# IMMUNOGENICITY AND PROTECTIVITY OF A LIVE SPORE *BACILLUS ANTHRACIS* VACCINE IN GOATS

BY

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# List of Abbreviations

| | |
|---|---|
| AUCC | Animal use and care committee |
| AVA | Anthrax vaccine adsorbed |
| AV-UK | Anthrax vaccine-United Kingdom |
| BC | Before Christ |
| BclA | Bacillus collagen-like protein of anthracis |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| CHO | Chinese hamster ovary |
| CMG-8 | Capillary morphogenesis protein-2 |
| $CO_2$ | Carbon dioxide |
| CV | Coefficient of variation |
| CWS | Cell wall skeleton |
| DNA | Deoxyribonucleic acid |
| EF | Oedema factor |
| EIA | Enzyme immunoassay |
| ELISA | Enzyme-linked immunosorbent assay |
| ET | Oedema toxin |
| FIS | Formaldehyde inactivated *Bacillus anthracis* spores |
| HCl | Hydrogen Chloride |
| IMHA | Indirect microhaemagglutination test |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |

| | |
|---|---|
| kDa | Kilodalton |
| KNP | Kruger national park |
| LDL-6 | Low density lipoprotein receptor-related protein-6 |
| $LD_{50}$ | Lethal dose 50% |
| LF | Lethal factor |
| LT | Lethal toxin |
| MTT | 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MAPKK | Mitogen-activated protein kinase kinases |
| $MgSO_4$ | Magnesium sulfate |
| MID | Minimum infective dose |
| MPL | Monophosphoryl lipd A |
| MTTD | Mean time to death |
| NALP1b | NLR family, pyrin domain containing 1 |
| NT 50 | 50% neutralization |
| OBP | Onderstepoort biological products |
| OD | Optical density |
| OIE | International office of epizootics |
| PA | Protective antigen |
| PBS | Phosphate buffered saline |
| PBST | PBS with Tween |
| PBSTM | PBST with skimmed milk powder |
| PGDA | Poly-gamma-D-glutamic acid |
| pXO1 | Plasmid XO 1 |

| | |
|---|---|
| pXO2 | Plasmid XO 2 |
| P-value | Probability values |
| SAVP | South African vaccine producers |
| SDS | Sodium dodecyl sulfate |
| SVG | Sterne vaccine group |
| Re | Per cent recovery |
| rPA | Recombinant protective antigen |
| TDM | Trehalose dimycolate |
| TEM-8 | Tumour endothelial marker-8 |
| TTD | Time to death |

# Thesis Summary

**Immunogenicity and protectivity of a live spore *Bacillus anthracis* vaccine in goats**

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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 only of a sporadic nature in developed countries due to effective vaccination and control measures. The current anthrax live spore veterinary vaccine was developed by Max Sterne and its introduction in the 1940s made the control of the disease possible.

The principal virulence factors of the *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. Attenuated strains lack either of the two virulence plasmids and consequently have reduced virulence. The Sterne 34F2 strain (tox+, cap-) therefore produces the PA, LF and EF components of the anthrax toxin but lacks the plasmid pXO2 encoding capsule formation and is therefore relatively safe, albeit with some residual virulence.

During the development of the avirulent Sterne vaccine, few studies were conducted on the immunogenicity of the vaccine in the target animals. *In vivo* immunity tests (pathogenicity test) using guinea pigs were mainly used due to few and less sensitive serological diagnostic tools being available at that time. The need for serology in relation to anthrax only became apparent during the development of a human vaccine much after the introduction of the veterinary vaccine. Consequently, though anthrax is recognized as a primary disease of herbivores, there are no records of an in depth study on the immunogenicity and protectivity of the widely accepted strain 34F2 Sterne spore vaccine in any ruminant species.

This study was undertaken to fill the present knowledge gap relating to the immunogenicity and duration of protectivity induced by vaccination with the Sterne live spore vaccine in a goat model, which are highly susceptible to the disease. Twenty-one age-matched Boer goats were procured and split into 3 groups of five animals each and vaccinated using different immunization regimens. Six goats served as negative controls utilized in determining the minimum infective dose (MID). Serum samples were collected at intervals for analysis in the laboratory. *Bacillus anthracis* virulent spore

challenge was done not earlier than 3 weeks after the last vaccination and survival was monitored for 14 days.

Our findings revealed the MID of virulent *B. anthracis* spores in naïve goats under experimental conditions to be below 36 spores using the subcutaneous route. Production of anti-anthrax immunoglobulins in the first month following Sterne vaccination was 400-fold higher than pre-vaccination levels with subsequent decline over time to a 50-fold difference, 14 months post vaccination. A similar trend was reflected in the toxin neutralizing antibody titres. There was a correlation between the toxin neutralizing antibody titres and protection against challenge with virulent anthrax spores ($P = 0.01$). Goats challenged 6 and 62 weeks after vaccination showed a survival rate of 60% and 80% respectively. Those revaccinated one year after the first vaccination were fully protected from virulent anthrax spore challenge. Early pre-validation data of a quantitative indirect ELISA for the detection of anti-PA antibodies was promising and should be further investigated as a tool for anthrax vaccine studies in goats.

# CHAPTER ONE

# INTRODUCTION

Anthrax is primarily a disease of herbivores affecting most warm-blooded mammalia. It is caused by the bacterium *Bacillus anthracis* and has a world-wide distribution. 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 which they 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. For example, in 1920, 1891 outbreaks of the disease were reported in South Africa alone, killing over 6000 cattle (Gilfoyle, 2006). With the introduction of the live attenuated Sterne 34F2 spore vaccine in the 1940s, controlling the disease became possible (Sterne et al., 1942). This period marked an important epoch in the control of anthrax not just in South Africa but in other parts of the world as the strain was adopted as the main veterinary vaccine against the disease. South Africa, as at 2005, was projected to have a livestock population of 13.8, 25.3 and 6.4 million cattle, sheep and goats respectively and the Sterne 34F2 live spore vaccine takes a vital position in protecting these highly susceptible species from the deadly anthrax disease.

Anthrax is a zoonosis and humans contract the disease through contact with animals, or animal products, that have died of the disease. Anthrax does not spread directly from one infected animal or person to another but is spread by spores. The spores can persist in the environment for very long periods as they are resistant to adverse environmental conditions.

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 respectively (Mikesell et al., 1983b, Uchida et al., 1986, Pezard et al., 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). Pasteur's vaccine, a variant of *B. anthracis,* was attenuated by cultivating the pathogen over a period of time at a high temperature. The reason for 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 $^{\circ}$C results in the partial loss of the toxin-encoding plasmid pXO1. The Pasteur vaccine provided low levels of protective immunity since the PA of *B. anthracis* is encoded by the lost pXO1 plasmid in the preparation of Pasteur type vaccines. Pasteur vaccines were displaced by the vaccine of Sterne utilising a toxin positive, capsule negative strain 34F2 (tox+, cap-) (Sterne et al., 1942). While this strain produces the protective factor (PA), lethal factor (LF) and oedema factor (EF) components of the anthrax toxin, it lacks plasmid pXO2 encoding capsule formation and is therefore relatively safe, albeit with some residual

virulence (Sterne, 1939, Turnbull et al., 1986, Cartwright et al., 1987, Welkos and Friedlander, 1988).

Following the development of the avirulent Sterne vaccine and subsequent field trials, there were few studies conducted on the immunogenicity of the vaccine by Max Sterne (Sterne, 1939), though the excellent efficacy and protectivity of the strain against anthrax infection has never been in doubt. *In vivo* immunity tests (pathogenicity test) using guinea pigs were mainly used due to few and less sensitive serological diagnostic tools being available at that time (Turnbull et al., 1992). I*n vivo* immunity tests of the Sterne vaccine mainly involved efficacy trials where guinea pigs were injected with different concentrations of vaccine spore suspensions to determine the minimum concentration that protected the guinea pigs against a challenge with a standard dose of virulent spores (Sterne, 1937). Furthermore, a field test to ensure that the vaccine did not induce adverse reactions was done on sheep using four times the standard vaccine spore concentration (Sterne, 1946). 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). The principal test was gel diffusion (Ouchterlony, 1953) that was very effective in the detection of antigens (and not antibodies) until the first application of the enzyme immunoassay (EIA) twenty years later using Dowex-I-XI-purified PA (Johnson-Winegar, 1984b). Presently, there are no records of any in-depth studies on the immunogenicity and protectivity of the Sterne vaccine strain 34F2 spores in a ruminant species. The current accepted immunization regimen of yearly re-vaccinations for

susceptible livestock in endemic areas has been based more on anecdotal rather than on empirical evidence (Turnbull, personal communication).

This dissertation thus describes studies comparing the immunogenicity, protective efficacy and safety of the commercial Sterne strain 34F2 spore vaccine in goats. These animals were used as the target specie since they have been reported to be highly susceptible to anthrax and exhibit adverse reactions to the vaccine (Sterne, 1946). The immunogenicity of the vaccine was studied using current accepted diagnostic tools in anthrax vaccine development namely; the enzyme-linked immunosorbent assay (ELISA) and anthrax toxin neutralization assay (TNA). Vaccine protection was assessed by virulent spore challenge.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 History of an ancient and modern disease

"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 the Egyptians in 1491 B.C. (Hambleton et al., 1984). All 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 bison population in Northwest Canada (Promed, 2012). Essentially a disease of herbivores (Beyer and Turnbull, 2009), it was largely responsible for 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, 1999, Hugh-Jones and Blackburn, 2009)

In the early 20th century, anthrax was a major cause of death in livestock within South Africa (1891 outbreaks reported in 1920) (Gilfoyle, 2006). The introduction of compulsory vaccination of livestock reduced the disease incidence. However, it still

remains endemic in sub-Saharan Africa, central Asia, parts of China, Indian sub-continent and Southern Europe (Beyer and Turnbull, 2009).

A clear description of the disease was first given in 1780 by Chabert (Wistreich and Lechtman, 1976) and the famous "Koch postulates" was based upon Robert Koch's work on anthrax. Davaine was the first person to show the "organisms" that was responsible for anthrax in susceptible animals (Davaine, 1863). He showed that the blood from an infected animal contained the causative organisms 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 aetiological agent of anthrax and not some contaminant or elements of the infective blood of a diseased animal (Pasteur and Joubert, 1877, Duclaux, 1896, Schwartz, 2009).

