COMPARATIVE STUDIES ON THE IMMUNOGENICITY OF THE LIVE SPORE ANTHRAX VACCINE VERSUS NON-LIVING VACCINE CANDIDATES IN GOATS AND PROTECTIVE CAPACITY OF IMMUNE SERA IN MICE

BY

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Dedication

To my mother, Roseline Ndumnego (Nwa Onitsha).

Your fruits blossomed.

Love you Mma.

Declaration

I, *Okechukwu Chinazo Ndumnego*, understand what plagiarism is and am aware of the University of Pretoria policy in this regard. I hereby declare that this thesis is my own original work. Where other people's work has been used (either from a printed source, internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements. I have not used work previously produced by another student or any other person to hand in as my own.

Neither the full thesis nor any part of it has been, is being, or is to be submitted for another degree at this or any other University.

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Summary

COMPARATIVE STUDIES ON THE IMMUNOGENICITY OF THE LIVE SPORE ANTHRAX VACCINE VERSUS RECOMBINANT PEPTIDE AND DNA VACCINE CANDIDATES IN GOATS AND PROTECTIVE CAPACITY OF IMMUNE SERA IN A PASSIVE MOUSE PROTECTION TEST

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Anthrax is a zoonotic disease affecting most warm-blooded mammals. Primarily recognized as a disease of herbivores, it is caused by a spore-forming, rod-shaped bacterium, *Bacillus anthracis*. The disease has a worldwide distribution though, mostly of sporadic nature in developed countries due to effective vaccination and control measures. This feat was largely due to the introduction of the Sterne 34F2 live spore vaccine in the 1940s. While proving effective in controlling anthrax, the Sterne vaccine has a number of problems which, it is

hoped, can be surmounted with the advent of recombinant peptides or DNA acellular alternatives. Such vaccines would overcome the issues of handling live *B. anthracis* during production and avoid batch to batch variation in content and immunogenicity and should improve the duration of immunity. Most importantly, these would also permit simultaneous anti-microbial treatment and vaccination of animals; allowing the early development of immunity in treated animals.

The principal virulence factors of *B. anthracis* are located on two plasmids pXO1 and pXO2. The pXO1 encodes the toxic factors; protective antigen (PA), lethal and oedema factors (LF and EF) respectively while pXO2 contains the encapsulation genes. The poly-ɣ-D-glutamic acid capsule is poorly immunogenic and assists in post-infection dissemination of the organism. The capsule enables the anthrax bacilli to evade immune surveillance mechanisms and enter the circulatory system where it proliferates systemically. PA combines with LF, a zinc metalloprotease, to form lethal toxin (LT) that inactivates most mitogen-activated protein kinase kinases (MAPKK) and inflammasome-activating NLR1B leading to the impairment and death of susceptible macrophages. Oedema toxin (ET), formed by the binding of PA to EF a calmodulin dependent adenylate cyclase, disrupts fluid homeostasis across the host cell membranes.

The antigens PA, BclA (Bacillus collagen-like protein of anthracis) and FIS (formaldehyde inactivated spores), alone or in combination are known for their protective efficacy from previous studies in laboratory rodents. However, it remains to be elucidated if these vaccines or their combinations will elicit a protective immune response in goats against anthrax infections. In this study, these antigens in addition to various adjuvants were administered to goats in combinations. Also, we assessed the immunogenicity and efficacy of plasmid DNA vaccine encoding immunodominant domains of PA, LF and BclA using a heterologous DNA prime/protein boost approach. The aims were to assess the immunogenicity of these acellular

vaccine candidates in goats and evaluate the protective capacity of the immune response in an *in vivo* A/J mouse passive protection model. Also, the immune response following simultaneous antibiotic treatment and immunization with either acellular or live spore vaccines were studied in goats. Attempts were made to compare the resulting immunity following booster vaccinations with the acellular or Sterne live spore vaccines.

Our findings indicated that the addition of FIS to recombinant PA and BclA vaccine candidates generated superior humoral immune responses compared to the recombinant peptides alone. Also, sera from goats vaccinated with the multi-component vaccine (rPA, rBclA and FIS) protected 73 % while the rPA+rBclA vaccinates' sera protected 68 % of A/J mice against Sterne 34F2 spores in the passive protection test. Sera from goats primed with the plasmid DNA vaccine and boosted with FIS failed to protect any of the A/J mice in the challenge studies. However, caprine sera obtained following plasmid DNA vaccination and rPA and rBclA boosting protected just over 40 % of challenged mice.

Importantly, our results showed that the simultaneous administration of acellular vaccine candidates with antibiotics did not negate the development of crucial anti-toxin and anti-spore antibodies in goats. The immune responses from the latter did not differ from that induced in goats treated with acellular or Sterne live spore vaccines alone. The simultaneous administration of penicillin G with Sterne live spore vaccination, while not fully blocking the development of antibody titres, did obviate the production of antibodies in 60% of treated animals. In summary, these studies demonstrate the potential of utilizing a non-living vaccine candidate in the prevention and treatment of anthrax infections in a ruminant model.

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List of Abbreviations

AVA	Anthrax vaccine adsorbed
AV-UK	Anthrax vaccine-United Kingdom
BC	Before Christ
BCA	Bicinchoninic acid
BclA	*Bacillus* collagen-like protein of *anthracis*
BclAD1D3	DNA vector encoding BclA domain 1 and domain 3
BrdU	bromodeoxyuridine
cAMP	Cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CIITA	Class II transactivator
CO_2	Carbon dioxide
cfu	colony forming unit
CWS	Cell wall skeleton
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
ECACC	European Collection of Authenticated Cell Cultures
EF	Oedema factor
ELISA	Enzyme-linked immunosorbent assay
ET	Oedema toxin
FCS	Fetal calf serum
FDA	Food and drug administration
FIS	Formaldehyde inactivated spores
HCl	Hydrogen chloride
IgG	Immunoglobulin G
IMHA	Indirect microhemagglutination test
kDa	Kilodalton
KNP	Kruger national park
LD_{50}	Lethal dose 50%
LT	Lethal toxin
LF	Lethal factor
LFD1PAD4	DNA vector encoding LD domain 1 and PA domain 4
LPA	Lipopeptide adjuvant
LSV	Live spore vaccine
LT	Lethal toxin
MAPKK	Mitogen-activated protein kinase kinases
MHC	Major histocompatibility complex
MID	Minimum infectious dose
mIPS1	Mouse interferon-ß promoter stimulator 1
MPL	Monophosphoryl lipid A
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCG	Negative control group
NLRP1B	Nucleotide-binding domain and leucine-rich repeat, pyrin domain 1
NLV	Non-living vaccine

NLV+Pen G	Non-living vaccine+Penicillin G
NT_{50}	50 % neutralization
OBP	Onderstepoort biological company
OIE	International office of epizootics
PA	Protective antigen
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST	PBS with Tween
PBSTM	PBST with skimmed milk powder
PCR	Polymerase chain reaction
pDNA	Plasmid DNA vector
PGDA	Poly-gamma-D-glutamic acid
pXO1	Plasmid XO 1
pXO2	Plasmid XO 2
p-value	Probability values
rBclA	Recombinant BclA
rPA	Recombinant PA
RPMI	Roswell park memorial institute
RT	Room temperature
SI	Stimulation index
SLSV	Sterne live spore vaccine
SLSV+Pen G	SLSV+Penicillin G
SVG	SLSV vaccinated group
TDM	Trehalose dicorynomycolate
TNA	Toxin neutralization assay
TPA	Tissue plasminogen activator
UPBRC	University of Pretoria biomedical research center

CHAPTER ONE

Introduction

In 1876 Robert Koch established *Bacillus anthracis* as the cause of anthrax. The disease is recognized primarily as a disease of herbivores but affecting most warm-blooded mammals. Despite research in anthrax being amongst the earliest in bacteriology, the disease remains a risk to animals and man today (Scorpio *et al.* 2006). The earliest record of the disease among susceptible herbivores in South Africa is obscure but by the mid-nineteenth century, it was already familiar to local farmers and pastoralists who called it "miltziekte" (spleen disease) because of the swollen spleen found on post mortem examination (Gilfoyle 2006). Between the nineteenth and twentieth century, anthrax was widely perceived as an increasingly serious and widespread hazard especially to the livestock population of Southern Africa. This was made worse by the zoonotic nature of the disease, with humans contracting the infection through contact with animals or contaminated animal products.

The principal virulence factors of *B. anthracis* are coded for on two plasmids, pXO1 and pXO2. The pXO1 encodes the toxic factors; protective antigen (PA), lethal and oedema factors (LF and EF) respectively (Mikesell *et al.* 1983, Uchida, Hashimoto & Terakado 1986, Pezard, Berche & Mock 1991) while pXO2 contains the encapsulation genes (Green *et al.* 1985, Makino *et al.* 1989). Pasteur and Greenfield were the first to vaccinate livestock against anthrax by administering cultures of vegetative bacilli (Tigertt 1980). During the original trial of the Pasteur vaccine at Pouilly-le-Fort in 1881, all vaccinated sheep were protected while every unvaccinated succumbed to lethal infection (Pasteur, Chamberland & Roux 1881). Being heat-attenuated, the vaccine suffered from declining potencies (Sterne,

Nicol & Lambrechts 1942) and unwholesome variations in virulence particularly to certain susceptible species (Sterne 1937, Turnbull 1991). This vaccine was replaced by a toxin positive, capsule negative strain, 34F2 ($pXO1^{+}/pXO2^{-}$) (Sterne, Nicol & Lambrechts 1942).

The introduction of the live attenuated Sterne 34F2 vaccine strain in the 1940s made the control of anthrax in livestock possible. Nevertheless, the vaccine has limitations due to its residual virulence in sensitive species (Sterne 1939, Cartwright, McChesney & Jones 1987), short term protection (Turnbull *et al.* 2004), ineffectiveness during outbreaks and incompatibility with antibiotics (Turnbull 1991, Fasanella *et al*. 2008, Webster 1973). These could be addressed with the advent of recombinant protein or DNA acellular alternatives. This will surmount issues of handling live *B. anthracis* during vaccine production as well as in the field, could enhance the standardization of vaccine production and avoid batch to batch variation in content and immunogenicity. Enhanced immunity can also be achieved with the concomitant use of improved adjuvants (Coffman, Sher & Seder 2010). Most importantly, it would permit simultaneous treatment and vaccination of animals; ensuring treated animals have better opportunity for early development of immunity.

Although the antigens of *B. anthracis* including the recombinant protective antigen (rPA), recombinant *Bacillus* collagen-like protein of *anthracis* (rBclA) and formaldehyde inactivated spores (FIS) are known for their protective efficacy from many published studies (Brossier, Levy & Mock 2002, Gauthier *et al*. 2009, Saile & Quinn 2011), it remains to be elucidated if these vaccines or their combinations will elicit a protective immune response in livestock against anthrax infection. In aiming to address this question, the objectives of this research are;

1. Evaluation of the immune and protective parameters in goats following single or booster vaccinations with the Sterne 34F2 live spore vaccine.

It is recognized that the development and duration of antibodies following immunization with the Sterne 34F2 vaccine have never been systematically studied in livestock (Fasanella *et al.* 2010). In a previous study, we had assessed the immunogenicity and protection induced by the vaccine in goats (Ndumnego 2012). The current objective attempts to evaluate the immune parameters in a goat host following single or booster vaccinations with the Sterne 34F2 live spore vaccine.

2. Feasibility of simultaneous antibiotic treatment and immunization with non-living vaccines in animals at risk of infection during an outbreak scenario.

We combined the rPA with the exosporium antigen, rBclA and FIS in a simultaneous vaccine formulation with penicillin G in order to assess the *in vivo* effect of the latter on immunogenicity of non-living vaccine candidates. Comparisons of specific immune titres following vaccination with either the Sterne 34F2 vaccine alone or in combination with penicillin G were also performed to ascertain any effect of the antibiotic on developing antibody titres.

3. Assessment of the immunogenicity of non-living vaccine candidates in goats and evaluation of the protective capacity of induced immune response in an *in vivo* A/J mouse challenge model.

I. Investigate if immunization with 75 µg of antigenic components consisting of toxin (rPA) and spore exosporium protein (rBclA), supplemented by 10^8 FIS and lipopeptide adjuvant (administered in three immunizations) can elicit a humoral immune response indicative for a protective status of a vaccinated animal.

II. Investigate if serum from three times immunized goats with acellular antigenic components will protect A/J mice against a lethal infection with spores of the Sterne 34F2 vaccine strain by intra-peritoneal administration.

III. Investigate the immune parameters of immunization with a DNA vaccine prime comprising the *pag* and *bclA* genes in goats and boosting combination with non-living antigens (FIS, rPA and rBclA).

IV. Investigate if serum of goats immunized with DNA vaccine followed by single or double booster vaccination with non-living vaccine (FIS, rPA and rBclA) will protect A/J mice against a lethal infection with spores of the Sterne 34F2 vaccine strain by intra-peritoneal route.

CHAPTER TWO

Literature Review

2.1 Introduction – History of anthrax

"Anthrax" was first coined for a disease characterized by the black colour of skin lesions and blood in the 5th century B.C. by Hippocrates, a famous Greek doctor (Schwartz 2009). It was believed to be the fifth plague which affected the livestock of Egyptians in 1491 B.C. (Hambleton, Carman & Melling 1984). Throughout history, the disease has remained a major cause of death for animals from the epidemic mentioned in Virgil's *Georgics* (70 - 19 B.C.) to the more recent outbreak in cattle population in Matabeleland Zimbabwe (ProMED 2015). Essentially a disease of herbivores (Beyer & Turnbull 2009), it was largely responsible for the pandemic called "black bane" which decimated over 60,000 humans and caused vast livestock losses in Europe between 17th to 19th century, almost wiping out half of the sheep population on the continent in the mid-eighteen century (Schwartz 2009). Though now mostly of a sporadic nature in developed countries due to effective vaccination and control measures, it remains an endemic problem in domestic and wild herbivorous populations in the developing world (Hugh-Jones & Blackburn 2009, Hugh-Jones 1999).

A clear description of the disease was given in 1780 by Philibert Chabert (Wistreich & Lechtman 1976) and the famous "Koch postulates" were based upon Robert Koch's work on anthrax. Davaine (1863) first found that the organism was responsible for anthrax in susceptible animals. He showed that the blood from an infected animal contained the causative agent and also transmitted the disease when injected into a previously healthy animal. Following the work of Davaine and Koch, Louis Pasteur refined and performed a conclusive experiment using a series of subcultures of an initial inoculum to prove that the bacterium, *Bacillus anthracis*, was the etiological agent of anthrax and not some contaminant

or elements of the infective blood of a diseased animal (Duclaux 1896, Pasteur & Joubert 1877, Schwartz 2009).

B. anthracis is a facultative, aerobic, endospore-forming, rod-shaped bacterium (OIE 2012). The rods measures 4 µm × 1 µm, stains positive on Gram staining and consistently appears in chains of two to a few cells in length under the microscope. The vegetative cells are square-ended (box-car shaped) and form spores in a nutrient deprived environment. The vegetative cells can release some of their polypeptide capsule when cultured under experimental conditions of high CO_2 partial pressure and presence of bicarbonate. The capsular nature of *B. anthracis* was first observed by M'Fadyean in 1904. Fully virulent strains of the bacillus give rise to whitish, rough, non-hemolytic colonies on blood agar (reviewed by Jayachandran 2002).

Successive investigations on the anthrax toxins in the early and mid-1900s culminated in the isolation and characterization of the tripartite and proteinaceous nature of the complex (reviewed by Schwartz, 2009). The toxin complex consists of three independent and innocuous factors namely; PA, EF and LF. These toxins are of the binary kind (A and B) with the A component (EF or LF) bearing the enzymatic activity. EF (89 kDa) is a calmodulin-dependent adenylate cyclase that increases intracellular cAMP concentration (Moayeri & Leppla 2009, Tang & Guo 2009) while LF (90 kDa) is a zinc-dependent metalloprotease cleaving specifically the N-terminus of most mitogen-activated protein kinase kinases (MAPKK) (Moayeri & Leppla 2009, Vitale *et al*. 2000). The B component, protective antigen (PA) so-called because of its immunogenic properties (Stanley & Smith 1963, Wright, Green & Kanode Jr 1954) is common for both toxins and involved in binding to the host cell receptor. PA is secreted as a 83 kDa protein (PA83) which following secretion, binds to host cell receptors; tumor endothelial marker-8 (TEM-8) and capillary morphogenesis protein-2 (CMG-2) (Bradley *et al*. 2001). Binding of PA83 to its receptors

allows its cleavage by a furin-like proteinase into two subunits of 63 and 20 kDa with the resulting release of the latter (Klimpel *et al.* 1992). PA63 then oligomerizes to form a heptameric structure that constitutes a channel in the host cell membrane through which the LF and EF are translocated into the cytosol. The PA-LF/PA-EF complex is internalized by a receptor-mediated endocytosis, and following the acidification of the endosome, the toxin(s) is translocated into the host cell cytosol where it exerts insidious enzymatic effects (Milne *et al.* 1994, Mogridge *et al.* 2002, Mogridge, Cunningham & Collier 2002). PA can exist as PA63 following cleavage by calcium-dependent serum protease and interact with LF/EF in the bloodstream (Ezzell & Abshire 1992, Ezzell *et al.* 2009). Studies by (Ezzell & Abshire 1992) show the PA63-LF complex to be present in the serum of wide ranges of animals including horses, goats, sheep, dogs, and cats with functional activity similar to free LF (Ezzell *et al.* 2009, Panchal *et al.* 2005). In contrast, (Moayeri, Wiggins & Leppla 2007) failed to detect PA protease activity in plasma from rats. The presence and relevance of PA63 in the bloodstream may vary among species.

Lethal toxin (LT) induces macrophage death by disrupting the MAPKK-dependent pathways that regulate prosurvival genes (Park *et al.* 2002, Turk 2007) and by increased levels of caspase-1 following activation by *NALP1b* genes present in susceptible cells (Cordoba-Rodriguez *et al.* 2004, Boyden & Dietrich 2006). The oedema toxin (ET) induced rise in cyclic AMP also inhibits phagocytosis, microbicidal activity, neutrophil chemotaxis and superoxide production (Turk 2007). ET has also been associated with the up-regulation of toxin receptors and impairment of toxin clearance, hence increasing the host sensitivity and prolonging the plasma half-life of the toxins (Maldonado-Arocho & Bradley 2009, Sastalla *et al.* 2012, Liu, Moayeri & Leppla 2014). It is suggested that the dual sub-lethal expression of LT and ET at the early spore germination stage following infection act synergistically to blunt the bactericidal activity of phagocytes, thereby promoting the survival and release of

engulfed bacilli from macrophages (Moayeri & Leppla 2004). The inhibition of the innate immune responses allows *B. anthracis* to evade host defences leading to extensive bacteraemia. However, several studies have suggested that animals which are resistant to LT are susceptible to challenge by *B. anthracis* spores and vice versa (Terra *et al.* 2010, Welkos, Keener & Gibbs 1986, Newman, Printz *et al.* 2010, Newman *et al.* 2010) This has been largely attributed to the pleomorphic nature of the *NLRP1* inflammasome pathway with the cleavage of the latter by LT leading to a rapid, caspase-1-dependant apoptosis (Terra *et al.* 2010, Park *et al.* 2002, Friebe *et al* 2016). The preponderance of LT-susceptible macrophages, rather than resistant ones, appeared to protect mice from *B. anthracis* infection (Terra *et al.* 2010, Moayeri *et al.* 2010). To the contrary, the rapid LT-induced death in Fischer rats correlated strongly with the sensitivity of its macrophages to LT (Newman *et al.* 2010, Beall *et al* 1962, Levinsohn *et al.* 2012).

The second virulence factor of *B anthracis*, the poly-γ-D-glutamic acid (PGDA) capsule, acts to disguise the germinated bacilli from immune surveillance through its poorly immunogenic and anti-phagocytic properties (Little & Ivins 1999). The PGDA capsule is polymerized on the peptidoglycan cell surface to produce a high molecular mass structure (Makino *et al.* 2002), this is then degraded to a lower molecular mass that is released from the bacterial cell surface and acts as a decoy to protect the bacteria from complement (Makino *et al.* 2002). The capsule is directly anchored to the underlying peptidoglycan cell surface by a covalent bond (Candela & Fouet 2005) and has been reported to enhance the activity of LT (Ezzell *et al.* 2009, Jang *et al.* 2011). This could possibly be attributed to the sensitization of macrophages to LT by the capsule and bacterial cell wall products (Moayeri & Leppla 2004). Stripping *B. anthracis* of the PGDA capsule appears to enhance the immune response against the bacilli (Scorpio *et al.* 2007, Scorpio *et al.* 2008, Negus *et al.* 2015). This is probably due to the induction of opsonizing antibodies that target bacilli to the host animal phagocytes

(Chabot *et al.* 2004, Scorpio *et al.* 2010, Chabot *et al.* 2015). This was achieved by treatment with capsule depolymerase (CapD), a membrane-associated PGDA-specific depolymerase (Candela & Fouet 2005). Recent studies have further implicated the PGDA in the pathogenesis of *B. anthracis* through the inducement of proinflammatory cytokines (Jeon *et al.* 2015, Lee *et al.* 2015). Lee *et al* showed that PGDA induces the production of nitric oxide (NO) in a dose-dependent manner. Massive release of NO in vascular smooth tissues leads to excessive vasodilation, hypotension and play a critical role in the pathogenesis of septicaemia (Moncada & Higgs 1993, Chandra *et al.* 2006, Bogdan 2015). With increasing bacteraemia, more PGDA are shed into the blood circulation exacerbating the systemic inflammation and septicaemia in the host (Lee *et al.* 2015, Coggeshall *et al.* 2013). The general mode of action and pathogenesis of the virulence factors in the extensively studied mice model provide important clues to understanding the role of anthrax toxins in herbivores.

2.2 Routes of infection

B. anthracis can be transmitted through the cutaneous, gastrointestinal or inhalational routes in animals. The bacilli are generally shed from the body orifices of infected animals during the terminal stages of illness or when infected carcasses are torn open by scavengers (Hambleton, Carman & Melling 1984, WHO 2008). The shed bacilli readily sporulate in soil at temperatures of 20-30 $^{\circ}$C. Livestock feeding on contaminated soil and/or foliage subsequently become infected and incidences of these increase in situations of drought or overgrazing when the likelihood of animals inhaling or ingesting spore-laden dust is higher (Hambleton, Carman & Melling 1984). The route by which the spores enter the body exerts an important influence in both experimental and natural infections. In nature or husbandry conditions, animals usually acquire anthrax by the ingestion of contaminated substances (feed, grass, water or infected carcasses) and thus are more likely to suffer from the

gastrointestinal form of the disease than any other (Bhatnagar & Batra 2001). Blowflies have also been incriminated in the disease transmission when these flies feed on body fluids of anthrax carcasses and then deposit highly contaminated faeces or vomit on the vegetation in the area. Browsing herbivores then may contract anthrax when they feed on leaves contaminated with *B. anthracis* spores (Braack & De Vos 1990, De Vos 1990, De Vos & Bryden 1996). Cutaneous anthrax can occur in animals through the bites of blood-sucking flies like tabanids as hypothesized in a recent study (Palazzo *et al*. 2012). In highly susceptible animals, such as ruminants, the disease is acute and runs a rapid course (Bhatnagar & Batra 2001).

Data on the natural infectious dose of *B. anthracis* spores in ruminants is not readily available. A minimum oral infectious dose (MID) of 5×10^8 spores is estimated for sheep, horses and cattle (Carter & Pearson 1999). Published LD_{50} of anthrax spores by the subcutaneous route were 100, 3000, 5000, 10^6, 10^9 and 5×10^{10} for sheep, rhesus monkeys, rabbits, rats, pigs and dogs respectively (Schlingman *et al*. 1956, Watson & Keir 1994). A respiratory MID of 3500 spores was estimated for sheep by Fildes in 1943 (Turnbull *et al*. 1998) whereas a subcutaneous dose of a 1000 spores was used as the minimum lethal dose for sheep and goats in a vaccine trial in 2007 (Jula & Jabbari 2007). It is important to point out that these LD_{50} or MIDs only provide a rudimentary guide to the likely or actual infectious dose in nature as factors such as *B. anthracis* strain, route(s) of infection, species, breed and state of health of animal(s) play an important role in its epidemiology (WHO 2008).

2.3 Clinical signs

Clinical signs in the highly susceptible ruminant species are non-specific and are rarely observed until shortly before the terminal stage. In cattle, there is a steep rise in temperature

and irritability which may be accompanied by dullness. Death usually occurs within 2-3 days with the affected animal showing cramp-like symptoms and shivering (Sterne 1959). Blood may ooze from body orifices and the urine might be blood-tinged (WHO 2008). Oedematous or carbuncular lesions can be seen in animals with waning immunity (Sterne 1959). Anthrax generally takes a peracute nature in goats (WHO 2008) which are regarded as among the most susceptible subset of animals within the vulnerable herbivore species. The initial sign of the disease in a flock is sudden death of one or more of the animals (Beyer & Turnbull 2009) after a 3-5 days incubation period. With horses, colic and diarrhoea may present in affected animals, which may show large oedema in the brisket, abdomen, neck and shoulders in cases transmitted by biting flies (Sterne 1959). Pigs are regarded as more resistant to anthrax than other domestic livestock. They usually show signs of severe oedema of the throat and the submandibular lymph nodes (Beyer & Turnbull 2009).

Affected animals show signs of distress at the terminal stages of infection before becoming comatose and dying within hours of showing these signs. The peracute course is characterized by sudden staggering and recumbency with convulsions occurring minutes before death. These signs can be modified in poorly immune animals (WHO 2008). Post mortem gross pathology includes extravasation of blood from body orifices, splenomegaly, dark poorly clotted blood and incomplete rigor mortis (Hambleton, Carman & Melling 1984).

2.4 Host immune response to *B. anthracis*

Theoretically, the development of antibodies to the three toxin components of anthrax should occur following the natural/experimental infection of a host animal. This has been confirmed in studies with ELISA using *B. anthracis*-specific antigens (Ivins & Welkos 1988, Mahlandt *et al*. 1966, Taft & Weiss 2008b, Turnbull *et al*. 1992, Turnbull *et al*. 2004). PA is the most essential component of live, attenuated or protein-based vaccines (Leppla *et al*.

2002) and *B. anthracis* strains cured of the toxin producing plasmid, pXO1, have been reported to be non-virulent and failed to confer protective immunity to animals, with exception to mice (Leppla *et al*. 2002, Welkos, Vietri & Gibbs 1993). Protective immunity to anthrax depends on two critical factors; resistance to establishment of infection and resistance to disease progression (intoxication) (Mahlandt *et al*. 1966). With the latter, production of anti-PA antibodies is crucial if the animal must survive the infection. The pathogenesis of the disease seen in susceptible animals is caused largely by the binding of EF and LF to PA to form oedema and lethal toxins respectively, and the translocation of these into the host cell cytosol (Leppla *et al*. 2002). Due to the essential role played by PA in anthrax pathogenesis, it is deemed to be the most essential part of any anthrax vaccine. Numerous studies have shown PA to be able to induce protective responses in experimental models of infection (see review by Cybulski Jr, Sanz & O'Brien 2009). Anti-PA antibodies have also been shown to suppress germination and enhance phagocytosis of spores by macrophages and thus preventing the establishment of infection (Welkos *et al*. 2001). The presence of anti-PA antibodies have been recorded in various wild herbivores (Cizauskas *et al.* 2014, Lembo *et al.* 2011). This is believed to be triggered by sub-lethal exposure to *B. anthracis* spores, which wanes rapidly overtime (Cizauskas *et al.* 2014). Similarly, during the sampling of goats for this study, we had detected anti-PA antibodies in some of the animals. Also, a significant proportion of open field-raised sheep had been found positive for anti-PA antibodies in Spain (Pierre Goossens, Personal Communication). Carnivores in anthrax enzootic areas are also known to possess these specific antibodies (Turnbull *et al*. 1992). While production of antibodies to LF and EF has been shown in animals, their roles in mediating protection remain less clear (Taft & Weiss 2008a). One could surmise that the presence of LF or EF antibodies in unvaccinated animals, especially in the more resistant carnivores, could be indicative of the prevalence of the disease in their localities (Turnbull *et*

al. 1988, Turnbull *et al*. 2001, Turnbull *et al*. 1992). Early studies reported that EF increased the immunizing capability of PA in guinea pigs while LF decreased the protection induced by the PA/EF mixture (Stanley & Smith 1963) while Mahlandt *et al*. (1966) showed that both LF and EF have an additive effect on the protectivity of PA in rats, mice and guinea pigs. Also, EF can act as an adjuvant to potentiate anti-PA responses (Quesnel-Hellmann *et al*. 2006) while a combination of functional domains of LF and PA showed enhanced protection compared to PA alone (Baillie *et al*. 2010). Contrastingly, other studies indicated that although PA-plus LF/EF vaccines induced high LF and EF antibody titres, the vaccines did not increase protection during *B. anthracis* spore challenge when compared to PA-alone vaccines (Little & Knudson 1986, Taft & Weiss 2008a).

2.5 Anthrax immunology: Evolving diagnostic tools

The monitoring and evaluation of the immune response induced by the administration of experimental vaccines is a crucial point in the development of new anthrax vaccines. Over the years, various methods have been employed in the assessment of antibodies specific against *B. anthracis* in the sera of vaccinates. Initially, there was no known serological method of measuring these specific antibodies and testing of new vaccines was through *in vivo* immunity tests on guinea pigs, sheep and other species (Auerbach & Wright 1955, Schlingman *et al*. 1956, Sterne 1937, Wright, Green & Kanode Jr 1954). Following the isolation, purification and production of the proteinaceous components of anthrax toxin and the increasing need for serological tools in context of the search for a potent human anthrax vaccine, additional immunological monitoring techniques were employed for the first time in the late 1950s (Belton & Strange 1954, Puziss & Wright 1954, Turnbull *et al*. 1992, Wright, Hedberg & Slein 1954). This and the subsequent improvements will be listed in the following subsections.

2.5.1 Agar-gel diffusion

Agar-gel diffusion was first developed by a Swedish physician, Organ Ouchterlony in 1953; it was first utilised in the determination of antigen and antibody concentrations in culture filtrates and antisera of vaccinated animals by Thorne & Belton (1957). In agar-gel diffusion, round wells were punched out in solidified agar in Petri dishes with cork borers and the inner wells were filled with undiluted antiserum to the antigen of interest. Following incubation overnight at 2˚C, outer reservoirs were then filled with the appropriate dilutions of the test antigens in saline. The plates will be inspected for lines of precipitation following 20-24 hours of incubation. These lines of precipitation occur when immune complexes precipitate following the diffusion and meeting of the antigen and antisera preparations from their respective wells. The highest dilution which produced a visible line of precipitation was taken as the endpoint. For determination of the antibody titre of a test serum, it is mixed with a standard antigen preparation and its ability to prevent the formation of a precipitation line with the standard antiserum in the agar diffusion plates evaluated (this was basically an inhibition format assay). Though relatively simple and inexpensive, it was not very sensitive with titres of 1/16 frequently obtained for supposedly hyper-immune antisera preparations (Thorne & Belton 1957) . It also lacked consistency and reproducibility when the visually determined end points of the same sera were observed by different technicians (Ray Jr & Kadull 1964).

2.5.2 Indirect microhaemagglutination test (IMHA)

Increasingly improved methods of purification of PA led to better yields (Fish *et al*. 1968, Wilkie & Ward 1967) and a new method for the detection of anthrax antibodies in sera was published in 1971 (Buchanan *et al*. 1971). The anthrax indirect microhaemagglutination (IMHA) was a great improvement over the agar-gel diffusion assay, more sensitive and provided a significant increase in the rapidity of testing (Johnson-Winegar 1984). Basically, it entailed the screening of complement-inactivated test sera to cause the agglutination of PA-

sensitized sheep red blood cells (Buchanan *et al*. 1971). Reactions are recorded as complete, partial or negative with the entire bottom of the microtitre wells covered with a smooth mat of red blood cells in complete haemagglutination. A serum sample is considered reactive to anthrax PA when it produced a haemagglutination of antigen-absorbed red cells at a titre four- fold higher than that produced by any of the controls. Though it became the test of choice in the 70s in the USA (Turnbull *et al*. 1986), it suffered from significant setbacks. Lack of reproducibility, variability in sheep erythrocytes and unstable erythrocyte-antigen preparations detracted from the merits of this assay (Johnson-Winegar 1984) and it was never fully accepted as a standard test for the assaying of anthrax antibodies in animal sera.

2.5.3 Enzyme-linked immunosorbent assay (ELISA)

In 1960, Rosaly Yalow and Solomon Berson described a method with which they measured insulin levels in human plasma based on the competition between epitopes on human insulin and radioactively-labelled beef insulin for insulin-binding antibodies in sera of guinea pigs immunized with beef insulin (Yalow & Berson 1960). In 1971, two independent researchers published techniques in which they replaced radioactively-labelled antigens/antibodies with enzyme-linked variants (Engvall & Perlmann 1971, Van Weeman & Schuurs 1971). ELISA has since come to be the gold standard assay utilised in the quantitation of specific antibodies/antigens at very low concentrations. The first application of ELISA to anthrax serology was in 1984 by Anna Johnson-Winegar (Turnbull *et al*. 1992). She adapted an ELISA for the detection of anthrax antibodies using the PA component of the anthrax toxin (Johnson-Winegar 1984). Microtitre plates (96-well) were coated with PA and incubated at specific temperatures and time before the addition of test sera. Enzyme conjugated anti-test sera antibodies were then added to bind to any antigen-antibody complexes in the plates. Finally, the reaction was visually quantified by the colour change following the addition of the enzyme substrate. ELISA provided a method of assaying anthrax antibodies which is

simple, rapid and highly sensitive. It also required lesser amounts of reagents compared to IMHA and the enzyme-linked conjugates and substrates are readily available (Johnson-Winegar 1984). Several studies have utilised ELISA in assessing the immune response following vaccination of animals (Cizauskas *et al.* 2014, Hahn *et al*. 2006, Lembo *et al.* 2011, Little *et al*. 2004, Turnbull *et al*. 1986). It has proved to be more sensitive and rapid than earlier tests and less subjective to operator(s) bias in interpreting results since simple machine-read endpoints are utilised (Williams *et al*. 1984). Due to the unavailability of commercial ELISA kits for assaying anti-anthrax antibodies in livestock (Cizauskas *et al.* 2014), the development and optimizing of specific ELISA systems for serology in these animals is unavoidable. Issues such as identifying and securing a constant supply of reagents, such as antibodies, enzyme-labelled analytes and substrates, may affect the overall choice of ELISA format to be used. Lot to lot variations in assay reagents may also affect reproducibility and standardised operating procedures (SOPs) for each assay will need to be established (Sittampalam *et al.* 1996). The key to optimisation of ELISA is the availability of key reagents required for the daily running of the assay. The availability of positive and negative control sera is of the essence and samples need be collected from representative population of animals for which the assay will be applied (Crowther 2009). These samples will be used to optimise other reagents and assay conditions throughout the development process. The control sera should give the expected results, preferably in one or more serological techniques other than the one being developed (Deshpande 1996). Also, the availability of control sera will assist in the estimation of intra- and inter-assay variation on different days (Crowther 2009). This will confirm the feasibility of the assay or will determined ultimately whether the test format should be abandoned.

Various adaptations of ELISA have been introduced since the development of enzyme immunoassays. The major systems/techniques are direct, indirect and sandwich ELISAs from which various techniques are derived. The direct ELISA enables the detected of antigens/antibodies immobilized on the walls of microtitre wells and using specific labelled primary antibody (Engvall & Perlmann 1971, Van Weeman & Schuurs 1971). The resulting labelled antibody-antigen/antibody complex is then detected by the addition of the enzyme substrate. The concentration of antigen/antibody in the serum is directly proportional to the colour change intensity (Engvall 2010). This method is regarded as the simplest form of ELISA and is very efficient when conjugated monoclonal antibodies are available (Crowther 2009). It is, however, limited by its inflexibility and low sensitivity due to minimal signal amplification (Aydin 2015). The indirect ELISA technique was developed in 1978 and differs from the direct method through the introduction of a secondary conjugated antibody (Lindström & Wager 1978). With this method, the solid phase-immobilized antibody-antibody complex is recognized by the addition of specific enzyme tagged secondary antibody. Then the enzyme substrate is added to the medium to produce some colour change, which correlates with the concentration of antigen-antibody complexes in the medium (Lindström & Wager 1978). It has high flexibility since a wide variety of labelled secondary antibodies can be introduced to the format. With the availability of non-species specific conjugated protein A/G in place of the labelled secondary antibody (Eliasson *et al.* 1988), the development and use of species-independent ELISA as an effective serological in many host species has blossomed (Inoshima *et al.* 1999, Bhide *et al.* 2004, Zhang *et al.* 2010, Schaefer *et al.* 2012, Al-Adhami & Gajadhar 2014, Nymo *et al.* 2013). A limitation of the indirect ELISA is the non-specificity of the antigen immobilisation step, which might cause some cross-reactivity with crude antigens (Gan & Patel 2013). In general, the enzyme

immunoassay technique is a continually evolving paradigm with increasingly more sensitive analytical platforms in development.

