Evaluation of veterinary non-living anthrax vaccine candidates in cattle

by

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Dedication

I dedicate this work to my late mom Mrs Juliana Jauro and to my unborn child.

Declaration

I, **Solomon Jauro**, hereby declare that this thesis is an original report of my research work, the entire thesis has been written by me. The experimental work is almost entirely my own work except points where the collaborative contributions have been duly acknowledged and clearly indicated in the references provided in the thesis. Neither the whole work nor part of it has been submitted for degree in any University in the world.

Sign:…  ……. Date:…15th October 2020……

Thesis Summary

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Anthrax has been the plague of animals and humans for centuries, even though it is primarily a disease of herbivores. Anthrax is caused by the spore-forming bacterium *Bacillus anthracis*, the virulence of *B. anthracis* is regulated by two plasmids pXO1 and pXO2. The pXO1 regulates the anthrax toxins by encoding the tripartite proteins, protective antigen (PA), oedema and lethal factors (EF and LF) while the pXO2 regulates the capsule formation by encoding a poly-D-glutamic acid capsule (PDGA) which is immunogenically weak. PA plays a catalytic role to form the anthrax toxins and is the main antigenic component of anthrax. Anthrax is one of the earliest bacterial diseases controlled by vaccination. The *B. anthracis* Pasteur's Duplex vaccine and Sterne 34F2 live spore vaccine (SLSV) are live

attenuated vaccines which renders them incompatible with accompanying antibiotic treatment in the phase of a disease outbreak. The use of non-living anthrax vaccine (NLAV) which include recombinant PA (rPA), could overcome the shortcomings associated with live attenuated anthrax vaccine by allowing the concurrent use of antibiotics. However, cost-effective production of rPA in a biologically active and up-scalable form is a major challenge. Attempts were made to produce a more cost-effective NLAV that compares with the SLSV.

The NLAV used in this study was formulated by improving upon NLAV's that use rPA, bacillus collagen-like antigen (BclA) and formaldehyde inactivated spores (FIS) in laboratory animals and goats. However, in our study, the NLAV comprised of two formulations of rPA and FIS; purified rPA + FIS (PrPA+FIS) and crude rPA + FIS (CrPA+FIS) both were adjuvanted with a combination of Emulsigen-D®/Alhydrogel®. A two-step vaccination schedule was employed in both vaccination categories and their immunogenicity in vaccinated cattle groups assessed with and without penicillin-G (Pen-G) treatment. Their immunogenicity was compared to cattle groups vaccinated with SLSV with and without Pen-G treatment. An Enzyme-linked immunosorbent assay (ELISA) for immunoglobulins; IgG, IgG1, IgG2 and IgM against rPA and FIS, a toxin neutralisation assay (TNA) and an opsonophagocytic assay were used to assess the immune response in all studies. The level of *in vivo* protection was determined using a passive mouse protection test.

Our findings amongst all groups vaccinated without Pen-G treatment revealed that the polyclonal IgGs, particularly IgG1 and IgM, showed a significant increase across all vaccine groups after the first vaccination. After the second vaccination all IgG subset titres significantly increased across all vaccine groups at week 5 and retained these titres at week 12 when compared to pre-vaccination titres. The toxin neutralisation titres (NT_{50}) among cattle groups vaccinated with the NLAV showed a similar pattern of response but had lower titres, compared with those observed with the ELISA titres. In addition, opsonising antibody responses showed significant spore phagocytosis of 75% (PrPA+FIS), 66% (CrPA+FIS) and 80% (SLSV) following spore opsonisation. In the passive protection test, A/J mice injected with purified polyclonal IgG from cattle vaccinated with PrPA+FIS+Emulsigen-

D®/Alhydrogel® and SLSV revealed 73% and 75% protection following challenge with *B. anthracis* strain 34F2 spores, whereas only 20% protection was recorded among cattle vaccinated with CrPA+FIS+Emulsigen-D®/Alhydrogel®.

Furthermore, our findings in the cattle groups vaccinated twice with NLAVs plus Pen-G treatment compared to cattle groups vaccinated twice with SLSV alone, and SLSV plus Pen-G revealed that the IgG titres against rPA for NLAVs plus Pen-G and SLSV without pen-G treatment displayed a significant increase, whereas the titres of the cattle groups immunised with SLSV plus Pen-G treatment was not significant. Contrarily, the IgG titres against FIS revealed a significant antibody titres level among all vaccine groups at week 3 and 5 compared to pre-vaccination titres. The IgM, IgG1 and IgG2 titres against rPA were significant for vaccine groups vaccinated with NLAVs plus Pen-G and SLSV without Pen-G at week 3 and 5 but insignificant for group vaccinated SLSV plus Pen-G. Similar patterns were also revealed for IgM, IgG1 and IgG2 titres against FIS. However, the group vaccinated with SLSV plus Pen-G treatment displayed significant titres against FIS. The toxin neutralisation titres (NT_{50}) was significant among the cattle group vaccinated with NLAVs plus Pen-G and SLSV but not for SLSV plus Pen-G at week 3 and 5 when compared to pre-vaccination titres. Our finding showed the effect of antibiotics on the immunogenicity of SLSV as compared to NLAV and reinforced that cocktails of immune response against both rPA and FIS complement each other thereby conferring better immune response than either rPA or FIS alone. Furthermore, the stimulation of both IgG1 and IgG2 implies a balance between Th1 and Th2 response. The spore opsonising antibodies at all dilution showed a significant opsonisation response among NLAVs plus Pen-G and SLSV without Pen-G vaccine group except at 1:10000 dilution. Our data support the important role of opsonising antibodies in the immune response against infection by presenting spores of *B. anthracis* to phagocytes. Hence, opsonisation can be adopted as a correlate for the protective immune response against *B. anthracis* infection. The passive mouse protection test demonstrated significant protection among mice that received polyclonal IgG from SLSV and PrPA+FIS plus Pen-G but not significant for SLSV plus Pen-G and CrPA+FIS plus Pen-G vaccine groups.

It is significant to indicate that, in both vaccination categories our findings revealed that there were equivalent to SLSV in protective immune response in cattle vaccinated twice with either the PrPA+FIS or PrPA+FIS plus Pen-G when compared to SLSV without Pen-G treatment. Importantly, our results further confirmed the incompatibility of SLSV with antibiotic treatment. Additionally, NLAV did not show any residual side effects in vaccinated cattle. Finally, our study revealed that NLAV can be used simultaneously with Pen-G antibiotics that will provide long term benefits for future anthrax outbreaks, feedlots and valuable breeding stock.

