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Construction of three foot-and-mouth disease virus peptide phage display libraries as a tool for the identification of important epitopes

By

Naledi Palesa Brilliantine Sekgobela

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae (Veterinary Science Tropical Diseases)

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MSc

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University of Pretoria

October 2023

Supervisor : Dr. Melanie Chitray

Co-supervisor : Dr. Pamela Opperman

DECLARATION

I, **Naledi Palesa Brilliantine Sekgobela** hereby declare that this dissertation, which I hereby submit for the Master of Science degree in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, to be my own work, unless otherwise indicated by a reference and has not been previously submitted by me for degree purposes at another tertiary institution.

_____ _____

Naledi Palesa Brilliantine Sekgobela Date: 05 October 2023

(15118968)

“*Therefore I tell you, whatever you ask for in prayer, believe that you have received it, and it will be yours. And when you stand praying, if you hold anything against anyone, forgive them, so that your Father in heaven may forgive you your sins.***”**

Mark 11:21-25.

For My Inamorato

Sibonelo Nkosi

I would not be where I am today without your care and support. No matter how many times I have failed, you have treated me like a winner.

I am thankful!

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“*No one who achieves success does so without acknowledging the help of others. The wise and confident acknowledge this help with gratitude***” Alfred North Whitehead**.

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LIST OF CONTENTS

LIST FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

α	Alpha
A	Absorbance
aa	Amino acid
ARC	Agricultural Research Council
β	Beta
BEI	Binary ethyleneimine
bp	Base pair
BSL3	Biosecurity level 3
ºC	Degrees Celsius
cDNA	Complementary deoxyribonucleic acid
CDR	Complementary determining region
CFU	Colony Forming Units
CPE	Cytopathogenic effect
DNA	Deoxyribonucleic acid
DC	Dendritic cells
dNTP	Deoxynucleoside-5'-triphosphate
dpi	Days post infection
E. coli	*Escherichia coli*
e.g.	Exempli gratia (for example)
eIF	eukaryotic initiation factor
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et al.	Et alia (and others)
EtBr	Ethidium bromide
etc	Et cetera
Fab	Antigen binding fragment
FBS	Foetal bovine serum
Fc	Fragment crystallizable
FAO	Food and Agriculture Organization of the United Nations
Ff	Filamentous phage
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
Fv	Fragment variable region
γ	Gamma
g	Gravitational force
GAG	Glycosaminoglycan
H	Heavy
hr	Hour
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
HS	Heparan sulphate

i.e.	Id est (that is)
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Lambda	λ
LB	Luria Bertani broth
kb	Kilobase pair
KNP	Kruger National Park
M	Molar
mM	Millimolar
MAbs	Monoclonal antibodies
MAR	Monoclonal antibody neutralization resistant
mg	Milligram
mins	Minutes
ml	Milliliter
mM	Millimolar
MOI	Multiplicity of Infection
µM	Micromolar
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium chloride
NCR	Non-coding region
NGS	Next Generation Sequencing
NK	Natural killer
nm	Nanometre
nt	Nucleotides
OD	Optical density
ORF	Open reading frame
OVR	Onderstepoort Veterinary Research
PBS	Phosphate-buffered saline
PBS/T	1× PBS containing 0.1% (v/v) Tween-20
PBS-0.05%T	1× PBS containing 0.05% (v/v) Tween-20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	Potential of hydrogen
PK	Pig kidney cells.
RGD	Arginine-Glycine-Aspartic acid
RNA	Ribonucleic acid
rpm	Revolutions per minute
RS	Instituto Biologico Renal Suino-5 (IB-RS-2) cells.
RT-PCR	Reverse transcriptase-polymerase chain reaction
sec	Seconds
SAT	Southern African Territories
scFv	Single chain variable fragment
TADP	Transboundary Animal Diseases Programme
TAE	Tris-acetate-EDTA

TFCA	Transfrontier conservation area
TPB	Tryptose phosphate broth
Tris	Tris-hydroxymethyl-aminomethane
TY	Tryptone-yeast extract-NaCl
TYE	Tryptone-yeast extract-NaCl-agar
U	Units
µg	Microgram
µl	Microlitre
v/v	Volume per volume
via	By way of
viz.	Videlicet ‘namely’
VP	Viral protein
VPg	Viral genome-linked protein
vRNA	Viral RNA
w/v	Weight per volume
WOAH	World Organisation for Animal Health
WRLFMD	World Reference Laboratory for Foot-and-Mouth Disease

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Transboundary Animal Diseases

for the degree MSc

Foot-and-mouth disease (FMD) is a transboundary animal disease (TAD) that severely affect the production of livestock and the World Organisation for Animal Health (WOAH) ranks FMD as an economically important infectious animal disease. There are seven

serologically distinct serotypes *i.e.,* A, O, C, Asia1 and the Southern African Territories (SAT) types: SAT1, SAT2 and SAT3. Five of the seven serotypes exist in sub-Saharan Africa and considering the virus maintenance host *i.e.,* the African buffalo, eradication is near impossible in Southern Africa. Thus, emphasis is mainly placed on control by vaccination and animal movement restriction. Due to the FMD virus' (FMDV) high antigenic variation, vaccination against one serotype does not confer protection against another and these variations commonly occur on the capsid coding (P1) region of the FMDV genome.

Knowledge of FMDV antigenic sites can be useful to produce recombinant FMD vaccines with broad and long-lasting immunological responses, therefore improving FMD control. Towards this end and to address the scarcity of knowledge regarding SAT antigenic sites, this study aimed to construct three FMDV serotype-specific, phage display peptide libraries using the fragmented P1 regions of SAT1, SAT2 and SAT3 viruses and biopanning these libraries against purified IgGs from FMDV infected bovine sera samples. To achieve this, the amplified respective P1 genomic regions underwent DNA shearing to fragment the P1 DNA. The DNA fragments were cloned into a pCVEP1585042 phagemid vector and transformed using *E. coli* TG1 competent cells. Three novel FMDV SAT serotype peptide phage display libraries were successfully constructed and these libraries were used to identify FMDV SAT3 potential antigenic regions.

From this research work through biopanning, one positive FMDV SAT3 36-mer peptide sequence *i.e.,* SAT3φ1 was identified, potentially containing part of a FMDV SAT3 epitope. The SAT3φ1 sequence aligned to the C-terminus of FMDV VP1, overlapping to the N-terminus of 2A (amino acid position 726 to 740 of the P1/2A region). Additionally, further analysis of the biopanning outputs for SAT3 revealed a total of nine potential FMDV SAT3 antigenic sites located throughout the P1/2A region, where a majority of these correlated to previously published FMD antigenic sites research. No known published research data for four of these sites *i.e.,* amino acid positions 35-67, 134-150, 300-334 and 500-533 of the FMDV SAT3 P1 region could be correlated to FMDV antigenic sites. Thus, this study proposes these amino acids as novel potential antigenic

sites for FMDV SAT3. This study has not only added important value to our FMD knowledge on FMDV SAT3 antigenic sites, but also significantly contributed towards FMD research *i.e.,* future development of improved recombinant vaccines by using reverse genetics technology. The availability of such improved vaccines is of significance, as they could reduce the frequency of livestock vaccination required by farmers as well as provide immune protection against multiple FMDV strains and serotypes. Such improved vaccines could also positively impact the economy of Southern Africa and the consequences of TADs, like FMD, by improving control of the disease, potentially elevating trade and livestock productivity, alleviating poverty through increased food security and consequently enhance the socioeconomic well-being of disadvantaged resource-limited communities/farmers.

CHAPTER 1: LITERATURE REVIEW

1.1. INTRODUCTION

Foot and mouth disease (FMD) is a highly contagious transboundary animal disease of domestic and wildlife cloven-hoofed animals (Anderson *et al.,* 1993; Grubman and Baxt, 2004). FMD continues to be a major impediment to progress and poverty reduction in the developing world for various reasons, including the high cost of control measures, production losses due to lower milk yields, and reduced live animal production (Hammond *et al*., 2021). This disease significantly impacts the economy of affected countries as it affects livestock *i.e.,* sheep, cattle, goat, pigs and other cloven hoof ruminants. Although the disease is rarely fatal in adults, it is frequently fatal in young animals due to myocarditis (Anderson *et al.,* 1993; Grubman and Baxt, 2004; WOAH, 2021). FMD is estimated to circulate in 77% of the global livestock populations in the Middle East, Africa, Asia and in defined areas of South America (Rweyemamu *et al.,* 2008a). Low income and lower-middle income countries sustain more than 70% of the costs associated to FMD prevention and control (WOAH, 2021). Africa is one of the regions that sustains the largest costs, accounting to 50% of the total worldwide costs (WOAH, 2021). There are seven distinct FMD serotypes *i.e.,* the Southern African territories (SAT1, SAT2, SAT3), Asia1, A, O and C that are endemic in different regions worldwide (Bachrach, 1968; Grubman and Baxt, 2004). As there is no long-lasting cross-protection between serotypes, animals that have developed an immune response against one serotype (through vaccination or infection) will not be protected against infection from the other serotypes (Grubman and Baxt, 2004). Furthermore, multiple strains exist within these serotypes that are classified into a large number of subtypes, such that a serotype specific vaccine (or infection) may not protect against all the strains or subtypes within that serotype (Jamal and Belsham, 2013; Diaz-San *et al.,* 2014). Therefore, vaccines need to be carefully matched to

currently circulating virus strains (Hammond, 2011) to be effective. Some countries have eradicated FMD, while it continues to be widespread in others. FMD-free countries (without vaccination) are nonetheless vulnerable to FMD outbreaks. Early detection and warning systems, as well as effective surveillance, are used to prevent FMD outbreaks (Knight-Jones and Rushton, 2013; Maree *et al*., 2014; WOAH, 2021).

FMD epidemiology in Sub-Saharan Africa is more complex compared to other regions worldwide. This complexity arises from the presence of five out of the seven FMD serotypes found in Africa. These serotypes are unevenly distributed across different regions (with the exception of Asia1), making disease control through vaccination more challenging (Brooksby, 1972; Ferris and Donaldson, 1992; Vosloo *et al*., 2002). It is believed that serotype C is most likely extinct as it was last reported in 2004, circulating in East Africa (Sangula *et al.,* 2011; Brito *et al.,* 2017; Belsham, 2020; Paton *et al.,* 2021). There are two cycles of FMD in Sub-Saharan Africa *i.e.,* one in which the virus circulates between wildlife hosts and domestic animals, and the other in which the virus spreads among domestic animals without the involvement of wildlife (Vosloo and Thomson, 2004). Infection with the FMD virus (FMDV) can lead to the establishment of a carrier state, where the virus can be identified in infected animals 28 days after infection (Sutmoller *et al.,* 1968; Grubman and Baxt, 2004). African buffalo (*Syncerus caffer*) herds have been found to be long-term maintenance hosts for the SAT serotypes of FMDV with no evident clinical illness (Gainaru *et al.,*1986; Thomson *et al.,* 2003). In Africa, wildlife plays a unique and crucial role in the disease's epidemiology (Van Bekkum *et al*., 1959; Alexandersen *et al*., 2002; Ayebazibwe *et al*., 2010). With the presence of buffaloes, eradication of the disease is nearly impossible, thus the focus is on control, mainly by vaccination (Vosloo *et al*., 2002; 2006). The existing vaccination methods alone have not been successful in preventing FMD outbreaks and therefore other control measures such as restricting animal movement (through fencing), where livestock and wildlife are separated from each other by veterinary cordon fences, have been implemented. The wildlife species are contained in reserves and routine FMD inspections of livestock are carried out in controlled areas adjacent to reserves (Thomson and Bastos, 2004; Lazarus, 2014).

1.2. FOOT-AND-MOUTH DISEASE VIRUS

Fracastorius reported an illness affecting cattle in Italy in 1514, with symptoms that were comparable to FMD as we know it today (Fracastorius, 1546). Only centuries later, in 1897, did Loeffler and Frosch demonstrate that FMD was caused by a filterable agent, namely the FMD virus (Loeffler and Frosch, 1897; Rueckert, 1996). FMDV belongs to the *Picornaviridae* family, *Aphthovirus* genus (Rowlands, 2003). Due to the absence of a proof-reading RNA polymerase in RNA replication, the virus has exhibited a significant degree of genetic and antigenic variation (Sobrino *et al*., 2001), with SAT 2 serotypes being particularly antigenically variable (Doel, 2003; Mahapatra and Parida, 2018). The capsid-coding region, or P1 region of the genome, is where these mutations most frequently occur (King *et al*., 1985; McCahon *et al*.,1985). The global distribution and geographic patterns of these FMDV serotypes appear to differ (Bastos *et al*., 2001; 2003a; 2003b; Bronsvoort *et al*., 2004) and are named after their place of origin (Waldmann and Trautwein, 1926). In 1922, Vallee and Carre were the first to discover serotypes O and A, while Waldmann and Trautwein discovered serotype C in 1926 (Vallee and Carre, 1922; Waldmann and Trautwein 1926). Later, three additional serotypes, SAT1, SAT2 and SAT3 were discovered in South African samples (Brooksby, 1958). In 1954, Asia-1, was discovered in a sample taken from a water buffalo in Okara, Punjab, Pakistan (Brooksby and Rogers, 1957; Brooksby,1958).

The FMDV's exceptional sensitivity to acidic pH results in virus inactivation once virions are exposed to slightly acidic pH (Newman *et al*., 1973). The mechanism of FMDV penetration in host cells has been linked to its acid sensitivity (Baxt,1987; Johns *et al*., 2009) and the current model supports the concept that acidification of endosomes, where FMDV virions are sorted, causes viral uncoating and genome release (O'Donnell *et al*., 2005). At neutral pH, the virus can live in lymph nodes and bone marrow, but at pH <6.0 *i.e.,* after *rigor mortis*, the virus is destroyed in muscles (WOAH, 2013). Apart from a neutral pH, temperatures below 50°C and relative humidity greater than 55% are ideal for virus survival (Bartley *et al*., 2002; Colenutt *et al*., 2018).

1.3. EPIDEMIOLOGY OF FMDV

1.3.1. DISTRIBUTION

In certain places, FMD has been eradicated, while it is still present in others. The disease's presence and rapid spread poses a constant threat to FMD-free areas (Knight-Jones and Rushton, 2013; Knight-Jones *et al.,* 2016; Metwally *et al.,* 2021). FMDV serotypes are distributed unevenly over the world and are categorized into pools. Each pool represents the FMDV serotypes that are currently circulating and emerging in different parts of the world (Rosso, 2018). Serotypes O and A are the most common FMDV serotypes in endemic areas, according to the World Organisation for Animal Health (WOAH) and the Food and Agriculture Organization of the United Nations (FAO), with the exception of Southern Africa (WRLFMD, 2021). The Asia-1 serotype is present in Asia's FMD-endemic areas, while the SAT serotypes are naturally found in the virus maintenance hosts *i.e.,* the African buffalo (Thomson *et al*., 2003). The most extensively distributed serotype in Africa is SAT2, which is also the serotype most frequently associated with outbreaks in cattle in southern Africa, followed by SAT1 and SAT3 respectively (Dyason, 2010; Hall *et al*., 2013). Viruses of the SAT3 serotype have the most limited distribution, occurring only in southern Africa (Bastos *et al*., 2003; WRLFMD, 2021). FMDV serotype C has not been detected since 2004 (in Kenya and Brazil), suggesting that it is no longer circulating and may be extinct (Brito *et al*., 2017; Belsham, 2020).

FMD has been geographically distributed into seven endemic pools, based on genetic and antigenic analyses (Figure 1.1). Pool 1 involves Southeast Asia, Central Asia and East Asia; pool 2 incorporates South Asia, while pool 3 covers the West Eurasia and the Middle East. Pools 1-3 consist of serotypes A, O and Asia1 (Figure 1.1). Africa can be divided into FMDV pools 4, 5 and 6 (Figure 1.1). The North and East of Africa having serotypes O, A, SAT1, SAT2 and SAT3 (pool 4); the West and Central Africa has serotypes O, A, SAT1 and SAT2 (pool 5) and pool 6 incorporating Southern Africa

includes SAT1- 3. Lastly, pool 7, which covers South America comprises of serotypes A and O (Figure 1.1; Paton *et al*., 2009; Di Nardo *et al*., 2011; Hammond *et al*., 2021).

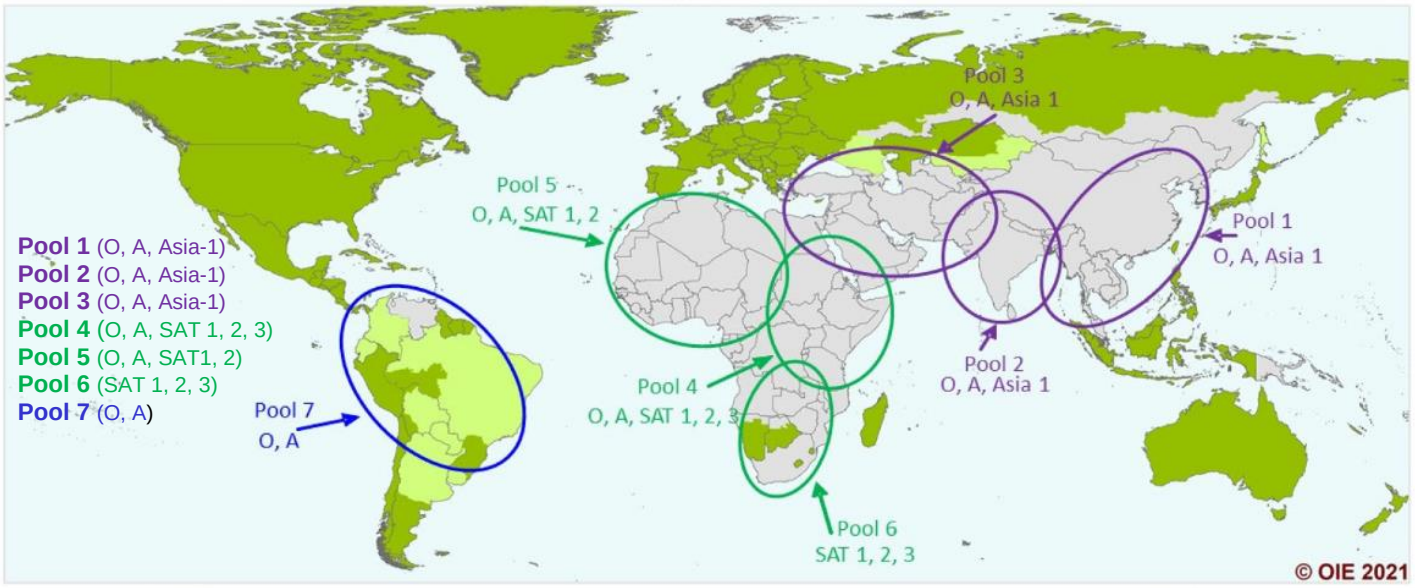


Figure 1.1: The geographical distribution of the seven endemic pools of foot and mouth disease. Adapted from Hammond *et al.,* (2021).

1.3.2. EPIDEMIOLOGICAL PATTERNS FOR FMDV IN AFRICA

When compared to serological procedures, molecular biology techniques give more precise results and have allowed virus strains to be characterized, allowing viruses isolated from outbreaks to be traced (Knowles and Samuel, 2003). Thus, countries can now be grouped into epidemiological clusters, based on the FMDV topotypes within each serotype that occurs. It is important to note that FMD epidemiological spread can change from a regional one to large-scale global spread (Knowles and Samuel, 2003; Hammond *et al.,* 2021). This is due to the results of globalization and has been witnessed with the type-O Pan-Asian lineage, which led to FMD outbreaks in Asia. These outbreaks subsequently spread to various regions of Europe and Africa, from 1998 to 2001 (Knowles *et al.,* 2005; Rweyemamu *et al*., 2008a).

Six of the seven serotypes (O, A, C, SAT1, SAT2 and SAT3) have been found in Africa, according to the cumulative incidence of FMDV serotypes (Donaldson, 1999; Valarcher

et al., 2004). Figure 1.2A-E depicts the distribution of five serotypes as well as the several topotypes. The virus distribution in Africa has been classified into three virus pools (distribution in Figure 1.1) based on genetic characterization and antigenic connection of FMDV. There have been periodical incursions of SAT1 and SAT2 from Africa into the Middle East, most likely due to animal movements (Donaldson, 1999; Valarcher *et al.,* 2004). In 1990 the SAT2 virus was reported to have spread to Yemen and Saudi Arabia, to Kuwait in 2000 and to the Palestinian Autonomous Territories and Bahrain in 2012 (Hall *et al.,* 2013).

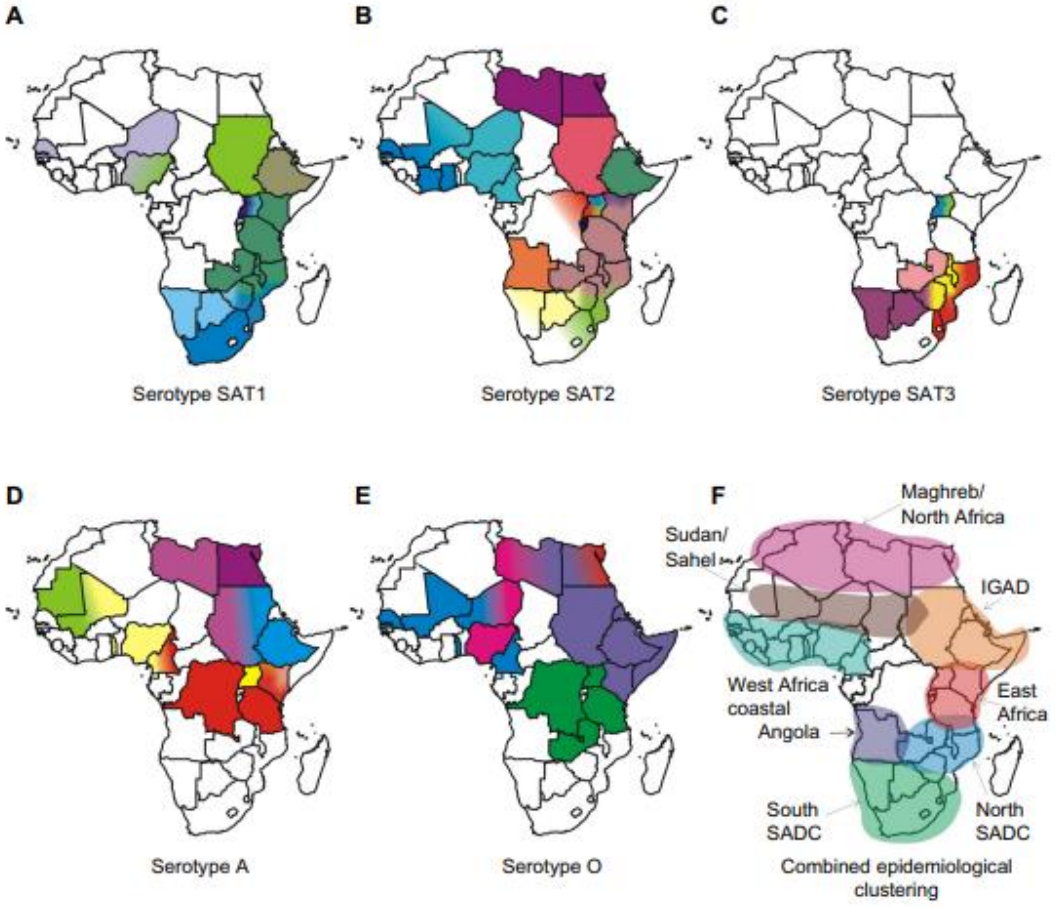


Figure 1.2: Serotype and topotype distribution maps for Africa. Colour coding was used to differentiate the topotypes. There is evidence of epidemiological clustering and the cluster on the maps (A-F) do not always correspond to country borders. Taken from Maree *et al.,* (2014).

To better comprehend the complexities of FMD epidemiology in Africa, as well as to aid decision-making and improve continental FMD control, the virus pools must be further divided into epidemiological clusters (Rweyemamu *et al.,* 2008a). Rweyemamu *et al.,* (2008a) proposed eight epidemiological clusters for Africa (Figure 1.2F) based on the distribution of serotypes and topotypes in different African areas (Figure 1.2A–E), animal movement patterns, wildlife impact and farming systems.

1.3.3. THE ROLE OF CARRIERS IN THE EPIDEMIOLOGY OF FMDV

Recent studies have shown that carriers can be found in cattle that have been vaccinated as early as 15 dpi and 21 dpi in unvaccinated cattle *i.e.,* neoteric subclinical infection (Stenfeldt *et al.,* 2016a). These animals do not exhibit any clinical symptoms (asymptomatic), while producing low levels of FMDV excretions. Ruminants with and without vaccination histories can both be carriers. Thus, exposure to FMDV causes the carrier state rather than vaccination (Salt, 1993; Van Bekkum *et al*., 1959; Sutmoller and Gaggero, 1965; Sutmoller *et al*., 2003). Studies have shown that a simultaneous subclinical FMD carrier combined with a subclinical neoteric infection result in super-carriers (Arzt *et al.,* 2021; Fish *et al.,* 2022; Palinski *et al.,* 2022). Therefore, the existence of FMD "carriers" complicates efforts to control and eradicate the disease, impacting global trade of animal products (Arzt *et al*., 2018). The African buffalo (can carry FMDV for up to five years) is the only species that has been shown to transmit FMDV to susceptible animals while in the carrier state and FMDV transmission from carrier to naïve buffalo occurs within two weeks of exposure (Straver *et al.,* 1970; Condy *et al.,* 1985; Vosloo *et al*., 1996; Tekleghiorghis *et al*., 2016; Bertram *et al*., 2018).

Bovine carriers can harbor FMDV for two years (Condy *et al*., 1985). Although the exact method by which FMD spreads from buffalo to cattle is unknown, it is known that these animals must come into direct contact with one another (Maree *et al.,* 2011). Infected cattle may also maintain SAT viruses independent of contact with buffalo (Thomson, 1994; Thomson *et al.,* 2003).

1.3.4. TRANSMISSION OF FMD

The FMD virus transmission is aided by the virus's expulsion and release from ruptured vesicles (Alexandersen *et al*., 2003) and in bodily secretions such as milk, urine, semen, and faeces (Hyde *et al.,* 1975; Donaldson, 1987). Infection can occur through various routes (Sellers, 1971), via direct contact through inhalation of the virus from infected animals or indirect contact by aerosol from contaminated products. Direct transmission occurs when the recipient and infected animals are in close proximity, while indirect transmission involves the environment, whereby the recipient animal is exposed to contaminated products, infected secretion and excretion (Velkers *et al*., 2012). Contaminated agricultural equipment, persons, cars, and feed for animals are among other recognized sources of indirect contact (Sellers, 1971). Furthermore, cold and humid weather favours airborne transmission, where the aerosols are carried by the wind (Donaldson, 1987). Infected pigs are a significant source of aerosolized FMDV, even though they have been demonstrated to be less vulnerable to airborne FMDV infection (Donaldson *et al.,* 1982; Alexandersen *et al.,* 2002; Brown *et al.,* 2022).The ability of the virus to survive outside the host, the amount of virus needed to initiate infection at the primary infection sites of exposed animals and the quantity, duration, and method by which the virus is released into the environment are all parameters that determine FMD transmission. The virus is stable in favourable conditions, such as cool temperatures, neutral or alkaline conditions, or organic material and its stability plays a role in FMDV transmission (Sellers, 1971; Chitray 2018; Paton *et al.,* 2018). High temperatures (above 50°C) and acidic environments can, however, inactivate the virus (Charleston *et al.,* 2011; Chase-Topping *et al.,* 2013).

1.4. CONTROL AND ERADICATION OF FMDV

According to the WOAH and FAO, FMD ranks as one of the most economically infectious animal diseases and poses a serious threat to global food security, particularly to the affected countries. Furthermore, this leads to major international restrictions on exports

of agricultural animal products from endemic regions and maintaining an FMD-free status signifies the progress and development of their veterinary infrastructure and disease control measures (Thomson *et al.,* 1992; Leforban, 1999; WOAH, 2009; Pattnaik *et al.,* 2012). Many countries around the globe that are free of FMD with or without vaccination, are threatened by the presence of FMD in surrounding countries (Pattnaik *et al.,* 2012). Several countries such as the United State of America, United Kingdom, Canada, Mexico and Brazil have managed to eradicate the disease through coordinated mass vaccination, control of animal movements, quarantine practices and strict zoo-sanitary measures (Ferguson *et al.,* 2001), however in large regions of Asia and Africa, FMD still persists (WOAH, 2022). The ultimate goal in Africa is the eradication of FMDV, but due to certain constraints (*i.e.,* wildlife maintenance host), it is doubtfully possible in the near future. Thus, emphasis is placed on control rather than eradication and there are several methods of managing FMD through implementing effective control measures and prevention strategies (Maree *et al.,* 2014). These measures include fencing to physically separate domestic animals from wildlife, limiting and controlling the movement of animals, carefully assessing the risk of FMDV introduction to disease-free areas and regular vaccination of livestock in endemic areas (Brückner *et al.,* 2002; Thomson *et al.,* 2003; Jori *et al.,* 2009). In FMD-free countries *e.g*., Argentina, Europe and Australia (Marcos and Perez, 2019; Stenfeldt *et al.,* 2015), strict restrictions of animal movements and their products from endemic regions, movement of contaminated products and culling is practiced, maintaining their FMD-free status (Paton *et al.,* 2005).

South Africa was one of the countries that had obtained a FMD free status without vaccination from the WOAH for certain zones within the country (Figure 1.3) (Vosloo *et al.,* 2002). Over the past few decades, the country has managed to increase its FMD control efforts in the same general manner as several other countries. They have chosen to use the zoning laws to separate areas where African buffalo are found from cattle-raising areas (Perry *et al.,* 2003). The KNP and neighboring game reserves, as well as two other game reserves in northern KwaZulu-Natal, are officially classified as FMD infected zones due to the buffalo being an endemic FMDV carrier in South Africa. The areas immediately around the infected zones are designated as FMD protection zones,

where cattle are vaccinated every 6 months with vaccines incorporating all three SAT serotypes (Vosloo *et al.,* 2002). This zone also separates the infected zones from the rest of the country, which was previously declared as FMD free (Figure 1.3) (DALRRD, 2014). Due to on-going FMD outbreaks in South Africa, during the years 2011 and 2019 and more recently 2021 and 2022 (DALRRD, 2022), the country's FMD free status has been temporarily suspended (DALRRD, 2019). The suspension was mainly as a result of FMDV serotype SAT2 outbreak that were recorded in 2019, after being detected in the FMD free zone (DALRRD, 2019; Lazarus *et al.,* 2019).

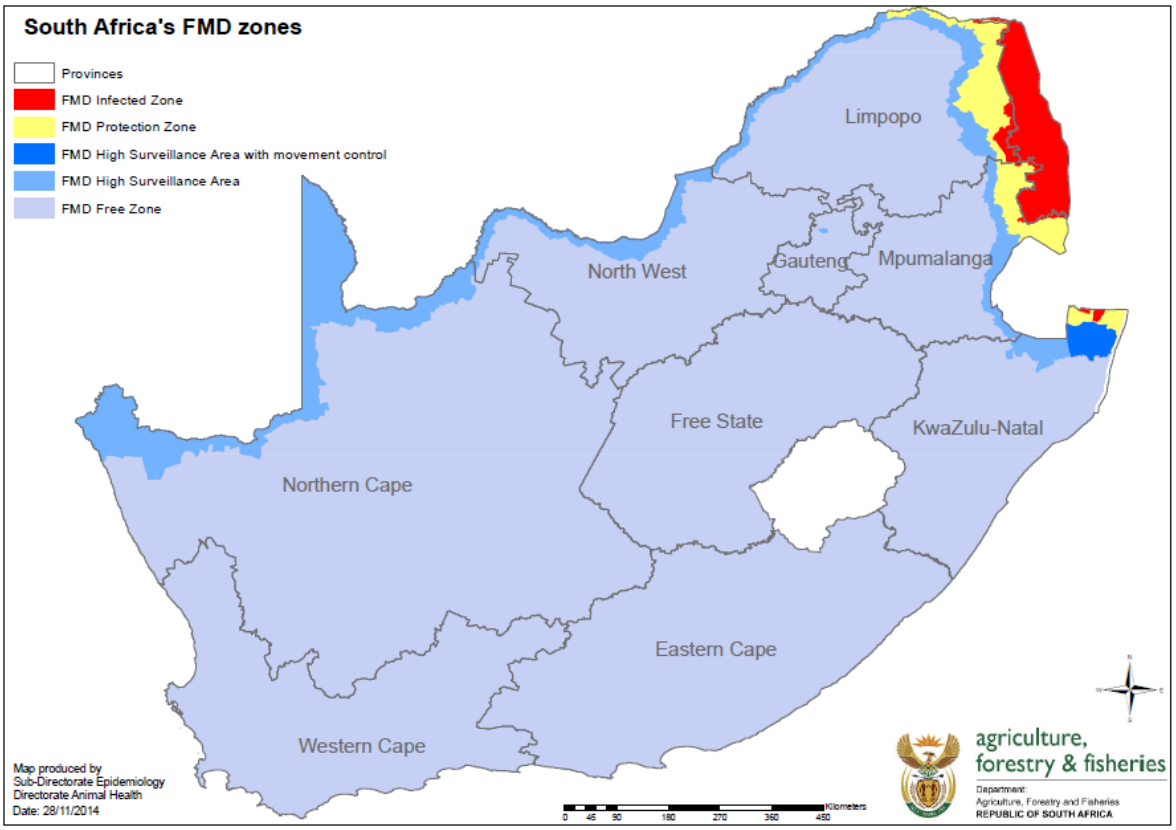


Figure 1.3: A South African map showing the FMD zones. Obtained from the Department of Agriculture, Land Reform and rural development (DALRRD, 2014).

Currently, South Africa has 183 active FMD outbreaks occurring in the previous FMD free zones (Figure 1.4) (DALRRD, 2023). These outbreaks affected the North-West, Limpopo, Mpumalanga, Gauteng, KwaZulu Natal (KZN) and Free State provinces (DALRRD, 2022;

DALRRD, 2023). The first epidemic to occur in May 2021 was caused by a SAT2 serotype, which affected the region of KwaZulu Natal. There was limited spread to the Free State province (DALRRD, 2023). This virus was associated to the SAT2 virus that caused the 2019 FMD outbreak in Limpopo and was last reported in September 2022 (DALRRD, 2022). A second outbreak occurred in the previous FMD free zone of the Limpopo province in March 2022 and by April 2022, it had spread to the northern Gauteng province, with the last positive case reported in June 2022. The third event involved outbreaks that originated in the North-West province (March 2022) and had spread to Gauteng, Free State and Mpumalanga province. These outbreaks were reported in April, July, August and September 2022 (DALRRD, 2022; DALRRD, 2023) and were caused by the SAT3 virus, which was unrelated to any recent outbreak viruses. It was thought to be believed that illegal animal movements from one province to another led to these outbreaks, which resulted in the movement of sheep, cattle and goat being prohibited in disease management areas of Limpopo, Free State and KZN provinces (DALRRD, 2022). In January 2023, the most recent outbreak was reported to be caused by a SAT3 virus and affected the North-West province. This outbreak was not a result of recent spread but was identified as part of the ongoing surveillance of neighboring areas (DALRRD, 2023).

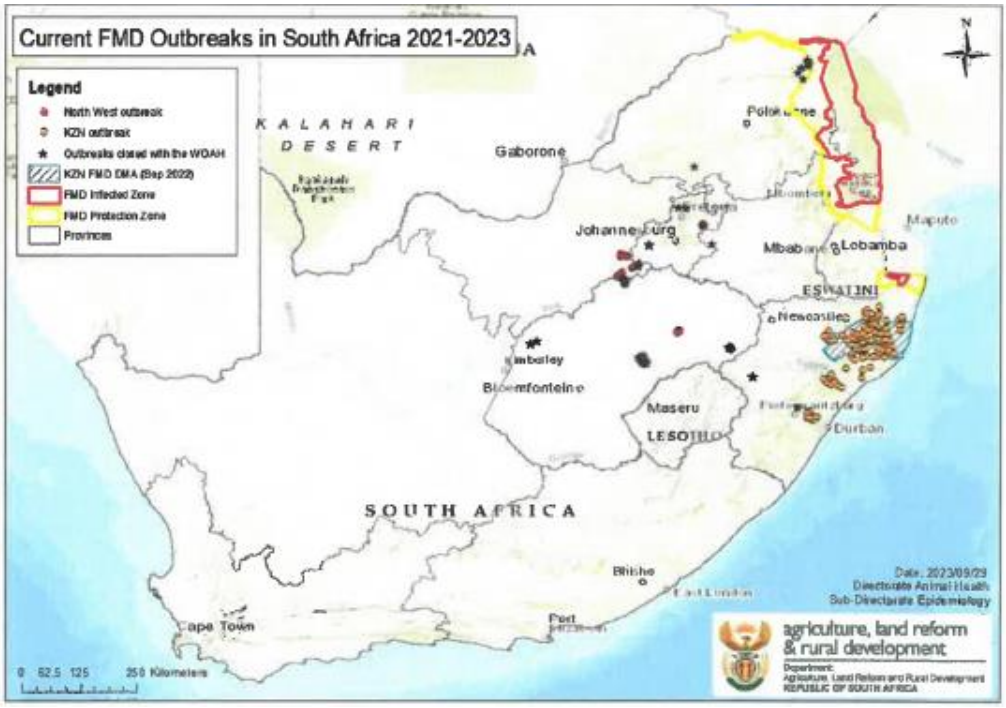


Figure 1.4: A map showing the FMD outbreaks reported in the previous South African FMD free zones in 2021 to 2022. Taken from the Department of Agriculture, Land Reform and rural development (DALRRD, 2023).

FMDV vaccines are of utmost importance in controlling the spread of FMD and effectively preventing infections caused by the virus. This is achieved by generating clinical protection against FMD virus, thus playing a crucial role in disease management (Orsel and Bouma, 2009). FMDV vaccines are detailed in section 1.11.

1.5. PATHOGENESIS OF THE DISEASE

The clinical signs, immune response and pathogenesis of FMD not only differ between serotypes and hosts but can also not be clinically differentiated from other vesicular diseases (Burrows *et al.,* 1981; WOAH, 2009). In natural infections, the respiratory tract serves as the main route of virus entry (Burrows *et al.,* 1981; Arzt *et al.,* 2010) and regardless of the method of infection, the pharyngeal area is the region where the FMDV first localizes and replicates in pigs and cattle (Pacheco *et al.,* 2010; Stenfeldt *et al.,*

2014a). During the primary infection phase, the pharynx epithelium, in particular, is where the initial virus replication takes places, producing primary vesicles (Burrows *et al.,* 1981). These produced vesicles affect the epithelial stratum espinosum (Salt, 1993). Within 1 to 2 days after infection the primary clinical phase of infection occurs which includes fever and viraemia, leading the virus to enter the bloodstream and virus spreads into several organs and tissues for secondary replication (Salt 1993; Arzt *et al.,* 2011). The acute disease phase takes about a week and slowly subsides with the appearance of a strong humoral response (Salt, 1993). Animals either can terminate the disease and recover or enter a persistent phase (>28 dpi) *i.e.,* carrier. However, research studies have challenged the conventional FMDV carrier state definition and shown that animals that clears the virus (one to two weeks) can be differentiated to carriers much earlier. As early as 21 dpi in non-vaccinated cattle and 10 dpi in vaccinated cattle (Stenfeldt and Belshem, 2012; Stenfeldt *et al.,* 2016a). As a result of these findings, the definition of transitional phase of FMDV infection corresponds to the time period in which the virus is cleared from cattle that do not develop a carrier state (Stenfeldt *et al.,* 2016a). In cattle, the pharyngeal epithelium is strongly linked to viral persistence (Pacheco *et al.,* 2015). Thus, determining how to eradicate the virus at the onset of invasion is important for minimizing the spread of FMDV (Longjam *et al.,* 2011).

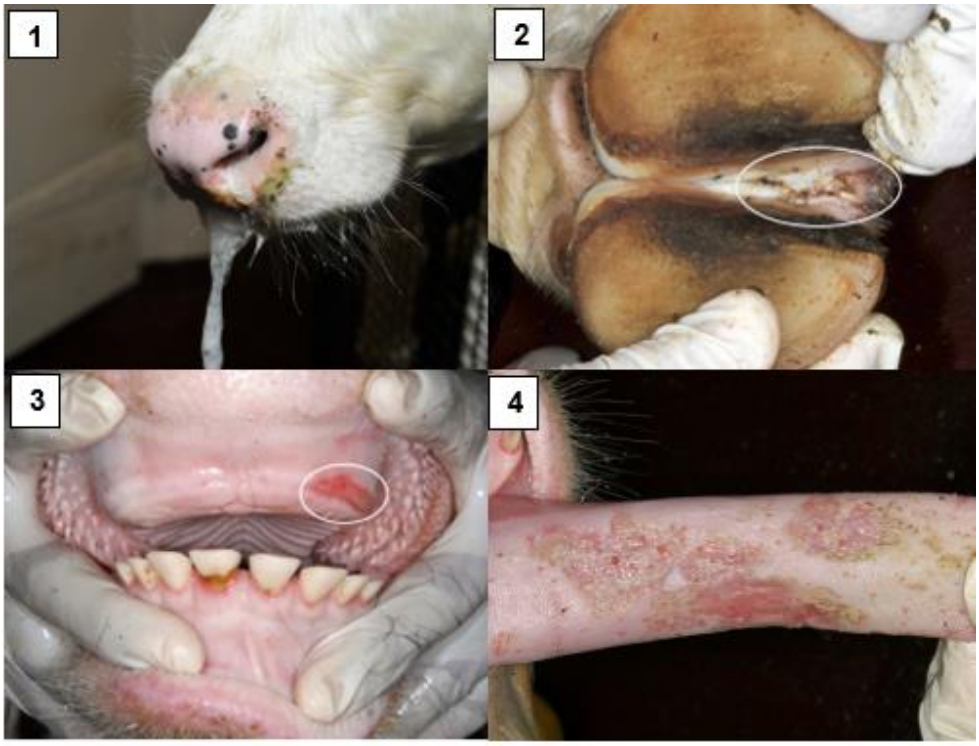


Figure 1.5: FMD clinical signs from infected cattle, showing (1) excessive salivation due to mouth lesions, (2) rupture of interdigital vesicles and granulation tissue formation, (3) linear erosions on dental pad with fibrin and (4) multifocal and coalescing erosions and ulcers with fibrin on dorsal surface of tongue [The Center for Food security and Public Health (CFSPH), 2015].

Fever, lameness and the presence of vesicles in the foot and mouth are among the most common symptoms of FMD in infected animals (Figure 1.5). FMDV is found in large quantities in the tissues, excretions, and secretions. Vesicles can be observed on the nipples, toes, and other hairless regions of infected calves around 3 to 4 dpi (Arzt *et al.,* 2017) and difficulties in swallowing and salivation is observed as a result of vesicular lesions in the mouth, throat, and nose (Stenfeldt *et al.,* 2016b). The morbidity and mortality of FMD vary by breed and age of the animal, with relatively low (2%) deaths in adults compared to young stock (20%) (Radostits *et al.,* 1994). Pigs are less susceptible to airborne infection but produce larger amounts of aerosolized virus compared to ruminants (Stenfeldt *et al.,* 2014b). In contrast to piglets, who experience gastroenteritis, calves show myocarditis (Buxton and Fraser, 1977). In sheep and goats, symptoms are typically less severe, making disease detection more challenging (Knowles *et al.,* 2001).

1.6. THE VIRION

The FMDV virion is a small virus ~30 nm in diameter and consists of a non-enveloped symmetrical capsid that encloses the genomic ribonucleic acid (RNA) (Bachrach, 1968; Mateu, 2017). The viral genome consists of a single-stranded positive–sense RNA genome of ~8.3 kb (Grubman, 1980) and is surrounded by 60 copies of each of the four structural capsid proteins, namely, VP1, VP2, VP3 and VP4 (Domingo *et al.,* 1985). Since the viral RNA is infectious, the virus particle protects the genome while it is outside of cells and aids in the transfer of the RNA to the cell's cytoplasm, where infection can begin. When compared to other picornaviruses, the surface of the virus particle is comparatively smooth due to the lack of peaks and depressions and its capsid is generally spherical, with icosahedral symmetry. (Bachrach *et al.,* 1964; Grubman *et al.,* 1979; Jamal and Belsham, 2013; Chitray 2018). The absence of a surface canyon, or pit, which has been demonstrated to be the receptor binding site for the entero- and cardioviruses, separates FMDV from other picornaviruses (Hogle *et al.,* 1985; Grubman and Baxt, 2004). Another characteristic of the virion is the existence of a channel at the fivefold axis that allows the entry of small molecules (*i.e.,* CsCl) into the capsid, which results in FMDV having the highest buoyant density of the picornaviruses (Acharya *et al.,* 1989; Jackson *et al.,* 2003). In contrast to other picornaviruses, the FMDV capsid dissociates into 12S pentameric subunits at pHs below 6.5 (Brown and Cartwright, 1961). The cluster of histidine (His) residues at the interface between VP2 and VP3 are thought to be the reason for this instability, as they become protonated at a low pH and the capsid is weakened through electrostatic repulsion (Curry *et al.,* 1995; Ellard *et al.,* 1999). Due to this low-pH-induced instability, the mechanism of the FMDV uncoating during cell infection differs from that of other picornaviruses (Grubman and Baxt, 2004).

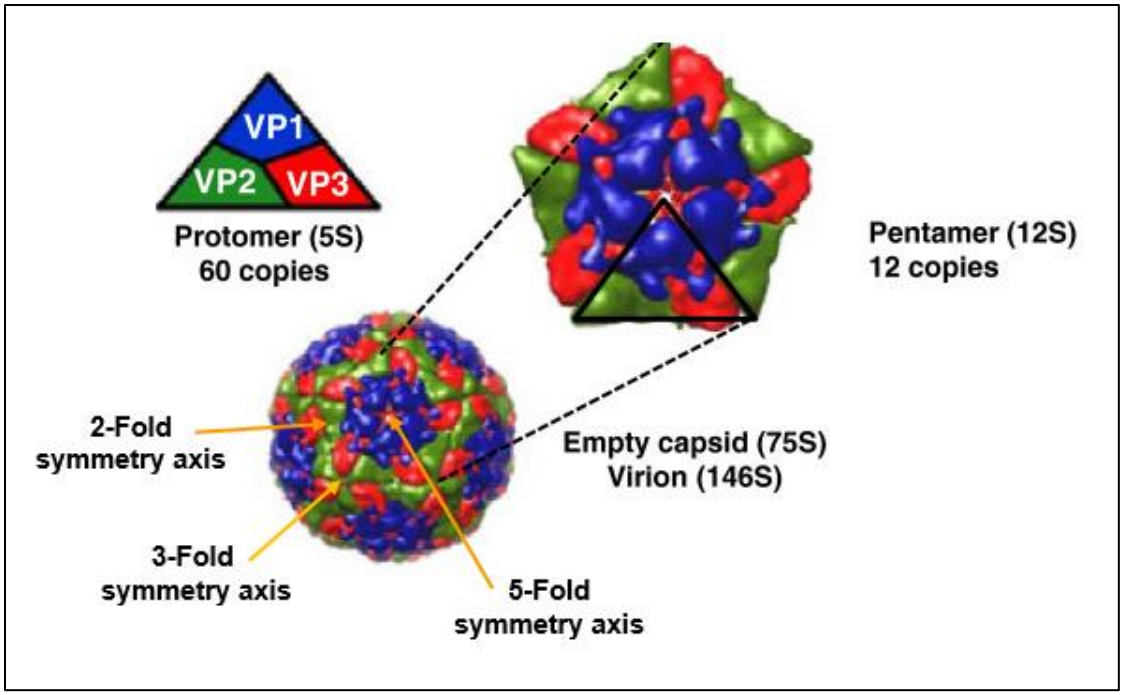


Figure 1.6: A schematic representation of the FMDV structure, displaying the surface capsid proteins, subunits and the virus capsid. The assembly of the virus particle from a protomeric subunit (with external capsid proteins VP1-3) to a pentameric subunit is outlined. VP4 is internal. Self-assembly of the empty capsid without the RNA genome is also possible. The 2-fold, 3-fold and the 5-fold symmetry axes are indicated on the capsid structure. Adapted from Jamal and Belsham, (2013) and Chitray, (2018).

