

# **Comparative analysis of the immunologic response induced by the Sterne 34F2 live spore *Bacillus anthracis* vaccine in a ruminant model**

Okechukwu C. Ndumnego<sup>a#</sup>, Susanne M. Köhler<sup>b\*</sup>, Jannie Crafford<sup>a</sup>, Henriette van Heerden<sup>a</sup>, Wolfgang Beyer<sup>b</sup>

Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110, Pretoria, South Africa<sup>a</sup>

Department of Livestock Infectiology and Environmental Hygiene, Institute of Animal Sciences, University of Hohenheim, Stuttgart, Germany<sup>b</sup>

<sup>#</sup>Corresponding author; Okechukwu C. Ndumnego, +27125460630,

okeyndumnego@gmail.com

O.C.N. and S.M.K. contributed equally to this work.

\* Current address: Robert Koch Institute, Nordufer 20, 13353, Berlin, Germany

## Highlights

- Anti-BclA IgG absent in goats immunized with the Sterne 34F2 live spore vaccine
- Booster vaccinations with Sterne vaccine induced robust humoral immunity in goats
- Preliminary challenge studies indicate infective dose of 36 spores in naïve goats
- Anthrax infection was peracute in naïve goats with incubation period of 2-3days
- Four in five goats protected against anthrax a year after single Sterne vaccination

## Abstract

The Sterne 34F2 live spore vaccine (SLSV) developed in 1937 is the most widely used veterinary vaccine against anthrax. However, literature on the immunogenicity of this vaccine in a target ruminant host is scarce. In this study, we evaluated the humoral response to the *B. anthracis* protective antigen (rPA), a recombinant *bacillus* collagen-like protein of *anthracis* (rBclA), formaldehyde inactivated spores (FIS) prepared from strain 34F2 and a vegetative antigen formulation prepared from a capsule and toxin deficient strain (CDC 1014) in Boer goats. The toxin neutralizing ability of induced antibodies was evaluated using an *in vitro* toxin neutralization assay. The protection afforded by the vaccine was also assessed in vaccinates. Anti-rPA, anti-FIS and lethal toxin neutralizing titres were superior after booster vaccinations, compared to single vaccinations. Qualitative analysis of humoral responses to rPA, rBclA and FIS antigens revealed a preponderance of anti-FIS IgG titres following either single or double vaccinations with the SLSV. Antibodies against FIS and rPA both increased by 350 and 300-fold following revaccinations respectively. There was no response to rBclA following vaccinations with the SLSV. Toxin neutralizing titres increased by 80-fold after single vaccination and 700-fold following a double vaccination. Lethal challenge studies in naïve goats indicated a minimum infective dose of 36 *B. anthracis* spores. Single and double vaccination with the SLSV protected 4/5 and 3/3 of goats challenged with > 800 spores respectively. An early

booster vaccination following the first immunization is suggested in order to achieve a robust immunity. Results from this study indicate that this crucial second vaccination can be administered as early as 3 months after the initial vaccination.

**Keywords:** Anthrax; Sterne vaccine; vaccination; immunity; goat; antibodies

## 1. Introduction

Anthrax is a primary disease of herbivores caused by the Gram-positive bacterium *Bacillus anthracis* (Hambleton et al., 1984, 125-132). The disease develops as a peracute or acute infection in ruminants following an incubation period of 3-5 days (Beyer and Turnbull, 2009, 481-489), which often elapses without clinical signs until shortly before death due to sudden septicemic shock. Clinical signs and course of infection are largely dependent on the species and the route of infection with ambiguous early signs (Hambleton et al., 1984, 125-132). The pathogen expresses two major plasmid encoded virulence factors, a gamma-linked poly-D-glutamic acid capsule [(PGDA) (coded by pX02)] and a tripartite toxin (coded by pX01) comprising of protective antigen (PA), lethal factor (LF) and edema factor (EF) (See review **Mourez, 2005, 135-164**). Virulence of *B. anthracis* is dependent on the presence of both plasmids (Green et al., 1985, 291-297).

The PDGA is weakly immunogenic and assists in post infection dissemination of *B. anthracis* (Candela and Fouet, 2005, 717-726). The capsule enables the anthrax bacilli to evade immune surveillance mechanisms and enter the circulatory system where it proliferates systemically (Sutherland et al., 2008, 899-906). PA combines with LF to form lethal toxin, a zinc metalloprotease that inactivates most mitogen-activated protein kinase kinases (MAPKK) and the inflammasome-activating NLRP1B leading to impairment and death of

susceptible macrophages (Friedlander, 1986, 7123-7126, Chavarría-Smith and Vance, 2013, e1003452). Edema toxin (ET), a calmodulin dependent adenylate cyclase formed by the binding of PA to EF, disrupts fluid homeostasis across the host cell membranes (Mourez, 2005, 135-164).

The current anthrax veterinary Sterne live spore vaccine (SLSV) is a non-encapsulated but toxigenic variant 34F2 that was developed in 1937 by Max Sterne (Sterne, 1937, 49-67). This vaccine is still extensively used in the control of anthrax in domestic animals (International Office of Epizootics, 2008, 94-95). Constraints that include limited duration of immunity, failure to induce protective immunity, variation in vaccine quality and adverse reactions in sensitive species, such as llamas (*Lama glama*) and goats (*Capra aegagrus hircus*) have been reported (Cartwright et al., 1987, 715-716, Turnbull, 1991, 533-539). The current OIE manual stipulates annual vaccination of animals against the disease (OIE, 2012, 135-144). Immune parameters and correlates to protection for anthrax in goats are limited in literature because serological methods were not readily available during earlier vaccine trials in domestic species. These trials mainly depended on clinical responses and level of protection after vaccination (Sterne, 1937, 49-67, Sterne et al., 1942, 53).

The principal immune response induced by vaccination of animals with the SLSV is the development of antibodies against PA (International Office of Epizootics, 2008, 94-95). Anti-PA antibodies prevent the development of lethal intoxication and protection against anthrax is mainly provided by the development of immunity to the antigen (Shakya et al., 2007, 5374-5377). The presence of antibodies against the spore (formaldehyde inactivated spores, FIS) and spore-associated antigens such as the *bacillus* collagen-like protein of *anthracis* (BclA) and vegetative antigens has been reported to augment the protection afforded to animals (Brossier et al., 2002, 661-664, Hahn et al., 2006, 4569-4571). Hence we sought to evaluate the humoral immune response

in Boer goats directed against these antigens following single or booster vaccinations with the SLSV. Antibody responses were assessed using ELISA. Also, the ability of induced antibodies to neutralize lethal toxin was measured using the *in vitro* toxin neutralization assay (TNA). The level of protection following vaccination was evaluated by challenge with virulent *B. anthracis* spores.

