Comparative studies on the immunological response to the live spore anthrax vaccine in goats and passive protection test in mice

By

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Master of Veterinary Science

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DECLARATION

I, P.H. Phaswana, hereby declare that the dissertation for the Master of Veterinary Science degree in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science at the University of Pretoria, hereby submitted by me, has not previously been submitted for a degree at this or any other university, and that it is my own work in design and execution and that all reference material contained therein has been duly acknowledged.

P. H. Phaswana

2 February 2016

DEDICATION

I dedicate this thesis to my late grandfather Ntshavheni Thomas Phaswana and the rest of my family (my parents Rembuluwani Paulina and Ambani Daniel, my wonderful son Wade Phumutshelo, my brother Ntsumbeni and my sisters Zwivhuya and Unarine).

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LIST OF ABBREVIATIONS

AEC	Animal ethics committee
AVA	Anthrax vaccine adsorbed
AV-UK	Anthrax vaccine-United Kingdom
B. anthracis	*Bacillus anthracis*
BSL-3	Biosafety level-3
cAMP	Cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CMG-2	Capillary morphogenesis protein-2
CO_2	Carbon dioxide
°C	Degrees Celsius
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
EF	Oedema factor
ELISA	Enzyme-linked immunosorbent assay
ET	Oedema toxin
FIS	Formaldehyde inactivated *Bacillus anthracis* spores
HCl	Hydrogen Chloride
IMHA	Indirect microhaemagglutination test
IgG	Immunoglobulin G
IL-1 β	Interleukin -1β

IP	Intraperitoneally
kDa	KiloDalton
LD_{50}	Lethal dose 50%
LF	Lethal factor
LT	Lethal toxin
MTT	3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MAP	Mitogen-activated protein
MAPKK	Mitogen-activated protein kinase kinases
MID	Minimum infective dose
MTTD	Mean time to death
µg	microgram
µl	microliter
ml	millilitre
ng	nanogram
OBP	Onderstepoort biological products
OIE	International office of epizootics
PA	Protective antigen
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	PBS with Tween
PBSTM	PBST with skimmed milk powder
PGA	Poly-D glutamic acid
pXO1	Plasmid XO1

pXO2	Plasmid XO2
P-value	Probability values
rPA	Recombinant protective antigen
SANS	South African National Standard for the Care and Use of Animals for Scientific Purpose
SC	Subcutaneous
SDS	Sodium dodecyl sulfate
TEM-8	Tumour endothelial marker-8
TNA	Toxin neutralization assay
TNF-α	Tumour necrosis factor- alpha
TTD	Time to death
UPBRC	University of Pretoria Biomedical Research Centre
USSR	Sanitary Technical Institute in Russia

Thesis Summary

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Anthrax caused by the spore-forming bacterium *Bacillus anthracis* is primarily a disease of herbivorous animals and to a lesser extent in humans. Anthrax commonly infects wild and domestic ruminants that ingest or inhale the spores. *Bacillus anthracis* has several major virulence factors encoded on plasmids (pXO1 and pXO2), including the toxin components namely lethal factor (LF), edema factor (EF) and protective antigen (PA). The three exotoxin components combine to form two binary toxins. LF in combination with PA gives rise to lethal toxin (LT) which is lethal in several animal models including mice and guinea-pigs, and EF combines with PA to give oedema toxin (ET). Both LT and ET are responsible for the characteristic signs and symptoms of anthrax. Since PA complexes with EF and LF and interacts with host cell receptors to translocate the toxins into the cytosol, it plays an essential role in anthrax pathogenesis.

Presently, the anthrax veterinary vaccine comprises of the live, attenuated *B. anthracis* 34F2 spores that was developed in 1937 by Max Sterne and known as the Sterne vaccine. The vaccine is a stable uncapsulated mutant that produces all three toxin components of *B. anthracis* (PA, LF and EF), but lacks plasmid pXO2 encoding capsule formation whilst still providing protection against *B. anthracis* infections. The Sterne vaccine is the most widely used veterinary vaccine, which provides safe and effective protection against anthrax in animals, by inducing immune

response against antigens of the spore, cell mediated response and the toxins including protective antigen (PA) of *B. anthracis*, a component of anthrax toxin. While being effective in controlling anthrax globally, the live spore vaccine requires the use of animals for safety and efficacy tests. A major obstacle to improved anthrax vaccine research has been the lack of a convenient and sensitive method of monitoring the antibody response induced when the vaccine was developed. In a previous challenge study, revaccinated Sterne goats were 100% protected against virulent *B. anthracis* challenge but due to the lack of biosafety facility, we investigated the use of passive protection test in mice to evaluate anthrax vaccine protection. The main focus of the study was to develop a model that correlates immune response induced by the live spore anthrax vaccine in goats with survival through the passive protection test in mice. Furthermore to investigate the performance of macrophage cell lines from target host in an existing toxin neutralization assay against the standard mice macrophage cell line in same assay.

Five Boer goats were vaccinated twice over 3 months (week 0 and week 12) with 1ml of the Sterne live spore vaccine (at spore concentration of 6×10^6 per dose) and a negative group (n=3) consisted of naive goats received only sterile saline (1 ml) administered subcutaneously. Enzyme-linked immunosorbent assay (ELISA) and anthrax toxin neutralization assays (TNA) were used to monitor the anti-PA and toxin neutralizing antibodies of vaccinated and non-vaccinated (naive) goat groups after two vaccinations (week 4 and week 17). The anti-PA ELISA antibody titres indicated no significant difference between unvaccinated and goats vaccinated one (week 0 and 4) whereas a significant difference in the titer could be seen after the second vaccination (week 0 and 17). The TNA titres after the first vaccination (week 4) were low and with two goats were nil. All goats developed toxin neutralizing titres after the second vaccination (week 17). The ELISA and TNA assay titres showed no correlation of vaccinated animals at 17 weeks after the initial vaccination ($r^2 = -0.177$). The TNA was done using mice macrophage cell line (BALB/c monocyte macrophage cell line J774A.1). The TNA assay could not be done using caprine, bovine (BOMAC) and canine (DH 82) macrophage cell lines as a continuous caprine macrophage cell line could not be obtained and the toxins had no effect on the bovine (BOMAC, host) and canine (DH 82, non-host) macrophage cell lines.

A/J mice were passively immunized with different dilutions of sera ranging from 1:1000, 1:100, 1:50, 1:10, 1:5 to undiluted from individual goats immunized with Sterne spore vaccine to investigate the protectivity of the Sterne vaccinates sera against anthrax. These were diluted (0.5 ml) and administered intraperitoneally (500 µl) to A/J mice (n = 3 for each goat serum) for each dilution from individual goat. The mice were challenged with 2 LD_{100} of live *B. anthracis* Sterne strain administered subcutaneously (SC). Mice receiving the undiluted sera showed the highest protection (86%) with the survival frequency directly correlating to serum dilution (r_s = 0.77 and p = 0.01). There was no correlation between anti-PA titers and protection in mice (passive protection test) (r_s = -0.20; p = 0.01) in comparison there was significant correlation between toxin neutralizing antibodies and passive protection test with virulent *B. anthracis* challenge in A/J mice (r_s = 0.59; *p* = 0.01). Findings indicate humoral antibodies are crucial in the protection of naïve animals from anthrax infection. Correspondingly, the protective antibody titres directly correlate to survival which is reflected in the in vivo toxin neutralizing assay with mouse macrophages.

CHAPTER 1

INTRODUCTION

Anthrax is a zoonotic disease caused by *Bacillus anthracis* that affect all mammals but primarily herbivorous mammals (Hambleton *et al*., 1984). The main virulence factors are encoded by two plasmids, pXO1 and pXO2. The toxin bearing plasmid, pXO1 is 182 kb in size and pXO2 is a smaller plasmid of 95 kb (Farrar, 1994; Koehler, 2002). The pXO1 that encode for tripartite protein toxin (anthrax toxin) namely protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa) and oedema factor (EF, 89 kDa) regulates the production of toxins (Stanley *et al*., 1960; Mikesell *et al*., 1983; Friedlander and Little, 2009), whereas pX02 encodes for a protein that synthesise an antiphagocytic poly-D glutamic acid (PGA) capsule (Thorne *et al*., 1952; Green *et al*., 1985; Uchida *et al*., 1985). This capsule allows *B. anthracis* to evade the host immune system by protecting itself from phagocytosis (Zwartouw and Smith, 1956; Keppie *et al*., 1963). The three exotoxin components combine to form two binary toxins. LF in combination with PA gives rise to lethal toxin (LT) that is lethal in several animal models including mice, and EF combines with PA to give oedema toxin (ET) which is responsible for the massive oedema in the skin at the site of injection and inhibits neutrophil functions (Leppla, 1982; Duesbery *et al*., 1998; Moayeri *et al*., 2003).

Both these toxins (LT and ET) are responsible for the characteristic signs and symptoms of anthrax (Turnbull, 1998). LT is the dominant virulence factor produced by *B. anthracis* and is the major cause of death in infected animals. The PA plays a fundamental role in the toxic action of *B. anthracis*. PA has the ability to elicit a protective immune response against anthrax in both experimental animals and humans, thus the term protective antigen was derived (Gladstone, 1946; Wright *et al*., 1954; Ivins *et al*., 1998). PA component of anthrax toxin is considered to be highly immunogenic and is therefore a necessary component of veterinary and human anthrax vaccines due to its presence in both the oedema and lethal toxins (Stanley and Smith, 1963; Hambleton and Turnbull, 1990; Little and Ivins, 1999). Complete virulence of *B. anthracis* is dependent on the presence of the antiphagocytic capsule and tripartite toxin (Mikesell *et al*., 1983; Makino *et al*., 1989; Brossier and Mock, 2001). If either plasmid is absent, there will be a

decrease in virulence and the organism will be attenuated (Hambleton *et al*., 1984; Welkos, 1991).

The current anthrax veterinary vaccine use spores from the live, attenuated *B. anthracis* 34F2 strain which was developed in 1937 by Max Sterne (Sterne, 1939). The vaccine is a stable uncapsulated mutant that produces all three toxin components of *B. anthracis* (PA, LF and EF), but it lacks plasmid pXO2 whilst still providing protection against anthrax infections (Sterne, 1939; Turnbull *et al*., 1986). Immunity develops 2-4 weeks after vaccination and revaccination is stipulated every 9-12 months with the Sterne vaccine to ensure protection (Anthrax vaccine leaflet, Onderstepoort Biological Products, South Africa, http://www.obpvaccines.co.za; OIE, 2012). *In vivo* immunity tests (pathogenicity test) of the Sterne vaccine mainly involved pathogenicity and efficacy testing where guinea pigs were vaccinated with Sterne vaccine and challenge with *B. anthracis* 17 JB strain (Pasteur II strain) (OIE, 2012). *In vivo* immunity tests (pathogenicity test) using guinea pigs were mainly used due to lack of sensitive serological diagnostic tool being available at that time (Turnbull *et al*., 1992). The need for serology in anthrax vaccine research and development first became apparent with the search for efficacious human vaccines.

The only data on the immunogenicity and/or protective efficacy of the Sterne spore vaccine in ruminants was done by Turnbull *et al.* (1992) until Ndumnego (2012) evaluated the immune response and survival after *B. anthracis* virulent challenge of Boer goats vaccinated with Sterne 34F2 vaccine. Ndumnego (2012) challenged Boer goats 6 and 58 weeks after vaccination and 4 weeks after revaccination respectively subcutaneously with 843 spores of a virulent *B. anthracis* strain. All the revaccinated goats (100%) survived the virulent challenge, whereas the Sterne vaccinated groups challenged 58 weeks and 6 weeks after vaccination had 80% and 60% survival following challenge, respectively (Ndumnego, 2012).

In this study, we investigated the possibility of establishing a model for assaying protection against anthrax. This was done by correlating protection and survival of Sterne vaccinated goats through passive protection test in mice with the immune responses using anti-PA IgG ELISA and TNA in the vaccinated animals. Furthermore immune response in vaccinated animals was compared using TNA in J774A.1 mice macrophage cell line with host and non-host macrophage

cell lines to investigate whether TNA using host cell line provided more accurate immune response. The study aims were investigated with the following objectives: (i) determine the immune response in Sterne hyper-immune (revaccinated) goats using ELISA and TNA in J774A.1 mice macrophage cell line; (ii) compare the immune response in revaccinated goats using TNA in mice macrophages with caprine, bovine and dog macrophages and; (iii) correlate the immune response in the vaccinated goats using TNA with a passive protection test in mice that might provide a standard (or model) against which the immunological parameters of vaccine candidates can be compared and which should give an idea of the efficacy and protectivity.