Structural proteins VP1 to VP3 have molecular weights (MW) of ca. 24kDa, which cooperate to form the external surface of the shell, while VP4 (MW 8.5 kDa) forms the interior of the virus particle (Acharya *et al.,* 1989; Belsham *et al.,* 1991; Domingo *et al.,* 1992). The structural capsid protein precursor, P1 is cleaved to produce VP0 (cleavage precursor of VP4 and VP2), VP3, VP1 and a 2A peptide (Jiang *et al.,* 2014; Kristensen and Belsham, 2019; Curry *et al.,* 1997), forming immature protomers by weak chemical bonds (Fry *et al.,* 2005a). In the final stages of virion formation, VP0 is cleaved into VP4 and VP2 and this cleavage is viral RNA dependent and autocatalytic (Arnold *et al.,* 1987; Basavappa *et al.*, 1994; Curry *et al.,* 1997; Grubman and Baxt, 2004). While VP2 and VP3 are located at the two- and three-fold axes of symmetry, five copies of VP1 are grouped around the five-fold symmetry (Figure 1.6) (Acharya *et al.,* 1990). A promoter is formed by combining VP3, VP1 and VP0. Thus, five protomers combine into a pentamer

and twelve pentamers combines with viral RNA particles to form a viral capsid structure (Figure 1.6) (Grubman and Baxt, 2004).

1.7. FMD VIRAL STRUCTURAL PROTEINS

The viral structural proteins, VP1 to VP3 fold into an eight-stranded wedge-shaped β-barrel that fits together to form the majority of the capsid structure (Acharya *et al.,* 1989) and the loops connecting the strands of the VP1-3 β-barrels forms the virion's outside surface (Jackson *et al.,* 2003). However, the majority of the identified antigen sites associated with the host immunological response, are primarily found in the βG-βH loop of VP1 (Abubakar *et al.,* 2018). The βG-βH loop has a hypervariable region, which may contribute to the high variability of VP1 (Fernandez-Sainz *et al.,* 2019). The VP4 has a myristyl group covalently linked to its N terminus and is concealed within the capsid (Chow *et al.,* 1987; Belsham *et al.,* 1991). These arrangements of the structural capsid proteins provide the antigenic regions that causes infection or vaccination immunological responses (Acharya *et al.,* 1989). Approximately 30-50% of the residues that makes up the capsid proteins are exposed on the virus surface and comprise of neutralizing epitopes (Reeve *et al.,* 2010; Lea *et al.,* 1995). The conservation of the four structural proteins varies: VP1 is highly variable [74% of its residues are variable (Carrillo *et al.,* 2005)], VP2 and VP3 are relatively conserved (Peng *et al.,* 2020) and VP4 being highly conserved across all serotypes (Feng *et al.,* 2018).

VP1 has 3 different antigenic sites. Site 1 (or Site A in Asia-1) contains a highly conserved motif of three residues (arginine, glycine, and aspartic acid; RGD), located at the apex of the GH loop (Carrillo *et al.,* 2005) whilst the surrounding residues are highly variable (Mateu, 1995; Grubman and Baxt, 2004). This is most likely due to its crucial role in binding integrin molecules on the host cell surface (Alcala *et al.,* 2001; Domingo *et al.,* 2002). The second antigenic site is formed by the C-terminal residues (Acharya *et al.,* 1989), while residues 43-45 and 48, located in the BC loop forms the antigenic site 3. However, only site 1 is found in all serotypes, while site 2 and 3 differs with serotypes

(Crowther *et al.,* 1993; Fry *et al.,* 2005a). The length of the VP1 varies according to the serotypes and ranges from 207 and 219 amino acids. This is mainly due to insertions and deletion around the GH loop (Logan *et al.,* 1993; Carrillo *et al.,* 2005).

In comparison of the VP2 to other picornaviruses, FMDV VP2 has 218 or 219 amino acids (Acharya *et al.,* 1989; Carrillo *et al.,* 2005). The N-terminal residues of three neighbouring pentamers are organized around the viral particle's three-fold symmetry axis. It is hypothesized that there is a calcium binding site at position six of the protein, which contains a conserved glutamic acid and mediates a crucial ionic interaction that supports the particle's structural integrity (Han *et al.,* 2015).

The size of VP3 ranges from 219 to 221 amino acid and 61% of its residues are highly variable (Carrillo *et al.,* 2005; Dill and Eschbaumer, 2020). Like VP2, VP3 is positioned around the viral capsid's threefold axis (Acharya *et al.,* 1990). Additionally, the pentamer protomers are connected by the N-termini of five copies of VP3 that are weaved together along the fivefold axis, forming an axial channel that permits the penetration of tiny molecules (*i.e.,* caesium ions) into the particle (Acharya *et al.,* 1989; Han *et al.,* 2015). Due to the presence of the substantially conserved amino acids phenylalanine, valine and cysteine at positions three, five, and seven, respectively on VP3, this pore structure is very hydrophobic (Han *et al.,* 2015; Dill and Eschbaumer, 2020). VP4 is a small, very hydrophobic protein (Yuan *et al.,* 2017), consisting of 29% variable amino acids (Carrillo *et al.,* 2005). Among the picornaviruses, FMDV has the longest VP4 protein, composed of 85 residues total (Acharya *et al.,* 1989; Han *et al.,* 2015).

1.8. FMDV GENOME

The FMDV genome is ~8300 nucleotides (nt) in length and bound at the 5’ end to a small covalently linked protein, VPg (Jackson *et al.,* 2003), while the 3’ end of the genome is polyadenylated (Bachrach, 1968, Figure 1.7). Like all picornaviruses, the virus contains a single long open reading frame (ORF) of about 7000 nt (Figure 1.6), flanked by the 5’ and 3’ untranslated regions (UTR) (Belsham, 2005; Carrillo *et al.,* 2005). The 5’ UTR is

~1300 nt (Belsham, 2005) and comprises of a short S-fragment (350-380 nt), a poly (C) tract (100-420 nt), *cis*-acting replication element (*cre*), several pseudoknots, the internal ribosome entry site (IRES) (Belsham, 2005) and a large (L) fragment with more than 700 nucleotides (Mason *et al.,* 2003). The IRES is roughly 450 nt and plays a role in viral protein synthesis. The short 3' UTR of ~90 nt long, folds into a stem-loop structure and is followed by a poly A tract. This UTR plays a crucial role in viral genome replication (Agol *et al.,* 1999; Belsham, 2005; Jamal and Belsham, 2013).

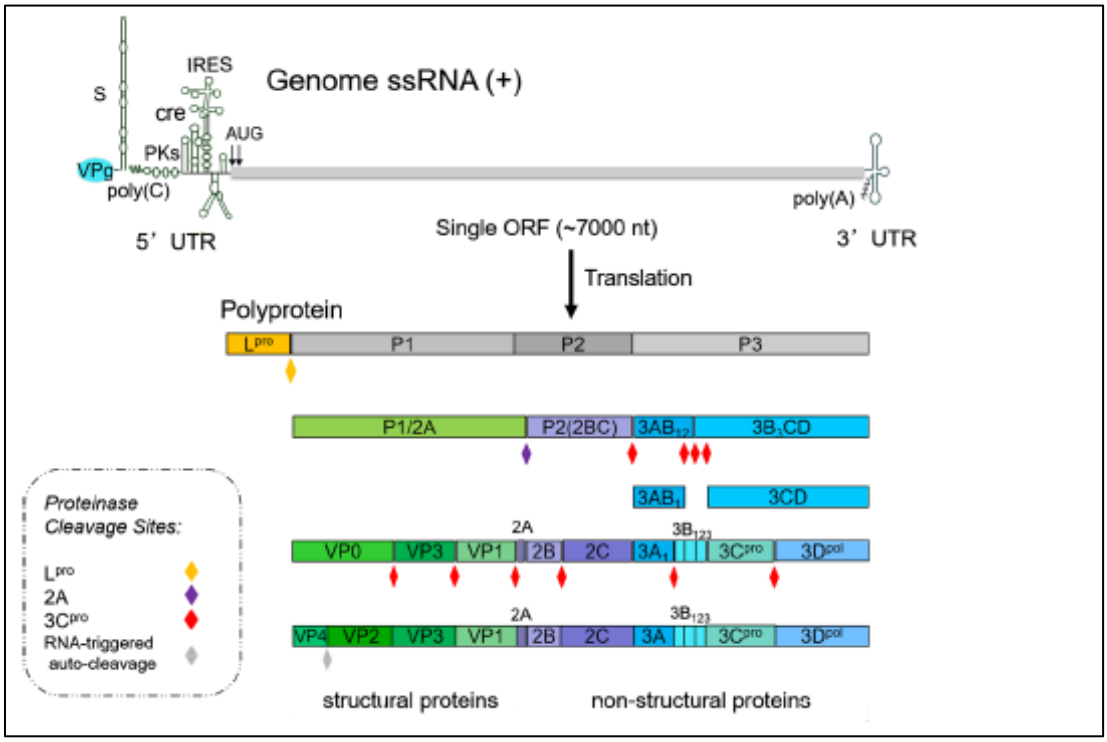


Figure 1.7: Schematic diagram of the FMDV genome, displaying the genome's functional components and the protein cleavage products. Obtained from Gao *et al.,* (2016).

There are three main regions that the genome (Figure 1.7) can be categorized into: (i) the 3' non-coding regulatory region, (ii) the 5' non-coding regulatory region, and (iii) the polyprotein coding region (subdivided into L, P1, P2, and P3) (Longjam *et al.,* 2011). Viral proteases cleave the viral genome, which is a single polypeptide, into the four structural capsid proteins namely; VP4 (1A), VP2 (1B), VP3 (1C) and VP1 (1D) and the non-

structural proteins include L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D (Ryan *et al.,* 1989). The P1 encodes the four structural surface proteins (Mateu *et al.,* 1995; Usherwood *et al.,* 1995), which plays a role in antigenicity as well as binding of cellular receptors (Jackson *et al.,* 2003). The L, P2 and P3 are precursors of non-structural proteins (Luke *et al.,* 2008; Kristensen and Belsham, 2019) and these non-structural proteins function in cellular regulation, virus replication as well as in FMDV-host interaction (Kristensen and Belsham, 2019).

1.9. VIRUS REPLICATION AND TRANSLATION

Viruses attach to particular cell surface receptors before entering host cells (Jackson *et al.,* 2022). The heparan sulfate proteoglycan (HSPG) (Biswal *et al.,* 2015), integrins (Xin *et al.,* 2018) and an additional unidentified receptor (Bai *et al.,* 2019) have all been reported as FMDV receptors. The FMDV binds to these receptors on the surface of host cells and enters by receptor-mediated endocytosis (Figure 1.8; Rothenburg and Brennan, 2020; Li *et al.,* 2021). The entire replication process occurs in the host cell cytoplasm (Rodriguez and Saiz, 2017). Firstly, the low pH of the endosome induces virus uncoating and transfer of the viral RNA, via the endosomal membrane to the cytoplasm (Jang *et al.,* 1988; O'Donnell *et al.,* 2005). The viral RNA initiates the infection cycle without the need of any viral proteins (Belsham and Martinez-Salas, 2004). Furthermore, this viral RNA carries all the information necessary to invade the cellular machinery and cause host molecular synthesis shut down in infected cells, where translation of viral products occurs by a cap-independent manner. RNA replication is carried out by the RNA-dependent RNA polymerase (3Dpol) and primed by VPg (3B), which transcribes the positive RNA strand into a complementary negative RNA strand molecule (Rodriguez and Saiz, 2017). The infectiousness of the viral RNA demonstrates that translation must be the first phase of the infection cycle, in order to produce the viral proteins within the cell (Belsham and Martinez-Salas, 2004). Thereafter, several positive RNA strands, produced by the 3Dpol, either undergoes a new translation cycle and RNA replication or are packed into capsid

proteins for the formation of a new virus particle, which are then released by cell lysis (Belsham and Martinez-Salas, 2004; Rodriguez and Saiz, 2017).

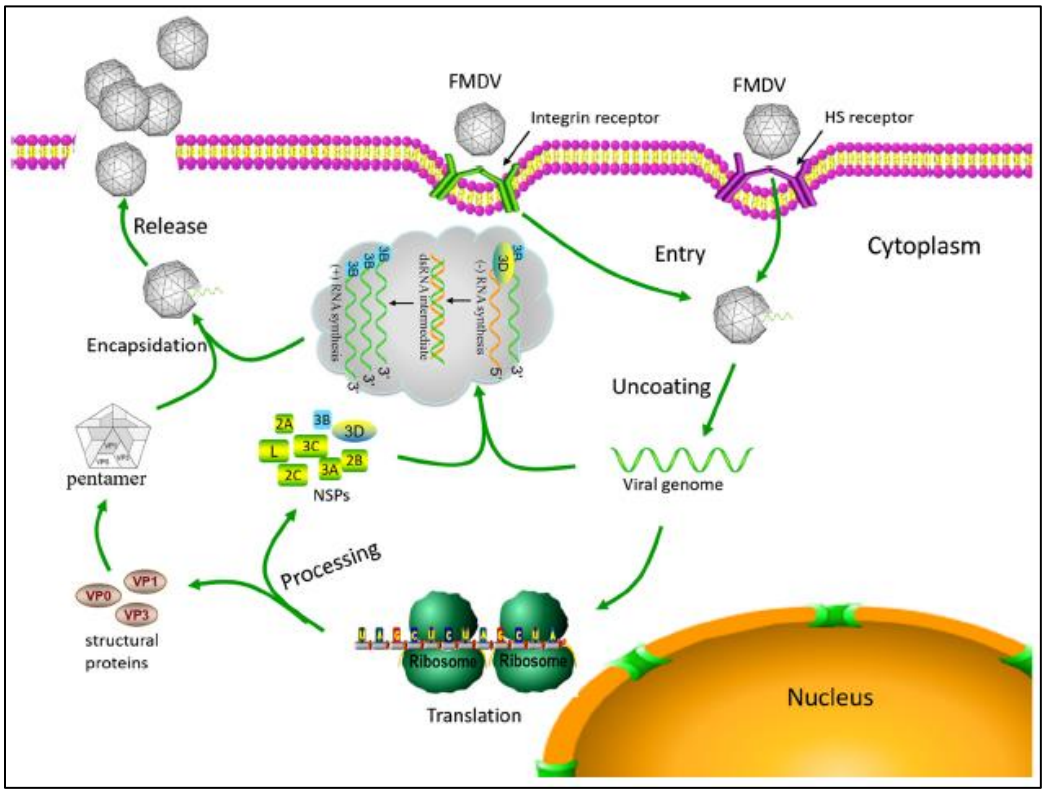


Figure 1.8: FMDV replication and translation in host cells. After FMDV RNA entry into the cytoplasm, RNA must be translated to produce the viral proteins needed for viral RNA replication. NSPs are non-structural proteins and HS is heparan sulfate. The orange line is the viral negative strand (-) and the green line is the viral (+) positive strand. Taken from Gao *et al.,* (2016).

Translation is initiated at two AUG codons (84 nt apart) succeeding the internal ribosomal entry site (IRES) (Ryan *et al.,* 1989; Longjam *et al.,* 2011). Due to the lack of the 5'cap in picornaviruses, the IRES act as a ribosomal entering site by binding to the ribosome to initiate protein synthesis (Jang *et al.,* 1988). A highly structured region is found on the IRES and is located more than a hundred nucleotides away from the genomic RNA's uncapped 5' end (Belsham and Martinez-Salas, 2004). Additionally, because of this 5' cap absence, all other known cellular translation factors for IRES translation initiation are required except for the cap-binding protein, eIF4E (Belsham, 2005; Sonenberg and Hinnebusch, 2009). The initiation factor eIF4F, recognizes a cap structure (m7GpppN.)

found in all cytoplasmic eukaryotic mRNAs, which plays a role in translation initiation (Gingras *et al.,* 1999; Hershey and Merrick, 2000). This trimeric complex (eIF4F) consists of an RNA helicase (eIF4A), a scaffold protein (eIF4G) and a cap-structure binding protein (eIF4E) (Dever, 1999; Rogers *et al.,* 2002). The latter has different binding sites, such that other proteins like poly(A) binding proteins (PABP), eIF3 (the small ribosomal subunit) and an eIF4E kinase (Mnk-1) can bind (Belsham and Martinez-Salas, 2004). The viral protein L^{Pro} cleaves the eIF4G, resulting in the removal of its N-terminus eIF4E binding site and weakening the host cell's cap-dependent protein synthesis (Medina *et al.,* 1993; Sonenberg and Hinnebusch, 2009). However, the binding site for eIF4A and eIF3 (multimeric factor) are retained on the C-terminus of eIF4G, which promotes the IRES activity (Yu *et al.,* 2011a; Martinez-Salas *et al.,* 2015). As the complex bound to the 40S ribosomal subunit is delivered to the mRNA with these factors, protein synthesis results (Sonenberg and Hinnebusch, 2009; Gao *et al.,* 2016).

1.10. THE CELLULAR RECEPTORS OF FMDV

Two cellular receptor families have been linked to the mediation of FMDV infection *i.e.,* integrins and HSPG (Jackson *et al.,* 1996; Neff *et al.,* 1998). While integrins binding to FMDV serotype A, O and C uses clathrin-dependent mechanism to infect host cells, through endocytosis (Berryman *et al.,* 2005; O'Donnell *et al.,* 2005; Martin-Acebes, 2007), HSPG uses an HS proteoglycan protein moiety dependent route via caveola-mediated endocytosis (Belting, 2003; O'Donnell *et al.,* 2008),

Integrins are proteins that serve as integral membrane receptors and play a role in cell adhesion, signal transduction, apoptosis and cell growth and development. They form part of a family of heterodimeric transmembrane glycoproteins, containing alpha (α) and beta (β) subunits bonded by non-covalent interactions. Integrin subunits are type I transmembrane proteins with a large extracellular domain [>940 (α) and >640 (β) residues], a small transmembrane domain and a cytoplasmic domain (González-Amaro and Sánchez-Madrid, 1999; Springer, 2002). There are at least 18 distinct α and β

subunits that have been identified, which associate to form 24 different αβ heterodimers (Hynes, 1999). Only eight integrins (αvβ1, α5β1, αvβ3, αvβ8, αvβ5, αvβ6, α8β1 and αIIbβ3) have been identified, to recognize the RGD sequence (Ruoslahti, 1996). From the eight integrins, at least four integrins can be used as FMDV receptors to mediate FMDV infection viz. αvβ3, αvβ1, αvβ6 and αvβ8 (Berinstein *et al.,* 1995; Jackson *et al.,* 2000; 2002; 2004).

Heparan sulfate (HS) is a highly sulfated glycosaminoglycan (GAG), composed of long unbranched polysaccharides with repeating disaccharide (*i.e.,* hexosamine, hexuronic acid, or galactose) units and negatively charged (Bernfield *et al.,* 1999). GAGs are carbohydrate components of a glycoprotein family known as proteoglycans. Proteoglycans are present on nearly all cell types in animal tissues, both as extracellular matrix components and as integral membrane components (Kjellen and Lindah, 1991; Jackson *et al.,* 1996). Heparan sulfate is the second molecule to act as a FMDV cellular receptor and have shown to inhibit FMDV infection in cultured cells (Jackson *et al.,* 1996) when FMDV was unable to infect HS-deficient cells (confirming that HS on the cell membrane is a FMDV receptor). Initially, HS was thought to be a co-receptor for FMDV serotype O used to enter host cells (Jackson *et al.,* 1996). Later, research revealed that other FMDV serotypes (Asia-1, A, C and SAT1) could bind to HS as well (Baranowski *et al.,* 2000). Heparan binding has no effect on the integrin recognition site and these two receptor binding sites appear to be independent of one another (Baranowski *et al.,* 2000). The functional relation between integrin and HS receptor is still lacking, due to different FMDV strains using different receptors (Baranowski *et al.,* 1998).

In addition to the integrin and HS, a third group of receptors for FMDV has been proposed but not yet defined. FMDV investigations have led to the discovery of FMDV infecting macrophages through Fc receptor-mediated adsorption (Baxt and Mason, 1995). The RGD mutations to RSD, REG, RGG, SGD, GGG or RDD in VP1 retains the infectivity of the virus (Rieder *et al.,* 2005; Li *et al.,* 2011; Wang *et al.,* 2015a). However, it is yet uncertain which receptors these mutants employ. Thus, it is generally believed that this third group of receptors is involved in adsorption and internalization of FMDV in distinct

host cells (Lawrence *et al.,* 2013). To find new receptors in these groups, the use of an immunoprecipitation assay and mass spectrometry can be a useful tool. Comparing structural proteins variation between viruses with different pathogenicity may also reveal some information about FMDV receptors (Li *et al.,* 2021).

1.11. FMDV VACCINES

In the 1950s and 1960s, after Sabin created an attenuated poliomyelitis vaccine, extensive research was conducted along the same lines for FMD. The basic procedure included passaging the FMD virus using *in vitro* cell culture systems in order to select a viral population that elicited an immune response without causing disease (Barteling, 2002). The production of attenuated (also known as 'modified-live') viral vaccines in cell cultures is extremely straightforward and inexpensive, since only a minimal amount of infectivity is required per dose of vaccine. However, challenges were encountered in achieving viral attenuation for all animal species (*i.e.,* breeds, species, ages and sexes) and reversion to virulence was another challenging issue (Olascoaga *et al.,* 1999, Barteling, 2002). Therefore, it was impossible to guarantee safe attenuation of the FMD virus for all types of animals that required vaccination. Due to these issues, the alternative was to employ chemically inactivated (*i.e.,* 'killed') viruses as the primary immunogen in FMD vaccines (Waldmann *et al.,* 1937; Barteling and Vreeswijk, 1991).

1.11.1. INACTIVATED VACCINES

The majority of commercially available FMD vaccines are inactivated vaccines produced by treatment with binary ethyleneimine (BEI), which inactivates the virus particle (Aarthi *et al.,* 2004; Kamel *et al.,* 2019). To enable the differentiation of infected from vaccinated animals (DIVA) through diagnostic testing, additional antigen purification steps are necessary in FMD vaccine production. These processes aim to eliminate non-structural proteins (NSPs) and other cellular contaminants from the vaccine formulation (Uddowla *et al.,* 2012; Park *et al.,* 2020). Thus, vaccinated animals will develop antibodies against

the viral structural proteins that are exposed on the virion surface, preventing further infection (Barteling, 2002; Parida, 2009). Furthermore, these vaccines are prepared in a suspension of baby kidney-21 (BHK-21) cells and can be either monovalent, bivalent or multivalent, however multivalent vaccines are common (Parida, 2009; Kamel *et al.,* 2019; Mignaqui *et al.,* 2019; Peta *et al.,* 2021). The vaccines have proven to be effective by reducing FMD in endemic regions and have been crucial to the success of the FMD control strategies in South America and Europe (Brown, 2003). Commonly, vaccines contain one or more virus strains as vaccination induces an immune response that is strain specific (Martinez *et al.,* 1988; Doel, 2003), thus, it is frequently necessary to combine different FMDV circulating strains to ensure protection. However, the cost of the vaccines increases with the number of virus strains included, limiting its use in many developing countries (Kitching *et al.,* 2007). The selection of FMD vaccines that provides cross-protective immunity in Africa is a challenge, due to the diversity of occurring FMD serotypes and their subtypes (Rweyemamu *et al.,* 2008b; Maree *et al.,* 2014).

FMD control strategies vary per country, based on the FMD status and resources available (Thomson *et al.,* 1994). Vaccination of livestock is an essential component of FMD control globally, with billions of vaccine doses utilized in disease control or eradication operations each year (Keeling *et al.,* 2003; Brito *et al.,* 2016). Effective vaccinations were used in the 1980s, to successfully eliminate FMD from Europe (Iyer *et al.,* 2001). However, eradication by vaccination is unfeasible in most African nations due to the prevalence of the African buffalo in game reserves, which act as the SAT serotypes maintenance hosts (Barsa *et al.,* 2008). In South Africa, cattle herds bordering the KNP game reserve are presently vaccinated up to four times yearly using vaccines, made from cell culture-derived chemically inactivated SAT 1, 2, and 3 antigens, adjuvanted with aluminum hydroxide and saponin (FMD Bulletin, 2010). However, even after increased vaccination frequency, it was clear that these vaccines are inadequately effective, as evidenced by recurring and persistent epidemics in vaccinated animals (Vosloo *et al.,* 2017; Peta *et al.,* 2021). The antigenic relatedness of vaccine strains to circulating field strains, vaccine potency and viral structural integrity (*i.e.,* the 146 S particle) can all influence the vaccines potential to elicit a protective immunity (Barteling, 2002). The

potency of a vaccine is a measurement of the number of protective doses (PD) in a vaccine based on the resistance to infectious virus challenge of animal groups vaccinated with varying vaccine doses (WOAH, 2018a). FMD vaccinations are categorised as "standard" or "higher" potency (WOAH, 2018b), with at least 3PD50 and >6PD50 per dose respectively (Stear, 2005; Parida, 2009; Elnekave *et al.,* 2016). A 50% protective dose (PD50) protects 50% of immunized animals from clinical FMD (WOAH, 2018a). As a result, conventional potency vaccines are appropriate for preventative vaccinations, since they are produced with antigen concentrations that result in a minimum potency level of 3PD50 (Peta *et al.,* 2021).

Modern FMD vaccinations, together with other zoo-sanitary measures, can be utilized to both eradicate endemic FMD and prevent FMD outbreaks in typically FMD-free regions (Barteling and Sutmoller, 2002; Leforban, 2002). However, the current inactivated vaccines have a short shelf life, require an adequate cold chain, high-containment facilities are needed for vaccine manufacture and certain serotypes and subtypes have proven difficult to adapt to cell cultures for vaccine production (Rodriguez and Grubman, 2009; Kamel *et al.,* 2019). Due to the short-lived immunity, naive animals receive two vaccine doses (3-4 weeks apart), followed by a re-vaccination every 4-6 months (Hunter, 1998; Cloete *et al.,* 2008). To overcome these challenges, new vaccines that provide a wide and long-lasting immune response are critical and urgently required, as vaccination against one serotype does not provide protection against another (Grubman and Baxt, 2004; Zhang *et al.,* 2011; Kamel *et al.,* 2019).

1.11.2. RECOMBINANT AND PEPTIDE VACCINES

Almost all vaccines under development are based on purified recombinant proteins or pathogen subunits (Perrie *et al.,* 2008). Certain genes of causative agents have been cloned, produced and purified for use in vaccines (Hansson *et al.,* 2000; Clark and Cassidy-Hanley, 2005). The use of recombinant protein vaccines allows the avoidance of numerous possible concerns associated with purified macromolecules-based vaccines,

i.e., the reversion to their toxigenic form (Sette and Rappuoli, 2010; Nascimento and Leite, 2012). The expression systems utilised can be bacteria, mammalian, yeast or insect cells. However, due to the high degree of expression, bacterial systems are mostly used (Hansson *et al.,* 2000; Clark and Cassidy-Hanley, 2005).

In the 1970s, researchers discovered that the capsid protein, VP1, had a prominent surface exposure (Laporte, 1969; Rowlands *et al.,* 1971). Based on this discovery, a variety of methods were used to create peptide vaccines as alternatives to the current inactivated vaccines. Bachrach *et al.,* (1975) initially isolated VP1 from purified viruses, which induced a neutralizing antibody response in pigs. The use of recombinant DNA technology demonstrates that VP1 produced in *E. coli* protected both cattle and pigs challenged with FMDV (Kleid *et al.,* 1981). Other studies demonstrated that parts of the VP1 might elicit a neutralizing antibody response and variations in these protein regions were observed in different FMDV strains (Acharya *et al.,* 1989). These protein regions represented the variable βG-βH loop located on the surface of the FMDV capsid (Acharya *et al.,* 1989) and the VP1 carboxy-terminal region. They also corresponded to B-cell epitopes (Bittle *et al.,* 1982; Rodriguez and Grubman, 2009). According to Giavedoni *et al.,* (1991) TrpE fusion proteins comprising parts of the C-terminal region of VP1 induced a neutralizing antibody response and test animals were protected against challenge. Later, Zhijun *et al.,* (2000) and Zhuang *et al.,* (2005) independently tested the immunological impact of fusion proteins containing T and B cell epitopes. The findings suggested that fusion proteins might elicit both humoral and cellular immune responses. In a thorough analysis of peptide vaccinations in cattle, Taboga *et al.,* (1997) discovered that they provided, at most, 40% protection and that virus escape mutants had amino acid alterations at the major antigenic sites represented in the peptide vaccines. The results from this study showed that peptide vaccines containing only a few number of antigenic sites or epitopes of the virus are unable to elicit significant protection. However, a recombinant FMD vaccine that expressed the fusion protein gal-FMDV of β-galactosidase and a portion of the VP1 protein, with amino acids (residue number 21-40 and 141-160), provided protection against infection in guinea pigs and swine (Zheng *et al.,* 1994, Huang *et al.,* 1999). Moreover, a study of Yi *et al.,* (2004) developed two bivalent vaccines by

fusing an immunogenic peptide comprised of the B- and T-cell epitopes of the VP1 gene of FMDV serotypes O and A, respectively. Their results demonstrated that both recombinant proteins induced a strong immune response in the vaccinated guinea pigs.

1.11.3. FMDV ATTENUATED VACCINES

Biotechnology has been used to develop attenuated vaccines that are designed to disrupt a region or a few oligonucleotides of the virus, but not continually cultured in a susceptible non-native host as the conventional attenuated vaccines (Rieder *et al.,* 1994; Zhang *et al.,* 2011). Previously, efforts to develop live attenuated FMD vaccines had minimal success due to the vaccine viruses exhibiting unstable phenotypes or differences in pathogenesis between species *i.e.,* attenuation in cattle but not in pig. Moreover, the reversion to virulence by mutation was a concern (Martin and Edwards, 1965; Mowat *et al.,* 1969). However, advancements in attenuated vaccines have addressed these challenges by ensuring stability, minimal toxicity and a reduced risk of reversion to virulence. The introduction of mutations or deletions at specific regions of the viral genome responsible for encoding virulence factors, sets these vaccines apart from previously developed attenuated vaccines (Uddowla *et al.,* 2012; de Los Santos *et al.,* 2018; Diaz-San *et al.,* 2021). A major concern in current vaccine production is the need to produce significant amounts of wild-type virus, during manufacturing. One safer alternative is to substitute the wild-type virus with attenuated derivatives that can grow in cell culture without causing disease or spreading if exposed to the natural host (de Los Santos *et al.,* 2018). In 1996, a live attenuated vaccine derived from a FMDV serotype A12 lacking the L^{pro}-coding region (*i.e.,* A12-LLV2) was developed (Brown *et al.,* 1996), which generated an effective protective immune response in cattle (Mason *et al.,* 1997; Chinsangaram *et al.,* 1998). Taking advantage of this knowledge, researchers used a leaderless infectious clone to create an improved inactivated vaccines platform (Uddowla *et al.,* 2012; de Los Santos *et al.,* 2018). This innovative infectious clone vaccine incorporates nuclease restriction sites, enabling convenient swapping of the capsid and enabling the swift development of vaccines against emerging FMDV strains (de Los

Santos *et al.,* 2018). These research studies show the effectiveness of a rationally designed attenuated FMDV vaccine (Zhang *et al.,* 2011).

1.11.4. EMPTY CAPSID VACCINES

The production of immunogens that incorporate the whole repertoire of immunogenic sites found on the intact virus particle, but excluding the genetic material, is an alternative approach to peptide or protein vaccines. These are empty viral capsids, which are normally synthesized in infected cells and are as immunogenic as virions (Rowlands *et al.,* 1975; Rweyemamu *et al.,* 1979; Rodriguez and Grubman, 2009). The immunogenicity and stability of FMDV empty particles was initially confirmed by Rweyemamu *et al.,* (1979). Mayr *et al.,* (2001) developed a replication-defective adenovirus 5 that encodes the FMDV capsid and 3C proteinase coding areas (*i.e.,* Ad5-FMDV3CWT), which can specify the production of empty capsids. Pigs that received the first vaccine with Ad5-FMDV3CWT, followed by a booster vaccine at four weeks, developed significant levels of FMDV-neutralizing antibodies (Mayr *et al.,* 2001). Furthermore, full protection against FMD was provided for five of the six pigs, while the disease was limited on the sixth pig. In a similar study, a single dose of the replication-defective human adenovirus type 5 vector expressing capsid proteins of FMDV strain A24, provided protection in challenged pigs and cattle as early as seven days post vaccination (Moraes *et al.,* 2002). Thus, these recombinant FMDV empty capsids have shown to be useful in developing novel FMD vaccines.

1.11.5. NUCLEIC ACID VACCINES

Nucleic acid vaccines have been used for a variety of bacterial, viral and parasite disease models (Khan, 2013), including cancer models. These vaccines use genetically modified DNA to elicit an immune response in inoculated animals. In comparison to traditional vaccines, nucleic acid vaccines have several benefits, such as long-term immunity, a long shelf life (Hu *et al.,* 2007) and induces a broader spectrum of immune response (Zhang

et al., 2011). Plasmids have been employed as ideal models to produce DNA vaccines. Plasmid constructs expressing FMDV structural and non-structural proteins induced immunological responses in mice and pigs, as well as protecting pigs against FMD (Wong *et al.,* 2000; Benvenisti *et al.,* 2001). The effect of cytokines is one of the main focuses on recent studies investigating DNA vaccines. Several cytokines such as interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) have been investigated as DNA vaccine adjuvants. Su *et al.,* (2008) used a eukaryotic construct expressing either IL-6 or TNF-α as adjuvants to co-vaccinate with a FMDV VP1 DNA vaccine. Both humoral and cellular responses were greatly improved in mice, following the administration of either adjuvant with the FMDV DNA vaccine (Su *et al.,* 2008). Thus, the cytokines IL-6 and TNF- α may act as an effective adjuvant in improving the adaptive immune response to FMDV DNA vaccinations (Su *et al.,* 2008).

Even though the majority of the novel vaccines are still under investigation and are very limited, they might be the solution to the current inactivated vaccines shortcomings and possibly aid in FMD eradication.

1.12. ANTIGENIC SITES

The knowledge of amino acid residues that comprises the antigenic determinants (epitopes) of FMDV would considerably increase our understanding of virus neutralization in *in vivo* studies (Dunn *et al.,* 1998). These known regions can be incorporated using reverse genetics technology to produce recombinant vaccines, thus producing improved immunogenic vaccines. The high antigenicity presented by FMDV serotypes has posed a challenge on FMD control by vaccination. Several studies have been performed to identify neutralizing epitopes, since the antigenic site located on the βG-βH loop of VP1 was believed to be the only FMDV antigenic site (Bachrach., 1975; Strohmaier *et al.,* 1982) due to abrogation of immunogenicity and FMDV cell attachment by trypsin cleavage of VP1 (Wild *et al.,* 1969). Nonetheless, the use of monoclonal antibodies (MAbs) and sequencing of MAb neutralization-resistant (MAR) mutants have played a

vital role in identifying amino acid residues, encoding the antigenic sites. Several antigenic sites for serotypes A, C, O and Asia-1 have been identified (Grazioli *et al.,* 2013), however, there is limited knowledge of the SAT serotypes antigenic sites (Maree *et al.,* 2011).

1.12.1. SEROTYPE O

Five antigenic regions have been identified for FMDV serotype O. A conformationally dependent and trypsin-sensitive site, site 1 is located on the βG-βH loop of VP1 and involves amino acid residues 144, 148, 154 and 208 (Mahapatra *et al.,* 2012; Opperman, 2013). Site 2-5 are all conformationally dependent and trypsin resistant (Mahapatra *et al.,* 2012). The major antigenic site of serotype O, site 2, is located on VP2, involving residues 70-73, 75, 77 and 131 (Mahapatra *et al.,* 2012). Site 3 (located on βG-βH loop of VP1) involves residue 43-44 and 48, while the β-B "knob" of VP3 (residues 56 and 58-59) constitutes site 4 (McCullough *et al.,* 1987; Barnett *et al.,* 1998). Site 5 is located on VP1, involving amino acid 149 (McCullough *et al.,* 1987; Barnett *et al.,* 1998; Aktas and Samuel, 2000; Opperman *et al.,* 2013).

1.12.2. SEROTYPE A

For FMDV serotype A, five antigenic sites have been identified (Thomas *et al.,* 1988a; Baxt *et al.,* 1989; Maree *et al.,* 2011; Mahapatra *et al.,* 2011) and are located on the surface of the capsid (Fry *et al.,* 2005b; Mahapatra *et al.,* 2011). Three antigenic sites located on VP1, VP2 and VP3 were revealed by MAR mutants of A_{10}, A_{12}, A_{22} and A_{24} (Thomas *et al.,*1988b; Baxt *et al.,* 1989). Site 1 is located on the βG-βH loop of VP1, includes amino acid residues 142-157 and has been identified for A_{10}, A_{12}, A_{22} and A_{24} (Thomas *et al.,* 1988b; Baxt *et al.,* 1989; Mahapatra *et al.,* 2011). Site 2 consists of amino acid residues 200-212 of VP1, as identified for FMDV A_{10} and A_{12} (Thomas *et al.,* 1988b; Baxt *et al.,* 1989). Site 3 involves amino acid residues 82-88 in the βB-βC loop of VP2, amino acid 58-61 in βB-βC loop of VP3, amino acid 136-139 (βE-βF loop of the VP3) and

amino acid 195 (βH-βI loop of the VP3) for A_{10} (Thomas *et al.,* 1988b). Both amino acid residues 169 and 175-179 (βH-βI loop of the VP1) constitutes site 4 of A_{10} (Thomas *et al.,* 1988b). The βB-βC loop of VP3 with amino acid residues 69-70, forms site 5. This site was analogous to site 5 of serotype O and was identified for A_{24} (Mahapatra *et al.,* 2011).

1.12.3. ASIA-1

FMDV serotype Asia-1 is similar to the A and O viruses and the antigenic sites have also been identified using MAR mutants (Sanyal *et al.,* 1997; Sanyal *et al.,* 2003). Recently, Grazioli *et al.,* (2013) identified four distinct antigenic sites using 24 FMDV isolates of Asia-1; three of those identified sites showed close similarity to the sites identified for serotypes A and O. Site 1, located on βG-βH loop of VP1 involves amino acid 140-142 and proceeds the RGD motif. This site is related to site 1 of serotype O and A, as well as site 1 of serotype C (Bolwell *et al.,* 1989; Mateu *et al.,* 1990; Mahapatra *et al.,* 2011). The VP1 C-terminus of Asia-1 has not been demonstrated to be antigenically significant, compared to serotype A, C and O viruses (Grazioli *et al.,* 2013). Site 2 involves amino acid residues 67-79 in the βB-βC loop of VP2 (Grazioli *et al.,* 2013) and is analogous to site 3 of serotype A (Thomas *et al.,* 1988a), site 2 of serotype O (Kitson *et al.,* 1990) and site D2 of serotype C (Lea *et al.,* 1994). Asia-1 antigenic site 4 (the third antigenic site) is situated within the β-B “knob” of VP3, with amino acid residue 58/59 (Grazioli *et al.,* 2013) and corresponds to site 3 of serotype A, site D3 of serotype C and site 4 of serotype O (Thomas *et al.,* 1988a; Kitson *et al.,* 1990; Lea *et al.,* 1994). A unique site for Asia-1, site 5 (the fourth antigenic site) is situated on the C-terminus (amino acid residue 218) of VP3 and is the first time such a FMDV site was described (Grazioli *et al.,* 2013).

1.12.4. SEROTYPE C

In contrast to the antigenic sites of serotype A, Asia-1 and O, the antigenic sites in serotype C are alphabetically labelled. Site A, situated within the βG-βH loop of VP1 involves amino acid residues 138-150 (Mateu *et al.,* 1989; Mateu *et al.,* 1990), while site

C comprises amino acid residues 192-209, forming the C-terminus of VP1 (Mateu *et al.,* 1990) Both sites A and C display topological independency (Mateu *et al.,* 1990). Site D is the dominant discontinuous site, comprising of loops in VP1 (subsite D1), VP2 (subsite D2) and VP3 (subsite D3) (Lea *et al.,* 1994). Critical residues at subsites D1, D2 and D3 have been identified involving amino acid residues 193 of VP1 (near C-terminus), amino acid residues 72, 74 and 79 and 58, respectively (Lea *et al.,* 1994).

1.12.5. SAT TYPES

In contrast to the abundance of knowledge on antigenic sites of FMDV serotypes A, O, C and Asia-1, there is limited information regarding the SAT serotypes antigenic sites. Only a few number of studies have been performed to identify antigenic sites by producing MAbs against SAT1 and SAT2 (Opperman, 2013). Five antigenic sites [1(a, b), 6, 7 and 8] have been identified thus far for SAT1. Site 1 is situated on the βG-βH loop of VP1 involving amino acid residues 146 and 148 (site 1b), upstream of the RGD motif and amino acid residues 154, 156-157 (site 1a), downstream of the RGD motif (Grazioli *et al.,* 2006; Opperman, 2013). These sites correspond to site 1 of serotypes A and O, as well as site A of serotype C. Site 6 involves a residue (amino acid 135 of VP3) not present on other FMDV antigenic sites, while site 7 comprises of two amino acid residues from two distinct proteins *i.e.,* amino acid residues 72 of VP2 and 181 of VP1 (Grazioli *et al.,* 2006; Opperman, 2013). It was noted that residue 72 of VP2 was analogous to the previously described site 2 of the Euro-Asian FMDV serotypes (Grazioli *et al.,* 2006). Site 8 involves the amino acid residue at position 111 of VP1 (Grazioli *et al.,* 2006).

Regarding SAT 2 serotype viruses, at least two antigenic sites have been identified within the VP1 region. Conformationally dependent amino acid residues 147-149, 154, 156 or 158, all make up antigenic site 1 and are located on the βG-βH loop of VP1 (Crowther *et al.,* 1993; Grazioli *et al.,* 2006). Site 2 is situated in the C terminus, positioned at amino acid residue 210 of VP1 (Grazioli *et al.,* 2006). Recently, Maake *et al.,* (2020) identified at least four locations as potential FMDV SAT 3 antigenic sites. This study indicated that

antigenic variation in VP1 was discovered at amino acid residues 83 and 169. Additionally, amino acid residue 169 is located near the C terminus of the VP1 βG-βH loop, whereas residue 83 forms an exposed cluster around the 5-fold axis (Maake *et al.,* 2020). Similarly, antigenic effects were related to amino acid residues 168 in VP3 and 134 in VP2, both of which were hypothesized to operate as conformational epitopes (Maake *et al.,* 2020). The VP3 amino acid residue 168 has not previously been characterized as a FMDV antigenic site, but amino acid residue 134 of VP2 corresponds to SAT2 and serotype O antigenic sites (Crowther *et al.,* 1993; Grazioli *et al.,* 2013; Maake *et al.,* 2020).

1.13. THE IMMUNE RESPONSE TO FMDV

The immune system is divided into two subsystems *i.e.,* the innate and adaptive immune systems. The innate immune system is the first line of defence against infection and has no memory of prior responses. This system involves physical barriers such as the skin, humoral components (soluble proteins) and cellular processes such as phagocytosis (Riera *et al.,* 2016). If a pathogen persists, despite the defence offered by innate immunity, then an adaptive immunity is recruited. An adaptive immune response, specifically to a particular antigen, provides long lasting immunity and creates memory for future response (Alberts *et al.,* 2016; Smith *et al.,* 2019). For an effective immune response to FMDV, both these systems are necessary.

1.13.1. THE INNATE IMMUNE SYSTEM

The innate immune system is primarily composed of macrophages, monocytes and dendritic cells. Monocytes and macrophages have the capacity to phagocytose and eliminate viral pathogens. It has been noted that FMDV-infected macrophages contribute to acute infection, serving as infectious carriers and spreading the virus to other body regions (Rigden *et al.,* 2002; Tewari and Jain, 2019). Macrophage activity plays a crucial

role in immune defence against FMDV (McCullough *et al.,* 1986; 1988; 1992). After antibody-mediated FMDV opsonization, macrophages can rapidly phagocytose the antibody-FMDV complex, using the Fc receptors (McCullough *et al.,* 1988), relating to efficient protection *in vivo* (McCullough *et al.,* 1986; 1988; 1992).

Dendritic cells (DCs), the most effective antigen-presenting cells (APC), are crucial in bridging innate and adaptive immunity (Weigel *et al.,* 2002; Rossi and Young, 2005). In *in vitro* studies, plasmocytoid DCs have been shown to be susceptible to FMDV infection (Guzylack-Piriou *et al.,* 2006). Although it has been demonstrated that the FMDV and APC interactions are abortive and no virions are generated (Rigden *et al.,* 2002; Diaz-San *et al.,* 2009), functional consequences influence the host response. In acute infection, FMDV induces DCs to produce interleukin (IL)-10, resulting in an adaptive immunity that favours a strong humoral response rather than a T-cell mediated response (Diaz-San *et al.,* 2009). The toll-like receptors (TLRs) allow DCs to directly detect and recognize pathogens, resulting in an increased expression of co-stimulatory molecules, chemokines, antigen presentation and pro-inflammatory cytokines (Fujii *et al.,* 2004; Iwasaki and Medzhitov, 2004; Sporri *et al.,* 2005). Through these processes, DCs can mature and have the capacity to induce an adaptive immune response (Munz *et al.,* 2005). Research of FMDV and DCs has been limited to porcine and murine DC. The first publication on DC infection by the virus was in 1995, which described FMDV's cytopathic effects following infection of porcine skin DCs (Gregg *et al.,* 1995). However, Bautista *et al.,* (2005) showed that these skin DCs are resistant to FMDV infection, due to constitutive production of interferon (IFN) type I in response to FMDV infection. Furthermore, IFN type III also demonstrated to be a critical component of innate immunity, functioning in limiting infection and was present in cattle plasmocytoid DCs (Reid and Charleston, 2014). Several subsets of porcine DC, *i.e.,* plasmacytoid DC (pDC), bone marrow-derived and monocyte-derived DC, were demonstrated to be susceptible to FMDV infection (Guzylack-Piriou *et al.,* 2006; Harwood *et al.,* 2008). Moreover, structural and non-structural viral proteins were detected as well as double stranded (ds) RNA up to 24 hours after infection (Harwood *et al.,* 2008).

Natural killer (NK) cells also play a vital role in the early stages of host response (Diaz-San *et al.,* 2006), through their cytokines production and cytolytic activity (Lanier *et al.,* 2008; Vivier *et al.,* 2008; Cook *et al.,* 2014). By binding to the Fc receptor of antibodies, NK cells kill virus-infected cells (target cells) as they release perforin and granzyme through antibody-dependent cellular cytotoxicity mechanism(Wang *et al.,* 2015b). The NK cells that were derived from FMDV re-stimulated bovine peripheral blood mononuclear cells (PBMCs) of vaccinated cattle and have demonstrated to be cytotoxic against FMDV-infected target cells (Amadori *et al.,* 1992). NK cells produce high amounts of IFN-γ during the initial phase of viral infection, which induces antiviral effects (Toka *et al.,* 2009; Li *et al.,* 2021). However, FMDV *in vivo* studies have shown that NK cells from infected swine have a decreased ability to lyse FMDV-infected cells and decreased IFN-γ secretion. Conversely, *in vitro* studies of porcine NK cells, utilizing pro-inflammatory cytokines induced lysis of target cell and an increased production of IFN-γ (Toka *et al.,* 2009). During the viremic phase of acute infection, the dysfunction of NK cells shows that their function can be blocked by FMDV, resulting in host immune system evasion and promoting FMDV replication and dissemination in host cell (Stenfeldt *et al.,* 2016b).

1.13.2. THE ADAPTIVE IMMUNE SYSTEM

Both the humoral and cell-mediated immunity forms part of the adaptive immune system. It involves the production of antibodies, cytokines and cytotoxic T-lymphocytes as well as the activation and proliferation of antigen- specific B- and T-cells (Tewari and Jain, 2019).

1.13.2.1. HUMORAL IMMUNE RESPONSES

In both infected and vaccinated animals, FMDV induces a rapid humoral response. Animals are protected against infection or re-infection by virus-specific antibodies, depending on virus serotypes (McCullough *et al.,* 1992; Salt, 1993). This protection is usually associated with high levels of neutralizing antibodies (McCullough *et al.,* 1992; Salt, 1993). Immunoglobulin (Ig) M is the first neutralizing antibody to appear, after 3 to 4

days of challenge, peaks 10–14 days later and declines thereafter (Sobrino *et al.,* 2001; Golde *et al.,* 2008). After 4 to 7 days of infection/vaccination, IgG is detected and becomes the predominant neutralizing antibody after 2 weeks (Collen *et al.,* 1989; Salt, 1996). According to Salt, 1993, isotype IgG1 was detected to have a higher titre than IgG2. The three main antibody subclasses found in the upper respiratory tract are IgM, followed by IgA and IgG (Salt, 1993). Furthermore, IgM and IgA neutralizing activity is detected in the pharyngeal fluid, 7 days post infection (Francis *et al.,* 1983). However, it was reported that the presence of IgA in the early phase of infection could be caused by tissue fluid and serum leakage, whereas its presence in the pharyngeal fluid (20 days post infection) is thought to be produced at mucosal surface (Tewari and Jain, 2019). In both mice (Borca *et al.,* 1986) and cattle (Borca *et al.,* 1986; Juleff *et al.,* 2009), antibody generation following FMDV infection has been shown to be T cell independent. The proliferation of FMDV-specific CD4 T-cells following infection or vaccination has showed that T cells are involved in the development of antibody responses in ruminants (Blanco *et al.,* 2001; Gerner *et al.,* 2007), which may induce a protracted immunological response in infected animals (Parida, 2009).

