SCIENTIFIC REPORTS

OPEN

Immunogenicity of anthrax recombinant peptides and killed spores in goats and protective efficacy of immune sera in A/J mouse model

Okechukwu C. Ndumnego [1,3], Susanne M. Koehler[2,4], Jannie E. Crafford[1], Wolfgang Beyer[2] & Henriette van Heerden[1]

Anthrax is primarily recognized as an affliction of herbivores with incubation period ranging from three to five days post-infection. Currently, the Sterne live-spore vaccine is the only vaccine approved for control of the disease in susceptible animals. While largely effective, the Sterne vaccine has several problems including adverse reactions in sensitive species, ineffectiveness in active outbreaks and incompatibility with antibiotics. These can be surmounted with the advent of recombinant peptides (non-living) next generation vaccines. The candidate vaccine antigens comprised of recombinant protective antigen (PA), spore-specific antigen (bacillus collagen-like protein of anthracis, BclA) and formaldehyde inactivated spores (FIS). Presently, little information exists on the protectivity of these novel vaccine candidates in susceptible ruminants. Thus, this study sought to assess the immunogenicity of these vaccine candidates in goats and evaluate their protectivity using an *in vivo* mouse model. Goats receiving a combination of PA, BclA and FIS yielded the highest antibody and toxin neutralizing titres compared to recombinant peptides alone. This was also reflected in the passive immunization experiment whereby mice receiving immune sera from goats vaccinated with the antigen combination had higher survival post-challenge. In conclusion, the current data indicate promising potential for further development of non-living anthrax vaccines in ruminants.

Anthrax, a disease widely recognized as a primary disease of ruminants[1], is caused by the Gram-positive, aerobic, spore-forming bacterium *Bacillus anthracis*. In nature the disease is mainly transmitted to susceptible herbivores by viable dormant spores present in contaminated foliage and soils. It progresses as a hyperacute infection in ruminants following an incubation period of three-five days[2]. The main virulence factors are encoded by two plasmids; pXO1, which codes for the three toxin components of protective antigen (PA), lethal factor (LF) and edema factor (EF)[3–5] whereas pXO2 encodes the anti-phagocytic poly-ɣ-D-glutamic acid capsule (PDGA)[6]. The full virulence of *B. anthracis* depends on the existence of both plasmids. PA bonds with LF, forming lethal toxin (LT), a zinc metalloprotease that inactivates several mitogen-activated protein kinase kinases (MAPKK) leading to impairment and death of susceptible macrophages and other immunocompetent cells[7,8]. Edema toxin (ET) is a calmodulin dependent adenylate cyclase formed by the combination of PA to EF and which catalyses the production of cyclic AMP from the host ATP leading to the disruption of fluidic homeostasis in the host cells[9,10].

The poorly immunogenic PDGA facilitates the dissemination of *B. anthracis* in the body of infected animals[11]. Masking of anthrax bacilli by the PDGA capsule enables it to avoid immune surveillance mechanisms and to proliferate systemically once inside the circulatory system[12,13]. Research has linked the PDGA with LT in the

[1]Department of Veterinary Tropical Diseases, University of Pretoria, Onderstepoort, South Africa. [2]Institute of Animal Science, Department of Livestock Infectiology and Environmental Hygiene, University of Hohenheim, Stuttgart, Germany. [3]Present address: Africa Health Research Institute, Durban, South Africa. [4]Present address: Robert Koch Institute, Berlin, Germany. Correspondence and requests for materials should be addressed to O.C.N. (email: okechukwu.ndumnego@ahri.org) or H.H. (email: Henriette.VanHeerden@up.ac.za)

1

Vaccine group		Pre-vaccination	Week 3	Week 6	Week 10
rPA + rBclA	Anti-PA IgG	2.41 ± 0.37	2.87 ± 0.55	$4.44 \pm 0.23^{***}$	$4.14 \pm 0.36^{***}$
	Anti-BclA IgG	2.42 ± 0.30	$2.85 \pm 0.22^{*}$	$3.28 \pm 0.19^{**}$	$3.19 \pm 0.24^{***}$
	Anti-spore IgG	0.71 ± 1.20	0.35 ± 0.98	1.07 ± 1.22	0.68 ± 1.16
	TNA[c]	n.d.	0.37 ± 1.04	$2.95 \pm 0.25^{***}$	$2.79 \pm 0.54^{***}$
rPA + rBclA + FIS	Anti-PA IgG	2.09 ± 0.22	$2.59 \pm 0.33^{*}$	$3.91 \pm 0.38^{***}$	$4.27 \pm 0.37^{***}$
	Anti-BclA IgG	2.06 ± 0.14	$2.51 \pm 0.23^{*}$	$3.10 \pm 0.35^{**}$	$3.19 \pm 0.48^{**}$
	Anti-spore IgG	1.16 ± 1.31	$3.05 \pm 0.42^{*}$	$3.32 \pm 0.23^{*}$	$2.99 \pm 0.18^{*}$
	TNA[c]	n.d.	0.34 ± 0.95	$2.61 \pm 0.33^{***}$	$2.92 \pm 0.47^{***}$

Table 1. Antibody titres[a][b] ($\log_{10}$) of goats vaccinated on weeks 0, 3 and 6 with recombinant proteins (rPA + rBclA) and lipopeptide adjuvant or recombinant proteins, inactivated spores and lipopeptide adjuvant (rPA + rBclA + FIS). [a]Mean $\log_{10}$ titres $\pm$ 95% confidence interval. [b]Titres were compared to the respective pre-vaccination titres ($^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$). [c]Lethal toxin neutralization titres rPA; Recombinant protective antigen 83. rBclA; Recombinant bacillus collagen-like protein of anthracis. FIS; Formaldehyde inactivated spore. n.d; Not detected.

blood of experimentally infected animals[14] and this was shown to significantly enhance the deleterious effects of LT in mice[15].

