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A novel method for performing antigenic vaccine matching for foot-and-mouth disease in absence of the homologous virus

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ABSTRACT

Foot-and-mouth-disease (FMD) is a highly contagious transboundary animal disease that has negative consequences on regional and international trade. Vaccination is an important approach for FMD control and an essential consideration is the degree of cross-protection conferred by the vaccine against currently circulating field viruses. The objective of this study was to evaluate a new vaccine matching technique that does not require knowledge concerning the homologous vaccine virus. As a proof of concept, the vaccine-match was assessed for 41 FMD field viruses isolated from southern Africa over a 25-year period.

A diverse group of 20 SAT1 and 21 SAT2 FMDV isolates collected from cattle and wildlife during 1991–2015 were selected for this study. Virus neutralization tests were performed against two sets of pooled sera for each serotype: vaccinated cattle sera (4–16 weeks post-vaccination) and convalescent cattle sera (3 weeks post-experimental challenge). Novel r_1-values were calculated as the ratio of the titre of the vaccinated sera to the titre for convalescent cattle sera. A validation r_1-value was calculated based on an assumption concerning the true homologous vaccine virus. There was a strong positive correlation between r_1-values for the novel and the validation methods for SAT1 viruses (Spearman's rho = 0.84, $P < 0.01$) and a very strong correlation for SAT2 viruses (Spearman's rho = 0.90, $P < 0.01$). In addition, there was moderate to good agreement between the novel and validation methods for both serotypes based on a r_1-value cut-off of 0.3, which is presumed to represent a good vaccine-match. The agreement between methods using prevalence-adjusted and bias-adjusted kappa (PABAK) was 0.67 and 0.84 for SAT1 and SAT2 viruses, respectively.

The new r_1-value method provides a feasible, alternative vaccine matching approach that could benefit FMD control in southern Africa.

1. Introduction

Foot-and-mouth-disease (FMD) is a highly contagious transboundary animal disease (TAD) that affects cloven-hoofed animals and reduces productivity of livestock [1]. The Southern African Development Community (SADC) has categorized FMD as one of the most important animal diseases within the region due to its effects on regional trade in livestock, wildlife and other agricultural products [2].

FMD causes high morbidity with vesicular lesions developing on the mouth and feet. High mortality can occur in young animals due to myocarditis [3]. The disease is caused by FMD virus (FMDV), which belongs to the genus *Aphthovirus*, family *Picornaviridae* [4]. The viral genome is a single-stranded positive sense RNA that has a high mutation rate. The structure of FMDV is non-enveloped, 20–30 nm in diameter and is composed of 60 copies of four capsid proteins named VP1, VP2, VP3 and VP4 [5].

There are seven serotypes of FMDV: O, A, C, Asia 1 and Southern African Territories (SAT) 1, 2 and 3 [6]. The SAT serotypes were historically restricted to sub-Saharan Africa, but in recent years SAT1 and SAT2 viruses have been identified in North Africa and the

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Middle East [7,8]. For the FMDV serotypes, major virus lineages have evolved separately and cluster according to their geographic location that can be subdivided into topotypes based on nucleotide differences of VP1 sequences [9,10]. Thirteen viral topotypes have been identified for SAT1, 14 for SAT2 and five for SAT3 [8,10,11].

Vaccination is practised to reduce virus transmission and the number of susceptible animals in a population; it is regarded as one of the most important approaches for FMD control [12]. The essential component of vaccination is the degree of cross-protection provided by the vaccine against currently circulating field viruses. Thus, the FMDV used to produce the vaccine must have similar antigenic characteristics as potential outbreak strains for vaccination to be effective [13]. Vaccination can induce protective immunity and prevent transmission in as short as four days but the effectiveness depends upon the potency of the vaccine, the match between vaccine and outbreak strain and the level of viral exposure [14].

Vaccine matching is performed to select either the most effective vaccine for a particular circumstance or to monitor the suitability of vaccines in antigen reserves. The lack of vaccine-induced protection in the field is the practical indicator that vaccine matching is required [15,16]. Direct vaccine matching is an *in vivo* cross-protection test, which is costly, time consuming, laborious and requires the use of live animals [17–19]. Indirect *in vitro* methods are practical alternatives and several indirect vaccine matching tests have been developed [20]. Indirect vaccine matching is typically performed by *in vitro* serological methods and assesses the serological relationship (r_1-value) between a field isolate and a vaccine virus. Enzyme-linked immunosorbent assay (ELISA) and virus neutralization test (VNT) can be used for serological vaccine matching [21–23]. The r_1-value is calculated as the ratio of the reciprocal titre of reference serum (from animals exposed to the vaccine virus) against field virus to the reciprocal titre of the same reference serum against the reference vaccine virus [20,24].

In South Africa, FMD is a controlled animal disease in accordance with the South African Animal Diseases Act (Act 35 of 1984) and the country had been classified by the OIE as having an FMD free zone without vaccination [25]. However, the FMD free status has been suspended due to a recent SAT2 outbreak [26]. FMD control in South Africa includes animal movement restrictions placed on cloven-hoofed species and products, prophylactic vaccination of cattle, clinical surveillance, and disease control fencing to prevent contacts between livestock and wildlife [27].

The control of FMD in the protection zone with vaccination of South Africa is complicated by the antigenic variability of SAT FMDV and the uncertainty surrounding protection by currently used vaccines. The objective of this study was to develop and evaluate a new vaccine matching technique that does not require the live homologous vaccine virus in the laboratory to perform the vaccine matching. A secondary objective was to estimate the serological vaccine match for 41 FMD field viruses isolated from southern Africa during 1991–2015.

2. Material and methods

2.1. Study area

FMD control areas in South Africa are divided into three primary FMD zones: infected, protection and free. The majority of the infected zone is comprised of the Kruger National Park (KNP) and adjacent wildlife conservation areas. The protection zone is adjacent to the infected zone and falls within the three provinces of Mpumalanga, Limpopo, and Kwazulu-Natal. The FMD protection zone is subdivided into two areas: the protection zone with and without vaccination. Cattle within the protection zone with vaccination are prophylactically vaccinated every four months using a trivalent vaccine (containing SAT serotypes 1, 2 & 3).