1.13.2.2. CELLULAR IMMUNE RESPONSES

T cells and B cells play a key role in the beginning of the adaptive immune response, which possesses the traits of immune memory, diversity, self-recognition, specificity and non-self-recognition (Subramaniam *et al.,* 2010). However, there is still some controversies regarding the role of cellular immunity in the defence of animals against FMD. Following infection or vaccination, specific T-cell antiviral responses involving CD4+ and CD8+ cells have been shown in cattle and pigs (Garcia-Valcarcel *et al.,* 1996; Childerstone *et al.,* 1999; Bautista *et al.,* 2003) and it has been proposed that cell-mediated immunity plays a role in clearing FMDV in persistently infected animals (Ilott *et al.,* 1997, Childerstone *et al.,* 1999). According to Carr *et al.,* (2013), *in vivo* studies showed that when cattle were immunized with inactivated FMDV vaccine, neutralizing antibody titres dropped as CD4+ cells decreased, however from vaccinated pigs, the

CD8+ cytotoxic lymphocytes (CTLs) can specifically kill FMDV infected cells, *in vitro* (Patch *et al.,* 2013). Since the T-cell response to certain specific FMDV epitopes seems to occur with different FMDV serotypes, epitope identification serves as a significant tool in the development of vaccines (Blanco *et al.,* 2001; Parida *et al.,* 2006). The combination of the T-cell epitope and the B-cell epitope in a peptide vaccine has shown to significantly reduce FMDV excretion after vaccination and challenge (Cubillos *et al.,* 2012). IFN-γ has been used to measure antigen-specific T-cell activation (Tassignon *et al.,* 2005). After re-stimulation with vaccine antigen, lymphocytes generated from FMDV-infected animals have been shown to proliferate and produce IFN-γ (Van Lierop *et al.,* 1992; Gerner *et al.,* 2007; Carr *et al.,* 2013) and the vaccinated cattle had memory CD4+ T-cells (Glass *et al.,* 1994; Naessens *et al.,* 1998). Other cytokines, besides IFNs may potentially contribute to the host response. Rigden *et al.,* (2003) showed that pigs vaccinated with a conventional FMD vaccine did not have a systemic inflammatory response but observed an increase in chemotactic activity of plasma on peripheral blood leukocytes, within week 1 of vaccination. Furthermore, after vaccination and/or challenge, levels of IL-6, IL-8 and IL-12 in plasma increased, suggesting the activation of macrophages or monocytes (Barnett *et al.,* 2002).

1.14. ANTIBODIES

Antibodies (abs), also known as Ig, are glycoproteins produced by B cells and participate in humoral immune responses by neutralizing foreign molecules *i.e.,* viruses, bacteria *etc.* (Burton,1990). Apart from a small part of the heavy chain, an antibody is identical to the B cell receptor that produces it. Antibodies are easily obtained and studied, due to their solubility and being produced in large quantities. As a result, the majority of the work on B cell receptors comes from studying antibodies (Janeway *et al.,* 2001; Murphy and Weaver, 2017). Polyclonal and monoclonal antibodies (MAbs) have not only been used to perform such studies but also as reagents in diagnostic tests. Polyclonal antibodies (PAbs) are a diverse group of antibodies (Lipman *et al.,* 2005), produced from many B cell clones. Thus, they have the affinity for the same antigen but detect different epitopes

(Nelson *et al.,* 2000). Conversely, MAbs are identical antibodies, produced from a single B-cell clone and specific to a single epitope (Nelson *et al.,* 2000). MAbs provides homogeneity and consistency, as they are more specific than PAbs. They can identify individual protein family members and their monospecific nature is useful in assessing changes in molecular structures, phosphorylation states and protein-protein interactions (Lipman *et al.,* 2005). MAbs are frequently produced through hybridoma technology (Köhler and Milstein, 1975) or by bio-panning with recombinant antibody libraries (Clackson *et al.,* 1991; Marks *et al.,* 1991; Basu *et al.,* 2019). Some of the limitations of hybridoma technology includes the necessity for specialized cell culture facilities, it requires a significant amount of time and is expensive. In addition, many molecules are not immunogenic in mice (are toxic) and therefore cannot be used as antigens (Harlow and Lane, 1988; Karu *et al.,* 1995). Through recombinant monoclonal antibody technology, many of the drawbacks associated with hybridoma antibody generation are overcome (Hoogenboom *et al.,* 1998; Chao *et al.,* 2006).

1.14.1. ANTIBODY STRUCTURE AND DIVERSITY

Antibodies are generally Y-shaped molecules (Figure 1.9) and their structure is sectioned into two parts *i.e.,* the variable (V) region, which defines antigen (Ag) binding characteristics and the constant (C) region that interacts with effector cells and chemicals (Janeway *et al.,* 2001; Murphy and Weaver, 2017). All antibodies are made of two heavy (H) polypeptide chains (*ca.* 50 kDa) and two light (L) polypeptide chains (*ca.* 25 kDa) and are connected by a disulphide bond, forming the arms of Y-shaped antibody structure (Figure 1.8). The Fragment, antigen binding (Fab) is located on each arm of the Y-shaped antibody (the heavy and the light chain) and consist of one constant and one variable domain (Edelman and Benacerraf, 1962; Janeway *et al.,* 2001; Murphy and Weaver, 2017). As a result, an antibody molecule contains two identical antigen-binding sites and may thus, bind to two identical structures simultaneously. Within this broad group, there are five distinct Ig classes namely; IgM, IgG, IgD, IgE and IgA, which can be differentiated by their C regions (Janeway *et al.,* 2001; Schroeder and Cavacini, 2010; Murphy and

Weaver, 2017). The light chain of an Ig can either be a lambda (λ) or kappa (κ), chain, never both (Janeway *et al.,* 2001; Wang *et al.,* 2007; Murphy and Weaver, 2017). The two variable domains (V_H and V_L) dimerize to form the fragment variable region (Fv), which contains the antigen binding site. Each variable domain contains six hypervariable loops (Wu and Kabat, 1970), also known as complementarity determining regions (CDR1, CDR2, and CDR3) (Wang *et al.,* 2007). Three of the loops are in the light chain (L1, L2 and L3), while the other three are located in the heavy chain (H1, H2 and H3) and all are supported by the conserved framework region (FR) of the β-sheet. The hypervariable loops are then brought together through the folding of the L and H chains, forming the paratope (Sela-Culang *et al.,* 2013). The heavy chain determines the antibody's effector function, with its domain C_H2 and C_H3 and forms the Fc (fragment crystallizable). The Fc region (Figure 1.8) plays a role in biological activity mediation (Janeway *et al.,* 2001; Sela-Culang *et al.,* 2013; Murphy and Weaver, 2017) and the hinge region, differ amongst antibody isotypes. However, the general arrangement of the domains is the same for all isotypes (Janeway *et al.,* 2001; Murphy and Weaver, 2017).

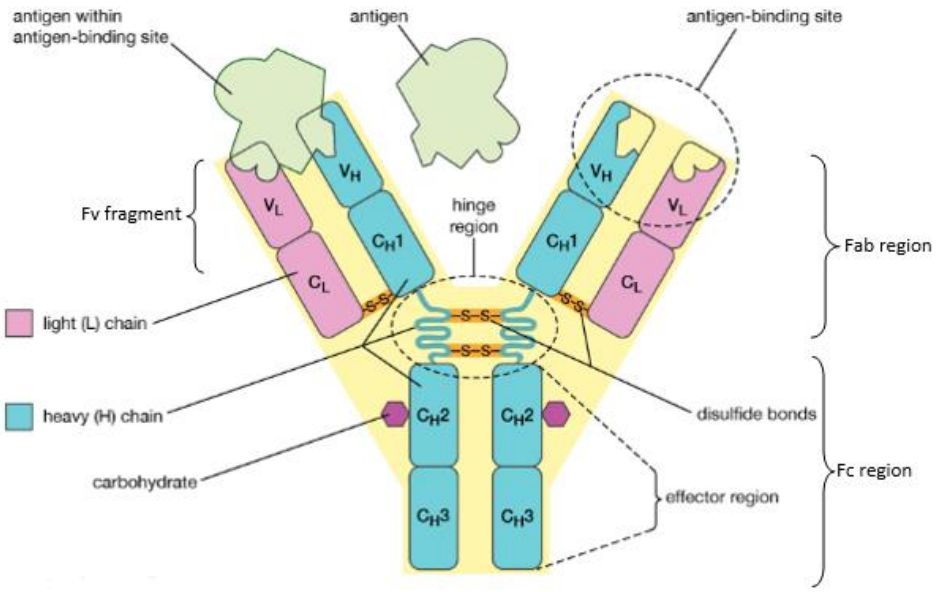


Figure 1.9: A general antibody structure. There are disulfide bonds connecting the two light polypeptide chains and two heavy polypeptide chains together. One variable region (V_H) and three constant regions (C_H1, C_H2, and C_H3) (indicated in blue) make up each heavy chain, whereas one variable region (V_L) and one constant region make up each light chain (C_L) (indicated in pink). The Fab region is located on each arm of the Y-shaped antibody structure and the antigen binding

site is shown. The Fv fragment and Fc region are also indicated. Adapted from https://www.britannica.com/science/antibody.

Antibodies serve two functions *i.e.,* to selectively and specifically bind their target antigen and to stimulate an immune response against the bound antigen by drawing in other cells and molecules. The interaction between an Ab and an Ag involves multiple non-covalent interactions between the binding site on an Ag (*i.e.,* epitope) and the binding site on the Ab (*i.e.,* paratope) (Sela-Culang *et al.,* 2013). Antibody epitopes, also called B-cell epitopes, are regions within an Ag that interact with an Ab paratope and are usually used in immunodiagnostics and vaccine development. The discovery of B-cell epitopes within an antigenic protein may aid in developing molecules (biologic or synthetic) that mimic epitopes and might be used in prophylactic or therapeutic vaccines (Amanna *et al.,* 2006; Sela-Culang *et al.,* 2013). Thus, boosting protective immunity (Sela-Culang *et al.,* 2013). Understanding how Abs recognizes their epitopes is crucial for the development of vaccines and the prevention of diseases (Yang and Yu, 2009). Existing techniques for identifying Ab epitopes include X-ray crystallography, phage display, expressed fragments, mass spectrometry and mutagenesis analysis (Xu *et al.,* 2010).

1.15. PHAGE DISPLAY TECHNOLOGY

1.15.1. INTRODUCTION TO PHAGE DISPLAY

Phage display can be used as an exceptionally strong *in vitro* technique (Frei and Lai, 2016), whereby peptides or proteins are fused to a bacteriophage coat protein and displayed on the virion's surface (Wu *et al.,* 2016). It can be defined as a technology that expresses peptides, antibody fragments or proteins at the phage particle surface (Smith 1985; Winter *et al.,* 1994; Kay and Hoess, 1996). Phage display technology has been used to map epitopes (protein-ligand interactions), identify cell surface receptors, determine protein modifying enzymes and substrate specificity of kinases and proteases (Kay, 1994; Ngubane *et al.,* 2013; Rebollo *et al,* 2014; Christiansen *et al.,* 2015). A gene encoding the desired protein is incorporated into a phage or phagemid genome as a

fusion to a gene encoding the surface phage coat protein, thereby providing a physical link between the phenotype and genotype (Hoogenboom, 1997; Hoogenboom *et al.,* 1998; Sparks *et al.,* 1996). This phage surface representation can lead to the identification of several peptides, with great affinity and specificity for a particular type of target (Arap, 2005). Typically, phage display libraries are screened through an affinity selection (or bio-panning) procedure, which involves the exposure of the phage populations to their targets (Hoogenboom, 1997; Hoogenboom *et al.,* 1998; Sparks *et al.,* 1996).

The production of monoclonal antibodies against antigens has traditionally been achieved through animal immunization and using hybridoma technology (Carmen and Jermutus, 2002). However, due to several limitations associated with hybridoma technology (Frenken *et al.,* 2000; Brien and Aitken, 2002; Lee *et al.,* 2007) (see section 1.14), a more radical approach that produces antibodies that cannot be acquired by any traditional vaccination (*i.e.,* Abs from non-immunogenic molecules) has been developed (Frenzel *et al.,* 2017). Over the last 10 years, developments in molecular biology have enabled the use of *Escherichia coli* (*E. coli*) (*i.e.,* antibody phage display) in *in vitro* studies to generate recombinant antibodies (Carmen and Jermutus, 2002; Lee *et al.,* 2007). The first phage display method was based on the lytic phage lambda, however it had minimal success (Huse *et al.,* 1989; Schirrmann *et al.,* 2011). Filamentous phage display is currently widely used (Schirrmann *et al.,* 2011). The oligopeptides are bound to the filamentous bacteriophage M13 protein III gene by fusion of the corresponding gene segments (Clackson *et al.,* 1991; Hoogenboom *et al.,* 1991). The output peptide-pIII fusion protein is then presented on the surface of the M13 phage, allowing peptide affinity purification (McCafferty *et al.,* 1990; Barbas *et al.,* 1991). However, for library generation, smaller antibody fragments such as Fab and the single chain variable fragment (scFv) are commonly used (Holt, 2003; Schirrmann *et al.,* 2011). This size restriction is due to the *E. coli* folding machinery, which can also accommodate the V_H domain antibodies (DAbs) (Bahara *et al.,* 2016). Phagemids are a more preferred form of vector for display since they carry gene III with appropriate cloning sites and need a helper phage such as M13 for production (Bass *et al.,* 1990; Clackson *et al.,* 1991; Garrard *et al.,* 1991; Hoogenboom *et al.,* 1991).

1.15.2. STRUCTURE AND BIOLOGY OF BACTERIOPHAGES

Bacteriophages, also known as phages, are single-stranded DNA viruses that infect a variety of gram-negative bacteria, including *E. coli*. The Ff filamentous phage particles that are commonly used in phage display technology include strains M13, Fd, f1 and ft (Russel, 1991; Arap, 2005). These phage particles are composed of a long, cylindrical protein capsid with dimensions of 900 nm and 6-7 nm (Webster, 1996; Makowski and Russel, 1997), enclosing a circular single-stranded DNA (ssDNA) genome with roughly 6400 (Fd) (Arap, 2005) or 6407 (M13) base pairs long. The genome consists of 11 genes, five of which are coat proteins. The major coat protein, gene protein (gp) 8 (gp8) is present in about 2700 copies and responsible for phage DNA encapsulation (Figure 1.10). Five copies of each g7p and g9p are located on the distal end of the phage particle, while the proximal end consist of 4 to 5 copies of each gp6 and gp3 (Carmen and Jermutus, 2002).

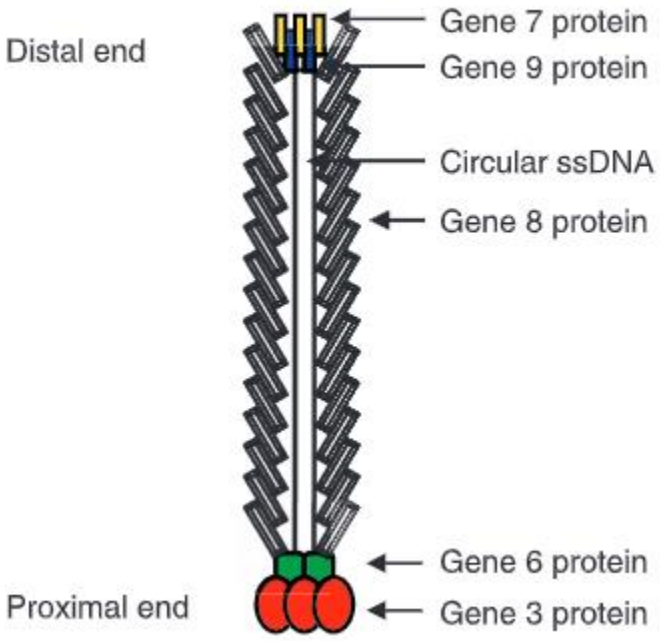


Figure 1.10: A schematic representation of M13 filamentous bacteriophage. Shown are the distal and proximal ends, along with the gene protein (gp) capped in each end. Taken from Stopar *et al*., (2003).

Only male bacteria (*i.e., E. coli* cells bearing the F-plasmid, encoding the F-pilus) are infected by the M13 bacteriophage and the interaction between the gp3 and the F-pilus facilitates infection (Carmen and Jermutus, 2002; Arap, 2005). The circular ssDNA of the phage enters the bacteria and is converted into a double-stranded replicative form (RF) by the host DNA replication machinery. The RF produces ssDNA through a rolling circle replication and also acts as a template for the expression of g3p and g8p proteins. These phage progenies are formed by packaging the ssDNA into protein coats and are released through the bacterial membrane (Russel, 1991; Arap, 2005). One advantage of filamentous phages is the ability to replicate and not lyse infected host cells. As a result, it is released from the cell membrane whilst the host cell keeps growing and dividing (Russel, 1991; Carmen and Jermutus, 2002; Arap, 2005).

1.16. ANTIBODY PHAGE TECHNOLOGY

The ability to produce specific scFv/Fab fragments to a given antigen within weeks, is one of the main advantages of phage display technology of antibody fragments compared to hybridoma technology (Carmen and Jermutus, 2002). Typically, the initial step is to construct an antibody library (*i.e.,* naïve or immune library), preferably, with a size of 10^9-10^{11} clones. These clones then undergo 2 to 3 rounds of selection (biopanning), to enrich for a high percentage of antibody fragments to a specific target antigen (Hoogenboom *et al.,* 1998; Carmen and Jermutus, 2002). A naive library is derived from natural unimmunized rearranged variable gene (V-gene) (Marks *et al.,* 1991), mostly IgMs, which have wide specificity to a variety of different antigens. Conversely, immune libraries are derived from naturally infected or immunized donors. After vaccination with target antigen, IgGs are obtained from spleen B cells and a range of V-genes (encodes for antibody variable domain) are modified and inserted into a phage library vector to generate an immune library. Moreover, immune libraries are usually smaller than the naïve, however, they are advantageous due to the *in vivo* affinity maturation process and high antibody affinity (Clackson *et al.,* 1991; Mondon *et al.,* 2008). A single naïve library serves as the animal donor's primary repertoire and can be utilized for antibody selection against a

broad range of antigen (Ponsel *et al.,* 2011; Opperman, 2013). Construction of these libraries involves mRNA reverse transcription of the antibody genes from the V_H and V_L chains. This is followed by polymerase reaction chain (PCR) amplification and restriction-based cloning to insert the antibody segments into a phagemid display vector. Bacterial transformation/electroporation is then performed to introduce the recombinant phagemid into competent *E. coli* cells (Azzazy and Highsmith, 2002; Carmen and Jermutus, 2002) and is crucial as it directly influences the library size. The phagemid containing bacterial cells are cultured and subsequently infected with a helper phage (*i.e.,* M13KO7 or VCS) to produce recombinant phages, displaying scFv antibody fragments fused to the phage coat proteins (Azzazy and Highsmith, 2002).

1.17. PEPTIDE PHAGE DISPLAY LIBRARY

In addition to phage display of antibody fragments, peptides or proteins can also be expressed on the surface of phage particles (Smith, 1985; Winter *et al.,* 1994; Kay and Hoess, 1996). A peptide phage library is an effective tool used for both specific peptide screening and the expression of a variety of random peptides (Zambrano-Mila *et al.,* 2020). Almost the same method of antibody library construction is followed for peptide libraries. The nucleotide sequence encoding the protein to be displayed is inserted into the genome of a phage or phagemid, as a fusion to a gene encoding a phage coat protein. (Carmen and Jermutus, 2002; Chitray, 2018). Phagemids are hybrids of phages and plasmid and are now a more popular vector for display. These vectors consist of the origin of replication (ori) for both *E. coli* and M13, as well as multiple cloning sites and antibiotic resistance genes (Mead and Kemper, 1988). However, any other structural and non-structural genes needed for phage generation are absent. Alternatively, phagemids can be packaged as recombinant M13 or cultured as plasmids, with the help of a helper phage (M13KO7 or VCSM13), which has a slightly defective ori and provides all the structural proteins that are necessary for producing a complete phage. This process is called ‘phage rescue’. Thus, the rescued phage particles may have either the polypeptide-pIII fusion protein, encoded by the phagemid or the g3p (pIII) from the helper phage (Azzazy and

Highsmith, 2002). As a result, after the phage particles are assembled, the protein of interest is displayed on the phage surface and the sequence encoding the protein is contained within the same phage particle (Carmen and Jermutus, 2002; Chitray, 2018).

After construction of the peptide phage library, a screening or selection process is performed. The bio-panning (also known as panning) process is one of the protocols used to obtain reliable phage libraries (Figure 1.11). This is a crucial step since the panning results are directly related to the quality of the library (Arap, 2005). The phage library is screened by binding the recombinant phages to the target antigen [Figure 1.11, (1)], usually immobilized on a solid substrate. Then unbound phages are washed-off (2). This is followed by the elution and amplification of antigen-specific phages by infecting with *E. coli* (3 and 4) (Azzazy and Highsmith, 2002). Usually, three to four biopanning rounds are performed for enrichment of potential target-specific binders (Koivunen *et al*., 1999; Arap, 2005). Following bio-panning, the enriched pools are then evaluated in an enzyme-linked immunosorbent assay (ELISA) (polyclonal ELISA), to determine the level of enrichment. (AC't Hoen *et al.,* 2012; Lim *et al.,* 2019). Phages from biopanning rounds that showed enrichment (outperformed) are then subjected to a monoclonal ELISA to select MAbs (clones) (Aghebati-Maleki *et al.,* 2016; Lim *et al.,* 2019). Thereafter, the selected MAbs (output clones) are sequenced to retrieve their sequences (Arap, 2005; AC't Hoen *et al.,* 2012; Aghebati-Maleki *et al.,* 2016; Lim *et al.,* 2019).

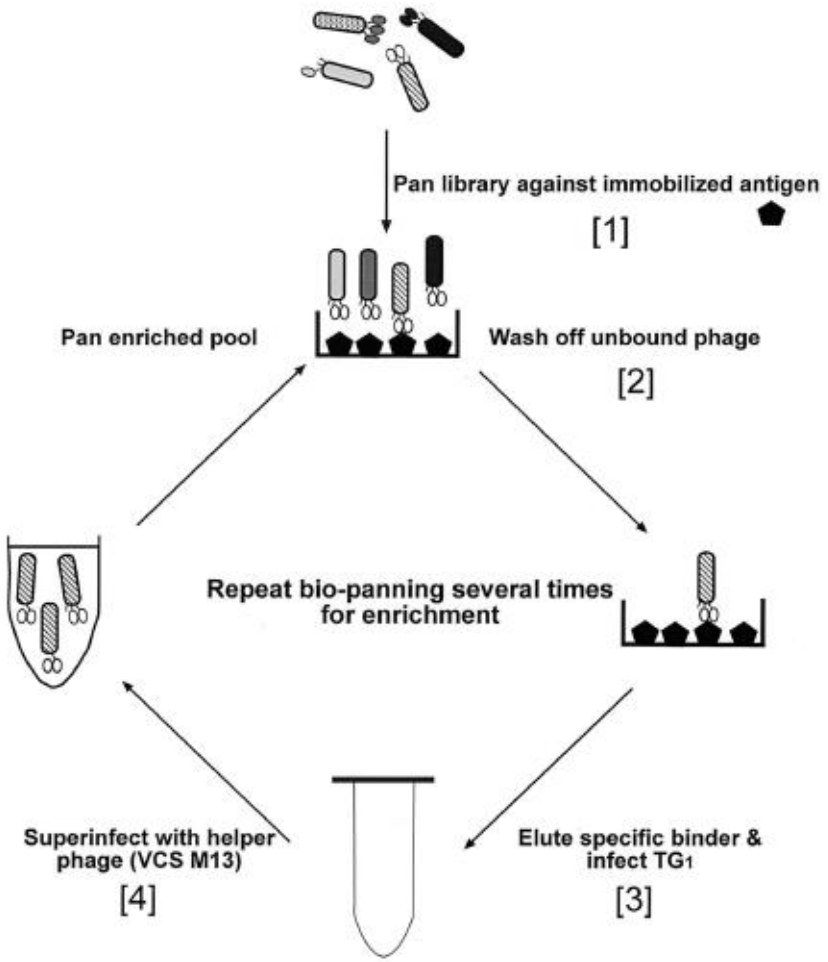


Figure 1.11: A biopanning process of phage display libraries. The phage libraries are screened as follows :(1) Binding of phages in the phage library to the target antigen. (2) Unbound phages are then washed-off. (3) The antigen-specific phages are eluted and infected with *E. coli*. (4) Amplification of antigen-specific phages by infecting with M13 helper phage, then repeating several times (usually 3 to 4 times) for target-specific binder enrichment. Taken from Azzazy and Highsmith, (2002).

Phage-borne peptides have shown to have a high ability to mimic linear, conformational and non-proteinaceous epitopes (Smith, 1991; Smith *et al.,* 1993) and peptide libraries have been used to identify epitopes. Peptide motifs are recognized by antibodies based on only three or four conserved residues (Scott and Smith, 1990). As a result, the motif revealed by phage display may be used to define the area of a protein recognized by an antibody (Scott and Smith, 1990). Phage display-identified peptide sequences have been

demonstrated to function as receptor agonists and antagonists (Doorbar and Winter, 1994). Moreover, peptides that neutralizes antibodies can be used as diagnostic reagents (Azzazy and Highsmith, 2002) or as therapeutic agents in autoimmune diseases (Blank *et al.,* 1999). Epitope mapping of monoclonal and polyclonal antibodies and peptide ligand identification can also be performed using random peptide libraries (Matthews and Wells, 1993; Ohkubo *et al.,* 2001). Lastly, the expression of small peptides on the phage particle surface can increase their immunogenicity and hence, their potential as vaccine candidates (Azzazy and Highsmith, 2002).

1.18. AIMS OF THE STUDY

In the sub-Saharan Africa, not only are the three SAT serotype viruses endemic, but the role of the buffalo in the epidemiology of the disease makes FMD unique in this region (Thomson, 1995). This uniqueness presented by the SAT serotypes and their involvement with the African buffalo is fundamental towards understanding, controlling and possibly the eradicating FMD (Thomson, 1995). FMDV serotypes displays a high degree of antigenic variation (Sobrino *et al.,* 2001), especially the SAT 2 serotype which is antigenically highly diverse (Doel, 2003). Most commonly, these variations occur on the capsid coding P1 region, which encodes the structural proteins, VP1, VP2, VP3 and VP4 (King *et al.,* 1985; Mateu *et al.,* 1995). Thus, the region of focus in this study. The arrangement of the structural proteins on the capsid, provides the antigenic regions that induce infection or vaccination responses (Acharya *et al.,* 1989) and the amino acid residues that make up these proteins are exposed on the virus surface, thereby determining the virus's antigenicity (Reeve *et al.,* 2010). A number of FMDV immunodominant neutralizing antigenic sites have been identified (Dunn *et al.,* 1998; Opperman, 2013), located on the three surface-exposed structural proteins. However, it was noted that even in the absence of an immune response to these neutralizing epitopes, an IgG specific protection can be obtained, which suggests the existence of other undiscovered neutralizing epitopes (Dunn *et al.,* 1998; Burton, 2002; Opperman, 2013). Information on the FMDV antigenic structure may contribute to a better

understanding of the virus-host interactions and may also aid in the development of improved FMDV vaccines.

Several antigenic sites have been reported from FMDV serotype A, O, Asia-1 and C, and very little is known for the SAT serotypes. Most frequently, monoclonal antibodies are used for this, however, recombinant antibodies produced from phage display libraries have also been successfully used to map epitopes for serotype O viruses (Harmsen *et al.,* 2007; Yu *et al.,* 2011b), the SAT2 serotype (Opperman *et al.*, 2012; Opperman, 2013) as well as other animal viruses, such as African swine fever virus (Wang and Yu, 2009) and Bluetongue virus (Wang *et al.,* 1995; Qin *et al.,* 2013). Phage display technology has proved to be a promising approach for providing new strategies, aiding in vaccine production and development (Aghebati-Maleki *et al.,* 2016). A phage display library, the 'Nkuku® library' was constructed by the new generation vaccines programme at the ARC-OVR (Van Wyngaardt *et al.,* 2004), which consists of the naïve immunoglobulin repertoire of the chicken and is diverse enough to recognize a various haptens, proteins and viruses (Fehrsen *et al.,* 2005; Rakabe, 2008; Sixholo, 2009; Wemmer *et al.,* 2010). The 'Nkuku® library' has proved to be a potential valuable resource to obtain recombinant antibodies against FMDV (Van Wyngaardt *et al.,* 2004; Opperman *et al.,* 2012) and previously used to successfully map FMDV SAT2 epitopes (Opperman *et al.,* 2012). An affinity selection process (biopanning) with purified IgGs from immunogenic sera with virus peptide phage libraries, is a valuable tool to determine virus-specific epitopes (Bachler *et al.,* 2013; Aghebati-Maleki *et al.,* 2016; Machado *et al.,* 2022). Through this phage display technique, immunogenic peptide sequences can be obtained and used to construct novel vaccines (Bazan *et al.,* 2012). Consequently, this study aims to identify epitopes on the three FMDV SAT serotypes by constructing FMDV SAT serotype specific peptide phage display libraries, using the fragmented capsid coding P1 regions. Thereafter, bio-panning the constructed libraries with purified IgG from FMDV bovine sera samples of infected animals. These constructed FMDV SAT peptide phage libraries are all novel.

The ultimate goal in Africa is the eradication of FMD, but due to certain constraints (*i.e.,* wildlife maintenance host), it is doubtfully possible in the near future. Thus, emphasis is

placed on control rather than eradication (Maree *et al.,* 2014). In South Africa, the majority of FMD outbreaks are a result of SAT2 viruses, followed by SAT1 and SAT3 (Maree *et al.,* 2011). A trivalent, inactivated vaccine containing combinations the SAT 1, SAT 2 and SAT 3 serotypes is routinely used to control FMD (Brückner *et al.,* 2002; Lazarus *et al.,* 2018), however, the numerous recent outbreaks have indeed highlighted that the current FMD vaccines are less effective. The vaccine provides immune protection against all three FMDV SAT serotypes, however, animals may be re-infected and still able to infect non-vaccinated animals, as vaccination does not elicit sterile immunity (Salt, 1993; Doel, 2003; Maree *et al.,* 2011). Moreover, the current vaccines have some limitations and concerns (*i.e.,* providing short-lived immunity), thus, there is a need to develop improved FMDV vaccines. To date, there is limited information available regarding the epitopes of SAT serotypes (Maree *et al.,* 2011) and knowledge thereof can be useful in producing recombinant FMD vaccines with broad immunogenic coverage, thereby improving FMD control. Thus, the identification of epitopes from this proposed study can be of future use in the production of improved recombinant FMDV vaccines. To address the scarcity of knowledge regarding the FMDV SAT serotype epitopes, the specific objectives for the study were:

1. To construct three FMDV peptide phage display libraries, using the fragmented capsid coding P1 region of selected FMD virus strains from SAT1, SAT2 and SAT3 serotypes.

2. To identify FMDV-specific antigenic determinant/s (epitopes) by purifying total IgG from known FMDV infected bovine sera samples and biopanning the constructed FMDV peptide libraries with the respective IgGs.

CHAPTER 2: MATERIALS AND METHODS

2.1. SELECTION OF FMDV SAT SEROTYPE VIRUSES

Representative FMDV viruses from the three SAT serotypes were chosen based on past suitable FMDV vaccine strains and phylogenetic data from the Agricultural Research Council, Onderstepoort Veterinary Research (ARC-OVR) Institute, Transboundary animal diseases (TADs) biobank. Specifically, FMDV strains SAT1/KNP/196/91, SAT2/KNP/19/89, and SAT3/SAR/01/06 were chosen for this study (Table 2.1). Additionally, these viruses were also selected based on the availability of relevant bovine serum samples at the TADs biobank for this study, which was essential for the biopanning process (section 3.6).

Table 2.1: FMDV strains used in this study.

FMDV Serotype	Virus strain	Passage History	Genbank accession number
SAT1	KNP/196/91	PK1RS5	DQ009716
SAT2	KNP/19/89	PK1RS5	DQ009735
SAT3	SAR/01/06	PK1RS5	MK415736

* PK = Pig kidney cells.
* RS = Instituto Biologico Renal Suino-5 (IB-RS-2) cells.

Animal and research (AEC and REC) regulatory approvals from the University of Pretoria (Faculty of Veterinary Sciences) were attained (REC180-19). Also, the Department of Agriculture, Land Reform and Rural Development (DALRRD) granted a section 20 (12/11/1/1) approval for this study and for working with FMDV and serum samples stored at the ARC-OVR-TADs biobank.

2.2. CELL CULTURE AND VIRUS

The SAT viruses (Table 2.1) were passaged using monolayer (T25 flasks) baby hamster kidney (BHK) cells, strain 21, clone 13 (ATCC CCL-10), which were maintained in Glasgow minimum essential medium (GMEM, Sigma), supplemented with 10% (v/v) foetal bovine serum (FBS, Hyclone), 1 mM L-glutamine (Invitrogen), 10% (v/v) tryptose phosphate broth (TPB, Sigma-Aldrich) and 1X antibiotic-antimycotic solution (Invitrogen).

Only one cell passage level was performed to confirm virus viability by attaining 100% cytopathogenic effect (CPE). Virus cultures were clarified by centrifugation at 3000 xg for 5 min and the supernatant was stored at - 80 °C until further use.

2.3. RNA EXTRACTION, cDNA SYNTHESIS AND PCR AMPLIFICATION

Using the QIAamp viral RNA kit (QIAGEN, Cat. No. 52904), as per the manufacturer's protocol, the respective FMDV SAT viral RNAs were extracted from the cell culture virus samples. A one-step reverse transcription polymerase chain reaction (RT-PCR) kit (QIAGEN, Cat. No. 210212) was then used to process the RNA (as per the manufacturer's instruction) to sequentially carry out complementary DNA (cDNA) synthesis and the amplification of the respective P1 genome region in the same tube. Respective gene specific forward and reverse primers (Table 2.2) and 1 µg of the viral RNA was used. Briefly, the viral RNA was reverse transcribed at 30 ºC for 30 minutes (min) for cDNA synthesis. Reverse transcriptase inactivation and cDNA template denaturation then followed, during the first PCR activation step at 95 ºC for 15 min. Thereafter, a three-step cycling procedure was performed, which involved denaturation at 94 ºC for 1 min, primer (Table 2.2) annealing at 55 ºC (SAT1 and SAT2) or 56 ºC (SAT3) for 3 min and an extension at 72 ºC for 4 min. These cycles were repeated ('cycled') 35 times, to exponentially produce the exact copies of the target DNA (P1 region). Lastly, a final extension step at 72 ºC for 5 min was performed.

Table 2.2: Oligonucleotides sequences used for the amplification of the FMDV P1 genome region.

Oligonucleotides	Sequences
Forward oligonucleotide SAT1 and SAT2 (NCR1)	5’ TACCAAGCGACACTCGGGATCT 3’
Forward oligonucleotide SAT3 (NCR2)	5’ GCTTCTATGCCTGAATAGG 3’
Reverse oligonucleotide (WDA)	5’ GAAGGGCCCAGGGTTGGACTC 3’

A 1% (w/v) agarose gel was prepared to separate all the SATs P1 PCR products and the Lambda/HindIII DNA marker (Promega) was used as a molecular weight standard to confirm the P1 DNA size. The P1 regions [~2300 base pairs (bp)] were excised from the agarose gel and purified using the Zymoclean Gel DNA Recovery Kit (ZymoResearch), as per the manufacturer’s instructions. The concentrations of the purified DNA were then measured at an absorbance of 260 nm (A_{260nm}) on a NanoDropND-1000 UV-Vis spectrophotometer (Thermo Scientific).

2.4. P1-CODING REGION FRAGMENTATION AND DEPHOSPHORYLATION

The purified P1 DNA products (SAT1, 2 and 3) were sheared using the Covaris Adaptive Focused Acoustics® technology (performed at the ARC-OVR Biotechnology Platform). Briefly, 100 µl of the purified DNA and 30 µl dH_2O was added into the covaris fragmentation instrument to yield fragments in the 50-200 bp range. A small aliquot was then analysed on a 2.5% (w/v) agarose gel using electrophoresis to confirm the fragmentation. Before cloning into a vector, DNA fragments underwent blunt ending. This

was performed according to Du Plessis and Jordaan (1996) with some minor alterations. Firstly, 100 ng/µl of the fragmented DNA was added to 10X T4 polymerase buffer (Invitrogen) with 2.5 mM dNTPs (Invitrogen), 5 U/µl T4 DNA polymerase (Invitrogen) and distilled water (dH_2O) to make up a 100 µl reaction. The reaction was incubated at 15 ºC for an hour (hr), then 1 U of Klenow fragment (Promega) was added, followed by incubation at 37 ºC for 30 min and 65 ºC for 10 min.

Dephosphorylation of the blunt ended fragments was performed to prevent self-fragment ligation and concatemers formation. This was undertaken by adding 10X Antarctic phosphatase rection buffer (NEB), 5 U Antarctic phosphatase (NEB) and dH_20. The reaction was then incubated at 37 ºC for 15 min and heat inactivated at 65 ºC for 5 min. Thereafter, the DNA was purified using the MSB® Spin PCRapace kit (Invitek Molecular), as per the manufacturer's protocol, followed by elution in microcentrifuge tubes and samples were stored at 4 ºC.

2.5. PHAGEMID PREPARATION AND PLASMID PURIFICATION

The phagemid vector, pCVEP1585042, was obtained from the ARC-OVR Immunology division, which was used to construct the three FMDV SAT peptide phage display libraries. To prepare stocks of the cloning vector, glycerol stocks of the vector in TG1 cells were inoculated into 25 ml Luria-Bertani broth (LB), supplemented with 100 µg/ml ampicillin and 2% (w/v) glucose and grown overnight at 37 ºC, shaking at 240 revolution per minute (rpm). To collect the cells, a centrifugation step was performed, at 13000 xg for 15 min. The pelleted cells were then subjected to plasmid purification.

A QIAprep spin miniprep kit (QIAGEN, Cat. No. 27104) was used to purify plasmid DNA. The cells obtained after phagemid preparations were purified as per the manufacturer's protocol. The eluted plasmid DNA was subjected to a NanoDropND-1000 spectrophotometer (Inqaba Biotechnology), to measure the concentrations at A_{260nm}.

2.6. DNA LIGATION

Prior to DNA fragment ligation, the pCVEP1585042 vector was digested with PmeI restriction enzyme (NEB) as per the manufacturer's instruction. A 10X cutsmart buffer (NEB), 1 µg of vector DNA, 10 U/µl PmeI enzyme and dH_2O were used for the reaction. The reaction was incubated at 37 °C for 15 min and visualized on a 0.7 % (w/v) agarose gel, stained with ethidium bromide.

The blunt-ended and dephosphorylated SAT P1 region fragments were ligated into the PmeI digested pCVEP1585042 vector. DNA ligation was performed separately for SAT1, SAT2 and SAT3 samples. A high insert to DNA vector ratio (*i.e.,* 5:2) was used. The ligation reaction of each FMDV SAT contained approximately (approx.) 500 ng DNA fragment (insert), 200 ng of the linearized vector, 10X T4 DNA ligase buffer (Promega), 1 U T4 DNA ligase (Promega) and dH_2O to make up a final volume of 20 µl. The reaction was incubated overnight at 25 ºC. The following day, the reaction was incubated at 16 ºC for 2 hrs and heat inactivated at 65 ºC for 5 min, preceding transformation.

2.7. TRANSFORMATION

The Mix & Go! *Escherichia coli* (*E. coli)* chemically competent TG1 cells (Zymo Research) were used for highly efficient DNA transformation. Transformation was undertaken separately for SAT1, SAT2 and SAT3 samples. This step was performed as per the manufacturer's protocol (Cat. No. T3001). Briefly, 5 µl ligated DNA (65 ng/µl) was mixed with 100 µl competent TG1 cells (on ice) and 10X dilutions were prepared in 2X TY media (16 g/L tryptone, 10 g/L yeast extract and 5 g/L NaCl) containing 100 µg/ml ampicillin and 2% (w/v) glucose. The dilutions were then plated out on a Tryptone yeast extract-NaCl (TYE) agar plates (petri dishes), containing 100 µg/ml ampicillin and 2% (w/v) glucose. All plates were incubated at 30 ºC overnight and colonies were counted the next morning. The library size was then calculated using the average colony forming unit (CFU): CFU x dilution factor x fraction plated. Colonies from dilution plates were randomly selected and the DNA fragment sizes containing the insert of interest were confirmed by colony PCR

(section 2.9). The remaining colonies were scraped (using a L-Shaped Spreader) with 2X TY medium, supplemented with 100 µg/ml ampicillin and 2% (w/v) glucose and was stored at - 70 ºC in tubes containing 15% (v/v) glycerol. The cloning procedure was repeated twice for all SAT libraries to increase the size of the libraries. SAT1 clonings were pooled into a single cryotube and the same was undertaken for SAT2 and SAT3 clonings respectively before phage rescue (section 2.11).

2.8. COLONY PCR

Ten colonies were randomly selected from the titration plates were added to dH_2O using an inoculating loop and the samples were heated at 100 ºC for 5 min in a digital dry bath (Labnet). The reaction was then cooled on ice for 2 min before centrifugation to pellet the cells. An aliquot of the supernatant was taken for PCR. The PCR reaction contained 2X GoTaq® green master mix (Promega), 10 µM of each forward and reverse primers (Table 2.3) and the prepared template DNA. The undigested phagemid vector, pCVEP1585042, was used as a control. The PCR conditions included denaturation at 92 ºC for 1 min, followed by primer annealing at 56 ºC for 30 sec and extension for 4 min at 72 ºC. The cycles were repeated 35 times for optimal amplification of the desired products.

Table 2.3: Primers used for colony PCR.

Primer	**Sequence**
M13 Forward	5’ GTAAACGACGGCCAG 3’
M13 Reverse	5’ CAGGAAACAGCTATGAC 3’

The PCR products were analysed on a 1% (w/v) agarose gel using electrophoresis. The gels were prepared using 1X TAE buffer and were stained with ethidium bromide (Thermofisher) to visualize the gel. Agarose gels were run at 120 volts (V) and 240 milliampere (mA) for 30 min and then visualized under a UV light gel viewer (Auto Chemi ® systems, UVP Bioimaging system). A PCR control (undigested phagemid vector) ~400 bp, was used to compare the PCR products containing the insert (> 400 bp). Sizes were estimated using the 1 kb hyperladder marker (Bioline). A program called GelAnalyzer 19.1 (www.gelanalyzer.com) was used to analyse the gel by automatically detecting the lanes and bands on the gel. The exact sizes of the DNA fragments were determined and the average insert fragment sizes, per library were calculated. Thereafter, the insert fragment sizes were used to calculate the theoretical recombinant size needed for library construction. The formula N = ln (1-P) / ln (1-I/G) was used to calculate the probability of having the desired DNA sequence represented in the libraries (theoretical library size), where, N is the total number of recombinant needed, P is the desired probability, I is the average insert fragment size (in bp) and G is the genome size (P1 region, in bp) (Clarke and Carbon,1976; Perez-Ortin *et al*., 1997).

The genome coverage (how well the P1 region was represented in each library) was also calculated by dividing the actual library sizes by the theoretical sizes needed.

2.9. MISEQ OF UNPANNED LIBRARIES

Phage rescue (section 2.11) and plasmid purification (QIAprep spin miniprep kit, QIAGEN, section 2.6) were performed on all three peptide phage libraries. Thereafter, their respective plasmid DNA were sent to Inqaba Biotechnical Industries (Pty) Ltd for MiSeq sequencing (section 2.16.2) to confirm the coverage of the FMDV SAT P1 genome.

2.10. PHAGE TITRE

Phage titre determined how many infectious particles were present in the FMDV peptide phage libraries. Briefly, a glycerol stock of TG1 cells was inoculated into an Erlenmeyer flask containing 2X TY medium and grown overnight at 37 ºC, shaking at 100 rpm. The next day, a 1:100 dilution of the overnight culture in 2X TY medium supplemented with 100 µg/ml ampicillin and 2% (w/v) glucose was prepared and incubated at 37 ºC (shaking at 196 rpm), until an OD_{600} of 0.5 - 0.6 was reached (mid-log phase) (UV-1800 Shimadzu Spectrophotometer). Ten-fold dilutions of the SAT peptide libraries phage stock (section 2.8) in 2X TY containing 100 µg/ml ampicillin and 2% (w/v) glucose were prepared and equal volumes of the exponentially growing TG1 cells and the phage dilutions were mixed. Incubation at 37 ºC for 30 min followed. Thereafter, an aliquot of each dilution was plated on TYE agar plates, containing 100 µg/ml ampicillin and 2% (w/v) glucose. Additionally, a TYE agar plate with only TG1 cells plated was used as a negative control and all plates were incubated overnight at 30 ºC. The phage titre was calculated as follows:

Phages/ml= [Number of colonies x dilution x 1 ml] / [volume plated x 2]

2.11. PHAGE RESCUE

The primary FMDV SATs libraries consist of phagemids in bacterial cells. Utilising the M13K07 (NEB) helper phage, each of the libraries were subjected to a process of phage rescue before biopanning. Each SAT serotype phage library rescue was performed separately and on different days to prevent cross contamination. A small volume of the pooled aliquot of a SAT library was added to 2X TY medium containing 100 µg/ml ampicillin and 2% (w/v) glucose until the OD_{600} was 0.05 - 0.06 (UV-1800 Shimadzu Spectrophotometer). The cells were incubated at 37 ºC, while shaking at 196 rpm, until the OD_{600} was 0.5 - 0.6 (approx. 2hrs). After incubation, 40% of the volume of the cells were transferred into an Erlenmeyer flask and the M13K07 (NEB) helper phage at a multiplicity of infection (MOI) of 1:20 (bacterial cells= 8×10^8 CFU/ml) was added. To

calculate the amount of M13K07 helper phage (concentration= 2 x 10^{12} PFU/ml) that was added, the formula:

[OD_{600} x Volume of phage culture x (8 x 10^8)] / [Concentration of helper phage]

was used and the cells were incubated at 37 ºC for 30 min (no shaking), followed by an incubation at 37 ºC for 30 min, while shaking at 100 rpm. Thereafter, the cells were centrifuged for 15 min at 31 000 xg (Eppendorf 5810 R) and the supernatant was discarded. The resultant pellet was resuspended in 2X TY media with 100 µg/ml ampicillin and 25 µg/ml kanamycin, followed by overnight incubation at 30 ºC, shaking at 196 rpm. The next day, the overnight culture was centrifuged for 15 min at 31 000 xg (Eppendorf 5810 R) and the pellet was discarded, while the supernatant (with the phages) was transferred into a sterile 50 ml centrifuge tube. Phages were precipitated by adding 1/5th volume of 20% (w/v) polyethylene glycol-600 (PEG) with 2.5 M NaCl to the supernatant and incubated for 1 hr on ice, while shaking the tube in 15 min intervals. Thereafter, phages were rescued by centrifuging at 31 000 xg (Eppendorf 5810 R) for 15 min. The supernatant was discarded and the pellet was resuspended in 1X phosphate buffered saline (PBS). These phages were stored at – 80 ºC for use in biopanning.

2.12. SOLID PHASE COMPETITION ELISA (SPCE) AND NONSTRUCTURAL PROTEIN (NSP) ELISA

A SPCE (OIE Terrestrial Manual) and NSP ELISA (PrioCHECK® FMDV NS) were performed to detect antibodies against FMDV from bovine sera samples. The basis of the SPCE relies on the competition between the serotype-specific guinea pig anti-FMDV antiserum and the antibodies present in the test serum. The FMDV serotype-specific rabbit anti-serum was immobilized on the maxisorp microtiter plates (Nunc) to capture the corresponding FMD serotype-specific antigen. Thereafter, the unbound antigen was discarded and the test serum, along with the specific guinea pig antiserum were added on to the ELISA plates. The plates were incubated at 37ºC for 1 hr. After incubation, a wash step (three times) with 0.05% (v/v) PBST was performed and the goat anti guinea-

pig IgG conjugated to horseradish peroxidase (Millipore- AQ108P) added. The plates were incubated at 37ºC, for 1 hr. Thereafter, a final wash step with 0.05% (v/v) PBST was performed and the plates were developed using a substrate solution consisting of 100 µl of 4 mM 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma-Aldrich) and 25 µl of 0.015% (v/v) H_2O_2 in a 10 ml substrate buffer (0.1 M tri-potassium citrate, 0.1 M citric acid monohydrate; pH 4.5). After 10 min, the colour reaction was stopped by 50 µl of 1M H_2SO_4 and a Labsystems Multiscan photometer (Thermo Scientific), was used to measure the absorbance values at 450 nm (A_{450nm}).