2.5.4 Toxin neutralization assay (TNA)

Following several studies depicting the effects of anthrax lethal and oedema toxins on susceptible cells (Leppla 1982, Friedlander 1986, Bhatnagar *et al*. 1989, Singh *et al*. 1989, Leppla 1991), the TNA technique was developed to measure the functional ability of antibodies in sera of immunized animals to neutralize LT/ET cytotoxicity for certain sensitive cell lines (Pitt *et al*. 2001, Reuveny *et al*. 2001). Three types of TNAs have been developed (Ngundi *et al*. 2010). The J774A.1 (J774) and RAW 264.7 (RAW) TNA are murine macrophage-like cell based assays which measure the ability of anti-PA antibodies to neutralize the cytocidal activity of LT (Pitt *et al*. 2001, Reuveny *et al*. 2001). The susceptibility of these cell lines has been linked to the presence of the polymorphic gene *Nlrp1b* on chromosome 11 (Roberts *et al.* 1998, Boyden & Dietrich 2006). Activation of the paralog, mNlrp1b, leads to assembly of the inflammasome, a multi-protein complex responsible for the activation of caspase-1 (Boyden & Dietrich 2006, Martinon, Burns & Tschopp 2002, Newman *et al.* 2010). The mNlrp1b inflammasome-mediated activation of caspase-1 is a prerequisite for the programmed death of susceptible macrophages (Fink, Bergsbaken & Cookson 2008, Sastalla *et al.* 2013).

The third assay format is the Chinese hamster ovary (CHO) cell-based TNA, which evaluates the ability of anti-PA antibodies to neutralize the ET-induced increase in intracellular cAMP levels (Zmuda *et al.* 2005). The principle of this assay is based on the ability of EF, an adenylate cyclase, to catalyse the synthesis of copious cAMP following activation by calcium-dependent calmodulin (Leppla 1982 & 1984, Kumar, Ahuja & Bhatnagar 2002). CHO cells have been shown to have increased tolerance to significant levels of cAMP, induced by incubation with ET (Leppla 1982, Voth *et al.* 2005). Clearly, the

ability of the CHO cells to survive intoxication while showing large increases in CHO levels is very critical to the CHO-based TNA.

The viability of the surviving cells in the J774 and RAW formats is measured by their ability to reduce 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, MTT) to purple coloured MTT formazan following their treatment with test sera-LT mixture (Abe & Matsuki 2000, Williamson *et al.* 2005, Weiss *et al.* 2006, Liu *et al.* 2009, Parreiras *et al.* 2009, Ngundi *et al.* 2012). The amount of ET-induced increase of cAMP in susceptible unprotected CHO cells is estimated using chemiluminescent cAMP ELISA (Zmuda *et al.* 2005, Zeng *et al.* 2006, Kulshreshtha & Bhatnagar 2011, Ngundi *et al.* 2012). TNA is highly versatile, species independent and has been standardized for use with multiple species (Hering *et al*. 2004, Omland *et al*. 2008, Zmuda *et al*. 2005, Fay *et al.* 2012, Ndumnego *et al.* 2013). This is a major advantage of the TNA compared to the conventional ELISA. The TNA also quantifies functional anti-toxin antibodies rather than total antibodies (Omland *et al*. 2008), but it is relatively more time consuming and elaborate in comparison to ELISA.

2.6 Anthrax veterinary vaccines

Current veterinary vaccines against anthrax are composed of attenuated strains of *B. anthracis*. These are broadly classified into three groups;

- Capsulated and atoxigenic – Pasteur strain
- Uncapsulated and toxigenic – Sterne strain
- Capsulated and toxigenic – Carbosap strain

2.6.1. Pasteur vaccine

This was the first vaccine adopted for use against anthrax and initially involved double inoculations two weeks apart (Turnbull 1991). The initial vaccination consisted of *B.*

anthracis cultures incubated at 42 °C for 21 days (Pasteur type I) which was pathogenic for mice. Pasteur vaccine type II spores were incubated for 14 days, less attenuated and pathogenic for both guinea pigs and mice. It is bereft of toxic factors and sero-analysis of vaccinated goats do not show specific antibodies (see review by Fasanella *et al*. 2010). The duplex vaccine was replaced by single vaccines consisting of type II spores suspended in glycerol and addition of saponin (1-10 %). This was surmised then to consist of mixed populations of $pXO1^{-}/pXO2^{+}$ and $pXO1^{+}/pXO2^{+}$ *B. anthracis* spores (Mikesell *et al*. 1983, Saile & Quinn 2011). However, a recent study described the presence of pXO1 in the attenuated Pasteur strain II using more sensitive molecular biology techniques (Liang *et al.* 2016). The results indicated that the high temperature-induced attenuation of the strain may have only reduced the total copy number of pXO1, but not completely eradicating the plasmid (Liang *et al.* 2016, Thorkildson, Kinney & AuCoin 2016). The use of this vaccine was mostly discontinued due to considerable residual virulence in livestock (Turnbull 1991).

2.6.2. Sterne vaccine

The Sterne vaccine was first introduced in 1939 and was obtained by cultivating *B. anthracis* spores on a medium with 50 % horse serum in 30 % CO_2 atmosphere overnight (Sterne 1937). The attenuation of this strain is due to the loss of the capsular synthesizing plasmid, pXO2 (Mikesell *et al*. 1983). The vaccine is formulated in 0.5 % saponin in 50 % glycerin-saline solution and is characterized by increased protective capacity compared to the Pasteur vaccine (Sterne 1939, Turnbull 1991). It is the most widely used vaccine against anthrax in animals today. Immune response in vaccinated animals is mainly induced by the toxic factors though other spore-associated antigens may contribute to induced immunity following vaccination (Fasanella *et al*. 2010, WHO 2008). Notwithstanding, care is advocated in vaccinating some sensitive species with this vaccine. During the development of the vaccine, Max Sterne advised caution in the use in goats as it can provoke idiopathic

adverse reactions under field conditions in these animals (Sterne 1939). In llamas, vaccination with the vaccine is not recommended due to the potential virulence in the species (Cartwright, McChesney & Jones 1987), while it has been reported to cause fatal immune-mediated vasculitis in miniature horses (Wobeser 2015). Currently, the OIE recommends a single subcutaneous dose of 1-5 × 10^6 culturable spores for small ruminants and 2-10 × 10^6 spores for cattle (OIE 2012). A two-dose initial vaccination is advised for horses as some may be slow to develop immunity following initial vaccination (OIE 2012). In goats, it is recommended that the single dose be administered in initial half doses on the inner thighs, in order to obviate adverse reactions (OBP 2016).

2.6.3. Carbosap vaccine

The Carbosap vaccine strain possesses both plasmids pXO1/pXO2 and was used in immunizing cattle and sheep (Adone *et al*. 2002, Fasanella *et al*. 2001). The precise basis for attenuation of the Carbosap strain is largely unknown (Adone *et al.* 2002, La Rosa *et al.* 2006) though whole genome sequencing of the strain revealed three chromosomal deletions of 29, 24 and 3.5 kb relative to fully virulent strains (Harrington *et al.* 2013). It consisted of *B. anthracis* spores suspended in 10 % saponin which was responsible for local inflammatory reactions seen with the use (Turnbull 1991). The vaccine was eventually discontinued due to its residual virulence.

2.7 Experimental anthrax vaccines

Since the introduction of the Sterne attenuated live spore vaccine in the 1940s, various studies aimed at the development of safer and more efficacious vaccines have been conducted. As far as the development of these improved vaccines were concerned, the first of

these advances was the ability to purify and define the anthrax toxin components, PA, LF and EF, which had first been separated and partially characterized from 1950-1960 (Stanley, Smith & Sargeant 1961, Stanley & Smith 1963). The improved purifications (Leppla 1982, Leppla 1988, Leppla 1991, Quinn *et al*. 1988) allowed the development of enzyme immunoassays which made it possible for the first time to monitor the response to the vaccines in humans and guinea pigs and to relate the response in the guinea pigs to protective immunity (Ivins *et al*. 1986, Turnbull *et al*. 1986, Turnbull *et al*. 1988, Ivins & Welkos 1988, Ivins *et al*. 1990, Turnbull *et al*. 1990). The second major advance with significant bearing on the development of improved vaccines was the discovery (Mikesell *et al*. 1983, Ezzell *et al*.1985) that the genes encoding the toxins lay on a large plasmid subsequently designated pXO1 and that curing *B. anthracis* of this plasmid resulted in non-toxigenic avirulent derivatives (Green *et al*. 1985). These developments led to the demonstrations of the need for the toxin (or some part) in a vaccine for induction of protective immunity (Ivins *et al*. 1986). It also indicated the ability of PA to produce effective protection in the absence of EF and LF (Ivins *et al*. 1986, Ivins & Welkos 1988, Ivins *et al*. 1990, Turnbull *et al*. 1990). Studies also showed that the addition of totally non-specific cellular entities to either the human vaccines or purified PA enhanced their protective effects (Turnbull *et al*. 1988, Turnbull *et al*. 1990). These progressive discoveries pointed the way to a number of approaches aimed at second generation improved vaccines that mainly included recombinant vaccines. Ivins *et al*. (1990) was able to transform the PA gene in *B. subtilis*. The PA gene has also been cloned and expressed by strains of *Salmonella*, baculovirus and vaccinia virus (Iacono-Connors *et al*. 1994, Garmory *et al*. 2003, Baillie *et al*. 2008). The recent availability of the complete DNA sequence of several strains of *B. anthracis* has enabled the search for other putative vaccine antigens (Read *et al*. 2003).

Protein vaccines against anthrax for use in humans were developed in the 1950s in the USA and UK (Wright, Green & Kanode Jr 1954, Brachman *et al*. 1962). Their efficacy has been established in laboratory animals, in particular on rabbits and rhesus monkeys (Fellows *et al*. 2001), but it is known that they induce only partial protection against fully virulent *B. anthracis* in guinea pigs and no protection in mice (Turnbull *et al*. 1986, Welkos & Friedlander 1988). Also, being essentially adsorbed or precipitated culture filtrates (Wright, Hedberg & Slein 1954, Brachman *et al.* 1962), the final products of these protein vaccines are poorly defined. Other shortcomings of these vaccines were the high number of boosters required and side effects such as erythema, soreness and swellings seen at site of injection (Pittman *et al.* 2001, Niu & Ball 2009, Garman *et al.* 2016). The administration of the AVA was recently reduced to three intramuscular doses over six months, which was said to reduce the frequency, severity and duration of the injection site reactions (Marano *et al.* 2008, Wright *et al.* 2014, Quinn *et al.* 2016, Schiffer, McNeil & Quinn 2016).

Several studies have looked at supplementing protein-based vaccines with an anti-bacterial component with promising results (Hahn, Boehm & Beyer 2006, Gauthier *et al*. 2009, Cote *et al*. 2012, Köhler, Baillie & Beyer 2015). In the case of naturally acquired immunity following recovery from infection, it is assumed that beside anti-toxin immunity, defence is also targeted at spores, spore germination and self-propagating vegetative pathogens (Delvecchio *et al.* 2006, Chitlaru *et al.* 2007, Ingram *et al.* 2010). It is therefore significantly more effective than vaccination with toxin antigens alone (Little & Knudson 1986, Cybulski *et al.* 2008, Gauthier *et al.* 2009, Vergis *et al.* 2013, Köhler, Baillie & Beyer 2015, Kempsell *et al.* 2015). These antigens may contribute to the increase in protection of a protein-based vaccine. Findings from research on the protectiveness of the rPA vaccine and formaldehyde inactivated *B. anthracis* spores (FIS) give this assumption strong backing (Brossier, Levy & Mock 2002, Gauthier *et al*. 2009). The combination was able to fully protect the trial animals

(mice and guinea pigs) against infection with virulent spores as compared to partial protection observed in animals immunized with rPA alone (Brossier, Levy & Mock 2002, Gauthier *et al*. 2009). However, these studies have never been done in any farm animal species since the trials were aimed at developing a human vaccine. The *B. anthracis* capsule, the second essential factor for complete virulence, consists of PGDA. It has poor immunogenicity and protects the vegetative cell from phagocytosis following opsonization and from destruction by complement activation (Ezzell Jr & Welkos 1999). As a prominent surface structure, it would be an ideal target for an antibacterial vaccine. Recent studies have shown it is possible to induce specific antibodies against the capsule by conjugating different lengths of PGDA with rPA, bovine serum albumin (BSA) or peptides (Rhie *et al*. 2003, Schneerson *et al*. 2003, Chabot *et al*. 2012, Chabot *et al*. 2015). Candela and co-workers also demonstrated the immunogenicity and protective effect of a preparation of peptidoglycan naturally linked to PGDA (GluPG) (Candela & Fouet 2005, Richter *et al*. 2009, Candela *et al*. 2012). A combined GluPG and PA vaccination conferred full protection against cutaneous anthrax with a fully virulent strain (Candela *et al*. 2012).

The induction of both humoral and cell mediated immune responses by applying the genetic information of antigenic determinants in eukaryotic cassettes (nucleic acid immunization) is another promising approach in anthrax vaccinology (Gu, Leppla & Klinman 1999). Published results on intramuscular vaccination of BALB/c mice with a PA encoding expression vector induced a mixed immune response (both humoral and cellular) and seven out of eight immunized animals were protected from a challenge with lethal toxin (Gu, Leppla & Klinman 1999), although it was noted that the published toxin doses did not result in the mortality rate expected in control animals. Hermanson *et al* (2004) published successful vaccination trials of rabbits with plasmid DNA encoding the full length PA in combination with the cationic adjuvant Vaxfectin. The PA gene was synthesized with a deleted furin

cleavage site, using the human codon usage (Hermanson *et al*. 2004). Two doses of this plasmid, given intramuscularly, protected 100% of rabbits from aerosol challenge with about 100 LD_{50} of *Bacillus anthracis* Ames strain. In recent trials (Hahn *et al*. 2006, Kim *et al*. 2015, Köhler, Baillie & Beyer 2015), the efficacy of DNA vaccines was tested in mice and sheep. By combining antitoxic (anti-PA and anti-LF) with antibacterial (anti-BclA) immunization and optimizing antigen presentation Hahn *et al*. (2006) and Köhler *et al*. (2015) showed the possibility of inducing long lasting immunity in sheep and protecting mice against infection with fully virulent *B. anthracis* Ames spores respectively.

Better protection by live spore vaccines compared to licensed protein vaccines, frequently accompanied by lower antibody ELISA titres has been attributed in particular to better stimulation of cell mediated immunity (Ivins *et al*. 1986, Klein *et al*. 1962, Little & Knudson 1986, Turnbull *et al*. 1986, Ivins & Welkos 1988). In a seminal work, (Glomski *et al.* 2007a), investigated the critical role cell mediated immunity play in protection against anthrax. Glomski and co-workers demonstrated that the stimulation of naïve splenic macrophages with *B. anthracis* spores induced the secretion of IFN-ɣ by NK cells (Glomski *et al.* 2007b) and that the transfer of splenocytes from FIS-immunised mice protected naïve mice against *B. anthracis* spores infection (Glomski *et al.* 2007a). Cell mediated immunity is important in tackling the early intracellular stage of *B. anthracis* pathogenesis since the spores are the infective forms of the organisms (Glomski *et al.* 2007a, Guidi-Rontani *et al.* 1999, Ascough, Ingram & Altmann 2012). It was demonstrated that LF specific IFN-ɣ producing $CD4^+$ T cells play vital roles in the generation of lasting cellular immunity to anthrax (Ingram *et al.* 2010). The T-cell responses in naturally infected humans were narrowed down to the IV domain of LF, a region responsible for the catalytic activity of the protein (Ingram *et al.* 2010, Comer *et al.* 2005, Paccani *et al.* 2005). The cellular immune responses observed were characterised by a more diverse cytokine profile compared to that induced by vaccination

with PA-based vaccines (Ingram *et al.* 2015). Exposure to cutaneous infection by *B. anthracis* induced a cytokine response encompassing a more global immune response compared to narrower responses in vaccinates (Ingram *et al.* 2015, Ascough, Ingram & Altmann 2012). While humans recovering from natural infection show robust long-term T-cell memory to LF, PA and EF epitopes (Wattiau *et al.* 2009, Ingram *et al.* 2010, Laws *et al.* 2016), Midha and Bhatnagar suggest that the IFN-ɣ-mediated T-cell pathway is either functional when LT is absent or neutralized by humoral anti-toxin antibodies (Midha & Bhatnagar 2009). This is due to the inhibition of $CD4^+$ T-lymphocytes activation following disruption of the MAPKK antigen-receptor signalling pathway by LT (Paccani & Baldari 2011, Midha & Bhatnagar 2009, Paccani *et al.* 2005, Fang *et al.* 2005, Comer *et al.* 2005). As a result, it has been suggested that the stimulation of both humoral and cellular arms of adaptive immunity should be important goals of future *B. anthracis* vaccine design (Ascough *et al.* 2012, Gauthier *et al.* 2009, Zhang *et al.* 2008).

It should be noted that in all these comparisons the same adjuvants were used (aluminium hydroxide or alum) and most of the trials were carried out using guinea pigs. As known guinea pigs only develop incomplete protection against a challenge with anthrax vaccine adsorbed (AVA) or anthrax vaccine-UK (AV-UK) vaccines in contrast to rabbits or rhesus monkeys (Ivins *et al.* 1992, Ivins, Fellows & Nelson 1994, Ivins *et al.* 1995, Fellows *et al.* 2001). Ivins *et al.* (1990, 1992, 1995) were able to show that adjuvants stimulating the innate response significantly improved the protective effect of PA immunization to afford protection to guinea pigs and mice against fully virulent strains of *B. anthracis*. Among the adjuvant combinations tested and found effective have been the complete Freund's adjuvant together with dead bacteria as well as the RIBI trimix combination of monophosphoryl lipid A, cell wall skeleton and trehalose dimycolate (Turnbull 1991). The use of the RIBI adjuvant, however, is contraindicated on account of known and significant side effects in some animals

(Lipman *et al*. 1992, Robuccio *et al*. 1995). Recent trials have therefore used a lipopeptide adjuvant, already validated in other contexts, in an anthrax protein vaccine and none of the animals showed clinically visible side effects with this adjuvant (Schild *et al*. 1991, Loleit *et al*. 1996, Köhler, Baillie & Beyer 2015).

It is pertinent to note that though most of the current vaccine development trials were conducted with animal subjects, the ultimate aim was the development of a safer and more efficacious human vaccine. The efficacy of these new acellular vaccines in protecting highly susceptible ruminants against anthrax and replacing the current Sterne live spore vaccines remains to be evaluated and thus the latter remains the sole veterinary vaccine of choice against anthrax infection in livestock.

2.8 References

ABE, K. & MATSUKI, N. 2000. Measurement of cellular 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction activity and lactate dehydrogenase release using MTT. *Neuroscience Research*, 38:325-329

ADONE, R., PASQUALI, P., LA ROSA, G., MARIANELLI, C., MUSCILLO, M., FASANELLA, A., FRANCIA, M. & CIUCHINI, F. 2002. Sequence analysis of the genes encoding for the major virulence factors of *Bacillus anthracis* vaccine strain Carbosap'. *Journal of Applied Microbiology*, 93:117-121

AL-ADHAMI, B.H. & GAJADHAR, A.A. 2014. A new multi-host species indirect ELISA using protein A/G conjugate for detection of anti-Toxoplasma gondii IgG antibodies with comparison to ELISA-IgG, agglutination assay and Western blot. *Veterinary parasitology*, 200:66-73

ASCOUGH, S., INGRAM, R.J. & ALTMANN, D.M. 2012. Anthrax lethal toxin and the induction of CD4 T cell immunity. *Toxins*, 4:878-899

AUERBACH, S. & WRIGHT, G.G. 1955. Studies on Immunity in Anthrax. *The Journal of Immunology*, 75:129

AYDIN, S. 2015. A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides*, 72:4-15

BAILLIE, L.W., HUWAR, T.B., MOORE, S., MELLADO-SANCHEZ, G., RODRIGUEZ, L., NEESON, B.N., FLICK-SMITH, H.C., JENNER, D.C., ATKINS, H.S., INGRAM, R.J.,

ALTMANN, D.M., NATARO, J.P. & PASETTI, M.F. 2010. An anthrax subunit vaccine candidate based on protective regions of *Bacillus anthracis* Protective Antigen and Lethal Factor. *Vaccine*, 28:6740-6748

BAILLIE, L.W., RODRIGUEZ, A.L., MOORE, S., ATKINS, H.S., FENG, C., NATARO, J.P. & PASETTI, M.F. 2008. Towards a human oral vaccine for anthrax: the utility of a *Salmonella Typhi* Ty21a-based prime boost immunization strategy. *Vaccine*, 26:6083-6091

BEALL, F.A., TAYLOR, M.J. & THORNE, C.B. 1962. Rapid lethal effect in rats of a third component found upon fractionating the toxin of *Bacillus anthracis*. *Journal of Bacteriology*, 83:1274-1280

BELLAN, S.E., CIZAUSKAS, C.A., MIYEN, J., EBERSOHN, K., KUSTERS, M., PRAGER, K.C., VAN VUUREN, M., SABETA, C. & GETZ, W.M. 2012. Black-backed jackal exposure to rabies virus, canine distemper virus, and Bacillus anthracis in Etosha National Park, Namibia. *Journal of wildlife diseases*, 48:371-381

BELTON, F. & STRANGE, R. 1954. Studies on a protective antigen produced *in vitro* from *Bacillus anthracis*: medium and methods of production. *British Journal of Experimental Pathology*, 35(2):144-152

BEYER, W. 2004. Impfstrategien zur Milzbrandprophylaxe (Strategies of vaccination against anthrax). http://vetline.de/245333/150/3130/70412

BEYER, W. & TURNBULL, P.C. 2009. Anthrax in animals. *Molecular Aspects of Medicine*, 30:481-489

BHATNAGAR, R. & BATRA, S. 2001. Anthrax toxin. *Critical Reviews in Microbiology*, 27:167-200

BHATNAGAR, R., SINGH, Y., LEPPLA, S.H. & FRIEDLANDER, A.M. 1989. Calcium is required for the expression of anthrax lethal toxin activity in the macrophage like cell line J774A. 1. *Infection and Immunity*, 57:2107-2114

BHIDE, M., CURLIK, J., TRAVNICEK, M. & LAZAR, P. 2004. Protein A/G dependent ELISA a promising diagnostic tool in Lyme disease seroprevalence in game animals and hunting dogs. *Comparative immunology, microbiology and infectious diseases*, 27:191-199

BOGDAN, C. 2015. Nitric oxide synthase in innate and adaptive immunity: an update. *Trends in immunology*, 36:161-178

BOYDEN, E.D. & DIETRICH, W.F. 2006. Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nature Genetics*, 38:240-244

BRAACK, L. & DE VOS, V. 1990. Feeding habits and flight range of blow-flies (*Chrysomyia* spp.) in relation to anthrax transmission in the Kruger National Park, South Africa. *Onderstepoort Journal of Veterinary Research*, 57:141-142

BRACHMAN, P.S., GOLD, H., PLOTKIN, S.A., FEKETY, F.R., WERRIN, M. & INGRAHAM, N.R. 1962. Field evaluation of a human anthrax vaccine. *American Journal of Public Health and the Nations Health*, 52:632-645

BRADLEY, K.A., MOGRIDGE, J., MOUREZ, M., COLLIER, R.J. & YOUNG, J.A.T. 2001. Identification of the cellular receptor for anthrax toxin. *Nature*, 414:225-229

BROSSIER, F., LEVY, M. & MOCK, M. 2002. Anthrax spores make an essential contribution to vaccine efficacy. *Infection and Immunity*, 70:661-664

BUCHANAN, T.M., FEELEY, J.C., HAYES, P.S. & BRACHMAN, P.S. 1971. Anthrax indirect microhemagglutination test. *The Journal of Immunology*, 107:1631

CANDELA, T., DUMETZ, F., TOSI-COUTURE, E., MOCK, M., GOOSSENS, P.L. & FOUET, A. 2012. Cell-wall preparation containing poly-γ-d-glutamate covalently linked to peptidoglycan, a straightforward extractable molecule, protects mice against experimental anthrax infection. *Vaccine*, 31:171-175

CANDELA, T. & FOUET, A. 2005. *Bacillus anthracis* CapD, belonging to the γ-glutamyltranspeptidase family, is required for the covalent anchoring of capsule to peptidoglycan. *Molecular Microbiology*, 57:717-726

CARTER, G.B. & PEARSON, G.S. 1999. British biological warfare and biological defence, 1925–45, in *Biological and Toxin Weapons: Research, Development and Use from the Middle Ages to 1945*, edited by E. Geissler & J. van Courtland Moon, Oxford: Oxford University Press: 168-189

CARTWRIGHT, M.E., MCCHESNEY, A.E. & JONES, R.L. 1987. Vaccination-related anthrax in three llamas. *Journal of American Veterinary Medical Association*, 191:715-716

CHABOT, D.J., SCORPIO, A., TOBERY, S.A., LITTLE, S.F., NORRIS, S.L. & FRIEDLANDER, A.M. 2004. Anthrax capsule vaccine protects against experimental infection. *Vaccine*, 23:43-47

CHABOT, D.J., RIBOT, W.J., JOYCE, J.G., COOK, J., HEPLER, R.W., NAHAS, D.D. & FRIEDLANDER, A.M. 2015. *Protection of Rhesus macaques against inhalational anthrax with a capsule conjugate vaccine*, The 7th International Conference on *Bacillus anthracis, B. cereus* and *B. thuringiensis*.New Delhi, India ed

CHABOT, D.J., JOYCE, J., CAULFIELD, M., COOK, J., HEPLER, R., WANG, S., VIETRI, N.J., RUTHEL, G., SHOOP, W., PITT, L., LEFFEL, E., RIBOT, W. & FRIEDLANDER, A.M. 2012. Efficacy of a capsule conjugate vaccine against inhalational anthrax in rabbits and monkeys. *Vaccine*, 30:846-852

CHANDRA, A., ENKHBAATAR, P., NAKANO, Y., TRABER, L.D. & TRABER, D.L. 2006. Sepsis: emerging role of nitric oxide and selectins. *Clinics*, 61:71-76

CHITLARU, T., GAT, O., GROSFELD, H., INBAR, I., GOZLAN, Y. & SHAFFERMAN, A. 2007. Identification of In Vivo-Expressed Immunogenic Proteins by Serological Proteome Analysis of the *Bacillus anthracis* Secretome . *Infection and immunity*, 75:2841-2852

CIZAUSKAS, C.A., BELLAN, S.E., TURNER, W.C., VANCE, R.E. & GETZ, W.M. 2014. Frequent and seasonally variable sublethal anthrax infections are accompanied by short-lived immunity in an endemic system. *Journal of Animal Ecology*, 83:1078-1090

COGGESHALL, K.M., LUPU, F., BALLARD, J., METCALF, J.P., JAMES, J.A., FARRIS, D. & KUROSAWA, S. 2013. The sepsis model: an emerging hypothesis for the lethality of inhalation anthrax. *Journal of Cellular and Molecular Medicine*, 17:914-920

COMER, J.E., CHOPRA, A.K., PETERSON, J.W. & KONIG, R. 2005. Direct inhibition of T-lymphocyte activation by anthrax toxins in vivo. *Infection and immunity*, 73:8275-8281

CORDOBA-RODRIGUEZ, R., FANG, H., LANKFORD, C.S.R. & FRUCHT, D.M. 2004. Anthrax lethal toxin rapidly activates caspase-1/ICE and induces extracellular release of interleukin (IL)-1β and IL-18. *Journal of Biological Chemistry*, 279:20563-20566

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 toxaemia. *Journal of Medical Microbiology*, 61:1380-1392

CROWTHER, J. 2009. The ELISA Handbook, in *Methods in Molecular Biology*, edited by J. Walker, Totowa, NJ: Humana Press: 79-109

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

CYBULSKI JR, R.J., SANZ, P. & O'BRIEN, A.D. 2009. Anthrax vaccination strategies. *Molecular Aspects of Medicine*, 30:490

DELVECCHIO, V.G., CONNOLLY, J.P., ALEFANTIS, T.G., WALZ, A., QUAN, M.A., PATRA, G., ASHTON, J.M., WHITTINGTON, J.T., CHAFIN, R.D., LIANG, X., GREWAL, P., KHAN, A.S. & MUJER, C.V. 2006. Proteomic profiling and identification of immunodominant spore antigens of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*. *Applied and Environmental Microbiology*, 72:6355-6363

DESHPANDE, S. 1996. *Enzyme immunoassays: from concept to product development*, 1st ed. New York, NY: Springer Science & Business Media

DAVAINE, C. 1863. Recherches sur les infuoires du sang dans la maladie de la pustule maligne. *Comptes Rendus I' Academie des Sciences*, 60:1296-1299

DE VOS, V. 1990. The ecology of anthrax in the Kruger National Park, South Africa. *Salisbury Medical Bulletin Supplementary*, 68:19-23

DE VOS, V. & BRYDEN, H. 1996. Anthrax in the Kruger National Park: temporal and spatial patterns of disease occurrence. *Salisbury Medical Bulletin*, 87:26-30

DUCLAUX, É. 1896. *Pasteur--histoire d'un esprit* http://gallica.bnf.fr/ark:/12148/bpt6k764468/f397.image.r=Emile+Duclaux+.langFR

ELIASSON, M., OLSSON, A., PALMCRANTZ, E., WIBERG, K., INGANAS, M., GUSS, B., LINDBERG, M. & UHLEN, M. 1988. Chimeric IgG-binding receptors engineered from staphylococcal protein A and streptococcal protein G. *The Journal of biological chemistry*, 263:4323-4327

ENGVALL, E. & PERLMANN, P. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry*, 8:871

ENGVALL, E. 2010. The ELISA, enzyme-linked immunosorbent assay. *Clinical chemistry*, 56:319-320

EZZELL, J.W. & ABSHIRE, T.G. 1992. Serum protease cleavage of *Bacillus anthracis* protective antigen. *Journal of general microbiology*, 138:543

EZZELL, J.W., ABSHIRE, T.G., PANCHAL, R., CHABOT, D., BAVARI, S., LEFFEL, E.K., PURCELL, B., FRIEDLANDER, A.M. & RIBOT, W.J. 2009. Association of *Bacillus anthracis* capsule with lethal toxin during experimental infection. *Infection and immunity*, 77:749-755

EZZELL JR, J. & WELKOS, S. 1999. The capsule of *Bacillus anthracis*, a review. *Journal of Applied Microbiology*, 87:250-250

EZZELL, J.W., MIKESELL, P., IVINS, B.E. & LEPPLA, S.H. 1985. The genetic basis of Pasteur's attenuation of *Bacillus anthracis*, in *World's Debt to Pasteur*, edited by H. Koprowski, & S.A. Plotkin, United States: The Wistar Symposium Series: 107-116

EZZELL, J.W., ABSHIRE, T.G., PANCHAL, R., CHABOT, D., BAVARI, S., LEFFEL, E.K., PURCELL, B., FRIEDLANDER, A.M. & RIBOT, W.J. 2009. Association of *Bacillus anthracis* capsule with lethal toxin during experimental infection. *Infection and Immunity*, 77:749-755

FANG, H., CORDOBA-RODRIGUEZ, R., LANKFORD, C.S. & FRUCHT, D.M. 2005. Anthrax lethal toxin blocks MAPK kinase-dependent IL-2 production in CD4+ T cells. *Journal of immunology (Baltimore, Md.: 1950)*, 174:4966-4971

FASANELLA, A., LOSITO, S., TROTTA, T., ADONE, R., MASSA, S., CIUCHINI, F. & CHIOCCO, D. 2001. Detection of anthrax vaccine virulence factors by polymerase chain reaction. *Vaccine*, 19:4214-4218

FASANELLA, A., GALANTE, D., GAROFOLO, G. & JONES, M.H. 2010. Anthrax undervalued zoonosis. *Veterinary Microbiology*, 140:318-331

FAY, M.P., FOLLMANN, D.A., LYNN, F., SCHIFFER, J.M., STARK, G.V., KOHBERGER, R., QUINN, C.P. & NUZUM, E.O. 2012. Anthrax vaccine-induced antibodies provide cross-species prediction of survival to aerosol challenge. *Science translational medicine*, 4:151ra126

FELLOWS, P., LINSCOTT, M., IVINS, B., PITT, M., ROSSI, C., GIBBS, P. & FRIEDLANDER, A. 2001. Efficacy of a human anthrax vaccine in guinea pigs, rabbits, and rhesus macaques against challenge by *Bacillus anthracis* isolates of diverse geographical origin. *Vaccine*, 19:3241-3247

FINK, S.L., BERGSBAKEN, T. & COOKSON, B.T. 2008. Anthrax lethal toxin and Salmonella elicit the common cell death pathway of caspase-1-dependent pyroptosis via distinct mechanisms. *Proc Natl Acad Sci U S A*, 105

FISH, D.C., MAHLANDT, B.G., DOBBS, J.P. & LINCOLN, R.E. 1968. Purification and Properties of In Vitro–produced Anthrax Toxin Components. *Journal of Bacteriology*, 95:907-918

FRIEBE, S., VAN DER GOOT, F GISOU & BÜRGI, J. 2016. The Ins and Outs of Anthrax Toxin. *Toxins*, 8:69

FRIEDLANDER, A.M. 1986. Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. *Journal of Biological Chemistry*, 261:7123-7126

GAN, S.D. & PATEL, K.R. 2013. Enzyme immunoassay and enzyme-linked immunosorbent assay. *Journal of Investigative Dermatology*, 133:1-3

GARMAN, L., SMITH, K., MUNS, E.E., VELTE, C.A., SPOONER, C.E., MUNROE, M.E., FARRIS, A.D., NELSON, M.R., ENGLER, R.J. & JAMES, J.A. 2016 (In Press). Unique Inflammatory Mediators and Specific IgE Levels Distinguish Local from Systemic Reactions After AVA Vaccination. *Clinical and vaccine immunology : CVI*, 10.1128/CVI.00092-16

GAUTHIER, Y.P., TOURNIER, J.N., PAUCOD, J.C., CORRE, J.P., MOCK, M., GOOSSENS, P.L. & VIDAL, D.R. 2009. Efficacy of a vaccine based on protective antigen and killed spores against experimental inhalational anthrax. *Infection and Immunity*, 77:1197-1207

GLOMSKI, I.J., CORRE, J.P., MOCK, M. & GOOSSENS, P.L. 2007a. Cutting edge: IFN-γ-producing CD4 T lymphocytes mediate spore-induced immunity to capsulated *Bacillus anthracis*. *The Journal of Immunology*, 178:2646-2650

GLOMSKI, I.J., FRITZ, J.H., KEPPLER, S.J., BALLOY, V., CHIGNARD, M., MOCK, M. & GOOSSENS, P.L. 2007b. Murine splenocytes produce inflammatory cytokines in a MyD88-dependent response to *Bacillus anthracis* spores. *Cellular microbiology*, 9:502-513

GREEN, B.D., BATTISTI, L., KOEHLER, T.M., THORNE, C.B. & IVINS, B.E. 1985. Demonstration of a capsule plasmid in *Bacillus anthracis*. *Infection and Immunity*, 49:291-297

GU, M.L., LEPPLA, S.H. & KLINMAN, D.M. 1999. Protection against anthrax toxin by vaccination with a DNA plasmid encoding anthrax protective antigen. *Vaccine*, 17:340-344

GUIDI-RONTANI, C., WEBER-LEVY, M., LABRUYÈRE, E. & MOCK, M. 1999. Germination of *Bacillus anthracis* spores within alveolar macrophages. *Molecular microbiology*, 31:9-17

HAHN, U.K., AICHLER, M., BOEHM, R. & BEYER, W. 2006. Comparison of the immunological memory after DNA vaccination and protein vaccination against anthrax in sheep. *Vaccine*, 24:4595-4597

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

HARRINGTON, R., ONDOV, B.D., RADUNE, D., FRISS, M.B., KLUBNIK, J., DIVIAK, L., HNATH, J., CENDROWSKI, S.R., BLANK, T.E., KARAOLIS, D., FRIEDLANDER, A.M., BURANS, J.P., ROSOVITZ, M.J., TREANGEN, T., PHILLIPPY, A.M. & BERGMAN, N.H. 2013. Genome Sequence of the Attenuated Carbosap Vaccine Strain of *Bacillus anthracis. Genome announcements*, 1:10.1128/genomeA.00067-12. Epub 2013 Jan 15

HERING, D., THOMPSON, W., HEWETSON, J., LITTLE, S., NORRIS, S. & PACE-TEMPLETON, J. 2004. Validation of the anthrax lethal toxin neutralization assay. *Biologicals*, 32:17-27

HERMANSON, G., WHITLOW, V., PARKER, S., TONSKY, K., RUSALOV, D., FERRARI, M., LALOR, P., KOMAI, M., MERE, R., BELL, M., BRENNEMAN, K., MATECZUN, A., EVANS, T., KASLOW, D., GALLOWAY, D. & HOBART, P. 2004. A cationic lipid-formulated plasmid DNA vaccine confers sustained antibody-mediated protection against aerosolized anthrax spores. *Proceedings of the National Academy of Sciences of the United States of America*, 101:13601-13606

HUGH-JONES, M. 1999. 1996–97 global anthrax report. *Journal of Applied Microbiology*, 87:189-191

HUGH-JONES, M. & BLACKBURN, J. 2009. The ecology of *Bacillus anthracis. Molecular Aspects of Medicine*, 30:356-367

IACONO-CONNORS, L.C., NOVAK, J., ROSSI, C., MANGIAFICO, J. & KSIAZEK, T. 1994. Enzyme-linked immunosorbent assay using a recombinant baculovirus-expressed *Bacillus anthracis* protective antigen (PA): measurement of human anti-PA antibodies. *Clinical and Diagnostic Laboratory Immunology*, 1:78-82

INGRAM, R.J., ASCOUGH, S., REYNOLDS, C.J., METAN, G., DOGANAY, M., BAILLIE, L., WILLIAMSON, D.E., ROBINSON, J.H., MAILLERE, B. & BOYTON, R.J.