## **2. Materials and methods**

### **2.1 Animals**

Eight-week old female BALB/c mice [(n=6) (South African Vaccine Producers, Sandringham, South Africa)] were used to confirm the virulence of the *B. anthracis* challenge strain (Welkos et al., 1986, 795-800). Twenty-six 1-year naïve old Boer goats were housed at Onderstepoort Biological Products (OBP), South Africa after screening for anti-rPA83 cross-reactive antibodies. Lethal challenge studies were conducted at a remote site in an endemic area of the Kruger National Park (KNP), South Africa. Animal experiments and clinical score sheets for monitoring experimental animals were drawn up according to the guidelines of the National Research Council of the USA (Clark et al., 1996, 21-34) and approved by the animal use and care committees of the South African national parks, OBP and University of Pretoria (Protocol numbers V041-10 and V065/12) respectively. Approval for Section 20 of the animal disease act 35 of 1984 was granted by the Directorate of Animal Health, Department of Agriculture, Forestry and Fisheries, South Africa (registration number 12/11/1/1/6).

### **2.2 *B. anthracis* vaccine and challenge strains**

Animals were vaccinated using the SLSV as recommended by the manufacturer [(OBP, Onderstepoort, South Africa) (OBP)]. Challenge was performed with a virulent South

African *B. anthracis* strain (20SD) isolated from a sheep in 2001. The presence of both plasmids, pXO1 and pXO2, was confirmed using real time PCR and sequencing (Lekota et al., 2015, 10.1128/genomeA.01313-15).

Spores from the challenge strain were prepared as previously described with minor modifications (Welkos et al., 2011, 4238-4250). Virulence of the spores was confirmed in BALB/c mice and naïve goats. Two groups of 3 mice received an intra-peritoneal challenge of ~500 and ~1000 spores respectively.

Two goats from each of the 3 negative control groups (NCG1-3) were challenged (subcutaneously in the thigh) with 36, 172 and 844 spores respectively. Spore numbers in the respective challenge doses were estimated by counting colony forming units (cfu) prepared from redundant doses. The highest dose of 844 spores was subsequently used for the challenge of the SLSV vaccinated goats (SVG1 to 3, Table 1). Death from anthrax was confirmed after microscopic demonstration of Gram-positive encapsulated rod-shaped bacilli in stained blood smears.

### **2.3. Experimental design**

The immunogenicity and protectiveness of the SLSV were evaluated in four scenarios using 5 goats per group [(SVG1 to 4) (Table 1)]. Two groups were vaccinated once and challenged after 6 (SVG1) and 62 (SVG2) weeks respectively. SVG3 was vaccinated twice at weeks 0 and 58 before lethal challenge 4 weeks later. A fourth group of goats (SVG4) was vaccinated at weeks 0 and 12 to evaluate the titre development in a shortened two vaccination schedule. However, ethical approval for lethal challenge was not obtained for this group. Blood for serum harvest was collected as indicated in Table 1 and stored at -20°C.

Following lethal challenge, the goats were monitored for a period of 14 days for signs of pyrexia (temperatures of  $\geq 40$  °C) and abnormal behaviour. Goats were euthanized by sodium pentobarbitone overdose (Eutha-naze®, Bayer, Isando, South Africa, 400 mg/kg body mass) as soon as bacilli rods were detected in blood smears. Survivors were treated with procaine benzylpenicillin (Depocillin®, Intervet, Spartan, South Africa, 20mg/kg body mass) and euthanized after confirmation of absence of bacilli rods in blood smears.

#### **2.4. Serology**

Sera were analysed with indirect ELISAs for specific antibodies against recombinant protective antigen (rPA83), recombinant bacillus collagen-like protein of anthracis (rBclA), formaldehyde inactivated spores (FIS) and a vegetative antigen formulation derived from a capsule and toxin deficient strain (CDC 1014). *Escherichia coli* BL21-CodonPlus-RIL cells (Stratagen, LaJolla, USA) harbouring the plasmid pREP4 (Qiagen, Venlo, Netherlands) and pQE-30 (Qiagen, Venlo, Netherlands) encoding either rPA83 or rBclA were grown and purified as previously described (Hahn et al. 2004) for the proteins used in the ELISA. The FIS suspension from the SLSV strain (34F2) was prepared as previously described (Brossier et al., 2002, 661-664) with some modifications. Spores ( $10^9$  spores/mL concentrations) were inactivated at 37 °C overnight in PBS with 4 % formalin solution. Following incubation, the spores were pelleted ( $4000 \times g$ , 15 min, room temperature) and washed four times with PBS in 0.1 % gelatine (Merck, Darmstadt, Germany). The final pellet was re-suspended in PBS and stored at -80 °C. An aliquot of the FIS preparation was tested for sterility by treatment with histidine (to neutralise any remaining formalin) and streaking on blood agar. For the vegetative antigen preparation, a *B. anthracis* strain (pX01–, pX02–) was grown in brain heart infusion broth with bicarbonate over night at 37 °C. The cells were pelleted at  $4000 \times g$  for 30 min at 4 °C and washed with PBS. The pellet was mechanically and chemically lysed by briefly

freezing in liquid nitrogen and subsequently thawing through addition of 20 ml PBS spiked with 1 % SDS. To further increase lysis the suspension was sonicated 10 times for 5 - 10 seconds on ice. Insoluble fractions were pelleted and discarded. The supernatant was passed through a filter (0.45  $\mu\text{m}$ ) and checked for sterility. Protein concentration was assessed through the Bicinchoninic acid (BCA) method (Walker, 2009, 11-15). ELISAs were essentially performed as previously described (Hahn et al., 2004, 35-44) with some modifications utilizing horseradish peroxidase-conjugated rabbit anti-goat IgG (Invitrogen, Camarillo, USA) as secondary antibody. Wells were coated with 0.5  $\mu\text{g}$  antigen/well or  $10^8$  FIS/well. Sera were serially diluted in two-fold steps. Endpoint titres of individual sera were defined as the reciprocal of the highest serum dilution giving an optical density of 0.1.

An *in vitro* toxin neutralising assay (TNA) was performed using the mouse macrophage cell line J774A.1 (European collection of cell cultures ECACC cat number 91051511) as previously described with slight modifications (Ndumnego et al., 2013, 265). Briefly, 96-well flat-bottomed tissue culture plates (Greiner bio one, Germany) containing 80 000 macrophages/well in DMEM and 10 % FCS were incubated overnight at 37 °C and 5 % CO<sub>2</sub>. Goat sera (starting dilution; 1:50) were doubly diluted in culture medium containing PA and LF (List Biological Laboratories Inc., Campbell, USA) at concentration of 500 ng/mL and 400 ng/mL (lethal toxin, LT) respectively. The sera/LT mixture was incubated for 1 h at 37 °C and 5 % CO<sub>2</sub> before adding to the overnight cultured cells (after discarding medium) and incubated for 3 h. Each serum sample was tested in duplicates. Following incubation, 25  $\mu\text{L}$  of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Invitrogen, USA) was added to each well and incubated in the dark at 37 °C and 5 % CO<sub>2</sub>. After 2 h incubation, the cells were lysed with pre-warmed (37 °C) acidified isopropyl alcohol (90% isopropyl alcohol, 0.5 % SDS (w/v), 25 mM HCl) to solubilize the formazan dye. The plates were

rested for 5 min and the absorbance readings taken at 540 nm with a Biotek power wave XS2 reader. Endpoint titres of individual sera were defined as the reciprocal of the highest serum dilution neutralizing 50 % of the LT cytotoxicity.