The study area included the FMD infected and protection zones in the provinces of Mpumalanga and Limpopo (Fig. 1). The study excluded the protection zone of Kwazulu-Natal Province because it is a recently designated protection area (2014) and no FMDV isolates had been obtained prior to the study. Ethical approvals were obtained from the Animal Ethics Committees of the University of Pretoria (No. v005-15) and Onderstepoort Veterinary Research of the South African Agricultural Research Council (No.25/04/P001).

2.2. Cattle immunization and sera collection

Vaccinated animal sera were collected in a previous study [28]. Cattle were vaccinated against FMD by the South African veterinary services using a trivalent inactivated-vaccine containing SAT1, SAT2 and SAT3 antigens (Aftovax®, Merial Animal Health Limited /Botswana Vaccine Institute, Gaborone). Cattle were longitudinally sampled and tested for antibodies against FMDV structural proteins using liquid-phase blocking ELISA [29]. One hundred and ninety-one sera samples from 136 cattle at least 4 weeks post-vaccination and with ELISA titres ≥ 2.2 $\log_{10}$ were selected to provide sufficient quantity of sera for the current study. Separate serum pools were created for SAT1 (92 samples of 0.5 ml each) and SAT2 (99 samples of 0.5 ml each) serotypes. Serum pools were negative for non-structural proteins (NSP) using the commercial PrioCHECK® FMDV NS Antibody ELISA Kit. Testing was performed following manufacturer instructions with slight modifications [30].

2.3. Cattle viral challenge and sera collection

Cattle were housed at the Animal containment BSL-3 facility at Trans-boundary Animal Diseases-Onderstepoort Veterinary Research (TAD-OVR). Convalescent sera were obtained from cattle 21 days post-infection with SAT1 and SAT2 field viruses by two consecutive FMDV challenge studies. Eight cattle in total were infected; two per serotype for the first and second passage, respectively. For the first passage, cattle were inoculated intradermolingually with three SAT1 viruses (SAR 08/10; SAR 10/10; SAR 21/10) isolated during a single outbreak in cattle. The three viruses were combined into a single pool prior to challenge. The same challenge procedures were performed with three SAT2 viruses (SAR 01/13; SAR 15/13; SAR 04/14) also from a single outbreak in cattle. FMDV from infected tissue collected from the first study was isolated and pooled and inoculated into a second set of cattle for a second passage. The experimental challenge dose was 10^4 to 10^6 median tissue culture infective dose ($TCID_{50}$) for both passages and serotypes.

Blood was collected at day 21 post-challenge (study termination) and serum samples stored at −70 °C until used. Convalescent sera from both passages were subsequently pooled independently by serotype and stored at −20 °C.

2.4. FMD virus selection

Eight (4 SAT1 and 4 SAT2) reference FMDV from the virus bank of the TAD-OVR FMD Reference Laboratory were included in the study (Supplemental Tables 3 and 4). Six of the 8 available reference strains were selected for sera standardization. Reference viruses were collected from South Africa and other southern African countries. Reference strains were possible vaccine strains, with four of the viruses historically used to control FMD along the borders of South Africa [31]. The FMDV used in this study were