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List of Abbreviations

aa	amino acid
ANTXR1	Anthrax toxin receptor 1
ANTXR2	Anthrax toxin receptor 2
APC	Antigen presenting cell
ATP	Adenosine triphosphate
atxA	Anthrax toxin trans-activator
AVA	Anthrax vaccine adsorbed
AVP	Anthrax vaccine precipitate
BclA	Bacillus collagen-like antigen
cAMP	cyclic adenosine monophosphate
ccdA	control of cell death A
ccdB	control of cell death B
CD8+	Cluster of difference 8+
CMG2	Capillary morphogenesis protein 2
CMI	Cell-mediated Immunity
CpG	Cytosine phosphate guanine
CrPA	Crude recombinant protective antigen
CSF	Classical swine fever
CWS	Cell wall skeleton
DDA	Dimethyl-dioctadecyl ammonium bromide
DMEM	Dulbecco's Modified Eagle Media
EA-1	Extractable antigen 1
ECACC	European collection of cell cultures
EF	Edema factor
ELISA	Enzyme linked immunosorbent assay
ET	Edema toxin
FBS	Foetal Bovine Serum
FCA	Freud's complete adjuvants
FCS	Foetal Calf Serum
FHV	Flock house virus
FIA	Freud's incomplete adjuvants
FIS	formaldehyde inactivated spores
FMD	Foot and mouth disease
HA	Heameagglutination
iELISA	Indirect enzyme linked immunosorbent assay
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IgG1	Immunoglobulin G 1
IgG2	Immunoglobulin G 2
IgM	Immunoglobulin M
IL-1	Interleukin-1

IL-10	Interleukin-10
IL-12	Interleukin-12
IL-18	Interleukin-18
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-23	Interleukin-23
IL-27	Interleukin-27
IL-4	Interleukin-4
IL-5	Interleukin-5
IPTG	Isopropyl β- d-1-thiogalactopyranoside
ISA	Incomplete SEPPIC Adjuvants
ISCOMs	Immune-stimulating complexes
LB	Luria-Bertani
LD_{50}	Lethal dose 50%
LDL	Low density lipoprotein
Lef	Lethal factor
LF	Lethal factor
LPS	Lipopolysaccharide
LRP	Receptor-related protein
LT	Lethal toxin
MAPKKs	Mitogen activating protein kinase kinases
MHC I	Major histocompatibility complex I
MHC II	Major histocompatibility complex II
MIDAS	Metal ion dependant adhesion site
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NegCtl	Negative control
NLAV	Non-living anthrax vaccine
NLAVs+Pen-G	Non-living anthrax vaccines + penicillin-G
NT_{50}	50% neutralisation
O/W	Oil-in-water
OIE	Office International des Epizooties
OVARU	Onderstepoort Veterinary Animal Research Unit
PA	Protective antigen
PA-D2	Protective antigen domain II
PA-D4	Protective antigen domain IV
PCV2	porcine circovirus type 2
PDGA	poly-D-glutamic acid
Pen-G	Penicillin-G
PrPA	Purified recombinant protective antigen
pXO1	Plasmid XO 1
pXO2	Plasmid XO 2
rPA	Recombinant protective antigen
Sap	Surface array protein

SLSV	Sterne live spore vaccine
SLSV+Pen-G	SLSV+penicillin-G
TDM	Trehalose dimycolate
TEM	Transmission electron microscope
TEM8	Tumour endothelium marker 8
Th1	T helper 1
Th2	T helper 2
TLR	Toll like receptor
TNA	Toxin neutralisation assay
TNF-α	Tumour necrosis factor-α
VLPs	Virus-Like Particle
VNP	Virus nanoparticle
VWA	von Wildebrand factor A
W/O	Water-in-oil
W/O/W	Water-in-oil-in-water
WHO	World health organisation

CHAPTER ONE

1.1 Introduction

Anthrax is a bacterial disease that infects almost all warm-blooded mammals, but it is primarily known to be a disease of herbivores (Hambleton et al., 1984). Ruminants get exposed to *B. anthracis* spore infection usually during browsing in contaminated vegetation and/or soil (Dragon et al., 2001, Hassan et al., 2015, OIE, 2018). Humans are a more resistant host but can be infected after exposure to infected animals or products (Hambleton et al., 1984, Manchee et al., 1981). Anthrax is caused by *Bacillus anthracis,* a gram-positive, endospore-forming, non-motile bacterium (Koch, 1937). The shedding of bacteria by infected animals occurs at death when the vegetative cells form (endo)spores and contaminate the environment (Hambleton et al., 1984, Manchee et al., 1981). The spores are resistant and can persist for years in soil and are the infective form of *B. anthracis* in the environment (WHO, 2008; Van Ness, 1971). Grazing livestock ingest spores, which germinate into the vegetative form and multiply rapidly in the infected host and release the virulence factors (Hambleton et al., 1984, Smith, 1960).

The virulence factors of *B. anthracis* are regulated by genes on two extrachromosomal plasmids; pXO1 and pXO2 (Green et al., 1985, Mikesell et al., 1983). The ability of the pathogen to escape phagocytosis by innate immune response is regulated by poly-D-glutamic acid (PDGA) capsule genes located on pXO2 (Green et al., 1985). The toxic activity of *B. anthracis* is regulated by tripartite proteins; protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa) and edema factor (EF, 89 kDa) which are encoded on pXO1. The anthrax toxins are formed when either EF and/or LF binds in a binary form with PA (Leppla, 1982, Lincoln and Fish, 1970). LF is a specific zinc metalloprotease which cleaves to the N-terminus of the members of Mitogen activating protein kinase kinases (MAPKK) family leading to increased production of cytokines; tumour necrosis factor-α (TNF-α), revamped cell signalling, interleukin-1β (Il-1β), and nitric oxide. Following the cleavage of MAPKK, it

activates the secretion of multiple protein complexes and caspases-1 that mediated cellular apoptosis (Duesbery et al., 1998, Vitale et al., 1998). The EF is a calcium/calmodulin-dependent adenylate cyclase which increases the intracellular cyclic adenosine monophosphate (cAMP) level inside the cell. The intracellular cAMP regulates the mechanism through which cytokines are produced and neutrophil function is hindered and at the same time cause characteristic oedema seen in the cutaneous form of anthrax by initiating imbalance in water homeostasis (Leppla, 1982). When LF binds to PA, they form lethal toxin (LT) whereas, EF binds to PA to form oedema toxin (ET) (Mikesell et al., 1983; Pezard et al., 1991). The competitive intracellular translocation of LT and ET to induce the toxic effect on the cells is facilitated by PA (Mogridge et al., 2002). PA has four different domains through which it binds to cell receptors either through capillary morphogenesis gene 2 (CMG2) or tumour endothelial marker 8 (TEM8) via its C-terminal (Bradley et al., 2001, Scobie et al., 2003). The toxin translocating activity by PA is executed proteolytically by activating furin-like proteases leading to fragmentation of PA83 at its N-terminal to release 20 kDa fragment. The complementary 63 kDa of PA is bound competitively by LF and EF to form the ring-shaped prepore during self-assembling of PA63. *B. anthracis* toxin complexes that are formed are endocytosed into the cell through the plasma membrane regions refer to as lipid rafts, this regions have high content of cholesterol and sphingomyelin (Abrami et al., 2003).