## **2.5. Data Analysis**

Means, standard deviations and coefficient of variation from ELISA and TNA data were computed using the Gen5 data analysis software (Biotek Instruments, Winooski, USA). Homogeneity of variances was preliminarily tested using the Levene's tests (IBM SPSS Statistics 23, Sandton, South Africa). Differences in antibody titres at specific time points were analysed using paired (intra-group comparison) or unpaired (inter-group comparison) student *t*-test, with a two-tailed *P*-value. Log-rank test was used to compare the survival times following virulent challenge. *P*-values of  $\leq 0.05$  were considered statistically significant.

## **3. Results**

### **3.1 Recombinant protective antigen (rPA83) ELISA and toxin neutralizing assay (TNA) titres**

A single vaccination with the SLSV induced high anti-rPA83 IgG and toxin neutralizing titres within four weeks (Figs 1 and 2). After the initial recorded peak, the titres declined to a constant level that was still significantly elevated ( $P \leq 0.017$ ) until either challenge (SVG2) or revaccination (SVG3) when compared to titres before vaccination. Revaccination at week 58 with the SLSV (SVG3) induced higher rPA83 and toxin neutralizing antibodies compared

to **SVG2 titres** as can be seen in Figures 1 and 2 (SVG3 ▼). These titres decreased slightly after lethal challenge with *B. anthracis* spores. In contrast, a titre increase was observed in survivors (SVG2 Δ).

### **3.2 Spore and vegetative antigen ELISA titres**

Mean anti-spore (FIS) IgG titres in the SLSV vaccinated goats were significantly increased (Fig 3,  $P = 0.002$ ) four weeks after the first immunization, then gradually declined until week 20, when the titres remained at a constant level until the next vaccination or challenge. No change in IgG titres could be measured against rBclA for any of the vaccinated animals on all the given time points (data not shown).

Titres against the vegetative antigen were increased 4 weeks after the first immunization (Fig 4) and remained at this elevated level throughout the year. The revaccination of SVG3 significantly increased the titres (compared to SVG2). Titres of survivors remained unchanged after challenge. There was no change in titres recorded for SVG1 for all time points (data not shown).

### **3.3 Single versus double vaccination – antibody responses to ELISA antigens and lethal toxin**

Qualitative analysis of humoral response to FIS, PA, and BclA antigens by ELISA revealed the preponderance of anti-FIS IgG titres following either single or double vaccinations. There was a 30-fold increase in anti-FIS titres following a single vaccination (Fig 5). This increased by 350-fold in goats that were vaccinated twice three months apart. Anti-PA IgG titres increased by 10-fold after a single vaccination and by 300-fold following double vaccinations. There was no response to rBclA following either

single or double vaccinations with the SLSV (not shown). The functional ability of induced antibodies to neutralize anthrax lethal toxin was assessed in the TNA. There were 80 and 700-folds increases in neutralizing titres following single and double vaccinations respectively.

### **3.4 Virulence and protectiveness**

A summary of the time to death of naïve goats challenged with different *B. anthracis* spore doses is presented in Table 2. Our study indicated that a subcutaneous dose of 36 *B. anthracis* spores is enough to establish a lethal infection in goats.

All goats vaccinated with the SLSV (SVG1 to 3) were challenged with 844 spores of a fully virulent *B. anthracis* strain. Goats in SVG1 were challenged 6 weeks after a single vaccination and demonstrated a 60% (3/5) survival rate. Animals challenged 62 weeks after a single vaccination (SVG2) had a survival rate of 80% (4/5) while all the goats of the twice-vaccinated group (SVG3) survived. The vaccinated groups (SVG1-3) were significantly better protected when compared to the naïve control groups [(NCG1-3) ( $P \leq 0.011$ )]. There was no significant difference in survival rate amongst the respective vaccination groups [ $P \geq 0.246$ ] (Fig 6)].

## Discussion

In the early 20<sup>th</sup> century, anthrax was a major cause of death in livestock within South Africa [(over 1800 outbreaks reported in 1920) (Gilfoyle, 2006, 465-490)]. The introduction of compulsory vaccination of livestock with the SLSV drastically reduced the disease incidence. However, little is known about the specific immunity induced by the vaccine in the target ruminant host. It should be noted that most of the extensive research done on anthrax vaccines were performed on laboratory rodents and non-human primates in attempts to improve the current human vaccine. In this study we evaluated and compared the humoral immune response in goats following single and double vaccinations. Booster vaccination with the SLSV delivered a qualitatively better immune response with high fold increases in ELISA titres and toxin neutralizing capacity. Also, survival data from our challenge studies indicated that a single vaccination with the SLSV protected  $\geq 60\%$  (3/5) of challenged animals. All the goats (3/3) vaccinated twice with the SLSV survived lethal challenge. However, more challenge studies with larger animal numbers will be needed to prove if double SLSV vaccinations offer significantly better protection against anthrax.

In anthrax endemic localities where regular vaccination is essential to protect susceptible animals against the disease, annual revaccinations are recommended by the OIE guidelines (OIE, 2012, 135-144). Results from our study indicate that boosting only after three months (SVG4) induces a more robust immune response against anthrax antigens compared to a single vaccination (SVG1). One could argue that, the optimal time point for the booster can be from as early as one month after the first vaccination as recommended for horses (OIE, 2012, 135-144) to three months based on empirical evidence from our study. Lethal challenge four weeks after revaccination (SVG3) did not increase any of the measured antibody titres and thus no post challenge anamnestic humoral response was detected. This was likely due

to the peaking of antibody production (or titres) following revaccination of the animals (SVG3) before challenge. The revaccination of animals should therefore be performed earliest when the antibody titres drop to minimal stable values for maximum effect. Antibody titres measured for SVG2 and 3 decreased shortly after the initial peak in week 4 and reached stable values in month 3 and 4. These values remained stable until either revaccination or challenge. This can be adduced to sustained antibody production by long-living plasma cells (Tizard, 2008, 152-169) engendered by antigen persistence following administration of the SLSV (Garman et al., 2014, 2424-2431). One can therefore assume that booster vaccinations should be applied no earlier than 3-4 months after an effective initial vaccination. To test this hypothesis, SVG4 was immunized twice three months apart with the Sterne live spore vaccine. The postponed second vaccination resulted in over 300-fold increase in anti-PA and anti-spore titres and a 700-fold increase in toxin neutralization titres compared to the baseline values (Fig 5). In all, a much improved antibody response was generated against these antigens following double vaccination with the SLSV.