CHAPTER 2

LITERATURE REVIEW

2.1 THE ORGANISM / AETIOLOGY

Bacillus anthracis is a rod-shaped, non-motile, facultatively anaerobic, capsulated, spore-forming gram-positive bacterium that causes the zoonotic disease anthrax (Koch, 1876; Hambleton *et al*., 1984; Beedham *et al*., 2001). It is primarily a disease of domestic and wild animals, though humans can contract the disease. Humans usually appear to be moderately resistant to acquiring infection. Humans can inhale viable spores through accidental or deliberate release of the spores. Infection can also be through ingestion of infected undercooked meat or entry of spores through skin wounds (Turnbull, 1991; Mock and Fouet, 2001). *Bacillus anthracis* is found in vegetative and spore forms. The vegetative form is the growing form, which produces the toxins (lethal and oedema toxins) (Brossier and Mock, 2001). The resulting toxaemia has systematic effects that lead to the death of the host (Smith and Keppie, 1954; Dixon *et al*., 1999; Ascenzi *et al*., 2002).

The vegetative forms of *B. anthracis* have specific nutrient requirements and appear to survive poorly outside the animal host (Sterne, 1959; Manchee *et al*., 1981; Turnbull *et al*., 1989). The bacterium requires a nutrient poor environment and the presence of free oxygen to undergoing the process of sporulation (Choquette and Broughton, 1981; Hugh-Jones and De Vos, 2002; Ghosh and Setlow, 2009), therefore factors such as climatic conditions, topography, certain chemicals and the bacterium itself may indirectly or directly affect the ability of *B. anthracis* to germinate and/or sporulate (Sterne, 1959; Sussman and Halvroson, 1966; Titball *et al*., 1991). In the absence of nutrients the organism is able to form spores which are highly resistant to harsh conditions like chemical disinfection, heat, cold etc. and also has the ability to survive in the natural environment/soil for a long period of time (Van Ness, 1971; Gould, 1977; Manchee *et al*., 1981).

2.2 VIRULENCE

The capacity of *B. anthracis* to cause the disease is dependent on both the expression of a tripartite toxin and the production of a polyglutamic acid capsule present on pXO1 and pXO2, respectively (Mikesell *et al*., 1983; Green *et al*., 1985; Brossier and Mock, 2001). The pXO1 encode the PA, LF and EF (Mikesell *et al*., 1983; Welkos *et al*., 1986). PA combines with LF to form LT, and ET is formed by the binding of PA together with EF, which together is referred to as anthrax toxin (Leppla, 1982; Klimpel *et al*., 1992; Collier and Young, 2003). The toxin complex exhibit the classical A-B enzyme model, where the A (EF or LF) and B (PA) components work together to effect a change in cell conformation (Dixon *et al*., 1999; Ascenzi *et al*., 2002; Collier and Young, 2003). The two exotoxins share PA as a common cell-receptor binding protein (Green *et al*., 1985; Beedham *et al*., 2001; Pitt *et al*., 2001).

Figure 2.1, depicts the mechanisation of the anthrax toxin at the molecular level. PA_{83} (83 kDa protein) binds to cell surface receptors, the tumour endothelium marker 8 (TEM8) or the capillary morphogenesis protein 2 (CMG2), both of which are widely expressed on many cell types, including immune cells and becomes proteolytically activated (Escuyer and Collier, 1991; Bradley *et al*., 2001; Hudson *et al*., 2008). Upon receptor binding by TEM8 and CMG2, PA is cleaved by a furin-like protein into two fragments PA_{20} and PA_{63} with the resulting amino terminal PA_{20} fragment dissociating from the cell surface leaving PA_{63} attached to the receptor (Klimpel *et al*., 1992). PA_{63} then oligomerizes into a heptamer pore and binds EF or LF. ET consists of PA_{63} heptamer and EF (89 kDa), which is a calcium and calmodulin-dependent adenylate cyclase that gives rise in intracellular cyclic-adenosine monophosphate (cAMP) levels (Leppla, 1982; Moayeri and Leppla, 2009). These high cAMP levels in the cell will upset water homeostasis resulting in massive oedema (Leppla, 1982, Dixon *et al*., 1999). LT consists of heptamer PA_{63} and LF (90 kDa), which is a zinc metalloprotease that inactivates members of the mitogen-activated protein kinase kinases (MAPKKs) family and other potential targets (Klimpel *et al*., 1994; Duesbery *et al*., 1998; Vitale *et al*., 2000; Pannifer *et al*., 2001). Moreover, LT disrupt cell cycle regulation and kills the cell to cause imbalance in the production of cytokines (Duesbery *et al*., 1998; Singh *et al*., 1999; Collier and Young, 2003).

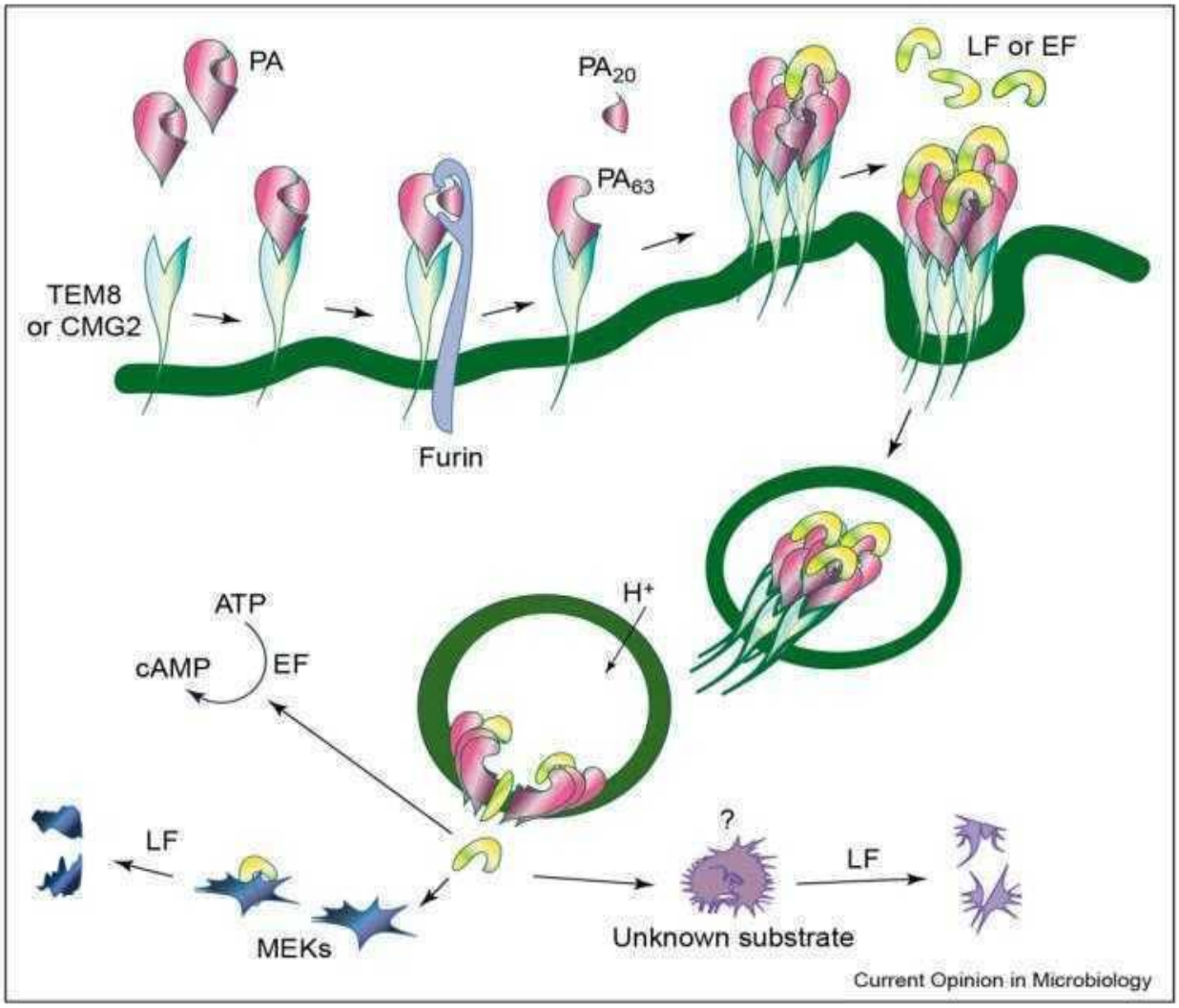


Figure 2.1: Anthrax toxin mechanism in cells (Moayeri and Leppla, 2004). Anthrax toxin compose of the cell-binding protective protein (PA) and two enzyme components (oedema factor (EF) and lethal factor (LF)) that act together to impart their physiological effects. The 83 kDa PA (PA_{83}) binds to two specific cell wall receptors tumour endothelium marker 8 (TEM8) or the capillary morphogenesis protein 2 (CMG2). Following binding to its receptors, it is then cleaved by a cell wall protease into two subunits PA_{20} and PA_{63}. PA_{63} oligomerizes to form a heptamer/ prepore that forms a channel in the host cell membrane through which the LF and EF are translocated into the cytosol. The acidic pH enables the release of lethal toxin (LT) and oedema toxin (ET). EF within the host protects bacteria from phagocytic destruction, and increases intracellular levels of cyclic AMP (cAMP) resulting in a severe oedema and death. LF cleaves members of the mitogen-activated protein kinase kinases (MAPKKs), there by disrupting cell cycle regulation and causing cell death in macrophages.

2.3 HOSTS

Practically all mammals can be affected by the disease. The disease commonly affects domestic animals including cattle, sheep, goats, horses, donkeys, mules, pigs, dogs and wild ruminants, such as kudu, roan antelope and impala (Koch, 1937; Hugh-Jones and De Vos, 2002; Fasanella *et al*., 2007). Anthrax varies considerably between the different animal species with the most susceptible being herbivores. Variability in host species susceptibility to anthrax is primarily influenced by the feeding behaviour, infection route (Pienaar, 1967; De Vos, 1990; Hugh-Jones and De Vos, 2002) and age. Adult animals are more vulnerable than the young animals (Brunsdon, 1968; De Vos, 1990; De Vos and Bryden, 1998a). Of the herbivores, browsers are more susceptible to the disease than the grazers (De Vos, 1990) whereas omnivores and carnivores are more resistant to the disease in contrast to herbivores (Pienaar, 1967; De Vos, 1990; Lindeque *et al*., 1998). The resistance in wild carnivores is enhanced by naturally acquired immunity that is as a result of frequent exposure from scavenging carcasses of anthrax victims (Turnbull *et al*., 1992). In general, carnivores and omnivores occasionally become infected after feeding on infected carcasses, despite being relatively resistant to anthrax (De Vos, 1990; De Vos and Bryden, 1998b).

Omnivorous species like pigs and humans are considered incidental host (Christie, 1980; Hugh-Jones and De Vos, 2002) and are resistant to anthrax (Smith, 1973). Carnivorous species are less commonly affected in anthrax enzootic areas (e.g. Etosha National Park in Namibia) due to naturally acquired specific antibodies (Turnbull *et al*., 1992) and outbreaks affecting large numbers of carnivores are very rare (WHO, 2008). Anthrax has been reported in cheetahs (*Acinonyx jubatus*) that rarely have natural anti-anthrax antibodies (Lindeque *et al*., 1998; Turnbull *et al*., 2004). Wild scavenging carnivores such as lions, hyenas and jackals, are less susceptible to the disease and require more spores that are found in infected meat to become infected and then disperse spores in their faeces (Pienaar, 1967; Ebedes, 1976; Turnbull *et al*., 1989). Among birds only ostriches are known to be susceptible to anthrax (Huchzermeyer, 1998). Birds including scavenging vultures and ravens seem to be resistant to anthrax as vegetative forms do not survive transit through the digestive tract of vultures (Houston and Cooper, 1975), but they can act as carriers by disseminating spores to pastures and drinking water holes (Pienaar, 1967; Turnbull, 1990; Hugh-Jones and Blackburn, 2009).

Variation in host resistance to *B. anthracis* has been extensively studied (Young *et al*., 1946; Klein *et al*., 1962; Jones *et al*., 1967). In highly susceptible animals, the disease is acute and runs a rapid course (Bhatnagar and Batra, 2001), where animals die without showing clinical signs before death. Actively immunized guinea pigs with live spore vaccines are considered to be partially protected against a virulent strain of *B. anthracis* challenge (Little and Knudson, 1986; Turnbull *et al*., 1986; Ivins *et al*., 1992), whereas mice have been reported to be susceptible and difficult to protect using live spore vaccines when challenged with virulent *B. anthracis* (Welkos and Friedlander, 1988; Welkos *et al*., 1990). The spore vaccine provides protection to immunized rabbits and monkeys when challenged with virulent *B. anthracis* (Ivins *et al*., 1996; Zaucha *et al*., 1998; Williamson *et al*., 2005). A combination of formaldehyde-inactivated spores (FIS) of *B. anthracis* and recombinant PA (rPA)/ alhydrogel vaccine provided protection against challenge with fully virulent spores in both guinea pigs and mice. The recombinant PA alhydrogel vaccine and FIS combination provides 100% protection to mice and guinea pigs against infection with 30 to 300 LD_{50} of fully virulent spores, as compared with 0 and 22% survival of the animals with rPA and alhydrogel respectively (Brossier and Mock, 2001; Brossier *et al.*, 2002).