*Bacillus anthracis* is an aerobic, facultative anaerobic, endospore-forming, rod-shaped bacterium (OIE, 2012, International Office of Epizootics, 2008). Measuring 4 µm by 1 µm, it 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 the presence of oxygen. The vegetative cells secrete their polypeptide capsule when cultured under experimental conditions of high $CO_2$ partial pressure, bicarbonate and absence of oxygen. The capsular nature of *B. anthracis* was first observed by M'Fadyean in 1904. Fully virulent strains of the bacillus give rise to

rough, non-haemolytic to mildly haemolytic colonies in blood agar while smooth mucoid colonies are seen with avirulent strains (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 and Leppla, 2009,Tang and Guo, 2009) while LF (90 kDa) is a zinc-dependent metalloprotease cleaving specifically the N-terminus of most mitogen-activated protein kinase kinases (MAPKK) (Vitale et al., 2000; Moayeri and Leppla, 2009). The B component, protective antigen (PA) so-called because of its immunogenic properties (Wright et al., 1954, Stanley and Smith, 1963) 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; tumour endothelial marker-8 (TEM-8) and capillary morphogenesis protein-2 (CMG-2) (Bradley et al., 2001). Recently, a third co-receptor named LDL receptor protein (LRP-6) has been proposed (Wei et al., 2006, Trescos and Tournier, 2012). Binding of PA83 to its receptors allows its cleavage by a furin-like protein 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 forms 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., 2002a, b).

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 and Dietrich, 2006). The oedema toxin (ET) induced rise in cyclic AMP also inhibits phagocytosis, microbicidal activity, neutrophil chemotaxis and superoxide production (Turk, 2007). It is suggested that the dual sublethal 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 and Leppla, 2004). The shutdown of the innate immune responses allows *B. anthracis* to evade host defences leading to extensive bacteraemia.

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 and 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. The capsule is directly anchored to the underlying peptidoglycan cell

surface by a covalent bond (Candela and Fouet, 2005) and has been reported to enhance the activity of lethal toxin (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 and Leppla, 2004). It should be noted that most of the extensive studies done on anthrax virulence factors were performed with mice as experimental model. Though widely acknowledged as a primary disease of herbivores, 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

Anthrax can be transmitted through the cutaneous, gastrointestinal or by inhalational routes. 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 et al., 1984, International Office of Epizootics, 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 et al., 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 and 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 contract anthrax when they feed on the contaminated leaves (Braack and De Vos, 1990, De Vos, 1990, De Vos and 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, the disease is acute and runs a rapid course (Bhatnagar and Batra, 2001).

Data on the natural infectious dose of anthrax spores in ruminants is not readily available. A minimum oral infectious dose (MID) of $5\times10^{8}$ spores is estimated for sheep, horses and cattle (International Office of Epizootics, 2008). Published $LD_{50}$ of anthrax spores by the subcutaneous route were 100, 3000, 5000, $10^{6}$, $10^{9}$ and $5\times10^{10}$ for sheep, rhesus monkeys, rabbits, rats, pigs and dogs respectively (Schlingman et al., 1956, Watson and 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 and Jabbari, 2007). It is important to point out that these $LD_{50}$ or MIDs only provide rudimentary guide to the likely or actual infection in nature as factors such as anthrax strain, route(s) of infection, species, breed and state of health of animal(s) play an important role in its epidemiology (International Office of Epizootics, 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 M., 1959). Blood may ooze from natural orifices and the urine might be blood-tinged (International Office of Epizootics, 2008). Oedematous or carbuncular lesions can be seen in animals with waning immunity (Sterne M., 1959). Anthrax generally takes a peracute nature in goats (International Office of Epizootics, 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 and 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 M., 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 (International Office of Epizootics, 2008).

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 immunized animals

(International Office of Epizootics, 2008). Post mortem gross pathology includes extravasation of blood from body orifices, splenomegaly, dark poorly clotted blood and incomplete rigor mortis (Hambleton et al., 1984).

## 2.4 Host immune response to *Bacillus 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 utilizing ELISA using *B. anthracis*-specific antibodies (Mahlandt et al., 1966, Ivins and Welkos, 1988, Turnbull et al., 1992, Turnbull et al., 2004, Taft and Weiss, 2008). PA is the most essential component of living, attenuated or protein-based vaccines (Leppla et al., 2002) and strains of *B. anthracis* 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 (Welkos et al., 1993; Leppla et al., 2002). 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 a protective response in an experimental model of infection (reviewed by Cybulski et al., 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). Herbivores rarely have natural anti-anthrax antibodies, while carnivores in anthrax enzootic areas are sometimes known to possess these specific antibodies (Turnbull et al., 1992). This phenomenon can be interpreted as being indicative of the susceptibility of the different species to infection. While production of antibodies to LF and EF have been shown in animals, their roles in mediating protection remain less clear (Taft and Weiss, 2008). 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 and 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. Recently, Quesnel-Hellmann et al. (2006) showed that EF acts as an adjuvant to potentiate the anti-PA response 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 and Knudson, 1986; Taft and Weiss, 2008,). 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, 1992, 2001).

## 2.5 Anthrax vaccinology: The story so far

Anthrax was one of the first bacterial diseases to be controlled by vaccination. The active component of Louis Pasteur's vaccine formulated in 1880 was *B. anthracis*, attenuated by serial culture at elevated temperature. The elevated temperature was shown more than 100 years later (Mikesell et al., 1983b) to result in the partial loss of the toxin-encoding plasmid pXO1 in the Pasteur vaccine. Pasteur vaccines therefore retain some residual virulence but the reduction of the principal PA also occured during attenuation and 50 years later the Pasteur vaccine was displaced by the vaccine of Sterne using a toxin positive, capsule negative strain 34F2 (Sterne, 1937; Sterne et al., 1942).

Since the development 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 et al., 1961, Stanley and Smith, 1963). The improved purifications (Leppla, 1982, 1986, Quinn et al., 1988, Leppla, 1991) allowed the development of enzyme immunoassays by which it became 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 (Turnbull et al., 1986, 1988, Ivins and Welkos, 1988, Ivins et al., 1986, 1990, Turnbull et al., 1990). The second major advance with significant bearing on the development of improved vaccines was the discovery (Mikesell et al., 1983a, b, 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. These developments led to the demonstrations of the need for the toxin, or some part of it, in a vaccine for induction of protective immunity (Ivins et al., 1986) and the ability of PA to produce effective protection in the absence of EF and LF (Ivins et al., 1986, Ivins and 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, 1990). These progressive discoveries, then, pointed the way to a number of approaches aimed at second generation improved vaccines that mainly included recombinant vaccines. Ivins et al. (1986, 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, 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 1954, Brachman 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. Further drawbacks include the high number of boosters required, variations in the bacterial cultures used and a number of clinically relevant side effects. Efforts are being made to develop improved alternative vaccines by using recombinant antigens, recombinant bacterial and viral vectors and plasmid vectors

and by assessing alternative testing for combinations with new adjuvants (Beyer, 2004). Special attention is being given to supplementing existing toxoid vaccines with an anti-bacterial component (Hahn et al., 2006b).

In the case of naturally acquired immunity following recovery from infection, it is assumed that defence is targeted at spores, spore germination and self-propagating vegetative pathogens. It is therefore significantly more effective than vaccination with toxin antigens. Other antigens may therefore also contribute to the increase of protectivity of a protein vaccine. Findings from research on the protectivity of the recombinant PA (rPA)/alhydogel vaccine and formaldehyde inactivated *B. anthracis* spores (FIS) give this assumption strong backing (Brossier and Mock, 2001). The combination was able to protect 100% of the trial animals (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 immunized with rPA and alhydrogel. These studies, however, have never been done in animal species for which the vaccine was intended. 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 opsonisation and from destruction by complement activation (Makino et al., 1989, Ezzell Jr and 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 (Schneerson et al., 2003, Rhie et al., 2003).

The induction of both humoral and cell mediated immune responses by applying the genetic information of antigenic determinants in eukaryotic cassettes (nucleic acid immunisation) is another promising approach in anthrax vaccinology (Gu et al., 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 immunised animals were protected from a challenge with the lethal toxin (Gu et al., 1999) Price et al. (2001) took these trials a step further using 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 immunised animals survived, 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. Two doses of this plasmid, given intramuscularly, protected 100% of rabbits from aerosol challenge with about 100 $LD_{50}$ of Ames spores. In recent trials (Hahn et al., 2004, 2006a, 2006b), the efficacy of DNA vaccines was tested in mice and sheep. By combining antitoxic (anti-PA) with antibacterial (anti-BclA) immunisation and optimising antigen presentation Hahn et al. (2006a) showed for the first time the possibility of protecting mice against infection with fully virulent spores of the Ames strain.

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 (Klein et al., 1962, Ivins et al., 1986, Little and Knudson, 1986, Turnbull et al., 1986, Ivins and Welkos, 1988). It should be noted that in all these comparisons the same adjuvants were used (aluminium hydroxide or alum) and that most of the trials were carried out using guinea pigs. As we know 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, 1994, 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 immunisation 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 MPL+CWS+TDM (Turnbull, 1991). The use of the RIBI adjuvant, however, is contraindicated on account of known and significant side effects in animals. 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 (Loleit et al., 1996, Schild et al., 1989, 1991, Wiesmüller et al., 1989)

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 seen and thus the latter remains the veterinary vaccine of choice against anthrax infection in livestock.

## 2.6 Anthrax immunology: Evolving diagnostic tools

The assessment of animal sera for the presence of anthrax-specific antibodies is mostly done for research purposes. The monitoring and evaluation of immune response induced by the administration of experimental vaccines is a crucial point in the development of new anthrax vaccines. The "Anthrax Euronet" protocols specify that the parameters being assessed should correlate with protection in the test animal (http://ec.europa.eu/research/fp6/ssp/anthrax_euronet_en.htm) and this is usually associated with the presence of antigen specific antibodies. Over the years, since the development of the Sterne veterinary vaccine in the 1940s, various methods have been employed in the assessment of anthrax antibodies in the sera of vaccinates. Initially, there was no known serological method of measuring these protective antibodies and testing of new vaccines was through *in vivo* immunity tests on guinea pigs, sheep and other species (Sterne, 1937, Wright et al., 1954, Auerbach and Wright, 1955, Schlingman et al., 1956). Following the isolation, purification and production of the proteinaceous toxic component of anthrax 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 and Strange, 1954, Puziss and Wright, 1954, Wright et al., 1954, Turnbull et al., 1992,). This and the subsequent improvements will be listed in the following subsections.