2015. Natural cutaneous anthrax infection, but not vaccination, induces a CD4 T cell response involving diverse cytokines. *Cell & bioscience*, 5:1. 10.1186/s13578-015-0011-4

INGRAM, R.J., METAN, G., MAILLERE, B., DOGANAY, M., OZKUL, Y., KIM, L.U., BAILLIE, L., DYSON, H., WILLIAMSON, E.D., CHU, K.K., ASCOUGH, S., MOORE, S., HUWAR, T.B., ROBINSON, J.H., SRISKANDAN, S. & ALTMANN, D.M. 2010. Natural exposure to cutaneous anthrax gives long-lasting T cell immunity encompassing infection-specific epitopes. *Journal of immunology (Baltimore, Md.: 1950)*, 184:3814-3821

INOSHIMA, Y., SHIMIZU, S., MINAMOTO, N., HIRAI, K. & SENTSUI, H. 1999. Use of protein AG in an enzyme-linked immunosorbent assay for screening for antibodies against parapoxvirus in wild animals in Japan. *Clinical and diagnostic laboratory immunology*, 6:388-391

IVINS, B., FELLOWS, P., PITT, L., ESTEP, J., FARCHAUS, J., FRIEDLANDER, A. & GIBBS, P. 1995. Experimental anthrax vaccines: efficacy of adjuvants combined with protective antigen against an aerosol *Bacillus anthracis* spore challenge in guinea pigs. *Vaccine*, 13:1779-1784

IVINS, B.E., FELLOWS, P.F. & NELSON, G.O. 1994. Efficacy of a standard human anthrax vaccine against *Bacillus anthracis* spore challenge in guinea-pigs. *Vaccine*, 12:872-874

IVINS, B.E. & WELKOS, S.L. 1988. Recent advances in the development of an improved, human anthrax vaccine. *European Journal of Epidemiology*, 4:12-19

IVINS, B.E., EZZELL, J.W., JEMSKI, J., HEDLUND, K.W., RISTROPH, J.D. & LEPPLA, S.H. 1986. Immunization studies with attenuated strains of *Bacillus anthracis. Infection and Immunity*, 52:454-458

IVINS, B.E., WELKOS, S.L., KNUDSON, G.B. & LITTLE, S.F. 1990. Immunization against anthrax with aromatic compound-dependent (Aro-) mutants of *Bacillus anthracis* and with recombinant strains of *Bacillus subtilis* that produce anthrax protective antigen. *Infection and Immunity*, 58:303-308

IVINS, B.E., WELKOS, S.L., LITTLE, S.F., CRUMRINE, M.H. & NELSON, G.O. 1992. Immunization against anthrax with *Bacillus anthracis* protective antigen combined with adjuvants. *Infection and Immunity*, 60:662-668

JANG, J., CHO, M., CHUN, J.H., CHO, M.H., PARK, J., OH, H.B., YOO, C.K. & RHIE, G.E. 2011. The poly-gamma-D-glutamic acid capsule of *Bacillus anthracis* enhances lethal toxin activity. *Infection and Immunity*, 79:3846-3854

JAYACHANDRAN, R. 2002. Anthrax: biology of *Bacillus anthracis. Current Science*, 82:1220-1226

JEON, J.H., LEE, H.R., CHO, M.H., PARK, O.K., PARK, J. & RHIE, G.E. 2015. The Poly-gamma-d-Glutamic Acid Capsule Surrogate of the *Bacillus anthracis* Capsule Is a Novel

Toll-Like Receptor 2 Agonist. *Infection and immunity*, 83:3847-3856JOHNSON-WINEGAR, A. 1984. Comparison of enzyme-linked immunosorbent and indirect hemagglutination assays for determining anthrax antibodies. *Journal of Clinical Microbiology*, 20:357-361

JULA, G.M. & JABBARI, A. 2007. Stability and potency studies of anthrax vaccine (*Bacillus anthracis* 34F2 Sterne strain) in Iran. *Archives of Razi Institute*, 62:145-149, 176

KEMPSELL, K.E., KIDD, S.P., LEWANDOWSKI, K., ELMORE, M.J., CHARLTON, S., YEATES, A., CUTHBERTSON, H., HALLIS, B., ALTMANN, D.M., ROGERS, M., WATTIAU, P., INGRAM, R.J., BROOKS, T. & VIPOND, R. 2015. Whole genome protein microarrays for serum profiling of immunodominant antigens of *Bacillus anthracis*. *Frontiers in Microbiology*, 6:747

KIM, N.Y., CHANG, D.S., KIM, Y., KIM, C.H., HUR, G.H., YANG, J.M. & SHIN, S. 2015. Enhanced Immune Response to DNA Vaccine Encoding *Bacillus anthracis* PA-D4 Protects Mice against Anthrax Spore Challenge. *PloS One*, 10:e0139671

KLEIN, F., DEARMON JR., I.A., LINCOLN, R.E., MAHLANDT, B.G. & FERNELIUS, A.L. 1962. Immunological studies of anthrax. II. Levels of immunity against *Bacillus anthracis* obtained with protective antigen and live vaccine. *Journal of Immunology (Baltimore, Md.: 1950)*, 88:15-19

KLIMPEL, K.R., MOLLOY, S.S., THOMAS, G. & LEPPLA, S.H. 1992. Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. *Proceedings of the National Academy of Sciences*, 89:10277

KÖHLER, S.M., BAILLIE, L.W. & BEYER, W. 2015. BclA and toxin antigens augment each other to protect NMRI mice from lethal *Bacillus anthracis* challenge. *Vaccine*, 33:2771-2777

KULSHRESHTHA, P. & BHATNAGAR, R. 2011. Inhibition of anthrax toxins with a bispecific monoclonal antibody that cross reacts with edema factor as well as lethal factor of *Bacillus anthracis*. *Molecular immunology*, 48:1958-1965

KUMAR, P., AHUJA, N. & BHATNAGAR, R. 2002. Anthrax edema toxin requires influx of calcium for inducing cyclic AMP toxicity in target cells. *Infection and immunity*, 70:4997-5007

LA ROSA, G., MUSCILLO, M., SALI, M., DE CAROLIS, E., MARIANELLI, C., CIUCHINI, F., FASANELLA, A. & ADONE, R. 2006. Molecular study of genes involved in virulence regulatory pathways in *Bacillus anthracis* vaccine strain "Carbosap". *The new microbiologica*, 29:307-310

LAWS, T.R., KUCHULORIA, T., CHITADZE, N., LITTLE, S.F., WEBSTER, W.M., DEBES, A.K., SAGINADZE, S., TSERTSVADZE, N., CHUBINIDZE, M. & RIVARD, R.G. 2016. A Comparison of the Adaptive Immune Response between Recovered Anthrax

Patients and Individuals Receiving Three Different Anthrax Vaccines. *PloS one*, 11:e0148713

LEE, H., JEON, J.H., PARK, O., CHUN, J., PARK, J. & RHIE, G. 2015. The poly-γ-d-glutamic acid capsule surrogate of the *Bacillus anthracis* capsule induces nitric oxide production via the platelet activating factor receptor signaling pathway. *Molecular immunology*, 68:244-252

LEMBO, T., HAMPSON, K., AUTY, H., BEESLEY, C.A., BESSELL, P., PACKER, C., HALLIDAY, J., FYUMAGWA, R., HOARE, R., ERNEST, E., MENTZEL, C., MLENGEYA, T., STAMEY, K., WILKINS, P.P. & CLEAVELAND, S. 2011. Serologic surveillance of anthrax in the Serengeti ecosystem, Tanzania, 1996-2009. *Emerging infectious diseases*, 17:387-394

LEPPLA, S.H. 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proceedings of the National Academy of Sciences*, 79:3162

LEPPLA, S.H. 1984. *Bacillus anthracis* calmodulin-dependent adenylate cyclase: chemical and enzymatic properties and interactions with eucaryotic cells. *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*, 17:189-198

LEPPLA, S.H. 1988. Production and purification of anthrax toxin. *Methods in Enzymology, 165:103-116*

LEPPLA, S.H 1991. The anthrax toxin complex, in *Sourcebook of Bacterial Protein Toxins*, edited by J. Alouf, United States: Academic Press: 277–302

LEPPLA, S.H., ROBBINS, J.B., SCHNEERSON, R. & SHILOACH, J. 2002. Development of an improved vaccine for anthrax. *Journal of Clinical Investigation*, 110:141-144

LEVINSOHN, J.L., NEWMAN, Z.L., HELLMICH, K.A., FATTAH, R., GETZ, M.A., LIU, S., SASTALLA, I., LEPPLA, S.H. & MOAYERI, M. 2012. Anthrax lethal factor cleavage of Nlrp1 is required for activation of the inflammasome. *PLoS pathogens*, 8:e1002638

LIANG, X., ZHANG, H., ZHANG, E., WEI, J., LI, W., WANG, B., DONG, S. & ZHU, J. 2016. Identification of the pXO1 plasmid in attenuated *Bacillus anthracis* vaccine strains. *Virulence*:1-9. 10.1080/21505594.2016.1164366

LINDSTRÖM, P. & WAGER, O. 1978. IgG Autoantibody to Human Serum Albumin Studied by the ELISA-Technique. *Scandinavian Journal of Immunology*, 7:419-425

LIPMAN, N.S., TRUDEL, L.J., MURPHY, J.C. & SAHALI, Y. 1992. Comparison of immune response potentiation and in vivo inflammatory effects of Freund's and RIBI adjuvants in mice. *Laboratory Animal Science*, 42:193-197

LITTLE, S., WEBSTER, W., NORRIS, S. & ANDREWS, G. 2004. Evaluation of an anti-rPA IgG ELISA for measuring the antibody response in mice. *Biologicals*, 32:62-69

LITTLE, S.F. & IVINS, B.E. 1999. Molecular pathogenesis of *Bacillus anthracis* infection. *Microbes and infection / Institut Pasteur*, 1:131-139

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. *Infection and Immunity*, 52:509-512

LIU, T.H., OSCHERWITZ, J., SCHNEPP, B., JACOBS, J., YU, F., CEASE, K.B. & JOHNSON, P.R. 2009. Genetic Vaccines for Anthrax Based on Recombinant Adeno-associated Virus Vectors. *Molecular Therapy: the Journal of the American Society of Gene Therapy*, 17:373-379

LIU, S., MOAYERI, M. & LEPPLA, S.H. 2014. Anthrax lethal and edema toxins in anthrax pathogenesis. *Trends in microbiology*, 22:317-325

LOLEIT, M., IHLENFELDT, H.G., BRUNJES, J., JUNG, G., MULLER, B., HOFFMANN, P., BESSLER, W.G., PIERRES, M. & HAAS, G. 1996. Synthetic peptides coupled to the lipotripeptide P 3CSS induce in vivo B and T(helper) cell responses to HIV-1 reverse transcriptase. *Immunobiology*, 195:61-76

MAHLANDT, B.G., KLEIN, F., LINCOLN, R.E., HAINES, B.W., JONES JR, W.I. & FRIEDMAN, R. 1966. Immunologic Studies of Anthrax IV. Evaluation of the Immunogenicity of Three Components of Anthrax Toxin. *The Journal of Immunology*, 96:727-733

MAKINO, S., WATARAI, M., CHEUN, H., SHIRAHATA, T. & UCHIDA, I. 2002. 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

MALDONADO-AROCHO, F.J. & BRADLEY, K.A. 2009. Anthrax edema toxin induces maturation of dendritic cells and enhances chemotaxis towards macrophage inflammatory protein 3beta. *Infection and immunity*, 77:2036-2042

MARANO, N., PLIKAYTIS, B.D., MARTIN, S.W., ROSE, C., SEMENOVA, V.A., MARTIN, S.K., FREEMAN, A.E., LI, H., MULLIGAN, M.J. & PARKER, S.D. 2008. Effects of a reduced dose schedule and intramuscular administration of anthrax vaccine adsorbed on immunogenicity and safety at 7 months: a randomized trial. *Jama*, 300:1532-1543

MARTINON, F., BURNS, K. & TSCHOPP, J. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-β. *Molecular cell*, 10:417-426

MIDHA, S. & BHATNAGAR, R. 2009. Anthrax protective antigen administered by DNA vaccination to distinct subcellular locations potentiates humoral and cellular immune responses. *European journal of immunology*, 39:159-177

MIKESELL, P., IVINS, B.E., RISTROPH, J.D. & DREIER, T.M. 1983. Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. *Infection and Immunity*, 39:371-376

MILNE, J.C., FURLONG, D., HANNA, P.C., WALL, J.S. & COLLIER, R.J. 1994. Anthrax protective antigen forms oligomers during intoxication of mammalian cells. *Journal of Biological Chemistry*, 269:20607-20612

MOAYERI, M., CROWN, D., NEWMAN, Z.L., OKUGAWA, S., ECKHAUS, M., CATAISSON, C., LIU, S., SASTALLA, I. & LEPPLA, S.H. 2010. Inflammasome sensor Nlrp1b-dependent resistance to anthrax is mediated by caspase-1, IL-1 signaling and neutrophil recruitment. *PLoS pathogens*, 6:e1001222

MOAYERI, M. & LEPPLA, S.H. 2004. The roles of anthrax toxin in pathogenesis. *Current Opinion in Microbiology*, 7:19-24

MOAYERI, M., WIGGINS, J.F. & LEPPLA, S.H. 2007. Anthrax protective antigen cleavage and clearance from the blood of mice and rats. *Infection and immunity*, 75:5175-5184

MOAYERI, M. & LEPPLA, S.H. 2009. Cellular and systemic effects of anthrax lethal toxin and edema toxin. *Molecular Aspects of Medicine*, 30:439-455

MOGRIDGE, J., CUNNINGHAM, K. & COLLIER, R.J. 2002. Stoichiometry of anthrax toxin complexes. *Biochemistry*, 41:1079-1082

MOGRIDGE, J., CUNNINGHAM, K., LACY, D.B., MOUREZ, M. & COLLIER, R.J. 2002. The lethal and edema factors of anthrax toxin bind only to oligomeric forms of the protective antigen. *Proceedings of the National Academy of Sciences*, 99:7045

MONCADA, S. & HIGGS, A. 1993. The L-arginine-nitric oxide pathway. *The New England journal of medicine*, 329:2002-2012

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

NEGUS, D., VIPOND, J., HATCH, G.J., RAYNER, E.L. & TAYLOR, P.W. 2015. Parenteral Administration of Capsule Depolymerase EnvD Prevents Lethal Inhalation Anthrax Infection. *Antimicrobial Agents and Chemotherapy*, 59:7687-7692

NEWMAN, Z.L., CROWN, D., LEPPLA, S.H. & MOAYERI, M. 2010. Anthrax lethal toxin activates the inflammasome in sensitive rat macrophages. *Biochemical and biophysical research communications*, 398:785-789

NEWMAN, Z.L., PRINTZ, M.P., LIU, S., CROWN, D., BREEN, L., MILLER-RANDOLPH, S., FLODMAN, P., LEPPLA, S.H. & MOAYERI, M. 2010. Susceptibility to anthrax lethal toxin-induced rat death is controlled by a single chromosome 10 locus that includes rNlrp1. *PLoS pathogens*, 6:e1000906

NGUNDI, M.M., MEADE, B.D., LIN, T., TANG, W. & BURNS, D.L. 2010. Comparison of Three Anthrax Toxin Neutralization Assays. *Clinical and Vaccine Immunology*, 17:895-903

NGUNDI, M.M., MEADE, B.D., LITTLE, S.F., QUINN, C.P., CORBETT, C.R., BRADY, R.A. & BURNS, D.L. 2012. Analysis of defined combinations of monoclonal antibodies in anthrax toxin neutralization assays and their synergistic action. *Clinical and vaccine immunology : CVI*, 19:731-739

NIU, M. & BALL, R. 2009. Adverse events after anthrax vaccination reported to the Vaccine Adverse Event Reporting System (VAERS), 1990-2007 [Vaccine 2009;27:290-297]. *Vaccine*, 27:6654-6655

NYMO, I.H., GODFROID, J., ASBAKK, K., LARSEN, A.K., DAS NEVES, C.G., RODVEN, R. & TRYLAND, M. 2013. A protein A/G indirect enzyme-linked immunosorbent assay for the detection of anti-Brucella antibodies in Arctic wildlife. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc*, 25:369-375

OBP. 2016. *Manufacturer's insert: Anthrax spore vaccine*, http://www.obpvaccines.co.za/products ed

OIE. 2012. Anthrax, in *Manual of diagnostic tests and vaccines for terrestrial animals.* France: 135-144

OMLAND, K.S., BRYS, A., LANSKY, D., CLEMENT, K., LYNN, F. & AND THE PARTICIPATING LABORATORIES. 2008. Interlaboratory Comparison of Results of an Anthrax Lethal Toxin Neutralization Assay for Assessment of Functional Antibodies in Multiple Species. *Clinical and Vaccine Immunology*, 15:946-953

PACCANI, S.R. & BALDARI, C.T. 2011. T Cell Targeting by Anthrax Toxins: Two Faces of the Same Coin. *Toxins (Basel)*, 3:660-671

PACCANI, S.R., TONELLO, F., GHITTONI, R., NATALE, M., MURARO, L., D'ELIOS, M.M., TANG, W.J., MONTECUCCO, C. & BALDARI, C.T. 2005. Anthrax toxins suppress T lymphocyte activation by disrupting antigen receptor signaling. *The Journal of experimental medicine*, 201:325-331

PALAZZO, L., DE CARLO, E., SANTAGADA, G., SERRECCHIA, L., ACETI, A., GUARINO, A., ADONE, R. & FASANELLA, A. 2012. Recent Epidemic-Like Anthrax Outbreaks in Italy: What Are the Probable Causes? *Open Journal of Veterinary Medicine*, 2:74-76

PANCHAL, R.G., HALVERSON, K.M., RIBOT, W., LANE, D., KENNY, T., ABSHIRE, T.G., EZZELL, J.W., HOOVER, T.A., POWELL, B., LITTLE, S., KASIANOWICZ, J.J. & BAVARI, S. 2005. Purified *Bacillus anthracis* lethal toxin complex formed in vitro and during infection exhibits functional and biological activity. *The Journal of biological chemistry*, 280:10834-10839

PARK, J.M., GRETEN, F.R., LI, Z.W. & KARIN, M. 2002. Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. *Science's STKE*, 297:2048

PARREIRAS, P.M., SIROTA, L.A., WAGNER, L.D., MENZIES, S.L. & ARCINIEGA, J.L. 2009. Comparability of ELISA and toxin neutralization to measure immunogenicity of Protective Antigen in mice, as part of a potency test for anthrax vaccines. *Vaccine*, 27:4537-4542

PASTEUR, R. & JOUBERT, J. 1877. Etude sur la maladie charbonneuse. *Comptes Rendus Hebdomadaires des*

PITT, M.L., LITTLE, S.F., IVINS, B.E., FELLOWS, P., BARTH, J., HEWETSON, J., GIBBS, P., DERTZBAUGH, M. & FRIEDLANDER, A.M. 2001. In vitro correlate of immunity in a rabbit model of inhalational anthrax. *Vaccine*, 19:4768-4773

PITTMAN, P.R., GIBBS, P.H., CANNON, T.L. & FRIEDLANDER, A.M. 2001. Anthrax vaccine: short-term safety experience in humans. *Vaccine*, 20:972-978

PROMED. 2015. *Anthrax - Zimbabwe (04): (Matabele South) human, bovine, vaccine mobilization*, http://promedmail.org/post/20151228.3896439 ed

PUZISS, M. & WRIGHT, G.G. 1954. STUDIES ON IMMUNITY IN ANTHRAX IV.: Factors Influencing Elaboration of the Protective Antigen of *Bacillus anthracis* in Chemically Defined Media. *Journal of Bacteriology*, 68:474

QUESNEL-HELLMANN, A., CLERET, A., VIDAL, D.R. & TOURNIER, J.N. 2006. Evidence for adjuvanticity of anthrax edema toxin. *Vaccine*, 24:699-702

QUINN, C.P., SABOURIN, C.L., SCHIFFER, J.M., NIEMUTH, N.A., SEMENOVA, V.A., LI, H., RUDGE, T.L., BRYS, A.M., MITTLER, R.S., IBEGBU, C.C., WRAMMERT, J., AHMED, R., PARKER, S.D., BABCOCK, J., KEITEL, W., POLAND, G.A., KEYSERLING, H.L., EL SAHLY, H., JACOBSON, R.M., MARANO, N., PLIKAYTIS, B.D. & WRIGHT, J.G. 2016. Humoral and Cell-Mediated Immune Responses to Alternate Booster Schedules of Anthrax Vaccine Adsorbed in Humans. *Clinical and vaccine immunology : CVI*, 23:326-338

QUINN, C.P., SHONE, C.C., TURNBULL, P. & MELLING, J. 1988. Purification of anthrax-toxin components by high-performance anion-exchange, gel-filtration and hydrophobic-interaction chromatography. *Biochemical Journal*, 252:753

RAY JR, J.G. & KADULL, P.J. 1964. Agar-gel precipitin technique in anthrax antibody determinations. *Applied Microbiology*, 12:349-354

READ, T.D., PETERSON, S.N., TOURASSE, N., BAILLIE, L.W., PAULSEN, I.T., NELSON, K.E., TETTELIN, H., FOUTS, D.E., EISEN, J.A. & GILL, S.R. 2003. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature*, 423:81-86

REUVENY, S., WHITE, M.D., ADAR, Y.Y., KAFRI, Y., ALTBOUM, Z., GOZES, Y., KOBILER, D., SHAFFERMAN, A. & VELAN, B. 2001. Search for correlates of protective immunity conferred by anthrax vaccine. *Infection and Immunity*, 69:2888-2893

RHIE, G.E., ROEHRL, M.H., MOUREZ, M., COLLIER, R.J., MEKALANOS, J.J. & WANG, J.Y. 2003. A dually active anthrax vaccine that confers protection against both bacilli and toxins. *Proceedings of the National Academy of Sciences of the United States of America*, 100:10925-10930

RICHTER, S., ANDERSON, V.J., GARUFI, G., LU, L., BUDZIK, J.M., JOACHIMIAK, A., HE, C., SCHNEEWIND, O. & MISSIAKAS, D. 2009. Capsule anchoring in *Bacillus anthracis* occurs by a transpeptidation reaction that is inhibited by capsidin. *Molecular microbiology*, 71:404-420

ROBUCCIO, J.A., GRIFFITH, J.W., CHROSCINSKI, E.A., CROSS, P.J., LIGHT, T.E. & LANG, C.M. 1995. Comparison of the effects of five adjuvants on the antibody response to influenza virus antigen in guinea pigs. *Laboratory Animal Science*, 45:420-426

ROBERTS, J.E., WATTERS, J.W., BALLARD, J.D. & DIETRICH, W.F. 1998. Ltx1, a mouse locus that influences the susceptibility of macrophages to cytolysis caused by intoxication with *Bacillus anthracis* lethal factor, maps to chromosome 11. *Molecular microbiology*, 29:581-591

SAILE, E. & QUINN, C.P. 2011. Anthrax vaccines, in *Bacillus anthracis and Anthrax*, edited by H.N. Bergman, : Wiley Online Library: 269-293

SASTALLA, I., TANG, S., CROWN, D., LIU, S., ECKHAUS, M.A., HEWLETT, I.K., LEPPLA, S.H. & MOAYERI, M. 2012. Anthrax edema toxin impairs clearance in mice. *Infection and immunity*, 80:529-538

SASTALLA, I., CROWN, D., MASTERS, S.L., MCKENZIE, A., LEPPLA, S.H. & MOAYERI, M. 2013. Transcriptional analysis of the three Nlrp1 paralogs in mice. *BMC Genomics*, 14:1-10

SCHAEFER, J.J., WHITE, H.A., SCHAAF, S.L., MOHAMMED, H.O. & WADE, S.E. 2012. Chimeric protein A/G conjugate for detection of anti-Toxoplasma gondii immunoglobulin G in multiple animal species. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc*, 24:572-575

SCHIFFER, J.M., MCNEIL, M.M. & QUINN, C.P. 2016. Recent developments in the understanding and use of anthrax vaccine adsorbed: achieving more with less. *Expert review of vaccines*:1-12

SCHILD, H., DERES, K., WIESMÜLLER, K.H., JUNG, G. & RAMMENSEE, H.G. 1991. Efficiency of peptides and lipopeptides for in vivo priming of virus-specific cytotoxic T cells. *European Journal of Immunology*, 21:2649-2654

SCHLINGMAN, A.S., DEVLIN, H.B., WRIGHT, G.G., MAINE, R.J. & MANNING, M.C. 1956. Immunizing activity of alum-precipitated protective antigen of *Bacillus anthracis* in cattle, sheep, and swine. *American Journal of Veterinary Research*, 17:256-261

SCHNEERSON, R., KUBLER-KIELB, J., LIU, T., DAI, Z., LEPPLA, S.H., YERGEY, A., BACKLUND, P., SHILOACH, J., MAJADLY, F. & ROBBINS, J.B. 2003. Poly(γ-d-glutamic acid) protein conjugates induce IgG antibodies in mice to the capsule of *Bacillus anthracis*: A potential addition to the anthrax vaccine. *Proceedings of the National Academy of Sciences*, 100:8945-8950

SCHWARTZ, M. 2009. Dr. Jekyll and Mr. Hyde: a short history of anthrax. *Molecular Aspects of Medicine*, 30:347-355

SCORPIO, A., CHABOT, D.J., DAY, W.A., HOOVER, T.A. & FRIEDLANDER, A.M. 2010. Capsule depolymerase overexpression reduces *Bacillus anthracis* virulence. *Microbiology*, 156:1459-1467

SCORPIO, A., CHABOT, D.J., DAY, W.A., O'BRIEN, D.K., VIETRI, N.J., ITOH, Y., MOHAMADZADEH, M. & FRIEDLANDER, A.M. 2007. Poly-Î³-Glutamate Capsule-Degrading Enzyme Treatment Enhances Phagocytosis and Killing of Encapsulated *Bacillus anthracis*. *Antimicrobial Agents and Chemotherapy*, 51:215-222

SCORPIO, A., TOBERY, S.A., RIBOT, W.J. & FRIEDLANDER, A.M. 2008. Treatment of experimental anthrax with recombinant capsule depolymerase. *Antimicrobial Agents and Chemotherapy*, 52:1014-1020

SINGH, Y., LEPPLA, S.H., BHATNAGAR, R. & FRIEDLANDER, A.M. 1989. Internalization and processing of *Bacillus anthracis* lethal toxin by toxin-sensitive and -resistant cells. *Journal of Biological Chemistry*, 264:11099-11102

SITTAMPALAM, G.S., SMITH, W.C., MIYAKAWA, T.W., SMITH, D.R. & MCMORRIS, C. 1996. Application of experimental design techniques to optimize a competitive ELISA. *Journal of immunological methods*, 190:151-161

STANLEY, J. & SMITH, H. 1963. The three factors of anthrax toxin: their immunogenicity and lack of demonstrable enzymic activity. *Journal of General Microbiology*, 31:329-337

STANLEY, J., SMITH, H. & SARGEANT, K. 1961. Purification of factor I and recognition of a third factor of the anthrax toxin. *Journal of General Microbiology*, 26:49

STERNE, M. 1959. Anthrax, in *InfectiousDiseases of Animals, Diseases Due to Bacteria*, edited by A.W. Stableforth & Galloway I.A., United Kingdom: Academic Press: 16-52

STERNE, M. 1939. The use of Anthrax Vaccines Prepared from Avirulent (Uncapsulated) Variants of *Bacillus anthracis*. *Onderstepoort Journal of Veterinary Research*, 13:307

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

TAFT, S.C. & WEISS, A.A. 2008a. Neutralizing activity of vaccine-induced antibodies to two *Bacillus anthracis* toxin components, lethal factor and edema factor. *Clinical and Vaccine Immunology*, 15:71-75

TAFT, S.C. & WEISS, A.A. 2008b. Toxicity of Anthrax Toxin Is Influenced by Receptor Expression. *Clinical and Vaccine Immunology*, 15:1330-1336

TANG, W.J. & GUO, Q. 2009. The adenylyl cyclase activity of anthrax edema factor. *Molecular Aspects of Medicine*, 30:423-430

TERRA, J.K., COTE, C.K., FRANCE, B., JENKINS, A.L., BOZUE, J.A., WELKOS, S.L., LEVINE, S.M. & BRADLEY, K.A. 2010. Cutting edge: resistance to *Bacillus anthracis* infection mediated by a lethal toxin sensitive allele of Nalp1b/Nlrp1b. *Journal of immunology (Baltimore, Md.: 1950)*, 184:17-20

THORKILDSON, P., KINNEY, H.L. & AUCOIN, D.P. 2016. Pasteur revisited: an unexpected finding in *Bacillus anthracis* vaccine strains. *Virulence*, 10.1080/21505594.2016.1186325:00-00

THORNE, C. & BELTON, F. 1957. An agar-diffusion method for titrating *Bacillus anthracis* immunizing antigen and its application to a study of antigen production. *Journal of General Microbiology*, 17:505

TURK, B. 2007. Manipulation of host signalling pathways by anthrax toxins. *Biochemical Journal*, 402:405-417

TURNBULL, P.C.B., LEPPLA, S., BROSTER, M., QUINN, C. & MALLING, J. 1988. Antibodies to anthrax toxin in humans and guinea pigs and their relevance to protective immunity. *Medical Microbiology and Immunology*, 177:293-303

TURNBULL, P., RIJKS, J., THOMPSON, I., HUGH-JONES, M. & ELKIN, B. 2001. *Seroconversion in bison (Bison bison) in northwest Canada experiencing sporadic and epizootic anthrax* in 4th International Conference on Anthrax pp 10-13

TURNBULL, P., LINDEQUE, P., LE ROUX, J., BENNETT, A. & PARKS, S. 1998. Airborne movement of anthrax spores from carcass sites in the Etosha National Park, Namibia. *Journal of Applied Microbiology*, 84:667-676

TURNBULL, P., QUINN, C., HEWSON, R., STOCKBRIDGE, M. & MELLING, J. 1990. Protection conferred by microbially-supplemented UK and purified PA vaccines. *Salisbury Medical Bulletin*, 68:89-91

TURNBULL, P.C., BROSTER, M.G., CARMAN, J.A., MANCHEE, R.J. & MELLING, J. 1986. Development of antibodies to protective antigen and lethal factor components of

anthrax toxin in humans and guinea pigs and their relevance to protective immunity. *Infection and Immunity*, 52:356-363

TURNBULL, P.C., DOGANAY, M., LINDEQUE, P.M., AYGEN, B. & MCLAUGHLIN, J. 1992. Serology and anthrax in humans, livestock and Etosha National Park wildlife. *Epidemiology and Infection*, 108:299-313

TURNBULL, P.C., TINDALL, B.W., COETZEE, J.D., CONRADIE, C.M., BULL, R.L., LINDEQUE, P.M. & HUEBSCHLE, O.J. 2004. Vaccine-induced protection against anthrax in cheetah (*Acinonyx jubatus*) and black rhinoceros (*Diceros bicornis*). *Vaccine*, 22:3340-3347

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

VAN WEEMAN, B. & SCHUURS, A. 1971. Immunoassay using antigen-enzyme conjugates. *FEBS Letter*, 15:232-235

VERGIS, J.M., COTE, C.K., BOZUE, J., ALEM, F., VENTURA, C.L., WELKOS, S.L. & O'BRIEN, A.D. 2013. Immunization of mice with formalin-inactivated spores from avirulent *Bacillus cereus* strains provides significant protection from challenge with Bacillus anthracis Ames. *Clinical and vaccine immunology : CVI*, 20:56-65

VITALE, G., BERNARDI, L., NAPOLITANI, G., MOCK, M. & MONTECUCCO, C. 2000. Susceptibility of mitogen-activated protein kinase kinase family members to proteolysis by anthrax lethal factor. *Biochemical Journal*, 352:739

VOTH, D.E., HAMM, E.E., NGUYEN, L.G., TUCKER, A.E., SALLES, I.I., ORTIZ-LEDUC, W. & BALLARD, J.D. 2005. *Bacillus anthracis* oedema toxin as a cause of tissue necrosis and cell type-specific cytotoxicity. *Cellular microbiology*, 7:1139-1149

WATSON, A. & KEIR, D. 1994. Information on which to base assessments of risk from environments contaminated with anthrax spores. *Epidemiology and Infection*, 113:479-490

WATTIAU, P., GOVAERTS, M., FRANGOULIDIS, D., FRETIN, D., KISSLING, E., VAN HESSCHE, M., CHINA, B., PONCIN, M., PIRENNE, Y. & HANQUET, G. 2009. Immunologic Response of Unvaccinated Workers Exposed to Anthrax, Belgium. *Emerging infectious diseases (Print)*, 15:1637-1640

WEISS, S., KOBILER, D., LEVY, H., MARCUS, H., PASS, A., ROTHSCHILD, N. & ALTBOUM, Z. 2006. Immunological Correlates for Protection against Intranasal Challenge of *Bacillus anthracis* Spores Conferred by a Protective Antigen-Based Vaccine in Rabbits. *Infection and Immunity*, 74:394-398

WELKOS, S.L., KEENER, T.J. & GIBBS, P.H. 1986. Differences in susceptibility of inbred mice to *Bacillus anthracis*. *Infection and immunity*, 51:795-800

WELKOS, S.L. & FRIEDLANDER, A.M., 1988. Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. *Microbial pathogenesis*, 5(2): 127-139.

WELKOS, S., VIETRI, N. & GIBBS, P. 1993. Non-toxigenic derivatives of the Ames strain of *Bacillus anthracis* are fully virulent for mice: role of plasmid pX02 and chromosome in strain-dependent virulence. *Microbial Pathogenesis*, 14:381-388

WELKOS, S., LITTLE, S., FRIEDLANDER, A., FRITZ, D. & FELLOWS, P. 2001. 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

WILKIE, M.H. & WARD, M.K. 1967. Characterization of anthrax toxin. *Federation Proceedings*, 26(5):1527-1531

WILLIAMS, J.E., GENTRY, M.K., BRADEN, C.A., LEISTER, F. & YOLKEN, R.H. 1984. Use of an enzyme-linked immunosorbent assay to measure antigenaemia during acute plague. *Bulletin of the World Health Organization*, 62:463

WILLIAMSON, E.D., HODGSON, I., WALKER, N.J., TOPPING, A.W., DUCHARS, M.G., MOTT, J.M., ESTEP, J., LEBUTT, C., FLICK-SMITH, H.C., JONES, H.E., LI, H. & QUINN, C.P. 2005. Immunogenicity of Recombinant Protective Antigen and Efficacy against Aerosol Challenge with Anthrax. *Infection and immunity*, 73:5978-5987

WISTREICH, G.A. & LECHTMAN, M.D. 1976. *Microbiology and human disease*: Glencoe Press

WOBESER, B.K. 2015. Anthrax vaccine associated deaths in miniature horses. *The Canadian veterinary journal.La revue veterinaire canadienne*, 56:359-360

WORLD HEALTH ORGANIZATION & INTERNATIONAL OFFICE OF EPIZOOTICS. 2008. Anthrax in humans and animals, edited by P. Turnbull, : World Health Organization: 94-95

WRIGHT, G., GREEN, T.W. & KANODE JR, R. 1954. Studies on immunity in anthrax. V. Immunizing activity of alum-precipitated protective antigen. *Journal of Immunology*, 73:387

WRIGHT, G.G., HEDBERG, M.A. & SLEIN, J.B. 1954. Studies on immunity in anthrax. III. Elaboration of protective antigen in a chemically defined, non-protein medium. *Journal of Immunology*, 72:263-269

WRIGHT, J.G., PLIKAYTIS, B.D., ROSE, C.E., PARKER, S.D., BABCOCK, J., KEITEL, W., EL SAHLY, H., POLAND, G.A., JACOBSON, R.M., KEYSERLING, H.L., SEMENOVA, V.A., LI, H., SCHIFFER, J., DABABNEH, H., MARTIN, S.K., MARTIN, S.W., MARANO, N., MESSONNIER, N.E. & QUINN, C.P. 2014. Effect of reduced dose schedules and intramuscular injection of anthrax vaccine adsorbed on immunological response and safety profile: A randomized trial. *Vaccine*, 32:1019-1028

YALOW, R.S. & BERSON, S.A. 1960. Immunoassay of endogenous plasma insulin in man. *Journal of Clinical Investigation*, 39:1157

ZENG, M., XU, Q., HESEK, E.D. & PICHICHERO, M.E. 2006. N-fragment of edema factor as a candidate antigen for immunization against anthrax. *Vaccine*, 24:662-670

ZHANG, D., WANG, Z., FANG, R., NIE, H., FENG, H., ZHOU, Y. & ZHAO, J. 2010. Use of protein AG in an enzyme-linked immunosorbent assay for serodiagnosis of *Toxoplasma gondii* infection in four species of animals. *Clinical and vaccine immunology : CVI*, 17:485-486 ZHANG, Y., QIU, J., ZHOU, Y., FARHANGFAR, F., HESTER, J., LIN, A.Y. & DECKER, W.K. 2008. Plasmid-based vaccination with candidate anthrax vaccine antigens induces durable type 1 and type 2 T-helper immune responses. *Vaccine*, 26:614-622

ZMUDA, J.F., ZHANG, L., RICHARDS, T., PHAM, Q., ZUKAUSKAS, D., PIERRE, J.L., LAIRD, M.W., ASKINS, J. & CHOI, G.H. 2005. Development of an edema factor-mediated cAMP-induction bioassay for detecting antibody-mediated neutralization of anthrax protective antigen. *Journal of Immunological Methods*, 298:47-60

CHAPTER THREE

General Methods

3.1 *Bacillus anthracis* vaccine and challenge strain preparation

B. anthracis 34F2 Sterne spores (Batch No. 86) utilised for goat immunization and *in vivo* mouse challenge were sourced from Onderstepoort Biological Products (OBP, Onderstepoort, South Africa). For caprine immunization studies, 1 mL dose was split into two and injected subcutaneously in the inner thighs of each animal (OBP 2016). For the *in vivo* A/J mouse challenge experiments, the spores were prepared by the dilution and centrifugation of the spores in sterile PBS with 0.1 % glycerine (PBS-glycerine) solution at 3000 × *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.

