Small Ruminant Research 224 (2023) 106982

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Preliminary validation of a single-spot version of a solid-phase competition ELISA for the detection of southern African territories foot-and-mouth disease serotype exposure in goats

Moses Gobiye [a], Geoffrey T. Fosgate [b,*], Livio Heath [c], David D. Lazarus [b,d], LaToya Seoke [b,c], Pamela A. Opperman [b,e]

[a] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa
[b] Department of Production Animal Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa
[c] Transboundary Animal Diseases Programme of Onderstepoort Veterinary Research, Agricultural Research Council, Onderstepoort, South Africa
[d] Epidemiology and Training Programme of Onderstepoort Veterinary Research, Agricultural Research Council, Onderstepoort, South Africa
[e] Vaccines and Diagnostics Development Programme, Onderstepoort Veterinary Research, Agricultural Research Council, Onderstepoort, South Africa

ARTICLE INFO

Keywords:
FMD
Goats
SAT
Serology
SPCE
Vaccination
Validation

ABSTRACT

The objective of this study was to perform a preliminary validation of a solid-phase competition ELISA (SPCE) in goats exposed to foot-and-mouth disease virus (FMDV) southern African territories (SAT) serotypes through vaccination or experimental infection. Thirty-nine goats were vaccinated with a FMDV vaccine and 37 subsequently challenged with a SAT1 virus serotype. Blood was collected every 7 days until termination at 14 days post-challenge. Single-spot SAT1 virus serotype SPCE (ss-SPCE) was performed in duplicate at two time points and a half-titration version was performed after a variable time of long-term storage. Coefficient of variation (CV) was calculated and accuracy of the ss-SPCE was estimated relative to a half-titration SPCE log10 titer of 1.6 using mixed-effect logistic regression. Additionally, sensitivity and specificity were estimated based on serological results 14-days post-challenge and at study enrolment, respectively. Three hundred and forty-two serum samples were tested in duplicate on two non-consecutive days. The median (interquartile range (IQR)) CV for the ss-SPCE for SAT1 was 2.1% (0.5, 14.3%) and 2.5% (0.6, 12.8%) for the two testing days, respectively. Median (IQR) inter-assay (different day) CV was 10.6% (2.5, 42.5%). Specificity and sensitivity of the ss-SPCE relative to the log10 titer using a 70% percentage inhibition positive threshold were 83.4% (95% confidence interval, 77.7–87.9) and 95.8% (90.7–98.2), respectively. Specificity was estimated as 100% (92.6, 100) and sensitivity as 97.3% (87.4, 99.9) when only considering serum tested at the beginning and end of the study, respectively. The SAT1 ss-SPCE is repeatable and accurate for determining FMDV serological status in goats.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious and widely distributed disease affecting cloven-hoofed animals and camelids (Grubman and Baxt, 2004) that is caused by infection with FMD virus (FMDV), which belongs to the *Aphthovirus* genus within the Picornaviridae family (Kitching et al., 2005). The virus has a single-stranded positive-sense RNA genome of approximately 8500 nucleotides with a non-enveloped icosahedral capsid (Domingo et al., 2002). FMDV exists as seven immunologically distinct serotypes identified as O, A, C, Asia-1, southern African territories (SAT) 1,2 and 3 (Knowles and Samuel, 2003) with all except Asia-1 having multiple antigenically distinct topotypes (Knowles et al., 2016). Serotypes A and O are well established in most FMD endemic regions except southern Africa where outbreaks due to these serotypes are less common (Rweyemamu et al., 2008; Sirdar et al., 2021; Chimera et al., 2022). Serotype O is responsible for the majority of outbreaks globally (Ranaweera et al., 2019) and is becoming increasingly important in southern Africa (Banda et al., 2022). Asia-1 predominates in Asia, and there have been no recent reports of serotype C (Sangula et al., 2011; Brito et al., 2017). The SAT serotype viruses predominate in southern Africa and constitute a distinct lineage separate from the A, O, C and Asia-1 serotypes (Vosloo et al., 2009). African buffalo (*Syncerus caffer*) are a natural host for the SAT virus serotypes (Thomson et al., 2003; Hughes et al., 2017; Blignaut et al., 2020) and

* Corresponding author.
E-mail address: geoffrey.fosgate@up.ac.za (G.T. Fosgate).

https://doi.org/10.1016/j.smallrumres.2023.106982
Received 27 September 2022; Received in revised form 22 February 2023; Accepted 19 April 2023
Available online 20 April 2023
0921-4488/© 2023 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

FMDV serotypes SAT1 and SAT2 are distributed throughout Africa, while SAT3 is limited to southern and a small area in eastern Africa (Bastos et al., 2003).

FMD affects all cloven-hoofed livestock but there are few reports of clinical disease in small ruminants (Gibson and Donaldson, 1986; Barnett and Cox, 1999; Hughes et al., 2002; Elnekave et al., 2016). Goats have been experimentally infected with FMDV (McVicar and Sutmoller, 1968; Anderson et al., 1976; Lazarus et al., 2020; Muthukrishnan et al., 2020) but there are limited data concerning natural infections. Goats have the potential to become FMDV carriers (McVicar and Sutmoller, 1968, 1972; Anderson et al., 1976) and this species has been incriminated in the introduction of FMDV into previously disease-free countries through illegal trade (Kitching and Hughes, 2002). In South Africa, small stock including goats are sometimes moved from FMD control areas without first obtaining official movement permits (Lazarus et al., 2021).

The typical clinical signs of FMD do not vary by host species or serotype and the major clinical features include fever, lameness, and the appearance of vesicles and ulcerative lesions in the mouth, feet, teats and mammary glands (Arzt et al., 2011). Pain and discomfort from the vesicles and ulcerative lesions can lead to depression, anorexia, excessive salivation, lameness, and reluctance to move or stand (Kitching and Hughes, 2002). Host species, breed, pre-existing immunity, FMDV serotype and dose all influence disease severity (Singh et al., 2019). FMD clinical signs range from subclinical to severe and laboratory confirmation is required due to the possibility of other ulcerative diseases (Alexandersen et al., 2003) and for serotype determination.

Viral isolation and methods that detect viral antigens, nucleic acids, and antibodies are core techniques for the laboratory diagnosis of FMD (Longjam et al., 2011). Routine serological methods performed in South Africa include the liquid-phase blocking ELISA (LPBE), solid-phase competition ELISA (SPCE), non-structural protein ELISA, and the virus neutralization test (VNT). These tests detect anti-FMDV antibodies produced by the host against structural or non-structural FMDV proteins. A SPCE measures the competition for FMDV binding sites between serotype-specific rabbit or guinea pig anti-FMDV antiserum and antibodies present in the test serum (Mackay et al., 2001). Conventional SPCEs have been developed to detect multiple FMDV serotype exposure for a number of species including cattle, sheep, and pigs (Mackay et al., 2001; Paiba et al., 2004; Li et al., 2012; Kang et al., 2018; Wong et al., 2020). Goats have also been included in a multiple species evaluation of a recombinant capsid polyprotein SPCE for the detection of serotype O FMDV infection (Biswal et al., 2015) but the authors are not aware of a formal SPCE validation for SAT serotypes in this species.

The objective of the current study was to perform a preliminary validation of a conventional single-spot SPCE for use in goats exposed to FMDV SAT serotypes through vaccination or experimental infection.