Considering that there was no difference in SVG3 anti-PA titres 4 weeks after the first and second immunization, the anti-FIS, anti-vegetative and TNA titres were significantly higher ( $P \leq 0.019$ ) after the second immunization. This may be important, given that for evaluation of anthrax vaccines, anti-PA ELISA titres are considered a main indicator of protection (Turnbull, 1991, 533-539). With anti-PA titres essentially equal after first and second immunization, this misconception could fuel the notion that there is virtually no difference in protection, as the vaccine is advertised to convey full protection after a single immunization. However, larger trials will be needed in order to establish if animals stand a better chance of surviving an outbreak after a second immunization.

The anti-FIS and vegetative titres after revaccination at week 58 (of SVG3) were approximately 10-fold higher than the titres measured after the first vaccination. This

anamnestic response induced by the 34F2 vaccine is not seen when vaccinated animals are challenged with virulent spores as observed in pre and post-challenge titres of SVG2 animals (Fig 3). The absence of reaction against the vegetative antigens might be due to capsule formation in the fully virulent challenge strain thus obscuring the respective antigens. This was not totally unexpected as the capsule PGDA layer has been reported to obscure germinated *B. anthracis* from immune surveillance (Candela and Fouet, 2005, 717-726). Surprisingly, we found no change in anti-BclA titres for any group immunized with SLSV or challenged with a fully virulent strain of *B. anthracis*. BclA, being the most immunogenic structure of the exosporium, has been shown to be immunogenic in other species (Steichen et al., 2003, 1903-1910). Likewise, immunization with SLSV in other species resulted in detectable anti-BclA titres (Hahn et al., 2006, 4569-4571, ChunQiang et al., 2008, 774-782). The absence of antibodies against BclA in the immunized goats in our study was unforeseen and contradicts the current assumption on the importance of BclA as an antigen. Recent studies exposed BclA as not being the only relevant immunogen of the spore and further defined its role rather as means of masking these other, possibly much more relevant spore antigens (Cybulski et al., 2008, 4927-4939, Cote et al., 2012, 1380-1392). Taking the lack of reactivity against BclA in goats into account, this might be a much more pressing issue in this animal model and should be considered in future live and acellular vaccine trials.

As previously documented (OIE, 2012, 135-144), the disease progressed as a peracute septicaemia with few clinical signs in naïve animals. The incubation period ranged from 2-3 days in unvaccinated goats to 2-10 days in vaccinates. The absence of pronounced clinical signs following lethal challenge confirms the peracute nature of the disease in **goats**. Use of the SLSV has led to occasional field reports of swelling at the injection sites and occasional mortalities (Cartwright et al., 1987, 715-716). However, this has not been observed in this or earlier studies (Shakya et al., 2007, 5374-5377) and is only indicated in

studies concerning the increased sensitivity of certain inbred mice strains and guinea pigs towards the Sterne live vaccine (Little and Knudson, 1986, 509-512, Welkos et al., 1986, 795-800). Goats could be more sensitive to the vaccine strain as a result of adverse effects due to the choice of inoculation site or are possibly more prone to concurrent infections generating adverse effects to vaccination with the live spore vaccine. During this study, two of the original five animals of SVG3 and one of the original three NCG1 animals were diagnosed with heartwater (*Ehrlichia ruminantium*) and were excluded from the challenge study phase. Such undetected and related co-infections, frequently present in goats could play a role in the record of adverse reactions in these animals and in cases of fatalities could easily lead to the misconception as vaccine-related cause of death.

In summary, this is the first study that describes the antibody dynamics over a year period in a ruminant species vaccinated with the Sterne 34F2 vaccine. The results of this study indicate that booster vaccination improves humoral immunity and may also increase the chances of survival against a lethal infection.

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## References

- Beyer, W., Turnbull, P.C., 2009. Anthrax in animals. *Mol. Aspects Med.* 30, 481-489.
- Brossier, F., Levy, M., Mock, M., 2002. Anthrax spores make an essential contribution to vaccine efficacy. *Infect. Immun.* 70, 661-664.
- Candela, T., Fouet, A., 2005. *Bacillus anthracis* CapD, belonging to the  $\gamma$ -glutamyltranspeptidase family, is required for the covalent anchoring of capsule to peptidoglycan. *Mol. Microbiol.* 57, 717-726.
- Cartwright, M.E., McChesney, A.E., Jones, R.L., 1987. Vaccination-related anthrax in three llamas. *J. Am. Vet. Med. Assoc.* 191, 715-716.
- Chavarría-Smith, J., Vance, R.E., 2013. Direct proteolytic cleavage of NLRP1B is necessary and sufficient for inflammasome activation by anthrax lethal factor. *PLoS Pathog* 9, e1003452.
- ChunQiang, L., Nuttall, S.D., Tran, H., Wilkins, M., Streltsov, V.A., Alderton, M.R., 2008. Construction, crystal structure and application of a recombinant protein that lacks the collagen-like region of BclA from *Bacillus anthracis* spores. *Biotechnol. Bioeng.* 99, 774-782.
- Clark, J., Baldwin, R., Bayne, K., Brown, M., Gebhart, G., Gonder, J., Gwathmey, J., Keeling, M., Kohn, D., Robb, J., 1996. Guide for the care and use of laboratory animals. Washington, DC: Institute of Laboratory Animal Resources, National Research Council 125, 21-34.
- Cote, C.K., Kaatz, L., Reinhardt, J., Bozue, J., Tobery, S.A., Bassett, A.D., Sanz, P., Darnell, S.C., Alem, F., O'Brien, A.D., Welkos, S.L., 2012. Characterization of a multi-component anthrax vaccine designed to target the initial stages of infection as well as toxemia. *J. Med. Microbiol.* 61, 1380-1392.
- Cybulski, R.J., Sanz, P., McDaniel, D., Darnell, S., Bull, R.L., O'Brien, A.D., 2008. Recombinant *Bacillus anthracis* spore proteins enhance protection of mice primed with suboptimal amounts of protective antigen. *Vaccine* 26, 4927-4939.
- Friedlander, A.M., 1986. Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. *J. Biol. Chem.* 261, 7123-7126.