The Sterne live spore vaccine is currently the only vaccine of choice for the control of anthrax in domestic animals. It is an attenuated pXO1+, pXO2− strain (34F2)[16] known to induce good levels of immunity without clinical signs of the disease. However, some of the limitations of this vaccine include possible adverse reactions in some sensitive species[17–19], short term protection[20], ineffectiveness in active outbreaks and incompatibility with antibiotics[21,22]. Thus, the development of an alternative vaccine that can be produced quickly in the face of an anthrax outbreak, safe to administer and compatible with antibiotics is invaluable.

Induction of antibodies against PA is the main immune response following vaccination of animals with the Sterne live spore vaccine[23–25]. The anti-PA antibodies prevent the development of lethal intoxication and is vital for protection against germinating virulent anthrax bacilli[26]. Adding other anthrax antigens to PA-based vaccine candidates has been reported to improve the protection afforded to laboratory animals challenged with virulent anthrax spores[27]. An ideal non-living recombinant protein-based anthrax vaccine should be able to induce broad spectrum immunity targeting both toxaemia and bacteraemia. In the current study, an acellular vaccine formulation comprising of recombinant PA (rPA) and two other antigens; Bacillus collagen-like protein of anthracis (rBclA) and formaldehyde inactivated spores (FIS) were administered in a goat model and the resulting immune response and protection evaluated. BclA is an immunodominant glycoprotein found on the surface of the *B. anthracis* exosporium[28,29], Previously, the addition of BclA to PA constructs had offered superior protection against virulent challenge in mice[30,31] while FIS with PA-based vaccines significantly augmented the protection afforded to mice and guinea pigs[32,33]. We assessed the antibody response to rPA, rBclA, FIS and a lipopeptide adjuvant following vaccination in goats. The adjuvant comprised of *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R*, *S*)-propyl]-(*R*)-cysteinyl-seryl-(lysyl)$_3$-lysine (Pam$_3$Cys-SK$_4$), a potent TLR2/1 activator admixed to Pam$_3$Cys conjugated to FISEAIIHVLHSRHPG, a T-helper cell epitope from the sperm whale myoglobin[34–36]. We have previously shown that 70% of NMRI mice vaccinated with a combination of PA, BclA and lipopeptide adjuvant were protected against virulent challenge with *B. anthracis* Ames strain spores[37]. The adjuvant is well-defined, superior to conventional preparations and shows no untoward effects in animals[38].

Results

Humoral immune response to non-living anthrax antigens in goats.
Five goats each were vaccinated subcutaneously with rPA + rBclA+ lipopeptide adjuvant or rPA + rBclA + FIS+ lipopeptide adjuvant on weeks 0, 3 and 6. The jugular blood of each animal was sampled before each vaccination and again on week 10. Goats vaccinated twice with the Sterne live spore vaccine and vaccine diluent served as controls. Prepared sera were tested for IgG against PA, BclA, whole spores and lethal toxin neutralizing ability. Mean IgG titres against PA rose significantly after the first vaccination with rPA + rBclA + FIS+ lipopeptide adjuvant ($P = 0.023$) and increased after the second vaccination (Table 1). Significant changes in levels of anti-PA IgG were observed after the second vaccination with rPA + rBclA+ lipopeptide adjuvant. The highest increases in titres were observed after a second vaccination with either of the antigen combinations. A third vaccination only effected marginal changes in the recorded titres. The pre-challenge titres were equivalent with those observed following vaccinations with the Sterne live spore vaccine (Fig. 1).

A significant increase of anti-BclA IgG was observed in goats vaccinated with rPA + rBclA and rPA + rBclA + FIS ($P \leq 0.031$) after the first vaccination. Generally, the anti-BclA titres were a log lower than anti-PA titres after the second or third vaccination (Table 1). These antibodies were insignificant in the Sterne-vaccinated goats throughout the trials (Fig. 2). Conversely, the anti-spore IgG response in the Sterne-vaccinated group was higher when compared with any of the groups vaccinated with the non-living vaccine candidates ($P \leq 0.002$), though also significantly elevated in the FIS-containing group (Fig. 3). While production of anti-spore IgG was evident following the first vaccination with rPA + rBclA + FIS, none was observed throughout the study in the rPA + rBclA-vaccinated animals (Table 1 and Fig. 3).