Direct goat challenge studies were 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). Spores from the challenge strain were prepared as previously described in the "Stuttgart" method (Welkos *et al.* 2011) in a BSL2+ laboratory. This laboratory, in addition to BSL-level 2 requirements, has the following facilities; 1) Physical separation from access doors 2) Self-closing, double-door access 3) Non-circulation of exhausted air 4) Entry through airlock 5) Hand-washing sink near laboratory exit (https://www.cdc.gov/biosafety/publications/bmbl5/bmbl5_sect_iv.pdf). Bacterial strains are stored in access-controlled fridges/freezers. For the challenge spores preparation, a pure inoculum of virulent *B. anthracis* (20SD strain) cultured overnight on sheep blood agar was suspended in sterile PBS. This was used to inoculate meat yeast agar in cell culture (Roux) flasks and incubated at 30 °C for ten days. Evidence of sporulation was evaluated on the third

day using phase contrast microscopy. On the tenth day, spores were harvested by flushing with distilled water and pelleted by centrifugation at 4000 × g for 15 mins at 20 °C. This was repeated four times with the supernatant aspirated away and pellets carefully re-suspended in distilled water each time before centrifugation. The final spore suspension was treated at 65 °C for 30 mins to kill any remaining vegetative cells before counting and storage in sterile PBS/gelatine (0.1 % wt/vol) solution. The purity of the spore preparation was evaluated by microscopy after staining. Spore numbers were determined by plating appropriate dilutions on blood agar and counting overnight-cultured colony forming units. Before each challenge experiments, spore aliquots were heated at 65° C for 30 min to ensure only dormant spores were used.

Formaldehyde inactivated spore (FIS) suspensions from Sterne vaccine strain (34F2) used for vaccination and serology were prepared as follows;

Vaccine 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. Following incubation, the spores were pelleted (4 000 × *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 streaking overnight on blood agar after treatment with histidine to neutralize any remaining formalin (Neuberger 1944).

3.2. Preparation and purification of recombinant proteins

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 (recombinant PA 83) or rBclA were grown and purified as previously described (Hahn *et al.* 2004) in the laboratory of Dr Wolfgang Beyer, University of

Hohenheim, Germany. Proteins used for ELISA received no further treatment whilst proteins used for vaccination were tested for endotoxin using the Limulus Amoebocyte Lysate Endochrome-K test kit (Charles River, Wilmington, USA) as described by the manufacturer. Endotoxin removal was carried out via EndoTrap blue endotoxin removal system (Hyglos, Bernried, Germany).

3.3. ELISA

Sera were analysed for specific immunoglobulins using ELISA as previously described by (Hahn *et al.* 2004) with some modifications. The PA, BclA and FIS antigen for ELISA were prepared as described earlier. For the vegetative antigen, avirulent CDC 1014 *B. anthracis* strain spores (pX01−, pX02−) were grown in brain heart infusion broth with bicarbonate overnight at 37 °C. The cells were pelleted at 4000 x 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 ten times for 5 - 10 seconds on ice. Insoluble fractions were pelleted and discarded. The supernatant was passed through a filter (0.45 µm) and checked for sterility. Protein concentration was assessed through the bicinchoninic acid (BCA) method (Walker 2009).

Individual wells of 96-well microtitre plates (Nunc immunoplate Maxisorp, Germany) were coated with 0.5 µg of PA, BclA, vegetative antigen or 10^7 FIS in bicarbonate buffer (0.1 M, Ph 9.2) and incubated overnight at 4 °C. The plates were washed twice with PBS containing 0.05 % Tween (PBST) using a PW40 washer (Biorad, Mamesla-Coquette, France) and blocked in 200 µL of PBST containing 5 % skimmed milk powder (PBSTM) and incubated for 1 h at room temperature (RT). The plates were washed twice, the test sera were diluted in PBSTM and two-fold dilutions (starting dilution; 1:50) made across the plates in duplicates.

Every 96-well plate contained one duplicate negative control serum (unvaccinated goat), six blanks (diluent) and a single row 2-fold diluted positive control serum (hyper-immunised goat). Thereafter, the plates were incubated for 30 min on a rotatory shaker (Titretek® flow labs, Irvine, 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 five times, the plates were developed with 2,2' azino bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma, Steinheim, 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.

3.4. Toxin neutralization assay

An *in vitro* toxin neutralizing assay (TNA) was performed using the mouse macrophage cell line J774A.1 (European collection of cell cultures ECACC cat nr 91051511) as previously described with slight modifications (Hering *et al.* 2004).The 96-well flat-bottomed tissue culture plates (Greiner bio one, Frickenhausen, Germany) containing 80 000 macrophages/well in DMEM and 10 % FCS were incubated overnight at 37 °C and 5 % CO_2. 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 % CO2 before adding to overnight cultured cells (after discarding medium) and incubated for 3 h. Each serum sample was tested in duplicates. Following incubation, 25 µL 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_2. 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) by vigorously pipetting up and down to solubilize the formazan dye.

The plates were rested for five min and the absorbance readings taken 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:

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

and expressed as the reciprocal of the highest serum dilution neutralising 50 % of the LT cytotoxicity. Neutralization titres (NT 50) were expressed as the reciprocal of the serum dilution neutralising 50 % of the LT cytotoxicity and obtained using the Gen5 data analysis software (Biotek Instruments, Winooski, USA).

3.5. Lymphocyte proliferation assay

Proliferative responses of caprine peripheral blood mononuclear cells (PBMCs) isolated from the jugular vein were measured as described previously with modifications (Andrianarivo *et al.* 1999). Briefly, PBMCs were isolated from heparinized blood and cultured at 10^7 cells/mL in 96-wells tissue culture plates using DMEM:RPMI-1640 culture medium (Life Technologies, CA, USA). The medium was supplemented with penicillin (50 U/mL), streptomycin (100 µg/mL) and amphotericin B (0.2 µg/mL) and 10 % FCS (all from Life Technologies, CA, USA) in the presence of rPA (2 µg/mL). The latter was replaced by 2

µg/mL of concavalin A (ConA, positive control wells) and culture medium only (negative control wells) to determine spontaneous proliferation. These were incubated for 48 h to determine cell proliferation, before adding 20 µL/well of bromodeoxyuridine (BrdU) labelling agent and re-incubated for another 4 h. Subsequent fixing and staining of cells were done according to the manufacturer's instructions (Roche cell proliferation ELISA for BrdU, Roche, Mannheim, Germany). Absorbance was measured at 450 nm with a reference wavelength at 690 nm using a Biotek power wave XS 2 reader (Winooski, USA). All proliferation assays were performed in triplicates and expressed as stimulation indices (SI) determined with the formula:

$$SI = \frac{Mean\ PA\ stimulated\ cells - Mean\ unstimulated\ cells}{Mean\ Con\ A\ stimulated\ cells - Mean\ unstimulated\ cells} \times 100$$

SI values below zero were ascribed arbitrary values of 0.01 for statistical purposes.

3.6 References

ANDRIANARIVO, A., CHOROMANSKI, L., MCDONOUGH, S., PACKHAM, A. & CONRAD, P. 1999. Immunogenicity of a killed whole *Neospora caninum* tachyzoite preparation formulated with different adjuvants. *International journal for parasitology*, 29:1613-1625

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. *International journal of medical microbiology : IJMM*, 294:35-44

HERING, D., THOMPSON, W., HEWETSON, J., LITTLE, S., NORRIS, S. & PACE-TEMPLETON, J. 2004. Validation of the anthrax lethal toxin neutralization assay. *Biologicals*, 32:17-27

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 announcements*, 3:10.1128/genomeA.01313-15

NEUBERGER, A. 1944. The reaction between histidine and formaldehyde. *The Biochemical journal*, 38:309-314

OBP. 2016. *Manufacturer's insert: Anthrax spore vaccine*, http://www.obpvaccines.co.za/products ed

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

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. *Antimicrobial Agents and Chemotherapy*, 55:4238-4250

CHAPTER FOUR

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

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4.1. Introduction

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). This vaccine is still extensively used in the control of anthrax in domestic animals (WHO 2008). Anomalies 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*), goats and horses (*Capra aegagrus hircus*) have been reported (Cartwright, McChesney & Jones 1987, Sterne 1939, Turnbull 1991, Wobeser 2015). The reasons for these reactions are unknown and no conclusive explanation has been described in scientific literature (Wobeser 2015). A once-off vaccine dose per year is currently recommended for ruminants (OIE 2012). 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. During initial tests of the vaccine, 6/8 and 8/8 sheep subcutaneously vaccinated with ~ 300,000 34F2 spores survived challenge with virulent spores (Sterne 1937). Max Sterne and co-workers will go ahead to describe systematic immunization of 2.5 million cattle and several thousand horses and sheep with only slight reactions in field tests (Sterne 1937, Sterne, Nicol & Lambrechts 1942). However, they noted

that though large doses were harmless under laboratory conditions, far smaller doses provoked swellings and occasional deaths in goats (Sterne 1937). The only reason cited for this observation was that goats might be more affected by the uncapsulated strains than other domestic animals (Sterne 1937).

The principal immune response induced by vaccination of animals with the SLSV is the development of antibodies against PA which prevent the development of lethal intoxication (Shakya, Hugh-Jones & Elzer 2007). 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) has been reported to augment the protection afforded to animals (Brossier, Levy & Mock 2002, Cybulski *et al.* 2008). 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. Boer goats (*Capra aegagrus hircus*) are short-haired breeds with white bodies and light-coloured red heads (Campbell 2003). The breed was developed in South Africa in the early 1900s and raised widely due to its ease of adaptability to intensive or harsh conditions and meat production (Malan 2000).

4.2. Materials and methods

4.2.1. 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. Twenty-six healthy one-year naïve old Boer goats (females and emasculated males)

were housed at Onderstepoort Biological Products (OBP), South Africa after screening for background anti-rPA83 cross-reactive antibodies by ELISA. Lethal challenge studies were conducted at a remote site in an endemic area of the Kruger National Park (KNP), South Africa. Animal experiments were performed according to the guidelines of the National Research Council of the USA (Clark *et al*. 1996) 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).

Animals were vaccinated using the SLSV by subcutaneous injection in the inner thigh, as stipulated for goats (OBP). Challenge was performed with a virulent South African *B. anthracis* strain (Lekota *et al*. 2015) prepared as indicated in the methods section. Virulence of the spores was confirmed in BALB/c mice and naïve goats. Two groups of three mice received an intra-peritoneal challenge of ~500 and ~1000 spores respectively and monitored till death. Cause of death was confirmed by re-isolation of *B. anthracis* colonies from liver and spleen cultures made on sheep blood agar.

Two goats from each of the three negative control groups (NCG1-3) were challenged (subcutaneously in the thigh) with 36, 172 and 844 spores respectively (Table 4.1). 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 4.1). Death from anthrax was confirmed after microscopic demonstration of Gram-positive encapsulated rod-shaped bacilli in stained blood smears. All virulent challenge (mice and goats) were performed in the anthrax-endemic region of the Kruger National Park (KNP). Decontamination of challenge environment was carried out with 4 % formalin after incineration of animals and bedding

materials. Following decontamination, soil samples were collected from the incineration and challenge sites to verify if free of *B. anthracis* spores contamination.

4.2.2. Experimental design

The immunogenicity and protectiveness of the SLSV were evaluated in four scenarios using five goats per group (SVG1 to 4) (Table 3.1). 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. The rationale was to evaluate the protection elicited by this vaccine one month (SVG1), one year (SVG2) after single vaccinations and one month after a double (year apart) vaccination (SVG3). 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 direct lethal challenge was not obtained for this group due to unavailability of the challenge facility. Blood for serum harvest was collected as indicated in Table 3.1 and stored at -20 ˚C.

Following lethal challenge, the goats were monitored for a period of 14 days for signs of pyrexia (temperatures of ≥ 40 °C) and abnormal behavior. Goats were euthanized by sodium pentobarbitone overdose (Eutha-naze®, Bayer, Isando, South Africa, 400 mg/kg body mass) as soon as pyrexia and bacilli rods were detected in blood smears. Survivors were treated with procaine benzylpenicillin (Depocillin®, Intervet, Spartan, South Africa, 20 mg/kg body mass) and euthanized after confirmation of absence of bacilli rods in blood smears. This was a condition stipulated by the animal health authorities, as a precautionary measure against environmental dispersion of the challenge strain (even though animals protected by vaccination should have no detectable *B. anthracis* at this stage).

Table 4. 1. Vaccination and lethal challenge study design

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

[a] Only 3 goats challenged due to incidental deaths from heartwater
[b] Only 2 goats challenged due to incidental death from heartwater
[c] Challenge dose 844 spores
[d] Challenge dose 172 spores
[e] Challenge dose 36 spores
[f] Unchallenged
[g] Sampling time-point of survivors
SLSV-Sterne live spore vaccine (1 mL per dose, subcutaneous)
SVG-SLSV vaccinated group
NCG-Negative control group

4.2.3. Serology

Sera were analysed with indirect ELISAs for specific antibodies against recombinant protective antigen (rPA83); recombinant bacillus collagen-like protein of anthracis (rBclA) (Steichen *et al*. 2003); formaldehyde inactivated spores (FIS) (Brossier, Levy & Mock 2002) and a vegetative antigen formulation derived from a capsule and toxin deficient strain (CDC 1014) as described in the methods section.

Neutralizing antibody titres were assessed using an *in vitro* toxin neutralizing assay (TNA) as described in methods section.

4.2.4. 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 ≤ 0.05 were considered statistically significant.

4.3. Results

4.3.1. 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 4.1 and 4.2). 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 much higher rPA83 and toxin neutralizing antibodies as can be seen in Figs 4.1 and 4.2 (SVG3▼). These titres decreased slightly after lethal challenge with *B. anthracis* spores. In contrast, a titre increase was observed in survivors (Figs 4.1 and 4.2 SVG2 Δ).

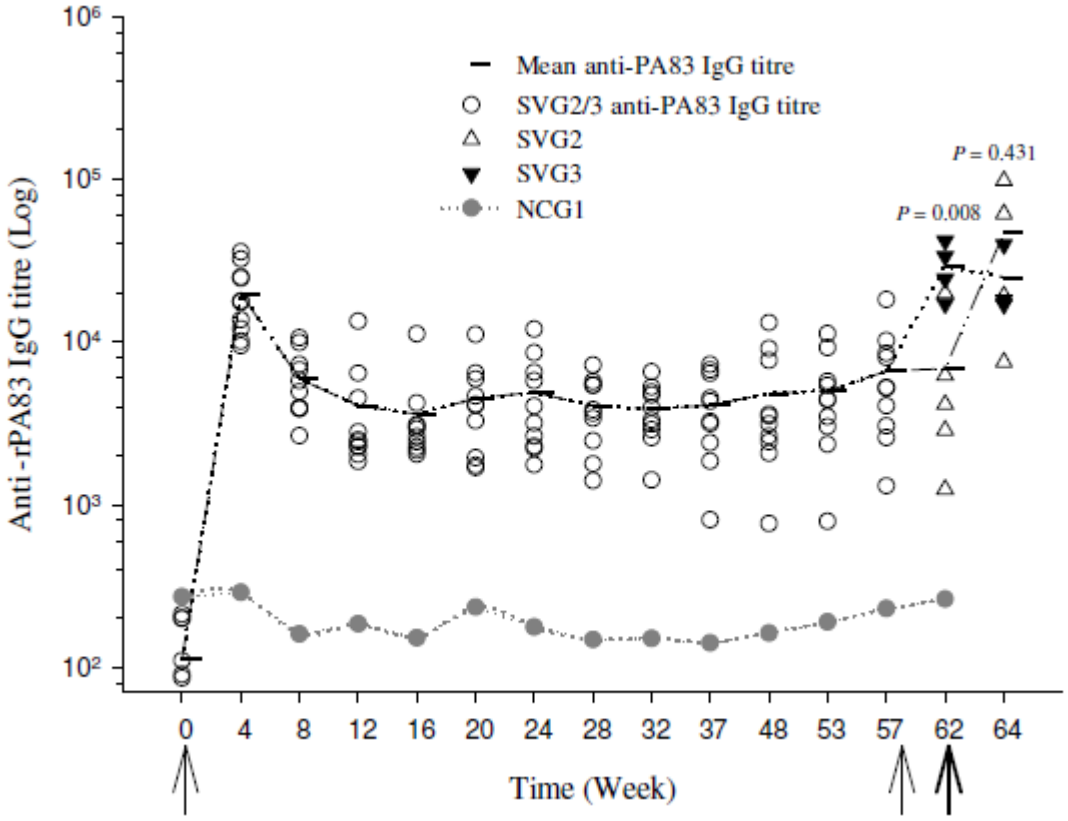


Fig 4. 1.: Anti-rPA83 (recombinant protective antigen 83kDa) titres following Sterne live spore vaccine immunizations (light arrow) and virulent *Bacillus anthracis* spores challenge (**dark arrow**) in goats. SVG2 was vaccinated once on week 0, SVG3 was vaccinated twice on weeks 0 and 58. SVG2, SVG3 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.

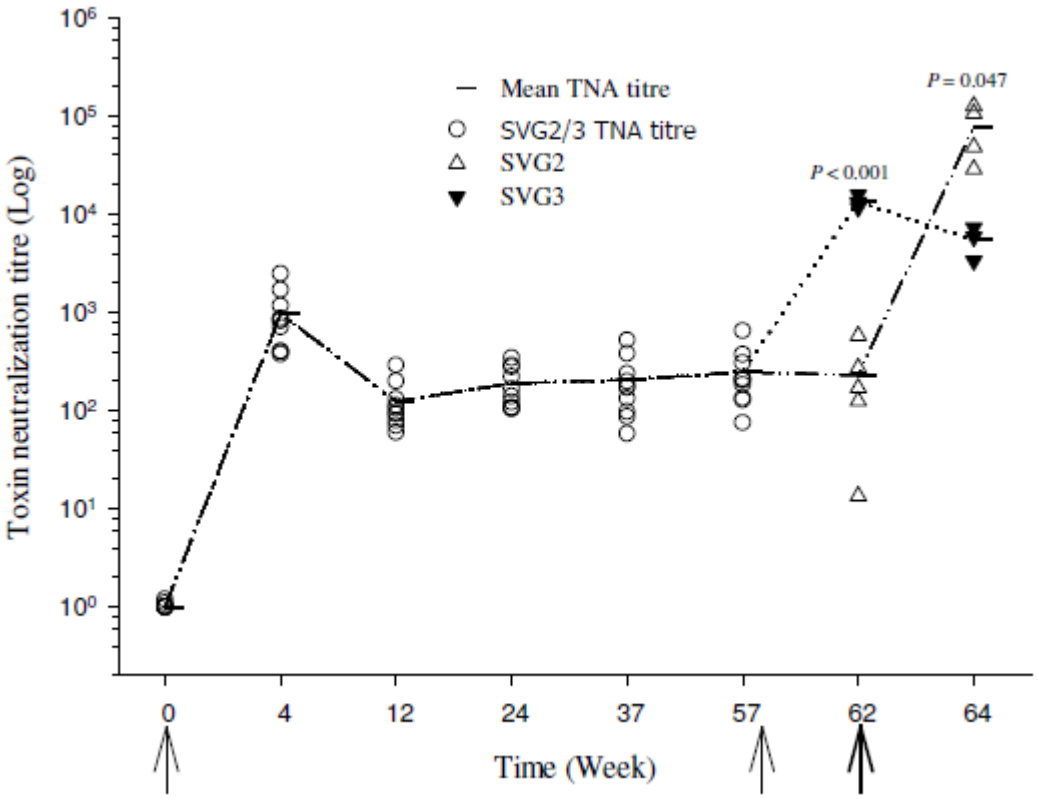


Fig 4. 2.: Toxin neutralizing titres following Sterne live spore vaccine immunizations (light arrow) and virulent *Bacillus anthracis* spores challenge (**dark arrow**) in goats. SVG2 (vaccinated once on week 0) and SVG3 (vaccinated twice on weeks 0 and 58) were challenged on week 62. Individual titres 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.

4.3.2 Spore and vegetative antigen ELISA titres

Mean anti-spore (FIS) IgG titres in the SLSV vaccinated goats were significantly increased (Fig 4.3, 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).

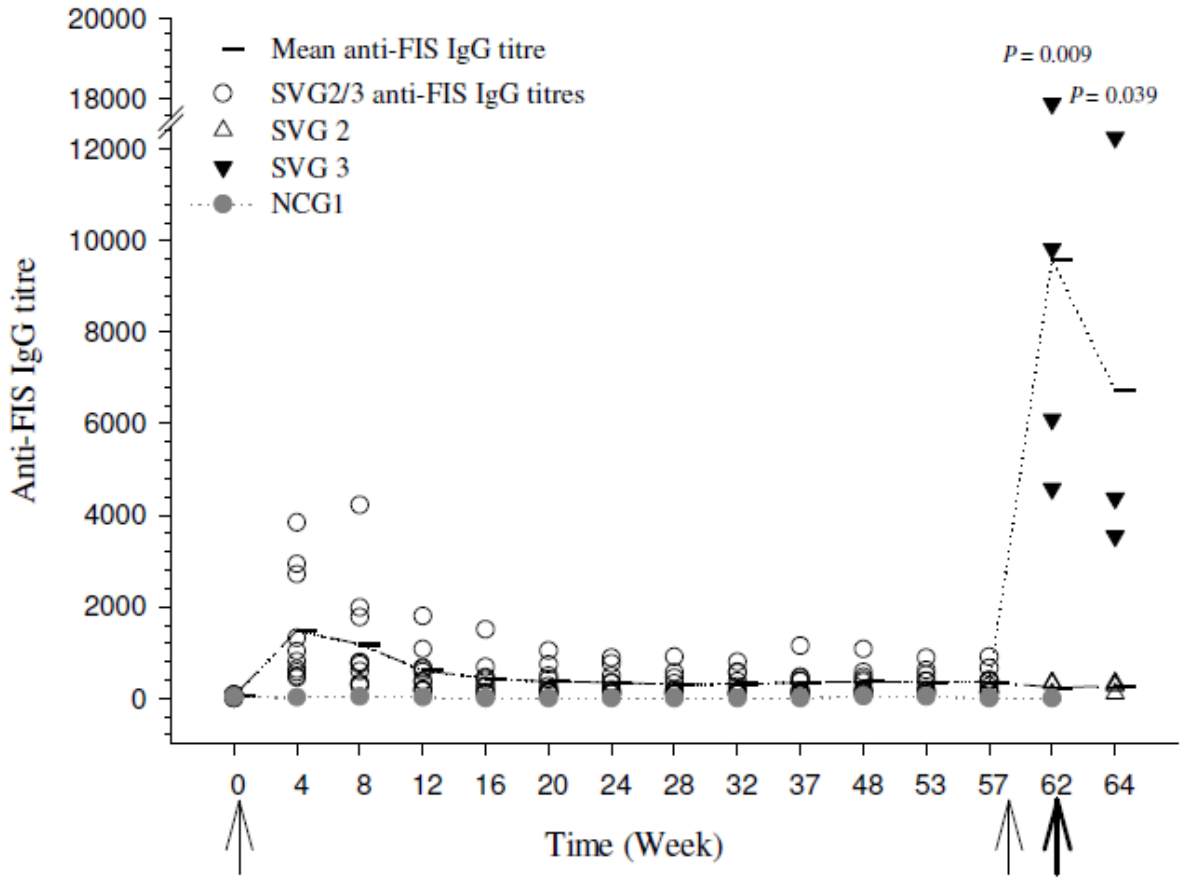


Fig 4. 3.: Anti-FIS (formaldehyde inactivating spores) IgG titres following Sterne live spore vaccine immunizations (light arrow) and virulent *Bacillus anthracis* spores challenge (**dark arrow**) in goats. SVG2 was vaccinated once on week 0, SVG3 was vaccinated twice on weeks 0 and 58. SVG2, SVG3 and 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.

Titres against the vegetative antigen were increased 4 weeks after the first immunization (Fig 4.4) 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).

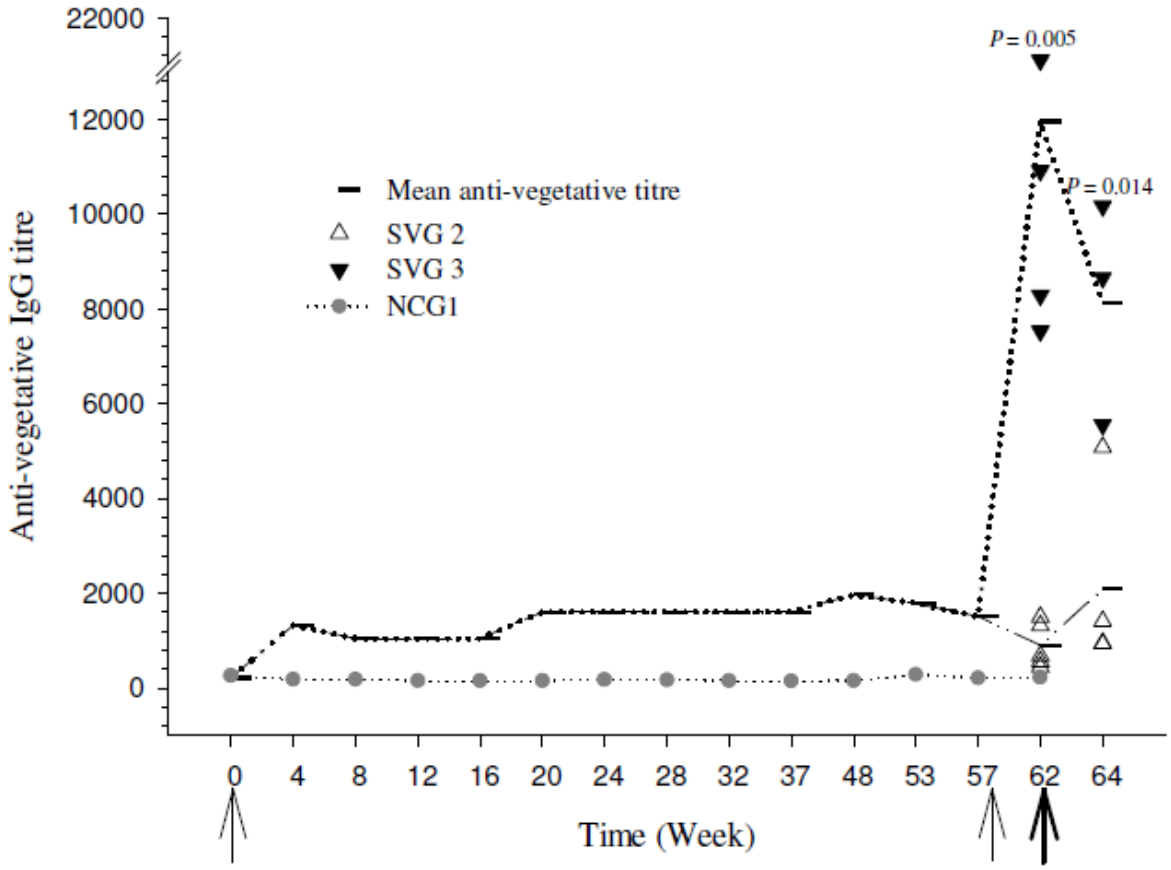


Fig 4. 4.: Anti-vegetative antigen (of a chemically and mechanically lysed pXO1 and pXO2 deficient strain) IgG titres in pooled group sera following Sterne live spore vaccine immunization (light arrow) in goats at week 0 (SVG2 and SVG3), revaccination at week 58(SVG3) and virulent *Bacillus anthracis*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

4.3.3 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 4.5). 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.

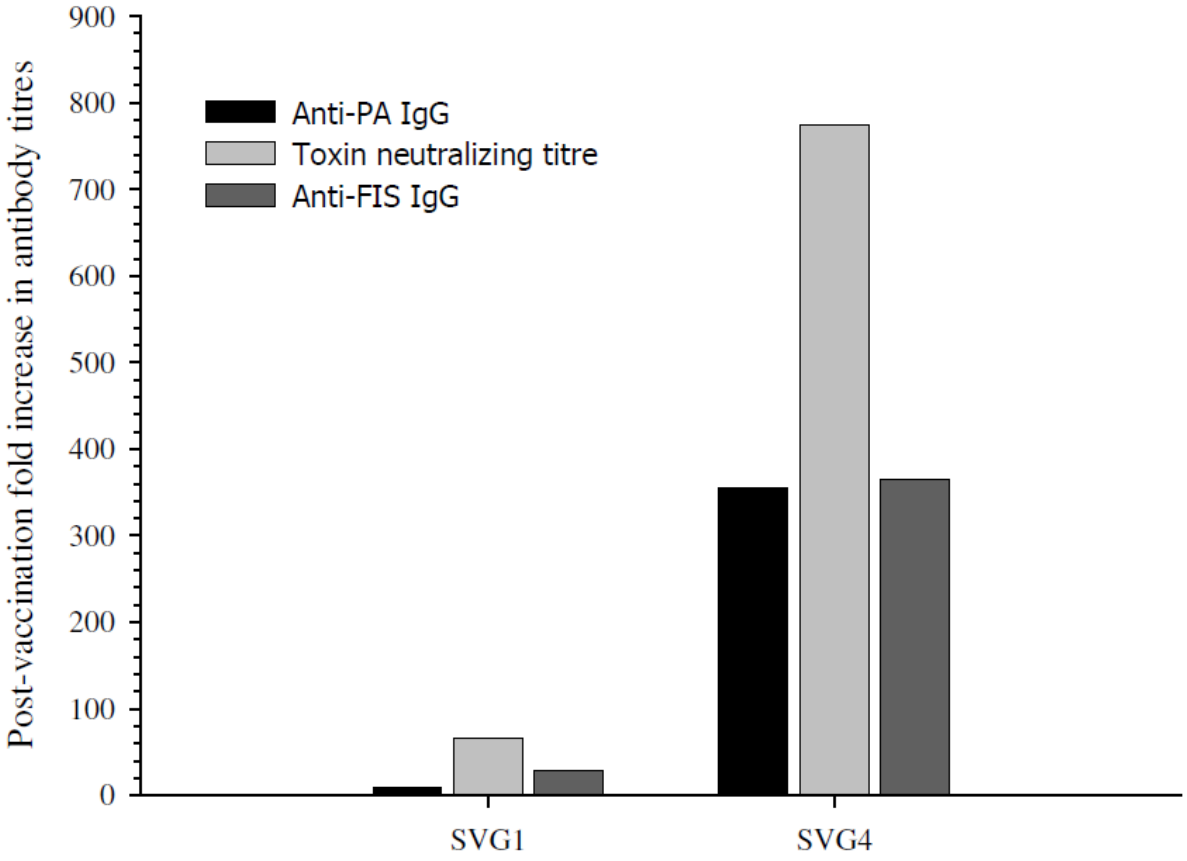


Fig 4. 5.: Immune titre increases six weeks after a single vaccination (SVG1, n = 5) or booster vaccinations at week 0 and 12 (SVG4, n = 5) with the Sterne live spore vaccine in goats.

4.3.4 Virulence and protectiveness

All the mice inoculated with either 500 or 1000 spores of the challenge strain died within two days of infection. A summary of the time to death of naïve goats challenged with different *B. anthracis* spore doses is presented in Table 4.2. Our study indicated that a subcutaneous dose of 36 *B. anthracis* spores is enough to establish a lethal infection in goats.

Table 4. 2. Time to death of naive Boer goats challenged with different doses of *B. anthracis* spores

Groups	Challenge dose[a]	Time to death in hours (mean)[b]
NCG1	36 spores	57;83
NCG2	172 spores	68;64
NCG3	844 spores	38;64

[a] Actual challenge dose as established by plate counts
[b] Two animals per group
Negative control group (NCG)

All goats vaccinated with the SLSV (SVG1 to 3) were challenged with ~ 844 spores of a fully virulent *B. anthracis* strain. The aim was to use a dose of about 1000 virulent *B. anthracis* spores dose for the lethal challenge study. However, after the lethal challenge and enumeration of redundant doses, the average count of ~ 844 spores was recorded. 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). There was no significant difference in survival rate amongst the respective vaccination groups ($P \geq 0.246$) (Fig 4.6).

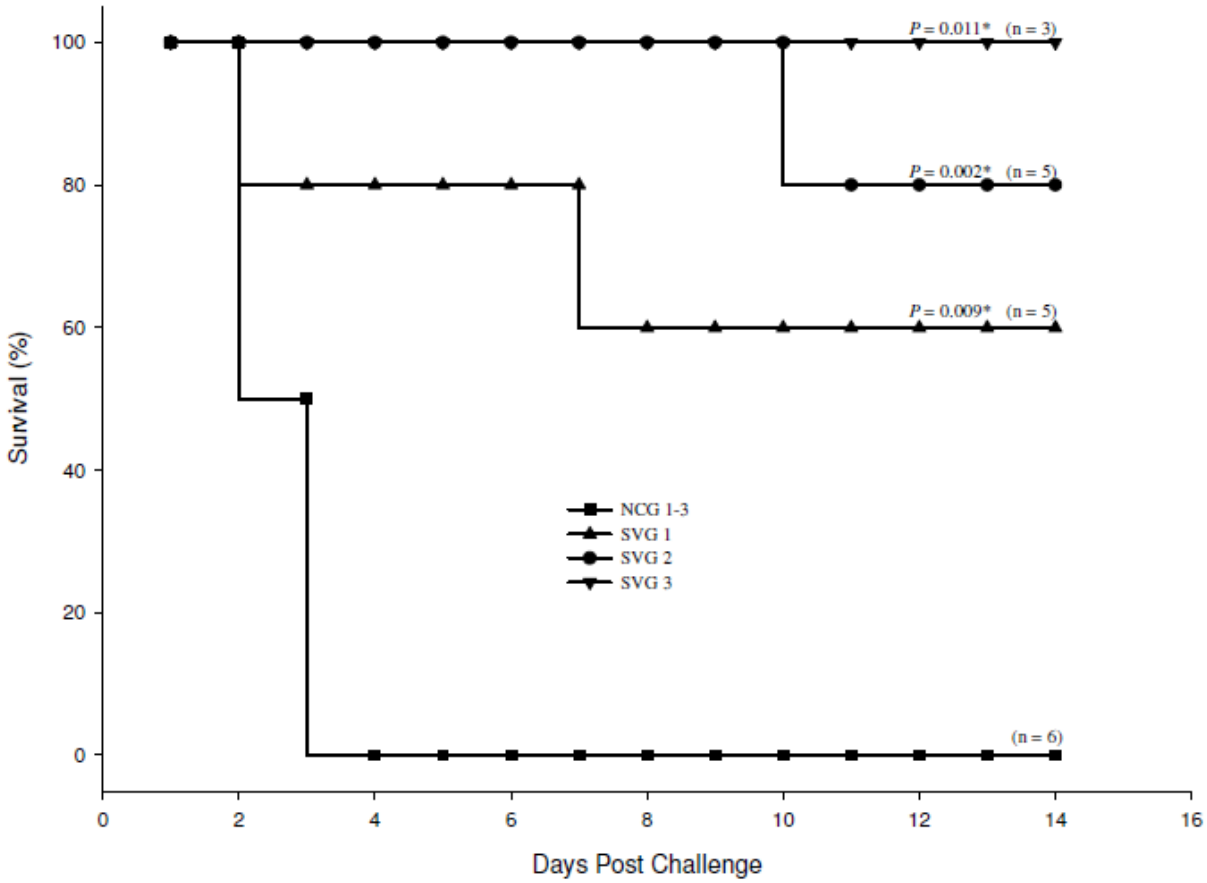


Fig 4. 6.: Kaplan-Meyer plots comparing survival times of Sterne live spore vaccine vaccinated and control groups after challenge with 844 virulent *Bacillus anthracis spores*. Spore vaccinated group (SVG) 1 and 2 were respectively challenged at 6 and 62 weeks post vaccination. SVG3 was vaccinated twice 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

4.4 Discussion

In the early 20th 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 ≥ 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 SVG4 data (revaccinated after 3 months). 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 4.5). 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. Previously, anti-PA ELISA titres were considered a main indicator of protection for evaluation of anthrax vaccines (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 4.3). 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.

