Development and validation of a foot-and-mouth disease virus SAT serotype-specific 3ABC assay to differentiate infected from vaccinated animals

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Abstract

The effective control of foot-and-mouth disease (FMD) requires sensitive, specific and rapid diagnostic tools. However, the control and eradication of FMD in Africa is complicated by, among other factors, the existence of five of the seven FMD virus (FMDV) serotypes, including the SAT-serotypes 1, 2 and 3 that are genetically and antigenically the most variable FMDV serotypes. A key diagnostic assay to enable a country to re-gain its FMD-free status and for FMD surveillance, is the 3ABC or the non-structural protein (NSP) enzyme-linked immunosorbent assay (ELISA). Although many kits are available to detect 3ABC antibodies, none has been developed specifically for the variable SAT serotypes. This study designed a SAT-specific NSP ELISA and determined whether this assay could better detect NSP-specific antibodies from FMDV SAT-infected livestock. The assay's performance was compared to validated NSP assays (PrioCheck®-NSP and IZSLER-NSP), using panels of field and experimental sera, vaccinated and/or infected with FMDV SAT1, SAT2 or SAT3. The sensitivity () of the SAT-NSP was estimated as 76% (70%, 81%) whereas the specificity was 96% (95%, 98%) at a 95% confidence interval. The sensitivity and specificity were comparable to the commercial NSP assays, PrioCheck®-NSP (82% and 99%, respectively) and IZSLER-NSP (78% and 98%, respectively). Good correlations were observed for all three assays.

Keywords: FMD; ELISA; NSP; SAT; 3ABC

1. Introduction

Foot-and-mouth disease (FMD) is one of several contagious transboundary diseases that can spread rapidly within livestock populations with a devastating effect on the economy of a country or region. The causative agent, FMD virus (FMDV), an *Aphthovirus* in the family *Picornaviridae*, though clinically indistinguishable, exist as seven distinct serotypes (Knowles and Samuel, 2003). The epidemiology of FMD in Africa is unique in the sense that five of the seven serotypes of FMDV [South African Territories [(SAT) 1, 2, 3, A and O)], with the exception of types C and Asia-1, occur. Another unique feature is the two different epidemiological patterns in Africa i.e. a cycle involving wildlife, in particular the African buffalo (*Syncerus caffer*), and an independent cycle maintained within domestic animals (Vosloo *et al*., 1996). The presence of large numbers of African buffalo provides a potential source of sporadic spill-over to domestic livestock (Hedger, 1972; Vosloo *et al.*, 1996). Although the precise mechanism of transmission of FMDV from buffalo to cattle is not well understood, it is facilitated by direct contact between these two species. Once cattle are infected they may maintain FMDV infections without the further involvement of buffalo (Dawe *et al.*, 1994). Outbreaks of the disease can cause high mortality of young animals due to myocarditis, as well as decreased production of milk and meat in older animals (Grubman and Baxt, 2004).

Considering the complex epidemiology of FMDV in Africa, in the Southern African Development Community (SADC) emphasis is placed on control rather than eradication of the disease. Countries implement control strategies to separate wildlife and livestock creating areas free of FMD, either through physical separation and movement restrictions or by creating an immunological 'barrier' via repeated vaccination of cattle herds potentially exposed to wildlife. The costs of control are substantial and trade restrictions severely affect economies that are reliant on agricultural production (reviewed in Maree *et al.*, 2014).

In southern Africa, where an increase in the incidence of outbreaks in livestock has been experienced over the last 10 years, a fast and reliable assay to distinguish between infected and vaccinated animals is essential in the decision making for the implementation of control measures. Direct detection methods of FMDV, including virus isolation, reverse-transcription polymerase chain reaction (RT-PCR), real-time RT-PCR and nucleotide sequencing are available (Reid *et al.*, 2003; Jamal *et al.,* 2013; Samuel and Knowles 2001, Knowles and Samuel 2003). Secondary detection methods include conventional FMDV enzyme-linked immunosorbent assay (ELISA) such as the liquid-phase blocking ELISA (LPBE), solid-phase competition ELISA (SPCE) and the virus neutralization test (VNT). These are useful for detecting antibodies to the FMDV structural proteins following infection or vaccination (The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 7th Edition, 2012). In addition, previous studies have shown that serum antibodies specific for the non-structural protein (NSP), 3ABC, is a reliable marker of FMD virus replication in infected cattle (De Diego *et al.*, 1997; Mackay *et al.*, 1998; Sorensen *et al.*, 1998; Brocchi *et al.*, 2006). Thus, this serves as a way to differentiate between FMDV vaccinated and infected animals (DIVA).

A variety of 3ABC ELISA kits, containing expressed 3ABC or 3AB polyproteins in either *Escherichia coli* or *Spodoptera frugiperda* cells using baculovirus, or peptides of the polyprotein are available, which have been validated mostly outside Africa. Additionally, the expressed 3ABC polyprotein or peptides have been derived from the classical "European/South American" types (A, O and C) (Sorensen *et al.*, 1998; Clavijo *et al*., 2004; Brocchi *et al.,* 2006) or Asia-1 serotype (Sharma *et al.,* 2014). However, the use, development and validation of DIVA tests in a region should take into account factors such as the viruses circulating in the region, in addition to vaccine quality, coverage and economy. The high genetic heterogeneity of the FMDV 3ABC polypeptide of the SAT serotype viruses (Van Rensburg *et al*., 2002; Nsamba *et al.,* 2015), prompted us to design a 3ABC ELISA using SAT-derived antigens. In

this study, we developed a SAT-specific NSP-ELISA and compared its performance with those of the commercially available PrioCheck®-NSP, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER)-NSP ELISA (De Diego *et al.*, 1997 and Brocchi *et al*., 2006). The PrioCheck®-NSP and IZSLER-NSP are tests that have already been validated; however, in this study, useful information on the comparative performance of the tests as well as essential information on the validity of the two tests and a SAT-specific NSP ELISA in the SADC cattle population for FMDV is provided.