FMD specific antibodies, in the positive test sera, bind to the antigen, inhibiting the binding of the specific guinea pig antiserum and consequently, the anti-guinea-pig immunoglobulin-horseradish peroxidase conjugate. Hence a reduced colour reaction. Duplicate tests were performed and optical density (OD) values averaged. The percentage inhibition (PI) values were calculated as follows:

PI = 100 – [100 x (OD test serum mean/OD strong positive control mean)]. Test sera samples with PI <50% were classified as negative, while those showing ≥50% were considered a positive serological response (Paiba *et al.,* 2004).

The same serum samples were tested for the presence of antibodies against FMDV 3ABC non-structural proteins (NSP) using the PrioCHECK® FMDV NS (Prionics, Lelystad, Netherlands) as a confirmatory assay. Samples with PI<50% were classified as negative (antibodies against NSP considered absent) and samples with PI≥50% were classified as positive (Sorensen *et al.,* 1998).

2.13. FMDV SERA FOR IMMUNOGLOBULIN (Ig) PURIFICATION

The FMDV bovine sera was selected from the ARC-OVR-TAD biobank and were from previous animal trials that were performed for FMD research projects. Relevant bovine sera to use for IgG purification followed by the biopanning process were selected and obtained from experimentally FMDV infected animals Table 2.4.

Table 2.4: Serotype and virus strain-specific bovine sera samples.

*Virus Strains	Bovine sera samples (Lab animal number)	Period serum collected (in days post infection, dpi)
SAT1/KNP/196/91	15-0840 15-0847 15-0856 15-0897 15-0804	28
SAT2/KNP/19/89	12-0852 12-0891 12-0833	21
SAT3/SAR/1/06	1067 1060 1051 1070a 1070b 1018a 1018b.	21

* The same virus strains were used for the phage peptide library construction.

The bovine sera were diluted 5X in 20 mM sodium phosphate, pH 7 (binding buffer). The total immunoglobulin (IgG) was then purified from the bovine sera (each SAT serotype specific sera purified separately), using the protein G HP spin trap kit (GE healthcare Life Sciences) as per the manufacturer's protocol. A NanodropND-1000 spectrophotometer (Inqaba Biotechnology) was used to measure the eluted IgG concentration at an absorbance of 280 nm (A_{280nm}). According to Bialuk *et al*., 2011, the absorbance of 1 mg/ml of IgG at 280 nm is 1.35. Thus, all IgG concentrations were calculated as follows: [1 mg/ml x A_{280}]/1.35. Purified IgGs were then stored in 50 µl aliquots at – 20 ºC.

2.14. AFFINITY SELECTION

The three FMDV SAT developed peptide phage display libraries were used in affinity selection *i.e.,* biopanning against the purified IgGs to select for specific peptides that bind

to the target (positive binders). For the first biopanning round, each SAT1, SAT2 and SAT3 derived IgG was biopanned against the corresponding rescued (section 2.11) SAT1, SAT2 and SAT3 peptide phage display library (input). Five selection rounds of biopanning were performed. Phages rescued from the prior biopanning round, became the input for the next round (*i.e.,* round 2 output was used as round 3 input) (Chitray *et al.,* 2020). Two milk powder (MP) controls were used; one was used as a negative control (wells coated with IgG but MP added instead of the phage libraries). The other MP control was used to assess the specificity of the FMDV peptides in the phage libraries to MP (coated with MP followed by the addition of phage libraries). The affinity screening was performed using maxisorp microtiter (Nunc) 96-well immunoplates and was performed as follows: respective plate wells were coated with 40 µg/ml IgG in 1X PBS and 2% (w/v) milk powder in PBS (MPBS), then incubated overnight at 4 °C. The following day, unbound IgG was discarded and 2% (w/v) MPBS was used to block any non-specific binders for 1 hr at room temperature. Thereafter, the plate was washed three times with 1X PBS. The rescued phage libraries (section 2.11) were pre-incubated in 2% (w/v) MPBS with 0.1% (v/v) Tween-20 for 20 min and (50 µl) was then added to each well. To allow phages containing the FMDV peptides to bind to a correlating IgG, the plate was incubated at 37 °C for 1 hr. Unbound and non-specific phages were washed off using a 0.1% (v/v) PBST solution (20 times), followed by a wash step of 1X PBS solution (20 times). Phages were then eluted by addition of the elution buffer (0.1 M Glycine, pH 2.2) to each well and incubated at room temperature for 15 min. The eluate was neutralised by adding a neutralising buffer (1 M Tris-HCl, pH 9). The eluate was then used to infect exponentially growing TG1 cells with an OD_{600} of 0.5 – 0.6. Infection occurred by incubation at 37 ºC for 30 min, followed by another 37 °C incubation while shaking at 100 rpm for 30 min. After incubation, the cells were centrifuged for 15 min at 31 000 xg (Eppendorf 5810 R). The pellet was then resuspended in 2X TY medium and plated onto 150 mm TYE agar plates (input plates) with 100 µg/ml ampicillin and 2% (w/v) glucose. A 10X dilution of the rescued phages in 2X TY was performed and were plated on 90 mm petri dishes (titration plates). All the plates were then incubated at 30 ºC overnight. Colonies on the titration plates were counted the following day to calculate the number of

viable phages [colony forming units (CFU)]. From the 150 mm input plates, colonies were scraped off using 2X TY medium, containing 100 µg/ml ampicillin and 2% (w/v) glucose. This scraped bacterial solution was added to 60% (v/v) glycerol, carefully mixed and stored at – 80 ºC. This process of biopanning was repeated for five selection rounds, where the output of the previous selection round was the input phages for the next selection round. After every biopanning round, the number of phages released was calculated as follows: (Total volume/volume plated) x number CFU x dilution factor.

2.15. POLYCLONAL PHAGE ELISA

To check for phage-displayed peptide enrichment after each selection round of biopanning, a polyclonal ELISA (Van Wyngaardt *et al*., 2004; Mathebula, 2015) was performed. A 96-well nunc maxisorp microtiter (Themofisher), 'test', plate was coated with 40 µg/ml purified IgG (from respective FMDV SAT serotype viruses) in 1X PBS. A second plate was coated with 2% (w/v) MPBS (two MP controls, see section 2.13). The samples were triplicated in wells for consistency and plates were incubated at 4 ºC overnight. The following day, the test and control plate were washed with 0.1% (v/v) PBST, to remove unbound IgG and both plates were blocked with 2% (w/v) MPBS. Thereafter, the plates were incubated at 37 ºC for 1 hr, followed by a wash step [three times with 0.1% (v/v) PBST]. The biopanning inputs, rescued phages, were 10X diluted in 4% (w/v) MPBST, for each round and added into the test and control ELISA plates. Following incubation at 37 ºC for 1 hr, plates were washed three times with 0.1% (v/v) PBST. The addition of, and incubation at 37 ºC for 1 hr with a 1:1000 dilution of the mouse monoclonal filamentous phages M13 fd F1 antibody, B62-FE2 (Acuris antibodies, Germany) in 4% (w/v) MPBST allowed for the detection of phage-displayed peptides. The B62-FE2 MAb binds to an epitope on g8p (phage coat protein). Thereafter, the plates were washed three times with 0.1% (v/v) PBST and a polyclonal rabbit anti-mouse immunoglobulins/HRP, P0260 (Dako, Denmark) (1:1000 in 4% (w/v) MPBST), was added followed by incubating the plates for 1 hr at 37 ºC. A final wash step with 0.1% (v/v) PBST was performed and the plates were developed using a substrate solution consisting of 100 µl of 4 mM 3,3',5,5'-

Tetramethylbenzidine (TMB) (Sigma-Aldrich) and 25 µl of 0.015% (v/v) H_2O_2 in a 10 ml substrate buffer (0.1 M tri-potassium citrate, 0.1 M citric acid monohydrate; pH 4.5). After 10 min, the colour reaction was stopped by 50 µl of 1M H_2SO4 and the plates were placed on a Labsystems Multiscan photometer (Thermo Scientific), to measure the absorbance values at 450nm (A_{450nm}). For the controls used: the negative control showing MP ELISA signals (wells were coated overnight with MP followed by the addition of the phage libraries the next day) were subtracted from the test ELISA result (inputs) to give the final value on the polyclonal ELISA graph. However, the second control was used to determine if there were any binders against MP since MP was used in the biopanning process. For this control, MP ELISA signals (wells coated with IgG overnight, but MP added the next day instead of the phage libraries) were plotted on the graph.

2.16. MONOCLONAL ELISA

Following the fifth round of panning, individual phage clones were randomly selected from the titration plates of each selection round and inoculated into wells of a 96-well tissue culture plate (Nunc, Themofisher) containing 2X TY medium supplemented with 100 µg/ml ampicillin and 2% (w/v) glucose. The clones were grown at 30 ºC overnight, shaking at 100 rpm. Using a metal 96-well plate inoculator (Sigma-Aldrich), bacterial cells were transferred from the overnight plate to a new 96-well tissue culture plate containing 2X TY medium, with 100 µg/ml ampicillin and 2% (w/v) glucose. This was followed by an incubation of the inoculated plate at 37 ºC for 2.5 hrs, shaking at 100 rpm. The overnight plate, with 60% (v/v) glycerol added to it, was stored at – 80 ºC. Following the 2.5 hrs incubation, 2X TY medium containing 100 µg/ml ampicillin, 2% (w/v) glucose and 2 x 10^9 pfu/ml of M13K07 helper phage was added to each well and the plate was incubated at 37 ºC for 30 min. Thereafter, the plate was centrifuged at 1700 xg for 10 min and the supernatant was discarded. The resulting pellet was resuspended with 2X TY medium, supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin. The plate was incubated at 30 ºC overnight, while shaking at 100 rpm to be used in the monoclonal phage ELISA. Simultaneously, a 96-well nunc maxisorp microtiter (Themofisher) plate

was coated with the relevant 40 µg/ml purified IgG (Table 2.4) in 1X PBS and with 2% (w/v) MPBS. The plate was incubated at 4 ºC overnight, in preparation for the monoclonal phage ELISA (section 2.15.1).

2.16.1. MONOCLONAL PHAGE ELISA

The 50 µl overnight IgG coated maxisorp plate was washed using 0.1% (v/v) PBST buffer and the wells were then blocked with the addition of 300 µl of 2% (w/v) MPBS, followed by incubation at 37 ºC for 1 hr, shaking at 100 rpm. After incubation, the maxisorp plate was washed with 0.1% (v/v) PBST and 25 µl of 4% (w/v) MPBST added into each well. Concurrently, the overnight plate containing the phage-displayed peptides was centrifuged at 1700 xg for 10 min. Following centrifugation, 25 µl of the supernatant was then added to the maxisorp ELISA test plate. Following incubation, the plates were washed three times with 0.1% (v/v) PBST, followed by the addition of 1:1000 dilution of the mouse monoclonal antibody B62-FE2 (Acuris antibodies, Germany) in 4% (w/v) MPBST. The phage-displayed peptides were detected by the B62-FE2 MAb as it binds to an epitope on g8p (phage coat protein). Again, the plates were incubated at 37 ºC for 1 hr. After washing the plates three times with 0.1% (v/v) PBST, a polyclonal rabbit anti-mouse immunoglobulins/HRP, P0260 (Dako, Denmark) (1:1000) was added and incubated for an hour at 37 ºC. The control plate for this ELISA underwent all the same steps as the test plate, except it was coated with 2% (w/v) MPBS overnight. A final wash step with 0.1% (v/v) PBST was performed and the plates were developed using a substrate solution consisting of 100 µl of 4 mM 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma-Aldrich) and 25 µl of 0.015% (v/v) H_2O_2 in a 10 ml substrate buffer (0.1 M tri-potassium citrate, 0.1 M citric acid monohydrate; pH 4.5). After 10 min, the colour reaction was stopped by the addition of 50 µl of 1M H_2SO4 and the plates were placed on a Labsystems Multiscan photometer (Thermo Scientific), to measure the absorbance values at 450nm (A_{450nm}). The absorbance values from the 2% (w/v) MPBS control plate were subtracted from the test plate absorbances to obtain the final result. Potential

“positive” clones (with absorbance values greater than 1) were further processed and sent to Inqaba biotechnology for Sanger sequencing for confirmation.

2.17. SEQUENCING

Phage rescue (section 2.11) and plasmid purification (QIAprep spin miniprep kit, QIAGEN, section 2.6) were performed on the output phage selection and potential positive binders (clones). Thereafter, their respective plasmid DNA were sent to Inqaba Biotechnical Industries (Pty) Ltd for Sanger and MiSeq sequencing to confirm the FMDV SAT3 coverage. The potential clones were only sequenced on a Sanger sequencer to determine if sequences correlated with the SAT P1 genomes.

2.17.1. SANGER SEQUENCING

The plasmid DNA (10 ng/µl) were PCR amplified using a OneTaq 2X mastermix with standard buffer (NEB) and 10 µM M13 forward (5’ GTAAACGACGGCCAG 3’) and reverse primers (5’ CAGGAAACAGCTATGAC 3’). The first PCR activation step was at 94 ºC for 5 min. Thereafter, a three-step cycling procedure was performed, which involves denaturation at 94 ºC for 30 sec, primer annealing at 50 ºC for 30 sec and an extension at 68 ºC for 1 min. These cycles were repeated 35 times, to exponentially produce the exact copies of the target DNA (segmented P1 region). Lastly, a final extension step at 68 ºC for 10 min was performed. PCR amplicons’ integrity was visualized on a 1% agarose gel stained with E-Z vision®Bluelight DNA dye and the fast ladder (NEB) was used as a standard marker on all gels. Thereafter, the amplified DNA was excised from the agarose gel and enzymatically purified using exonuclease I (20 U/µl) and shrimp alkaline phosphatases (1 U/µl) (Exo/SAP) master mix (NEB) as per the manufacturers’ protocol. Following purification, using the Zymo Research, ZR DNA sequencing clean-up kit, amplicons were sequenced, using the BrilliantDye™ Terminator cycle sequencing kit (3V.1, BRD3-100/1000, Nimagen) and the ABI 3730*xl* genetic analyser (Applied Biosystems, Thermo Fisher Scientific) with a 50 cm array.

The sequenced data (FMDV SAT3 potential positive clone nucleotide sequences) were analysed using the BioEdit Alignment (Hall, 1999) Sequence Editor software (https://bioedit.software.informer.com/) by inserting and deleting gaps to align to the pCVEP1585042 vector nucleotide sequence. The edited clone sequences were subjected to the NCBI nucleotide to search against a database of FMDV SAT3 published nucleotide sequences. BioEdit was used to translate nucleotide sequences into in-frame amino acid sequences (protein) and identify correct clones.

2.17.2. MiSeq SEQUENCING

Amplicons used for Sanger sequencing were used for Illumina libraries preparation. The Illumina libraries were prepared using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina (NEB), as per the manufacturer's protocol. Thereafter, amplicons were then sequenced on the NextSeq 500 platform (Illumina), using a NextSeq 300 cycle kit (Inqaba Biotech). Data of 300 Mb (2 x 150 bp long paired end reads) were produced for each sample. The sequenced data was analysed using CLC Genomics Workbench v9.5.2 software (Qiagen, Asrhus) and the forward and reverse end match were paired. The paired reads were aligned to their respective reference sequences [National Centre for Biotechnology Information (NCBI)] and the following alignment settings: match score 1, mismatch cost 2, insertion cost 3, deletion cost 3, length fraction 0.8 and similarity fraction 0.8 were used to generate the CLC genomic workbench read mapping graphs (coverage) for the libraries and output phage selection.

CHAPTER 3: RESULTS

3.1. CONSTRUCTION OF FMDV PEPTIDE PHAGE DISPLAY LIBRARIES

3.1.1. FMDV DNA FRAGMENT PREPARATION

To construct the FMDV peptide phage libraries, RT-PCR was used and all three FMDV SAT serotype P1 regions were successfully amplified with sizes of 2322 base pairs (bp) for SAT1/KNP/196/91 and 2220 bp for both SAT2/KNP/19/89 and SAT3/SAR/1/06 (Figure 3.1). The DNA products of interest were purified from the agarose gel and individually pooled (per serotype) for DNA shearing/fragmentation.

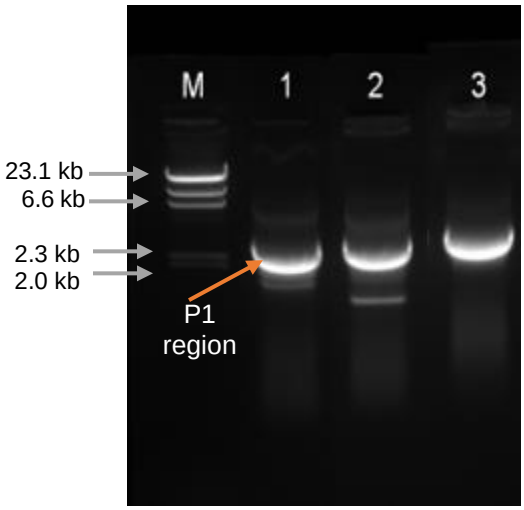


Figure 3.1: A 1% (w/v) agarose gel showing the PCR amplification products of the FMDV SAT1/KNP/196/91, SAT2/KNP/19/89 and SAT3/SAR/1/06 virus strain's, P1 regions that were used in peptide phage display library construction. Lane M in the figure is the Lambda DNA/HindIII marker (Promega) in kilobases (kb). Lanes labelled 1, 2 and 3 represent SAT1, SAT2 and SAT3, respectively. The FMD SAT viruses P1 regions are ~2.2 kilo-bases (kb).

The purified P1 DNA products with concentrations of 174 ng/µl to 222 ng/µl were randomly fragmented. The DNA was mixed with dH_2O and inserted into the covaris fragmentation instrument. To confirm the fragmentation, the sheared DNA was analysed

on a 2.5% (w/v) agarose gel by electrophoresis. A yield of DNA fragments in the 50-200 bp range was observed (Figure 3.2). This fragment range of the FMDV SATs P1 DNA indicated sufficient shearing for the next step in peptide library construction. Unpaired or overhanging bases were removed from the fragments (blunt ended) and the fragments were further dephosphorylated and precipitated before ligation.

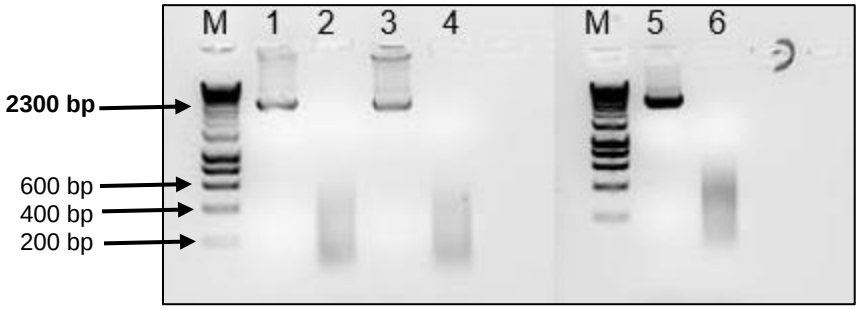


Figure 3.2: A 2.5% (w/v) agarose gel subjected to electrophoresis showing the fragmentation of the FMDV SAT serotypes P1 DNA products. Sheared DNA fragments of 50-200 bp range were observed. Lane M is a 1Kb marker (Bioline). Lanes 1, 3 and 5 represent the unsheared SAT1, SAT2 and SAT3 P1 DNA whilst lanes 2, 4 and 6 represent the sheared SAT1, SAT2 and SAT3 P1 DNA.

3.1.2. PHAGEMID VECTOR PREPARATION

The restriction enzyme, PmeI was used to digest the 3.4 kb phagemid vector, pCVEP1585042, resulting in blunt ends that allowed DNA fragments with blunt ends to be ligated. Agarose gel electrophoresis was performed to confirm the digestion of the vector by PmeI (Figure 3.3). The restriction site (cleaved by PmeI) permits cloning of foreign DNA fragments and the DNA fragments cloned into the phagemid are displayed at the phage surface as a fusion protein to the major protein coat (pVIII) encoded by gene VIII of the M13 filamentous phage.

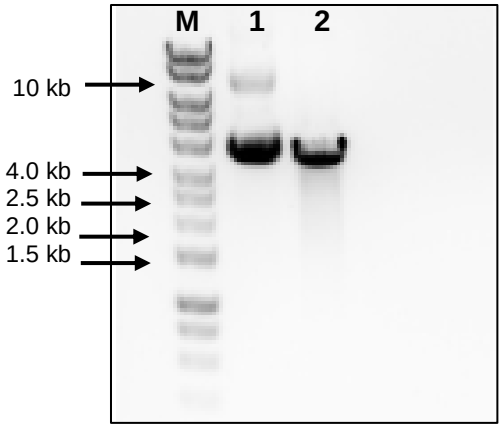
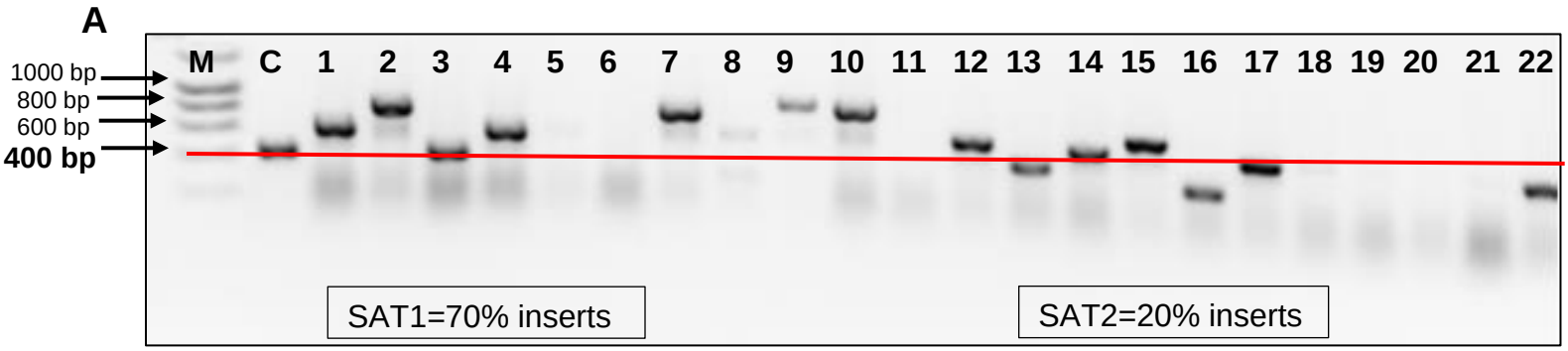


Figure 3.3: A 0.7% agarose gel subjected to electrophoresis showing the vector, pCVEP1585042, restriction enzyme digested with PmeI. Lane M represents the 1 kilobase (kb) marker (Bioline), while lanes 1 and 2 represents the PmeI digested and undigested vector, respectively.

3.1.3. CLONING: DNA LIGATION AND COLONY PCR

To construct the libraries, the blunt ended and dephosphorylated P1 DNA fragments were ligated into the linearized phagemid vector, followed by transformation into TG1 cells (The Mix & Go! *E. coli*, Zymo Research). Colony PCRs were performed, to confirm if the insert of interest were cloned into the vector by comparing the size range of the clones (Figure 3.4).



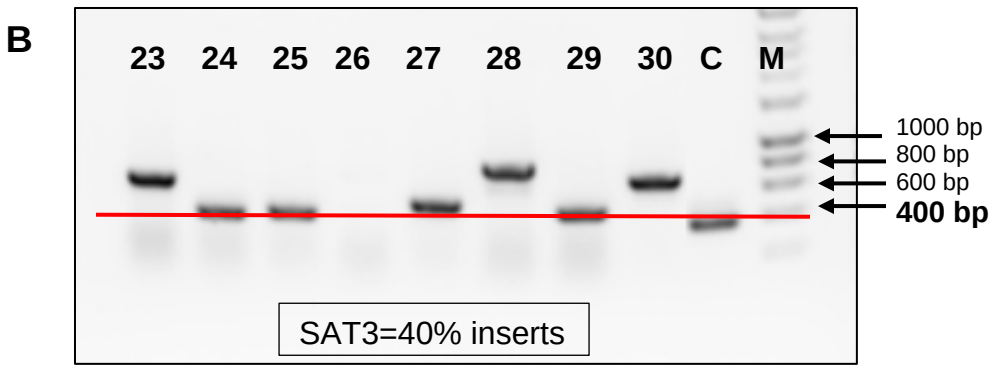


Figure 3.4: A representation of the colony PCR results where the products were separated on a 1% (w/v) agarose gel by electrophoresis (A and B). The insert of interest is present in clones larger than 400 bp in size (red line indicates the 400 bp mark), which included 10 PCR products for each serotype (all results not shown). The control vector is represented by a C (approximately 400 bp), while lane M is the 1kb hyperladder marker (Bioline) in bp. The numbers on the gels represent the lanes, (**A**) lanes 1-10 are SAT1 clones, 11-20 are SAT2 clones; 21-22 are SAT3 clones and (**B**) lanes 23-30 are SAT3 clones.

The control vector, pCVEP1585042 represented by C (Figure 3.4A and B), had no insert and was approximately 400 bp in size, when amplified using the M13 forward and reverse primers. All clones amplified using the same primer set that were bigger than 400 bp in size, indicated clones with inserts (Figure 3.4A and B). The percentage of the insert of interest (> 400 bp) present in 10 PCR products of each serotype was calculated (cloning efficiencies). Taking this result into account, different cloning efficiencies were observed from the libraries *i.e.,* 70% for SAT1; 20% for SAT2 and 40% for SAT3. The cloning was successful and the library size and clone efficiency correlated. The GelAnalyzer result for the average size of the inserts obtained were 339 bp, 107 bp and 196 bp for SAT1, SAT2 and SAT3 respectively. Utilising these results, the number of recombinants needed to cover the P1 region of each FMDV SAT serotype was determined (Table 3.1). Table 3.1 shows the three novel FMDV SAT peptide phage library sizes (in CFU), obtained after library construction.

Table 3.1: The three novel FMDV SAT peptide phage library sizes compared to the theoretically required library sizes for a 99.9% probability representation of the genome.

FMDV serotype	Theoretical library sizes required	Actual library sizes (CFU)	*Genome coverage (P1-capsid coding region)
SAT1	5.19×10^2	1.3×10^3	3X
SAT2	1.74×10^3	7.1×10^2	0.41X
SAT3	9.30×10^2	1.1×10^3	1.2X

* Genome coverage = Actual library size/ Theoretical library size

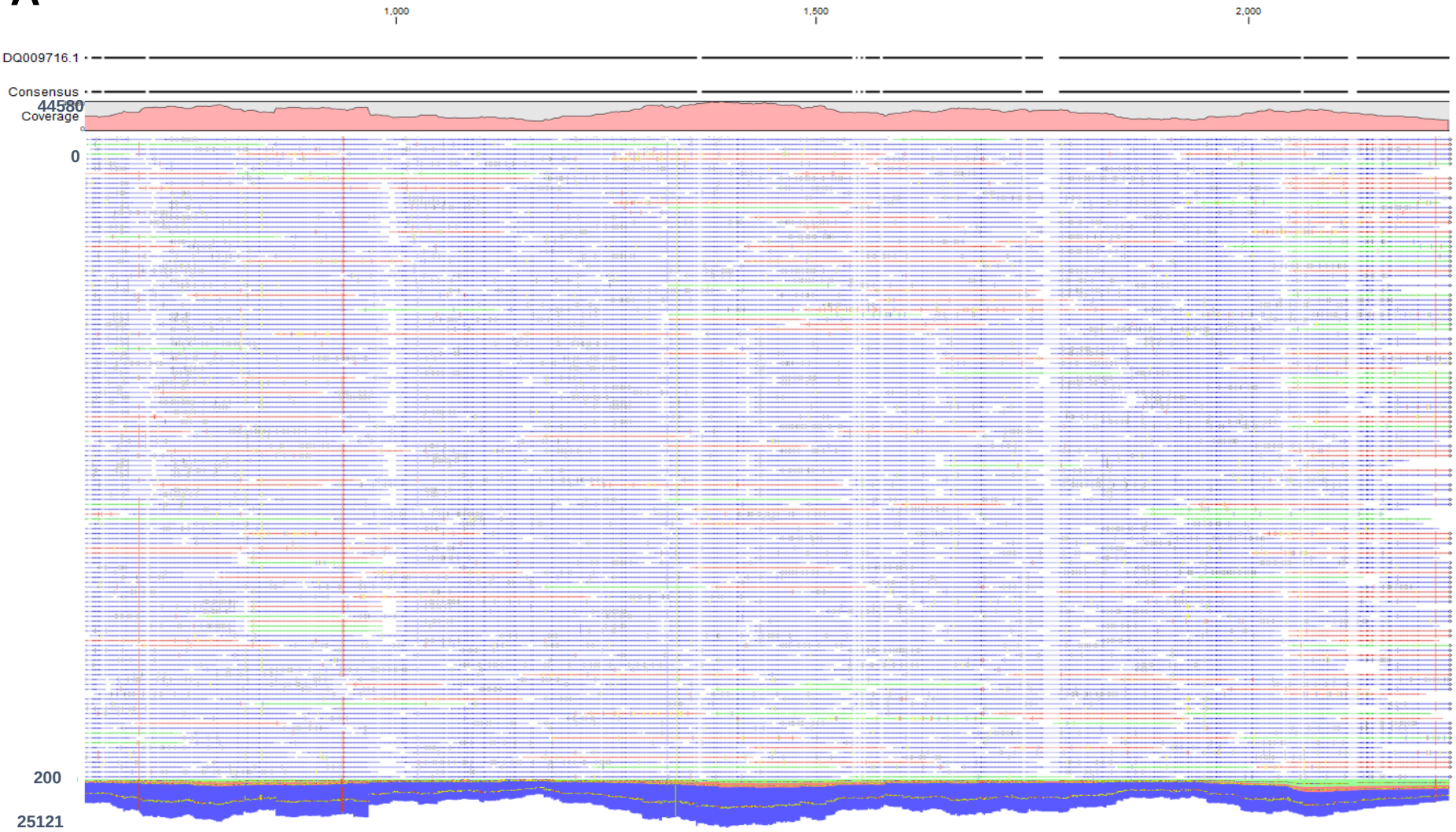
The genome coverage was calculated by dividing the actual library sizes by the theoretical sizes needed. The library sizes and genome coverage were sufficient for a peptide phage library. Amongst the three libraries, SAT1 had the largest P1 region coverage *i.e.,* 3X (1.3×10^3 CFU) that of the theoretical size calculated (Table 3.1). The SAT3 library was the second largest and was 1.2 times (1.1×10^3 CFU) more than the minimum theoretical size needed (genome coverage) (Table 3.1). Furthermore, the SAT2 actual library size of 7.1×10^2 CFU was 0.41 times less than the theoretical library size calculated, which was a minimal difference (Table 3.1).

3.2. MISEQ OF UNPANNED LIBRARIES

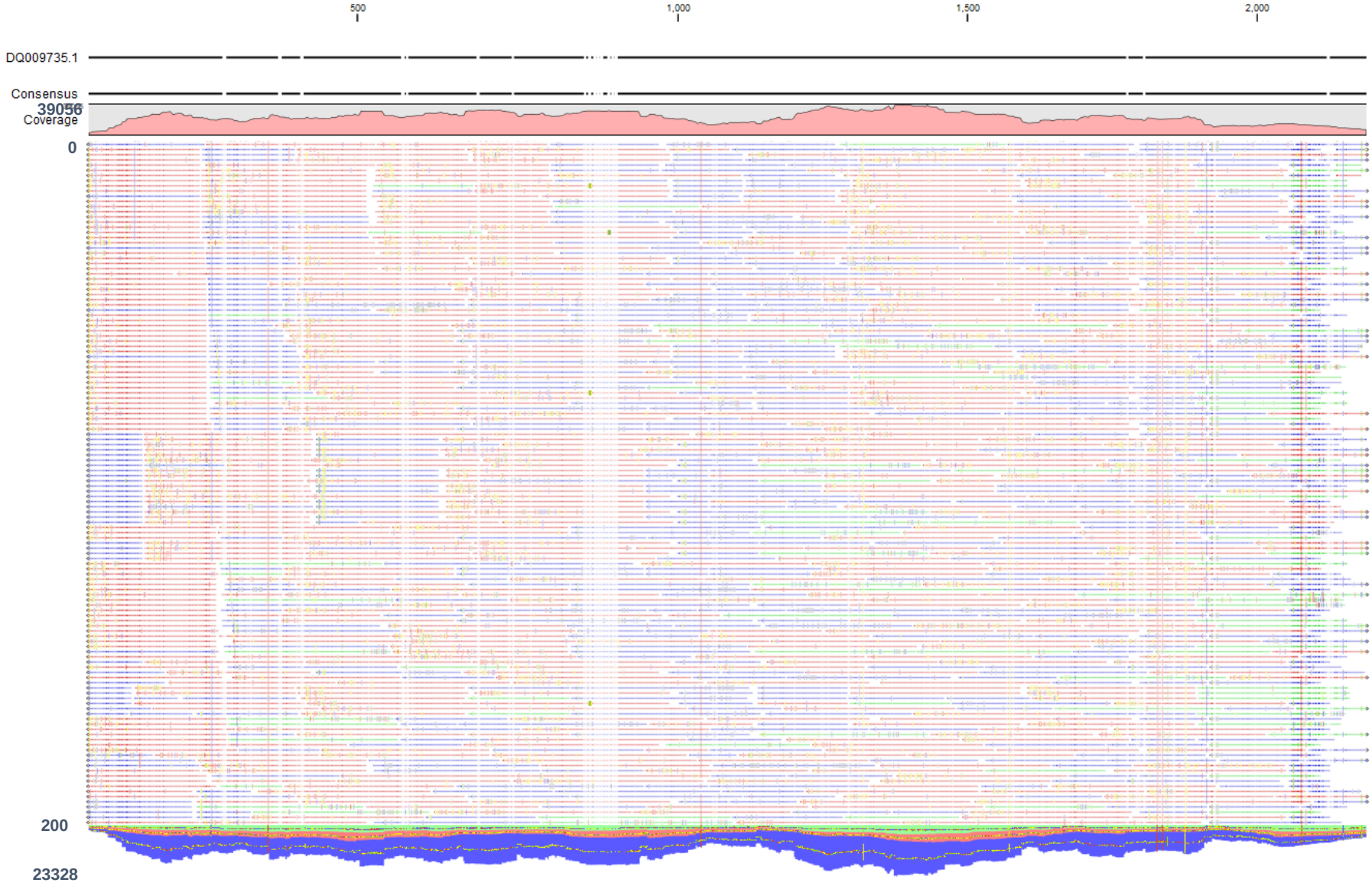
All three FMDV SAT peptide phage libraries contained sufficient clones to proceed to affinity selection. The libraries were first sequenced by high throughput sequencing to further confirm that the entire P1 region was sufficiently represented.

The MiSeq sequencing data for each constructed library was analysed and aligned to their respective FMDV SAT P1 sequence (GenBank) used for the construction. Analysis of this MiSeq alignment revealed library coverage of 44580, 39056 and 48184 for SAT1, SAT2 and SAT3 respectively across the entire SAT P1 regions (Figure 3.5A - C). Additionally, the maximum sequence depth was 25121, 23328, 28845 for SAT1, SAT2 and SAT3 respectively. Thus, it can be concluded that the SAT3 peptide library had the highest library coverage and sequence depth (Figure 3.5A – C).

A


1,000
1,500
2,000
DQ009716.1
Consensus
44580
Coverage
0
200
25121

B


500
1,000
1,500
2,000
DQ009735.1
Consensus
39056
Coverage
0
200
23328

C

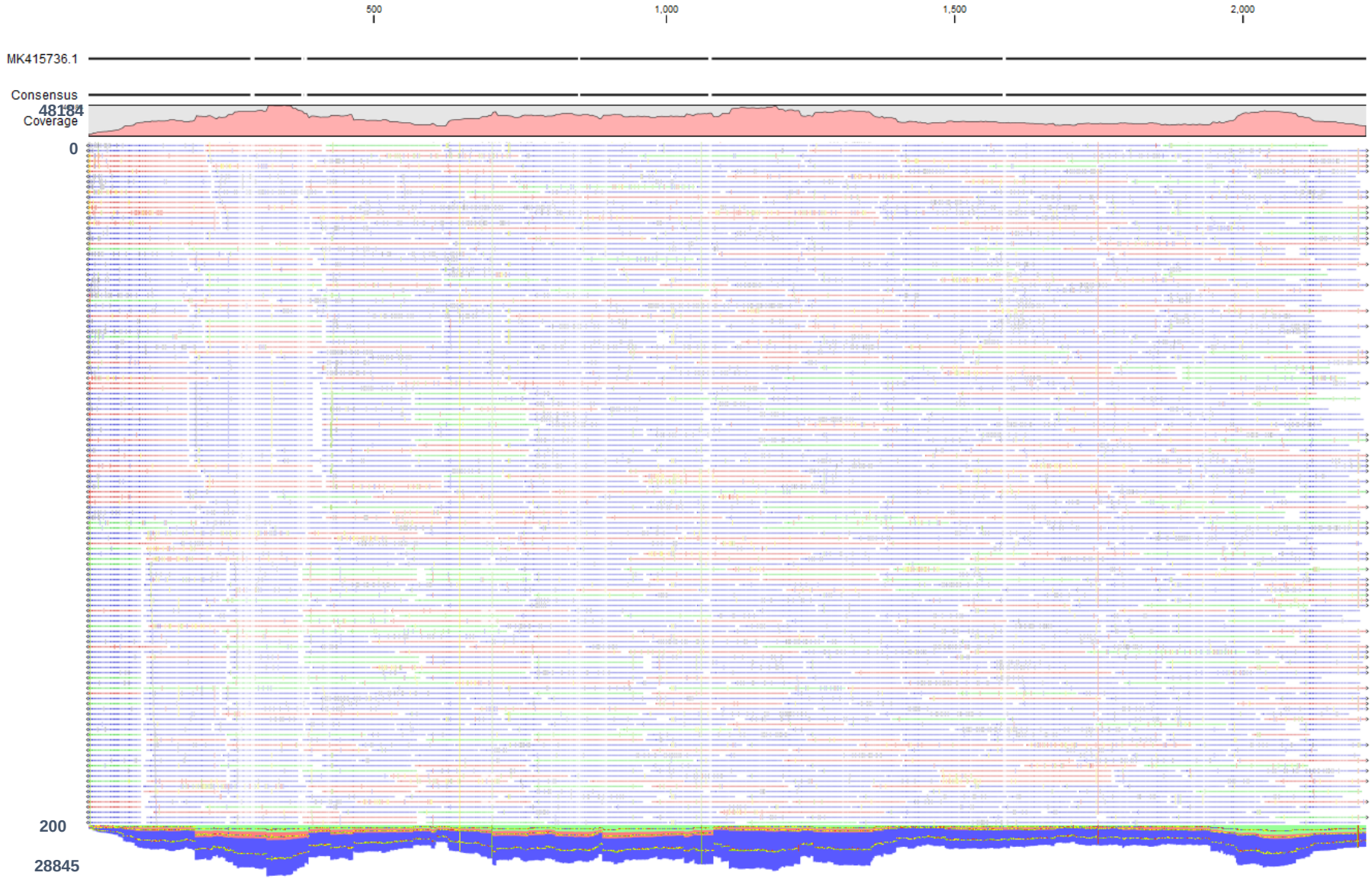

500
1,000
1,500
2,000
MK415736.1
Consensus
48184
Coverage
0
200
28845

Figure 3.5: A CLC genomic workbench read mapping representation of the unpanned FMDV constructed peptide phage libraries aligned against their respective FMDV SAT P1 reference sequence, (**A**) FMDV SAT1, (**B**) FMDV SAT2 and (**C**) FMDV SAT3. The P1 reference sequence is indicated as a bold line at the top. The blue colour indicates a paired-end read, while the green and red colours are the forward and reverse read match, respectively. The sequences from all the libraries revealed library coverage ranging from 39056-48184 and sequence depth of 23328-28845 across the entire SAT P1 regions. Numbers in the top left indicates library coverage, while those in the bottom left represents sequence depth.

Further analysis of the MiSeq data revealed a mapping summary indicating the percentage of mapped reads (Table 3.2) of the constructed FMDV unpanned libraries to be 58, 43 and 51 % for SAT1, 2 and 3 respectively. Moreover, the average mapped reads length ranged from 204 to 217 bp, correlating to the GelAnalyzer results for the average size of clones during library construction (section 3.1.3). The percentage of paired reads ranged from 35 to 53, amongst the FMDV SAT libraries (Table 3.2). The CLC genomic workbench read mapping representation of the three libraries and the mapping summary report correlated with the theoretical library size calculations and clone efficiencies.

Table 3.2: Unpanned FMDV SAT libraries mapping summary report.

FMDV SAT libraries mapping summary	SAT1	SAT2	SAT3
Percentage of mapped reads (%)	58	43	51
Percentage of reads in pairs (%)	53	35	43
Average mapped reads length (bp)	204	217	215

3.3. SOLID PHASE COMPETITION ELISA (SPCE) AND NONSTRUCTURAL PROTEIN (NSP) ELISA

Prior to biopanning, a SPCE and NSP ELISA were performed to detect the presence of antibodies against FMDV structural proteins (SP) and antibodies against the FMD 3ABC non-structural proteins (NSP) in bovine serum samples, respectively (Table 3.3). Animals with an active FMDV infection will produce

antibodies against the SP and NSPs, therefore the NSP ELISA was used as a confirmatory test whilst the SPCE was used to confirm serotype specific infectivity. Additionally, the FMD diagnostic SPCE and NSP ELISA tests were performed to determine the antibody titres of all the FMDV SAT serotype sera samples (Table 3.3) against their respective serotype viruses, before biopanning to ensure the viability of the samples to use for the study. The SPCE results revealed a percentage inhibition (PI) greater than 60 for all sera tested, indicating FMDV sero-positive results. All the SAT1 and SAT2 infected sera samples tested positive for NSP and the SPCE results had a PI range of 62 to 68 and 79 to 81 respectively. For FMDV SAT3, all sera samples tested positive for SPCE, with a PI range of 77 to 83 and only serum from animal lab number 1051 tested negative for NSP. The SPCE positive results confirmed that the sera samples have antibodies against the structural proteins of FMDV (*i.e.,* positive serological response) and positive antibodies against FMDV NSP confirmed exposure to FMDV infection. All tested sera samples were used for IgG purification before biopanning.

Table 3.3: SPCE and NSP ELISA results of the respective FMDV SAT bovine sera samples. The percentage inhibition, (PI) >60 was considered positive.

***LABORATORY ANIMAL IDENTIFICATION NUMBER**	**FMD SPCE DIAGNOSTIC RESULT**			**FMDV NSP DIAGNOSTIC RESULT**
	SEROTYPE	**PI**	**RESULT**	**RESULT**
15-0840	SAT1	65	**POS**	**POS**
15-0847	SAT1	66	**POS**	**POS**
15-0856	SAT1	65	**POS**	**POS**
15-0804	SAT1	62	**POS**	**POS**
15-0897	SAT1	68	**POS**	**POS**
12-0852	SAT2	81	**POS**	**POS**
12-0891	SAT2	78	**POS**	**POS**
12-0833	SAT2	79	**POS**	**POS**
1070	SAT3	83	**POS**	**POS**
1067	SAT3	77	**POS**	**POS**
1060	SAT3	78	**POS**	**POS**

1051	SAT3	78	**POS**	**NEG**

***** Animals were experimentally infected (intradermolingually, previous research study) with the following FMDV strain:
SAT1: SAT1/KNP196/91; SAT2: SAT2/KNP/19/89 and SAT3: SAT3/BOT/6/98. Biobank sera stocks of SAT3/SAR/1/06 were not available at the time of biopanning, thus the available SAT3/BOT/6/98 sera stocks were used instead for biopanning of the SAT3 library.

3.4. BOVINE SERA AND IgG PURIFICATION

Total IgG was extracted from samples confirmed by diagnostic serological analysis as FMDV positive (Table 3.4). The total IgG was purified and yielded good concentrations from all FMDV SAT sera samples (Table 3.4).

Table 3.4: Total IgG concentrations of bovine sera after purification. The sera were obtained from animals experimentally infected with the FMDV SAT serotypes and collected at 28 days post infection (dpi) for SAT1 and 21 dpi for SAT2 and SAT3.

***Virus strains**	**FMDV SAT bovine sera lab animal identification number**	**Total IgG concentration (mg/ml)**	**FMDV infected (intradermolingually)**
SAT1/KNP/ 196/91	15-0840	2	✓
	15-0847	1.17	
	15-0856	1.21	
	15-0804	1.14	
	15-0897	1.08	
SAT2/KNP/ 19/89	12-0852	2.28	✓
	12-0891	1.67	
	12-0833	3.09	
SAT3/BOT/ 6/98	1070	1.86	✓
	1067	2.85	
	1060	2.44	
	1051	1.84	

*The FMD SAT virus strains used to infect the animals (previous research study), where the subsequent sera used to obtain IgG for the biopanning process.

The concentrations obtained ranged from 1.08 mg/ml to 3.09 mg/ml (Table 3.4). These purified total IgGs were individually used to affinity select (biopanning) potential binders from the respective constructed FMDV serotype-specific peptide phage libraries.

3.5. PEPTIDE PHAGE TITRES

Prior to biopanning, the libraries' phage concentrations (number of infectious particles in each peptide phage library) were determined (Table 3.5).

Table 3.5: FMDV SAT peptide phage titres calculated in phages/ml.

FMDV SAT peptide phage library	Phage titre (phage/ml)
SAT1	3.5×10^{12}
SAT2	4.2×10^{12}
SAT3	4.0×10^{12}

All three FMDV SAT libraries had high phage titres, *i.e.,* SAT1 peptide library had a phage titre of 3.5×10^{12} phage/ml, whilst SAT2 and SAT3 libraries had slightly higher titres of 4.2 and 4.0 $\times 10^{12}$ phage/ml respectively. No contamination was observed on the control plate (with TG1 cells) and all three peptide phage libraries had sufficient infectious phages to proceed with the biopanning process.

3.6. AFFINITY SELECTION (BIOPANNING)

The three constructed FMDV serotype-specific SAT peptide phage displayed libraries were screened separately for FMDV specific binders against individually purified IgGs from the bovine sera. Serotype-specific IgGs were panned with the corresponding FMDV serotype peptide phage library. Three to five rounds of biopanning were performed and the number of phages rescued (output phages) after each round was calculated (Table 3.6) to confirm enrichment (the phage increase in subsequent rounds). Enrichment was observed for all the SAT1 IgGs biopanned (Table 3.6). There was no enrichment for the SAT2 IgGs biopanned whilst for the SAT3 IgGs biopanning, only one IgG (SAT3 IgG 1070) showed enrichment (Table 3.6).

Table 3.6: Output phage titres (phage/ml) from biopanning rounds of FMDV SATs peptide phage libraries against the respective purified IgGs. Three to five biopanning selection rounds were performed with each FMDV SAT library, against each IgG.

IgGs Biopanned	Output Phage Titres (Phage/ml) at each biopanning round				
	Round 1	Round 2	Round 3	Round 4	Round 5
SAT1 IgG 15-0840	2.00×10^7	1.50×10^8	1.61×10^8	**	**
SAT1 IgG 15-0847	1.50×10^4	1.04×10^8	4.34×10^7	**	**
SAT1 IgG 15-0856	1.52×10^8	1.46×10^8	2.56×10^8	**	**
SAT1 IgG 15-0804	5.23×10^7	6.60×10^7	6.42×10^7	**	**
SAT1 IgG 15-0897	6.44×10^8	6.90×10^8	2.14×10^8	**	**
SAT2 IgG 12-0852	1.30×10^2	1.24×10^2	1.33×10^2	**	**
SAT2 IgG 12-0891	2.54×10^2	3.12×10^2	3.61×10^2	**	**
SAT2 IgG 12-0833	1.56×10^2	1.50×10^2	1.62×10^2	**	**
SAT3 IgG 1070	1.68×10^6	1.20×10^6	3.50×10^6	3.72×10^6	4.23×10^6
SAT3 IgG 1067	2.43×10^3	1.95×10^2	1.87×10^3	**	**
SAT3 IgG 1060	3.91×10^2	2.58×10^2	3.10×10^2	**	**
SAT3 IgG 1051	1.30×10^2	1.77×10^2	1.93×10^2	**	**

******Biopanning rounds not performed.

3.7. POLYCLONAL PHAGE ELISA

The input phages for each biopanning selection round were used in the polyclonal ELISA. This ELISA further confirms the enrichment observed from biopanning and was performed only for the output titres that showed enrichment (Table 3.6).