2.6.1 Agar-gel diffusion: Agar-gel diffusion was first developed by a Swedish physician, Organ Ouchterlony in 1953, it was first utilized in the determination of antigen and antibody concentrations in culture filtrates and antisera of vaccinated animals by Thorne and 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 and 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 and Kadull, 1964).

2.6.2 Indirect microhaemagglutination test (IMHA): With increasingly improved methods of purification of PA which led to better yields (Wilkie and Ward, 1967, Fish et al., 1968), a new method for the detection of anthrax antibodies in sera was published in

1971 (Buchanan et al., 1971). The anthrax 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, 1984a). 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 fourfold 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, 1984a) and it was never fully accepted as a standard test for the assaying of anthrax antibodies in animal sera.

2.6.3 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 and Berson, 1960). In 1971, two independent researchers published techniques in which they replaced radioactively-labelled antigens/antibodies with enzyme-linked variants (Engvall and Perlmann, 1971, Van Weeman and Schuurs, 1971). ELISA has since come to be the gold standard assay utilized 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, 1984a). 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 more rapid, simple 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, 1984a). Various versions and adaptations of the ELISA have been developed in various studies to assess the immune response following vaccination of animals (Turnbull et al., 1986, Little et al., 2004, Hahn et al., 2006a). 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 utilized (Williams et al., 1984).

2.6.4 Toxin neutralization assay (TNA): Following several studies depicting the effects of anthrax lethal and oedema toxins on sensitive 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 (Reuveny et al., 2001,Pitt et al., 2001). Three types of TNAs have been developed (Ngundi et al., 2010). The J774A.1

(J774) and RAW 264.7 (RAW) are murine macrophage-like cell based assays which measure the ability of anti-PA antibodies to neutralize the cytocidal activity of LT (Reuveny et al., 2001, Pitt et al., 2001). 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 (Ngundi et al., 2010). 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 (Mosmann, 1983, Abe and Matsuki, 2000). The amount of ET-induced increase of cAMP in susceptible unprotected CHO cells is estimated using chemiluminescent cAMP ELISA. TNA is highly versatile, species independent and has been standardized for use with multiple species (Hering et al., 2004, Zmuda et al., 2005, Omland et al., 2008). This is a major advantage of the TNA compared to the conventional ELISA. The TNA also quantifies functional anti-anthrax antibodies rather than total antibodies (Omland et al., 2008), but it is relatively more time consuming and elaborate in comparison to ELISA.

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# CHAPTER THREE

# Evaluation and efficacy studies on immunogenicity and protectivity of the Sterne live spore *Bacillus anthracis* vaccine in goats

## 3.1 Introduction

Anthrax, a primary disease of herbivores (Hambleton et al., 1984), is caused by *Bacillus anthracis*. In nature, the disease is transmitted to herbivores by viable resistant spores present in contaminated foliage and soils. The disease progresses as a peracute or acute infection in ruminants after an incubation period of 3-5 days (Beyer and Turnbull, 2009). The main virulence factors are encoded by two plasmids; pXO1 which codes for the three toxin components of protective antigen (PA), lethal factor (LF) and oedema factor (EF) (Vodkin and Leppla, 1983, Robertson and Leppla, 1986, Mock et al., 1988) whereas pXO2 encodes the anti-phagocytic poly-ɣ-D-glutamic acid capsule (PDGA) (Uchida et al., 1985) The virulence of *B. anthracis* is dependent on the expression of both plasmids. PA, LF and EF are coded independently by pXO1 and PA combines with LF and EF to form the lethal toxin and oedema toxin (LT and ET) of anthrax respectively (Gauthier et al., 2009b). The toxins are characterized by an A moiety (LT and/or ET) that acts within the cytosol of the target cells and a B moiety (PA) that binds target cells and translocate the A moiety into the cytosol (Mock and Fouet, 2001) After secretion, the three proteins assemble on the target cell surface where PA (83 kDa) binds to the ubiquitous anthrax toxin receptors coded by the *tumour endothelial marker-8* (TEM-8) and *capillary*

*morphogenesis gene-2* (CMG2) (Bradley et al., 2001, Scobie et al., 2003). PA is cleaved by a furin-like protease and the resulting amino-terminal 20 kDa fragment (PA20) dissociates from the receptor bound carboxy-terminal 63 kDa fragment (PA63) and is released into the extracellular compartment. The receptor-bound PA63 then spontaneously oligomerizes into a heptamer and binds EF and/or LF. The assembled toxic complexes are internalized by receptor-mediated endocytosis and translocated into the host cell cytosol, where it exerts cytotoxic effects (Young and Collier, 2007). LT is a zinc metalloprotease that inactivates most mitogen-activated protein kinase kinases leading to impairment and death of susceptible macrophages (Friedlander, 1986, Banks et al., 2005) while ET is a calmodulin dependent adenylate cyclase which catalyzes the activities of cyclic AMP, leading to the disruption of fluidic homeostasis in the host cell (Leppla, 1982).

The PDGA is weakly immunogenic and assists in the dissemination of *B. anthracis* in the body of infected animals (Candela and Fouet, 2005). Recent research has linked PDGA with LT in the blood of experimentally infected animals and this has been shown to significantly enhance the deleterious effects of the lethal toxin in mice (Jang et al., 2011). Disguisement of the anthrax bacilli by PDGA capsule enables it to evade immune surveillance mechanisms, and to enter the circulatory system where it proliferates systemically (Makino et al., 2002, Sutherland et al., 2008).

The current anthrax veterinary live spore vaccine is a non-capsulated but toxinogenic variant which was developed in 1937 by Max Sterne by growing a virulent strain on serum agar in carbon dioxide (Sterne, 1937). The Sterne vaccine has been utilized widely in the control of anthrax in domestic animals since its development (Hambleton et al., 1984) and had proved to be safer than the earlier heat-attenuated capsulated vaccines (Sterne, 1939). Currently, revaccination every 9-12 months is advocated to ensure year-long protection (Anthrax vaccine leaflet, Onderstepoort Biological Products, South Africa). Concerns still remain on the safety of the vaccine in species like goats which were first indicated by Sterne (Sterne, 1939) to be more affected by the uncapsulated strains than other domestic animals. Sterne's trials with the anthrax vaccine in different domestic species had involved mainly protection trials following vaccination (Sterne, 1937, Sterne et al., 1942), without much elucidation on the immune response in the target animals due to the unavailability of more specific tests at the time. The need for serology in anthrax vaccine research and development became apparent in relation to the search for efficacious human vaccines (Thorne and Belton, 1957, Ray and Kadull, 1964, Turnbull et al., 1992). Presently, few data are available on the immune response stimulated by the Sterne live spore vaccine in a ruminant specie and its relevance to protectivity.

Two types of assays, an antigen-specific ELISA (Pombo et al., 2004) and a lethal toxin neutralization assay (Hering et al., 2004) are available to measure antibodies after vaccination with a candidate anthrax vaccine. The ELISA is utilized in the measurement of antibodies that bind PA. Anti-PA immunoglobulins have been shown to be essential

for protection against challenge with virulent anthrax spores and correlated with protection in different animal models (Ivins and Welkos, 1988, Turnbull et al., 1988, Little et al., 2004b). The TNA measures the ability of elicited anti-toxin immunoglobulins to protect the susceptible mouse macrophages from the lethal effects of the anthrax toxin. It detects a functional subset of the anti-anthrax antibodies and is considered to be species independent (Hering et al., 2004, Omland et al., 2008, Ngundi et al., 2010).

This study evaluated the immunogenicity and protective efficacy of the Sterne live spore vaccine licensed for livestock use in South Africa and neighboring countries. The animal experiments were carried out with Boer goats which are known to show adverse reactions to the vaccine. We determined the minimum infective dose of spores from a virulent strain of *B. anthracis* in naive goats. We also evaluated the immune response after vaccination with the Sterne vaccine using current serological tools. Attempts were also made to establish possible correlates of protection detected by these serological tools.

## 3.2 Materials and Methods

### 3.2.1. *Bacillus anthracis* strains

The Sterne live spore vaccine consisting of glycerine suspension of *B. anthracis* strain 34F2 at a spore concentration of 6 x $10^{6}$ per dose as produced by Onderstepoort Biological Products (OBP, South Africa). This immunization was done according to the manufacturer's instruction which stipulates the subcutaneous injection of 1 mL of the suspension for all ruminants and pigs, irrespective of species, breed or age. The challenge strain was isolated from a 2002 ovine carcass that was confirmed positive for anthrax by bacteriology at the Veterinary faculty, University of Pretoria, South Africa. The *B. anthracis* strain was confirmed to contain both pXO1 and pXO2 plasmids using molecular typing described by Keim et al. (2000) and Lista et al. (2006).

### 3.2.2. Animals

Eight-week old BalB/c mice (n=6) procured from SAVP (Sandringham, South Africa) were used to confirm the virulence of the *B. anthracis* challenge strain. These mice were housed in autoclavable cages enriched with shredded tissue paper holders. Healthy age-matched Boer goats which were confirmed to be PA-reactive antibody negative by PA-ELISA and kept at the experimental animal facility of OBP during the immunization period. These animals comprised of female and emasculated male goats. They were treated against round and tapeworms and blue ticks using a combination albendazole/niclosamide and ivermectin respectively. The goats were housed in a fenced,

outdoor facility with concrete floors throughout the trial. For the challenge studies, the animals were transported to the anthrax endemic region of the Kruger National Park (KNP), Sanparks, South Africa. All animal experiments were approved by the animal use and care committees of Sanparks, Onderstepoort Biological Products and University of Pretoria and also by the Directorate of Animal Health, South Africa under section 20 of Act 34 of 1984 (Protocol number V41-10). Clinical score sheets for monitoring experimental animals were drawn up according to the principles stated in the guide for the care and use of laboratory animals, National Research Council of the USA (1996).

