Evaluation of the solid-phase competition ELISA for detecting SAT foot-and-mouth disease virus vaccination and infection in goats

By

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Declaration

I, Moses Gobiye hereby declare that the research entitled: **Evaluation of the solid-phase competition ELISA for detecting Southern African Territories foot-and-mouth disease virus in goats** presented in this dissertation, in partial fulfilment of the requirements for the Master of Science in Tropical Animal Health degree was executed by me, with guidance from my supervisors. This dissertation has not been submitted in the past, or is to be submitted for a degree at the University of Pretoria or any other university.

Moses Gobiye

Date

09 September 2021

Abbreviations

BEI	Binary ethyleneimine
BHK	Baby hamster kidney cell line
BTY	Bovine thyroid primary cell line
CFT	Complement fixation test
COV	Coefficient of variation
CI	Confidence Interval
ELISA	Enzyme linked-immunosorbent assay
FMD	Foot and mouth disease
FMDV	Foot and mouth disease virus
IB-RS-2	Instituto Biologico-Renal Suino-2
IQR	Interquartile range
KNP	Kruger National Park
LK	Lamb kidney cell line
LFD	Lateral flow device
LPBE	Liquid-phase blocking ELISA
MAb	Monoclonal antibody
MVPK-1	Pig kidney cell line
NSPs	Non-structural proteins
OD	Optical density
OIE	Office International des Epizooties
PCR	Polymerase chain reaction
PI	Percentage inhibition
pH	Negative log of hydrogen ion concentration
RNA	Ribonucleic acid
ROC	Receiver operating characteristics
RT-PCR	Reverse transcriptase polymerase chain reaction
SADC	Southern African Development Community
SAT	South African Territories
SE	Sensitivity
SP	Specificity
SPCE	Solid Phase Competition ELISA
SS-SPCE	Single Spot Solid Phase Competition ELISA

TAD	Transboundary Animal Disease
TADP	Transboundary Animal Disease Programme
$TCID_{50}$	Tissue culture infective dose 50
T-SPCE	Titration-Solid Phase Competition ELISA
VNT	Virus neutralisation test
VP	Variable protein
ZZ-R 127	Goat foetal tongue cell line

Abstract

Title: **Evaluation of the solid-phase competition ELISA for detecting SAT foot-and-mouth disease virus vaccination and infection in goats**

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Foot-and-mouth disease (FMD) is a highly contagious and widely distributed disease affecting cloven-hoofed animals. The exact role played by goats in the epidemiology of the Southern African Territories (SAT) serotypes is an area currently under investigation. In this study, diagnostic properties of the solid phase competition ELISA (SPCE) were estimated using serum samples collected from goats that had participated in a FMD vaccination and challenge study. The goats were vaccinated with a pentavalent vaccine containing two SAT1 viruses, two SAT2 viruses and one SAT3 virus. After vaccination, the goats were challenged with a pool of three field SAT1 viruses. The SPCE were performed initially using a single-spot version (SS-SPCE) that was followed by a half-titration SPCE (T-SPCE) performed specifically for this study. The two independent runs (SS-SPCE and T-SPCE) were conducted in duplicate on two separate occasions. The repeatability of the assay was estimated from the duplicate wells (intra-aassay) and also from mean percentage inhibition from the separate tests (inter-assay). The coefficients of variations calculated from the duplicate percentage inhibitions from each of the two test runs were used to calculate within run repeatability while the mean percentage inhibitions for each run were used to measure inter-assay repeatability. The mean percentage inhibitions from the independent SPCE runs were used to estimate the level of correlation, agreement and relationship between them. The mean percentage inhibitions from each of the two SPCE runs were also compared to titer and the correlation, agreement and relationships were estimated. The ROC curve and area under the curve were used to estimate the accuracy of the SPCE and the optimum threshold cut off of the SPCE method across all three SAT serotypes was determined based on the Youden index ($Y = \max c\,(Se\,(c) + Sp\,(c) - 1)$). Approximately 80% of all intra-assay and 60% of all inter-assay SPCE results across all SAT serotypes had a good repeatability (<20% coefficient of variation). There was a very strong

correlation between the two SPCE test runs and the titer results (Spearman's rho=0.8 to 0.97). The agreement between the both SPCE runs was substantial across all serotypes, with kappa values ranging from 0.74 to 0.82. However, when the individual SPCE runs were compared with titer, the agreement was not as strong and differed according to serotype. SAT 1 agreement was moderate to substantial (k=0.592 and 0.612), SAT2 was moderate (k=0.423) and SAT3 was fair (k=0.24 and 0.309). The area under the T-SPCE ROC curve for SAT1, SAT2 and SAT3 was 0.98, 0.979 and 0.953 respectively, indicating a high diagnostic accuracy. The optimum SPCE percentage inhibition cut off based on the Youden index was found to be 75.63% PI for SAT1, 76.6% PI for SAT2 and 71.6% PI for SAT3. The cut off determined by this study were significantly higher when compared to the one normally used for this assay.

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1 Literature review

1.1 General introduction

Foot-and-mouth disease (FMD) is a highly contagious and widely distributed disease affecting cloven-hoofed animals. The major clinical features of the disease include fever, lameness, and the appearance of vesicles and ulcerative lesions in the mouth, feet, teats and mammary glands (Arzt et al., 2011; Horsington et al., 2018). Pain and discomfort from the vesicles and ulcerative lesions lead to other signs of disease such as depression, anorexia, excessive salivation, lameness, and reluctance to move or stand (USDA, 2021). Several factors including species, breed, pre-existing immunity, virus type and infecting dose influence FMD morbidity in a susceptible herd (Singh et al., 2019). The morbidity rate can approach 100% in naive populations, but transmission of some FMD viruses ceases after infecting only a relatively low percentage of the animals (Spickler, 2021). Mortality from FMD is low in adult livestock, with the case fatality percentage approximately 1-5% for most strains. However, higher mortality occurs in juveniles because of the affinity of the virus for cardiac tissues (Grubman & Baxt, 2004; Spickler, 2021). Clinical presentation of diseases including vesicular stomatitis and vesicular exanthema is similar to FMD and can only be differentiated based on laboratory confirmation (Alexandersen et al., 2003; Spickler, 2021). Other diseases or conditions that cause the development of ulcers/vesicles/blisters in the mouth or feet require further diagnostics to rule out FMD.

Routinely applied FMD outbreak control measures include (a) prompt detection and identification of the virus type, (b) establishment of outbreak extent, (c) quarantining the affected areas,(d) where applicable, culling of the infected and in-contact animals, (e) monitoring all livestock movements in the quarantined area, (f) conducting forward and backwards tracing of all movements of susceptible livestock and possible fomites in the quarantined area, (g) vaccination, (h) conducting general extension and awareness to all livestock stakeholders in the affected region and (i) notification of all local and international trade partners (Thomson & Bastos, 2004; Vosloo et al., 2002). Such a multidisciplinary disease control operation requires a well-coordinated and committed approach with adequate resources (Alexandersen et al., 2003; Thomson & Bastos, 2004; Vosloo et al., 2002).

1.2 Aetiology and viral characteristics

Foot-and-mouth disease virus (FMDV); (family *Picornaviridae*, genus Aphtovitus) infection causes an acute and highly contagious disease of cloven-hoofed animals (Thomson & Bastos,

2004). The virus particle comprises a non-enveloped icosahedral shaped protein capsid surrounding a single-stranded, positive-sense RNA genome that is approximately 8400 nucleotides in length (Jamal & Belsham, 2018). The genome encodes four proteins (VP1, VP2, VP3, and VP4) that make up the structure of the viral capsid (structural proteins) and several non-structural proteins (NSPs; L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D) (Gao et al., 2016). Three structural proteins, VP1-3, are located on the virion surface, whereas VP4 is within the virus particle (Jamal & Belsham, 2018). Non-structural proteins do not form part of the FMDV virion (Gao et al., 2016).

1.3 Diversity of FMD viruses

FMDV exists as seven immunologically distinct serotypes identified as O, A, C, Asia-1, Southern African Territories (SAT 1,2 and 3). Each of the seven serotypes has a spectrum of antigenically distinct subtypes (OIE, 2021a; Samuel & Knowles, 2001). Serotypes A and O are well established in most FMD endemic regions except southern Africa where incursions only occur sporadically (Sikombe et al., 2015). Serotype O is responsible for the majority of the outbreaks globally (OIE, 2021a). Asia-1 serotype circulation predominates in Asia. There have been no reports, anywhere, of disease due to the serotype C FMDV since 2004 and is thought to be extinct (Belsham, 2020; Brito et al., 2017; Sangula et al., 2011).

The African buffalo (*Syncerus caffer*) is the natural host for the SAT serotypes. FMD serotypes SAT1 and SAT2 are distributed throughout Africa, while SAT3 is limited to southern and a small area in eastern Africa (Thomson & Bastos, 2004). The only serotype yet to be reported in Africa is Asia-1 (Kerfua et al., 2018; Vosloo et al., 2002; Wungak et al., 2016). The SAT serotype viruses predominate in Southern Africa and constitute a distinct lineage separate from the A, O, C and Asia-1 viruses (Vosloo et al., 2009).

This wide global variation and genetic polymorphism of circulating FMDV arise from the high mutation rate during RNA genome replication due to the viral RNA–dependent RNA polymerase's inability to proofread (Ayelet et al., 2009).

1.4 Characteristics of the virus

The virus is made up of a single-stranded positive-sense RNA genome of approximately 8500 nucleotides and structurally similar to all picornaviruses, consisting of a non-enveloped icosahedral capsid, with a diameter of about 20–30 nm diameter and 60 asymmetrical protomers (Domingo et al., 2002) The virion consists of approximately 70% protein, 30% RNA and a small quantity of lipids. It has a relative molecular mass of about 8.5 x 10^6 and sedimentation constant of 146S (Sobrino et al., 2001). The capsid surface is relatively smooth,

with a protruding G-H loop in VP1 (Saiz et al., 2002; Sobrino et al., 2001; Thomson & Bastos, 2004). This large loop contains a highly conserved Arg–Gly–Asp (RGD) triplet, a cell recognition site critically involved in virus attachment through its interaction with cell surface receptors (Mateu et al., 1996). The immunogenicity of FMDV particles is associated with peptides on the surface of the capsid (Sobrino et al., 2017; Paton et al., 2005; Saiz et al., 2002). The FMDV particle is labile at pH slightly below neutrality (Bachrach et al., 1957; Newman et al., 1973; Yuan et al., 2017). At pH 6, the rate of FMDV inactivation is 90% per minute while at pH 5, it is 90% per second (Saiz et al., 2002). FMDV is relatively heat resistant with the resistance varying depending on the virus type and strain (Thomson & Bastos, 2004). However, temperatures above 43°C causes rapid inactivation of aerosolised viral particles. (Sobrino et al., 2001; Thomson & Bastos, 2004).

1.5 Significance of FMD to Southern Africa

An estimated 75% of about 150 million livestock in Southern Africa is under traditional smallholder farming system where primitive animal husbandry methods are practised (SADC, 2012). Sixty per cent of land in southern Africa is suitable for livestock farming, and boosting animal production can significantly improve the livelihoods and economies of regional countries (SADC, 2012).