Anthrax vaccines preparation for animal use have always been through attenuation of live spores of *B. anthracis*. The first *B. anthracis* spore attenuation was done by Louis Pasteur in 1881 (Hambleton et al., 1984). Pasteur produced a duplex vaccine consist of two different batches of attenuated *B. anthracis* attenuated separately (Hambleton et al., 1984, Turnbull, 1991). Pasteur batch I was incubated at 42-43 °C for 15-20 days and the second batch (Pasteur II) was incubated at 42-43 °C for 10-12 days to obtained Pasteur's Duplex anthrax vaccine which are all formulated and administered separately (Hambleton et al., 1984). Even though Pasteur's Duplex vaccine was effective for over 50 years, the vaccine suffered some setback such as the reduction in potencies and variation in virulence, and it leads to

occasional losses among vaccinated animals, It could not be administered safely to certain susceptible animal species (Hambleton et al., 1984, Mikesell et al., 1983). To improve on the limitations of Pasteur's duplex vaccine, Max Sterne developed Sterne live spore vaccine (SLSV) from avirulent non-encapsulated *B. anthracis* 34F2 strain. This strain was a subculture of an isolate from a bovine case of anthrax that was attenuated by incubation on horse serum nutrient agar (50%) for 24 hours under 30% CO_2 atmosphere (Sterne, 1937). Since its introduction in the 1930s, SLSV has proved to be safer and more effective and has been adopted as a livestock anthrax vaccine of choice in most countries (OIE, 2018). However, SLSV have some shortcomings and the prominent limitation is the live vaccine's incompatibility with antibiotics. This is paramount in an outbreak situation where antibiotic treatment must be administered for immediate protection or as prophylaxis in an environment where anthrax is endemic or animals suspected to come down with anthrax. After the withdrawal period of the antibiotic the animals can be vaccinated for long term protection with the vaccine (Webster, 1973, Stepanov et al., 1996, Fasanella et al., 2008).

Stanley and Smith in the 1950-1960s purified and characterised *B. anthracis'* pXO1 and its components PA, LF and EF (Stanley and Smith, 1963, Stanley et al., 1961, Stanley et al., 1960). This is after Gladstone (1946) proofed that the acellular anthrax vaccine from *B. anthracis* culture filtrate provided protection to sheep and rodents against anthrax (Gladstone, 1946). This initiated new investigation using PA to produce acellular anthrax vaccines for human in the UK (Anthrax Vaccine Precipitate (AVP)) and USA (Anthrax Vaccine Adsorbed (AVA)) (Wright et al., 1954). The human AVA is prepared from an unencapsulated *B. anthracis* V770 strain adjuvanted with aluminium hydroxide and AVP preparation consists of the cell-free culture supernatant of the Sterne 34F2 strain and adjuvanted with aluminium precipitated are commercially available in the USA and UK respectively (Mahlandt et al., 1966).

Even though PA is the major immunogen in AVA and AVP, investigations have shown traces of LF and EF from the culture filtrates that could results in toxicity in vaccinated individuals

(Turnbull et al., 1986, Turnbull et al., 1988). Other disadvantages include the extensive dose regimen with lots of boosters to confer protection, nonspecific composition and variation in batches (Brachman et al., 1962, Puziss and Wright, 1963). Hence, the search for alternative ways to obtain purified PA for better and safe anthrax vaccine continues. The answer seems to come from recombinant technology and its advances. PA was cloned and expressed in *Bacillus subtilis,* and investigation showed that the recombinant PA (rPA) is immunogenic (Turnbull et al., 1986, Turnbull et al., 1988, Turnbull et al., 1990, Turnbull, 1991). Even though this is the way forward for safer vaccines, the expression of recombinant protein especially rPA may post challenges such as the overproduction of proteins. The overproduction can lead to the accumulation of inclusion bodies within the bacteria host; production of soluble protein and; the solubilisation of insoluble protein may influence the biological activity of the protein negatively.

Furthermore, the de- and re-naturing process to solubilise the protein is not cost-effective (San-Miguel et al., 2013, Suryanarayana et al., 2016). Large scale production is a major setback with some expression system since the host may lose the vector during the fermentation process (Pierce and Gutteridge, 1985, Rai and Padh, 2001, Sodoyer et al., 2012). Numerous studies are now focusing on how to resolve problems with recombinant technology like improving the process of soluble protein expression, ensuring the biological activity and stability of the protein, as well as, using an expression system that can be upscaled. Furthermore, the purification process of the recombinant protein should be easy and cost-effective (Miroux and Walker, 1996, Nguyen et al., 2004, Page et al., 2004, Peti and Page, 2007).

The use of rPA conjugated with various *B. anthracis* spore components such as BclA (Bacillus collagen-like antigen), putative basal layer protein (BxpB) and/or the whole spore in the inactivated form (formaldehyde inactivated spores; FIS) is believed to augment rPA immunogenicity in laboratory animals and goat (Brossier et al., 2002, Koehler et al., 2017, Majumder et al., 2018, Majumder et al., 2019, Ndumnego et al., 2018). In previous studies,

rPA and FIS of a vaccine formulated with rPA, FIS, BclA and lipoprotein adjuvant enhanced the immune response and provided protection against *B. anthracis* in virulent challenge studies conducted in laboratory animals (mice) and goats (Koehler et al., 2017, Ndumnego et al., 2018). Therefore, this project investigated the immune response and protection of non-living anthrax vaccines consisting of rPA expressed using two cost-effective approaches and conjugated to FIS and formulated with Emulsigen-D®/Alhydrogel adjuvant (1:1) for veterinary use according to the objectives itemised below:

1. **Cost-effective production and purification of *Bacillus anthracis* recombinant protective antigen *in Escherichia coli***

The rPA expressed in an up-scaleable vector (pStaby1.2) and produced using two cost-effective methods (crude and purified) were used to formulate the vaccines by conjugating with FIS and the Emulsigen-D®/Alhydrogel® (1:1) adjuvant.

2. **Immunogenicity of non-living anthrax vaccine candidates in cattle and protective efficacy immune sera in A/J mouse model compared to the Sterne live spore vaccine**

Cattle were vaccinated with either of the non-living anthrax vaccines (purified recombinant PA (PrPA) or crude rPA (CrPA)) and FIS formulated with Emulsigen-D®/Alhydrogen ®adjuvants and SLSV. The immunogenicity of the NLAVs and SLSV were assessed in cattle using ELISA, toxin neutralisation assay (TNA) and opsonophagocytosis testing. The passive protection of serum from cattle immunised twice with the non-living vaccine (PrPA and CrPA, FIS and adjuvant) was evaluated by injecting A/J mice with antibodies purified from the serum collected from vaccinated cattle and challenging mice with the *B. anthracis* 34F2 (Sterne) strain spores via the intraperitoneal route.

3. Immunogenicity and protective efficacy of a non-living anthrax vaccine versus a live spore vaccine with simultaneous penicillin G treatment in cattle

Cattle were two-step vaccinated with NLAVs candidates and SLSV with simultaneous antibiotic treatment as well as SLSV (alone) and the immunogenicity and protective potential of the antibodies were determined. ELISA, TNA and opsonophagocytic assay were used to determine the specific humoral immune. The protective efficacy was determined using the passive mouse protection test with purified polyclonal IgG from the vaccinated cattle and lethal challenged with *B. anthracis* 34F2 spores.

CHAPTER TWO

Literature review

2.1 Historical evolution of anthrax

Anthrax has been documented in history since ancient times. The term anthrax was derived from a Greek word *anthrakites*, which means coal-like and this denotes the typical black eschar seen in the cutaneous form of human anthrax following infection (Dirckx, 1981, Virgil, 1956). Anthrax has been linked to the fifth (death of livestock) and sixth (boils) plagues of Egypt documented in the Bible in the ninth chapter of Exodus by some scholars (Hambleton et al., 1984). Even the famous plague of Athens that occured in 430-427 BC was also suggested to be an epidemic of inhalation anthrax (Sternbach, 2003). The pestilence of anthrax of both animals and human continued through the middle ages to the epidemic of anthrax in the 19th century that resulted in death of the half of the domestic livestock across Europe (Dirckx, 1981) and over a million sheep in Iran in 1945 (Kohout et al., 1964). This is recorded as the highest epizootic in the history of anthrax disease. Anthrax endemic persists to the present time killing cattle and impala with the large outbreak in the 1980s involving both humans and cattle was reported in Zimbabwe (Wilson et al., 2016).

