjugate horse radish peroxidase which will eventually stopped by addition of sulphuric acid. The optical density of the developed colour is measured using spectrophotometer and results are expressed as reciprocal antibody titres. Determined antibody titres of heterologous and homologous from the plate results are used to calculate the antigenic relationship r_1-value to establish the degree of antigenic similarity between vaccine strain and field isolate (Brehm et al., 2008, Kitching et al., 1989). Establishment of r_1-value using LPBE involves the determination of total antibody-antigen binding titre which are determined following LPBE (Hamblin et al., 1986).

An r_1-value of above 0.4 -1.0 indicates a close antigenic relationship towards the field isolate and the use of the vaccine against the field strain will adequately confer immunity (Ferris and Donaldson, 1992, OIE., 2021). An r_1-value of 0.2- 0.39 illustrates a partial antigenic relatedness and the use of the vaccine will provide inadequate protection (Ferris and Donaldson, 1992, OIE., 2021). An r_1-value of below 0.2 indicate a distant antigenic relatedness between the vaccine strain and field isolate and the vaccine will not provide protection against the field isolate (Ferris and Donaldson, 1992). The advantage of using the LPBE as a vaccine matching tool is that it is sensitive, specific, rapid, reproducibly , requires the use of inactivated virus and uses small volumes of sera; in contrast to the VNT (Hamblin et al., 1986, Maradei et al., 2008). The VNT and LPBE have relatively good correlation with homologous protection (Reeve et al., 2010, Rweyemamu et al., 2008) and several validated LPBE's have indicated reliable thresholds for protection (Periolo et al., 1993, Van Maanen and Terpstra, 1989, Paton et al., 2019) and perform better in deciding the suitable vaccine to use in an outbreak situation than VNT (Tekleghiorghis et al., 2016). One limitation of the LPBE is its inadequate specificity in identifying low-positive samples, which may cause inconsistencies in the quantification of antibody titres and consequently compromise the accuracy of the calculated r_1-value.

Furthermore, a contributing factor is the inadequate robustness observed when analyzing bulk samples, especially those containing overlapping antigenic sites and inactivated antigen with variable stability; the LPBE has identified sites that may not be pertinent to protection (Reeve et al., 2016). As a result of the limitations, SPCE was created, which can detect samples with low antibody concentrations and is suitable for bulk testing (Mackay et al., 2001)

1.8.1.4. Solid phase competition elisa (SPCE)

Solid phase competition ELISA is an *in vitro* method that has been advanced and validated for the detection of FMD virus antibodies using a wide range of sera from cattle, pigs, goats and sheep (Chénard et al., 2003, Mackay et al., 2001, Paiba et al., 2004). SPCE consist of all the qualities of LPBE and the advantage over the LPBE is the improved specificity to distinguish the low positive samples and being robust in determining antibody titre since the single dilution serum is used for the entire plate since the LBPE have limited single dilution of serum. These improved qualities can be used to improve the consistency and reliability of determining antibodies titres using SPCE which will enhance the accuracy of calculating the r_1-value used as determinant for vaccine matching. Persistence challenge of VNT and LPBE tests determining variable antibody titres from the same sera lead to incomparable titres that establish r_1-value that does not correlate with *in vivo* potency test in terms of cross-protection (Brehm et al., 2008, Brito et al., 2014, Mattion et al., 2009). Mackay at., and Chenard et.al., developed the SPCE and optimised for bulk FMD routine diagnostic by Mackay at. and Chenard et.al (Chénard et al., 2003, Mackay et al., 2001). The SPCE entail the coating of plate with pre-determined concentration of serotype specific rabbit antiserum for defined period illustrated in **figure 1**. A prepared antigen derived from virus of interest, is optimal diluted and added to plate coated with homologous rabbit antisera and incubated for prescribed period. Following incubation and a series of washes, the test serum is added to a plate followed by instant addition of guinea pig antiserum which is homologous to added antigen. The two added sera will compete for binding to trapped antigen on solid phase or surface of the plate during incubation. The antibodies contained in the test serum will compete for binding with antibodies of guinea pig antisera that are homologous to the trapped antigen The test serum antibody will compete with guinea pig antisera to interact with trapped antigens based on antigenic relatedness. These two steps of adding sera to the solid surface and binding competition between the two added sera based on antigenic similarity of contained antibody set the SPCE apart from LPBE and makes it robust to determine the antigenic relatedness of vaccine strain and field isolate. Detection of attached of guinea pig antisera is detected by addition of rabbit anti-guinea pig immunoglobulins conjugate which will followed by addition of substrate or

horseradish peroxidase to initiate the colour development and the development colour will be terminated by addition of sulphuric acid. Results of the binding of either test serum antibodies nor guinea pig antisera antibodies and the quantification of antibodies interacted with trapped antigen will be distinguished by the colour developed and colour intensity, which will be determined using spectrophotometer. Spectrophotometer will measure the optical density (OD) for each plate well reaction (homologous and heterologous strain) and the results are analysed in terms of percentage inhibitions which will be used to determine the log serum titre. Determined serum titres of heterologous and homologous will be used to calculate r_1-value to established indirectly antigenic similarity between the vaccine strain and field isolate.

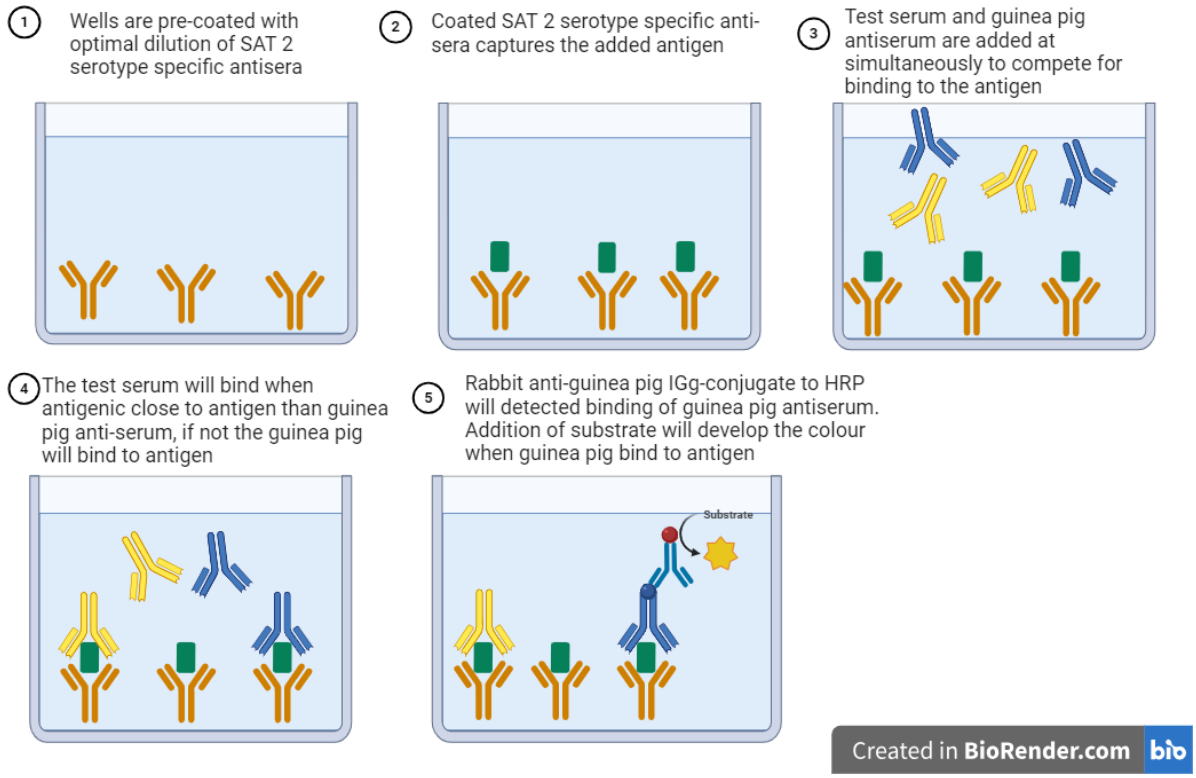


Figure 1: *Description of SPCE determining the cross reactivity of antigen against the competing test sera and guinea-pig antiserum.*

SPCE demonstrates a level of performance similar to LPBE in detecting antibodies utilized in serum titre calculations. Notably, it surpasses the VN test in sensitivity and ability to detect minimal quantities of antibodies (Mackay et al., 2001, Paiba et al., 2004). In contrast to the LPBE, the specificity of the SPCE is comparable to the VNT (Mackay et al., 2001). Therefore, it offers a better antibody detection technique that is sensitive, specific, robust, rapid, and easy to use (Chénard et al., 2003, Mackay et al., 2001), which are the qualities required to improve the vaccine matching tool for better correlation with the *in vivo* potency test.

2. Problem statement

Foot and mouth disease, a highly contagious viral disease that affects cattle, sheep, pigs, and goats, affects subsistence farming and agricultural production worldwide (Alexandersen and Mowat, 2005). FMD has been eradicated in developed countries like the US and Europe through vaccination, animal movement control, slaughtering infected animals, and trade barriers (Bachrach, 1968, Santos et al., 2018), while is still present in parts of South America, Asia, Middle East and Africa (Rweyemamu et al., 2014, Vosloo and Thomson, 2017). Since the 1960s, certain regions of the African continent have witnessed the success of FMD control measures, including animal movement control and the use of inactivated vaccines. Furthermore, numerous countries, including Egypt, South Africa, Botswana, and Namibia, have succeeded with vaccine campaigns that incorporate systematic vaccination (Capstick, 1978, Paton et al., 2005, Rweyemamu et al., 2014, Vosloo and Thomson, 2017). Implementing mass slaughter of infected livestock as a means to control and eradicate FMDV in Africa appears unfeasible for the following reasons: economic impracticability, personnel capacity constraints, logistics shortfall, and the FMD virus's persistent circulation in African buffaloes (Hunter, 1998). It is inconceivable to eradicate FMD in Africa through the annihilation of wildlife species, specifically African buffaloes, due to the irreplaceable and distinctive nature of these animals. Moreover, nature-based tourism provides economic benefits to the continent, especially in eastern and southern regions (Biggs et al., 2004, Cumming, 2008, Thomson et al., 2013).

The utilization of inactivated vaccines as a vaccination strategy to manage FMD in an endemic environment has proven to be effective in southern Africa (Hunter, 1998). However, there has been an increase in the frequency of FMD outbreaks in domestic livestock in the southern Africa region since the year 2000. This has resulted in the ineffectiveness of the existing vaccine (FMD-Bulletin, 2010, Penrith and Thomson, 2012, Jori et al., 2016a, Lazarus et al., 2018, OIE., 2021). An increase in FMD outbreaks and the emergence of novel strains of FMD SAT serotypes exhibiting antigenic variation have been indicative of a lack of antigenic relatedness with vaccine strains and of the ineffectiveness of the existing FMD vaccine in southern Africa (Bastos et al., 2003, Maree et al., 2011, Maree et al., 2015, Vosloo and Thomson, 2017). Serotypes SAT 1 and 2 of FMD are accountable for the majority of outbreaks in southern Africa due to their greater antigenic variation (FMD-Bulletin, 2010, Maree et al., 2011, Vosloo and Thomson, 2017). Currently, the SAT 2 serotype is primarily accountable for FMD outbreaks, affecting an estimated half of the outbreaks in cattle. The remaining half is attributed to the SAT 1 and SAT 3 serotypes (FMD-Bulletin, 2010, Vosloo and Thomson, 2017,

OIE., 2021). In the southern African region, a trivalent FMD vaccine consisting of SAT 1, SAT 2, and SAT 3 strains is routinely administered. However, this vaccine has exhibited insufficient efficacy in terms of preventing and restricting the transmission of FMD, indicating a deficiency in broader antigenic coverage (FMD-Bulletin, 2010, Lazarus et al., 2018, Maree et al., 2015) Antigenic variables of SAT serotypes, which are subdivided into a number of topotypes and new emerging variants that are spatially and temporally distributed, can affect the efficacy of vaccines, necessitating frequent improvements in vaccine usage

In order to address the restricted antigenic coverage of the existing vaccine against SAT 2 outbreak strains, the Agricultural Research Council – Onderstepoort Veterinary Research (ARC-OVR) has formulated a pentavalent FMD vaccine. The effectiveness of a vaccine relies on the cautious selection of the appropriate vaccine strain into vaccine development. The implementation of rapid vaccine matching against the field or outbreak virus is imperative in order to enhance the certainty surrounding the selection of the proper vaccine during an outbreak (Brehm et al., 2008). The VNT, the presently employed vaccine matching technique, is characterized by suboptimal sensitivity, time-consuming process, and inadequate responsiveness when assessing suitability against the field isolate that is specific to a given outbreak (Mackay et al., 2001). The liquid phase blocking ELISA is rapid, reproducible, easy to used, and correlates favourably with VNT in terms of protection contrast (Chénard et al., 2003, Hamblin et al., 1986) A limited specificity, on the other hand, renders the test unreliable for mass screening of samples and frequently yields negative results. As a consequence of these constraints, the SPCE is favoured over the LPBE, which possesses the advantageous characteristics of robust and enhanced specificity (Chénard et al., 2003, Mackay et al., 2001, Paton et al., 2019).

This study will optimize an SPCE to indirectly determine antigenic similarity between the selected prevalent SAT 2 FMD viruses circulating in South Africa and neighbouring countries and sera induced from cattle vaccinated with an ARC-OVR pentavalent vaccine. SPCE will measure antibodies to determine sera titres. Sera titre will be used to calculate r_1-values, which is the ratio of serum titre against heterologous and homologous viruses, to establish antigenic relationships for vaccine matching (Brehm et al., 2008, Kitching et al., 1989, Paton et al., 2019). A close antigenic relationship between the vaccine strain and field isolate is indicated by a r_1-value above 0.4 -1.0, a partial antigenic similarity by 0.2-0.39, and a distant similarity by 0.2 or less (Ferris and Donaldson, 1992, OIE, 2021). The result will demonstrate whether or not the ARC FMD pentavalent vaccine provides protection against SAT2 virus strains that are prevalent. The development of such a SPCE for vaccine matching will enable rapid screening

of circulating field isolates to identify the most appropriate vaccine strain to be used in the formulation of FMD.

3. Hypothesis

3.1. Null hypothesis:

H_0 = The antigenic relationship of the pentavalent vaccine and selected SAT 2 strains does not have an r_1-value greater than 0.4 and the vaccine strain is not antigenically related to the field strain and will not provide adequate protection against the selected SAT 2 strains.

3.2. Alternative Hypothesis:

H_1 ≠ The pentavalent vaccine is antigenically related to selected SAT 2 strains with a r_1-value greater than 0.4 and the pentavalent vaccine is highly likely to provide adequate protection against the against the selected SAT 2 strains.

4. Aims and objectives

4.1. Aims

Determine if the current pentavalent FMD vaccine, developed by the ARC-OVR will adequately protect against prominent SAT 2 strains using an optimised serological assay.

4.2. Objectives

1. To determine the most contemporary prevalent SAT 2 strains within topotype-1 that are circulating and/or caused FMD outbreaks in South Africa and neighbouring countries.
2. Optimisation of an ELISA assay to determine r_1-values.
3. To estimate the antigenic relatedness between the ARC-OVR pentavalent vaccine and the selected SAT 2 FMD strains.

5. Materials and Methods

5.1. Ethical clearance

Permission to conduct the study was granted by the Animal Ethics Committee of Agricultural Research Council - Onderstepoort Veterinary Research, South Africa (Reference: AEC 19.10)

and Research Ethics Committee (REC) and Animal Ethics Committee (AEC) of the Faculty of Veterinary Science, University of Pretoria, South Africa (Reference: REC: 263-19). In South Africa, foot and mouth disease is a controlled animal disease and in accordance with the South African Animal Disease Act (Act no: 35 of 1984), prior commencement of FMD studies requires approval from the Department of Agriculture Land Reform and Rural Development (DALRRL) as such the study was granted approval under the reference (12/11/1/1/8 (1638KL)).