2. Materials and Methods

2.1 Cloning and expression of SAT2/ZIM/7/83 truncated 3ABC polypeptide

A soluble truncated version of the SAT2/ZIM/7/83 virus 3ABC polypeptide (SAT-Tr3ABC) was generated by removing the coding sequence of the C-terminal 52 amino acid residues of the 3C protease, including critical amino acids in the 3C active site. Sequence-specific primers with 5' and 3' introduced unique restriction enzyme sites (*Kpn*I and *Bam*H1) i.e. 5'-ggtaccatggctATTTCCATTCCTTCCCAAAAGTCC and 5'-ccaaggatccAACCTTagcCCCAGCGCGGTACGC was utilised in an optimised PCR reaction containing dNTPs, buffer, $MgCl_2$ and Takara Ex Taq^{TM} enzyme. The ca. 1,160 bp amplicon was agarose gel purified and digested with *Kpn*I and *Bam*H1 to allow cloning into the pET29a plasmid.

The SAT-Tr3ABC was cloned into the pET29a (Novagen) expression vector using standard procedures (Sambrook and Russell, 2001) and recombinant plasmids were used to transform competent BL21 *E.Coli* cells by heat shock (Sambrook and Russell, 2001). Expression of the SAT-Tr3ABC protein was induced by IPTG according to the pET System Manual (2003), 10th Ed., Novagen. Briefly, 1ml aliquots of Luria broth (LB) containing 100ug/ml of carbenicillin was inoculated with single colonies from the transformation. The

inoculant was incubated overnight at 37℃, shaking at 220-250rpm. Thereafter, 500ul of the overnight cultures were inoculated into 10ml LB containing no selection antibiotics and incubated with shaking at 220-250rpm at 37℃ until the optical density (OD) was OD_{600}~0.5-1.0 (approximately 2 hours). To induce the target protein, isopropyl-B-D-Thiogalactoside (IPTG, Roche) was added to the cultures to obtain a final concentration of 1mM and incubated at 37℃ shaking at 220-250rpm for 4 hours. To determine the protein expression, the samples were analysed via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel. To obtain the soluble fraction of the protein, the BugBuster™ Protein Extraction Reagent (Novagen) was utilised to lyse the cells. The crude lysate extract obtained was utilised as the SAT-Tr3ABC antigen for the SAT-specific NSP ELISA.

2.2 SDS-polyacrylamide gel electrophoresis and Immunoblotting

Crude bacterial lysates were prepared as described, mixed with an equal volume of Protein Solvent Buffer (PSB: 125 mM Tris-HCl [pH 6.8]; 4% [w/v] SDS; 20% [v/v] glycerol; 10% [v/v] 2-mercaptoethanol; 0.002% [w/v] bromophenol blue). Proteins were resolved by 10% (w/v) SDS-PAGE and transferred to Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech AB) with a semi-dry electro-blotter (SemiPhor, Hoefer Scientific Instruments) using standard protocols (Sambrook and Russell, 2001). The membrane was incubated with a 1:200 dilution of anti-SAT2/ZIM/7/83 infected bovine antisera. After washing, the membrane was incubated with horseradish peroxidase-conjugated mouse anti-bovine monoclonal antibody for detection. Bands corresponding to the *ca.* 37kDa protein were captured onto film and developed in a dark room.

2.3 NSP ELISAs

A SAT-specific 3ABC ELISA (SAT2/ZIM/7/83 truncated 3ABC) and two commercially available NSP ELISAs *i.e.* the PrioCheck®- NSP and the IZSLER-NSP 3ABC-monoclonal antibody (MAb) trapping ELISA were profiled against a bovine serum panel.

A detailed test protocol of the PrioCheck®-NSP blocking ELISA (Sørensen *et al.,* 1998) is available on the Thermofisher website. Briefly, the test plates of the kit contained FMDV NSP antigen captured by the coated MAb. The test is performed by dispensing the test samples to the wells of a test plate, after incubation the plate is washed, and the conjugate is added. The results for all samples were expressed as a percentage inhibition (PI) relative to the OD_{450} max and samples showing ≥ 50% inhibition are considered positive.

The IZSLER-NSP ELISA is described in detail in De Diego *et al*., 1997, with the modification reported in Brocchi *et al*., 2006. Briefly, this test uses an anti-3A specific MAb coated to the solid phase to trap the recombinant 3ABC polypeptide expressed in *E. coli*. After incubation of test sera the specific antibodies bound to the 3ABC are detected using a peroxidase-conjugated anti-species immunoglobulin. Results are interpreted as percent positivity in relation to the OD generated by a positive control serum, with the threshold fixed at 10% (or OD serum/OD positive control = 0.1), as per the manufacturer's protocol.

The SAT-NSP ELISA uses the same capturing and detector anti-species immunoglobulin MAb as the IZSLER-NSP ELISA, however the O1 Kaufbeuren 3ABC antigen was replaced with the FMDV SAT2/ZIM/7/83 truncated 3ABC. The SAT-Tr3ABC antigen was used at the pre-determined optimal 1/50 dilution in dilution buffer (1 x PBS pH 7.4, containing 3% non-fat dried milk and 0.05% Tween 20). ELISA plates were washed with PBS containing 0.05% (vol/vol) Tween 20 and the ELISA plates were developed using substrate-chromogen solution, consisting of 4mM 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) in substrate buffer (0.1M citric acid monohydrate, 0.1M, tripotassium citrate; pH 4.5) and 0.015% (vol/vol) H_2O_2. The OD was read at 450 nm using a Labsystems Multiskan Plus photometer. The test samples

were done in duplicate and the OD value calculated as an average of the two values for each test sample. Thereafter the percentage positivity was calculated by dividing the average test sample value by the average of the strong positive control OD value. The percentage positivity result of 10-14 is considered as a weak positive result, whilst a value ≥15 is considered strong positive. Any value <10 is considered negative.

