Evaluation of a DAS-ELISA for quantification of foot-and-mouth disease virus 146S antigen during vaccine preparation

by

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Submitted in partial fulfilment of the requirements for the degree MSc TAH (Tropical Animal Health) in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Sciences, University of Pretoria

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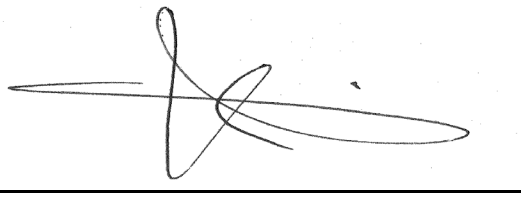
October 2023

DECLARATION

I, **Kabelo Tlaka**, do hereby declare that this dissertation entitled **"Evaluation of a DAS-ELISA for quantification of foot-and-mouth disease virus 146S antigen during vaccine preparation"**, which I hereby submit for the degree of Master of Science in Tropical Animal Health at the University of Pretoria, is my work and has not previously been submitted in part or full by me for a degree at this or any other tertiary institution. All sources cited or quoted in this research paper are indicated and acknowledged with a comprehensive list of references.



Kabelo Tlaka

October 2023

ETHICS STATEMENT

I, **Kabelo Tlaka**, the author of this dissertation have obtained for the research described in this work the applicable research ethics approval. I declare that I have observed the ethical standards required in terms of the University of Pretoria's code of ethics for researchers and the policy guidelines for responsible research. The ethics approval reference number is REC060-20

ACKNOWLEDGEMENTS

To have achieved this degree, I would like to express my sincere gratitude to the following:

- First, the almighty God for giving me the strength, health, knowledge and perseverance to never give up.
- My family for their patience and unwavering moral support.
- My research supervisor Prof Melvyn Quan and co-supervisor Dr Livio Heath for your guidance, understanding, encouragement and suggestions you provided during this research study.
- To Dr Faith Peta for providing me with cells, viruses, reagents and allowing me to use the FMD vaccine production laboratory for my studies.
- My colleagues in the FMD vaccine production facility for providing insightful inputs and reviews to this research study.
- The FMD diagnostic staff for their ELISA training and allowing me to utilize their laboratory services.
- The Institute of Tropical Medicine in Antwerp for a partial FA4 bursary to attend the MSc induction week.
- Finally, the Agricultural Research Council, for not only providing the funding which allowed me to undertake this research study but also for allowing me the opportunity to attend lecture classes and to meet many academically inspiring individuals.

ABSTRACT

The inactivated foot-and-mouth disease (FMD) vaccine's protection is dependent on the intact component of the FMD virus (FMDV) antigen, the 146S antigen particle. Sucrose density gradient (SDG) centrifugation is the standardised method for quantifying 146S antigen during FMD vaccine formulation. However, because it is operator-dependent, this approach is labour-intensive and produces varied outcomes. As a result, the polyclonal double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was developed as an alternative approach to quantify the intact 146S antigen of both FMDV South African Territories (Mahapatra and Parida) -1 & 2 serotypes.

The polyclonal DAS-ELISA performance was compared to the SDG centrifugation test to evaluate the assay as an alternative technique for quantifying the intact 146S antigen of both SAT serotypes. In BHK-21 cells, the FMDV 146S antigen of both SAT serotypes was generated. For each serotype, sixteen samples were examined in duplicates using the SDG (10-30%) centrifugation and polyclonal DAS-ELISA at varied time intervals (0, 5, 10, 15 minutes) and dissociation conditions (Intact 146S, Temperature, pH, complete dissociation (CD)). The SDG method identified the intact 146S antigen particles at 254 nm using a Type 11 Optical Unit for a UA-6 absorbance detector, whereas polyclonal DAS-ELISA detected FMDV antigen particles at OD 450 nm using a microplate ELISA reader.

The SDG was more specific to the Immunogenically intact 146S antigen, whereas polyclonal DAS-ELISA measured an equivalent reactivity to the intact 146S antigen and the 12S protein components in both SAT serotypes. The polyclonal DAS-ELISA technique was not suitable for quantifying the intact 146S antigen and so could not be used for quantification of the immunogenic 146S antigen component during vaccine production.

Key terms: FMDV, Sucrose Density Gradient (SDG), polyclonal DAS-ELISA, 146S antigen, 12S protein subunits, complete dissociation (CD)

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LIST OF ABBREVIATIONS

AEC	Animal Ethics Committee
ALP	Alkaline phosphatase
AUFS	Absorption units full-scale
BHK	Baby hamster kidney
BEI	Binary ethylenimine
CD	Complete dissociation
CF	Complement fixation
CO_2	Carbon dioxide
DALRRD	Department of Agriculture, Land Reform and Rural Development
DVTD	Department of Veterinary Tropical Diseases
ELISA	Enzyme-linked immunosorbent assay
FMD	Foot and mouth disease
FMDV	Foot and mouth disease virus
GMEM	Glasgow's minimum essential medium
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
IgM	Immunoglobulin M
IRES	Internal ribosome entry site
LAH	Lactalbumin hydrolysate
NaOH	Sodium hydroxide
NK	Natural killer cells
NSPs	Non-structural proteins
NBS	Normal bovine serum
OD	Optical density
ORF	Open reading frame
OVR	Onderstepoort Veterinary Research
PAb	Polyclonal antibody
PBS	Phosphate-buffered saline
PD_{50}	50% Protective dose
PEG	Polyethylene glycol
pH	Potential of hydrogen
PKs	Pseudo knots
pNPP	P-nitro-phenyl phosphate
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
REC	Research Ethics Committee
SAT	Southern African territories
SDG	Sucrose density gradient centrifugation
TADP	Trans-Boundary Animal Disease Programme
TPB	Tryptose Phosphate Broth
USA	United States of America
UTR	Untranslated region
VPg	Viral protein genome
Vv	Volume/volume.

CHAPTER 1. INTRODUCTION

1.1. Introduction

Foot and mouth disease (FMD), caused by the Foot-and-mouth disease virus (FMDV), is one of the most infectious transboundary viral diseases of cloven-hoofed ungulates. The disease has a broad geographical distribution with diverse host species, primarily in domesticated livestock (cattle, goats, sheep, pigs) and susceptible wild artiodactyls with African buffalo (*Syncerus caffer*) serving as the persistent carrier for Southern African Territories (Mahapatra and Parida) serotypes within Southern African regions (Paton et al., 2018, Wekesa et al., 2015, Condy et al., 1985).

FMDV infections are characterized by episodes of high morbidity and low mortality in mature livestock but higher mortality in much younger livestock (Abu-Elnaga et al., 2015, Knight-Jones and Rushton, 2013). Because of the enormous costs associated with disease control, prevention, eradication, and the imposition of strict measures on the movement of both local and international meat, meat products, and animal trade markets, outbreaks can have devastating indirect socio-economic implications (Rodriguez and Gay, 2011, Mahapatra and Parida, 2018). Direct negative economic impacts are due to the significant reduction in milk production, reduced animal growth and spontaneous abortions in affected animals (Delgado et al., 2001, Ellis and Putt, 1981).

Apart from culling, stamping-out, movement control and monitoring of infected and infectious animals, zoning and constant surveillance, ongoing mass vaccination with inactivated whole virus vaccine against FMD is one of the most viable and effective approaches for preventing, controlling, and eliminating FMD outbreaks (Depa et al., 2012, Sutmoller et al., 2003, McKenna et al., 1995). The virus that causes foot-and-mouth disease can be genetically and serologically characterized (or classed) into seven immunologically variable serotypes (O, A, C, Asia-1, SAT-1, SAT-2, and SAT-3) (Vallée and Carré, 1922, Waldmann and Trautwein, 1926, Brooksby and Roger, 1957, Brooksby, 1958). FMDV has high genetic variability, which has resulted in the existence of several diverse antigenic subtypes and topotypes within different serotypes (Bari et al., 2014, Mittal et al., 2005).

1.2. Foot and mouth disease history

Five centuries ago in 1514, an Italian physician monk Hieronymi Fracastorii reported a disease that affected cattle in the city of Venice, which had similar symptoms to that of FMD as we know it today. Animals affected with the disease displayed a lack of appetite, oral mucosal inflammation and the development of vesicular lesions in the oral cavity and around the cloven hooves (Jamal and Belsham, 2013). It was only at the end of the 19th century in Greifswald, Germany where it was demonstrated by Friedrich Loeffler and Paul Frosch that FMD was caused by tiny ultra-filterable particles, which were infectious and identified as FMD virus (FMDV) particles (Loeffler and Frosch, 1897). Regarded as one of the most highly contagious and transmissible diseases caused by viruses among animals (Pereira, 1981), FMDV was recognized as the first virus that could cause disease in animals (Loeffler and Frosch, 1897).

1.3. Overview of foot-and-mouth disease virus

1.3.1. Structure and genome

The aetiological agent of FMD, FMDV, according to the International Committee on Taxonomy of Viruses (ICTV) belongs to the *Picornaviridae* family and is the prototype member of the genus *Aphthovirus* (Zell et al., 2017). It is a small viral particle of about 25 - 30 nm in diameter with a roughly spherical capsid.(Bachrach, 1968, Forss et al., 1984). A single virion contains a non-enveloped, single-stranded, positive-sensed RNA genome that is approximately 8.5 kilobases (Abu-Elnaga et al.) long and that is enclosed in a capsid with icosahedral symmetry (Belsham, 2005, Jamal and Belsham, 2013). The 5′ untranslated region (UTR) is about 1,300 nucleotides long and the 3′ UTR is shorter at nearly 100 nt long (Tuthill et al., 2010, Belsham, 2005). The 5′ end is covalently bonded to smaller protein molecules of about 24 to 25 nt long encoded within the 3B region of the open reading frame (ORF) termed the viral protein genome (VPg). These VPgs exist in three small forms (VPg1 (3B1), VPg2 (3B2) and VPg3 (3B3) and are homologous (Mason et al., 2003, Sangar et al., 1977). The VPgs are uridylated by the viral RNA dependant RNA polymerase (RdRp; 3D^POL) to form (VPgpU (Pu). This form of the protein will actively serve as a primer for the synthesis of both positive (+'ve) sense and negative (-'ve) antisense strands required for the

replication of viral genome (Nayak et al., 2006, Paul and Wimmer, 2015, Nayak et al., 2005).