Animals are used to determine protection and efficacy of vaccines with laboratory animals most prominently used as they are more economical due to reduction in handling and biosafety expenses of the animals. A number of species used to test efficacy and protection of anthrax vaccines include guinea pigs (Sterne, 1937; Little and Knudson, 1986; Ivins *et al*., 1994), mice (Welkos *et al*., 1986; Ivins and Welkos, 1988; Pezard *et al*., 1995), rabbits (Zaucha *et al*., 1998; Pitt *et al*., 2001; Fellows *et al*., 2001) and rhesus monkeys (Ivins *et al*., 1998; Williamson *et al*., 2005). Research has shown that toxin negative, capsule positive variants (pXO1-; pXO2+) of *B. anthracis* retained a greater virulence than corresponding toxin positive, capsule negative (pXO1+; pXO2-) strains in inbred mice (Welkos, 1991). The efficacy of Sterne live spore vaccine and PA based human vaccines has been tested in various animal models (laboratory and domestic animals) (Sterne, 1939; Hambleton *et al*., 1984; Little and Knudson, 1986). The PA based vaccines efficacy has been established in laboratory animals, in particular on rabbits and rhesus monkeys (Ivins *et al*., 1996; 1998; Fellows *et al*., 2001), but it is known that they induce only partial protection against virulent *B. anthracis* in guinea pigs and no protection in mice

(Turnbull *et al*., 1986; Ivins and Welkos, 1988; Welkos *et al*., 1990). Welkos *et al.* (1986) found inbred mice to differ in susceptibility to virulent *B. anthracis* and distinctly resistant or susceptible to lethal infection of Sterne vaccine strain. A/J inbred mice have a LD_{50} of 10^3 *B. anthracis* Sterne spores since the mice lacks the C5 genes, a complement factor which plays a role in the opsonisation and phagocytosis allowing lethal challenge with avirulent Sterne spore vaccine strain (Ooi and Colten, 1979; O'Brien *et al*., 1985; Welkos *et al*., 1988). Virulent studies with *B. anthracis* restrict experiment to biosafety level-3 (BSL-3) facilities. Therefore Turnbull *et al.* (2004) used serum from host animals in passive protection test in A/J mice challenged with *B. anthracis* Sterne strain to conduct experiments without the need for a BSL-3 facility (Welkos *et al*., 1986; Welkos and Friedlander, 1988). The passive protection test enables protection testing of host using avirulent Sterne strain which is lethal to A/J mice.

2.4 MODE OF INFECTION

Anthrax disease can occur in both animals and humans but manifests itself in three different ways depending on the route of entrance in the host (Welkos *et al*., 1986; Mock and Fouet, 2001). In herbivores and wild ruminant animals, anthrax usually manifest as peracute or apoplectic, acute, and subacute to chronic forms (Wilson and Miles, 1966; Hambleton *et al*., 1984; Hugh-Jones and De Vos, 2002). When spores are inhaled, ingested, or come into contact with a skin lesion on a host, they may become reactivated and multiply rapidly to produce vegetative cells (Barnes, 1947; Ross, 1957; Gundi-Ronti *et al*., 1999). Once in the host the vegetative bacilli produce the toxin and kill the host (Leppla, 1982; Mock and Fouet, 2001). Anthrax commonly infects herbivorous mammals that ingest or inhale the spores while grazing (Young *et al*., 1946; Hambleton *et al*., 1984; Akula *et al*., 2005).

Human can contract anthrax in laboratory accidents, through biological warfare or by handling infected animals or their products (Brachman, 1980; Leppla *et al*., 1985; Mock and Fouet, 2001; WHO, 2008). Cutaneous anthrax (most common in human) develops when infection occurs through the skin wound. Therefore resulting in swelling at the area of infection, which progress to the formation of large black "coal" scars (black eschar) (Ascenzi *et al*., 2002; Spencer, 2003). Inhalation anthrax (more severe but rare) occurs through inhalation of spores (Laforce, *et al*., 1969; Borts, 1972; WHO, 2008).

In animals, cutaneous anthrax has been suggested to occur through the bites of blood-sucking flies like tabanids (Davies, 1983; Turell and Knudson, 1987; Palazzo *et al*., 2012). Gastrointestinal anthrax occurs in animals when the spores are ingested with plants contaminated with *B. anthracis* spores and is believed to be the primary route of infection of grazing herbivores. Gastrointestinal infections usually lead to acute septicemia, which often results in death (Lindeque and Turnbull, 1994).

The course of infection and clinical manifestations are depicted in Figure 2.2. Endospores enter the body by inhalation, ingestion or through abrasions. Upon entry into the host body, *B. anthracis* spores, are engulfed by macrophages and carried to regional lymph nodes where they germinate (Hanna, 1998; Dixon *et al*., 1999). Once germination occurs, the organism may multiply and localise in lymph nodes and spread to the blood stream or other organs through the efferent lymphatics and result in septicaemia (Keppie *et al*., 1955; Friedlander *et al*., 1993; Mock and Fouet, 2001). Replicating bacteria secrete exotoxins (Dixon *et al*., 1999). ET causes swelling of the affected areas by altering cyclic AMP levels. These high cAMP levels inhibit water homeostasis and resulting in massive oedema (Leppla, 1982; Dixon *et al*., 1999).

LT inactivates mitogen-activated protein kinase (MAPK) 1 and 2 and eventually leading to cell death. Lethal toxin is responsible for evoking the release of tumour necrosis factor α (TNF-α) and interleukin -1β (IL-1 β) resulting in shock and death as the result of the action of a tripartite toxin produced by the bacterium (Smith and Keppie, 1954; Hanna, 1999; Inglesby *et al*., 2002). In domestic livestock and wild animal populations, transmission occurs mostly by ingestion of spore, and possibly inhalation, although entry through skin lesions cannot be ruled out. Herbivores are thought to be particularly susceptible due to inhalation and ingestion of spores while grazing on contaminated land (Young *et al*., 1946; De Vos 1990).

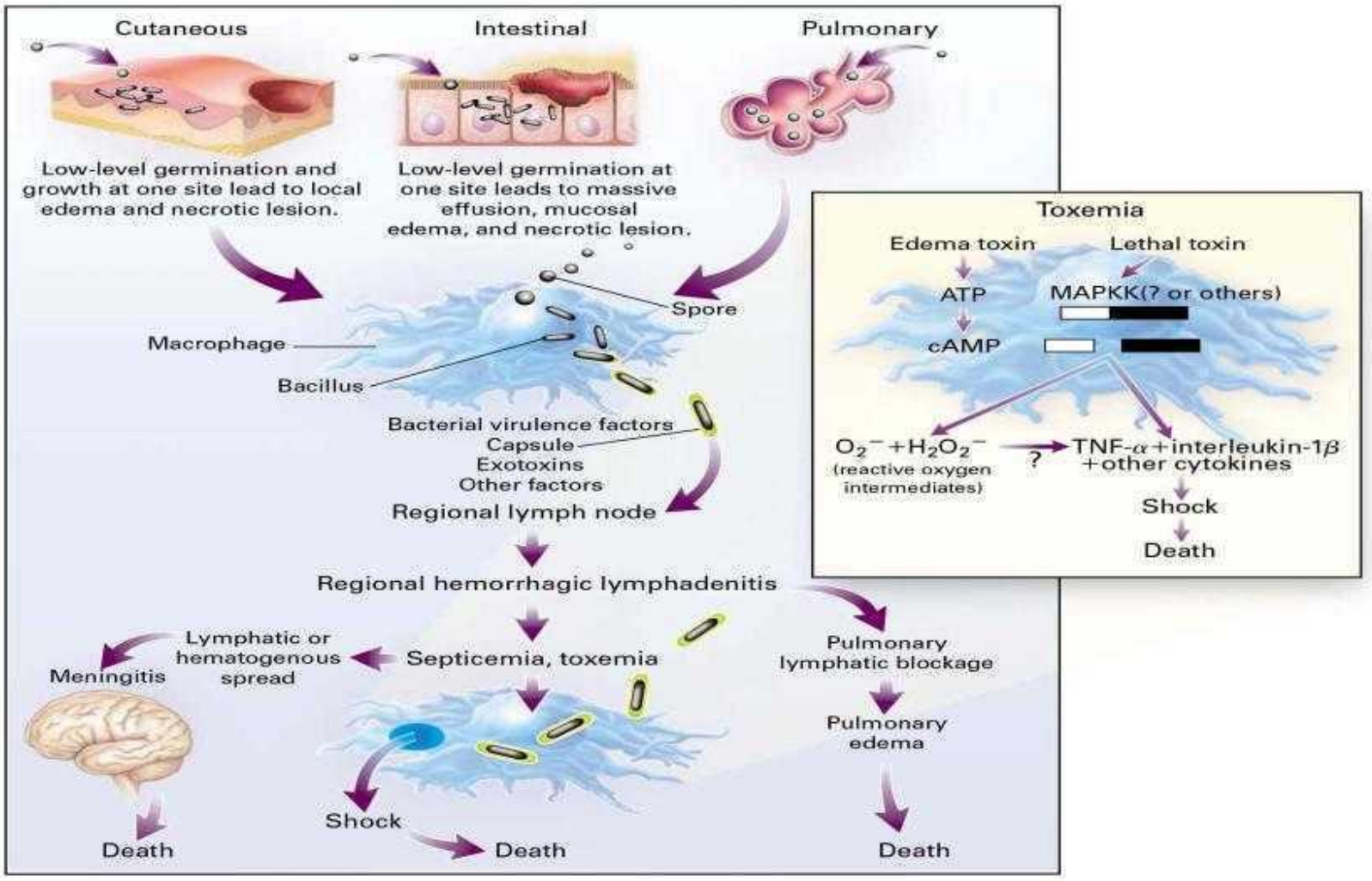


Figure 2.2: Pathophysiology of anthrax as shown by Dixon *et al*. (1999). A *B. anthracis* infection is initiated by the entry of spores into the host body via the inhalation, cutaneous, and gastrointestinal routes. Following entry, the *B. anthracis* spores are engulfed by local macrophages and carried to regional lymph nodes. Within macrophages, *B. anthracis* spores germinate and the vegetative bacilli grow in the lymph node, creating regional haemorrhagic lymphadenitis. Once germination occurs, they multiply and release toxin. The vegetative bacilli are then spread into blood stream through lymph nodes. The bacteria build up rapidly in the blood causing severe septicaemia which results death of the host due to toxin action. The toxemia due to toxin action indicates that the oedema toxin increase the cyclic AMP levels that results in massive oedema. Lethal toxin inactive mitogen-activated protein kinase (MAPK) causing death of macrophages and evoke the release of tumour necrosis factor α (TNF-α) and interleukin -1β (IL-1 β) that results in shock and death.

2.5 CLINICAL SIGNS

Clinical signs in animals differ among species and the route of exposure influences the extent of clinical signs. Herbivorous mammals are mostly affected by ingestion form and possibly inhalation of spores. Symptoms in the highly susceptible species are non-specific and are rarely observed until shortly before the terminal stage. Therefore the first sign of anthrax is a dead animal. The clinical history usually describes the animal to be in good health a few hours before death without any previous evidence of illness (Wilson and Miles, 1964; Hugh-Jones and De Vos, 2002; Beyer and Turnbull, 2009). Dark blood may ooze from the mouth, nostrils, and anus with marked bloating and rapid body decomposition. Generally incomplete or absent rigor mortis, rapid decomposition of the carcass, exudation of blood through natural openings and dark poorly clotted blood is common for post mortem gross pathology in ruminants (Gleiser, 1967; Marcato, 1981; De Vos, 1994). Herbivores such as cattle, sheep, goats and most wild ruminants such as kudu, roan antelope and impala mainly show peracute and acute symptoms (Hugh-Jones and De Vos, 2002). Horses, donkeys and zebra exhibit the acute form whereas omnivores and carnivores suffers from subacute to chronic form which may involve an intestinal form with gastroenteritis or swelling of the glands of the mouth and throat due to ingestion of large volumes of infected meat (Davies, 1982; Hambleton *et al*., 1984, Fasanella *et al*., 2010).