The development of significant lethal toxin neutralizing antibodies was only seen after the second vaccination with either of the non-living vaccine combinations (Table 1). Thereafter it followed the pattern as seen with
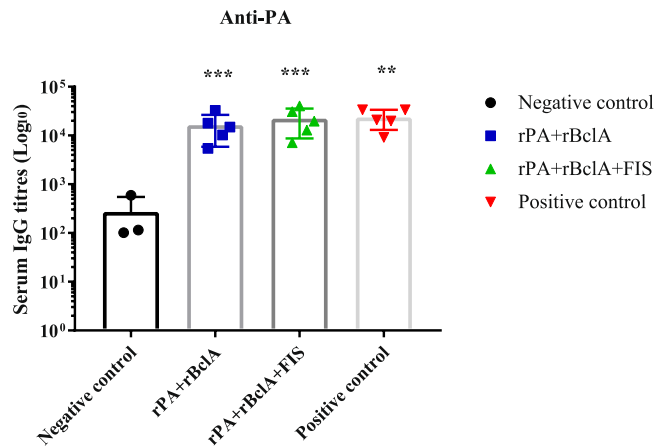
Figure 1. Individual anti- protective antigen (PA) IgG titres in goats (with mean bars and SD). The animals were either vaccinated thrice with rPA+rBclA (n =5) and rPA+rBclA+FIS (n =5) on weeks 0, 3 and 6 (sera collected for analyses on week 10) in combination with lipopeptide adjuvant or twice with Sterne live spore (n = 5) on weeks 0 and 12 (sera collected for analyses on week 17). The naïve controls (n = 3) received the vaccine diluent. IgG titres of each group were compared to the respective pre-immune titres (**P < 0.005; ***P < 0.0005). rPA; Recombinant protective antigen 83rBclA; Recombinant bacillus collagen-like protein of anthracisFIS; Formaldehyde inactivated spore.
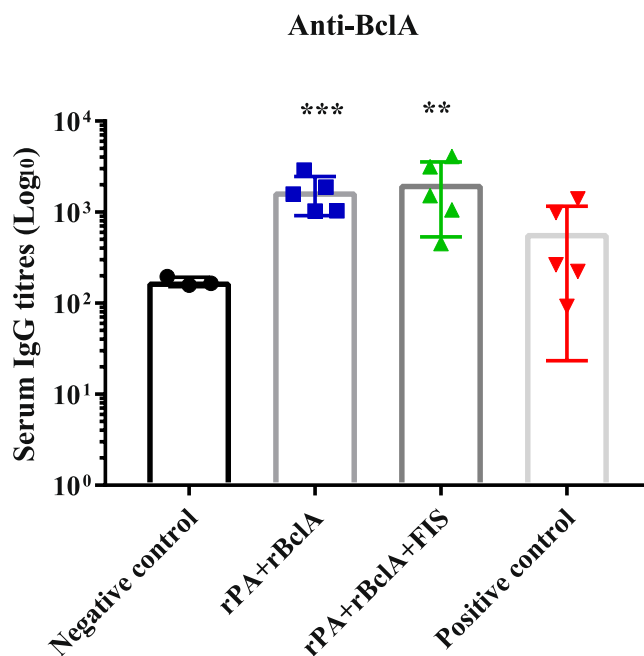
Figure 2. Individual anti-BclA IgG titres in goats (with mean bars and SD). The animals were either vaccinated thrice with rPA+rBclA (n = 5) and rPA+rBclA+FIS (n = 5) together with lipopeptide adjuvant on weeks 0, 3 and 6 (sera collected for analyses on week 10) or twice with Sterne live spore (n = 5) on weeks 0 and 12 (sera collected for analyses on week 17). The naïve controls (n = 5) received the vaccine diluent. IgG titres of each group were compared to the respective pre-immune titres (*P < 0.05; **P < 0.005; ***P < 0.0005). rPA; Recombinant protective antigen 83rBclA; Recombinant bacillus collagen-like protein of anthracisFIS; Formaldehyde inactivated spore.

anti-PA IgG titres but at a lower level. There was no difference in the TNA titres after the second or third vaccination ($P \geq 0.152$). Following the stabilization of the neutralizing antibody levels after the second vaccination, the titres remained steady till the end of the experiment and were equivalent to the Sterne-vaccinated controls (Fig. 4).

Protection conferred on A/J mice by caprine immune sera. To assess the protective efficacy of the immune response generated by vaccination of goats with anthrax recombinant proteins and inactivated spores, a

Anti-spore
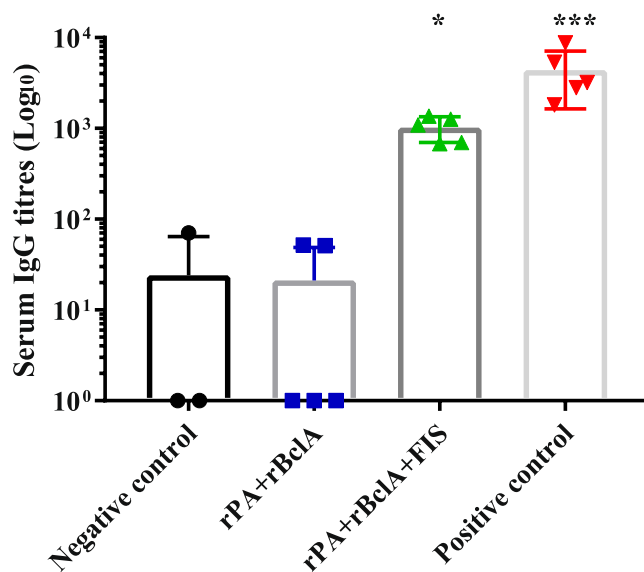
Figure 3. Individual anti-spore IgG titres in goats (with mean bars and SD). The animals were either vaccinated thrice with rPA+rBclA (n = 5) and rPA+rBclA+FIS (n = 5) together with lipopeptide adjuvant on weeks 0, 3 and 6 (sera collected for analyses on week 10) or twice with Sterne live spore (n = 5) on weeks 0 and 12 (sera collected for analyses on week 17). The naïve controls (n = 5) received the vaccine diluent. IgG titres of each group were compared to the respective pre-immune titres (*P < 0.05; **P < 0.005; ***P < 0.0005). rPA; Recombinant protective antigen 83rBclA; Recombinant bacillus collagen-like protein of anthracisFIS; Formaldehyde inactivated spore.