History has shown that anthrax has been and remains to be a problem in South Africa despite using the vaccine, especially in the wildlife setting. The first reliable report of anthrax in South Africa was made in the 1880s by Hutcheon when he disclosed typical anthrax features following microscopic examination of blood from sheep (Viljoen et al., 1928). The disease was poorly reported and misdiagnosed in South Africa where Bradford (1876) mistook anthrax ("*Miltziekte*") for gallsickness and Wiltshire in 1877-1882 that stated that anthrax might be confused with sponssiekte, redwater and horsesickness.

The bacterial agent of anthrax was a myth, until Robert Koch, a German physician, and microbiologist became the first to identify and describe the whole life cycle of anthrax and formation of spore that remain in the environment for very long periods. In addition, he

stated that animals could only acquire anthrax when bacilli are transmitted from another infected host (Koch, 1937). Furthermore Koch isolated the bacterium responsible for anthrax, named it *B. anthracis* and challenged healthy animals by inoculating them with the bacterial cultures (Koch, 1937). The finding was the very first successfully established connection between microorganisms and the disease and led to the dogma of pathogenic microbiology, known as Koch's postulates (Carter, 1985). A few years later, J. H. Bell observed that the frequency of infection is high in mill workers having regular contact with animal fibres contaminated with *B. anthracis* spores. Even though it was misleading in the perception that infection was commonly seen in personnel who are in regular contact with goat or alpaca hair, this observation by Bell deduced woolsorters' disease was caused by *B. anthracis* (Laforce, 1978).

2.2 Biology of *B. anthracis*

B. anthracis is primarily a pathogen of herbivores but can infect virtually all mammals (Hambleton et al., 1984). It is a member of the Group 1 bacilli, genus *Bacillus*, a gram-positive, non-motile, rod-shaped, spore-forming, facultative aerobic bacterium that is also found in the soil. The vegetative cells of *B. anthracis* measure between 1 µm by 4-8 µm and occur in a chain with a bamboo-like or box-car appearance with the capsule visualised microscopically using India ink stain (Koch, 1937). *B. anthracis* forms a long, convoluted chain at log phase of growth in brain heart agar, but in infected tissues, it forms short chains (made of 2-3 cells) (Lincoln et al., 1964). *B. anthracis* grows optimally at 37 °C on sheep blood agar by producing non-haemolytic colonies with a doubling time of 30 to 60 minutes (Leise et al., 1959). The colonial appearance of *B. anthracis* on agar is typically 4-5 mm rough ground glass, white colonies sometimes with a characteristic comma shape or tail often referred to as "curly-hair" or "medusa head" colonies (Pearce and Powell, 1951). The colonies stick tightly to the surface of the agar and collection of colony/colonies using loop can result in strings of cells that can be drawn up and remain perpendicular to the agar surface (Koehler, 2009).

B. anthracis spore is usually formed at the central or paracentral part of the bacterium without swelling on the bacterium (Battisti et al., 1985). Sporulation occurs in *B. anthracis* as a means of survival to withstand adverse conditions, that may be associated with nutrients depletion which happens after the host is dead and exposed to air (Van Ness, 1971). This is also evident with most laboratory-based sporulation protocols, which deny nutrients to the *B. anthracis* culture at 37°C to initiate the sporulation (Tarr, 1933). The spore is made up of lamellar protein coat which covers the cortex (Kornberg et al., 1968, Warth et al., 1963). The ability of the spore to withstand environmental challenges including microbial predators is due to the presence of the protein coat covering the spore, even though the spores are resistant to physical insults but the coat is flexible (Van Ness, 1971). Spores of *B. anthracis* are highly resistant to different types of environmental changes. They can survive indefinitely in water, air, vegetation and soil despite desiccation, ultraviolet exposure, chemical treatment, intense heat or cold (Manchee et al., 1981). The highly resistant nature of the spore helps in the persistence of the disease pathogen in the harsh environmental condition (Minett, 1950). After infection of the host, the spores germinate to vegetative cells that replicate in the body tissues. The virulence factors are produced after germination in the host. Sirard et al. (1994) found that the highest level of toxins and capsule are produced at 37°C. The differentiating feature of *B. anthracis* spores and other *Bacillus* spp. is the presence of the exosporium. The exosporium is the outermost layer present in *B. anthracis* spore but absent in the spores of many *Bacillus* spp (Koch, 1937, Kramer and Roth, 1968) when viewed under crystallography and atomic force microscope (Kailas et al., 2011). The exosporium proteins are made up of BclA, which is an external hair-like nap layer extension of collagen-like glycoprotein (Fox et al., 2003). This has been identified as an immunogenic antigen for use in vaccine and a spores detection ligands in recent years (Fox et al., 2003, Tournier et al., 2009). Studies by Majumder et al. (2018) and Majumder et al. (2019) have shown that BclA stimulates better immune response in mice. However, studies by Ndumnego et al. (2018) and Koehler et al. (2017) have reported that the BclA failed to stimulate adequate immune response when compared to rPA and FIS in

goat. There may be proteinaceous parts of *B. anthracis* exosporium that are not defined due to the variation in exosporium structure and composition observed amongst *Bacillus* spp (Aronson and Fitz-James, 1976, Holt and Leadbetter, 1969). Hence the used of rPA and FIS with the exception of BclA as the anthrax vaccine antigen in this study has shown good immune response in cattle. Furthermore, our findings have confirmed that BclA is not a compulsory antigen in non-living anthrax vaccines (NLAV) to stimulate significant antibody response and to confer protection.

The sporulation process of *B. anthracis* in natural settings is stimulated by environmental factors such as shortage of phosphorus, nitrogen and/or carbon with the high aerobic condition. These sporulation conditions are lacking within the host, resulting in support of vegetative form survival (Errington, 2003, Losick et al., 1986). *B. anthracis* will survive as spores in nutrient-poor or extreme environmental conditions. *B. anthracis* requires at least 8 hours completely transform from vegetative cell to spore (Steichen et al., 2003). However, the specific factor that provokes sporulation is still not well defined, but it is believed that signal transduction network linked to available nutrient, presence and/or absence of certain environmental factors and stage in the life cycle (Turnbull, 2002).

Sporulation has seven stages based on their appearance under the microscope and genetic analysis of its elements as they evolve. During stage 0, which is also called the pre-divisional stage, their morphology is similar to the vegetative cells (LeDeaux et al., 1995). But toward the end of this stage the central appearance of the nucleoid extension forming bulky mass indicating preparation for division (Piggot and Coote, 1976). The transformation led to stage I, when the axial filament is formed by pole to pole extension of the nucleoid (Ryter et al., 1966), at this stage the chromosome copies are two thereby indicating each division will inherit the whole genome copy (Piggot and Coote, 1976). Stage II is initiated by splitting the polar septum into two compartments consisting of the mother cell and the spore-precursor, the spore-precursor take in the genome through translocation (Wu and Errington, 1998). This occur at almost the second hour of sporulation (Driks, 1999). The spore-precursor

engulfed by the mother cell (Driks, 1999, Ryter, 1965) commence stage III at about third hour of sporulation. Following complete shrouding of the spore-precursor and the mother cell exterior part, which is joined detach their attachment from the polar (Warth and Strominger, 1972). This frees the spore-precursor in the cytoplasm of the mother cell with the mother cell exterior part becoming the spore-precursor external membrane (Warth and Strominger, 1972). Stage IV and V initiate the formation of spore cortex by the synthesis of the peptidoglycan layer (Levin et al., 1993, Price and Losick, 1999). The cortex maintain the dehydration of the spore (Driks, 1999). The assembling of the spore cortex complex structure consisting of more than fifty different proteins by the mother cell is carried out in stage VI (Dion and Mandelstam, 1980, Driks, 1999, Jenkinson et al., 1980). The released of the spore occur after the degradation of the mother cell in stage VII (Driks, 1999, Piggot and Coote, 1976).