In horses the clinical manifestation involves oedematous subcutaneous swelling of the neck, shoulders, high fever, depression, chest, abdomen and death if spores are ingested (Sterne, 1959). In pigs, there is a haemorrhagic oedematous swelling of the mucosa and sub-mucosa of the pharynx which may extend to the neck, throat, depression and death occurs due to occlusion of the airway (Henning, 1956; Ferguson, 1981). This species is more resistant and the disease is usually subclinical (Smith, 1973). In wild and domestic carnivores a subacute to chronic form occurs with a clinical signs similar to that of pigs. Carnivores such as dogs show a severe inflammation of the tongue, throat, stomach and intestine (Davies, 1982). It manifests and is localized in anthrax angina or in the intestine. Carnivores show signs of acute gastroenteritis and oro-pharyngitis when affected, which is due to the uptake of large volume of infected meat. Recovery is not uncommon to many of the carnivores due to natural resistance to anthrax (Davies, 1982).

The peracute form is most common observed in ruminants, including cattle, sheep and goats occurring at the beginning of an outbreak. The latter species is regarded as among the most susceptible within the vulnerable herbivore species (Hambleton *et al*., 1984; OIE, 2008). In peracute anthrax, the course of the clinical disease is usually less than two hours. In animals where clinical signs are observed the peracute form is characterized by fever, dyspnoea and muscle tremors for 1 to 2 hours before death (WHO, 2008), followed by recumbency with convulsions occurrence minutes before death if seen. Sudden death of one or more of the animals may be the only initial sign of the disease in a flock after a 3-5 days incubation period (Beyer and Turnbull, 2009) with rapid bloating, bleeding from natural body openings and incomplete rigor mortis as evidence of rapid post-mortem (Hambleton *et al*., 1984; Hugh-Jones and De Vos, 2002). A blood-stained discharge from the mouth, nose, and anus may be observed within a few hours after death (Christie, 1980; Hambleton *et al*., 1984; OIE, 2008), although this is not always observed. Sheep are less resistant than cattle and the disease develops faster. The fulminant form is the most common in sheep. The animal shows signs of fever, muscle tremors, dyspnoea and, the appetite are lost thereafter the animal die in few minutes with convulsions (OIE, 2008; Fasanella *et al*., 2010).

In the acute form, the course of the disease may be apparent for 1 to 3 days with some animals surviving a week or more (Sterne, 1959). The acute form has a more prolonged prodromal period. In this form, fever and excitement may be followed by depression, stupor, disorientation, muscle tremors, dyspnoea, and congested mucous membranes, which may be observed up to 48 hours before death (Davies, 1982; Hambleton *et al*., 1984). The abrupt rise in body temperature may reach as high as 41- 42 ºC in acute anthrax. There may be bloody discharges from the natural body openings. Some infections are characterized by localized, subcutaneous, oedematous swelling that can be quite extensive due to insect bite or skin abrasion. Extensive swelling seen in most frequently areas including the ventral neck, thorax, abdomen, mammary glands and be followed by death in 48 to 96 hours (WHO, 2008). Symptoms include fever, shock, and multiple organ failure; discharge/bleeding from various orifices, cardiac distress, respiratory distress and swelling of the tongue and throat (Hugh-Jones and De Vos, 2002; OIE, 2008). Affected animals show signs of distress at the terminal stages of infection before becoming comatose and dying within hours of showing these signs (OIE, 2008). Development of

subacute anthrax may be seen with prolonged illness lasting several days. Subacute anthrax is not always fatal and is characterized by localized oedematous swelling and may less involve the gastrointestinal tract (WHO, 2008).

2.6 TREATMENT AND PREVENTION

B. anthracis can be killed in the host by treatment with antibiotics as it is susceptible to a wide variety of antibiotics (Inglesby *et al*., 2002). *B. anthracis* is penicillin sensitive and can be effectively treated with a range of antimicrobials including tetracycline, chloramphenicol and streptomycin (Lightfoot *et al*., 1990; Odendaal *et al*., 1991; Baillie, 2001). Antibiotics therapy must be initiated shortly after exposure since the efficacy diminishes as toxaemia progresses (Smith and Keppie, 1954). Vaccination represents the most cost-effective and least costly method of reducing susceptibility to infection (Friedlander and Brachman, 1998; Baillie, 2001). The development of successful livestock vaccines has significantly reduced the disease throughout the world over the past half century (Sterne, 1937). The introduction of compulsory vaccination of livestock implemented since the 1940s in South Africa reduced the disease incidence (De Vos and Turnbull, 2004). Although effective and safe vaccines have been available for decades, the disease still remains a problem causing livestock mortality in the developing world or certain endemic countries due to lack of proper vaccination and control policy (Hugh- Jones, 1999; Baillie 2001).

2.7 VACCINES

Anthrax was one of the first bacterial diseases to be controlled by vaccination. While the first veterinary vaccine was developed by Pasteur in 1881 (Pasteur, 1881), the human vaccines did not emerge until the middle of the 20th century. The veterinary vaccine used by Louis Pasteur in 1881 to immunise livestock consisted of an attenuated strain of *B. anthracis* which resulted from repeated culture over a period of time at elevated temperatures (Pasteur, 1881). The basis of the attenuation remained a mystery for over 100 years until Mikesell *et al*. (1983) were able to show that increasing the incubating temperature to 42 °C resulted in the partial loss of the toxin-encoding plasmid pXO1. The Pasteur vaccine provided low levels of protective immunity since the *B. anthracis* PA is encoded by the absent pXO1 plasmid. Pasteur vaccine provide effective protection, but suffered from problems of declining potencies, retained some residual virulence,

required repeated dosing and have been associated with transient side-effects which led occasionally to the death of animals (Sterne *et al*., 1942; Turnbull, 1991; Mock and Fouet, 2001). Therefore it could not be administered safely to certain species (Sterne, 1937). The Pasteur vaccine remained in use until it was replaced by the attenuated live spore vaccine developed by Max Sterne (Mikesell *et al*., 1983).

The effective and safe Sterne vaccine developed in in the late 1930s (Sterne, 1939; Hambleton *et al*., 1984; Ivins and Welkos, 1988) is used for prevention of anthrax in animals (Mock and Fouet, 2001). The Sterne strain lacks pXO2 encoding capsule formation and is therefore relatively safe, albeit with some residual virulence (Sterne, 1939, Turnbull *et al*., 1986, Cartwright *et al*., 1987). *Bacillus anthracis* Sterne strain is toxigenic but non-encapsulated, this vaccine is based on an avirulent non-encapsulated strain 34F2 derived from the subculture of an isolate from a case of bovine anthrax (Sterne, 1937, Sterne *et al*., 1942). The animal vaccines that use strain 34F2 consist of approximately 10^7 spores per ml suspended in 0.5% saponin in 50% glycerine saline (Sterne, 1939; Turnbull, 2008; OBP vaccine insert, http://www.obpvaccines.co.za). The live spore animal vaccine 34F2 strain has proved to be extremely safe and effective for many years but has side effects in some species, suffered from declining potencies and retained a residual virulence in some immunized animals (Sterne *et al*., 1939; Welkos *et al*., 1986; Turnbull, 1991). Goats have been reported to be highly susceptible species to the deadly anthrax disease and exhibit adverse reactions to the vaccine (Sterne, 1946).

2.8 VACCINE EFFICACY

Established infectious doses clearly depend on several factors such as route(s) of infection, species, breed, state of health of animal(s) and virulence of the infecting anthrax strain (OIE, 2008). These factors play an important role in the disease susceptibility following exposure to the spores. The effectiveness of spore based anthrax vaccines is dependent on the induction of anti-PA antibodies (Reuveny *et al*., 2001; Beedham *et al*., 2001) and varies in the different animal models (Pitt *et al*., 2001) that are assessed by virulent spore challenge. Evidence for the efficacy of Sterne vaccine and PA based vaccines comes from several studies in laboratory animals, including guinea pigs (Little and Knudson, 1986; Ivins *et al*., 1992; Turnbull *et al*., 1990), mice (Ivins and Welkos, 1988; Pezard *et al*., 1995), rabbits (Belton and Strange, 1954;

Wright *et al*., 1954; Pitt *et al*., 2001) and rhesus monkeys (Fellows *et al*., 2001) using different routes (subcutaneous, intravenous or intramuscular) of administration. Vaccine efficacy has been tested by virulent challenge of *B. anthracis* in vaccinated guinea pigs (Little and Knudson, 1986; Turnbull *et al*., 1986; 1990), rabbits (Belton and Strange, 1954; Wright *et al*., 1954; Fellows *et al*., 2001), mice (Welkos and Friedlander, 1988; Welkos *et al*., 1990; Pezard *et al*., 1995) and goats (Ndumnego, 2012). Vaccinated guinea pigs and rabbits developed a complete protection against a virulent challenge (Little and Knudson, 1986; Ivins *et al*., 1992; Turnbull *et al*., 1990), whereas vaccinated mice were not protected and have been found to be particularly susceptible and difficult to protect (Welkos and Friedlander, 1988; Welkos *et al*., 1990). Annual Sterne live spore vaccination is reported to provide effective immunity for 9-12 months (OBP vaccine insert, http://www.obpvaccines.co.za; Sterne, 1939; Turnbull *et al*., 1988). However no documented study could be found that proved the previous statement. More recently, Ndumnego (2012) tested the effective immunity and protectivity of the Sterne live spore vaccine in goats.

With human vaccination, most vaccines use toxin components. However, immunization with live spores in humans has been used and limited to the former USSR (Sanitary Technical Institute in Russia) and China (Knop and Abalakin 1986; Dong 1990; Turnbull, 1991). In most other countries, live spore vaccines are not licensed for use in humans due to fears concerning residual pathogenicity (Hambleton *et al*., 1984; Turnbull, 1991). Human anthrax vaccines were developed in the 1950s in the USA and UK (Wright *et al*., 1954; Brachman 1962). The current UK (British) vaccine (Darlow *et al*., 1956) consists of an alum-precipitated cell free culture filtrate of the no-capsulated Sterne strain of *B. anthracis* 34F2 (Belton and Strange, 1954). In the USA, anthrax vaccine adsorbed (AVA) is the only anthrax vaccine licensed for human immunisation. The AVA is a subunit vaccine in that it is a cell-free extract. The USA vaccine (AVA) is an aluminium hydroxide-adsorbed cell free culture filtrate of a non-capsulated strain (V770-NP1-R), containing mostly PA (Puziss and Wright, 1963; Ivins and Welkos, 1988; Ivins *et al*., 1994). AVA induces protective immunity primarily by stimulating the production of antibodies against PA, a critical component of the tripartite anthrax toxin. Numerous studies have shown that antibodies to PA can confer protection in both animals and humans against virulent *B. anthracis* challenge due to the ability to exert a protective immune response (Ivins *et al*., 1998; Welkos *et al*., 2001; Ngudi *et al*., 2010).

Welkos *et al.* (1993) found the PA vaccines confer only limited protection in mice and PGA capsule appears to be primary virulence factor. Moreover there is no direct correlation between anti-PA titres and protection in mice and hamsters (Welkos *et al*., 1993; Fellows *et al*., 2002). Further disadvantage of human vaccines include high rates of mild and severe adverse reactions likely due to residual toxicity in AVA or other contaminants. These mainly include fatigue, muscle ache, headache, myalgia, vomiting, allergic or inflammatory reactions at the injection site and anaphylaxis (Joellenbeck *et al*., 2002; Marano *et al*., 2008; Wright *et al*., 2014). The development of new vaccines rests on these issues. The efficacy of licensed and experimental vaccines and measurable parameters, which correlate with protection, appear to be dependent on which animal model is used (Ivins *et al*., 1992; Pitt *et al.*, 2001; Fellows *et al*., 2002). The differences could be due to changes in host susceptibility or the relative importance of various mechanism of immunity to anthrax amongst different animal species (Beedham *et al*., 2001). Various studies aimed at the development of safer and more efficacious vaccine have been conducted most often using guinea pigs. Guinea pigs only develop incomplete protection against a challenge with AVA or anthrax vaccine-UK (AV-UK) vaccines in contrast to rabbits or rhesus monkeys that develop complete protection (Ivins *et al*., 1992; 1994; Fellows *et al*., 2001).

A number of investigations using guinea pigs have shown that protection against anthrax infection cannot be correlated with antibody titre against PA (Ivins *et al*., 1994; Little *et al*., 1997; Turnbull *et al*., 1998). Several studies have shown that immunization of live Sterne spores vaccines provide better protection in guinea pigs than immunization with PA-based vaccines even when vaccines elicit stronger anti-PA responses. The PA-based vaccines confer only limited protection against all virulent strains of *B. anthracis* in mice (Little and Knudson, 1986; Turnbull *et al*., 1986; 1990; Ivins *et al*., 1992). However, it is also important to note that the lives spore vaccines induce lower anti-PA titres than the PA-base vaccine (Little and Knudson, 1986; Ivins *et al*., 1992). Investigators have shown that protection against anthrax in the susceptible host is dependent on the development of antibodies to the PA component of the anthrax toxin (Reuveny *et al*., 2001; Weiss *et al*., 2006).