4.5. References

BROSSIER, F., LEVY, M. & MOCK, M. 2002. Anthrax spores make an essential contribution to vaccine efficacy. *Infection and immunity*, 70:661-664

CAMPBELL, Q. 2003. The origin and description of southern Africa's indigenous goats. *S Afr J Anim Sci*, 4:18-22

CANDELA, T. & FOUET, A. 2005. *Bacillus anthracis* CapD, belonging to the γ-glutamyltranspeptidase family, is required for the covalent anchoring of capsule to peptidoglycan. *Molecular microbiology*, 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

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. *Biotechnology and bioengineering*, 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 toxaemia. *Journal of medical microbiology*, 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

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. *Medical history*, 50:465-490

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

INTERNATIONAL OFFICE OF EPIZOOTICS. 2008. Anthrax in humans and animals, in Geneva, Switzerland: World Health Organization: 94-95

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. *Infection and immunity*, 52:509-512

MALAN, S. 2000. The improved Boer goat. *Small Ruminant Research*, 36:165-170

OBP. 2016. *Manufacturer's insert: Anthrax spore vaccine*, http://www.obpvaccines.co.za/products ed

OIE, A.H.S. 2012. Anthrax, in *Manual of diagnostic tests and vaccines for terrestrial animals (OIE Terrestrial Manual 2008)*Paris: OIE: 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. *Journal of Bacteriology*, 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

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

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

VERGIS, J.M., COTE, C.K., BOZUE, J., ALEM, F., VENTURA, C.L., WELKOS, S.L. & O'BRIEN, A.D. 2013. Immunization of mice with formalin-inactivated spores from avirulent *Bacillus cereus* strains provides significant protection from challenge with *Bacillus anthracis* Ames. *Clinical and vaccine immunology : CVI*, 20:56-65

WELKOS, S.L., KEENER, T.J. & GIBBS, P.H. 1986. Differences in susceptibility of inbred mice to *Bacillus anthracis*. *Infection and immunity*, 51:795-800

WELKOS, S.L. & FRIEDLANDER, A.M. 1988. Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. *Microbial pathogenesis*, 5:127-139

WOBESER, B.K. 2015. Anthrax vaccine associated deaths in miniature horses. *The Canadian veterinary journal.La revue veterinaire canadienne*, 56:359-360

CHAPTER FIVE

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

5.1. Introduction

The Sterne live spore vaccine (SLSV) is currently the most widely used vaccine of choice for the control of anthrax in animals (OIE 2008). It is an attenuated $pXO1^{+}$/ $pXO2^{-}$ strain (Sterne 1937) 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 (Sterne 1939, Cartwright, McChesney & Jones 1987), short term protection (Turnbull *et al.* 2004), ineffectiveness during active outbreaks and incompatibility with antibiotics (Turnbull 1991, Fasanella *et al*. 2008). Thus, the development of a safe alternative vaccine, which can be rapidly deployed in outbreaks and compatible with antibiotics, is invaluable.

The principal immune response induced by vaccination of animals with the Sterne vaccine is the development of antibodies against PA (WHO 2008). Protection against anthrax is mainly provided by the development of antibodies to PA as these antibodies prevent lethal intoxication. (Pitt *et al*. 2001). 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 (Cybulski Jr, Sanz & O'Brien 2009). An ideal non-living recombinant anthrax vaccine should be able to induce broad spectrum immunity targeting both toxaemia and bacteraemia. The addition of BclA to PA constructs offered superior protection against virulent challenge in mice (Hahn, Boehm & Beyer 2006, Brahmbhatt *et al.* 2007, Cote *et al.* 2012) while FIS with PA-based vaccines significantly augmented the protection afforded to

mice and guinea pigs (Brossier, Levy & Mock 2002, Gauthier *et al*. 2009). Other factors that have significantly affected the quality of immune response include (i) the route of administration (Hanly, Artwohl & Bennett 1995, Wright *et al.* 2010), (ii) the number of and interval between administrations (Wright *et al.* 2010), (iii) amount of antigen and solvent type, i.e. oily or aqueous (Fasanella *et al.* 2008, Mohanan *et al.* 2010) and (iv) adjuvants (Ivins *et al.* 1992, Little *et al.* 2007).

In this study, we assessed the antibody response to PA, BclA, formaldehyde inactivated spores (FIS) and a lipopeptide adjuvant following vaccination in goats. Previously, 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 (Köhler, Baillie & Beyer 2015). The lipopeptide adjuvant used in the study was sourced from EMC Microcollections, Tubingen, Germany. It comprised of *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R*, *S*)-propyl]-(*R*)-cysteinyl-seryl-$(lysyl)_3$-lysine (Pam_3Cys-SK_4), a potent TLR2/1 activator admixed to Pam_3Cys conjugated to FISEAIIHVLHSRHPG, a T-helper cell epitope from the sperm whale myoglobin (Wiesmüller, Jung & Hess 1989, Hoffmann *et al.* 1997, Spohn *et al.* 2004). The adjuvant is well-defined, superior to conventional preparations and shows no untoward effects in animals (Mittenbuhler *et al.* 2003). It has been speculated that vaccination with spores elicits cell-mediated immunity (CMI) (Brossier, Levy & Mock 2002, Gauthier *et al.* 2009). A study by (Glomski *et al.* 2007) revealed that FIS-immunized mice showed protective cellular immune responses mediated by IFN-ɣ producing CD^+4 T lymphocytes. Furthermore, humans exposed to natural spore infection or vaccinated with PA were recorded to show CMI responses (Doolan *et al.* 2007, Ingram *et al.* 2010, Quinn *et al.* 2016). Limited CMI responses were assessed in this study by measuring proliferative responses in isolated peripheral blood mononuclear cells (PBMCs) using a lymphocyte proliferation assay (Andrianarivo *et al.* 1999). The lymphocyte proliferation assay measures

the ability of lymphocytes to proliferate in response to *in vitro* stimulation by a foreign antigen (Sitz & Birx 1999). The protection elicited by the caprine immune sera was evaluated using an *in vivo* A/J mouse protection test (Turnbull *et al.* 2004). This infection model was described in earlier studies, and followed by studies showing the protection of A/J mice against Sterne spores by passive antibody treatment (Welkos, Keener & Gibbs 1986, Welkos & Friedlander 1988)The latter measures the ability of sera from vaccinated goats to protect the susceptible A/J mice against anthrax ensuing from Sterne 34F2 spores challenge.

5.2. Experimental Procedures

5.2.1. 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 pens, the goats were randomly allocated to designated vaccine groups and immunized as indicated (Table 5.1). Female 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). These mice are susceptible to *B. anthracis* 34F2 spores (Welkos, Keener & Gibbs 1986). Experiments with animals were conducted in compliance with ethical principles and guidelines approved by the animal use and care committees of the UPBRC, OBP and University of Pretoria respectively (Protocol number V065/12). Approval for Section 20, 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).

For the passive protection tests, sera (500 µl) from vaccinated goats and controls (naive and hyperimmune goats) were collected on week 10 (rPA+BclA, rPA+rBclA+FIS and negative control) or week 17 (positive control) and injected intraperitoneally into the naïve A/J mice.

This was followed 24 h later by lethal challenge with a subcutaneous injection of ~1.92 x 10^5 Sterne 34F2 spores. The rodents were monitored for 14 days following inoculation. Humane endpoints were strictly observed so that mice displaying clinical signs indicating lethal infection (piloerection, immobility, hunched posture, weight loss) were euthanized. Mice surviving at the end of the experiments or succumbed to lethal infection were aseptically dissected and liver and spleen cultures made on sheep blood agar to confirm presence or absence of Sterne 34F2 spores. The aim was to use five mice per serum sample for the designated groups but some of the mice reacted adversely to the serum and either died within hours of injection or were euthanized for humane reasons, as will be discussed later. Hence sera of three goats were assessed with four, three and two mice in rPA+rBclA group (Table 5.1). In the rPA+rBclA+FIS group, sera from two goats were not tested. Sera from three goats were tested with five, five and one mice respectively (11 in total).

Table 5. 1. Vaccine group designations, dosage and schedule

Group	Number of goats	Dosage (Subcutaneous)	Vaccination, weeks	Number of Mice (*in vivo* challenge)
rPA+rBclA × 3	5	rPA = 75 µg/dose rBclA = 75 µg/dose Lipopeptide adjuvant = 500 µg/dose	0, 3, 6	19†
rPA+rBclA+FIS × 3	5 (3)*	rPA = 75 µg/dose rBclA = 75 µg/dose FIS = 10^8 spores/dose Lipopeptide adjuvant = 500 µg/dose	0, 3, 6	11†
Negative (naive) × 1	3	1 mL of vaccine diluent	0	9 (3 mice tested/goat)
Positive × 2	4	Goats vaccinated twice with 1mL Sterne live spore vaccine	0, 12	12 (3 mice tested/goat)

†Some mice reacted adversely from *in vivo* goat serum transfer and either died or euthanized.
rPA; Recombinant protective antigen 83
rBclA; Recombinant bacillus collagen-like protein of anthracis
FIS; Formaldehyde inactivated spore
*Sera of three goats assessed by *in vivo* challenge due to adverse reactions in mice

5.2.2. Serology

Sera were analysed for specific immunoglobulins against PA, BclA and FIS as indicated in the methods chapter. Toxin neutralizing antibodies were measured using the *in vitro* neutralization assay (TNA) as indicated.

5.2.3. Lymphocyte proliferation determination

Proliferative responses of caprine peripheral blood mononuclear cells (PBMCs) isolated from the jugular vein were determined as described in the methods chapter.

5.2.4. 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 homogeneity of variances preliminarily tested using the Levene's tests (IBM SPSS Statistics 23). Differences in antibody titres between groups at specific time points were analysed using unpaired student *t*-test, with a two-tailed *P*-value. Baseline and pre-challenge ELISA, TNA and lymphocyte proliferation 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. *P*-values of ≤ 0.05 were considered statistically significant.

5.3. Results

5.3.1. Humoral immune response

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 neutralising 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 5.2). 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 double vaccinations (on weeks 0 and 12) with the Sterne live spore vaccine and sera collected on week 17 (Fig 5.1).

Table 5. 2. Antibody titres[ab] (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)

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 ± 0.23***	4.14 ± 0.36***
	Anti-BclA IgG	2.42 ± 0.30	2.85 ± 0.22*	3.28 ± 0.19**	3.19 ± 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 ± 0.25***	2.79 ± 0.54***
rPA+rBclA+FIS	Anti-PA IgG	2.09 ± 0.22	2.59 ± 0.33*	3.91 ± 0.38***	4.27 ± 0.37***
	Anti-BclA IgG	2.06 ± 0.14	2.51 ± 0.23*	3.10 ± 0.35**	3.19 ± 0.48**
	Anti-spore IgG	1.16 ± 1.31	3.05 ± 0.42*	3.32 ± 0.23*	2.99 ± 0.18*
	TNA[c]	n.d.	0.34 ± 0.95	2.61 ± 0.33***	2.92 ± 0.47***

[a] Mean log_{10} titres ± 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

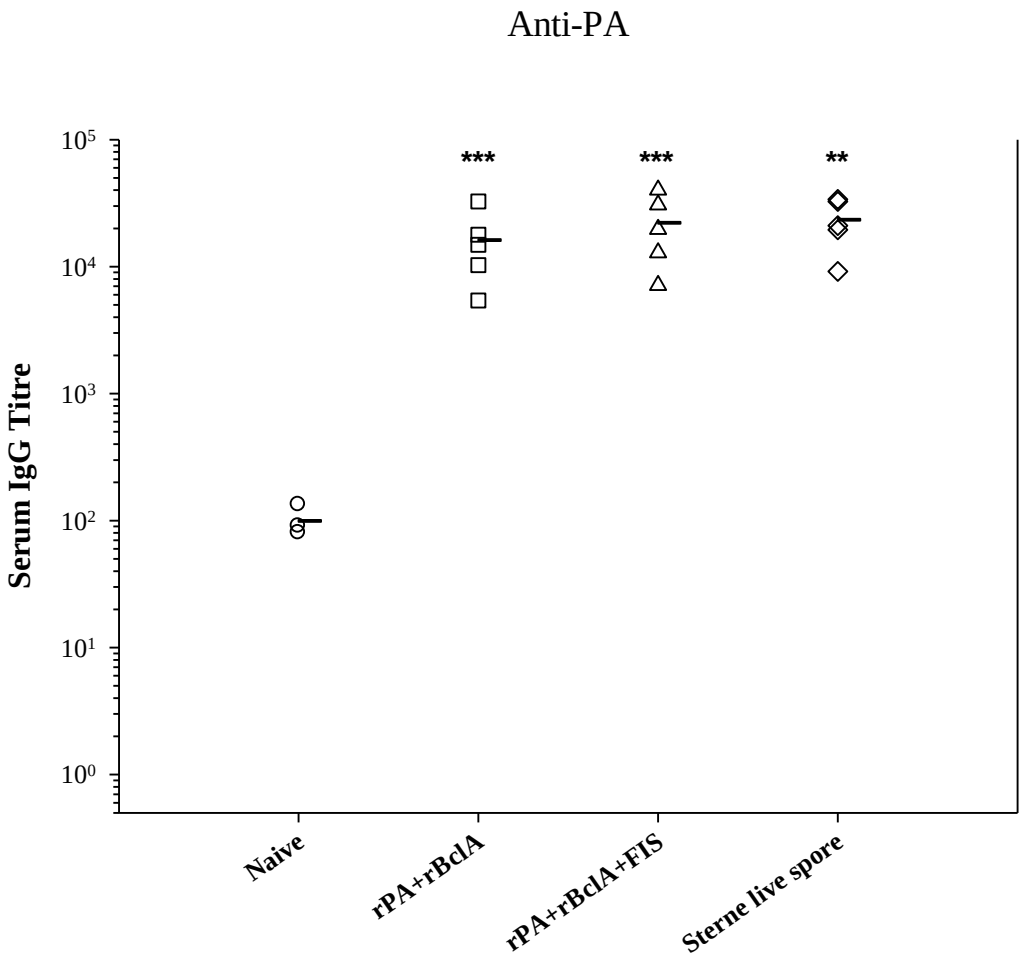


Fig 5. 1.: Anti- protective antigen (PA) IgG titres in goats. The animals were either vaccinated thrice with rPA+rBclA (n =5) and rPA+rBclA+FIS (n =5) on weeks 0, 3 and 6 (sera collected for analyses on week 10) in combination with lipopeptide adjuvant or twice with Sterne live spore (n = 5) on weeks 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 83
rBclA; Recombinant bacillus collagen-like protein of anthracis
FIS; Formaldehyde inactivated spore

The significant inducement 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 5.2). These antibodies were absent in the Sterne-vaccinated goats throughout the trials (Fig 5.2A). 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*

≤ 0.002), though also significantly elevated in the FIS-containing group. 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 5.2 and Fig 5.2B).

The development of significant lethal toxin neutralising antibodies was only seen after the second vaccination with either of the non-living vaccine combinations (Table 5.2). Thereafter it followed the pattern as seen with 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 neutralising 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 5.3).

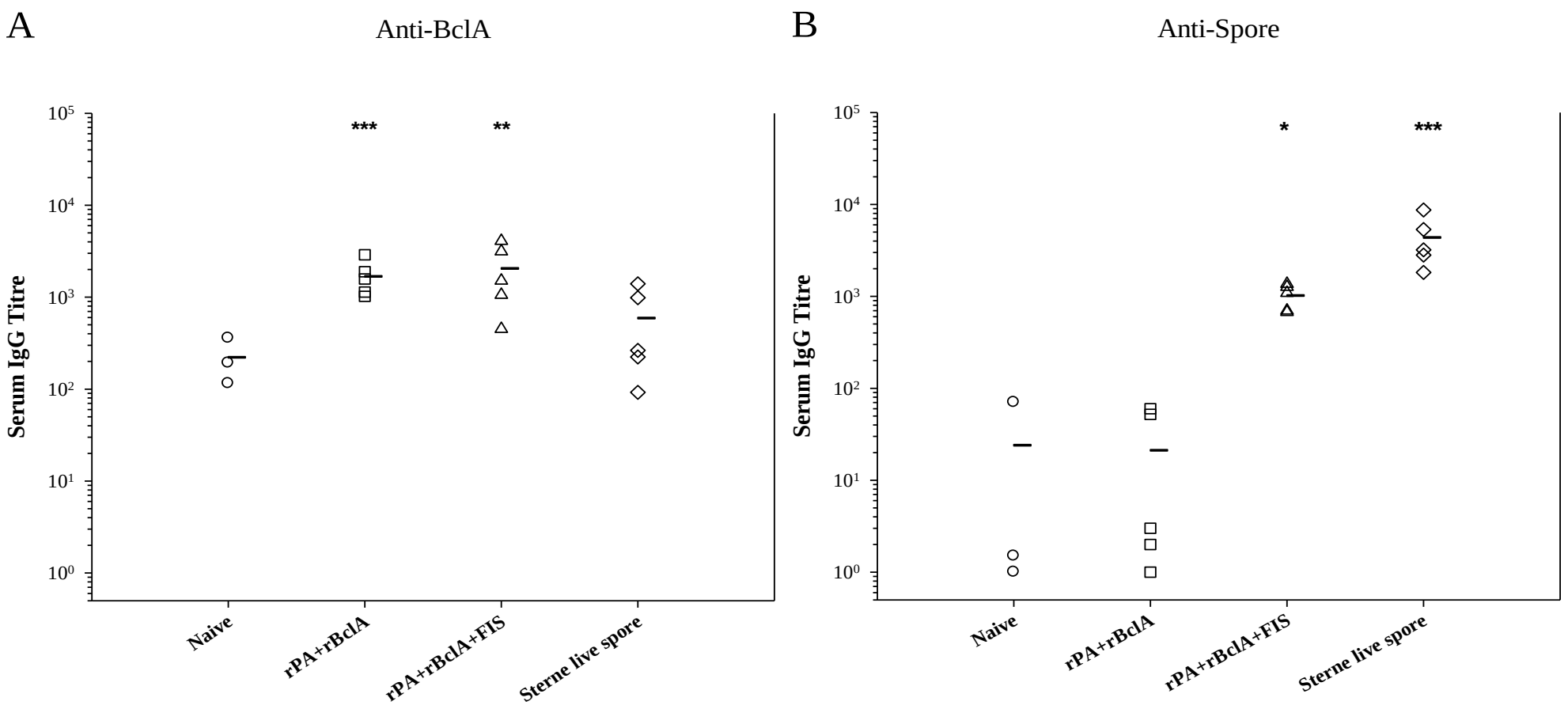


Fig 5. 2.A-B. : Anti-BclA (A) and anti-spore (B) IgG titres in goats. The animals were either vaccinated thrice with rPA+rBclA (n = 5) and rPA+rBclA+FIS (n = 5) together with lipopeptide adjuvant on weeks 0, 3 and 6 (sera collected for analyses on week 10) or twice with Sterne live spore (n = 5) on weeks 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 83
rBclA; Recombinant bacillus collagen-like protein of anthracis
FIS; Formaldehyde inactivated spore

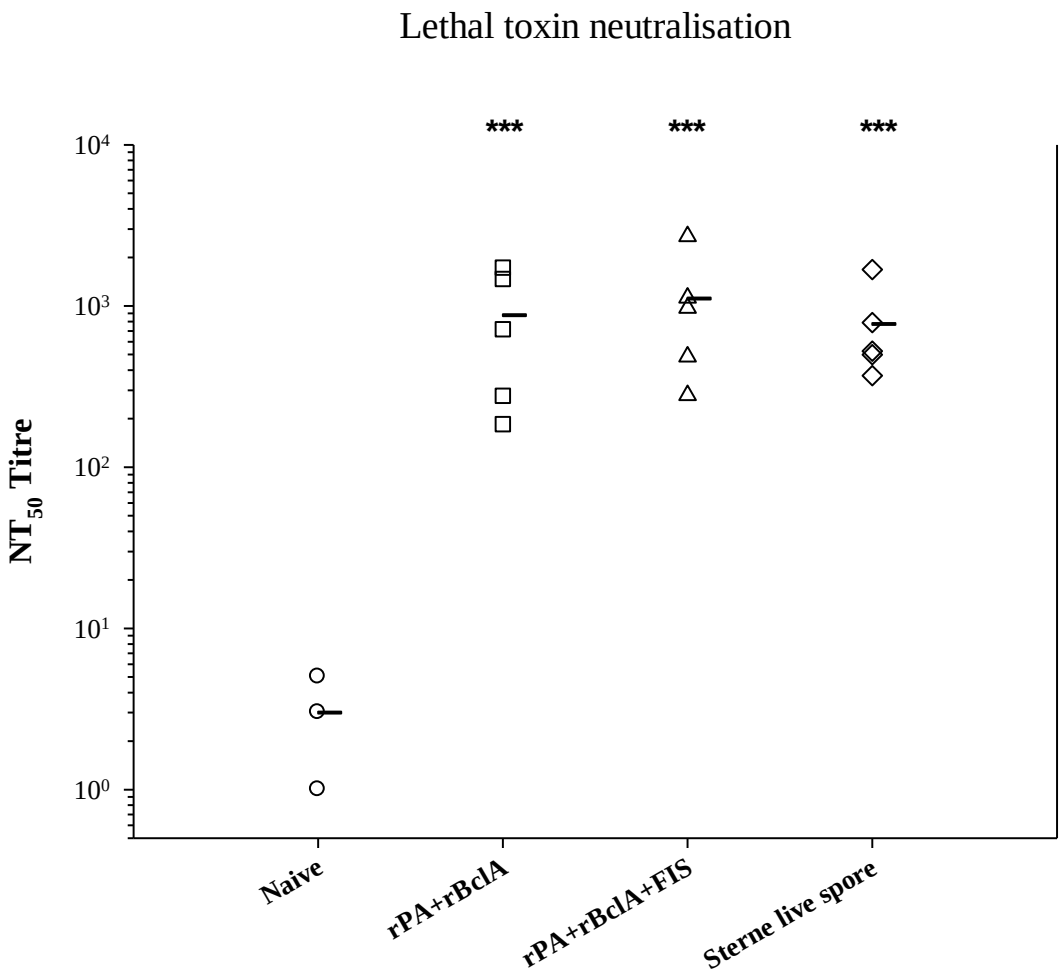


Fig 5. 3.: Anthrax lethal toxin neutralization in goats. The animals were either vaccinated thrice with rPA+rBclA (n = 5) and rPA+rBclA+FIS (n = 5) with lipopeptide adjuvant on weeks 0, 3 and 6 (sera collected for analyses on week 10) or twice with Sterne live spore (n = 5) on weeks 0 and 12 (sera collected 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 83
rBclA; Recombinant bacillus collagen-like protein of anthracis
FIS; Formaldehyde inactivated spore

5.3.2. Proliferative responses in lymphocytes

Lymphocyte proliferation assays were carried out on PBMCs isolated from jugular blood samples of the goats. These cells were rPA-stimulated with ConA and culture medium serving as positive and negative control stimuli respectively. The individual mean proliferation for each goat was calculated as the ratios (%) between the mean O.D. of cells cultured with PA and the mean O.D. of cells cultured with conA, corrected for cells cultured

with medium alone. The proliferative responses of the caprine PBMCs to ConA stimulation were consistently high as expected. Wide ranging individual animal stimulation index (SI) values were observed post-stimulation with PA (Fig 4.4). There was no difference in the mean SI values of the vaccinated and naïve groups ($P \geq 0.328$).

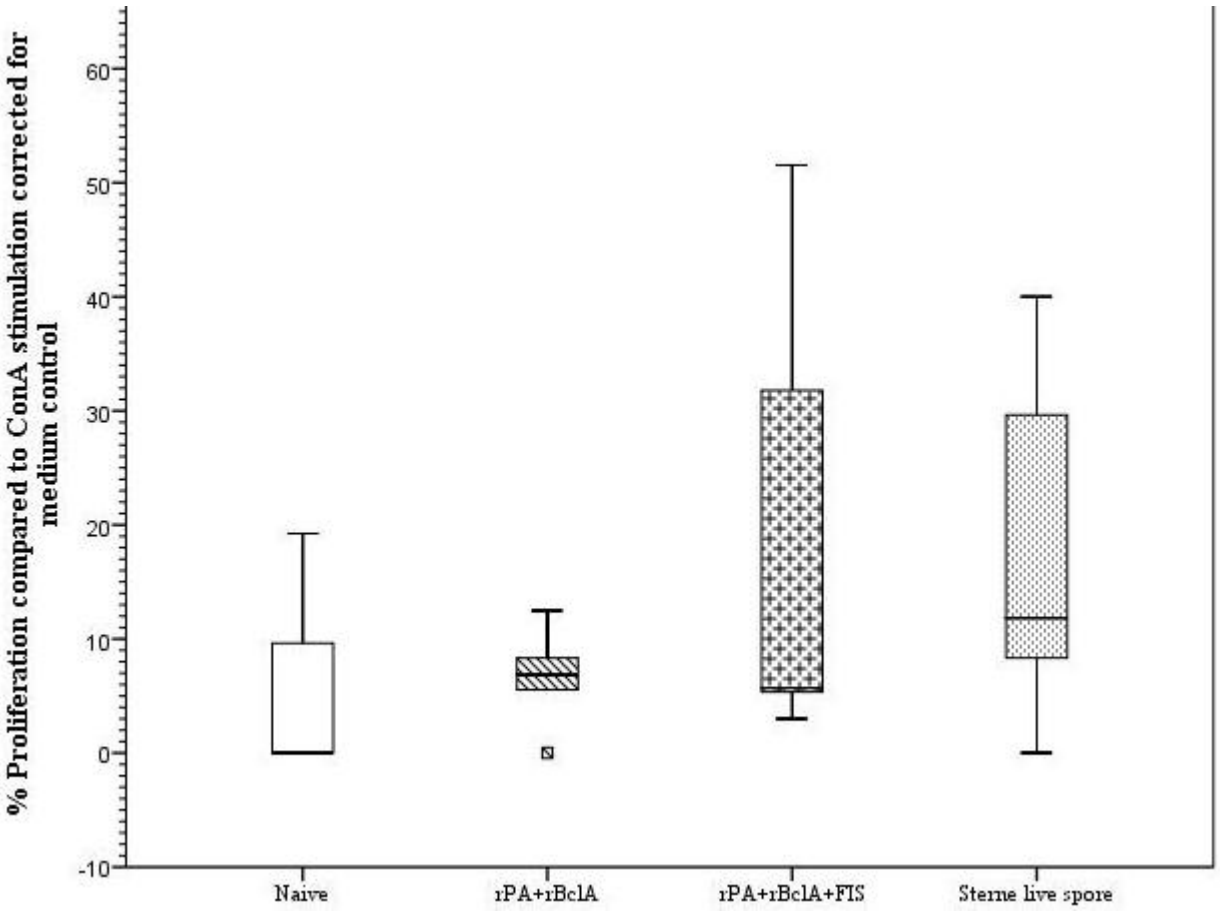
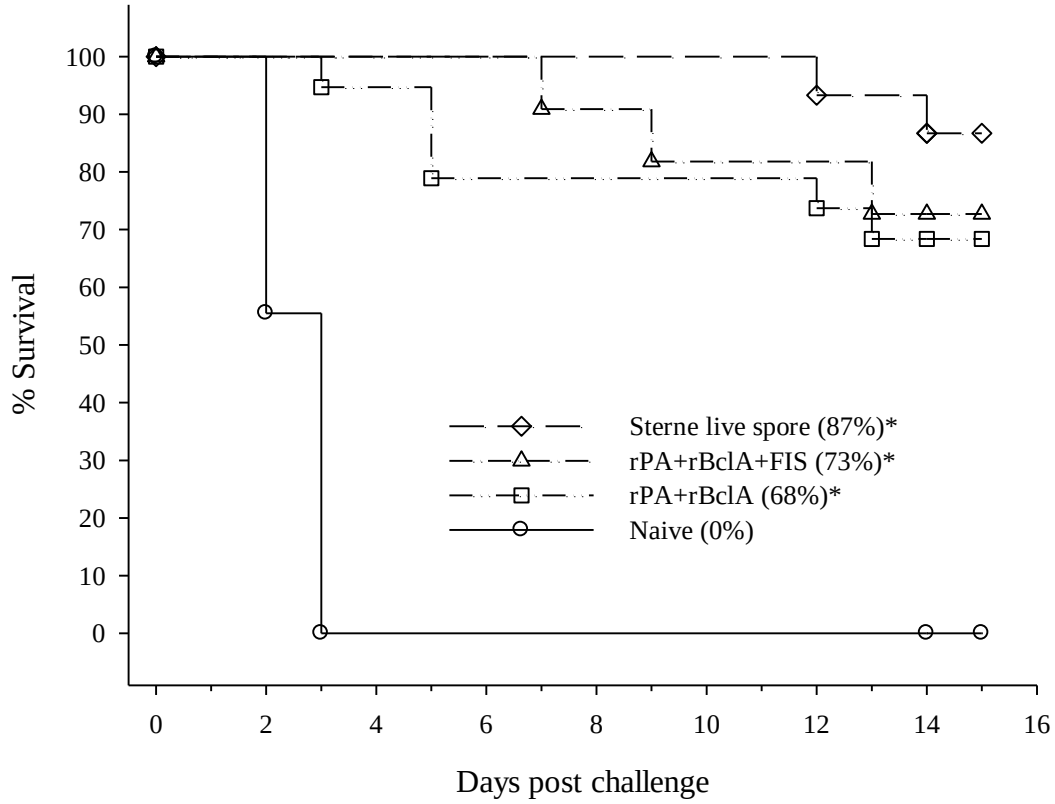


Fig 5. 4.: Lymphocyte proliferation following in vitro rPA stimulation of PBMCs isolated from jugular blood in goats. The animals were either vaccinated thrice with rPA+rBclA (n = 5) and rPA+rBclA+FIS (n = 3) with lipopeptide adjuvant on weeks 0, 3 and 6 (PBMCs isolated for analyses on week 10) or twice with Sterne live spore (n = 3) on weeks 0 and 12 (PBMCs isolated for analyses on week 17). The naïve controls (n = 3) received the vaccine diluent.

rPA; Recombinant protective antigen 83
rBclA; Recombinant bacillus collagen-like protein of anthracis
FIS; Formaldehyde inactivated spore
PBMC; Peripheral blood mononuclear cells
⧅ Outlier

5.3.3. Protection conferred on A/J mice by caprine immune sera

In order to assess the protective efficacy of the immune response generated by vaccination of goats with anthrax recombinant proteins and inactivated spores, a passive *in vivo* mouse protection experiment was carried out. Sera from the vaccinated goats were transferred into A/J mice by the intra-peritoneal route and lethal challenge performed 24 h later with Sterne strain of *B. anthracis*. The experimental design was to use a total number 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]. 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. In rPa+rBclA group, two goat sera were tested with five mice each with four, three and two mice for the rest 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. Following lethal challenge with ~1.92 x 10^5 Sterne 34F2 *B. anthracis* spores, sera from the naïve goats failed to protect the susceptible mice with all the challenged mice dying within 3 days (Fig 4.5). 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 vaccinees 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 ($P \geq 0.189$). On the other hand, these groups showed increased survival times compared to the naïve (negative) controls ($P < 0.001$).



rPA; Recombinant protective antigen 83 **Fig 5. 5.**: Passive protection of A/J mice following in vivo transfer of immune sera from goats and lethal challenge with ~1.92 x 10^5 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 = 12) 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 ($P \geq 0.189$).

rBclA; Recombinant bacillus collagen-like protein of anthracis
FIS; Formaldehyde inactivated spore

To determine 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 neutralising antibody titres and survival in the mice (Table 5.3). There was no significant correlative association between anti-BclA IgG titres and survival in the challenged mice.

Table 5. 3. Pearson's correlation analysis of relationship between survival times of passively challenged A/J mice and caprine sera antibody titres

		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

[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)

5.4. Discussion

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 (Cybulski *et al.* 2009), it remains to be elucidated if these antigens will elicit a similar response in a ruminant model of anthrax infection. Anthrax is chiefly a disease of herbivorous animals with ruminants being most susceptible (Hambleton *et al.* 1984, Beyer & Turnbull 2009). The disease is largely controlled by the use of the Sterne live spore vaccine which is an unencapsulated but toxinogenic strain (Sterne, Nicol & Lambrechts 1942, Hugh-Jones & De Vos 2002). The vaccine retains some residual virulence, though attenuated and has limited effectiveness in the face of an active anthrax outbreak (Turnbull 1991, Stepanov *et al.* 1996). With the advent of multicomponent recombinant anthrax antigens capable of stimulating broad spectrum immune response in vaccinees, questions have risen about the potential usefulness of such vaccine candidates in the control of the disease in livestock. Previously (chapter 4), approval was obtained from the relevant government authorities to carry out direct virulent challenge trials in an anthrax-endemic region of Kruger National Park. However, for inexplicable reasons, we were made to understand that this approval will not be extended to subsequent trials. Hence, the decision was made to use a passive mice protection test instead. This model has been used previously albeit in homogenous mouse sera transfer

(Beedham, Turnbull & Williamson 2001) or adoptive heterogenous immune sera transfer from Sterne live spore vaccinates (Turnbull *et al*. 2004). 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.

PA is responsible for the production of toxin neutralising antibodies which is vital for protection against anthrax infections (Pitt *et al*. 2001, Welkos *et al*. 2001). It is the principal immunogen of the licensed human vaccine (Pitt *et al*. 1999) and forms the primary component of numerous recombinant anthrax candidate vaccines. The presence of anti-PA and toxin neutralising antibodies peaked after a second vaccination with either of rPA+rBclA or rPA+rBclA+FIS and lipopeptide adjuvant. This was also replicated in the production of anti-BclA and anti-spore (rPA+rBclA+FIS only) antibodies. The results indicated that these recombinant vaccines were able to induce strong immune responses which were comparable to the Sterne live spore vaccinated controls. The humoral response to BclA in the Sterne vaccinated controls was poor, which was noted previously (Chapter 4). These were unexpected as previous studies in mice (Köhler, Baillie & Beyer 2015) and rabbits (unpublished data) had recorded antibody titres against the BclA used in this study. Notwithstanding, almost 90% of challenged mice were protected by Sterne vaccinates sera implying the insignificant role of anti-BclA antibodies in protection against anthrax in these animals, a fact also observed in the direct challenge of Sterne vaccinated goats (Chapter 4). The addition of FIS to the rPA+rBclA vaccine combination increased survival (not significant) by 5 % (Fig 5.5). In a different study using the same vaccine candidates, 50 % of goats vaccinated with rPa+rBclA were protected against direct virulent *B. anthracis* spores challenge compared to 80 % survival observed in rPA+rBclA+FIS vaccinated goats (see appendix 1). This suggests an additional role of FIS in protection against anthrax in goats. Though not significant, the FIS, BclA and PA preparation were more effective in protecting

the challenged mice than PA and BclA alone. A correlative study of the association between antibody titres and survival reinforced this assumption with anti-PA, anti-spore and toxin neutralization titres showing positive correlations (Table 5.3). A possible "best-mix" combination of possible vaccine candidates (for future trials) based on our results will be a PA and FIS vaccine. Not only will this combination be less complex, but also potentially easier to mass produce. A similar PA and FIS vaccine combination was shown to afford better protection in mice and guinea pig models previously (Brossier *et al*. 2002, Gauthier *et al*. 2009).

With the exception of the anti-spore response, there was little difference in the humoral response of the recombinant vaccinated animals. The *in vivo* A/J mouse protection model evaluates the level of protection afforded by induced humoral antibodies following vaccination (Beedham, Turnbull & Williamson 2001, Turnbull *et al.* 2004, Albrecht *et al.* 2007). In this model, the toxin-neutralizing efficacy of these antibodies in protection of the susceptible A/J mice against lethal challenge with *B. anthracis* Sterne strain (toxin+ capsule−) (Welkos & Friedlander 1988). This, in essence, makes the level of protective antibody titre in the immune sera very significant. The ~ 70 % protection observed following vaccination with rPA+rBclA and rPA+rBclA+FIS in this study demonstrates the potential protective capacity in the donor animal. The lipopeptide adjuvant used in this study, $Pam_3Cys\text{-}SK_4$ represents a highly efficient immunoadjuvant used in peptide/protein vaccination (Mittenbuhler *et al.* 2003). $Pam_3Cys\text{-}SK_4$ has been reported severally to enhance the humoral immune response to antigens in various species (Mittenbuhler *et al.* 2003, Bessler *et al.* 2003, Hope *et al.* 2003, Wedlock *et al.* 2008, Wedlock *et al.* 2011). Co-administration of the adjuvant with the recombinant proteins was well tolerated by the goat hosts throughout the trials. The choice of 500 µl as the volume of passively transferred immune sera was based on previous studies (Beedham, Turnbull & Williamson 2001,

Turnbull *et al.* 2004) and after consultations with PCB Turnbull. The adverse reactions in some of the mice following intraperitoneal serum transfer were unexpected. The post mortem reports on the affected mice indicated possible anaphylactic reactions (serum sickness) due to the serum infusion (not shown). On hindsight, the use of affinity-purified antibodies from immune sera could have prevented these reactions.

Preliminary data from the proliferation of lymphocytes following vaccination with either the recombinant or live spore vaccines revealed varied and inconsistent responses (Fig 4.4). The lymphocyte proliferation assay measures the ability of lymphocytes to proliferate in response to *in vitro* stimulation by a foreign antigen (Sitz & Birx 1999). Typically, $CD4^+$ lymphocytes multiply in response to antigenic peptides in association with class II major histocompatibility complex (MHC) molecules on antigen-presenting cells (Alberts *et al.* 2002). We had hoped the assay will give a limited measure of the cell-mediated immune responses following vaccination with the recombinant or live spore vaccines. Data obtained following re-stimulation of isolated PBMCs (from different vaccinated goat groups) with rPA indicated group-wide variations in individual SI values.

In summary, our study revealed the potential of a non-living anthrax vaccine in inducing a protective immune response in vaccinated goats. Our results indicate the protective capacity induced in caprine sera following vaccination with either rPA+rBclA or rPA+rBclA+FIS in combination with a lipopeptide adjuvant. However anti-BclA IgG titres had no visible role in protection of the mice against lethal challenge and call the inclusion of this protein in future trials into question.

