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Evaluation of immune responses of stabilised SAT2 antigens of foot-and-mouth disease in cattle

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ABSTRACT

Foot-and-mouth disease (FMD) vaccines with improved stability and less reliant on a cold-chain are needed to improve the longevity of immune responses elicited in animals. This is especially so for serotypes O and SAT2 which are unstable in mildly acidic pH conditions or at elevated temperatures leading to dissociation of the capsid (146S particle) and loss of immunogenicity. Previously, stabilised SAT2 viruses were generated by reverse genetic approaches and assessed *in vitro* and *in vivo* with a guinea pig trial. Here we investigated the efficacy and comparative immunological responses of two thermostable and wild-type SAT2 vaccines over 5 months followed by challenge. We assessed humoral immune responses elicited in cattle in terms of total and neutralizing antibodies and IgG1/2 isotyping; and cell-mediated responses of IFN-γ as *in vitro* markers of protection. Whilst there were significant differences in total and neutralizing antibodies for the vSAT2-93H group compared to other vaccinated groups after the first vaccination, there were no significant differences after the second immunization. Following intra-dermolingual challenge all vaccinated groups were fully protected as determined by the absence of generalized lesions. These results provide proof that two vaccine doses, consisting of SAT2 antigen combined with ISA206B adjuvant, administered 4–6 weeks apart were able to protect animals up to 5 months pv. Additionally, vSAT2-93Y had significantly higher levels of IFN-γ after challenge and had a lower clinical score indicative of better protection compared to other vaccinated groups and the importance of cell mediated responses and antigen stability in protection.

1. Introduction

Foot-and-mouth disease (FMD) virus (FMDV), an *Aphthovirus* within the family *Picornaviridae*, is highly infectious and one of the most economically important diseases of cloven-hoofed livestock and other artiodactyl species. In the developing world FMDV is widely distributed, especially in Africa, Asia and South America [1,2]. In Africa, FMDV is maintained by the African buffalo (*Syncerus caffer*), in a cycle involving wildlife, and independently within domestic animals [3–6]. In Southern Africa, sporadic infection of livestock and wildlife with the three Southern African Territories (SAT) serotypes, i.e. SAT1, SAT2 and SAT3, with multiple topotypes [3,7–10] readily occurring from buffalo transmission [11–13]. Therefore, FMD control in sub-Saharan Africa relies on regular vaccination of cattle in high risk areas; fences separating animals at the wildlife-livestock interface in control zones; movement restriction and constant surveillance.

In developing countries effective administration of vaccines which are capable of inducing effective immunity are lacking. This has been linked to factors including poor duration of immunity [14], vaccine potency [15], biophysical (temperature and pH) stability of the antigen [14], and poor cross-protection due to multiple variants in these regions [16]. The poor duration of immunity has been linked to the temperature liability of viruses belonging to the SAT serotypes [14]. One of the foremost factors which influences the potency of vaccine preparations and permits the induction of a protective antibody response is the structural integrity of the intact virion typified by a sedimentation rate of 146S [14]. Due to poor duration of immunity, countries have to rely on a multiple dose yearly vaccination schedule.

Thus, the development of stabilised FMD vaccines with improved immunity are a necessity in Africa. Recent advances in

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the manipulation of the biological properties of field or laboratory strains [17–19] and structural analyses of the capsid have allowed for the design of thermostable mutations [20,21] integrated into reverse genetic approaches [22,23]. Several studies have shown that chimeric vaccines successfully induce protective immune responses and protect FMD host species against live virus challenge [23–25]. Additionally, the SAT capsid can be engineered to encode antigens required for vaccines in specific geographic localities [25] and for improved cell adaptation properties [26,27].

In many remote areas of Africa, the reliable maintenance of an adequate cold-chain for FMD vaccines from the manufacturer to the field is not possible. Therefore, more stable FMDV vaccines could improve the duration of immune responses in animals and additionally be less reliant on the cold-chain [14]. FMD is inherently unstable, but especially for O and SAT2 serotypes [14] at raised temperatures or when exposed to mildly acidic pH, which has been linked to loss of immunogenicity due to dissociation of the 146S particle.

The capsids are held together by electrostatic interactions, hydrogen bonds and weak hydrophobic interactions between the inter-pentameric subunits [21,28]. The inter-pentameric interface residues and their associated interactions are responsible for stabilisation and infectivity of the virion [21,28]; however, experimental studies on the molecular interactions relating to capsid assembly, disassembly, and stability are still very limited. Furthermore, mutations nearby the FMDV interfaces can affect conformational stability [29–32]. We recently showed that using reverse genetic approaches, we could improve stability by introducing stabilising mutations into a SAT2 infectious clone and recovering stabilised viruses [20]. Residue substitutions were predicted by comparing crystallography structures, sequence data, *in silico* calculations and modelling of the inter-pentameric interfaces between the thermostable A serotype and the more unstable O and SAT2 serotype viruses [20].