2.4 Serum Panel

A panel of bovine serum samples (n= 1977), obtained from the Agricultural Research Council, Onderstepoort Veterinary Institute, Transboundary Animal Diseases (ARC-OVI-TAD), was constituted for the study. The panel consisted of sera from FMD naïve bovines (n=617), bovines vaccinated with a trivalent vaccine containing SAT1, SAT2 and SAT3 antigen (n=1145) and experimentally FMDV infected cattle (samples collected from day 3 to 146 days post-infection from cattle intra-dermolingually challenged with either a SAT1/KNP/196/91, SAT1/NIG/5/81, SAT2/KNP/19/89, SAT2/UGA/2/02 or SAT3/KNP/10/90 virus) (n=215). The vaccinated sera were samples obtained during surveillance in the FMD control zone in the Mpumalanga region of South Africa (Fig. 1) where the ARC-OVI-TAD confirmed a SAT1 outbreak occurring at that time. This group of sera consisted of 592 cattle that were negative on a SAT1, SAT2 and SAT3 liquid-phase blocking ELISA (LPBE) and therefore was classified into the group designated vaccinated and LPBE negative (VLN). Additionally, from the vaccinated group of sera, 553 were positive on the SAT1 LPBE, which were classified as vaccinated and LPBE positive (VLP). The naïve bovine sera originated from the FMD free Northern Cape region in South Africa (Fig. 1). Each serum sample was aliquoted in triplicate for the three NSP assays that were performed. The SAT-NSP was performed at the ARC-OVI-TAD institute in South Africa whilst the IZSLER-NSP was undertaken at the IZSLER institute

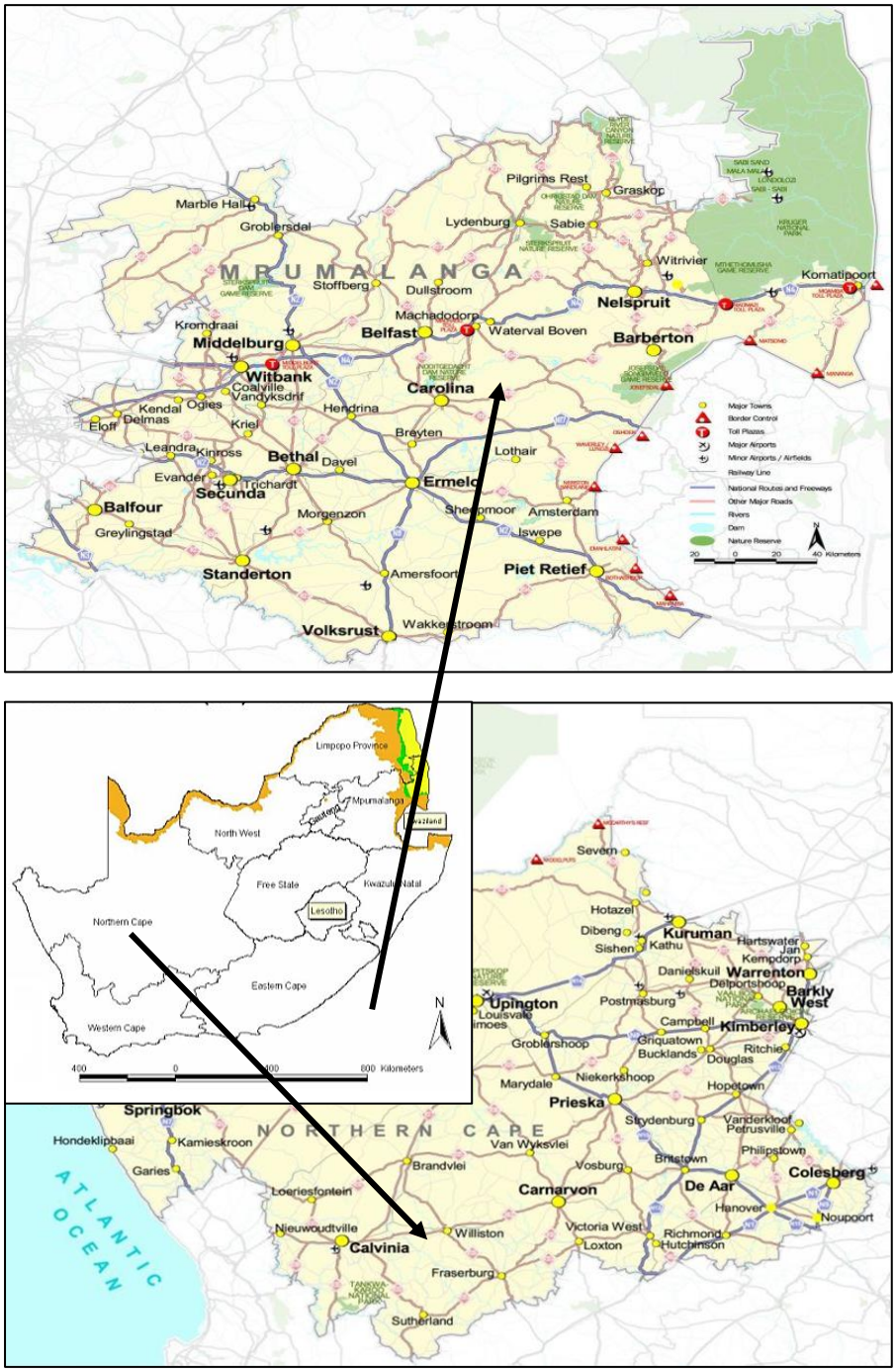


Fig. 1.: Map showing the provinces of South Africa (inset), highlighting the Mpumalanga (top) and Northern Cape regions (below) in detail. The vaccinated group of bovine sera originated from the FMD control zone region of Mpumalanga whilst the naïve bovine sera originated from the Northern Cape region.

in Brescia, Italy and the PrioCheck®-NSP kit was completed at the CODA-CERVA-VAR institute in Belgium for all samples.