### 3.2.3. Vaccination Regimen

Two groups of five animals each were vaccinated subcutaneously in the inner thighs with 1 ml of the Sterne live spore veterinary vaccine (OBP) on day 0 and challenged with virulent *B. anthracis* 6 and 62 weeks later (Sterne vaccine group 1, SVG1 and Sterne vaccine group 2, SVG2 respectively). A third group of goats (n=3) were vaccinated on day 0, revaccinated 58 weeks with the same vaccine, route and dose as above. The latter was challenged four weeks after the second vaccination (Sterne vaccine group 3, SVG3). Negative control goats (n=3) received only 1 ml of sterile saline.

### 3.2.4. Minimum infective dose (MID) and animal challenge

The challenge dose of *B. anthracis* spores was prepared according to a validated in-house protocol, developed as part of the "Anthrax Euronet" project and the virulence assessed *in vivo* in BalB/c mice before use in the trial. Briefly, the spores were prepared

by culturing the spore isolate on blood agar at 30 °C overnight. Meat yeast agar (with $MgSO_4$) in culture flasks was inoculated with a pure colony from the overnight-cultured colonies. This was incubated at 30°C for ten days. Spores were harvested by flushing with distilled water and the resulting solution washed by centrifugation at 4000rpm for 15 minutes at 20°C. The final spore suspension was treated at 65°C for 30 minutes in a water bath to kill remaining vegetative cells before counting and storage in sterile 0.1% PBS/glycerine solution (pH 7.4) at 4°C. The purity of the spore preparation was confirmed using the hot spore (Rakette) staining method. To confirm the virulence of the prepared spore inoculum, two groups of Balb/c mice (n=3) were challenged intraperitoneally with 500 and 1000 spores (12.5 and 25 $LD_{50}$) respectively (Welkos et al., 1986).

The spore preparation was suspended in physiological saline with 0.1% gelatine and spore concentration was determined by serial dilution and plating on blood agar before the challenge. Six naïve goats were used to determine the minimum infective dose (MID). Three groups of two goats were each challenged with concentrations of 847, 170 and 36 spores respectively by the subcutaneous route on the inner thighs. After each animal challenge, the spore concentration was confirmed as above using aliquots of the inoculum. The challenged goats were monitored up to the terminal stage and cause of death was confirmed by positive blood smears. The Sterne-strain vaccinated animals were challenged as above with ≈20MID. Animals were monitored for 14 days after challenge and survivors were euthanized following confirmation of anthrax-negative blood smears.

3.2.5. Enzyme-linked immunosorbent assay (ELISA)

Blood was collected from the animals and serum harvested from the blood before vaccination and 1, 3, 6, 9, 12 and 14 months thereafter. An indirect ELISA was used to assess for anti-protective antigen immunoglobulin G (IgG) antibodies in the collected sera. The ELISA was performed as previously described (Hahn et al., 2004) with some modifications. Briefly, individual wells of 96-well microtitre plates (Nunc-immuno plate Maxisorp, Germany) were coated with 0.5 µg of rPA83 The anti-PA ELISA was performed using recombinant PA (obtained from W. Beyer, University of Hohenheim, Stuttgart, Germany) in bicarbonate buffer (0.05M, pH 9.5) and incubated overnight at 4 ˚C. The plates were washed twice with PBS containing 0.05% Tween-20 (PBST) using a Biorad PW40 washer (Marnes-La-Coquette, France) and blocked with 200 µl of PBST containing 10% skimmed milk powder (PBSTM) for one hour at room temperature. The plates were washed twice and the test sera and controls diluted in PBSTM. A two-fold dilution series (1:50 to 1:6400 or 1:1000 to 1:128000 for hyper-immune sera) was made across the plates in duplicates and incubated for 30 minutes on a rotatory shaker (Titretek® flow labs, UK). Following incubation, the plates were washed five times and 100 µl of horseradish peroxidase-conjugated rabbit anti-goat IgG (H+L) (Invitrogen, Camarillo, USA) diluted to 1:4000 in PBSTM was added to every well and incubated for 30 minutes 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, Germany) and absorbance readings taken at 405 nm using a Biotek powerwave XS2 reader (Winooski, USA). Endpoint titres of mean serum duplicates were defined as the

reciprocal of the highest serum dilution that resulted in an absorbance greater than two standard deviations above the mean of a pool of pre-vaccination sera from same group of animals. Titres of <50 were ascribed an arbitrary value of 0.

### 3.2.6. Toxin Neutralization Assay (TNA)

An *in vitro* toxin neutralizing assay (TNA) was performed using the mouse macrophage cell line J774A.1 (European collection of cell cultures ECACC cat# 91051511) as previously described (Hering et al., 2004) with slight modifications. 96-well flat-bottomed tissue culture plates (Greiner Bio One, Germany) containing 80000 macrophages/well in DMEM (Life Technologies, USA) and 10% FCS (Life Technologies, USA) were incubated overnight at 37 °C and 5% $CO_2$. Goat sera were doubly diluted (1:50 to 1:6400) in culture medium containing PA and LF (List Biological Laboratories Inc., Campbell, CA) at concentration of 500 ng/ml and 400 ng/ml (lethal toxin, LT) respectively. The sera/LT mixture was incubated for one hour at 37 ˚C and 5% $CO_2$ before adding to overnight cultured cells (after discarding medium) and incubated for three hours. Each serum sample was tested in duplicate. Following incubation, 25 µl of 5 mg/ml MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Life Technologies, USA) was added to each well and incubated in the dark at 37 ˚C and 5% $CO_2$. After two hours incubation, the cells were lysed with prewarmed (37 ˚C) acidified isopropanol (90% isopropanol, 0.5% SDS w/v, 25 mM HCl, pH 4.7) by vigorously pipetting up and down to solubilize the formazan dye. The plates were rested for five minutes 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. 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).

### 3.2.7. Data Analysis

Means, standard deviations and CV data from ELISA and TNA was computed using the Gen5 data analysis software. The percent neutralization titre of each test serum was calculated by: NT 50 = (sample – toxin control) / (medium control – toxin control) × 100 and expressed as the reciprocal of the serum dilution neutralizing 50% of the LT cytotoxicity. NT 50 data were obtained using the Gen5 data analysis software (Biotek Instruments, USA). Curve fitting and logistic regression values were obtained using SigmaPlot (Systat Software Inc., San Jose, USA) and Gen5 respectively. Antibody titres collected at different time intervals from the different groups were compared using unpaired Student *t* test, with a two-tailed *P* value. One-way analysis of variance was used to analyze individual group means and we used Spearman's rank correlation to test for correlation between PA-specific antibody titres and survivability. *P*-values of $< 0.05$ were considered statistically significant.

## 3.3 Results

### 3.3.1 Virulence assessment of *B. anthracis* spores in Balb/c mice

Following the intraperitoneal challenge of two groups of Balb/c mice with 500 and 1000 spores of the challenge strain of *B. anthracis*, all the mice died within two days (Table 3.1).

**Table 3.1: Mean time to death of Balb/C mice challenged with different doses of *B. anthracis* spores. This data gives an indication of the virulence of the spores.**

| **Challenge Dose** | **Mean TTD (Hours)**[b] |
|---|---|
| 1000 spores (3)[a] | 32 |
| 500 spores (3) | 40 |

[a] Number of animals challenged s.c. per dose are shown

[b] Mean time to death

### 3.3.2 Clinical signs and minimum infective dose (MID) in goats

Six naïve goats were infected with varying doses of the virulent *B. anthracis* spores as shown (Table 3.2).

**Table 3.2 Mean time to death of Boer goats challenged with different doses of *B. anthracis* spores. This data gives an indication of the minimum infective dose of the challenge strain.**

| Challenge dose[a] | Time to death (Hours)[b] |
|---|---|
| 843 spores | 38/64 (51) |
| 170 spores | 68/57 (62.5) |
| 36 spores | 57/83 (70) |

[a]Two animals per group

[b]Mean time to death in parentheses

The individual means time to death (MTTD) recorded for the three groups (n = 2) of naïve goat groups (n=2) ranged from 38 to 83 hours (Table 3.2). The data from the above table indicated that the MID of *B. anthracis* spores in goats is less than 50 spores. Clinical manifestations observed in goats following *B. anthracis* challenge were generally rare and non-specific up to few hours before death. Lethargy, recumbency and mild inappetence were observed in some of the challenged animals still incubating the infection. There were no apparent consistent rise in body temperatures of animals that succumbed to inoculation with few having 40 °C or slightly higher. Few hours to occurrence of death, the subjects hyperventilated and bleated incessantly. These signs were usually followed by sudden convulsions and death generally ensued. Fulminating bacteremia was consistently observed in blood smears taken within the last hour before death. The disease followed a peracute course in all the animals with no evidence of blood extravasation from any of the body orifices.

### 3.3.3 Anti-PA antibody production in Sterne-vaccinated animals

Sera from the vaccinated animals (SVG 1-3) were assayed for PA-specific antibody using ELISA. Anti-PA IgG titres taken six weeks after vaccination of (SVG 1) ranged from 4000 to 58000 with mean titres around 20000 (Fig 1a). Sera collected at monthly intervals for SVG 2 (Fig 1b), which consist of goats monitored for approximately 14 months (62 weeks) after first vaccination, showed that mean anti-PA IgG titres decreased from 43000 four-weeks post vaccination to just below 3000 before challenge (62nd week). The same decrease in anti-PA IgG was observed in the revaccinated group (SVG 3) that had a mean titre of just below 4000 before revaccination (Fig 1c). There was no significant difference in titres between animals in SVG 2 and SVG 3 animals (before revaccination of animals in SVG 3, $P = 0.275$). The anti-PA titre of SVG 3 increased to 81000 four weeks after revaccination (Week 62), which was significantly different from sera collected at weeks 4 ($P = 0.0041$) and 58 ($P = 0.0003$) respectively (Fig 1c).