5.5. References

ALBERTS, B., JOHNSON, A., LEWIS, J., RAFF, M., ROBERTS, K. & WALTER, P. 2002. T cells and MHC proteins, in *Molecular Biology of the Cell*New York: Garland Science

ALBRECHT, M.T., LI, H., WILLIAMSON, E.D., LEBUTT, C.S., FLICK-SMITH, H.C., QUINN, C.P., WESTRA, H., GALLOWAY, D., MATECZUN, A., GOLDMAN, S., GROEN, H. & BAILLIE, L.W. 2007. 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

ANDRIANARIVO, A., CHOROMANSKI, L., MCDONOUGH, S., PACKHAM, A. & CONRAD, P. 1999. Immunogenicity of a killed whole *Neospora caninum* tachyzoite preparation formulated with different adjuvants. *International journal for parasitology*, 29:1613-1625

BEEDHAM, R., TURNBULL, P. & WILLIAMSON, E. 2001. Passive transfer of protection against *Bacillus anthracis* infection in a murine model. *Vaccine*, 19:4409-4416

BESSLER, W.G., MITTENBÜHLER, K., ESCHE, U. & HUBER, M. 2003. Lipopeptide adjuvants in combination treatment. *International immunopharmacology*, 3:1217-1224

BEYER, W. & TURNBULL, P.C. 2009. Anthrax in animals. *Molecular aspects of medicine*, 30:481-489

BRAHMBHATT, T.N., DARNELL, S.C., CARVALHO, H.M., SANZ, P., KANG, T.J., BULL, R.L., RASMUSSEN, S.B., CROSS, A.S. & O'BRIEN, A.D. 2007. 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

BROSSIER, F., LEVY, M. & MOCK, M. 2002. Anthrax spores make an essential contribution to vaccine efficacy. *Infection and immunity*, 70:661-664

CARTWRIGHT, M.E., MCCHESNEY, A.E. & JONES, R.L. 1987. Vaccination-related anthrax in three llamas. *J Am Vet Med Assoc*, 191:715-716

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 toxaemia. *Journal of medical microbiology*, 61:1380-1392

CYBULSKI JR, R.J., SANZ, P. & O'BRIEN, A.D. 2009. Anthrax vaccination strategies. *Molecular aspects of medicine*, 30:490

DOOLAN, D.L., FREILICH, D.A., BRICE, G.T., BURGESS, T.H., BERZINS, M.P., BULL, R.L., GRABER, N.L., DABBS, J.L., SHATNEY, L.L., BLAZES, D.L., BEBRIS, L.M., MALONE, M.F., EISOLD, J.F., MATECZUN, A.J. & MARTIN, G.J. 2007. The US capitol bioterrorism anthrax exposures: clinical epidemiological and immunological characteristics. *The Journal of infectious diseases*, 195:174-184

FASANELLA, A., TONELLO, F., GAROFOLO, G., MURARO, L., CARATTOLI, A., ADONE, R. & MONTECUCCO, C. 2008. Protective activity and immunogenicity of two recombinant anthrax vaccines for veterinary use. *Vaccine*, 26:5684-5688

GAUTHIER, Y.P., TOURNIER, J.N., PAUCOD, J.C., CORRE, J.P., MOCK, M., GOOSSENS, P.L. & VIDAL, D.R. 2009. Efficacy of a vaccine based on protective antigen and killed spores against experimental inhalational anthrax. *Infection and immunity*, 77:1197-1207

GLOMSKI, I.J., CORRE, J.P., MOCK, M. & GOOSSENS, P.L. 2007. Cutting edge: IFN-γ-producing CD4 T lymphocytes mediate spore-induced immunity to capsulated *Bacillus anthracis*. *The Journal of Immunology*, 178:2646-2650

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

HANLY, W.C., ARTWOHL, J.E. & BENNETT, B.T. 1995. Review of Polyclonal Antibody Production Procedures in Mammals and Poultry. *ILAR journal / National Research Council, Institute of Laboratory Animal Resources*, 37:93-118

HOFFMANN, P., LOLEIT, M., MITTENBUHLER, K., BECK, W., WIESMULLER, K.H., JUNG, G. & BESSLER, W.G. 1997. 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

HOPE, J.C., WHELAN, A.O., HEWINSON, R., VORDERMEIER, M. & HOWARD, C.J. 2003. Maturation of bovine dendritic cells by lipopeptides. *Veterinary immunology and immunopathology*, 95:21-31

HUGH-JONES, M. & DE VOS, V. 2002. Anthrax and wildlife. *Revue Scientifique et Technique-Office International des Epizooties*, 21:359-384

INGRAM, R.J., METAN, G., MAILLERE, B., DOGANAY, M., OZKUL, Y., KIM, L.U., BAILLIE, L., DYSON, H., WILLIAMSON, E.D., CHU, K.K., ASCOUGH, S., MOORE, S., HUWAR, T.B., ROBINSON, J.H., SRISKANDAN, S. & ALTMANN, D.M. 2010. Natural exposure to cutaneous anthrax gives long-lasting T cell immunity encompassing infection-specific epitopes. *Journal of immunology (Baltimore, Md.: 1950)*, 184:3814-3821

INTERNATIONAL OFFICE OF EPIZOOTICS. 2008. Anthrax in humans and animals, in Geneva, Switzerland: World Health Organization: 94-95

IVINS, B.E., WELKOS, S.L., LITTLE, S.F., CRUMRINE, M.H. & NELSON, G.O. 1992. Immunization against anthrax with *Bacillus anthracis* protective antigen combined with adjuvants. *Infection and immunity*, 60:662-668

KÖHLER, S.M., BAILLIE, L.W. & BEYER, W. 2015. BclA and toxin antigens augment each other to protect NMRI mice from lethal *Bacillus anthracis* challenge. *Vaccine*, 33:2771-2777

LITTLE, S., IVINS, B., WEBSTER, W., NORRIS, S. & ANDREWS, G. 2007. Effect of aluminum hydroxide adjuvant and formaldehyde in the formulation of rPA anthrax vaccine. *Vaccine*, 25:2771-2777

MITTENBUHLER, K., V D ESCHE, U., HEINEVETTER, L., BESSLER, W.G. & HUBER, M. 2003. Lipopeptides: adjuvanticity in conventional and genetic immunization. *FEMS immunology and medical microbiology*, 37:193-200

MOHANAN, D., SLÜTTER, B., HENRIKSEN-LACEY, M., JISKOOT, W., BOUWSTRA, J.A., PERRIE, Y., KÜNDIG, T.M., GANDER, B. & JOHANSEN, P. 2010. Administration routes affect the quality of immune responses: a cross-sectional evaluation of particulate antigen-delivery systems. *Journal of Controlled Release*, 147:342-349

OIE TERRESTRIAL MANUAL. 2008. OIE listed diseases and other diseases of importance to international trade. Part-2, Chapter 2.1. 1. *Anthrax.pp-135-144*

PITT, M., LITTLE, S., IVINS, B., FELLOWS, P., BOLES, J., BARTH, J., HEWETSON, J. & FRIEDLANDER, A. 1999. In vitro correlate of immunity in an animal model of inhalational anthrax. *Journal of applied microbiology*, 87:304-304

PITT, M.L., LITTLE, S.F., IVINS, B.E., FELLOWS, P., BARTH, J., HEWETSON, J., GIBBS, P., DERTZBAUGH, M. & FRIEDLANDER, A.M. 2001. In vitro correlate of immunity in a rabbit model of inhalational anthrax. *Vaccine*, 19:4768-4773

QUINN, C.P., SABOURIN, C.L., SCHIFFER, J.M., NIEMUTH, N.A., SEMENOVA, V.A., LI, H., RUDGE, T.L., BRYS, A.M., MITTLER, R.S., IBEGBU, C.C., WRAMMERT, J., AHMED, R., PARKER, S.D., BABCOCK, J., KEITEL, W., POLAND, G.A., KEYSERLING, H.L., EL SAHLY, H., JACOBSON, R.M., MARANO, N., PLIKAYTIS, B.D. & WRIGHT, J.G. 2016. Humoral and Cell-Mediated Immune Responses to Alternate Booster Schedules of Anthrax Vaccine Adsorbed in Humans. *Clinical and vaccine immunology : CVI*, 23:326-338

SITZ, K.V. & BIRX, D.L. 1999. Lymphocyte proliferation assay, in *HIV Protocols*: Springer: 343-353

SPOHN, R., BUWITT-BECKMANN, U., BROCK, R., JUNG, G., ULMER, A.J. & WIESMÜLLER, K. 2004. Synthetic lipopeptide adjuvants and Toll-like receptor 2—structure–activity relationships. *Vaccine*, 22:2494-2499

STEPANOV, A., MARININ, L., POMERANTSEV, A. & STARITSIN, N. 1996. Development of novel vaccines against anthrax in man. *Journal of Biotechnology*, 44:155-160

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. 1939. The use of Anthrax Vaccines Prepared from Avirulent (Uncapsulated) Variants of *Bacillus anthracis*. *Onderstepoort Journal of Veterinary Research*, 13:307

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

TURNBULL, P.C., TINDALL, B.W., COETZEE, J.D., CONRADIE, C.M., BULL, R.L., LINDEQUE, P.M. & HUEBSCHLE, O.J. 2004. Vaccine-induced protection against anthrax in cheetah (*Acinonyx jubatus*) and black rhinoceros (*Diceros bicornis*). *Vaccine*, 22:3340-3347

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

WEDLOCK, D.N., ALDWELL, F.E., VORDERMEIER, H.M., HEWINSON, R.G. & BUDDLE, B.M. 2011. 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

WEDLOCK, D.N., DENIS, M., PAINTER, G.F., AINGE, G.D., VORDERMEIER, H.M., HEWINSON, R.G. & BUDDLE, B.M. 2008. 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

WELKOS, S.L., KEENER, T.J. & GIBBS, P.H. 1986. Differences in susceptibility of inbred mice to *Bacillus anthracis*. *Infection and immunity*, 51:795-800

WELKOS, S. & FRIEDLANDER, A. 1988. Pathogenesis and genetic control of resistance to the Sterne strain of *Bacillus anthracis*. *Microbial pathogenesis*, 4:53-69

WELKOS, S., LITTLE, S., FRIEDLANDER, A., FRITZ, D. & FELLOWS, P. 2001. 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

WIESMÜLLER, K.H., JUNG, G. & HESS, G. 1989. Novel low-molecular-weight synthetic vaccine against foot-and-mouth disease containing a potent B-cell and macrophage activator. *Vaccine*, 7:29-33

WRIGHT, J.G., QUINN, C.P., SHADOMY, S.V., MESSONNIER, N. & CENTERS FOR DISEASE CONTROL AND PREVENTION (CDC). 2010. *Use of anthrax vaccine in the United States: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2009*: Department of Health and Human Services, Centers for Disease Control and Prevention

CHAPTER SIX

Immunogenicity of the Sterne live spore vaccine versus non-living anthrax vaccine candidates in combination with simultaneous penicillin G treatment in goats

Submitted to PLOSONE (Under review).

6.1. Introduction

Most countries use the *B. anthracis* strain 34F2 for the prevention of anthrax in animals (WHO 2008). This strain was derived from a virulent bovine isolate cultured on serum agar in carbon dioxide (Sterne 1937). It is a non-encapsulated but toxinogenic spore variant ($pXO1^{+}/pXO2^{-}$) which has been utilised widely in the control of anthrax in domestic animals since development (Hambleton, Carman & Melling 1984). Vaccination with the Sterne live spore vaccine (SLSV) induces the production of protective antibodies without clinical symptoms of the disease (WHO 2008) with some exceptions (Sterne 1939, Cartwright, McChesney & Jones 1987). A drawback of the SLSV is the incompatibility with antibiotics, especially when susceptible animals are suspected of incubating infection during outbreaks (warranting prophylactic antibiotic treatment). In the past, vaccination with the SLSV and treatment with penicillin had been used in an attempt to curb outbreaks in wildlife populations (De Vos, Van Rooyen & Kloppers 1973) though contraindicated. The apparent immunity from such scenarios could be illusory as the germinated or germinating *B. anthracis* spores are susceptible to penicillin, a drug of choice in treatment of anthrax (Webster 1973). Moreover, as it takes about eight days to develop sufficient immunity following vaccination with the SLSV (Fasanella *et al.* 2008); it is of little use in protecting already infected animals. Hence it is recommended that animals at risk be treated with long-

acting antibiotics 8-14 days before vaccination with the SLSV (WHO 2008). For valuable stock and endangered wildlife, the rapid induction of high levels of protective antibodies using vaccines that are compatible with antibiotics will assist in curbing anthrax outbreaks in the affected populations. This has become attainable with the advent of recombinant and non-living anthrax vaccine candidates (NLV) (Turnbull 1991).

Findings from many studies indicate that a broader protective immune response is elicited following vaccination with live spore than with protein-based vaccines (Klein *et al.* 1962, Little & Knudson 1986, Welkos & Friedlander 1988). Alluding to this, the addition of inactivated spores or spore-based antigens conferred higher protection against virulent spores challenge than PA alone in mice (Brossier, Levy & Mock 2002, Gauthier *et al.* 2009, Cote *et al.* 2012, Köhler, Baillie & Beyer 2015). These studies suggest that a synergistic relationship exists between the anti-toxin and anti-spore antibodies induced by PA and spore-based antigens. Therefore, using a conceptual approach, we sought to evaluate the immunogenicity of anthrax recombinant peptides with formaldehyde inactivated spores (FIS) given simultaneously with or without antibiotic treatment in goats. Also, for the first time, humoral responses following treatment with penicillin and vaccination with the SLSV were evaluated in a ruminant model (goats). Specific antibody responses were detected using the toxin neutralization assay (TNA) and ELISA. Cell-mediated immune responses in treated animals were assessed with a lymphocyte proliferation assay.

6.2. Materials and Methods

6.2.1. Reagents

The recombinant PA, BclA and FIS antigens were prepared as described in the methods section. The *B. anthracis* Sterne 34F2 vaccine was sourced from the manufacturer

(Onderstepoort Biological Products, OBP, South Africa) and kept at 4 °C. Procaine penicillin (Pen G) was purchased from Intervet, Pretoria, South Africa.

6.2.2. Animals and vaccine groups

Twenty-three naïve Boer goats were sourced from farms adjoining the Pretoria region, South Africa after initial screening for anti-PA cross-reactive antibodies. These comprised of a heterogenous group of both castrated male and female goats of approximately 12 months. They were randomly distributed into designated vaccine groups (Table 5.1), dewormed and kept at the experimental animal facility of OBP throughout the duration of the study. Feeding was provided *ad libitum*. Pen G was administered (intramuscularly) at the same time with the vaccines in indicated groups (NLV+Pen G) and (SLSV+Pen G) (De Vos, Van Rooyen & Kloppers 1973). Titres induced following treatment with the Sterne vaccine and Pen G (SLSV+Pen G) were compared to titres induced after initial single vaccination with Sterne vaccine alone (SLSV).

Table 6. 1. Vaccine group designations, dosage and schedule

Group	Number of goats	Vaccine used (SC)	Dosage (Subcutaneous)	Schedule
(1) NLV×3	5	rPA + rBclA + FIS + Lipopeptide adjuvant (LpA)	rPA = 75 µg/dose rBclA = 75 µg/dose LpA = 500 µg/dose FIS = 10^8 spores/dose	Thrice with 3 weeks intervals
(2) NLV+Pen G ×3	5	rPA + rBclA + FIS + LpA + simultaneous treatment with penicillin G	rPA = 75 µg/dose rBclA = 75 µg/dose LpA = 500 µg/dose FIS = 10^8 spores/dose Pen G = 300 mg/dose	Thrice with 3 weeks intervals

(3) SLSV+Pen G ×1	5	Sterne live spore vaccine + simultaneous treatment with penicillin G	1 mL of Sterne live spore vaccine Pen G = 300 mg/dose	Once
(4) SLSV ×2	5	Sterne live spore vaccine (Positive control)	1 mL of Sterne live spore vaccine	Twice with 12 weeks interval.
(5) Negative control ×1	3	Negative control	1 mL of saline	Once

SC – Subcutaneous
NLV – Non-living vaccine
rPA – Recombinant protective antigen
rBclA – Recombinant bacillus collagen-like protein of anthracis
LpA- Lipopeptide adjuvant
FIS – Formaldehyde inactivated spore
Pen G – Penicillin G
SLSV – Sterne live spore vaccine

6.2.3. Enzyme-linked immunosorbent assay (ELISA)

Sera were analysed for antibodies against PA, BclA and FIS using ELISA as described in the methods section.

6.2.4. Toxin neutralization assay (TNA)

The presence of lethal toxin neutralizing antibodies in treated animals was assessed using the *in vitro* TNA as described previously.

6.2.5. Lymphocyte proliferation determination

Proliferative responses of PBMCs isolated from the jugular vein were determined as described previously with the lymphocyte proliferation assay.

6.2.6. Data Analysis

Box and whisker plots were used to depict data showing individual vaccine group quartile ranges with error bars indicating 95 % confidence intervals (Krzywinski & Altman 2014). Homogeneity of variances was preliminarily tested using the Levene's tests (IBM SPSS Statistics 23). Differences in antibody titres between groups at specific time points were analysed using unpaired student *t*-test, with a two-tailed *P*-value. Baseline and post-

vaccination titres within vaccine groups were compared using a paired Student's *t* test. *P*-values of ≤ 0.05 were considered statistically significant.

6.3. Results

6.3.1. Serology

6.3.1.1. ELISA

Antibody titres induced after vaccination with NLV, NLV+Pen G or SLSV are summarised in Table 6.2. A third vaccination with the NLVs did not significantly increase the anti-PA titres ($P \geq 0.113$). The mean titres induced by the NLVs after two or three vaccinations with three week intervals were similar to the mean titres in animals that received two SLSV vaccinations (without Pen G) (Table 6.2). The anti-PA titres after two vaccinations with the NLVs were significantly higher than titres observed following a single SLSV vaccination without Pen G ($P < 0.05$). However, a second vaccination with SLSV increased the titres to the same level as for the NLV vaccinated groups ($P \geq 0.871$). The concurrent administration of Pen G and NLVs had no effect on the development of anti-PA titres ($P \geq 0.185$). There was only limited seroconversion in goats given one dose of SLSV+Pen G. Elevated anti-PA titres were observed in only two of five goats, i.e. by week 2 in D2 and week 4 in D11. Four of the five goats vaccinated with SLSV showed seroconversion two weeks after vaccination. The anti-PA and TNA titres declined by week 12 in most of the goats vaccinated with SLSV showed seroconversion two weeks after vaccination. The anti-PA and TNA titres declined by week 12 in most of the goats vaccinated with SLSV, with or without Pen G. However, these titres remained higher than pre-vaccination titres when measured 12 weeks after vaccination ($P = 0.041$).

The concurrent administration of Pen G and NLVs had no effect on the development of anti-BclA titres (Table 6.2, $P \geq 0.827$). The immunization of goats with SLSV or SLSV+Pen G

did not induce anti-BclA IgG titre as shown in Table 6.4. Also, the concurrent administration of Pen G and NLVs had no effect on the development of anti-FIS titres ($P \geq 0.138$). Though there was significant seroconversion to the FIS antigen at four weeks after the administration of SLSV+Pen G ($P < 0.01$), the titres were generally lower than those seen after single vaccination with SLSV alone (Table 6.4). The anti-FIS titres observed 12 weeks after treatment in both SLSV groups, though much declined, remained significantly higher than the pre-vaccination titres ($P \leq 0.001$) (Table 6.4).

6.3.1.2. TNA

The neutralizing antibody titres to lethal toxin were determined with the TNA. The mean toxin neutralizing titres of NLV, NLV+Pen G and SLSV groups did not vary significantly following booster vaccinations ($P \geq 0.253$, Table 6.2). A third vaccination with the NLVs only caused ~2 fold increases in neutralizing titres of vaccinated animals ($P \geq 0.226$). The two animals (D2 and D11) in the SLSV+Pen G group, which responded to the PA antigen in the ELISA, also demonstrated seroconversion to lethal toxin in the TNA (Table 6.3). Similarly, the animals in the SLSV group (four out of five) that seroconverted to the PA antigen in the ELISA also seroconverted to lethal toxin in the TNA by week 4. The titres remained elevated in only two out of five animals by week 12.

Table 6. 2. Comparing mean humoral antibody titres in goats after different doses of treatment with non-living vaccine (NLV), NLV and Penicillin G (NLV+Pen G) and Sterne live spore vaccine (SLSV)†.

Vaccine group/number of dose	Anti-PA IgG[a]	Anti-FIS[a]	Anti-BclA[a]	TNA[a]
(1) NLV×2	(130±50) 9700± 6200*	(50±50) 2300 ± 900*	(120±30) 1500±800*	470 ± 280
(2) NLV+Pen G×2	(240±110) 23000 ± 20000*	(50±10) 1800 ± 1200	(70±50) 1600±1600*	900 ± 1000
(4) SLSV×1	(70±90) 1400 ± 1000	(40±10) 1100 ± 600	(190±50) 320±260	200 ± 240
(1) NLV×3	22000 ± 13000	1020 ± 300	2100±1500	1100 ± 950
(2) NLV+Pen G×3	30000 ± 19000	5300 ± 5800	1600±2200	1600 ± 1500
(4) SLSV×2	23000 ± 10000	4400 ± 2700	600±600	770 ± 530

[a] Mean ± Standard deviation
NLV; rPA+rBclA+FIS
NLV+Pen G; rPA+rBclA+FIS+Penicillin G
SLSV; Sterne live spore vaccine
TNA; Lethal toxin neutralization titres (pre-vaccination titres absent)
*Titres were compared to the respective SLSV titres ($P < 0.05$)
† Pre-vaccination titres are depicted in brackets.

Table 6. 3. Anti-PA IgG and lethal toxin neutralization titres in goats vaccinated once with Sterne livespore vaccine (SLSV) or SLSV and Penicillin G treatment (SLSV+Pen G)

		Anti-PA IgG titre				Lethal toxin neutralization titre			
Vaccine group	Animal	Pre-vaccination	Week 2	Week 4	Week 12	Pre-vaccination	Week 2	Week 4	Week 12
SLSV	D5	0	5980	2330	1880	0	290	80	0
	D6	210	10200	1170	1020	0	550	260	70
	D20	50	270	70	200	0	0	0	0
	D29	70	17260	2430	2140	0	1210	600	100
	D31	0	15940	850	630	0	170	70	0
SLSV+Pen G	D2	270	2440	3660	1300	0	330	70	50
	D18	120	120	360	310	0	0	0	0
	D11	270	330	2370	830	0	170	70	0
	D8	400	420	290	480	0	0	0	0
	D1	230	280	280	370	0	0	0	0

Table 6. 4. Anti-FIS IgG and anti-BclA IgG titres in goats vaccinated once with Sterne livespore vaccine (SLSV) or SLSV and Penicillin G treatment (SLSV+Pen G)

		Anti-FIS IgG titre			Anti-BclA IgG titre	
Vaccine group	Animal	Pre-vaccination	Week 4	Week 12	Pre-vaccination	Week 4
SLSV	D5	60	2120	350	190	210
	D6	0	700	340	250	420
	D20	0	990	330	140	130
	D29	0	820	360	140	730
	D31	0	900	280	210	110
SLSV+Pen G	D2	60	650	190	220	180
	D18	0	420	140	320	180
	D11	0	860	240	240	190
	D8	0	360	150	110	130
	D1	0	460	150	150	170

BclA; Bacillus collagen-like protein of anthracis

6.3.1.3. Lymphocyte proliferative responses

To investigate the cell-mediated immune response, lymphocyte proliferation assays were performed on PBMCs. There was a large variation in pre- and post-vaccination SI values within all the vaccinated groups Fig 6.1 below depicts the SI ranges (with standard deviation, SD) following the final vaccinations in the respective groups. There was no significant difference between any of the vaccine groups following either double or triple vaccinations ($P \geq 0.154$) even though the NLV+Pen G group had the highest mean SI value (19.47 ± SD10.49) followed by the SLSV (17.97 ± SD16.40) and NLV (13.29 ± SD21.45). PBMCs from negative control animals (unvaccinated) had mean SI value of 6.42 ± SD11.11.

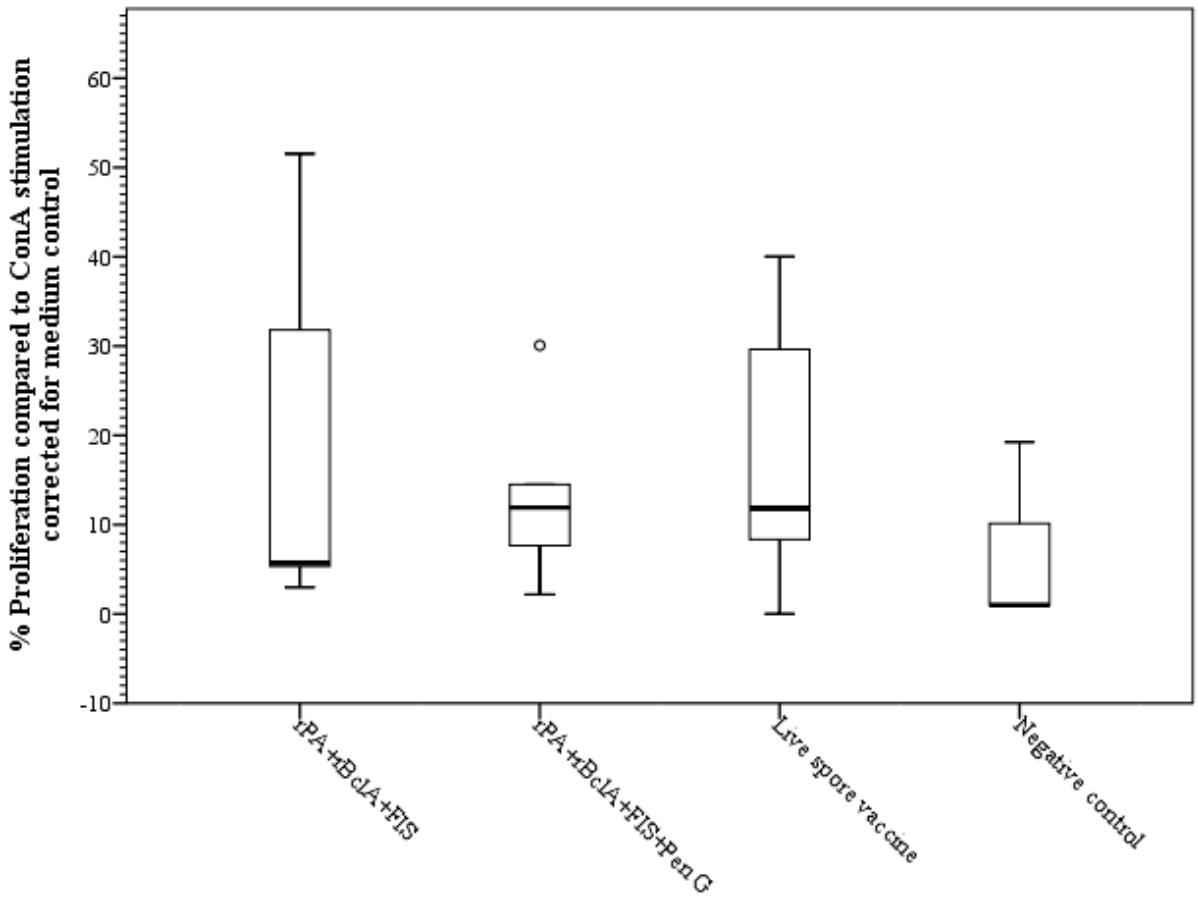


Fig 6. 1.: Lymphocyte proliferation from PBMCs (n = 5 goats) following vaccination with non-living vaccine candidates alone and in combination with Penicillin G as well as the Sterne live spore vaccine. The non-living vaccines were administered at week 0 and 3 and antibody was measured 3 weeks later. The Sterne vaccine was given at week 0 and 12 and antibody was measured 5 weeks later.

o Outlier (D37)

rPA – Recombinant protective antigen
rBclA – Recombinant bacillus collagen-like protein of anthracis
FIS – Formaldehyde inactivated spore
Pen G – Penicillin G
PBMCs – Peripheral blood mononuclear cells

6.4. Discussion

The introduction of the SLSV in the 1940s heralded a significant reduction in the number of occurrences and magnitude of anthrax outbreaks in livestock (Hugh-Jones & De Vos 2002). The vaccine remains the best option for the routine control of anthrax in animals especially when administered early enough for adequate development of protective immunity before the

outbreak seasons (Fasanella *et al.* 2008). However, the effectiveness of the SLSV is limited in the face of an active outbreak which warrants the use of anti-microbial therapy (Stepanov *et al.* 1996). Such situations require the use of long-acting antibiotics to provide protective cover to animals. During this time (~ 8-14 days) the use of SLSV is contraindicated (WHO 2008). The simultaneous administration of vaccine and an antibiotic will be a cheaper and more efficient approach especially during outbreak situations. The employment of a NLV (recombinant peptides and formaldehyde inactivated spores in our case) can be a feasible option in such scenarios. Recently, the rPA and in conjunction with mutant LF and EF protected 100 % of challenged rabbits when administered as a single dose vaccine emulsified in oil-based adjuvants (Fasanella *et al.* 2008). In our proof of concept study, we combined the rPA with the exosporium antigen, BclA, and FIS (NLV) in a simultaneous formulation with Pen G in order to assess the *in vivo* effect of the latter on immunogenicity of the vaccine candidates. Comparisons of specific immune titres following vaccination with either SLSV alone or SLSV and Pen G were performed to ascertain any effect of the latter on developing antibody titres. The NLV+Pen G vaccine combination did not negate the development of crucial anti-toxin and anti-spores antibodies. There was no significant difference in mean titres developed in goats treated with NLV and SLSV. The simultaneous administration of Pen G with the SLSV inhibited the magnitude and duration of titres in some (but not all) of the treated animals when compared to SLSV vaccinates.

The substantive role of rPA (Iacono-Connors *et al.* 1991, Welkos *et al.* 2001, Fasanella *et al.* 2008), rBclA (Hahn, Boehm & Beyer 2006, Brahmbhatt *et al.* 2007, Köhler *et al.* 2015) and FIS (Brossier *et al.* 2002, Gauthier *et al.* 2009, Vergis *et al.* 2013) antigens in stimulating protective antibodies to anthrax have been shown previously. The anti-bacterial properties of antibiotics in combination with the anti-toxin and anti-spore benefits of such multi-

component vaccine should terminate incubating anthrax infections while inducing a protective immune response.

A third vaccination within three weeks with the NLVs induced less than 3-fold increases in immune titres (Table 6.2). There was no difference between NLV and NLV+Pen G treated groups. We observed that the two vaccinations with the NLVs (with or without Pen G) gave similar titre response to vaccinating twice (12 weeks apart) with the SLSV (Table 6.2). The twelve weeks interval between the SLSV vaccinations was to allow the development of adequate immune response as it has been shown that more than one initial dose (preferably >8 weeks apart) is required for the development of durable immunity (Turnbull *et al.* 2004). The induction of lethal toxin neutralising antibodies by vaccinating with NLV (with or without Pen G) was evident three weeks after the second vaccination. These antibodies are functional subsets of anti-PA and LF antibodies and have been shown to be a reliable predictor of survival against lethal spores challenge in various species (Fowler *et al.* 1999, Reuveny *et al.* 2001, Weiss *et al.* 2006). A third vaccination slightly increased neutralising titres, though not significant. Importantly, this indicates that two vaccinations with the NLV antigens could be sufficient to stimulate a robust immune response, as currently advocated for the SLSV (WHO 2008, Turnbull *et al.* 2004). However, further studies will be needed to optimize the schedule and number of doses.

A concurrent SLSV vaccination and treatment of guinea pigs with Pen G had failed to protect the animals from virulent spores challenge in a previous work by Webster (1973). In order to understand the immune response to various antigens following a single simultaneous treatment with SLSV and Pen G, we compared SLSV vaccination alone and SLSV+Pen G vaccination/treatment. This is, especially, important, as the vaccination/treatment approach had been attempted in the past (De Vos, Van Rooyen & Kloppers 1973). In our study, SLSV vaccination induced higher mean anti-PA titres ($P = 0.041$) 12 weeks after (Table 6.3). Mean

anti-PA titres following treatment with SLSV+Pen G remained similar throughout this time ($P \geq 0.088$). However, while four out of five goats showed seroconversion following vaccination with SLSV, two of the SLSV+Pen G treated animals seroconverted. Simultaneous administration of Pen G and SLSV may have reduced the production of PA leading to the lower antibody levels when compared to pre-vaccination titres ($P = 0.041$). Although we observed the development of neutralizing titres in the TNA after SLSV vaccination, the post-vaccination titres were not significantly high (Table 6.3). This could be ascribed to individual variation and the small sample size. Also, the titres developed in the SLSV+Pen G group ebbed faster than were observed in the SLSV vaccinated animals.

The addition of Pen G to SLSV appeared to affect the level of induced anti-FIS antibodies when compared to SLSV vaccination alone, though not significant. However, the titres remained significantly higher 12 weeks after treatment with SLSV+Pen G ($P = 0.001$). The presence of Pen G kills the bacteria as soon as germination is triggered (Pierre Goossens, Personal communication) but did not influence the immunogenicity of the spores. This will remain speculative as there is no record of study measuring the level of anti-spores antibodies after simultaneous antibiotic treatment and spores vaccination/challenge (Kelly *et al.* 1992, Friedlander *et al.* 1993, Altboum *et al.* 2002, Vietri *et al.* 2009, Klinman & Tross 2009, Ionin *et al.* 2013, Sivko *et al.* 2016). The SLSV vaccinated group had twice the anti-spore titres at 12 weeks after treatment (Table 6.4) when compared to the SLSV+Pen G vaccinated animals. All the SLSV+Pen G treated goats showed some seroconversion to FIS, whereas only two animals had anti-PA or TNA titres. This suggests the role of the Sterne spores as persistent immunogen even in the presence of Pen G.

Our results indicated that while the NLV candidates were able to induce modest levels of antibodies against BclA, vaccination with SLSV failed to induce any significant anti-BclA antibodies even after a booster vaccination. BclA is a major filamentary component of the

spore exosporium. It has been the focus of numerous novel vaccination approaches and has been shown to be immuno-dominant in mice and guinea pigs (Cote *et al.* 2012, Brahmbhatt *et al.* 2007, Cybulski *et al.* 2008). In our study, we could not detect any cross-reacting antibody to the rBclA that might have been induced by the SLSV. This indicates that antibodies generated against the SLSV spore in goats have no activity against the exosporium-based BclA. Also, the modest levels of anti-BclA antibodies in the NLV treatment groups may indicate poor immunogenicity of the rBclA antigen component in goats and hence the need for evaluation on a species-specific basis.

Preliminary studies on the proliferation of lymphocytes following vaccination with either the SLSV or NLV (with/without Pen G) was carried out using the lymphocyte proliferation assay. Although we anticipated the presence of a cell-mediated immune response in vaccinated animals we were unable to demonstrate any significant response in NLV or SLV vaccinated animals. Results were inconclusive and need further research. Currently, we are studying the up regulation/down regulation of immune-related genes and cytokines expression in goats following vaccination with these vaccine candidates, which could provide further insight. To the best of our knowledge, the molecular mechanisms underlying the activation and development of immune responses to either SLSV or NLV vaccination in caprine or any other ruminant species have never been studied. Goats are among the most susceptible ruminant species to the pathogen and understanding the transcriptional responses underpinning protection against anthrax in these animals is important. Results from this study should provide insight on the role of innate and acquired immune related genes in vaccine-instigated protective responses.

In summary, though a once-off treatment of goats with SLSV and Pen G did not completely negate immune response, goats vaccinated once with the SLSV alone developed superior antibody responses compared to SLSV+Pen G. Simultaneous treatment and vaccination with

the NLV+Pen G induced strong anti-toxin and anti-spore immune responses in vaccinated goats. This indicates the potential of utilizing a non-living vaccine and anti-microbial combination in nipping incubating infections in vulnerable ruminants. Recently the FDA approved the pre- and post-exposure prophylactic use of the PA-based AVA and recommended antibiotic treatment in humans with suspected or confirmed exposure to *B. anthracis* (FDA 2015). Components of the NLV candidates in this study have been tested and evaluated exhaustively in laboratory rodents and non-human primates in original researches aimed at improving the current human vaccine (Ivins *et al.* 1992, Cohen *et al.* 2000, Hahn, Boehm & Beyer 2006, Gauthier *et al.* 2009, Cote *et al.* 2012, Kachura *et al.* 2016). Our work presents a case for the contingent use of these NLVs and antibiotics in the treatment of animals in the face of acute anthrax outbreaks. More research in this area will be needed before this objective can be realized. The vaccine-antibiotic combination will require evaluation in a representative target animal live challenge model. Currently, a multicomponent vaccine Supavax®, is produced by MSD Animal Health® for the vaccination of livestock against anthrax, botulism and blackleg. The vaccine consists of the Sterne 34F2 *B. anthracis* spores and clostridial toxoids (http://www.msd-animal-health.co.za/products/supavax/020_product_details.aspx). The potential and feasibility of combining these anthrax NLVs with such toxoids will be an interesting prospect. Finally, it must be highlighted that the administration of antibiotics with the NLVs will be in specific circumstances and not for routine use. The deployment will be under the direction and supervision of veterinarians and animal health workers.