5.2. Selection criteria for contemporary prevalent SAT 2 viruses and their origin.

The origin of the virus samples used in this study were derived from various areas within South Africa, including Mhala and Mutale districts in Limpopo province; communal cattle inspection points in Bushbuckridge municipality of Mpumalanga; Kruger National Park (KNP); and neighboring country, Zimbabwe, in Drysdale, Umguza Matebeleland North in Zimbabwe. The samples were dispatched to OVR - Transboundary Animal Disease (TAD) for the purpose of diagnosing FMD.

On arrival, samples are registered in the OVR-TAD institute database, processed for diagnostic testing, and stored in the -70 ˚C biobank freezers for reference and research purposes. The prevalence of contemporary FMD SAT 2 serotype viruses used in this study was obtained from the records of the OVR-TAD institute database. South Africa and its neighbours were areas of interest for SAT 2 viruses, and topotype-1 viruses were chosen. The FMD SAT 2 viruses that emerged from South Africa, Botswana, Malawi, Mozambique, Tanzania, Zambia, and north west Zimbabwe are referred as topotype-1 viruses (Vosloo and Thomson, 2017). By utilization the OVR-TAD database, six topotype-1 SAT 2 viruses responsible for FMD outbreaks in South Africa and neighbouring country Zimbabwe were selected. Five of six selected SAT 2 viruses that occurred in South Africa are SAR/1/2001; SAR/1/2003; SAR/1/2013; SAR/15/2013 and KNP/12/2008; while one SAT 2 virus, ZIM/2/2013 occurred in Zimbabwe.

SAR/01/2001 virus was derived from cattle during an outbreak in Mhala district in Limpopo province (previously known as Northern province during the time) in South Africa between the 1st of February and the 30th of May 2001 (Vosloo et al., 2002, Bruckner et al., 2002). SAR/1/2003 virus was derived from cattle in 2003 outbreak in Masisi, Mutale Vhembe district municipality, Limpopo province in South Africa (Jori et al., 2016a), while SAR/1/2013 and SAR/15/2013 virus originated from cattle during an outbreak on 14th August 2013 to 3rd June 2014 at the inspection communal point in the FMD protection zone adjacent to Kruger National

Park, Bushbuckridge municipality in Mpumalanga province in South Africa (Blignaut et al., 2020). KNP/12/2008 virus was from buffalo in the Kruger National Park during FMD surveillance studies while the ZIM/2/2013 virus was from cattle during an outbreak in April 2013 in Drysdale, Umguza Matebeleland North in Zimbabwe. Viruses' selection process used was a convenient or purposive non-probability method based on the knowledge that the selected viruses are responsible for contemporary FMD outbreaks in South Africa and neighbouring countries.

5.3. Determination of SAT 2 viruses' lineage.

5.3.1. Ribonucleic acid (RNA) extraction

RNA was extracted from BHK 21 cell cultures infected with SAT 2 viruses using the Quick-RNA™ Viral kit according to the manufacturer's instructions (Zymo Research, United States). Briefly the SAT 2 infected BHK 21 cell cultures of 200 µl, were lysed with double volumes (400µl) of viral RNA buffer containing guanidinium isothiocyanate (GuITC) and sodium iodide solution. The mixture was transferred to spin columns (silica-based matrix that bind the nucleic acids) with collection tubes and centrifuge for 2 minutes at 13 000 rpm. Following centrifugation, wash buffer was added to the column and once again centrifuged for 30 seconds at 13 000 rpm to rinse the columns. A further wash step was performed by the addition of ethanol and centrifugation for 1 minute at 13 000 rpm. Bound RNA was eluted from the silica-based matrix column by the addition of 15 µl DNase/RNase free water, followed by centrifugation at 13 000 rpm for 30-60 seconds and stored at -70˚C.

5.3.2. cDNA synthesis and amplification of SAT 2 P1 region

The purified viral RNA was reversed transcribed to synthesise complementary DNA (cDNA) and the P1 region of FMD virus was amplified using the One-Taq one-step kit protocol (Biolab®Inc), according to manufactures instructions. The reaction mixture consisted of 25 µl reverse transcriptase buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 0.01 % IGEPAL, 0.5 mM DTTP, 0.4 mM oligonucleotides, 10 mM DNTP); 2 µl Reverse Transcriptase and 2 µl of 10 pmol of random primers followed with 2 µl 10 pmol of each of the forward (NCR1 - 5'-TACCAAGCGACACTCGGATCT-3') and reverse (WDA 5'-GAAGGGCCCAGGGTTGGACTC-3') primers described by in the study by Chitray et al and 5 µl (Chitray et al., 2020). The RNA template of 5 µl was include and ionized H_2O used to adjust to the volume to 50 µl. The tubes were place in the GeneAmp® 9700 thermal cycler (Applied Bioscience) and reverse transcription and gene amplification was conducted in a single tube using the conditions stated in table 1. Gene amplification of the P1 region was set for 35 cycles.

Table 1: Conditions of reverse transcription and amplification of P1 region for 35 cycles

Steps	Temperature	Time
Reverse transcription	48 ˚C	30 minutes
Initial Denaturation	94 ˚C	1 minute
Denaturation	94 ˚C	15 seconds
Annealing	55 ˚C	30 seconds
Extension	68 ˚C	2.30 minutes
Final extension	68 ˚C	5 minutes
Hold/Storage	4 ˚C	Until recovered

5.3.3. PCR amplicon analysis and sequencing of VP1 region of FMDV

Using 1 x TAE buffer (40 mM Tris-acetate, 2 mM EDTA; pH 8) and 0.5 µg/mL ethidium bromide, a 1.5% (w/v) agarose gel was prepared. The PCR amplicons were loaded into wells of an agarose gel containing 1 x TAE buffer, and the electrophoresis was conducted for 30 minutes at 120 volts. The GeneRuler 1kb plus DNA marker from Thermofisher Scientific was used on the agarose gel to determine the size of the DNA amplicons. The expected size of the bands was around 2.2 kilo base pairs (kbp). The DNA bands were excised and cleaned using the gel DNA recovery kit (Zymo Research) following the manufactures's instructions The DNA bands were excised and cleaned using the gel DNA recovery kit (Zymo Research) following the manufactures instructions. The purified DNA amplicons were inactivated at 55 °C for 30 minutes after purification before being sent to Inqaba Biotechnical Industries for VP 1 gene sequencing. The sequencing reaction and Sanger sequencing were prepared and performed by Inqaba Biotechnical Industries.

5.3.4. VP1 gene sequence analysis by phylogenetic tree

The nucleotide sequences of the SAT 2 VP1 gene in both the forward and reverse directions were examined, edited, aligned, and used to generate consensus sequences using BioEdit version 7.2.5 (Hall, 1999). The SAT 2 viruses consensus sequences were confirmed using the BLAST (Basic Local Alignment Search Tool) software provided by the National Centre for Biotechnology Information. A phylogenetic tree was generated using the distance-based approach and neighbour-joining algorithm implemented in the MEGA 10 Software (Hall, 2013, Tamura et al., 2013, Kumar et al., 2018). The substitution model utilized for determining the evolutionary relationship of the SAT 2 partial VP1 involved the application of the nucleotide

Kimura 2-parameter. This was achieved by comparing the homologous sequences and calculating the number of base substitutions per site. The reliability of the phylogenetic tree was assessed using a statistical re-sampling process called bootstrapping. This involved generating 1,000 bootstrapping replicates. Values equal to or greater than 65% were deemed to be statistically significant.

5.4. Media preparation for cells and virus cultivation

The growth medium used for cell cultivation was constituted of supplemented Glasgow minimum essential medium (GMEM, Sigma Aldrich™) with 10 % (v/v) Bovine Sera (Cell-Sera Australia™), 10 % (v/v) Tryptose Phosphate broth (TPB, Becton, Dickson™ [BD] and Company, USA) and 3 % (v/v) lactalbumin hydrolysate (LAH, Becton, Dickson™ [BD] and Company, USA) with no antibiotics added and the composition was considered as complete cell media. The virus cultivation medium contained GMEM and nutrient supplements similar to the cell complete medium, except the bovine sera concentration was 1 % (v/v) and the titration test complete media was 2 % (v/v).

5.5. Cultivation of BHK 21 cells for virus inoculation

The baby hamster kidney (BHK) cell line is known to be susceptible to FMDV (Macpherson and Stoker, 1962) and BHK cell strain 21 (BHK 21) monolayer, clone 13 (American Tissue Culture Collection CCL-10) was cultivated for the propagation of SAT 2 viruses. An ampoule of 1-2 ml containing BHK 21 cells maintained in complete cell media was sourced from the repository of OVR-TAD Tissue Culture Unit. The ampoule was stored in liquid nitrogen and cells were resuscitated by gradually defrosting the ampoule on ice at room temperature. The defrosted ampoule of BHK 21 cells was transferred into a 5 ml centrifuge tube and centrifuged for one minute at 1 500 rpm, the supernatant was decanted. The cell pellet was reconstituted with 2 ml of complete cell medium, briefly centrifuged again to remove excess cryoprotective agent (dimethyl sulfoxide) and reconstituted once again with 5 ml of complete cell medium for cell seeding. A cell seeding rate of 0.05 x 10^6 cell/cm^2 was used to seed a 175 cm^2 T-flask (Corning™ Inc., United States) and topped with complete cell media to 50 ml.

The cell media with seeded cells' pH was adjusted using medical CO_2, altering the cell media colour from reddish to orange yellow, which is estimated to be between pH 6.5 and 7.5, and the flask was incubated at 37 °C. Cell growth was monitored daily, and by day three, cell confluence was 90 –100%. The flask's spent growth medium was decanted and rinsed with Mg2+ and Ca+-free PBS to passage cells. For cell detachment, the trypsin solution (10% v/v; ATV, Merck™, South Africa) constituted in PBS was briefly added into the flask, covering the

flask surface. A minute after incubation, the trypsin solution was decanted, and 20 ml cell complete media was added to homogenize the cells. Six 175 cm^2 T-flasks were seeded with 0.05 x 10^6cell/cm^2 for each virus. After 48 hours at 37 °C, flasks had 100% cell confluence for virus inoculation.

5.6. Inoculation of FMD SAT 2 viruses to BHK 21 cells

From the -70 °C ultra-freezer biobank, six FMD SAT 2 virus aliquots (SAR/1/2001; SAR/1/2003; SAR/1/2013; SAR/15/2013; KNP/12/2008; ZIM/2/2013) were retrieved and thawed on ice. A 500 µl aliquot of each virus was blindly inoculated into one of six 175 cm^2 T-flasks containing BHK 21 cells. Prior virus inoculation, the spent growth media was decanted from the flasks, and replenished with 50 ml of virus cultivation media (1 % bovine sera) and incubated at 37 °C for one hour to reacclimatize cells. After cell acclimatation, the inoculated cultures were incubated at 35 ˚C, and the flasks were observed daily using inverted microscope (light microscope® CKX31, Olympus™) for signs of cytopathic effect (CPE) as an indicator for virus infection and replication in the cells. Following 72 hours post-infection (hpi), a CPE ranging from 70% to 80% was observed. The virus culture, which was in suspension, was harvested using pipetting. The attached monolayer cells that were infected with the virus were harvested through rapid freezing and thawing. The harvested virus was pooled together (suspension harvest and freeze and thaw harvest) and added 20 % (v/v) glycerol (Merck™, South Africa) to maintain the stability of FMD virus stock. This was applied to all SAT 2 viruses cultivated and the virus harvests were aliquoted into 5 ml cryotubes and stored at -20 °C for further virus adaptation and virus titre determination. This procedure was applied to all SAT 2 viruses cultivated and the virus harvests were aliquoted into 5 ml cryotubes and stored at -20 °C for further virus adaptation and virus titre determination.

5.7. Adaptation of SAT 2 viruses to BHK 21 cells and titres determination

The SAT 2 viruses (SAR/1/2001; SAR/1/2003, SAR/1/2013, SAR/15/2013, KNP/12/2008, and ZIM/2/2013) previously preserved in Instituto-Biologico - Rim-Suino-2 (IB-RS-2) cells were cultured and adapted to BHK 21 cells. The viral titers were determined by conducting virus titration to assess the viruses' adaptation to the BHK 21 cells. Titrating of SAT 2 viruses, 2 x 175 cm2 T-flasks (Corning™ Inc. United States) of BHK 21 cells were cultivated as described in section 5.4 (**Cultivation of BHK 21 cells for virus inoculation**). Upon reaching 100 % cell confluency, the BHK 21 cells were harvested by decanting spent growth media and adding trypsin solution (10 % (v/v); ATV, Merck™, South Africa). Following a brief incubation of one-minute, the trypsin was decanted, and the cells resuspended with 20 ml of complete titration

cell media (2 % [v/v] bovine sera). Cell count was determined using Neubauer chamber and the cell concentration was adjusted to 1.0 x 10^5 cells/ml using the titration cell media.

A virus titration was performed by aliquoting a cell suspension of 1.0 x 10^5 cells/ml concentration into nine tubes (10^{-1} to 10^{-9} tubes). Cultivated SAT 2 virus volume of 300 µl was added to 10^{-1} tube, making a 1:10 dilution, and a tenfold serial dilution was performed by by titrating 300 µl from 10^{-1} tube throughout the tubes until 10^{-9} tube as illustrated in **figure 2**. Virus titration of 10^{-2} tube to 10^{-9} tube dilution, 200 µl volumes were added to flat-bottom 96 microwell titration plate (Thermofisher Scientific™) wells into row A-H, column 1-12 as illustrated in **figure 2**. This virus titration was performed for all the SAT 2 cultivated viruses and the positive control of SAT 3 strain (KNP/10/1990) virus and negative control of uninfected BHK 21 cells were included. Plates were placed in a 5 % CO_2 incubator at 37 ˚C and were observed daily under an inverted microscope (light microscope® CKX31, Olympus™) for signs of CPE. After 72 hours the plates were retrieved for results readout and each row (A-H) on the plate was examined for CPE signs. The presence of CPE was recorded indicated by a positive symbol while the absence of CPE was indicated by a negative symbol. The statistical excel-sheet formula was used to input the recorded results and the titre results were expressed as $\log_{10}$ $TCID_{50}$/ml (50 % tissue culture infective dose). All the SAT 2 viruses were repeatedly sub-cultured and virus titration was carried out until the viruses were adapted to BHK 21 cells, where 100 % CPE was observed within 24 hours with a virus titre of above $\log_{10}^{7.0}$ $TCID_{50}$/ml reached.