Lethal toxin neutralization
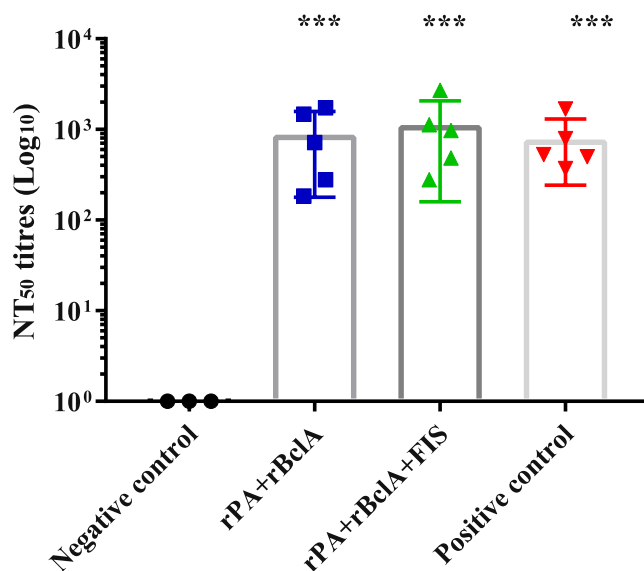
Figure 4. Individual anthrax lethal toxin neutralization titres in goats (with mean bars and SD). The animals were either vaccinated thrice with rPA+rBclA (n = 5) and rPA+rBclA+FIS (n = 5) with lipopeptide adjuvant on weeks 0, 3 and 6 (sera collected for analyses on week 10) or twice with Sterne live spore (n = 5) on weeks 0 and 12 (sera collected for analyses on week 17). The naïve controls (n = 3) received the vaccine diluent. IgG titres of each group were compared to the respective pre-immune titres (***P < 0.0005). rPA; Recombinant protective antigen 83rBclA; Recombinant bacillus collagen-like protein of anthracisFIS; Formaldehyde inactivated spore.
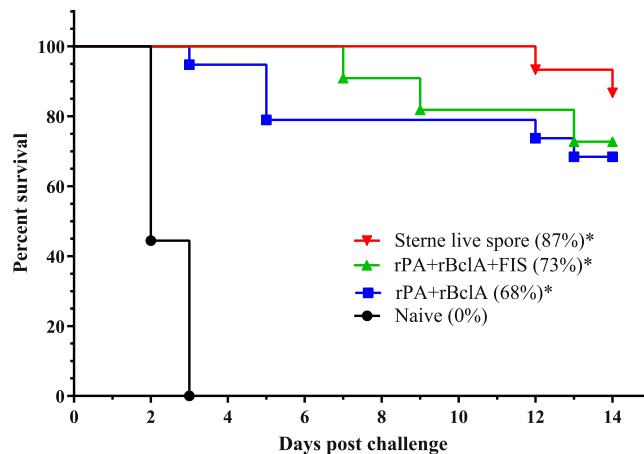
Figure 5. Passive protection of A/J mice following in vivo transfer of immune sera from goats and lethal challenge with ~1.92 × 105 Sterne 34F2 spores. The rPA+rBclA+FIS (n = 11) and rPA+rBclA (n = 19) groups with lipopeptide adjuvant received sera from goats vaccinated thrice with the respective vaccine candidates (also see Table 1). The Sterne live spore group (n = 15) sera were collected from goats vaccinated twice with Sterne strain spores. Naive (n = 9) sera were from goats injected with vaccine diluent (saline). *** denotes significantly increased survival as compared to the negative control (P < 0.001). There was no difference between the survival times of the Sterne live spore, rPA+rBclA+FIS and rPA+rBclA vaccinated groups (P ≥ 0.189). rPA; Recombinant protective antigen 83rBclA; Recombinant bacillus collagen-like protein of anthracisFIS; Formaldehyde inactivated spore.

		Anti-PA IgG	Anti-spore IgG	TNA[a]	Anti-BclA IgG
Survival	Pearson Correlation	0.460**	0.357**	0.336*	0.251
	Significance (2-tailed)	<0.001	0.008	0.013	0.067

Table 2. Pearson's correlation analysis of relationship between survival times of passively challenged A/J mice and caprine sera antibody titres. [a]Lethal toxin neutralization titres. *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).

passive *in vivo* mouse protection experiment was carried out after adoptive caprine sera transfer. Following lethal challenge with ~1.92 × 10^5 Sterne 34F2 *B. anthracis* spores, sera from naïve goats failed to protect the susceptible mice with all the challenged mice dying within 3 days (Fig. 5). Sera from rPA + rBclA-vaccinated goats protected 68% of the challenged mice and 73% were protected by rPA + rBclA + FIS immune sera. Sera from the Sterne vaccinates protected 87% of challenged A/J mice. Analysis of the survival data from the groups showed no difference in survival between the rPA + rBclA, rPA + rBclA + FIS and Sterne vaccinated groups (*P* ≥ 0.189). On the other hand, these groups showed increased survival times compared to the naïve (negative) controls (*P* < 0.001).

To ascertain if there is any association between the measured antibody titres and survival against anthrax in the A/J mice, a Pearson's correlation study was performed. This revealed a strong positive relationship between anti-PA, anti-spore and toxin neutralizing antibody titres and survival in the mice (Table 2). There was no significant correlative association between anti-BclA IgG titres and survival in the challenged mice.

Discussion

In addition to performing the trials in this study under more controlled conditions, evaluation of the protective capacity of immune sera was performed using an *in vivo* mouse challenge model. This model has been used previously albeit in homogenous mouse sera transfer[39] or adoptive heterogenous immune sera transfer from Sterne live spore vaccinates[20]. To the best of our knowledge, this study represents the first attempt to assess a recombinant anthrax vaccine candidate in a ruminant model using a passive mice protection model. Immune sera from goats vaccinated with combinations of rPa + rBclA + FIS and rPA + rBclA were able to protect 73% and 68% of A/J mice from lethal anthrax challenge respectively. Immune titres peaked following a single booster vaccination with the non-living vaccine candidates, indicating the expendability of a second booster vaccination.