Unfortunately, the southern and east African region has more trade-sensitive transboundary animal diseases (TADs) than any other part of the world because of the abundant wildlife which play a critical role in their maintenance (Bengis et al., 2004). The SAT serotypes of FMDV predominate in southern Africa and are endemic to most African buffalo populations (Bengis et al., 2004; Rweyemamu et al., 2008; Thomson & Bastos, 2004). Interaction between wildlife and cattle at wildlife/ livestock interfaces increases the chances of their transmission to livestock (Thomson et al., 2013). Effective control of FMD and other TADs in southern Africa has focused on the separation of wildlife and livestock (Thomson et al., 2013) by erecting and maintaining fences around game reserves. Specific FMD control measures on livestock populations at the wildlife/livestock interface including vaccination, disease surveillance and livestock identification and movement tracing have allowed some countries in the region to establish and maintain FMD free zones (Thomson et al., 2013). Namibia, Botswana, South Africa and Zimbabwe maintained recognition with the World Organisation for Animal Health (OIE) as having a 'FMD free zone without vaccination' within their territory for more than 20 years (Thomson et al., 2013). From early 2000, the FMD situation in the Southern African Development Community (SADC) region started deteriorating. Currently, the only countries

that still have OIE the FMD free zone status are Botswana and Namibia (OIE, 2021). Declining standards of control on the part of the respective Veterinary Services is the major driver to this heightened FMD activity and transboundary spread in the SADC region. It has been demonstrated that the quality and efficacy of vaccines used against the SAT serotypes of FMDV in the SADC region have been declining (Thomson et al., 2013) and vaccination programmes are not being adhered to so as to generate and maintain adequate levels of herd immunity (Thomson et al., 2013). Disease surveillance and laboratory diagnostic capacities activities have also steadily declined. Illegal transboundary movement of livestock and their products is not being closely monitored. Furthermore, the increase in census of wildlife in the FMD infected zones and a corresponding increase in communal livestock activities at areas adjacent to the infected zones has resulted in increased wildlife/livestock interaction (Thomson et al., 2013). This pressure and conflict at the wildlife/livestock interface has resultantly led to frequent damages to fences surrounding FMD infected zones (Thomson et al., 2013).

Another factor making FMD control in southern Africa challenging is that SAT serotypes have a wide array of antigenic variants compared to other FMDV serotypes, rendering vaccination against FMD caused by SAT serotypes less effective than is the case for FMD elsewhere in the world (Maree et al., 2014).

There are fifteen reference laboratories for FMD diagnostics worldwide, and the two on the African continent are in Southern Africa (OIE/FAO, 2020). Their main objective is to improve the diagnosis of FMD globally. However, the diagnosis of FMD is challenging due to its broad host range and a wide array of antigenically distinct serotypes and subtypes that require uniquely and fully validated assays for definitive diagnosis.

1.6 Transmission

Foot-and-mouth disease is transmitted when a susceptible animal is exposed to viral particles either when in contact with an infected animal shedding viral particles or indirectly by coming into contact, ingesting contaminated material or inhaling resuspended viral particles (Colenutt et al., 2020; Thomson & Bastos, 2004). Shedding of FMDV can occur from all secretions and excretions of infected animals. These include exhaled breath, mouth vesicles, nasal discharges, saliva, ruptured vesicles on feet, milk, semen, urine, faeces, vaginal discharges, aborted fetuses and embryo washings (Grubman & Baxt, 2004; OIE, 2021a; Thomson & Bastos, 2004). Blood and animal products from infected animals can also indirectly transmit the infection (Sutmoller, 2001).

The major infection pathway is through the respiratory system. The viral particle is transmissible in aerosols generated either when an animal breathes or resuspension from infected material. Factors that determine aerosol transmissibility include the quantity of airborne virus emitted from an infected holding, the strain of virus, weather and topography (Donaldson & Alexandersen, 2002).

1.7 Clinical signs

The incubation period of FMD in livestock varies from two to fourteen days depending on the infecting dose, virus strain and host susceptibility (Kitching, 2002). Cattle and pigs tend to show more apparent clinical signs when compared to small ruminants (Watson, 2004). The clinical picture of the disease in wildlife is similar to domesticated livestock. Wild populations of some species such as mountain gazelles (*Gazella gazelle*), impala (*Aepyceros melampus*) and saiga antelope (*Saiga tatarica*) show more apparent clinical signs compared to African buffalo (Thomson & Bastos, 2004). Pyrexia is usually the first clinical sign (approximately 40°C) lasting for 24 to 48 hours followed by the development of vesicles on the tongue, hard palate, dental pad, lips, gums, muzzle, coronary band, interdigital spaces and on teats of lactating animals. Young livestock might die due to myocarditis before the appearance of apparent clinical signs because the virus invades and destroys heart muscle cells (Grubman & Baxt, 2004). Acutely infected livestock can salivate profusely and develop lameness due to painful oral and feet lesions. In severe cases, the whole tongue epithelium and hoof can slough (Grubman & Baxt, 2004).

1.8 Laboratory diagnosis

The severity of FMD clinical signs varies with the strain of virus, exposure dose, age and breed of animal, host species, and degree of host immunity. FMD clinical signs range from inapparent (subclinical) to severe (OIE, 2021a). The clinical signs displayed by FMD are broad and disease confirmation requires laboratory diagnosis. Laboratory diagnostic methods also enable serotype differentiation (OIE, 2021a).

Viral isolation and methods that detect viral antigens, nucleic acids, and antibodies are the core repertoire of techniques used for the laboratory diagnosis of FMD (OIE, 2021a).

FMD antigen detection methods include:

(a) Virus isolation, achieved by observation of cytopathic effects (CPE) on cell culture systems or animal models inoculated with a suspension containing FMDV,

(b) Nucleic acid recognition methods that amplify genome fragments of FMDV in diagnostic materials and

(c) Immunological methods such as antigen detecting Enzyme-linked immunosorbent assay (ELISA), CFT and Pen-side, lateral flow devices that detect antigenic particles within the test sample.

Routinely used FMDV antibody detection serological methods include:

(a) Liquid Phase Blocking ELISA (LPBE),

(b) Solid Phase Competition ELISA (SPCE),

(c) Non-Structural Protein (NSP) ELISA and

(d) Virus Neutralisation Test (VNT).

The basis of these antibody detection methods hinges on the ability to detect anti-FMDV antibodies produced by the host against structural or non-structural FMDV proteins (OIE, 2021a). The LPBE, SPCE and VNT detect the antibodies against the structural proteins, while the NSP tests detect the non-structural proteins (OIE, 2021a).

1.8.1 FMDV antigen detection (Virus isolation)

Despite the development of more advanced rapid tests and nucleic acid amplification assays for quick identification and diagnosis of FMDV infection, isolation of viruses by the cell culture systems through observation of cytopathic effects (CPE) induced by virus infection remains the gold standard for FMD diagnosis because it identifies the live virus, and produces higher virus titers for use in further tests such as serotyping (Jamal & Belsham, 2013).

Blood, epithelium tissues of intact or ruptured vesicles, vesicular fluid and oropharyngeal scrapings are the routine samples for FMDV isolation (Alexandersen et al., 2003; OIE, 2021a). Viral isolation is sensitive when applied to samples collected during early clinical disease stages because viral excretion and load will be at their highest levels. A sharp decline in viral excretion and viremia occurs around day 4–5 of clinical disease when a significant circulating antibody response is detectable (OIE, 2021a). The likelihood of virus isolation from samples collected after day 10 of clinical appearance is very low (Alexandersen et al., 2003).

Virus isolation uses cells for virus propagation. FMDV replicates in a wide range of primary and continuous *in-vitro* cell cultures. However, the sensitivity of cell cultures differs according to FMDV serotypes (Kabelo et al., 2020). Primary bovine thyroid (BTY) cells are the most sensitive cell culture for isolation of FMDV (House & Yedloutschnig, 1982; Kabelo et al., 2020). Continuous cell cultures also susceptible to FMDV infection include baby hamster kidney (BHK), lamb kidney (LK), pig kidney cell lines IB-RS-2 and MVPK-1. The goat fetal tongue cell line (ZZ-R 127) has a sensitivity equivalent to BTY (Brehm et al., 2009).

The quality and type of cells used, sample quality and FMDV serotype influence the sensitivity of virus isolation (Conlan et al., 2008). BTY cell types tend to lose sensitivity when passaged and frozen (Fukai et al., 2015), hence the need for newly prepared thyroid tissue cells for each virus isolation. Production of cell lines is expensive, time-consuming and laborious.

1.8.2 FMD antigen detection (Sandwich ELISA)

Antigen capture sandwich ELISA, diagrammatically illustrated in Fig1, is used for the detection of virus antigen and is the preferred procedure for detecting FMDV antigen and viral serotyping (OIE, 2021a). The test is based on an indirect sandwich ELISA where different rows in multiwell plates are coated with rabbit polyclonal antisera (capture sera) specific to each of the seven FMDV serotypes. Virus antigens present in sample suspensions bind to the capturing antibodies are detected by the serotype-specific tracing antibody. The colour reaction is developed by tracing antibody specific conjugated antibody and substrate solution (OIE, 2021a).

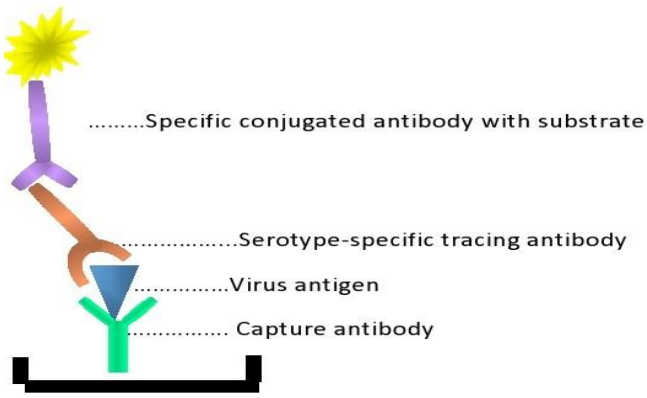

Fig 1: Diagram showing the Sandwich ELISA

Antigen-capture ELISA does not use live viruses and employs robust technology hence applicable for use in environments with low technology. The FMDV antigen-capture ELISA is now preferred instead of complement fixation test for the primary diagnosis FMD and serotyping (Ma et al., 2011). Samples collected from fresh lesions with higher viral particles are preferred but those found to be unsuitable for virus isolation can still be tested this ELISA method (Lazarus et al., 2010). Though sandwich ELISA is a much faster approach to detect viral antigens, its analytical sensitivity is low, and primarily used for FMDV confirmation after isolation (Remond et al., 2002).

1.8.3 Lateral flow device

Lateral flow devices (LFD) for detecting FMDV have been developed (Ferris et al., 2010). The principle of LFD is immuno-chromatography, where soluble antigens flow through a porous strip and binds labelled antibody conjugate to form an immune complex. The fluid then flows through a zone where an immobilised antibody against the antigen detects the immune complex (Ferris et al., 2010).

The LFD is a very simple, and rapid procedure and is applicable as pen-side test in field conditions for diagnosis and serotyping once fully developed and validated for use (Ferris et al., 2010; Ferris et al., 2009).

1.8.4 Molecular techniques for nucleic acid detection

Polymerase chain reaction (PCR) is a powerful amplification technique that generates an ample supply of a specific DNA segment (i.e., an amplicon) from a small amount of starting template. The technique is based on the in vitro ability of the enzyme DNA polymerases to synthesise DNA molecules (Lorenz, 2012). Templates of RNA viruses must be converted to complementary DNA by the enzyme reverse transcriptase before amplification (Kasanga et al., 2014). The reverse transcription-polymerase chain reaction (RT-PCR) amplifies cDNA (Kasanga et al., 2014; Lorenz, 2012). The elongation and amplification of the template DNA or cDNA employs a three-step cycling process: 1) heat-induced denaturation of double-stranded DNA to separate the two complementary strands, 2) temperature reduction for hybridisation and, 3) use of DNA polymerase to produce a complimentary copy of the target DNA sequence (Kasanga et al., 2014; Lorenz, 2012).

The products of each reaction cycle serve as a template for the subsequent cycles, and the result is an exponential amplification of the targeted DNA fragment. PCR can also amplify multiple DNA fragments in the same reaction chamber (Lorenz, 2012).

Electrophoresis is the most widely used method for the analysis of PCR products. The PCR products are visualised either by staining the amplified DNA product with a chemical dye such as ethidium bromide, which intercalates between the two strands of the duplex or labelling the PCR primers or nucleotides with fluorescent dyes (Garibyan & Avashia, 2013). PCR products are separated by size (Lorenz, 2012).