This paper describes the evaluation of immune responses of two stabilised antigens in comparison to the wild-type SAT2/ZIM/7/83 antigen as a vaccine in cattle. We assessed the serological profile generated in cattle, using total and neutralizing antibodies, IgG1/2 isotyping and cell-mediated responses of IFN-γ as *in vitro* markers of protection [33,34]. The two SAT2 stabilised viruses were selected for their potential use as a vaccine strain based on (i) *in vitro* studies that showed improved temperature stability and (ii) initial data showing their ability to elicit an improved immune response with longer duration and shelf-life than wild-type in guinea pigs [20].

2. Materials and methods

2.1. Cells, viruses and plasmids

The SAT2 FMDV vaccine strain Zimbabwe (ZIM)/7/83 is a bovine virus originating from an outbreak in western Zimbabwe during 1983 [35] and was maintained at the Transboundary Animal Diseases (TAD) of the Agricultural Research Council (ARC) (South Africa). The plasmid pSAT2, a genome-length infectious cDNA clone of SAT2/ZIM/7/83 [36], was used as the genetic backbone in the construction of recombinant cDNA clones with amino acid substitutions S2093Y and S2093H in VP2 [20]. The cloning, transfection and recovery of viruses were performed as described [20] and were termed vSAT2-wt, vSAT2-93Y and vSAT2-93H.

Baby hamster kidney (BHK) cells, strain 21, clone 13 (ATCC CCL-10) and foetal goat tongue (ZZ-R CCLV-RIE127) cells were maintained as described previously [37]. Mutant viruses were passaged as follows $BHK_2ZZ\text{-}R_2BHK_5$ to produce a vaccine master seed stock. Virus stocks were titrated by plaque assays in BHK-21 cells as described previously [15]. Following the recovery of viable viruses, the presence of the mutation was verified with automated sequencing. The viruses were passaged four times and used for antigen production.

IB-RS-2 (Instituto Biológico renal suino) cells were maintained in RPMI medium (Sigma) supplemented with 10% FCS (Delta Bioproducts), and were used for virus isolations and as the indicator system in the virus neutralization test (VNT).

2.2. Production of plasmid-derived chimeric FMDV antigen and vaccine formulation

BHK-21 cell-culture infected fluids from vSAT2-wt, vSAT2-93Y and vSAT2-93H were harvested, inactivated and purified as described before [23]. The inactivated 146S fractions were quantified as described previously [38] and used for vaccine formulation. Three separate vaccines (vSAT2-wt, vSAT2-93Y and vSAT2-93H) of 6–8 μg each were formulated as double oil emulsions with Montanide ISA 206B (Seppic) as described [23].

2.3. Cattle immunizations and viral challenge

Twenty-three Nguni cattle 6–9 months of age or 150 kg were divided randomly into 3 groups (n = 7) and a control group (n = 2). Groups had balanced numbers of females and males. The animal ethics were approved by the ARC-OVI Animal Ethics Committee (25.12), University of Pretoria AEC (V060-15) and Department of Agriculture, Forestry and Fisheries (DAFF, South Africa) Section 20 permit (10/04/2013). After an acclimatization period, the cattle were vaccinated intramuscularly on days 0 and 42 with 2 ml of 6–8 μg per dose of (1) vSAT2-wt (wild-type); (2) vSAT2-93Y or (3) vSAT2-93H vaccine. Two control animals were left unvaccinated. Blood samples (clotted and heparinised) were collected every second day from days 0 to 14, 21, thereafter every second week from 28 to 160 days post-vaccination (dpv). The animals were allowed to roam freely in a 0.3 ha camp for 150 days.

At 150 dpv the cattle were brought into the high-containment animal facility at ARC-Onderstepoort Veterinary Institute (ARC-OVI) and each group housed separately. Animals were acclimatized for 12 days. On 162 dpv the three immunized and control groups were inoculated intra-dermolingually at two sites each with 1 ml of 10^4 $TCID_{50}$ SAT2/ZIM/7/83 challenge virus as recommended by the Office International des Epizooties (OIE) [39]. Oropharyngeal (OP) fluid, tonsil swabs (TS) and blood were collected on 0, 2, 4, 7 and 10, 12, 14 days post-challenge (dpc). The animals were examined daily for fever (mild = 39.5–40 °C, severe ≥40 °C) and clinical signs (small lesion/healing vesicle = 1, moderate vesicles = 2; severe lesions = 3) and sedated as described [23].

2.4. Liquid phase blocking ELISA

Antibody titres in cattle vaccinated with the 3 antigens were detected by a SAT2-specific liquid-phase blocking ELISA (LPBE) on days 0, 2, 4, 7, 9, 11, 14, 21, 28 thereafter every 2 weeks until 162 dpv when challenge commenced and sampled 0–14 dpc. The LPBE was essentially carried out as described in the OIE Manual [39]. The optical density (OD^{405nm}) was measured with a Multiskan EX. Samples with serum titres >1/50 (>$1.6\log_{10}$) were considered positive.

2.5. Virus neutralization test (VNT)

Neutralizing antibodies against SAT2/ZIM/7/83 in serum samples collected at 0, 7, 21, 28, 42, 56, 70, 84, 98, 112, 134, 155, 162 dpv from cattle were measured with a VNT, according to the method described in the OIE Manual [39] using IB-RS-2 cells in

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microtitre plates. The 50% end-point serum titres were calculated according to the method of Kärber [40].