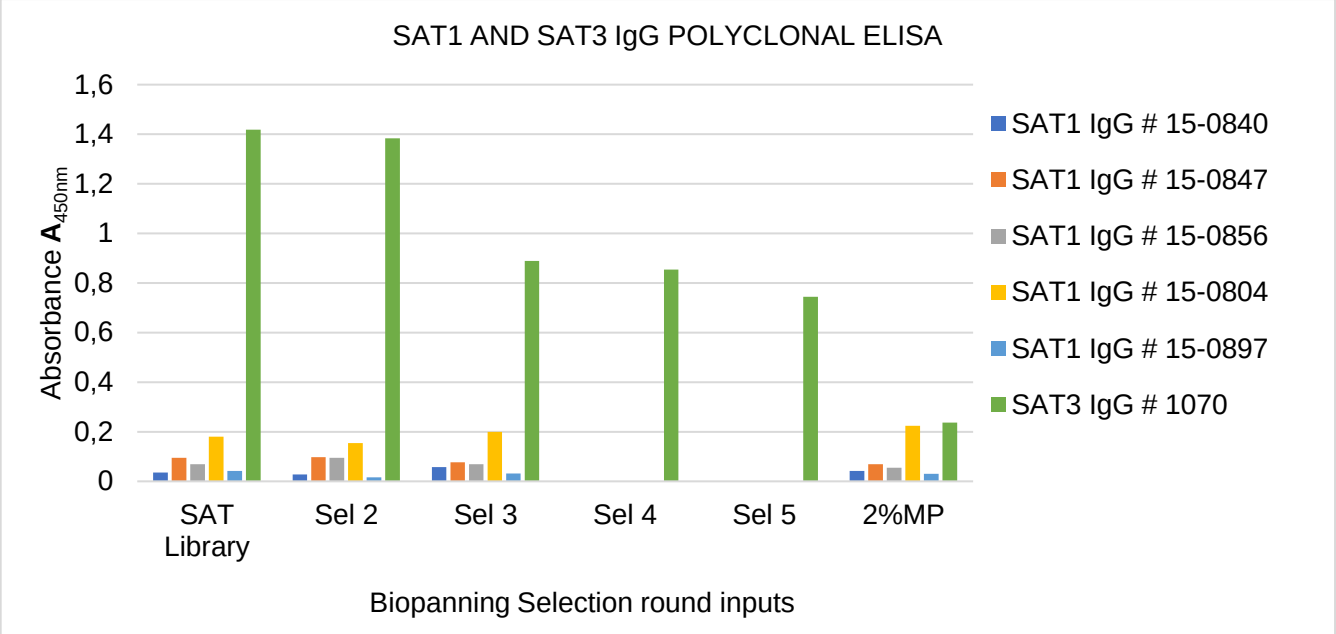


Figure 3.6: Enrichment of phage-displayed FMDV peptides using a polyclonal ELISA. Phage displayed FMDV peptides from the input phages that bound to corresponding IgGs (SAT library biopanning round) and was amplified in subsequent affinity selection (labelled sel) rounds are shown (colour key indicated). Five SAT1 IgGs screened for three affinity selection rounds is shown, as well as one SAT3 IgG screened for five affinity selection rounds. The unpanned aliquot of either the SAT1 or SAT3 peptide phage library (labelled SAT library) was a non-enriched control. The negative control used was 2% milk powder (labelled 2% MP). The ELISA signals were measured at absorbance A_{450nm}.

No enrichment was observed for FMDV SAT1 phage library screened against the respective IgGs where ELISA signals ranged from 0.0165 to 0.2 (Figure 3.6). However, good enrichment was observed for SAT3 IgG 1070 biopanned against the SAT3 peptide library (A_{450nm} 1.42). This IgG had high ELISA signals measured at A_{450nm} and ranged from 0.7 to 1.4 with increased affinity selection. These ELISA results correlate with the biopanning output calculations (Table 3.6). The MP control plotted on the graph (Figure 3.6) had low/negative A_{450nm} signals indicating no MP binders. Due to these results, only SAT3 IgG 1070 was used going forward in this study.

3.8. POTENTIAL FMDV BINDERS

In order to identify the individual positive binders (the phages displaying the FMDV peptides recognized by the SAT3 IgG 1070 and enriched for, during biopanning), a monoclonal ELISA was performed. Individual phage colonies from output phage titration petri dish plates (biopanning rounds) were individually and randomly selected and tested.

A total of 384 phage clones were screened against the SAT3 IgG 1070 and a sample of the results (96 clones) of the monoclonal phage ELISA signals obtained is shown in Figure 3.7. The FMDV SAT3 IgG 1070 ELISA signals A_{450nm} ranged from 0.2 to 1.5 for the phage clones. Phage clones with a strong ELISA signal (1 or higher) were considered as potential positive binders *i.e.,* 19 out of the 384 clones screened.

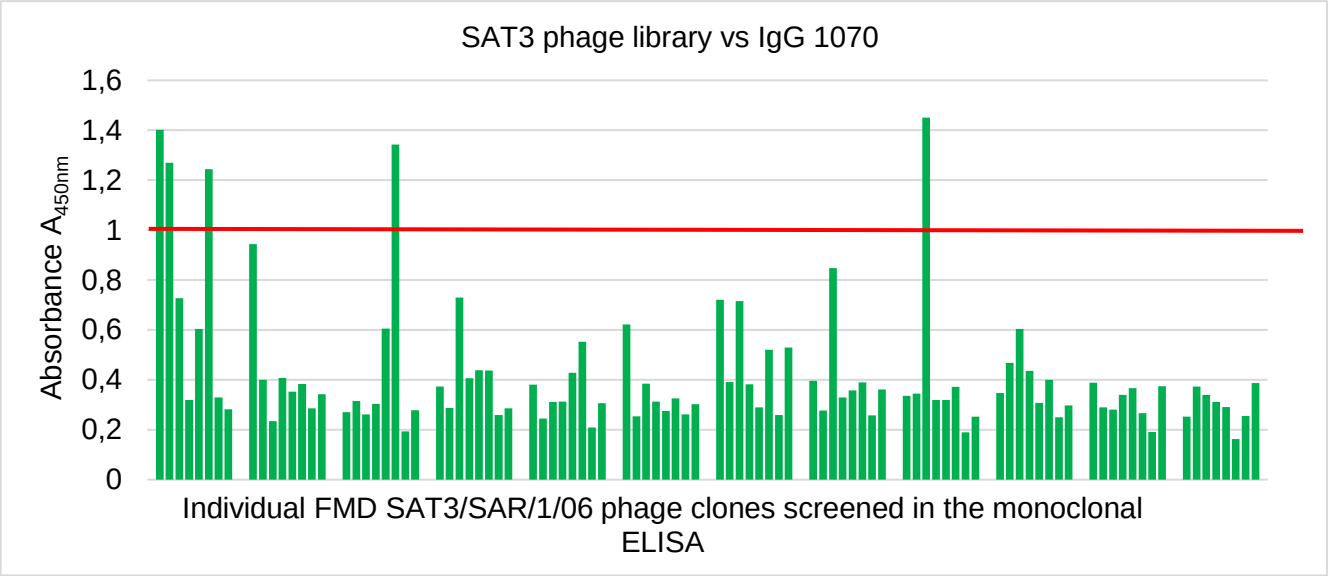


Figure 3.7: Monoclonal ELISA of single phage clones randomly selected following biopanning round five and four titration plates (only one ELISA plate result shown) to identify monoclonal SAT3/SAR/1/06-specific binders. Clones with ELISA signals of ≥1 were chosen as potential positive clones. ELISA signals were measured at absorbance A_{450nm}. These clones were named according to the respective position (well) on the 96-well ELISA plate.

3.9. CONFIRMATION OF POSITIVE BINDERS

To determine the genomic region on the FMDV SAT3 P1 to which the SAT3 IgG 1070 clones bind to, the plasmid DNA of the potential positive binders underwent Sanger sequencing. From the analysis on these sequencing results, three out of the 19 potential positive clones obtained from the monoclonal phage ELISA, aligned to the FMDV SAT3 P1 region. Additionally, these three clones *i.e.,* G2, C8 and B1 (Figure 3.8), had the same sequence. Thus, sequencing confirmed one positive clone obtained for FMDV SAT3 named SAT3φ1.

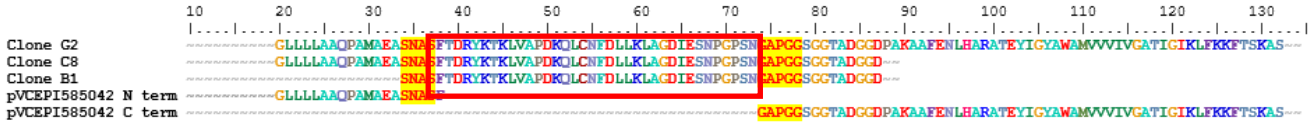


Figure 3.8: Alignment of FMDV SAT3 binders *i.e.,* G2, C8 and B1 sequences to the pVCEPI585042 phagemid vector (BioEdit Alignment Sequence Editor). All three SAT3 binder sequences had the same sequence. The red box shows the sequence of the SAT3 FMDV peptide inserted into the pVCEPI585042 vector during cloning. The sequences flanking the red box for each binder indicates the vector sequence where the restriction enzyme (*PmeI*) cleavage site is highlighted in yellow.

The nucleotide sequences were blasted on the NCBI and the SAT3φ1 sequence matched to 36 FMDV SAT3 published sequences (with various sequence % coverages), where 5 of the 36 had >80% peptide sequence coverage (Figure 3.9). Thus, confirming FMDV SAT3φ1 from this study as a FMDV SAT3 peptide.

Figure 3.9: A graphic overview of the NCBI database of FMDV SAT3 sequences matching and aligning to the FMDV SAT3φ1 sequence when conducting a blast search (NCBI). The horizontal-coloured coded bars represent sequence alignment by % scores and shows the extent match of the alignment to the SAT3φ1 sequence. Alignment scores are colour coded and are shown above the sequence distribution overview.

Since SAT3φ1 was identified through the affinity selection process, it is thus recognized by an FMDV specific antibody. Therefore, SAT3φ1 potentially forms part of a FMDV epitope (Figure 3.8).

Further sequence analysis was done where the sequence of SAT3φ1 obtained after Sanger sequencing analysis was aligned to the SAT3/SAR/1/06 and SAT3/BOT/6/98 virus strains (Figure 3.10). Strain SAT3/SAR/1/06 was used in construction of the peptide phage libraries and for the CLC data alignment, whilst SAT3/BOT/6/98 virus strain was used to infect the animals from which the sera were obtained and subsequently IgG purified and used for the biopanning. The alignment revealed that the SAT3φ1 sequence corresponded to the C-terminus of VP1 encompassing amino acids 726 to 740 of P1, with an overlap to the N-terminus 2A region (amino acids 741 to 761 of P1/2A) (Figure

3.10). Only the P1 sequences for SAT3/SAR1/06 and SAT3/BOT6/98 virus strains are available on GenBank, therefore the N-terminus 2A sequences are not observed in Figure 3.10.

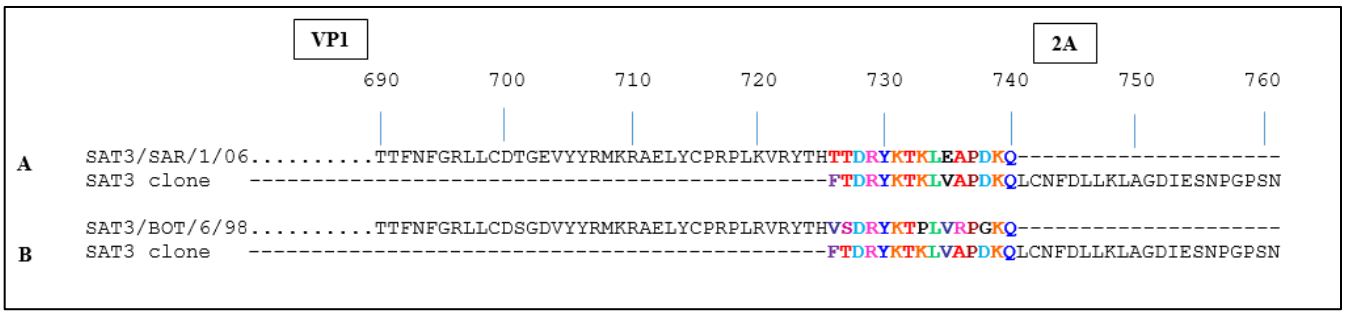


Figure 3.10: Partial FMDV genome P1/2A amino acid sequence alignment of the FMDV SAT3φ1 to published FMD SAT3 virus strains. The SAT3φ1alignment to **(A)** SAT3/SAR/1/06 and **(B)** SAT3/BOT/6/98 virus strains are shown (partial C-terminus P1 sequences, N-terminus 2A sequence not observed). The dots (.) represents a continuous sequence, while the dash (-) represents absent amino acids.

3.10. ILLUMINA SEQUENCING

Illumina MiSeq sequencing was used to confirm the coverage (the number of sequencing reads that were uniquely mapped to the SAT3 reference strain, SAT3/SAR/1/06, sequence and “covered” a known part of the genome) across the FMDV SAT3 P1/2A region from the total output affinity selection round from which SAT3φ1 was obtained. Regions with peaks and coverage of ≥20000 indicated the highest number of reads that aligned to that specific part of the P1/2A region (Figure 3.11).

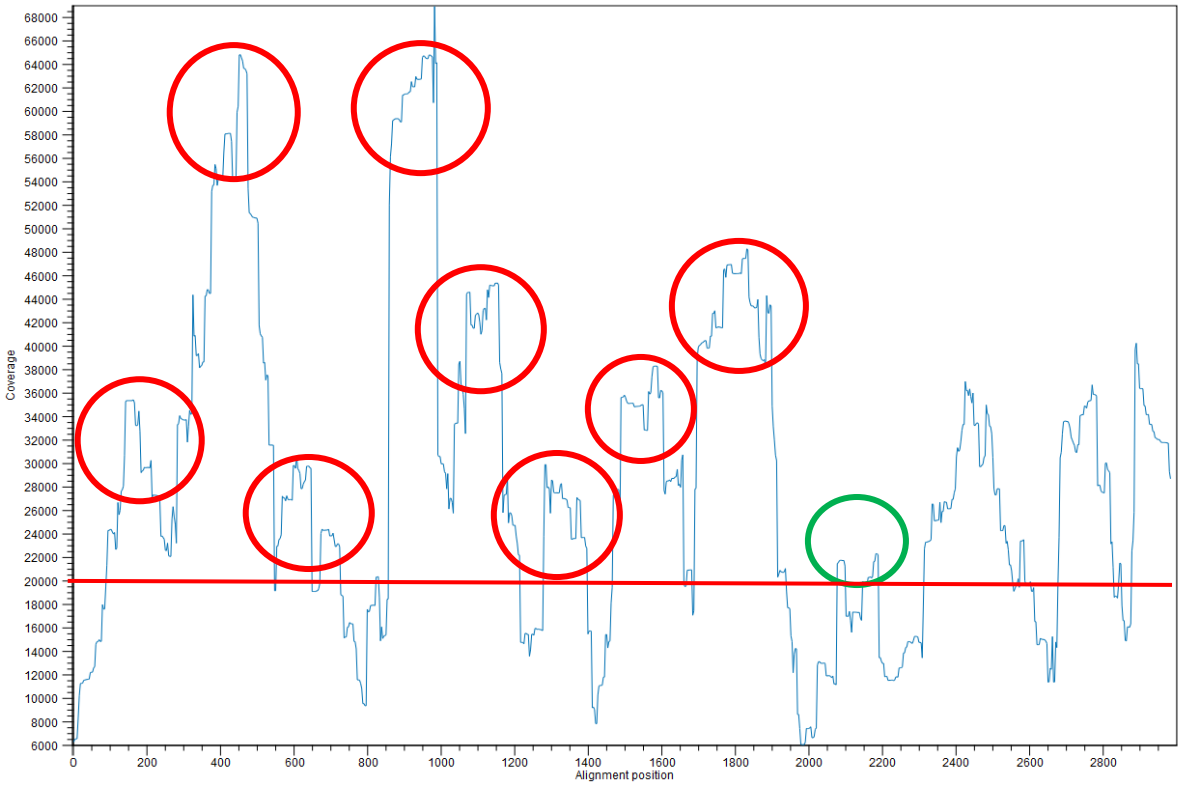


Figure 3.11: The CLC analysis of the Illumina data, showing the coverage across the FMDV SAT3 P1/2A region from the output selection round. Regions with the highest coverage (≥20000) indicate potential epitopic regions. This includes the sequence for SAT3φ1, nucleotide positions at 2178 to 2220 (green circle). Other potential epitopic regions (red circle) include nucleotide position regions (bp) 105 to 200, 401 to 450, 550 to 701, 900 to 1001, 1050 to 1101, 1301 to 1401, 1500 to 1600 and 1701 to 1900 of the FMDV SAT3 P1 region.

These regions are potential FMDV peptides that are recognized by host antibodies during FMDV infection. The peptide regions are thus potential epitopic regions located throughout the P1/2A region. A total of nine potential antigenic sites were identified and the number of amino acids ranged from 14 to 66 amino acids (Table 3.7).

Table 3.7: Potential antigenic sites located on the FMDV SAT3 P1 region.

Genome position	Nucleotide/alignment positions (bp)	Amino acid positions	Number of amino acids
*VP1	1701-1900	567-633	66
VP1	2178-2220	726-740	14

VP2	401-450	134-150	16
VP2	550-701	183-234	51
VP2 to VP3	900-1001	300-334	34
VP3	1050-1101	350-367	17
VP3	1301-1401	434-467	33
VP3 to VP1	1500-1600	500-533	33
VP4	105-200	35-67	32

*VP=Viral protein

No known published research data for amino acid positions 35-67, 134-150, 300-334 and 500-533 (Table 3.7) of the FMDV SAT3 P1 region could be correlated to FMDV antigenic sites. Thus, this study proposes these amino acids as novel potential antigenic sites for FMDV SAT3. Five of the nine potential epitope sites identified corelated in most instances to previously published research (Table 3.8).

Table 3.8: Comparison of identified potential FMDV SAT3 antigenic sites to known published antigenic sites for FMDV.

***Potential antigenic sites and amino acid positions**	**Known antigenic sites**	**FMDV serotypes**
Amino acids 567-633 of P1 (42-108 of VP1)	Site 3 amino acid residues 43-45 and 48 of VP1 (Barnett *et al.,* 1998; Kitson *et al.,* 1990; Grazioli *et al.,* 2013).	O
Amino acids 726-740 of P1 (201-215 of VP1)	Residues 200-213 of the C-terminus of VP1 for serotype O (Xie *et al.,* 1987; Parry *et al.,* 1989; Grazoili *et al.,* 2013) and serotype C (Mateu *et al.,* 1990; Grazoili *et al.,* 2013).	O, C
Amino acids 183-234 of P1 (98-149 of VP2)	Amino acid 208 of VP1 (Mahapatra *et al.,* 2012; Opperman, 2013). VP2 residue 134 (SAT3) (Maake *et al.,* 2020; Mukonyora, 2015). SAT2 and serotype O (Crowther *et al.,* 1993; Grazioli *et al.,* 2013; Maake *et al.,* 2020).	O, SAT2 and SAT3
	Amino acid residues 56 and 58-59, located at the β-B “knob” of VP3	

Amino acids 350-367 of P1 (46-63 of VP3)	(McCullough *et al.,* 1987; Barnett *et al.,* 1998) of serotype O and serotype A, Asia1 and site D3 of serotype C (Thomas *et al.,* 1988; Kitson *et al.,* 1990; Lea *et al.,* 1994; Grazioli *et al.,* 2013).	O, A, Asia1 and C
Amino acids 434-467 of P1 (132-165 of VP3)	Amino acid residue 139, was for Serotype A, (Thomas *et al.,* 1988) and amino acid residue at 135 of VP3 for SAT1 viruses (Grazioli *et al.,* 2013; Maake *et al.,* 2020).	A and SAT1

* Detailed P1/2A amino acid alignment positions are shown in Appendix C.

The sequence alignment of SAT3φ1 with published SAT3 virus strain sequences as well as the potential antigenic sites from Table 3.8 above are shown in Appendix C It is significant to note that SAT3φ1 identified from this study is an important antigenic region (Table 3.8, Appendix C).

CHAPTER 4: DISCUSSION

FMD continues to pose a challenge in cloven-hoofed animals globally (Sobrino *et al.,* 2001). FMD-free countries face constant threat of FMD introduction (Metwally *et al.,* 2021) whilst countries with FMD and especially those that are endemic, find it challenging to control and/or eradicate the disease (Maree *et al.,* 2014). In South Africa alone, there has been 183 outbreaks since 2021 and the country has not re-gained its FMD-free status (DALRRD, 2019; Sirdar *et al.,* 2021; DALRRD, 2023). Thus, there is an immediate need to develop effective vaccines or improve on current vaccines (inactivated vaccines), to avoid the incidence and spread of FMD (Wang *et al.,* 2018). An aspect rendering FMD vaccines less efficient in controlling FMD is the fact that FMDV serotypes display a high degree of antigenic variation (Sobrino *et al.,* 2001). This is practically true for the SAT2 serotype which is antigenically highly diverse (Doel, 2003) and this diversity results in virus variants such that vaccines become less likely to protect against emerging new strains or strains genetically different from the ones utilised for vaccine production (Hwang *et al.,* 2023). Most commonly, the genetic variations occur on the FMDV capsid coding P1/2A region, which encodes the structural proteins, VP1, VP2, VP3 and VP4 (King *et al.,* 1985; Mateu *et al.,* 1995) and was the region of focus in this study. The presentation and arrangement of the viral structural proteins (VP1-VP3) on the outer capsid surface (Acharya *et al.,* 1989) results in the amino acid residues that make up these proteins to be surface exposed and are antigenic regions *i.e.,* the regions that induces an immune response due to FMDV infection or vaccination, thereby determining virus antigenicity (Reeve *et al.,* 2010). The high antigenicity presented by FMDV serotypes leads to the evasion of the host's immune response, posing a challenge on FMD control by vaccination (Rweyemamu *et al.,* 2008b; Maree *et al.,* 2015). Furthermore, knowledge of these amino acid residues that comprises the antigenic determinants (epitopes) of FMDV, would increase our understanding of virus neutralization in *in vivo* studies (Dunn *et al.,* 1998). This can be achieved as these known regions can be incorporated, using reverse genetics technology, to produce recombinant vaccines. Recombinant vaccines that include the identified FMDV epitopes will

allow for a rapid immune response and potentially longer lasting protection, which will be beneficial for FMD control, especially in endemic regions. Producing such recombinant vaccines still needs to be investigated in future studies to improve current FMD vaccines. Thus, towards such research, in this study, the identification of epitopes for the FMDV serotypes plaguing South Africa was of importance. Several studies have been conducted to identify neutralizing epitopes and it was previously believed that the antigenic site situated on the βG-βH loop of VP1 was the sole FMDV antigenic site (Bachrach, 1975; Strohmaier *et al.,* 1982). This conclusion was drawn based on the observation that trypsin cleavage of VP1 resulted in the loss of immunogenicity and the ability of FMDV to attach to host cells (Wild *et al.,* 1969). Nonetheless, the use of monoclonal antibodies (MAbs) and sequencing of MAb neutralization-resistant (MAR) mutants have played a vital role in the identification of amino acid residues responsible for encoding the antigenic sites (Grazioli *et al.,* 2013) of FMDV serotype A, O, Asia-1 and C (Sanyal *et al.,* 1997; Barnett *et al.,* 1998; Thomas *et al.,* 1998a, 1998b; Grazioli *et al.,* 2013; Mahapatra *et al.,* 2011, 2012). Moreover, recombinant antibodies produced from phage display libraries have also been successfully used to map epitopes for serotype O viruses (Harmsen *et al.,* 2007; Yu *et al.,* 2011b) as well as other animal viruses, such as African swine fever virus (Wang and Yu, 2009) and Bluetongue virus (Wang *et al.,* 1995; Qin *et al.,* 2013).

Phage display technology has proven to be a promising approach for providing new strategies, aiding in vaccine production and development (Aghebati-Maleki *et al.,* 2016). This technology involves a gene encoding the desired protein being incorporated into a phage or phagemid genome as a fusion to a gene encoding surface phage coat protein, thereby providing a physical link between the phenotype and genotype (Sparks *et al.,* 1996; Hoogenboom, 1997; 1998). This phage surface representation leads to the identification of several peptides, with great affinity and specificity for any type of target (Arap, 2005). Typically, phage display libraries are screened through an affinity selection (or biopanning) procedure, which involves the exposure of the phage populations to their targets (Sparks *et al.,* 1996; Hoogenboom, 1997; 1998). To determine

the level of enrichment, the enriched biopanning pools are evaluated in a polyclonal ELISA (AC't Hoen *et al.,* 2012; Lim *et al.,* 2019). Subsequently, the phages from the output biopanning rounds are subjected to a selectivity test *i.e.,* monoclonal ELISA, which evaluates the affinity and specificity of the selected phage clones (Pande *et al.,* 2010). The selected phage clones are analyzed by DNA sequencing to identify the targeted region (Arap, 2005; Pande *et al.,* 2010; Lim *et al.,* 2019). In this study, towards the aim of identifying FMDV epitopes, the same phage display strategy was followed where the FMDV P1 known antigenic region was utilised to construct peptide phage libraries for the SAT serotypes.

To date, there is limited information of the FMDV SAT serotypes epitopes (Maree *et al.,* 2011) and this study aimed to address this scarcity of knowledge. The study was successful in constructing three FMDV SAT serotype (SAT1, SAT2 and SAT3) peptide phage display libraries and all three libraries are novel. The construction of peptide phage display libraries can facilitate the discovery of epitopes recognized by antibodies generated during FMDV infection. In order to identify FMDV-specific antigenic determinant/s (epitopes), total IgG was purified from known FMDV infected bovine sera samples and this was used as the target during biopanning (affinity selection) against the constructed FMDV SAT peptide phage libraries. The results have shown the successful use of the constructed peptide phage libraries to identify FMDV SAT epitopic regions.

These FMDV libraries were constructed by cloning the randomly sheared FMDV SAT P1 DNA into a phage vector and transfecting susceptible *E. coli* cells, thus enabling the FMDV peptides to be displayed on the surface of the phage particles. As mentioned above, a phage or phagemid vector is utilised for the peptide library construction *i.e.,* pCVEP1585042 (O'Connell *et al.,* 2002). These vectors are advantageous due to their smaller size, making them more convenient to handle, maintain and propagate. Moreover, phagemids generally enable higher transformation efficiencies in bacterial host cells (Clackson and Lowman, 2004; Peltomaa *et al.,* 2019).

By utilizing these phage libraries, it is possible to identify peptides that mimic epitopes (Aghebati-Maleki *et al.,* 2016) or represent regions of the FMDV capsid proteins, which are the primary targets for neutralizing antibodies (Lea *et al.,* 1995; Reeve *et al.,* 2010). In the construction of such libraries, the DNA shearing step is crucial for generating fragments that can be further used in the library construction process. In this study, Covaris Adaptive Focused Acoustics® technology was employed for DNA shearing, which uses focused acoustic energy to fragment DNA and provides controlled DNA shearing (Apone *et al.,* 2017; Covaris®, 2019). Contrary to other DNA shearing techniques *i.e.,* enzymatic digestion, Covaris technology stands out, with the ability to generate more uniform (no fragmentation biasness) and precise fragment sizes (Covaris®, 2019). Thus, the constructed FMDV SAT libraries will fully represent the randomly fragmented P1 DNA.

The three constructed SAT peptide libraries sizes ranging from 10^2 to 10^3 CFU were considered “small” libraries as Alejaldre *et al.,* (2021) defined small libraries to be less than 10^3 and medium sized libraries to be between 10^3 and 10^5. Theoretically, the bigger and more diverse a library is, the increased possibility there is to identify genes of interest (Ling, 2003; Peltomaa *et al.,* 2019; Bashir and Paeshuyse, 2020). However, although small libraries have limited sequence diversity, they are usually used in a focused screening approach, to select improved variants of known binders (Quartararo *et al.,* 2020). Thus, in order to achieve successful phage library construction with sufficient representativeness of the target peptide, it is imperative that the actual library size exceeds the theoretical library size. Taking this into account, the constructed SAT peptide libraries were considered more than sufficient in size for the purpose of this study as they were on average 1.5 X bigger than their theoretical size.

Ten clones were screened for each SAT peptide phage library constructed and different cloning efficiencies were observed *i.e.,* for SAT1, seven of 10 clones had the insert (70%), whilst 20% of clones (two of 10) for SAT2 and 40% of clones (four of 10) for SAT3 incorporated the insert. The cloning efficiency of SAT2 and SAT3 libraries could have been influenced by the ligation efficiency,

DNA to insert ratio and the concentration of vector or insert. Ligation efficiency refers to the effectiveness of joining the vector and insert DNA fragments together and if the ligation efficiency is low, it can result in a lower cloning efficiency. Moreover, if the DNA to insert ratio is not optimal or the concentration of vector or insert is low, it may lead to lower cloning efficiencies. Thus, it is important to optimize these factors during the cloning process to maximize the efficiency of cloning for a peptide library. For this study, the cloning efficiencies of the SAT2 (20%) and SAT3 (40%) libraries may seem low, but this result is actually attributed to the low number of clones (n=10) tested for each serotype (randomly selected FMDV clones for colony PCR). It is important to note that testing limited number of clones reduces the chances of identifying successful clones with desired peptide inserts and may then underestimate cloning efficiencies, by inadequately assessing the overall representation and diversity of the peptide library. Thus, more clones from each serotype library should be tested to definitively conclude the cloning efficiency. According to Azzazy and Highsmith (2002), the library sizes are directly influenced by the ligation and bacterial transformation step, hence a correlation between library sizes and clone efficiency was observed in this study. Similar to other selection techniques, the success of phage display also relies on the quality or diversity of libraries (Huang *et al.,* 2012). Library quality can be assessed by its performance in selection rounds by calculating the phage increase in subsequent (*i.e.,* enrichment) biopanning round (Carmen and Jermutus, 2002), as well as by checking the number of phages obtained (*i.e.,* clones whose phagemid contains the insert and represents the desired peptide sequences) (Azzazy and Highsmith, 2002). A study of Waterkamp *et al.,* (2006) suggested that library diversity could be increased by enhancing cloning efficiency and transformation techniques or by simply increasing the amount of DNA (DNA concentration) used for cloning. This further provides evidence supporting the correlation between library size, diversity and cloning efficiencies observed in this study for the constructed libraries. The SAT1 library had the highest cloning efficiencies of the three libraries and was the biggest library (in size), with the largest P1 library coverage as well. The contrary was observed for the SAT2 peptide library. Furthermore, the calculated actual library sizes for SAT1 and

SAT3 exceeded the expected theoretical library sizes and was similar for SAT2, indicating that the desired target size was achieved. Another way to more accurately confirm whether the constructed peptide libraries contained the entire respective desired FMDV P1 region, which was applied in this study, is using next generation MiSeq sequencing. This technology enables the determination of the P1 region representation in the constructed libraries.

Illumina high-throughput sequencing (also known as Next Generation Sequencing or NGS) and Sanger sequencing are commonly used molecular biology techniques for DNA or peptide sequencing. However, NGS, particularly Illumina high-throughput sequencing, is favoured because of its unique capability to allow massively parallel DNA sequencing (AC't Hoen *et al.,* 2012; Matochko *et al.,* 2012; Juds *et al.,* 2020; Sloth *et al.,* 2023). This allows for high throughput analysis of multiple samples simultaneously (Liu *et al.,* 2020) and at a lower cost compared to Sanger sequencing (Mardis, 2011; Young and Gillung, 2020). Such an analysis for each unpanned library revealed sufficient coverage and depth across the entire FMDV SAT P1 regions, with minimal gaps (Figure 3.5). In accordance with Sims *et al.,* (2014) and Xu *et al.,* (2017), sequence coverage is commonly defined as the average number of the reference genome/region covered by alignment of reads from a reconstructed sequence. The coverage of the constructed libraries was determined by comparison to the respective reference FMDV P1 region. Furthermore, the read mapping representation and library sequence coverage of the three libraries coincided with the theoretical library sizes and clone efficiencies, with the FMDV SAT2 library having the least P1 coverage over the 2220 bp region. A study of Xu *et al.,* 2017 further indicated that sample size should be increased to improve library coverage. For the peptide phage libraries in this study a single FMD virus strain from each serotype was utilized for construction. Within each FMDV serotype, there is a range of variants (*i.e*., topotypes, subtypes and strains) that exists and have their own distinct antigenic, epidemiological and biological properties (Jamal and Belsham, 2013). Thus, the possibility of identifying more epitopes for the SAT serotypes using peptide phage libraries can be increased by using more FMDV P1 DNA from a variety of antigenically

different strains within a FMDV serotype. Investigating phylogenetic data will assist in choosing strains that are genetically different to include those in future peptide phage library construction. Utilising one viral strain or topotype for phage library construction, increases the possibility of missing epitopes that are unique to other strains or topotypes, within the serotype. This may explain the low number of positive binders for the SAT3 biopanning and no enrichment for the SAT1 and SAT2 biopanning. Homologous SAT1 and SAT2 strains were used for peptide library construction and IgG biopanning, however, for SAT3, heterologous strains were used *i.e*., SAT3/SAR/1/06 for library construction and SAT3/BOT/6/98 sera stocks for biopanning (SAT3/SAR/1/06 biobank sera stocks were not available at that time of this study). It is important to note that the two SAT3 strains utilised are genetically very different as per the VP1 phylogenetic comparisons (results not shown). Additionally, SAT3/SAR/1/06 was an outbreak virus strain in 2006 in South Africa, whilst SAT3/BOT/6/98 occurred in Botswana in 1998. Interestingly, although these strains are so diverse, the SAT3 affinity selection results were successful in this study, proving that peptide libraries should incorporate various strains and should be biopanned against different FMDV serotypes' seropositive sera, to increase the possibilities of epitope identification, especially for an antigenically diverse virus as FMDV, where epitopes can be the same within and between serotypes.

Another crucial parameter to consider for NGS is the sequence depth (or the depth of coverage). This refers to the quantification of how many times a specific nucleotide has been read or sequenced in the sequencing reaction (Alekseyev *et al.,* 2018). In the context of this study, the sequencing depth corresponds to the number of reads that incorporates the displayed peptide-encoding sequence. The three constructed peptide library coverage depths were high and ranged from 2.3×10^4 to 2.9×10^4 X (Figure 3.5A-C). Generally, a higher sequencing depth offers a more representative library coverage, while enhancing the accuracy in characterizing the library composition (Sims *et al.,* 2014). Consequently, this leads to an in-depth understanding of library quality and the involved selection rounds (biopanning) (Sloth *et al.,* 2023). Furthermore, a recent study (Sloth *et al.,* 2023) compared two NGS platforms

with different sequencing depths (high- and low-throughput sequencing), to evaluate their capacity in characterizing the composition, quality and diversity of the unselected Ph.D.TM-12 phage display peptide library. Their results showed that high-throughput sequencing provided an increased library heterogeneity, broader distribution of peptide frequencies and better differentiation between peptides. Thus, concluding that a higher sequencing depth (x 10^6) enabled a more comprehensive understanding of library composition, quality and diversity (Sloth *et al.,* 2023). This shows a direct proportional relationship between library depth and coverage and taking into account the high coverage and depth of all the SAT peptide libraries constructed for the P1 region, it can be concluded that the construction of all three libraries was a successful outcome.

Only a few number of studies have been performed to identify antigenic sites using monoclonal antibodies (MAbs) against SAT1, SAT2 and SAT3 viruses. This approach involves selecting viral mutants that can resist MAb neutralization and subsequent identification of specific amino acid changes responsible for the mutations. The neutralizing MAbs identified four independent antigenic sites for SAT1 and two sites for SAT2 (Grazioli *et al.,* 2006). Moreover, Opperman *et al.,* (2012) and Opperman, (2013) used the Nkuku® phage display naïve single-chain variable fragment (scFv) library to identify a neutralizing epitope (a known FMDV immunogenic region located on the C-terminus of the βG-βH loop) and another potential antigenic sites of VP1 βG-βH loop of SAT2. Another study by Chitray, (2018), utilizing the same scFv library identified additional scFv binders, including one for SAT1, two for SAT3 and nine for serotype A of FMDV and proved that scFvs can be used to identify antigenic regions (Chitray *et al*., 2020). Therefore, in this study it was aimed to expand the phage display work to identify antigenic sites for all three SAT serotypes and use a peptide phage display approach instead of an antibody phage display library. During the enrichment process using the constructed phage libraries against SAT1/KNP/196/91, SAT2/KNP/19/89 and SAT3/SAR/01/06 FMD viruses, one peptide binder was confirmed for SAT3 (named SAT3φ1). Studies have shown that following affinity selection against

other viruses, it is not uncommon to observe a low number of identified binders in phage display, whether using peptide or antibody phage libraries. For instance, a study focused on defining ligands that mimic structural epitopes on the allergen Bet v 1 for a murine monoclonal antibody (BIF4) using peptide phage-displayed libraries, resulted in one positive binder out of 112 tested (Jensen-Jarolim *et al.,* 1998). Similarly, in other studies utilizing the Nkuku® phage-display library, three binders were identified against the *M. tuberculosis* antigen (Sixholo *et al.,* 2011) and two novel scFvs-specific to Bluetongue virus (BTV) were identified (Fehrsen *et al.,* 2005). A limitation of this study that may have contributed to not attaining more binders during affinity selection could be due to the limited availability of animal sera for IgG purification. Future FMD studies should include sera samples not only from FMDV infected animals, but from FMDV vaccinated and infected/challenged animals, as well as increasing the number of animals used. Due to sera samples availability at the ARC-OVR biobank, only FMDV infected sera IgG was biopanned against the three peptide phage libraries. In addition, the sera used were from experimental studies undertaken in the years 2010 and 2011 for SAT1, 2020 for SAT2 and 2015 for SAT3, thus the sera samples were outdated and may mean that FMDV-specific IgG were not present at the time of IgG purification. This might be due to the thaw-freeze cycles over the years and the prolonged refrigeration, as it may reduce serum' stability and performance (R&D Systems, 2023).

Another attribute to consider is the timing of sera collection for the samples used, which was 21 and 28 days post infection (dpi) after experimentally challenging animals with FMDV intradermolingually. According to Pega *et al.,* (2013), the isotype IgG1 titres reaches its peak at 9 dpi (high titres) and remains stable from day 11 to 14, in FMDV infected cattle through aerosol exposure. Similar results were obtained by Collen *et al*., (1989), Salt, (1996), Grumb and Baxt, (2004) and Eschbaumer *et al.,* (2016) for FMDV infected animals. On the other hand, isotype IgG2 levels remained low at 4 to 14 dpi (Pega *et al.,* 2013). Consequently, the collection time points of the sera used in this study may have missed the peak of FMDV-specific IgG response (low concentration), potentially affecting the biopanning process and may have contributed to the low or no

enrichment results in this study. However, for future studies, the selected time points of sera collection, 21 and 28 dpi, could be suitable for studies involving FMDV vaccinated animals, as indicated by Pega *et al*., (2015), where IgG1 and IgG2 isotypes peaked at 21 days post-vaccination (dpv) and remained stable until 29 dpv. It is important to note that the suitability of the sera samples were also validated for the study by performing a FMDV diagnostic SPCE and NSP ELISA. For the purpose of detecting the presence of antibodies against FMDV, a SPCE to detect FMDV structural proteins (SP), whilst a NSP ELISA to detect antibodies against the FMDV 3ABC non-structural proteins (NSP) was performed on the bovine serum samples selected for use in this study. FMDV seropositive animals will produce antibodies against the SP and NSPs, which is what was observed from the results, validating their use in the study (Table 3.3).

Furthermore, the laboratory technique of shearing the FMDV P1 DNA was used to fragment it before cloning into the phage vector. This random DNA fragmentation meant that specific FMDV peptide regions that mimic epitopes (mimotopes) (Muller, 1999) could have been disrupted. Additionally, Barry *et al.*, (2004) showed that one drawback of random fragmentation is, its potential to disrupt conformational epitopes. Subsequently, during affinity selection, if the peptide sequence recognized by a specific IgG purified from the serum sample is not present (in its correct form) in the peptide phage library, no binding will be possible. Thus, although serum samples tested positive for FMD, it is possible that the phage library used did not have the specific epitope region sequence presented during affinity selection. This sequence disruption could explain the identification of one SAT3φ1 in this study. An alternative approach *i.e.,* enzyme-based fragmentation, where DNA cleavage occurs at specific recognition sequences/sites (Apone *et al.,* 2017) could be used. Interestingly, despite the SAT3 peptide phage library not being the largest constructed library, phage enrichment was observed. This observation suggests that the random fragmentation might have had a relatively minor effect on the epitope regions of the SAT3 strain, thus identifying one binder specific to FMDV SAT3.

The infectious phages per ml for each library after phage rescue was high and similar *i.e.,* 3.5-4.2 x 10^{12}, indicating that the same concentration of phage library was used for all affinity selections, thus there was no bias in this regard affecting the biopanning and ELISA outputs. No phage increase (*i.e.,* phage enrichment) was observed from biopannings of SAT1 and SAT2 libraries, thus the polyclonal ELISA was used to further confirm the increase of strong binders following the SAT3 IgG 1070 biopanning process. However, a pattern of increase-decrease was observed from affinity selection round one to five. The first selection round had an output phage titre of 1.68 x 10^6 phage/ml and there was a decrease (in phage/ml) from round one to two, followed by an increase in subsequent rounds. This was not expected as an increase of strong binders from round one to five was expected. However, observations of an increase-decrease trend are not uncommon and have been documented by Roman *et al.,* (2006). The documented biopanning of a phage library against voltage-dependent anion channel (VDAC) liposome (in biosensor by fractionation) resulted in a decrease in output titre following a certain round of selection, followed by a slight increase with the subsequent rounds and verse visa. Following polyclonal ELISA, the monoclonal phage ELISA revealed multiple individual clones from the SAT3 IgG 1070 biopanning selection rounds with ELISA A_{450nm} of 1 or higher representing potential positive binders. These signals were at least three times greater than those observed for the negative control (milk powder) at A_{450nm}. Comparable ELISA results were obtained from a study by Bagheri *et al.,* (2017), where the 4-1BB scFv-phage clones (surface glycoprotein) were scored as positive when the ELISA signals were at least three times higher than those on BSA-coated plates (control). The selected potential positive peptide binders were further analysed by Sanger sequencing and the results confirmed one positive FMDV SAT3 36-mer peptide sequence. The SAT3φ1 thus forms part of the potential epitope binding footprint on the FMDV SAT3 P1 to which the FMDV-specific IgG binds. The nucleotide sequence blast of SAT3φ1 revealed matches to 36 FMDV SAT3 published sequences where five of these sequences had >80% peptide sequence coverage (Figure 3.9), proving that SAT3φ1 identified is a potential part of a FMDV SAT3 epitope. Additionally, the SAT3φ1 sequence aligned to the C-

terminus of the FMDV capsid-coding P1 region of SAT3/SAR/1/06 and SAT3/BOT/6/98 virus strains, the strains specifically used for library construction and IgG purification (Figure 3.10). Specifically, the aligned sequence was to the C-terminus of VP1 and overlapped to the N-terminus of 2A (amino acid position 726 to 740 of P1/2A region). The study results coincide with previous research which demonstrated the C-terminus of VP1 as an epitope for FMDV serotypes A, O, Asia1, SAT1 and SAT2 (Grazioli *et al.,* 2006; Grazioli *et al.,* 2013). However, further studies are needed to confirm whether the C-terminus of VP1 is an epitope for SAT3, as this study is the first known to propose that the C-terminus of VP1 may be an epitope for SAT3. These studies include mapping and characterization of epitopes by analysis in competitive ELISAs, virus neutralization tests and mapping by mass spectrometry (Harmsen *et al.,* 2023). Moreover, the SAT3φ1 sequence overlaps to the N-terminus of the 2A region. Kristensen and Belsham (2019) have reported that the C-terminus of P1/2A includes five residues (YCPRP), which show remarkable conservation, not only among all FMDVs but also across other picornaviruses. Mutant FMDV P1-2A precursors that had single amino acid substitutions within this specific motif displayed a high level of resistance to cleavage at internal junctions (Kristensen and Belsham, 2019). These substitutions also led to the loss of virus infectivity. This particular motif was also observed in the peptide sequence. Consequently, it can be inferred that this motif plays a crucial role in maintaining the correct structure of picornavirus capsid precursors prior to processing and subsequent capsid assembly. This site associated with the P1/2A region, may potentially be involved in interactions with cellular chaperones (Kristensen and Belsham, 2019) and hence of importance. Notably, no antigenic sites have been identified in association with the P1/2A junction and could potentially indicate the presence of a new FMDV SAT3 antigenic site in this region.

Hundreds of clones potentially need to be screened and sequenced before other positive binders (FMDV peptides of immune importance) can be identified, thus, another approach to identify binders for the peptide phage libraries, is to conduct NGS. Therefore, the output selection round from which

the SAT3φ1 was selected underwent IIumina MiSeq sequencing to identify more potential epitopic regions. Illumina sequencing does not provide a specific guideline for the ideal sequencing coverage level. Instead, users typically assess the required coverage based on various factors, such as the nature of the study, gene expression levels, the size of the reference genome, published research literature and established best practices within the scientific community (Illumina, 2014). Thus, based on the size of the reference genome (P1 region ~2220 bp), regions with peaks and coverage of ≥20000 were considered as good coverage, indicating the highest number of reads that aligned to that specific part of the P1/2A region and are possible FMDV peptides enriched from biopanning.

A total of nine potential antigenic sites were identified and these regions are potential SAT3 FMDV peptides that may be recognized by host antibodies during FMDV infection. The peptide regions thus may contain potential epitopic sites located throughout the P1/2A region. Majority of these identified potential epitope sites (five of the nine) correlated in most instances to previously published research. A potential antigenic site at amino acids 42-108 of VP1 (567-633 of P1) correlated with serotype O site 3 that was mapped at amino acid residues 43-45 and 48 of VP1 (Barnett *et al.,* 1998; Kitson *et al.,* 1990; Grazioli *et al.,* 2013). Based on the location of these two sites of VP1 (H-I and B-C loop respectively), it was suggested that these sites could be related (Grazioli *et al.,* 2013). Amino acids 175-209 of VP1 (726-740 of P1) coincided with residues 200-213 of the C-terminus of VP1 that has been reported to contribute to the formation of the discontinuous site coupled with site 1 of serotype O (Xie *et al.,* 1987; Parry *et al.,* 1989; Grazioli *et al.,* 2013). The same site was identified in serotype C (site C) (Mateu *et al.,* 1990; Grazioli *et al.,* 2013). Furthermore, a conformationally dependent and trypsin-sensitive site (site 1 of serotype O), which is located on the βG-βH loop of VP1 also involves amino acid 208 (Mahapatra *et al.,* 2012; Opperman, 2013).

Another potential antigenic site at amino acids 98-149 of VP2 (183-234 of P1), correlated with a known SAT3 antigenic site at residue 134 of VP2 (KNP/1/03 and ZIM/5/91) (Maake *et al.,* 2020). The same site was also identified by

Mukonyora (2015), using *in silico* predictions programmes. This site was previously identified as an antigenic site for FMDV SAT2 and serotype O (Crowther *et al.,* 1993; Grazioli *et al.,* 2013; Maake *et al.,* 2020). Amino acid 46-63 of VP3 (350-367 of P1), coincided with an antigenic site at amino acid residues 56 and 58-59 (located at the β-B “knob” of VP3) that constitutes site 4 of serotype O (McCullough *et al.,* 1987; Barnett *et al.,* 1998). This antigenic site corresponds to site 4 of serotype A, Asia1 and site D3 of serotype C (Thomas *et al.,* 1988a; 1998b; Kitson *et al.,* 1990; Lea *et al.,* 1994; Grazioli *et al.,* 2013). Lastly, amino acids 132-165 of VP3 (434-467 of P1), corresponded to a serotype A identified antigenic site at residue 139 of VP3, forming part of site 4 (Thomas *et al.,* 1988b). Moreover, the use of MAb resistant (MAR) mutants identified amino acid residue at 135 of VP3 to be also of antigenic importance for SAT1 viruses (Grazioli *et al.,* 2013; Maake *et al.,* 2020).

No previously published research information could be correlated with the identified potential antigenic sites from this study at amino acid positions 35-67, 134-150, 300-334 and 500-533 of the FMDV SAT3 P1 region. Thus, this study proposes these amino acids as novel potential antigenic sites for FMDV SAT3.

This study was an advancement in FMDV research to address the scarcity of knowledge regarding the FMDV SAT serotype epitopes by using phage display technology successfully *i.e.,* the construction of three FMDV peptide phage display libraries for SAT1, SAT2 and SAT3 serotypes. This is the first study to use such an approach for the FMDV SAT serotypes. Further studies are underway in other research projects to confirm the potential epitopes identified.