Different types of PCR have been developed, but their basis of operation remains the same. Advantages of the PCR method include higher analytical sensitivity, a shorter testing period of about 4-8 hours and relatively cheaper when compared to virus isolation (Kasanga et al., 2014). However, PCR has a few shortcomings compared to virus isolation and these include lower

specificity due to factors including non-specific primer binding resulting in the amplification of an undesired sequence, ease of contamination and inability to identify the viability of identified organism. It is also expensive to setup PCR testing station (Garibyan & Avashia, 2013).

1.8.5 FMD antibody detection

Antibody detection methods detect anti-FMDV antibodies produced by the host against structural or non-structural FMDV proteins (OIE, 2021a). Routinely used FMDV antibody detection serological methods include liquid-phase blocking ELISA (LPBE), solid-phase competition ELISA (SPCE), non-structural protein (NSP) ELISA and the virus neutralisation test (VNT) (OIE, 2021a).

1.8.5.1 Virus neutralisation test

The VNT is a serotype-specific serological test for FMDV that quantifies the ability of antibodies in test serum to neutralise the biological activity of FMDV when mixed in vitro (Lazarus et al., 2010). Should the test serum sample contain specific FMDV-antibodies, the known virus in the test matrix will be blocked from infecting cells thus preventing CPE. The titer of the virus must be known when added to cultures of susceptible cells. The titer of the serum is defined as the dilution of serum that can neutralize 100 $TCID_{50}$ of virus in 50% of individual inoculated cell monolayers (Golding et al., 1976; OIE, 2021a).

VNT is highly sensitive when the virus or antigen used in the test is closely matched with the strain in the test serum (Golding et al., 1976) and is the prescribed test for international trade (gold standard). However, this method is prone to variability due to different sensitivity levels of cell cultures to the test matrix. The live virus must be closely related to the targeted antibodies in the test serum sample. VNT is also susceptible to contamination and testing should be performed in bio-containment with cell culture facilities. The VNT is also time-consuming and testing takes several days to complete (OIE, 2021a).

1.8.5.2 Liquid-phase blocking ELISA(LPBE)

This method is based on the blocking of FMDV antigen by specific antibodies in test serum within the liquid phase (Hamblin et al., 1986).

The LPBE is serotype-specific but can be designed to detect antibodies against all seven FMDV serotypes. FMDV specific polyclonal rabbit and guinea pig antibodies are often used to detect residual FMDV antigen following *in-vitro* incubation of test serum and FMDV antigen within the liquid phase. The LPBE can be used as an in-vitro method to estimate protection against FMDV challenge and as an alternative to the VNT (Hamblin. et al., 1987; Kang et al., 2018).

LPBE has low specificity when compared to other serological methods and therefore tends to produce false-positive results (Clavijo et al., 2004; Mackay et al., 2001; Wong et al., 2020). The test is time-consuming (on average two days), requires virus containment facilities and cannot differentiate vaccinated from convalescing animals. Though recommended for detecting FMDV-specific antibodies by the OIE (OIE, 2021a), its current usage across FMD diagnostic laboratories is declining.

1.8.5.3 Solid-phase competition ELISA

A solid-phase FMD competition ELISA (SPCE) evaluates competition between serotype-specific guinea pig anti FMDV antiserum and antibodies present in the test serum for binding to FMDV antigen. Like the LPBE, the SPCE is also serotype-specific and has been developed for the testing of all seven FMDV serotypes (Mackay et al., 2001). SPCE is more rapid than the LPBE and results can be obtained in the same day (4 – 5 hours). SPCE is also more robust with higher sensitivity and specificity when compared to the LPBE (Li et al., 2012; Mackay et al., 2001; Paiba et al., 2004). For these reasons, the SPCE is preferred over LPBE at most FMD diagnostic facilities.

1.8.5.4 Non-structural protein assays

The majority of FMD vaccines in commercial production contain inactivated virus as antigen. FMD vaccines are produced, under high bio-containment conditions, by replicating infectious virus within baby hamster kidney (BHK) cells in suspension culture followed by chemical inactivation using binary ethyleneimine (BEI) (Belsham, 2020). Vaccine preparations can then be purified to remove non-structural viral proteins (Belsham, 2020). This enables the usage of non-structural protein assays to differentiate vaccinated from infected animals.

NSP ELISA employs purified recombinant NSP antigens adsorbed onto microplates to trap antibodies present in samples of infected animals (DeDiego et al., 1997; Mackay et al., 2001; Sorensen et al., 1998). NSP assays are not serotype-specific and a positive reaction indicates antibody present to any of the seven serotypes (Mackay et al., 2001). Detection of antibodies to the NSPs of FMDV can represent past or recent infections (OIE, 2021a).

Several FMD-NSP ELISA kits have been developed and are recommended by the OIE for use in FMD diagnosis. However, their use is more applicable to the herd level because of their lower diagnostic sensitivity (Paton et al., 2010).

1.9 FMD Control in Southern Africa

Some countries in southern Africa (e.g. Namibia, Botswana and South Africa) have obtained an OIE recognised FMD freedom status for zones within their countries. Lesotho and Eswatini

have FMD freedom for the whole country (Thomson et al., 2013). Unfortunately, in January 2019, South Africa recently lost the OIE recognised FMD free zone status, due to recent outbreaks of the disease (OIE, 2019) The other Southern African countries either lost their OIE recognised FMD free-zone status and never regained it or have not yet applied for the status. Zimbabwe had an OIE recognised status up to early 2000 but never regained it thereafter (Thomson et al., 2013).

FMD is mainly transmitted by direct contact between infected and susceptible animals. The epidemiology of FMD in Southern Africa is unique because of two distinct but overlapping situations that contribute to the effective transmission of FMDV to susceptible livestock (Thomson & Bastos, 2004). The first is cattle to cattle transmission and the second being the association of the disease with wildlife, especially African buffalo (Thomson & Bastos, 2004). The establishment of trans-frontiers conservation areas that transcend the borders of South Africa, Mozambique and Zimbabwe further complicate the application of effective FMD control in southern Africa.

The FMD control measures applied by member countries include:

a) Effective separation of wildlife from the infected game reserves (FMD infected zone) by erection and thorough maintenance of game proof fences to minimise incursion of the disease to livestock,

b) Routine vaccinations of cattle at the livestock/wildlife interface (protection zone) with a vaccine closely matched to the topotypes of FMDV prevalent in the livestock and buffalo populations in the vicinity to induce herd immunity,

c) Identification and tracking of livestock movements in the infected and protection zones, and

d) Regular surveillance (both clinical and serological) on livestock in the infected and protection zones (Rweyemamu et al., 2008; Thomson & Bastos, 2004; Vosloo et al., 2002).

FMD is a controlled disease in all countries in southern Africa and government veterinary services are actively involved in its management. Should an outbreak of FMD outside the FMD infected zones occur, the affected area is placed under quarantine, and disease surveillance measures are intensified to establish the extent of infection. This is usually followed by vaccination of all cattle in a specified radius around the infected or outbreak area. Cattle within the infected area are also vaccinated. Local and international trading partners and the general public are updated on the outbreak progression till resolution (DALRRD, 2019).

Wildlife is a natural resource in southern Africa. The elimination of FMD in persistently infected buffalo is not an option and thus FMD is unlikely to be eradicated (Vosloo et al.,

2002). A regional approach is imperative for the sustainable control of FMD in the region (Maree et al., 2014) .

1.10 Justification and study benefits

Globally, the OIE has 15 designated laboratories in different countries to research scientific and technical problems related to FMD. These laboratories are mandated to make available accurate and timely data to support global surveillance and control of FMD. They participate in inter-laboratory testing schemes and proficiency testing with other designated laboratories so that their diagnostic methods remain relevant for the early detection of a possible disease outbreak. The laboratories are also encouraged to conduct in-house intra-laboratory testing to ascertain the repeatability and reproducibility of their assays (OIE, 2021a).

The OIE designated laboratories also constantly develop and validate different FMD diagnostic methods to enable prompt detection of the circulating serotypes and incursions by other serotypes from other regions.

The FMD diagnostic laboratory in South Africa, Transboundary Animal Diseases Programme –Onderstepoort Veterinary Research (TAD-OVR) is one of the 15 global OIE reference laboratories and one of only two in Africa. The SPCE is the primary serological test performed for FMD at TAD-OVR and is validated for testing FMDV-specific antibodies in cattle and African buffalo sera. The validation of the SPCE method for use in other susceptible species is in progress. Results from this study will contribute towards validation of the SPCE for detection of antibodies against FMD in goats.

Most African countries, especially those in the Southern African Development Community (SADC), send FMD samples for testing to the TAD-OVR laboratory for confirmation. Currently, five FMDV serotypes are in circulation in the SADC region, namely the prevalent SAT serotypes and the recently detected O and A in central Zambia. Thus, there is a need to improve the performance of FMD testing methods at TAD-OVR through complete validation and routine performance monitoring for them to be applicable for use in diagnosing FMD from the various susceptible species and for all serotypes, especially to those prevalent in southern Africa.

This study will help towards the validation of the SPCE in goats and also avail more information regarding the overall performance of the assay. The diagnosis of FMD is challenging due to its non-specific clinical signs and the existence of many antigenically distinct serotypes (Grubman & Baxt, 2004). Increasing the number and variety of test methods

available for FMD diagnosis enables a more informed result interpretation after interrogating results from different assays.

The display of competence by TAD-OVR to accurately diagnose by having a wide verify of fully validated FMD assays will not only strengthen FMD controls but also boost confidence of the regional and international community to open trade opportunities in livestock and livestock products with Southern African countries.
TAD-OVR clients will also have a wide selection of fully validated FMD diagnostic tests.

1.12 Aims and objective

The primary objective is to determine the repeatability of the single dilution SPCE assay and secondly to determine the correlation between the single dilution SPCE and the titration SPCE assay. The optimal positive threshold of the SPCE method will also be determined in vaccinated goats subsequently infected with SAT1 FMDV.

2 Materials and Methods

2.1 Ethical Clearance

The Animal Ethics Committee (REC 171-19) of the Faculty of Veterinary Science at the University of Pretoria approved this study. Permission to conduct this study was obtained from the Department of Agriculture, Forestry and Fisheries, Directorate: Animal Health (Application Number 12/11/1/1) of the Republic of South Africa.

2.2 Sample origin

Aliquots of stored serum samples previously collected from goats that had participated in a FMDV challenge study (Lazarus et al., 2020) and tested in duplicate for antibodies against structural FMDV proteins using the SPCE were included in the study. Serum samples were retrieved from the TAD-OVR biobank where they had been kept for approximately five months at -80°C.

2.3 Brief background of the sample origin

A group of 40 indigenous South African goats (6-12 months of age) of mixed sexes were sourced from livestock farms within the FMD free-zone of South Africa before the 2019 FMD SAT2 outbreak (DALRRD, 2019). The goats participated in a study to evaluate the efficacy of a candidate pentavalent FMD vaccine in goats against heterologous challenge with a pool of field SAT1 FMDV. The TAD-OVR personnel had determined the effective dose of the candidate inactivated pentavalent FMD vaccine containing serotypes SAT1, SAT2 and SAT3 in cattle as 2 ml (Lazarus et al., 2020) and the aim of the previous study was a preliminary evaluation to determine the effective dose of the same vaccine in goats.

The 40 goats were randomly allocated to five treatment groups. Goats in group 1 (allocated five goats) were vaccinated with a full cattle dose, while those in groups 2, 3 and 4 (with ten goats each) were vaccinated with reduced cattle doses: 1/3rd (0.67 ml), 1/6th (0.33 ml), 1/12th (0.16 ml) respectively. Five goats were allocated to group 5 and were the unvaccinated placebo control.

Goats were vaccinated with an inactivated pentavalent FMD vaccine containing serotypes SAT1, SAT2 and SAT3 on day 0 and received a booster vaccination at day 20 post-initial vaccination. All goats, except two in treatment group 3(1/6th (0.33 ml) of the cattle dose), were challenged by tongue inoculation at day 41 post-vaccination using $10^{4.57}$ 50% tissue culture infective dose ($TCID_{50}$) FMDV SAT1 pool. The occurrence of natural transmission of the SAT1 virus was assessed in the two unchallenged goats, kept close to those that were challenged (Lazarus et al., 2019).