- Garman, L., Smith, K., Farris, A.D., Nelson, M.R., Engler, R.J., James, J.A., 2014. Protective Antigen-Specific Memory B Cells Persist Years after Anthrax Vaccination and Correlate with Humoral Immunity. *Toxins* 6, 2424-2431.
- Gilfoyle, D., 2006. Anthrax in South Africa: economics, experiment and the mass vaccination of animals, c. 1910-1945. *Med. Hist.* 50, 465-490.
- Green, B.D., Battisti, L., Koehler, T.M., Thorne, C.B., Ivins, B.E., 1985. Demonstration of a capsule plasmid in *Bacillus anthracis*. *Infect. Immun.* 49, 291-297.
- Hahn, U.K., Alex, M., Czerny, C.P., Bohm, R., Beyer, W., 2004. Protection of mice against challenge with *Bacillus anthracis* STI spores after DNA vaccination. *Int. J. Med. Microbiol.* 294, 35-44.
- Hahn, U.K., Boehm, R., Beyer, W., 2006. DNA vaccination against anthrax in mice-combination of anti-spore and anti-toxin components. *Vaccine* 24, 4569-4571.
- Hambleton, P., Carman, J.A., Melling, J., 1984. Anthrax: the disease in relation to vaccines. *Vaccine* 2, 125-132.
- International Office of Epizootics, 2008. Anthrax in humans and animals. In: Anonymous , World Health Organization, Geneva, Switzerland, pp. 94-95.
- Lekota, K.E., Mafofo, J., Madoroba, E., Rees, J., van Heerden, H., Muchadeyi, F.C., 2015. Draft Genome Sequences of Two South African *Bacillus anthracis* Strains. *Genome Announc* 3, 10.1128/genomeA.01313-15.
- Little, S.F., Knudson, G.B., 1986. Comparative efficacy of *Bacillus anthracis* live spore vaccine and protective antigen vaccine against anthrax in the guinea pig. *Infect. Immun.* 52, 509-512.
- Mourez, M., 2005. Anthrax toxins. *Rev. Physiol. Biochem. Pharmacol.*, 135-164.
- Ndumnego, O.C., Crafford, J., Beyer, W., Van Heerden, H., 2013. Quantitative anti-PA IgG ELISA; assessment and comparability with the anthrax toxin neutralization assay in goats. *BMC veterinary research* 9, 265.
- OBP, Manufacturer's insert: Anthrax spore vaccine 2012.
- OIE, A.H.S., 2012. Anthrax. In: Anonymous Manual of diagnostic tests and vaccines for terrestrial animals (OIE Terrestrial Manual 2008), OIE, Paris, pp. 135-144.
- Shakya, K.P., Hugh-Jones, M.E., Elzer, P.H., 2007. Evaluation of immune response to orally administered Sterne strain 34F2 anthrax vaccine. *Vaccine* 25, 5374-5377.
- Steichen, C., Chen, P., Kearney, J.F., Turnbough, C.L., Jr, 2003. Identification of the immunodominant protein and other proteins of the *Bacillus anthracis* exosporium. *J. Bacteriol.* 185, 1903-1910.

Sterne, M., Nicol, J., Lambrechts, M., 1942. The effect of large scale active immunization against anthrax. *JS Afr.Vet.Med.Assoc* 13, 53.

Sterne, M., 1937. The Effects of Different Carbon Dioxide Concentrations on the Growth of Virulent Anthrax Strains. Pathogenicity and Immunity Tests on Guinea-Pigs and Sheep with Anthrax Variants derived from Virulent Strains. *Onderstepoort Journal of Veterinary Science* 9, 49-67.

Sutherland, M.D., Thorkildson, P., Parks, S.D., Kozel, T.R., 2008. In vivo fate and distribution of poly- $\gamma$ -d-glutamic acid, the capsular antigen from *Bacillus anthracis*. *Infect. Immun.* 76, 899-906.

Tizard, I., 2008. B Cells and their response to antigens. In: *Veterinary immunology. An introduction.*, Elsevier Health Sciences, St. Louis, Missouri, pp. 152-169.

Turnbull, P.C.B., 1991. Anthrax vaccines: past, present and future. *Vaccine* 9, 533-539.

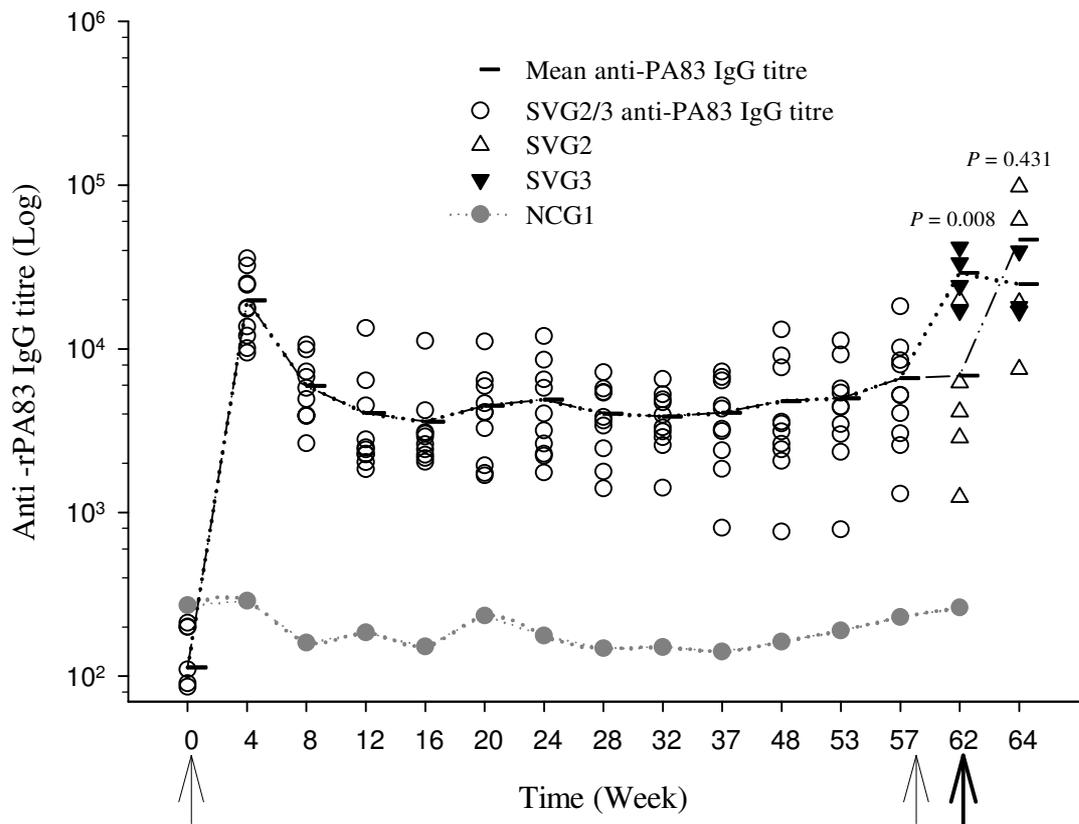
Walker, J.M., 2009. The bicinchoninic acid (BCA) assay for protein quantitation. In: *Anonymous The Protein Protocols Handbook*, Springer, pp. 11-15.

Welkos, S.L., Keener, T.J., Gibbs, P.H., 1986. Differences in susceptibility of inbred mice to *Bacillus anthracis*. *Infect. Immun.* 51, 795-800.