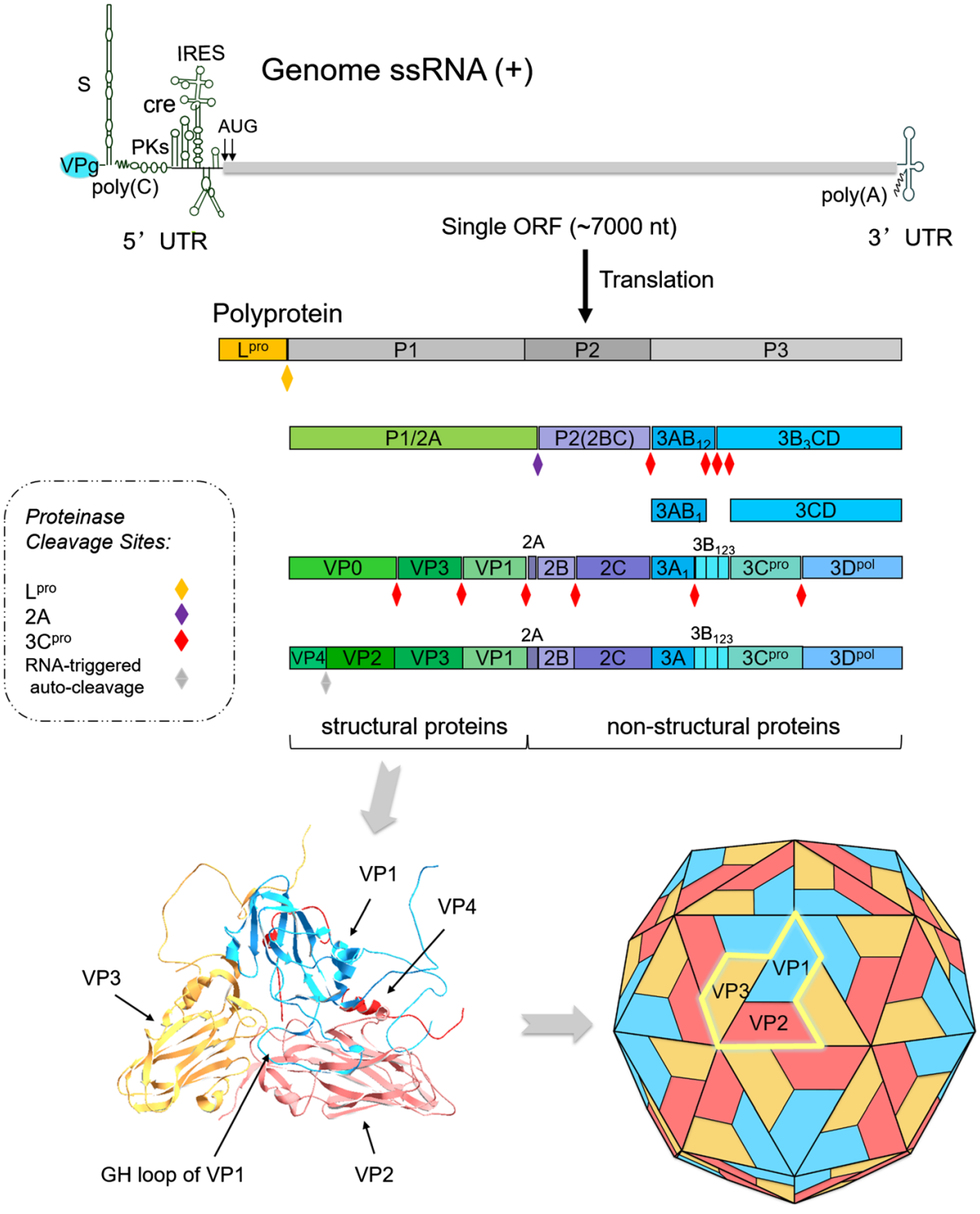

Figure 1.1. A schematic diagram of FMDV genome, processing of viral polypeptide and conformation of the structural proteins. The genome contains a single open reading frame (ORF) of about 7 kb of RNA with two alternative initiation sites. The ORF is flanked by a long 5'-untranslated region (5'-UTR) with a 3B (VPg) covalently bound to its 5' end and a short 3'-UTR. The ORF region is divided into four functional areas (L, P1, P2 and P3) due to the different functions of mature polypeptides. The ORF-

encoded polyprotein is processed into four products: Lpro, P1-2A, 2BC and P3 by Lpro, 2A and 3Cpro. The precursors P1-2A, 2BC and P3 are further processed into mature viral proteins and some cleavage intermediates with relative stability, such as VP0 or 1AB, 3ABC, 3BCD, 3AB, and 3CD by 3Cpro (Gao et al., 2016).

The longer 5' UTR end can be subdivided into five functional segments as in Figure 1. The first part is the S fragment, about 350 - 360 nt long and is believed to fold into a long hairpin structure. It is assumed to inhibit host exonuclease activity in order to preserve viral genome integrity and replication (Gao et al., 2016, Clarke et al., 1987, Escarmís et al., 1992). The second segment is a poly-C tract of about 150 - 250 nt long, made of not less than 90% C residues and essential for live virus rescue with no evidence of association to virus virulency. The third segment, the L fragment of approximately 700 nt, is made of multiple pseudo knots (PKs) of about four to five structures (Brown et al., 1974, Harris and Brown, 1977). The fourth part represents the *cis*-acting replication element (*cre*/*bus*), forming a stable stem-loop structure of about 55 nt. Within its stem-loop, the structure contains a highly conserved AAACA motif that acts as a template during the uridylylation process of VPg protein making it essential in initiating the RNA replication process with the aid of $3D^{pol}$ (Mason et al., 2002, McKnight and Lemon, 1996, Mcknight and Lemon, 1998). The last segment is the internal ribosome entry site (IRES) of type II with a length of about 450 nt. It plays an essential role to initiate cap-independent translation of the viral genome (Jang et al., 1988, García-Nuñez et al., 2014). As a whole component, the entire 5' UTR plays an integral part in initiating viral polyprotein cap-independent translation as well as replication of the viral genome (de Quinto and Martinez-Salas, 1997).

Following the 5' UTR, is the translated large ORF of about 7 kb long that produces the polyprotein, which is cleaved virally by two enzymatic proteases (leader (L^{pro}) and 3C protease ($3C^{pro}$)) to yield the resulting structural proteins and non-structural proteins (NSP) (Gao et al., 2016, Conda-Sheridan et al., 2014, Jamal and Belsham, 2013, Strebel and Beck, 1986). The L^{pro} in its viral proteinase format is the first protein produced after its self-cleavage at the C terminus of the polyprotein. It is a papain-like cysteine protease and exists in two protein formats, the Lab and the Lb protein format that results from the initiation of translation at two start codons (AUGs) located 84 nt apart, in which the Lab protein contains a highly variable N-terminal extension of about ~28 amino acids compared to the Lb protein. The start codon of the Lb protein is, however, preferably used more than the start codon of the Lab protein during

translation (Esteban-Torres et al., 2015, Pöyry and Jackson, 2011). Some of the vital roles played by the L^{pro} during translation include acting as a viral virulence factor to inhibit protein synthesis of the host (Grubman and Baxt, 2004). The L^{pro} also cleaves eIF4G and its derivatives (eIF4GI and eIF4GII) which are essential for the initiation of translation in the host, resulting in shutting off host mRNA cap-dependent translation (Devaney et al., 1988).

Immediately downstream closer to the L region is the P1 region of the polyprotein. The P1 is cleaved by the $3C^{pro}$ into three mature capsid-forming structural proteins (VP0, VP3, and VP1) (Vakharia et al., 1987). The VP0 in the presence of the RNA genome is cleaved further to yield VP4 and VP2 during the process of genome encapsulation. This results in four structural proteins contained within P1 in which the VP4 is internally enclosed within virus particles while the remaining VP1, VP2 and VP3 are expressed on the outer surface of the virus capsid (Lea et al., 1994, Thomas et al., 1988b). A single copy of each structural viral protein (VP) makes up one promoter, the 5S, five promoters together make one pentamer, the 12S and 12 pentamers together make a complete capsid, the 75S. A mature single viral capsid is icosahedral in shape and made up of 60 copies of the capsomers self-assembled into 12 pentamers in which each capsomer encompasses the four proteins with structural functions (VP1, VP2, VP3 and VP4) along with the single viral RNA genome, the intact 146S. All this takes place during the process of viral capsid assembly (Acharya et al., 1989, Han et al., 2015, Fry et al., 2005, Vasquez et al., 1979).

Following the P1 region are the P2 and the P3 regions respectively. The P2 is located at the centre of the genome while the P3 is at the 3’ end just before the 3′ UTR. This region encodes 2A, 2B, and 2C, whereas the P3 region encodes 3A, 3B (3B1, 3B2 and 3B3), $3C^{pro}$ and $3D^{pol}$ non-structural viral proteins. The 2A serves as a precursor protein for the cleavage of a P1-2A junction, together with the 2B they are essential for the process of virus replication and contain virulence factors that are involved in inhibiting the functions of the host cell, which includes both RNA transcription and protein synthesis. The $3C^{pro}$, a “chymotrypsin-like cysteine protease” serves as a primary enzyme for the proteolytic processing of the full-length viral polyprotein and as an RNA binding polyprotein (Klump et al., 1984, Vakharia et al., 1987, Birtley et al., 2005, Neeta and Tayo, 2012). The $3D^{pol}$ (RdRp) is involved in viral genome replication

and lacks proofreading ability (Rai et al., 2017, Cowan and Graves, 1966). The last segment of the genome following the termination codon of ORF is the 3' UTR. This segment is made of two parts, which include a short ~ 90 nt stem forming loop structure and a length variable poly-A tail (Serrano et al., 2006, Mulcahy et al., 1990, Dorsch-Häsler et al., 1975). The 3' UTR play a role in viral infection and genome replication. It stimulates an IRES-dependent activity required for translation within the cytoplasm while determining the virulency of the FMD virus (Saiz et al., 2001, López de Quinto et al., 2002).

1.3.2. Serotypes and distribution

FMDV exists within seven immunologically variable serotypes. These include O, A, C, Asia-1, SAT-1, SAT-2, and SAT-3 serotypes. Serotypes O and A were originally discovered by Vallee and Carre in 1922 after cattle which had previously recovered from FMDV infection of serotype O from French origin, became re-infected from coming in contact with cattle that were recently infected with FMDV serotype A from German origin. In the late 1920s in Europe, Waldmann and Trautwein discovered the C serotype which was found to be circulating mainly in bovine and swine species, however causing less outbreaks than O and A serotypes. In 1954 Brooksby and Rogers, identified the Asia-1 serotype, after its detection from clinical samples collected from Asian water buffalo (*Bubalus bubalis)* in the city of Okara, Punjab province, Pakistan. Brooksby identified the last recognised serotypes SAT-1, SAT-2 and SAT-3 in 1958, isolated from clinical samples of South African origin (Paton et al., 2021, Vallée and Carré, 1922, Waldmann and Trautwein, 1926, Brooksby, 1958, Brooksby and Roger, 1957). In Southern Africa, the SAT serotypes are maintained in African buffalo (*Syncerus caffer*), which serve as a reservoir host (Thomson et al., 1992). The virus has high genetic variability due to frequent genome replication that results in a high rate of mutations, genetic recombination and formation of quasispecies. This has, over time, resulted in the existence of multiple diversified antigenic subtypes and topotypes within different serotypes (Robson et al., 1977, Domingo and Holland, 1997, Mittal et al., 2005).

FMDV serotypes are unevenly distributed globally, with serotypes O and A the most widely distributed on several continents. This includes Africa, Asia, the Middle East and South America, with some isolated appearances in some parts of Europe.

Serotype O was initially classified into 10 to 11 antigenic subtypes by Davie, (1964) while serotype A displays greater antigenic diversity than any other serotypes amongst the Eurasian serotypes by having up to 32 subtypes (Pereira, 1976). Previous records indicate that serotype C has long been restricted to the Indian subcontinent after disappearing from both the European and South American continents. However, the serotype was recently isolated in 2004 in the Amazonas state of Brazil and Kenya, Africa. Originally, this serotype was classified into five antigenic subtypes C1- C5 (Rweyemamu et al., 2008, Sangula et al., 2011). Asia-1 is a prevalent serotype on the Asian continent, with isolated appearances in Europe and the Middle East. It is one of the antigenically less diverse serotypes among Eurasian serotypes, having been characterized into only three antigenic subtypes (Davie, 1964). The SAT-1, -2, and -3 serotypes are prevalent across the African continent, particularly in sub-Saharan Africa with several outbreaks of both SAT-1 and -2 recorded in some countries within the Middle East (Saudi Arabia and Kuwait). In one incident, the SAT-1 serotype was isolated in Greece (Knowles and Samuel, 2003, Grazioli et al., 2020, Sangula, 2006).

1.3.3. FMDV antigenicity

Among the four FMDV structural proteins, VP1 illustrated in Figure 1 contains the main “neutralizing antigenic site” that can illicit the required neutralizing antibodies (Gao et al., 2016, Laporte, 1973). The VP1 protein contains highly variable linear epitopes on its amino acids residues 135 to 155 and on its carboxyl terminus (amino acids residues 200 to 213). These are areas of major and minor antigenic activities respectively. Beta overlapping G-H loops located within these residues induce infectivity, neutralizing and non-neutralizing antibody reactions. This trait is shared among different serotypes of the FMDV and accounts for its poor cross-reaction across the seven serotypes. The VP1 protein contains trypsin-sensitive regions between the two residues 138 to 154 and 200 to 212. Exposure to these proteolytic enzymes results in reduced immunity and infectious abilities of the capsid protein (Strohmaier et al., 1982, Saiz et al., 1991, Thomas et al., 1988a, Wild et al., 1969).