Anthrax is chiefly a disease of herbivorous animals with ruminants being most susceptible[1,2]. The disease is largely controlled using the Sterne live spore vaccine which is an unencapsulated but toxinogenic strain[40,41]. The vaccine retains some residual virulence, though attenuated and has limited effectiveness in the face of an active anthrax outbreak[22,42]. With the advent of multicomponent recombinant anthrax antigens capable of stimulating broad spectrum immune response in vaccinates, questions have risen about the potential usefulness of such vaccine candidates in the control of the disease in livestock. While the antigens of *B. anthracis* rPA, rBclA and FIS, alone or in combination are recognized for their protective efficacy from many laboratory rodent studies[27], it remains to be elucidated if these antigens will elicit a similar response in a ruminant model of anthrax infection.

The FIS suspension from Sterne vaccine strain (34F2) done as previously[53] with some modifications. Spores were incubated at 37 °C overnight in PBS at concentrations containing at least 10^9 spores/mL and formaldehyde at a final concentration of 4% formalin. Subsequently, after pelleting at $4000 \times g$ for 15 min (room temperature), the spores were washed four times with PBS in 0.1% gelatine. This was followed by resuspension in PBS and storage at −80 °C. Aliquots of the FIS suspension was tested for sterilization by streaking on blood agar after treatment with histidine (to neutralize any remnant formalin).

B. anthracis 34F2 Sterne spores (batch no. 86) utilized for goat immunization and *in vivo* mouse challenge were sourced from Onderstepoort Biological Products (OBP, Onderstepoort, South Africa). The spores were prepared by the dilution and centrifugation of the spores with sterile PBS/glycerin (0.1%) solution at $3000 \times g$ for 15 min at 4 °C. The final spore suspension was treated at 65 °C for 30 min in a water bath before counting and storage at 4 °C in sterile PBS/glycerine solution.

Immunization experiments and passive protection tests. Goats were screened for PA-reactive antibodies using the conventional PA ELISA. Following arrival and acclimatization at the OBP experimental animal facility, the goats were randomly allocated to designated vaccine groups and immunized as indicated in Table 3. A/J mice (Jackson Laboratories, Bar Harbor, USA) were procured for the *in vivo* challenge study performed at the University of Pretoria Biomedical Research Centre (UPBRC), South Africa. The A/J mouse strain is deficient in the C5 complement component which renders it vulnerable to infection by vaccine-strain Sterne spores[54,55]. These mice develop lethal systemic anthrax following infection with Sterne spores in the absence of protective antibodies[20,25,39]. Experiments with animals were conducted in accordance with ethical principles and guidelines provided by the animal use and care committees of the UPBRC, OBP and University of Pretoria respectively (Protocol number V065/12). Section 20, Act 35 of 1984 permission was granted by the Directorate of Animal Health, Department of Agriculture, Forestry and Fisheries, South Africa (registration number 12/11/1/1/6).

For the passive protection tests, sera (500 μl) from vaccinated goats and controls (naive and hyperimmune goats) were injected intraperitoneally into naïve A/J mice. Following the transfer of caprine sera into the A/J mice, lethal challenge was performed 24 h later with *B. anthracis* Sterne strain. In the experimental design, a total of 5 mice per goat serum (for the protein-vaccinated groups) and 3 mice per serum [for the Sterne-vaccinated (positive) and naïve (negative) controls] were used. However, due to the unexpected adverse reactions shown by some of the mice following the adoptive transfer of sera and subsequent exclusion from the trials, the challenge experiment was performed with reduced mice number for some of the serum samples (Table 3). In rPA + rBclA group, two goat immune sera were tested with five mice each while sera from three goats were assessed with four, three and two mice respectively (19 mice in total). Two caprine sera were assessed with five mice each in rPA + rBclA + FIS group and a serum sample was tested with one mouse (three sera samples were assessed in this group, 11 in total). Consequently, the challenge and survival data were pooled for each vaccine group.

Measurement of serum IgG titres. Sera were analysed for specific immunoglobulins using ELISA as previously described by[52] with some modifications. Individual wells of 96-well microtitre plates (Nunc immuno-plate Maxisorp, Germany) were coated with 0.5 μg of antigen (or 10^7 FIS) in bicarbonate buffer and incubated overnight at 4 °C. The plates were washed twice with PBS containing 0.05% Tween (PBST) using a Biorad PW40 washer (Mamesla-Coquette, France) and blocked in 200 μL PBST containing 5% skimmed milk powder (PBSTM) and incubated for 1 h at room temperature (RT). The plates were washed twice, and the test sera and controls diluted in PBSTM. A two-fold dilution (starting dilution; 1:50) was made across the plates in duplicates and incubated for 30 min on a rotatory shaker (Titretek® flow labs, UK). Following incubation, the plates were washed 5 times and 100 μL of horseradish peroxidase-conjugated rabbit anti-goat IgG (Invitrogen, Camarillo, USA) diluted to 1:4000 in PBSTM was added to every well and incubated for 30 min on the plate shaker. After washing 5 times, the plates were developed with 2,2′ azino bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma, Germany) and absorbance readings taken at 405 nm using a Biotek powerwave XS2 reader (Winooski, USA). Endpoint titres of individual serum were defined as the reciprocal of the highest serum dilution giving an optical density of 0.1. Titres of <50 were ascribed an arbitrary value of 0.

Toxin neutralization assay (TNA). An *in vitro* toxin neutralizing assay (TNA) was carried out with J774A.1 mouse macrophage cell line (ECACC cat no 91051511) as done previously with modifications[24,56]. The 96-well flat-bottomed tissue culture plates (Greiner bio one, Germany) seeded with 80 000 macrophages per well in Dulbecco's modified eagle media (DMEM) and 10% foetal calf serum (FCS) were incubated overnight at 37 °C and 5% CO_2. Test sera were diluted 2-fold (starting dilution; 1:50) in culture medium with PA and LF (List Biological Laboratories Inc., Campbell, USA) at concentrations of 500 ng/mL and 400 ng/mL respectively. The serum/lethal toxin mixture was incubated for 1 h at 37 °C and 5% CO2 before addition to overnight-cultured cells and incubation for 3 h. Every serum sample was tested in duplicates. Subsequently, addition of 25 μL of 5 mg/mL MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Invitrogen, USA) per well and incubation in the dark at 37 °C and 5% CO_2 was performed. Two hours after incubation, cells were lysed with pre-warmed (37 °C) acidified isopropyl alcohol (90% isopropyl alcohol, 0.5% SDS, 25 mM HCl) by vigorously pipetting up and down to solubilize the formazan dye.