6.5. References

ALTBOUM, Z., GOZES, Y., BARNEA, A., PASS, A., WHITE, M. & KOBILER, D. 2002. Postexposure Prophylaxis against Anthrax: Evaluation of Various Treatment Regimens in Intranasally Infected Guinea Pigs. *Infection and immunity*, 70:6231-6241

BRAHMBHATT, T.N., DARNELL, S.C., CARVALHO, H.M., SANZ, P., KANG, T.J., BULL, R.L., RASMUSSEN, S.B., CROSS, A.S. & O'BRIEN, A.D. 2007. 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

BROSSIER, F., LEVY, M. & MOCK, M. 2002. Anthrax spores make an essential contribution to vaccine efficacy. *Infection and immunity*, 70:661-664

CARTWRIGHT, M.E., MCCHESNEY, A.E. & JONES, R.L. 1987. Vaccination-related anthrax in three llamas. *J Am Vet Med Assoc*, 191:715-716

COHEN, S., MENDELSON, I., ALTBOUM, Z., KOBILER, D., ELHANANY, E., BINO, T., LEITNER, M., INBAR, I., ROSENBERG, H., GOZES, Y., BARAK, R., FISHER, M., KRONMAN, C., VELAN, B. & SHAFFERMAN, A. 2000. Attenuated Nontoxinogenic and Nonencapsulated Recombinant *Bacillus anthracis* Spore Vaccines Protect against Anthrax. *Infection and immunity*, 68:4549-4558

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 toxaemia. *Journal of medical microbiology*, 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

DE VOS, V., VAN ROOYEN, G. & KLOPPERS, J. 1973. Anthrax immunization of free-ranging roan antelope Hippotragus equinus in the Kruger National Park. *Koedoe*, 16:11-25

FASANELLA, A., TONELLO, F., GAROFOLO, G., MURARO, L., CARATTOLI, A., ADONE, R. & MONTECUCCO, C. 2008. Protective activity and immunogenicity of two recombinant anthrax vaccines for veterinary use. *Vaccine*, 26:5684-5688

FDA. 2015. *Press Announcements > FDA approves vaccine for use after known or suspected anthrax exposure* , http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm474027.htm ed

FOWLER, K., MCBRIDE, B., TURNBULL, P. & BAILLIE, L. 1999. Immune correlates of protection against anthrax. *Journal of applied microbiology*, 87:305-305

FRIEDLANDER, A.M., WELKOS, S.L., PITT, M.L., EZZELL, J.W., WORSHAM, P.L., ROSE, K.J., IVINS, B.E., LOWE, J.R., HOWE, G.B. & MIKESELL, P. 1993. Postexposure prophylaxis against experimental inhalation anthrax. *The Journal of infectious diseases*, 167:1239-1243

GAUTHIER, Y.P., TOURNIER, J.N., PAUCOD, J.C., CORRE, J.P., MOCK, M., GOOSSENS, P.L. & VIDAL, D.R. 2009. Efficacy of a vaccine based on protective antigen

and killed spores against experimental inhalational anthrax. *Infection and immunity*, 77:1197-1207

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

HUGH-JONES, M. & DE VOS, V. 2002. Anthrax and wildlife. *Revue Scientifique et Technique-Office International des Epizooties*, 21:359-384

IACONO-CONNORS, L.C., WELKOS, S.L., IVINS, B.E. & DALRYMPLE, J.M. 1991. Protection against anthrax with recombinant virus-expressed protective antigen in experimental animals. *Infection and immunity*, 59:1961-1965

INTERNATIONAL OFFICE OF EPIZOOTICS. 2008. Anthrax in humans and animals, in Geneva, Switzerland: World Health Organization: 94-95

IONIN, B., HOPKINS, R.J., PLEUNE, B., SIVKO, G.S., REID, F.M., CLEMENT, K.H., RUDGE, T.L.,JR, STARK, G.V., INNES, A., SARI, S., GUINA, T., HOWARD, C., SMITH, J., SWOBODA, M.L., VERT-WONG, E., JOHNSON, V., NABORS, G.S. & SKIADOPOULOS, M.H. 2013. Evaluation of immunogenicity and efficacy of anthrax vaccine adsorbed for postexposure prophylaxis. *Clinical and vaccine immunology : CVI*, 20:1016-1026

IVINS, B.E., WELKOS, S.L., LITTLE, S.F., CRUMRINE, M.H. & NELSON, G.O. 1992. Immunization against anthrax with *Bacillus anthracis* protective antigen combined with adjuvants. *Infection and immunity*, 60:662-668

KACHURA, M.A., HICKLE, C., KELL, S.A., SATHE, A., CALACSAN, C., KIWAN, R., HALL, B., MILLEY, R., OTT, G., COFFMAN, R.L., KANZLER, H. & CAMPBELL, J.D. 2016. A CpG-Ficoll Nanoparticle Adjuvant for Anthrax Protective Antigen Enhances Immunogenicity and Provides Single-Immunization Protection against Inhaled Anthrax in Monkeys. *Journal of immunology (Baltimore, Md.: 1950)*, 196:284-297

KELLY, D.J., CHULAY, J.D., MIKESELL, P. & FRIEDLANDER, A.M. 1992. Serum concentrations of penicillin, doxycycline, and ciprofloxacin during prolonged therapy in rhesus monkeys. *The Journal of infectious diseases*, 166:1184-1187

KLEIN, F., DEARMON JR., I.A., LINCOLN, R.E., MAHLANDT, B.G. & FERNELIUS, A.L. 1962. Immunological studies of anthrax. II. Levels of immunity against *Bacillus anthracis* obtained with protective antigen and live vaccine. *Journal of immunology (Baltimore, Md.: 1950)*, 88:15-19

KLINMAN, D.M. & TROSS, D. 2009. A single-dose combination therapy that both prevents and treats anthrax infection. *Vaccine*, 27:1811-1815

KÖHLER, S.M., BAILLIE, L.W. & BEYER, W. 2015. BclA and toxin antigens augment each other to protect NMRI mice from lethal *Bacillus anthracis* challenge. *Vaccine*, 33:2771-2777

KRZYWINSKI, M. & ALTMAN, N. 2014. Points of significance: Visualizing samples with box plots. *Nature methods*, 11:119-120

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. *Infection and immunity*, 52:509-512

REUVENY, S., WHITE, M.D., ADAR, Y.Y., KAFRI, Y., ALTBOUM, Z., GOZES, Y., KOBILER, D., SHAFFERMAN, A. & VELAN, B. 2001. Search for correlates of protective immunity conferred by anthrax vaccine. *Infection and immunity*, 69:2888-2893

SIVKO, G., STARK, G., TORDOFF, K., TAYLOR, K., GLAZE, E., VANRADEN, M., SCHIFFER, J., HEWITT, J., QUINN, C. & NUZUM, E. 2016. Evaluation of early immune response-survival relationship in cynomolgus macaques after Anthrax Vaccine Adsorbed vaccination and *Bacillus anthracis* spore challenge. *Vaccine*

STEPANOV, A., MARININ, L., POMERANTSEV, A. & STARITSIN, N. 1996. Development of novel vaccines against anthrax in man. *Journal of Biotechnology*, 44:155-160

STERNE, M. 1939. The use of Anthrax Vaccines Prepared from Avirulent (Uncapsulated) Variants of *Bacillus anthracis*. *Onderstepoort Journal of Veterinary Research*, 13:307

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

TURNBULL, P.C., TINDALL, B.W., COETZEE, J.D., CONRADIE, C.M., BULL, R.L., LINDEQUE, P.M. & HUEBSCHLE, O.J. 2004. Vaccine-induced protection against anthrax in cheetah (*Acinonyx jubatus*) and black rhinoceros (*Diceros bicornis*). *Vaccine*, 22:3340-3347

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

VERGIS, J.M., COTE, C.K., BOZUE, J., ALEM, F., VENTURA, C.L., WELKOS, S.L. & O'BRIEN, A.D. 2013. Immunization of mice with formalin-inactivated spores from avirulent *Bacillus cereus* strains provides significant protection from challenge with *Bacillus anthracis* Ames. *Clinical and vaccine immunology : CVI*, 20:56-65

VIETRI, N.J., PURCELL, B.K., TOBERY, S.A., RASMUSSEN, S.L., LEFFEL, E.K., TWENHAFEL, N.A., IVINS, B.E., KELLOGG, M.D., WEBSTER, W.M., WRIGHT, M.E. & FRIEDLANDER, A.M. 2009. A short course of antibiotic treatment is effective in

preventing death from experimental inhalational anthrax after discontinuing antibiotics. *The Journal of infectious diseases*, 199:336-341

WEBSTER, A. 1973. Letter: Inhibiting effect of antibiotics on anthrax vaccination. *Australian Veterinary Journal*, 49:545

WEISS, S., KOBILER, D., LEVY, H., MARCUS, H., PASS, A., ROTHSCHILD, N. & ALTBOUM, Z. 2006. Immunological Correlates for Protection against Intranasal Challenge of *Bacillus anthracis* Spores Conferred by a Protective Antigen-Based Vaccine in Rabbits. *Infection and immunity*, 74:394-398

WELKOS, S., LITTLE, S., FRIEDLANDER, A., FRITZ, D. & FELLOWS, P. 2001. 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

WELKOS, S.L. & FRIEDLANDER, A.M. 1988. Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. *Microbial pathogenesis*, 5:127-139

CHAPTER SEVEN

Immunogenicity of a DNA prime/recombinant protein boost and DNA prime/formaldehyde inactivated spores boost formulations in goats and protective efficacy of immune goat sera in an A/J mouse model

7.1. Introduction

The induction of humoral and cell mediated immune responses by applying the genetic information of antigenic determinants in eukaryotic cassettes has been shown previously in anthrax vaccinology (Gu, Leppla & Klinman 1999, Price *et al.* 2001, Hermanson *et al.* 2004, Luxembourg *et al.* 2008, Livingston *et al.* 2010, Albrecht *et al.* 2012, Kim *et al.* 2015). During plasmid DNA vaccination, genes encoding the antigens of interest are delivered into host cells, enabling *in vivo* antigen production (Tang, DeVit & Johnston 1992, Kim *et al.* 2015). The gene sequence, which is generated synthetically or by PCR, is enzymatically inserted into the multiple cloning region of a plasmid backbone (Kutzler & Weiner 2008). Following purification, the DNA plasmid is inoculated into the skin, subcutaneum or muscle (Fynan *et al.* 1993) and taken up by the transfected muscle and resident antigen presenting cells [(APCs) (Wolff *et al.* 1990, Raz *et al.* 1994)]. In the nucleus of the affected myocytes and APCs, the plasmid DNA initiates gene transcription, followed by protein production in the cytoplasm and formation of foreign antigens as proteins or peptide strings (Raz *et al.* 1994, Tighe *et al.* 1998). The host cell carries out post-translational modifications to the antigens, reproducing native peptide conformations in a manner similar to pathways induced by live infections (Kutzler & Weiner 2008). The generation and processing of the foreign

proteins are driven by the host cell machinery and these proteins become associated with the MHC I or MHC II molecules of the APCs (Flingai *et al.* 2013). This affords these cells the ability to prime naïve T cells in the draining lymph nodes in combination with signalling by co-stimulatory molecules (Ulmer *et al.* 1993, Corr *et al.* 1996, Doe *et al.* 1996) eventually leading to immune response and expansion of T cells. Recognition of peptide-MHC I complexes on APCs by $CD8^+$ T cells stimulates the differentiation of the cells into cytotoxic T cells, while recognition of peptide-MHC II by $CD4^+$ T cells stimulate their activation and differentiation into T helper cells and mediate interactions between antigen-specific B cells and T helper cells (Roche & Furuta 2015).

DNA vaccines are amongst the next generation anthrax vaccines in development (Ferrari, Hermanson & Rolland 2004). These vaccines are safe to produce and can be designed to target specific cellular compartments in order to modulate immune responses in target animals (Midha & Bhatnagar 2009). The subcellular localization of antigens can determine the magnitude and quality of humoral immune response and also target the response to $CD8^+$ or $CD4^+$ cells (da Costa Godinho, Rodrigo Maciel *et al.* 2014). Hence, targeting of antigens to different cellular compartments enhance its presentation by MHC I or MHC II molecules and improve specific immune response (da Costa Godinho, Rodrigo Maciel *et al.* 2014, Nuchtern, Biddison & Klausner 1990, Kovacsovics-Bankowski & Rock 1995, Kaur *et al.* 2009). Antigen routed to the endoplasmic reticulum, which carries out initial folding and glycosylation, ensures an improved antibody response (Walter & Johnson 1994, Rice *et al.* 1999) whereas antigen targeted to the cytosol gain direct access to the proteasomal degradation machinery for induction of peptide-specific $CD8^+$ T cell responses (Price *et al.* 2009). Effective routing of antigens is accomplished by the incorporation of specific signal sequences that guide the antigen secretion to particular subcellular compartments (Boyle,

Koniaras & Lew 1997, Li *et al.* 1999, Fernandes, Vidard & Rock 2000, Rock *et al.* 2002, Midha & Bhatnagar 2009).

A potential key strength of the deployment of DNA vaccines is the employment of a heterologous prime-boost strategy. This involves the priming of the host animal's immune system to a target antigen using a vector and boosting the ensuing response by re-administering the antigen using a different vector (Woodland 2004). These studies had entailed priming immunizations with plasmid DNA and protein boosting with recombinant PA to induce higher anti-PA IgG and toxin neutralising levels in mice and rabbits (Williamson *et al.* 1999, Galloway & Baillie 2004, Cybulski, Sanz & O'Brien 2009). The underlying principle behind the heterologous prime-boost approach is the elicitation of both humoral and cell-mediated immune responses (Lu 2009). DNA vaccines induce cell-mediated immune responses which is also very effective in priming antigen-specific memory cells (Gu, Leppla & Klinman 1999, Kaech, Wherry & Ahmed 2002). On the other hand, protein vaccines directly stimulate the antigen-specific memory B cells to differentiate into antibody-secreting cells (Alekseeva *et al.* 2009, Williamson *et al.* 1999). This successive immunization approach with different vectors is capable of inducing high levels of $CD8^+$ and $CD4^+$ T cells compared to sequential vaccination with homologous vectors (Dunachie & Hill 2003, Kardani, Bolhassani & Shahbazi 2016).

The combination of plasmids encoding toxins (LF and PA) and spore proteins significantly protected mice against challenge with fully virulent Ames strain spores (Hahn, Boehm & Beyer 2006, Köhler, Baillie & Beyer 2015). However, to the best of our knowledge, the efficacy of such vaccines has never been evaluated in a ruminant model. Consequently, we evaluated the immunogenicity of a DNA vaccine using a DNA prime with a heterologous protein/spore boost strategy. Also, we investigated the protection of A/J mice against Sterne 34F2 spores challenge by sera from susceptible goats vaccinated with the DNA/protein/spore

vaccine combinations. These mice are NOD deficient, lack the *Hc* gene encoding for complement component 5 (C5) and are susceptible to the nonencapsulated but toxigenic Sterne strain of *B. anthracis* (Welkos & Friedlander 1988, Lamkanfi *et al.* 2007).

7.2. Materials and Methods

7.2.1. Vaccines

The DNA plasmids vaccines consisting of vector backbones (pDNAVaccUltra and NTC7382) with signal sequences (Köhler *et al.* 2015) were utilised for cloning of respective gene sequences. Purification of the constructs was performed by Nature Technology Corporation (Lincoln, NE, USA). The DNA plasmids were constructed with genes encoding the integrated antigens; PA (domain 4), LF (domain 1) and spore surface glycoprotein (BclA domains 1 and 3) (Baillie *et al.* 2010). PA comprises of four functionally independent domains with domain 4 known to display the most dominant protective epitopes with the host cell receptor binding site (Singh *et al.* 1991, Little *et al.* 1996, Flick-Smith *et al.* 2002). Domain 1 of LF, on the other hand, contains the binding site for PA prior to membrane translocation (Pannifer *et al.* 2001, Chichester *et al.* 2007). A combination of both domains have been shown to enhance the degree of protection against virulent *B. anthracis* spores challenge compared to individual antigens (Baillie *et al.* 2010, Köhler, Baillie & Beyer 2015). BclA, the bacillus collagen-like protein of anthracis, is an abundant immunodominant protein which forms hair-like projections on the spore surface (Sylvestre, Couture-Tosi & Mock 2002, Sylvestre, Couture-Tosi & Mock 2003, Boydston *et al.* 2005). The deletion of the collagen-like region (domain 2) has no detrimental effect on immunogenicity (ChunQiang *et al.* 2008) and results in the smaller peptide (domains 1 and 3, BclAD1D3).

Separate vectors encoding for a positive MHCII-regulator (CIITA) and interferon β promoter stimulator (mIPS1) served as adjuvants for the DNA vaccines (Köhler *et al.* 2015) and were acquired from Genecopoeia (Rockville, MD, USA). CIITA is a non-DNA binding coactivator that serves as the master control factor for MHCII upregulation and expression (Ting & Trowsdale 2002, LeibundGut-Landmann et al. 2004). IPS1 is an adaptor protein that stimulates endogenous promoters of genes encoding type I interferons in a TLR-independent manner (Kawai et al. 2005). The tissue plasminogen activator which facilitates the secretion of encoded antigen was utilized to target the MHC II pathway (Li *et al.* 1999, Nuchtern, Biddison & Klausner 1990) while lysosome-associated membrane protein (LAMP1) serve to direct proteins to endosomes (Drake *et al.* 1999). Previous studies have shown the ability of these domains (LFD1PAD4-mIPS1 or TPA-BclAD1D3-LAMP1) to provide protection against anthrax in mice, either alone or in combination (Baillie *et al.* 2010, Köhler, Baillie & Beyer 2015).

The protein vaccines consisted of PA83, BclA, formalin-inactivated spores (FIS) and a lipopeptide adjuvant (Pam_3Cys) in various combinations (Brossier, Levy & Mock 2002, Hahn *et al.* 2004, Ghielmetti *et al.* 2005).The DNA vaccines were administered using the intramuscular route while all protein/FIS boosts were given via the subcutaneous route.

7.2.2. Animals and vaccine groups

The goats in this study were sourced from same herd and were screened for anthrax PA-reactive antibodies before purchase. Animals from the entire herd showing the least ODs in the PA-ELISA were selected. Following arrival and acclimatization at the Onderstepoort Biological Products (OBP) experimental animal facility, the goats were randomly allocated to designated vaccine groups (Table 7.1). A/J mice (Jackson Laboratories, ME, USA) were procured for the *in vivo* challenge study performed at the University of Pretoria biomedical research centre (UPBRC), South Africa. Experiments with animals were conducted in

compliance with ethical principles and guidelines approved by the animal use and care committees of the OBP and University of Pretoria (protocol number V065/12) respectively. Approval for Section 20, Act 35 of 1984 (animal disease act) was granted by the Directorate of Animal Health, South Africa (registration number 12/11/1/1/6).

Table 7. 1. Vaccine group designations, dosage and schedule

Group	Number of goats	pDNA/antigens injected	Dosage	Vaccination, weeks	Number of Mice (*in vivo* challenge)
I	5	Combination of LFD1PAD4-mIPS1, TPA-BclAD1D3-LAMP1 + CIITA (once, IM) & twice (SC) with rPA +rBclA + Lipopeptide adjuvant (LpA) (DNA vaccine × 1, protein booster × 2)	dLFD1PAD4-mIPS1 = 1000 µg/dose dTPA-BclAD1D3-Lamp1 = 1000 µg/dose CIITA = 1000 µg/dose rPA = 75 µg/dose rBclA = 75 µg/dose LPA = 500 µg/dose	0, 3, 6*	21 (5 mice per goat)†
II	5	Combination of LFD1PAD4-mIPS1, TPA-BclAD1D3-LAMP1 + CIITA (once, IM) & once (SC) with FIS (DNA vaccine × 1, FIS booster × 1)	dLFD1PAD4-mIPS1 = 1000 µg/dose dTPA-BclAD1D3-Lamp1 = 1000 µg/dose CIITA = 1000 µg/dose FIS = 10^8 spores/dose	0, 3*	22 (5 mice per goat) ‡
III	3	Saline (SC)	1 mL of saline (Unvaccinated)	0*	9 (3 mice per goat)
IV	4	Sterne live spore vaccine (twice, SC) and challenged with virulent *B. anthracis* spores [(SC), chapter three study]	Sera for *in vivo* protection tests collected from survivors (two weeks after virulent challenge on week 62)	0, 58	12 (3 mice per goat)

†Four mice reacted adversely from *in vivo* goat serum transfer and were euthanized.
‡Three mice reacted adversely from *in vivo* goat serum transfer and were euthanized.
* Sera were collected for mouse *in vivo* protection tests on week 7 (II) and week 10 (I and III), i.e. four weeks after the last vaccination (I and II).
IM; Intramuscular/ SC; Subcutaneous

7.2.3. Serology – ELISA and toxin neutralization assay

Sera were analysed for antibodies against PA, BclA and FIS using ELISA as described in the methods section. Toxin neutralizing antibodies were measured using the *in vitro* neutralization assay (TNA) as indicated.

7.2.4. Lymphocyte proliferation determination (BrdU assay)

Proliferative responses of PBMCs isolated from the jugular vein were determined as described previously with the lymphocyte proliferation assay.

7.2.5. *In vivo* mice challenge

Sera (500 µl) from goats vaccinated with DNA vaccine constructs and control groups were injected in naïve A/J mice using the intra-peritoneal route. Immune sera from goats which had survived challenge with virulent *B. anthracis* spores following a double vaccination with the Sterne live spore vaccine (Chapter 3) served as the positive controls while the negative control sera were sourced from the unvaccinated controls. This was followed 24 h later by subcutaneous injection of ~1.92 x 10^5 Sterne 34F2 spores (175 LD_{50}) based on previous *B. anthracis* susceptibility study (Welkos, Keener & Gibbs 1986). The rodents were monitored for 14 days following inoculation and mice succumbing to infection were dissected aseptically and liver and spleen smear cultures made on sheep blood agar to confirm presence of *B. anthracis* Sterne 34F2 bacilli.

7.2.6. Statistics

Box and whisker plots were used to depict antibody response data showing individual vaccine groups quartile ranges with error bars indicating 95 % confidence intervals (Krzywinski & Altman 2014). 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). Pre- and post-vaccination ELISA, TNA and lymphocyte proliferation data within vaccine groups were compared using a paired Student's *t* test (IBM SPSS Statistics version 23 for Windows, Sandton, South Africa).

Kaplan-Meier (product limit estimation) plots were used to compute the mean survival times of challenged mice. Survival curves were compared between treatment groups using the Mantel-Cox (Log Rank) test. *P*-values of ≤ 0.05 were considered statistically significant.

7.3. Results

7.3.1. Antibody responses following priming with a DNA vaccine and boosting with recombinant proteins or FIS

Immune responses following intramuscular vaccination with a pDNA vaccine encoding domains 1 and 4 of LF and PA respectively and boosting twice with rPA83 and rBclA were assessed using an indirect ELISA and TNA. The pDNA vaccine was administered on week 0 and protein vaccines on weeks 3 and 6. Sera for analysis were collected before each vaccination. The animals had high anti-PA background titres before pDNA vaccination on week 0 (Fig 7.1A). However, none of the animals showed any increase in anti-PA titres following pDNA and initial protein vaccinations on weeks 0 and 3 respectively ($P \geq 0.544$). Three out of five animals seroconverted four weeks after the third (second protein) vaccination (Fig 7.1A). Similarly, the toxin neutralising antibodies remained absent three weeks after the second vaccination. Three animals developed toxin neutralising titres four weeks after the second vaccination with the recombinant proteins (Fig 7.1B). Only two out of five animals seroconverted to BclA three weeks after the first vaccination with the recombinant proteins and none showed any seroconversion 4 weeks after the second vaccination (Figs 7.1C and 7.1D).

Conversely, animals primed with the pDNA and boosted with FIS showed strong anti-spore IgG titres ($P = 0.011$) after a single boost-vaccination with FIS (Fig 7.2B). For ELISA, no

seroconversion could be demonstrated against rPA and rBclA (Figs 7.2A and 7.2C) following either vaccination. Also, toxin neutralising titres were absent at all the measured time points (not shown).

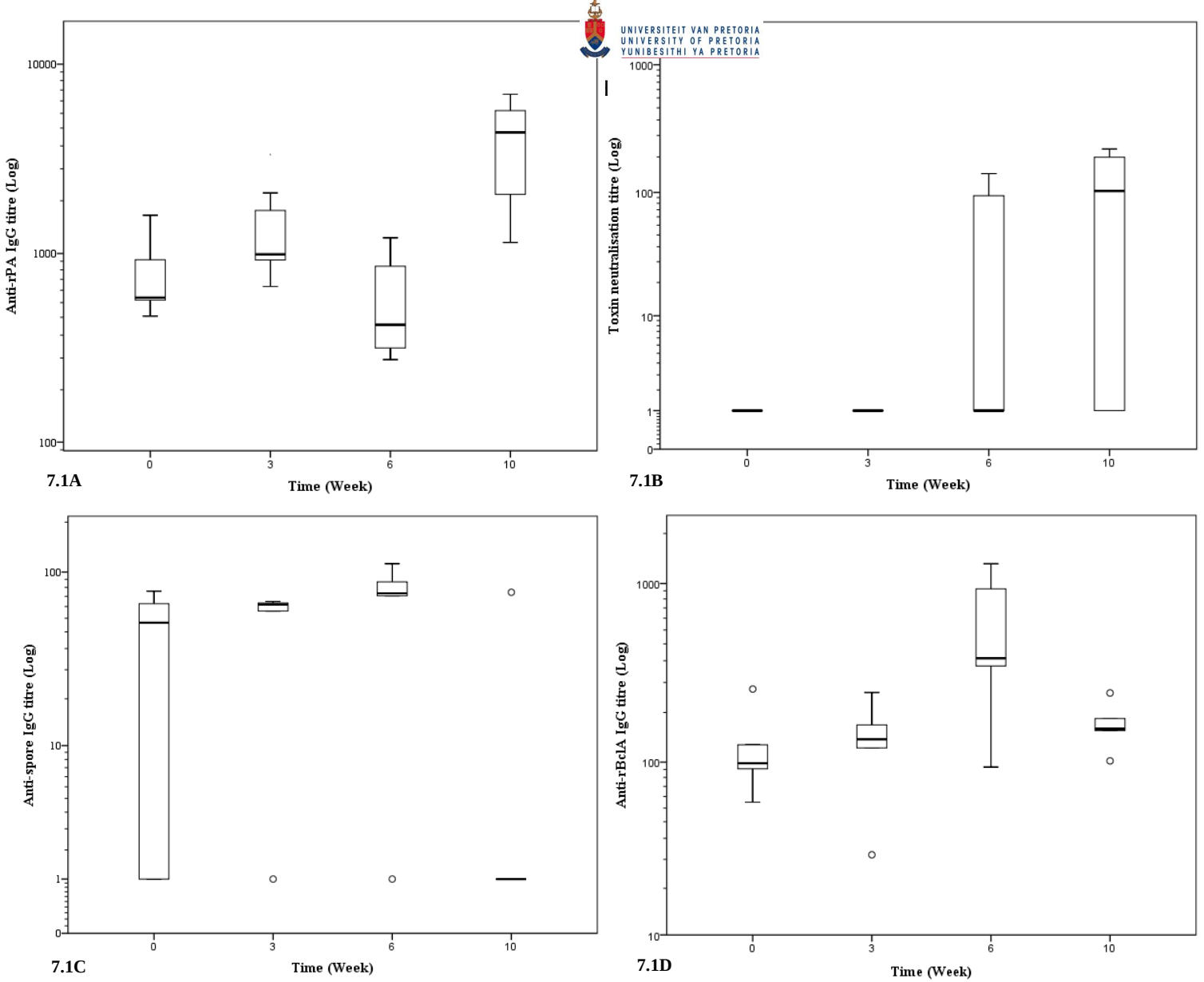


Fig 7. 1.: Immune response in goats (n = 5) vaccinated with a combination of LFD1PAD4-mIPS1+TPA-BclAD1D3-LAMP1+CIITA (once at week 0) and rPA+rBclA+Lipopeptide adjuvant (at weeks 3 and 6). Sera were collected before every vaccination and four weeks after last vaccination (week 10) for analyses.
O Outliers
LFD1PAD4-mIPS1 – DNA vector encoding domain 1 of LF and domain 4 of PA
TPA-BclAD1D3-LAMP1 –DNA vector encoding domains 1 and 3 of BclA

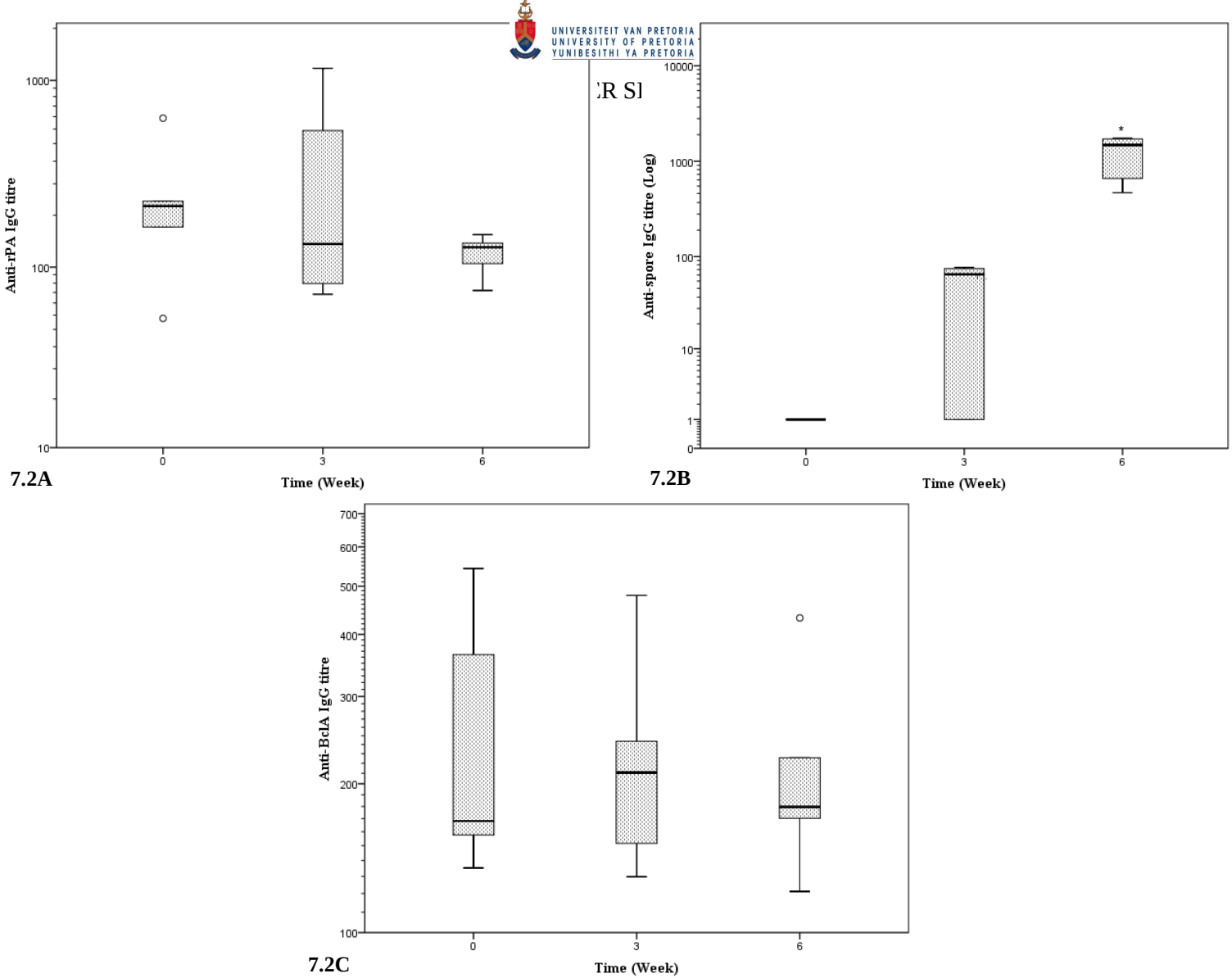


Fig 7. 2.A-C.: Immune response in goats (n = 5) vaccinated with a combination of LFD1PAD4-mIPS1+TPA-BclAD1D3-LAMP1+CIITA (once at week 0) and formaldehyde inactivated spores (FIS) (once at week 3). No toxin neutralizing titres developed following each vaccination, hence not shown. Titres significantly different ($P < 0.05$) from pre-vaccination titres are indicated with an *. Sera were collected before every vaccination and three weeks after last vaccination (week 6) for analyses.

^{O} Outliers
LFD1PAD4-mIPS1 – DNA vector encoding domain 1 of LF and domain 4 of PA
TPA-BclAD1D3-LAMP1 –DNA vector encoding domains 1 and 3 of BclA

7.3.2. Assessment of proliferative responses of lymphocytes following vaccination

The ability of lymphocytes isolated from jugular blood to proliferate following stimulation with rPA antigen was assessed three weeks following each vaccination. The animals did not show any difference in proliferative response following vaccination with the pDNA vaccine (Figs 7.3A and 7.3B). The responses only increased slightly following booster vaccinations with recombinant proteins (double vaccination) and FIS ($P \geq 0.296$). Only two out of five animals showed increase in lymphocyte proliferation following a second vaccination with rPA and rBclA while three out of five goats showed increased proliferation following a single booster vaccination with FIS.

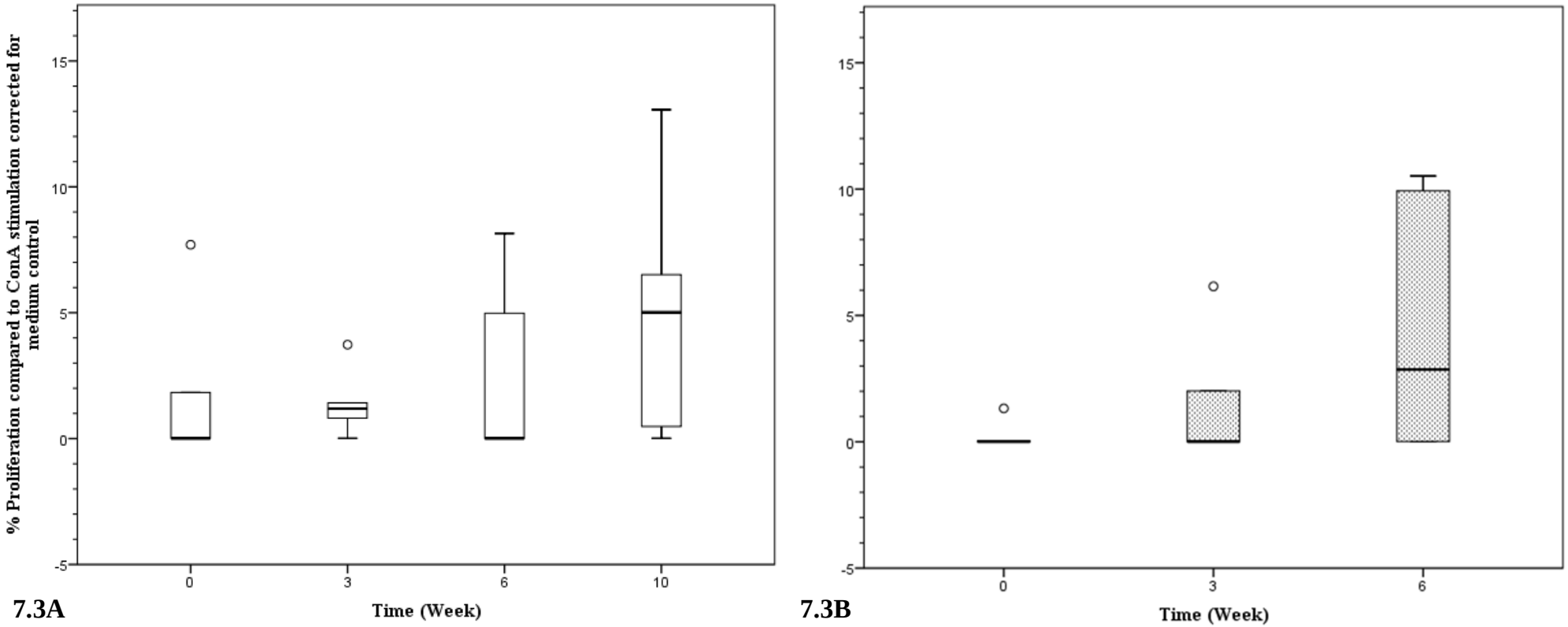


Fig 7. 3.A-B.: Lymphocyte proliferation from PBMCs isolated from jugular blood in goats. **Fig 7.3A** Goats (n = 5) were vaccinated with LFD1PAD4-mIPS1+TPA-BclAD1D3-LAMP1+CIITA (once at week 0) and rPA+rBclA+Lipopeptide adjuvant (at weeks 3 and 6). **Fig 7.3B** Goats (n = 5) were vaccinated with LFD1PAD4-mIPS1+TPA-BclAD1D3-LAMP1+CIITA (once at week 0) and formaldehyde inactivated spores (FIS) (once at week 3).
O Outliers
LFD1PAD4-mIPS1 – DNA vector encoding domain 1 of LF and domain 4 of PA
TPA-BclAD1D3-LAMP1 –DNA vector encoding domains 1 and 3 of BclA
PBMCs – Peripheral blood mononuclear cells

7.3.3. Protective efficacy of sera from goats primed with pDNA vaccine and boosted with recombinant proteins or FIS

In order to ascertain the protective capacity of the humoral response observed in the vaccinated goats, *in vivo* transfer of the goats immune sera to A/J mice and lethal challenge of the mice with Sterne 34F2 spores were performed. All the positive control mice survived while the negative control mice (received unvaccinated goat sera) succumbed to infection within 3 days of challenge (Fig 7.4). None of the mice receiving sera from goats vaccinated with pDNA prime/FIS boost survived virulent challenge. Boosting twice with the rPA83 and rBclA protected 43 % (9/21) mice, which was significantly different from the negative control ($P < 0.001$).