2. Materials and methods

2.1. Study design

A group of 40, 6–12 month old indigenous South African goats of both sexes were obtained from farms within the FMD free-zone of South Africa before the 2019 FMD SAT2 outbreak and subsequent suspension of the FMD free-zone in the country. Goats were obtained to evaluate the efficacy of a pentavalent FMD vaccine to protect against heterologous challenge with SAT1 FMDV (Lazarus et al., 2020) and were confirmed FMD-free prior to enrolment using liquid-phase blocking ELISA. Briefly, goats were randomly allocated into five treatment groups based on decreasing vaccine dosages including an unvaccinated placebo control group. The experimental vaccine was an inactivated pentavalent FMD vaccine containing serotypes SAT1 ($n = 2$ viruses), SAT2 ($n = 2$) and SAT3 ($n = 1$). Vaccinations were administered on day 0 and repeated on day 20 with the FMDV experimental challenge occurring on day 41. All except two goats were challenged by intradermolingual inoculation with a $10^{4.57}$ 50% tissue culture infective dose ($TCID_{50}$) of FMDV SAT1. Natural transmission occurred in the two unchallenged goats (Lazarus et al., 2019) and FMDV infection was confirmed through the development of lesions and shedding of the virus detected by reverse transcriptase PCR (Lazarus et al., 2020). All goats also had evidence of seroconversion at the end of the study based on non-structural protein ELISA (Lazarus et al., 2020). Blood samples were collected from the jugular vein into plain evacuated tubes (Vacutainer®, BD Becton, Dickinson and Company, USA) on day 0 and every subsequent 7 days (with the exception of day 20, which was a 6 day interval) until study termination at 14 days post-challenge (day 55). Whole blood was allowed to clot at room temperature and harvested sera were stored at − 20 °C until testing at the completion of the study and then relocated to − 80 °C for long-term storage. A second serological testing was performed after a variable number of days of long-term storage (median of 84 days; range, 78–282). More details concerning the animal methods have been presented elsewhere (Lazarus et al., 2020). Additionally, 329 banked serum samples collected from goats for a peste des petits ruminant surveillance project were included in the study. These goats were sampled from the Northern Cape Province (FMD-free zone) prior to the suspension of the free zone in 2019 due to the SAT2 FMD outbreak in Limpopo Province.

The study protocol was approved by the Research Ethics Committee of the Faculty of Veterinary Science at the University of Pretoria (REC 171–19) and the Animal Ethics Committee of the Onderstepoort Veterinary Institutes (AEC 6.17). Permission to conduct this study was also obtained from the Department of Agriculture, Land Reform and Rural Development (Application Number 12/11/1/1).

2.2. Solid-phase competition ELISA (SPCE)

2.2.1. Single-spot SPCE

Single-spot SPCE (ss-SPCE) for FMDV SAT1–3 were performed on all experimental sera specimens following standard procedures (Mackay et al., 2001; Paiba et al., 2004). Field specimens from the Northern Cape were only tested on the SAT1 ss-SPCE on a single day. The first testing of specimens from experimental animals was batched and performed the day following study completion. The ss-SPCE was performed on an initial 1:10 serum dilution thus with a final working dilution of 1:20. FMDV serotype-specific rabbit anti-serum was diluted at a predetermined optimum concentration in carbonate/bicarbonate coating buffer (pH 9.6) and then 100ul were added to each well of a 96-well NUNC Maxisorp™ micro-titer plate. Plates were incubated overnight at room temperature followed by a 3-cycle wash with washing buffer using an automatic plate washer. Coated plates were stored at − 20ºC until required. After every incubation step, plates were washed four times with phosphate buffered saline containing Tween®20 (PBST) wash solution on a micro-titer plate washer except after the addition of the substrate solution. FMDV antigen homologous to the coated rabbit anti-serum was diluted then added to the plates and incubated at 37ºC for one hour. After capture of the antigen, the test serum, negative and positive control sera were added to the micro-titer plates together with the specific guinea pig antiserum and incubated for one hour at 37ºC. Following incubation, the micro-titer plates were washed, and anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase was added. The plates were incubated for one hour at 37ºC followed by washing. FMDV antigen, guinea-pig antiserum and conjugate were diluted to predetermined working concentrations in PBST containing 5% non-fat skimmed milk powder and phenol red indicator. Tetramethylbenzidine (TMB) substrate solution was added to all wells and the plates were incubated at room temperature for 10 min to allow for color development. The reaction was stopped by the addition of sulphuric acid and the optical densities (OD) were read at 450 nm in a spectrophotometer.

Tests were performed in duplicate and percentage inhibition (PI) was calculated for each well as: PI = 100 – ((mean OD test or control serum / median OD competition control) x 100). Samples with a percentage

inhibition ≥ 50% were classified as positive (Paiba et al., 2004). Assay results were accepted if the OD value of the antigen control was in the range 0.8 – 1.4, the PI of the negative control was < 50%, and the PI for the strong positive control was > 80%.

2.2.2. Half-titration SPCE

Half-titration SPCE (t-SPCE) for all three SAT serotypes were performed in the same manner as the ss-SPCE test but using 1:5, 1:10, 1:20, and 1:40 initial serum dilutions. The initial 1:10 serum dilution was used as a replicate of the original single-spot testing. Samples were tested in duplicate and testing was performed after 78–282 days of long-term storage. SPCE antibody titers were calculated using the Kärber method (Kärber, 1931; Ramakrishnan, 2016) and samples with antiserum titers equal to or greater than 1.6 log10 were considered positive based on laboratory internal validation data.

2.3. Statistical analysis

Intra-assay (within plate) repeatability of raw optical density (OD) values and calculated PI was assessed by calculating the coefficient of variation (CV) as the standard deviation divided by the mean of replicates and expressed as a percentage. Inter-assay (between different days) repeatability was estimated by first determining the mean PI from each assay run and then calculating the CV of these two values. Data were descriptively presented as scatter and box plots using the ggplot2 package (Wickham, 2009) within R (R Development Core Team, 2017). Spearman's rho was calculated as a measure of correlation and kappa was calculated as an estimate of agreement between the ss-SPCE at the 50% PI positive threshold and the calculated SPCE titer at the log10 1.6 threshold. Repeatability, correlation, and agreement assessments were performed using data from all three SAT FMDV serotypes in experimental goats because all were included in the administered vaccine. The accuracy of the ss-SPCE was estimated relative to a SPCE log10 titer of 1.6 and descriptively presented using a 2-line receiver-operating characteristic curve created in ggplot2. The assessment of accuracy was restricted to SAT1 SPCE results because this was the experimental challenge strain and also restricted to the second testing day in experimental goats because this was when the t-SPCE was performed. The optimal positive ss-SPCE threshold was determined by evaluating the Youden index (sensitivity + specificity – 1) with an emphasis on maximizing sensitivity for the expected use of the ss-SPCE as a screening test. Mixed-effect logistic regression models were fit as described previously for a different analytical situation (Annandale et al., 2021) to estimate sensitivity and specificity while accounting for the repeated measures study design. Intercept-only mixed-effect models were fit that included a random effect term for animal and restricted to SAT1 titer positive (≥1.6 log10) and titer negative results to estimate sensitivity and specificity, respectively. Sensitivity, specificity, and 95% confidence intervals for these relative accuracy estimates were calculated by converting exponentiated regression coefficients into probability values. Diagnostic accuracy was also estimated relative to the known disease status for the goats in a subset of available specimens. Specificity was estimated as the simple proportion of animals that tested negative on the ss-SPCE at the start of the study prior to vaccination or challenge (n = 39 due to the loss of one goat) and as the proportion of goats sampled from the FMD free zone that tested negative. Sensitivity was calculated as the simple proportion of animals that were seropositive 14 days after experimental challenge with the SAT1 FMDV (n = 37 due to the loss of three goats). Kappa was calculated to estimate the agreement between t-SPCE titer positive and evaluated ss-SPCE PI positive thresholds. All statistical analyses were performed using commercially available software (IBM SPSS Statistics Version 27, International Business Machines Corp.) unless stated otherwise. Significance was assessed at $P < 0.05$.