Finally, plates were rested for 5 min and the absorbance read at 540 nm with a Biotek power wave XS2 reader. Each assay included a single dilution series of positive control serum from a goat hyper-immunized with the Sterne live spore vaccine serving as a positive control and for assay reproducibility. Three wells in each assay receiving LT served as blanks, another triplicate of wells (with cells) received only LT as toxin control while only culture media was placed in two wells (medium control). The neutralization of each serum sample was calculated using the formula:

7

$$NT_{50} = (sample - toxin\ control) \div (medium\ control - toxin\ control) \times 100$$

Neutralization titres (NT_{50}) were expressed as the reciprocal of the highest serum dilution at which the macrophage survival yielded 50% and obtained using the Gen5 data analysis software (Biotek Instruments, USA).

Statistics. For determination of ELISA and TNA titres, 4-parametre logistic regression curves were generated from serial dilution data using the Gen 5 data analysis software (Biotek Instruments, Winooski, USA). Data obtained were log-transformed and variance equality were tested with the Levene's tests (IBM SPSS Statistics 23). Antibody titre differences between groups at particular time points were analysed using unpaired student t-test, with a two-tailed P-value. Baseline and pre-challenge ELISA and TNA data within vaccine groups were compared using a paired Student's t test. Kaplan-Meier (product limit estimation) plots were used to compute the mean survival times of challenged mice (pooled per treatment group). Survival curves were compared between each treatment group and controls using the Mantel-Cox (Log Rank) test. The strength of association between the survival time following lethal challenge and specific immune titres was measured using the Pearson's correlation coefficient. Results having a p value of ≤ 0.05 were considered statistically significance.

Data Availability
The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

References
1. Hambleton, P., Carman, J. A. & Melling, J. Anthrax: the disease in relation to vaccines. *Vaccine* **2**, 125–132, https://doi.org/10.1016/0264-410X(84)90003-3 (1984).
2. Beyer, W. & Turnbull, P. C. Anthrax in animals. *Molecular aspects of medicine* **30**, 481–489, https://doi.org/10.1016/j.mam.2009.08.004 (2009).
3. Mock, M., Labruyère, E., Glaser, P., Danchin, A. & Ullmann, A. Cloning and expression of the calmodulin-sensitive Bacillus anthracis adenylate cyclase in Escherichia coli. *Gene* **64**, 277–284, https://doi.org/10.1016/0378-1119(88)90342-3 (1988).
4. Robertson, D. L. & Leppla, S. H. Molecular cloning and expression in Escherichia coli of the lethal factor gene of Bacillus anthracis. *Gene* **44**, 71–78, https://doi.org/10.1016/0378-1119(86)90044-2 (1986).
5. Vodkin, M. H. & Leppla, S. H. Cloning of the protective antigen gene of Bacillus anthracis. *Cell* **34**, 693–697, https://doi.org/10.1016/0092-8674(83)90402-6 (1983).
6. Uchida, I., Sekizaki, T., Hashimoto, K. & Terakado, N. Association of the encapsulation of Bacillus anthracis with a 60 megadalton plasmid. *Journal of general microbiology* **131**, 363–367, https://doi.org/10.1099/00221287-131-2-363 (1985).
7. Banks, D. J., Barnajian, M., Maldonado-Arocho, F. J., Sanchez, A. M. & Bradley, K. A. Anthrax toxin receptor 2 mediates Bacillus anthracis killing of macrophages following spore challenge. *Cellular microbiology* **7**, 1173–1185, https://doi.org/10.1111/j.1462-5822.2005.00545.x (2005).
8. Friedlander, A. M. Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. *Journal of Biological Chemistry* **261**, 7123–7126 (1986).
9. Tang, W. J. & Guo, Q. The adenylyl cyclase activity of anthrax edema factor. *Molecular aspects of medicine* **30**, 423–430, https://doi.org/10.1016/j.mam.2009.06.001 (2009).
10. Mourez, M. Anthrax toxins. *Reviews of physiology, biochemistry and pharmacology*, 135–164, https://doi.org/10.1007/s10254-004-0028-2 (2005).
11. Candela, T. & Fouet, A. Bacillus anthracis CapD, belonging to the γ-glutamyltranspeptidase family, is required for the covalent anchoring of capsule to peptidoglycan. *Molecular microbiology* **57**, 717–726, https://doi.org/10.1111/j.1365-2958.2005.04718.x (2005).
12. Makino, S., Watarai, M., Cheun, H., Shirahata, T. & Uchida, I. Effect of the lower molecular capsule released from the cell surface of Bacillus anthracis on the pathogenesis of anthrax. *Journal of Infectious Diseases* **186**, 227–233, https://doi.org/10.1086/341299 (2002).
13. Sutherland, M. D., Thorkildson, P., Parks, S. D. & Kozel, T. R. In vivo fate and distribution of poly-γ-d-glutamic acid, the capsular antigen from Bacillus anthracis. *Infection and immunity* **76**, 899–906, https://doi.org/10.1128/IAI.01176-07 (2008).
14. Ezzell, J. W. et al. Association of Bacillus anthracis capsule with lethal toxin during experimental infection. *Infection and immunity* **77**, 749–755, https://doi.org/10.1128/IAI.00764-08 (2009).
15. Jang, J. et al. The poly-gamma-D-glutamic acid capsule of Bacillus anthracis enhances lethal toxin activity. *Infection and immunity* **79**, 3846–3854, https://doi.org/10.1128/IAI.01145-10 (2011).
16. Sterne, M. 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 (1937).
17. Cartwright, M. E., McChesney, A. E. & Jones, R. L. Vaccination-related anthrax in three llamas. *J Am Vet Med Assoc* **191**, 715–716 (1987).
18. Sterne, M. The use of Anthrax Vaccines Prepared from Avirulent (Uncapsulated) Variants of Bacillus anthracis. *Onderstepoort Journal of Veterinary Research* **13**, 307 (1939).
19. Wobeser, B. K. Anthrax vaccine associated deaths in miniature horses. *The Canadian veterinary journal.La revue veterinaire canadienne* **56**, 359–360 (2015).
20. Turnbull, P. C. et al. Vaccine-induced protection against anthrax in cheetah (Acinonyx jubatus) and black rhinoceros (Diceros bicornis). *Vaccine* **22**, 3340–3347, https://doi.org/10.1016/j.vaccine.2004.02.037 (2004).
21. Fasanella, A. et al. Protective activity and immunogenicity of two recombinant anthrax vaccines for veterinary use. *Vaccine* **26**, 5684–5688, https://doi.org/10.1016/j.vaccine.2008.08.026 (2008).
22. Turnbull, P. C. B. Anthrax vaccines: past, present and future. *Vaccine* **9**, 533–539, https://doi.org/10.1016/0264-410X(91)90237-Z (1991).
23. Turnbull, P. C. B., Leppla, S. H., Broster, M. G., Quinn, C. P. & Malling, J. Antibodies to anthrax toxin in humans and guinea pigs and their relevance to protective immunity. *Medical microbiology and immunology* **177**, 293–303 (1988).
24. Ndumnego, O. C., Köhler, S. M., Crafford, J., van Heerden, H. & Beyer, W. Comparative analysis of the immunologic response induced by the Sterne 34F2 live spore Bacillus anthracis vaccine in a ruminant model. *Veterinary immunology and immunopathology* **178**, 14–21, https://doi.org/10.1016/j.vetimm.2016.06.005 (2016).
25. Phaswana, P. H. et al. Use of the mice passive protection test to evaluate the humoral response in goats vaccinated with Sterne 34F2 live spore vaccine. *Veterinary research* **48**, 46, https://doi.org/10.1186/s13567-017-0451-4 (2017).