FMDV particles can be divided into several specific antigenic particles based on their sedimentation coefficient in sucrose density gradient centrifugation (Barteling and Meloen, 1974, Fayet et al., 1971). First, the intact 146S/140S virion is a whole virus particle made of 12 pentameric structures which contain one copy of a single-stranded

RNA molecule with a molecular weight of about 2.6 million daltons. The virus is made up of 60 copies of each structural protein VP1, VP2, and VP3 expressed on the outer surface of the capsid with a combined molecular weight of about 24,000 daltons and a VP4 structural protein that is located within the internal surface of the capsid with a molecular weight of about 8,000 daltons.

The efficacy and immunogenicity of FMD vaccines depend entirely on the wholeness and integrity of the antigenic 146S particle. It is highly immunogenic in that it is mainly responsible for stimulating neutralizing antibodies, and producing precipitation and complex fixation antibodies. This is believed to be due to the configuration of antigenic sites on the outer surface of the intact 146S antigen particle, resulting in the antigen particle having epitopes that are both conformation-dependent and conformation-independent.

The 75S particle is referred to as “natural empties”. It is an empty viral capsid particle, which contains no RNA molecule. It is made of 60 copies of each VP1, VP3 and VP0, an uncleaved precursor of VP2 and VP4 structural proteins. The 75S particle antigenic properties are said to be similar, if not identical to those of the 146S particle. It is also able to induce neutralizing antibodies however of a lesser quantity compared to the intact particle, the 146S.

The 45S antigen particle is related to peptides of which not much has been reported. The 12S antigen is a pentameric subunit made of five copies of structural proteins VP1, VP2 and VP3, but lacks the VP4 protein. It is regarded as the “crypto-antigen” as it is only available after a slight disruption of the 146S antigen by acid exposure at a pH of around 6.5 or through heat exposure at a temperature of about 56°C in a process that is irreversible. The 12S subunits are single pentameric pieces, which when assembled in a group of 12 together form the intact 146S virion. The 12S antigen subunits can elicit reduced amounts of neutralizing antibodies however able to stimulate precipitating and complement fixation antibody production as for the intact 146S particle. The 12S subunits share several epitope similarities to those of the 146S particle, hence some of the monoclonal and polyclonal antibodies targeting the 146S antigen epitopes may cross-react. Because the 12S antigenic sites are not present on the complete particle's outer surface, they exhibit a separate epitope conformation, resulting in diminished immunogenic activity.

The 3.5S antigen is a virus infection associated antigen, which is associated with the RNA polymerase with a molecular weight of 56,000 (Crowther, 1986, Doel and Chong, 1982, McCullough et al., 1987, Cartwright et al., 1980, Feng et al., 2016, Kim et al., 2020, Hussein et al., 2015, Denoya et al., 1978, Brown and Newman, 1963, Laporte, 1973).

1.3.4. FMDV stability and dissociation

Among the seven existing FMDV serotypes, the temperature and acid stability of the capsid protein are variable, with both the O and SAT serotypes being highly sensitive to acid and temperature variations (Doel and Baccarini, 1981). The FMD viral capsid stability depends on several identified factors, including the electrostatic repulsive interactions (hydrogen bonds, salt bridges, van der Waals forces, covalent bonds, electrovalent bonds and disulphide bonds) that occur between the capsid subunits and residues of amino acids within the nucleic acid. Exposure to variable environmental pH and temperatures easily affects these electrostatic interactions, resulting in the dissociation of the intact viral capsid into subunits and rendering the virion's immunogenicity inactive. (Caridi et al., 2015, Curry et al., 1995, Mateo et al., 2008, Martín-Acebes et al., 2010, Rincón et al., 2014). Because FMDV particles have the highest acid and temperature lability among other members within the *Picornaviridae* family, exposure to acid or temperature variations will lead to capsid dissociation. Pentameric subunits will form as a result of capsid dissociation due to exposure to pH slightly below neutral values (Newman et al., 1973, Yuan et al., 2017). During the process of capsid dissociation into pentameric subunits, the highly hydrophobic protein VP4, which has myristoylation abilities is released together with the genomic RNA (Belsham et al., 1991). The subsequent release of this internal VP4 protein plays an integral role in the ionization and permeability of the cell membrane in which genomic RNA is released via the endosomal channel (Danthi et al., 2003, Davis et al., 2008).

During the uncoating of genomic material into the endosome of the infected host cell through the endosomal pathway, the FMDV acid-labile character is required to start virus replication, however, during formulation and storage of the inactivated FMDV vaccine using capsid protein, the integrity of the intact capsid is a prerequisite. Thus a balance is needed between pH sensitivity and stability to produce an effective inactivated vaccine' (O'Donnell et al., 2005, Yuan et al., 2020). To date, several amino

acid residues relevant for FMDV capsid acid sensitivity when pH is changed have been discovered (Yuan et al., 2017). The negatively charged histidine residues (H142 and H145) located within the VP3 protein colonise the inter-pentameric interface of the capsid's subunits in a high density which triggers the acid-mediated induction of capsid dissociation after protonation in the acidic environment of the endosome. This phenomenon may even occur at a neutral pH level, which may be due to the electrostatic repulsive interactions that occur in between these protonated histidine residues (Acharya et al., 1989, Rincón et al., 2014).

In a quest to determine alternative methods of improving the acid stability and sensitivity of FMDV, it has been determined that the substitution of a single amino acid residue in the capsid protein can enhance stability and increase acid sensitivity of the virus (Martín-Acebes et al., 2010). To date, about 11 amino acid residues when substituted have proven to be able to improve the acid stability of the FMDV particle during inactivation (Yuan et al., 2017). It was revealed during investigation using FMDV serotype C, O, and Asia-1 that the substitution of amino acids residues N17D located within the VP1 N terminus can increase acid resistance, improve alkali stability and maintain thermal-stability of the antigen (Martín-Acebes et al., 2011, Liang et al., 2014, Vázquez-Calvo et al., 2014). However, the substitution of amino acids residue N17D of VP1 resulted in a decreased ability to replicate *in vitro* in a BHK-21 cell line and resulted in a mild increase in virulence *in vivo* in suckling mice. In contrast, the substitution of amino acids residue D86H of VP2 had no adverse effect on the virulence of the virus (Yuan et al., 2020).

1.4. FMDV vaccination

1.4.1. Vaccine history

Research and development of the FMD vaccine began early during the 20th century with the development of an experimental vaccine made from mucosal lesions of FMD-infected cattle in saline buffer, filtered and inactivated using formaldehyde (Vallée et al., 1926). It was around this time when Belin (1927) experimented on the attenuation of FMDV but it was only in 1973 at the German Institute of Reims Island in the Baltic Sea where the first practical vaccine was developed using a virus harvested from the epithelium and vesicular fluid from the tongue of a cow that was deliberately infected.

The harvested virus was inactivated using formaldehyde at a lower concentration of 0.05% and maintained pH levels greater than nine while keeping the temperature at around 25°C for about 48 hours (Lombard et al., 2007). Aluminium hydroxide gel was used as an adjuvant during vaccine formulation. The aluminium hydroxide adjuvant facilitates virus inactivation while being involved in improving the immunogenicity of the final product (Waldmann et al., 1937). Years later in Chile, it was discovered that saponins are an effective adjuvant when mixed with the aluminium hydroxide gel (Espinet, 1951).

Because of the undesirability of harvesting the starting biological material from deliberately infected cattle, a Dutch scientist (Frenkel, 1947) from the Amsterdam Veterinary Institute adapted epithelial suspension cells *in vitro*. The cells were obtained from the tongue of healthy cattle that were recently slaughtered. Some challenges came with the maintenance, preparation and collection of adequate epithelial cells from the tongue of cattle. A new cell line, BHK-21 clone 13 monolayer cells that were developed previously by Macpherson and Stoker (1962) was therefore adapted to grow and titrate FMDV (Mowat and Chapman, 1962). To produce FMDV antigen and vaccine on a large industrial scale, BHK-21 clone 13 monolayer cells were adapted to grow as a suspension of cells at Pirbright Laboratory in the UK, 1962 (Capstick et al., 1965). Once adapted, the BHK-13 suspension cells were then produced in larger fermenters, an industrialized FMD vaccine production system that is still operational to date (Telling and Elsworth, 1965).

1.4.2. Vaccine types

Because of the great genetic and antigenic variation of FMDV, vaccination or previous infections with one serotype does not confer immunity against new infections with any of the other six other serotypes and may also fail to provide full protection against subtypes within the same serotype (Diaz-San Segundo et al., 2014, Doel, 2005, Paton et al., 2005). Therefore, currently formulated FMDV vaccines are either monovalent (protective against a single strain), bivalent (protective against two strains), trivalent, pentavalent or multivalent vaccines that contain several different types of strains (Lombard and Füssel, 2007, Parida, 2009). The combination of strains in the formulation of FMDV vaccines depends on the requirements of the species to be vaccinated and the epidemiological situation of the area where the vaccine is to be

applied. Vaccines in countries with high endemic levels of FMDV tend to include more than one viral strain in their combination (Parida, 2009, Doel, 2003). Just like many other available vaccines, immunisation with FMDV vaccines does not elicit a sterilising immune response, however, replication of the virus at mucosal-epithelial surfaces may occur resulting in carrier state development in vaccinated animals after being challenged with live virus. The vaccine is however capable of preventing clinical infection against live FMDV challenge (Doel, 2003, Parida, 2009).

Ideally, FMD vaccines should be safe to use and elicit rapid but prolonged protective immunity even after a single dose of inoculation, with no permanent carrier state. The vaccine hould provide serotype and subtype cross-protection, remain potent and be able to stimulate humoral and cellular immune responses. The vaccine should be cost effective to produce, vaccine productions should be scalable, and preferably a cold chain should not be required for distribution of the vaccine. The vaccine should contain a stable antigen that closely matches circulating field strains, be sterile, non-infectious and free of adventitious agent, but most importantly have properties to allow naturally infected and deliberately vaccinated subjects to be distinguished (differentiation of infected from vaccinated animals (DIVA)). Multivalent vaccines are ideal in that they offer protection against several FMDV serotypes and their respective subtypes (Kamel et al., 2019, Park, 2013, Yang et al., 2017).

Most currently used FMD vaccines that are produced commercially depend on the integrity of whole virion particles: the intact 146S antigens that contain the most relevant protein, viz. VP1. These vaccines are produced from the propagation of live FMDV of the desired serotypes in suspension tissue culture (BHK-21 cells), That are then inactivated through binary ethyleneimine (BEI) and purified by antigen purification with either polyethylene glycol (PEG) or ultrafiltration. The aluminium hydroxide (which can boost the immune system response) together with gel or mineral oil is used as an adjuvant depending on the produced serotype (Doel, 1999, Doel, 2003, Sáiz et al., 2002). This is referred to as traditional/conventional inactivated FMD vaccines (Tang et al., 2012, Parida, 2009). To date, several new types of FMDV vaccines have been developed which include DNA vaccines, attenuated vaccines, recombinant vaccines, subunit vaccines and peptide vaccines (Kamel et al., 2019, Hardham et al., 2020).

1.4.3. Vaccine advantages and limitations

Different types of FMD vaccines have different advantages and limitations. Traditionally inactivated vaccines can be highly concentrated and reach the equivalence of three to six times the required 50% protective dose (PD_{50}) to enhance vaccine potency. These vaccines allow longer preservation periods in liquid nitrogen while maintaining the required potency (Brehm et al., 2008, Golde et al., 2005). Several limitations exist with this type of vaccine, that includes the requirement of a biosafety level III containment facility to prevent the escape of infectious material to the outside environment during production. The inclusion of multi-serotypes into multivalent vaccines induces stress effects in the immune system of the inoculated animal. The requirement of cold chain containment to avoid heat denaturation of the vaccine, the inability to provide full or cross-protection at all times against different strains including those of the same serotype and failure to prevent persistent infections remains an additional challenge. The possibility of false-positive results due to the present of the minimal amounts of residual NSP in traditional vaccines, incomplete inactivation in the vaccine, and the need to revaccinate with additional doses to boost immune longevity are some of the limitations (Commission and Committee, 2008, Hyslop et al., 1963, Robiolo et al., 2006, Behura et al., 2016, Hardham et al., 2020).