2.6. Solid phase competition ELISA (SPCE)

Rabbit anti-serum raised to SAT2 serotype specific FMDV was absorbed onto micro-titre plates and used to capture the FMD type-specific antigen. The SPBE test based on the competition between guinea pig anti-FMDV-SAT2 (GPS) antiserum and antibodies present in the test serum was performed as described in OIE Manual [39]. Positive test sera binding to the antigen that prevents the GPS and conjugate from binding, resulted in a decreased colour reaction.

2.7. Non-structural protein (NSP) ELISA

The Priocheck® NSP FMDV ELISA [41] was used for the detection of antibodies against the 3ABC polypeptide. Samples with percent inhibition of <50% are negative (antibodies against FMDV NS protein are absent) and positive >50% (antibodies are present).

2.8. IgG1 and IgG2 isotyping ELISA

The IgGl and IgG2 isotyping ELISA was performed according to Capozzo et al. [33] with slight modification. Maxisorp 96-well plates (Nunc) were directly coated with 100 ng/well solution of SDG purified 146S ZIM/7/83 particles in 50 mM carbonate/bicarbonate buffer pH 9.6. Two-fold dilutions of test sera were prepared from 1:50 to 1:3200. Sheep anti-bovine IgG1 or IgG2 HRP-conjugated antibodies (BD-Serotec) at a dilution of 1:750 and 1:1500 respectively were used. Titres were expressed as the inverse dilution reaching the cut off value (0.2) calculated as mean OD + 2SD achieved by the FMDV-negative Nguni bovine serum samples (n = 23).

2.9. Whole blood re-stimulation and bovine Interferon gamma (IFN-γ) ELISA

Whole blood assays [42] were performed using 1.5 ml aliquots of heparinised blood, incubated in 24-well sterile cell culture plates from animals every two weeks from 14 to 84, 164 and 171 dpv. For each animal, duplicate wells were stimulated with 10 μg/ml Pokeweed mitogen (PWM) as a positive control stimulator of all IFN-γ, 10 μg/ml purified inactivated 146S FMDV ZIM/7/83 or phosphate buffer saline (PBS) as negative control. Plates were incubated for 48 h with 5% CO_2 and plasma collected. The Bovine Interferon-γ Specific ELISA Assay Kit (MCA5638KZZ, Bio-rad) using two different mouse anti-bovine IFN-γ monoclonal antibodies and recombinant bovine IFN-γ as a standard (0.025–50 ng/ml) was performed.

2.10. Virus isolation and plaque assays

FMDV in OP fluid, tonsil swabs and whole blood was detected by the inoculation of IB-RS-2 monolayer cells as described by the OIE Manual [39]. The supernatant was blind passaged at least twice or until cytopathic effect (CPE) was observed.

Plaque assays were performed in duplicate by infecting IB-RS-2 monolayer cells in 35 mm cell culture plates (Nunc™) for 1 h, followed by the addition of 2 ml tragacanth overlay [43] and incubated at 37 °C for 48 h. Staining with 1% (w/v) methylene blue in 10% (v/v) ethanol and 10% (v/v) formaldehyde in phosphate buffered saline, pH 7.4 was performed.

2.11. Viral RNA detection by real-time quantitative RT-PCR

The viral RNA in OP fluid, tonsil swabs and whole blood was detected using a one-step real-time RT-PCR assay targeting the 3D region [44], tested in duplicate. Total RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's specifications and used for cDNA synthesis. Positive test and control samples had a Ct value <30; Ct values 30–40 are designated as weak positive whilst samples Ct values ≥40 negative.

2.12. Data analysis

Time-course titres obtained by LPBE, SPCE, VNT and IgG-isotype ELISAs were plotted and results between the two experimental groups were compared by ANOVA 2-factor repeated measures followed by Bonferroni multiple comparisons test. Mann–Whitney test was used when data from two groups were compared. The confidence interval was 95%. Statistical analyses were carried out using GraphPad Prism v5.0 (GraphPad Software).

3. Results

3.1. Characterization of vSAT2-wt, vSAT2-93Y and vSAT2-93H viruses

The construction of the wild-type and thermo-stabilised $vSAT2^{ZIM7/83}$ viruses have been described [20]. The vSAT2-wt, vSAT2-93Y and vSAT2-93H viruses were already adapted to BHK-21 cells. The vSAT2-93Y virus grew slower in BHK-21 monolayers however differences in recovery of antigen were negligible.

3.2. Antibody kinetics of the vSAT2-wt, vSAT2-93Y and vSAT2-93H vaccines in Nguni cattle

Sera collected from 0 to 162 dpv were tested by LPBE, SPCE and VNT's to assess total antibody and neutralizing antibody responses to vaccination (Fig. 1). Total antibody titres from LPBE peaked at 9 dpv for vSAT2-wt and −93H groups and at 14 dpv for the vSAT2-93Y group (Fig. 1A). From 42 to 162 dpv (after the second vaccination) there was no significant difference ($p < 0.05$) in the total antibody titres from LPBE (Fig. 1A) and SPCE (Fig. 1B) between the vaccinated groups. A few animals from each of the vaccinated groups still had positive titres ≥1.6 at 162 dpv (Fig. 1A). After challenge, the circulating antibodies were consumed 0–6 dpc, as animals cleared the virus completely both vaccinated and non-vaccinated cattle elicited high levels of total antibodies (Fig. 2A and B).