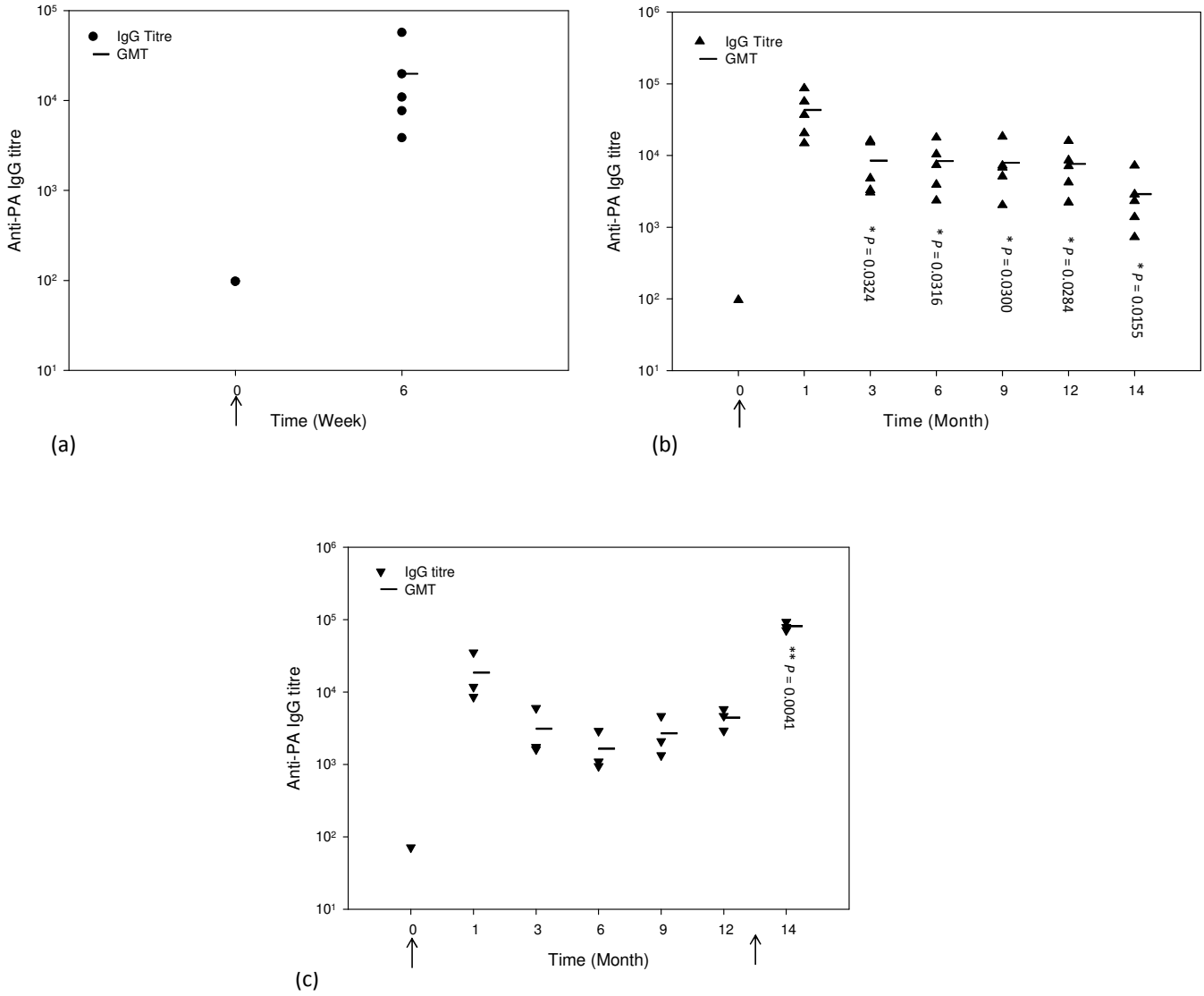
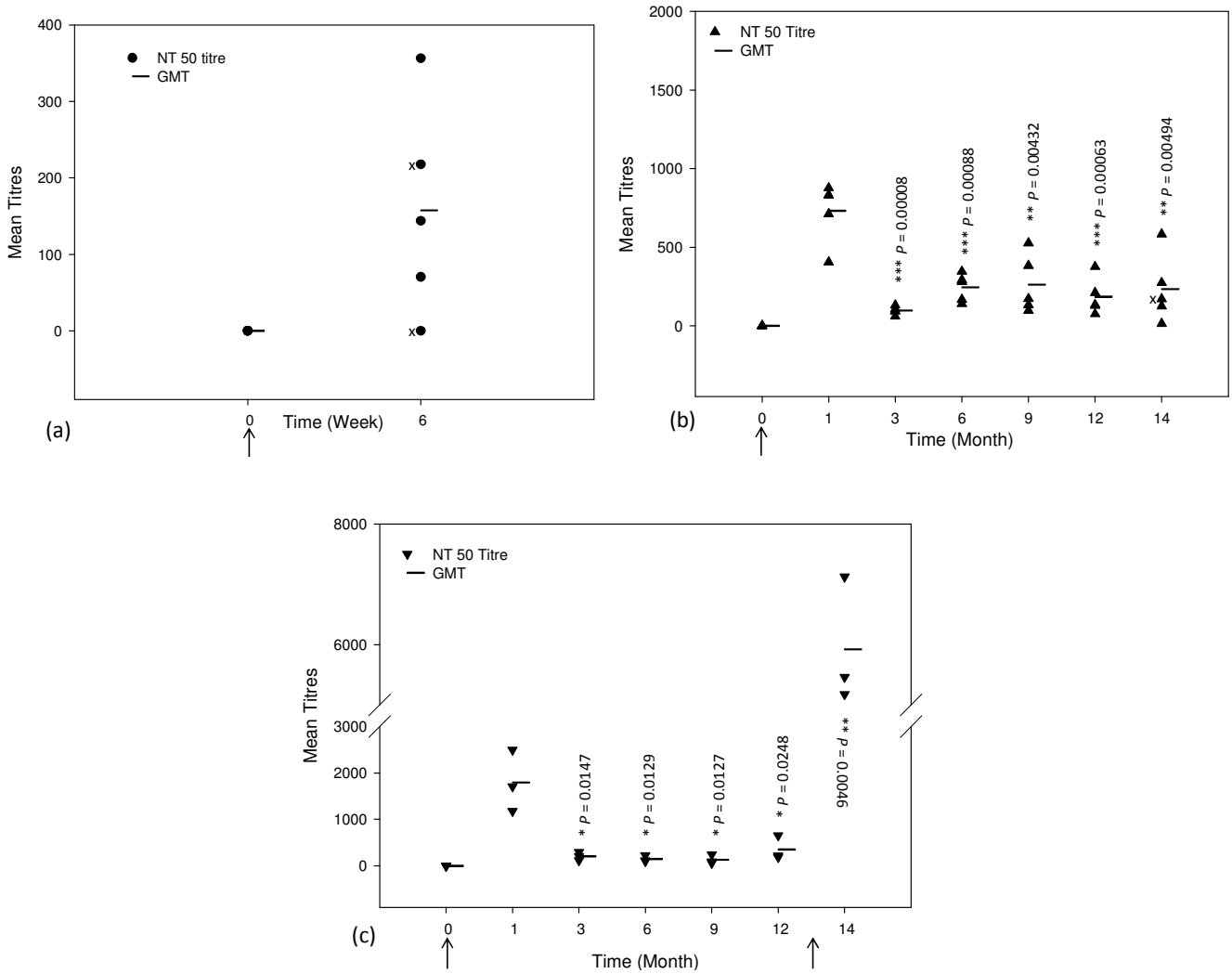



**Figure 1a-1c**; Anti-PA IgG production following Sterne live spore vaccine vaccination in goats consisting of (1a) the Sterne vaccine group 1 (SVG 1, n=5) six weeks after immunization, (1b) the SVG 2, n=5) 62 weeks after immunization and (1c) the SVG 3 (n=3) four weeks after re-immunized after 58 weeks. Titers at time points significantly different from month-1 are indicated. According to the *P* value, the difference was significant (*), very significant (**), or extremely significant (***).

### 3.3.4 *In vitro* protection of mouse macrophages from lethal toxin cytotoxicity

Following vaccination, individual caprine sera was assayed for their ability to protect J774A.1 cells from the deleterious effects of lethal toxin. Six weeks post-vaccination goat sera (SVG 1, Fig 2a) had a mean TNA titre of 160, while that of sera collected one year after vaccination was 230 (SVG 2, Fig 2b). There was no significant difference between SVG 1 and SVG 2 titres ($P$ = 0.744). Mean TNA titres of SVG 3 animals was 350 one year after vaccination. The titre increased to 6000 following revaccination which was significantly different from sera last collected before revaccination ($P$ = 0.0009, Fig 2c).




↑ indicates vaccination time points

**Figure 2a-2c.** The mean toxin neutralization titres (NT 50) indicating the induction of lethal toxin neutralizing antibodies following (Fig. 1a) the Sterne vaccine group 1 (SVG 1, n=5) six weeks after immunization, (Fig. 1b) the SVG 2 (n=5) 62 weeks after immunization and (Fig. 1c) the SVG 3 (n=3) four weeks after re-immunized after 58 weeks. Titres of subsequent months that are significantly different from month-1 sera are indicated. Non-survivors are marked with ×, *P* values are as indicated above.

### 3.3.5 Challenge with virulent *B. anthracis* spores

In order to assess the *in vivo* protective ability of the immune response elicited by the Sterne vaccine, the immunized goats were challenged with ~20MID *B. anthracis* spores 6 (SVG 1) and 62 weeks following vaccination (SVG 2) and 4 weeks after revaccination with the Sterne vaccine (SVG 3). The animals were monitored over a period of 14 days following challenge and exact time to death (TTD) noted (Fig 3).