The DNA vaccine for FMDV have been proven to protect vaccinated animals from the development of lesions at inoculation sites (Mason et al., 1997). The DNA vaccines are easily manufactured, and the formulated vaccines contain no infectious material so are safe for use. DNA vaccines can contain coding regions for multiple antigenic sites and can stimulate both T and B cell immune responses, without inducing any stress-related effects on the immune system of the host.. Because of their great stability, costly cold-chain containment are not necessary (Leitner et al., 1999, Li et al., 2001). Some of the limitations of the DNA vaccine include the requirement of large DNA amounts to be injected and that multiple booster doses are required to elicit optimal protective immunity against FMDV (Pisetsky, 1998).

Peptide vaccines are made of singular peptides that are amino acid chains, however, a mixture of several peptides together targeting several antigenic epitopes has proven to illicit better immunogenic response than the singular peptides (Bachrach et al., 1975, Shao et al., 2011). These vaccines are stable and can be easily manufactured

at a large scale at relatively lower costs, without using highly infectious FMDV during production (Bachrach et al., 1975).

1.4.4. Vaccine potency

Maintaining the intact FMDV 146S antigen particle in its whole Intact form increases and maintains the potency of the vaccine while its degradation leads to the reduction of vaccine potency (Doel and Chong, 1982, Doel, 2005). However, the correlation between the concentration of the 146S antigen component and its formulated vaccine potency does not seem to obey a linear function (Rweyemamu et al., 1982). The first method to measure FMD vaccine potency is the 50% Protective Dose (PD_{50}), which was introduced by Henderson and Galloway (1953) after performing an experimental cattle test challenged via the intra-dermo-lingual route. To date, the golden standard method for FMDV vaccine potency testing is the challenge test, which is performed *in vivo* on animals of the targeted species that were vaccinated over a certain period with several vaccine doses. The PD_{50} test and the South American Protection against Generalized test (PG) are commonly used testing methods.The PG is test used to evaluate the efficacy of a vaccine in protecting against the FMDV in cattle.The test involves vaccinating a group of cattle with a single field dose of the vaccine and then exposing them to the disease-causing agent (challenging them) after a certain period of time. The objective of the PG test is to determine whether the vaccine provides a sufficient level of protection against FMDV. The pass requirement for the vaccine was set at a threshold of 75% protection (Vianna Filho et al., 1993, Robiolo et al., 2010, Belsham, 2020, Doel, 2003).

1.4.5. Vaccine immunity

Most FMDV vaccines depend on the humoral immune response from circulating antibodies/neutralizing antibodies (Nabs) to offer protection against virus exposure. The interaction of B lymphocytes with Th lymphocytes results in the production of circulating antibodies (Pay and Hingley, 1987, McCullough et al., 1992). The components of the innate immune response are involved in the early immune response of FMDV infections. First, macrophages are involved in acute infection by serving as phagocytes engulfing virus pathogens, however, they may also serve as a virus carrier and disseminate the virus within the body (Rigden et al., 2002). The

FMDV-infected macrophages are cleared from the infection within 10 to 14 hours through opsonization, which leads to the destruction of the infectious virus. Secondly, natural killer cells will induce cytotoxic effects against the targeted FMDV-infected cells while γδ T-cells proliferate to induce cytokine effects against FMDV (Amadori et al., 1992, Takamatsu et al., 2006). During the humoral immunity of the adaptive immune response, immunoglobulin M (IgM) is the first neutralising antibody that will appear three to four days post natural infection or vaccination, increasing in quantity during the 10th to 14th day then declining thereafter (Sobrino et al., 2001, Golde et al., 2008). Immunoglobulin G (IgG) antibodies appear four to seven days post-infection or after vaccination and begin to become dominant two weeks later. The IgG1 isotype is said to be more dominant than IgG2. IgM, IgA and IgG are found in the upper respiratory tract while only IgM and IgA are found in the pharyngeal fluid of the infected host seven days post-exposure (Collen et al., 1989, Salt et al., 1996, Salt, 1993, Francis et al., 1983).

1.4.6. FMDV diagnosis

Besides the diagnosis of FMD based on clinical signs, there are several reliable laboratory-based methods available for diagnosis. They include virus isolation by use of susceptible cell culture or nucleic acid amplification through reverse transcription-polymerase chain reaction and RT-qPCR. For detection of FMD viral antigens, virus-specific antibodies and antibodies to viral NSP can be diagnosed through serological assays which include lateral flow devices (LFD), the outdated complement fixation (CF) and the more specific and sensitive enzyme-linked immunosorbent assay (ELISA) (Aydin, 2015).

ELISA is a quantitative immunoassay that measures the concentration of biological molecules through colour-changing signals that result from the reaction of the antigen and antibody interaction with the aid of enzyme-linked conjugate together with the enzyme substrate (Hornbeck, 1992). Microtitre plates are utilized as the solid phase in which antigen and antibody molecules will be bound to the surface. Enzymes such as glucose oxidase, horseradish peroxidase (HRP), or alkaline phosphatase (ALP) are conjugated to secondary antobody such as goat anti-guinea pig IgG F(ab')2) conjugate then according to their substrate compatibility, immunological colour reactions is induced during interactions. The interaction of ALP with p-Nitro-phenyl

Phosphate (pNPP) substrate yields a yellow positive colour reaction, while the interaction of 5-amino salicylic acid or ortho-phenylene-diamine produces a brown positive colour reaction (Engvall, 2010). After completion of the enzyme-substrate reaction, either sodium hydroxide (NaOH), hydrochloric acid (HCl) or sulfuric acid (H_2SO_4) is added to stop the reaction. Once the reaction is stopped, results are read using a spectrophotometer at a wavelength between 400 - 600 nm range. Several ELISA techniques exist which include direct ELISA, indirect ELISA, sandwich ELISA and competitive ELISA depending on their combination of antigen and antibody (Hornbeck, 1992, Aydin, 2015).

The sandwich ELISA, discovered previously by (Kato et al., 1977) is called so because the target antigen is "sandwiched" between the capture and detection antibodies. it is known for its high sensitivity and specificity due to its dual antibody binding. This ELISA begins when microplate wells are coated with a primary "capture" antibody that is specific to the target antigen. The coated plates are covered and incubated overnight at low temperatures. This allows the primary capture antibody to adhere to the plate surface (Tabatabaei and Ahmed, 2022). Post the incubation, the plates are washed with wash buffere to remove any unbound capture antibodies. The coated wells are then blocked by using blocking agent such bovine serum albumin (BSA) or any other to prevent non-specific binding of other molecules (Kohl and Ascoli, 2017). The sample (containing the target antigen) is added to the microplate wells. The target antigen in the sample binds to the capture antibody that is immobilized on the plate. The microplate is incubated, allowing the antigen to bind to the capture antibody. After incubation, the plate is washed to remove any unbound components in order to reduce background noise. A second, "detection" antibody which is specific to a different epitope (binding site) on the target antigen in added. This detection secondary antibody is then conjugated to enzyme such as glucose oxidase, horseradish peroxidase (HRP), or alkaline phosphatase (ALP). The detection antibody binds to the antigen, forming a "sandwich" complex with the capture antibody. The plate is again washed to remove any unbound detection antibodies. An enzyme substrate is then added to the wells. If the enzyme-conjugated antibodies are bound to the antigen, they will then produce a detectable signal (usually a color change) when they come into contact with the substrate. The color change resulting from the enzymatic reaction is

measured using a spectrophotometer or a similar instrument. The intensity of the signal is proportional to the amount of antigen present in the sample.

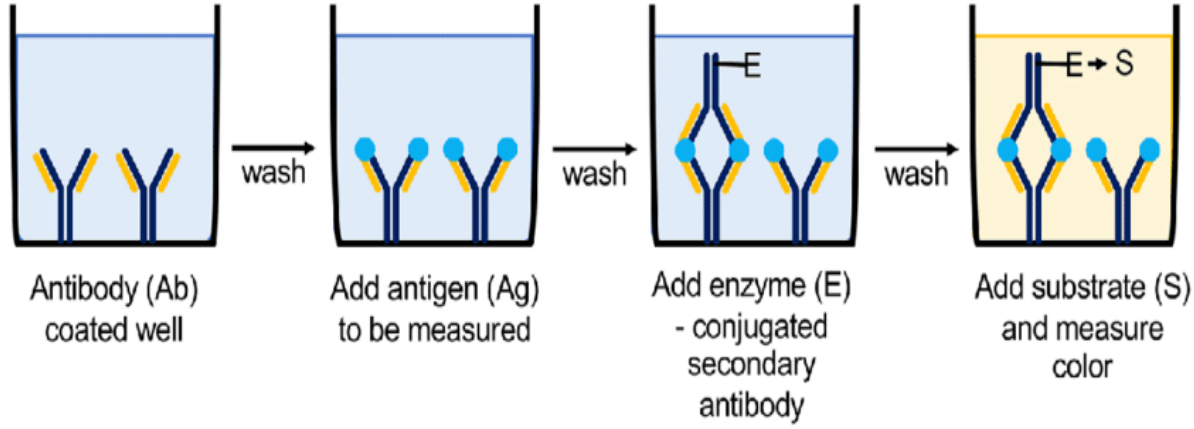

Figure 1.2. Schematic presentation of sandwich ELISA (enzyme-linked immunosorbent assay) principle (Boguszewska et al., 2019).

1.5. Problem statement

Because immunization against one serotype of FMD does not give protection against the other seven serotypes and subtypes (Feng et al., 2016), the immunogenicity of the presently employed inactivated FMD vaccine depends on the completeness of the infective and intact component of antigen, called the 146S antigen particle (Cartwright et al., 1980, Crowther et al., 1995). A single 146S particle is made up of a single-stranded RNA molecule containing 60 copies of each VP1, VP2, VP3 and VP4 structural protein (Brown and Crick, 1959). The VP1 capsid protein, situated on the outer surface of the 146S virus particle can produce enough neutralizing antibodies to elicit appropriate immunity and produce protection against FMD infections (Acharya et al., 1989, Bachrach et al., 1975). The recommended testing method to quantify 146S antigen particles during FMD vaccine formulation is through sucrose density gradient centrifugation (SDG) (Barteling and Meloen, 1974).

Even though the SDG can quantify the desired antigen yield, the test is highly variable because it is dependent primarily on the operator, requires expensive instrumentation for operation, only quantifies one sample every five to seven minutes, is labour intensive and the detecting instrument cannot be automated (Feng et al., 2016). Importantly the test cannot indicate whether the immunogenic epitopes on the outer

capsid protein (VP1) of 146S antigen have remained intact, have not degraded or have been cleaved by proteolytic enzymes and trypsin effects throughout the vaccine formulation processes (Doel and Collen, 1982, Baxt et al., 1984).

The use of a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) to detect the 146S antigen provides higher sensitivity and specificity and it is much simpler to run than SDG. The DAS-ELISA testing is extremely fast, robust, reproducible, time-efficient and allows for the quantification of a greater number of samples in a shorter amount of time. Most notably, the DAS-ELISA when deployed using monoclonal antibodies (mAb) have proven to have the ability to assess the epitope integrity of the VP1 protein on the 146S antigen's outer surface, w:hich is critical for vaccination potency (Van Maanen and Terpstra, 1990, Feng et al., 2016).

1.6. Research question

Is it possible to use a DAS-ELISA to quantify the intact 146S antigen of FMDV SAT-1 & 2 serotypes?

1.7. Aims

- To develop a DAS-ELISA-based method for the quantification of the intact 146S antigen of FMDV SAT-1 & 2 serotypes.
- To compare the DAS-ELISA assay method to the SDG fractionation method in quantifying the intact 146S antigen of FMDV SAT-1 & 2 serotypes.

1.8. Objectives

- Produce the intact 146S antigen of FMDV SAT-1 & 2 serotypes.
- Maintain the intact 146S antigen of FMDV SAT-1 & 2 serotypes at 4 °C temperature and pH 7.5.
- Dissociate the intact 146S antigen of FMDV SAT-1 & 2 serotypes using temperature of 56 °C while at pH of 7.5.
- Dissociate the intact 146S antigen of FMDV SAT-1 & 2 serotypes using acid at 6.5 pH while at 4 °C temperature.
- Completely dissociate (CD) the intact 146S antigen of FMDV SAT-1 & 2 serotypes using both acidic at 6.5 pH and 56 °C temperature.