2.3 *Bacillus anthracis* virulence factors

The virulent strains of *B. anthracis* consist of two large extrachromosomal plasmids, namely pXO1 (182 kb) and pXO2 (96 kb), and they are regarded as the primary virulence factors of *B. anthracis.* Deletion of any of these plasmids attenuates the bacterium rendering it nonvirulent (Sterne, 1937, Sterne, 1939, Turnbull, 1991). The pXO2 carries genes coding for capsule biosynthesis to produce PDGA capsule regarded as poorly immunogenic. However, it plays a vital role in establishing infection in the host by conferring protection to the vegetative cells of *B. anthracis* from the phagocytic activity of the host's immune cell, complement system and other components of sera that could be bactericidal to the vegetative cells (Little and Ivins, 1999). The pXO1 encodes the tripartite proteins complex toxin that encodes for lethal factor (LF 90 kDa), edema factor (EF 89 kDa) and protective antigen (PA 83 kDa). These proteins were earlier designated as factor I (EF), factor II (PA) and factor III (LF) (Harris-Smith et al., 1958, Smith et al., 1956, Stanley et al., 1961). The tripartite protein complex toxin plays A-B catalytic moiety activity that is complementary to each other. EF and LF act as the A catalytic moiety which must bind to the B moiety that is the PA to be active and toxic to the cell otherwise, individually the proteins are non-toxic

and are separately non-covalently bond (Mogridge et al., 2002). When the B catalytic moiety binds to either of the A moiety (EF or LF) the two major anthrax exotoxins are formed. When B moiety binds to LF it gives rise to the lethal toxin (LT), and when it binds to EF, it forms edema toxin (ET) (Friedlander, 1986). The PA, after binding to either EF or ET, play the major role of translocating them into the cell where they exact their toxic function (Mogridge et al., 2002).

2.3.1 Anthrax toxins components

2.3.1.1 Protective antigen (PA)

PA is the major component of anthrax toxin, which was previously called factor II. PA is the most extensively characterised component of anthrax toxin (Stanley et al., 1960). PA was the first to be identified, cloned and sequenced of the genes that encode the proteins regulated by pXO1 plasmid. The pXO1 plasmid, which is formerly known as pBA1 housed the *pag* locus that encoded for PA (Bhatnagar and Batra, 2001). The receptor moiety that conducts protein delivery of EF and LF into the cytosol to perform their cytotoxic activity is the PA. The PA binds to tumour endothelium marker 8 (TEM8) variant 1 and 2 which is regarded as the anthrax toxin receptor 1 (ANTXR1), and capillary morphogenesis protein (CMG2) they are also regarded as the ANTXR2 (Bradley et al., 2001). The TEM8 (ANTXR1) and CMG2 (ANTXR2) are cellular receptors where PA bind and translocate the proteins (EF and LF) in the cytosol (Figure 2.1). The PA and EF/LF complex are internalised into the cell cytoplasm by receptor-mediated endocytosis this is facilitated by the interaction of the cells receptors with low density lipoprotein (LDL) receptor-related protein (LRP) 6 (Orth et al., 1992, Stefansson et al., 1995).

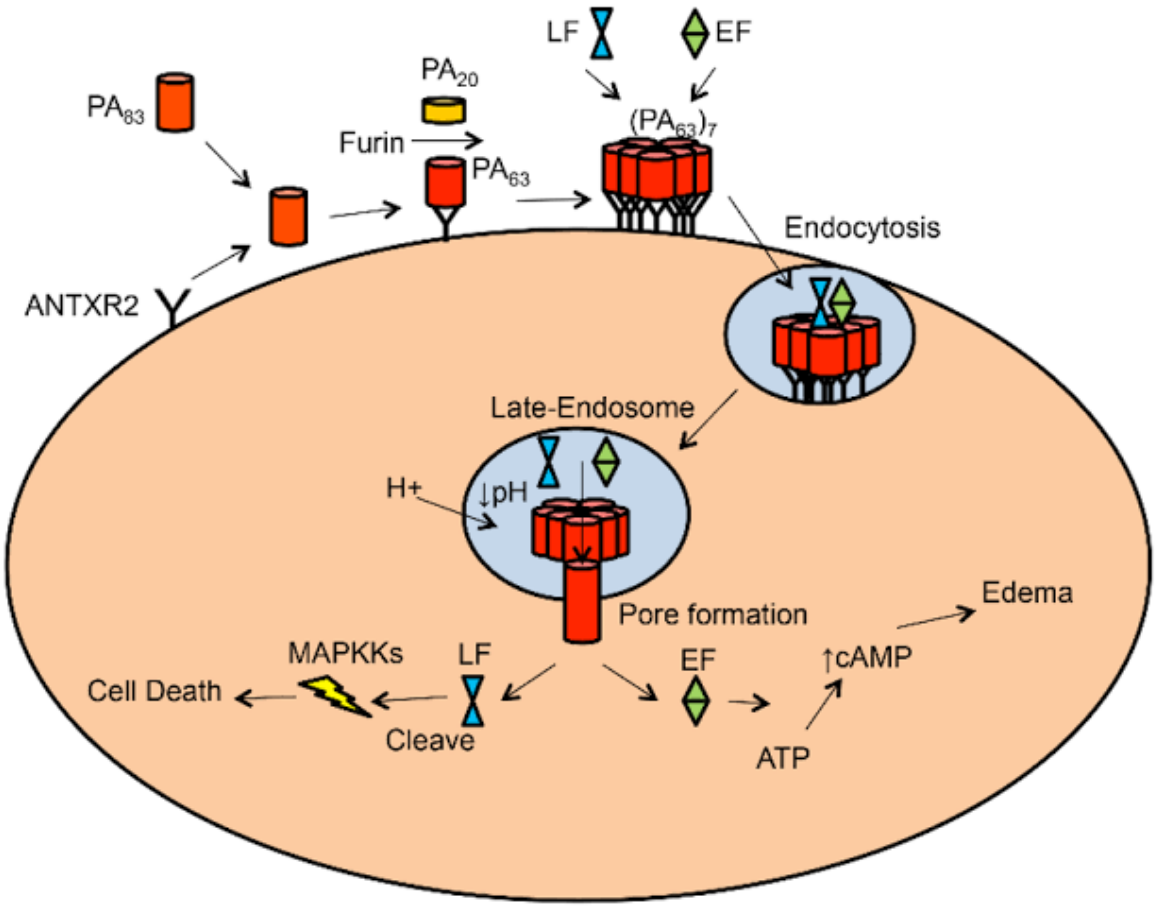

Figure 2. 1: Anthrax toxin-mediated intoxication of the host cell. The cleavage of PA83 into PA63 and PA20 by furin. The assembling of PA63 into a heptameric prepore (PA63)7, onto which LF/EF binds. The intracellular internalisation of the complex of PA63/EF/LF by the receptor-mediated endocytosis via the PA pore. EF elevates cAMP and LF cleaves MAPKK in the cytosol. (Sun and Jacquez, 2016). ANTXR2; Anthrax toxin receptor 2, ATP; Adenosine triphosphate, cAMP; cyclic adenosine monophosphate, EF; Edema factor, H+; Hydrogen ion, LF; Lethal factor, MAPKKs; Mitogen activating protein kinase kinases, PA20; protective antigen 20 kDA, PA63; protective antigen 63 kDA, PA83; protective antigen 83 kDA.