Blood samples were collected into plain evacuated tubes (Vacutainer®, BD Becton, Dickinson and Company, USA) on day 0 before vaccination and after every seven days until study termination at day 56 (14 days post-infection). The collected blood was allowed to clot at room temperature and sera harvested and stored at -20°C until testing. Four goats died at various stages before study termination. A total of 342 serum samples were available for the study.

After study termination, aliquots of the collected serum samples were tested in duplicate for FMDV SAT serotypes using the SPCE method by TAD-OVR. The remainder of the unused serum samples remained in long storage under similar conditions as specified above.

2.4 Laboratory analysis of specimens

2.4.1 Solid-phase competition ELISA (SPCE)

A SPCE for FMDV SAT serotypes was performed on the retrieved samples collected on days (0, 7,14,20,28,34,41,48 and 55) following standard procedures (Mackay et al., 2001; Paiba et al., 2004). The principle of this method is based on the competition between serotype specific guinea pig anti-FMDV antiserum and antibodies present in the test serum. FMDV serotype specific rabbit anti-serum adsorbed onto micro-titer plates captures the FMD type-specific antigen. After discarding the FMD antigen, the test serum was added to the micro-titer plates together with the specific guinea pig antiserum and incubated for one hour at 37ºC. After incubation, the micro-titer plates were washed and anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase is added. The plate was further incubated for one hour, during which the anti-guinea-pig immunoglobulin-horseradish peroxidase conjugate will bind to the specific guinea pig antiserum. This step was followed by washing of the plate and the final step being the addition of an indicator tetramethylbenzidine (TMB) substrate solution which produces a colour when it binds to the horse radish peroxidase conjugated to anti-guinea-pig immunoglobulin. Positive test sera bind to the antigen and prevent the specific guinea pig antiserum and therefore also the anti-guinea-pig immunoglobulin-horseradish peroxidase conjugate from binding, hence resulting in a decreased colour reaction.

Tests were performed in duplicate on the micro-titer plate and percentage inhibition (PI) values were calculated for each well, using the formula:

PI = 100 – (100 x (OD test serum mean/OD strong positive control mean)), representing the competition between the test sera and the guinea-pig anti-FMDV antisera for the FMDV antigen on the ELISA plate.

Samples with a percentage inhibition <50% were classified as negative and those ≥50% were considered a positive (Paiba et al., 2004).

The TAD-OVR laboratory provided a copy of the duplicate results (TAD-OVR) and allowed for them to be included in the analysis.

2.4.2 Titration SPCE method

The titration SPCE was conducted in the same manner as the single dilution SPCE test. Samples were tested in duplicate, in a two-fold dilution series starting from an initial 1:20 dilution. Three dilutions were performed per serum sample as well as on the positive and negative controls. The negative control was made up of the blocking buffer and negative serum sample. Samples with antiserum titers equal to or greater than ≥1.6 log10 were considered positive.

2.5 Description of data sets

The initial SPCE testing done by TAD-OVR in October and November 2018 as part of (Lazarus et al., 2020) vaccine efficacy study will be referred to as single spot SPCE. The second titrated SPCE testing conducted in March and April 2019 will be referred to as titration SPCE (T-SPCE) and titrated to determine antibody titer levels.

2.6 Data analysis

2.6.1 Coefficient of variation calculations for the separate test runs

Repeatability of the SPCE was measured for both runs (SS-SPCE and T-SPCE) using duplicate percentage inhibitions for each tested sample (within -run). Repeatability was also assessed between runs using average percentage inhibitions for each of the two test runs. A coefficient of variation below 20% was considered an indication of adequate repeatability (Couto et al., 2013; Jacobson, 1998; Jaworski et al., 2011).

The coefficients of variations were calculated per serotype for each of the 342 samples for each test run using duplicate percentage inhibitions. The coefficient of variation was calculated using the following formula (Canchola et al., 2017).

$$COV=100.\frac{\delta}{\mu}$$

Where δ is the standard deviation between the duplicate percentage inhibitions and μ is the mean of the duplicate percentage inhibitions.

The mean, median, interquartile range and standard deviation of the 342 coefficients of variations were calculated for each of the 3 SAT serotypes tested. The number of duplicate tests with coefficient of variances under 20 % were also recorded for each of the three

serotypes. This analysis also applied to both runs using means of individual run percentage inhibitions.

2.6.2 Correlation determination

The correlation and agreement between both SPCE runs (SS- SPCE and T-SPCE) and also of individual SPCE runs to titres (obtained from titration of the T-SPCE) were analysed. The mean percentage inhibitions for similar samples from both SPCE runs were compared to each other and also to titre. Correlations and agreement were conducted per serotype.

Correlation and agreement between mean percentage inhibitions for both runs as well as to the titration SPCE titer were described by calculating Spearman's rho as the measure of correlation and Cohen's Kappa as the measure of agreement respectively. The absolute value of the correlation categorised as: <0.19, 0.20-0.39, 0.40-0.59, 0.60-0.79, and ≥0.80 were classified as negligible, weak, moderate, strong, and very strong correlations, respectively (Evans, 1996). The strength of agreement as measured by Cohen's kappa (Landis & Koch, 1977) were classified as: none ($\kappa \leq 0$), slight ($0 < \kappa \leq 0.20$), fair ($0.21 < \kappa \leq 0.40$), moderate ($0.41 < \kappa \leq 0.60$), substantial ($0.61 < \kappa \leq 0.80$), and near-perfect agreement ($0.81 < \kappa \leq 1.0$) (McHugh, 2012). Statistical evaluations were performed in commercially available software (IBM SPSS Statistics Version 26, International Business Machines Corp., Armonk, NY, USA) and results were interpreted at the 5% level of significance.

2.6.3 Sensitivity and specificity

The accuracy of the SPCE method in detecting FMDV antibody levels was assessed by measuring its sensitivity and specificity when compared to titer results. Sensitivity and specificity of the SPCE method was achieved through comparing the T-SPCE percentage inhibitions and antibody titre results obtained from its titration. Antibody titer levels are considered a more accurate indicator when compared to percentage inhibitions hence were used as the gold standard. Antiserum titres ≥1.6 log10 were considered as positive.

The Vassarstats online tool (Lowry, 2004) was used to calculate sensitivity (SE) and specificity (SP) with 95% confidence intervals.

2.6.4 Scatter plots

The relationships between mean percentage inhibitions for both SPCE runs were compared to each other and titration SPCE titers using scatter plots in statistical software IBM SPSS Statistics Version 26, International Business Machines Corp., Armonk, NY, USA).

2.6.5 Receiver operating characteristics (ROC) and Youden Index calculation.

The overall diagnostic accuracy of the SPCE test, when compared to the titer result, was evaluated using a receiver-operating characteristics (ROC) curve analysis. The area under the ROC curve (AUC) ranges from 0 to 1 with a value of 0.5 indicating an inaccurate test and a value of 1 reflects an accurate test. A value between 0 and 0.5 indicates that the direction of the cut-off was selected in error. For example, the cut-off of ≥50% PI should be reversed to ≤50% PI instead. The optimal positive threshold for the diagnosis of FMD SAT1 infection in goats was determined based on the calculation of the Youden Index. The Youden index is determined as = max$_c$ (Se (c) + Sp (c) – 1). The cut-point that achieves this maximum is referred to as the optimal cut-point (c*) because it is the cut-point that optimizes the SPCE's differentiating ability when equal weight is given to sensitivity (Se) and specificity (Sp) (Faraggi, 2000; Ruopp et al., 2008; Youden, 1950). The statistical software (IBM SPSS Statistics Version 26, International Business Machines Corp., Armonk, NY, USA) was used for analysis

2.6.6 Descriptive plot of mean titer over time by treatment group

The mean titers for goats in each of the five treatment groups was calculated and graphically displayed over time with error bars using Microsoft Excel.

3 Results

3.1 Repeatability measurement

Three hundred and forty-two samples were tested in duplicate for SAT1, SAT2 and SAT3. The coefficients of variations were calculated for each sample tested (using duplicate percentage inhibitions). The COV's were grouped according to the serotype tested and test run. The following parameters were evaluated COV's for each serotype: mean COV, median COV, quartile 1 COV, quartile 3 COV, standard deviation of all the 342 COV's and number of individual COV's below 20% (Table 1 = SS-SPCE; Table 2=T-SPCE).

Of the 342 duplicate (percentage inhibitions) PI's analysed by the SS-SPCE (Table 1), 273 (79.8%) of SAT1; 303 (88.6%) of SAT2; and 302 (88.3%) of SAT3 had good repeatability with COV's below 20%. The median COV, quartile 1 and quartile 3 COV values for all the three serotypes were below the 20% threshold marker for good repeatability. The mean of coefficients of variations was calculated per serotype, and it was highest for SAT1 (44.11) while SAT2 and SAT3 had means of 14.3 and 10.03 respectively (Table 1). The standard of deviation for the coefficients of variations was also highest for SAT1 (296.93), followed by SAT2 (72.75) and SAT3 had the lowest value of (24.12).

Table 1 : Analysis of coefficients of variation on duplicate percentage inhibitions from SS-SPCE.

Serotype	Number COV's Under 20%	Median COV	Quartile 1 COV	Quartile 3 COV	Mean of COV	Standard deviation of COV
SAT1	273	2,08	0.50	14.24	44,11	296,93
SAT2	303	2,69	0.90	9.76	14,30	72,75
SAT3	302	3,19	1.21	7.62	10,03	24,12

Of the 342 duplicate PI analysed by the T-SPCE (Table 2), 278 (81.2%) of SAT1; 291 (85.08%) of SAT2; and 283 (82.74%) of SAT3 had good repeatability with COV's below 20%. The median COV, quartile 1 and quartile 3 COV values for all the three serotypes were below the 20% threshold marker for good repeatability. The mean of coefficients of variations was calculated per serotype, and it was highest for SAT1 (30.31) while SAT2 and SAT3 had means of 14.61 and 23.06 respectively (Table 2). The standard of deviation for the coefficients of variations was also highest for SAT1 (188.16), followed by SAT3 (96.38) and SAT2 had the lowest value of (47.73).

Table 2: Analysis of coefficient of variation on duplicate percentage inhibitions from T-SPCE.

Serotype	Number COV's Under 20%	Median COV	Quartile 1 COV	Quartile 3 COV	COV Interquartile Range	Mean of COV	Standard deviation of COV
SAT1	278	2,55	0.64	12.68	12,04	30,31	188,16
SAT2	291	3,45	1.04	11.34	10,30	14,61	47,73
SAT3	283	3,31	1.17	11.28	10,10	23,06	96,38

The COV for mean PI from both SPCE runs (Table 3) were also evaluated and is shown in table 3. Of the 342 duplicate PI analysed by the T-SPCE 216 (63.16%) of SAT1; 219 (64%) of SAT2; and 234 (68.4%) of SAT3 had good repeatability with COV's below 20%. The median COV and quartile 1 COV values for all the three serotypes were below 20% but significantly higher compared to the within run analysis. The quartile 3 COV value for all the three serotypes were above the 20% threshold marker for good repeatability. The mean of coefficients of variations was calculated per serotype, and were all above 20%, the highest was for SAT1 (164.82) while SAT2 and SAT3 had means of 32.48 and 30.97 respectively (Table 3). The standard of deviation for the coefficients of variations was also highest for SAT1 (1465.03) followed by SAT2 (136.38) and SAT3 had the lowest value (70.27).

Table 3: Analysis of coefficient of variation on mean percentage inhibitions for both SPCE runs one and two.