3. Results

3.1. Repeatability

Forty goats were obtained but one goat died during the acclimation period and two other goats died prior to FMDV experimental infection. Deaths were attributed to pre-existing conditions unrelated to vaccination or other study protocols. Three hundred and forty-two serum samples from 39 goats were tested in duplicate for all three SAT serotypes on the ss-SPCE over two different days. On the first day of testing 200 (58%), 188 (55%), and 211 (62%) were ss-SPCE positive (PI > 50%) for SAT1–3, respectively. On the second day, 204 (60%), 188 (55%), and 210 (61%) were ss-SPCE positive for SAT1–3, respectively. Intra-assay (within plate) repeatability on the optical density scale was generally good with a small number of extreme CV (Supplemental Fig. 1). The median (interquartile range (IQR)) CV for the PI on the ss-SPCE calculated on the first testing were 2.1% (0.5, 14.3%), 2.7% (0.9, 9.8%), and 3.2% (1.2, 7.6%) for serotypes SAT1–3, respectively. The corresponding median (IQR) CV for the PI on the testing performed after variable storage times were 2.5% (0.6, 12.8%), 3.5% (1.0, 11.4%), and 3.3% (1.2, 11.3%) for serotypes SAT1–3. Median (IQR) inter-assay (different day) CV for the PI were 10.6% (2.5, 42.5), 12.2% (3.6, 34.0%), and 11.6% (4.7, 28.6%) for serotypes SAT1–3 (Fig. 1) and appeared to vary based on the magnitude of the serological response (Supplemental Fig. 2).

3.2. Correlation and agreement

There was an apparent strong linear relationship between the PI calculated from the duplicate samples on the first testing with Spearman's rho of 0.985 ($P < 0.001$), 0.978 ($P < 0.001$), and 0.975 ($P < 0.001$) for SAT1–3, respectively (Fig. 2). The estimated correlations on the second testing after a variable storage time were 0.979 ($P < 0.001$), 0.982 ($P < 0.001$), and 0.967 ($P < 0.001$) for SAT1–3 (Fig. 3). Correlation between mean PI on the two different testing days were 0.892 ($P < 0.001$), 0.866 ($P < 0.001$), and 0.791 ($P < 0.001$) for SAT1–3 (Fig. 4). The correlations between the ss-SPCE PI and the SPCE antibody titer calculated for the same testing after the variable storage time were 0.960 ($P < 0.001$), 0.970 ($P < 0.001$), and 0.947 ($P < 0.001$) for SAT1–3 (Fig. 5). Kappa (95% CI) as the measure of agreement between the ss-SPCE PI at the 50% positive threshold and the t-SPCE at the 1.6 log10 positive threshold was 0.592 (0.516, 0.668), 0.432 (0.356, 0.508), and 0.309 (0.242, 0.376) for SAT1–3.

3.3. Diagnostic accuracy

The 2-line ROC curve (Fig. 6) and calculation of the Youden index suggested that a PI positive threshold of 70% would be optimal for use of the ss-SPCE for screening goats for FMDV exposure (vaccination or infection). Kappa (95% CI) as an estimate of agreement between the ss-SPCE and the t-SPCE antibody titer was estimated as 0.775 (0.708, 0.842) at the 70% PI positive threshold. The sensitivity (95% CI) and specificity (95% CI) of the ss-SPCE at the 70% PI positive threshold were 95.8 (90.7, 98.2) and 83.4 (77.7, 87.9), respectively (Table 1). Estimation of sensitivity and specificity using knowledge of the true infection status of the goats suggested higher estimates that did not vary based on the two evaluated positive thresholds for the ss-SPCE.

4. Discussion

Goats are an important livestock species in South Africa (Braker et al., 2002) and are often raised in close proximity to FMDV-infected wildlife reservoirs (Lazarus et al., 2021). Goats are susceptible to FMDV infection but frequently show minimal or no clinical signs (Kitching and Hughes, 2002; Lazarus et al., 2019; Lazarus et al., 2020) or behavioral changes (Wolf et al., 2020) but are still capable of

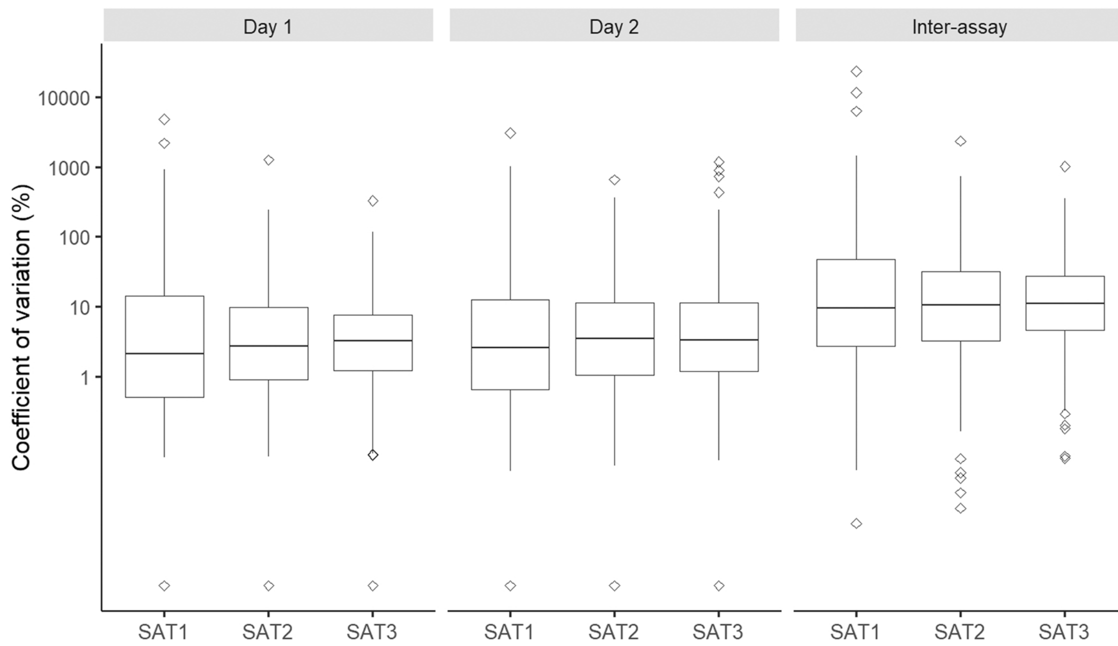

Fig. 1. Intra-assay (within plate) and inter-assay (between days) coefficient of variation on the percentage inhibition calculated for a single-spot version of a foot-and-mouth disease (FMD) solid-phase competition ELISA (SPCE) in 342 serum samples collected from 39 goats vaccinated against southern African territories serotypes 1–3 (SAT1–3) FMDV and subsequently experimentally challenged with a pool of field SAT1 viruses. Data presented with a log10 y-axis due to the small number of extreme values.