At 21 dpv (Fig. 1C) high positive anti-SAT2/ZIM/7/83 neutralizing antibody titres were observed from vSAT2-wt ($2.3–3.2\log_{10}$) and vSAT2-93H ($2.7–3.2\log_{10}$) groups which were statistically significant ($p < 0.05$) to the vSAT2-93Y group (Fig. 1D). At 42 dpv VNT titres of vSAT2-wt group remained statistically significant ($p < 0.05$) to the vSAT2-93H and -93Y groups (Fig. 1C and D). At 56–113 dpv (after the second vaccination) there were no significant differences ($p < 0.05$) in the neutralizing antibody titres of the vaccinated groups with mean group titres $>2.0\log_{10}$ (Fig. 1C and D). Vaccinated animals had high levels of neutralizing antibodies at 2 dpc ($2.74–3.07\log_{10}$), whereas non-vaccinated animal levels were low ($1.25 \pm 0.07\log_{10}$). There was no significant difference ($p < 0.05$) between vaccinated groups from 2 to 9 dpc (Fig. 2E).

3.3. IgG iso-typing and interferon gamma (IFN-γ) responses

IgG1 and IgG2 anti-FMDV serum titres were observed for 21, 42, 56 dpv (Fig. 1D) and for 0–9 dpc (Fig. 2C and D), with an increase

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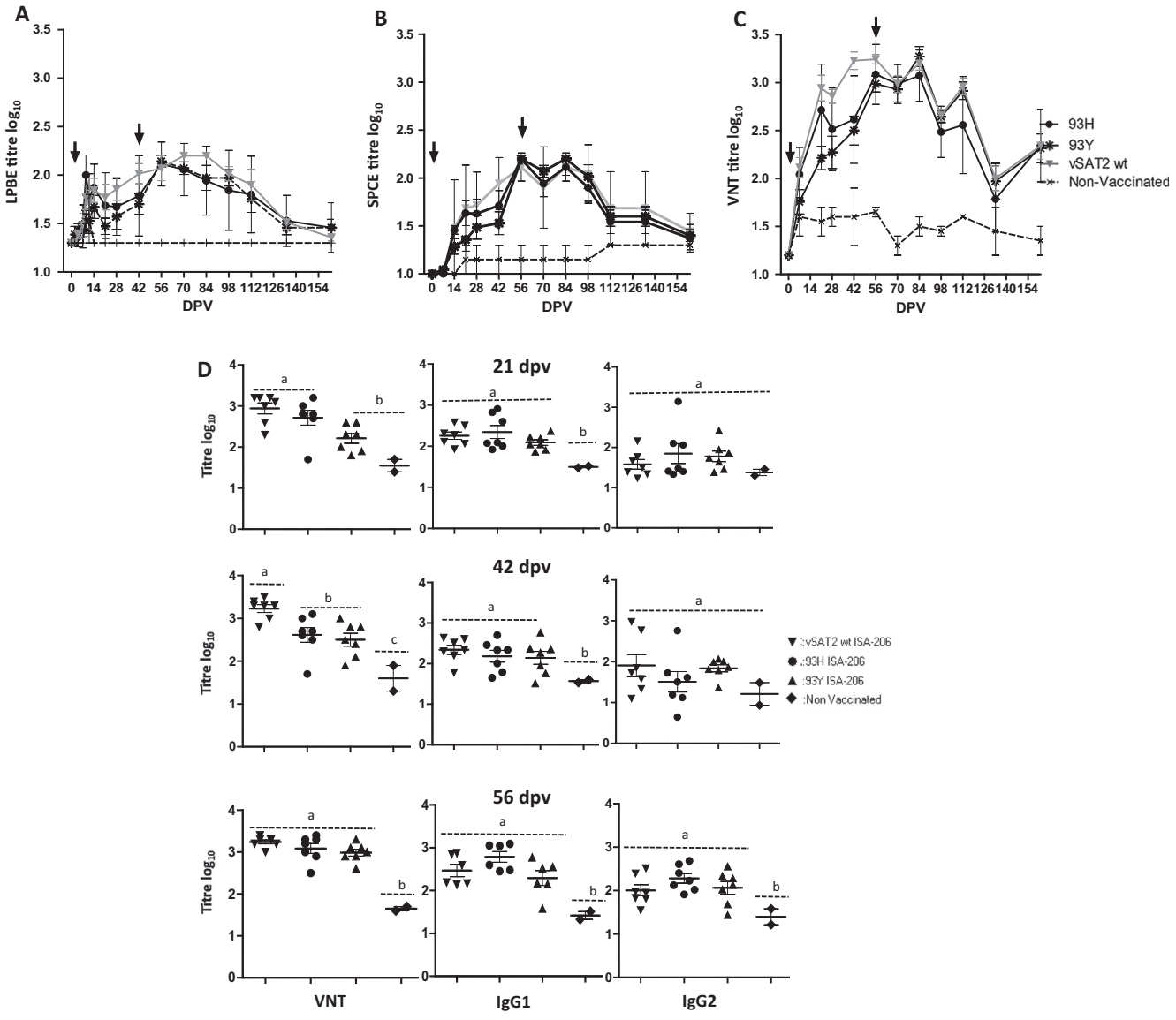