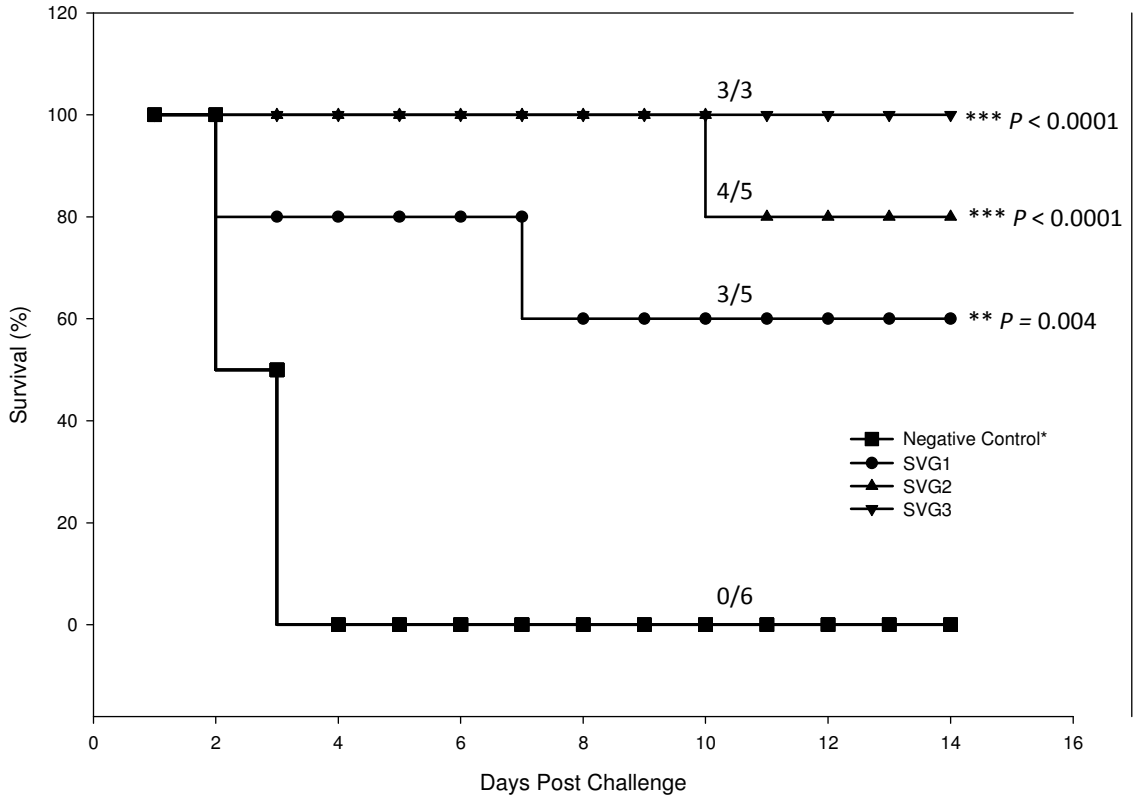



**Figure 3:** Survival rates of Boer goats vaccinated with Sterne live spore vaccine. Goats were challenged subcutaneously with 843 spores of a virulent *B. anthracis* strain 6 and 62 weeks after vaccination and 4 weeks after revaccination (SVG1, SVG2 and SVG3) respectively. Negative control animals had received only sterile saline. The animals were observed for 14 days following inoculation. The number of survivors in all the vaccinated groups were significantly greater that in the control group ($P \leq 0.004$).

Goats revaccinated after one year (SVG 3, n=3) were fully protected from challenge. The Sterne vaccinated groups challenged 58 weeks (14 months, SVG 2) and 6 weeks (SVG 1) after vaccination had 80% (4/5) and 60% (3/5) survival following challenge, respectively. None of the naïve goats survived inoculation with virulent spores concentration ranging from 843 to 36 spores.

Analysis of antibody titres in sera collected just before challenge showed significant correlation between toxin neutralizing antibodies and survival ($P = 0.01$). This correlation was absent between survival and anti-PA ELISA titres. There was no significant difference in survivability data between SVG 2 and SVG 3 ($P = 0.4816$). This was also the case for SVG 3 and SVG 1 ($P = 0.2909$) and SVG 1 and SVG 2 respectively ($P = 0.2920$).

## 3.4 Discussion

The Sterne live spore vaccine represents the gold standard in livestock vaccination against anthrax infections and has been in use for over 70 years. This study attempts to re-visit the development and trials of this veterinary vaccine using current diagnostic methods in an animal model recognized by Sterne himself for its idiosyncrasies against the vaccine (Sterne, 1939). Understanding the development and kinetics of the caprine immune response following vaccination with a live spore vaccine plays a pivotal role in the search for possible protection correlates, and further development and/or introduction of safer acellular vaccines in this animal and related species.

Our findings revealed that the MID of virulent *B. anthracis* spores in naïve goats under experimental conditions was less than 36 spores for the isolate used in this study. Two goats receiving a dose of 36 spores per animal died within three days following infection. Sheep receiving 55 spores of the Vollum strain had survived (Keppie, personal communication, In International Office of Epizootics, 2008a) though the $LD_{50}$ of the disease in sheep might fall within similar range when using the same challenge strain. It is important to note that variation in the $LD_{50}$ or MIDs of anthrax spores in either different or same species is also influenced by the strain of the challenge spores and genetic variations of the animals (International Office of Epizootics, 2008b). The nature of the disease produced in the challenged animals was peracute, characterized by a short course and few clinical signs. Anthrax has been described as primarily a disease of

herbivores (Hambleton et al., 1984) and results from this study did indicate goats as typical anthrax-susceptible species.

Though immunoglobulin M (IgM) is the major immunoglobulin produced during a primary immune response, IgG has the highest concentration in the blood of mammals and plays the major role in antibody-mediated defence mechanisms (Tizard, 2009). Both IgM and IgG are also produced during a secondary immune response but the former tends to be masked by the more specific IgG. Notwithstanding, IgM is known to be more efficient (on a molar basis) than IgG at complement activation, opsonisation and agglutination thus aiding protective cellular immune mechanisms. Production of anti-PA IgG(H+L) in the first month following Sterne vaccination was 400-fold higher than pre-vaccination levels followed by a rapid decline. The anti-PA IgG levels varied with a 50-fold decrease from a month after vaccination to 14 months post vaccination though the later was still significantly higher than pre-vaccination levels. This trend was also reflected in the toxin neutralizing antibody titre. One could attribute this to the prolonged production and presentation of low doses of antigen to the immune system due to the persistent cycles of germination, PA secretion and cell killing of the newly formed vegetative cells. This exposes the immune system to continuous stimulus by PA over a prolonged time, thus providing effective immunity (Cohen et al., 2000, Mendelson et al., 2005). Despite the production of anti-PA IgG in the vaccinated animals, there was no correlation between antibody titres to PA and protection against virulent anthrax spores challenge. This, in itself, is not a new phenomenon as previous studies have shown that correlation between anti-PA titres and protection against challenge can vary depending

on the specie model employed (Ivins et al., 1992, Pitt et al., 2001, Flick-Smith et al., 2002, Fellows et al., 2002, Little et al., 2004a). In contrast, our findings indicate a correlation between toxin neutralizing antibodies and protection against challenge with a virulent strain of anthrax spores (*P* 0.01). Neutralization titres as low as 300 have been found to be sufficient in protecting guinea pigs against challenge with 40 $LD_{50}$ of virulent Vollum strain (Reuveny et al., 2001, Peachman et al., 2006).

Better protection by live spore vaccines compared to licensed protein vaccines, has been attributed in particular to better stimulation of cell mediated immunity (Klein et al., 1962, Ivins et al., 1986, Little and Knudson, 1986, Turnbull et al., 1986, Ivins and Welkos, 1988). Attenuated spores have been shown to be very effective antigen presenting vehicles for PA, in addition to other spore-based antigens (Cohen et al., 2000). Antibody development against other *B. anthracis* antigens and enhancement of cellular immunity by these humoral antibodies could have possibly played important roles in the protection of vaccinates in this study. Data from SVG 1 and SVG 2 animals showed a slightly higher survival (though not significant) for the latter. One could hypothesize that cellular immunity in addition to higher affinity antibodies generated by later B cells and memory cells were crucial in the survival of the vaccinated animals following virulent spores challenge. More in-depth studies will be required to conclusively assert the reasons for these differences between SVG1 and SVG2 animals.

The ability of live spore vaccines to generate antibodies against the spore components could be vital in the development of protective immunity in the vaccinated animals (Cohen et al., 2000, Gauthier et al., 2009a). These antibodies have been shown to hamper germination, increase phagocytic uptake and possibly accelerate phagocyte-mediated spore destruction (Welkos et al., 2004, Cote et al., 2008). Though antibodies generated against BclA, an immunodominant spore glycoprotein, in this study was insignificant (not shown), other spore antigens like the BxpB and p5303 in addition to the former has been shown to enhance phagocytic spore uptake (Cybulski et al., 2008). These immunogens were generated from the Sterne vaccine strain as well as from isogenic mutants lacking BclA. We could assume that an enhanced phagocytosis of spores, a delay in vegetative outgrowth in addition to a spore-induced activation of the cell-mediated response (Glomski et al., 2007) will lead to a heightened immune response in these Sterne vaccinates.

In summary, this study is the first study to describe the immunological response of the Sterne vaccine in goats using current serological techniques. This study clearly indicates the strong role toxin neutralizing antibodies might play in protecting a caprine model against challenge with virulent anthrax spores. This is very important with respect to anthrax vaccine improvement and animal protection studies in the veterinary field especially while considering the ethical and logistical challenges associated with large animal trials. An infective dose of 36 spores of a virulent wild-type *B. anthracis* spores was shown to cause a rapidly fatal infection in naïve goats.

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# CHAPTER FOUR

# Quantitative Anti-anthrax IgG ELISA in Goats; Development, Optimization and Feasibility.

## 4.1 Introduction

*Bacillus anthracis* is a spore-forming bacterium that causes anthrax primarily in herbivorous animals but also affecting other mammalia including humans to a lesser extent (Hambleton et al., 1984). The virulence factors of *B. anthracis* are encoded on the pXO1 and pXO2 plasmids. The plasmid (pXO1) carries the genes *pagA*, *lef*, and *cya* that encode the protective antigen (PA), lethal factor (LF), and oedema factor (EF), respectively (Koya et al., 2005). The term "protective antigen" was derived because of the protein's ability to elicit a protective immune response against anthrax (Wright et al., 1954). Individually, none of these proteins are toxic, but PA combines with EF to form the oedema toxin (ET). Similarly, PA in combination with LF forms the anthrax lethal toxin (LT) (Ascenzi et al., 2002, Collier and Young, 2003, Koya et al., 2005). The pXO2 plasmid codes for the anti-phagocytic poly-ɣ-D-glutamic acid (PGDA) capsule which protects the bacteria against phagocytosis, or consumption by defensive cells of the immune system. Without its capsule, the bacteria can be phagocytized and destroyed (Ezzell et al., 2009, Jang et al., 2011). Attenuated strains that lack either of the plasmids have a reduced virulence (Hambleton et al., 1984).