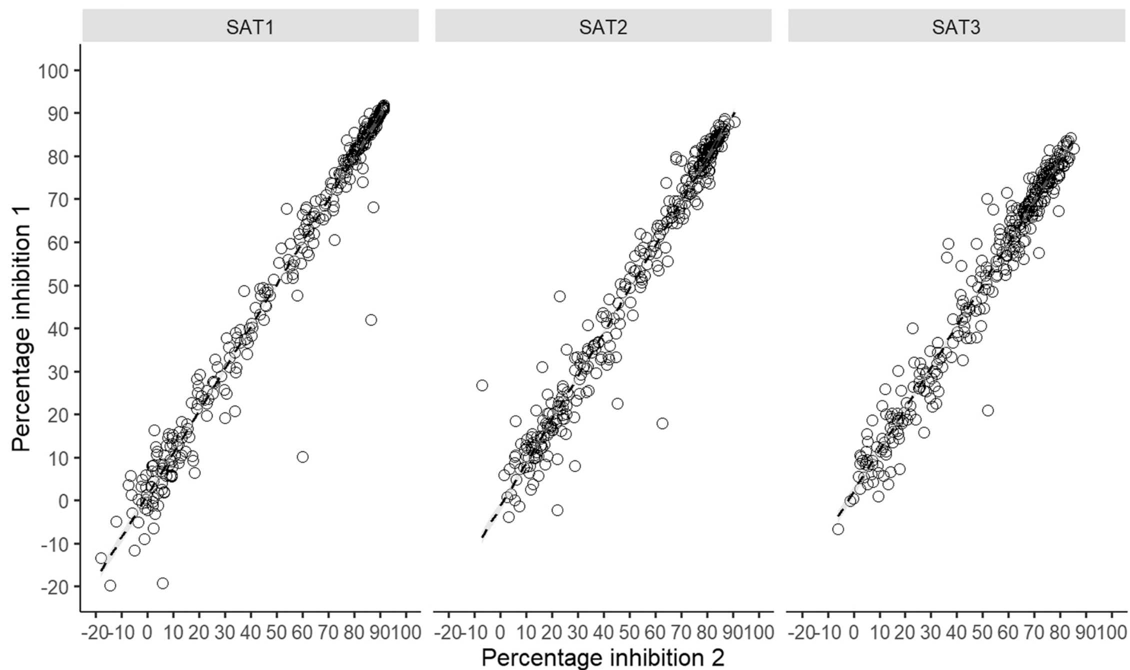

Fig. 2. Correlation between percentage inhibition calculated from replicate samples on the same plate on the first testing for a single-spot solid-phase competition ELISA (ss-SPCE) in 342 serum samples collected from 39 goats vaccinated against southern African territories serotypes 1–3 (SAT1–3) FMDV and subsequently experimentally challenged with field SAT1 viruses.

transmitting infection to in-contact animals (Lazarus et al., 2019). Subclinical infection and transmission are a concern because FMD surveillance in endemic regions typically target clinically affected livestock. South Africa, for example, only performs routine serological surveillance in cattle. Cattle are also the most common target species for FMD surveillance and assay validation is less common for other susceptible species. The objective of the current study was to perform a preliminary validation of a conventional SPCE for use in goats as a screening tool for exposure to SAT serotypes of FMDV. Published data concerning SAT serotypes are limited especially related to exposure of small ruminants. These data are required prior to the implementation of serological screening programs of goats designed at reducing FMD outbreaks in Africa.

Serological screening programs should be designed based on the use of validated assays and analytical validation is one of the first steps of the World Organisation for Animal Health prescribed validation

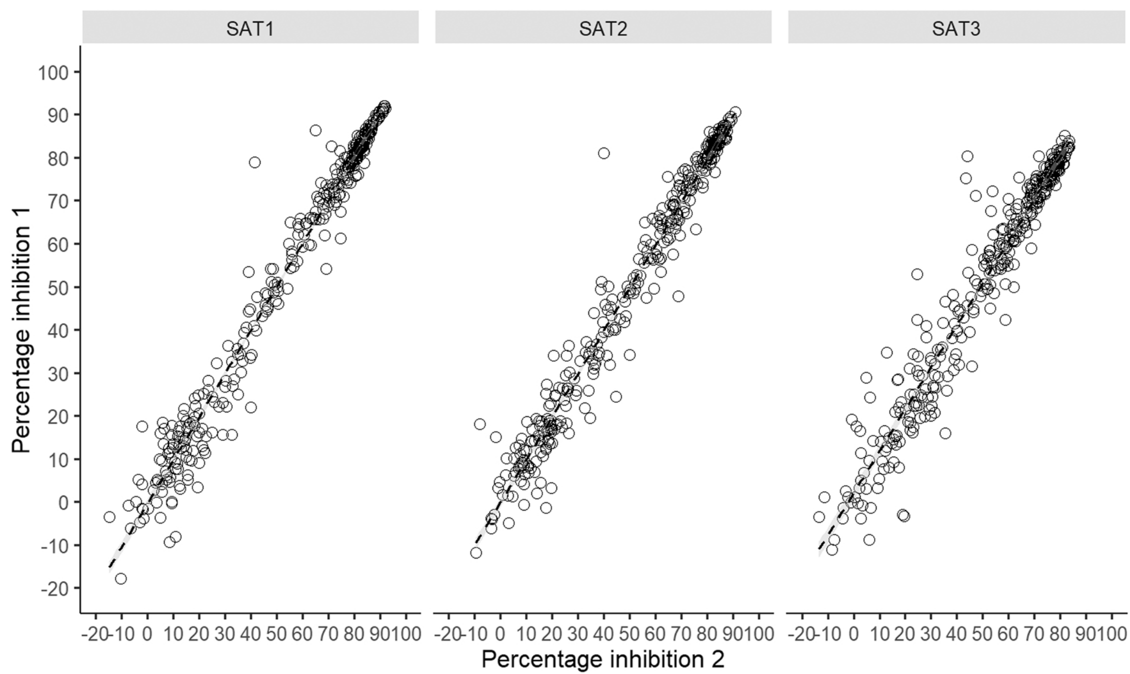

Fig. 3. Correlation between percentage inhibition calculated from replicate samples on the same plate after a 78–282 day storage time for a single-spot solid-phase competition ELISA (ss-SPCE) in 342 serum samples collected from 39 goats vaccinated against southern African territories serotypes 1–3 (SAT1–3) FMDV and subsequently experimentally challenged with field SAT1 viruses.

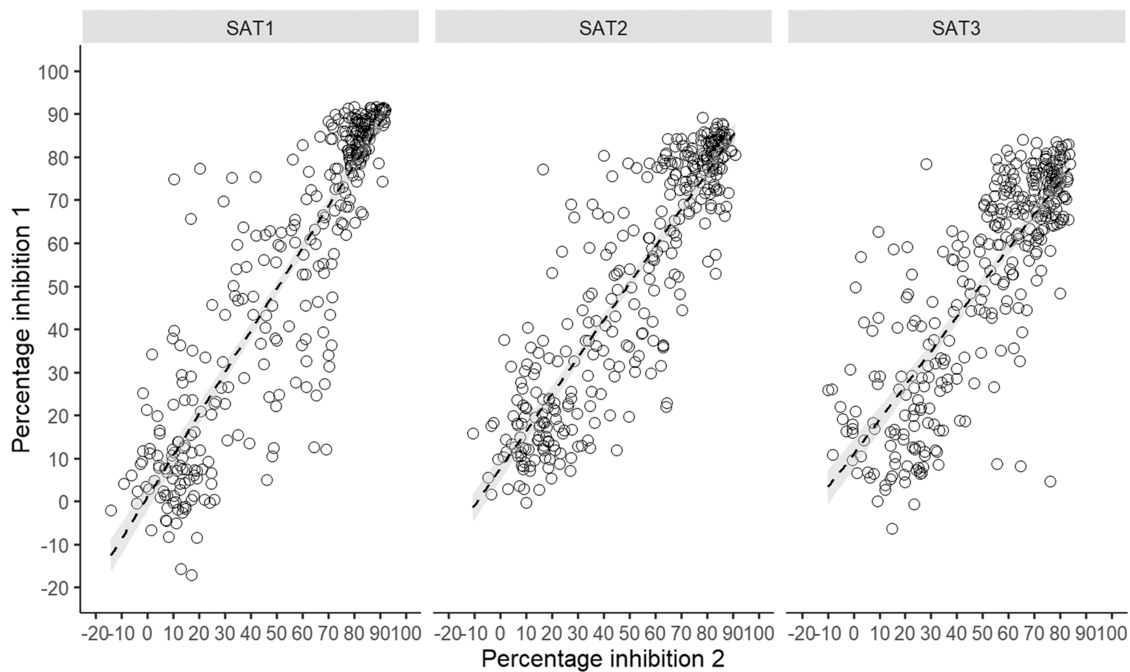

Fig. 4. Correlation between mean percentage inhibition calculated from two independent assay runs (after a variable amount of storage time) for a single-spot solid-phase competition ELISA (ss-SPCE) in 342 serum samples collected from 39 goats vaccinated against southern African territories serotypes 1–3 (SAT1–3) FMDV and subsequently experimentally challenged with field SAT1 viruses.