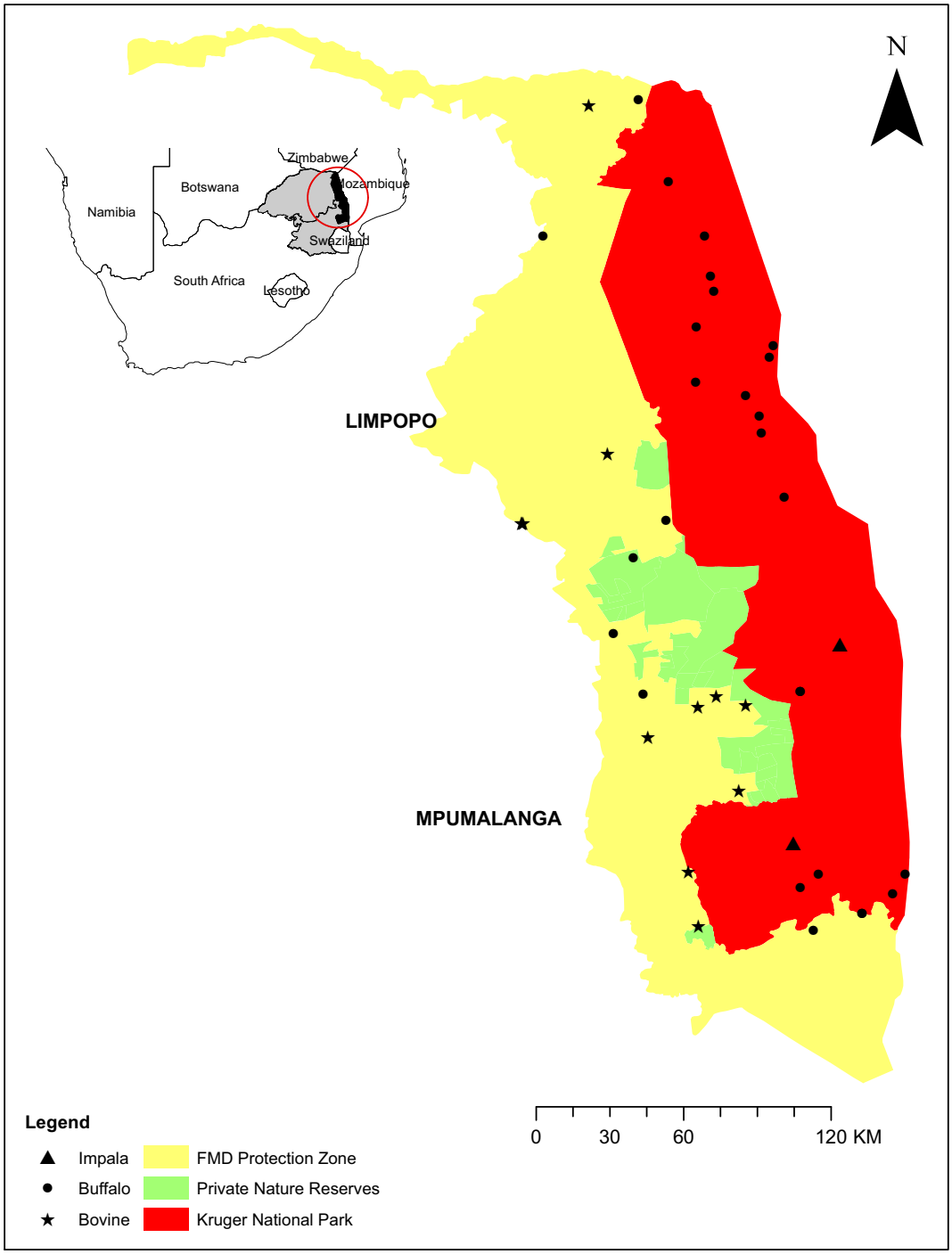

Fig. 1. Location and animal species of FMD study viruses isolated in South Africa between 1991 and 2015.

either isolated in bovine thyroid cells (BTY) or primary pig kidney cells (PK). They were further propagated and passaged in baby hamster kidney-21 cells clone 13 (BHK-21) and Instituto Biologico Renal Suino-2 cells (IB-RS-2) respectively.

A diverse group of 20 SAT1 and 21 SAT2 field viruses were chosen for antigenic vaccine matching (Supplemental Tables 3 and 4). The FMDV were purposely selected to represent all cattle reported outbreaks during the study period and to include genetically diverse viruses based on available VP1 sequence data. Selected viruses were isolated from the KNP and the South African FMD protection zones. Viruses were propagated in IB-RS-2 cells to a titre of >4.5 $\log_{10}$/ml (3–8 passages) and stored at −70 °C until used.

2.5. Serological testing

Virus neutralization tests (VNT) were performed as described in the 2018 OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (adopted 2017) [24]. The assay was performed in microtitre plates on IB-RS-2 cells with the end-point titre calculated as the reciprocal of the last dilution of serum to neutralize 100 $TCID_{50}$ in 50% of the wells [32]. Neutralization titre determinations were performed in duplicate and the test was repeated when the coefficient of variation between duplicates was ≥30%. VNT were performed for field and reference viruses against both the challenged/convalescent and vaccinated cattle sera pools.

2.6. Sera pool standardization

Prior to performing antigenic vaccine matching, VNT were performed for three SAT1 and three SAT2 reference viruses to standardize the FMDV antibody concentrations between the challenged/convalescent and vaccinated cattle sera pools. It was expected that convalescent sera would have higher antibody concentrations and therefore the initial plan was to dilute the convalescent pool to match the antibody concentration of the vaccinated cattle pool. However, the standardization procedure suggested that both pools had similar antibody concentrations and consequently the vaccine matching analysis was performed without diluting the sera.

2.7. Antigenic vaccine matching

One-way antigenic relationships (r_1-value) were calculated [33] with r_1-values greater than 0.3 indicating that the vaccine is likely to confer protection against challenge with the field isolate [24]. Two approaches to vaccine matching were employed.

- An "anticipated r_1-value" was calculated following the usual method by making and assumption concerning the true vaccine (homologous) virus. The available reference viruses were screened against vaccinated animal sera and the virus with the highest recorded titre was chosen as the presumed homologous vaccine virus.
- A "novel r_1-value" was developed as a newly proposed method to calculate r_1-values by comparing the titre from pooled vaccinated sera to a standardized positive control.

The "anticipated" r_1-value was calculated based on an assumed homologous vaccine virus using the following formula:

$$\frac{\text{titre of field isolate against vaccinated animal sera}}{\text{titre of assumed homologous virus against vaccinated animal sera}}$$

"Novel" r_1-values were calculated by substituting the usual r_1-value denominator with a standardized positive control. This approach is similar to the use of a sample to positive ratio, which has been described previously [34,35]. The novel r_1-value was calculated as the ratio between the heterologous virus against the vaccinated animal sera and the homologous challenge virus against challenge sera:

$$\frac{\text{titre of field isolate against vaccinated animal sera}}{\text{titre of challenged animal virus against challenged animal sera}}$$

2.8. Statistical analysis

The genetic diversity of selected SAT1 and SAT2 FMD viruses was evaluated using phylogenetic analysis. Partial VP1 sequences published for the viruses were assembled and aligned using BioEdit 5.0.9 and MEGA version 5 software packages, respectively [36]. A neighbor-joining tree was constructed in MEGA 5 using the p-distance method with bootstrap values of the phylogenetic nodes being calculated out of 1000 replicates [37].

The correlation between novel and anticipated r_1-values were assessed using scatter plots and Spearman's rank correlation coefficients. Correlations were categorized as $\leq$0.35 low or weak correlation, 0.36–0.67 modest or moderate correlation, 0.68–0.89 strong correlation and $\geq$0.9 very strong correlation [38]. The agreement between the novel and anticipated r_1-value methods was assessed using Cohen's kappa based on a r_1-value cut-off of 0.3. Prevalence-adjusted bias-adjusted kappa (PABAK) was calculated as another index of agreement [39]. Bias and repeatability of the novel r_1-value method were assessed using Bland and Altman diagrams [40]. Scatter plots and coefficient of variations were used to assess day-to-day variation of the titre for the standardised positive control.

All statistical procedures were performed using IBM SPSS Statistics (Version 25, International Business Machines Corp., Armonk, New York, USA) and results were interpreted at the 5% level of significance.

3. Results

Eight FMD reference strains (4 SAT1 and 4 SAT2), 20 SAT1, and 21 SAT2 field isolates were included in the study. Six of the 8 reference strains (3 SAT1 and 3 SAT2), were used for sera standardization. The majority of viruses (66% SAT1 and 40% SAT2) were isolated between 2001 and 2010. The largest number of isolates was from cattle, buffalo and impala in South Africa (Table 1; Fig. 1). The SAT reference strains and field isolates clustered according to serotype and revealed distinct genetic variants. Although some SAT1 reference viruses clustered separately from the SAT1 isolates, the majority of isolates formed one group. In contrast, SAT2 viruses were genetically diverse forming several genetic clusters (Fig. 2).