2.9 CURRENT ACCEPTED IMMUNOLOGICAL TOOLS IN ANTHRAX VACCINE DEVELOPMENT

Protective immunity against anthrax is thought to be primarily antibody mediated (Beedham *et al*., 2001). Serologic assays for anthrax have primarily been applied for the evaluation of immune response to anthrax vaccine in epidemiologic investigation of the disease in animals and in confirmatory diagnosis of anthrax in humans (Shlyakhov *et al*., 1973; Harrison *et al*., 1989). The need for anthrax serology only became apparent during the development of a human vaccine in the 1950s (Thorne and Belton, 1957; Ray Jr and Kadull, 1964). A variety of serological test have been used to detect serum antibodies to anthrax, including the agar-gel diffusion, indirect microhaemagglutination test (IMHA), ELISA and TNA (Thorne and Belton, 1957, Willkie and Ward, 1967; Johnson-Winegar, 1984; Reuveny *et al*., 2001).

Serology to detect the presence of specific antibodies to anthrax is mostly done for research purposes. The ELISA detect anthrax antibodies using the PA component of anthrax toxin (Johnson-Winegar, 1984; Pombo *et al*., 2004) whereas TNAs measure the functional ability of antibodies in sera of immunized animals to neutralize toxin (Reuveny *et al*., 2001; Hering *et al*., 2004). Anna Johnson-Winegar adapted an ELISA for the detection of anthrax antibodies using the PA component of the anthrax toxin, which was the first application of ELISA to anthrax serology (Johnson-Winegar, 1984; Turnbull *et al*., 1992). Since then ELISA have been widely used to measure specific antibodies/antigens at very low concentrations (Hering *et al*., 2004; Pombo *et al*., 2004) and is therefore a useful diagnostic tool for testing sera samples. However, the anti-PA ELISA is limited to measuring all antibodies that bind PA. Well established and improved ELISA methods have been developed by many studies to assess the immune response and confirm anthrax in animals and humans (Turnbull *et al*., 1986, Little *et al*., 2004; Hahn *et al*., 2006; Ndumnego *et al*., 2013). The benefit of using ELISA provided a method of assaying anthrax antibodies which is rapid, simple and highly sensitive.

TNA was developed to measure the functional ability of antibodies in sera of immunized animals to neutralize *B. anthracis* lethal toxin cytotoxicity (Reuveny *et al*., 2001; Pitt *et al*., 2001; Hering *et al*., 2004). TNA is highly versatile, not species specific and has been standardized for use with multiple species (Hering *et al*., 2004). TNA may be more useful in predicting vaccine efficacy,

since it measures the neutralizing activity of sera against the cytotoxic effect of the toxin formed by the combination of PA and LF. However, Chen *et al*. (2014) found that TNA was statistically significantly correlated with survival but did not provide the same good level of accuracy as anti-PA IgG in nonhuman primates (NHP). Furthermore, anti-PA IgG and TNA levels in NHP were highly correlated (Quinn *et al*., 2012; Chen *et al*., 2014). Ngundi *et al*. (2010) used different macrophage cell lines to measure the antibody levels elicited by anthrax vaccine namely J774A.1, RAW 264.7 and CHO cell lines. The authors detected differences in the Fc receptor-mediated neutralization associated with the J774A.1 and RAW 264.7 cell-base assays that may account for some of the species dependence of the assays. Macrophage cell lines such as J774A.1 cells are widely used as they are easy to grow and provide fast, convenient models to measure LT activity. In addition to their use in research studies, the J774A.1 cell line, have been used to assay serum sample from clinical vaccine studies and to measure antibody levels in clinical samples.

CHAPTER 3: MATERIALS AND METHODS

3.1 MATERIAL AND METHODS

3.1.1 STERNE LIVE SPORE VACCINE

The Sterne live spore vaccine is produced by Onderstepoort Biological Products (OBP) in South Africa and consists of *B. anthracis* strain 34F2 ($pXO1^+$, $pXO2^-$) spores suspended in glycerine at a spore concentration of 6×10^6 per dose.

3.1.2 EXPERIMENTAL ANIMALS

Healthy, age-matched naive Boer goats as well as 6-8 weeks old female A/J mice were utilized in this study. The Boer goats were confirmed to be anthrax PA antibody negative by anti-PA ELISA and kept at the experimental animal facility of OBP. The animals comprising of female and emasculated male goats were fed pellets and lucerne with water ad libitum. They were dewormed following arrival and kept in a fenced, outdoor facility with concrete floors throughout the trial.

A/J mice were obtained from Jackson Laboratories, USA and maintained in pathogen-free conditions at the laboratory animal facilities of the University of Pretoria Biomedical Research Centre (UPBRC) according to the South African National Standard for the Care and Use of Animals for Scientific Purpose (SANS). These mice were housed in autoclavable cages enriched with shredded tissue paper holders. Irradiated Epol rodent pellets and reverse osmosis water were provided ad libitum. All studies were approved by the animal ethics committee (AEC) of the University of Pretoria, South Africa (protocol approval number V083/13) and the Department of Agriculture, Forestry and Fisheries (DAFF), South Africa under the animals disease act (Act 36 Section 20, 1984).

3.1.3 VACCINATION REGIMEN

The experimental group consisted of hyper-immune Boer goats (n=5), immunized twice with the live spore vaccine (Table 3. 1). These were vaccinated subcutaneously at week 0 and week 12 with 1 ml of the vaccine according to the manufacturer's instruction (OBP) and monitored throughout the duration of the trial. A negative control group of Boer goats (n=3) received only sterile saline (1 ml) administered subcutaneously on week 0 and week 12. The animals were bled before the initial vaccination at week 0, week 4 and week 17 (5 weeks after the second vaccination).

Table 3. 1: Goat group designation, immunization and lethal challenge of passive protection in A/J mice.

Vaccine Group	Amount of A/J mice	Treatment
Positive control (Sterne hyper-immunized) (pre-virulent challenge sera; n= 4)	12	Individual serum administered (0.5 ml, IP) into 3 mice. Mice were challenged with Sterne vaccine strain 24 hours later (subcutaneous, SC).
Sterne hyper-immunized dilutions of protective serum (n=5)	90	Different dilutions (undiluted, 1:5, 1:10, 1:50, 1:100, 1:1000) of serum (0.5 ml) from Sterne vaccinated individual goats administered (IP) into 3 mice each. Mice were challenged with Sterne vaccine strain 24 hours later (SC).
Negative control (Naïve sera; n=3)	9	Individual serum (0.5 ml) administered (IP) into 3 mice. Mice were challenged with Sterne vaccine strain 24 hours later (SC)

3.1.4 ANTI-PA IgG ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Sera collected at weeks 0, 4 and 17 were analysed for PA-specific antibody response using ELISA. Sera from a hyper-immune goat surviving virulent *B. anthracis* challenge and a naive goat were used as positive control and negative control group respectively. The serum samples were tested in duplicates using the ELISA as previously described by Hahn *et al*. (2004) and Pombo *et al*. (2004) with some modifications. Briefly, individual wells of 96-well microtitre plates (Maxisorp Nunc-immuno plate) were coated with 0.5 µg of rPA83 (obtained from W. Beyer, University of Hohenheim, Stuttgart, Germany) in bicarbonate buffer (0.05M, pH 9.5) followed by overnight incubation at 4 °C. The wells were washed twice with PBS containing 0.05% Tween-20 (Merck) (PBST) using a Biorad PW 40 washer (Marnes-La Coquette) and blocked with 200 µl of PBST containing 5% skimmed milk powder (Oxoid, PBSTM) for one hour at room temperature. The plates were washed twice with washing buffer (PBST) and the test sera and controls diluted in PBSTM. Test sera were applied to the first column of each row in duplicate and serially diluted with two fold dilution series until last column of the row and incubated for 30 minutes on a rotatory shaker (Titretek®). Following incubation, the plates were washed five times with PBST and PA-specific antibodies detected using 100 µl of horseradish peroxidase-conjugated rabbit anti-goat IgG (H+L) (Invitrogen) and incubated for 30 minutes on the plate shaker. After washing five times with PBST, colour was developed using 2, 2’ azino bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma) and the absorbance taken at 405 nm using a Biotek power wave XS2 reader (Biorad).

3.1.5 MACROPHAGE CELL LINES

3.1.5.1 CAPRINE MACROPHAGE CELL LINE

Caprine (Boer goat) peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation of heparinated blood using the Ficoll method as previously described by Wardley *et al*. (1980) with and without some adaptations. Fresh blood was collected by venipuncture into four heparinized 10 ml blood collection tubes from negative (naïve) control group Boer goats. For the isolation of PBMCs, 15 ml volumes of whole blood were placed over equal volumes of Histopaque® -1077 (Sigma) and centrifuged at 4500 xg for 15 min at 4 °C. Buffy coats were collected and washed three times by centrifugation in culture medium RPMI-

HEPES/ L-glutamine (Life Technologies) with and without 2-β–mercapthoethanol (2 mL) in combination with 10 % foetal calf serum (FCS, Life Technologies), penicillin (10000 units/mL) streptomycin (10000 ug/mL) and amphotericin B (250 ug/mL) at 3500 xg for 10 minutes at 20 °C and resuspended to a final concentration at 5×10^6 cells/ml. Cells were then transferred to 25 cm^2 culture flasks (Corning Incorporated) and incubated overnight at 37 °C. Over the next 7-14 days, cells were maintained in sterile polystyrene flask at 37 °C and non-adherent cells with medium removed every day and replaced with fresh medium.

3.1.5.2 ESTABLISHED MACROPHAGE CELL LINES

BALB/c mouse monocyte macrophage cell line J774A.1, (European collection of cell cultures (ECACC)) (Ralph and Nakoinz, 1977), canine macrophage cell line DH 82 (Wellman *et al.*, 1988) and bovine macrophage cell line (BOMAC from the Friedrich-Loeffler-Institute, Germany, Stabel and Stabel, 1995) were obtained, cultured and utilized in the study. Macrophage cell lines were grown in monolayer culture in standard tissue culture flasks (25 cm^2) using a commercially available medium, DMEM and RPMI (Life Technologies) supplemented with 10% FCS (Life Technologies) and penicillin (10000 units/ml) streptomycin (10000 ug/ml) and amphotericin B (250 ug/ml). The cells are gradually expanded by transferring the confluent cell layers to progressively larger culture flasks (75 cm^2). All the mammalian cell lines were grown and maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells were routinely observed to ensure they are healthy and growing as expected. When the adherent cells reached confluency, the cultures were passaged and used for *in vitro* TNA.

3.1.6 TOXIN NEUTRALIZATION ASSAY (TNA)

An *in vitro* TNA was performed using the J774A.1, DH 82 and BOMAC macrophage cell lines as previously described by Hering *et al*. (2004) with slight modifications. In brief, cells were washed with sterile PBS and harvested after trypsinization (Wellman *et al.,* 1988) essentially to detach all the cells without appreciable loss. Cells were plated in 96-well flat bottomed tissue culture plates (Greiner Bio One) at a density of 4 x 10^5 cells/well in 200 µl of complete medium (DMEM and 10% FCS, (Life Technologies)) a day before the assay and allowed to grow over

night at 37 °C with 5% CO_2. Duplicate sera samples (collected from goat groups at week 0, 4 and 17 were diluted (1: 50; 1: 100; 1: 200 and 1: 1000) in culture medium (DMEM, (Life Technologies)) mixed with PA and LF (List Biological) (sera/LT mixture) at concentrations of 500 ng/ml and 400 ng/ml respectively, then pre-incubated for one hour at 37 °C in 5 % CO_2. A plate- to plate transfers from the titration plate to the overnight cultured cells (after discarding medium) was then performed and incubated for 3 hours. Following incubation, 25 µl of 5 mg/ml MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Life Technologies)) was added to each well and incubated in the dark at 37 °C and 5 % CO_2. After 2 hours incubation, the cells were lysed with pre-warmed (37 °C) acidified isopropanol (90% isopropanol, 0.5% SDS w/v, 25 mM HCl, pH 4.7), vigorously pipetting to solubilize the formazan dye. The plates were rested for 5 minutes and the absorbance readings taken at 540 nm with a Biotek power wave XS2 reader (Biorad). Each assay included a single dilution series of positive control serum from a Sterne hyper-immunized goat which survived a virulent spore's challenge (positive control). Three wells (without cells) 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 with cells (medium control).