pathway (Jacobson, 1998; Gardner et al., 2021). Analytical sensitivity including repeatability and limit of detection are two important aspects of analytical validation. Serial dilution experiments were not performed to assess the limit of detection since SPCE has been validation for use in other species. However, the intra-assay (within plate) repeatability of the evaluated ss-SPCE was very good within the evaluated goat samples. This repeatability appeared descriptively more variable for SAT1 testing as demonstrated by the wider interquartile ranges as can be observed in Fig. 1. Fig. 2 and 3 also show a lower negative range of PI values, which might be the source of the increased variability. This might be an indication that the competition control for the SAT1 version of the SPCE might not represent the true maximum reaction and therefore requires reassessment.

All SAT virus serotype versions of the ss-SPCE had a few very extreme CV values when assessing the intra-assay (within plate) repeatability that were more common with virus serotype SAT1. These extreme values

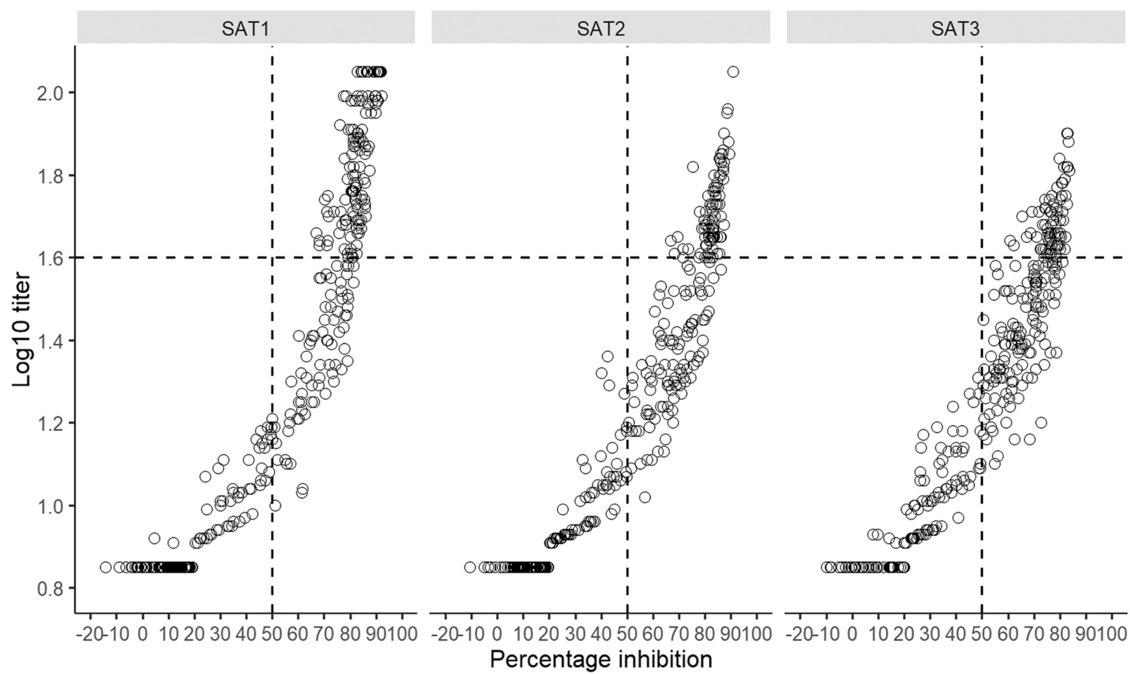

Fig. 5. Correlation between single-spot percentage inhibition and log10 titer for a sold-phase competition ELISA (SPCE) performed on the same day after a 78–282 day storage time in 342 serum samples collected from 39 goats vaccinated against southern African territories serotypes 1–3 (SAT1–3) FMDV and subsequently experimentally challenged with field SAT1 viruses. Dashed lines represent the usual thresholds for classifying positive reactions.

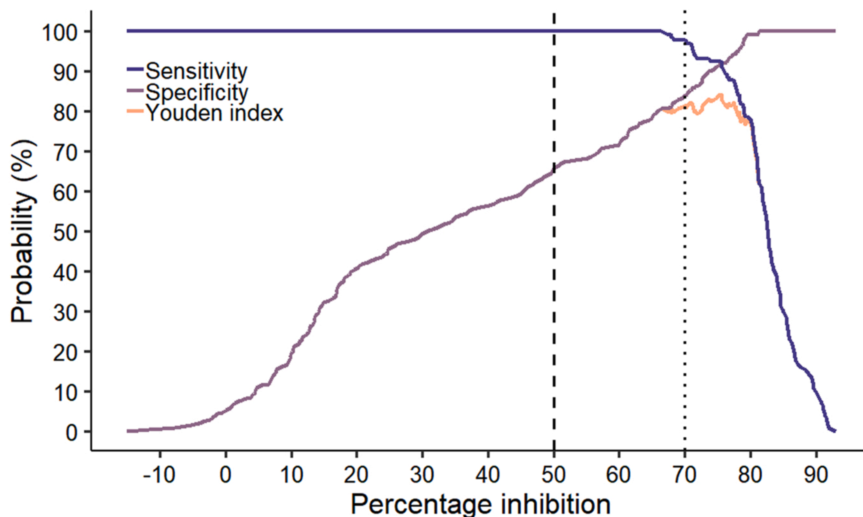

Fig. 6. Two-line receiver-operating characteristic plot for the accuracy of a foot-and-mouth disease (FMD) virus serotype southern African territories 1 (SAT1) single-spot solid-phase competition ELISA (ss-SPCE) relative to a log10 titer of 1.6 for diagnosis of SAT1 exposure in 39 vaccinated goats subsequently experimentally challenged. Dashed line indicates the typical 50% percentage inhibition positive threshold and the dotted line indicates the 70% percentage inhibition positive threshold with improved overall accuracy based on the Youden index (sensitivity + specificity – 1).

were associated with low PI values (Supplemental Fig. 2) as might be expected due to small absolute changes in the lower test result ranges having a larger influence on the proportional variability. The apparent low repeatability within this range of assay results would not affect the outcome classification as negative and thus not negatively influence the use of the assay for serological screening. The intra-assay (within plate) repeatability close to the typical positive threshold of 50% was very good for all three serotypes in general. The good intra-assay repeatability was further evidenced by the strong linear relationship and near perfect correlation when assessed for both testing time points (Fig. 2 and 3). The variable time of long-term storage also had very little apparent impact on these correlations.