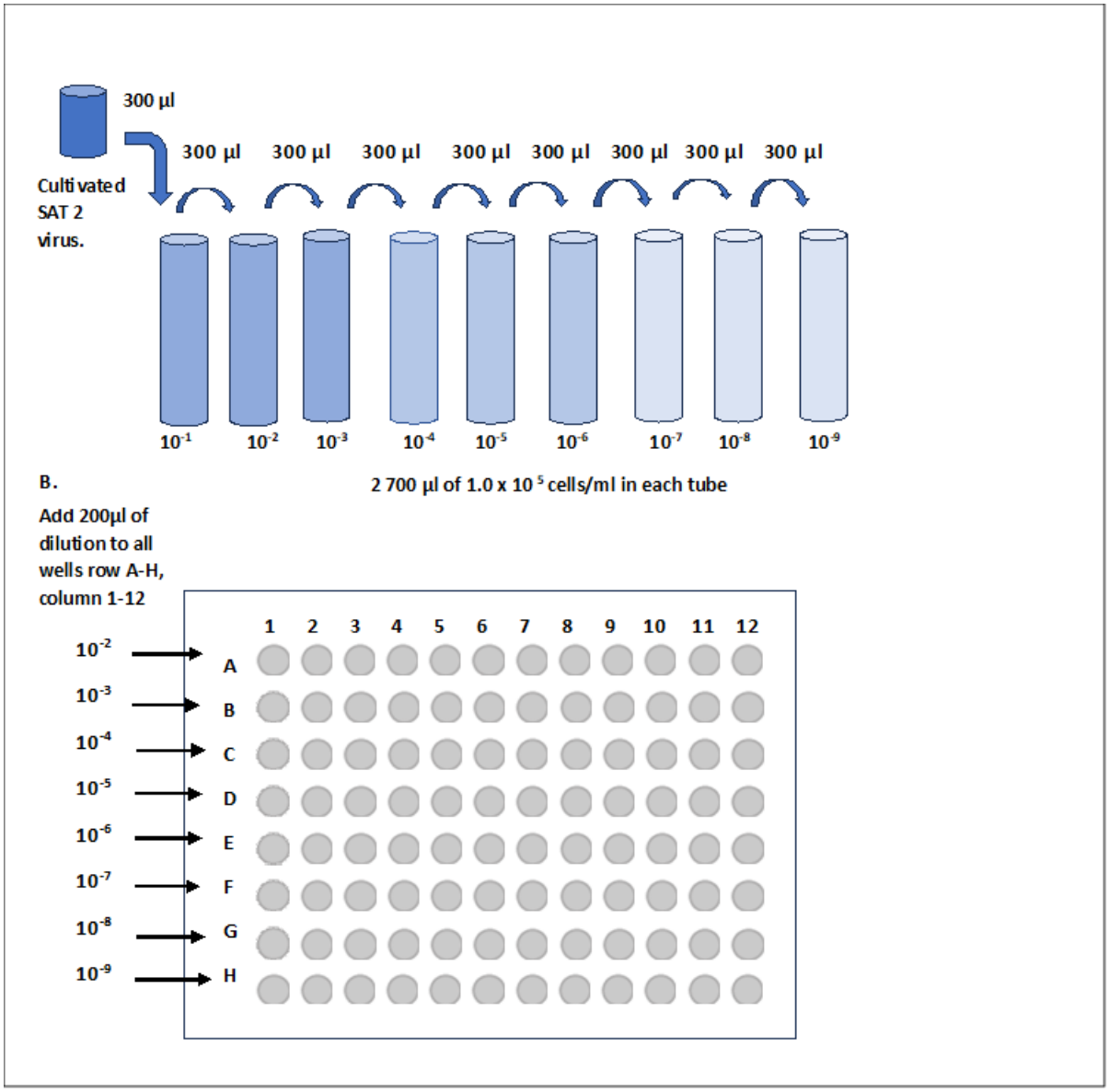


Figure 2. *A. Illustration the virus titration of SAT 2 viruses; SAR/1/2001; SAR/1/2003; SAR/1/2013; SAR/15/2013; KNP/12/2008; ZIM/2/2013 that were each titrated in nine tubes (10^{-1} to 10^{-9} tubes) using ten-fold dilution. The tubes contained 2.7 ml of BHK 21 cells adjusted to 1.0 x 10^5 cell concentration and 300 µl of cultivated SAT 2 virus was added to 10^{-1} tube. Tenfold dilution was carried throughout the tubes with volume of 300 µl from 10^{-1} to 10^{-9} tubes. B. Flat-bottom 96 well micro titration plate were virus titration of 10^{-2} to 10^{-9} tubes were added to the plate with 200 µl volume added to row A-H, column 1-12.*

5.8. Antigen preparation of SAT 2 viruses and precipitation

SAT 2 viruses SAR/1/2001; SAR/1/2003; SAR/1/2013; SAR/15/2013; KNP/12/2008 and ZIM/2/2013 all with virus titres of above log $10^{7.0}$ were used for the preparation of antigens. Each virus was inoculated into a set of 5 x 850 cm^2 roller bottles (Corning™ Inc. United States) cultivated with BHK 21 monolayer cells. The multiplicity of infection (MOI) ratio of 1: 50

$TCID_{50}$/cells were used for virus inoculation and roller bottles were incubated at 35 °C until the occurrence of CPE was observed.

Once 90% CPE was obtained, the virus culture of the five roller bottles (for each virus) was pooled into **bottle A**, as shown in **figure 3,** and excess cells on the roller bottles were removed by rapid freezing and thawing. Chloroform (0.3 % (v/v), (Merck™ company, South Africa) was added to the virus harvest and stirred at 26 ˚C for 30 minutes. For inactivation of virus harvest, 5 mM binary ethyleneimine (BEI, Merck™ Company, South Africa) was added in two parts (Bahnemann, 1975). First part, 2.25 mM BEI was added to the virus harvest and stirred for 4 hours at 26 °C. Virus harvest was centrifuged at 2 000 rpm for 10 minutes and supernatant was transferred into **bottle B**. Subsequently, an additional 2.25 mM of BEI was promptly introduced, and the mixture was left to incubate overnight while stirring at a temperature of 26 °C. After 24 hours of BEI inactivation, a solution of sodium thiosulfate (2% [w/v], Becton, Dickson™ [BD] and Company, USA) was introduced to halt the inactivation process (Ferris et al., 1990). Thereafter, the inactivated virus was further clarified by centrifugation at 5000 rpm for 30 minutes at 4 ˚C.

The supernatant with complete virus particles (146 particles), which is referred as antigen, were precipitated from the recovered suspension with a double treatment of 50 % (w/v) polyethylene glycol (PEG) and diatomaceous earth (DE) mixture (Merck™ company, South Africa) in two-part doses 8% and 6% displayed in **figure 3**. The first final concentration of 8 % was attained by adding a mixture of 50% (w/v) PEG plus DE solution to the suspension in **bottle C**. The suspension was then incubated at 4 °C for two hours with gentle stirring. The suspension was then centrifuged (30 minutes at 3000 rpm), and the supernatant was discarded while the pellet (PEG containing antigen) was suspended in 5% (v/v) Tris-phosphate buffer (pH 7.6) (Merck™ Company, South Africa). The suspension was centrifuged (30 minutes at 3000 rpm) once again, and the supernatant containing antigen was collected and stored at 2 °C – 4 °C. The pellet was further resuspended in 1 % (v/v) Tris-phosphate buffer (pH 7.6) and centrifuged for 30 minutes at 3000 rpm. Supernatant was collected and pooled together with the previous collected antigen. A second dose of PEG plus DE solution was added to the collected supernatant **bottle D** to achieve a final concentration of 6%. The mixture was then incubated and centrifuged as previously described. The two collected supernatants were pooled and resulted in purified 146 S virus particles, to which 20 % (v/v) glycerol (Merck™ company, South Africa) was added and stored at -20 °C

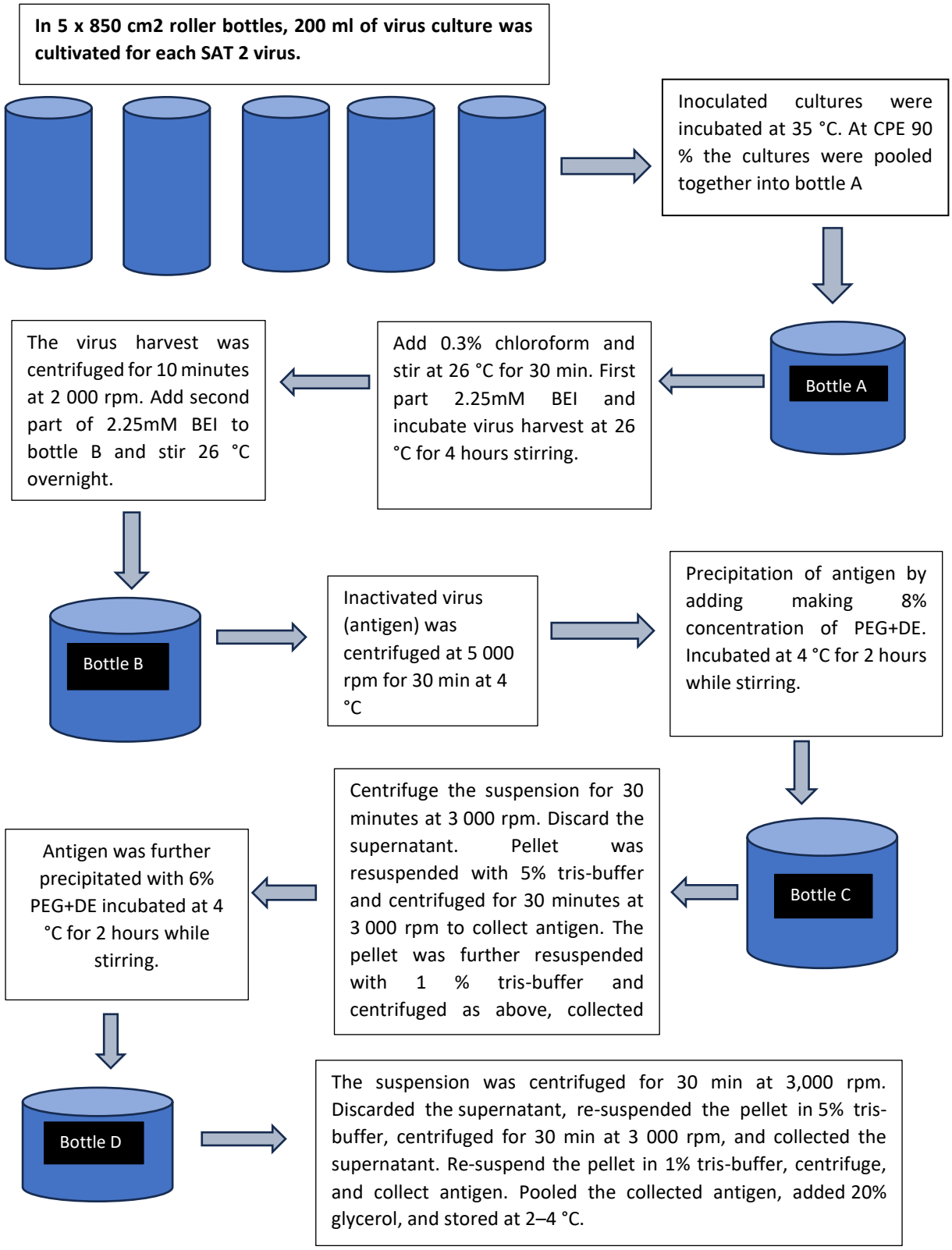
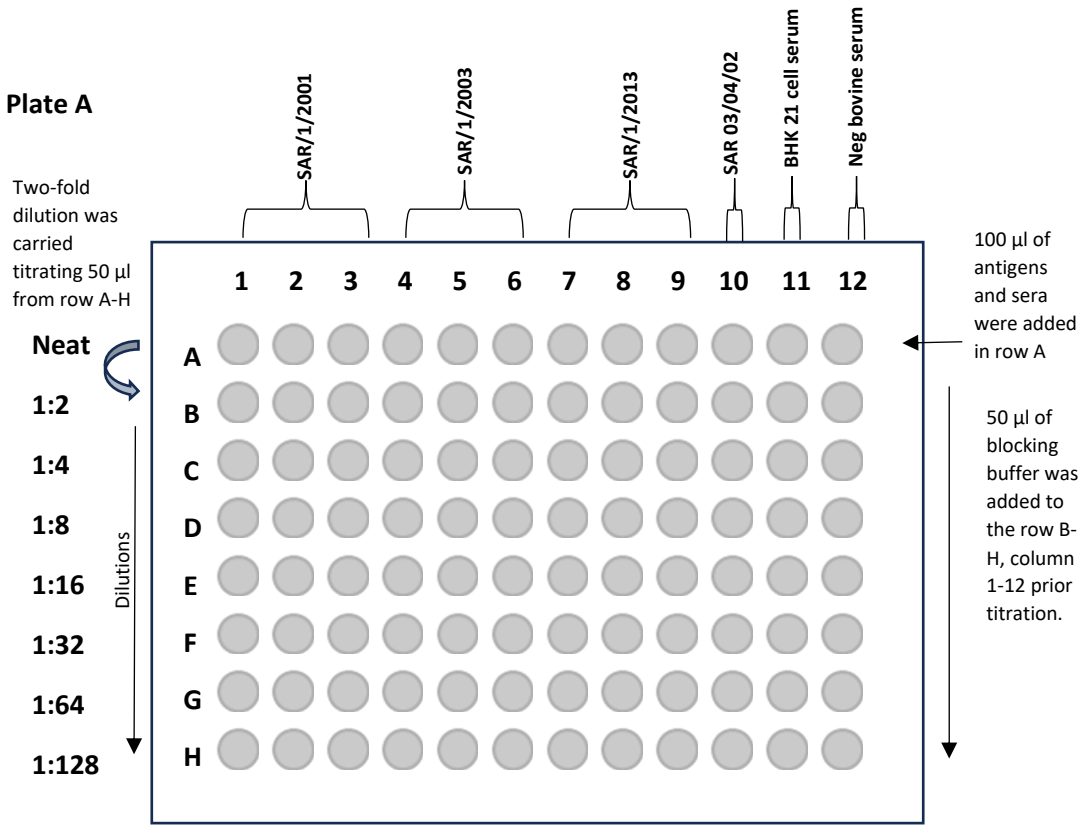


Figure 3. *Flow diagram illustration the inactivation and precipitation of cultivated SAT 2 viruses in 5 X 850 cm² roller bottles. SAT 2 viruses, SAR/1/2001, SAR/1/2003, SAR/1/2013, SAR/15/2013, KNP/12/2008 and ZIM/2/2013, each were cultivated in a set of 5 x 850 cm2 roller bottles. Virus cultures were harvested and pooled together into bottle A per virus and added 0.3 % chloroform which followed by inactivation with 2 x 2.25 mM BEI in bottle A and B respectively. Inactivated virus was precipitated with 8% PEG+DE in bottle C and 6% in bottle D, and antigens were collected and stored at 2–4 °C.*

5.8.1. Determination of antigen concentration using titration test.

The concentration of the SAT 2 antigens prepared from each of the six virus isolates and the suitable antigen dilution for use in the solid-phase competition enzyme ELISA (SPCE) were determined using the indirect sandwich ELISA. Flat-bottom Maxisorp™ 96-microwell plates (Nunc™) were coated with a pre-determined optimal dilution (1:50) of SAT 2 serotype-specific rabbit antisera in 50 mM carbonate/bicarbonate (pH 9.6) and incubated at room temperature overnight. The plates were washed three times with phosphate buffer saline (Brehm et al., 2008, Paton et al., 2005) (PBS/T) using an automated plate washer and stored at -20 ˚C until required. A volume of 50 µl was used throughout the ELISA, unless otherwise specified. Two Maxisorp™ 96 well plates (Nunc™) were used for titration of the six SAT 2 antigens. Per 96-microwell plate, three antigens were added in triplicate with 100 µl volume on row A (column 1-9) as illustrated in **figure 4**. The plates contained a positive control (SAR 03/04/02) in column 10 and negative controls in column 11 (BHK 21 cell serum) and column 12 (negative bovine serum) as illustrated in **figure 4**. In each well of row B- to- H, 50 µl of blocking buffer (PBS 0.1 M, pH 7.6, containing 2% [w/v] milk powder) was added and a two-fold dilution was performed on each well (neat to 1:128) across rows A–H. The plates were incubated for 1 hour at 37 ˚C on a rotary shaker and washed three times with PBS/T as described above. SAT 2 specific guinea pig antiserum diluted in blocking buffer (1: 60) was added to all the wells in each plate and the plates were incubated again for 1 hour at 37 °C. Following incubation, the plates were washed three times with PBS/T. The plates were subsequently added with horseradish peroxidase-conjugated rabbit anti-guinea pig immunoglobulin (IgG) (Sigma Aldrich™) diluted in blocking buffer (1:20,000). The plates were incubated at 37 °C for one hour. Following the final wash, the 100 µl of substrate/chromogen solution consisting of 4 mM 3,3',5,5'-Tetramethylbenzidine (Sigma-Aldrich) in substrate buffer (0.1 M citric acid monohydrate, 0.1 M tri-potassium citrate; pH 4.5) and 0.015% (v/v) H_2O_2, was added. The reaction was terminated after a duration of 10 minutes using 50 µl of 1M sulphuric acid (H_2SO_4). The absorbance measurements were obtained using a Labsystems Multiscan Plus photometer at 450 nm. The mean values were computed for the absorbance measurements performed in triplicate for each dilution, and the results were presented in the form of optical density (OD). The background interference caused by non-specific binding, represented by the average optical density (OD) values of BHK 21 cells (cultured tissue cells), was subtracted from the OD values of the antigens. The calculated OD results were plotted on a scatter plot to ascertain

the antigen concentration and determine the optimal dilution that can be utilized for all antigens in SPCE.