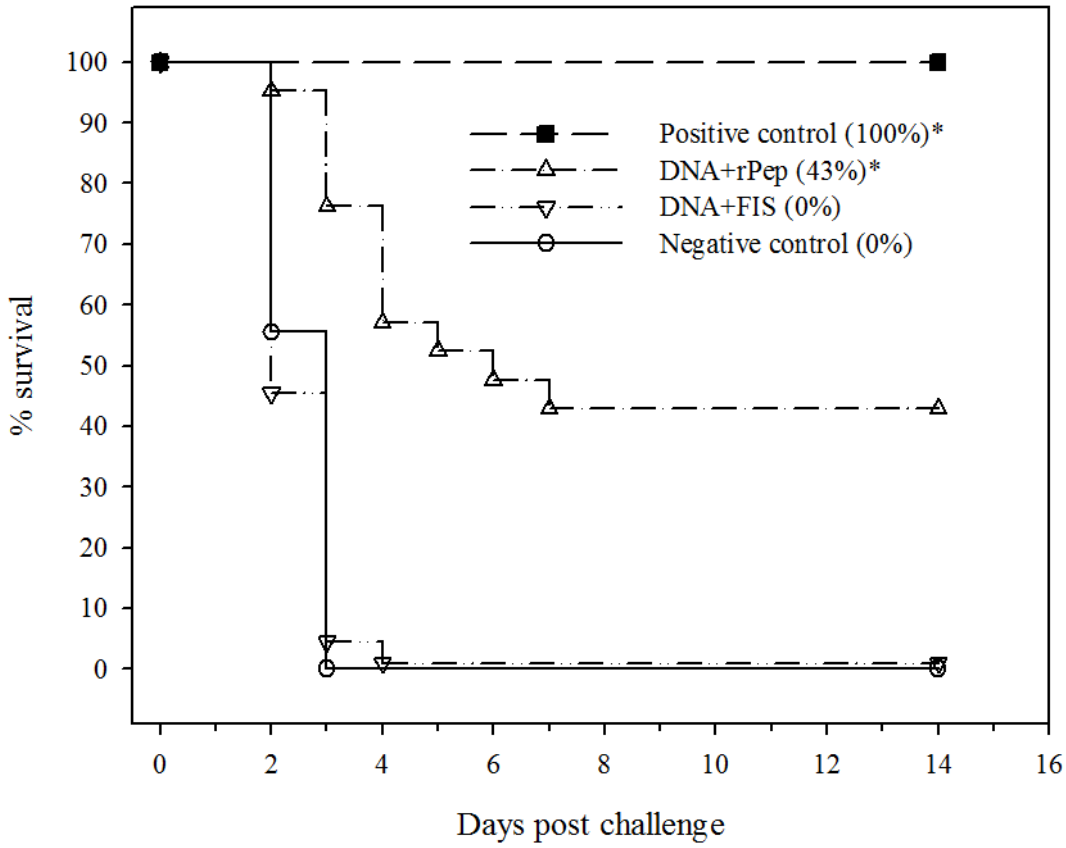


Fig 7. 4.: Passive protection of A/J mice following in vivo transfer of immune sera from goats and lethal challenge with 175 LD_{50} of Sterne strain spores. DNA and recombinant proteins (n = 21) and DNA and FIS (n = 22) groups received sera from goats vaccinated with the respective vaccine candidates as indicated in Table 6.1. Positive control (n = 12) sera were collected from goats which were vaccinated twice with Sterne strain spores and subsequently survived challenge with a fully virulent wild-type strain of *Bacillus anthracis*. Negative control (n = 9) sera were from goats injected with vaccine diluent (saline).

* denotes significantly increased survival as compared to the negative control ($P < 0.001$).

7.4. Discussion

In this study we evaluated the efficacy of the humoral immune response induced by a heterologous vaccine prime-boost approach. Goats were primed using a DNA vaccine construct that encodes protective domains of PA, LF and BclA (Baillie *et al.* 2010, Köhler *et al.* 2015) and boosted with either recombinant proteins (PA and BclA) or FIS. Protection against anthrax following DNA immunization has been shown in previous studies with laboratory animals, but this has not been replicated in goats. A previous study had evaluated the immune responses in sheep vaccinated with plasmid DNA encoding PA_{83} or PAD4, but did not investigate the protective efficacy of the elicited immunity (Hahn *et al.* 2006). The DNA vaccine utilised in the present work protected 90 % of NMRI mice following a lethal *B.anthracis* challenge in a previous study (Köhler *et al.* 2015). Though DNA vaccines have shown potential in laboratory rodents it has been less than promising in larger animal models (Babiuk *et al.* 2003, Tollefsen *et al.* 2003, Perkins *et al.* 2005). Thus we have adopted a DNA prime-protein boost strategy in an attempt to enhance the immune response induced by DNA vaccination.

Analysis of individual animal titres showed no visible increase following a single DNA vaccination. In the previous mice study a good antibody response was detected following DNA vaccination with a similar vaccine (Köhler *et al.* 2015). However, the vaccine was administered with a gene gun application in the mouse model. While DNA vaccination has been recognized to prime for a strong anamnestic response (Scheerlinck *et al.* 2001, De Rose *et al.* 2002, Kennedy *et al.* 2006), there is the possibility of only few muscle cells being transfected following our DNA immunization. Insufficient transfection of target cells invariably leads to sub-optimal antigen production (Tollefsen *et al.* 2003), thus failing to prime for discernible immune response in the vaccinated animals. This is more likely to be

the case in our study as we used a direct needle injection method. This can be circumvented through intramuscular electroporation, a process that has been shown to increase gene transfer and expression (Babiuk *et al.* 2004, Bruffaerts *et al.* 2015). Electroporation involves the application of short electric pulses to the vaccination site following injection of plasmid DNA (Flingai *et al.* 2013). This leads to inflammatory response and accompanying cellular infiltrations enhancing the immune response in the process (Babiuk *et al.* 2004, Flingai *et al.* 2013). Evident seroconversion was observed after the second vaccination with the recombinant proteins as seen in Figs 7.1A-B, though not significantly higher than pre-vaccination background titres (P = 0.052). The antibody titres detected were probably only from the second protein vaccination. The background anti-PA titres observed these goats were inexplicable; however, it is pertinent to note the animals in this study were from the same herd and locality. There is a possibility the animals could have been exposed to a similar *Bacillus spp* in the environment. These animals are highly susceptible to virulent *B. anthracis* spores and sub-lethal exposure to the organism could be possible but improbable. This warrants further study, especially to advance understanding on the ecology of anthrax in the wild. None of the animals showed any toxin neutralizing after the first (DNA) or second (protein) vaccination (Fig 7.1B). In previous studies, we had observed the poor ability of goats in producing anti-BclA IgG following immunization with either the recombinant antigen (rBclA) or FIS (Chapters 5 and 6). Likewise, the seroconversion to rBclA after protein vaccination was poor in the goat vaccinates in this study. The absence of anti-BclA titres were also observed in goats surviving a virulent *B. anthracis* challenge (Chapter 4) indicating that these antibodies are not produced and therefore not necessary in order to mount a protective immune response in these animals. Goats vaccinated with the DNA vaccine followed by a FIS boost only seroconverted to FIS. The absence of titres against PA and BclA indicates the single DNA vaccination had no effect on immune response in the

animals. An advantage of DNA vaccination is the ability to induce cell mediated immune response (Babiuk *et al.* 2002), however we only observed increased lymphocyte proliferation in two or three animals following recombinant protein or FIS vaccination respectively (Fig 7.3A-B). This could be due to poor immune priming as a result of the route of administration.

In order to assess the protective capacity of immune sera, the passive transfer of sera from caprine vaccinates to naïve A/J mice was performed before exposure to Sterne spores. The sera of DNA vaccination/recombinant protein twice-boosted group protected 43 % (9/21) of the challenged mice while none of the DNA vaccination/FIS single-boosted group sera inoculated mice survived (Fig 7.4). The partial protection seen in DNA vaccine followed by recombinant protein boost group could possibly be attributed to the booster components. We had shown previously that sera of goats vaccinated thrice with similar antigens protected 68% of challenged mice (Chapter 5). Also, our results reflect that the anti-FIS antibodies elicited following DNA priming and FIS boosting is not protective against anthrax using the A/J mouse Sterne strain challenge model. The significantly increased anti-spore titres observed in DNA vaccine and FIS boost group animals failed to protect or increase the survival time of the mice following lethal challenge. This was likely due to anthrax toxin being the major virulence factor in the A/J mouse Sterne strain challenge model.

7.5. References

ALBRECHT, M.T., EYLES, J.E., BAILLIE, L.W. & KEANE-MYERS, A.M. 2012. Immunogenicity and efficacy of an anthrax/plague DNA fusion vaccine in a mouse model. *FEMS immunology and medical microbiology*

ALEKSEEVA, E., SOMINSKAYA, I., SKRASTINA, D., EGOROVA, I., STARODUBOVA, E., KUSHNERS, E., MIHAILOVA, M., PETRAKOVA, N., BRUVERE, R., KOZLOVSKAYA, T., ISAGULIANTS, M. & PUMPENS, P. 2009. Enhancement of the expression of HCV core gene does not enhance core-specific immune response in DNA immunization: advantages of the heterologous DNA prime, protein boost immunization regimen. *Genetic Vaccines and Therapy*, 7:7

BABIUK, L., PONTAROLLO, R., BABIUK, S. & LOEHR, B. 2003. Induction of immune responses by DNA vaccines in large animals. *Vaccine*, 21:649-658

BABIUK, S., BACA-ESTRADA, M.E., FOLDVARI, M., MIDDLETON, D.M., RABUSSAY, D., WIDERA, G. & BABIUK, L.A. 2004. Increased gene expression and inflammatory cell infiltration caused by electroporation are both important for improving the efficacy of DNA vaccines. *Journal of Biotechnology*, 110:1-10

BABIUK, S., BACA-ESTRADA, M.E., FOLDVARI, M., STORMS, M., RABUSSAY, D., WIDERA, G. & BABIUK, L.A. 2002. Electroporation improves the efficacy of DNA vaccines in large animals. *Vaccine*, 20:3399-3408

BAILLIE, L.W., HUWAR, T.B., MOORE, S., MELLADO-SANCHEZ, G., RODRIGUEZ, L., NEESON, B.N., FLICK-SMITH, H.C., JENNER, D.C., ATKINS, H.S., INGRAM, R.J., ALTMANN, D.M., NATARO, J.P. & PASETTI, M.F. 2010. An anthrax subunit vaccine candidate based on protective regions of *Bacillus anthracis* Protective Antigen and Lethal Factor. *Vaccine*, 28:6740-6748

BOYDSTON, J.A., CHEN, P., STEICHEN, C.T. & TURNBOUGH, C.L.,JR. 2005. Orientation within the exosporium and structural stability of the collagen-like glycoprotein BclA of *Bacillus anthracis*. *Journal of Bacteriology*, 187:5310-5317

BOYLE, J.S., KONIARAS, C. & LEW, A.M. 1997. Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. *International immunology*, 9:1897-1906

BROSSIER, F., LEVY, M. & MOCK, M. 2002. Anthrax spores make an essential contribution to vaccine efficacy. *Infection and immunity*, 70:661-664

BRUFFAERTS, N., PEDERSEN, L.E., VANDERMEULEN, G., PRÉAT, V., STOCKHOFE-ZURWIEDEN, N., HUYGEN, K. & ROMANO, M. 2015. Increased B and T Cell Responses in *M. bovis* Bacille Calmette-Guérin Vaccinated Pigs Co-Immunized with Plasmid DNA Encoding a Prototype Tuberculosis Antigen. *PloS one*, 10:e0132288

CHICHESTER, J.A., MUSIYCHUK, K., DE LA ROSA, P., HORSEY, A., STEVENSON, N., UGULAVA, N., RABINDRAN, S., PALMER, G.A., METT, V. & YUSIBOV, V. 2007. Immunogenicity of a subunit vaccine against *Bacillus anthracis*. *Vaccine*, 25:3111-3114

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. *Biotechnology and bioengineering*, 99:774-782

CORR, M., LEE, D.J., CARSON, D.A. & TIGHE, H. 1996. Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *The Journal of experimental medicine*, 184:1555-1560

CYBULSKI, R.J.,JR, SANZ, P. & O'BRIEN, A.D. 2009. Anthrax vaccination strategies. *Molecular aspects of medicine*, 30:490-502

DA COSTA GODINHO, RODRIGO MACIEL, MATASSOLI, F.L., DE OLIVEIRA LUCAS, CAROLINA GONÇALVES, RIGATO, P.O., GONÇALVES, J.L.S., SATO, M.N., MACIEL JR, M., PEÇANHA, L.M.T., AUGUST, J.T. & DE AZEVEDO MARQUES JR, ERNESTO TORRES. 2014. Regulation of HIV-Gag expression and targeting to the endolysosomal/secretory pathway by the luminal domain of lysosomal-associated membrane protein (LAMP-1) enhance Gag-specific immune response. *PloS one*, 9:e99887

DE ROSE, R., TENNENT, J., MCWATERS, P., CHAPLIN, P.J., WOOD, P.R., KIMPTON, W., CAHILL, R. & SCHEERLINCK, J.Y. 2002. Efficacy of DNA vaccination by different routes of immunisation in sheep. *Veterinary immunology and immunopathology*, 90:55-63

DOE, B., SELBY, M., BARNETT, S., BAENZIGER, J. & WALKER, C.M. 1996. Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells. *Proceedings of the National Academy of Sciences of the United States of America*, 93:8578-8583

DRAKE, J.R., LEWIS, T.A., CONDON, K.B., MITCHELL, R.N. & WEBSTER, P. 1999. Involvement of MIIC-like late endosomes in B cell receptor-mediated antigen processing in murine B cells. *Journal of immunology (Baltimore, Md.: 1950)*, 162:1150-1155

DUNACHIE, S.J. & HILL, A.V. 2003. Prime-boost strategies for malaria vaccine development. *The Journal of experimental biology*, 206:3771-3779

FERNANDES, D.M., VIDARD, L. & ROCK, K.L. 2000. Characterization of MHC class II-presented peptides generated from an antigen targeted to different endocytic compartments. *European journal of immunology*, 30:2333-2343

FERRARI, M.E., HERMANSON, G. & ROLLAND, A. 2004. Development of anthrax DNA vaccines. *Current opinion in molecular therapeutics*, 6:506-512

FLICK-SMITH, H.C., WALKER, N.J., GIBSON, P., BULLIFENT, H., HAYWARD, S., MILLER, J., TITBALL, R.W. & WILLIAMSON, E.D. 2002. A Recombinant Carboxy-Terminal Domain of the Protective Antigen of *Bacillus anthracis* Protects Mice against Anthrax Infection. *Infection and immunity*, 70:1653-1656

FLINGAI, S., CZERWONKO, M., GOODMAN, J., KUDCHODKAR, S.B., MUTHUMANI, K. & WEINER, D.B. 2013. Synthetic DNA vaccines: improved vaccine potency by electroporation and co-delivered genetic adjuvants. *Frontiers in immunology*, 4:354

FYNAN, E.F., WEBSTER, R.G., FULLER, D.H., HAYNES, J.R., SANTORO, J.C. & ROBINSON, H.L. 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proceedings of the National Academy of Sciences of the United States of America*, 90:11478-11482

GALLOWAY, D.R. & BAILLIE, L. 2004. DNA vaccines against anthrax. *Expert Opin Biol Ther*, 4:1661-1667

GHIELMETTI, M., RESCHNER, A., ZWICKER, M. & PADOVAN, E. 2005. Synthetic bacterial lipopeptide analogs: structural requirements for adjuvanticity. *Immunobiology*, 210:211-215

GU, M.L., LEPPLA, S.H. & KLINMAN, D.M. 1999. Protection against anthrax toxin by vaccination with a DNA plasmid encoding anthrax protective antigen. *Vaccine*, 17:340-344

HAHN, U.K., AICHLER, M., BOEHM, R. & BEYER, W. 2006. Comparison of the immunological memory after DNA vaccination and protein vaccination against anthrax in sheep. *Vaccine*, 24:4595-4597

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. *International journal of medical microbiology : IJMM*, 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

HERMANSON, G., WHITLOW, V., PARKER, S., TONSKY, K., RUSALOV, D., FERRARI, M., LALOR, P., KOMAI, M., MERE, R., BELL, M., BRENNEMAN, K., MATECZUN, A., EVANS, T., KASLOW, D., GALLOWAY, D. & HOBART, P. 2004. A cationic lipid-formulated plasmid DNA vaccine confers sustained antibody-mediated protection against aerosolized anthrax spores. *Proceedings of the National Academy of Sciences of the United States of America*, 101:13601-13606

KAECH, S.M., WHERRY, E.J. & AHMED, R. 2002. Effector and memory T-cell differentiation: implications for vaccine development. *Nature Reviews Immunology*, 2:251-262

KARDANI, K., BOLHASSANI, A. & SHAHBAZI, S. 2016. Prime-boost vaccine strategy against viral infections: Mechanisms and benefits. *Vaccine*, 34:413-423

KAUR, M., CHUG, H., SINGH, H., CHANDRA, S., MISHRA, M., SHARMA, M. & BHATNAGAR, R. 2009. Identification and characterization of immunodominant B-cell epitope of the C-terminus of protective antigen of *Bacillus anthracis*. *Molecular immunology*, 46:2107-2115

KENNEDY, N.J., SPITHILL, T.W., TENNENT, J., WOOD, P.R. & PIEDRAFITA, D. 2006. DNA vaccines in sheep: CTLA-4 mediated targeting and CpG motifs enhance immunogenicity in a DNA prime/protein boost strategy. *Vaccine*, 24:970-979

KIM, N.Y., CHANG, D.S., KIM, Y., KIM, C.H., HUR, G.H., YANG, J.M. & SHIN, S. 2015. Enhanced Immune Response to DNA Vaccine Encoding *Bacillus anthracis* PA-D4 Protects Mice against Anthrax Spore Challenge. *PloS one*, 10:e0139671

KÖHLER, S.M., BAILLIE, L.W. & BEYER, W. 2015. BclA and toxin antigens augment each other to protect NMRI mice from lethal *Bacillus anthracis* challenge. *Vaccine*, 33:2771-2777

KOVACSOVICS-BANKOWSKI, M. & ROCK, K. 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science*, 267:243

KRZYWINSKI, M. & ALTMAN, N. 2014. Points of significance: Visualizing samples with box plots. *Nature methods*, 11:119-120

KUTZLER, M.A. & WEINER, D.B. 2008. DNA vaccines: ready for prime time?. *Nature Reviews Genetics*, 9:776-788

LAMKANFI, M., AMER, A., KANNEGANTI, T.D., MUNOZ-PLANILLO, R., CHEN, G., VANDENABEELE, P., FORTIER, A., GROS, P. & NUNEZ, G. 2007. The Nod-like receptor family member Naip5/Birc1e restricts *Legionella pneumophila* growth independently of caspase-1 activation. *Journal of immunology (Baltimore, Md.: 1950)*, 178:8022-8027

LI, Z., HOWARD, A., KELLEY, C., DELOGU, G., COLLINS, F. & MORRIS, S. 1999. Immunogenicity of DNA vaccines expressing tuberculosis proteins fused to tissue plasminogen activator signal sequences. *Infection and immunity*, 67:4780-4786

LITTLE, S.F., NOVAK, J.M., LOWE, J.R., LEPPLA, S.H., SINGH, Y., KLIMPEL, K.R., LIDGERDING, B.C. & FRIEDLANDER, A.M. 1996. Characterization of lethal factor binding and cell receptor binding domains of protective antigen of *Bacillus anthracis* using monoclonal antibodies. *Microbiology*, 142:707-715

LIVINGSTON, B.D., LITTLE, S.F., LUXEMBOURG, A., ELLEFSEN, B. & HANNAMAN, D. 2010. Comparative performance of a licensed anthrax vaccine versus electroporation based delivery of a PA encoding DNA vaccine in rhesus macaques. *Vaccine*, 28:1056-1061

LU, S. 2009. Heterologous prime–boost vaccination. *Current opinion in immunology*, 21:346-351

LUXEMBOURG, A., HANNAMAN, D., NOLAN, E., ELLEFSEN, B., NAKAMURA, G., CHAU, L., TELLEZ, O., LITTLE, S. & BERNARD, R. 2008. Potentiation of an anthrax DNA vaccine with electroporation. *Vaccine*, 26:5216-5222

MIDHA, S. & BHATNAGAR, R. 2009. Anthrax protective antigen administered by DNA vaccination to distinct subcellular locations potentiates humoral and cellular immune responses. *European journal of immunology*, 39:159-177

NUCHTERN, J.G., BIDDISON, W.E. & KLAUSNER, R.D. 1990. Class II MHC molecules can use the endogenous pathway of antigen presentation

PANNIFER, A.D., WONG, T.Y., SCHWARZENBACHER, R., RENATUS, M., PETOSA, C., BIENKOWSKA, J., LACY, D.B., COLLIER, R.J., PARK, S. & LEPPLA, S.H. 2001. Crystal structure of the anthrax lethal factor. *Nature*, 414:229-233

PERKINS, S.D., FLICK-SMITH, H.C., GARMORY, H.S., ESSEX-LOPRESTI, A.E., STEVENSON, F.K. & PHILLPOTTS, R.J. 2005. Evaluation of the VP22 protein for enhancement of a DNA vaccine against anthrax. *Genetic Vaccines and Therapy*, 3:3

PRICE, B.M., LINER, A.L., PARK, S., LEPPLA, S.H., MATECZUN, A. & GALLOWAY, D.R. 2001. Protection against Anthrax Lethal Toxin Challenge by Genetic Immunization with a Plasmid Encoding the Lethal Factor Protein. *Infection and immunity*, 69:4509-4515

PRICE, P.N., SOHN, M.D., LACOMMARE, K.S. & MCWILLIAMS, J.A. 2009. Framework for evaluating anthrax risk in buildings. *Environmental science & technology*, 43:1783-1787

RAZ, E., CARSON, D.A., PARKER, S.E., PARR, T.B., ABAI, A.M., AICHINGER, G., GROMKOWSKI, S.H., SINGH, M., LEW, D. & YANKAUCKAS, M.A. 1994. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proceedings of the National Academy of Sciences of the United States of America*, 91:9519-9523

RICE, J., KING, C.A., SPELLERBERG, M.B., FAIRWEATHER, N. & STEVENSON, F.K. 1999. Manipulation of pathogen-derived genes to influence antigen presentation via DNA vaccines. *Vaccine*, 17:3030-3038

ROCHE, P.A. & FURUTA, K. 2015. The ins and outs of MHC class II-mediated antigen processing and presentation. *Nature Reviews Immunology*, 15:203-216

ROCK, K.L., YORK, I.A., SARIC, T. & GOLDBERG, A.L. 2002. Protein degradation and the generation of MHC class I-presented peptides. *Advances in Immunology*, 80:1-70

SCHEERLINCK, J.Y., CASEY, G., MCWATERS, P., KELLY, J., WOOLLARD, D., LIGHTOWLERS, M.W., TENNENT, J.M. & CHAPLIN, P.J. 2001. The immune response to a DNA vaccine can be modulated by co-delivery of cytokine genes using a DNA prime-protein boost strategy. *Vaccine*, 19:4053-4060

SINGH, Y., KLIMPEL, K.R., QUINN, C.P., CHAUDHARY, V.K. & LEPPLA, S.H. 1991. The carboxyl-terminal end of protective antigen is required for receptor binding and anthrax toxin activity. *The Journal of biological chemistry*, 266:15493-15497

SYLVESTRE, P., COUTURE-TOSI, E. & MOCK, M. 2002. A collagen-like surface glycoprotein is a structural component of the *Bacillus anthracis* exosporium. *Molecular microbiology*, 45:169-178

SYLVESTRE, P., COUTURE-TOSI, E. & MOCK, M. 2003. 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

TANG, D., DEVIT, M. & JOHNSTON, S.A. 1992. Genetic immunization is a simple method for eliciting an immune response. *Nature*, 356:152-154

TIGHE, H., CORR, M., ROMAN, M. & RAZ, E. 1998. Gene vaccination: plasmid DNA is more than just a blueprint. *Immunology today*, 19:89-97

TOLLEFSEN, S., VORDERMEIER, M., OLSEN, I., STORSET, A., REITAN, L., CLIFFORD, D., LOWRIE, D., WIKER, H., HUYGEN, K. & HEWINSON, G. 2003. DNA injection in combination with electroporation: a novel method for vaccination of farmed ruminants. *Scandinavian Journal of Immunology*, 57:229-238

ULMER, J.B., DONNELLY, J.J., PARKER, S.E., RHODES, G.H., FELGNER, P.L., DWARKI, V.J., GROMKOWSKI, S.H., DECK, R.R., DEWITT, C.M. & FRIEDMAN, A. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science (New York, N.Y.)*, 259:1745-1749

WALTER, P. & JOHNSON, A.E. 1994. Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. *Annual Review of Cell Biology*, 10:87-119

WELKOS, S.L., KEENER, T.J. & GIBBS, P.H. 1986. Differences in susceptibility of inbred mice to *Bacillus anthracis*. *Infection and immunity*, 51:795-800

WELKOS, S. & FRIEDLANDER, A. 1988. Pathogenesis and genetic control of resistance to the Sterne strain of *Bacillus anthracis*. *Microbial pathogenesis*, 4:53-69

WILLIAMSON, E.D., BEEDHAM, R.J., BENNETT, A.M., PERKINS, S.D., MILLER, J. & BAILLIE, L.W. 1999. Presentation of protective antigen to the mouse immune system: immune sequelae. *Journal of applied microbiology*, 87:315-317

WOLFF, J.A., MALONE, R.W., WILLIAMS, P., CHONG, W., ACSADI, G., JANI, A. & FELGNER, P.L. 1990. Direct gene transfer into mouse muscle in vivo. *Science (New York, N.Y.)*, 247:1465-1468

WOODLAND, D.L. 2004. Jump-starting the immune system: prime–boosting comes of age. *Trends in immunology*, 25:98-104

CHAPTER EIGHT

General Conclusions

The first aim of this study sought to evaluate the humoral immune response in goats following single or booster vaccinations with the Sterne live spore vaccine (SLSV). The level of protection afforded by this vaccine was also assessed by challenge with virulent *Bacillus anthracis* spores. Anti-PA, anti-spore and lethal toxin neutralizing titres were higher 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 and 700-fold following single and double vaccination respectively. 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 immune response. Results from this study indicate that this crucial second vaccination can be administered as early as 3 months after the initial vaccination.

The second objective aimed to assess the immunogenicity of NLV candidates (rPA, rBclA and FIS) given simultaneously with or without penicillin G treatment in goats. The humoral immune response following simultaneous treatment with penicillin G and vaccination with the SLSV were also evaluated. This was compared with SLSV vaccination alone. The NLV

and penicillin G combination did not negate the development of crucial anti-toxin and anti-spores antibodies in goats. The immune responses were not different from that observed in goats vaccinated with only the NLV or SLSV. Simultaneous administration of penicillin G with the SLSV retarded the development of antibody titres in some of the treated animals.

The final objectives studied the immunogenicity of non-living vaccine candidates (NLV) in goats and used an *in vivo* A/J mouse model to evaluate the protective capacity of the immune goat sera. Findings indicated that these NLVs are able to induce strong immune responses in goats which were comparable with SLSV vaccinated animals. Survival data from passively challenged A/J mice and a correlative study of the association between antibody titres and survival showed the importance of anti-PA, anti-spore and toxin neutralizing titres in protection against anthrax. Vaccination of goats with formulations including rBclA showed poor seroconversion to rBclA. The anti-BclA IgG titres developed in the vaccinated goats appeared to play no role in protecting mice against anthrax, indicating the inclusion of rBclA in the vaccine formulation might be unnecessary. Also, we investigated the immunogenicity of a DNA vaccine using a heterologous DNA prime/protein boost approach. The DNA vaccine consisted of vector plasmids with gene sequences expressing PA and LF antigens (domains 4 and 1 respectively) and BclA (domains 1 and 3). The protein boost vaccine consisted of rPA and rBclA or FIS alone. Goats vaccinated once with the DNA vaccine and twice with the recombinant proteins (rPA and rBclA) only showed increased immune titres after the second protein boost and only to PA. The sera harvested from these animals protected 43 % of passively challenged mice. However, sera from goats primed with the DNA vaccine and boosted with FIS failed to protect any of the mice in this study.

This study indicates the potential of utilizing a non-living vaccine candidate in preventing anthrax infections in a ruminant host. An additional advantage of this vaccine is the compatibility with a prescribed antibiotic therapy. A possible best-mix combination of non-

living antigens for future trials based on our results will be a PA-FIS vaccine candidate. A group of animals vaccinated with PA alone can be included, in order, to fully ascertain the benefits of adding FIS to the construct. Also, a group of animals vaccinated with FIS alone should be included to prove if both PA and FIS are needed for protection against anthrax. The development of anti-toxin/anti-spore antibodies induced by this vaccine candidate can be evaluated in a representative target animal challenge model. Also the feasibility of combining the NLV with similar vaccines against related diseases (*Clostridium spp*) should be evaluated.

In summary, preliminary studies will be needed in order to establish the efficacy and safety of the test vaccine in goats and other related species. The constituents of the proposed vaccine candidate fall under Category 1 in the OIE listing of requirements for veterinary vaccine production (http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/). Category 1 vaccine biologics are classified as non-viable or killed products with negligible risk to the environment and presenting no safety concerns. Due to the biosafety concerns associated with virulent challenge studies, alternative methods need be assessed /developed for future protective efficacy studies. These can include: 1) Substituting a lethal toxin-only challenge for a virulent *Bacillus anthracis* challenge strain (though not truly reflecting the natural scenario). 2) Using a more attenuated *B. anthracis* strain, such as the 17JB strain (of intermediate virulence, pXO2+, pXO1+), in place of a fully virulent strain. 3) Indirect assessment using *in vivo* challenge models (following immune sera transfer) in addition to *in vitro* assays i.e. TNA and ELISA. However, initial studies to define optimal challenge conditions/doses will be needed with regards to these options. Guidelines for the development and testing of new veterinary vaccines have been drawn up recently by The International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (www.vichsec.org). These guidelines stipulate the basic

requirements/standards for the quality, safety and efficacy needed for the authorisation of veterinary vaccines.

Appendix 1

Protection of farm goats from a lethal challenge with *Bacillus anthracis* under field conditions by combinations of recombinant peptides and formalin inactivated spores

Approach

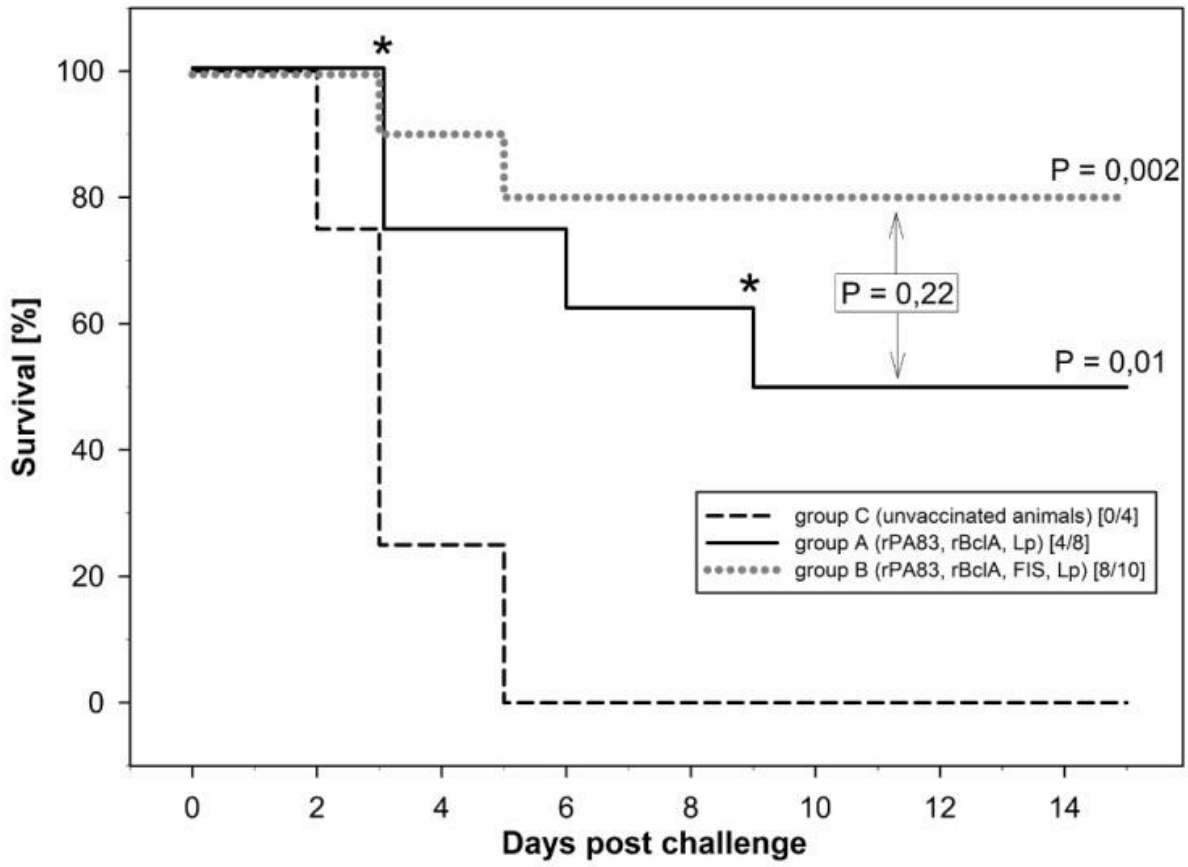
The goats were sourced from local farmers and comprised of a heterogeneous group of both male and female goats of ages around 1 year and of mixed breeds. These were tested for anti-PA antibodies by ELISA and only animals with low titres were enrolled. The animals were split into three groups; group A (n = 8) received a vaccine formulation of rPA, rBclA and lipopeptide adjuvant, group B (n = 10) received rPA, rBclA, FIS and lipopeptide adjuvant while group C (n = 4) served as unvaccinated controls. Groups A and B were vaccinated three times with three weeks intervals by subcutaneous injection. All animals were challenged subcutaneously with approximately 1000 virulent *B. anthracis* spores and monitored for 15 days. The challenge strain was isolated from the spleen of a deceased bovine in the Kars region (strain K-136, collection of the Institute of Microbiology, Kafkas University, Turkey). The presence of both virulence plasmids pXO1 and pXO2 was tested by PCR from 10 single colonies randomly picked from an overnight culture of the spore suspension on blood agar. The virulence of the challenge strain was confirmed in mice at Erciyes University (Hakan Cetinsaya Animal Experimentation Laboratory), Kayseri/Turkey.

To assess the health status of challenged animals, the general disposition was checked and anal temperature was measured twice daily post-challenge. In cases of temperatures of ≥ 40 °C a smear to determine the presence of encapsulated bacilli in the blood was made from ear capillary blood. An aliquot was taken to the laboratory for culture on sheep blood agar. Animals with negative blood smears were monitored more frequently. Positive animals were

treated with penicillin/streptomycin. The day of treatment was recorded as "time of death" for the analysis of protectiveness.

Results

The time to death curves are depicted in appendix Fig. 1 taking only full days into consideration. Both vaccine groups A and B showed significantly increased survival rate as compared to the unvaccinated controls (group C). Although group B had a better protection with 80 % (8/10) survival, it was not significantly different from group A (50 %, 4/8).



Appendix 1.: Survival data of goats immunized with protein component vaccines with or without FIS and challenged subcutaneously with fully virulent wild type strain *Bacillus anthracis* K-136. Survival was monitored up to 15 days. Significance was tested via a log-rank test. *P*-values are as compared to the unvaccinated animals if not indicated otherwise. The time of death was either the day the animal was found dead or the day of antibiotic treatment, as was the case for 2 goats of group A. The time points of their antibiotic treatment are labelled with * in the figure.

rPA83 – Recombinant protective antigen 83
rBclA – Recombinant *Bacillus* collagen-like protein of *anthracis*
FIS – Formaldehyde inactivated spores
LP – Lipopeptide adjuvant

Appendix 2

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Comparative studies on the immunogenicity of the live spore anthrax vaccine versus recombinant peptide and DNA vaccine candidates in goats and mice**
PROJECT NUMBER	**V065-12 (Amendment 2)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. O Ndumnego**

STUDENT NUMBER (where applicable)	**100 215 24**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Goats**	**Mice**
NUMBER OF ANIMALS	**36**	**250**
Approval period to use animals for research/testing purposes		**May 2013-September 2014**
SUPERVISOR	**Dr H van Heerden**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date **29 July 2013**
CHAIRMAN: UP Animal Ethics Committee	Signature

APPENDIX 2

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Comparative studies on the immunogenicity of the live spore anthrax vaccine versus recombinant peptides and DNA vaccine candidates in goats and mice**
PROJECT NUMBER	**V065-12 (Amend 3)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. O Ndumnego**

STUDENT NUMBER (where applicable)	**100 215 24**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Mice**	
NUMBER OF ANIMALS	**4**	
Approval period to use animals for research/testing purposes		**November 2013-November 2014**
SUPERVISOR	**Dr. H van Heerden**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	25 November 2013
CHAIRMAN: UP Animal Ethics Committee	Signature	