2.5 Comparison of the SAT-NSP ELISA and statistical analysis

Diagnostic test results for the three NSP ELISAs were described using box and whisker plots for unvaccinated cattle sampled from the FMD free zone (naïve), vaccinated cattle sampled during a SAT 1 outbreak that were VLN, cattle sampled during a SAT1 outbreak that were VLP, and cattle experimentally infected with one of the three SAT serotypes. Correlation between the diagnostic test results was described by calculating Spearman's rho. Test agreement was evaluated at the recommended positive thresholds by measuring absolute agreement and calculating the kappa statistic. Statistical evaluations were performed in commercially available software (MINITAB Statistical Software, Release 13.32, Minitab Inc, State College, PA, USA and IBM SPSS Statistics Version 23, International Business Machines Corp., Armonk, NY, USA) and results were interpreted at the 5% level of significance.

Sensitivity and specificity of the three NSP ELISAs were analysed using the LPBE results together with subsets of the experimentally infected animals as well as the naïve animal sera where the prevalence of NSP positives in this dataset was 28.9% (n=823). The sensitivity, specificity and accuracy of confidence limits rely on binomial distribution, Youden index confidence limits rely on normal approximation (Wald method for likelihoods) and the AUC (area under the ROC curve) is estimated by trapezoidal method (Simel *et al*., 1991, Zhou *et al*., 2002). Confidence intervals for the test sensitivity and specificity were calculated using the Exact method (Clopper and Pearson, 1934). Calculations were performed using the Epi library in the R program (http://www.rproject. org, The R Foundation for Statistical Computing, R Version2.7.2, 2008-08-25).

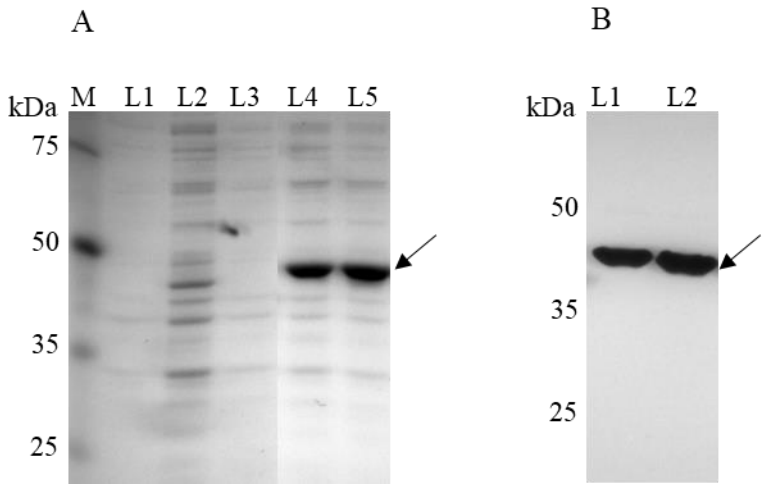


Fig. 2.: (A): SDS-PAGE analysis of the SAT-Tr3ABC protein expression. Lane M is the broad range protein marker (Promega) where the molecular weights are indicated, Lane L1 shows the non-induced, non-recombinant SAT-3ABC protein, L2 is the induced non-recombinant SAT-3ABC protein, L3 is the non-induced recombinant SAT-Tr3ABC protein whilst lanes L4 and L5 indicates successful expression of the recombinant SAT-Tr3ABC protein at *ca.* 37kDa (indicated by an arrow), by two clones. (B) Lanes L1 and L2 shows the western blot analysis of two clones of the expressed SAT-Tr3ABC protein. The position of the protein marker relative to the western blot is indicated.

3. Results

3.1 Expression of the SAT-Tr3ABC protein and optimization of the SAT-NSP ELISA

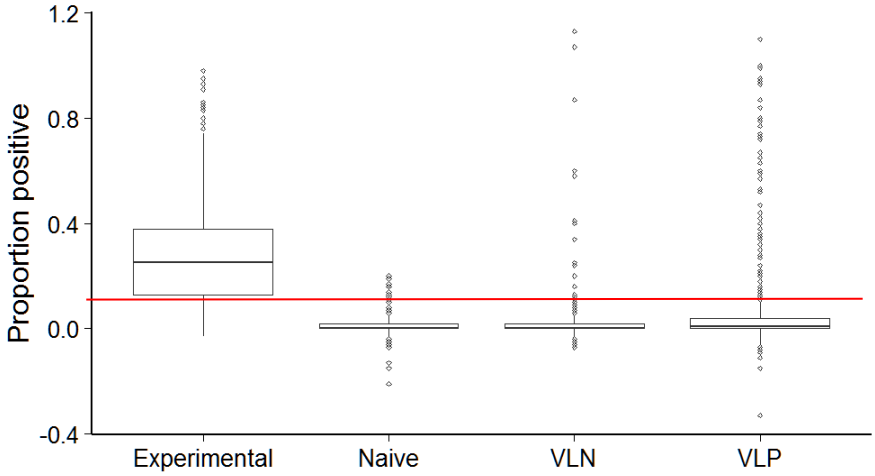
A truncated 3ABC coding sequence was recovered from SAT2/ZIM/7/83 viral RNA by reverse transcriptase PCR and directionally cloned into the pET29a bacterial expression vector. Expression was induced by IPTG and SDS-PAGE analysis (Fig. 2A) showed an abundant protein of *ca.* 37kDa produced from induced *E.coli* cells. To confirm the integrity of the protein an immunoblot was performed, showing polyclonal sera from SAT2/ZIM/7/83 infected animals specifically react to the SAT-Tr3ABC protein (Fig. 2B). This crude bacterial lysate was used as the antigen for the SAT-NSP ELISA at a 1:50 dilution.