SAT1 convalescent and vaccinated antibody concentrations were comparable with an average $\log_{10}$ titre for the reference viruses of 2.68 and 2.67 respectively. SAT2 vaccinated animal pooled sera had lower $\log_{10}$ antibody titres compared to the convalescent sera pool. However, the $\log_{10}$ VNT titres for the reference virus assumed to be a vaccine strain (ZIM/07/83) were similar between the two sera pools (Supplemental Table 1). Therefore, sera pools for SAT1 and SAT2 serotypes were not diluted prior to the vaccine matching analysis.

There was a strong positive correlation between the novel and anticipated r_1-values for SAT1 viruses (Spearman rho = 0.84). The positive correlation was even stronger between the novel and anticipated r_1-values for SAT2 viruses (Spearman rho = 0.90)

Table 1
Description of the FMD viruses included in the study of a novel r_1-value calculation technique.

		SAT1 (*n* = 24)	SAT2 (*n* = 25)
Isolation years	1980–1990[1]	**1** (04%)	**3** (12%)
	1991–1995	**3** (13%)	**4** (16%)
	1996–2000	**4** (17%)	**2** (08%)
	2001–2005	**8** (33%)	**4** (16%)
	2006–2010	**8** (33%)	**6** (24%)
	2011–2015	**0** (00%)	**6** (24%)
Species	Cattle	**07** (29%)	**09** (36%)
	Buffalo	**16** (67%)	**13** (52%)
	Impala	**01** (04%)	**03** (12%)
Location	South Africa Kruger National Park	**11** (46%)	**12** (48%)
	South Africa FMD protection zone	**11** (46%)	**10** (40%)
	Other southern African countries	**02** (08%)	**03** (12%)
Virus type	Reference strains	**4** (16.6%)	**4** (16%)
	Field isolates	**17** (70.8%)	**18** (72%)
	Other field isolates (challenge strains)	**3** (12.5%)	**3** (12%)

[1] Only reference strains were isolated during this period.

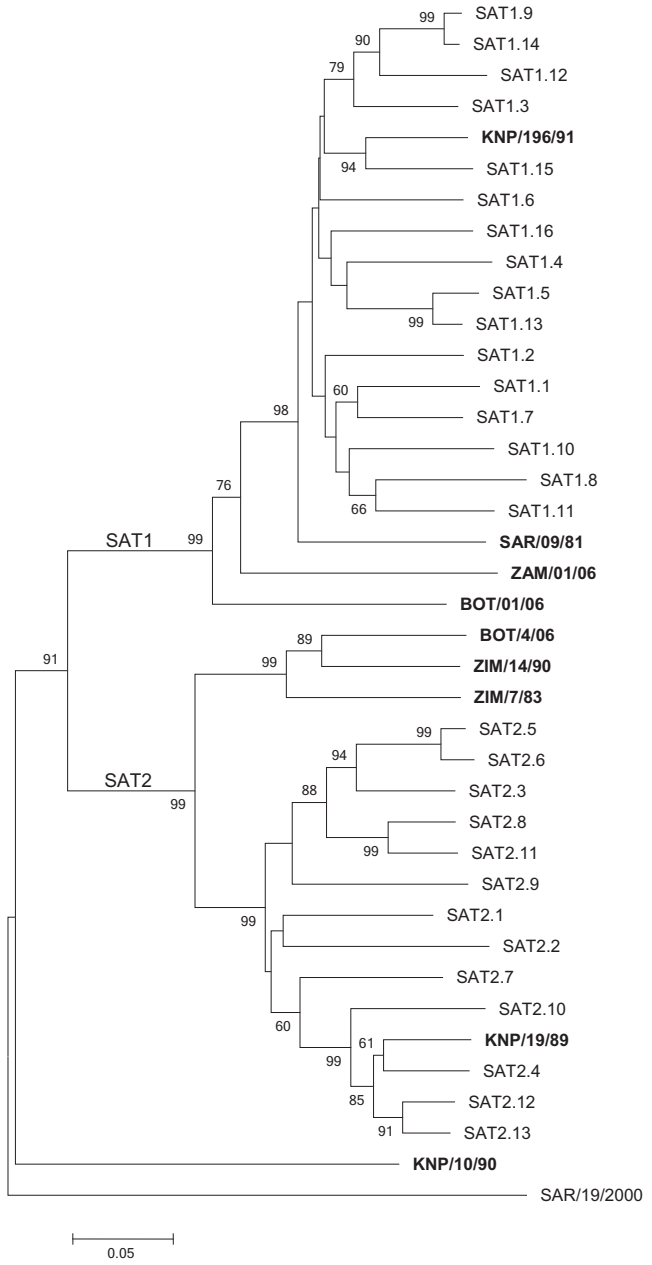

Fig. 2. A phylogenetic tree depicting the relationships of SAT1 and SAT2 FMD viruses isolated in South Africa between 1991 and 2015 based on partial VP1 sequences.

(Figs. 3a and 3b; Supplemental Tables 2 and 3). The distribution of novel r_1-values for SAT1 field viruses was more variable within the Bland-Altman plot (Fig. 4a). The mean difference between novel SAT1 r_1-values and the validation criterion of the Bland-Altman plot was also substantially different from zero. In contrast, the mean differences between r_1-value for SAT2 viruses were close to zero and the distribution within the Bland-Altman plot was less variable (Fig. 4b). The agreement (kappa) between novel and anticipated r_1-values at the 0.3 cut-off were 0.64 (95% CI, 0.34–0.94) and 0.47 (95% CI, 0.00–1.00) for SAT1 and SAT2 viruses respectively. The agreement estimated by PABAK between the two methods was 0.67 and 0.84 for SAT1 and SAT2 viruses, respectively. There was no apparent time trend within the standardized positive control titres and the coefficient of variation over time (different testing days) was less than 6% for both serotypes (Figs. 5a and 5b).