The functional region of PA comprises of four domains which were analysed and defined previously by fragmentation using chymotrypsin and trypsin cleavage (Duesbery and Vande Woude, 1999). Domain I is made up of tightly bound calcium ions resulting from the cleavage around ^{161}RKKR^{167} by furin or furin-like proteases that occur at the N-terminus of PA83 after binding to TEM8 or CMG2 during activation of proteolysis (Moayeri et al., 2015). This process leads to the cleavage of 20 kDa (PA20) from the amino-terminal (N-terminal) of PA 83 kDa, which is released into the extracellular domain (Figure 2.1). The carboxyl-

terminal (C-terminal) is the 63 kDa fragment of the PA, which forms the ring shaped heptamer by remaining bound to the cell's receptors. Studies have shown that the PA63 heptamer subunit interfaces with LF and EF by binding to 3 molecules of either LF or EF or even bind both LF and EF simultaneously under saturated condition. Therefore, up to nine different toxic complexes compositionally through the interplay between the three components of anthrax toxins (Abrami et al., 2005). It has been noted that EF and LF do not bind to monomeric PA63 but will bind oligomerised monomeric PA63 to form the ring shape heptamer (Petosa et al., 1997). The oligomerisation of PA63 has shown to stimulate the translocation of the toxin complex into the endosomes through a lipid rift-mediated clathrin-dependent process (Petosa et al., 1997). With a decrease in pH PA63 heptamers form a channel through which the EF and LF translocate to the cytosol in the endosomal membranes (Milne et al., 1994). The binding of domain II and IV of PA with von Willebrand factor A (VWA) which is a domain between extracellular regions of various cell surface protein (Bradley et al., 2001). This is mediated through metal ion dependant adhesion site (MIDAS) and the presence of several long β-strands in domain II forms the root of the membrane inserted channel (Duesbery and Vande Woude, 1999). It also has a large flexible loop which is implicated in membrane insertion and channel formation. Domain III appears to have a role in the oligomerisation of PA63, and this is involved in protein to protein binding since it contains the hydrophobic region. The other three domains are loosely associated with Domain IV, which is responsible for receptor binding (Bhatnagar and Batra, 2001). In addition, Kintzer et al. (2010) reported that PA heptameric oligomers are form at the pH 8.0 where octamers are form at the 7.3 at physiologic temperature. Indicating that the entire shift in PA is pH-dependant.

2.3.1.2 Lethal factor (LF)

LF, also called factor III, forms the other moiety of anthrax toxin along with EF (Hambleton et al., 1984, Stanley et al., 1961). LF is encoded by the *lef* located on pXO1 plasmid and is a zinc-metalloprotease with the ability to cleave and activate LF cellular targets which are MAPKKs (Figure 2.1). The *lef* expression is regulated through the *atxA* regulon (Hoffmaster

and Koehler, 1997). The protein LF has four domains (Pannifer et al., 2001), the LF domain 1 amino acid (aa) terminus share sequence homologue with EF's domain 1 N-terminus. The similarities expedite the binding of LF and EF to PA, the remaining morsel of LF shows no sequence similarity with any known protein (Arora and Leppla, 1993, Pannifer et al., 2001). The domains 2 and 3 of LF are relevant for binding of substrates, and domain 4 is a zinc-binding catalytic domain (Klimpel et al., 1994). LF binds to PA to form LT, which attribute majorly to cytotoxicity during anthrax disease (Hanna, 1999). Experiment by Arora and Leppla (1994) have shown that LF aa sequence from 1-254 aa allows the fusion of various proteins. The region is sufficient to allow their entry to cell cytosol via PA dependant route irrespective of the region it fused at aa or carboxyl terminus (Figure 2.1). The primary tissue target by LT is macrophages even though other cells are also affected, but macrophages are more prompt to the lysing effect of LT (Hanna, 1999). Majority of LF studies were built on the *in vitro* study by Friedlander (1986), where he discovered that most of the macrophages derived cell lines undergoes lysis after been exposed to the LT. Even though various cell lines show variation in sensitivities to LT, J774A.1 cell shows high sensitivity to LT, but IC21 cells are not, despite the fact that each of these cells can bind to PA and proteolytically initiate the cleavage to PA63. Only J774A.1 cell line gets lysed when the LF is introduced to the cytoplasm directly by lysis of the pinosomes by osmotic means. This implies that PA is not necessary for the activity of LF except for the fact that PA is always required to translocate LF to the cytoplasm of the cells (Park et al., 2002). Alteration in the genetic make-up of the *B. anthracis* such as deficiency in *lef* gene (encoding for LF) resulted in 3-fold higher LD_{50} whereas *cya* gene (encoding for EF) disruption resulted in 10-fold LD_{50} increase in *in vivo* mice studies (Pezard et al., 1991).

LT exacts its effects on innate and adaptive immune response by suppressing the immune response and cell signalling pathway disruption. LT exact its effect on cytokines by interfering with the MAPKK signalling, thereby interrupting with macrophages activation and neutrophils response (Duesbery et al., 1998, Moayeri et al., 2003). The *in vitro* experiments had shown many immune cells such as TNF-α and IL-10 been affected by LT by

reducing their production after exposing dendritic cells to LT, the secretion of IL-2 also declined after exposing CD4+ lymphocytes to LT (Comer et al., 2005, Tournier et al., 2005).

2.3.1.3 Edema factor (EF)

EF was previously called factor I (Hambleton et al., 1984, Stanley et al., 1961, Stanley et al., 1960). EF is the first anthrax component revealed to have an enzymatic effect, and that is adenylate cyclase (Bhatnagar and Batra, 2001, Mikesell et al., 1983). The fusion of EF to PA leads to the formation of ET. EF is a very efficient adenylate cyclase during active bacterial growth, which is facilitated by the catalytic and stimulatory activity that dependent on calmodulin presence. This is enhanced by the interaction between calmodulin of the host molecule and calcium (Hoeflich and Ikura, 2002). The structure and activity of the adenylate cyclase seen in anthrax resemble that in *Bordetella pertussis* (Gordon et al., 1989). EF enzymatic activity activation depends on host molecules calmodulin and calcium ion interaction (Leppla, 1984). Once EF gains entry into the cell it causes a 30° rotation of the helical structural domain as a result of binding of the native EF by calmodulin, this allows the protein to bind to a calcium ion and 3'dATP molecule (Leppla, 1982). The complex formed by the binding of EF to calcium ion as well as 3'dATP activates the enzyme's catalytic site, which leads to the conversion of the EF bound 3'dATP complex to cAMP (Leppla, 1984). The cAMP is a pervasive secondary cell messenger which take part in many of the cellular process regulation. cAMP can affect the host cell's ATP stores by rapidly consuming the cells' energy. In 60 seconds, EF converts up to half of the cell's ATP to a concentration of cAMP to reach 1000 of the normal concentration (Hoeflich and Ikura, 2002). The accumulation of fluids in the surrounding tissue and the characteristics edema experience during *B. anthracis* infection results from water and ion loss from the cells. This is triggered by an increase in the level cAMP in the cell and the loss of water and ions from the cell (Leppla, 1984). Even though EF's extent of cytotoxicity still deliberated, but the enzymes contributes to the virulence differently. ET is a strong inhibitor of neutrophil chemotaxis and directly reduces the phagocytic ability and oxidative burst (Yeager et al., 2009). It causes cell migration as a result of critical pro-inflammatory cytokines expression thereby causing