Fig. 1. Antibody kinetics of vaccination responses to wildtype and stabilised antigens. Mean serum titres (log_{10}) measured by (A) SPCE; (B) LPBE; (C) VNT and (D) distribution titres for VNT, IgG1, IgG2 antibody titres at 21, 42 and 56 dpv of Nguni cattle immunized twice with a 6–8 μg dose of either vSAT2-wt (n = 7), vSAT2-93H (n = 7) or vSAT2-93Y (n = 7) BEI-inactivated, SDG-purified antigens with Montanide ISA206 adjuvant. Arrows indicate the time of first vaccination (0 dpv) or second vaccination (42 dpv). Error bars represent the standard deviation. Animals regarded as negative had log_{10} titres ≤1.6 as shown by the unvaccinated control animals (n = 2). a, b or c denotations refer to statistically significant groupings where a is statistically significant to b and c, and b is statistically significant to c.

observed for all vaccinated animals and no statistical difference for the vaccinated groups ($p < 0.05$) (Fig. 1D). The kinetics of IgG1 and IgG2 titres at 21, 42 and 56 dpv were comparable between the vaccinated groups (Fig. 1D). At 7 dpc IgG1 titres increased, peaking at 9 dpc (Fig. 2C) for vaccinated animals, however there was no statistical difference ($p < 0.05$) in IgG1 and IgG2 titres between all groups 0–9 dpc. The ratio of IgG1/IgG2 titres were higher at all the time points.

IFN-γ levels measured from whole blood are extremely variable [45], however validation and implementation of the test was based on previous findings [42]. Blood was incubated with PBS (negative control <1 ng/μl), SAT2-FMDV and PWM (positive control >3 ng/μl). Stimulated blood of both naïve and vaccinated animals were tested with a commercial kit which includes an IFN-γ standard 0.025–50 ng/μl. IFN-γ responses peaked at 28 dpv with vSAT2-93H (8.55 ± 7.89 ng/μl) followed by vSAT2-93Y (4.86 ± 5.17 ng/μl) and vSAT2-wt (2.74 ± 4.55 ng/μl) (Fig. 3). IFN-γ responses before challenge (84 dpv) showed high systemic IFN-γ levels for vSAT2-wt and vSAT2-93Y. After challenge (2 dpc) IFN-γ levels induced by the vSAT2-93Y vaccine were significantly higher ($p < 0.05$) than the rest.

3.4. Protection of vaccinated cattle against live SAT2/ZIM/7/83 virus challenge

The 3 groups (n = 7), that received the vSAT2-wt, vSAT2-93Y and vSAT2-93H vaccines, were protected against systemic spread of FMD after the intra-dermolingual challenge of SAT2/ZIM/7/83 virus as observed by the absence of generalized lesions on their hooves, whilst mild fever (39.5–40 °C) was present in animals vaccinated with vSAT2-wt (n = 1) and vSAT2-93H (n = 2) 48 h pc. (Fig. 4B). There was no statistical difference in clinical scores between the vaccinated groups ($p < 0.05$). This was in contrast to the unvaccinated control animals (n = 2) which developed severe pyrexia and severe lesions that were generalized to all four hooves within 48–72 h of challenge (Fig. 4B). The vSAT2-93Y group had