26. Pitt, M. L. *et al. In vitro* correlate of immunity in a rabbit model of inhalational anthrax. *Vaccine* **19**, 4768–4773, https://doi.org/10.1016/S0264-410X(01)00234-1 (2001).
27. Cybulski, R. J., Sanz, P. & O'Brien, A. D. Jr. Anthrax vaccination strategies. *Molecular aspects of medicine* **30**, 490–502, https://doi.org/10.1016/j.mam.2009.08.006 (2009).
28. Sylvestre, P., Couture-Tosi, E. & Mock, M. Polymorphism in the collagen-like region of the Bacillus anthracis BclA protein leads to variation in exosporium filament length. *Journal of bacteriology* **185**, 1555–1563, https://doi.org/10.1128/JB.185.5.1555-1563.2003 (2003).
29. Rety, S. *et al.* The crystal structure of the Bacillus anthracis spore surface protein BclA shows remarkable similarity to mammalian proteins. *The Journal of biological chemistry* **280**, 43073–43078, M510087200 [pii] (2005).
30. Brahmbhatt, T. N. *et al.* Recombinant exosporium protein BclA of Bacillus anthracis is effective as a booster for mice primed with suboptimal amounts of protective antigen. *Infection and immunity* **75**, 5240–5247, https://doi.org/10.1128/IAI.00884-07 (2007).
31. Hahn, U. K., Boehm, R. & Beyer, W. DNA vaccination against anthrax in mice-combination of anti-spore and anti-toxin components. *Vaccine* **24**, 4569–4571, https://doi.org/10.1016/j.vaccine.2005.08.031 (2006).
32. Brossier, F., Levy, M. & Mock, M. Anthrax spores make an essential contribution to vaccine efficacy. *Infection and immunity* **70**, 661–664, https://doi.org/10.1128/IAI.70.2.661-664.2002 (2002).
33. Gauthier, Y. P. *et al.* Efficacy of a vaccine based on protective antigen and killed spores against experimental inhalational anthrax. *Infection and immunity* **77**, 1197–1207, https://doi.org/10.1128/IAI.01217-08 (2009).
34. Wiesmüller, K. H., Jung, G. & Hess, G. Novel low-molecular-weight synthetic vaccine against foot-and-mouth disease containing a potent B-cell and macrophage activator. *Vaccine* **7**, 29–33, https://doi.org/10.1016/0264-410X(89)90007-8 (1989).
35. Hoffmann, P. *et al.* Induction of an epitope-specific humoral immune response by lipopeptide-hapten conjugates: enhancement of the anti-melittin response by a synthetic T helper (Th)-cell epitope. *FEMS immunology and medical microbiology* **17**, 225–234, doi:S0928-8244(97)00010-2 [pii] (1997).
36. Spohn, R. *et al.* Synthetic lipopeptide adjuvants and Toll-like receptor 2—structure–activity relationships. *Vaccine* **22**, 2494–2499, https://doi.org/10.1016/j.vaccine.2003.11.074 (2004).
37. Köhler, S. M., Baillie, L. W. & Beyer, W. BclA and toxin antigens augment each other to protect NMRI mice from lethal Bacillus anthracis challenge. *Vaccine* **33**, 2771–2777, https://doi.org/10.1016/j.vaccine.2015.04.049 (2015).
38. Mittenbuhler, K., v d Esche, U., Heinevetter, L., Bessler, W. G. & Huber, M. Lipopeptides: adjuvanticity in conventional and genetic immunization. *FEMS immunology and medical microbiology* **37**, 193–200, doi:S0928824403000695 [pii] (2003).
39. Beedham, R. J., Turnbull, P. C. B. & Williamson, E. D. Passive transfer of protection against Bacillus anthracis infection in a murine model. *Vaccine* **19**, 4409–4416, https://doi.org/10.1016/S0264-410X(01)00197-9 (2001).
40. Sterne, M., Nicol, J. & Lambrechts, M. C. The effect of large scale active immunization against anthrax. *JS Afr.Vet.Med.Assoc* **13**, 53 (1942).
41. Hugh-Jones, M. E. & De Vos, V. Anthrax and wildlife. *Revue Scientifique et Technique-Office International des Epizooties* **21**, 359–384 (2002).
42. Stepanov, A. V., Marinin, L. I., Pomerantsev, A. P. & Staritsin, N. A. Development of novel vaccines against anthrax in man. *Journal of Biotechnology* **44**, 155–160, https://doi.org/10.1016/0168-1656(95)00092-5 (1996).