- Develop a polyclonal antigen-based DAS-ELISA to quantify the intact 146S antigen produced during a vaccine production run.
- Prepare and quantify the intact 146S antigen versus the dissociated SAT-1 & 2 antigens (heat, pH and CD) using the standardised SDG and the newly developed polyclonal antigen-based DAS-ELISA.
- Compare the performance of the polyclonal antigen-based DAS-ELISA to the SDG results.

CHAPTER 2. MATERIALS AND METHODS

2.1. Production of FMDV 146S antigen

2.1.1. Cell culture preparation

BHK-21 suspension cells, as described by Capstick et al., (1962), were supplied by the tissue culture section of the Onderstepoort Veterinary Research Campus-Trans-Boundary Animal Disease Programme (ARC-OVR) sub-cultured and re-suspended as a start-up culture at passage level 3 (P+3) with an initial concentration of 0.1 x 10^6 cells/ mL into an 850 cm^2 roller bottle containing Glasgow's minimum essential medium (GMEM) cell culturing growth medium (Merck KGaA, Darmstadt, Germany), supplemented with 10% volume/volume (v/v) gamma irradiated adult bovine serum (Cell Sera, cat: 102131610), 10% v/v tryptose phosphate broth (Bacto, cat: 260200) and 3% v/v lactalbumin hydrolysate (Bacto, cat: 259961). An appropriate level of CO_2 was added to the cell culturing medium and the roller bottle was incubated at 37°C for 72 hours (hrs) on a high-speed (Frenkel) roller cart with a rotating speed of 30 revolutions per minute (RPM). After 72 hrs of incubation, cells were harvested and counted for viability and a total estimate of 2.78 x 10^6 cells/ mL was obtained. Another series of passages followed, in which P+4 cells were seeded at 0.10 x 10^6 cells/mL concentration in 1x 850 $cm^{2'}$ for 72 hrs and a harvest of 3.04 x 10^6 cells/ml concentration was obtained. Passage P+5 was seeded at 0.10 x 10^6 cells/ mL concentration for 72 hrs in three 1070 cm^2 Corning® polystyrene roller bottles (Merck KGaA, Darmstadt, Germany) each containing 1,500 mL of the GMEM cell culture growth medium. At harvest, cells were allowed to settle overnight and the spent media were decanted leaving behind about 500 mL of cell culture suspension from each Corning® roller bottle that were pooled together into a Pyrex bottle. About 500 mL of the virus-seeding media was added to the pool to a total of about 2,000 mL of suspension cell culture. A total of 5.83 x 10^6 cells/ml were estimated from the 2,000 mL pool, which was split into two halves. About 1,000 mL of virus seeding medium was added into each half of 1,000 mL making a total of 2x 2,000 mL'of suspension cell culture for virus propagation.

2.1.2. Virus propagation in cell culture

2.1.2.1. Propagation of FMDV SAT-1 and SAT-2 viral serotypes

Concentrations of 2.84 x 10^6 cells/mL and 2.20 x 10^6 cells/mL were estimated from each 2x 2,000 mL suspension cell culture grown in the laboratory for inoculation with the FMDV SAT-1 serotype and SAT-2 serotype respectively, which were obtained from the OVI-TADP vaccine production facility. The amount of virus required for both SAT-1 and SAT-2 serotype to inoculate into suspension cells at a specified MOI and titre was determined using the formula: (cell count (c/mL) x culture volume (mL) x MOI) ÷ virus titre in which for SAT-1 serotype, 9 uL of virus with a titre of $10^{8.8}$ $TCID_{50}$/mL was inoculated into 2000 mL of cells at an MOI of 0.001. For SAT-2 serotype, 1.75 ml of virus with a titre of $10^{7.4}$ $TCID_{50}$/mL was inoculated into 2000 mL cells at an MOI of 0.01. The cultivated FMDV SAT-1 serotype culture was incubated at 37°C for 15 hrs on an HS roller cart rotating at 30 rpm while the cultivated FMDV SAT-2 serotype culture was incubated at 37°C for 16 hrs also on an HS roller cart with a rotating speed of 30 rpm. At harvest, both cultures were centrifuged for 10 minutes (Feng et al.) at 4°C and 2626 relative centrifugal force (g) (Hettich, Germany). The supernatant containing the antigen was transferred into 10 x 50 mL and 3 x 500 mL aliquots, which were labelled and stored at -70°C.

2.1.2.2. The 146S antigen particle dissociation design of both SAT-1 & 2 serotypes

For the dissociation of the 146S antigen particle, four different dissociation conditions were set with four different sampling time intervals. The first condition was normal short-term storage at 4°C and pH 7.5 termed the “Intact 146S”, the second condition was dissociation with a higher temperature at 56°C and pH 7.5 termed “Temperature”, the third condition was dissociation with strong acid at 6.5 pH and 4°C termed “pH” and the fourth condition was dissociation with both, acidic at pH 6.5 and temperature of 56°C termed complete dissociation “CD”. A high temperature treatment was achieved using a water bath set at 56°C while an acidic treatment was achieved using 1M HCL. During dissociation, sampling was done at zero, five, ten and fifteen minutes for each condition. Samples were aliquoted into a series of 4 x 1.5 mL cryo-tubes and labelled accordingly. The series of aliquots were used in SDG antigen purification and ELISA testing.

2.1.2.3. The 146S antigen particle determination by SDG of both SAT-1 & 2 serotypes

The SDG ultracentrifugation method as described by Barteling and Meloen, (1974) was used in sample preparation for antigen quantification. A linear 10 - 30% (w/w) concentration of SDG solution was prepared in polypropylene centrifuge tubes (Beckman Coulter, CA, USA) using a model B108-2 gradient master (BioComp Instruments, Fredericton, Canada). To remove cell debris, samples were centrifuged for 1 min at 252 g using a Neofuge 15R/15 centrifuge (Heal Force, Shanghai, China). Samples were tested in duplicate, in which 20 µL of the centrifuged sample was layered on top of the 10 - 30% (w/w) sucrose density gradient solutions in polypropylene centrifuge tubes. The tubes were carefully loaded into an SW 55 Ti Swinging-Bucket Rotor, which was attached to a Beckman Coulter Optima™ XPN-90 ultracentrifuge (Beckman Coulter, CA, USA) drive hub. Samples were centrifuged for 50 min and 303 8000 g at 4°C hold. After centrifugation, the precipitated 146S particle fractions in the gradient were scanned at 254 nm wavelength using a Type 11 Optical Unit for UA-6 absorbance detectors (Teledyne Isco, Nebraska, USA). A peak forming 146S particle was calculated and measured in µg/ml using peak integration Clarity™ chromatography software (DataApex, Prague 5, The Czech Republic). Previously purified 146S particles with known antigen concentration were used as the positive control while BHK-21 suspension cell culture with no antigen was used as a negative control of the test.

2.2. Development of a polyclonal antigen-based DAS-ELISA

2.2.1. Coating of microtitre plates with the capture antibody

To coat the 96-well microtitre flat-bottom plates (Nunc™MaxiSorp™, Thermo Fisher Scientific, USA), rabbit antisera (coating serum) specific for FMDV SAT-1 and -2 serotypes was immobilized as the capture antibody. The capture antibody was diluted to a working concentration of 1/2500 carbonate/bicarbonate coating buffer, pH 9.6). An amount of 100 µL of the diluted coating serum was added to each well of the 96-well microtitre plate using a 12-channel micro-pipette and the coated plates were incubated overnight at room temperature between 18°C - 25°C. Post incubation, the plates were washed with washing buffer (PBS containing 0.05% Tween-20) using an

automated plate washer for three wash cycles, to remove the unbound capture antibodies. The plates were then stored at -20°C until further use.

2.2.2. Preparation of the detection antibody

The guinea-pig antiserum of the same specificity as the capture antibody was prepared as a detecting antibody. The guinea-pig serum was pre-blocked with normal bovine serum (50% v/v) by mixing 10 mL of guinea pig with 10 mL of the bovine serum and 80 mL of 0.5% PBS-skim milk was added to the mix ture. The detecting antibody mixture (working stock) was aliquoted in 10 mL volumes and stored at -20°C.

2.2.3. DAS-ELISA sample testing design

For each serotype, sixteen samples of different time intervals (0, 5, 10, 15 min) and dissociation conditions (Intact 146S, Temperature, pH and CD) were tested in duplicate from column 1 to column 10. The duplicates were titrated in two-fold dilution series from row A to row H. Test controls were tested in duplicate in columns 11 and 12 and serially diluted two-fold, with the negative control (blocking buffer/0.5% PBS-skim milk) titrated from rows A to D and the positive control (SAT antigen specific to either serotype 1 or 2) titrated from rows E to H for each testing plate.

		Intact 146S 0 MIN	Intact 146S 0 MIN	Intact 146S 5 MIN	Intact 146S 5 MIN	Intact 146S 10 MIN	Intact 146S 10 MIN	Intact 146S 15 MIN	Intact 146S 15 MIN	TEMP 0 MIN	TEMP 0 MIN	Negative control -C	Negative control -C		
		1	2	3	4	5	6	7	8	9	10	11	12		
1	A	1	1	9	9	17	17	25	25	33	33	-C	-C	1	
1/2	B	2	2	10	10	18	18	26	26	34	34	-C	-C	1/2	
1/4	C	3	3	11	11	19	19	27	27	35	35	-C	-C	1/4	
1/8	D	4	4	12	12	20	20	28	28	36	36	-C	-C	1/8	
1/16	E	5	5	13	13	21	21	29	29	37	37	+C	+C	1	Positive control +C
1/32	F	6	6	14	14	22	22	30	30	38	38	+C	+C	1/2	
1/64	G	7	7	15	15	23	23	31	31	39	39	+C	+C	1/4	
1/128	H	8	8	16	16	24	24	32	32	40	40	+C	+C	1/8	

		TEMP 5 MIN	TEMP 5 MIN	TEMP 10 MIN	TEMP 10 MIN	TEMP 15 MIN	TEMP 15 MIN	pH 0 MIN	pH 0 MIN	pH 5 MIN	pH 5 MIN	Negative control -C	Negative control -C		
		1	2	3	4	5	6	7	8	9	10	11	12		
1	A	1	1	9	9	17	17	25	25	33	33	-C	-C	1	
1/2	B	2	2	10	10	18	18	26	26	34	34	-C	-C	1/2	
1/4	C	3	3	11	11	19	19	27	27	35	35	-C	-C	1/4	
1/8	D	4	4	12	12	20	20	28	28	36	36	-C	-C	1/8	
1/16	E	5	5	13	13	21	21	29	29	37	37	+C	+C	1	Positive control +C
1/32	F	6	6	14	14	22	22	30	30	38	38	+C	+C	1/2	
1/64	G	7	7	15	15	23	23	31	31	39	39	+C	+C	1/4	
1/128	H	8	8	16	16	24	24	32	32	40	40	+C	+C	1/8	

		pH 10 MIN	pH 10 MIN	pH 15 MIN	pH 15 MIN	CD 0 MIN	CD 0 MIN	CD 5 MIN	CD 5 MIN	CD 10 MIN	CD 10 MIN	Negative control -C	Negative control -C		
		1	2	3	4	5	6	7	8	9	10	11	12		
1	A	1	1	9	9	17	17	25	25	33	33	-C	-C	1	
1/2	B	2	2	10	10	18	18	26	26	34	34	-C	-C	1/2	
1/4	C	3	3	11	11	19	19	27	27	35	35	-C	-C	1/4	
1/8	D	4	4	12	12	20	20	28	28	36	36	-C	-C	1/8	
1/16	E	5	5	13	13	21	21	29	29	37	37	+C	+C	1	Positive control +C
1/32	F	6	6	14	14	22	22	30	30	38	38	+C	+C	1/2	
1/64	G	7	7	15	15	23	23	31	31	39	39	+C	+C	1/4	
1/128	H	8	8	16	16	24	24	32	32	40	40	+C	+C	1/8	

		CD 15 MIN	CD 15 MIN	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Negative control -C	Negative control -C		
		1	2	3	4	5	6	7	8	9	10	11	12		
1	A	1	1	9	9	17	17	25	25	33	33	-C	-C	1	
1/2	B	2	2	10	10	18	18	26	26	34	34	-C	-C	1/2	
1/4	C	3	3	11	11	19	19	27	27	35	35	-C	-C	1/4	
1/8	D	4	4	12	12	20	20	28	28	36	36	-C	-C	1/8	
1/16	E	5	5	13	13	21	21	29	29	37	37	+C	+C	1	Positive control +C
1/32	F	6	6	14	14	22	22	30	30	38	38	+C	+C	1/2	
1/64	G	7	7	15	15	23	23	31	31	39	39	+C	+C	1/4	
1/128	H	8	8	16	16	24	24	32	32	40	40	+C	+C	1/8	

Figure 2.1. Plate layout diagram of both SAT 1&2 antigen dilutions, including controls and the blank indicated in the appropriate wells.