the innate immune response interference. This effect can be seen in monocytes culture exposed to EF by serious inhibition of expression of tumour necrosis factor (TNF)-α inducible by lipopolysaccharide (LPS) but elevates the levels of secretion of interleukin (IL)-6 (Hoover et al., 1994). BALB/c mice exposed to ET showed a toxic response to a low dose (Firoved et al., 2005). Fluid accumulation in various tissues such as kidneys, myocardium, adrenal glands, gastrointestinal tract and secondary lymphoid organs was observed during post-mortem analysis on BALB/c mice exposed to ET. This revealed that ET intoxication leads to multiple organs failure (Duesbery et al., 1998, Firoved et al., 2005). The surge in the concentration of cAMP in cardiac pacemaker cells has also been involved in bradycardia and cardiac failure, which was the effect of ET in the rat (Cui et al., 2007). In another study the effect of ET on the pathogenesis of wild type Ames *B. anthracis* and isogenic mutant *B. anthracis* that lacks other virulence factors was evaluated in rabbit. The rabbit that was infected with the wild type Ames *B. anthracis* or isogenic mutant containing ET recorded 100% lethality. Whereas, those infected with isogenic mutant *B. anthracis* containing LT recorded 80% lethality. Thereby, indicating that *B. anthracis* require the presence of just one of the toxins to be lethal (Lovchik et al., 2012).

2.3.2 Capsule and S-Layer

The *B. anthracis'* capsule is comprised of repeating polymers of D-glutamic acid, which is negatively charged with a sheath that serves as the outer layer of vegetative cells. This facilitates antiphagocytic activity by the bacterium against innate immune response. In addition, the structure and length of the glycoprotein further make the bacterium difficult to be phagocytosed (Goodman and Nitecki, 1966). These features allow lucrative immune dodging leading to the persistence of the infection. But the PDGA residues are said to be poorly immunogenic due to the simplicity of its repeating nature (Goodman and Nitecki, 1966).

The plasmid pXO2 encode the capsule operon, which consists of five genes (*capA, capB, capC, capD and capE*). *capBCA* drives the production of PDGA as well as convey and enhance

attachment of the capsule polymers to the cell wall of the bacteria (Drysdale et al., 2005). The polymerisation of PDGA is carried out by the complex integration of *capB* and *capC*. *capB* is said to contain ATP-binding (Walker-A motif) that is envisioned to bind glutamic acid onto PDGA via utilisation of ATP. Even though the function of *capC* remain anonymous, but it collaborates with *capB* to expedite the activity of ATPase (Drysdale et al., 2005, Lekota et al., 2018). Whereas, *capA* and *capE* are part of the *capBCAE* network that conveys the PDGA out of the cell following the synthesis of PDGA in the cytoplasm (Drysdale et al., 2005, Goodman and Nitecki, 1966, Green et al., 1985, Lekota et al., 2018). Capsule depolymerase (CapD) encodes PDGA or ϒ-glutamyl transpeptidase responsible for covalently attaching the capsule to the *B. anthracis*. Thus, isolates that is deficient of CapD yield that is not PDGA covalently attached to the carrier protein, thereby rendering the *B. anthracis* less virulent or nonvirulent (Candela and Fouet, 2005). In addition, PDGA can be depolymerised by utilising the water acceptor property of CapD this feature can be explored for anthrax treatment as well (Mogridge, 2007). The pXO2 also encodes three genes, namely *acpA* ,*acpB,* and *atxA* along with certain bicarbonate concentration regulates the formation of the capsule (Drysdale et al., 2004, Vietri et al., 1995, Wilson et al., 2008).

The capsule synthesis operon was shown to contribute greatly to the movement of *B. anthracis* during infection toward the regional lymph nodes (Wu et al., 2009). The capsule is thus necessary for virulence in inhalational challenges of mice by equipping the pathogen with anti-phagocytic attributes. Jang et al. (2011) demonstrated the capsule cytotoxic effect by administering fragments of the capsule, which enhanced the cytotoxicity of lethal toxin in J774A.1 mouse macrophage. Study by Scorpio et al. (2008) demonstrated the effect of host phagocytes on CapD co-treated *B. anthracis* bacilli to mimic natural infection scenario in BALB/c challenged 6 hours later. All the seven animals co-treated with CapD survived the challenge whereas those co-treated with placebo only thress out of seven survived. Scorpio et al. (2008) went futher to determine the effect of CapD treament on the virulent strain in different Swiss Webster mice groups then challenged with 4000 CFU of *B. anthracis* Ames bacilli. All the mice coinjected with active CapD survived the challenge for at least 28 h,

three among them survived further to 42 h and two for 66 h, the mice coinjected with heat-activated CapD all succumbed by 18 h. They further confirmed that enzymatic removal of CapD exposes *B. anthracis* bacilli to phagocytosis despite the ability of CapD to increase protection in Ames strain of *B. anthracis* spore (Scorpio et al., 2007, Scorpio et al., 2008). When pXO2 is removed from an isolate, either experimentally or naturally, it renders the isolate less virulent or fully attenuated (Baillie and Read, 2001). This is notably seen with the *B. anthracis* Sterne strain, which lacks pXO2 and is widely used as Office International des Epizooties (OIE) recommended anthrax vaccine (Mikesell et al., 1983, Sterne, 1939, Turnbull, 1991). However, some researchers have evaluated the *B. anthracis* capsule as a vaccine, but the success was not promising (Goodman and Nitecki, 1966, Smith et al., 1953). As claimed by some studies to be weakly immunogenic considered not a suitable vaccine candidate to stimulate protective immune response (Goodman and Nitecki, 1966, Gupta et al., 1993, Guidi-Rontani et al., 1999, Little and Ivins, 1999, Vogel, 1995).