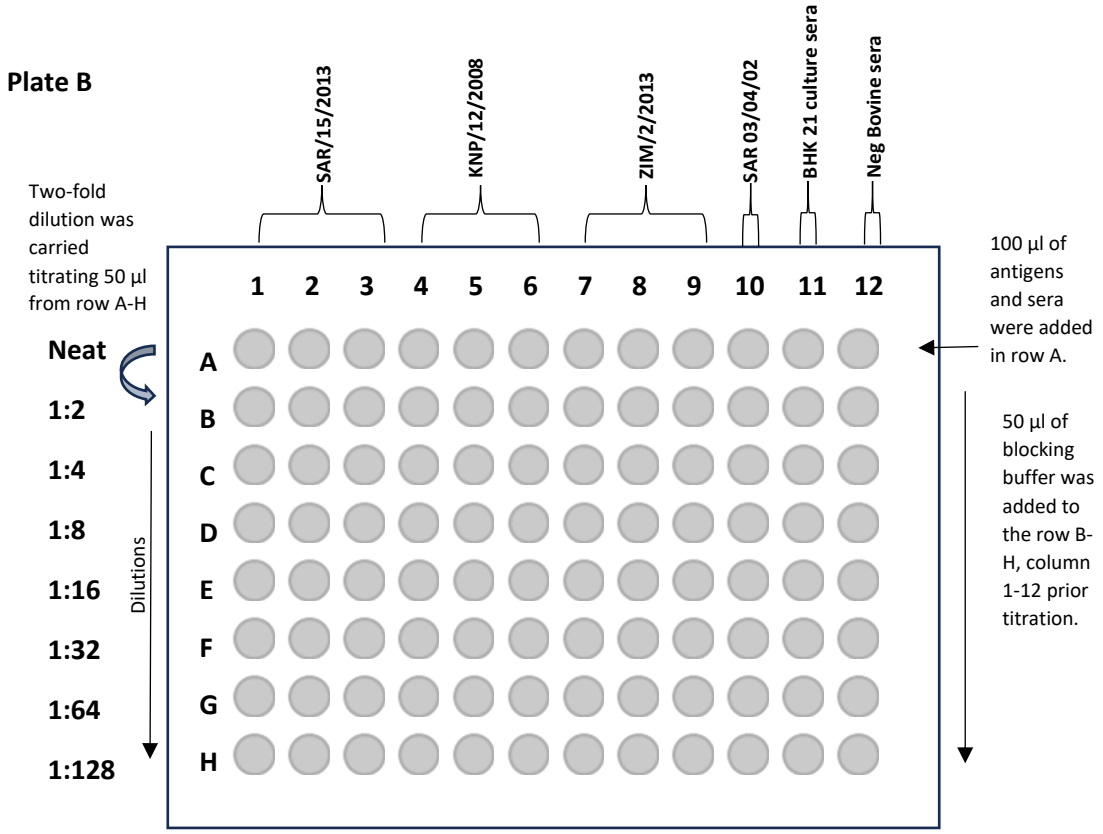


Figure 4: *Diagram titration of SAR/1/2001; SAR/1/2003; SAR/1/2013; SAR/15/2013; KNP/12/2008; and ZIM/2/2013, which are all prepared SAT 2 antigens. The titration of antigens was conducted using two Maxisorp™ well plates (A and B). Each plate had three antigens added in triplicate in row A, with a volume of 100 µl of undiluted antigen (neat). The positive control SAR 03/4/02 was included, along with the negative controls BHK 21 culture sera and Negative bovine sera. A blocking buffer of 50 µl was added to rows B-H. A two-fold dilution was performed by titrating 50 µl from row A-H, resulting in dilutions ranging from neat to 1:128.*

5.9. Vaccine matching test

5.9.1. Origin of bovine post-vaccinal sera

Bovine post-vaccinal sera used in the study for vaccine matching were obtained from cattle vaccine trial study conducted at the OVR-TAD Biosecurity level 3 (BSL-3) containment facility. The study approval was granted by the ARC-OVR Animal Ethics Committee (Reference no: AEC: 23.18) and permission to conduct the research was obtained from the South African Department of Agriculture, Land Reform and Rural Development under section 20 of the Animals Diseases Act, 1984 (Act No 35 of 1984) with permit no: 12/11/1/6. The cattle were vaccinated with the OVR-TAD pentavalent vaccine, comprised of five FMD SAT strains namely SAT 1 (BOT/ 1/2006 and SAR/9/1981); SAT 2 (SAR/3/2004 and KNP/1/2010) and SAT 3 (KNP/10/1990). The antigen payload was 3.0 µg/ml for each SAT 1 and SAT 3 and 6.0 µg/ml for each SAT 2 strain. The antigens were pooled and formulated into pentavalent vaccine (Peta et al., 2021).

Calves of 6 month were used in the vaccine trial, where 2.0 ml of vaccine dose was administered intramuscularly per cattle and bovine post vaccinal sera were collected during the study at 0, 7-, 14-, 21- and 28-days post vaccination (dpv) and stored in the -70 ˚C biobank freezer. Seven samples of bovine sera collected 28 days post-vaccination (dpv) were utilized in this investigation. Five of these samples (Serum 1, Serum 2, Serum 3, Serum 4, and Serum 5) originated from pentavalent vaccine-vaccinated cattle. The two remaining samples (serum 6 and serum 7) were obtained from unvaccinated control cattle. Indirect vaccine matching (antigenic matching) was performed using the optimised SPCE to determine cross-reactivity between bovine post vaccinal sera against the antigens prepared from field virus isolate (six SAT 2 viruses that were responsible for outbreaks in South Africa and neighbouring country Zimbabwe). The cross-reactivity results were used to establish antigenic relationship between pentavalent vaccine strains and SAT 2 viruses using the r_1-value method.

5.9.2. SAT 2 Solid Phase Competition Enzyme linked Immunosorbent assay (SPCE)

Dilutions of the test sera and the SAT 2 guinea pig anti-FMDV antiserum were used to optimize the SPCE, with the antiserum competing for antigen binding. The dilution of 1:4 dilution of test sera and dilution of 1:60 for SAT 2 guinea pig anti-FMDV antiserum in blocking buffer were the optimal dilution for targeted antigens.

Optimised SPCE for FMDV serotype SAT 2 was performed following the standard procedure (Mackay et al., 2001, Paiba et al., 2004) using the prepared SAT 2 antigens and the bovine post-vaccinal sera (test serum). The principle of the test is based on the competition between the antibodies present in the test serum and serotype SAT 2 guinea pig anti-FMDV antiserum. Flat-bottom Maxisorp™ 96-microwell plates (Nunc™) coated with SAT 2 serotype specific rabbit antisera (trapping antibody) prepared as indicated in section 5.7.1, were retrieved, and thawed at room temperature. Two Maxisorp™ 96-microwell plates (Nunc™) were used for each test serum and 50 µl volumes of undiluted SAT 2 antigens were added in duplicate wells. Plate-1 was prepared by adding specific antigens to different columns. SAR/1/2003 antigen was added to column 1 and 2, SAR/1/2013 antigen to column 3 and 4, SAR/1/2001 antigen to column 5 and 6, and SAR/15/2013 antigen to column 7 and 8. Column 9 was left empty. Columns 10-12 were reserved for antigen control, negative control, and positive control, respectively. In these columns, the SAR 03/04/2 antigen was added. In Plate-2, KNP/12/2008 antigen was added to column 1 and 2, ZIM/2/2013 antigen to column 3 and 4, and SAR 03/04/2 (homologous antigen) to column 5 and 6. The controls were added to columns 7-9, following the same procedure as described in plate-one. The empty columns were treated with blocking

buffer and the plates were incubated for 1 hour at 37 °C on a rotary shaker. Subsequently, the plates were washed three times with PBS/T using an automated plate washer. Test serum (20 µl) and 80 µl blocking buffer (1:4 dilution) were added to antigen wells in row A of plates. In row A, the antigen control received 100 µl of blocking buffer, the negative control received 100 µl of negative bovine, and the positive control received 100 µl of SAR 3/04/2 sera. Two-fold dilutions ranging from 1:4 to 1:512 were carried out on the plates. Then, 50 µl of guinea pig antiserum specific to SAT 2, diluted in blocking buffer at a ratio of 1:60, was immediately added to all the wells of the plates. The plates were then incubated at 37 °C for 1 hour. Three washes with PBS/T were performed on the plates. Following that, horseradish peroxidase (HRP) conjugate rabbit anti-guinea pig immunoglobulin (IgG), diluted at a ratio of 1:50 in blocking buffer, was added to each well in a volume of 50 µl. The plates were subsequently incubated at 37 °C for an additional hour. Following the last washing step, 100 µl of the substrate/chromogen solution was added. The substrate solution consisted of 0.1 M citric acid monohydrate, 0.1 M tri-potassium citrate with a pH of 4.5, and 0.015% (v/v) hydrogen peroxide and 4 mM 3,3',5,5'-Tetramethylbenzidine (Sigma-Aldrich). The color reaction was terminated after 10 minutes by adding 50 µl of 1 M sulphuric acid (H_2SO_4). Subsequently, the absorbance of the plates was measured at 450 nm using a Labsystems Multiscan Plus photometer.

The duplicate absorbance results were averaged and expressed as mean OD. The mean OD of test serum well divided with the mean OD antigen control well were used to calculate the percentage inhibition (PI %) of each serial dilution using the following formula:

$$\textbf{Percentage Inhition (PI \%)} = \textbf{100} - \left(\frac{\textbf{mean OD of test serum well}}{\textbf{mean OD of antigen control well}}\right)\textbf{x 100 \%}$$

During two-fold serial dilution, PI % showed how much test serum inhibited SAT 2 specific guinea pig antiserum from interacting with the antigen. The calculated PI % on row A (1:4 serum dilution) was referred as P1, row B (1:8 serum dilution) as P2 until row H as P8 (1:512 serum dilution). The distinction between positive and negative reactions was established by using a cut-off value for the PI %, which was determined by comparing the highest PI % value of negative serum to the lowest PI % value of the test serum. Determined P1-to-P8 percentage inhibitions were used to calculate sera titres against the SAT 2 antigens where the 50 % endpoint titres were determined following the Spearman-Kärber method (Kärber, 1931, Spearman, 1908). The serum titres were expressed as a reciprocal of the final dilution of serum with 50 % inhibition using the formula below. The standard deviation was calculated to distribution of serum titres and determine the intra-repeatability and reproducibility of the SPCE.

$$\textbf{Log serum titer} = \left[\left(\left(\frac{\sum \textbf{PI} + \textbf{P2} \ldots + \textbf{P8}}{\textbf{100}}\right) - \textbf{0.5}\right)\right] \textbf{X log serial dilution (2)} + \textbf{log starting dilution (1)}$$

Figure 5*: Description: Spearman-Karber method used to calculate the log*$_{10}$ *serum titre at 50 % end point dilution. Log serum titres against SAT 2 viruses were determined by summing the percentage inhibition of P1 to P8 and divided with the highest percentage inhibition (100) and subtracted with 50 % endpoint dilution (- 0.5). The product was multiplied with a log serial dilution factor of 2 which is the two-fold serial dilution carried out for the test serum while the log starting dilution (1) is the reciprocal of the highest dilution which the percentage inhibition is positive.*

5.10. Determination of r_1-value to estimate antigenic relatedness.

The serum titre of vaccine strain (homologous strain) and the field isolate (heterologous strain) were determined to establish the r_1-value to establish the antigenic relatedness between vaccine strain and circulating strain. The antigenic relatedness can be correlated to the potential protection that can be afforded by the vaccine and the following formula is used to calculate the r_1-value:

$$\textbf{r1 value} = \frac{\textbf{Titre of post vaccinal serum against the heterologous virus (SAT 2 virus strain)}}{\textbf{Titer of post vaccinal serum against the homologous virus (vaccine strain)}}$$

5.10.1. Interpretation of r_1-value results

The interpretation of r_1-value results, based on the recommended guidelines of the WOAH (OIE, 2021) and Ferris and Donaldson (Ferris and Donaldson, 1992), are as follows.

0.4–1.0: Close antigenic relationship between field isolate and vaccine strain. The vaccine strain is potent and will afford protection against the field virus.

0.2–0.39: The field virus is antigenically related to the vaccine strain. The vaccine strain might be appropriate to use against field virus if no closer match is available, on the basis that animals are immunised more than once.

<0.2: The field isolate is distantly related to the vaccine strain and the vaccine strain is unlikely to protect against challenge with the field isolate.

6. Results

6.1. Phylogenetic tree analysis of characterized VP1 region sequences of SAT 2 viruses

In the effort to understand the spread and epidemiology of FMD virus, the VP1 coding region of the virus has been systematically explored by sequencing and analysis to characterize the FMD viruses. Sequencing of VP1 coding region has remain the leading tool used for comparison and classification of the FMD viruses (Knowles et al., 2016).

In this study, six SAT 2 field viruses from topotype-I were selected from the database of ARC-OVR TAD, OIE reference laboratory. The P1 region of the FMD viruses which comprise of structural proteins (VP1-4) region (Domingo et al., 2002, Rowlands, 2008) were amplified and analysed in **figure 6**. The amplicons of all six SAT 2 viruses exhibited a size consistency with the anticipated 2.2 kbp for the P1 region. The VP1 region, which contains the dominant antigenic sites of FMD virus (Grazioli et al., 2006) , was sequenced from the P1 region. Using BLAST, the VP1 sequences were confirmed as being FMD SAT 2 viruses.

The phylogenetic tree was constructed using neighbour-joining model with 1000 bootstrap replicates (**Figure 7**), where nucleotides similarities were compared among six SAT 2 field viruses ARC SAT 2 vaccine strain (SAR/03/2004); and two reference SAT 2 prototype which are SA/106/59 and ZIM/14/2002 strain. Inclusion of references The SAT 2 prototype strains were based on that; the strains are clustered within the topotype-1 and indicated relatedness based on the clustering in the phylogenetic tree. Phylogenetic analysis of VP1 sequences showed viruses being grouped into four distinct genotypes (**Figure 7**). The ARC SAT 2 vaccine strain (SAR/03/2004) clustered into different genotype which was separate from all the six SAT 2 viruses including the reference SAT 2 prototype strains. Based on the phylogenetic tree clustered, the ARC SAT 2 indicated distant genetic relatedness with the selected SAT 2 viruses. Clustering of the SAR/15/2013 and SAR/1/2013 viruses into a single genotype suggests that the two viruses share close genetic similarity based bootstrap value of 100. The apparent similarity of the viruses can be attributed to the fact that they were collected from various cattle inspection sites during the 2013 outbreak in the municipality of Mpumalanga. Genetic similarity was observed between the virus's SAR/1/2001, KNP/12/2008, and ZIM/2/2013 and the reference SAT 2 protyotype strain SA/106/59, which indicated genetic distinct from the ARC SAT 2. Based on 100 bootstrap values, the KNP/12/2008 and ZIM/2/2013 indicated a high degree of genetic similarity, which may indicate that the viruses emerge from common ancestral strains through evolution. The genetic similarity between the viruses was

inferred from the clustering of the SAR/01/2003 and SAT 2 prototype strains ZIM/14/2002 into a single genotype; These apparent nucleotide similarities proposed that, the source of SAR/01/2003 outbreak in Masisi might have emerged from prototype strain ZIM/14/2002. However, the viruses exhibited a distant genetic relationship with ARC SAT 2.