2.2.4. Polyclonal DAS-ELISA for FMDV SAT-1 and 2 antigen quantification

The coated microtitre plates stored at -20°C were thawed at room temperature and residual moisture contents were removed by tapping the plates upside down on a lint-free absorbance surface. One hundred µL of FMD test antigen was loaded in duplicates in each well from columns 1 to 10 and titrated at a two-fold serial dilution (1:2 - 1:128) in blocking buffer (0.5% PBS-skim milk) by transferring 50 µL from row A to H of the plate. From columns 11 to 12, 100 µL of the negative control (blocking buffer) were loaded in duplicate and titrated at a two-fold serial dilution (1:2 - 1:8) by transferring 50 µL from row A to D, while the positive control (SAT antigen specific to serotype 1 or 2) was titrated at a two-fold serial dilution (1:2 - 1:8) by transferring 50 µL from row E to H of each well. The plates were incubated for 1 hr at 37°C while shaking continuously on an orbital shaker. After incubation plates were washed with a washing buffer (PBS containing 0.05% Tween-20) for three wash cycles followed by the addition of 50 µL of the detecting antibody/guinea-pig antisera diluted 1:50 in blocking buffer. The plates were then incubated for 1hr at 37°C while shaking continuously on an orbital shaker, followed by washing with a wash buffer for three wash cycles. Following the washing step, 50 µL of secondary antibody (goat anti-guinea pig IgG F(ab')2) conjugated to horseradish peroxidase (HRP) enzyme") (Sigma, St. Louis, USA) diluted 1:60 in blocking buffer were distributed in each well and incubated for 1hr at 37°C while shaking continuously on an orbital shaker. After incubation, the plates were washed with a washing buffer for three wash cycles. One hundred µL of freshly prepared substrate/chromogen solution [30 ml substrate solution, 300 µL TMB and 23 µL H_2O_2] enough for 3 x 96 wells was added to all wells and incubated for 15 min on a bench at room temperature. To stop the colour reaction, 50 µL of 1 M (mol/L) H_2SO_4 was added to each well and plates were analysed using Thermo Scientific Multiskan EX (Thermo Fisher Scientific, Massachusetts, USA) microplate photometer ELISA reader that reads wavelength at 450 nm.

	rabbit antisera	rabbit antisera	rabbit antisera	rabbit antisera	rabbit antisera	rabbit antisera	rabbit antisera	rabbit antisera	rabbit antisera	rabbit antisera	rabbit antisera	rabbit antisera
	1	2	3	4	5	6	7	8	9	10	11	12
A	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
B	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
C	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
D	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
E	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
F	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
G	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
H	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL

1. Wells were coated with 100 µL of rabbit antisera capture antibody in which pates were incubated overnight at room temperature between 18°C - 25°C.

		Intact 146s 0 Min	Intact 146s 0 Min	Intact 146s 5 Min	Intact 146s 5 Min	Intact 146s 10 Min	Intact 146s 10 Min	Intact 146s 15 Min	Intact 146s 15 Min	Temperature 0 Min	Temperature 0 Min	Blocking buffer/0.5% PBS-skim milk (-C)	Blocking buffer/0.5% PBS-skim milk (-C)		
		1	2	3	4	5	6	7	8	9	10	11	12		
1	A	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	1	
1/2	B	50	50	50	50	50	50	50	50	50	50	50	50	1/2	
1/4	C	25	25	25	25	25	25	25	25	25	25	25	25	1/4	
1/8	D	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	1/8	
1/16	E	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	100	100	1	SAT 1/2
1/32	F	3.127	3.127	3.127	3.127	3.127	3.127	3.127	3.127	3.127	3.127	50	50	1/2	antigen (+C)
1/64	G	1.5625	1.5625	1.563	1.563	1.563	1.563	1.563	1.563	1.563	1.563	25	25	1/4	
1/128	H	0.78125	0.78125	0.0781	0.0781	0.078	0.078	0.078	0.078	0.078	0.078	12.5	12.5	1/8	

2. One hundred (100) µL of FMD test antigen (SAT 1/2) together with the test controls (negative control, blocking buffer) and positive control (SAT antigen specific to serotype 1 or 2) were bound to the capture antibody. The plates were incubated for 1 hr at 37°C.

	guinea-pig antiserum	guinea-pig antiserum	guinea-pig antiserum	guinea-pig antiserum	guinea-pig antiserum	guinea-pig antiserum	guinea-pig antiserum	guinea-pig antiserum	guinea-pig antiserum	guinea-pig antiserum	guinea-pig antiserum	guinea-pig antiserum
	1	2	3	4	5	6	7	8	9	10	11	12
A	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
B	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
C	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
D	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
E	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
F	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
G	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
H	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

3. Fifty (50) µL of guinea-pig antisera was added to the mixture as the detecting antibody in which plates were then incubated for 1 hr at 37°C.

Figure 2.2. Plate layout diagram (steps 1-3) showing all the steps performed during the DAS-ELISA for both SAT 1 &2 atigens.

	goat anti-guinea pig IgG F(ab')2 horseradish peroxidase (HRP)	goat anti-guinea pig IgG F(ab')2 horseradish peroxidase (HRP)	goat anti-guinea pig IgG F(ab')2 horseradish peroxidase (HRP)	goat anti-guinea pig IgG F(ab')2 horseradish peroxidase (HRP)	goat anti-guinea pig IgG F(ab')2 horseradish peroxidase (HRP)	goat anti-guinea pig IgG F(ab')2 horseradish peroxidase (HRP)	goat anti-guinea pig IgG F(ab')2 horseradish peroxidase (HRP)	goat anti-guinea pig IgG F(ab')2 horseradish peroxidase (HRP)	goat anti-guinea pig IgG F(ab')2 horseradish peroxidase (HRP)	goat anti-guinea pig IgG F(ab')2 horseradish peroxidase (HRP)	goat anti-guinea pig IgG F(ab')2 horseradish peroxidase (HRP)	goat anti-guinea pig IgG F(ab')2 horseradish peroxidase (HRP)
	1	2	3	4	5	6	7	8	9	10	11	12
A	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
B	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
C	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
D	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
E	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
F	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
G	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
H	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

4. Fifty (50) µL of secondary antibody (goat anti-guinea pig IgG F(ab')2) conjugated to horseradish peroxidase (HRP) enzyme was added in each well and incubated for 1 hr at 37 °C.

	TMB chromogen solution	TMB chromogen solution	TMB chromogen solution	TMB chromogen solution	TMB chromogen solution	TMB chromogen solution	TMB chromogen solution	TMB chromogen solution	TMB chromogen solution	TMB chromogen solution	TMB chromogen solution	TMB chromogen solution
	1	2	3	4	5	6	7	8	9	10	11	12
A	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
B	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
C	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
D	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
E	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
F	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
G	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
H	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL

5. One hundred µL of TMB chromogen solution/ substrate was added to all wells and incubated for 15 min on a bench at room temperature.

	1 M (mol/L) H2SO4	1 M (mol/L) H2SO4	1 M (mol/L) H2SO4	1 M (mol/L) H2SO4	1 M (mol/L) H2SO4	1 M (mol/L) H2SO4	1 M (mol/L) H2SO4	1 M (mol/L) H2SO4	1 M (mol/L) H2SO4	1 M (mol/L) H2SO4	1 M (mol/L) H2SO4	1 M (mol/L) H2SO4
	1	2	3	4	5	6	7	8	9	10	11	12
A	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
B	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
C	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
D	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
E	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
F	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
G	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL
H	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

6. To stop the colour reaction, 50 µL of 1 M (mol/L) H_2SO_4 was added to each well.
7. The plates were then analysed using Thermo Scientific Multiskan EX (Thermo Fisher Scientific, Massachusetts, USA) microplate photometer ELISA reader that reads wavelength at 450 nm.

Figure 2.3. Plate layout diagram (steps 4-7) showing all the steps performed during the DAS-ELISA for both SAT 1 &2 atigens.

2.3. Data analysis

Serological data was captured and analysed in a Microsoft Excel spreadsheet. Samples were tested in duplicate and the mean and standard deviation of the $OD_{450\ nm}$ value results were calculated.

CHAPTER 3. RESULTS

3.1. FMDV SAT-1 serotype

3.1.1. The intact 146S antigen quantification by sucrose density gradient fractionation

The FMDV SAT-1 146S antigen concentrations under four different conditions (Intact 146S, Temperature, pH and complete dissociation “CD”) over 15 min are shown in Figure 3.1 below.

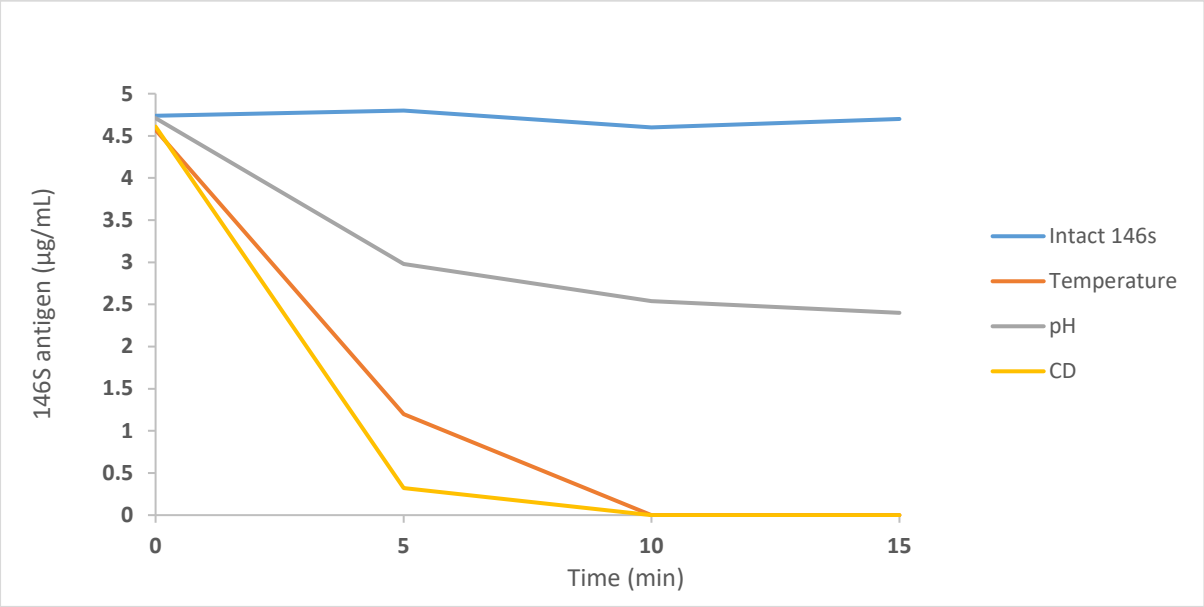

Figure 3.1. Quantification of FMDV SAT-1 146S antigen concentrations under four conditions using a Type 11 Optical Unit absorbance detector at 254 nm after sucrose density gradient (SDG) fractionation.

3.1.2. Polyclonal antigen-based double-antibody sandwich ELISA.

First, the two-fold dilution series of FMDV SAT-1 antigen concentration from samples under four different conditions (Intact 146S, Temperature, pH and CD) was conducted to achieve the linearity (concentration where optimal antigen signal is obtained using minimum antigen quantity) through a polyclonal DAS-ELISA as shown in Figure 3.2. The linearity (an optimal amount of antigen) was achieved successfully at dilution point 0.25 dilution for the four conditions shown in Figure 3.2. Hence the dilution point 0.25 dilution was selected for quantification of the SAT-1 antigen samples as shown in Figure 3.3 below.