The variable time of long-term storage might have had an impact on the measures of inter-assay (between day) repeatability as the CV were descriptively higher than for the intra-assay repeatability (Fig. 1). There was a strong correlation between the PI estimated on the different testing days but the correlations were weaker for all SAT virus serotypes and there was more variability around the predicted regression lines (Fig. 4). Poorer inter-assay (between day) repeatability compared to intra-assay (within plate) repeatability is not unexpected and it has been reported previously (Ran et al., 2019). Despite the increased variability, the median CV were still within acceptable limits as evidenced by Fig. 4. A CV of less than 20% is considered acceptable whereas a value of 30% or more in a majority of tests would be a reason for concern (Jacobson, 1998). A higher proportion of SAT1 tests had CV greater than 30% relative to the other evaluated serotypes. For example, approximately 30% of the SAT1 results had inter-assay CV greater than 30%. It is possible that long-term storage and antibody decay are responsible for some of the measured variability. However, this is unlikely to be the major source of the variability due to the scatter plots suggesting that the

Table 1
Accuracy of a foot-and-mouth disease (FMD) virus serotype southern African territories 1 (SAT1) single-spot solid-phase competition ELISA (ss-SPCE) for diagnosis of SAT1 virus exposure in goats. Accuracy estimated for the ss-SPCE relative to a log10 titer of 1.6 and 39 goats experimentally challenged with a South African field SAT1 viral pool.

Reference standard	Positive threshold (PI)	Sensitivity-% (95% CI)	Specificity-% (95% CI)
1.6 log10 titer	50	100 (NA)	65.4 (58.7, 71.6)
	70	95.8 (90.7, 98.2)	83.4 (77.7, 87.9)
Experimental challenge	50	97.3 (87.4, 99.9)	100 (92.6, 100)
	70	97.3 (87.4, 99.9)	100 (92.6, 100)
FMD-free zone goats	50	NA	100 (99.1, 100)
	70	NA	100 (99.1, 100)

PI = percentage inhibition. CI = confidence interval. NA = Not able to calculate

PI from the second testing were frequently higher than the first. Other sources of variability to consider would be due to the operator, which was different, and changes in test reagents indicating quality control issues. Furthermore, extreme differences might be due to mislabelling of samples or errors in loading plates breaking the true relationship between sample ID and results. Testing was performed manually without the assistance of robotics and automated data entry. Errors in data entry and plate loading are a potentially important source of the reduced inter-assay (between day) variability and appear more important than variable storage times. The sources of inter-assay variability require further investigation to identify the relative influence of laboratory and assay-specific factors. The reproducibility and robustness of the ss-SPCE assay for FMDV serological diagnosis in goats depends upon the contribution of each potential source of variability.

There was a very strong curvilinear correlation between the PI for the ss-SPCE and the log10 titer calculated from the same test day for all SAT serotypes (Fig. 5). The strong correlation suggests that the ss-SPCE could be used as an accurate predictor of the FMDV antibody titer, which would be more rapid and economical. However, kappa estimated based on the typical positive thresholds descriptively varied by serotype and only suggested fair (SAT3) to moderate (SAT1&2) agreement (Landis and Koch, 1977) between the ss-SPCE PI and the calculated titer. Fig. 5 also demonstrates the large proportion of results that are classified in the PI positive (PI $\geq$ 50%) but titer negative (titer $<$ 1.6) quadrant. This suggests that the ss-SPCE has potential as an accurate screening test but that the PI $\geq$ 50% positive threshold is too low and likely should be increased for goats.

The 2-line ROC analysis suggested that ss-SPCE might be accurate relative to a 1.6 log10 titer but that the positive threshold of 50% PI is not optimal (Fig. 6), which is consistent with the interpretation of the scatter plot findings. The Youden index was still in the ascending phase at the 50% PI positive threshold and started to plateau at approximately 70% PI before declining again after 80% PI. An approximate 70% PI positive threshold would be preferable due to the approximate maximum of the Youden index with the highest estimated sensitivity. The evaluation of the ss-SPCE at the 70% PI positive threshold had good sensitivity (96%) and adequate specificity (83%) as well as having substantial agreement (kappa = 0.78) with classification of SPCE log10 titers $\geq$ 1.6 suggesting a possible role as a screening test for SAT1 virus serotype exposure (vaccination or infection) in goats.

The diagnostic accuracy of the currently evaluated SPCE in goats is lower than most reported estimates of sensitivity ($\geq$ 96%) and specificity ($\geq$ 99%) for multiple serotype exposures in other species (Wong et al., 2020). Literature concerning FMD diagnosis specifically for goats is scarce and often the FMD epidemiology in goats is described in conjunction with sheep (Kitching and Hughes, 2002). Sheep have received more attention in the peer-review literature presumably due to their relative importance as commercial livestock in developed countries. The SPCE has been reported to have a sensitivity and specificity of 100% and 99%, respectively when evaluated against sheep experimentally exposed to a serotype O virus (Paiba et al., 2004). Another study evaluated a SPCE for the diagnosis of exposure to serotypes A, C, Asia-1, and SAT1–3 in sheep and the specificities were reported to be $>$ 99% for all evaluated serotypes when all species were combined (Li et al., 2012). However, sensitivities were not reported nor were sheep-specific estimates of specificity. Goat-specific estimates of diagnostic accuracy of a conventional SPCE could not be identified in the available English-language literature.

The 70% PI positive threshold of the ss-SPCE appears to be an effective screening test; however, this high of a positive threshold might no longer be within the linear range of the assay. Scatter plots between the log10 titer and the PI described a curvilinear relationship in which the slope of a tangential line progressively increased with the PI once it surpassed 50%. This relationship implies that smaller changes in the PI have larger effects on the titer at higher PI values. Imperfect precision of the test would therefore tend to yield more changes in the predicted titer values possibly causing changes in diagnostic decisions. It might therefore be worthwhile to investigate a higher initial serum dilution of the assay for use in goats rather than simply using the cattle dilution and increasing the PI threshold. Further research is required to identify an optimal starting serum dilution to employ in this assay.

The estimation of diagnostic accuracy of the ss-SPCE relative to a predicted log10 antibody titer calculated from multiple dilutions of the same assay is not an optimal approach for the estimation of diagnostic accuracy. Diagnostic accuracy should be estimated relative to an appropriate reference (gold) standard (Gardner et al., 2019) but perfect reference standards are often unavailable. In addition to estimation relative to the log10 antibody titer, sensitivity and specificity were also estimated relative to the known FMDV infection status at the beginning of the study (specificity) and two weeks after experimental infection (sensitivity). Based on this approach, the ss-SPCE appeared to have perfect specificity (100%) and excellent sensitivity (97%), which was more consistent with the current literature related to SPCE accuracy in other species. This is also the typical study design approach used to estimate diagnostic accuracy. However, such estimates might not be accurate representations of what would be expected in field situations because vaccination and experimental challenge are unlikely to mimic natural exposure to the virus. Latent class analyses (Fosgate et al., 2017) based on the Hui-Walter paradigm (Hui and Walter, 1980) using data collected in a field situation might be a better alternative in effort to reduce the selection bias (Fosgate, 2021) that can be introduced when experimental animals are utilized to estimate diagnostic accuracy. Banked specimens from goats sampled from a FMD-free area were included in the study to provide a field validation component. These specimens provided further evidence of near perfect specificity of the SAT1 ss-SPCE, but sensitivity could not be estimated. The lack of a complete field validation of the ss-SPCE is a limitation of the current study and an area that requires future research.

The findings of the current study might also be limited because formal sample size calculations were not performed (Fosgate, 2009) and serum specimens from a relatively small number of goats from previous research were conveniently selected for study. Additional limitations of the current study also include the vaccination with a pentavalent vaccine containing antigen for all three SAT virus serotypes, the evaluation of goats vaccinated at different dosage levels, experimental infection of goats with only serotype SAT1 viruses, and the variable long-term storage duration prior to the second testing of specimens. Virus neutralization tests would have also been a more appropriate reference standard for the evaluation of the ss-SPCE.