Serotype	Number COV's Under 20%	Median COV	Quartile 1 COV	Quartile 3 COV	COV Interquartile Range	Mean of COV	Standard deviation of COV
SAT1	216	9,58	2.70	47.17	44,48	164,82	1465,03
SAT2	219	10,56	3.24	31.53	28,29	32,48	136,38
SAT3	234	11,14	4.57	27.30	22,70	30,97	70,27

3.2 Correlation and agreement

3.2.1 SS-SPCE and T-SPCE percentage inhibitions

The correlation between mean SS-SPCE and T-SPCE percentage inhibition was strong for SAT3 (Spearman's rho = 0.791, and even stronger for SAT1 and SAT2 that a Spearman's rho correlation of 0.892 and 0.866, ($p<0.00.1$) respectively (Table 4).

Table 4: Spearman's rho and Cohen's kappa correlation **p<0.001**

	Spearman's rho			Cohen's kappa		
	SAT1	SAT2	SAT3	SAT 1	SAT 2	SAT 3
Correlation mean SS-SPCE PI to mean T-SPCE PI	0.892	0.866	0.791	0.782	0.746	0.771
Correlation mean SS-SPCE PI to mean T-SPCE titer	0.911	0.853	0.818	0.612	0.432	0.285
Correlation mean T-SPCE PI to T-SPCE titer	0.960	0.970	0.970	0.592	0.432	0.309

The correlation and relationships were further analysed and displayed as scatter plots. The pattern of a scatter plot indicates the correlation and strength of the relationship Linear relationships show a strong correlation and relationship.

Scatter plots displaying the relationship between mean percentage inhibitions of SS-SPCE and T-SPCE for SAT1 (Fig.1), SAT2 (Fig.2) and SAT3 (Fig.3) are not displaying a simple linear relationship and the correlation is not as strong when compared with Spearman's rho. The scatter plots display a closer correlation only when percentage inhibitions approach 80%.

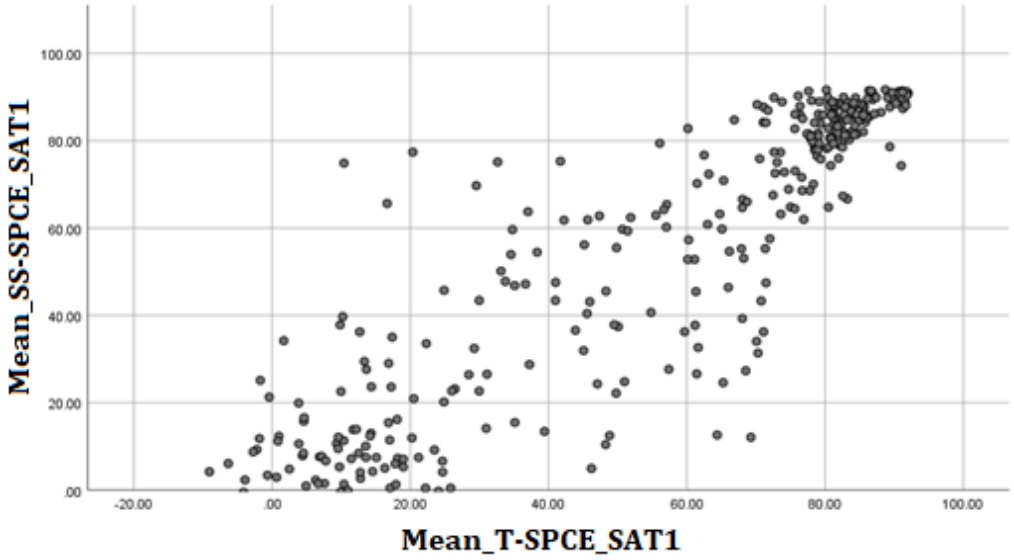

Fig 2: Relationship between mean SAT 1 percentage inhibitions for SS-SPCE and T-SPCE

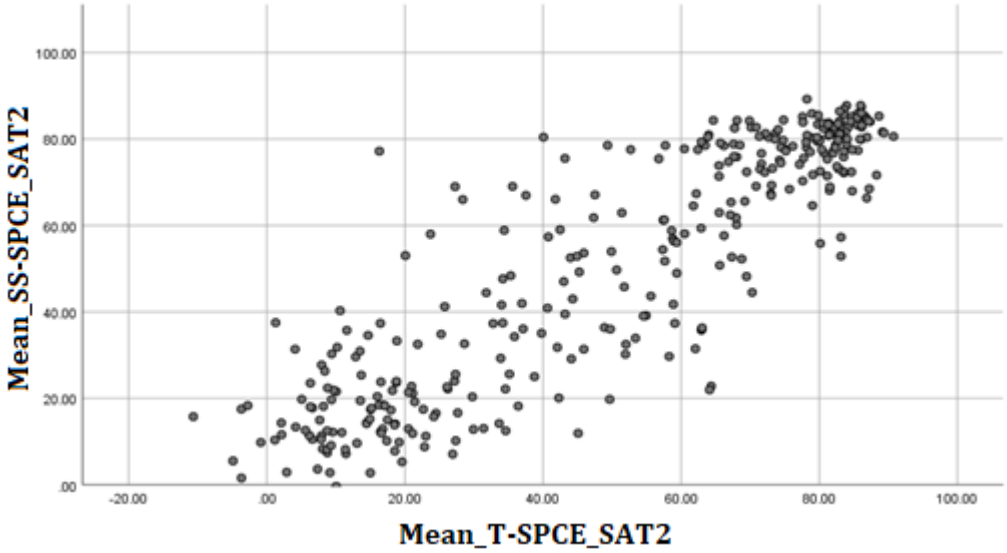

Fig 3: Relationship between mean SAT 2 percentage inhibitions for SS-SPCE and T-SPCE

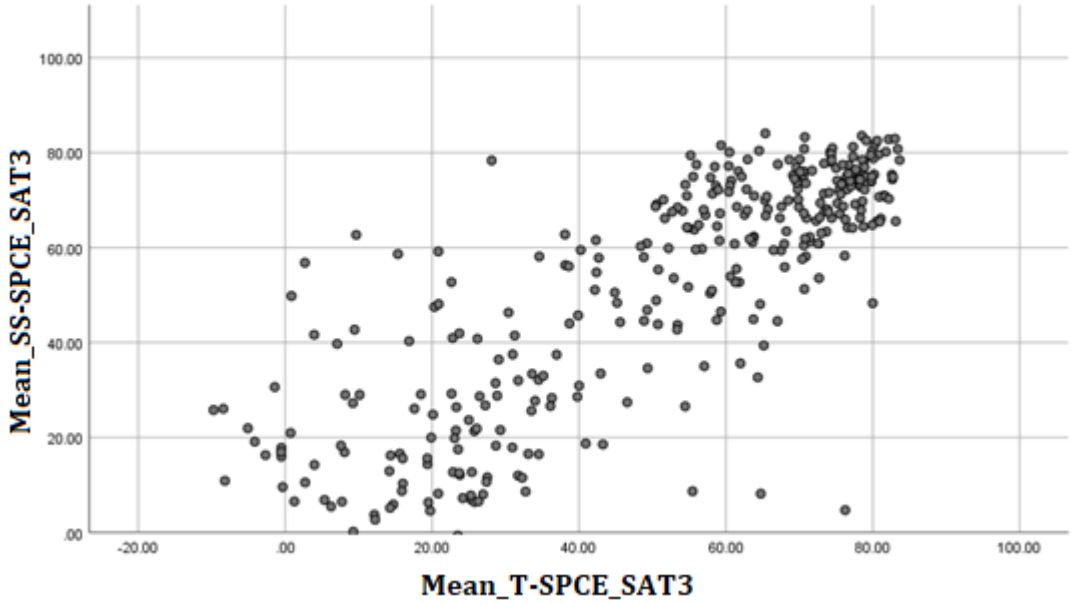

Fig 4: Relationship between mean SAT 3 percentage inhibitions for SS-SPCE and T-SPCE

The agreement between SS-SPCE and T-SPCE percentage inhibition was substantial across all 3 SAT serotypes (Table 1) with SAT1 having the highest agreement (Cohen kappa (k) = 0.782), and followed by SAT3 (k=0.771) and lowest being SAT2 (k=0.746), ($p<0.00.1$).

3.3.2 SS-SPCE mean percentage inhibition and titer

The correlation between SS-SPCE mean percentage inhibition and titer (Table 4) was very strong across all 3 SAT serotypes, SAT1 (rho=0.911), SAT2 (rho=0.853) and SAT3 (rho=0.818). This correlation was further analysed and displayed as scatter plots as shown in Fig 4, Fig 5 and Fig 6 for SAT1, SAT2 and SAT3 respectively. The scatter plots display did not suggest a strong correlation and the variability seem to slightly decrease at higher titer and PI inhibition levels.

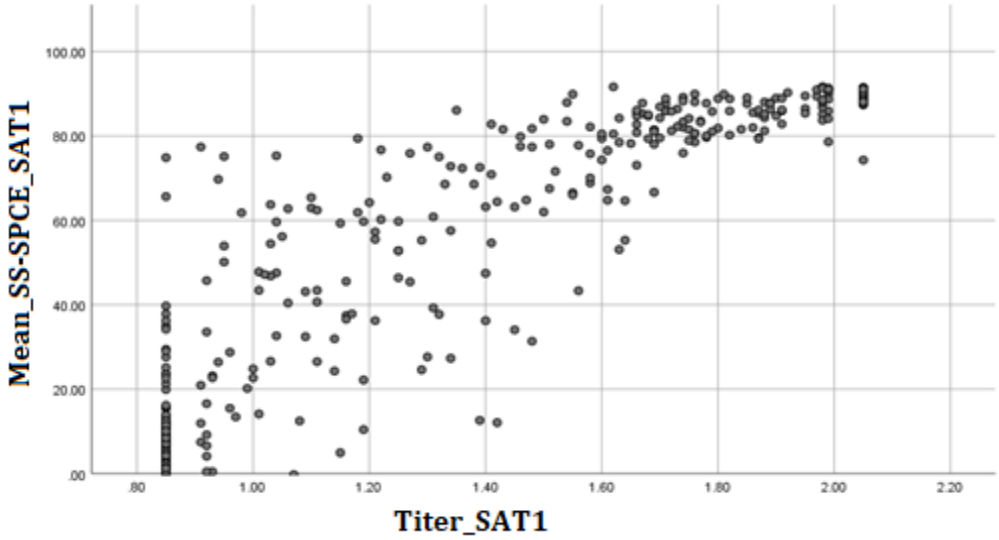

Fig 5: Relationship between mean SAT 1 percentage inhibitions for SS-SPCE and titer

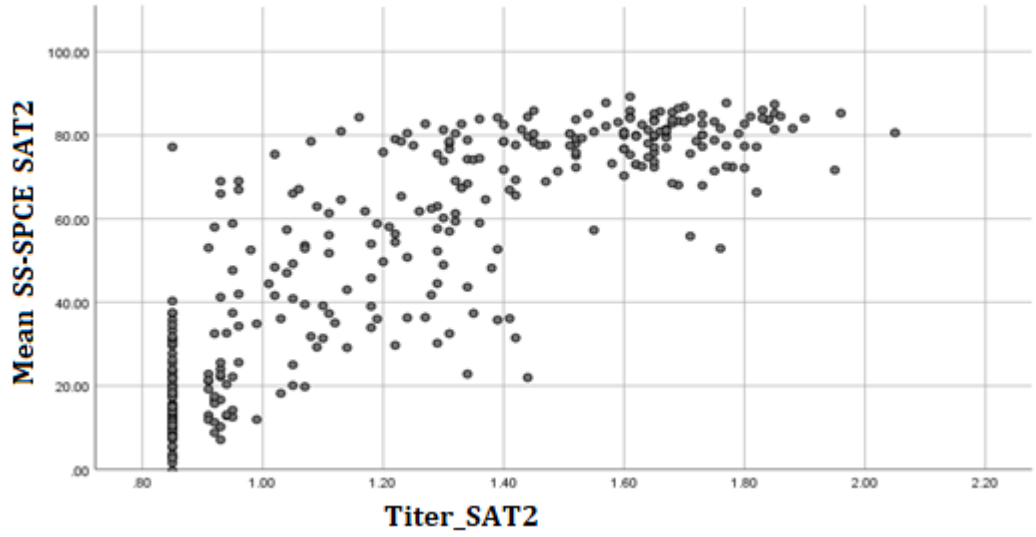

Fig 6: Relationship between mean SAT 2 percentage inhibitions for SS-SPCE and titer