CHAPTER 5: CONCLUDING REMARKS AND FUTURE PROSPECTS

Foot and mouth disease (FMD) has either been effectively managed or eradicated in many parts of the world through preventive vaccination approaches, such as chemically inactivated vaccines (Doel, 2003; Rodriguez and Grubman, 2009). However, FMD is endemic in Southern Africa and there are challenges faced when it comes to control, one of which being the presence of various genetic and antigenic variations within the South African Territories (SAT) serotype viruses (Vosloo *et al.,* 1995; Bastos *et al.,* 2001; Opperman, 2013). Antigenic variation is brought about by alterations in one or more amino acids in the exposed loops of the viral capsid (antigenic drift) or conformational changes (antigenic shift) (Chen and Deng, 2009; Opperman, 2013). Due to the discovery of recombinant vaccines, much research has been placed on the knowledge and identification of specific amino acid residues that constitute the antigenic determinants (epitopes) of FMDV (Mahapatra *et al.,* 2011; Opperman, 2013; Maree *et al.,* 2014; Chitray *et al.,* 2020). Such knowledge is beneficial to enhance our understanding of virus neutralization *in vivo* (Dunn *et al.,* 1998) and is essential for designing recombinant vaccine seed viruses that antigenically matches prevalent emerging viruses (Opperman, 2013; Bari *et al.,* 2014). Producing such vaccines will be achieved by incorporating multiple antigenic sites, leading to the development of vaccines that induces a broad immunogenic response and provide improved, longer-lasting protection in vaccinated animals (Shao *et al.,* 2011; Opperman, 2013; Du *et al.,* 2021). Furthermore, there is still limited information concerning the FMDV SAT serotypes antigenic sites (Maree *et al.,* 2011; Chitray, 2018), with SAT3 in particular having exceedingly limited available information. Therefore, to address this lack of knowledge, this study aimed to construct three FMDV peptide phage display libraries using the fragmented P1 regions of one virus strain of each of the FMDV SAT1, SAT2 and SAT3 serotypes and biopanning them against purified IgGs from FMDV infected bovine sera samples to identify epitopic/antigenic regions. This conclusion will focus on how the aims of the study were achieved, briefly summarizing the new information that was

discovered, the study contribution to FMD research and future proposed research.

Phage library construction was achieved by cloning the randomly sheared FMDV SAT P1 DNA into a phage vector and transfecting susceptible *E. coli* cells. As a result, the FMDV peptides were displayed on the surface of the phage particles. Phage display has been shown to be an exceptionally strong selection technique (Wu *et al.,* 2016) and has been used to map epitopes (Rebollo *et al.,* 2014; Christiansen *et al.,* 2015). For this study, three FMDV SAT serotype (SAT1, SAT2 and SAT3) peptide phage display libraries were successfully constructed and all three libraries are novel. Phage-borne peptides have shown to have a high ability to mimic linear, conformational and non-proteinaceous epitopes (Smith,1991; 1993). Thus, by utilizing these libraries, it is possible to identify peptides that mimic epitopes (Aghebati-Maleki *et al.,* 2016) or represent regions of the FMDV capsid proteins, which are the primary targets for neutralizing antibodies (Lea *et al.,* 1995; Reeve *et al.,* 2010). In depth analysis using Illumina high-throughput sequencing (Miseq) of the constructed phage libraries demonstrated sufficient coverage of the P1 region for each SAT library. Moreover, the library sizes and high throughput analysis for each unpanned library revealed sufficient coverage and sequence depth across the entire FMDV SAT P1 regions. Consequently, this confirmed the effective use of phage display technology to construct peptide phage libraries, with the purpose to identify FMDV epitopes.

Several studies have identified neutralizing antigenic sites for serotype O (Kitson *et al.,* 1990; Crowther *et al.,* 1993), A (Thomas *et al.,* 1988a; Baxt *et al.,* 1989; Bolwell *et al.,* 1989; Mahapatra *et al.,* 2011), C (Mateu *et al.,* 1990) and Asia-1 (Grazioli *et al.,* 2013). However, knowledge regarding epitopes for the SAT serotypes which are geographically restricted to occur in the continent of Africa, remains limited. A few number of studies have been performed to identify antigenic sites of SAT1, SAT2 and SAT3 viruses, using monoclonal antibodies (MAbs). This approach involves selection of viral mutants capable of resisting MAb neutralisation and subsequent identification of specific amino acid changes responsible for the mutations. Notably, four independent antigenic

sites for SAT1 and two sites for SAT2 were identified through this approach (Grazioli *et al.,* 2006). Moreover, Opperman *et al.,* (2012) and Opperman, (2013) used the Nkuku® naïve phage-display single-chain variable fragment (scFv) (small recombinant antibody) library to generate three novel scFv binders specific to the SAT2 serotype, which showed neutralization of one SAT2 virus and resulted in the identification of a known FMDV immunogenic region (C-terminus of the βG-βH loop). Chitray, (2018), also utilized the same scFv library, identifying additional scFv binders including one for SAT1, two for SAT3 and nine for serotype A of FMDV and proved that scFvs can be used to identify antigenic regions. The Nkuku library is an antibody library, whereas this study employs a peptide library. The advantage of utilizing immune sera and biopanning them against virus-specific peptide libraries is that affinity maturation has already occurred in immunized animals and the recognized epitope regions are identifiable. Therefore, this study was aimed to expand the phage display work to identify antigenic sites for all three SAT serotypes, using a peptide phage display as an alternative, opposite approach.

All three constructed FMDV SAT peptide phage libraries were used for biopanning against FMDV serotype specific IgGs purified from FMDV infected sera samples. Through this process and after several biopannings against a number of serotype specific IgGs, no binders resulted for SAT1 and SAT2 biopannings but three positive binders for the SAT3 serotype was obtained. These three SAT3 binders shared an identical nucleotide sequence, thereby confirming one binder sequence for SAT3. Further analysis by Sanger sequencing and performing a nucleotide blast against published FMDV SAT3 sequences, validated SAT3φ1 to be a positive FMDV SAT3 36-mer peptide sequence. Due to the fact that this peptide bound to FMDV serotype specific IgG, it can be concluded that this region is in an antigenic region containing an epitope. SAT3φ1 sequence aligned to the C-terminus of VP1, overlapping to the N-terminus of 2A (amino acid position 726 to 740 of P1/2A region). These research findings are consistent with previous FMD studies that demonstrated the C-terminus of VP1 as an epitope for FMDV serotypes A, O, Asia1, SAT1 and SAT2 (Grazioli *et al.,* 2006; Grazioli *et al.,* 2013). However, it is important

to note that further studies are necessary to conclusively determine whether amino acid position 726 to 740 of the P1/2A region is an immunogenic region. This study is the first to propose this potential result for SAT3 and forms the basis for subsequent research investigations on SAT3 epitopes.

The output biopanning selection round from which the SAT3φ1 was selected, underwent IIumina MiSeq sequencing with the aim of identifying additional potential epitopic regions. The results revealed a total of nine potential antigenic sites/regions at specific positions within the FMDV SAT3 viral proteins: 42-108 and 201-215 of VP1; 49-65, 98-149 and 215-249 of VP2; 46-63, 132-165 and 198-221 of VP3 and 35-67 of VP4. Parts of five of the nine potential FMDV SAT3 antigenic sites correlated with previously published research identifying antigenic sites for different FMDV serotypes. These correlations were observed for serotypes O (Barnett *et al.,* 1998; Kitson *et al.,* 1990; Grazioli *et al.,* 2013), Asia1 (Thomas *et al.,* 1988a; Kitson *et al.,* 1990; Lea *et al.,* 1994; Grazioli *et al.,* 2013), C (Mateu *et al.,* 1990; Grazioli *et al.,* 2013), SAT1 (Grazioli *et al.,* 2013; Maake *et al.,* 2020), SAT2 (Crowther *et al.,* 1993; Grazioli *et al.,* 2013; Maake *et al.,* 2020) and SAT3 (Mukonyora, 2015; Maake *et al.,* 2020). Conversely, the remaining four potential antigenic sites did not show any correlation to known published FMDV antigenic sites. Notably, a portion of the SAT3φ1 peptide overlapped with the N-terminus of the FMDV 2A genomic region. This finding might suggest the possibility of new discoveries regarding antigenic sites of FMDV SAT3.

In future research, focus on identifying new or additional FMDV SATs antigenic sites is proposed by peptide phage library construction using various FMD virus strain from each serotype. It is worth noting that within each FMDV serotype, there is a range of variants *(i.e.,* topotypes, subtypes and strains) that exists and have their own distinct antigenic, epidemiological and biological properties (Jamal and Belsham, 2013). Thus, to increase the possibility of identifying more epitopes for the SAT serotypes, the use of FMDV P1 DNA from a variety of antigenically different strains within a FMDV serotype could be of importance. Moreover, investigating phylogenetic data will assist in the selection of genetically distinct strains and currently circulating strains to include in these

future peptide phage library constructions. It is also proposed that various constructed FMDV SAT peptide libraries should be biopanned against a variety of different IgGs purified from a number of FMDV SAT positive sera samples. It is also crucial to consider the timing and date of sera collection for samples. Utilizing recently collected FMDV infected/vaccinated sera samples at 9-14 dpi and 21-28 dpv is recommended to increase the likelihood of purifying FMDV specific IgG.

Additionally, to prevent disruption with the specific epitope sequences during affinity selection, alternative fragmentation approaches *i.e.,* restriction enzyme digestion may be employed, as this method involves cleaving DNA at specific recognition sequences or sites (Apone *et al.,* 2017). Since this study is the first to propose that the C-terminus of VP1 may be an epitope for SAT3 (P1/2A region), further studies are necessary to confirm whether the C-terminus of VP1 is a SAT3 epitope. These investigative studies include mapping and characterization of epitopes by analysis through methods like competitive ELISAs with synthetic peptides representing the potential epitope, virus neutralization tests and mapping by mass spectrometry (Harmsen *et al.,* 2023). Thus, the novel discoveries in this study points towards exciting directions for future FMD research exploration. In conclusion, as previously mentioned regarding the limited information available on FMDV SAT3 antigenic sites, this study was successful and has made significant contributions towards FMD research as three novel peptide phage libraries were constructed, offering the potential for the screening of other FMDV-specific IgGs and the identification of more antigenic sites and epitopes. Furthermore, the identification of potential antigenic sites specific to FMDV SAT3 in this study has greatly increased our knowledge on FMDV SAT3 antigenic sites and host-virus interactions. This knowledge provides a step forward not only in facilitating the selection of antigenically appropriate vaccines but can be advantageous in the production of improved FMD vaccines. FMD continues to pose a threat in South Africa, as is evident from the recent outbreaks from SAT3 and SAT2 viruses (last outbreak in January 2023). Thus, this study providing evidence for SAT3 antigenic regions is an important contribution for future work towards controlling FMD

outbreaks through recombinant vaccines. Consequently, these vaccines could offer a broad immunogenic response and protection. The availability of such improved vaccines holds significant importance, as it would reduce the frequency of livestock vaccination required by farmers. Given the crucial role of livestock farming in the economy of Southern Africa and the impact of Transboundary Animal Diseases (TADs) like FMD on productivity and trade, these improved vaccines could bring about substantial positive changes. They have the potential to elevate livestock productivity, alleviate poverty and consequently enhance the socioeconomic well-being of disadvantaged communities. As a result, disease control efforts would be positively influenced, offering far-reaching benefits for both livestock and the livelihoods of resource-limited farmers.

REFERENCES

Aarthi, D., Ananda R. K., Robinson, R. and Srinivasan, V. A. (2004). Validation of binary ethyleneimine (BEI) used as an inactivant for foot and mouth disease tissue culture vaccine. *Biologicals: Journal of the International Association of Biological Standardization*. **32**:153-156.

Abubakar, M., Manzoor, S. and Ahmed, A. (2018). Interplay of foot and mouth disease virus with cell-mediated and humoral immunity of host. *Reviews in Medical Virology*. **28**:e1966.

AC't Hoen, P., Jirka, S. M., Ten Broeke, B. R., Schultes, E. A., Aguilera, B., Pang, K. H., Heemskerk, H., Aartsma-Rus, A., van Ommen, G. J. and den Dunnen, J. T. (2012). Phage display screening without repetitious selection rounds. *Analytical Biochemistry*. **421**:622-631.

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

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

Aghebati-Maleki, L., Bakhshinejad, B., Baradaran, B., Motallebnezhad, M., Aghebati-Maleki, A., Nickho, H., Yousefi, M. and Majidi, J. (2016). Phage display as a promising approach for vaccine development. *Journal of Biomedical Science*. **23**:66.

Agol, V. I., Paul, A. V. and Wimmer, E. (1999). Paradoxes of the replication of picornaviral genomes. *Virus Research*. **62**:129–147.

Aktas, S. and Samuel, A. R. (2000). Identification of antigenic epitopes on the foot-and-mouth disease virus isolate O1/Manisa/Turkey/69 using monoclonal antibodies. *Revue scientifique et technique International Office of Epizootics*. **19**:744-753.

Alberts, B., Johnson, A. and Lewis J. (2002). The adaptive immune system. In: *Molecular Biology of the Cell*. 4th edition. New York: Garland Science. Chapter 4.

Alcala, P., Feliu, J. X., Aris, A. and Villaverde, A. (2001) Efficient accommodation of recombinant, foot-and-mouth disease virus RGD peptides to cell-surface integrins. *Biochemical and Biophysical Research Communication*. **285**:201–206.

Alejaldre, L., Pelletier, J. N. and Quaglia, D. (2021). Methods for enzyme library creation: Which one will you choose? A guide for novices and experts to introduce genetic diversity. *BioEssays*. **43**:e2100052.

Alekseyev, Y. O., Fazeli, R., Yang, S., Basran, R., Maher, T., Miller, N. S. and Remick, D. (2018). A next-generation sequencing primer—how does it work and what can it do?. *Academic Pathology*. **5**:2374289518766521.

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

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

Amadori, M., Archetti, I. L., Verardi, R. and Berneri, C. (1992). Isolation of mononuclear cytotoxic cells from cattle vaccinated against foot-and mouth disease. *Archives of Virology*. **122**:293-306.

Amanna, I. J., Slifka, M. K. and Crotty, S. (2006). Immunity and immunological memory following smallpox vaccination. *Immunology Reviews*. **211**:320–337.

Anderson, E. C., Foggin, C., Atkinson, M., Sorensen, K. J., Madekurozva, R. L. and Nqindi, J. (1993). The role of wild animals, other than buffalo, in the current epidemiology of foot-and-mouth disease in Zimbabwe. *Epidemiology & Infection*, **111**:559–563.

Apone, L., Dimalanta, E. and Stewart, F. (2017). *Improving enzymatic dna fragmentation for next generation sequencing library construction*. New England Biolabs. Available from: https://www.neb-online.de/wp-content/uploads/2017/11/Expressions_FeatureArticle_2017_11_DE_UltraII-FS.pdf. [Accessed on 12 February 2023].

Arap, M. A. (2005). Phage display technology: Applications and innovations. *Genetics and Molecular Biology*. **28**:1-9.

Arnold, E., Luo, M., Vriend, G., Rossmann, M. G., Palmenberg, A. C., Parks, G. D, Nicklin, M. J. and Wimmer. E. (1987). Implications of the picornavirus capsid structure for polyprotein processing. *Proceedings of the National Academic of Sciences*. **84**:21–25.

Arzt, J., Pacheco, J. M. and Rodriguez, L. L. (2010). The early pathogenesis of foot-and-mouth disease in cattle after aerosol inoculation: Identification of the nasopharynx as the primary site of infection. *Veterinary Pathology*. **47**:1048-1063.

Arzt, J., Baxt, B., Grumbman, M. J., Jackson, T., Juleff, N., Rhyan, J., Rieder, E., Waters, R. and Rodriguez, L. L. (2011). The pathogenesis of foot and mouth disease II; viral pathways in swine, small ruminants and wildlife; myotropism, chronic syndromes and molecular virus-host interactions. *Transboundary Emerging Diseases*. **58**:305-326.

Arzt, J., Pacheco, J. M., Stenfeldt, C. and Rodroguez, L. L. (2017). Pathogenies of virulent and attenuated foot and mouth disease virus in cattle. *Virology Journal*. **14**:1-5.

Arzt, J., Belsham, G. J., Lohse, L., Bøtner, A., and Stenfeldt, C. (2018). Transmission of foot-and-mouth disease from persistently infected carrier cattle to naive cattle via transfer of oropharyngeal fluid. *mSphere*. **3**:e00365-18.

Arzt, J., Fish, I. H., Bertram, M. R., Smoliga, G. R., Hartwig, E. J., Pauszek, S. J., Holinka-Patterson, L., Diaz-San, Segundo, F. C., Sitt, T., Rieder, E. and

Stenfeldt, C. (2021). Simultaneous and staggered foot-and-mouth disease virus coinfection of cattle. *Journal of Virology*. **95**:e0165021.

Ayebazibwe, C., Mwiine, F. N., Tjornehoj, K., Balinda, S. N., Muwanika, V. B., Okurut, A. R. A., Belsham, G. J., Normann, P., Siegismund, H. R. and Alexandersen, S. (2010). The role of African buffalos (*Syncerus caffer*) in the maintenance of foot-and-mouth disease in Uganda. *BMC Veterinary Research*. **6**:1-13.

Azzazy, H. M. and Highsmith Jr, W. E. (2002). Phage display technology: clinical applications and recent innovations. *Clinical Biochemistry*. **35**:425-445.

Bachler, B.C., Humbert, M., Palikuqi, B., Siddappa, N.B., Lakhashe, S.K., Rasmussen, R.A. and Ruprecht, R.M. (2013). Novel biopanning strategy to identify epitopes associated with vaccine protection. *Journal of Virology*. **87**:4403-4416.

Bachrach, H. L., Trautman, R. and Breese, S. S. (1964). Chemical and physical properties of virtually pure foot-and-mouth disease virus. *American Journal of Veterinary Research*. **25**:333–342.

Bachrach, H. L. (1968). Foot-and-mouth disease. *Annual Review of Microbiology*. **22**:201–244.

Bachrach, H. L., Moore, D. M., McKercher, P. D. and Polatnick, J. (1975). Immune and antibody responses to an isolated capsid protein of foot-and-mouth disease virus. *Journal of Immunology*. **115**:1636-1641.

Bagheri, S., Yousefi, M., Safaie-Qamsari, E., Riazi-Rad, F., Abolhassani, M., Younesi, V., Dorostkar, R., Movassaghpour, A. A. and Sharifzadeh, Z. (2017). Selection of single chain antibody fragments binding to the extracellular domain of 4-1BB receptor by phage display technology. *Tumour Biology*. **39**:1010428317695924.

Bahara, N. H. H., Chin, S. T., Choong, Y. S. and Lim, T. S. (2016). Construction of a semisynthetic Human VH single-Domain antibody library and selection of

domain antibodies against α-crystalline of mycobacterium tuberculosis. *Journal of Biomolecular Screening*. **21**:35-43.

Bai, X. W., Bao, H. F., Li, P. H., Ma, X. Q., Sun, P., Bai, Q. F., Zhang, M., Yaun, H., Chen, D. D., Li, K. and Chen, Y. L. (2019). Engineering responses to amino acid substitutions in the vp0-and vp3-coding regions of panasia-1 strains of foot-and-mouth disease virus serotype O. *Journal of Virology*. **93**:e02278-18.

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

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

Barbas, C. F., Kang, A. S., Lerner, R. A. and Benkovic, S. J. (1991). Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proceedings of the National Academic of Sciences*. **88**:7978-7982.

Bari, F. D., Parida, S., Tekleghiorghis, T., Dekker, A., Sangula, A., Reeve, R., Haydon, D. T., Paton, D. J. and Mahapatra, M. (2014). Genetic and antigenic characterisation of serotype A FMD viruses from East Africa to select new vaccine strains. *Vaccine*. **32**:5794-5800.

Barnett, P. V., Samuel, A. R., Pullen, L., Ansell, D., Butcher, R. N. and Parkhouse, R. M. (1998). Monoclonal antibodies, against O1 serotype foot-and-mouth disease virus, from a natural bovine host, recognize similar antigenic features to those defined by the mouse. *Journal of General Virology*. **79**:1687-1697.

Barnett, P. V., Cox, S. J., Aggarwal, N., Gerber, H. and McCullough, K. C. (2002). Further studies on the early protective responses of pigs following immunization with high potency foot and mouth disease vaccine. *Vaccine.* **20**:3197–3208.

Barry, M. A., Howell, D. P., Andersson, H. A., Chen, J. L. and Singh, R. A. (2004). Expression library immunization to discover and improve vaccine antigens. *Immunological reviews*. **199**:68-83.

Barsa, M., Catley, A., Machuchu, D., Laqua, H., Pout, E., Tap-Kot D. and Ikiror, D. (2008). Foot and mouth vaccination in Sudan: benefit-cost analysis and livelihoods impact. *Transboundary Emerging Diseases*. **55**:339–351.

Barteling, S. J. and Vreeswijk, J. (1991). Developments in foot-and-mouth disease vaccines- Review. *Vaccine*. **9**:75-88.

Barteling, S. J. (2002). Development and performance of inactivated vaccines against foot and mouth disease. *Revue scientifique et technique-Office international des epizooties*. **21**:577-583.

Barteling, S. J. and Sutmoller, P. (2002). Culling versus vaccination: challenging a dogma in veterinary (FMD) science. In: *Report of the research group of the standing technical committee of the European commission for the control of foot-and-mouth disease*. 17-20 September, Izmir, Turkey. Food and Agriculture Organization, Rome (in press).

Bartley, L. M., Donnelly, C. A. and Anderson, R. M. (2002). Short communications—Review of foot-and-mouth disease virus survival in animal excretions and on fomites. *Veterinary Record*. **151**:667–669.

Basavappa, R., Syed, R., Flore, O., Icenogle, J. P., Filman, D. J. and. Hogle, J. M. (1994). Role and mechanism of the maturation cleavage of VP0 in poliovirus assembly: structure of the empty capsid assembly intermediate at 2.9 A resolution. *Protein Science*. **3**:1651–1669.

Bashir, S. and Paeshuyse, J. (2020). Construction of antibody phage libraries and their application in veterinary immunovirology. *Antibodies*. **9**:21.

Bass, S., Greene, R. and Wells, J. A. (1990). Hormone phage: an enrichment method for variant proteins with altered binding properties. *Proteins: Structure, Function, and Bioinformatics*. **8**:309-314.

Bastos, A. D., Haydon, D. T., Forsberg, R., Knowles, N. J., Anderson, E. C., Bengis, R. G., Nel, L. H. and Thomson, G. R. (2001). Genetic heterogeneity of SAT-1 type foot-and-mouth disease viruses in Southern Africa. *Archives of Virology*. **146**:1537–1551.

Bastos, A. D., Haydon, D. T., Sangare, O., Boshoff, C. I., Edrich, J. L. and Thomson, G. R. (2003a). The implications of virus diversity within the SAT 2 serotype for control of foot-and-mouth disease in sub-Saharan Africa. *Journal of General Virology*. **84**:1595–1606.

Bastos, A. D, Anderson, E. C., Bengis, R. G., Keet, D. F., Winterbach, H. K. and Thomson, G. R. (2003b). Molecular epidemiology of SAT3-type foot-and-mouth disease. *Virus Genes*. **27**:283–290.

Basu, K., Green, E. M., Cheng, Y. and Craik, C. S. (2019). Why recombinant antibodies-benefits and applications. *Current Opinion in Biotechnology*. **60**:153-158.

Bautista, E. M., Ferman, G. S. and Golde, W. T. (2003). Induction of lymphopenia and inhibition of T cell function during acute infection of swine with foot and mouth disease virus (FMDV). *Veterinary Immunology and Immunopathology*. **92**:61–73.

Bautista, E. M., Ferman, G. S., Gregg, D., Brum, M. C., Grubman, M. J. and Golde, W. T. (2005). Constitutive expression of alpha interferon by skin dendritic cells confers resistance to infection by foot-and-mouth disease virus. *Journal of Virology*. **79**:4838–4847.

Baxt, B. (1987). Effect of lysosomotropic compounds on early events in foot-and-mouth disease virus replication. *Virus research*. **7**:257–271.

Baxt, B., Vakharia, V., Moore, D. M., Franke, A. J. and Morgan, D. O. (1989). Analysis of neutralizing antigenic sites on the surface of type A12 foot-and-mouth disease virus. *Journal of Virology*. **63**:2143-2151.

Baxt, B. and Mason, P. (1995). Foot-and-mouth disease virus undergoes restricted replication in macrophage cell cultures following Fc receptor-mediated adsorption. *Journal of Virology.* **207**:503–509.

Bazan, J., Całkosiński, I. and Gamian, A. (2012). Phage display—A powerful technique for immunotherapy. *Human Vaccines and Immunotherapeutics*. **8**:1829–1835.

Belsham, G. J., Abrams, C. C., King, A. M. Q., Roosien, J. and Vlak, J. M. (1991). Myristoylation of foot-and-mouth disease virus capsid protein precursors is independent of other viral proteins and occurs in both mammalian and insect cells. *Journal of General Virology*. **72**:747–751.

Belsham, G. and Martinez-Salas, E. (2004). Genome organization, translation and replication of foot-and-mouth disease virus. *Foot-and-Mouth Disease: Current Perspectives*. 19-52.

Belsham, G. J. (2005). Translation and relation of FMDV RNA. *Current Topics in Microbiology and Immunology*. **288**:43-70.

Belsham, G. J., (2020). Towards improvements in foot-and-mouth disease vaccine performance. *Acta Veterinaria Scandinavica*. **62**:1-12.

Belting, M. (2003). Heparan sulfate proteoglycan as a plasma membrane carrier. *Trends in Biochemical Sciences*. **28**:145–151.

Benvenisti, L., Rogel, A., Kuznetzova, L., Bujanover, S., Becker, Y. and Stram, Y. (2001). Gene gun-mediate DNA vaccination against foot-and- mouth disease virus. *Vaccine*. **19**:3885-3895.

Berinstein, A., Roivainen, M., Hovi, T., Mason, P. W. and Baxt. B. (1995). Antibodies to the vitronectin receptor (integrin V3) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. *Journal of Virology*. **69**:2664–2666.

Bernfield, M., Götte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J and Zako, M. (1999). Functions of cell surface heparin sulfate proteoglycans. *Annual Review of Biochemistry*. **68**:729–777.

Berryman, S., Clark, S., Monaghan, P. and Jackson, T. (2005). Early events in integrin v6 mediated cell entry of foot-and-mouth disease virus. *Journal of Virology*. **79**:8519–8534.

Bertram, M. R., Vu, L. T., Pauszek, S. J., Brito, B. P., Hartwig, E. J., Smoliga, G. R., Hoang, B. H., Nguyen, T., Phuong, N. T., Stenfeldt, C., Fish, I. H., Hung, V. V., Delgado, A., VanderWaal, K, Rodriguez, L. L., Long, N. T., Dung, D. H. and Arzt, J. (2018). Lack of transmission of foot-and-mouth disease virus from persistently infected cattle to naïve cattle under field conditions in Vietnam. *Frontiers in Veterinary Science*. **5**:174.

Biswal, J. K., Mohapatra, J. K., Bisht, P., Subramaniam, S., Sanyal, A., Pattnaik, B. (2015). A positively charged lysine residue at VP2 131 position allows for the enhanced adaptability of foot-and-mouth disease virus serotype A in BHK-21 cells. *Biologicals*. **43**:71–78.

Bittle, J. L., Houghten, R. A., Alexander, H., Shinnick, T. M., Sutcliffe, J. G., Lerner, R. A., Rowlands, D. J. and Brown, F. (1982). Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. *Nature*. **298**:30–33.

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

Blank, M., Shoenfeld, Y., Cabilly, S., Heldman, Y., Fridkin, M. and Katchalski, K. E. (1999). Prevention of experimental antiphospholipid syndrome and endothelial cell activation by synthetic peptides. *Proceedings of the National Academic of Sciences*. **96**:5164–5168.

Bolwell, C., Clarke, B. E., Parry, N. R., Ouldridge, E. J., Brown, F. and Rowlands, D. J. (1989). Epitope mapping of foot-and-mouth disease virus with neutralizing monoclonal antibodies. *Journal of General Virology*. **70**:59-68.

Borca, M. V., Fernandez, F. M., Sadir, A. M., Braun, M., and Schudel, A. A. (1986). Immune response to foot-and-mouth disease virus in a murine experimental model: effective thymus-independent primary and secondary reaction. *Immunology*. **59**:261-267.

Brien, P. M. and Aitken, R. (2002). Antibody phage display: methods and protocols. In: *Methods in molecular biology*. Edited by Robert, A. New York: Humana Press. pp 178.

Britannica. (2022). *Antibody*. Available from: https://www.britannica.com/science/antibody. [Accessed on 02 November 2022]

Brito, B. P., Jori, F., Dwarka, R., Maree, F. F., Heath, L. and Perez, A. M. (2016). Transmission of foot-and-mouth disease SAT2 viruses at the wildlife-livestock interface of two major transfrontier conservation areas in Southern Africa. *Frontier Microbiology*. **7**:528.

Brito, B. P., Rodriguez, L. L., Hammond, J. M., Pinto, J., Perez, A. M. (2017). Review of the global distribution of foot-and-mouth disease virus from 2007 to 2014. *Transboundary and Emerging Diseases*. **64**:316–332.

Bronsvoort, B. M., Sørensen, K. J., Anderson, J., Corteyn, A., Tanya, V. N., Kitching, R. P. and Morgan, K. L. (2004). Comparison of two 3ABC enzyme-linked immunosorbent assays for diagnosis of multiple serotype foot-and-mouth disease in a cattle population in an area of endemicity. *Journal of Clinical Microbiology*. **42**:2108–2114.

Brooksby, J. B. and Rogers, J. (1957). Methods used in typing the virus of Foot-and mouth disease at Pirbright, 1950–55. In: *Methods of typing and cultivation of foot-and-mouth disease virus*. Project No. 208. Paris: European Productivity Agency of the Organization of European Cooperation (OEEC).

Brooksby, J. B. (1958). The virus of foot-and-mouth disease. *Advances in Virus Research*. **5**:1–37

Brooksby, J. B. (1972). Epizootiology of foot and mouth disease in developing countries. *World Animal Review*. **3**:10-13.

Brown, C. C., Piccone, M. E., Mason, P. W., McKenna, T. S. and Grubman, M. J. (1996). Pathogenesis of wild-type and leaderless foot-and-mouth disease virus in cattle. *Journal of Virology.* **70**:5638–5641.

Brown, E., Nelson, N., Gubbins, S. and Colenutt, C. (2022). Airborne transmission of foot-and-mouth disease virus: a review of past and present perspectives. *Viruses*. **14**:1009.

Brown, F. and Cartwright, B. (1961). Dissociation of foot-and-mouth disease virus into its nucleic acid and protein components. *Nature*. **192**:1163–1164.

Brown, F. (2003). The history of research in foot and mouth disease. *Virus Research*. **91**:3-7.

Brückner, G. K, Vosloo, W., Du Plessis, B. J., Kloeck, P. E., Connoway, L., Ekron, M. D., Weaver, D. B., Dickason, C. J., Schreuder, F. J., Marais, T. and Mogajane, M. E. (2002). Foot-and-mouth disease: the experience of South Africa. *Revue scientifique et technique-Office international des epizooties*. **21**:751–764.

Burrows, R., Mann, J. A. and Garland, A. J. M. (1981). The pathogenesis of natural and simulated natural foot-and-mouth disease infection in cattle. *Journal of Comparative Pathology*. **91**:599–609.

Burton, D. R. (1990). Antibody: The flexible adaptor molecule. *Trends Biochemistry Science*. **15**:64-69.

Burton, D.R. (2002). Antibodies, viruses and vaccines. *Nature Reviews Immunology*. **2**:706-713.

Buxton, A. and Fraser, G. (1977). *Animal microbiology*. Vol. 2. United Kingdom: Oxford Blackwell Scientific Publications. Available from: https://books.google.co.za/books?id=sBQSzQEACAAJ.

Carmen, S. and Jermutus, L. (2002). Concepts in antibody phage display. *Briefings in Functional Genomics*. **1**:189-203.

Carr, B. V., Lefevre, E. A., Windsor, M. A., Inghese, C., Gubbins, S., Prentice, H., Juleff, N. D. and Charleston, B. (2013). CD4+ T – cell responses to foot and mouth disease virus in vaccinated cattle. *Journal of Virology*. **94**:97-107.

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

Chao, G., Lau, W. L. Hackel, B. J., Sazinsky, S. L., Lippow, S. M. and Wittrup, K. D. (2006). Isolating and engineering human antibodies using yeast surface display. *Nature Protocols*. **1**:755-768.

Charleston, B., Bankowski, B. M., Gubbins, S., Chase-Topping, M. E., Schley, D., Howey, R., Barnett, P. V., Gibson, D., Juleff, N. D. and Woolhouse, M. E. (2011). Relationship between clinical signs and transmission of an infectious disease and the implications for control. *Science*. **332**:726-729.

Chase-Topping, M. E., Handel, I., Bankowski, B. M., Juleff, N. D., Gibson, D., Cox, S. J., Windsor, M. A., Reid, E., Doel, C., Howey, R., Barnett, P. V., Woolhouse, M. E. and Charleston, B. (2013). Understanding foot-and-mouth disease virus transmission biology: identification of the indicators of infectiousness. *Veterinary Research*. **44**:46.

Chen, J. and Deng, Y.M. (2009). Influenza virus antigenic variation, host antibody production and new approach to control epidemics. *Virology Journal*. **6**:1-3.

Childerstone, A. J., Cedillo-Baron, L., Foster-Cuevas, M. and Parkhouse, R. M. (1999). Demonstration of bovine CD8 T-cell responses to foot-and mouth disease virus. *Journal of General Virology*. **80**:663–669.

Chinsangaram, J., Mason, P. W. and Grubman, M. J. (1998). Protection of swine by live and inactivated vaccines prepared from a leader proteinase-deficient serotype A12 foot-and-mouth disease virus. *Vaccine*. **16**:1516–1522.

Chitray, M. (2018). *Improvement of foot-and-mouth disease virus vaccines and diagnostics through structural design.* Doctor of Philosophy (Veterinary Science) Dissertation. University of Pretoria. Pretoria.

Chitray, M., Opperman, P. A., Rotherham, L., Fehrsen, J., van Wyngaardt, W., Frischmuth, J., Rieder, E., Maree, F. F. (2020). Diagnostic and epitope mapping potential of single-chain antibody fragments against foot-and-mouth disease virus serotypes A, SAT1, and SAT3. *Frontiers in Veterinary Science*. **7**:475.

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

Christiansen, A., Kringelum, J. V., Hansen, C. S., Bogh, K. L., Sullivan, E., Patel, J., Rigby, N. M., Eiwegger, T., Szepfalusi, Z., de Masi, F., Neilsen, M. M., Lund, O. and Dufva, M. (2015). High-throughput sequencing enhanced phage display enables the identification of patient-specific epitope motifs in serum. *Scientific Reports*. **5**:12913.

Clackson, T., Hoongenboom, H. R., Griffiths, A. D. and Winter, G. (1991). Making antibody fragments using phage display libraries. *Nature*. **352**:624-628.

Clark, T. G. and Cassidy-Hanley, D. (2005). Recombinant subunit vaccines: potentials and constraints. *Developmental Biology*. **121**:153-163.

Clarke, L. and Carbon, J. (1976). A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire *E. coli* genome. *Cell*. **9**:91-99.

Cloete, M., Dungu, B., Van Staden, L. I., Ismail-Cassim, N. and Vosloo, W. (2008). Evaluation of different adjuvants for foot-and-mouth disease vaccine containing all the SAT serotypes. *Onderstepoort Journal of Veterinary Research*. **75**:17-31.

Colenutt, C., Brown, E., Nelson, N., Wadsworth, J., Maud, J., Adhikari, B., Chapagain, K. S., Upadhyaya, M., Kafle Pandey, K. S., Paton, D. J., Sumption, K. and Gubbins, S. (2018). Evaluation of environmental sampling as a low technology method for surveillance of foot-and-mouth disease virus in an endemic area. *Applied and Environmental Microbiology*. **84**:AEM.00686-18.

Collen, T., Pullen, L. and Doel, T. R. (1989). T cell-dependent induction of antibody against foot-and-mouth disease virus in a mouse model. *Journal of General Virology*. **70**:395-403.

Condy, J. B., Hedger, R. S., Hamblin, C. and Barnett, I. T. (1985). The duration of the foot-and-mouth disease virus carrier state in African buffalo in the individual animal and in a free-living herd. *Comparative Immunology, Microbiology and Infectious Diseases*. **8**:259–265.

Cook, K. D., Waggoner, S. N. and Whitmire, J. K. (2014). NK cells and their ability to modulate t cells during virus infections. *Critical Reviews in Immunology*. **34**:359.

Covaris®. (2019). *DNA shearing for next generation sequencing (NGS) with the m220 focused-ultrasonicator*. Available from: https://www.covaris.com/wp-content/uploads/M020013.pdf. [Accessed on 12 February 2023].

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

Cubillos, C., De La, T. B., Barcena, J., Andreu, D., Sobrino, F., Blanco, E. (2012). Inclusion of a specific T cell epitope increases the protection conferred

against foot-and-mouth disease virus in pigs by a linear peptide containing an immunodominant B cell site. *Virology Journal*. **9**:66.

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

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

de Los Santos, T., Diaz-San, S. F. and Rodriguez, L. L. (2018). The need for improved vaccines against foot-and-mouth disease. *Current Opinion in Virology*. **29**:16-25.

Department of Agriculture, Land Reform and Rural development (DALRRD). (2014). *South Africa's FMD zones*. Available from: http://www.old.dalrrd.gov.za/vetweb/epidemiology/Disease%20Maps/FMD%20zones%202014.pdf. [Accessed on 21 June 2022].

Department of Agriculture, Land Reform and Rural development (DALRRD). (2019). *Foot and Mouth Disease: Molemole outbreak follow up report (27 November 2019)*. Available from: https://www.mpo.co.za/wp-content/uploads/2019/11/FMD-_-Molemole-outbreak-follow-up-report-27-11-2019.pdf. [Accessed on 26 June 2022].

Department of Agriculture, Land Reform and Rural development (DALRRD). (2022). *Foot and mouth disease outbreak and surveillance update report (26 September 2022)*. Available from: http://www.old.dalrrd.gov.za/docs/media/2022-09-26%20FMD%20Outbreak%20Follow-up%20Report..pdf. [Accessed on 26 June 2022].

Department of Agriculture, Land Reform and Rural Development (DALRRD). (2023). *Foot and mouth disease outbreak report (2021-2023)*. Available from:

https://nahf.co.za/wp-content/uploads/2023/03/2023-03-28-FMD-Outbreak-Technical-Report.pdf. [Accessed on 28 June 2023].

Dever, T. E. (1999). Translation initiation: adept at adapting. *Trends in Biochemical Sciences*. **24**:398-403.

Di Nardo, A., Knowles, N. J. and Paton, D. J. (2011). Combining livestock trade patterns with phylogenetics to help understand the spread of foot and mouth disease in sub-Saharan Africa, the Middle East and Southeast Asia. *Revue scientifique et technique International Office of Epizootics*. **30**:63–85.

Diaz-San, S. F., Salguero, F. J., de Avila, A., de Marco, M. M., Sanchez-Martin, M. A. and Sevilla, N. (2006). Selective lymphocyte depletion during the early stage of the immune response to foot-and-mouth disease virus infection in swine. *Journal of Virology*. **80**:2369–2379.

Diaz-San, S. F., Rodriguez, C. T., de Avila, A. and Sevilla, N. (2009). Immunosuppression during acute infection with foot-and-mouth disease virus in swine is mediated by IL-10. *PLoS One*. **4**:e5659.

Diaz-San, S. F., Medina, G. N., Grubman, M. J. and de los Santos, T. (2014). Animal health: foot-and-mouth disease. In: *Encyclopedia of agriculture and food systems*. Edited by Van Alfen, N. K. United State of America: Academic Press. pp 327-345.

Diaz-San, S. F., Medina, G. N., Spinard, E., Kloc, A., Ramirez-Medina, E., Azzinaro, P., Mueller, S., Rieder, E. and de Los Santos, T. (2021). Use of synonymous deoptimization to derive modified live attenuated strains of foot and mouth disease virus. *Frontiers in Microbiology*. **11**:610286.

Dill, V. and Eschbaumer, M. (2020). Cell culture propagation of foot-and-mouth disease virus: adaptive amino acid substitutions in structural proteins and their functional implications. *Virus Genes*. **56**:1–15.

Doel, T. R. (2003). FMD vaccines. *Virus Research*. **91**:81-99.

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

Domingo, E., Escarmıs, C., Martinez, M. A., Martinez-Salas, E. and Mateu, M. G. (1992). Foot-and-mouth disease virus populations are quasispecies. *Current Topics in Microbiology and Immunology*. **176**:33–47.

Domingo, E., Baranowski, E., Escarmis, C. and Sobrino, F. (2002) Foot-and-mouth disease virus. *Comparative Immunology, Microbiology and Infectious Diseases*. **25**:297–308.

Donaldson, A. I., Gloster, J., Harvey, L. D. J. and Deans, D. H. (1982). Use of prediction models to forecast and analyse airborne spread during the foot-and-mouth disease outbreaks in Brittany, Jersey and the Isle of Wight in 1981. *Veterinary Record*. **110**:53–57.

Donaldson, A. I. (1987). Foot-and-mouth disease: the principal features. *Iris Veterinary Journal*. **41**:325-327.

Donaldson, A. I. (1999). *The global status of foot-and-mouth disease and its relevance to control and eradication efforts in South-East Asia*. Report of 33rd session of the European Commission for the Control of Foot-and-Mouth Disease (EUFMD Commission). Appendix 9. Rome.

Doorbar, J. and Winter, G. (1994). Isolation of a peptide antagonist to the thrombin receptor using phage display. *Journal of Molecular Biology*. **244**:361–369.

Du Plessis, D. H. and Jordaan, F. (1996). Phage libraries displaying random peptides derived from a target sequence. In: *Phage Display of Peptides and Proteins: A Laboratory Manual*. Edited by Kay, B. K., Winter, J. and McCafferty, J. San Diego: Academic Press. pp 141-150.

Du, L., Hou, L., Yu, X., Cheng, H., Chen, J., Zheng, Q. and Hou, J. (2021). Pattern-recognition receptor agonist-containing immunopotentiator cvc1302 boosts high-affinity long-lasting humoral immunity. *Frontiers in Immunology*. **12**:697292.

Dunn, C. S., Samuel, A. R., Pullen, L. A. and Anderson, J. (1998). The biological relevance of virus neutralisation sites for virulence and vaccine protection in the guinea pig model of foot-and-mouth disease. *Virology*. **247**:51-61.

Dyason, E. (2010). Summary of foot-and-mouth disease outbreaks reported in and around the Kruger National Park, South Africa, between 1970 and 2009. *Journal of South African Veterinary Association*. **81**:201–216.

Edelman, G. M. and Benacerraf, B. (1962). On structural and functional relations between antibodies and Proteins of the gamma-system. *Proceedings of the National Academic of Sciences*. **48**:1035-1042.

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

Elnekave, E., Dekker, A., Eble, P., van Hemert-Kluitenberg, F., Gelman, B., Storm, N. and Klement, E. (2016). The serological response against foot and mouth disease virus elicited by repeated vaccination of dairy cattle. *Vaccine*. **34**:4920-4926.

Eschbaumer, M., Stenfeldt, C., Rekant, S. I., Pacheco1, J. M., Hartwig, E. I., Smoliga, G. R., Kenney, M. A., Golde, W. T., Rodriguez, L. L. and Arzt, J. (2016). Systemic immune response and virus persistence after foot-and-mouth disease virus infection of naïve cattle and cattle vaccinated with a homologous adenovirus vectored vaccine. *BMC Veterinary Research*. **12**:205.

Fehrsen, J., Van Wyngaardt, W., Mashau, C., Potgieter, A. C., Chaudhary, V. K., Gupta, A., Jordaan, F. A. and Du Plessis, D. H. (2005). Serogroup-reactive

and type-specific detection of bluetongue virus antibodies using chicken scFvs in inhibition ELISAs. *Journal of Virological Methods*. **129**:31-39.

Feng, H. H., Zhu, Z. X., Cao, W. J., Yang, F., Zhang, X. L., Du, X. L., Zhang, K. S., Liu, X. T. and Zheng, H. X. (2018). Foot and mouth disease virus induces lysosomal degradation of NME1 to impair p53-regulated interferon-inducible antiviral genes expression. *Cell Death and Disease*. **9**:885.

Ferguson, N. M., Donnelly, C. A. and Roy, M. (2001). Transmission intensity and impact of control policies on the foot and mouth epidemic in Great Britain Anderson. *Nature* **413**:542–548.

Fernandez-Sainz, I., Gavitt, T.D., Koster, M., Ramirez-Medina, E., Rodriguez, Y.Y., Wu, P., Silbart, L.K., de Los Santos, T. and Szczepanek, S.M. (2019). The VP1 GH loop hypervariable epitope contributes to protective immunity against Foot and Mouth Disease Virus in swine. *Vaccine*. **37**:3435-3442.

Ferris, N. P. and Donaldson, A. I. (1992). The World Reference Laboratory for foot and mouth disease: a review of thirty-three years of activity (1958-1991). *Revue scientifique et technique International Office of Epizootics.* **11**:657-684.

Fish, I., Stenfeldt, C., Spinard, E., Medina, G. N., Azzinaro, P. A., Bertram, M. R., Holinka, L., Smoliga, G. R., Hartwig, E. J., de Los Santos, T. and Arzt J. (2022). Foot-and-mouth disease virus interserotypic recombination in superinfected carrier cattle. *Pathogens*. **11**:644.

FMD Bulletin. (2010). *Bulletin Foot-and-Mouth Disease: Southern Africa*. Available from: http://www.afrivip.org/education/livestock/diseasebulletins/fmd-bulletin/2014. [Accessed on 18 May 2021].

Fracastorius, H. (1546). De alijs differentijs contagionis. *De Sympathia et Antipathia Rerum Liber Unus. Decontagione et Contagiosis Morbis et Eorum Curatione (libri III)*, Venice: Heirs of L. A. Junta Book 1. pp 36-38.

Francis, M. J., Ouldridge, E. J. and Black, L. (1983). Antibody response in bovine pharyngeal fluid following foot-and-mouth disease vaccination and/or, exposure to live virus. *Research in Veterinary Science*. **35**:206-210.

Frei, J. C. and Lai, J. R. (2016). Protein and antibody engineering by phage display. *Methods in enzymology*. **580**:45-87.

Frenken, L. G., van der Linden, R. H. J., Hermans, P. W. J., Bos, J. W., Ruuls, R. C., de Geus, B. and Verrips, C. T. (2000). Isolation of antigen specific llama VHH antibody fragments and their high-level secretion by Saccharomyces cerevisiae. *Journal of Biotechnology*. **78**:11-21.

Frenzel, A., Kügler, J., Helmsing, S., Meier, D., Schirrmann, T., Hust, M., and Dübel, S. (2017). Designing Human Antibodies by Phage Display. *Transfusion Medicine Hemotherapy*. **44**:312–318.

Fry, E. E., Stuart, D. I., Rowlands, D. J. (2005a). The structure of foot-and-mouth disease virus. *Current Topics in Microbiology and Immunology*. **288**:71–101.

Fry, E. E., Newman, J. W., Curry, S., Najjam, S., Jackson, T., Blakemore, W., Lea, S. M., Miller, L., Burman, A., King, A. M. and Stuart, D. I. (2005b). Structure of foot-and-mouth disease virus serotype $A10_{61}$ alone and complexed with oligosaccharide receptor: receptor conservation in the face of antigenic variation. *Journal of General Virology*. **86**:1909–1920.

Fujii, S. I., Liu, K., Smith, C., Bonito, A. J. and Steinman, R. M. (2004). The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 co-stimulation. *Journal of Experimental Medicine*. **199**:1607–1618.

Gainaru, M.D., Esterhuysen, J.J., Bruce, W., Pini, A. and Bengis, R.G., (1986). Foot-and-mouth disease and the African buffalo (*Syncerus caffer*) II. Virus excretion and transmission during acute infection. *Onderstepoort Journal of Veterinary Research*. **53**:75-85.

Gao, Y., Sun, S. and Guo, H. (2016). Biological function of foot-and-mouth disease virus non-structural proteins and non-coding elements. *Virology Journal*. **13**:107.

Garcia-Valcarcel, M., Doel, T., Collen, T., Ryan, M. and Parkhouse, R. M. (1996). Recognition of foot-and-mouth disease virus and its capsid protein VP1 by bovine peripheral T lymphocytes. *Journal of General Virology*. **77**:727–735.