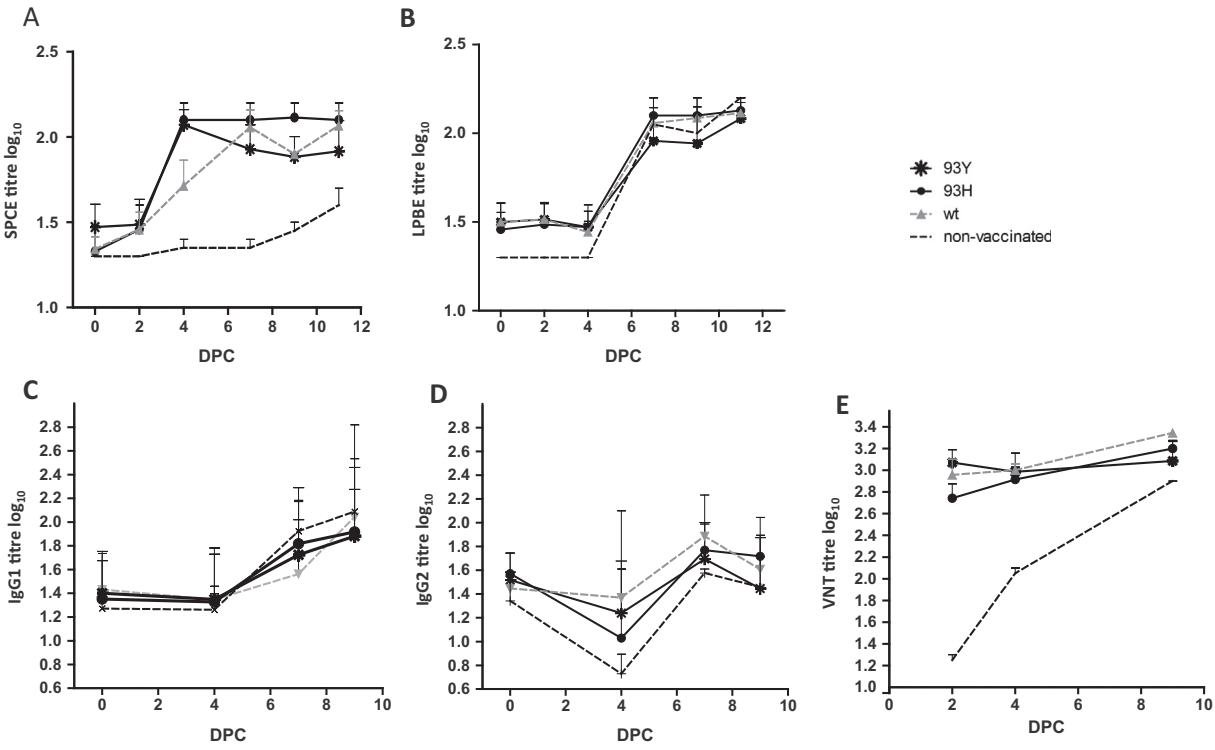

Fig. 2. Antibody kinetics of challenge responses to wildtype and stabilised antigens. Mean serum titres (log_{10}) of cattle challenged with live SAT2/ZIM/7/83 virus at 0–12 days post challenge (dpc) measured by (A) SPCE; (B) LPBE; (C) IgG1; (D) IgG2 and (E) VNT after being vaccinated twice with either vSAT2-wt (n = 7), vSAT2-93H (n = 7) or vSAT2-93Y (n = 7) antigens and unvaccinated control animals (n = 2). Error bars represent the standard deviation.

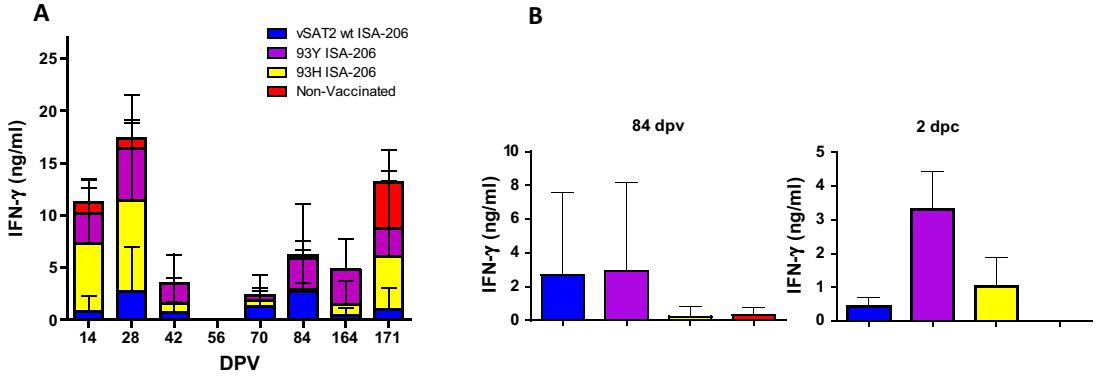

Fig. 3. Cell mediated IFN-γ responses. (A) Mean serum titres of IFN-γ (ng/ml) of Nguni cattle after being vaccinated twice with either vSAT2-wt, vSAT2-93 H or vSAT2-93Y antigens 14–84 dpv. Error bars represent the standard deviation. Arrows indicate the time of first vaccination (0 dpv) or second vaccination (42 dpv) and challenge 162–171 dpv (0–9 days post challenge). (B) Comparison of IFN-γ (ng/ml) levels at 84 dpv and 2 dpc, showing significantly higher levels ($p < 0.05$) of vSAT2-93Y at 2 dpc compared to other antigens.

the shortest period of 2–4 days of subclinical infection compared to 2–7 days with the other vaccinated groups. Additionally, less animals showed subclinical infection in the vSAT2-93Y group (n = 3) compared to vSAT2-wt (n = 6) and vSAT2-93H (n = 5).

3.5. Virus isolation and the presence of viral RNA

The presence of non-structural protein (NSP) antibodies were positive using the NSP ELISA indicative of presence of live FMDV in challenged animals, but was negative during the 5 months of vaccination. FMDV was recovered from cell culture and FMDV RNA was detected by real-time RT-PCR (positive sample Ct value <32) in OP, tonsil swab (TS) samples from 0 to 11 dpc and from whole blood on days 2–4 dpc (Fig. 4A, Suppl Fig. 1).