3.1.7 PASSIVE TRANSFER OF SERUM AND CHALLENGE IN A/J MICE

Passive protection tests as previously described by Turnbull *et al*. (2004) were conducted using naïve A/J mice. Briefly, individual serum from hyper-immunized goats (week 17) were diluted (0.5 ml) ranging from 1:1000, 1:100, 1:50, 1:10, 1:5 to undiluted. These were administered intraperitoneally (500 µl) to A/J mice (n = 3 for each goat serum) for each dilution from individual goat (Table 3. 1). Negative control mice received undiluted sera from the naïve group (Table 3. 1). The mice were challenged after 24 hours by the subcutaneous route with 2 LD_{100} Sterne 34F2 *B. anthracis* vaccine strain (1.92×10^5 spores) and monitored for survival over 14 days. Death due to anthrax was confirmed by re-isolation of *B. anthracis* Sterne spore strain from liver and spleen smears on sheep blood agar. Survivors were euthanized using barbiturate inhalation overdose and confirmed free of Sterne spores infection following culture of liver/spleen smears.

3.1.8 COMPARISON BETWEEN ELISA, TNA AND PASSIVE PROTECTION TEST

We compared the immune response in Sterne vaccinated goats using ELISA and TNA with the passive protection test in mice. The relationship between ELISA, TNA antibody titres and time of survival was investigated using the Pearson's correlation coefficient. *P*-values of < 0.05 were considered statistically significant. For determination of the survival time for mice receiving different diluted serum dose. Data were analyzed using the Kaplan–Meier method in SPSS statistics with the statistical software package SPSS Version 21 (IBM SPSS Statistics; IBM Corporation, Armonk, New York, USA). The anti-PA and TNA linear plot was done Sigma Plot (Systat software Inc, San Jose, USA).

Chapter 4: RESULTS

4.1 Anti-PA IgG ELISA

ELISA was performed to determine the serum IgG antibody levels against PA from individual sera collected at weeks 0, 4 and 17. The anti-PA titres of each hyper-immunized individual animal (week 17), before vaccination (week 0) and after first vaccination (week 4) is indicated in Figure. 4.1. There was significant difference in titres between week 0 and 17 (P = 0.0088). No significant differences were found in titres between week 0 and 4 (P = 0.0913), but there was significant difference between week 4 and 17 titres (P = 0.0192).

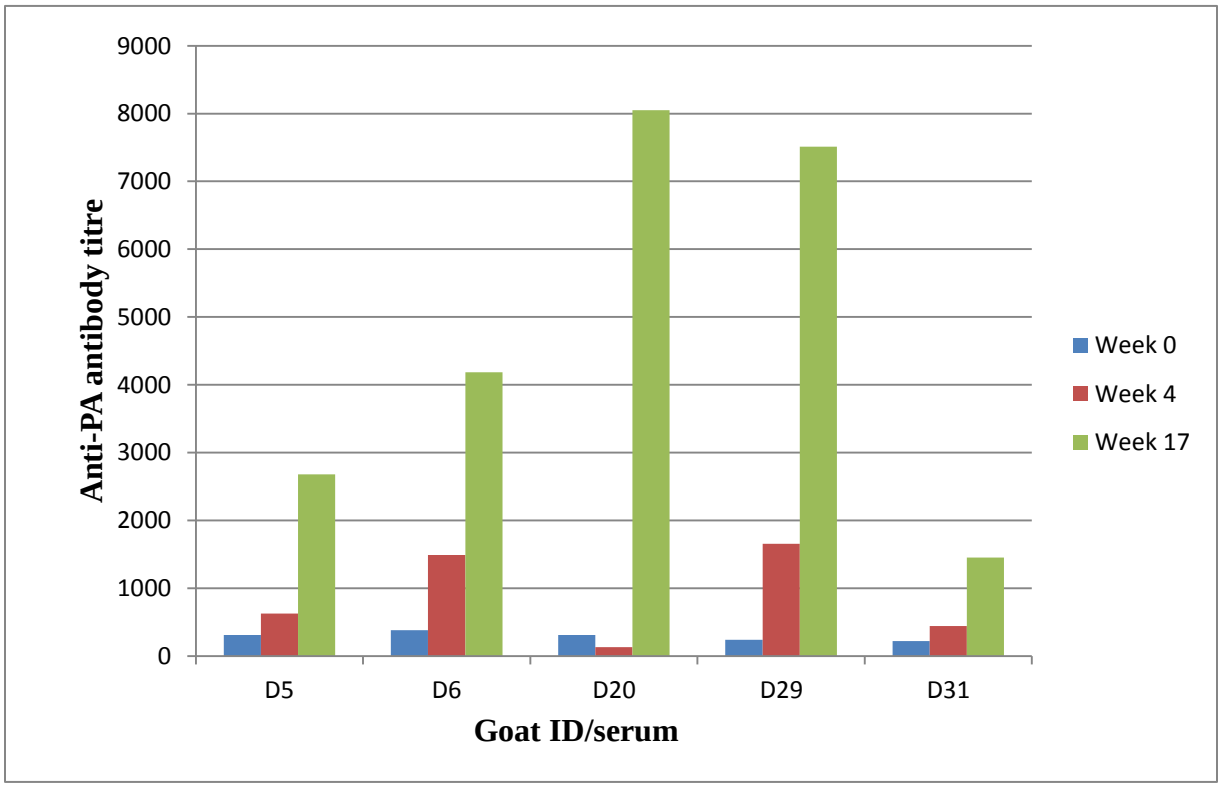


Figure 4.1: Anti-protective antigen IgG production following Sterne live spore vaccination of goats at weeks 0, 4 and 17. Serum samples of individual goats (D5, D6, D20 and D31) were collected and analysed before vaccination (week 0), four weeks after initial vaccination (week 4) and five weeks after second vaccination (week 17).

4.2 ESTABLISHMENT OF GOAT (CAPRINE) MACROPHAGES CELL LINE

Attempts to establish a caprine macrophage cell line from goat PBMCs using the method developed by Wardley *et al*. (1980) were unsuccessful, despite optimization and alterations to the protocol. During cultivation, the cells were semi-adherent as expected but monolayers maintained for 3 weeks by changing the medium 2-3 times a week failed to reach confluency. Alterations investigated to obtain continuous macrophage cell lines culture including the use of increased RPMI-HERPES or RPMI-L-glutamine, with and without 2-β–mercapthoethanol, increased fetal calf serum concentration and the replacement of the latter with goat serum were without any visible improvement.

4.3 TOXIN NEUTRALIZATION ASSAY

An *in vitro* toxin TNA was performed to assess toxin neutralizing antibodies in sera of vaccinated goats using murine J774A.1, canine DH 82 and bovine BOMAC macrophage cell lines. Using the standard J774A.1 cell line, neutralizing antibody titer measured by the TNA assay varied among the individual animals. All five Sterne vaccinated goat sera were able to neutralize the macrophage cytotoxicity of the anthrax LT. No serum anti-PA antibody titres were detectable in naïve animals (animals injected with a control sterile saline). There were no detectable neutralizing titres in sera collected at week 0 (before vaccination). The TNA titres at week 4 were low and even non-existent in some animals. Goat D6 and D20 did not give antibody titres after the first vaccination but all goats induced toxin neutralizing antibodies after the second vaccination (Figure 4.2). Goat D31 sera (vaccinated twice with Sterne vaccine 12 weeks apart) with neutralizing antibody titres of 238 conferred protection (100% at undiluted and 33% at 1:5; 1:1000 dilution folds respectively) to A/J mice against 2 LD_{100} *B. anthracis* Sterne strain spores challenge despite relatively low titre levels. No significant differences were found between TNA titres at week 0 and 4. There was no difference between week 0 and 4 titres (P = 0.2175), in contrast to the differences seen in titres between week 0 and 17 (P = 0.0044) and week 4 and 17 titres (P = 0.0245). Exposure of the DH82 macrophage and BOMAC cell lines to the anthrax LT (PA and LF at 500 ng/ml and 400 ng/ml, respectively) had no effect on the viability of the cells (data not shown).