Experimental samples positive on SAT2 LPBE were used for optimisation of the SAT-NSP ELISA. OD_{450} readings were ~0.3 higher for the IZSLER-NSP ELISA than the SAT-NSP ELISA OD values (data not shown) and were therefore comparable. The OD value for cut off between positive and negative samples were found to be the same for the SAT-NSP and the IZSLER-NSP (De Diego *et al.,* 1997) ELISAs and were taken as an OD_{450} reading of 0.2.

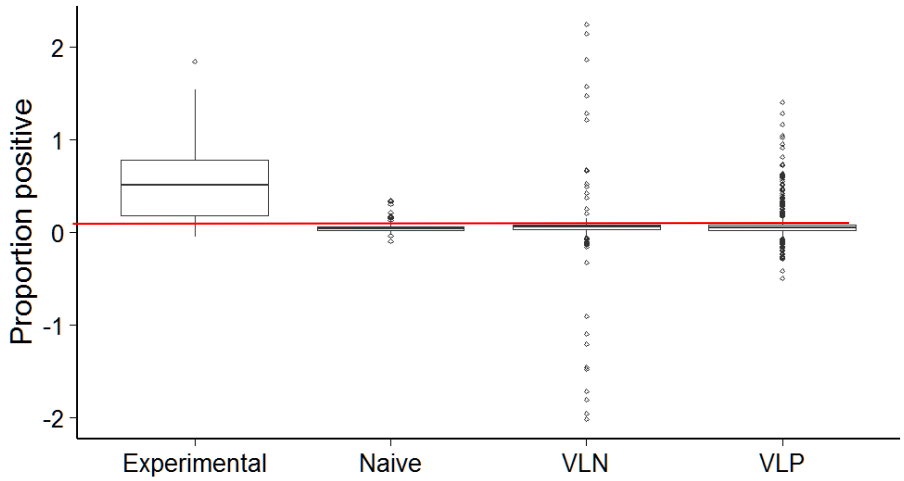
3.2 Comparison of the SAT-NSP, IZSLER-NSP and PrioCheck®-NSP kit

The performance of the three NSP ELISAs, presented in the boxplots in Fig. 3, were compared using 617 naïve cattle sera, 592 VLN sera, 553 VLP sera and 215 sera from cattle experimentally infected with either a SAT1, SAT2 or SAT3 virus (see section 2.4) and LPBE positive. All three NSP ELISAs showed similar results for the naïve sera group, with a few false-positives showing borderline values. Results for the vaccinated groups, originated from an area where a SAT 1 outbreak was confirmed, were more variable, although the three assays were in agreement that the majority of VLN and VLP sera was negative for NSP antibodies. For the VLN group the SAT-NSP and IZSLER-NSP detected 1.6% of the samples NSP positive, comparable to the PrioCheck®-NSP with 0.7%. For the VLP group, the three assays performed

A


Proportion positive
1.2
0.8
0.4
0.0
-0.4
Experimental
Naive
VLN
VLP

B


Proportion positive
2
1
0
-1
-2
Experimental
Naive
VLN
VLP

C

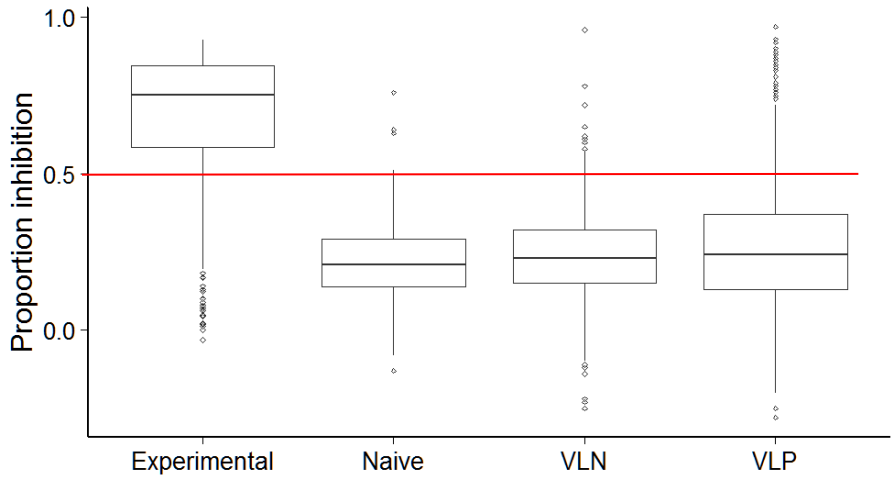

Proportion inhibition
1.0
0.5
0.0
Experimental
Naive
VLN
VLP

Fig.3.: Summary of the distribution of results for the (A) IZSLER-NSP, (B) SAT-NSP and (C) PrioCheck®-NSP tests from 617 cattle sampled within the FMD free zone (FMD naive), 1145 vaccinated cattle sampled during a SAT 1 outbreak of which 592 were test negative on a SAT1 liquid-phase blocking ELISA (VLN) and 553 were test positive on a SAT1 liquid-phase blocking ELISA (VLP), and 215 samples collected from FMDV experimentally challenged cattle (Experimental). The positive threshold for the IZSLER-NSP and SAT-NSP tests is 0.1 proportion positive and the PrioCheck®-NSP is considered positive at or above 0.5 proportion inhibition. The thresholds position is indicated by a red line in each graph. Outliers are plotted as individual points *.