4. Discussion

Appropriate vaccine strain selection is an important component of FMD control programmes in endemic settings [13]. One of the main challenges for disease control is the identification of a vaccine antigen that will protect against currently circulating viruses [41]. In the South African FMD Protection Zone with Vaccination, cattle are vaccinated against FMD every four months by South African veterinary services using a trivalent inactivated vaccine containing SAT1, SAT2 and SAT3 antigens (Aftovax®, Merial Animal Health Limited /Botswana Vaccine Institute, Gaborone).

The SAT1 and SAT2 field isolates included in this study represented a diverse group of viruses to account for this expected variability. At least one virus from each of the FMD outbreaks within South Africa during the period 1991–2015 was included in the study. The evaluated viruses included current as well as historical viruses as a broad representation of the FMDV occurring in South Africa. Several SAT1 and SAT2 partial VP1 sequence data for the chosen viruses of this study were previously published [42–49].

Typical r_1-value calculations require the homologous vaccine virus to be known [20]. However, vaccine manufacturers might not reveal information regarding the viruses used for commercial vaccine production. The novel r_1-value method was developed as a modification of a sample-to-positive ratio, often employed for the interpretation of ELISA results [34,35]. This approach would be important in situations where it is not possible to obtain the homologous vaccine virus.

The novel r_1-values were strongly correlated to the anticipated r_1-values for SAT1 viruses and very strongly correlated to SAT2 viruses. The SAT1 regression line had a positive y-intercept indicating an overestimation of r_1-values. This suggests a bias of the method within the SAT1 viruses. This likely can be attributed to the virus that was chosen as the assumed homologous virus for r_1-value calculation (BOT/01/06). Although the prediction based on the assumed homologous virus was quite good, it is unlikely that this virus was the true vaccine strain. Evidence of this is that four of the field viruses had r_1-values above 1 (SAR 10/10; SAR 07/03; SAR 09/03; KNP 10/03). In contrast, the novel and anticipated r_1-values calculated for SAT2 viruses were more strongly correlated and there was little evidence of bias in the estimation. The mean difference between novel and anticipated r_1-values was close to zero and there was no obvious pattern suggestive of a systematic error. It therefore appears that the SAT2 virus selected as the assumed homologous virus might be the actual virus in the commercial vaccine (ZIM/07/83). Alternatively, the assumed homologous virus has a very close antigenic relationship to the true vaccine virus. A previous study [44] identified the chosen virus as an inactivated vaccine strain used along the borders of South Africa. The unadjusted agreement between the novel and anticipated SAT2 r_1-values may have been biased by the low prevalence of field viruses with a good vaccine match. The SAT2 novel and anticipated r_1-values agreement increased substantially from 47 to 84% when adjusting for prevalence and possible bias. Imprecision in Kappa estimates was evidenced by wide confidence intervals due to few discordant results. PABAK might be similarly imprecise, but the method only adjusts the point estimate of the agreement and not the corresponding confidence intervals.

Virus neutralization methods typically have high variability caused by differences in cell batches viability, susceptibility and viral variability. Variation in viral doses can introduce variation in serum titres, which might introduce error in r_1-value calculations [33]. The intra and inter assay microneutralization variability is considered acceptable at 18–26% and 28–30% respectively [50]. An acceptable coefficient of variation for day-to-day variation within VNT is 15% [51]. The day-to-day variation within the

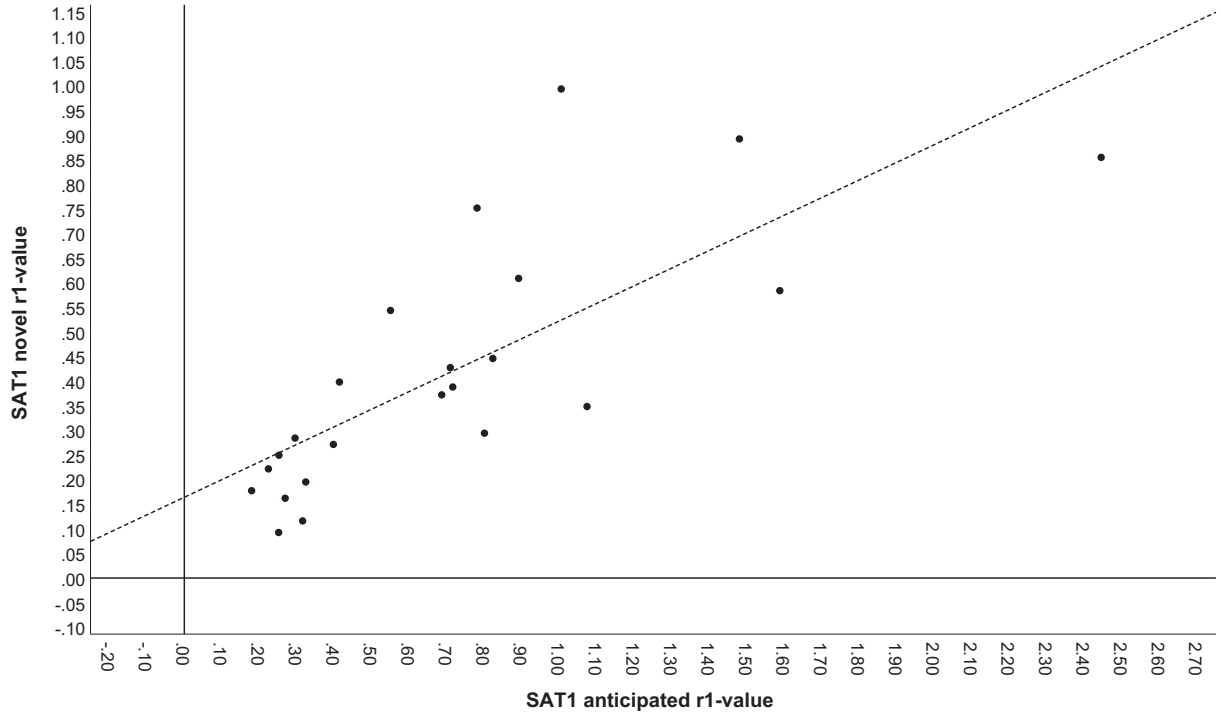

Fig. 3a. Spearman correlation of anticipated and novel r_1-values for SAT1 (Spearman rho = 0.84; P < 0.01) FMD virus isolates from South Africa (1991–2015).