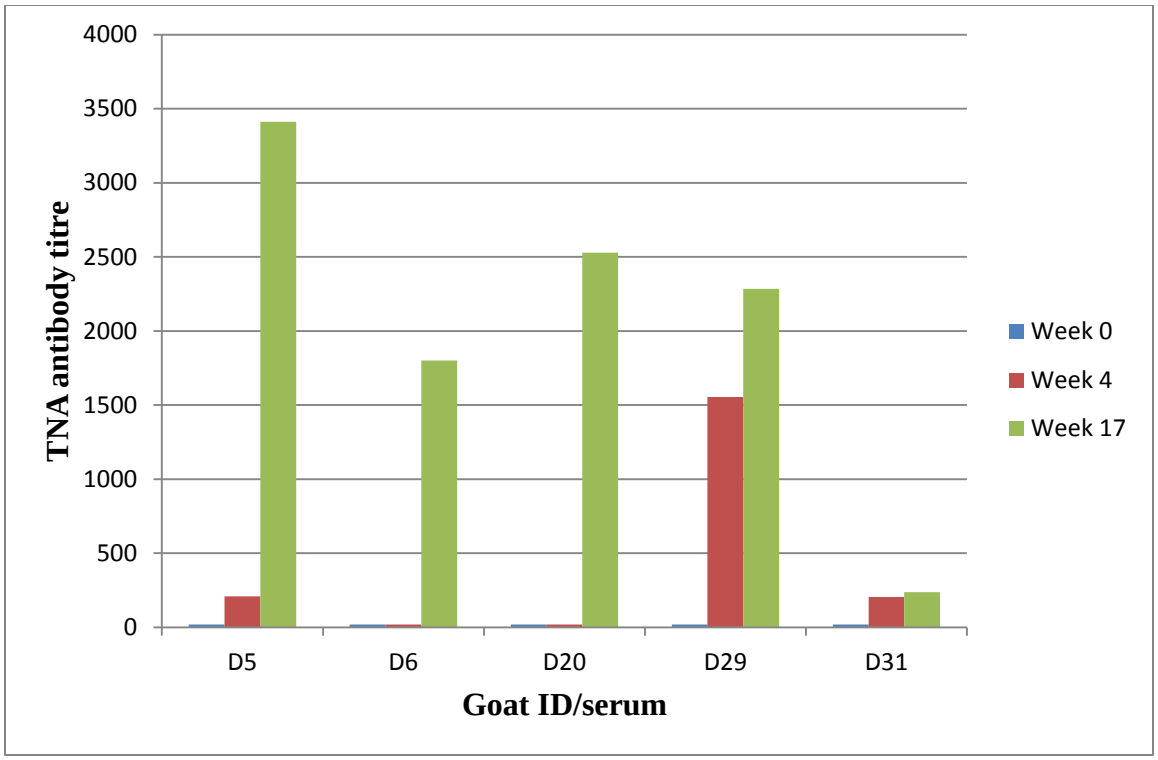


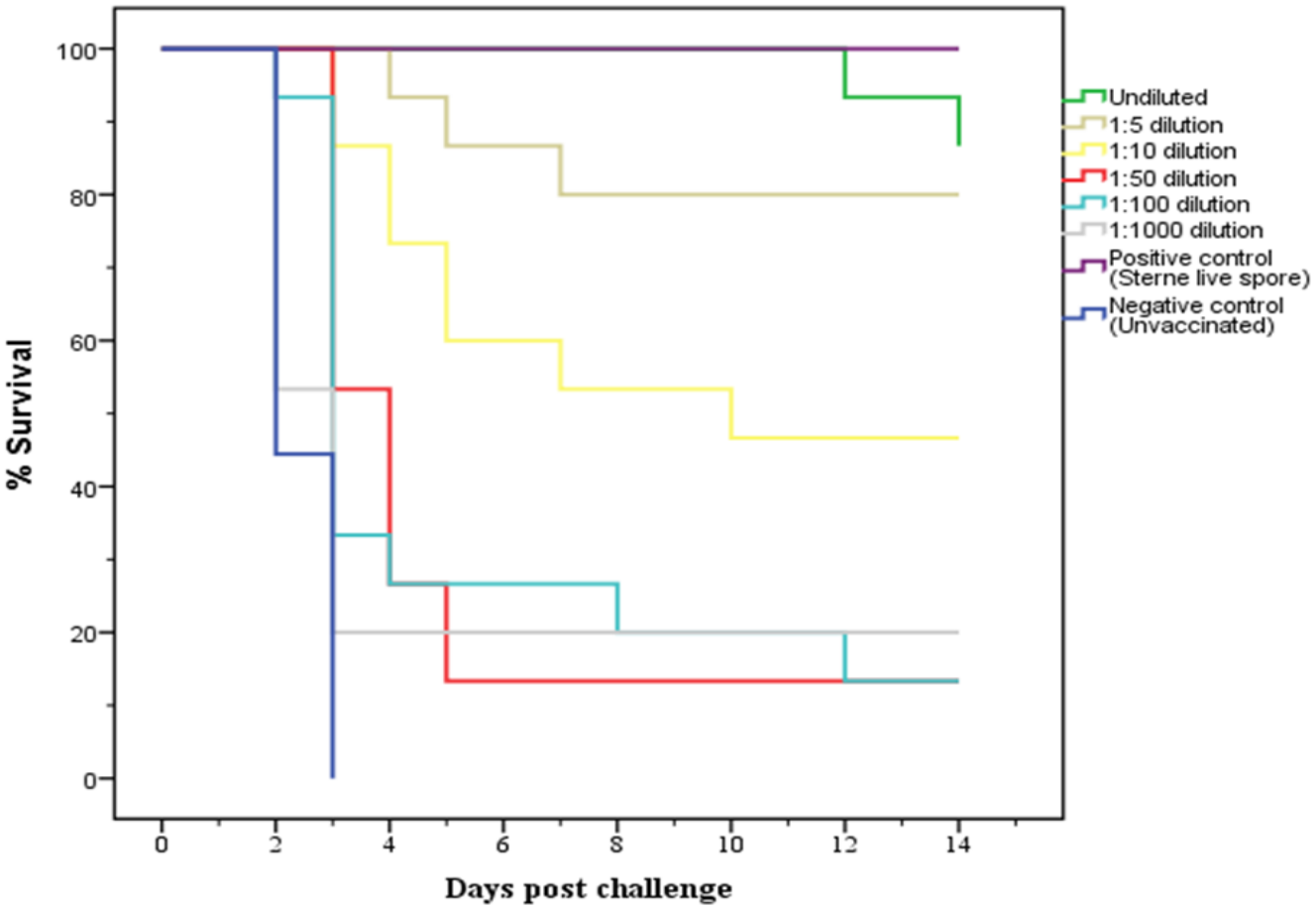
Figure 4.2. The toxin neutralisation titres (TNA) indicating the induction of toxin neutralisation antibodies for each goat following Sterne live spore vaccination of goats at weeks 0, 4 and 17. Serum samples of individual goats (D5, D6, D20, D29 and D31) were collected and analysed before vaccination (week 0), four weeks after initial vaccination (week 4) and five weeks after second vaccination (week 17).

4.4 PASSIVE PROTECTION TEST

A/J mice were passively immunized with undiluted and diluted sera collected at week 17 from the individual goats each immunized with Sterne vaccine. The protective effect of Sterne vaccinates sera was assessed following challenge with *B. anthracis* 34F2 spores by subcutaneous injection. Administration of goat hyper-immune sera to A/J mice resulted in protection against the lethal Sterne spores challenge, while sera from the naïve goats failed to protect the mice following challenge (Table 4.1). All nine A/J mice that received negative (naive) goat control group sera died within 3 days of lethal challenge, whereas all A/J mice that received hyper-immune confirmed positive Sterne vaccinated sera survived for 14 days (Table 4.1). All mice

injected with positive control sera (pre-challenge) from Sterne hyper-immunized vaccinated goats that survived lethal *B. anthracis* challenge (Ndumnego, 2012) survived (12/12) Sterne challenge (Table 4.1). Mice receiving Sterne vaccinated goat sera with higher dilutions showed a lower protection than those receiving sera with lower dilutions.

Table 4.1. Passive protection test results of A/J mice injected with hyper-immune and naive goat serum and challenged with *Bacillus anthracis* Sterne strain.

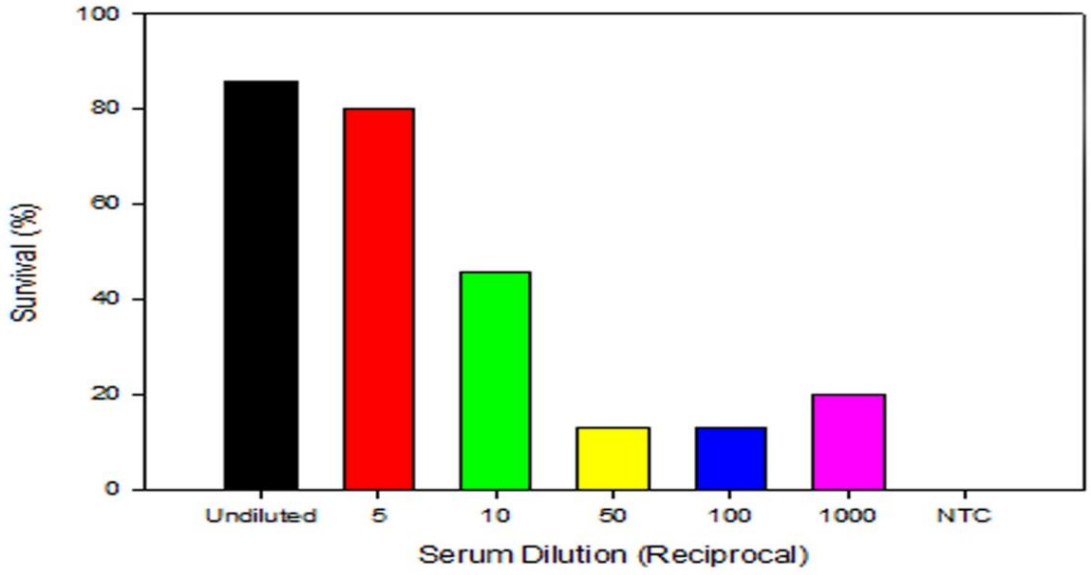


Overview challenge sera dilution groups vs days to death of challenged mice.

The animals were monitored over a period of 14 days following challenge and exact time to death (TTD) noted.

Of the undiluted goat sera, 13 of the 15 (86 %) mice injected with sera from vaccinated goats survived the challenge. Two A/J mice injected with undiluted sera from vaccinated goats D6 and D20 only died on day 12 (euthanized) and 14, respectively (Table 4.1). Both mice were confirmed dead due to anthrax by isolation of *B. anthracis* Sterne spores from mice spleen/liver. The undiluted group had a mean survival of 13.9 days compared to the dilution groups that varied 5-12 days depending on the dilution concentration (Table 4.1). The 1:5, 1:10, 1:50, 1:100 and 1:1000 dilutions had 80%, 47%, 13%, 13% and 20% survival respectively with most of the mice dying within the first 5 days (Table 4.1, Figure 4.3a and b). The clinical signs of partially /unprotected injected A/J mice with vaccinated and naive goat sera observed just before death included sub-mandibular and sub-scapular edema, loss of general body condition, inactivity and weight loss that were generally observed before death.

a



b

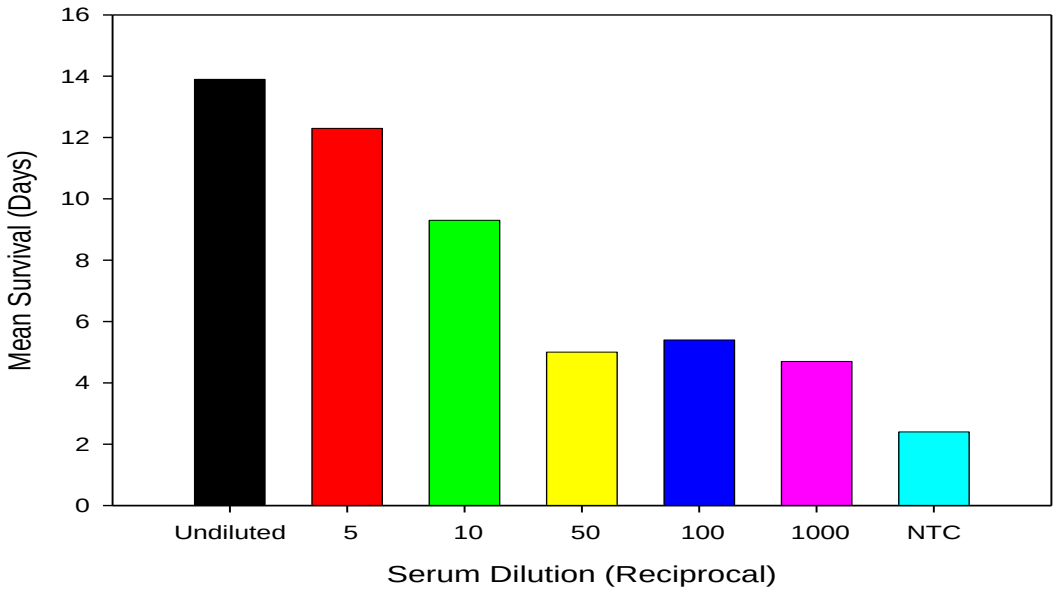


Figure 4.3. (a) A/J mice survival at different dilution of vaccinated goat sera injected into mice followed by lethal challenge of *Bacillus anthracis*, (b) Average time to death (mean survival in days) of A/J mice at different dilution of vaccinated goat sera injected into mice followed by lethal challenge of *Bacillus anthracis*.

4.5 COMPARISON OF ELISA, TNA AND PASSIVE PROTECTION TEST RESULTS

The ELISA and TNA titres of sera from individual goats at week 17 (5 weeks after second vaccination), which can be visualized in Figure 4.4, showed no correlation (r^2 = -0.177). The titres of anti-PA antibody measured in sera of the vaccinated goats did not correlate with passive protection in A/J mice (r_s = -0.20; *P* = 0.01). A significant positive correlation between toxin neutralizing antibodies and passive protection test (A/J mice survival following virulent *B. anthracis* challenge) was observed (r_s = 0.59; *P* = 0.01) as well as between TNA and time to death (TTD) (r_s = 0.61; *P* = 0.01). There was high correlation between mean survival days of challenged mice and dilutions (r_s = 0.79 and *P* = 0.01).

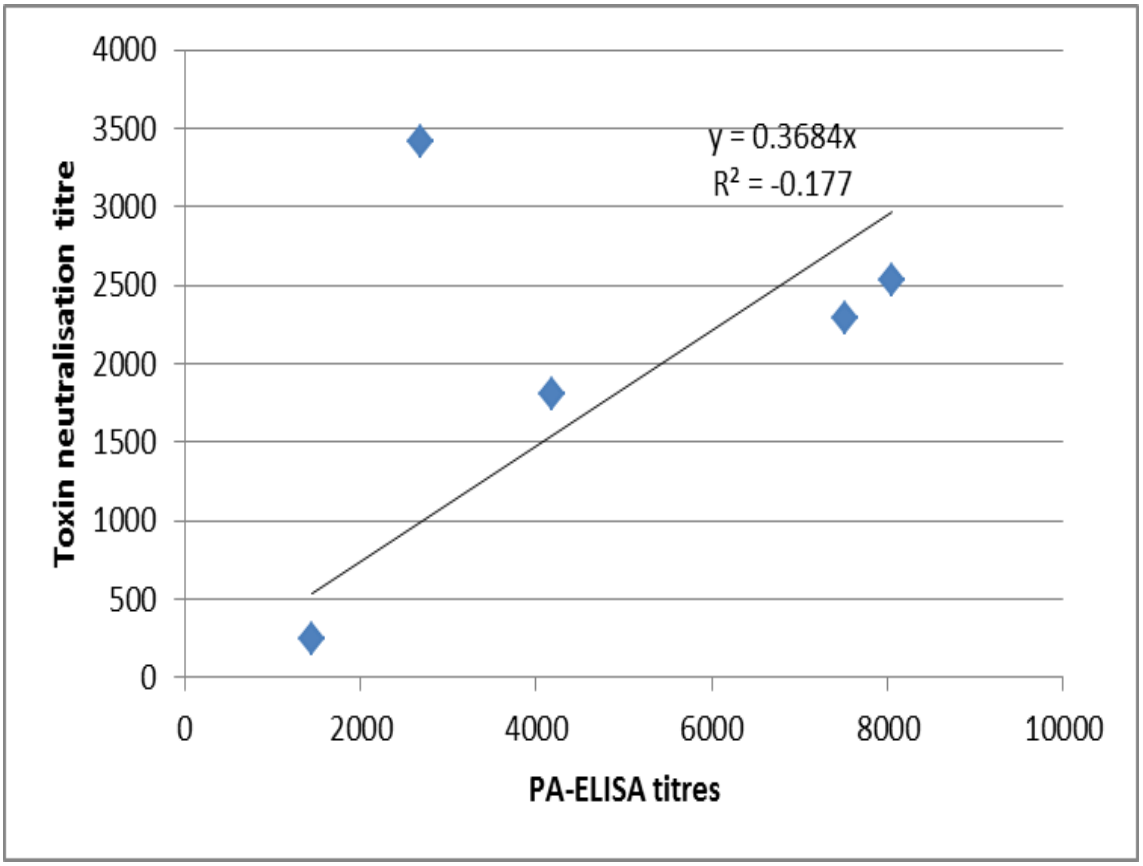


Figure 4.4. Antibody titres as measured by ELISA and toxin neutralization assay (TNA) for each goat five weeks after second vaccination (week 17). The *x*-axis represents the PA-ELISA with the *y*-axis indicating the toxin neutralizing titres respectively.