Garrard, L. J., Yang, M., O'Connell, M. P., Kelley, R. F. and Henner, D. J. (1991). Fab assembly and enrichment in a monovalent phage system. *Biotechnology*. **9**:1373–1377.

Gerner, W., Carr, B. V., Wiesmuller, K. H., Pfaff, E., Saalmuller, A. and Charleston, B. (2007). Identification of a novel foot-and-mouth disease virus specific T-cell epitope with immunodominant characteristics in cattle with MHC serotype A31. *Veterinary Research*. **38**:565-572.

Giavedoni, L. D., Kaplan, G., Marcovecchio, F., Piccone M. E. and Palma L. E. (1991). Protection conferred by TrpE fusion proteins containing portions of the C -terminal region of capsid protein VP1 of foot -and-mouth disease virus. *Journal of General Virology*. **72**:967-971.

Gingras, A. C., Raught, B. and Sonenberg, N. (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annual Review of Biochemistry*. **68**:913-963.

Glass, E. J. and Millar, P. (1994). Induction of effective cross-reactive immunity by FMDV peptides is critically dependent upon specific MHC peptide-T cell interactions. *Immunology*. **82**:1-8.

Golde, W. T., Nfon, C. K. and Toka, F. N. (2008). Immune evasion during foot-and-mouth disease virus infection of swine. *Immunological Reviews*. **225**:85-95.

González-Amaro, R. and Sánchez-Madrid, F. (1999). Cell adhesion molecules: selectins and integrins. *Critical Reviews in Immunology*. **19**:389–429.

Grazioli, S., Moretti, M., BarbierI, I., Crosatti, M. and Brocchi, E. (2006). Use of monoclonal antibodies to identify and map new antigenic determinants involved in neutralization of FMD viruses type SAT 1 and SAT 2. *Paphos, Cyprus: Food and Agriculture Organization.* 287-297.

Grazioli, S., Fallacara, F. and Brocchi, E. (2013). Mapping of antigenic sites of foot-and-mouth disease virus serotype Asia 1 and relationships with sites described in other serotypes. *Journal of General Virology*. **94**:559-569.

Gregg, D. A., Schlafer, D. H. and Mebus, C. A. (1995). African swine fever virus infection of skin-derived dendritic cells in vitro causes interference with subsequent foot-and-mouth disease virus infection. *Journal of Veterinary Diagnostic Investigation*. **7**:44–51.

Grubman M. J., Baxt, B. and Barach, H. L. (1979). Foot-and-mouth disease virion RNA: studies on the relation between the length of its 3'-poly(A) segment and infectivity. *Virology*. **97**:22-31.

Grubman, M. J. (1980). The 5' end of foot-and-mouth disease virion RNA contains a protein covalently linked to the nucleotide pUp. *Archives of Virology*. **63**:311–315.

Grubman, M. J. and Baxt, B. (2004). Foot-and-mouth disease. *British Journal of General Practice the Journal of the Royal College of General Practitioners*. **17**:418–432.

Guzylack-Piriou, L., Bergamin, F., Gerber, M., McCullough, K.C. and Summerfield, A. (2006). Plasmacytoid dendritic cell activation by foot-and mouth disease virus requires immune complexes. *European Journal of Immunology*. **36**:1674–1683.

Hall, M., Knowles, N., Wadsworth, J., Rambaut, A. and Woolhouse, M. (2013). Reconstructing geographical movements and host species transitions of foot-and-mouth disease virus serotype SAT 2. *American Society for Microbiology*. **4**:e00591-13

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

Hammond, J. (2011). *OIE/FAO FMD Reference Laboratory Network Annual Report*. Available from: http://www.wrlfmd.org/ref_labs/ref_lab_reports/OIE-FAO%20FMD%20Ref%20Lab%20Network%20Report%20201l.pdf. [Accessed on 02 December 2020].

Hammond, J. M., Maulidi, B. and Henning, N. (2021). Targeted FMD vaccines for Eastern Africa: The Ag results foot and mouth disease vaccine challenge project. *Viruses*. **13**:1830.

Han, S. C., Guo, H. C. and Sun, S. Q. (2015). Three-dimensional structure of foot-and-mouth disease virus and its biological functions. *Archives of Virology*. **160**:1–16.

Hansson, M., Nygren, P. A. and Stahl, S. (2000). Design and production of recombinant subunit vaccines. *Biotechnology and Applied Biochemistry*. **32**: 95-107.

Harlow, E. and Lane, D. (1988). *Antibodies: A laboratory Manual*. New York: Cold Spring Harbor Laboratory. pp 241.

Harmsen, M. M., Van Solt, C. B., Fijten, H. P. D., Van Keulen, L., Rosalia, R. A., Weerdmeester, K., Cornelissen, A. H. M., De Bruin, M. G. M., Eble, P. L. and Dekker, A. (2007). Passive immunization of guinea pigs with llama single-domain antibody fragments against foot and mouth disease. *Veterinary Microbiology*. **120**:193-206.

Harmsen, M. M., Li, H., Sun, S., van der Poel, W. H. and Dekker, A. (2023). Mapping of foot-and-mouth disease virus antigenic sites recognized by single-domain antibodies reveals different 146S particle specific sites and particle flexibility. *Frontiers in Veterinary Science*. **9**:1040802.

Harwood, L. J., Gerber, H., Sobrino, F., Summerfield, A. and McCullough, K.C. (2008). Dendritic cell internalization of foot-and-mouth disease virus: influence of heparan sulfate binding on virus uptake and induction of the immune response. *Journal of Virology*. **82**:6379–6394.

Hershey, J. W. B. and Merrick, W. C. (2000). Pathway and mechanism of initiation of protein synthesis. In: *Translational control of gene expression*. Edited by Sonenberg, N., Hershey, J. W. B. and Mathews, M. B. New York: Cold Spring Harbor Laboratory Press. pp 33-88.

Hogle, J. M., Chow, M. and Filman, D. J. (1985). Three-dimensional structure of poliovirus at 2.9 A resolution. *Science*. **229**:1358–1365.

Holt, L. J., Herring, C., Jespers, L. S., Woolven, B. P. and Tomlinson, I. M., (2003). Domain antibodies: proteins for therapy. *Trends in Biotechnology*. **21**:484-490.

Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P. and Winter, G. (1991). Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Research*. **19**:4133–4137.

Hoogenboom, H. R. (1997). Designing and optimising library selection strategies for generating high-affinity antibodies. *Trends in Biotechnology*. **15**:62–70.

Hoogenboom, H. R., De Bruine, A., Hufton, S. E., Hoet, R. M., Arends, J. and Roovers, R. C. (1998). Antibody phage display technology and its applications. *Immunotechnology.* **4**:1-20.

Hu, Y., Jin, H., Du, X., Xiao, C., Luo, D., Wang, B. and She, R. (2007). Effects of chronic heat stress on immune responses of the foot-and-mouth disease DNA vaccination. *DNA Cell Biology*. **26**:619-626.

Huang, H., Yang, Z., Xu, Q., Sheng, Z., Xie, Y., Yan, W., You, Y. Sun, L. and Zheng, Z. (1999). Recombinant fusion protein and DNA vaccines against foot

and mouth disease virus infection in guinea pig and swine. *Viral Immunology*. **12**:1–8.

Huang, J. X., Bishop-Hurley, S. L. and Cooper, M. A. (2012). Development of anti-infectives using phage display: biological agents against bacteria, viruses, and parasites. *Antimicrobial Agents and Chemotherapy*. **56**:4569-4582.

Hunter, P. (1998). Vaccination as a means of control of foot-and-mouth disease in Sub-Saharan Africa. *Vaccine*. **16**:261-264.

Huse, W. D., Sastry, L., Iverson, S.A.; Kang, A. S., Alting-Mees, M., Burton, D.R., Benkovic, S. J. and Lerner, R. A. (1989). Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science*. **246**:1275-1281.

Hwang, S. Y., Shin, S. H., Kim, H. M., Shin, S., Lee, M. J., Kim, S. M., Lee, J. S. and Park, J. H. (2023). Evaluation of vaccine strains developed for efficient, broad-range protection against foot-and-mouth disease type O. *Vaccines*. **11**:271.

Hyde, J. L., Blackwell, J. H. and Callis, J. J. (1975). Effect of pasteurization and evaporation on foot-and-mouth disease virus in whole milk from infected cows. *Canadian Journal of Comparative Medicine*. **39**:305-309.

Hynes, R. O. (1999). Cell adhesion: old and new questions. *Trends in Cell Biology*. **15**:33–37.

Illumina. (2014). *Estimating Sequencing Coverage*. Available from: https://www.illumina.com/documents/products/technotes/technote_coverage_calculation.pdf. [Accessed on 26 June 2023].

Ilott, M. C., Salt, J. S., Gaskell, R. M. and Kitching, R. P. (1997). Dexamethasone inhibits virus production and the secretory IgA response in oesophageal-pharyngeal fluid in cattle persistently infected with foot-and-mouth disease virus. *Epidemiology Infection*. **118**:181–187.

Iwasaki, A. and Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. *Nature Immunology*. **5**:987–995.

Iyer, A. V., Ghosh, S., Singh, S. N. and Deshmukh, R. A. (2001). Evaluation of the three ready to formulate oil-adjuvants for foot and mouth disease vaccine production. *Vaccine*. **19**:1097–1105.

Jackson, C. B., Farzan, M., Chen, B. and Choe, H. (2022). Mechanisms of SARS-CoV-2 entry into cells. *Nature reviews Molecular cell biology*. **23**:3-20.

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

Jackson, T., Sheppard, D., Denyer, M., Blakemore, W. and King, A. M. (2000). The epithelial integrin alphavbeta6 is a receptor for foot-and-mouth disease virus. *Journal of Virology*. **74**:4949–4956.

Jackson, T., Mould, A. P., Sheppard, D. and King, A. M. (2002). Integrin alphavbeta1 is a receptor for foot-and-mouth disease virus. *Journal of Virology*. **76**:935–41.

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

Jackson, T., Clark, S., Berryman, S., Burman, A., Cambier, S., Mu, D., Nishimura, S. and King, A. M. (2004). Integrin alphavbeta8 functions as a receptor for foot-and-mouth disease virus: role of the beta-chain cytodomain in integrin-mediated infection. *Journal of Virology*. **78**:4533–4540.

Jamal, S. M. and Belsham, G. J. (2013). Foot-and-mouth disease: Past, present and future. *Veterinary Research*. **44**:1–14.

Janeway, C. A., Travers, P., Walport, M. and Shlomchik, M. J., (2001). *Immunobiology: The immune system in health and disease*. 5th edition. New York: Garland Science. Chapter 3.

Jang, S. K., Krausslich, H. G., Nicklin, M. J. H., Duke, G. M., Palmenberg, A. C. and Wimmer, E. (1988). A segment of the 5' non-translated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *Journal of Virology*. **62**:2636–2643.

Jensen-Jarolim, E., Leitner, A., Kalchhauser, H., Zürcher, A., Ganglberger, E., Bohle, B., Scheiner, O., Boltz-Nitulescu, G. and Breiteneder, H. (1998). Peptide mimotopes displayed by phage inhibit antibody binding to bet v 1, the major birch pollen allergen, and induce specific IgG response in mice. *FASEB Journal*. **12**:1635-1642.

Jiang, P., Liu, Y., Ma, H.C., Paul, A. V. and Wimmer, E. (2014). Picornavirus morphologenesis. *Microbiology and Molecular Biology Reviews*. **78**:418-437.

Johns, H. L., Berryman, S., Monaghan, P., Belsham, G. J. and Jackson, T. (2009). A dominant-negative mutant of rab5 inhibits infection of cells by foot-and-mouth disease virus: implications for virus entry. *Journal of Virology*. **83**:6247–6256.

Jori, F., Vosloo, W., Du Plessis, B., Bengis, R., Brahmbhatt, D., Gummow, B. and Thomson, G. R. (2009). A qualitative risk assessment of factors contributing to foot and mouth disease outbreaks in cattle along the western boundary of the Kruger National Park. *Revue scientifique et technique International Office of Epizootics*. **28**:917–931.

Juds, C., Schmidt, J., Weller, M. G., Lange, T., Beck, U., Conrad, T. and Börner, H. G. (2020). Combining phage display and next-generation sequencing for materials sciences: a case study on probing polypropylene surfaces. *Journal of the American Chemical Society*. **142**:10624-10628

Juleff, N., Windsor, M., Lefevre, E. A., Gubbins, S., Hamblin, P., Reid, E., McLaughlin, K., Beverley, P. C., Morrison, I. W. and Charleston, B. (2009). Foot-and-mouth disease virus can induce a specific and rapid CD4+ T-cell-independent neutralizing and isotype class-switched antibody response in naive cattle. *Journal of Virology*. **83**:3626-3636.

Kamel, M., El-Sayed, A. and Vazquez, H. C. (2019). Foot-and-mouth disease vaccines: recent updates and future perspectives. *Archives of Virology*. **164**:1501–1513.

Karu, A. E., Bell, C. W. and Chin, T. E. (1995). Recombinant Antibody technology. *ILAR Journal*. **37**:132-141.

Kay, B. K. (1994). Biological displayed random peptides as reagents in mapping protein-protein interactions. *Perspective in drug discovery and design*. **2**:251-268.

Kay, B. K. and Hoess, R. (1996). Principles and applications of phage display. In: *Phage display of peptides and proteins, a laboratory manual*. Edited by Kay, B. K., Winter, J. and McCafferty, J. California: Academic Press. pp 21–34.

Keeling, M. J., Woolhouse, M. E. J., May, R. M., Davies, G. and Grenfell, B. T. (2003). Modelling vaccination strategies against foot-and-mouth disease. *Nature*. **421**:136–142.

Khan, K.H. (2013). DNA vaccines: roles against diseases. *Germs*. **3**:26-35.

King, A. M., McCahon, D., Saunders, K., Newman, J. W., and Slade, W. R. (1985). Multiple sites of recombination within the RNA genome of foot-and mouth disease virus. *Virus Research*. **3**:373–384.

Kitching, P., Hammond, J., Jeggo, M., Charleston, B., Paton, D., Rodriguez, L. and Heckert, R. (2007). Global FMD control—Is it an option?. *Vaccine*. **25**:5660–5664.

Kitson, J. D.A., McCahon, D. and Belsham, G. J. (1990). Sequence analysis of monoclonal antibody resistant mutants of type O foot and mouth disease virus; evidence for the involvement of the three-surface exposed capsid proteins in four antigenic sites. *Virology*. **179**:26-34.

Kjellen, L. and Lindah, U. (1991). Proteoglycans: structures and interactions. *Annual Review of Biochemistry*. **60**:443–475.

Kleid, D. G., Yansura, D., Small, B., Dowbenko, D., Moore, D. M., Grubman, M. J., McKercher, P. D., Morgan, D. O., Robertson, B. H. and Bachrach, H. L. (1981). Cloned viral protein vaccine for foot-and-mouth disease: responses in cattle and swine. *Science*. **214**:1125–1129.

Knight-Jones, T. J. D. and Rushton, J. (2013). The economic impacts of foot and mouth disease-What are they, how big are they and where do they occur?. *Preventive Veterinary Medicine*. **112**:161–173.

Knight-Jones, T. J. D., McLaws, M. and Rushton, J. (2016). Foot and mouth disease impact on smallholders- what do we know, what don't we know and how we can find out more?. *Transboundary and Emerging Diseases*. **64**:1079-1094.

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

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

Knowles, N. J., Samuel, A. R., Davies, P. R., Midgley, R. J. and Valarcher, J. F. (2005). Pandemic strain of foot and mouth disease virus serotype O. *Emerging Infectious Diseases*. **11**:1887-1893.

Köhler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. **256**:495-497.

Koivunen, E., Arap, W., Rajotte, D., Lahdenranta, J. and Pasqualini, R. (1999). Identification of receptor ligands with phage display peptide libraries. *Journal of Nuclear Medicine.* **40**:883-888.

Kristensen, T. and Belshem, G. J. (2019). Identification of a short, highly conserved, mortif required for picornavirus capsid precursor processing at distal sites. *PLoS Pathogens*. **15**:e1007509.

Lanier, L. L. (2008). Evolutionary struggles between NK cells and viruses. *Nature Reviews Immunology*. **8**:259–268.

Laporte, J. (1969). The structure of foot-and-mouth disease virus protein. *Journal of General Virology*. **4**:6314.

Lawrence, P., LaRocco, M. and Baxt, B. and Rieder, E. (2013). Examination of soluble integrin resistant mutants of foot-and-mouth disease virus. *Virology Journal*. **10**:1-13.

Lazar, I. Jr. and Lazar, I. Sr. *GelAnalyzer 19.1*. Available from: www.gelanalyzer.com (App Downloaded). [Accessed on 12 April 2021].

Lazarus, D. D. (2014). *Foot-and-Mouth Disease (FMD) vaccination and control in cattle at the wildlife/livestock interface of the Mnisi communal area.* Master of Science (Veterinary science) Dissertation. University of Pretoria.

Lazarus, D. D., Van Schalkwyk, O. L., Burroughs, R. E. J., Mpehle, A., Reininghaus, B., Rikhotso, O., Heath, L., Maree, F. F., Blignaut, B. and Fosgate. G. T. (2018). Serological responses of cattle inoculated with inactivated trivalent foot and-mouth disease vaccine at the wildlife-livestock interface of the Kruger National Park, South Africa. *Preventive Veterinary Medicine*. **158**:89–96.

Lazarus, D. D., Mutowembwa, P. B., Sirdara, M. M., Rametse, T. M., Heath, L., Oppermana, P. A., Burroughsa, R. E. J. and Fosgatea, G. T. (2019), Clinical presentation of FMD virus SAT1 infections in experimentally challenged indigenous South African goats. *Small Ruminant Research*. **180**:15–20.

Lea, S., Hernandez, J., Blakemore, W., Brocchi, E., Curry, S., Domingo, E., Fry, E., abu-ghazaleh, R., King, A., Newman, J., Stuart, D. and Mateu, M. G. (1994). The structure and antigenicity of a type C foot-and-mouth disease virus. *Structure*. **2**:123-139.

Lea, S., Abu-Ghazaleh, R., Blakemore, W., Curry, S., Fry, E., Jackson, T., King, A., Logan, D., Newman, J. and Stuart, D. (1995). Structural comparison of two

strains of foot-and-mouth disease virus subtype O1 and a laboratory antigenic variant, G67. *Structure*. **3**:571–580.

Lee C. M., Lorno, N., Sierro, F. and Christ, D. (2007). Selection of human antibody fragments by phage display. *Nature Protocols*. **2**:3001–3008.

Leforban, Y. (1999). Prevention measures against foot-and-mouth disease in Europe in recent years. *Vaccine*. **17**:1755–1759.

Leforban, Y. (2002). How predictable were the outbreaks of foot and mouth disease in Europe in 2001 and is vaccination the answer? In Foot and mouth disease: facing the new dilemmas (G.R. Thomson, ed.). *Revue scientifique et technique International Office of Epizootics*. **21**:549-556.

Li, K., Wang, C., Yang, F., Cao, W., Zhe, Z. and Zheng, H. (2021). Virus-host interactions in foot and mouth diseases virus infection. *Frontiers in Immunology*. **12**:571509.

Li, P., Lu, Z., Bao, H., Li, D., King, D.P., Sun, P., Bai, X., Cao, W., Gubbins, S., Chen, Y. and Xie, B. (2011). In-vitro and in-vivo phenotype of type Asia 1 foot-and-mouth disease viruses utilizing two non-RGD receptor recognition sites. *BMC Microbiology*. **11**:1-12.

Lim, C., Choong, Y. S. and Lim, T. (2019). Cognizance of Molecular Methods for the Generation of Mutagenic Phage Display Antibody Libraries for Affinity Maturation. *International Journal of Molecular Sciences*. **20**:1861.

Ling, M. M. (2003). Large antibody display libraries for isolation of high-affinity antibodies. *Combinatorial Chemistry and High Throughput Screening*. **6**:421−432.

Lipman, N. S., Jackson, L. R., Trudel, L. J. and Weis-Garcia, F. (2005). Monoclonal versus polyclonal antibodies: Distinguishing characteristics, applications and information resources. *ILAR Journal*. **46**:258-268.

Liu, T., Chen, CY., Chen-Deng, A. Chen, Y., Wang, J., Hou, Y. and Lin, M. (2020). Joining Illumina paired-end reads for classifying phylogenetic marker sequences. *BMC Bioinformatics*. **21**:105.

Loeffler, F. and Frosch, P. (1897). Summarischer Bericht uber die Ergebnisse der Untersuchungen zur Erforschung der Maul- und Klauenseuche. Zentbl. *Bakteriol. Parasitenkd Abt. I.* **22**:257–259.

Logan, D., Abu-Ghazaleh, R., Blakemore, W., Curry, S., Jackson, T., King, A., Lea, S., Lewis, R., Newman J, Parry, N. and Rowlands, D. (1993) Structure of a major immunogenic site on foot-and-mouth disease virus. *Nature* **362**:566–568.

Longjam, N., Deb, R., Sarmah A. K., Tayo, A., Awachat, V. B. and Saxena, V. K. (2011). A brief review on diagnosis of foot and mouth disease of livestock: conventional to Molecular tools. *Veterinary Medicine International*. 1-17.

Luke, G. A., Felipe, P. D., Lukashev, A., Kallioinen, S. E., Bruno, E. A., Ryan, M. D. (2008). Occurrence, function and evolutionary origin of '2A-like' sequences in virus genome. *Journal of General Virology*. **89**:1036-1042.

Maake, L., Harvey, W.T., Rotherham, L., Opperman, P., Theron, J., Reeve, R. and Maree, F.F., (2020). Genetic basis of antigenic variation of SAT3 Foot-And-Mouth Disease viruses in Southern Africa. *Frontiers in Veterinary Science*. **7**:568.

Machado, L. F. A., Santos, F. A. A., Siravenha, L. Q., Silva, A.N. M. R., Queiroz, M. A. F., Vallinoto, A. C. R., Ishak, M. O. G. and Ishak, R. (2022). Bioprospection and selection of peptides by phage display as novel epitope-based diagnostic probes for serological detection of HTLV-1 and use in future vaccines. *Frontiers in Medicine* **9**:884738.

Mahapatra, M., Seki, C., Upadhyaya, S., Barnett, P. V., La Torre, J. and Paton, D. J. (2011). Characterisation and epitope mapping of neutralising monoclonal antibodies to A24 Cruzeiro strain of FMDV. *Veterinary Microbiology*. **149**:242-247.

Mahapatra, M., Hamblin, P. and Paton, D. J. (2012). Foot-and-mouth disease virus epitope dominance in the antibody response of vaccinated animals. *Journal of General Virology*. **93**:488-493.

Mahapatra, M. and Parida, S. (2018). Foot and mouth disease vaccine strain selection: current approaches and future perspectives. *Expert Review of Vaccines*. **17**:577-591.

Makowski, L. and Russel, M. (1997). Structure and assembly of filamentous bacteriophages. In: *Structural biology of viruses*. Edited by Kay, B., Winter, G. and McCafferty, J. New York: Oxford University Press. pp. 352–380.

Marcos, A. and Perez, A. M. (2019). Quantitative risk assessment of foot-and-mouth disease (FMD) virus introduction into the FMD-free zone without vaccination of Argentina through legal and illegal trade of bone-in beef and unvaccinated susceptible species. *Frontiers in Veterinary Science*. **6**:78.

Mardis, E. R. (2011). A decade's perspective on DNA sequencing technology. *Nature*. **470**:198–203.

Maree, F. F., Blignaut, B., Esterhuysen, J. J., de Beer, T. A. P., Theron, J., O'Neill, H. G. and Rieder, E. (2011). Predicting antigenic sites on the foot-and-mouth disease virus capsid of the South African Territories types using virus neutralization data. *Journal of General Virology*. **92**:2297–2309.

Maree, F. F., Kasanga, C. J., Scott, K. A., Opperman, P. A., Chitray, M., Sangula, A. K., Sallu, R., Sinkala, Y., Wambura, P. N., King, D. P., Paton, D. J., Rweyemamu, M. M. (2014). Challenges and prospects for the control of foot and-mouth disease: an African perspective. *Veterinary Medicine: Research and Reports*. **5**:119-138.

Maree, F. F., Nsambac, P., Mutowembwaa, P., Rotherhama, L. S., Esterhuysen, J. and Scott, K. A. (2015). Intra-serotype SAT2 chimeric foot-and-mouth disease vaccine protects cattle against FMDV challenge. *Vaccine*. **33**:2909-2916.

Marks, J. D., Hoongenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D. and Winter, G. (1991). By-passing immunization: human antibodies from V-gene libraries displayed on phage. *Journal of Molecular Biology*. **222**:581-597.

Martin, W. B. and Edwards, L. T. (1965). A field trial in South Africa of an attenuated vaccine against foot-and-mouth disease. *Research in Veterinary Science*. **6**:196–201.

Martin-Acebes, M. A., Gonzalez-Magaldi, M., Sandvig, K., Sobrino, F. and Armas-Portela, R. (2007). Productive entry of type C foot-and-mouth disease virus into susceptible cultured cells requires clathrin and is dependent on the presence of plasma membrane cholesterol. *Virology*. **369**:105–118.

Martinez, M. A., Carrillo, C., Plana, J., Mascarella, R., Bergada, J, Palma, E. L., Domingo E. and Sobrino, F. (1988). Genetic and immunogenic variations among closely related isolates of foot-and-mouth disease virus. *Gene*. **62**:75-84.

Martinez-Salas, E., Francisco-Velilla, R., Fernandez-Chamorro, J., Lozano, G. and Diaz-Toledano, R. (2015). Picornavirus IRES elements: RNA structure and host proteins interactions. *Virus Research*. **206**:62-73.

Mason, P. W., Piccone, M. E., McKenna, T. S., Chinsangaram, J. and Grubman, M. J. (1997). Evaluation of a live-attenuated foot-and-mouth disease virus as a vaccine candidate. *Virology*. **227**:96–102.

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

Mateu, M. G., Martinez, M. A., Rocha, E., Andreu, D., Parejo, J., Giralt, E., Sobrino, F. and Domingo, E. (1989). Implications of a quasispecies genome structure: effect of frequent, naturally occurring amino acid substitutions on the antigenicity of foot-and-mouth disease virus. *Proceedings of the National Academic of Sciences*. **86**:5883-5887.

Mateu, M. G., Martinez, M. A., Capucci, L., Andreu, D., Giralt, E., Sobrino, F., Brocchi, E. and Domingo, E. (1990). A single amino acid substitution affects multiple overlapping epitopes in the major antigenic site of foot-and-mouth disease virus of serotype C. *Journal of General Virology*. **71**:629-37.

Mateu, M. G. (1995) Antibody recognition of picornaviruses and escape from neutralization: a structural view. *Virus Research*. **38**:1–24.

Mateu, M. G. (2017). The foot and mouth disease virion: Structure and function. In: *Foot and Mouth Disease Virus; current research and emerging trends*. Edited by Sobrino, F. and Domingo, E. United Kingdom: Caister Academic Press. pp 62.

Mathebula E. (2015). *Identifying B-cell epitopes of African horse sickness virus serotype 4 recognised by antisera of immunised horses.* Master of Science (Veterinary Science) Dissertation. University of Pretoria.

Matochko, W. L., Chu, K., Jin, B., Lee, S. W., Whitesides, G. M. and Derda, R. (2012). Deep sequencing analysis of phage libraries using Illumina platform. Methods. **58**:47–55.

Matthews, D. J. and Wells, J. A. (1993). Substrate phage: selection of protease substrates by monovalent phage display. *Science*. **260**:1113–1117.

Mayr, G. A., O'Donnell, V., Chinsangaram, J., Mason, P. W. and Grubman, M. J. (2001). Immune responses and protection against foot-and-mouth disease virus (FMDV) challenge in swine vaccinated with adenovirus-FMDV constructs. *Vaccine*. **19**:2152–2162.

McCafferty, J., Griffiths, A. D., Winter, G., Chiswell, D. J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature*. **348**:552-554.

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

McCullough, K. C., Crowther, J. R., Butcher, R. N., Carpenter, W. C., Brocchi, E., Capucci, L. and De Simone, F. (1986). Immune protection against foot-and-mouth disease virus studied using virus-neutralizing and non-neutralizing concentrations of monoclonal antibodies. *Immunology.* **58**:421–428.

McCullough, K. C., Crowther, J. R., Carpenter, W. C., Brocchi, E., Capucci, L., De Simone, F., Xie, Q. and McCahon, D. (1987). Epitopes on foot-and-mouth disease virus particles. *Virology*. **157**:516-525.

McCullough, K. C., Parkinson, D. and Crowther, J. R. (1988). Opsonization enhanced phagocytosis of foot-and-mouth disease virus. *Immunology*. **65**:187–191.

McCullough, K. C., De Simone, F., Brocchi, E., Capucci, L., Crowther, J. R. and Kihm, U. (1992). Protective immune response against foot-and-mouth disease. *Journal of Virology*. **66**:1835–1840.

Mead, D. A. and Kemper, B. (1988). Chimeric single-stranded DNA phage-plasmid cloning vectors. *Biotechnology*. **10**:85–102.

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

Metwally, S., Charleston, B. and Lyons, N. A., (2021). Foot and mouth disease. In: Veterinary Vaccines: Principles and Applications. 1st edition. Edited by Metwally, S., Viljoen, G. and Idrissi, A. E. United Kingdom: FAO and John Willey & Sons Limited. pp 207.

Mignaqui, A. C., Ruiz, V., Durocher, Y. and Wigdorovitz, A. (2019). Advances in novel vaccines for foot and mouth disease: focus on recombinant empty capsids. *Critical Review in Biotechnology*. **39**:306-320.

Mondon, P., Dubreuil, O., Bouayadi, K. and Kharrat, H. (2008). Human antibody libraries: a race to engineer and explore a larger diversity. *Frontiers in Bioscience*. **13**:1117-1129.

Moraes, M. P., Mayr, G. A., Mason, P. W. and Grubman, M. J. (2002). Early protection against homologous challenge after a single dose of replication-defective human adenovirus type 5 expressing capsid proteins of foot-and-mouth disease virus (FMDV) strain A24. *Vaccine*. **20**:1631–1639.

Mowat, G. N., Barr, D. A. and Bennett, J. H. (1969). The development of an attenuated foot-and mouth disease virus vaccine by modification and cloning in tissue cultures of BHK21 cells. *Archiv fur die gesamte Virusforschung*. **26**:341–54.

Mukonyora, M. (2015). *The in-silico prediction of foot and mouth disease virus (FMDV) epitopes on the South African territories (SAT)1, SAT2 and SAT3 serotypes.* Master of Science (Life Sciences) Dissertation. University of South Africa.

Muller, S. (1999). Chapter 1: Molecular dissection of protein antigens and the prediction of epitopes. In*: Laboratory Techniques in Biochemistry and Molecular Biology*. Volume 28. Edited by: Van Regenmortel, M. H. V. and Muller, S. New York: Elsevier. pp 1-78.

Munz, C., Steinman, R. M. and Fujii, S. I (2005). Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. *Journal of Experimental Medicine*. **202**:203–207.

Murphy, K. M. and Weaver, C. (2017). *Janeway's immunobiology*: An introduction to immunobiology and innate immunity. 9th edition. New York: Garland science. Chapter 1.

Naessens, J., Scheerlinck, J. P., De Buysscher, E. V., Kennedy, D. and Sileghem, M. (1998). Effective in vivo depletion of T cell subpopulations and loss of memory cells in cattle using mouse monoclonal antibodies. *Veterinary Immunology Immunopathology*. **64**:219-234.

Nascimento, I. P. and Leite, L. C. C. (2012). Recombinant vaccines and the development of new vaccine strategies. *Brazilian Journal of Medical and Biological Research*. **45**:1102-1111.

National Center for Biotechnology Information (NCBI). *FMDV P1 regions*. Available from: https://www.ncbi.nlm.nih.gov/nucleotide/. [Accessed on 03 January 2020].

Neff, S., Sa-Carvalho, D., Rieder, E., Mason, P. W., Blystone, S. D., Brown E. J. and Baxt, B. (1998). Foot-and-mouth disease virus virulent for cattle utilizes the integrin avb3 as its receptor. *Journal of Virology*. **72**:3587–3594.

Nelson, P. N., Reynolds, G. M., Waldron, E. E., Ward, E., Giannopoulos, K. and Murray, P. G. (2000). Monoclonal antibodies. *Molecular Pathology*. **53**:111-117.

Newman, J. F., Rowlands, D. J. and Brown, F. (1973). A physico-chemical sub-grouping of the mammalian picornaviruses. *Journal of General Virology*. **18**:171–180.

Ngubane, N. A. C., Gresh, L., Loerger, T. R., Sacchettini, J. C., Zhang, Y. J., Rubin, E. J., Pym, A. and Khati, M. (2013). High-Throughput sequencing enhanced phage display identifies peptides that bind mycobacteria. *PLoS One*. **8**:e77844.

O'Donnell, V., LaRocco, M., and Baxt, B. (2008). Heparan Sulfate-Binding Foot-and-Mouth Disease Virus Enters Cells via Caveola-Mediated Endocytosis. *Journal of Virology*. **82**:9075–9085.

O'Donnell, V., LaRocco, M., Duque, H. and Baxt, B. (2005). Analysis of foot-and-mouth disease virus internalization events in cultured cells. *Journal of Virology*. **79**:8506–8518.

O'Connell, D., Becerril, B., Roy-Burman, A., Daws, M. and Marks, J. D. (2002). Phage versus phagemid libraries for generation of human monoclonal antibodies. *Journal of Molecular Biology*. **321**:49-56.

Ohkubo, S., Miyadera, K., Sugimoto, Y., Matsuo, K., Wierzba, K. and Yamada, Y. (2001). Substrate phage as a tool to identify novel substrate sequences of proteases. *Combinatorial Chemistry and High Throughput Screening*. **4**:573–583.

Olascoaga, R. C., Gomes, I., Rosenberg, F.J., De Mello, P.A., Astudillo, V. M. and Magallanes, N. (1999). *Fiebre aftosa*. San Paulo: Editorial Atheneu. pp 58.

Opperman, P. (2013). *Antigenic site determination on a SAT2 foot-and-mouth disease virus using a chicken antibody phage display library*. Doctor of Philosophy (Natural and Agricultural Sciences) Dissertation. University of Pretoria.

Opperman, P., Maree, F. F., van Wyngaardt, W. and Vosloo, W. (2012). Mapping of antigenic determinants on a SAT2 foot-and-mouth disease virus using a chicken single-chain antibody fragment. *Virus Research*. **167**:370-379.

Orsel, K. and Bouma, A. (2009). The effect of foot-and-mouth disease (FMD) vaccination on virus transmission and the significance for the field. *Canadian Veterinary Journal*. **50**:1059-1063.

Pacheco, J. M., Butler, J. E., Jew, J., Ferman, G. S., Zhu, J. and Golde, W. T. (2010). IgA antibody response of swine to foot-and-mouth disease virus infection and vaccination. *Clinical and Vaccine Immunology*. **17**:550–558.

Pacheco, J. M., Smoliga, G. R., O'Donnell, V., Brito, B. P., Stenfeldt, C., Rodriguez, L. L. and Artz, J. (2015). Persistent foot and mouth disease virus infection in the nasopharynx of cattle: tissue-specific distribution and local cytokine expression. *PloS one*. **10**:e0125698.

Paiba, G. A., Anderson, J., Paton, D. J., Soldan, A. W., Alexandersen, S., Corteyn, M., Wilsden, G., Hamblin, P., MacKay, D. K. J. and Donaldson, A. I. (2004). Validation of a foot-and-mouth disease antibody screening solid-phase competition ELISA (SPCE). *Journal of Virological Methods*. **115**:145-158.

Palinski, R. M., Brito, B., Jaya, F. R., Sangula, A., Gakuya, F., Bertram, M. R., Pauszek, S. J., Hartwig, E. J., Smoliga, G. R., Obanda, V., Omondi, G. P., VanderWaal, K. and Arzt, J. (2022). Viral Population Diversity during Co-Infection of Foot-And-Mouth Disease Virus Serotypes SAT1 and SAT2 in African Buffalo in Kenya. *Viruses*. **14**:897.

Pande, J., Szewczyk, M. M. and Grover, A. K. (2010). Phage display: concept, innovations, applications and future. *Biotechnology advances*. **28**:849-858.

Parida, S., Oh, Y., Reid, S. M., Cox, S. J., Statham, R. J., Mahapatra, M., Anderson, J., Barnett, P. V., Charleston, B. and Paton, D. J. (2006). Interferon-gamma production *in vitro* from whole blood of foot-and-mouth disease virus (FMDV) vaccinated and infected cattle after incubation with inactivated FMDV. *Vaccine*. **24**:964-969.

Parida, S. (2009). Vaccination against foot-and-mouth disease virus: strategies and effectiveness. *Expert Review of Vaccines*. **8**:347–365.

Park, Y. S., Lee, J., Ko, Y. J., Kim, A., Lee, H. S., Park, S. H., Lee, S., Kim, H., Kim, J., Park, J., Yoon, H., Ko, Y. and Park, C. (2020). Efficient removal of non-structural protein using chloroform for foot and mouth vaccine production. *Vaccines*. **8**:483.

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

Patch, J. R., Kenny, M., Pacheco, J. M., Grubman, M. J. and Golde, W. T. (2013). Characterization of cytotoxic T lymphocyte function after foot and mouth disease virus infection and vaccination. *Viral Immunology*. **26**:239-249.

Paton, D. J., Valarcher, J. F., Bergmann, I., Matlho, O. G., Zakharov, V. M., Palma, E. L., Thomson, G. R. (2005). Selection of foot and mouth disease vaccine strains–a review. *Revue scientifique et technique International Office of Epizootics*. **24**:981–993.

Paton D. J., Sumption, K. J., and Charleston, B. (2009). Options for control of foot and mouth disease: knowledge, capability and policy. *Philosophical Transactions of the Royal Society B: Biological Sciences.* **364**:2657-2667.

Paton, D. J., Gubbins, S. and King, D. P. (2018). Understanding the transmission of foot-and-mouth disease virus at different scales. *Current Opinion in Virology*. **28**:85–91.

Paton, D. J., Di Nardo, A., Knowles, N. K., Wadsworth, J., Pituco, E. J., Cosivi, O., Rivera, A. M., Kassimi, L. B., Brocchi, E., de Clercq, K., Carrillo, C., Maree, F. F., Singh, R. K., Vosloo, W., Park, M., Sumption, K. J., Ludi, A. B. and King, D. P. (2021). The history of foot-and-mouth disease virus serotype C: the first known extinct serotype?. *Virus Evolution*. **7**:veab009.

Pattnaik, B., Subramaniam, S., Sanyal, A., Mahapatra, J. K., Dash, B. B., Ranjan, R. and Rout, M. (2012). Foot-and-mouth disease: Global status and future road map for control and prevention in India. *Agricultural Research*. **1**:132–147.

Pega, J., Bucafusco, D., Di Giacomo, S., Schammas, J. M., Malacari, D., Capozzo, A. V., Arzt, J., Pérez-Beascoechea, C., Maradei, E., Rodríguez, L. L., Borca, M. V., Pérez-Filgueira, M. (2013). Early adaptive immune responses in the respiratory tract of foot-and-mouth disease virus-infected cattle. Journal of Virology. **87**:2489-2495.

Pega, J., Di Giacomo, S., Bucafusco, D., Schammas, J. M., Malacari, D., Barrionuevo, F., Capozzo, A. V., Rodríguez, L, L., Borca, M. V. and Pérez-Filgueira, M. (2015). Systemic foot-and-mouth disease vaccination in cattle promotes specific antibody-secreting cells at the respiratory tract and triggers local anamnestic responses upon aerosol infection. *Journal of Virology*. **89**:9581-9590.

Peltomaa, R., Benito-Peña, E., Barderas, R. and Moreno-Bondi, M. C. (2019). Phage display in the quest for new selective recognition elements for biosensors. *ACS Omega*. **4**:11569-11580.

Peng, J., Yi, J., Yang, W., Ren, J., Wen, Y., Zheng, H. and Li, D. (2020). Advances in Foot-and-Mouth Disease Virus Proteins Regulating Host Innate Immunity. *Frontiers in Microbiology*. **11**:2046.

Perez-Ortin, E. J., Olmo, M. L. D., Matallana, E. and Torderm, V. (1997). Making your own gene library. *Biochemical Education*. **25**:237-241.

Perrie, Y., Mohammed, A. R., Kirby, D. J., McNeil, S. E. and Bramwell, V. W. (2008). Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens. *International Journal of Pharmaceutics*. **364**:272-280.

Perry, B. D., Randolph T. F., Ashley, S., Chimedza, R., Forman, T., Morrisson, J., Poulton, C., Sibanda, L., Stevens, C., Tebele, N., Yngström, I. (2003). *The impact and poverty reduction implications of foot and mouth disease control in southern Africa, with special reference to Zimbabwe.* Kenya: International Livestock Research Institute (ILRI). pp 14.

Peta, F. R., Sirdar, M. M., van Bavel, P., Mutowembwa, P. B., Visser, N., Olowoyo, J., Seheri, M. and Heath, L. (2021). Evaluation of potency and duration of immunity elicited by a multivalent fmd vaccine for use in South Africa. *Frontiers in Veterinary Science*. **8**:750223.

Ponsel, D., Neugebauer, J., Ladetzki-Baehs, K. and Tissot, K. (2011). High affinity, developability and functional size: the holy grail of combinatorial antibody library generation. *Molecules*. **16**:3675-3700.

Qin, Y. L., Sun, E. C., liu, N. H., Yang, T., Xu, Q. Y., Zhao, J., Wang, W. S., Wei, P., Feng, Y. F., Li, J. P. and Wu, D. L. (2013). Identification of a linear B-cell epitope within the Bluetongue virus serotype 8 NS2 protein using phage-displayed random peptide library. *Veterinary Immunology and Immunopathology*. **154**:93-101.

Quartararo, A.J., Gates, Z.P., Somsen, B.A., Hartrampf, N., Ye, X., Shimada, A., Kajihara, Y., Ottmann, C. and Pentelute, B. L. (2020). Ultra-large chemical libraries for the discovery of high-affinity peptide binders. *Nature Communications*. **11**:3183.

R&D systems, Inc. (2023). *Methods for storage, thawing and freezing of serum products.* Available from:

https://resources.rndsystems.com/images/site/Storage-Thawing-Freezing-Serum-Protocol.pdf. [Accessed on 20 September 2023].

Radostits, O. M., Blood, D. C. and Gay, C. C. (1994). *Veterinary medicine, A textbook of the disease of cattle, sheep, pigs, goats and horses*. Foot and mouth disease. 8th edition. London: Bailliere Tindall Ltd. pp 1015-1026.

Rakabe, M. (2008). *Selection of chicken single-chain antibody fragments directed against recombinant VP7 of bluetongue virus*. Master of Science (Veterinary Science) Dissertation. University of Pretoria.

Rebollo, I. R., Sabisz, M., Baeriswyl, V. and Heinis, C. (2014). Identification of target binding peptide motifs by high-throughput sequencing of phage-selected peptides. *Nucleic Acids Research*. **42**:e169.

Reeve, R., Blignaut, B., Esterhuysen, J. J., Opperman, P., Matthews, L., Fry, E. E., de Beer, T. A., Theron, J., Rieder, E., Vosloo, W., O'Neill, H. G., Haydon, D. T., Maree, F. F. (2010). Sequence-based prediction for vaccine strain selection and identification of antigenic variability in foot-and-mouth disease virus. *PLoS Computational Biology*. **6**:e1001027.

Reid, E. and Charleston, B. (2014). Type I and III interferon production in response to RNA viruses. *Journal of Interferon Cytokine Research*. **34**:649-658.

Rieder, E., Baxt, B., Lubroth, J. and Mason, P. W. (1994). Vaccines prepared from chimeras of foot-and-mouth disease virus (FMDV) induce neutralizing antibodies and protective immunity to multiple serotypes of FMDV. *Journal of Virology*. **68**:7092-7098.

Rieder, E., Henry, T., Duque, H. and Baxt, B. (2005). Analysis of a foot-and-mouth disease virus type A24 isolate containing an SGD receptor recognition site in vitro and its pathogenesis in cattle. *Journal of Virology*. **79**:12989–12998.

Riera, R. M, Perez-Martinez, D. and Castillo, F. C. (2016). Innate immunity in vertebrates: an overview. *Immunology*. **148**:125–139.

Rigden, R. C., Carrasco, C. P., Summerfield, A. and McCullough, K. C. (2002). Macrophage phagocytosis of foot-and-mouth disease virus may create infectious carriers. *Immunology*. **106**:537-548.

Rigden, R. C., Carrasco, C. P., Barnett, P. V., Summerfield, A. and McCullough K. C. (2003). Innate immune responses following emergency vaccination against foot-and-mouth disease virus in pigs. *Vaccine*. **21**:1466–1477.

Rodriguez, L. L. and Grubman, M. J. (2009). Foot-and-mouth disease virus vaccines. *Vaccine*. **27**: D90–D94.

Rodriguez, P. M. and Saiz, M. (2017). Molecular mechanisms of foot and mouth disease virus targeting the host antiviral responses. *Frontiers in Cellular and Infection Microbiology.* **7**:252.

Rogers, J. G. W., Komar, A. A. and Merrick, W. C. (2002). eIF4A: the godfather of the DEAD box helicases. *Progress in Nucleic Acid Research and Molecular Biology*. **72**:307-331.

Roman, I., Figys, J., Steurs, G. and Zizi, M. (2006). Hunting interactomes of a membrane protein: obtaining the largest set of voltage-dependent anion channel-interacting protein epitopes. *Molecular and Cellular Proteomics*. **5**:1667-1680.

Rossi, M. and Young, J. W. (2005). Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. *Journal of Immunology*. **175**:1373–1381.

Rosso, F. (2018). *Foot and mouth disease situation (Monthly report).* Food and Agriculture Organisation of the United Nations. Available from: https://www.fao.arg/fileadmin/user_upload/eufmd/FMD_reports_GMR/November_GMR_2018.pdf. [Accessed on 17 September 2021].

Rothenburg, S. and Brennan, G. (2020). Species-specific host–virus interactions: implications for viral host range and virulence. *Trends in Microbiology*. **28**:46–56.

Rowlands, D. J. (2003). Foot and mouth disease. *Virus Research*. **91**:1–161.

Rowlands, D. J., Sangar, D. V. and Brown F. (1971). Relationship of the antigenic structure of foot-and-mouth disease virus to the process of infection. *Journal of General Virology*. **13**:85–93.

Rowlands, D. J., Sangar, D. V. and Brown F. (1975). A comparative chemical and serological study of the full and empty particles of foot-and mouth disease virus. *Journal of General Virology*. **26**:227–238.

Rueckert, R. R. (1996). Picornaviridae: The viruses and their replication, In: *Fields virology*. 3rd edition. Edited by Fields, B. N., Knipe, D. M. and Howley, P. H. Philadelphia: Lippincott-Raven. pp. 609–654.

Ruoslahti, E. (1996). RGD and other recognition sequences for integrins. *Annual Review of Cell Developmental Biology*. **12**:697-715.

Russel, M. (1991). Filamentous phage assembly. *Molecular Microbiology*. **5**:1607-1613.

Russel, M., Clackson, T. and Lowman, H. B. (2004). Introduction to phage biology and phage display. In: *Phage Display: A Practical Approach*. No. 266. Edited by Clackson, T. and Lowman, H. B. New York: Oxford University Press. pp 1-19.

Rweyemamu, M., Terry, G. and Pay, T. W. (1979). Stability and immunogenicity of empty particles of foot-and-mouth disease virus. *Archives of Virology.* **59**:69–79.

Rweyemamu, M., Roeder, P., Mackay, D., Sumption, K., Brownlie, J., Leforban, Y., Valarcher, J. F., Knowles, N. J. and Saraiva, V. (2008a). Epidemiological patterns of foot-and mouth disease worldwide. *Transboundary and Emerging Diseases*. **55**:57–72.

Rweyemamu, M., Roeder, P., MacKay, D., Sumption, K., Brownlie, J. and Leforban, Y. (2008b). Planning for the progressive control of foot-and-mouth disease worldwide. *Transboundary Emerging Diseases*. **55**:73–87.

Ryan, M. D., Belsham, G. J., and King, M. Q. (1989). Specificity of enzyme-substrate interactions in foot-and-mouth disease virus polyprotein processing. *Virology*. **173**:35–45.

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

Salt, J. S., Mulcahy, G. and Kitching, R. P. (1996). Isotype-specific antibody responses to foot-and-mouth disease virus in sera and secretions of "carrier' and "non-carrier' cattle. *Epidemiology and Infection*. **117**:349-360.

Sangula, A. K., Siegismund, H. R., Belsham, G. J., Balinda, S. N., Masembe, C., Muwanika, V. B. (2011). Low diversity of foot-and-mouth disease serotype C virus in Kenya: evidence for probable vaccine strain re-introductions in the field. *Epidemiology and Infection*. **139**:189-196.