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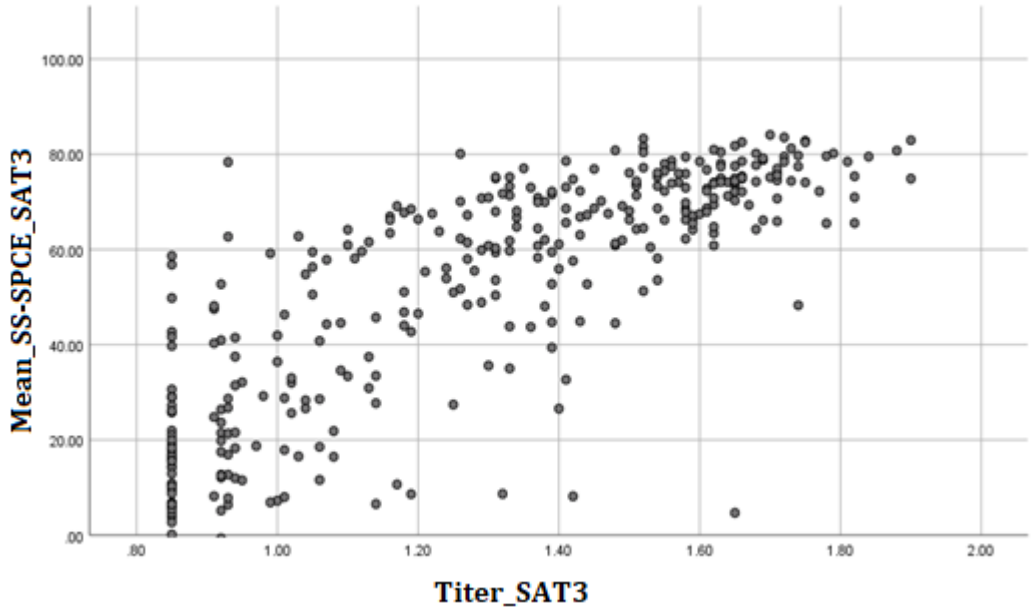

Fig 7: Relationship between mean SAT 3 percentage inhibitions for SS-SPCE and titer

The agreement between SS-SPCE mean percentage inhibition and titer was substantial for SAT1 (k=0.612), moderate for SAT2 (k=0.432) and fair for SAT 3 (K=0.285), (p<0.00.1).

3.3.3 T-SPCE mean percentage inhibition and titer

The correlation between T-SPCE mean percentage inhibition and titer (Table 4) was very strong across all three SAT serotypes, SAT1 (rho=0.960), SAT2 (rho=0.970) and SAT3 (rho=0.970). This correlation was further analysed and displayed as scatter plots as shown in Fig 7, Fig 8 and Fig 9 for SAT1, SAT2 and SAT3 respectively. The scatter plots displayed a better linear correlation but still the increased variability in the mid-section was noted.

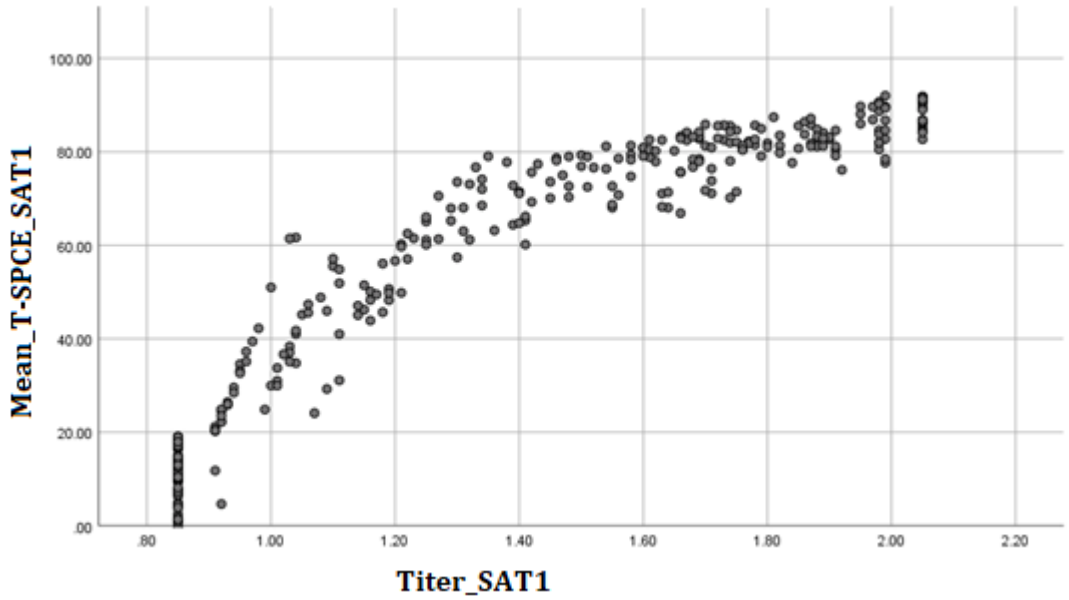

Fig 8: Relationship between mean SAT 1 percentage inhibitions for T-SPCE and titer

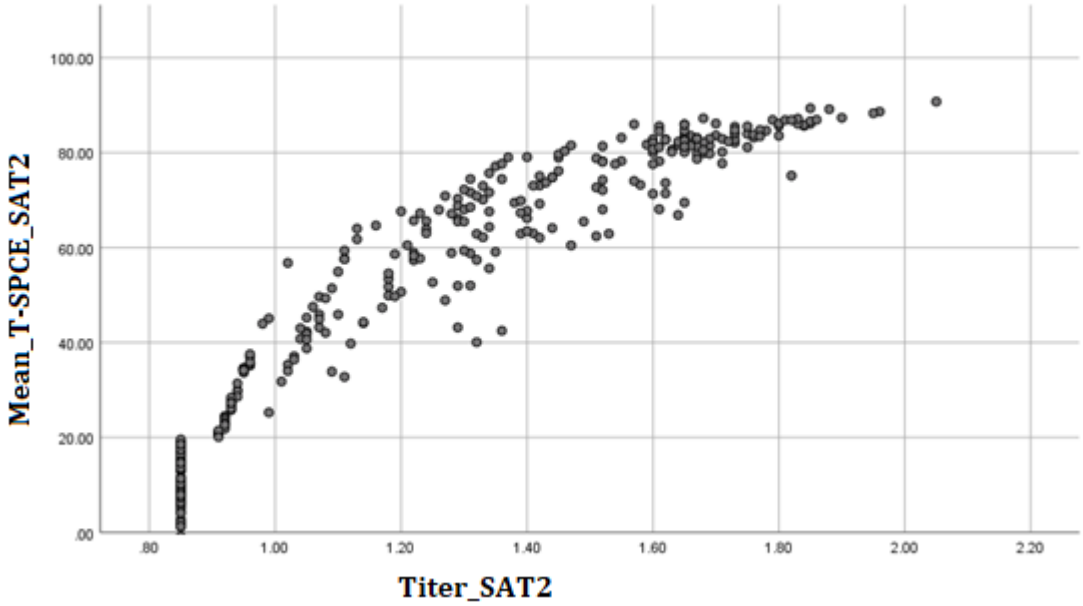

Fig 9: Relationship between mean SAT 2 percentage inhibitions for T-SPCE and titer

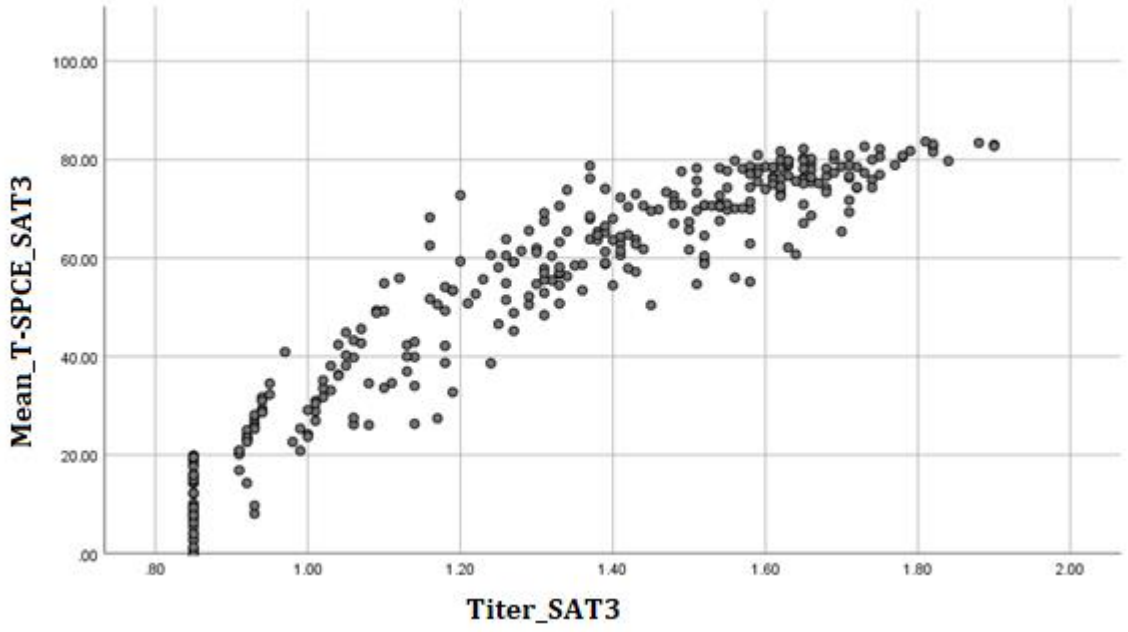

Fig 10: Relationship between mean SAT 3 percentage inhibitions for T-SPCE and titer

The agreement between T-SPCE mean percentage inhibition and titer was moderate for SAT1 (k=0.592) and SAT2 (k=0.432) while SAT 3 was fair (k=0.309), (p<0.00.1).

3.4 Diagnostic sensitivity and specificity

The titer results obtained from T-SPCE were used as gold standard to calculate the diagnostic sensitivity and diagnostic specificity for T-SPCE percentage inhibition. Titers ≥ 1.6 and percentage inhibitions ≥ 50 were considered positive (Mackay et al., 2001). The sensitivity of the T-SPCE was 100% (CL 95%) for all three SAT serotypes, but the specificity was substantially lower at 65.4% (CL 95%) for SAT1, 60.15% (CL 95%) for SAT2 and the lowest 49.8% (CL 95%) for SAT3.

3.5 SPCE diagnostic accuracy and determination of optimum percentage inhibition cut off

The area under the T-SPCE ROC curve for SAT1, SAT2 and SAT3 was 0.98, 0.979 and 0.953 respectively, indicating a test with a high diagnostic accuracy. The T-SPCE optimal percentage inhibition cut off as determined by the Youden index was 75.63 for SAT1; 77.6 for SAT2 and 71.6 for SAT3.

3.6 Mean antibody titers for goats in each of the five treatment groups

The mean FMD antibody titers for goats obtained from weekly samples for each of the five treatment groups made up of one unvaccinated group and four that were vaccinated with varying doses of the cattle dose (1/12,1/6,1/3 and a full dose) are displayed in Fig 10, 11 and 12 for SAT 1, SAT2 and SAT3 respectively. Goats in all treatments groups (except for 2 in the 1/6 vaccination group) were challenged with a pool of three field SAT1 viruses at day 41 of the study.