43. Koehler, S. M. *et al.* Protection of farm goats by combinations of recombinant peptides and formalin inactivated spores from a lethal Bacillus anthracis challenge under field conditions. *BMC Veterinary Research* **13**, 220, https://doi.org/10.1186/s12917-017-1140-2 (2017).
44. Welkos, S., Little, S., Friedlander, A., Fritz, D. & Fellows, P. The role of antibodies to Bacillus anthracis and anthrax toxin components in inhibiting the early stages of infection by anthrax spores. *Microbiology (Reading)* **147**, 1677–1685, https://doi.org/10.1099/00221287-147-6-1677 (2001).
45. Pitt, M. L. M. *et al. In vitro* correlate of immunity in an animal model of inhalational anthrax. *Journal of applied microbiology* **87**, 304–304, https://doi.org/10.1046/j.1365-2672.1999.00897.x (1999).
46. Albrecht, M. T. *et al.* Human monoclonal antibodies against anthrax lethal factor and protective antigen act independently to protect against Bacillus anthracis infection and enhance endogenous immunity to anthrax. *Infection and immunity* **75**, 5425–5433, https://doi.org/10.1128/IAI.00261-07 (2007).
47. Welkos, S. L. & Friedlander, A. M. Pathogenesis and genetic control of resistance to the Sterne strain of Bacillus anthracis. *Microbial pathogenesis* **4**, 53–69, https://doi.org/10.1016/0882-4010(88)90048-4 (1988).
48. Bessler, W. G., Mittenbühler, K., Esche, U. & Huber, M. Lipopeptide adjuvants in combination treatment. *International immunopharmacology* **3**, 1217–1224, https://doi.org/10.1016/S0264-410X(01)00197-9 (2003).
49. Hope, J. C., Whelan, A. O., Hewinson, R. G., Vordermeier, M. & Howard, C. J. Maturation of bovine dendritic cells by lipopeptides. *Veterinary immunology and immunopathology* **95**, 21–31, https://doi.org/10.1016/S0165-2427(03)00104-1 (2003).
50. Wedlock, D. N. *et al.* Enhanced protection against bovine tuberculosis after coadministration of Mycobacterium bovis BCG with a Mycobacterial protein vaccine-adjuvant combination but not after coadministration of adjuvant alone. *Clinical and vaccine immunology: CVI* **15**, 765–772, https://doi.org/10.1128/CVI.00034-08 (2008).
51. Wedlock, D. N., Aldwell, F. E., Vordermeier, H. M., Hewinson, R. G. & Buddle, B. M. Protection against bovine tuberculosis induced by oral vaccination of cattle with Mycobacterium bovis BCG is not enhanced by co-administration of mycobacterial protein vaccines. *Veterinary immunology and immunopathology* **144**, 220–227, https://doi.org/10.1016/j.vetimm.2011.09.005 (2011).
52. Hahn, U. K., Alex, M., Czerny, C. P., Bohm, R. & Beyer, W. Protection of mice against challenge with Bacillus anthracis STI spores after DNA vaccination. *International journal of medical microbiology: IJMM* **294**, 35–44, https://doi.org/10.1016/j.ijmm.2003.12.002 (2004).
53. Guidi-Rontani, C., Weber-Levy, M., Labruyère, E. & Mock, M. Germination of Bacillus anthracis spores within alveolar macrophages. *Molecular microbiology* **31**, 9–17, https://doi.org/10.1046/j.1365-2958.1999.01137.x (1999).
54. Welkos, S. L., Keener, T. J. & Gibbs, P. H. Differences in susceptibility of inbred mice to Bacillus anthracis. *Infection and immunity* **51**, 795–800 (1986).
55. Welkos, S., Trotter, R., Becker, D. & Nelson, G. Resistance to the Sterne strain of B. anthracis: phagocytic cell responses of resistant and susceptible mice. *Microbial pathogenesis* **7**, 15–35, https://doi.org/10.1016/0882-4010(89)90108-3 (1989).
56. Hering, D. *et al.* Validation of the anthrax lethal toxin neutralization assay. *Biologicals* **32**, 17–27, https://doi.org/10.1016/j.biologicals.2003.09.003 (2004).

Acknowledgements

Author Contributions

O.C.N. and S.M.K. performed the experiments. H.H. and W.B. conceived the study, all authors designed and interpreted the experiments. O.C.N. wrote the manuscript and all authors reviewed the final draft.

Additional Information

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.