similarly, and diagnosed 18.5%, 16.0% and 15.2% as positive for NSP for the SAT-NSP, IZSLER-NSP and the PrioCheck®-NSP, respectively (results not shown). In both VLN and VLP groups, the samples positive to NSP were outliers in each test, ranging up to values significantly higher than the false-positive observed in the naive group and thus suggesting that these NSP positive samples are true-positive results from animals exposed to infection. The results for the samples of the experimentally challenged group are mostly positive with each NSP-test and distributed over a range of positive values (according to the tests principles and respective thresholds) that reflects variable antibody levels.

The agreement between the three NSP assays as well as the strength of correlation was determined based on the results obtained for the four sera groups/populations (Table 1). Results on the three NSP tests were not significantly correlated within the FMD naïve and VLN groups, but there was substantial absolute agreement (> 93%) for all the assays combinations. The correlation among assay results was substantially larger in the VLP group. Correlation among the three NSP assays was strongest in the group of cattle that were experimentally infected but agreement was descriptively similar to what was observed in the VLP group of cattle.

Out of 1145 FMD vaccinated cattle, 1020 had complete test results for the three NSP assays and the SAT 1 LPBE. Detailed results on this set of samples are shown in Table 2. Forty-seven percent (484/1020) were positive on LPBE but only 11% (110/1020) of them were also positive on one or more of the NSP assays. Taking into consideration the epidemiological scenario in this population, the 374 samples LPBE SAT 1 positive and NSP-negative most probably correspond to animals vaccinated but not exposed to infection. Sera with a single NSP test positive, irrespective of the output of the SAT 1 LPBE (26 for PrioCheck, 22 for IZSLER-NSP and 27 SAT-NSP out of 1020 tested), can be interpreted as NSP-false positive and reflect the assays specificity performance, as described below. Finally, there are 79 sera with 2 or 3 NSP assays positive, that should be evidence of exposure to infection in these animals. Of them,

Table 1. Correlation and agreement among three FMD non-structural protein ELISA evaluated in cattle from South Africa.

Sub-population	Test 1	Test 2	rho	P value	Agreement (%)	Kappa	P value
FMD free and unvaccinated	SAT-NSP	PrioCheck®	-0.027	0.510	95.8	-0.012	1.0
n. sera 617	SAT-NSP	IZSLER	0.063	0.127	95.1	0.098	0.013
	PrioCheck®	IZSLER	0.010	0.804	97.6	0.116	0.001
Vaccinated, LPBE negative	SAT-NSP	PrioCheck®	0.129	0.002	95.2	0.317	<0.001
n. sera 592	SAT-NSP	IZSLER	0.020	0.654	96.1	0.441	<0.001
	PrioCheck®	IZSLER	0.098	0.023	93.7	0.117	0.033
Vaccinated, LPBE positive	SAT-NSP	PrioCheck®	0.218	<0.001	89.9	0.608	<0.001
n. sera 553	SAT-NSP	IZSLER	0.288	<0.001	92.6	0.723	<0.001
	PrioCheck®	IZSLER	0.360	<0.001	91.9	0.659	<0.001
Experimental infections*	SAT-NSP	PrioCheck®	0.613	<0.001	85.4	0.532	<0.001
n. sera 215	SAT-NSP	IZSLER	0.508	<0.001	85.5	0.574	<0.001
	PrioCheck®	IZSLER	0.638	<0.001	92.8	0.777	<0.001

LPBE = liquid-phase blocking ELISA for structural proteins
*Includes all virus serotypes (SAT1-3) and sampling periods

Table 2. Cross-classified test results for 1020 FMD vaccinated cattle sampled during a SAT 1 outbreak and evaluated using three non-structural protein (NSP) ELISA and a SAT 1 liquid-phase blocking ELISA (LPBE) in South Africa.

	NSP assay			LPBE	
	PrioCheck®-NSP	IZSLER-NSP	SAT-NSP	Negative	Positive
1	N	N	N	492	374
2	N	N	P	7	20
3	N	P	N	10	12
4	N	P	P	6	15
5	P	N	N	14	12
6	P	N	P	4	2
7	P	P	N	0	2
8	P	P	P	3	47

N = Negative test result. P = Positive test result.

Table 3. FMD prevalence and diagnostic accuracy estimates for 823 animals tested with three non-structural protein ELISAs in experimentally infected FMDV cattle and naïve animals.

Population or test	Measure	Median (95% CI)	AUC_{ROC}	Youden Index	Accuracy
PrioCheck®-NSP	Sensitivity	0.823 (0.770, 0.867)	0.91	0.82	0.94
	Specificity	0.993 (0.983, 0.997)			
IZSLER-NSP	Sensitivity	0.777 (0.720, 0.826)	0.88	0.76	0.92
	Specificity	0.979 (0.965, 0.988)			
SAT-NSP	Sensitivity	0.760 (0.702, 0.810)	0.86	0.72	0.91
	Specificity	0.964 (0.946, 0.976)			

CI = confidence interval.

50 are concordant positive in the three tests; among the remaining 29 the highest concordance level is observed between IZSLER-NSP and SAT-NSP tests (21 concordant sera), that share the same principle and the same reagents with exception of the antigen derivation.