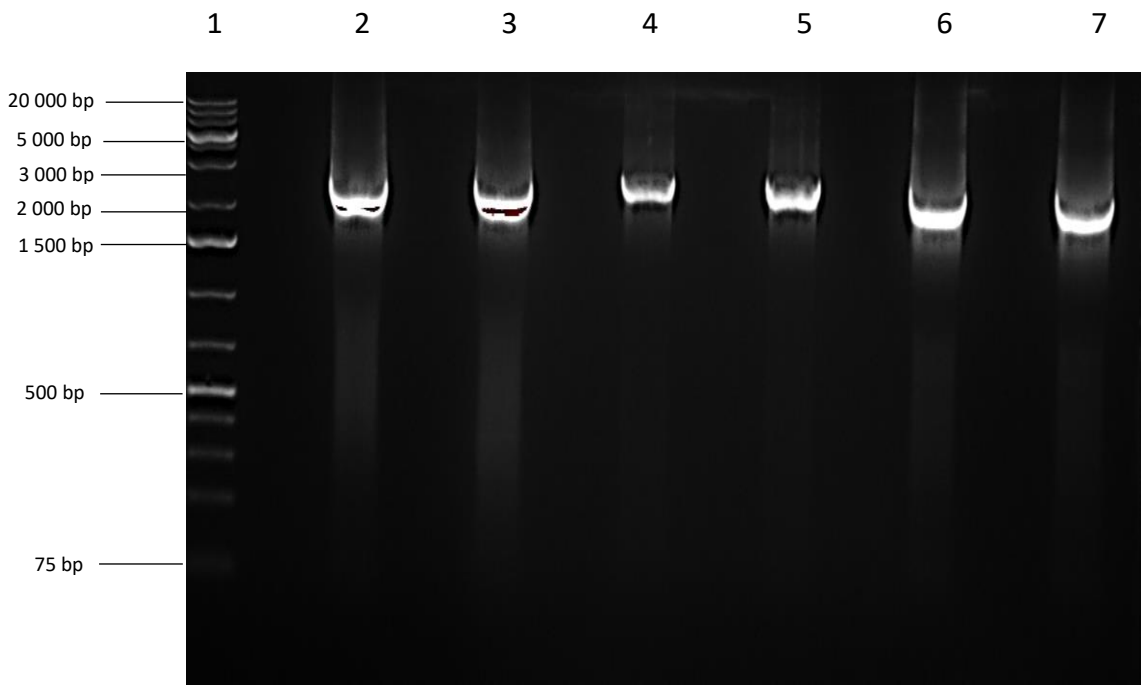


Figure. 6*: Agarose gel electrophoretic analysis of the amplicons obtained by RT-PCR amplification of the P1 capsid-encoding region of six SAT 2 isolates using primer set NCR1 and WDA. An amplicon of 2.2 kbp, estimated size according to the marker used, was observed for all the SAT 2 isolates. Lane 1: GeneRuler 1 Kb Plus DNA molecular weight marker. The amplified P1 regions of SAR 1/01/2 (lane 2); SAR 1/03/2 (lane 3); SAR 15/13/2 (lane 4); SAR 01/13/2 (lane 5); KNP 12/08/2 (lane 6) and ZIM 02/13/2 (lane 7).*

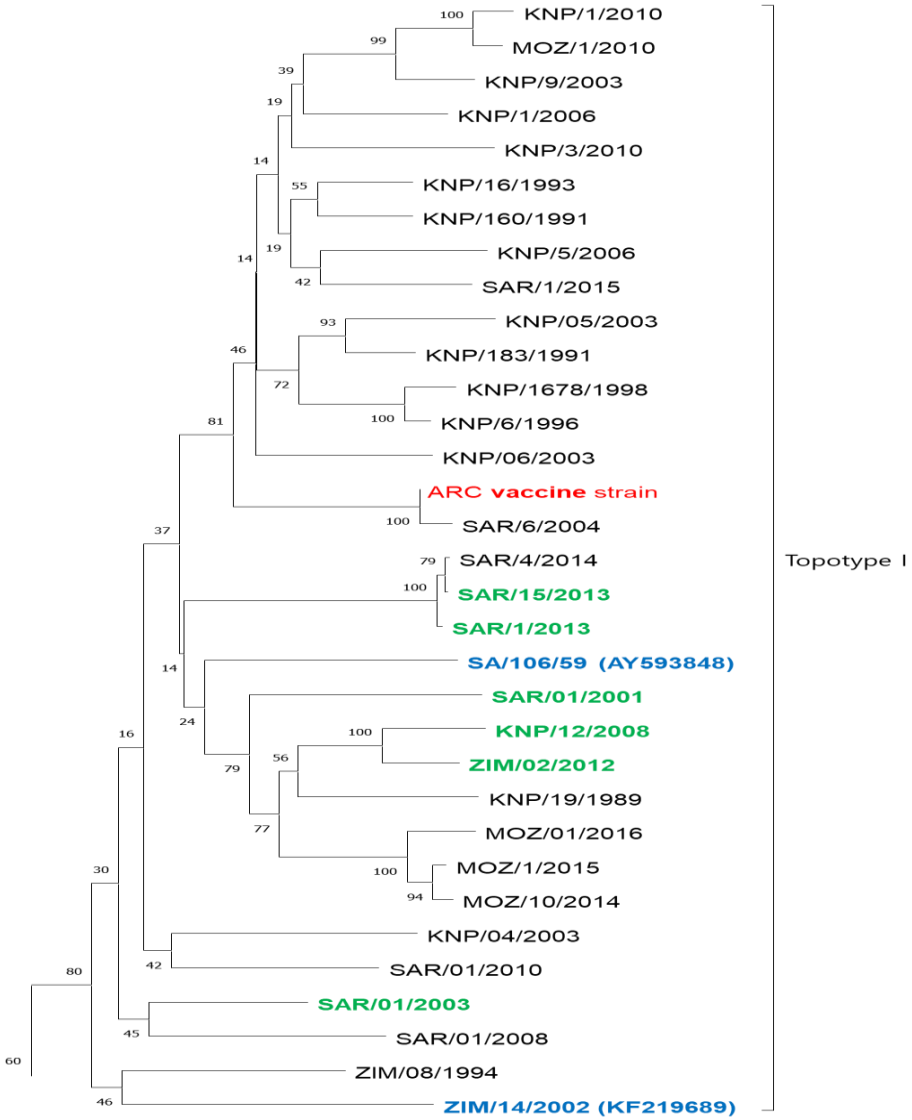


Figure 7*: Neighbour-joining tree depicting the relationships between nucleotide sequences of the VP1 regions of the six SAT 2 FMDV viruses that were in circulation within South Africa and neighbouring country Zimbabwe. The six SAT 2 viruses (green) were responsible for FMD outbreaks in different period between 2001 to 2013. The phylogenetic tree included the FMDV SAT 2 prototype strains SA/106/59 and ZIM/14/2002 in blue that have full/partial genome assigned accession numbers, ARC vaccine strain (SAR/3/2004) in red and relevant SAT 2 viruses within the topotype-1 in black. The six viruses were clustered in four distinct genotypes: ARC vaccine genotype; the SAR/15/2013 and SAR/1/2013 genotype;. SAR/01/2001; KNP/12/2008 and ZIM/02/2013 clustered within SA/106/59 genotype and SAR/01/2003 with ZIM/14/2002. The bootstrap of 1000 replicates and P distance 0.05 and the outgroup of the clustered are attached in annexure.*

6.2. Determined titres of adapted SAT 2 viruses to BHK 21 cells

During outbreaks in South Africa and Zimbabwe, SAT 2 field viruses were identified in cattle, whereas during FMD surveillance in South Africa, they were detected in buffalo. Following from virus isolation and confirmation of samples being FMD viruses, they were maintained in IB-RS-2 cells. The antigen preparation in the research necessitated the adaptation of the SAT

2 viruses to the BHK 21 monolayer cells, a standard cell culture used in the manufacturing of FMD antigen on a large scale (Doel, 2003). BHK 21 cells were inoculated at a multiplicity of infection of 1:50 (virus-to-cell). After 72 hours of incubation with the first virus passage, CPE was 70-80%; within 18-24 hours, the second virus passage achieved 80-90%. Virus titres were determined through virus titration method after each virus passage and virus titre of above log $10^{7.0}$ $TCID_{50}$/ml was considered as criteria for virus adaption within 24 hpi. The increase in virus titres coincided with continuous virus passage, which was observed during BHK 21-virus passage-1 to passage-3, where titres of above log $10^{7.0}$ $TCID_{50}$/ml were obtained for all viruses (Table 2).). SAR/15/2013, which had a virus titre log of $10^{7.20}$ $TCID_{50}$/ml, was the initial virus to successfully acclimate to BHK 21 cells throughout the initial virus passage. This was followed by four viruses: SAR/01/2001 with a virus titre of log $10^{7.07}$ $TCID_{50}$/ml; SAR/01/2003 with a virus titre of log $10^{7.81}$ $TCID_{50}$/ml; SAR/01/2013 with a virus titre of log $10^{7.28}$ $TCID_{50}$/ml; and ZIM/02/2013 with a virus titre of log $10^{7.20}$ $TCID_{50}$/ml in BHK 21-virus passage-2. KNP/12/2008 virus derived from buffalo was the adapted to the BHK 21 cells, following passage 3, attaining a virus titre of log $10^{7.41}$. Acquired virus titres were in line with the recommendation of ideal titres of at least 10^6 pfu/ml within 24 hours (Rweyemamu et al., 1978). The FMDV SAT 3 (KNP/10/1990), with a known virus titre of log $10^{8.0}$ $TCID_{50}$/ml was used as a positive control. KNP/10/1990 titres obtained during virus titration were titres of log $10^{8.08}$ $TCID_{50}$/ml, log $10^{8.13}$ $TCID_{50}$/ml, and log $10^{8.13}$ $TCID_{50}$/ml acquired in BHK-21 virus passage-1, BHK-21 virus passage-2 and BHK-21 virus passage-3 respectively. KNP/10/1990 acquired titres were consistent with known determined titre of log $10^{8.0}$ $TCID_{50}$/ml. This titre consistency of KNP/10/1990 throughout the titration test ensured the accuracy and dependability of the SAT 2 virus titration test that was performed. Furthermore, the consistency of virus titres of KNP/10/1990 between BHK-21 virus passage-1 to BHK-21 virus passage-3 suggested that under a minimal passage of three, the virus is likely to attain slight margin of virus titre. Continual virus passaging with intention to attain higher virus titre has a potential to cause modification to antigenic structure and growth characteristics (Pay, 1983, van Bekkum, 1978a). Such changes to antigenic structure and nucleotides alteration could have a negative/skewed impact on the determination of antigenic similarities in the study. However, the virus passages were limited to three passages after acquiring the virus titres of above log $10^{7.0}$ $TCID_{50}$/ml. Uninfected BHK 21 cells were used as a negative control during virus titration, and no virus replication, cell overgrowth, or deformation was observed.

Table 2: The titres of SAT 2 viruses acquired during virus adaptation to BHK 21 cell.

FMD SAT 2 virus	Species origins	History of passage	1st passage titre on BHK 21 cells (Log $_{10}$ $TCID_{50}$)	2nd passage titre on BHK 21 cells (Log $_{10}$ $TCID_{50}$)	3rd Passage titre on BHK 21 cells (Log $_{10}$$TCID_{50}$)
SAR/1/2001	Cattle	IB-RS-2[1]	6.00	7.07	NP
SAR /1/2003	Cattle	IB-RS-2[1]	6.70	7.81	NP
KNP/12/2008	Buffalo	IB-RS-2[1]	6.20	6.84	7.88
SAR/1/2013	Cattle	IB-RS-2[1]	6.48	7.28	NP
SAR /15/2013	Cattle	IB-RS-2[1]	7.20	7.42	NP
ZIM /2/2013	Cattle	IB-RS-2[1]	6.91	7.20	NP
Positive control **SAT 3 (KNP/10/90)**	-	BHK 21[2]	8.08	8.13	8.13

IB-RS-2[1] = First passage in IB-RS-2 cells.
BHK 21[2] = Second passage in BHK 21 cells
NP = No further passage

6.3. Determined SAT 2 antigens concentration using indirect sandwich ELISA

For antigen preparation, all SAT 2 viruses were inoculated on BHK 21 monolayer cells with virus titre above log log $10^{7.0}$ $TCID_{50}$/ml. A plot graph was used to plot and display the ODs of each antigen against reciprocal dilutions and the linear model downward slopes were established as illustrated in **figure 8**. Antigens were not substantially concentrated, and the highest undiluted antigen of SAR/1/2013 with OD_{450nm} of 1,40. This was followed by KNP/12/2008 with OD_{450nm} of 1,30; SAR/1/2001 and KNP/12/2008 with OD_{450nm} of 1,20 and ZIM/2/2013 and SAR/1/2001 were the least concentrated antigens with OD_{450nm} of 1,00. Since antigen concentrations decreased during serial dilution, no specific dilution would be desirable across antigens, according to the linear downward slope graph. Antigens may have been diluted by 20% glycerol added as a preservative. Undiluted antigens were preferred for the SPCE test due to lack of equal concentration. The uninfected BHK 21 cells (TCC) were used to measure the general non-specific binding interference, and an average background optical density (OD) of 0.18 was obtained. The uninfected BHK 21 cells (TCC) were included to

determine background interference of general non-specific binding and a background average OD of 0.18 was determined. In order to mitigate the influence of non-specific interference introduced by the BHK 21 cells, which were utilised in the antigen preparation process, the background average OD value was subtracted from the computed ODs.

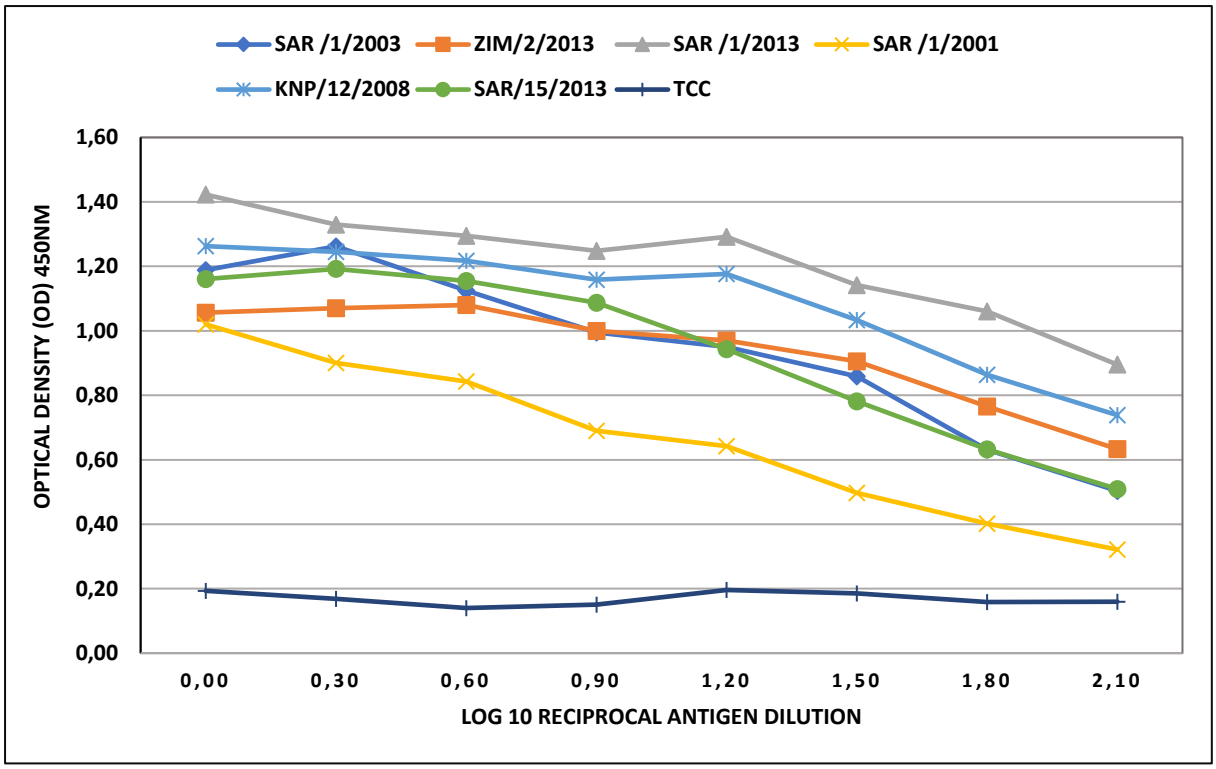


Figure. 8: *A descriptive plot graph illustrates the mean optical density (OD) of titrated antigens using the indirect sandwich ELISA that was measured at 450 nm absorbance. The OD results of the SAT 2 antigen concentrations of SAR/1/2003, ZIM/2/2013, SAR/1/2013/2, SAR/1/2001, KNP/12/2008, SAR/15/2013, and TCC [tissue cell culture (uninfected BHK 21 cells)] were plotted against the log 10 reciprocal antigen dilutions, producing a linear model of downward slopes of antigen concentration. The SAR/1/2013 virus was the leading concentrated antigen, and SAR 1/01/2 and ZIM 2/13/2 were the least concentrated antigens. The background interference of the indirect test was determined by the negative control TCC with mean OD of 0.18.*

6.4. Vaccine matching

6.4.1. Serological assay

Figure 9 illustrates the percentage inhibition of the test sera against the SAT 2 antigens designated as SAR/01/2001; SAR/01/2003; SAR/01/2013; SAR/15/2013; KNP/12/2008; ZIM/02/2013; and SAR/03/04. The SAR/01/2001 antigen **(image 1)** showed serum 1-3 with percentage inhibitions above and around 80 % through the dilutions (1/20 to 1/2560). High percentage inhibitions showed high antibody levels in test sera that were antigenically similar to SAR/1/2001, outperforming SAT 2 guinea pig anti-FMDV antiserum. This significant percentage inhibition was similar in serum 1-3 dilutions (1/20 to 1/160) for SAT 2 antigens, SAR/1/2013 (**image 2**), SAR/15/2013 (**image 3**), SAR/1/2003 (**image 4**), ZIM/2/2013 (**image**

5), and KNP/12/2008 (**image 6**). Serums 1–3 exhibited a notable percentage of inhibition, indicating that the cattle from which they were derived exhibited a suitable and adequate immune response to the pentavalent FMD vaccine. The percentage of inhibition in serum 4-5 was moderate and varied across the six SAT-2 antigens as the dilutions were performed. At a dilution of 1/20, the test sera exhibited the higher percentage inhibitions of 60% for SAR/1/2001 **(image 1**) and SAR/15/2013 (**image 3**). The antigens SAR/1/2013, ZIM/2/2023, SAR/1/2003, and KNP/12/2008 exhibited percentage inhibitions below 60% at a test sera dilution of 1/20 and below 40% from a test sera dilution of 1/320 across all six antigens. The pentavalent vaccine in cattle showed a moderate level of inhibition in serum 4 and 5, indicating an average cross-reactivity and a modest immune response.