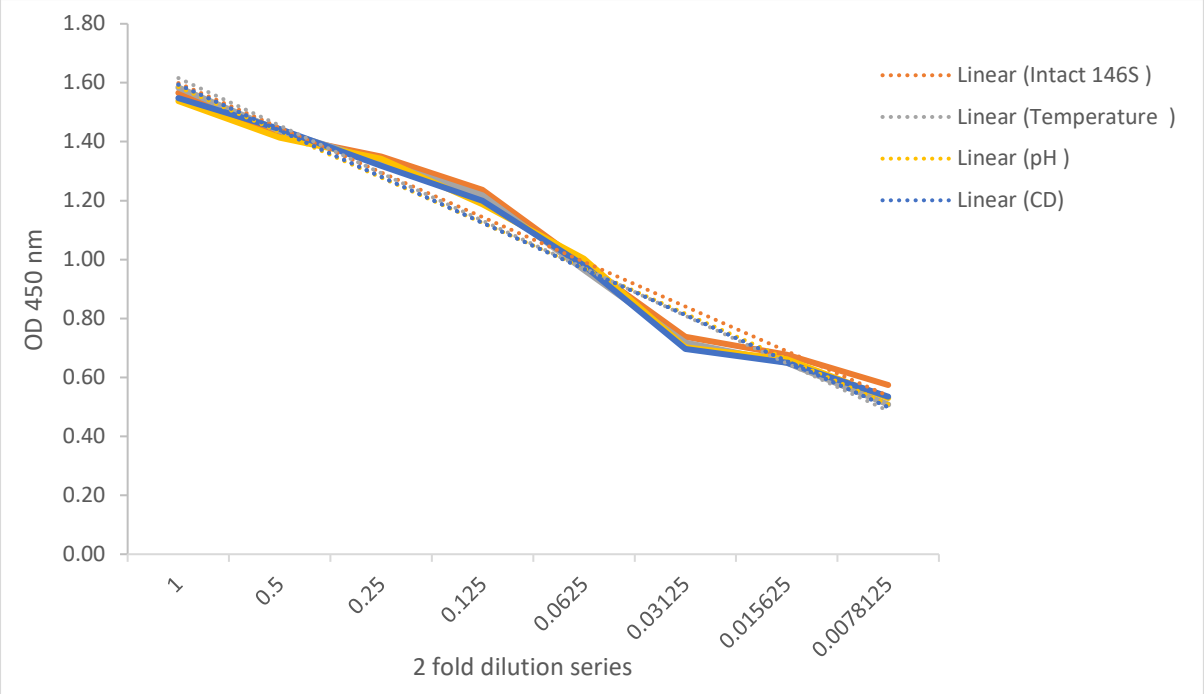

Figure 3.2. Two-fold serial dilutions of FMDV SAT-1 antigen using a polyclonal DAS-ELISA.

The FMDV SAT-1 antigen concentrations at 0.25 dilution from samples under four different conditions (Intact 146S, Temperature, pH and CD) at different time intervals. The results are shown in Figure 3.3 below.

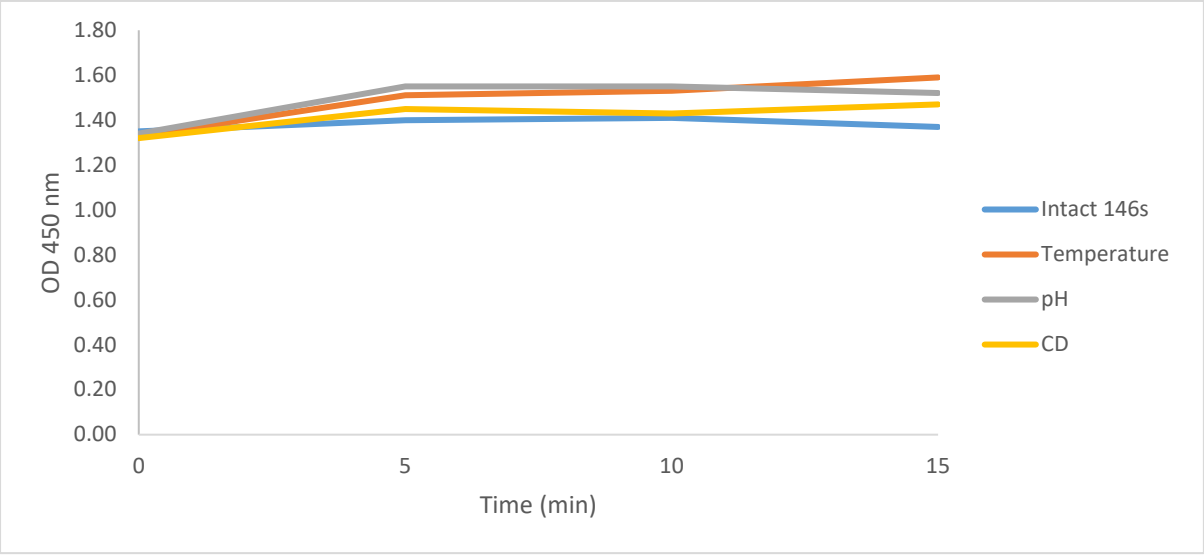

Figure 3.3. Quantification of FMDV SAT-1 antigen using a polyclonal DAS-ELISA at a 0.25 dilution under four conditions.

3.2. FMDV SAT-2 serotype.

3.2.1. The intact 146S antigen quantification by sucrose density gradient fractionation

FMDV SAT-2 146S antigen concentrations under four different conditions (Intact 146S, Temperature, pH and CD) over 15 min are shown in Figure 3.4 below.

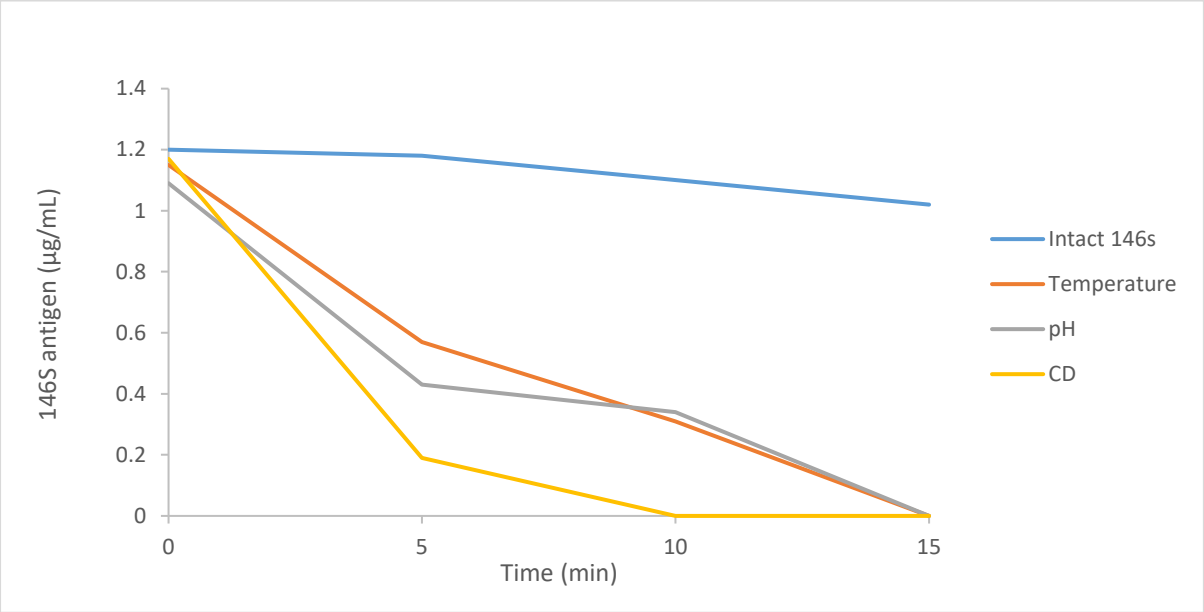

Figure 3.4. Quantification of FMDV SAT-2 146S antigen concentrations under four conditions using a Type 11 Optical Unit absorbance detector at 254 nm after sucrose density gradient (SDG) fractionation.

3.2.2. Polyclonal antigen-based double-antibody sandwich ELISA.

The two-fold dilution series of FMDV SAT-2 antigen concentration from samples under four different conditions (Intact 146S, Temperature, pH and CD) was conducted to achieve the linearity (concentration where optimal antigen signal is obtained using minimum antigen quantity) through a polyclonal DAS-ELISA as shown in . The linearity (an optimal amount of antigen) was achieved successfully at dilution point 0.25 dilution for the four conditions shown in Figure 3.5. Hence the dilution point 0.25 dilution was selected for quantification of the SAT-2 antigen samples as shown in Figure 3.6. below.

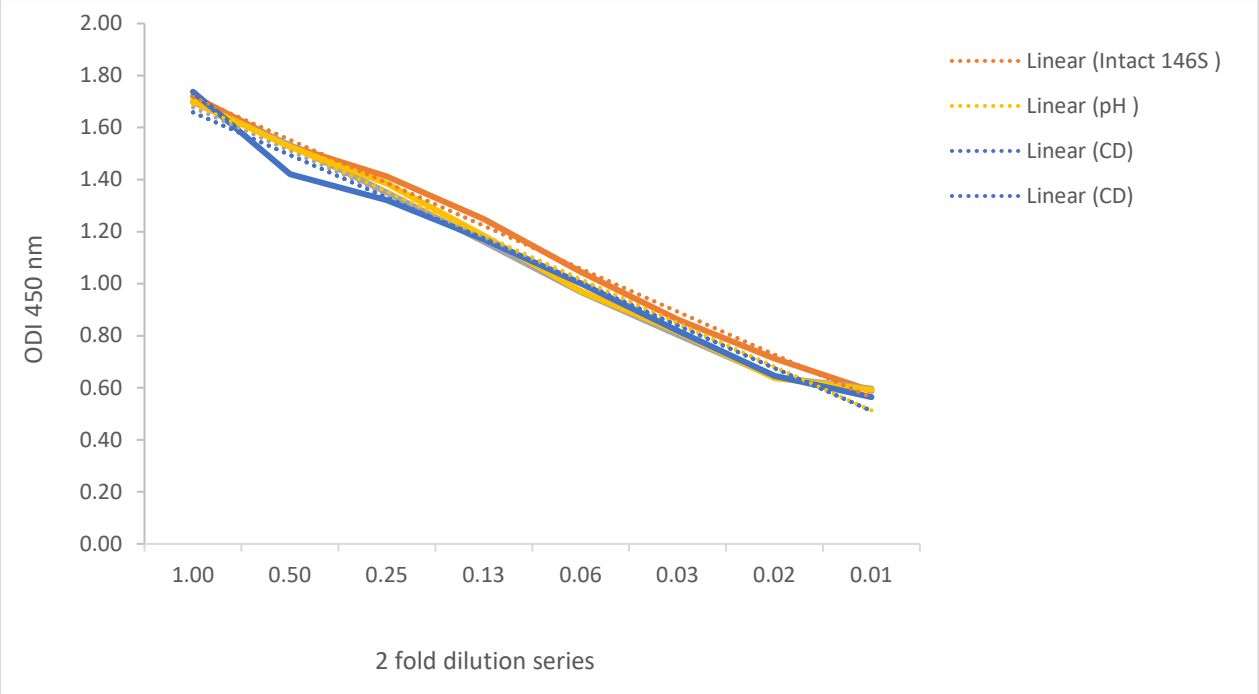

Figure 3.7. Two-fold serial dilutions of FMDV SAT-2 antigen using a polyclonal DAS-ELISA.

The FMDV SAT-2 antigen concentrations at 0.25 dilution from samples under four different conditions (Intact 146S, Temperature, pH and CD) at different time intervals. The results are shown in below.