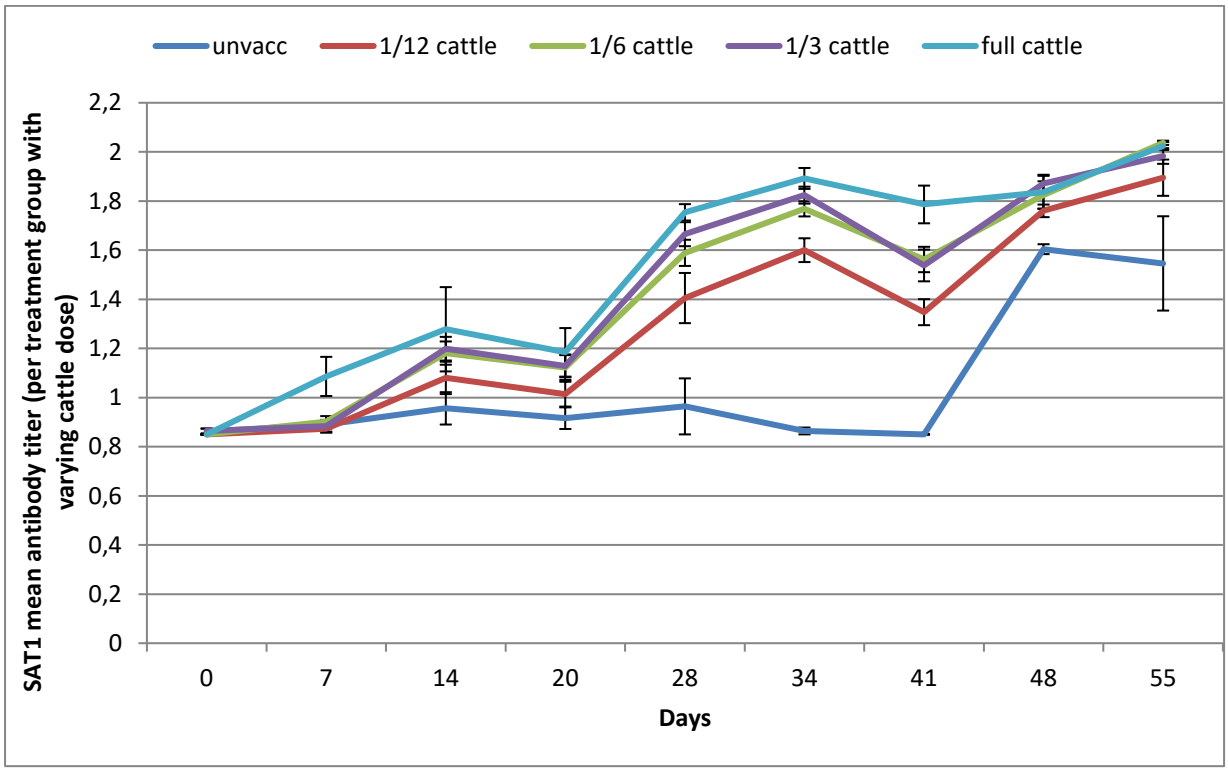

Fig 11: Descriptive plot mean SAT 1 titer per treatment group

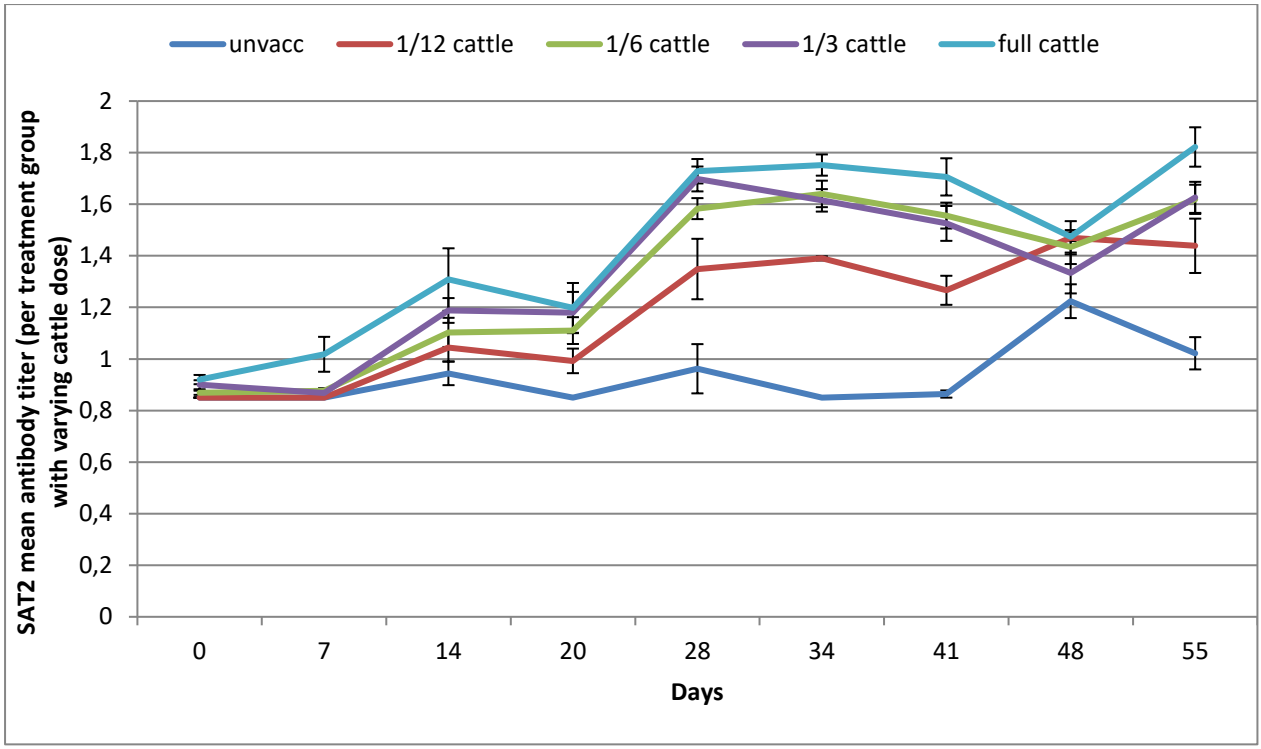

Fig 12: Descriptive plot mean SAT 2 titer per treatment group

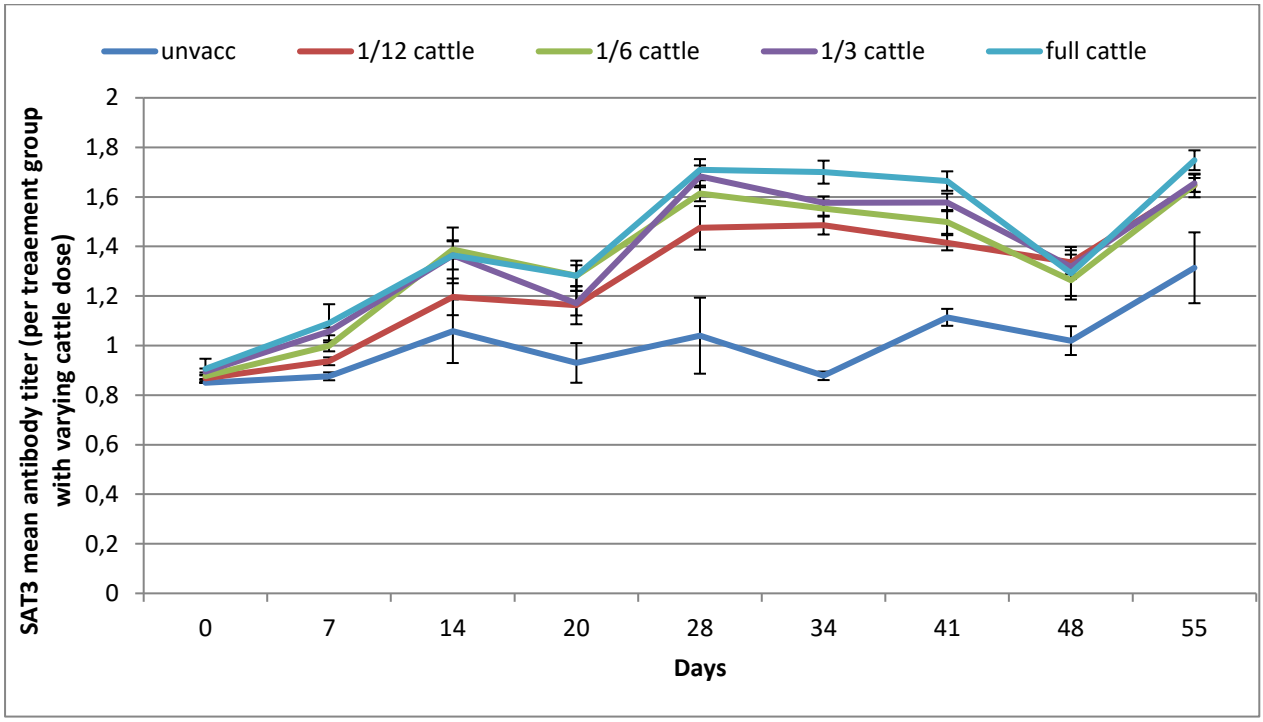

Fig 13: Descriptive plot mean SAT 3 titer per treatment group

4 Discussion

The aim of this study was to perform a preliminary validation of the SPCE diagnostic method when used for testing for anti-foot and mouth disease antibodies in goats. The samples were subjected to two separate testing runs (SS-SPCE and T-SPCE) for the presence of FMD antibodies by the SPCE method. Both test runs were conducted in duplicate and the second run was further titrated to determine antibody titer.

The following assay performance indicators were evaluated (a) repeatability, which was assessed by comparing the coefficient of variations of 'within run' duplicate percentage inhibitions and average percentage inhibitions for between run analysis. (b) correlations, agreements and relationships when both rest runs were compared to each other and also to the titers (Spearman's rho, Cohen's kappa and simple scatter plots), (c) Diagnostic sensitivity and specificity (SPCE PI's greater than or equal to 50% were considered positive) were compared to titer results (titers equal or greater than 1.6 were considered positive) as the reference standard and (d) determination of new assay thresholds (optimum cut offs).

Approximately 80% of all intra-assay SPCE tests across all SAT serotypes tested in both runs had COV values below 20%. This demonstrates that the SPCE method has a good 'within run' repeatability. The median COVs for both runs across all SAT serotypes ranged between 2.08% (SAT1) and 3.45% (SAT2). COV values below 5% indicate a very high repeatability (Fujimura et al., 2013). The high 'within run' repeatability of the SPCE method was further demonstrated by quartile 3 COV values from both test runs and across all serotypes being under 20%.

When corresponding COV's for SS-SPCE and T-SPCE were compared, approximately 60% of average percentage inhibitions had good repeatability. This was about 20% less when compared to intra-assay repeatability. Several other researches including Ran and co-workers have reported such a decrease where inter-assay variability is greater than intra-assay variability (Ran et al., 2019). In this study, both tests were performed in the same laboratory, but there was a time gap of about 5 months between the tests. The serum samples were stored at a temperature of -80°C which is suitable for long term storage hence this variation could be explained as being caused by other factors like human and equipment failure that influence test variability. The median COVs for both the SS-SPCE and T-SPCE across all SAT serotypes ranged between 9.58% (SAT 1) and 3.45% (SAT3). The median COV was substantially higher compared to the intra-analysis. It was however noted that SAT1 had the least median COV from both intra-and inter-assay comparisons and this could be because a SAT 1 virus was used

when the animals were challenged, resulting in the stimulation of a maximum production of SAT1 antibodies. SAT3 median COV was highest for the SS-SPCE and inter-assay comparison, and this could be as a result that a pentavalent vaccine which has two viruses for SAT1 and SAT2 and only one virus for SAT3 was used. Having only one SAT3 virus in the vaccine could have led to decreased SAT3 stimulation possibly resulting in increased variability.