The SAT 2 antigen SAR/3/2004 (**image 7**) displayed a distinct pattern in serum 1-5, diverging from the SAT 2 antigens, with the exception of SAR/01/2001. The observed percentage inhibitions ranged from approximately 80% for dilutions of 1/20 to 1/160, to 60% to 70% for dilutions of 1/320 to 1/2560. The high level of inhibition was anticipated because the pentavalent vaccine SAT 2 strain (SAR/3/2004) and SAT 2 antigen (SAR/3/2004) are antigenic close related.

As anticipated, the percentage inhibition of SAT 2 antigens in serum 6-7 derived from unvaccinated cattle (the control group) was minimal. This was due to the absence of antibody in the sera that could compete with the serotype SAT 2 guinea pig anti-FMDV antiserum. The antigens exhibited a range of inhibition percentages from 0% to 15.5%. General, non-specific interference may account for the negative percentage of inhibition observed in the negative serum. Previous research has also reported negative percentage inhibition values specific to the negative control (Mackay et al., 2001), suggesting that these values may be prevalent in the negative control samples as well. The cut-off value, which differentiates between positive and negative percentage inhibition, was determined based on the titration curves of the positive and negative controls. A 20% cut-off value for percentage inhibition was established to serve as a distinguishing characteristic between positive and negative percentage inhibition. The highest percentage inhibition of serum 6-7, which was 15.5%, was observed in the SAR/3/2004 (image 7) dilution (1/1280). This value serves as the cut-off point, with any value above 15.5% considered appropriate.

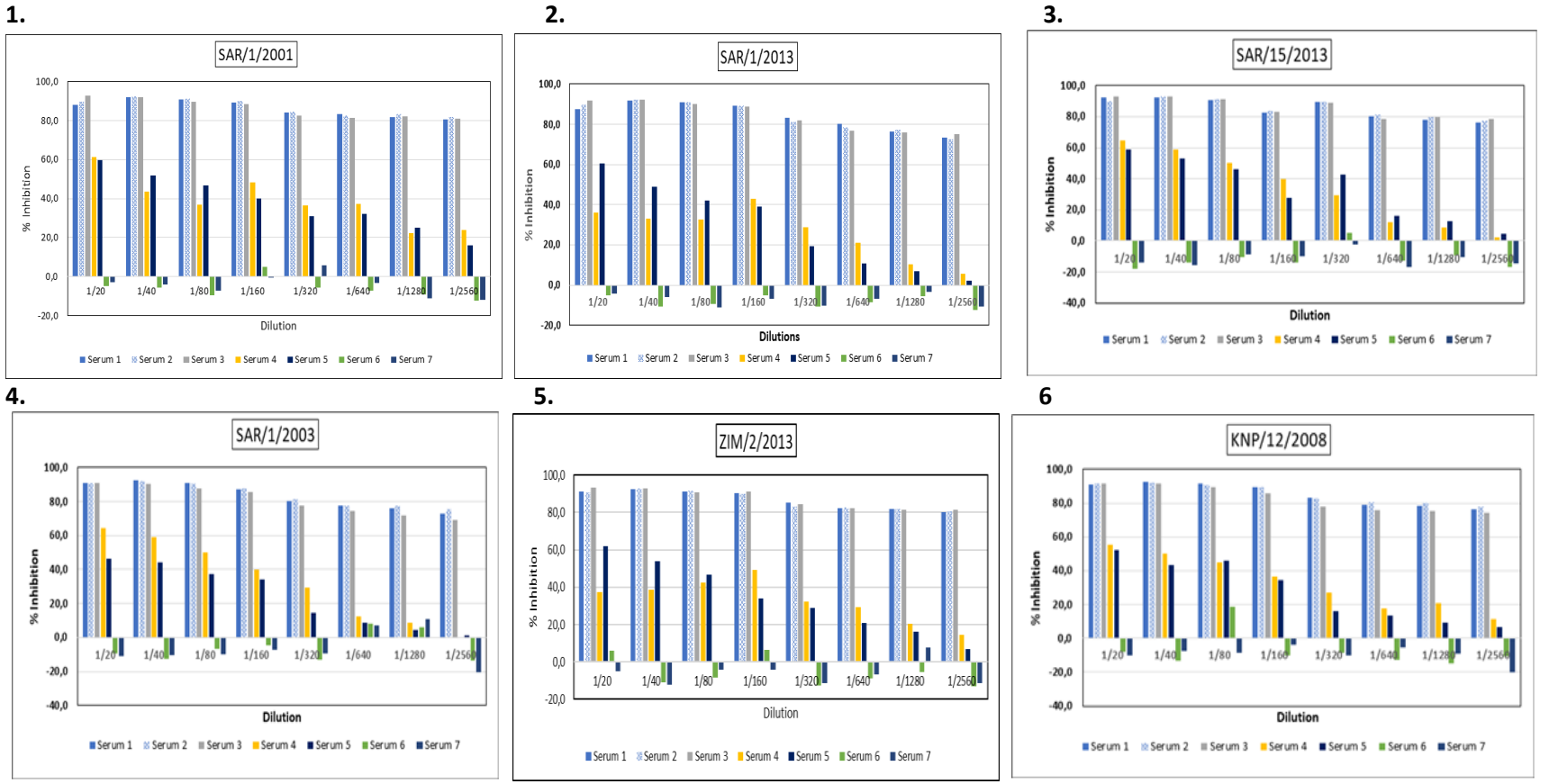


Figure 9: *The distribution of percentage inhibitions of test sera against the serotype SAT 2 guinea pig anti-FMDV antiserum for SAT using SPCE in dilution 1/20 to 1/2560. The antigens used in the study; SAR/12001; SAR/1/2013; SAR/15/2013; SAR/1/2003; ZIM/2/2013; KNP/12/2008 and SAR/3/2004. Test sera sample (serum 1-5) were derived from cattle induced with ARC-FMD pentavalent vaccine 28-day post-vaccination and sera sample (6-7) were from control group not induced. SAR/01/2001 (image 1) exhibited significant inhibition exceeding 80% in serum sample 1-3 dilution ranging from 1/20 to1/2560. SAR/1/2013 (image 2); SAR/15/2013 (image 3); SAR/1/2003 (image 4); ZIM/2/2013 (image 5); and KNP/12/2008 (image 6) showed a high degree of inhibition in serum 1-3 and moderate degree in serum 4-5. SAR3/2004 (image 7) showed a greater degree of inhibition in serum 1-5. The serum 6-7 exhibited low percentage inhibition across the antigens. The titration curve of both positive and negative control was utilised to established the cut-off value 20%*

6.4.2. Determination of serum titre against SAT 2 antigens for vaccine matching

Determination of vaccine matching using the r_1-value (indirect vaccine matching) which is essential for estimation of antigenic relationship requires the determination of homologous titres and heterologous titres. The percentage inhibition calculated from cross-reactivity of antigens and antibodies were used to determine the sera titres for both homologous and heterologous titres.

In the study, the log_{10} titres of serum 1-5 (vaccinated animals) and serum 6-7 (unvaccinated animals) were determined against the six SAT 2 antigens (heterologous strain) and SAR/3/2004 (homologous strain). The titres of serum (1-3) were between log_{10} 2.8 and log_{10} 3.1 against the SAT 2 antigens, while the serum 4 and 5 were moderate ranging similarly between log_{10} 1.4 and log_{10} 1.8. The presence of high to moderate levels of antibodies in the serum samples (serum 1-3 for high levels and serum 4-5 for moderate levels) indicated that the antibodies were able to react with all six SAT 2 antigens. This suggests that there is a similarity between the six SAT 2 viruses and the SAT 2 vaccine strains in terms of their antigens. Serum 1-5 against the SAR/03/2004 (ARC vaccine strain) which is homologous to the vaccine strain, demonstrated significant serum titres ranging between log_{10} 2.5 and log_{10} 3.0 which was essentially similar with serum 1-3 against the SAT 2 antigens. Serum 6-7 obtained serum titres of between log_{10} 0.3 to log_{10} 0.8. and was expected to achieved low serum titres across the SAT 2 antigens and SAR/03/2004 due to the sera derived from unvaccinated cattle. Lowest serum titre of serum (1-5) which was log_{10} 1.4 and highest serum titre serum between serum 4 and 5 was log_{10} 0.8. This was used to determine the cut-off value between vaccinated and unvaccinated sera. Therefore, serum titre of cut-off value of log_{10} 1.4 was selected. The standard deviation were calculated for the titres to determine the disperse of the titres and standard deviation between 0 – 0.4 were observed for all serum titres indicating high reproducibility

6.4.3. Antigenic matching using r_1-value method to estimate protection.

In the study, antigenic relationships between heterologous viruses (six contemporary SAT 2 viruses: SAR/1/2001, SAR/1/2013, ZIM/2/2013, SAR/15/2013, SAR/1/2003 and KNP/12/2008) and a homologous virus (SAT 2 SAR/3/2004: vaccine strain of pentavalent vaccine) were determined using the r_1-values method. R_1-values of SAT 2 viruses against each serum (serum 1-5: vaccinated cattle and serum 6-7: unvaccinated cattle) were calculated and were presented in **Table 3**.

R_1-values for each heterologous/homologous virus pair in serum 1-5 indicated significant antigenic relationships amongst the vaccine strain and the SAT 2 antigens despite being variable. R_1-value of SAR/1/2001 with the vaccine strain ranged between 0.65 to 1.19; SAR/1/2013 with the vaccine strain ranged between 0.54 to 1.17; ZIM/2/2013 with the vaccine strain ranged between 0.60 to 1.20; SAR/15/2013 with the vaccine strain ranged between 0.59 to 1.18; SAR/1/2003 with vaccine strain range between 0.50 to 1.14; and KNP/12/2008 with the vaccine strain ranged between 0.54 to 1.15. R_1-values of the vaccine strain/homologous strain were 1.00 which was expected due to antigenic similarity existence between the strains. Obtained r_1-values results according to the OIE antigenic matching interpretation based on liquid phase Blocking ELISA proposed that, r_1-value higher than 0.4 to 1.0 indicates sufficient antigenic match between the vaccine strain and the field virus (OIE, 2021). Therefore, the vaccine strain has the prospect of conferring protection against the field virus. R_1-values between 0.2 - 0.39 have the likelihood of an antigenic match between the field virus and the vaccine strain, while r_1-values less than 0.2 indicate a distant antigenic match between the field virus and the vaccine strain and, therefore, the vaccine strain will unlikely confer protection against the field virus (Ferris and Donaldson, 1992, OIE, 2021).

Following the WOAH recommendations, the analysis of mean r_1-values ranges between 0.63 to 1.15 indicating a high antigenic matching among the six contemporary SAT 2 field viruses and the vaccine strain. Despite the variation in antibody titres elicited by the FMD pentavalent vaccine in the vaccinated cattle, the antibodies triggered were suitable and capable to cross-react with the SAT 2 viruses leading to high r_1-values for the vaccine strain and the SAT 2 field viruses. The standard deviation of r_1-values across the SAT 2 viruses were 0.03 for serum-1, 0.01 for serum-2, 0.06 for serum-3, 0.14 for serum-4 and 0.16 for serum-5. This showed less variability and consistence of r_1-values obtained across the SAT 2 viruses within each serum. R_1-values of serum 6 and 7 (unvaccinated cattle) were mainly below 0.2, although SAR/1/2001 and SAR/1/2013 were 0.20 and 0.24 respectively. The virus r_1-value above 0.2 was unexpected since no cross -reactive antibodies were present in the sera, however the mean r_1-value of both serum 6 and 7 across the SAT 2 viruses were below 0.2. Serum 6 and 7 mean r_1-values provided a differentiator between vaccinated and unvaccinated sera and clearly demonstrates a distant antigenic relationship. Standard deviation was 0.03 for both serum-6 and serum-7 showing less variability of r_1-value across the SAT 2 viruses. This demonstrates that the determined r_1-values were consistent although the variation observed was minimal.

Table 3: Determined r_1-values

Determined r_1-values coefficient to estimate the antigenic relationship between the six contemporary SAT 2 field viruses and the vaccine strain. Heterologous titres (six SAT 2 field viruses) and homologous titres (SAR/03/2004: vaccine strain) used to calculate r_1-values were derived from serum 1-5 (vaccinated cattle) and serum 6-7 (unvaccinated cattle). Determined r_1-values indicated the antigenic relationship between the SAT 2 viruses and vaccine strain (SAR/03/2004).

	The r_1-values of the six SAT 2 viruses and vaccine strain						
	Serum 1	**Serum 2**	**Serum 3**	**Serum 4**	**Serum 5**	**Serum 6**	**Serum 7**
SAR /1/2001	1,07	0,99	1,19	0,65	0,65	0,15	0,20
SAR /1/2013	1,05	0,97	1,17	0,53	0,54	0,10	0,24
ZIM /2/2013	1,07	0,99	1,20	0,60	0,61	0,12	0,15
SAR/15/2013	1,12	0,98	1,18	0,59	0,60	0,05	0,19
SAR /1/2003	1,04	0,97	1,14	0,60	0,50	0,14	0,16
KNP/12/2008	1,06	0,98	1,15	0,64	0,54	0,12	0,18
SAR/03/2004 (ARC vaccine strain)	1,00	1,00	1,00	1,00	1,00	0,16	0,19
Min. titre	1,00	0,97	1,00	0,53	0,50	0,05	0,15
Max. titre	1,12	1,00	1,20	1,00	1,00	0,16	0,24
Mean	**1,06**	**0,98**	**1,15**	**0,66**	**0,63**	**0,12**	**0,19**
Standard Deviation	**0,03**	**0,01**	**0,06**	**0,14**	**0,16**	**0,03**	**0,03**

6.4.4. Estimation of protection using r_1-value

The r_1-values obtained for the SAT 2 field viruses against the homologous strain (SAR/3/2004:ARC vaccine strain) were plotted in a radar graph (**figure 9**). Radar graphs demonstrating the series of sera (serum 1-7) and their performance metrics against the variables (six SAT 2 field viruses), to correlate and conceptualise the protection based on cross-reactivity of sera against SAT 2 viruses.