The following results were found for OP samples (Fig. 4A): vSAT2-wt group (n = 7): virus and/or viral RNA was detected 2–7 dpc (n = 4) whilst virus or viral RNA (n = 1), low viral RNA (n = 2) only detected 4 dpc; vSAT2-93Y group (n = 7): virus and/or viral RNA detected 2 dpc (n = 2) whilst virus or viral RNA (n = 1), viral RNA (n = 3) or low viral RNA (n = 1) detected from 4 dpc. Overall there was low detection of RNA or virus compared to other vaccinated groups and detection was later from only 4 dpc; vSAT2-93H group (n = 7): virus and/or viral RNA was detected 2–7 dpc (n = 6) whilst viral RNA (n = 1) detected 4 dpc; and the unvaccinated group (n = 2): virus and viral RNA detected from 2 to 4 dpc and virus or RNA from 7 to 9 dpc (n = 2) and also developed severe symptoms with lesions on the hooves. No animals from the vaccinated groups that were positive on virus isola-

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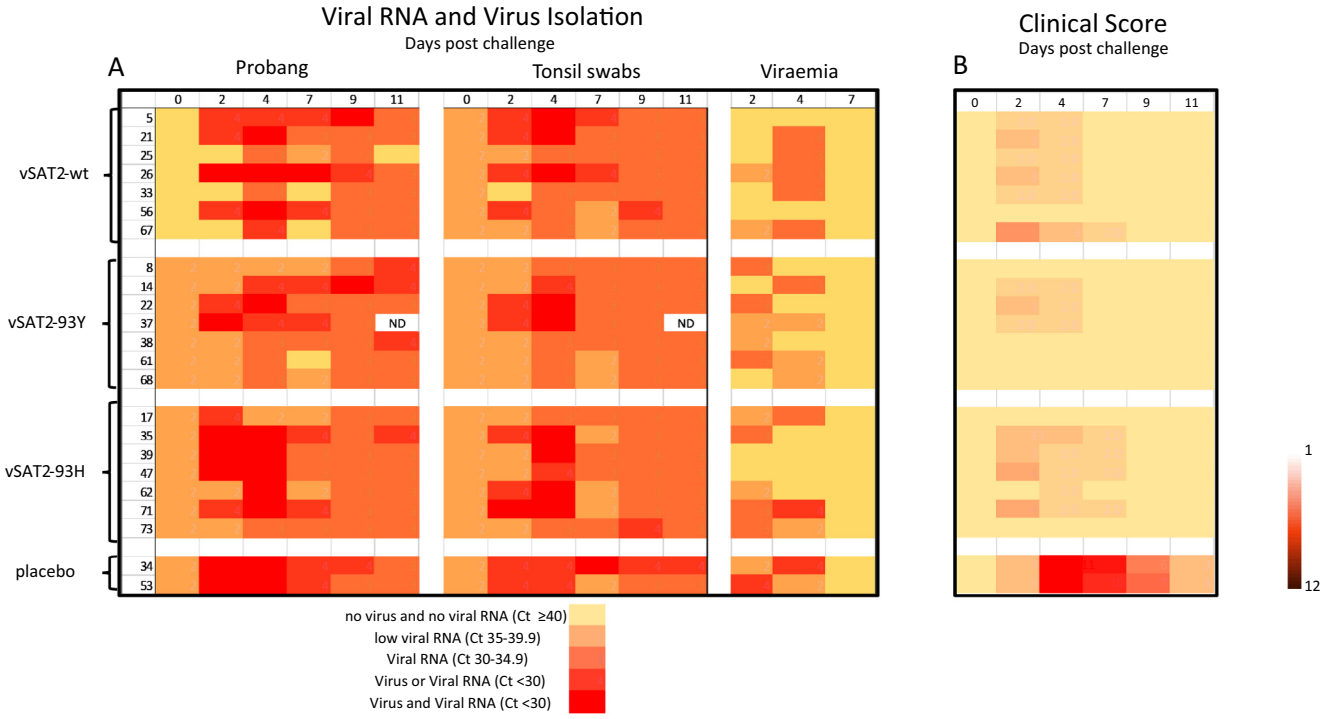

Fig. 4. Clinical scores and presence of FDMV and viral RNA 0–14 days post challenge. Summary of the (A) presence of viral RNA (determined by real-time RT-PCR) and virus isolation in oropharyngeal fluid (probang), tonsil swab and whole blood samples and (B) clinical scores of animals vaccinated with vSAT2-wt (n = 7), vSAT2-93 H (n = 7) or vSAT2-93Y (n = 7) antigens and non-vaccinated controls (n = 2) for 162 days. Thereafter challenged with SAT2/ZIM/7/83 virus. The clinical scores were calculated as described in materials and methods, 0–12 indicating low to high severity.

tion developed any lesions at sites other than site of inoculation. TS samples showed a similar result as OP samples (Fig. 4A, Suppl Fig. 1).

Viraemia was assessed 2-7dpc and virus was only isolated in the unvaccinated group (n = 2) on 2–4 dpc, with a corresponding high viral RNA load. Viral RNA was detected in blood later at 4 dpc for the vSAT2-wt group compared to the earlier presence of viral RNA from 2 dpc in the vSAT2-93Y and -93H groups (Fig. 4A, Suppl Fig. 1).

4. Discussion

In Africa, the duration of immunity and improved stability of SAT2 vaccines [14] are important considerations for FMD vaccines to be less reliant on a cold-chain in many remote areas in Africa [45]. The control of SAT2 in Africa is dependent on: (i) the selection of cross-protective strains to protect against the high antigenic diversity circulating strains and (ii) stable vaccines with intact 146S particles that will induce a strong and sustainable serological response [46]. Although SAT2 antibody responses were observed following vaccination of cattle in Mnisi, South Africa, the proportion of cattle with significant titres to previous vaccination was low suggesting that the interval between vaccinations was too long to maintain adequate antibody response [46].