The current anthrax veterinary vaccine is the attenuated *Bacillus anthracis* 34F2 strain which was developed in 1937 by Max Sterne at Onderstepoort in South Africa (Sterne, 1937). Sterne derived a rough variant of virulent *B. anthracis* following culture on serum agar in elevated $CO_2$ atmosphere which subsequently lost its ability to form a capsule as it was later shown to have lost the pX02 plasmid. This strain was named 34F2 (Sterne) strain. Compared with normal wild type *B. anthracis* strains, the Sterne strain is relatively avirulent but immunization of animals with the strain was able to stimulate a protective immune response. The Sterne vaccine consist of a 1-5 x $10^6$ spores per dose suspended in glycerine and is administered subcutaneously (Personeus et al., 1956). Ivins et al. 1986 in his study concluded that the nontoxigenic Pasteur vaccine lacking the pXO1 plasmid do not provide protection and that attenuated, live *B. anthracis* strains must produce the toxin components to enable successful immunization. Presently, the Sterne live spore vaccine is the most widely used strain for animal anthrax vaccine production.

Preliminary tests of the Sterne vaccine efficacy were mostly by immunity/challenge tests on sheep, guinea pigs, cattle, horses, goats and rabbits, which were tedious and not very comparable since the vaccine dose, challenge dose and strains varied in the different animal species (Hambleton et al., 1984). Furthermore certain species like goats appear to be more susceptible to ill effects from the vaccine than any other domestic herbivores (Turnbull, 1991) and Sterne (1939a) reported adverse reaction and sensitivity to high vaccine doses in goats. Lincoln et al (1967) indicated that the susceptibility of animal species to anthrax is proportional to its susceptibility to the anthrax toxin. This focused research on development and improvement of serological tests to assess protection

provided by anthrax vaccines. Serological tests before the 1980s lacked sensitivity and/or specificity (Turnbull et al., 1992b). This problem was surmounted by the purification of the protective antigen (PA) component of the anthrax toxin (Belton and Strange, 1954,Puziss and Wright, 1954,Turnbull et al., 1992a) and the application of the enzyme-linked immunosorbent assay (ELISA) that was highly sensitive in detecting anthrax antibodies and has been widely employed in the diagnosis and development of new anthrax vaccines ( Johnson-Winegar, 1984, Turnbull et al., 1992a). However very little data is currently available on the acquisition and enumeration of anthrax antibodies in vaccinated livestock resulting in the lack of information on the level and duration of immunity induced by veterinary vaccines in target animals. This prompted us to develop a quantitative indirect ELISA for quantitating anti-PA immunoglobulins in goats vaccinated with the Sterne live spore vaccine. This study attempts to address the feasibility of the use of a quantitative anti-PA antibody ELISA in evaluating the antibody kinetics in immunized domestic herbivore species.

## 4.2 Materials and Methods

### 4.2.1. Recombinant PA (rPA)

Purified recombinant antigen (rPA83) (Koehler et al., 2011) with concentration of 1450 µg/ml in bicarbonate buffer (0.05M, 9.5pH) stored at -20 °C was obtained from Dr Wolfgang Beyer, Institute of environmental and animal hygiene, University of Hohenheim, Stuttgart.

### 4.2.2 Immunization

Ten naïve, age-matched Boer goats were housed at the experimental animal facility of Onderstepoort Biological Products (OBP), South Africa where they were placed on appropriate ruminant feed and water. After an acclimatization period of 14 days, the animals were vaccinated subcutaneously on day 0 with 1 ml of the commercial *Bacillus anthracis* Sterne 34F2 spores ($1 \times 10^6$ spores/ml) according to the manufacturers' instruction (OBP) and monitored throughout the duration of the trial. Blood was collected on days 0, 28 and 140 respectively. The harvested sera were aliquoted into 1 ml cryovials and stored at -20 °C till use. The animal research was approved by the animal use and care committee (AUCC) of the University of Pretoria, Onderstepoort, South Africa with protocol approval number V41-10.

4.2.3. Quantitative indirect anti-PA IgG ELISA

An antibody reference standard that consisted of affinity-purified caprine polyclonal anti-anthrax PA83 IgG fraction (10 mg/ml) was obtained from Innate Therapeutics (Auckland) and stored at -20 °C until used. This indirect ELISA measured anti-PA83 specific IgG utilizing microtitre wells coated with rPA83 as the capture antigen. Each 96-well microtitre plate (Maxisorp Nunc-immuno plate, Germany) contained one duplicate negative control (from unvaccinated goat), three duplicates positive control (from goat hyper-immunized with Sterne vaccine) at high, medium and low concentrations, four blanks and 20 test sera in triplicates at a 1:400 dilution. The reference standard was titrated in a 2-fold dilution series (5µg/ml to 0.0098 µg/ml).

Individual wells of the plates were coated with 100 µl of rPA diluted to 5 µg/ml (Turnbull et al., 2004) in bicarbonate buffer. After 24 hours incubation at 4 °C, the plates were washed twice with PBS containing 0.05% Tween 20 (Merck, Pretoria, South Africa) (PBST) using a Biorad PW 40 washer (Marnes-La-Coquette, France). Plates were blocked with 200 µl of PBST containing 10% skimmed milk powder (Oxoid, Hampshire, England) (PBSTM) and incubated for 1 hour at room temperature. The plates were washed as before and 100 µl of the test sera pre-diluted in PBSTM was added to the respective wells. Blank wells received only 100 µl of PBSTM. Each plate contained a 10 two-fold dilution of reference standard (in duplicates). The plates were then incubated for 30 minutes on a rotatory plate shaker (200 rpm) (Titretek® Flow Labs, Irvine, UK). After five washes, 100 µl of horseradish peroxidase-conjugated rabbit anti-goat IgG (Invitrogen, Camarillo, CA, USA) diluted to 1:4000 in PBSTM was added to every well

and incubated for 30 minutes as before. After the incubation, the plates were washed five times before adding 100 µl of the enzyme substrate 2,2-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma, Steinheim, Germany) to each well. Plates were incubated for 40 minutes in the dark. Absorbance was measured at 405 nm using a Biotek power wave XS 2 reader (Winooski, USA). Plates were blanked on the wells containing PBSTM only. The anti-PA83 IgG concentration of the sera were calculated from the corresponding reference standard curve on the respective ELISA plates using the 4-parameter logistic regression equation in the Gen5 data analysis software (Biotek Instruments).

### 4.2.4. Statistical Analysis

For determination of assay precision, five assays were done in duplicates on different days for each serum sample. Mean absorbance values, standard deviation and coefficient of variation (CV) for each duplicate dilution of test samples, controls and *R2 of* standard curve were calculated using the Gen5 software. Descriptive statistics were performed with the Microsoft Excel software (Microsoft 2010) and curve fitting was done using SigmaPlot (Systat software Inc, San Jose, USA).

## 4.3. Results

### 4.3.1 Evaluation of characteristics of reference standard 4-parameter curve

The Gen5 program 4-parameter logistic curve is delineated by the formula; $Y = (A-D) / (1+ (X/C)^B) + D$ where 'Y' is the optical density (OD) of the test/control sample, 'A' is the response at zero concentration, 'B' is the measure of the slope curve at its inflection point, 'C' is the value of X at inflection point and 'D' is the response at infinite concentration. This equation defines the relationship between obtained absorbance values and the known concentrations of a reference standard (O'Connell et al., 1993, Little et al., 2004). Data from 22 reference standard curves made up of 10, two-fold serial dilutions from 5µg/ml to 0.0098µg/ml were calculated to be A = 0.0270 ± 0.0132; B = 1.151 ± 0.0467; C = 0.4268 ± 0.1576 and D = 3.473 ± 0.1154 (±SD). A sound standard calibration curve is essential in the development and assessment of quantitative assay characteristics such as accuracy and precision (Findlay and Dillard, 2007). The key factor is the level of agreement of known standard calibrator concentrations with back-fitted concentrations with the latter read off the fitted standard curve as if they were unknown samples (Karnes and March, 1991, Smith and Sittampalam, 1998). In evaluating the assay standard curve, we expressed the predicted standard concentrations as a percentage recovery (%Re) at each concentration level, %Re = 100(BC/NC), where BC and NC represent the back-calculated and nominal (known) concentrations respectively (Fig 4.1). A good %Re was recorded for the nominal concentrations with the only exception being at the lower asymptote (89%) for the 0.0098µg/ml concentration though well within the established acceptable range of 80% for lower limit of quantitation (DeSilva et al., 2003).

The mean correlation coefficient ($R^2$) of the standard curves was 0.9998 (Fig 4.2) with ranges between 0.9998 and 1.0000.

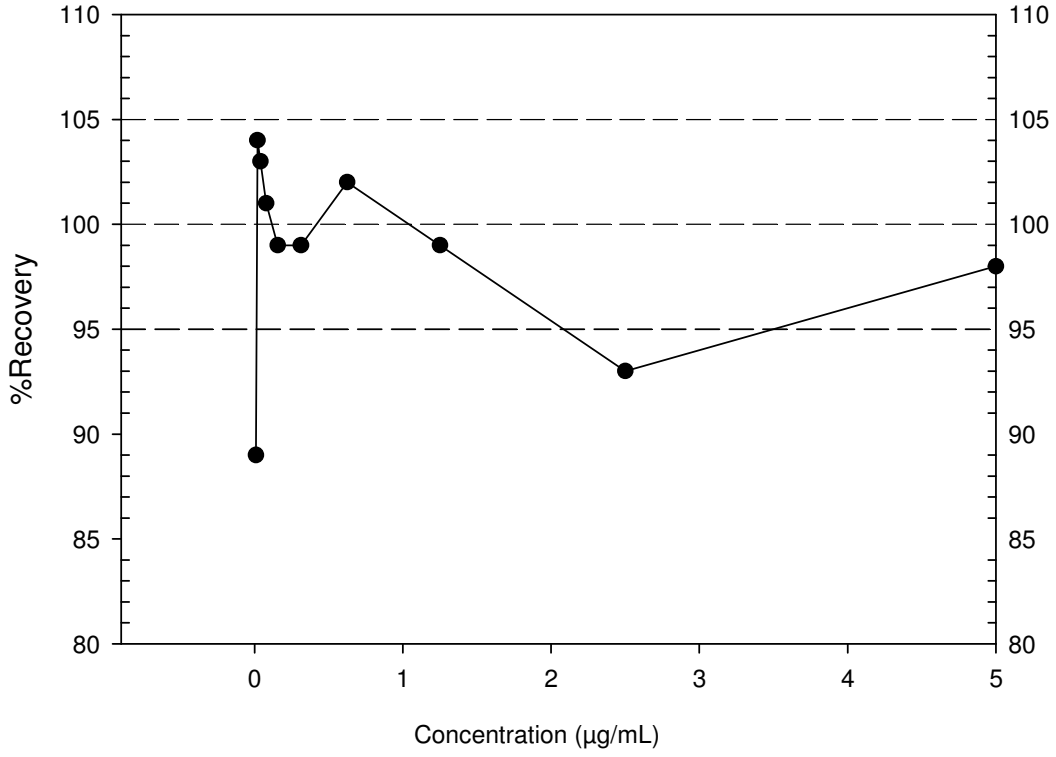


**Figure 4. 1** %Re plots for nominal concentrations of a reference anti-PA IgG standard using a 4-parameter logistic curve model. %Re indicates percentage recovery for known IgG concentrations of the standard in the ELISA. (%Re = 100(BC/NC), where BC and NC represent the back-calculated and nominal (known) concentrations respectively).