5. Conclusion

Goats are potentially important in the transmission of FMDV in

southern Africa but limited data are available concerning the role this species plays in the epidemiology of FMDV in the region. The current study provides novel data concerning the accuracy of a ss-SPCE for the detection of FMDV SAT serotype exposure (vaccination and infection) in goats. Serological screening of goats is recommended in regions suffering from SAT serotype outbreaks and the described ss-SPCE could be used in the design of screening programs. However, more research is necessary to optimize currently available FMD serological assays for use in goats.

CRediT author contribution statement

M Gobiye: Data curation, Formal analysis, Investigation, Writing – original draft. **GT Fosgate**: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – review & editing. **L Heath**: Conceptualization, Investigation, Methodology, Project administration, Resources, Writing – review & editing. **DD Lazarus**: Data curation, Investigation, Writing – review & editing. **L Seoke**: Data curation, Investigation, Methodology, Writing – review & editing. **PA Opperman**: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Conflicting Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgements

The authors want to thank Dr Paidamwoyo Mutowembwa and Thapelo Rametse of the Agricultural Research-Onderstepoort Veterinary Research, Transboundary Animal Diseases for their role in animal care and handling during the trial. All other staff in the animal containment facility that assisted with the project are highly acknowledged. We also acknowledge Mr Henry Harris and Hanlie Jones of the Diagnostic Section, Transboundary Animal Diseases, for their support in sample analysis, and data acquisition and capture in the laboratory.

This research was funded by the National Research Foundation (NRF), South Africa (Grant Number 90578 and 76734) with additional funding from the Peace Parks Foundation, under a contract agreement (Project A0U199). MSc studies of M. Gobiye supported by South African Agricultural Sector Education and Training Authority (AgriSETA) awarded to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. The funders did not have a role in the study design, interpretation of data, writing of the report, or in the decision to submit the article for publication.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.smallrumres.2023.106982.

References

Alexandersen, S., Zhang, Z., Donaldson, A.I., Garland, A.J.M., 2003. The pathogenesis and diagnosis of foot-and-mouth disease. J. Comp. Pathol. 129, 1–36.

Anderson, E.C., Doughty, W.J., Anderson, J., 1976. The role of sheep and goats in the epizootiology of foot-and-mouth disease in Kenya. J. Hyg. (Lond.) 76, 395–402.

Annandale, A., Fosgate, G.T., Eksteen, C.A., Kremer, W.D.J., Bok, H.G.J., Holm, D.E., 2021. Influence of an exercise program, muscle strength, proprioception, and arm length on veterinary students' bovine pregnancy diagnosis accuracy. J. Vet. Med Educ. 48, 196–210.

Arzt, J., Juleff, N., Zhang, Z., Rodriguez, L.L., 2011. The pathogenesis of foot-and-mouth disease I: viral pathways in cattle. Transbound. Emerg. Dis. 58, 291–304.

Banda, F., Shilongo, A., Hikufe, E.H., Khaiseb, S., Kabajani, J., Shikongo, B., Set, P., Kapapero, J.K., Shoombe, K.K., Zaire, G., Kabilika, S., Quan, M., Fana, E.M., Mokopasetso, M., Hyera, J.M.K., Wadsworth, J., Knowles, N.J., Nardo, A.D., King, D. P., 2022. The first detection of a serotype O foot-and-mouth disease virus in Namibia. Transbound. Emerg. Dis. 69, e3261–e3267.

Barnett, P.V., Cox, S.J., 1999. The role of small ruminants in the epidemiology and transmission of foot-and-mouth disease. Vet. J. 158, 6–13.

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

Biswal, J.K., Bisht, P., Mohapatra, J.K., Ranjan, R., Sanyal, A., Pattnaik, B., 2015. Application of a recombinant capsid polyprotein (P1) expressed in a prokaryotic system to detect antibodies against foot-and-mouth disease virus serotype O. J. Virol. Methods 215–216, 45–51.

Blignaut, B., van Heerden, J., Reininghaus, B., Fosgate, G.T., Heath, L., 2020. Characterization of SAT2 foot-and-mouth disease 2013/2014 outbreak viruses at the wildlife-livestock interface in South Africa. Transbound. Emerg. Dis. 67, 1595–1606.

Braker, M.J.E., Udo, H.M.J., Webb, E., 2002. Impacts of intervention objectives in goat production within subsistence farming systems in South Africa. South Afr. J. Anim. Sci. 32, 185–191.

Brito, B.P., Rodriguez, L.L., Hammond, J.M., Pinto, J., Perez, A.M., 2017. Review of the global distribution of foot-and-mouth disease virus from 2007 to 2014. Transbound. Emerg. Dis. 64, 316–332.

Chimera, E.T., Fosgate, G.T., Etter, E.M.C., Jemberu, W.T., Kamwendo, G., Njoka, P., 2022. Spatio-temporal patterns and risk factors of foot-and-mouth disease in Malawi between 1957 and 2019. Prev. Vet. Med 204, 105639.

Domingo, E., Baranowski, E., Escarmis, C., Sobrino, F., 2002. Foot-and-mouth disease virus. Comp. Immunol. Microbiol Infect. Dis. 25, 297–308.

Elnekave, E., van Maanen, K., Shilo, H., Gelman, B., Storm, N., Abed El Khaliq, M., Sharir, B., Berke, O., Klement, E., 2016. Prevalence and risk factors for foot and mouth disease infection in cattle in Israel. Prev. Vet. Med 130, 51–59.

Fosgate, G.T., 2009. Practical sample size calculations for surveillance and diagnostic investigations. J. Vet. Diagn. Invest 21, 3–14.

Fosgate, G.T., 2021. Study design synopsis: Bias can cast a dark shadow over studies. Equine Vet. J. 53, 205–216.

Fosgate, G.T., Motimele, B., Ganswindt, A., Irons, P.C., 2017. A Bayesian latent class model to estimate the accuracy of pregnancy diagnosis by transrectal ultrasonography and laboratory detection of pregnancy-associated glycoproteins in dairy cows. Prev. Vet. Med 145, 100–109.

Gardner, I.A., Colling, A., Caraguel, C., Crowther, J.R., Jones, G., Firestone, S.M., Heuer, C., 2021. Introduction - validation of tests for OIE-listed diseases as fit-for-purpose in a world of evolving diagnostic technologies. Rev. Sci. Tech. 40, 19–28.

Gardner, I.A., Colling, A., Greiner, M., 2019. Design, statistical analysis and reporting standards for test accuracy studies for infectious diseases in animals: Progress, challenges and recommendations. Prev. Vet. Med 162, 46–55.

Gibson, C.F., Donaldson, A.I., 1986. Exposure of sheep to natural aerosols of foot-and-mouth disease virus. Res Vet. Sci. 41, 45–49.

Grubman, M.J., Baxt, B., 2004. Foot-and-mouth disease. Clin. Microbiol Rev. 17, 465–493.

Hughes, G.J., Mioulet, V., Kitching, R.P., Woolhouse, M.E., Alexandersen, S., Donaldson, A.I., 2002. Foot-and-mouth disease virus infection of sheep: implications for diagnosis and control. Vet. Rec. 150, 724–727.

Hughes, K., Fosgate, G.T., Budke, C.M., Ward, M.P., Kerry, R., Ingram, B., 2017. Modeling the spatial distribution of African buffalo (Syncerus caffer) in the Kruger National Park, South Africa. PLoS One 12, e0182903.