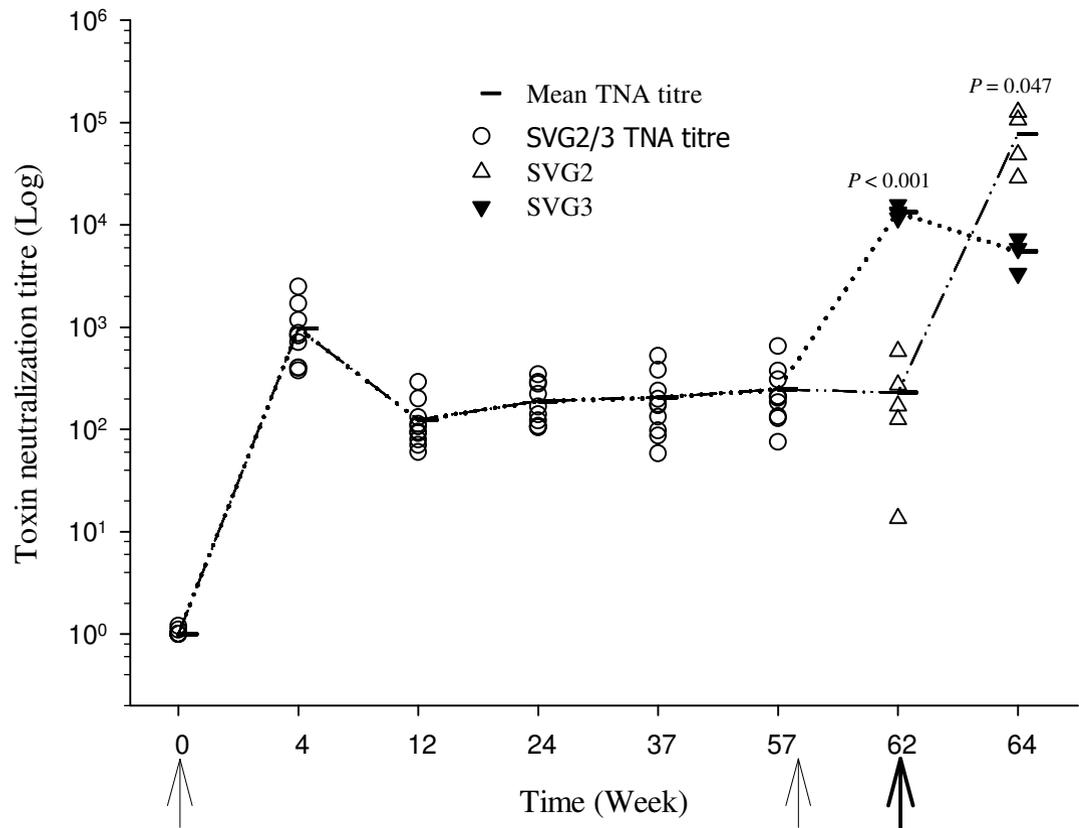
Welkos, S., Cote, C.K., Hahn, U., Shastak, O., Jedermann, J., Bozue, J., Jung, G., Ruchala, P., Pratikhya, P., Tang, T., Lehrer, R.I., Beyer, W., 2011. Humanized theta-defensins (retrocyclins) enhance macrophage performance and protect mice from experimental anthrax infections. *Antimicrob. Agents Chemother.* 55, 4238-4250.

## List of Figures and captions

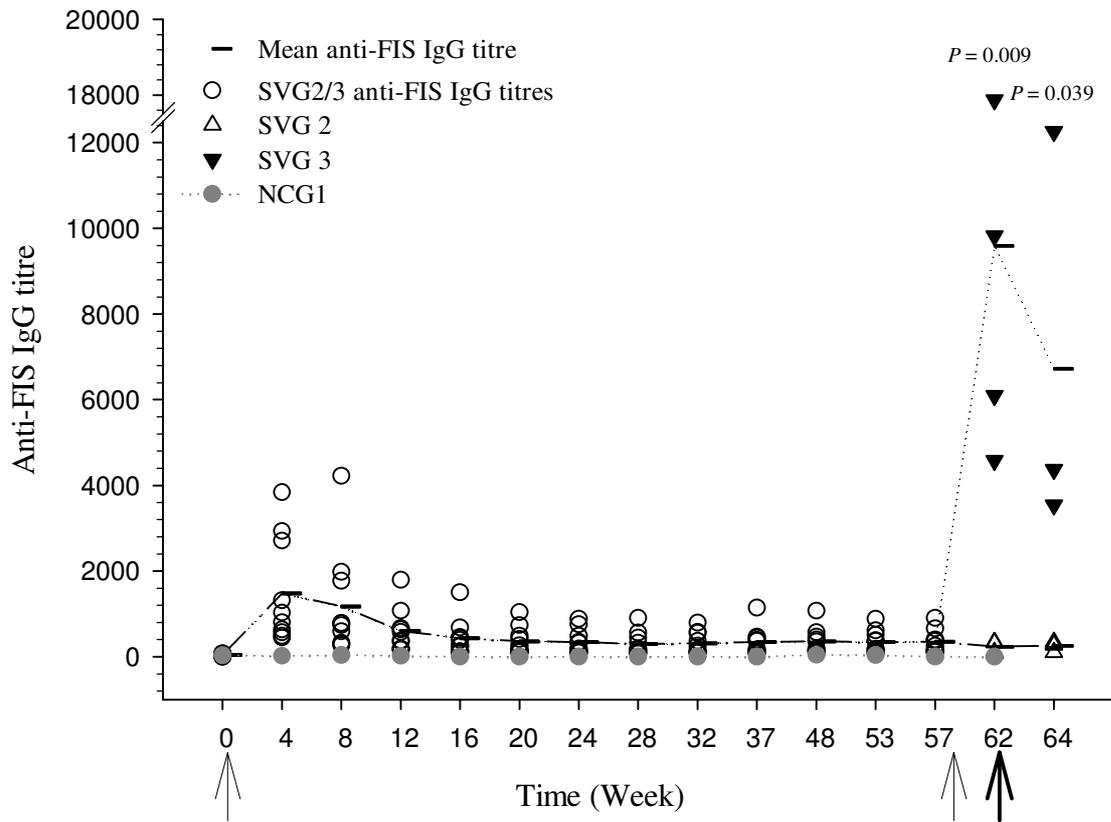
**Fig 1:** Anti-rPA83 (recombinant protective antigen 83kDa) titres following Sterne live spore vaccine immunizations (light arrow) and virulent *Bacillus anthracis* spores challenge (**dark arrow**) in goats. SVG2 was vaccinated once on week 0, SVG3 was vaccinated twice on weeks 0 and 58. SVG2, SVG3 and the unvaccinated negative control (NCG1) were challenged on week 62. Individual titres for all animals in SVG2 and SVG3 are indicated with “O” for the first 57 weeks. *P*-values indicate differences in titres between SVG2 and SVG3 before and after lethal challenge (weeks 62 and 64) respectively.