Sanyal, A., Venkataramanan, R. and Pattnaik, B. (1997). Antigenic features of foot-and-mouth disease virus serotype Asia1 as revealed by monoclonal antibodies and neutralization escape mutants. *Virus Research*. **50**:107-117.

Sanyal, A., Gurumurthy, C. B., Venkataramanan, R., Hemadri, D. and Tosh, C. (2003). Antigenic characterization of foot-and-mouth disease virus serotype Asia1 field isolates using polyclonal and monoclonal antibodies. *Veterinary Microbiology*. **93**:1-11.

Schirrmann, T., Meyer, T., Schütte., M, Frenzel A and Hust, M., (2011). Phage Display for the Generation of Antibodies for Proteome Research, Diagnostics and Therapy. *Molecules*. **16**:412-426.

Schroeder, H. W. J. and Cavacini, L. (2010). Structure and function of immunoglobulins. *Journal of Allergy and Clinical Immunology*. **125**:S41–S52.

Scott, J. K. and Smith, G. P. (1990). Searching for peptide ligands with an epitope library. *Science*. **249**:386-390.

Sela-Culang, I., Kunik, V. and Ofran, Y. (2013). The structural basis of antibody-antigen recognition. *Frontiers in Immunology*. **4**:302.

Sellers, R. F. (1971). Quantitative aspects of the spread of foot-and-mouth disease. *Veterinary Bulletin*. **41**:431-439.

Sette, A. and Rappuoli, R. (2010). Reverse vaccinology: developing vaccines in the era of genomics. *Immunity*. **33**:530-541.

Shao, J. J., Wong, C. K., Lin, T., Lee, S. K., Cong, G. Z., Sin, F. W. Y., Du, J. Z., Gao, S. D., Liu, X. T., Cai, X. P. and Xie, Y. (2011). Promising multiple-epitope recombinant vaccine against foot-and-mouth disease virus type O in swine. *Clinical and Vaccine Immunology*. **18**:143-149.

Sims, D., Sudbery, I., Ilott, N. E., Heger, A. and Ponting, C. P. (2014). Sequencing depth and coverage: key considerations in genomic analyses. *Nature Reviews Genetics*. **15**:121-132.

Sirdar, M. M., Fosgate, G. T., Blignaut, B., Mampane, L. R., Rikhotso, O. B., Du Plessis, B. and Gummow, B. (2021). Spatial distribution of foot-and-mouth disease (FMD) outbreaks in South Africa (2005-2016). *Tropical Animal Health Production*. **53**:376.

Sixholo, J. (2009). *Engineering recombinant chicken antibodies for improved characteristics.* Master of Science (Veterinary Science) Dissertation. University of Pretoria.

Sixholo, J., Van Wyngaardt, W., Mashau, C., Frischmuth, J., Du Plesis, D. H. and Fehrsen, J. (2011). Improving the characteristics of a mycobacterial 16akDa-specific chicken scFv. *Biologicals*. **39**:110-116.

Sloth, A. B., Bakhshinejad, B., Stavnsbjerg, C., Rossing, M. and Kjaer, A. (2023). Depth of sequencing plays a determining role in the characterization of phage display peptide libraries by NGS. *International Journal of Molecular Sciences*. **24**:5396.

Smith, G. P. (1985). Filamentous phage fusion: novel expression vectors that display cloned antigens on the surface of the virion. *Science*. **228**:1315–1317.

Smith, G. P. (1991). Surface presentation of protein epitopes using bacteriophage expression systems. *Current Opinion in Biotechnology*. **2**:668–673.

Smith, G. P., Schultz, D. A. and Ladbury, J. E. (1993). A ribonuclease S-peptide antagonist discovered with a bacteriophage display library. *Gene*. **128**:37–42.

Smith, N. C., Rise, M. L. and Christian, S. L. (2019). A comparison of the innate and adaptive immune systems in cartilaginous fish, ray-finned fish, and lobe-finned fish. *Frontiers in Immunology*. **10**:2292.

Sobrino, F., Sáiz, M., Jiménez-Clavero,M. A., Núñez, J. I., Rosas, M. F., Baranowski, E., and Ley, V. (2001). Foot-and-mouth disease virus: A long-known virus, but a current threat. *Veterinary Research*. **32**:1-30.

Sonenberg, N. and Hinnebusch, A. G. (2009). Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell*. **136**:731-745.

Sorensen, K. J., Madsen, K. G., Madsen, E. S., Salt, J. S., Nqindi, J. and Mackay, D. K. J. (1998). Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. *Archives of Virology*. **143**:1461-1476.

Sparks, A. B., Adey, N. B., Cwirla, S. and Kay, B. K. (1996). Screening phage-displayed random peptide libraries. In: *Phage display of peptides and proteins*. Edited by Kay, B.K., Winter J. and McCafferty, J. San Diego, CA: Academic Press. pp. 227–253.

Sporri, R. and Reis e Sousa, C. (2005). Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nature Immunology*. **6**:163-170.

Springer, T. A. (2002). Predicted and experimental structures of integrins and -propellers. *Current Opinions in Structural Biology*. **12**:802–813.

Stear, M. J. (2005). OIE manual of Diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). *Parasitology*. **130**:727.

Stenfeldt, C. and Belsham, G. J. (2012). Detection of foot-and-mouth disease virus RNA in pharyngeal epithelium biopsy samples obtained from infected cattle: investigation of possible sites of virus replication and persistence. *Veterinary Microbiology*. **154**:230–239.

Stenfeldt, C., Pacheco, J. M., Rodriguez, L. L. and Arzt, J. (2014a). Early events in the pathogenesis of foot and mouth disease in pigs; identification of oropharyngeal tonsils as sites of primary and sustained viral replication. *PLos One*. **9**:e106859.

Stenfeldt, C., Pacheco, J. M., Rodriguez, L. L. and Arzt, J. (2014b). Infection dynamics of foot and mouth disease virus in pigs using two novel simulated-natural inoculation methods. *Research in Veterinary Science*. **96**:396-405.

Stenfeldt, C., Eschbaumer, M., Pacheco, J. M., Rekant, S. I., Rodriguez, L. L. and Arzt, J. (2015). Pathogenesis of primary foot-and-mouth disease virus infection in the nasopharynx of vaccinated and non-vaccinated cattle. *PLoS One*. **10**:e0143666.

Stenfeldt, C., Eschbaumer, M., Rekant, S. I., Pacheco, J. M., Smoliga, G. R., Hartwig, E. J., Rodriguez, L. L. and Arzt, J. (2016a). The foot-and-mouth disease carrier state divergence in cattle. *Journal of Virology*. **90**:6344–6364.

Stenfeldt, C., Diaz-San, S. F., de los Santos, T., Rodriguez, L. L. and Arzt, J. (2016b). The pathogenesis of foot-and-mouth disease in pigs. *Frontiers in Veterinary Science*. **3**:41.

Stopar, D., Spruijt, R. B., Wolfs, C.J. and Hemminga, M.A. (2003). Protein–lipid interactions of bacteriophage M13 major coat protein. *Biochimica et Biophysica Acta*. **1611**:5-15.

Straver, P. J., Bool, P. H., Claessens, A. M. J. M. and Van Bekkum, J. G. (1970). Some properties of carrier strains of foot-and-mouth disease virus. *Arch Gesamte Virusforsch*. **29**:113–126.

Strohmaier, K., Franze, R. and Adam, K. H. (1982). Location and characterization of the antigenic portion of the FMDV immunizing protein. Journal of General *Virology*. **59**:295-306.

Su, B., Wang, J., Wang, X., Jin, H., Zhao, G., Ding, Z., Kang, Y. and Wang, B. (2008). The effects of IL-6 and TNF-alpha as molecular adjuvants on immune responses to FMDV and maturation of dendritic cells by DNA vaccination. Vaccine. **26**:5111-5122.

Subramaniam, R., Dassanayake, R. P., Norimine, J., Brown, W. C.and Srikumaran, S. (2010). Molecular cloning, characterization and *in vitro* expression of SERPIN B1 of bighorn sheep (*Ovis canadensis*) and domestic sheep (*Ovis aries*) and comparison with that of other species. *Veterinary Immunology and Immunopathology*. **137**:327–331.

Sutmoller, P. and Gaggero, A. (1965). Foot-and mouth diseases carriers. *Veterinary Record*. **77**:968–969.

Sutmoller, P., McVicar, J. W. and Cottral, G. E. (1968). The epizootiological importance of foot-and-mouth disease carriers. *Arch Gesamte Virusforsch*. **23**:227–235.

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

Taboga, O., Tami, C., Carrillo, E., Nunez, J. I., Rodriguez, A., Saiz, J. C., Blanco, E., Valero, M. L., Roig, X., Camerero, J. A. and Andreu, D. (1997). A largescale evaluation of peptide vaccines against foot-and-mouth disease: lack of solid protection in cattle and isolation of escape mutants. *Journal of Virology.* **71**:2606–2614.

Tassignon, J., Burny, W., Dahmani, S., Zhou, L., Stordeur, P., Byl, B., (2005). Monitoring of cellular responses after vaccination against tetanus toxoid: Comparison of the measurement of IFN-gamma production by ELISA,

ELISPOT, flow cytometry and real-time PCR. *Journal of Immunological Methods*. **305**:188-198.

Tekleghiorghis, T., Moormann, R. J., Weerdmeester, K. and Dekker, A. (2016). Foot-and-mouth disease transmission in Africa: implications for control, a review. *Transboundary Emerging Diseases*. **63**:136–151.

Tewari, A. and Jain, B. (2019). Antiviral immunity evoked post foot-and-mouth disease virus (fmdv) infection and vaccination. *Journal of Antiviral and Antiretrovirals*. **11**:185.

The Center for Food security and Public Health (CFSPH). (2015). Foot and mouth disease (FMD)- Pocket guide, cattle. Available from: https://www.cfsph.iastate.edu/pdf/foot-and-mouth-disease-pocket-guide-cattle. [Accessed on 13 November 2021].

Thomas, A. A., Woortmeijer, R. J., Barteling, S. J. and Meloen, R. H. (1988a). Evidence for more than one important, neutralizing site on foot-and-mouth disease virus. *Archives of Virology*. **99**:237-242.

Thomas, A. A., Woortmeijer, R. J., Puijk, W. and Barteling, S. J. (1988b). Antigenic sites on foot-and-mouth disease virus type A10. *Journal of Virology*. **62**:2782-2789.

Thomson, G. R., Vosloo, W., Esterhuysen, J. J., and Bengis, R. G. (1992). Maintenance of foot and mouth disease viruses in buffalo (*Syncerus caffer Sparrman*, 1779) in Southern Africa. *Revue scientifique et technique International Office of Epizootics*. **11**:1097-1107.

Thomson, G. R. (1994). Foot and mouth disease. In: *infectious diseases of livestock with special reference to Southern Africa*. Edited by Coetzer J. A. W., Thomson, G. R. and Tustin, R. C. United Kingdom: Oxford University Press. pp 825–852.

Thomson, G. R. (1995). Overview of foot and mouth disease in Southern Africa. *Revue scientifique et technique International Office of Epizootics*. **14**:503-520.

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

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

Toka, F. N., Nfon, C., Dawson, H., Golde, W. T. (2009). Natural killer cell dysfunction during acute infection with foot-and-mouth disease virus. *Clinical and Vaccine Immunology*. **16**:1738–1749.

Uddowla, S., Hollister, J., Pacheco, J. M., Rodriguez, L. L. and Rieder, E. (2012). A safe foot-and-mouth disease vaccine platform with two negative markers for differentiating infected from vaccinated animals. *Journal of Virology*. **86**:11675-11685.

Usherwood, E. J., Johnston, I. C. D., Lovelidge, L. J., Tonks, P. and Nash, A. A. (1995). Lymphocyte recognition elements on the VP1 protein of Theiler's virus. *Immunology*. **85**:190–197.

Valarcher, J. F., Knowles, N., Fernandez, R., Statham, B., Davies, P. R., Midgley, R. J., Hutchuings, G., Newman, B. J., Ferris, N. P. and Paton, D. J. (2004). Global FMD situation 2003–2004. In: *Report of the session of the research group of the standing technical committee of the European commission for the control of foot-and-mouth*. Chania, Greece. 137–148.

Vallee, H. and Carre, H. (1922). Sur la pluralité du virus aphteux. *Comptes rendus de l'Académie des sciences Paris*. **174**:1498–1500.

Van Bekkum, J. G., Frenkel, H. S., Frederiks, H. H. J. and Frenkel, S. (1959). Observations on the carrier state of cattle exposed to foot-and-mouth disease virus. *Tijdschr. Diergeneeskd*. **84**:1159–1164.

Van Lierop, M. J., Van Maanen, K., Meloen, R. H., Rutten, V. P., De Jong, M. A. and Hensen, E. J. (1992). Proliferative lymphocyte responses to foot-and-

mouth disease virus and three FMDV peptides after vaccination or immunization with these peptides in cattle. *Immunology*. **75**:406-413.

Van Wyngaardt, W., Malatji, T., Mashau, C., Fehrsen, J., Jordaan, F., Miltiadou, D. and Du Plessis, D. H. (2004). A large semi-synthetic single-chain Fv phage display library based on chicken immunoglobulin genes. *BMC Biotechnology*. **4**:1-14.

Velkers, F. C., Boums, A., Stegeam, J. A. and de Jong, M. C. M. (2012). Oocyst output and transmission rates during successive infections with Eimeria acervuline in experimental broiler flocks. *Veterinary Parasitology*. **187**:63-71.

Vivier, E., Tomasello, E., Baratin, M., Walzer, T. and Ugolini, S. (2008). Functions of natural killer cells. *Nature Immunology*. **9**:503–510.

Vosloo, W., Kirkbride, E., Bengis, R. G. and Keet, D. F., (1995), Genome variation in the SAT types of foot-and-mouth disease viruses prevalent in the buffalo (*Syncerus caffer*) in the Kruger Nation Park and other region of the Southern Africa. *Epidemiology and Infection*. **114**:203-218.

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

Vosloo, W., Bastos, A. D., Sangare, O., Hargreaves, S. K. and Thomson, G. R. (2002). Review of the status and control of foot and mouth disease in sub-Saharan Africa. *Revue scientifique et technique International Office of Epizootics*. **21**:437–449.

Vosloo, W. and Thomson, G. R. (2004). Natural habitats in which foot-and-mouth disease viruses are maintained. In: *Natural habitats in which foot-and-mouth disease viruses are maintained*. Edited by Domingo, E. and Sobrino, F. Norfolk: Horizon Bioscience. pp 384–410.

Vosloo, W., Dwarka, R. M., Bastos, A. D. S., Esterhuysen, J. J., Sahle, M., Sangare, O. (2006). Molecular epidemiological studies of foot and mouth disease virus in sub-Saharan Africa, indicate the presence of large numbers of topotypes; implication for loca and international control, *Proceedings of the 11th International Symposium on Veterinary Epidemiology and Economics*. Appendix 22. pp 149-158.

Vosloo, W. and Thomson, G. R. (2017). Natural habitat in which foot-and-mouth disease viruses are maintained. In: *Foot and Mouth Disease Virus Current Research and Emerging Trends*. Edited by Domingo, E. and Sobrino, F. Norfolk: Caister Academic Press. pp 179–210.

Waldmann, D., Kobe, K. and Pyl, G. (1937). Die aktive Immunisierung des Rindes gegen Maul- und Klausenseuche. *Zentralbl. Bakteriol*. *Orig*. **138**:401.

Waldmann, O. and Trautwein, K. (1926). Experimentalle untersuchungen ueber die pluralitet des maul-und klauenseuche virus. *Berlin Tierarztl Wochenschr*. **42**:569–571.

Wang, L. F., Du Plessis, D. H., White, J. R., Hyatt, A. D. and Eaton, B. T. (1995). Use of a gene-targeted phage display random epitope library to map an antigenic determinant on the bluetongue virus outer capsid protein VP5. *Journal of Immunological Methods.* **178:**1-12.

Wang, W., Singh, S., Zeng, D. L., King, K. and Nema, S. (2007). Antibody Structure, Instability and Formulation. *Journal of Pharmaceutical Sciences*. **96**:1-26.

Wang, L. F. and Yu, M. (2009). Epitope mapping using phage-display random fragment libraries. *Methods in Molecular Biology.* **524:**315-332.

Wang, G., Wang, Y., Shang, Y., Zhang, Z. and Liu, X. (2015a). How foot-and-mouth disease virus receptor mediates foot-and-mouth disease virus infection. *Virology Journal*. **12**:1-7.

Wang, W., Erbe, A. K., Hank, J. A., Morris, Z. S. and Sondel, P. M. (2015b). NK Cell-Mediated Antibody-Dependent Cellular Cytotoxicity in Cancer Immunotherapy. *Frontiers in Immunology*. ***6***:368-368.

Wang, G., Liu, Y., Feng, H., Chen, Y., Yang, S., Wei, Q., Wang, J., Liu, D. and Zhang, G. (2018). Immunogenicity evaluation of MS2 phage-mediated chimeric nanoparticle displaying an immunodominant B cell epitope of foot-and-mouth disease virus. *PeerJ*. **6**:e4823

Waterkamp, D. A., Müller, O.J., Ying, Y., Trepel, M. and Kleinschmidt, J. A. (2006). Isolation of targeted AAV2 vectors from novel virus display libraries. *Journal of Gene Medicine*. **8**:1307-1319.

Webster, R. E. (1996). Biology of the filamentous bacteriophage. In: *Phage Display of Peptides and Proteins*. Edited by Kay, B. R., Winter, J. and McCafferty, J. New York: Academic Press. pp. 1–20.

Weigel, B. J., Nath, N., Taylor, P. A., Panoskaltsis-Mortari, A., Chen, W., Krieg, A. M., Brasel, K. and Blazar, B. R. (2002). Comparative analysis of murine marrow-derived dendritic cells generated by Flt3L or GM-CSF/IL-4 and matured with immune stimulatory agents on the in vivo induction of antileukemia responses. *Blood*. **100**:4169–4176.

Wemmer, S., Mashau, C., Fehrsen, J., Van Wyngaardt, W. and Du Plessis, D. H. (2010). Chicken scFvs and bivalent scFv-CH fusions directed against HSP65 of *Mycobacterium bovis*. *Biologicals*. **38**:407-414.

Wild, T. F., Burroughs, J. N. and Brown, F. (1969). Surface structure of foot-and-mouth disease virus. *Journal of General Virology*. **4**:313-320.

Winter, G., Griffiths, A.D., Hawkins, R.E. and Hoogenboom, H. R. (1994). Making antibodies by phage display technology. *Annual Review of Immunology*. **12**:433–455.

Wong, H. T., Cheng, S. C. S., Chan, E. W. C., Sheng, Z. T., Yan, W. Y., Zheng, Z. X. and Xie, Y. (2000). Plasmids encoding foot-and-mouth disease virus VP1

epitopes elicited immune responses in mice and swine and protected swine against viral infection. *Virology*. **278**:27-35.

World Organisation for Animal Health (WOAH), founded as OIE. (2009). *OIE Terrestrial Manual. Chapter 2.1.5: Foot and mouth disease*. Available from: https://www.oie.int/eng/A_FMD2012/docs/2.01.05_FMD.pdf. [Accessed on 16 February 2020].

World Organisation for Animal Health (WOAH), founded as OIE. (2012). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. In: *OIE Terrestrial manual 2012*. Available from: https://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/. [Accessed on 20 February 21].

World Organisation for Animal Health (WOAH), founded as OIE. (2013). *Foot and mouth disease.* Available from: https://www.woah.org/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Disease_cards/FOOT_AND_MOUTH_DISEASE.pdf. [Accessed on 17 May 2021].

World Organisation for Animal Health (WOAH), founded as OIE. (2018a). Foot and Mouth Disease. Chapter 3.1.8 (POTENCY). In: *OIE Terrestrial Manual*. pp 442. Available from: https://www.oie.int/app/uploads/2021/03/foot-and-mouth-disease.pdf. [Accessed on 22 July 2021].

World Organisation for Animal Health (WOAH), founded as OIE. (2018b). *Foot and Mouth Disease. OIE Manual of Diagnostic Tests and Vaccines for terrestrial Animals*. Available from: https://www.oie.int/app/uploads/2021/03/foot-and-mouth-disease.pdf. [Accessed on 05 May 2021].

World Organisation for Animal Health (WOAH), founded as OIE. (2021). *Foot and mouth disease*. Available from: https://www.oie.int/en/disease/foot-and-mouth-disease/. [Accessed on 05 June 2022].

World Organisation for Animal Health (WOAH), founded as OIE. (2022). *Foot and mouth disease*. Available from: https://www.woah.org/en/disease/foot-and-mouth-disease/. [Accessed on 14 July 2022].

World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD). (2021). *Foot and mouth disease-Occurrence*. Available from: https://wrlfmd.org/foot-and-mouth-disease/occurence. [Accessed on 07 July 2021].

Wu, C. H., Liu, I. J., Lu, R. M. and Wu, H. C. (2016). Advancement and applications of peptide phage display technology in biomedical science. *Journal of Biomedical Science*. **23**:1-14.

Wu, T. T. and Kabat, E. A. (1970). An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *Journal of Experimental Medicine*. **132**:211–250.

Xie, Q. C., McCahon, D., Crowther, J. R., Belsham, G. J. and McCullough, K. C. (1987). Neutralization of foot-and-mouth disease virus can be mediated through any of at least three separate antigenic sites. *Journal of General Virology*. **68**:1637–1647.

Xin, X., Wang, H. L., Han, L. L., Wang, M. Z., Fang, H., Hao, Y., Li, J., Zhang, H., Zheng, C. and Shen, C. (2018). Single-cell analysis of the impact of host cell heterogeneity on infection with foot-and-mouth disease virus. *Journal of Virology*. **92**:e00179-18.

Xu, C., Wu, K., Zhang, J. G., Shen, H. and Deng, H. W. (2017). Low-, high-coverage and two-stage DNA sequencing in the design of the genetic association study. *Genetic Epidemiology*. **41**:187-197.

Xu, T. L., Sun, J., Liu, Q., Wang, X., Xu, T., Zhu, R., Wu, D. and Cao, Z. (2010). Evaluation of spatial epitope computational tools based on experimentally confirmed data set for protein antigens. *Chinese Science Bulletin*. **55**:2169–2174.

Yang, L., Li, C., Zhai, S., Zheng, H., Fu, L. and Li, D. (2016). An improved plating assay for determination of phage titer. *African Journal of Biotechnology*. **15**:1078-1082.

Yang, X. D. and Yu, X. L. (2009). An introduction to epitope prediction methods and software. *Review in Medical Virology*. **19**:77–96.

Yi, Z. J., Lui, M. Q., Zhu, C. Z., Zhang, Q., Sheng, Z., Du, Q., Yan, W. and Zheng, Z. (2004). Recombinant bivalent vaccines against foot and mouth disease virus serotype O/A infection in guinea pig. *Acta Biochimica et Biophysica Sinica*. **36**:589-596.

Young, A. D. and Gillung, J. P. (2020). Phylogenomics -principles, opportunities and pitfalls of big-data phylogenetics. *Systematic Entomology*. **45**:225-247.

Yu, Y., Abaeva, I. S., Marintchev, A., Pestova, T. V. and Hellen, C. U. (2011a). Common conformational changes induced in type 2 picornavirus IRESs by cognate trans-acting factors. *Nucleic Acids Research*. **39**:4851-4865.

Yu, Y., Wang, H., Zhao, L., Zhang, C., Jiang, Z. and Yu, L. (2011b). Fine mapping of a foot-and-mouth disease virus epitope recognized by serotype-independent monoclonal antibody 4B2. *Journal of Microbiology*. **49**:94-101.

Yuan, H., Li, P., Ma, X., Lu, Z., Sun, P., Bai, X., Zhang, J., Bao, H., Cao, Y., Li, D., Fu, Y., Chen, Y., Bai, Q., Zhang, J. and Liu, Z. (2017). The pH stability of foot-and-mouth disease virus. *Virology Journal*. **14**:233.

Zambrano-Mila, M., S., Blacio, K. E. S. and Vispo, N. S. (2020). Peptide phage display: Molecular principles and biomedical applications. *Therapeutic Innovation and Regulatory Science*. **54**:308-317.

Zhang, L., Zhang, J., Chen, H., Zhou, J., Ma, L., Ding, Y., Liu, Y. (2011). Research in advance for FMD novel vaccines. *Virology Journal*. **8**:268.

Zheng, Z. X., Xu, Q. X., Mao, C. Q., Zhao, H. X., Jin, J. P., You, Y. J., Yan, W. Y. (1994). Biosynthetic peptide vaccine against foot-and-mouth disease virus.

In: *Recombinant and synthetic vaccines.* Edited by Talwar, G. P., Rao, K. V. S. and Chauhn, V. S. New Delhi: Narosa Publishing House. pp 30–35.

Zhijun, Y., Yan, L., Guangjin, L., Weiyao, Y., Quanxing, X., Yongjin, Y. and Zhaoxing, Z. (2000). Recombinant vaccine containing both b cell and t cell epitopes induces guinea pig in immune responses against type o foot-and-mouth disease virus. *Journal of Fudan University. Natural Sciences*. **39**:564-568.

Zhuang, J., Cao, X., Cheng, B., Rao, Z., Pan, J., Xu, Q. and You, Y. (2005). The immune responses of the fusion protein including two copies of T cell and B cell epitopes of Food-and-mouth disease VP1. *Chinese Journal of Preventive Veterinary Medicine*. **27**:494-503.

APPENDICES

APPENDIX A: STOCK SOLUTIONS AND BUFFERS

20 mM Sodium phosphate, pH 7 (Binding buffer)

Dissolve 1.37 g of $Na_2HPO_4*2H_2O$ and 1.92 g of $NaH_2PO_4*2H_2O$ in 800 ml dH_2O. Adjust pH to 7 and make final volume to 1 L with dH_2O.

0.1 M Glycine, pH 2.2 (Elution buffer)

Dissolve 7.51 g of glycine in 800 ml dH_2O and adjust pH to 2.2 with concentrated HCl. Make up buffer solution to volume of 1 L with dH_2O.

1 M Tris-HCl, pH 9 (Neutralizing buffer)

Dissolve 12.11 g of Tris base in 800 ml dH_2O and adjust pH to 9 with concentrated HCl. Make up solution to a final volume of 1 L with dH_2O.

1X TAE

Mix 200 ml of the 50X Tris-Acetic acid-EDTA, pH 8 (buffer stock solution) with 9800 ml of dH_2O to make a 1X TAE working solution buffer (1 L).

20% PEG, 2.5 M NaCl

Dissolve 200g of polyethylene glycol (PEG)-6000 and 146.10 g of NaCl in 800 ml dH_20. Adjust volume to 1 L with dH_2O and autoclave.

APPENDIX B: MEDIA

LB medium

Dissolve 10 g bacto-tryptone, 5 g yeast extract and 10 g NaCl in 800 ml dH_2O. Make up to a final volume of 1 L with dH_2O and autoclave.

100 µg/ml Ampicillin

Dissolve 1 g ampicillin powder in 8 ml dH2O. Adjust final volume to 10 ml and filter sterilize.

25 µg/ml Kanamycin

Dissolve 1 g kanamycin powder in 8 ml dH2O. Adjust final volume to 10 ml and filter sterilize.

TYE agar plates

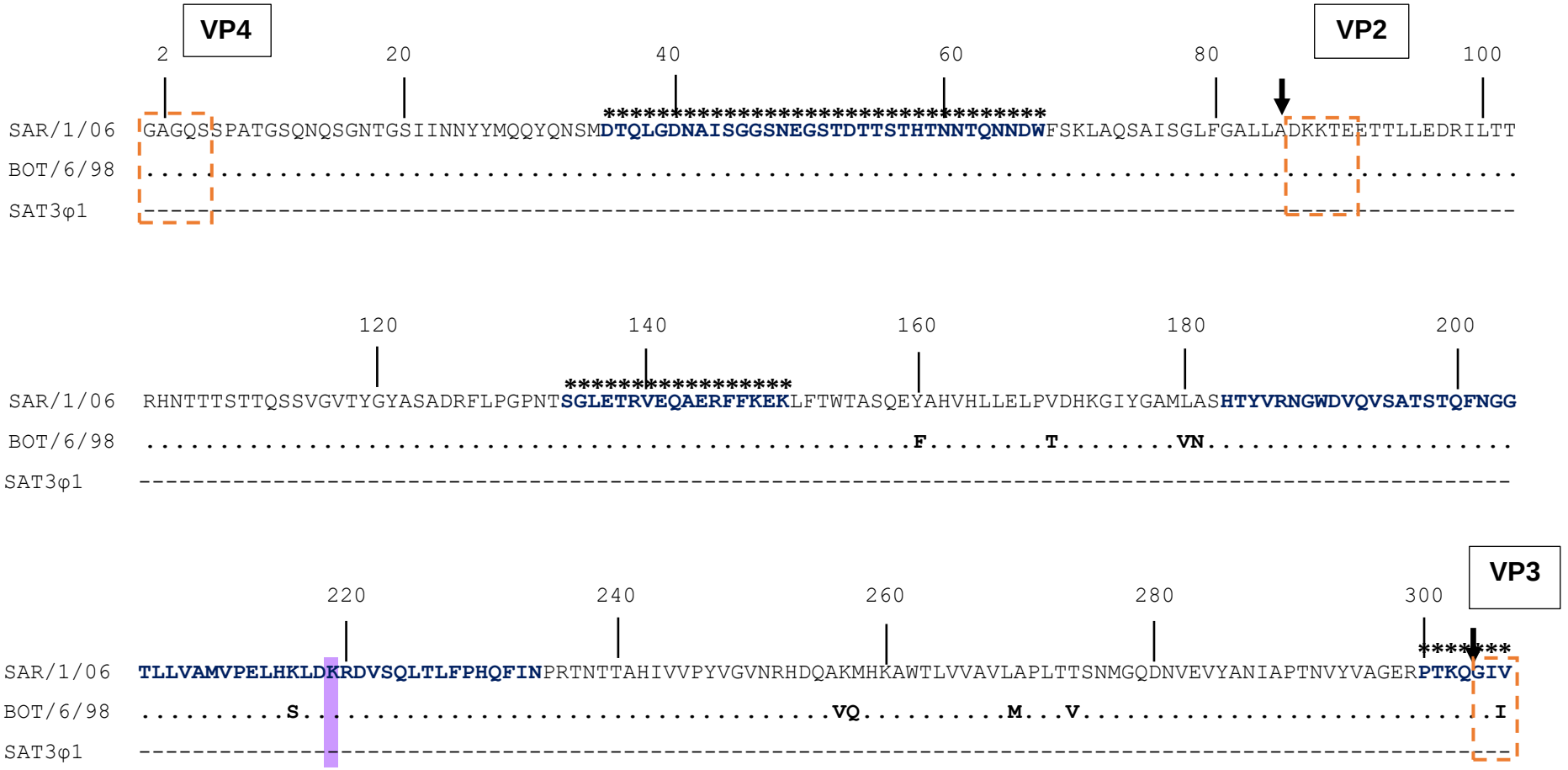
Dissolve 15 g agar, 8 g NaCl, 10 g tryptone, 5 g yeast extract in 800 ml dH2O and make final volume to 1 L. Autoclave and cool to 50 °C and add 100 µg/ml ampicillin and 2% glucose. Pour on plates (petri dishes) and store at 4 °C.

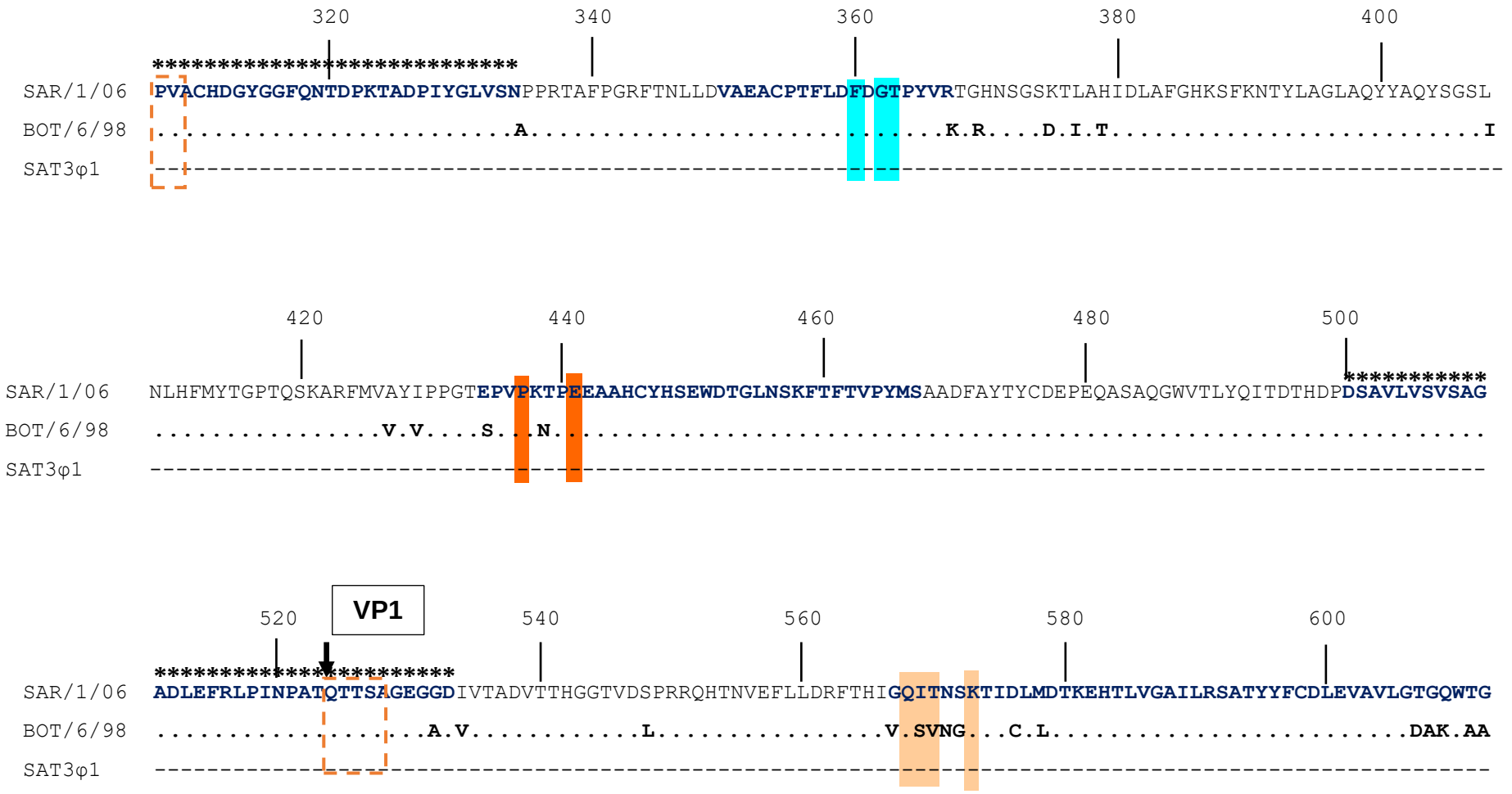
2X TY medium

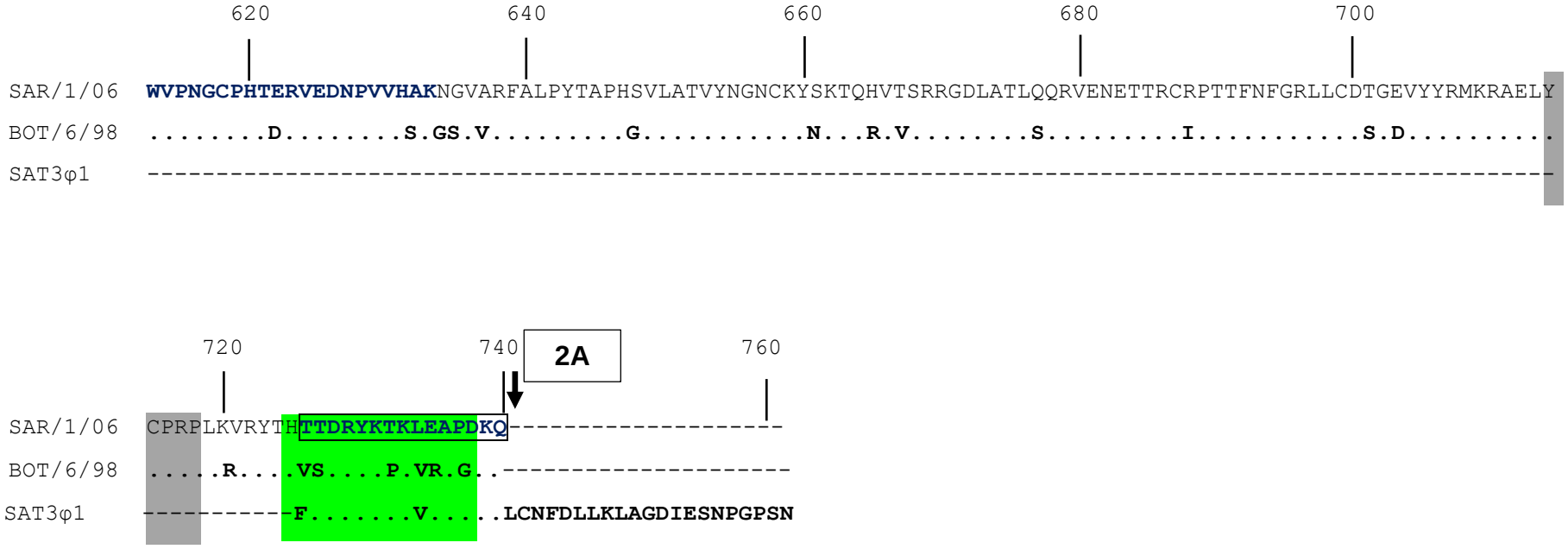
Dissolve 5 g NaCl, 16 g tryptone, 10 g yeast extract in 800 ml dH2O and make final volume to 1 L. Autoclave and cool to 50 °C and add 100 µg/ml ampicillin and 2% glucose or 25 µg/ml kanamycin.

APPENDIX C: SEQUENCE ALIGNMENTS

Amino acid alignment of the P1 region of two FMDV SAT3 virus strains and the FMDV SAT3 clone (SAT3φ1), including the 2A junction overlap of SAT3φ1. Identified potential FMDV SAT3 antigenic sites are indicated in **bold blue**. Known FMDV antigenic sites that correlates to potential antigenic sites from this study are shown (colour-coded). The dot (**.**) represents similarity of amino acids, whilst a dash (**-**) represents the absence of amino acids. The ⬚ represents the amino acid residues at the N-terminus of the FMD virus structural proteins of P1 *i.e.,* VP4, VP2, VP3 and VP1 respectively.




320
340
360
380
400
SAR/1/06 PVACHDGYGGFQNTDPKTADPIYGLVSNPPRTAFPGRFTNLLDVAEACPTFLDFDGTPYVRTGHNSGSKTLAHIDLAFGHKSFKNTYLAGLAQYYAQYSGSL
BOT/6/98 .....A.....K.R....D.I.T.....I
SAT3φ1
420
440
460
480
500
SAR/1/06 NLHFMYTGPTQSKARFMVAYIPPGTEPVPKTPEEAAHCYHSEWDTGLNSKFTFTVPYMSAADFAYTYCDEPEQASAQGWVTLYQITDTHDPDSAVLVSVSAG
BOT/6/98 .....V.V....S...N.
SAT3φ1
VP1
520
540
560
580
600
SAR/1/06 ADLEFRLPINPATQTTSAGEGGDIVTADVTTHGGTVDSPRRQHTNVEFLLDRFTHIGQITNSKTIDLMDTKEHTLVGAILRSATYYFCDLEVAVLGTGQWTG
BOT/6/98 .....A.V.....L.....V.SVNG...C.L.....DAK.AA
SAT3φ1


620
640
660
680
700
SAR/1/06 WVPNGCPHTERVEDNPVVHAKNGVARFALPYTAPHSVLATVYNGNCKYSKTQHVTSRRGDLATLQQRVENETTRCRPTTFNFGRLLCDTGEVYYRMKRAELY
BOT/6/98 .....D.....S.GS.V.....G.....N...R.V.....S.....I.....S.D.....
SAT3φ1 ----------
720
740
2A
760
SAR/1/06 CPRPLKVRYTHTTDRYKTKLEAPDKQ----------
BOT/6/98 .....R....VS....P.VR.G..----------
SAT3φ1 ----------F.....V.....LCNFDLLKLAGDIESNPGPSN

The identified potential antigenic sites with known FMDV antigenic sites (Refer to alignment sequence):

Potential antigenic site [Amino acids 350-367 of P1 (46-63 of VP3)]

Amino acid residues 56 and 58-59, located at the β-B “knob” of VP3 constitutes as site 4 (McCullough *et al.,* 1987; Barnett *et al.,* 1998) of serotype O was identified as an antigenic site. This antigenic site corresponds to site 4 of serotype A, Asia1 and site D3 of serotype C (Thomas *et al.,* 1988; Kitson *et al.,* 1990; Lea *et al.,* 1994; Grazioli *et al.,* 2013).

Potential antigenic site [Amino acids 434-467 of P1 (132-165 of VP3)]

A distant position amino acid residue 139, was also an antigenic site identified for Serotype A, forming part of site 4 (Thomas *et al.,* 1988).

Using MAb resistant (MAR) mutants, amino acid residue at 135 of VP3 was identified to be of importance for the antigenicity of SAT1 viruses (Grazioli *et al.,* 2013; Maake *et al.,* 2020).

Potential antigenic site [Amino acids 183-234 of P1 (98-149 of VP2)]

VP2 residue 134 has been identified as an antigenic site associated with SAT3 (KNP/1/03 and ZIM/5/91) (Maake *et al.,* 2020). The same site was identified by Mukonyora (2015) using in silico predictions programmes. This site was previously identified as an antigenic site for FMDV SAT2 and serotype O (Crowther *et al.,* 1993; Grazioli *et al.,* 2013; Maake *et al.,* 2020).

Potential antigenic site [Amino acids 567-633 of P1 (42-108 of VP1)]

Serotype O site 3 was mapped at amino acid residues 43-45 and 48 of VP1 (Barnett *et al.,* 1989; Kitson *et al.,* 1990; Grazioli *et al.,* 2013). Based on their location (H-I and B-C loop respectively), these sites were suggested to be related (Grazioli *et al.,* 2013).

Potential antigenic site [Amino acids 726-740 of P1 (201-215 of VP1)]

A conformationally dependent and trypsin-sensitive site (site 1 of Serotype O), which is located on the βG-βH loop of VP1 also involves amino acid residue 208 (Mahapatra *et al.,* 2012; Opperman, 2013). Residues 200-213 of the C-terminus of VP1 has been reported to form part of the discontinuous site, with site 1 of serotype O (Xie *et al.,* 1987; Parry *et al.,* 1989; Grazoili *et al.,* 2013). The same site was identified in serotype C (site C) (Mateu *et al.,* 1990; Grazoili *et al.,* 2013).

The conserved motif (Amino acid 185-190 of VP1 C-terminus)

An important motif for correct structure maintenance of picornavirus capsid precursors (P1-2A), before processing and subsequent capsid assembly. This site may represent interacts with cellular chaperones (Kristensen and Belsham, 2019).

*****No known published research data for amino acid positions **35-67**, **134-150**, **300-334** and **500-533** of the FMDV SAT3 P1 region could be correlated to FMDV antigenic sites. Thus, this study proposes these amino acids as novel potential antigenic sites for FMDV SAT3.

APPENDIX D: APPROVAL DOCUMENTS

RESEARCH ETHICS APPROVAL

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Research Ethics Committee

19 September 2022

LETTER OF APPROVAL

Ethics Reference No	**REC180-19**
Protocol Title	**DEVELOPMENT OF A FOOT-AND-MOUTH DISEASE VIRUS PEPTIDE PHAGE DISPLAY LIBRARY FOR THE IDENTIFICATION OF EPITOPES RECOGNISED BY IMMUNE SERA**
Principal Investigator	**Ms NPB Sekgobela**
Supervisors	**Mrs M Chitray**

Dear Ms NPB Sekgobela,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC180-19) 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.

We wish you the best with your research.

Yours sincerely

Mrs. MR Watson-Kriek
Chairperson (acting): Research Ethics Committee

Room 0-6, Arnold Theiler Building
University of Pretoria, Faculty of Veterinary Science
Private Bag X04, Onderstepoort, 0110, South Africa
Tel: +27 (0)12 529 8380
Email: marie.watson-kriek@up.ac.za
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Faculty of Veterinary Science
Fakulteit Veeartsenykunde
Lefapha la Disaense tSa Bongakadiruiwa

ANIMAL RESEARCH ETHICS APPROVAL

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

13 July 2022

Approval Certificate
Annual Renewal
(EXT2)

AEC Reference No.: REC180-19 Line 2
Title: DEVELOPMENT OF A FOOT-AND-MOUTH DISEASE VIRUS PEPTIDE PHAGE DISPLAY LIBRARY FOR THE IDENTIFICATION OF EPITOPES RECOGNISED BY IMMUNE SERA
Researcher: Ms NPB Sekgobela
Student's Supervisor: Mrs M Chitray

Dear Ms NPB Sekgobela,

The **Annual Renewal** as supported by documents received between 2022-03-01 and 2022-06-27 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-06-27.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Approved
Cattle -	
Samples	Approved
Bovine - Serum – ARC/OVR TAD (Stored/Historic retrospective)	3

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-07-13.
3. Please remember to use your protocol number (REC180-19) 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. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

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 Disaense tša Bongakadimelo

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 & Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Ms Marna Laing • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail:MarnaL@Dalrrd.gov.za
Reference: 12/11/1/1/MG (2413)

Dr Melanie Chitray
ARC-OVR (Transboundary Animal Diseases Programme)
100 Old Soutpan Road
Onderstepoort
0110
E-mail: chitraym@arc.agric.za

Dear Dr Melanie Chitray,

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

Your application dated 19 May 2022, 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 by any other act of the Republic of South Africa;
2. The study is approved as per the application and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to MarnaL @Dalrrd..gov.za;
3. All potentially infectious material utilised, collected or generated during the study are to be destroyed at the completion of the study. A registered waste removal company must dispose the material generated from the study. Records must be kept for five years for auditing purposes;
4. Only serum samples stored within the BSL3 facility at TAD-OVR must be used in this study;
5. No samples are to be outsourced to other laboratories for testing and storage;
6. Any deviation from the conditions listed in this permit must be permitted by the Director Animal Health prior to inception;

7. 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 approval;

Title of research/study: Development of a foot-and-mouth disease virus peptide phage display library for the identification of epitopes recognised by immune sera.

Researcher:	Dr Melanie Chitray,
Institution:	ARC-Onderstepoort Veterinary Research Institute
Our ref Number:	12/11/1/1/MG (2413)
Your ref:	
Expiry date:	31 December 2025

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date:

ARC ANIMAL ETHICS APPROVAL

AEC 19-21

ARC • LNR

Onderstepoort Veterinary Institute

ARC • LNR

Animal Ethics

Decision of the Animal Ethics Committee for the use of living vertebrates

PROJECT PERIOD
STARTING DATE: 31 January 2019 ENDING DATE: 31 December 2021

for research, diagnostic procedures and product development

PROJECT NUMBER:	P10000148			
PROJECT TITLE:	Development of a foot-and-mouth disease virus peptide phage display library for the identification of epitopes recognised by immune sera.			
PROJECT LEADER:	Dr Melanie Chitray			
DIVISION:	Transboundary Animal Diseases			
CATEGORY:	A			
SPECIES OF ANIMAL:	N/A			
NUMBER OF ANIMALS:	N/A			

RECOMMENDATIONS BY ANIMAL ETHICS COMMITTEE

Date of AEC meeting for consideration:	Action Taken:	SIGNATURE: AEC-Chairperson Dr P. Mutowembwa
2019.09.18	Approved APPROVED	

PLEASE NOTE: Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit a revised animal ethics clearance form to the animal ethics committee for approval before commencing with the experiment

CONFERENCE CONTRIBUTION

Sekgobela, N. P. B., Chitray, M., Opperman, P. A., Fehrsen, J. and Maree, F. F. (Poster presentation). Construction of three foot-and-mouth disease virus peptide phage display libraries as a tool for the identification of important epitopes. SASVEPM 2021, 24-27 August 2021, Bela Bela, South Africa.

Sekgobela, N. P. B., Chitray, M., Opperman, P. A., Fehrsen, J. and Maree, F. F. (Oral presentation). Construction of three foot-and-mouth disease virus peptide phage display libraries as a tool for the identification of important epitopes. University of Pretoria, Faculty of Veterinary Science, Faculty Day 2021, 21 October 2021, Pretoria, South Africa.