Hui, S.L., Walter, S.D., 1980. Estimating the error rates of diagnostic tests. Biometrics 36, 167–171.

Jacobson, R.H., 1998. Validation of serological assays for diagnosis of infectious diseases. Rev. Sci. Tech. 17, 469–526.

Kang, Y.L., Jeong, J.Y., Choi, H.Y., Zhang, Y., Liu, Y., Lee, H.J., Choi, J.C., Lee, S.H., Lee, B.J., Lee, S.W., Lee, J.B., Cho, K.H., Park, S.Y., 2018. Evaluation and optimization of a conventional SPCE for FMD post-vaccination monitoring. BMC Vet. Res 14, 371.

Kärber, G., 1931. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Naunyn-Schmiede Arch. für Exp. Pathol. und Pharmakol. 162, 480–483.

Kitching, R.P., Hughes, G.J., 2002. Clinical variation in foot and mouth disease: sheep and goats. Rev. Sci. Tech. 21, 505–512.

Kitching, R.P., Hutber, A.M., Thrusfield, M.V., 2005. A review of foot-and-mouth disease with special consideration for the clinical and epidemiological factors relevant to predictive modelling of the disease. Vet. J. 169, 197–209.

Knowles, N.J., Samuel, A.R., 2003. Molecular epidemiology of foot-and-mouth disease virus. Virus Res 91, 65–80.

Knowles, N.J., Wadsworth, J., Bachanek-Bankowska, K., King, D.P., 2016. VP1 sequencing protocol for foot and mouth disease virus molecular epidemiology. Rev. Sci. Tech. 35, 741–755.

Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biometrics 33, 159–174.

Lazarus, D.D., Mutowembwa, P.B., Sirdar, M.M., Rametse, T.M., Heath, L., Opperman, P. A., Burroughs, R.E.J., Fosgate, G.T., 2019. Clinical presentation of FMD virus SAT1 infections in experimentally challenged indigenous South African goats. Small Rumin. Res 180, 15–20.

Lazarus, D.D., Opperman, P.A., Sirdar, M.M., Wolf, T.E., van Wyk, I., Rikhotso, O.B., Fosgate, G.T., 2021. Improving foot-and-mouth disease control through the evaluation of goat movement patterns within the FMD protection zone of South Africa. Small Rumin. Res 201, 106448.

Lazarus, D.D., Peta, F., Blight, D., Van Heerden, J., Mutowembwa, P.B., Heath, L., Blignaut, B., Opperman, P.A., Fosgate, G.T., 2020. Efficacy of a foot-and-mouth

disease vaccine against a heterologous SAT1 virus challenge in goats. Vaccine 38, 4006–4015.
Li, Y., Swabey, K.G., Gibson, D., Keel, P.J., Hamblin, P., Wilsden, G., Corteyn, M., Ferris, N.P., 2012. Evaluation of the solid phase competition ELISA for detecting antibodies against the six foot-and-mouth disease virus non-O serotypes. J. Virol. Methods 183, 125–131.
Longjam, N., Deb, R., Sarmah, A.K., Tayo, T., Awachat, V.B., Saxena, V.K., 2011. A brief review on diagnosis of foot-and-mouth disease of livestock: conventional to molecular tools. Vet. Med Int 2011, 905768.
Mackay, D.K., Bulut, A.N., Rendle, T., Davidson, F., Ferris, N.P., 2001. A solid-phase competition ELISA for measuring antibody to foot-and-mouth disease virus. J. Virol. Methods 97, 33–48.
McVicar, J.W., Sutmoller, P., 1968. Sheep and goats as foot-and-mouth disease carriers. Proc. Annu Meet. U S Anim. Health Assoc. 72, 400–406.
McVicar, J.W., Sutmoller, P., 1972. Experimental foot-and-mouth disease in sheep and goats: an epizootiological model. Arch. Gesamt Virus 38, 85–96.
Muthukrishnan, M., Singanallur Balasubramanian, N., Villuppanoor Alwar, S., 2020. Experimental Infection of Foot and Mouth Disease in Indian Sheep and Goats. Front Vet. Sci. 7, 356.
Paiba, G.A., Anderson, J., Paton, D.J., Soldan, A.W., Alexandersen, S., Corteyn, M., Wilsden, G., Hamblin, P., MacKay, D.K., Donaldson, A.I., 2004. Validation of a foot-and-mouth disease antibody screening solid-phase competition ELISA (SPCE). J. Virol. Methods 115, 145–158.
R Development Core Team, 2017. R A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
Ramakrishnan, M.A., 2016. Determination of 50% endpoint titer using a simple formula. World J. Virol. 5, 85–86.
Ran, X., Yang, Z., Bai, M., Zhang, Y., Wen, X., Guo, H., Sun, S., 2019. Development and validation of a competitive ELISA based on bacterium-original virus-like particles of serotype O foot-and-mouth disease virus for detecting serum antibodies. Appl. Microbiol. Biotechnol. 103, 3015–3024.
Ranaweera, L.T., Wijesundara, U.K., Jayarathne, H.S., Knowles, N., Wadsworth, J., Mioulet, V., Adikari, J., Weebadde, C., Sooriyapathirana, S.S., 2019. Characterization of the FMDV-serotype-O isolates collected during 1962 and 1997 discloses new topotypes, CEY-1 and WCSA-1, and six new lineages. Sci. Rep. 9, 14526.
Rweyemamu, M., Roeder, P., Mackay, D., Sumption, K., Brownlie, J., Leforban, Y., Valarcher, J.F., Knowles, N.J., Saraiva, V., 2008. Epidemiological patterns of foot-and-mouth disease worldwide. Transbound. Emerg. Dis. 55, 57–72.
Sangula, A.K., Siegismund, H.R., Belsham, G.J., Balinda, S.N., Masembe, C., Muwanika, V.B., 2011. Low diversity of foot-and-mouth disease serotype C virus in Kenya: evidence for probable vaccine strain re-introductions in the field. Epidemiol. Infect. 139, 189–196.
Singh, R.K., Sharma, G.K., Mahajan, S., Dhama, K., Basagoudanavar, S.H., Hosamani, M., Sreenivasa, B.P., Chaicumpa, W., Gupta, V.K., Sanyal, A., 2019. Foot-and-mouth disease virus: immunobiology, advances in vaccines and vaccination strategies addressing vaccine failures-an indian perspective. Vaccines 7.
Sirdar, M.M., Fosgate, G.T., Blignaut, B., Mampane, L.R., Rikhotso, O.B., Du Plessis, B., Gummow, B., 2021. Spatial distribution of foot-and-mouth disease (FMD) outbreaks in South Africa (2005-2016). Trop. Anim. Health Prod. 53, 376.
Thomson, G.R., Vosloo, W., Bastos, A.D., 2003. Foot and mouth disease in wildlife. Virus Res 91, 145–161.
Vosloo, W., Thompson, P.N., Botha, B., Bengis, R.G., Thomson, G.R., 2009. Longitudinal study to investigate the role of impala (Aepyceros melampus) in foot-and-mouth disease maintenance in the Kruger National Park, South Africa. Transbound. Emerg. Dis. 56, 18–30.
Wickham, H., 2009. Ggplot2: elegant graphics for data analysis.
Wolf, T.E., Lazarus, D.D., Opperman, P., Heath, L., Ganswindt, A., Fosgate, G.T., 2020. Impact of foot-and-mouth-disease on goat behaviour after experimental infection with serotype SAT1 virus. Prev. Vet. Med 176, 104912.
Wong, C.L., Yong, C.Y., Ong, H.K., Ho, K.L., Tan, W.S., 2020. Advances in the diagnosis of foot-and-mouth disease. Front Vet. Sci. 7, 477.