In **figure 10**, serum 1-3 exhibited being in close proximity towards all six SAT 2 viruses and the homologous strain. This demonstrates a strong cross-reactivity of the sera towards the SAT 2 viruses and homologous strain due to high antigen-antibody complex formation. Strong antigenic match indicated by high r_1-values similarly express strong cross-reactivity. Since the humoral antibody is the main mechanism to afford protection for cattle and pigs against FMDV (Rweyemamu, 1984), therefore, strong cross-reactivity preceding to plenty antigen-antibody complex formation between vaccine sera and SAT 2 viruses demonstrated a high potential level of cross protection afforded by vaccine strain. The performance of serum 4-5 displayed an intermediate position towards the six SAT 2 field viruses. Average r_1-values, which indicate an average formation of antigen-antibody complexes, demonstrated that sera and the field virus exhibited moderate cross-reactivity. This average antigen-antibody complex formation correlates to a moderate potential level of cross-protection. Performance of serum 6-7 cross-reactivity and r_1-values were minimum, shown by a distant position towards SAT 2 field viruses. This demonstrates no/less antigen-antibody complex formation which indicates no cross-protection. This was expected since the sera were derived from unvaccinated cattle which were not exposed to vaccine strain. Therefore, the ability of serum 1-5 to cross-react with SAT 2 field viruses demonstrated by moderate to high r_1-values, express the potential correlation of the cross protection afforded by the vaccine strain that raised the sera, which was the SAT 2 vaccine strain (SAR/3/2004) that consisted in the ARC pentavalent vaccine.

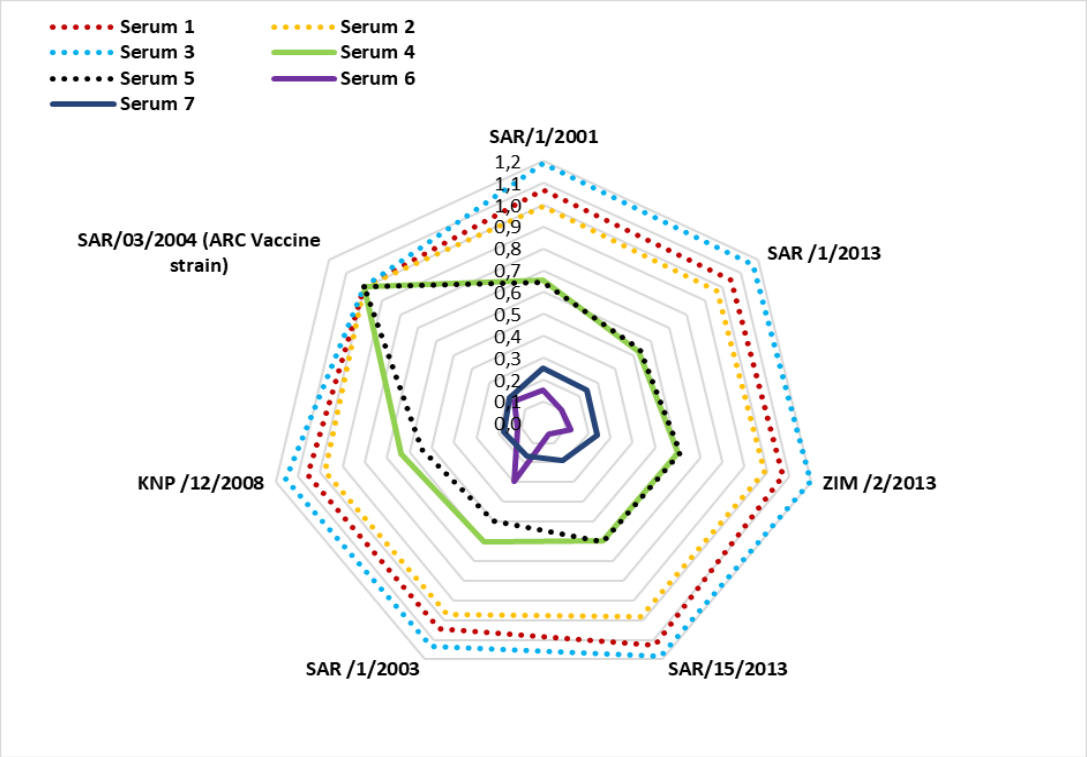


Figure. 10: *The radar graph presents antigenic matching expressed by the r_1-values of sera against the SAT 2 field viruses. Serum 1-5 were derived from vaccinated cattle with ARC pentavalent vaccine and serum 6-7 were derived from unvaccinated cattle. The r_1-values were plotted and the proximity and distance of serum towards the SAT 2 field virus and homologous strain (SAR/3/2004) were used to correlate and conceptualised the level of protection afforded by vaccine strain. The r_1-value of 0.4-1.0 indicated antigenic similarity, 0.2-0.39 potential antigenic similarity while 0.2 and below indicated distant antigenic similarity of vaccine strain to field virus.*

7. Discussion

The overall aim of the study was to determine if the pentavalent FMD vaccine developed by the Agricultural Research Council – Onderstepoort Veterinary Research (ARC-OVR) will adequately confer protection against prevalent FMD SAT 2 strains using an optimised SPCE assay. The ARC-OVR developed the pentavalent FMD vaccine since the currently used trivalent FMD vaccine has shown to be ineffective in preventing or minimising transmission of FMD viruses (Lazarus et al., 2018).

The trivalent vaccine demonstrated a restricted antigenic range towards circulating SAT 2 strains (Lazarus et al., 2018), and the ARC-OVR pentavalent FMD vaccine was designed to mitigate the limited antigenic coverage of the current vaccine toward SAT 2 strains in circulation. The SAT 2 serotype was the subject of the study due to its significant antigenic variation (Bastos et al., 2001, Bastos et al., 2003, Maree et al., 2011). It has been implicated in the majority of FMD outbreaks in southern Africa since 2000, responsible for an estimated half of these outbreaks, with SAT 1 and SAT 3 contributing the remaining half (FMD-Bulletin, 2010,

OIE., 2021, Vosloo and Thomson, 2017). In addition, South Africa has experienced a myriad of FMD outbreaks since 2021, the majority of which were attributed to the SAT 2 serotype (Department of Agriculture, 2022, OIE., 2021).

A significant proportion of FMD outbreaks in South Africa originate in Kruger National Park (KNP), where the emerged virus variant infiltrates and cause outbreaks in cattle within the adjacent protected buffer zone region (Jori et al., 2016a, Vosloo and Thomson, 2017). It is worth noting that biannual vaccinations are administered to the cattle residing in this area. This is evidence enough that new variants within serotypes are capable of evading immunity afforded by the vaccine and has prompted the need to identify prevalent SAT 2 strains circulating in South Africa and neighbouring countries and determine if the ARC pentavalent vaccine will protect against them. The research identified six prevalent contemporary SAT 2 field viruses that emerged between 2001 and 2013, as determined by the OVR-TAD database. FMD outbreaks in South Africa and the neighboring country, Zimbabwe, were attributed to a diverse group of SAT 2 topotype-1 viruses, with the exception of the KNP/12/2008 virus, which was identified during an FMD surveillance.

The selected viruses were confirmed to be FMD SAT 2 serotype through amplification and sequencing of the P1 region of the virus (**Figure 6**). The sequences of the VP1 region derived from the P1 region were confirmed to be SAT 2 viruses using the NCBI BLAST software tool. Through VP1 sequences analysis of viruses, genetic relatedness of SAT 2 viruses was used to characterise the viruses against the ARC vaccine strain (SAT 2: SAR/03/2004), reference prototype SAT 2 strains and cluster of related topotype-1 viruses. The overall genetic similarity based on the phylogenetic tree observation (**figure 7**), the ARC vaccine strain and SAT 2 field viruses were clustered in four distinct genotypes (clades) indicating genetic differences in comparison. The ARC SAT 2 vaccine strain (SAR/03/2004) was clustered into different genotype which was separate from all the six SAT 2 viruses including the reference SAT 2 prototype strains. The diversity in nucleotide sequences and how distributed the SAT 2 field viruses are, generally indicated by the antigenic distinction that exist within the SAT 2 topotype-1. Antigenic diversity can limit the strength of cross-protective immunity afforded by the vaccine strain. This was in agreement with other studies that indicated that minor mutations can cause huge impacts on virus antigenicity (Crowther et al., 1993, Rweyemamu and Ouldridge, 1982). The genotype location of five SAT 2 field viruses; the SAR/15/2013 and SAR/1/2013 where classified within one genotype while the SAR/1/2001; KNP/12/2008; and ZIM/2/2013) clustered in the same group; and indicated a heterogeneity of ≤ 15 %, which is the standard considered to classify viruses in genotypes (Vosloo et al., 1992). However, the

five SAT 2 field viruses (SAR/15/2013; SAR/1/2013; SAR/1/2001; KNP/12/2008; and ZIM/2/2013) based on phylogenetic tree observation indicated that, the two genotypes converge to the same root contrast to ARC vaccine strain and SAR/01/2003. This suggest that the five SAT 2 might derived from a common ancestral lineage. Results of the five SAT 2 field viruses suggests that most SAT 2 FMD outbreaks in South Africa are derived from a single strain and the strain is efficiently maintained within multiple hosts primarily the buffalo, in each geographic location where it evolves separately. This statement is supported by a previous study where VP1 analysis of the SAR/1/2001 outbreak in Mhala District, which borders the KNP indicated that the virus was similar to viruses that were previously isolated from buffalo in the adjacent KNP (Bruckner et al., 2002). Furthermore, the sequence analysis of SAR/15/2013 and SAR/1/2013 in our study, indicated being clustered closely together in the phylogenetic tree supported by bootstrap value of 100. This results was supported by previous study where the results of SAR/15/2013 and SAR/1/2013 viruses indicating ≥ 98.7 % nucleotide identity with 0.8 % genetic distance within a period of less than a year between sampling during the outbreak of 2013 and 2014 in Mpumalanga province (Blignaut et al., 2020). This suggesting that FMD outbreaks in cattle are derived from a single source of infection which is the buffalo (Blignaut et al., 2020, Jori et al., 2016a).

The phylogenetic tree clustered the KNP/12/2008 and ZIM/02/2013 sequences closely together with bootstrap of 100. These similarities predicts that the KNP/12/2008 derived from buffalo in KNP during surveillance studies has the possibility of being responsible for the ZIM/02/2013 outbreak in Drysdale, Umguza Matebeleland North in Zimbabwe. The association of the viruses due to vast distance between two areas which are KNP and the Drysdale farm in Matebeleland seems unlikely, however, due to ecological pressure such as landscape heterogeneity, environmental changes, the buffaloes movement of 40 – 1 000 km^2 has been reported in Sabi Sand nature reserve and KNP (Funston et al., 1994, Pienaar, 1969). Buffaloes have been known to escape the KNP through the broken cordon fences surrounding the KNP interacting with domestic cattle (Bruckner et al., 2002), where virus dissemination could occur. Based on this proposition, this would imply that due to similarity of KNP/12/2008 and ZIM/02/2013 viruses, the buffalo herds might have been efficient in maintaining genetically related virus within KNP, Limpopo National Park (Mozambique) and Gonarezhou National Park (Zimbabwe). However, no direct link was made that KNP/12/2008 was responsible for ZIM/02/2013 outbreak. In another study, a successful link of a genetically related virus was made between a SAT 2 outbreak in the Gaza province of Mozambique and a SAT 2 virus

circulating in the Gonarezhou National Park which was attributed to livestock movement by railway between Zimbabwe and Mozambique (Jori et al., 2016b).

In contrast, the SAR/01/2003 field virus was clustered in a genotype with prototype SAT 2 strain ZIM/14/2002, which was a separate cluster to where the five SAT 2 field viruses and ARC vaccine strain were located. The outbreak of the SAR/01/2003 virus which occurred in Masisi village in Limpopo province (South Africa), indicated close nucleotides relatedness with prototype SAT 2 strain ZIM/14/2002, which is the probable source of the SAR/01/2003 virus outbreak. Probability is based on that, Zimbabwe experienced massive widespread FMD outbreaks during 2001 and 2003 as a result of land reform and resettlement which incapacitated the veterinary control programme's ability to control the outbreaks (Jori et al., 2016a, Mavedzenge et al., 2008). Masisi village where the virus was isolated, is on the peripheral border between South Africa and Zimbabwe, separated by Limpopo river. Lack of movement control of domestic animals could have occurred leading to the dissemination of the virus in various hosts. Similar event of virus spread across countries due to uncontrol animal movement was reported during the outbreak of SAT 2 in Mozambique, which was attributed to unrestricted animal movement between Zimbabwe and Mozambique (Jori et al., 2016a).

The distribution and possible origin of SAT 2 outbreaks are complex based on interacting of domestic and wild animals, the diverse range of SAT 2 viruses and multiple hosts involved. However it has been realised that it is difficult to relate nucleotides sequences data directly to antigenic similarity (Knowles and Samuel, 2003) and conclusions of antigenic similarity to estimate cross-protection will not be used on the basis of close nucleotides sequence similarity.

Therefore, the prevalent selected six SAT 2 viruses were used to prepare antigens which were required to be used in the determination of indirect antigenic similarity between bovine post-vaccinal sera raised by ARC-OVR pentavalent FMD vaccine. SAT serotypes, in particular SAT 2 serotype have been reported for poor adaption in BHK 21 cells in comparison to FMD serotype O, A and C (Esterhuysen et al., 1988, Pay et al., 1978). During this study, all six SAT 2 viruses were able to be adapted to the BHK 21 monolayer cells within virus passage 3, acquiring virus titres of above log $10^{7.0}$ $TCID_{50}$/ml within 24 hours, which was considered as the criteria for successful virus adaption (**Table 2**). Swift virus infectivity and cell susceptibility in our study, indicated the virus viability and compatibility of virus RGD motif sequence with cellular receptor integrins which are primary components to facilitate virus-cell adsorption (Duque and Baxt, 2003, Fox et al., 1989). Additionally, the prior maintenance of SAT 2 viruses

in IB-RS-2 cell line might have contributed to this virus's efficient adaptation. Our adaption of SAT 2 field viruses results was considered rapid in comparison to related study by Esterhuysen et al,., where 33 of 34 virus which were a combination of serotypes SAT 1-3 achieved virus titre log $10^{6.5}$ $TCID_{50}$/ml at 6^{th} passaged under 22 hours (Esterhuysen et al., 1988). A study by Preston et al, experienced challenges in adapting a SAT 2 virus strain, where only one virus strain out of 27 strains was reported to have adapted to BHK 21 monolayer cell obtaining a virus titre of 10^6 p.f.u/ml at BHK 21 virus passage-3 along with a serotype A and SAT 1 viruses (Preston et al., 1982). A study by Maree et al , demonstrated efficient adapting of SAT 1 and SAT 2 viruses in BHK 21 monolayer cells , which further revealed changes in amino acids sequence in virus (VP1-3) equating to 11 amino acids varying between low and high virus passages (Maree et al., 2010). However, the differences in virus's adaption rate and success in these studies shows that the ability of virus infection and rate of replication vary according to serotypes. Furthermore it suggests that, for efficient adaption, viruses might rely on rapid changes in the amino acids sequence in VP1-3 virus capsid which result in positively charged capsid structure causing adherence to negatively charged cellular receptors of BHK 21 cells (Maree et al., 2010). These study results also support the virus titre of log $10^{7.0}$ $TCID_{50}$/ml being used as a criterion for virus adaptation which was similarly recommended by Rweyemamu et al (1978) indicating the ideal virus adaption condition as virus titres of at least 10^6 pfu/ml with 24 hours (Rweyemamu et al., 1978).