In the present study, the efficacy and comparative immunological responses of two thermostable and wild-type SAT2 vaccines were assessed. Viruses generated by reverse genetic approaches with modified residues identified through *in silico* modeling [20,47] that improve stability provided the basis for the structural engineering of two improved SAT2 vaccines. The stabilised SAT2 146S antigens were previously formulated as vaccines and elicited improved immune responses with longer duration and shelf-life than wild-type in guinea pigs [20].

Cattle vaccinated with vSAT2-wt and vSAT2-93H produced strong neutralizing antibodies titres (>2.3log$_{10}$) at 21 dpv whereas the neutralizing antibody titres to vSAT2-93Y were delayed, however after second vaccination (56–113 dpv) there was no significant difference ($p > 0.1$) between the vaccinated groups. By 162 dpv all vaccinated animals still had neutralizing antibodies titres between 1.7 and 2.4log$_{10}$, correlative to protection [34,47].

Total antibody titres peaked earlier at 9 dpv for the vSAT2-wt and −93H groups compared to 14 dpv for −93Y group, however there was no difference in titre after the second vaccination. Even though by 162 dpv total antibody titres were <1.6 for most animals, which is suggestive of poor protection there were however a few animals from each group that still maintained >1.6 titres. However, following intra-dermolingual challenge all vaccinated groups were fully protected as determined by the absence of generalized lesions even though virus excretion in the oropharynx was detected 2–4 dpc. As expected unvaccinated animals developed pyrexia 1 dpc, viraemia 2–4 dpc and generalized lesions within 2 dpc. These results also provide proof that two vaccine doses, consisting of SAT2 antigen combined with ISA206 adjuvant, administered 4–6 weeks apart were able to protect animals up to 5 months pv as advised by the OIE [39]. It has been reported that FMD vaccine immunity is short-lived and cattle in endemic areas require revaccination at regular intervals of 4–6 months to ensure protective levels of antibodies [16,49]. We have not tested the duration of immunity afforded by the stabilised vaccines in cattle after 6 months, this will be tested in the future.

There were no significant differences ($p < 0.05$) between IgG1 and IgG2 titres of the vaccinated groups with IgG1 > IgG2 titres across all time points. The role of the IgG1 isotype in protection of cattle from disease has been reported [33] and a predominance of IgG1 over IgG2 antibodies in animals receiving infectious or inactivated virus [50]. T-cell responses were measured by IFN-γ (cytokine) [34] which is mainly produced by activated cells [34]

in anamnestic responses. SAT2-specific IFN-γ responses peaked at 28 dpv with vSAT2-93H having the highest concentrations followed by vSAT2-93Y. Moreover, IFN-γ anamnestic T-cell responses were higher at 84 dpv for vaccinated animals even when total Ab responses had decayed. IFN-γ responses were boosted at 2 dpc, with higher levels for vSAT2-93Y than −93H and -wt vaccinated animals. Previously it was shown that correlation with increased structural stability of the whole A24 virus and the ability to induce greater IFN-γ [42]. This data may explain the improved ability of the 93Y vaccine to have a shorter subclinical period or no symptoms at all and the importance of IFN-γ T-cell responses in vaccine-mediated protection [42].

Although we have not exposed the thermostable and wild-type vaccines to any period of shelf-life storage or any break in the cold-chain to truly test the differences in stability and immunogenicity of the two antigens it is not unreasonable to expect from the *in vitro* stability data and guinea pig trial [20] that the two antigens will outperform the wild-type antigen during long term storage. Previously it was shown that ISA 206B oil adjuvant was the most suitable adjuvant for SAT serotypes and able to elicit immune response in cattle for >50 weeks pv, however when stored for 6 months at 4 °C, it failed to protect the cattle against a homologous infection even though the vaccine's pH level and emulsion were still of acceptable quality at the time of vaccination [48]. Improved storage of SAT2 vaccines will be advantageous for the vaccine producer, which currently has to increase the antigen load to make up for the rapid decrease during storage [15].

After the second vaccination (>52 dpv) there were no differences observed between vaccinated groups. Interestingly vSAT2-93Y had significantly higher levels of IFN-γ after challenge compared to other vaccines indicative of the importance of cell mediated responses and a shorter or no subclinical period. We did not measure antibody avidity in this study, however it is not unrealistic to assume that after the second vaccination cattle developed high avidity antibodies through affinity maturation [25].

In conclusion, we have shown that two thermostable and the wild-type SAT2/ZIM7/83 vaccines are capable of eliciting strong antibody titres and IFN-γ with full protection more than 5 months pv, this is the first step in proof of concept to now advance our knowledge on where problems of SAT2 stability occur from vaccine production to the administration in the field. We intend to follow up this work with thermostable vaccination trials assessing different shelf-life conditions, disturbances in cold-chain maintenance and potency to improve stability and SAT2 control.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.02.003.

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