The diagnostic sensitivity (dsn) (95% CI) and diagnostic specificity (dsp) (95% CI) of the SAT-NSP ELISA was estimated as 76% (70%, 81%) and 96% (95%, 98%), respectively (Table 3), which was similar to the IZSLER-NSP ELISA with 78% (72%, 83%) dsn and 98% (96%, 99%) dsp (Table 3). The PrioCheck®-NSP, however, exhibited a greater dsn of 82% (77%, 87%) and a greater dsp of 99% (98%, 99%). These results correlated with the AUC_{ROC} of 0.91, 0.88 and 0.86 for the PrioCheck®-NSP, IZSLER-NSP and SAT-NSP respectively (Table 3). The accuracies of the three tests were calculated as between 91% and 94% (Table 3).

The time to first detection of positive NSP antibody responses in animals experimentally infected with FMDV differed for each serotype, but all three ELISAs detected NSP in SAT2-infected cattle at 5 days post infection (DPI) (Table 4). Similarly, all three assays detected NSP antibodies at 5-7 DPI with SAT1 or SAT3 viruses with the exception of SAT1/NIG/5/81-infected animal, which was positive at 14 DPI.

4. Discussion

It is essential for countries with FMD outbreaks to regain the advantageous FMD-free status as soon as possible. In order to achieve this, affected countries need to confirm absence of disease and infection in livestock by undergoing clinical and serological surveillance. This makes the use of NSP assays vital. For this study, three NSP assays: one commercially available, one IZSLER in-house assay as well as one in-house assay developed in a South African laboratory, were compared for their ability to detect a marker of FMDV replication. Moreover, this study is unique due to the high number of sera collected from the field, allowing for a more accurate representation of the NSP test performance for South Africa, where a

Table 4. Comparison of NSP ELISA assay detection of 3ABC positive samples from the experimentally infected cattle sera

Serotype	ELISA*	Virus	Animal number	Days post infection							
				0	**2**	**5**	**7**	**9**	**11**	**14**	**21**
SAT1	PrioCheck®-NSP	SAT1/KNP/196/91	8011	0	ND	2	76	73	76	88	90
	IZSLER-NSP		8011	0	ND	2	21	28	86	84	98
	SAT-NSP		8011	0	ND	9	64	82	83	82	84
	PrioCheck®-NSP	SAT1/NIG/5/81	ZPO2 0805	0	ND	ND	13	0	28	72	74
	IZSLER-NSP		ZPO2 0805	0	ND	ND	0	1	2	23	48
	SAT-NSP		ZPO2 0805	0	ND	ND	5	6	5	51	40
SAT2	PrioCheck®-NSP	SAT2/KNP/19/89	1125	0	25	70	50	69	78	79	82
	IZSLER-NSP		1125	0	2	21	10	29	37	14	21
	SAT-NSP		1125	0	7	16	17	29	33	47	38
	PrioCheck®-NSP		1111	0	35	70	69	75	77	ND	ND
	IZSLER-NSP		1111	0	4	33	35	54	52	ND	ND
	SAT-NSP		1111	0	8	30	40	46	34	ND	ND
SAT3	PrioCheck®-NSP	SAT3/KNP/10/90	0102	0	25	73	84	81	75	81	78
	IZSLER-NSP		0102	0	7	36	54	85	85	74	50
	SAT-NSP		0102	0	4	21	28	67	42	31	28
	PrioCheck®-NSP		1124	0	17	10	59	67	69	68	61
	IZSLER-NSP		1124	0	8	3	33	91	91	95	74
	SAT-NSP		1124	0	7	8	55	72	61	55	51

* Priocheck ELISA results are indicated as % inhibition (>50% = positive), IZSLER-NSP and SAT-NSP results are indicated as % positivity (>10% = positive)

population of both vaccinated and FMDV infected animals co-exist. Additionally, this validation study provides useful information on the comparative performance of three NSP assays and the validity of a particular test result in a given population.

From the investigations of correlation and agreement amongst the SAT-NSP, PrioCheck®-NSP and IZSLER-NSP performances for all cattle sera, the NSP assays performed similarly for the various population groups. However, to expect a flawless performance of the NSP assays is not a realistic assumption, therefore, a small number of false negative and false positive results are likely to occur depending on the FMDV immune status. All three NSP assays had similar high specificity values within the naïve population. The NSP positive results obtained for the vaccinated and LPBE negative group i.e. 1.6% for the SAT-NSP and IZSLER-NSP and 0.7% for the PrioCheck®-NSP, could be due to the fact that the NSP ELISA assay can also detect previous exposure to FMDV regardless of the serotype of virus involved (De Diego *et al.*, 1997). Additionally, the occurrence of false-positive results should not be excluded and NSP antibodies may decline in some animals later than the structural protein antibodies (personal communication, E. Brocchi). It is known that the 3ABC antibodies can be detected for up to 1 year after FMDV infection (De-Diego *et al*., 1997, Kitching 2002, Sørensen *et al.,* 1998), which explains those samples that were found to be LPBE negative, but 3ABC positive. Furthermore, several studies have shown that multiple vaccinations or vaccinations and exposure of cattle to FMD in the control zones neighbouring National Wildlife Parks in southern Africa, revealed a variation in NSP serological incidence (Sammin *et al*., 2007; Miguel *et al*., 2013; Jori *et al*., 2016). Cattle in the communal area neighbouring the Kruger National Park are vaccinated three times per year with a trivalent SAT1, SAT2 and SAT3 vaccine. Lee *et al*. (2006) showed that multiple vaccinations resulted in a significant increase in anti-3ABC antibodies in calves. Additionally, Elnekave *et al*., 2015

showed that anti-NSP antibodies persist in naturally infected and repeatedly vaccinated cattle for more than three years.