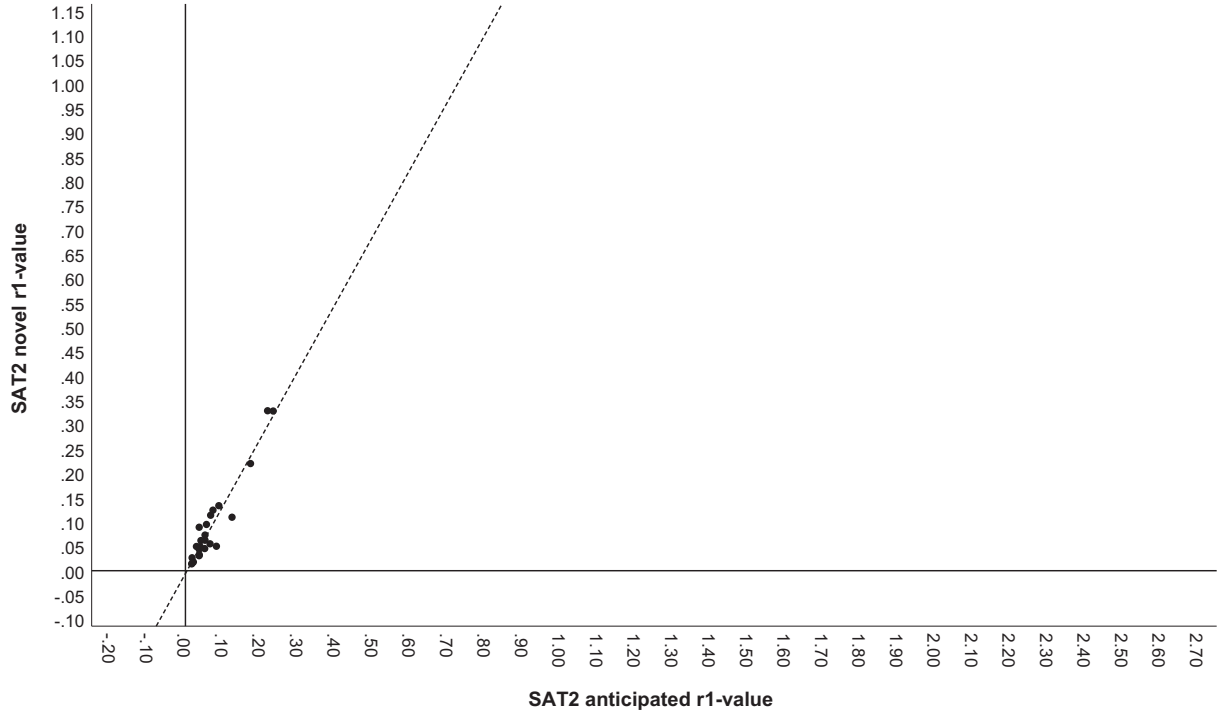

Fig. 3b. Spearman correlation of anticipated and novel r_1-values for SAT2 (Spearman rho = 0.90; P < 0.01) FMD virus isolates from South Africa (1991–2015).

current study was well within the acceptable range for the standardized positive control (challenge virus against challenge sera pool). Therefore, neither day-to-day variation in VNT titres nor improved proficiency in VNT application techniques over time can be the source of the different results between SAT1 and SAT2 viruses.

Fifty-eight percent (14/24) of the evaluated SAT1 viruses (reference and field) had an adequate vaccine match based on the novel r_1-value method. In contrast, only 12% (3/25) of the SAT2 isolates were antigenically similar to the vaccine strain based on the novel r_1-value calculation method. This difference is likely a reflection of the high variability of SAT2 viruses. SAT2 viruses have more VP1 genetic sequence variation compared to other serotypes [28,52,53]. A previous study also reported that SAT2 viruses from the region did not have a good antigenic match when tested