CHAPTER 5: DISCUSSIONS

In this study we demonstrated that the passive protection test using A/J mice is a valuable and worthwhile prospective test for assaying the protective potential of the antibody response elicited in goats vaccinated with the Sterne live spore vaccine. The results demonstrated that an antibody response is important in protection against *B. anthracis* infection as we observed that goat sera injected into A/J mice were protective following lethal challenge as well as dilution of the sera resulted in depletion in protection. We also perceived that hyper-immunized goat sera (goats vaccinated twice with Sterne vaccine 12 weeks apart) with neutralizing antibody titres of about 200 conferred protection to A/J mice against 2 LD_{100} *B. anthracis* Sterne strain spores challenge. The amount of goats (n=5) as well as mice (n=3) used in this study limited statistical analysis and should be only viewed as preliminary. However this study provide valuable insight in the potential use of immunological data and passive protection tests to measure protection conferred following hyper-immunization (revaccination) with the Sterne vaccine.

Anti-PA response measured by ELISA and/or TNA was investigated as antibodies have been shown to confer protection against anthrax (Beedham *et al*., 2001; Pitt et *al*., 2001; Reuveny *et al*., 2001). ELISA has been widely applied for the evaluation of immune response to development of new anthrax vaccine and in confirmatory diagnosis of anthrax both in animals and humans (Shlyakhov *et al*., 1973; Johnson-Winegar, 1984; Harrison *et al*., 1989). In our study the anti-PA ELISA antibody titres did not correlate with the degree of protection, a fact previously observed by Ndumnego (2012) also using the Sterne vaccine, recombinant and DNA vaccines in goat whereas various other studies indicated a positive correlation in different species with recombinant vaccines (Pitt *et al*., 2001; Quinn *et al.*, 2004; Merkel *et al.*, 2013). It appears this depends on the animal species in use and whether the authors considered if ELISA is capable of detecting accurate changes in antigen quantity and quality. Merkel *et al.* (2013) reported a positive correlation between anti-PA IgG ELISA titre and TNA titre for PA-based vaccines, but expressed concern about the applicability of the TNA titre to dependable correlate protection against inhalation anthrax as rabbits vaccinated with PA-based vaccine surviving a greater than 200 LD_{50} inhalations spore challenge but did not have any detectable toxin neutralizing antibodies. In our study we also observed non detectable toxin neutralizing antibody titres

following the first vaccination. Price *et al*. (2001) reported protection following immunization against the LF antigen alone for the first time. They have used a DNA vaccine encoding LF that was applied with a gene gun and the animals were protected from a challenge intravenously with 5 LD_{50} of lethal toxin. All the immunized animals survived. The resulting anti-LF antibodies following DNA-based immunization were capable of inhabiting the binding between PA and LF in vivo, thus preventing the formation of the LT complex (Price *et al*., 2001). Numerous studies have shown PA by itself to be able to produce a sufficient protection in the absence of EF and LF other *B. anthracis* antigens to protect experimental animals against anthrax (Ivins *et al*., 1986, Ivins and Welkos, 1988). Due to the essential role played by PA in anthrax pathogenesis, PA alone has been the primary target for any anthrax vaccine studies. When evaluating the LF and PA antigens, LF antigen have been shown to be more immunogenic and produce an immune response which lasts much longer than the response to the PA antigen. Thus, they initially hypothesized that it is feasible to use a DNA-based immunization strategy against anthrax and that any future vaccine against anthrax should considerer incorporation of a mutated version of the LF antigen (Price *et al*., 2001).

Anthrax TNA is a technique designed to measure the ability of elicited anti-PA immunoglobulin to protect certain susceptible cell lines from the lethal effects of anthrax toxin (Reuveny *et al*., 2001; Pitt *et al*., 2001). TNA is not species dependent and has been standardized for use with multiple species (Hering *et al*., 2004; Ngundi *et al*., 2010). This study clearly indicates the strong role toxin neutralizing antibodies might play in protecting a caprine model against challenge with virulent anthrax spores. The protective antibody titres directly correlated to the passive protection test (survival) in mice as it has been shown in various animal species (Little *et al*., 2004; Weiss *et al*., 2006; Peachman *et al*., 2006). We found that TNA titres of approximately 200 can confer protection in the passive protection test performed in this study. However, one notable observation is that sera from goat D31 provided 100% protection to A/J mice in the passive protection test for undiluted sera (Table 4.1) despite relatively low TNA antibody levels (Figure 4.2) but did not provide protections when diluted 1:10; 1:50 and 1:100 (Table 4.1). The TNA and ELISA tires of D31 was the lowest which is reflected in the passive protection dilution experiment as indicated (where undiluted provided protections but other dilutions did not provide protections as most of the mice died).

Our study provides additional evidence to support the observed protective efficacy with toxin neutralization assay titres in guinea pigs, where Reuveny *et al.* (2001) showed that TNA titres as low as 220 will provide some level of protection (guinea pigs were subcutaneously immunized with PA vaccine and TNA titres of 220 fully protected the guinea pigs against lethal challenge). With our results, we can propose that neutralizing antibody titres of > 200 in revaccinated (hyper-immune) goats could strongly correlate with protective efficacy of the vaccine and is likely sufficient to confer protection in goats against lethal challenge. In related experiments, Ndumnego (2012) have observed neutralizing antibody titres of 230 to be sufficient in protecting Boer goats against challenge with 20MID (Minimum infective dose) of virulent *B. anthracis* spores. Boer goats were vaccinated subcutaneously with Sterne live spore vaccine and challenged subcutaneously with 843 spores of a virulent *B. anthracis* strain 58 weeks after vaccination. In agreement with previous studies, our findings are in contrast, support and give Reuveny *et al*. and Ndumnego studies strong backing.

Low neutralization titres have previously been found to be sufficient in protecting guinea pigs against challenge with 40 LD_{50} of virulent Vollum strain (Reuveny *et al*., 2001), but these titers will differ between species employed as indicated by Fay *et al.* (2012) and other studies (Ivins *et al*., 1992; Pitt *et al*., 2001; Fellows *et al*., 2002). Statistical methods are applied to enable protection prediction using immune response, which requires minimal number of animals necessary to correctly interpret results. In this study, ethical and budget constraints limited the amount of animals (goats and specifically A/J mice used in passive protection test) and therefore limited the interpretation and conclusions of the results. The TNA antibody level in goats that confer protections needs to be further investigated using the passive protection test in different ruminant species and mice per animal before conclusive interpretations can be made.

Additionally, we investigated the potential use of a host specific macrophage cell line in the toxin neutralization assay to reflect protection or efficacy of the Sterne vaccine in host animals compared to the mouse J774A.1 cell line. The J774A.1 cells are the most commonly used cells in *in vitro* toxin neutralization assay. Other cell lines widely used in the TNA are RAW 264.7 (mouse leukaemic monocyte macrophage cell line) and Chinese hamster ovary (CHO) cell-based TNA (Ngundi *et al*., 2010). The effects of lethal toxin on the standard mouse macrophage cell

line (J774A.1) compared to caprine mouse macrophage (host specific), BOMAC (include as host-specific to be used with Sterne vaccinated bovine sera) and dog macrophage (non-host specific) cell lines were envisaged as part of this study. Therefore we tried to establish a goat macrophage cell line to be used in TNA. A continuous monocyte-macrophage cell line method using PBMC from caprine as obtained by Wardley *et al.* (1980) could not be established despite optimization attempts. Establishment of spontaneous monocyte/ macrophage cell lines is of particular interest because monocytes and macrophages could replicate *in vitro* (Lewis, 1925; Chang, 1976). We used Wardley *et al.* (1980) method because it is convenient and has been demonstrated to successfully yield continuous macrophage cell line which was not possible in this study.

Consequently, we obtained bovine (host) and dog (non-host) macrophage cell lines to serve as macrophage cell lines to test Sterne vaccinated and unvaccinated caprine/bovine sera (positive and negative controls) using the TNA. The murine macrophage cell line J774A1 used at present in TNA assay is susceptible to the lethal effects of toxin whereas both DH82 and BOMAC cells survived following exposure to similar concentrations. This shows DH82 and BOMAC cell lines are relatively inert to the deleterious effects of lethal toxin. The macrophage cell lysis induced by LT and release of associated intracellular cytokines (transient cytokine induction) observed in murine models of infection are important consequences of functional activity of *B. anthracis* lethal toxin. In fact, macrophage cell lines from several strains of murine and human macrophages are the target for the activity of LT (Prince, 2003). The macrophages from mouse strain such as Balb/C that is more sensitive to rapid lysis by LT than the C57BL/6 “resistant” mice strain, implies resistant macrophages may not undergo lysis and release cytokines (Friedlander *et al*., 1993; Prince, 2003). Several studies have, however, provided some evidence about the roles of CMG2 and TEM8 as anthrax toxin receptors *in vitro* (Bradley *et al.,* 2001; Scobie *et al*., 2003). In the absence of these receptors, host cells are resistant to anthrax toxins. These led us to suggest that the resistance of the BOMAC and DH82 macrophages to anthrax toxin might be due to the absence of these or similar receptors which needs further investigation.

A passive protection test was used to investigate the ability of the Sterne revaccinated goat sera to protect A/J mice against a subcutaneous challenge with the Sterne 34F2 *B. anthracis* vaccine

strain. We utilized A/J mice because of a deficiency in the complement system (the C5 genes) (Welkos and Friedlander, 1988) and associated susceptibility to toxigenic but nonencapsulated strains of *B. anthracis* such as Sterne or STI-1 (Beedham *et al*., 2001). A/J mice (complement-deficient) are significantly more susceptible to lethal toxins and succumb more rapidly to the deleterious effect of anthrax lethal toxins secreted during infection. In this study the percentage survival decreased as the dilution of sera injected in mice followed by Sterne strain challenge increased. The data obtained by the passive protection experiment indicated that mice receiving sera with lower dilutions were better protected than mice receiving higher serum dilutions.

This observation is supported by the fact that undiluted and 1:5 diluted sera showed the highest protection (86% and 80% respectively). Also, for non-survivors, the undiluted and 1:5 diluted sera groups had the highest TTD averages of 13.9 and 12.3 days respectively. Protective antibodies did not exist in the sera from unvaccinated (naive; negative control) goats. As expected, none of the mice injected with serum from naïve goats (unvaccinated control group) survived lethal challenge with Sterne spores, confirming that humoral anti-anthrax antibodies played a crucial role in protection against anthrax. The level of protective antibodies is very important with respect to anthrax vaccine improvement and animal protection studies in the veterinary field especially considering the ethical and logistical challenges associated with large animal trials. The use of passive protection test will prevent the use of higher species and also have the additional advantage of not requiring a fully virulent *B. anthracis* strain for the challenge and consequent high biosafety level specification.

Typically, the anti-PA IgG antibody titres were much higher than the toxin neutralizing titres since the latter is assumed to be a subset of the anti-PA IgG set in the serum. Based on previous studies, toxin-neutralizing PA-specific IgG antibodies have been identified as a correlate of protection (Reuveny *et al*., 2001; Little *et al*., 2004; Fay *et al*., 2012). It has also been noted previously that high anti-PA titres do not necessarily provide protection to challenge (Turnbull *et al*., 1986; Welkos and Friedlander, 1988). ELISA anti-PA antibody titres did not correlate with passive protection despite induction of high titres of anti-PA antibody while there was significant correlation between *in vitro* toxin neutralizing antibodies and passive protection test of mice. The antibodies detected by the TNA are indicative of the neutralizing effect of anti-PA antibodies

present in the vaccinated goats and could be used to determine the level of protection. Hence, a correlative relationship between TNA and a passive protection model can be utilized to determine possible protective capacity of the respective serum in question. While the results of this study are encouraging, further work is needed to improve protection. We can conclude that humoral antibodies are crucial in the protection of naïve animals from anthrax infection and that anti-PA neutralizing antibodies are the likely main mechanism of protection in goats using an A/J mouse passive protection model.

CHAPTER 6: REFERENCES

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Animal Ethics Committee

PROJECT TITLE	**Model to test protection against anthrax: Comparative studies on the immunological response of the live spore anthrax vaccine in goats and monitoring through passive protection test in mice**
PROJECT NUMBER	**V083-13**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Mr. PH Phaswana**

STUDENT NUMBER (where applicable)	**134 119 60**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Cattle**	
NUMBER OF ANIMALS	**300 (data already obtained)**	
Approval period to use animals for research/testing purposes		**November 2013–December 2013**
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	**28 October 2013**
CHAIRMAN: UP Animal Ethics Committee	Signature	