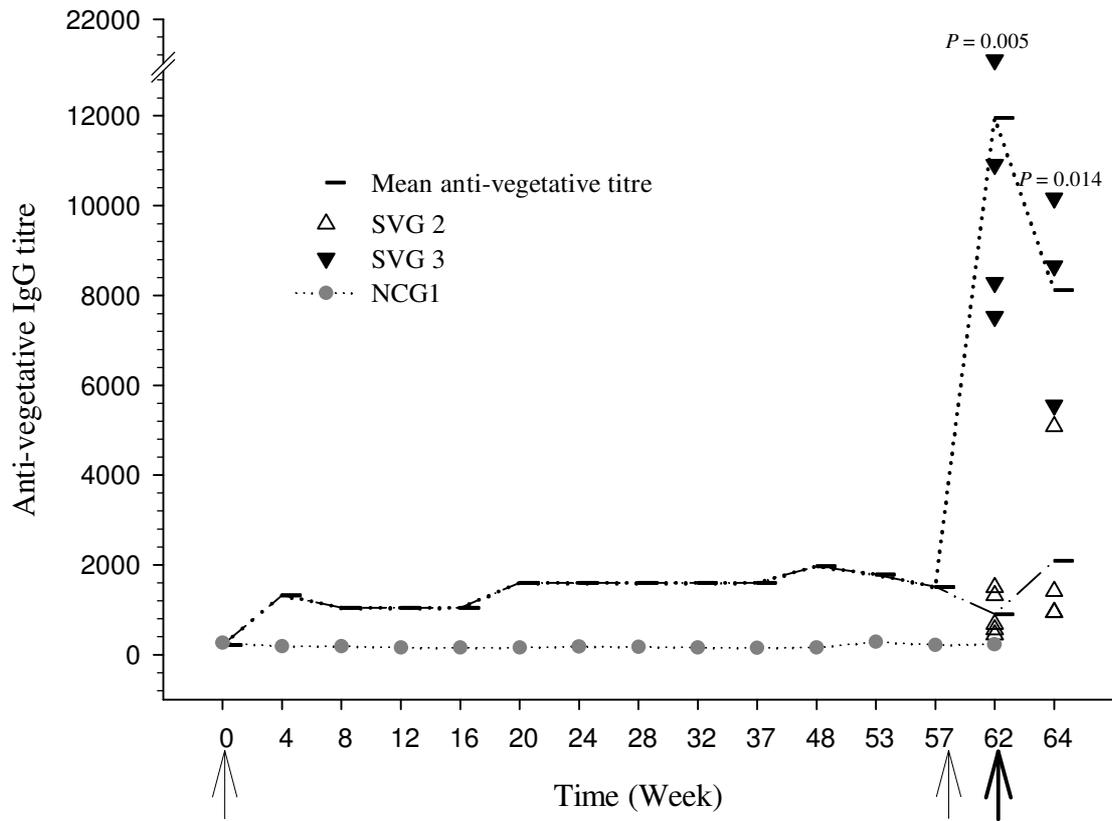
**Fig 2:** Toxin neutralizing titres following Sterne live spore vaccine immunizations (light arrow) and virulent *Bacillus anthracis* spores challenge (**dark arrow**) in goats. SVG2 (vaccinated once on week 0) and SVG3 (vaccinated twice on weeks 0 and 58) were challenged on week 62. Individual titres for all animals in SVG2 and SVG3 are indicated with “O” for the first 57 weeks. *P*-values indicate differences in titres between SVG2 and SVG3 before and after lethal challenge (weeks 62 and 64) respectively. **Toxin neutralizing titres were not detected for any of the negative control (unvaccinated) animals throughout the experiment.**



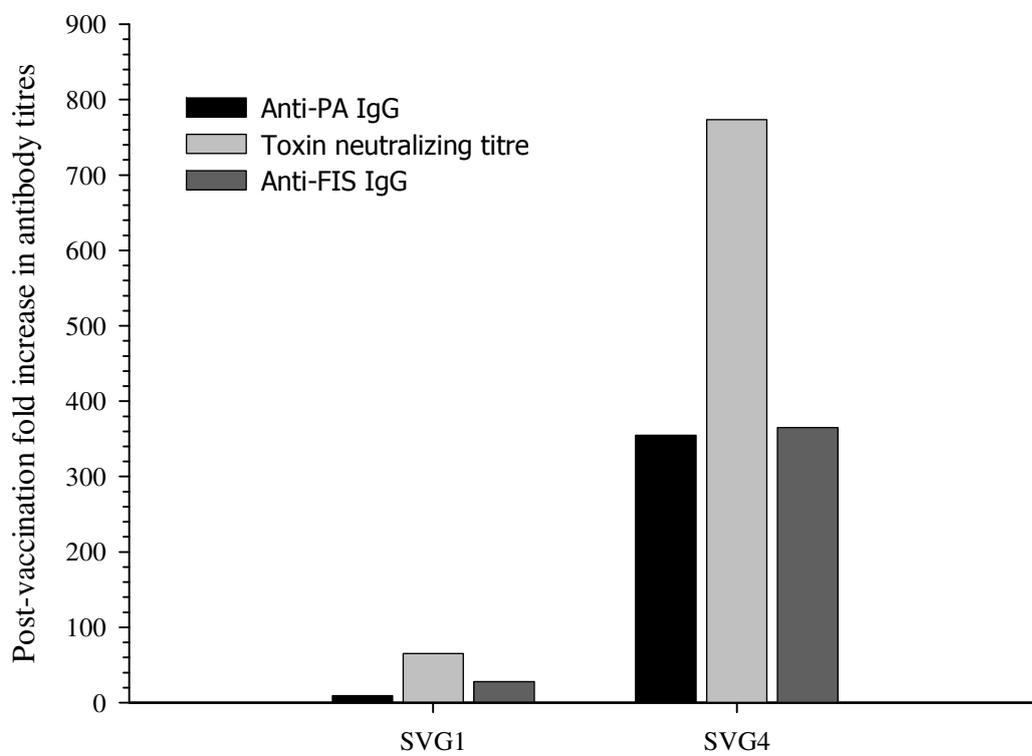
**Fig 3:** Anti-FIS (formaldehyde inactivating spores) IgG titres following Sterne live spore vaccine immunizations (light arrow) and virulent *Bacillus anthracis* spores challenge (**dark arrow**) in goats. SVG2 was vaccinated once on week 0, SVG3 was vaccinated twice on weeks 0 and 58. SVG2, SVG3 and the unvaccinated negative control group (NCG1) were challenged on week 62. Individual titres for all animals in SVG2 and SVG3 are indicated with “O” for the first 57 weeks. *P*-values indicate differences in titres between SVG2 and SVG3 before and after lethal challenge (weeks 62 and 64) respectively.