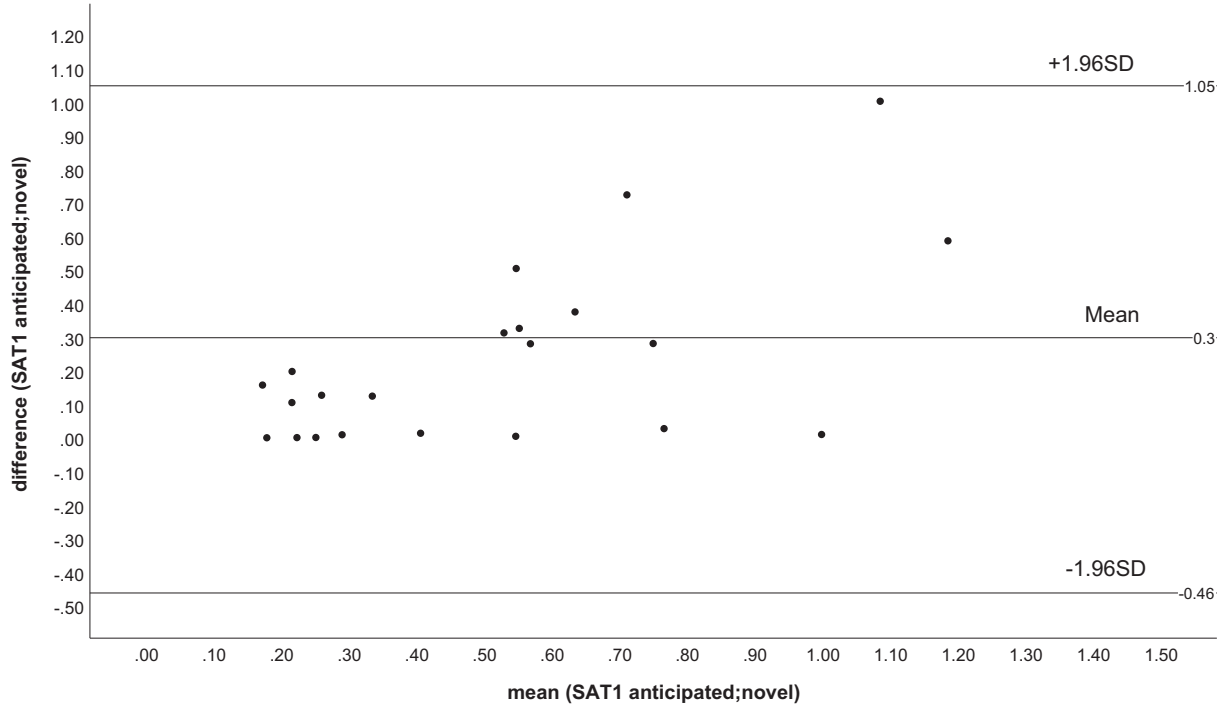

Fig. 4a. Bland-Altman Plot for SAT1 FMDV isolates from South Africa (1991–2015).

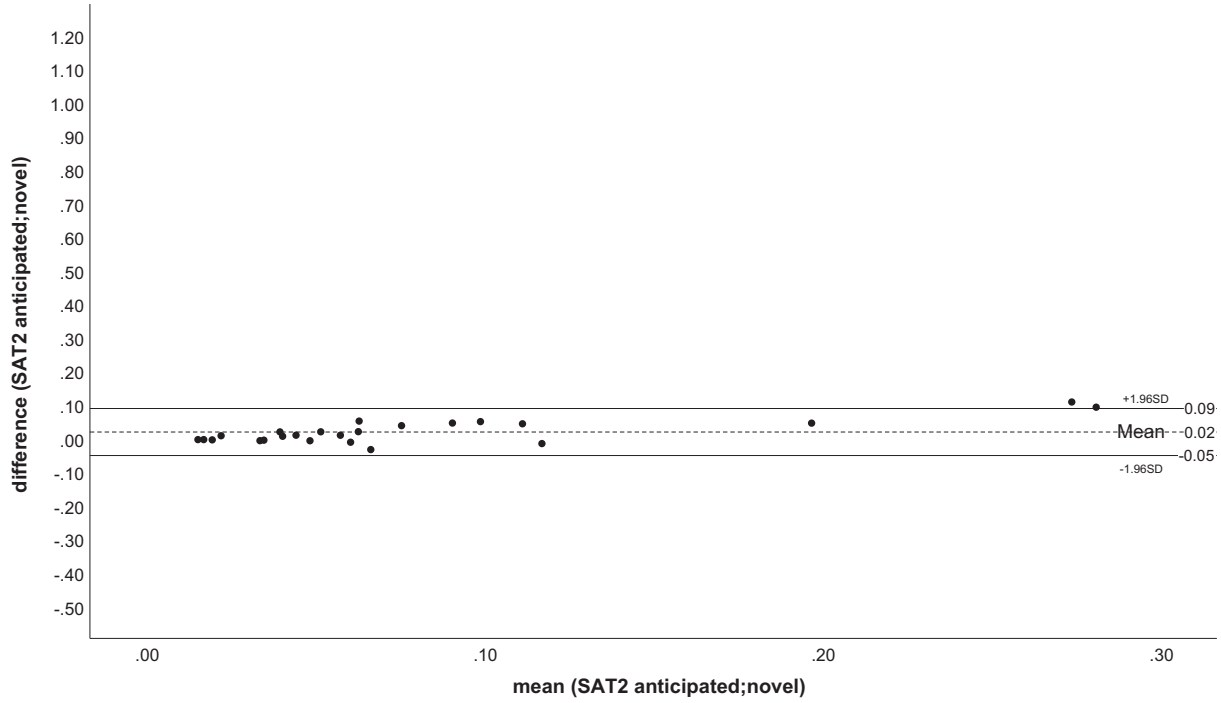

Fig. 4b. Bland-Altman Plot for SAT2 FMDV isolates from South Africa (1991–2015).

against the virus we used as the assumed homologous vaccine virus [44]. The extensive antigenic variation of SAT2 viruses has been known for over 30 years [54].

The novel r_1-value calculation method must have comparable precision and accuracy to the standard method to be an effective tool for use in vaccine matching. Serum titres obtained by different test systems cannot be compared directly; hence the novel method requires implementation of a rigorous validation protocol employing different FMDV serotypes. Standardization of the convalescent and vaccinated sera pools is a critical factor when conducting this type of analysis. It might be expected that titres from convalescent animals would be higher than vaccinated animals [55]. Calculated r_1-values can also vary depending on whether or not the sera were pooled prior to testing [51,56]. Pooling of serum samples reduces the inter-animal and inter-trial variation, irrespective of the number of serum samples in the pool (ranging from 2 to 16)

