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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.

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

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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 and Belsham, 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; Sørensen 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) (Sørensen 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™ 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°C, shaking at 220-250 rpm. Thereafter, 500 ul of the overnight cultures were inoculated into 10ml LB containing no selection antibiotics and incubated with shaking at 220-250 rpm at 37°C until the optical density (OD) was $OD_{600} \sim 0.5–1.0$ (approximately 2 h). 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°C shaking at 220–250 rpm for 4 h. 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 electroblotter (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 $\geq 50\%$ 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 × 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