The limited studies have been conducted to determine the antigenic match with the newly formulated ARC pentavalent FMD vaccine against the SAT 2 variants in circulation, where some caused outbreaks within South Africa and bordering countries. Various studies have explored the use of one-way antigenic relationship (r_1-value) as a predictive model for antigenic matching between homologous potency and heterologous potency where some success of antigenic match that correlated with protection was successful while in other instance correlation of protection was not accurate (Brehm et al., 2008, Maree et al., 2011, Sirdar et al., 2019). R_1-value method is the alternative rapid and cost-effective method for prediction of antigenic relationships, contrast to the *in vivo* potency method that involves the use of cattle and requires the use of a containment facility to conduct the experiments. Serological assay such as VNT are used to determine r_1-values which take three days to determine the results whereas the ELISA provides the r_1-values within a day (Tekleghiorghis et al., 2014).

In our study, the r_1-value method was applied to estimate the antigenic relationship or matching between the prevalent SAT 2 field viruses (Heterologous virus) against the pentavalent FMD vaccine, and this required the use of sera raised by vaccine. Therefore, sera collected during the study by Peta et.al of the pentavalent FMD vaccine trail (Peta et al., 2021), were used to determine cross-reactivity against SAT 2 antigens from prevalent SAT 2 field viruses. Analysis of the cross-reactivity generated by an optimized solid phase competition ELISA (SPCE) assay indicated moderate to high serum titres between homologous virus (SAR/03/2004) and heterologous viruses ranging between $\log_{10}$ 1.4 to $\log_{10}$ 3.1 from five sera samples (serum 1-5). Cross-reactivity of serum 1- 5 with homologous virus showed high serum titres ranging between $\log_{10}$ 2.5 and $\log_{10}$ 3.0. Titres values were significant in comparison to a study by Sirdar et.al where serum titre of $\log_{10}$ 2.2 was correlated with convalescent animals that were vaccinated and challenged with FMD virus during the study (Sirdar et al., 2019). The cross-reactivity of serum 1-5 against heterologous viruses indicated some variable serum titres in our study, however, the homologous titres were slightly similar across the serum 1-5. Additionally, the heterologous viruses showed a strong cross-reactivity with serum titres between $\log_{10}$ 2.8 to $\log_{10}$ 3.1 which is slightly higher than homologous virus reactivity range between $\log_{10}$ 2.5 to $\log_{10}$ 3.0.

This phenomenon was in agreement with another study that observed a heterologous virus that was phylogenetically distant to a homologous virus, but was able to exhibit higher antibody reactivity than the homologous virus that raised the sera (Barnett et al., 2001). Cross-reactivity of serum 4 and 5 against heterologous viruses showed different cross-reactivity in comparison to serum 1-3 obtaining serum titres range between $\log_{10}$1.4 to $\log_{10}$1.8. Variation in serum titres between serum 1-3 and serum 4-5 highlight the importance of harmonizing or pooling the sera, especially when derived from the same bovine post-vaccinal sera, this was supported by other studies were pooling of serum samples reduced the inter-animal and inter-trial variation (Brehm et al., 2008, Paton et al., 2005, Tom Willems et al., 2020). Determined sera titres indicated high antibody titres and the antibody is considered to be major component in humoral acquired immunity (McCullough et al., 1986). Overall serum titres established in our study were high which are suitable for the r_1-value determination as recommended previous study (Mattion et al., 2009).

Antigenic matching between pentavalent FMD vaccine strain (SAT 2: SAR/03/2004) indicated a good match with 100 % of r_1-values > 0.5 (**Table 3**). Strong cross-reactivity of serum 1-3 showed high r_1-values >0.97 across all heterologous viruses while serum 4 and 5 showed average cross-reactivity with r_1-values ranging between 0.50 to 0.65. Results were considered

a good vaccine match based on WOAH recommendation where r_1-value range between 0.40 -1.0 demonstrate a sufficient antigenic match. In addition, the SAR/1/2013 and SAR/15/2013 viruses (heterologous viruses) indicated being antigenically closely related to SAR/03/2004 (pentavalent FMD SAT 2 strain) with r_1-values ranging between 0.53 to 1.05 for SAR/1/2013 and 0.60 – 1.12 for SAR/15/2013. Results were in agreement with previous study, where SAR/3/2004 indicated r_1-value of approximately 1.0 for SAR/15/2013 and 0.8 for SAR/1/2013 (Blignaut et al., 2020). Although these antigenic predictions where similar with sera derived from different sources used between studies, the highlight was that our study used the optimised SPCE assay while study by Blignaut et.al., used the VNT. Therefore, use of optimised SPCE assay indicate potential to produce comparable results with VNT, however, the comparable test must experimentally conducted side by side.

The phenomenon of a r_1-value > 1.0 obtained in the heterologous virus (serum 1-3) expressing higher cross reactivity than a homologous virus despite heterologous virus being phylogenetically distant with vaccine strain was observed in previous study (Maake et al., 2020). In the study during the determination of antigenic relationship between SAT 3 viruses against homologous virus and heterologous viruses using r_1-value measured by VNT; the heterologous virus obtained high r_1-values > 1.0 than homologous virus (Maake et al., 2020).

Dilutions of the test sera and the SAT 2 guinea pig anti-FMDV antiserum were used to optimize the SPCE, with the antiserum competing for antigen binding. The dilution of 1:4 dilution of test sera and dilution of 1:60 for SAT 2 guinea pig anti-FMDV antiserum in blocking buffer were the optimal dilution for targeted antigens.

The findings in our study (i) the optimized of SPCE through optimal dilutions of 1:4 dilution for test sera and 1:60 for SAT 2 guinea pig anti-FMDV antiserum, the SPCE was used to determine the r_1-value to predict antigenic matching between SAT 2 field viruses and the vaccine strain; (ii) indicated R_1-values showing significant antigenic similarity between the selected prevalent SAT 2 field viruses and SAR/03/2004 (vaccine strain); (iii) showed that the pentavalent vaccine elicited broadly reactive antibodies in cattle; and (iv) antibodies elicited by the pentavalent FMD vaccine are capable of cross-reacting with SAT 2 viruses and can afford protection against the selected SAT 2 field viruses.

Due to limited available bovine post-vaccinal sera, our study could not conduct the parallel test in VNT or LPBE to compare if the r_1-values determined by these assays are comparable to those obtained with the SPCE assay. The study should have considered the inclusion of additional circulating SAT 2 strains to determine the antigenic variation of circulating strains

and predict their antigenic matching against the pentavalent FMD vaccine strain. Advantages of including more viruses and repeated testing provide statistical power to the SPCE and determine the robustness of the SPCE assay in terms of repeatability and reproducibility and its limits (Maradei et al., 2008, OIE, 2021). However, due to constraints with regards to the availability of sera, the addition of several circulating strains and other related serological assays would have not been possible. Therefore, optimized SPCE in the study was able to determine the cross-reactivity of SAT 2 field viruses against the serum raised by pentavalent FMD vaccine, which is crucial for the determination of serum titres and calculation of r_1-value. The r_1-values observed in the study are comparable to those previously reported. This indicates that the results of the SPCE assay showed a correlation with other serological assays, suggesting that the optimized SPCE has the potential to be a suitable antigen matching assay.

8. Conclusion

A SPCE assay was successfully optimised for antigenic matching of FMD SAT 2 viruses. The small-scale vaccine matching conducted using six prevalent SAT 2 viruses that were circulating within South Africa and neighbouring country Zimbabwe was successfully determined against the prevalent FMD vaccine strain. R_1-value outcome of related viruses where comparable with the r_1-values determined in another study (Blignaut et al., 2020) and this demonstrated the potential of the SPCE assay to determine the antigenic relation between a vaccine strain and field strains. The availability of a SPCE assay for vaccine matching will be valuable for the swift assessment of vaccine matching between a vaccine strain and circulating SAT 2 virus strains. This will provide immediate crucial information regarding the antigenic spectrum of the vaccine strain and whether the vaccine strain has the potential to confer cross-protection or requires optimization. Continuous FMD surveillance and rapid antigenic matching of viruses in circulation using the SPCE assay will contribute to minimize FMD outbreaks in South Africa, where the country has experienced numerous FMD outbreaks since 2021 (Department of Agriculture, 2022, OIE., 2021). While the current trivalent vaccine demonstrates being ineffective to prevent or minimised virus transmission within the vaccinated cattle (Lazarus et al., 2017), this newly ARC-OVR designed pentavalent FMD vaccine demonstrated the ability to confer protection on the six prevalent SAT 2 strains. South Africa relies mainly on vaccination to control the FMD and the vaccine being used must have similar antigenic characteristics with circulating virus or potential outbreak strain. Therefore, further studies are required to include several more SAT 2 strains in circulation to determine the antigenic coverage of the pentavalent FMD vaccine. The combination of SPCE and LPBE will be necessary to determining antibody titres that correlated with protection between the

assays and to gain further insight of reproducibility, comparability, and interpretation of r_1-values. In addition, the SPCE assay will require further rigorous testing to establish its capacity to determine r_1-values that will correlate with cross-protection (reliability), assay robustness and parameters which are prerequisites components to validate test assay (OIE, 2021). Although the optimised SPCE assay required further follow ups, however, the assay has demonstrated ability to determined r_1-values that are comparable with VNT assay in other studies and r_1-value of VNT have being correlated with protection (Barnett et al., 2003). Nevertheless, the qualities of SPCE were determined using few field viruses and the optimised SPCE indicate the potential to be suitable assay for vaccine matching.

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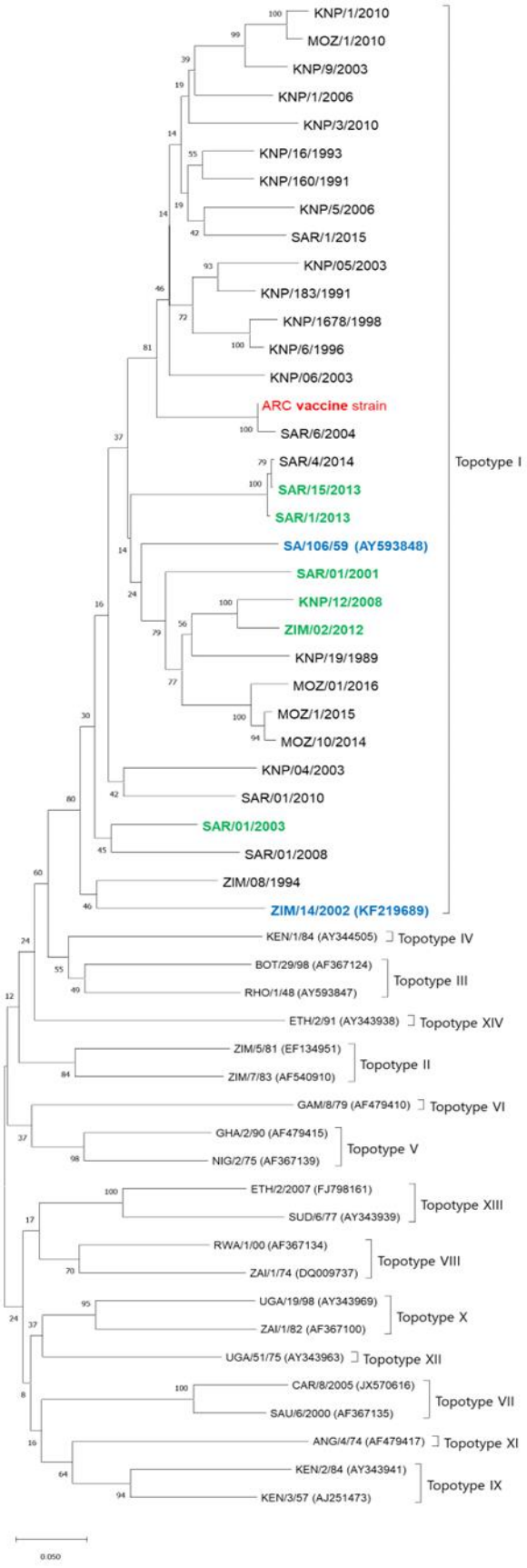
Annexes

Outlay of the Phylogenetic tree



Appendices

Research Ethics approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee 01 October 2020

CONDITIONALLY APPROVAL

Ethics Reference No	**REC263-19**
Protocol Title	**Development of a serological based vaccine matching technique for Foot-and-Mouth Disease (FMD) SAT 2 viruses.**
Principal Investigator	**Mr NT Malesa**
Supervisors	**Dr PA Opperman**

Dear Mr NT Malesa,

We are pleased to inform you that your submission has been conditionally approved by the Faculty of Veterinary Sciences Research Ethics committee, subject to other relevant approvals.

Please note the following about your ethics approval:

1. Please use your reference number (REC263-19) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application for post graduate studies (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

NOTES: Conditionally approved pending the following (and to ensure that rerouting to AEC is not delayed):
(i) Obtaining all other relevant approvals.
(ii) Upload of a letter of collaboration (or formal signed agreement, RCA) that describes the responsibilities and IP ownership of data.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS OF VETERINARY EDUCATION

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Faculty of Veterinary Science
Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadiruiwa

Animal ethics approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

4 November 2020

Approval Certificate
New Application

AEC Reference No.: REC263-19
Title: Development of a serological based vaccine matching technique for Foot-and-Mouth Disease (FMD) SAT 2 viruses.

Researcher: Mr NT Malesa
Student's Supervisor: Dr PA Opperman

Dear Mr NT Malesa,

The **New Application** as supported by documents received between 2020-07-22 and 2020-10-30 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-10-30.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Cattle	
Samples Serum (Historic/stored)	75 (ARC)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-11-04.
3. Please remember to use your protocol number (REC263-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. As part of your approval, the committee requires that you record a **short video footage** of major animal procedures approved in your study. The committee may request them for monitoring purposes at any later point.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiriswa

DARRLD Section 20 approval

agriculture, land reform
& rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development Private Bag X138, Pretoria 0001
Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@dalrrd.gov.za
Reference: 12/11/1/1/8 (1638KL)

Mr Ngwako Terrence Malesa
Transboundary Animal Diseases Programme
ARC-OVR
Tel: 012 529 9560
E-mail: MalesaN@arc.agric.za

Dear Mr Malesa

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Your undated application sent with the email on 2 September 2020 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. This permission is given upon finding the biosecurity of the research project as described to be acceptable to DAFF. This permission does not serve as any approval or endorsement by DAFF for the commercial use or registration of any Foot and mouth disease (FMD) vaccine for any purpose in South Africa;
3. The research project is approved as per the undated application form sent with the email on 2 September 2020 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the

– 1 –

conditions approved for this research project under this Section 20 permit. Please apply in writing to HerryG@dalrrd.gov.za;

4. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to HerryG@dalrrd.gov.za;
5. No live animals may be used in this research project under this Section 20 permit;
6. Samples collected in terms of the Section 20 permit issued on 28 November 2018 for the study entitled "*FMD oil vaccine formulated with the new suspension adapted SAT serotype antigens: Study of the immune response longevity and the determination of the best vaccination schedule to implement in the FMD control zone of South Africa*" or received by TADP for diagnostic or surveillance purposes may be used for this research project;
7. This research project must be conducted in the DALRRD approved ARC-OVR Transboundary Animal Diseases Programme (TADP) BSL 3 Animal Facility (DAFF-CQ03);
8. All potentially infectious material utilised or generated during or by the research project is to be destroyed at completion of the research project by incineration with the exception of:
    8.1. Serum samples as described in point 6 above that must be stored under access control at the DALRRD approved ARC-OVR Transboundary Animal Diseases Programme (TADP) BSL 3 Animal Facility (DAFF-CQ03). Serum samples may not be outsourced or used for further research without written permission from the Director: Animal Health;
9. It is the responsibility of the researcher and relevant laboratory or facility managers to ensure that the human safety aspects of this research project are adequately addressed;
10. All imported materials used in this study must have been legally imported according to all relevant South African legislation;
11. Records must be kept for five years for auditing purposes.

Title of research/study: Development of a serological based vaccine matching technique for FMD SAT 2 viruses
Researcher: Mr Ngwako Terrence Malesa
Institution: ARC-OVR Transboundary Animal Diseases Programme (TADP) BSL 3 Animal Facility (DAFF-CQ03)
Permit Expiry Date: 31 March 2021
Our ref Number: 12/11/1/1/8 (1638KL)
Your ref: ARC-AEC 19.10

Kind regards,

Name: Dr Mphoumaja
Reason: .
Date: 2020.09.30 09:45:10 CAT

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date:

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984) 12/11/1/1/8 (1638KL)*