Several conditions need to be considered related to sensitivity such as the immunological and infectivity status for vaccinated or non-vaccinated animals after infection with FMDV (Brocchi *et al*., 2006). A relative high estimate for sensitivity was achieved concerning diagnostic accuracy for the PrioCheck®-NSP (82%) whilst the SAT-NSP was 76% and IZSLER-NSP 78%, for the FMDV challenged group, showing that the PrioCheck®-NSP is more accurate for the detection of SAT-specific 3ABC antibodies. Furthermore, taking the kinetics of the immune response to 3ABC into account, the three assays showed the same sensitivity. Antibodies to NSP are found later in the infection cycle (6-8 days post infection) than antibodies to the structural proteins (De-Diego *et al*., 1997, Bruderer *et al*., 2004), therefore, sera samples from this study that were found to be FMDV positive on LPBE, may not necessarily be found 3ABC positive on the NSP assay, thus resulting in the higher number of 3ABC negative results found for the challenged group and also for the VLP classified group. Another possibility is that the VLP group of cattle had high antibody titres to FMDV due to immune response to repeated vaccinations, rather than infection, and therefore were found to be LPBE positive. Bruderer *et al*. (2004) describes the heterogeneity found in the 3ABC responses from various experiments in animals vaccinated and then challenged with FMDV, showing that in South African cattle, only 88.9% elicited anti-3ABC antibodies.

Approximately, 50% of vaccinated animals were found to be LPBE negative, most likely due to long inter-vaccination periods (ranging from 4-12 months). Chemically inactivated FMD vaccines induced short-lived antibody responses similar to other inactivated vaccines with antibody levels declining below the detection threshold two months post-vaccination (Hunter 1998; Cloete *et al*., 2008; Maree *et al.*, 2015; Lazarus *et al.*, 2017). The

lack of a sustained immune response beyond four months of vaccination of cattle populations in southern Africa is well documented (Massicame, 2012; Jori *et al.,* 2014).

Parida *et al*. (2005) showed that NSP seroconversion did not occur, despite a four-fold rise in virus neutralising antibody levels for vaccinated cattle exposed to FMDV-infected, in contact cattle. Furthermore, Bergmann *et al.* (2005) stated that a negative result to 3ABC antibodies cannot be taken as conclusive evidence that a single animal has not been exposed to FMDV, since the animal could be in the seroconversion phase which can be delayed in vaccinated animals exposed to infection. Thus, this could also explain the 3ABC negative results for the challenged and for the VLP groups in this study.

The ROC analysis was performed to determine the diagnostic accuracy of the three NSP assays in this study, where the accuracy is measured by the area under the curve. In summary, an area under the ROC curve of 1 represents a perfect test thus the closer a result is to 1, the more accurate a test is (Hanley and McNeil, 1982). Therefore, for the PrioCheck®-NSP, IZSLER-NSP and SAT NSP, the area under the ROC curve was 0.91, 0.88 and 0.86 correspondingly, indicating that all three assays were accurate for the detection of SAT 3ABC antibodies.

A limitation of this study included the sample collection and differentiation into the various groupings. It was challenging to collect good quality sera in large numbers and volumes and the VLP and VLN groups of sera were categorised based on the serology data from the ARC-OVI-TAD. Additionally, large numbers of FMDV infected only sera were difficult to source for the validation study and the sera collected i.e. the "experimental group" were samples that were collected from animal trials at the ARC-OVI-TAD.

Interestingly, although a high genetic variation has been shown for the FMDV SAT serotypes 3ABC polypeptide (Van Rensburg *et al*., 2002; Nsamba *et al.,* 2015); all three NSP kits tested in this study, irrespective of the origin of the 3ABC antigen, has proven to be reliable

and accurate for the detection of FMDV SAT 3ABC antibodies. The sensitivity of the SAT-NSP assay was comparable to the other two assays in detecting NSP-specific antibodies from SAT-infected livestock. The commercially available and widely used PrioCheck®-NSP kit proved to be an excellent NSP kit to use for the detection of SAT NSP antibodies whilst the IZSLER-NSP and SAT-NSP performed similarly and has also proven to be an accurate assay for FMDV NSP detection. The SAT-NSP assay is based on a SAT-specific 3ABC polyprotein with a truncation at the C-terminus as the antigen, which is captured onto ELISA plates by the 3A-specific monoclonal antibody used in the IZSLER-NSP ELISA, giving the advantage of using the antigen as a crude extract, thus avoiding laborious purification procedures. The SAT-NSP is therefore an advantageous test to be used routinely for detection of anti-3ABC antibodies in sub-Saharan Africa for the FMDV SAT serotypes.

Acknowledgements

This comparative study was performed by researchers at the ARC-OVI-TAD, IZSLER and CODA-CERVA-VAR institutes. Thus, special mention of thanks and appreciation must be made to all those who offered technical assistance at these institutes to ensure that the assays were performed accurately and timeously, in particular Dr Belinda Blignaut, Mrs Brenda Botha and Ms Tendai Mlingo. Thanks and appreciation to Dr Geoffrey Fosgate who assisted with the agreement statistics and for his review and statistical advice and expertise. Dr FF Maree received funding from the FAO (MTF/INT/003/EEC) and the IAEA (agreement #16085). The work at CODA-CERVA-VAR was funded by the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no 226556 (FMD-DISCONVAC) and the Veterinary and Agrochemical Research Centre (CODA-CERVA), Ukkel, Belgium. Special

mention of appreciation to Drs Otto Koekemoer and Sikhumbuzo Mbizeni (ARC-OVR) for reviewing this paper prior to publication.

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