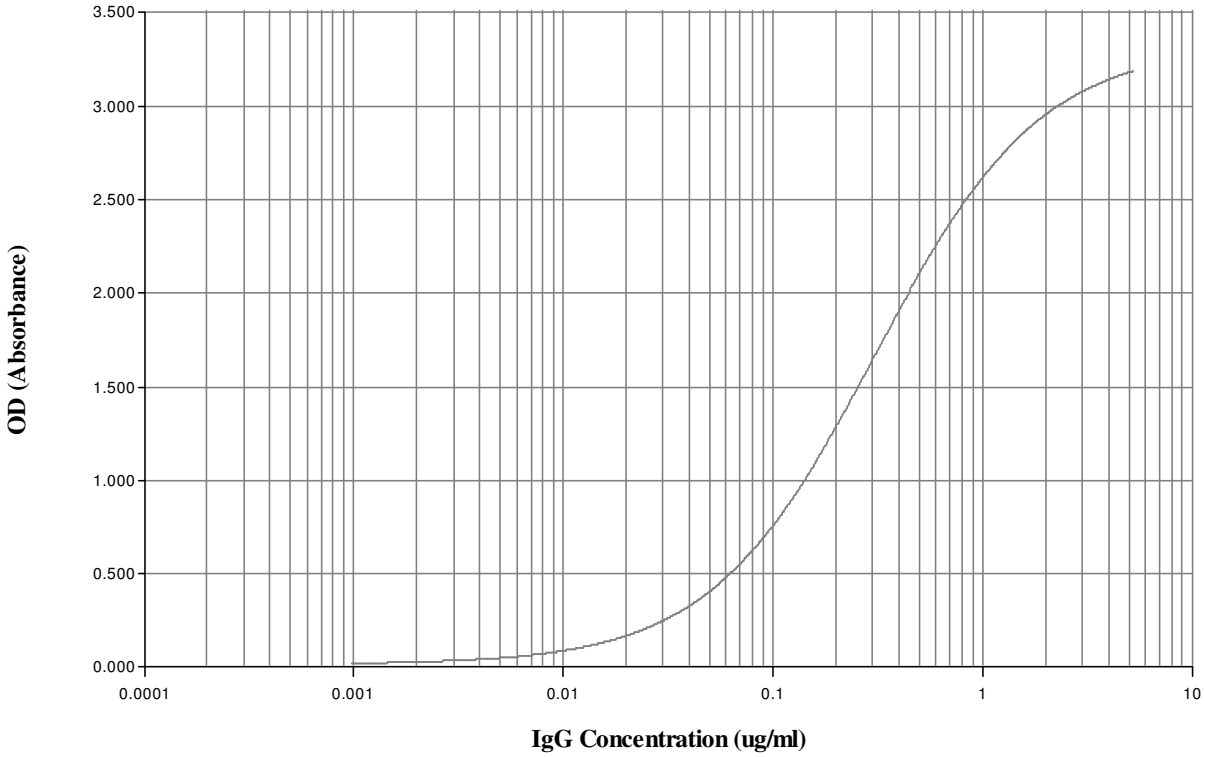


**Figure 4. 2** Anti-PA IgG 4-parameter logistic curve model derived for a quantitative indirect ELISA using the Gen 5 software.

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### 4.3.2 Precision of assay

Evaluation of the intra-assay coefficient of variation showed ranges from 0.23% to 16.9% and 0.40% to 12.46% for day 28 and 140 sera respectively. Average inter-assay CV was 18.53% for day 28 sera and 12.17% for day 140 sera collection (Table 4.1). Slightly higher CV values were observed with the day 28 sera compared to sera collected 140 days after vaccination.

**Table 4. 1:** Mean IgG concentrations for caprine sera collected on days 0, 28 and 140 days respectively [a]

| | Day 0 | Day 28 | Day 140 |
|---|---|---|---|
| **Animal** | **IgG µg/mL (CV)** | **IgG µg/mL (CV)** | **IgG µg/mL (CV)** |
| #8172 | 3.2534 (52) | 221.8 (16.8) | 56.1 (14.9) |
| #8173 | 1.1405 (11.5) | 719.6 (16.2) | 166.2 (15.1) |
| #8174 | 0 | 546.8 (16.3) | 102.7 (11.3) |
| #8175 | 2.3788 (65.5) | 585.9 (17.1) | 59.8 (9.9) |
| #8176 | 0.8115 (83.9) | 584.2 (19.0) | 106.3 (11.4) |
| #8178 | 1.7455 (14) | 464.4 (18.1) | 24.7 (13.1) |
| #8179 | 1.5884 (64.8) | 268.3 (20.1) | 55.9 (12.4) |
| #8180 | 0 | 227.2 (16.6) | 28.0 (14.5) |
| #8181 | 0.115 | 482.8 (22.3) | 41.8 (10.7) |
| #8182 | 0 | 719.0 (22.8) | 92.4 (8.44) |

[a] Five assays run in duplicates on different days for each serum sample

## 4.4 Discussion

In this study we have modified and adapted a previous immunoassay (Koehler et al., 2011) for the quantitation of anti-PA immunoglobulins in goats vaccinated with the Sterne live spore vaccine. The current study had looked at early feasibility/characteristics of an IgG-quantitative based indirect-ELISA with reference to sound IgG standard curve quality, precision and reproducibility of results from analysed samples. This approach has been employed in the field of vaccine development albeit with different model species (Little et al., 2004, Miura et al., 2008).

The current approach to evaluation of immune response in vaccinates is by a titre-based ELISA method and this has been utilized in vaccine potency and immunogenicity studies in the veterinary field (Hahn et al., 2006, Kelly et al., 2007, Shakya et al., 2007). This has largely replaced the potency test employed for testing the Sterne vaccine, which involved the challenge of guinea pigs with doses of a lethal *B. anthracis* strain following vaccination with a new batch of the Sterne vaccine (Sterne, 1937). Very little data is available on the kinetics of anthrax antibodies in vaccinated livestock and the duration of immunity. The use of immunoassays could also replace the potency tests for the Sterne and other vaccine types under development.

Protective antigen (PA) is the most essential component of living, inactivated or protein-based vaccines (Leppla et al., 2002) and strains of *B. anthracis* without the toxin antigen producing plasmid, pXO1, have failed to confer protective immunity to animals with exception to mice (Welkos et al., 1993, Leppla et al., 2002). Evaluation of anti-PA

antibodies in sera of immunized animals following vaccination is an important step in the evaluation of vaccine efficacy due to the essential role played by PA in anthrax pathogenesis. It is deemed to be the most essential part of any anthrax vaccine since numerous studies have shown it to be able to induce a protective response in experimental models of infection (Cybulski Jr et al., 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, Baillie et al., 2008). Proof of seroconversion and immune status in vaccinated herbivores can be assessed by means of quantitation of these antibodies.

Our study evaluated the quality of the standard curve on each ELISA plate and found this to be quite high with an average $R^2$ of 0.9998 (Figure 4.2) with high repeatability. The drifting of the %Re from the ideal 100% can be attributed to stronger effects of nonspecific binding at the lower asymptote as antibody concentration reduces dramatically. The same effect is seen at the upper asymptote possibly due to the near infinite antibody concentration (maximum response) though not as pronounced as seen in the lower asymptote. It can also be posited that the upper and lower limits of detection of antibodies of this assay are very close to the respective asymptotes (5 µg/mL and 0.0098 µg/mL respectively). Although %Re of the standard at both asymptotes is not optimal, this effect can be minimized by the limiting of the calibration to concentrations within the ideal 100% recovery level. Crucially, the assessment of the suitability of a standard curve for any immunoassay should be done early in the assay development, as a sound calibration curve is central to the development of sound assay characteristics (Findlay and

Dillard, 2007). This is also very important in the further development of the assay since the quantification of antibodies in test sera is derived from the standard curve (Plikaytis et al., 1991, Miura et al., 2008,). Moreover, the inclusion of a serially diluted standard reference on every ELISA plate serves as a normalization and/or internal control for the individual assays.

The $OD_{405}$ values of the assay blank wells also fell within the acceptable level for an early stage ELISA (averaging 0.069). The use of skimmed milk powder instead of the conventional fetal calf serum (Vogt et al., 1987) improved the specificity of the ELISA (low background noise). Non-specific binding or low background noise in an ELISA system has been attributed to the` use of sera as a blocking agent in ELISA systems (Vogt et al., 1987). This phenomenon was reduced with the use of skimmed milk powder as the blocking agent of choice.

More sera samples are also assessed on each ELISA plate using this method when compared to the conventional titre-based ELISA where end-point titrations for each serum sample are performed in rows on the plates. There was good reproducibility of the data obtained over five different assays with the CV within acceptable limits. The CV between different runs (performed on different days) was much lower for sera collected on day 140 when compared to samples collected on day 28. This could be due to the presence of more robust antibodies with higher antigenic affinities.

In summary, though not exhaustive in its approach, this study indicates that a reliable IgG quantitative ELISA can be employed for vaccine studies in goats. The assay has the

advantage of a reference standard in every plate which gives a measure of good internal control, in addition to the positive and negative controls. The feasibility of a full, long term validation of the assay looks favourable and should serve as the prelude to its deployment in vaccine research and production in goats.

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