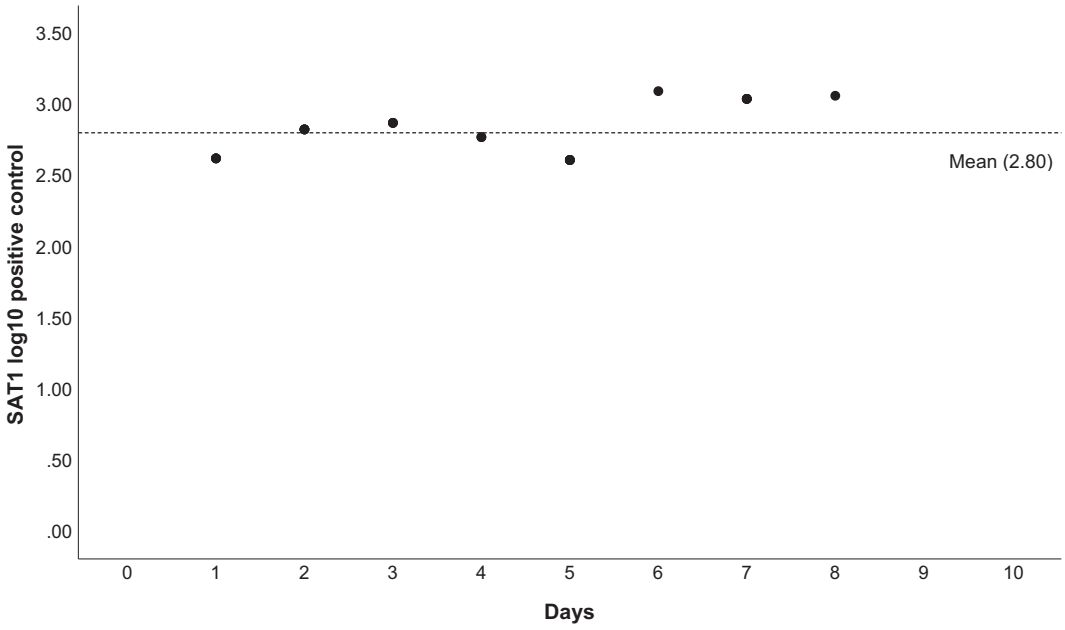

Fig. 5a. Scatter plot for SAT1 FMDV isolates from South Africa (1991–2015) to assess challenge virus against challenge sera (positive control) r_1-values day-to-day variation.

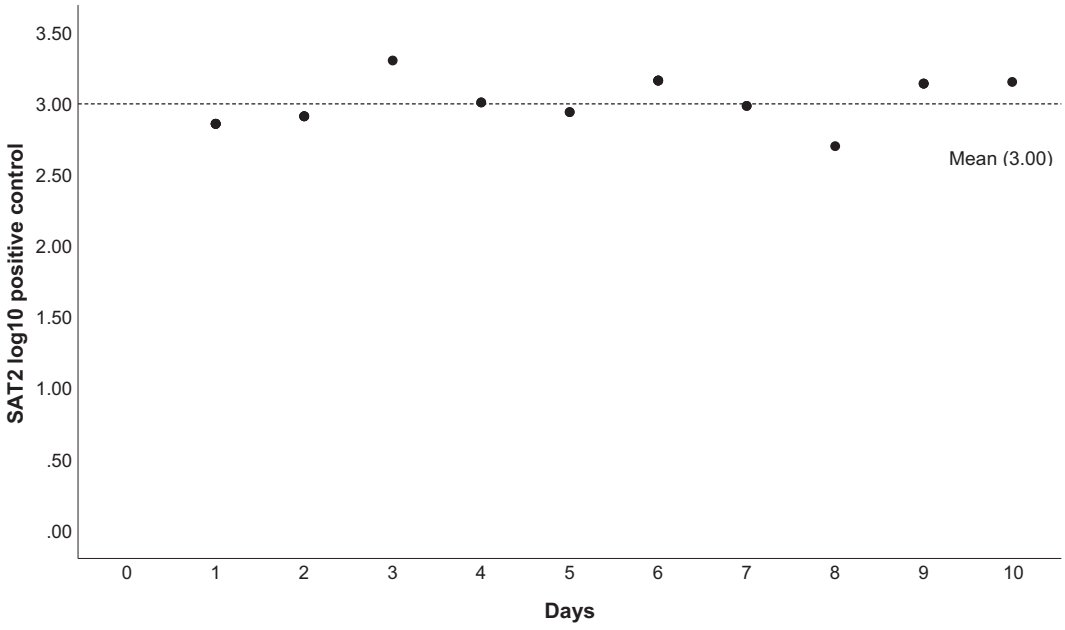

Fig. 5b. Scatter plot for SAT2 FMDV isolates from South Africa (1991–2015) to assess challenge virus against challenge sera (positive control) r_1-values day-to-day variation.

[19]. In the present study, sera were pooled from 50 vaccinated animals per serotype. Convalescent sera from the eight experimentally challenged animals were also pooled. Unprotected animals have lower serum titres and low titres are less suitable for r_1-value determination [19]. The current study selected sera from animals four to sixteen weeks post-vaccination with high/positive antibody titres of $\geq$2.2 $\log_{10}$ as determined previously by LPBE [28]. The employed methods reduced the likelihood of pooling and low titres influencing results and facilitated the standardization of vaccinated and challenge sera pools.

An advantage of the novel r_1-value method is that it makes vaccine matching possible in absence of knowledge concerning the homologous vaccine virus or having the strain available as diagnostic reagents in the laboratory. This approach is relevant when a vaccine manufacturer will not reveal information regarding the viruses used for commercial vaccine production. However, our results require a confirmatory evaluation in a study in which the true homologous virus is known with certainty. The availability of this information will allow rigorous and essential validation of the newly developed method. Our FMD group at the Transboundary Animal Diseases Programme at Onderstepoort Veterinary Research (TAD-OVR) are currently planning a follow-up study concerning a new experimental FMD vaccine under development at TAD-OVR. This follow-up will provide further validation of the novel method using the known homologous vaccine virus.

The preliminary results reported in this manuscript support the feasibility, validity and reliability of the new vaccine matching approach. In addition, the presented results are consistent with

SAT2 FMDV having high antigenic variability and the low proportion of viruses with a good match is a concern for FMD control in South Africa.

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Authors' contribution

All authors had full access to the data and had final responsibility to submit for publication. MMS, GTF, BB, LH & BG were involved with conceptualization and formal analysis, while MMS, DDL & BB conducted the data curation. Funding, resources and project administration was acquired by GTF and LH. The methodology, animal experiment and laboratory analysis were conducted by GTF, BB, MMS, BS, BM and DVM. The article was written by MMS and further reviewed and edited by GTF, BB, BG and LH.

Declaration of Competing Interest

'Declaration of Interest: none'.

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The funders had no role in study design; collection, analysis and interpretation of data; writing of the report; and decision to submit the article for publication.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.07.002.

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