The only mean COV that were below 20% were for the SS-SPCE (SAT2 and SAT3) and T-SPCE (SAT2). The mean for SS-SPCE (SAT1) was 44.1% and T-SPCE (SAT 1 and SAT3) were 30.31% and 23.06% respectively. The mean COV's for the inter-assay comparison were 164.2%, 32.4% and 30.97% for SAT1, SAT2 and SAT3 respectively. It was interesting to note that the median COV values were less than the means across all serotype assessments and for both runs indicating that they are positively skewed. The reasons for this observation could be the existence of a relatively small number of very high COV values and as such making the mean greater that the median. This demonstrates the need for the development of quality systems to evaluate SPCE results before they are released to clients. Errors including failure to load a reagent or sample into a well or failure of an equipment could lead to cases of a high COV. Errors may also arise during handling and processing of the optical density readings up to calculations of percentage inhibitions. This realisation further emphasised the need for SPCE tests to be run in duplicate and where possible correlating test results to the epidemiological picture. Data management systems must be developed and procedures that clearly guide the loading of samples on the multiwell testing plates and grouping of optical density readings must be developed. The processing of converting optical density readings to percentage inhibitions must also be designed to reduce possible sources of error.

The correlations between SS-SPCE and T-SPCE mean percentage inhibitions were found to be strong for SAT3 (Spearman's rho=0.791), and even much stronger for the other two serotypes. A very strong correlation across all serotypes (ranging from rho=0.818 to rho=0.967) was also observed when respective average percentage inhibitions were compared to titer. However, this strong correlation was not reflected on the scatter plots, where it was apparent that there was no clear linear relationship between respective percentage inhibitions from both runs (SS-SPCE and T-SPCE) and also between SS-SPCE and titer. The only linear relationship observed was between T-SPCE percentage inhibitions and titer. However, it should be noted that T-SPCE percentage inhibition evaluations and titrations were conducted simultaneously on the same plate and subjected to similar testing conditions.

Cohen's kappa results displayed no near perfect agreement across all serotypes. Cohens Kappa agreement was lowest when both SAT3 mean percentage inhibitions when compared to titer (k=0.285 for SS-SPCE and k=0.309 for T-SPCE) and was categorized as fair. Cohen's kappa agreement was moderate for both SAT2 mean percentage inhibitions when compared to titer (k=0.432 for both SS-SPCE and T-SPCE) and also for SAT1 when T-SPCE was compared with titer. However substantial Cohen's kappa agreement was noticed across all serotypes when SS-SPCS and T-SPCE mean percentage inhibitions were compared to each other, and also for SAT1 when SS-SPCE was compared with titer. The overall relatively low agreement of agreement when the test runs and methods were compared to each other further alludes to increased variability of the SPCE. The very low agreement noted with SAT3 serotype may further suggest the need for in-house assay standardization and optimization.

T- SPCE assay diagnostic sensitivity when compared to titer was found to be 100% for all serotypes. However diagnostic specificity was much reduced and lowest for SAT3 at 49.8%, followed by SAT2 at 60.15% and highest for SAT1 at 65.4%. This demonstrated that the SPCE has a very high sensitivity but a low specificity. This low specificity could be due many factors including to the complexity of the sera used in this study and possibilities cross reactions among different FMD serotypes as reported by other researchers including Li and co-workers (Li et al., 2012). In this study, the cross reactivity across different serotypes was not considered to be significantly contributing towards low specificity due to the fact that the same test was used as the reference standard but at multiple dilutions. A clearer indication of assay specificity may therefore require further research studies where animals are vaccinated with monovalent vaccines and challenged with a virus homologous to the vaccine or of a similar serotype. The optimum SPCE percentage inhibition cut off was found to be at 75.63% PI for SAT1, 76.6% PI for SAT2 and 71.6% PI for SAT3 when determined by the Youden index. The suggested positive cut off threshold is significantly higher when compared to the SPCE assay percentage inhibition cut off where values greater than or equal to 50% are considered positive (Mackay et al., 2001). Another earlier study (Kang et al., 2018) also identified that the SPCE PI cut off value may need to be revised upwards to improve assay specificity.

The mean titer per treatment group over time for vaccinated groups indicated a gradual increase in titer levels and plateauing off at about day 20 followed by a much higher increase after the administration of a booster vaccine and reaching a peak at about day 35. The titers then rose after challenge at day 41 with the SAT1 virus until study termination at day 55. There was however a momentarily decrease in titer levels on day 48 (one week after challenge) for SAT2

and SAT3 titers across most of the treatment groups except for SAT2 control and 1/12th group. This decrease may have arisen as a result of human, laboratory or reagent errors.

5 Conclusion

The application of molecular tests for the diagnosis of FMD is limited to early disease stages and samples must be handled in a very strict manner to avoid degradation and loss of virus viability. Application of molecular methods is further complicated when animals to be sampled are not close to a diagnostic laboratory. In certain species including goats, molecular diagnosis of FMD has limited use due to mild or in apparent clinical signs that makes it difficult to identify early disease stages. This makes serological methods critical for FMD in diagnosis in these species that tend to have subclinical infections.

Quality control measures must be strengthened for the serological methods to be useful for FMD control. In this study, the optimum threshold of the SPCE method was significantly higher than the one used currently. This emphasizes the need for regular evaluation of the performance of all FMD diagnostic methods so that they remain relevant and fit for purpose. Inter-laboratory comparisons and proficiency testing with other FMD laboratories must be encouraged. Regional OIE laboratories must improve the diagnostic capabilities of smaller FMD testing facilities.

The performance of serological tests is influenced by many factors and it is critical that the number of appropriately validated assays for FMD diagnosis be increased across all susceptible species. A wide range of FMD diagnostic tests will enable clients to choose their preferred tests and subsequently boosts confidence in the quality of results. Lastly, it would be beneficial for similar studies to be conducted for other FMD species especially the ones normally involved in SAT serotype outbreaks so that the repeatability and accuracy of the SPCE be compared across all FMD susceptible species. These study methods could also be incorporated into routine laboratory management procedures and applied to other serological methods for FMD diagnosis.

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7 Appendix

Appendix 1: Application for approval of title of dissertation Moses

UNIVERSITY OF PRETORIA

FACULTY OF VETERINARY SCIENCE

Application for approval of title of dissertation or thesis

PLEASE NOTE: This form must be completed in TYPING
Please attach a summary/abstract to this form

Name of candidate	Moses Gobiye
Student number	16393482
Degree	MSc (THP)
Course code	08251020
Department	PAS
Name of supervisor	Prof GT Fosgate
Name of co-supervisor(s)	Dr P Opperman

Protocol approved : Yes ☑ No **Date submitted/approved:** 18 July 2019
AEC approved : Yes No **AEC Number (Certificate):** N/A
Faculty Ethical Clearance : Yes No ☑ (pending)

Title of dissertation/thesis: Evaluation of the solid-phase competition ELISA for detecting Southern African Territories foot-and-mouth disease virus vaccination and infection in goats

SIGNED BY:	
CANDIDATE	DATE
SUPERVISOR	DATE 13 Sept 2019
CO-SUPERVISOR	DATE 13/9/2019
APPROVED BY HEAD OF DEPARTMENT	DATE
APPROVED BY CHAIR PGC/DEPUTY DEAN	DATE

Please submit this document to casalus.mnqomanu@up.ac.za or Student Administration

Appendix 2: REC171-19 Approval Certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee

Project Title	**Evaluation of the solid phase competition ELISA for detecting SAT1 foot-and-mouth disease virus infection in goats**
Project Number	**REC171-19**
Researcher / Principal Investigator	**Dr M Gobiye**

Dlissertation / Thesis submitted for	**Masters**

Supervisor	**Prof GT Fosgate**

APPROVED	Date: **2019-09-30**
CHAIRMAN: UP Research Ethics Committee	Signature:

Office of the Chairman: Research Ethics Committee
Room 2-24, Pathology Building, Onderstepoort
University of Pretoria, Private Bag X04
Onderstepoort 0110, South Africa
Tel +27 (0)12 529 8052
Email
www.up.ac.za

Appendix 3: REC171-19 Letter of support OVI 1

ARC • LNR

Enquiries / Navrae

Ref. no. / Verw. nr.

ARC-ONDERSTEPOORT VETERINARY INSTITUTE

LNR-ONDERSTEPOORT VEEARTSENYKUNDE-INSTITUUT

Private Bag / Privaatsak X05, Onderstepoort 0110, South Africa / Suid-Afrika
Tel: 012 529 9111 • Fax/Faks: 012 565 6573 (Int: +27 12)
E-mail / E-pos: ovi-info@arc.agric.za • Website / Webblad: www.arc.agric.za

22 July 2019

Prof N Duncan
Department of Paraclincal Sciences
Onderstepoort Veterinary Institute
University of Pretoria

Dear Prof Duncan,

RE: AVAILABILITY OF DIAGNOSTIC DATA TO BE USED IN THE RESEARCH PROJECT OF Dr M GOBIYE

This letter serves to confirm that the ARC-Onderstepoort Veterinary Research Institute (ARC-OVR) is aware and in support of Dr Gobiye's research project entitled 'Evaluation of the solid phase competition ELISA for detecting SAT1 foot and mouth disease virus infection in goats'. This project is to be undertaken in partial fulfilment of the Master of Science degree at the University of Pretoria, faculty of veterinary science. The project will be supervised Dr G Fosgate (UP) and Dr P Opperman (ARC/ UP).

Dr Gobiye will be given access to diagnostic and research data collected during a previous trial at the ARC-OVR where goats where vaccinated and challenged with foot and mouth disease. The use of data as part of this research project have been approved by the Department of Agriculture, Forestry and Fisheries under section 20 of the Act.

Please do not hesitate to contact me should any information relevant to this matter be required.

Yours Sincerely

Dr Livio Heath
Head of Laboratory: TAD Diagnostic Laboratory
RTM: Vaccine production
ARC-Onderstepoort Veterinary Research

Appendix 4: Section_20 Permit_for_V022-17

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Henry Goble • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@daff.gov.za
Reference: 12/11/1/8

Prof Geoffrey T. Fosgate
University of Pretoria
Faculty of Veterinary science
Department of animal Production
Onderstepoort
0110
Tel: 012 529 8257
Fax: 012 529 8315
E-Mail: Geoffery.fosgate@up.ac.za

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

Dear Professor Fosgate

Your application, 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. 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@daff.gov.za;
3. All potentially infectious material utilised or generated during or by the study is to be destroyed at completion of the study and only a registered waste disposal company may be used for the removal of waste generated during or by the study;

4. Records must be kept for five years for auditing purposes. A dispensation for the storage of samples is

5. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry date of the attached Section 20 permit. Please apply in writing to HerryG@daff.gov.za;

6. Subsequent to the completion of the study, all study animals must be euthanized and carcass material incinerated on site;

7. No animal to be used in the study may be purchased or originate from an area under any State Veterinary restriction;

8. No part of the study may begin until valid ethical approval has been obtained in writing from the relevant South African authority;

9. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82), the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act 1947 (Act no 36 of 47), the Medicines and Related Substances Control Act 1965 (Act 101 of 65) and the Genetically Modified Organisms Act, 1997 (Act No 15 of 1997);

Title of research/study: Epidemiology, vaccination and control of Foot-and-mouth disease (FMD) in goats at the greater KNP area South Africa
Researcher: Dr Dazhia Lazarus
Institution: University of Pretoria
Our ref Number: 12/11/1/1
Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2017 -10- 30

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

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Henry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: Henry@daff.gov.za
Reference: 12/11/1/1/8

Prof Geoffrey T. Fosgate
University of Pretoria
Faculty of Veterinary science
Department of animal Production
Onderstepoort
0110

Tel: 012 529 8257
Fax: 012 529 8315
E-Mail: Geoffery.fosgate@up.ac.za

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "Epidemiology, vaccination and control of Foot-and-mouth disease (FMD) in goats at the greater KNP area South Africa"

A dispensation is hereby granted on Point 3 of the Section 20 approval that was issued for the above mentioned study (attached):

i) Serum and clinical samples collected from the vaccinated and challenged animals may be stored in within the BSL 3 facility at TAD ARC-OVR under strict biosecurity. from;

ii) Stored samples from may not be outsourced or used for further research without prior written approval from Director: Animal Health.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2017 -10- 30