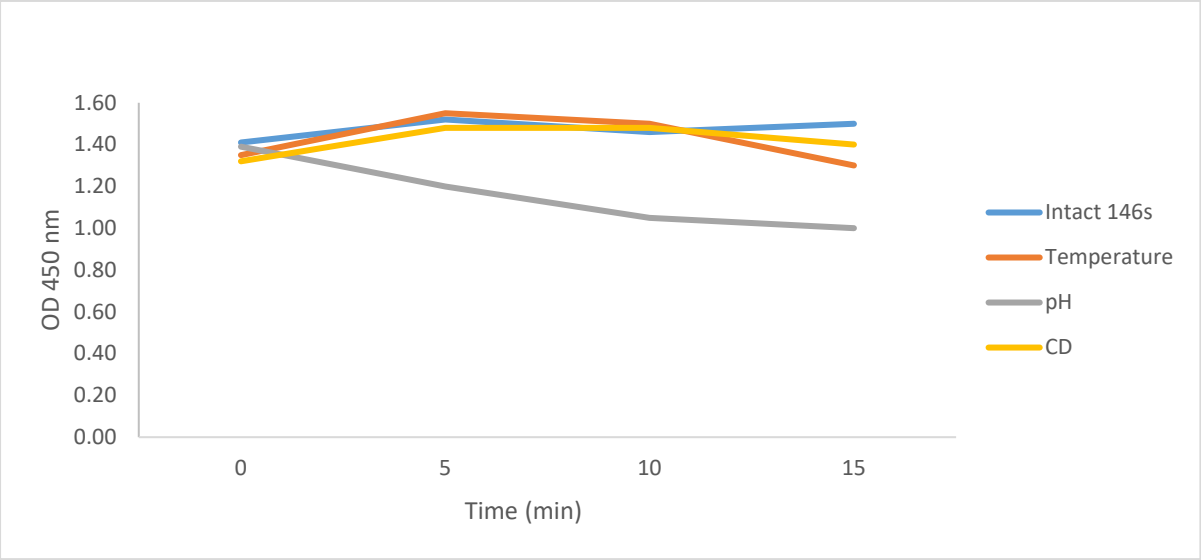

Figure 3.8. Quantification of FMDV SAT-2 antigen using a polyclonal DAS-ELISA at a 0.25 dilution under four conditions.

CHAPTER 4. DISCUSSION

In this research, we compared the SDG fractionation system to quantify FMDV 146S antigen particles at 254 nm using a Type 11 Optical Unit for UA-6 absorbance detector to the polyclonal DAS-ELISA, used to detect 146S antigen particles at OD 450 nm using a microplate photometer in samples under four different conditions: Intact 146S, Temperature, pH and Complete dissociation.

For the quantification of the FMDV SAT-1 serotype using the SDG fractionation system, our results showed that the Intact 146S antigen showed no sign of degradation when quantified at five minutes intervals over a 15 min period and retained its integrity over a 15 minutes period (Figure 3.1). The pH samples when compared to samples of the Intact 146S showed a slight degradation of the 146S antigen particles but remained detectable over the 15 minutes dissociation period when using the SDG fractionation system. This suggests a partial conversion of the 146S antigen into 12S protein subunits. The 146S antigen concentrations of samples under both Temperature and Complete dissociation conditions showed a significant decline after 5 minutes of exposure and there was no detection of the 146S antigen particles in samples between 10 – 15 minutes of exposure. While this indicated a complete degradation of the intact 146S antigen particles, it also suggested a successful conversion of the intact 146S antigen particles into the 12S protein subunits in samples after 10 minutes of exposure.

For the quantification of the FMDV SAT-2 serotype in Figure 3.4, results also indicated that the intact 146S antigen particles of SAT-2 retained their integrity over a 15 minutes period of dissociation. The 146S antigen concentration of both Temperature and pH conditions reduced significantly from 5 minutes to a point of no detection after 15 minutes exposure. This suggested a gradual dissociation of 146S antigen particles into 12S protein subunits between 5 - 10 minutes and a complete conversion by 15 minutes period. The 146S antigen concentration of the Complete dissociation condition indicated a significant decline after 5 minutes exposure to a point of no detection after 10 minutes exposure when compared to other treatment conditions. This result showed that the intact 146S antigen particles of the Complete dissociation condition dissociated completely after 10 minutes of exposure, suggesting a

successful conversion of the intact 146S antigen particles into the 12S protein subunits.

Quantification of other FMDV antigen particles, protein subunits and capsid polypeptides using the Type 11 Optical Unit for UA-6 absorbance detector is not possible since 75S, 12S and VP1 antigens are not detectable at the specified 254 nm absorbance. The 12S subunit has an absorbance of 260 nm and VP1 280 nm. Absorbance at 254 nm is therefore specific to the intact 146S antigen particles and able to quantify the antigen at the highest sensitivity of 0.002 absorption units. In total, there are ten available sensitivity ranges that includes 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 absorption units that are compatible to the 254 nm absorbance (Li et al., 2021, Rao et al., 1994).

The SDG centrifugation described by Barteling and Meloen (1974) is a standardised method and has been widely and successfully used over the past few decades to quantify whole virion (146S antigen) yield at 254 nm absorbance, but this method has several setbacks that limit its day to day operational practicality across different laboratories: The test yields highly variable results because of its dependence on the skills of an individual operator. It requires specialized, expensive instrumentation for operation. It is time-consuming and has a low sample testing capacity in that only one sample can be quantified in five to seven minutes. Finally, the test is labour-intensive and the antigen quantification instrument is neither fully nor semi-automated (Crowther et al., 1995). Importantly, the test cannot indicate whether the immunogenic epitopes on the outer capsid protein (VP1) of 146S antigen have remained intact, have not degraded or have been cleaved by proteolytic enzymes and trypsin effects during the vaccine formulation processes as the ELISA can (Crowther et al., 1995).

The use of a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) to detect a whole 146S antigen provides higher sensitivity and specificity when using monoclonal antibodies (mAbs) that recognises and bind only to the expressed epitopes of the intact 146S particle and not to the epitopes of the resulting protein subunits including the 12S which share common epitopes with the intact 146S (Van Maanen and Terpstra, 1990). The test is much simpler to run than the SDG centrifugation method. It is extremely fast, robust, reproducible, time-efficient and it allows for the quantification of a greater number of samples in a shorter amount of

time. Most notably, the DAS-ELISA can assess the epitope integrity of the VP1 protein on the intact 146S antigen's outer surface, which is critical in FMD vaccine formulation potency (Crowther et al., 1995).

Due to common epitopes that are serologically shared between the intact 146S antigen particles and the 12S protein subunits of FMDV, detection of the intact 146S antigen particles in the presence of the resulting 12S protein subunits using polyclonal antibody-based (pAbs) DAS-ELISA as in this study may result in cross-reaction (Harmsen et al., 2017). To avoid cross-reactivity between the whole antigen particle and the 12S protein subunit, dissociation of the intact 146S particles was achieved by exposure of the virus to either heat or acid or both heat and acid at once. The exposure of FMDV to heat above 65 °C and to acid with pH below 6.5, results in irreversible dissociation of the intact 146S antigen particles into 12S protein subunits (Harmsen et al., 2011). Other means of eliminating cross-reactivity involve the use of monoclonal antibody-based (mAbs) ELISA, which bind specifically to the intended and desired epitopes on either the 146S antigen or the resulting 12S subunits particles (Baxt et al., 1989, Harmsen et al., 2011).

According to validation of the solid-phase competition ELISA assay performed at ARC-FMDV diagnostic laboratory unit, the test is optimised to achieve an optimal antigen signal (presented in OD_{450} value) at 1:4 antigen quantity dilution. Hence the selection of the said dilution in testing of the samples, as illustrated in Figure 3.3 and Figure 3.6.

For SAT-1 serotype, The OD_{450} value results of between 0 to 15 minutes exposure indicated that the pAbs reacted equally to the intact 146S antigen particles and to other subunits produced in samples of other conditions (Temperature, pH and CD) as illustrated in Figure 3.3. This suggested that antigens present in these conditions (Temperature, pH and CD) shared common binding epitopes with epitopes on the intact 146S antigen particles. The indication of shared binding epitopes suggested the detection of the 12S subunits in these (Temperature, pH and CD) conditions.

Additionally, The trend was also observed with SAT-2 serotype in that the OD_{450} value results of between 0 to 15 minutes exposure showed that pAbs reacted equally to the intact 146S antigen particles and to subunits of the Temperature and CD conditions with the exception of the subunits in the pH condition as illustrated in Figure 3.6. This

suggests that antigens present in these conditions (Temperature, CD) shared common binding epitopes with epitopes on the intact 146S antigen particles. The shared binding epitopes also suggested detection of the 12S subunits in the Temperature and CD conditions. However, the OD_{450} value results of the pH condition in the SAT-2 serotype (Figure 3.6) showed a slight but non significant reduction in concentration of the detected subunits during the 15 minutes exposure when compared to other tested conditions (Intact 146S, Temperature and CD). This suggests that the 12S subunit detected in the pH condition was present at a reduced concentrations when compared to 12S subunit of other conditions (Intact 146S, Temperature and CD) (Figure 3.6).

CHAPTER 5. CONCLUSION AND RECOMMENDATIONS

Several methods have been developed for the quantification of the intact 146S antigen particles of the FMDV that is essential in the production of the inactivated FMD vaccine (Spitteler et al., 2011, Harmsen et al., 2011). Comparison of the SDG fractionation system with a pAbs DAS-ELISA assay for quantification of the intact 146S antigen demonstrated a higher specificity of the SDG fractionation assay to the intact 146S antigens for both SAT-1 & -2 serotypes. The pAbs DAS-ELISA demonstrated cross reactivity to the intact 146S antigen and the dissociated 12S protein subunits for both SAT-1 & -2 serotypes of FMDV. The pAbs DAS-ELISA assay was not specific only to the epitopes on the intact 146S antigen particles and therefore the quantification of 146S antigen particles using non-specific pAbs was not suitable. For a successful quantification of the intact 146S antigen particles of FMDV with less probability of cross reactivity, DAS-ELISA using monoclonal antibodies (mAbs) that recognize and bind specifically to the epitopes of the intact 146S antigen particles and not to the epitopes of the 12S subunits should be employed during routine production of the inactivated FMD vaccine (Harmsen et al., 2017). This was also proven in the studies of (Van Maanen and Terpstra, 1990, Krishna et al., 2019, Yang et al., 2008) whom managed to develop mAbs that recognised and bind specifically to the epitopes of the intact 146S antigen particles and to those of the 12S subunits.

CHAPTER 6. REFERENCES

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CHAPTER 7. APPENDIX

7.1. Section 20 permit

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: Mr Henry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@dalrrd.gov.za
Reference: 12/11/1/1(MG/1654)

Kabelo Tlaka
ARC-OVR Transboundary Animal Disease Programme (TADP)
100 Old Soutpan road
Onderstepoort
0110
E-mail:Tlakak@arc.agric.za

Dear Kabelo Tlaka

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

Your application sent per email requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The study is approved as per the application form dated 18 October 2019 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 HenryG@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 samples already stored in the BSL-3 facility at TADP must be used in this study;

5. Upon study completion, all remaining antigen samples must be stored within the BSL-3 facility at TADP;
6. Any further use or distribution of stored material is subject to obtaining a separate Section 20 approval;
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: Evaluation for alternative methods for quantification of foot and mouth disease virus (FMDV) 146S antigen concentration during vaccine preparations in South Africa.

Researcher: Kabelo Tlaka
Institution: ARC-OVR Transboundary Animal Disease Programme (TADP)
Our ref Number: 12/11/1/1(MG/1654)
Your ref:
Expiry date: 31 December 2022

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2021 -03- 0 8

SUBJECT: Evaluation for alternative methods for quantification of FMDV 146S antigen concentration during vaccine preparations in South Africa

7.2. Research ethics approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee 09 February 2021

CONDITIONALLY APPROVAL

Ethics Reference No	**REC060-20**
Protocol Title	**Evaluation of alternative method for quantification of Foot and Mouth Disease Virus 146S antigen concentration during vaccine preparations in South Africa.**
Principal Investigator	**Mr K Tlaka**
Supervisors	**Prof M Quan**

Dear Mr K Tlaka,

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

Please note the following about your ethics approval:

1. Please use your reference number (REC060-20) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application for post graduate studies (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

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

NOTES: Conditionally approved pending the following:
(i) Obtaining all other relevant approvals (including Section 20).
(ii) Upload of the BSL3 certificate.
(iii) Upload of a letter of collaboration (or formal signed agreement, RCA) that describes the responsibilities and IP ownership of data.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS OF VETERINARY EDUCATION

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