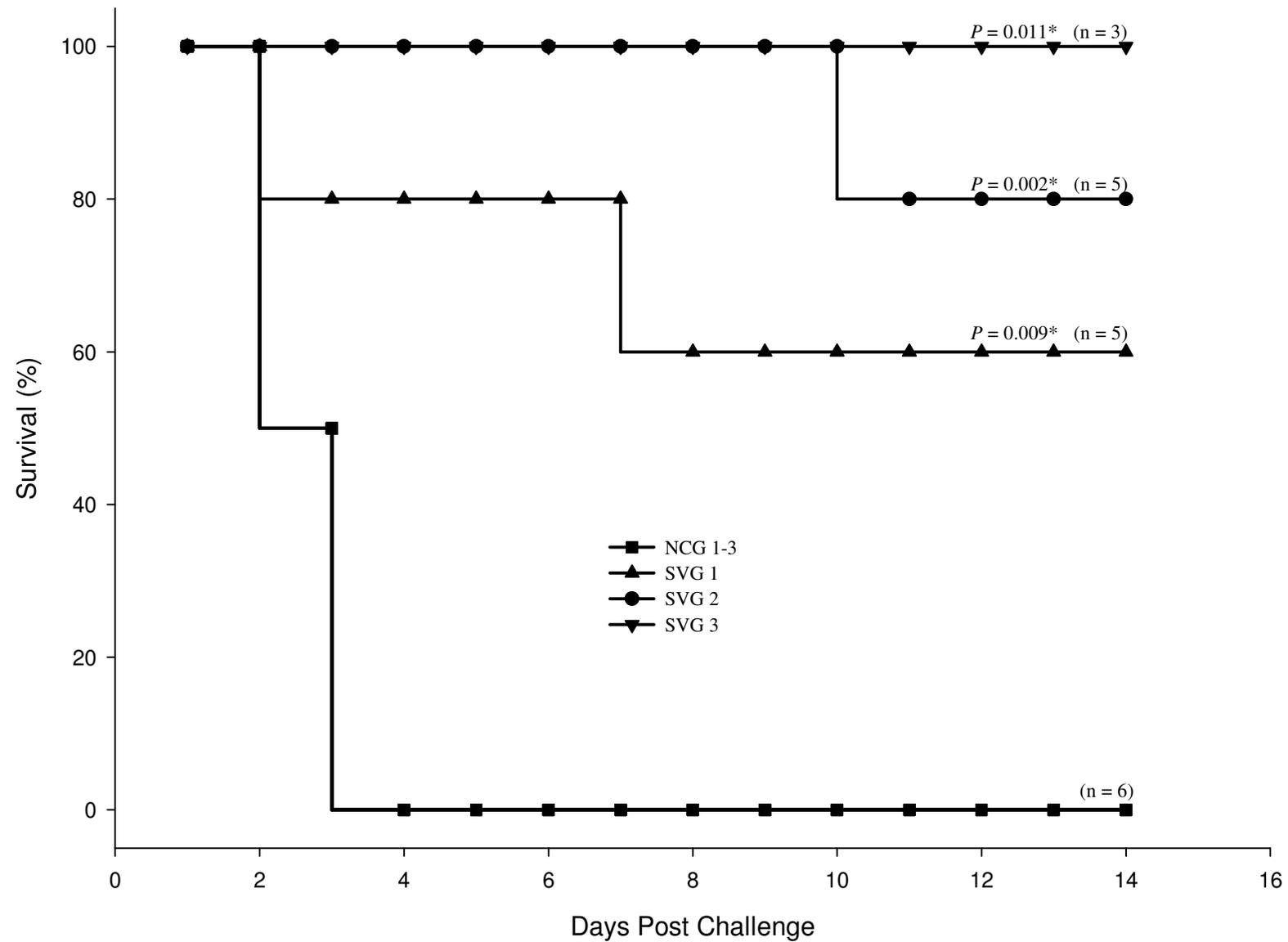
**Fig 4:** Anti-vegetative antigen (of a chemically and mechanically lysed pXO1 and pXO2 deficient strain) IgG titres in pooled group sera following Sterne live spore vaccine immunization (light arrow) in goats at week 0 (SVG2 and SVG3), revaccination at week 58 (SVG3) and virulent *Bacillus anthracis* spores challenge (**dark arrow**) at week 62. Negative control group animals (NCG1) were unvaccinated. *P* –values indicate differences between SVG2 and 3 at week 62 and 64.



**Fig 5:** Immune titre increases six weeks after a single vaccination (SVG1) or booster vaccinations at week 0 and 12 (SVG4) with the Sterne live spore vaccine in goats.



**Fig 6: Survival plots** of Sterne live spore vaccine vaccinated and control groups after challenge with 844 virulent *Bacillus anthracis* spores. **Sterne live spore vaccine** vaccinated group (SVG) 1 and 2 were respectively challenged at 6 and 62 weeks post vaccination. SVG3 was vaccinated twice on weeks 0 and 58 and challenged 4 weeks later (week 62). Negative control group (NCG) 1-3 (unvaccinated controls) were challenged with different doses of virulent spores (see table 2). All challenged animals were observed for 14 days following inoculation. There was no significant difference in survival amongst the SVG groups following virulent challenge ( $P \geq 0.246$ ). \*  $P$ -values as compared to the negative control group via log rank test.



**Table 1 Vaccination and lethal challenge study design**

Groups	Vaccine	No of animals	Immunization (week)		Serum collection time points for serology (week)															
					0	6 <sup>c</sup>	8 <sup>g</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-
SVG1	1x SLSV	5	0	-	0	6 <sup>c</sup>	8 <sup>g</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-
SVG2	1x SLSV	5	0	-	0	4	8	12	16	20	24	28	32	37	48	53	58	62 <sup>c</sup>	64 <sup>g</sup>	
SVG3	2x SLSV	5 <sup>a</sup>	0	58	0	4	8	12	16	20	24	28	32	37	48	53	58	62 <sup>c</sup>	64 <sup>g</sup>	
SVG4	2x SLSV	5	0	12	0	2	4	8	12	17	20	24 <sup>f</sup>								
NCG1	Unvaccinated	3 <sup>b</sup>	-	-	0	4	8	12	16	20	24	28	32	37	48	53	58	62 <sup>c</sup>	-	
NCG2	Unvaccinated	2	-	-	0	10 <sup>d</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	
NCG3	Unvaccinated	2	-	-	0	11 <sup>c</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	

<sup>a</sup> Only 3 goats challenged due to incidental deaths from heartwater

<sup>b</sup> Only 2 goats challenged due to incidental death from heartwater

<sup>c</sup> Challenge dose 844 spores

<sup>d</sup> Challenge dose 172 spores

<sup>e</sup> Challenge dose 36 spores

<sup>f</sup> Unchallenged

<sup>g</sup> Sampling time-point of survivors

SLSV-Sterne live spore vaccine

SVG-SLSV vaccinated group

NCG-Negative control group

**Table 2 Time to death of naive Boer goats challenged with different doses of *B. anthracis* spores**

<b>Groups</b>	<b>Challenge dose<sup>a</sup></b>	<b>Time to death in hours<sup>b</sup></b>
NCG1	36 spores	57;83
NCG2	172 spores	68;64
NCG3	844 spores	38;64

<sup>a</sup> Actual challenge dose as established by plate counts

<sup>b</sup> Two animals per group