The S-layer is a protein sheath found between the exterior capsule and the cell barrier of peptidoglycan. *B. anthracis* normal capsule formation does require S-layer but may result in structural modification. However, S-layer is not significant in *B. anthracis* virulence, it was reported that the deletion of the S-layer does not have any effect on the LD_{50} in mice ensuing virulent challenge (Kern and Schneewind, 2010). The S-layer is studied because it comprises of two proteins; the extractable antigen 1 (EA-1) and the surface array protein (Sap) that some researchers studied as prospective vaccine candidates (Kulshreshtha et al., 2012, Uchida et al., 2012). These proteins are either synthesised together or independently, and the bacterial growth phase is determined by the S-layer content (Mesnage et al., 1998, Mignot et al., 2002). The first S-layer protein to be assembled is Sap, and this is gradually outgrown during the static phase by EA-1 protein resulting in the complete replacement of Sap by EA-1 which is regarded as the developmental switch regulation of S-layer from Sap-S-layer to AE-1-S-layer (Mignot et al., 2002). The earlier mentioned interface between the capsule and bacterial membrane S-layer plays a role in preserving cell shape, inhibit cytolytic attack regulated by complement and mediated and evade the host macrophages

(Mesnage et al., 1998). S-layer may remain significant to the advancement of anthrax disease following infection due to its synergy with macrophages during phagocytosis of the bacterium and resistance to complement defence mediation (Mesnage et al., 1998). The S-layer has been demonstrated to stimulate an immune response. Hence it is considered to be a potential vaccine candidate against *B. anthracis* (Kulshreshtha et al., 2012, Uchida et al., 2012) even though there are paucity of data demonstrating the protective effect of S-layer in the animal trial study. S-layer was not consider as an antigen in this study due to the reports that only 50% of the identified S-layer protein is consider immunogenic based on serological assay (Ariel et al., 2003, Chitlaru et al., 2007, Gat et al., 2006) without strong evidence of protection in animal study based on our literature search.

2.4 Anthrax in animals

Anthrax is a zoonotic disease with a wide array of susceptible animal species ranging from domestic to wild animals. The susceptible host species primarily include herbivores (Hambleton et al., 1984). and are exposed to *B. anthracis*, mainly during grazing in contaminated vegetation and/or soil (Hambleton et al., 1984). Scavengers such as vulture, hyenas, and wild dogs have been implicated in the transmission of anthrax when they feed on infected carcasses and spread it to water holes and via their faeces (Bagamian et al., 2013, Fasanella et al., 2010, Moleon et al., 2014). Insects have been established experimentally to transmit anthrax and have also been implicated in the spreading of anthrax either mechanical or from the infected carcass to vegetation (WHO, 2008; Hugh-Jones & De Vos, 2002).

Anthrax, as a disease manifests differently in different animal species (Hambleton et al., 1984). In ruminants, the disease manifest as peracute, acute and subacute form (Hambleton et al., 1984). The peracute form of the disease in ruminant manifest clinically in animals by wobbling, dyspnoea, and convulsions. Death may occur with rapid advancement, without early signs of the disease. Often the animals are found dead without rigour mortis and bloated with unclotted blood from the orifices as a result of the *B. anthracis* toxin (Christie,

1969, Wilson and Miles, 1964). In the acute form, the animal shows signs such as pyrexia with the temperature reaching 47°C; depression due to respiratory and cardiac distress; cessation of rumination; convulsions; abortion; and drop in milk production and discolouration of the milk to blood-tinged or deep yellow (Hambleton et al., 1984). These symptoms are accompanied by haemorrhagic mucosa followed oedema of the tongue, throat, and abdomen with or without exudation of blood from all orifices followed by death within 48 to 72 hours. A post-mortem reveals pulpy spleen, unclotted blood dark in appearance, incomplete rigour mortis and expeditious carcass decay (Hambleton et al., 1984). The subacute signs are subtle in the animals, and the signs are oedematous, subcutaneous inflammation of the ventral aspect of the neck (brisket) as a result of the gland swelling and wounds in the oropharyngeal path (WHO, 2008, Hambleton et al., 1984). Furthermore, subacute sign characterised by gastroenteritis in the intestine also occurs (WHO, 2008, Hambleton et al., 1984). Septicaemia usually gets distributed to most vital organs leading to toxaemia along with all the subacute signs. The animal system is overwhelmed and eventually leads to the animal's death. In pregnant animals, the infection can induce abortion and *B. anthracis* can be isolated from the foetus (Hambleton et al., 1984).

2.5 Evolution of veterinary anthrax vaccine overtime

The anthrax vaccine used for immunisation in veterinary practice to date as well as humans in specific parts of the world consists of spores from attenuated *B. anthracis* strain (Turnbull, 1991, Knop and Abalakin, 1986). The evolution of the attenuated anthrax vaccine for veterinary use from its inception (Pasteur's duplex vaccine) to present are as follows: Pasteur's duplex vaccine (non-toxigenic and encapsulated pXO1-/pXO2+) (WHO, 2008, Mikesell et al., 1983); Sterne vaccine (toxigenic and non-encapsulated pXO1+/pXO2-); Carbosap/carbozoo vaccine (toxigenic and encapsulated pXO1+/pXO2+) (Adone et al., 2002, Fasanella et al., 2001); Experimental non-living anthrax vaccine that is devoid of the limitations such as residual virulence, batch to batch variation, risk of handling during production and incompatibility with antibiotics recorded by all the attenuated anthrax

vaccine. The search for experimental NLAVs were instigated after the discovery of PA as the immunogenic component of *B. anthracis* by Stanley and Smith (1963). Furthermore, the establishment that PA can be purified from *B. anthracis* followed by proving that it can be cloned to a vector of interest, transformed to the host of interest, then expressed and purified then be utilised for vaccines development and/or immunoassay purpose (Ivins et al., 1990, Mikesell et al., 1983, Stanley and Smith, 1963, Stanley et al., 1961). This advancement enables researchers to explore the pathogenesis of anthrax leading to the realisation that PA contributes greatly in the pathogenesis as the catalytic half necessary to form the two major anthrax toxins (LT and ET) and translocating the toxins into the cells (Milne et al., 1994, Stanley and Smith, 1963). In addition, PA was also realised to be the main protein for immunogenic response against anthrax toxins (Stanley and Smith, 1963). The later discovery led to the search for more anthrax vaccine alternatives that will be devoid of the afore-mentioned limitations witnessed with attenuated anthrax vaccines. Hence, PA became the focal point for the development of next-generation vaccines like the experimental NLAV as previously reported by Ivin et al. (1998) where they investigated the immunogenicity of PA compared AVA in Rhesus macaques. In addition, some studies reported that NLAV can consist of either rPA, BclA, FIS or a combination of the components such as rPA, BclA and FIS or rPA and BclA or rPA and FIS can confer more protection than any of the antigen alone (Brossier et al., 2002, Koehler et al., 2017, Ndumnego et al., 2018) and moving away from attenuated vaccines.

2.5.1 Pasteur's duplex vaccine

The first anthrax vaccine that proved to be effective and was able to reduce the virulence of anthrax in livestock was Pasteur's duplex anthrax vaccine (Pasteur, 1883). The vaccination schedule designed by Pasteur and his co-workers comprised of two injections at an interval of 2 weeks. The vaccine is termed a duplex vaccine because the vaccine consists of two different inoculums of *B. anthracis,* which were attenuated differently. The first vaccination consisted of *B. anthracis* cultured at 42-43°C for 15-20 days and the second vaccination was cultured at 42-43 °C for 10-12 days (Tournier et al., 2009, Turnbull, 1991).

Japan and Russia adopted Pasteur's attenuation conditions to generate attenuated anthrax vaccine known as Rentian II and Qiankefusiji II respectively (Suo, 1960). Investigations showed that the high temperature and incubation period of the culture cured *B. anthracis* of the pXO1 plasmid (Liang et al., 2016, Mikesell et al., 1983). It is well established that the genes encoding PA, EF and LF proteins form the anthrax toxins that are located on pXO1 (Okinaka et al., 1999). Therefore, the reduction in virulence of *B. anthracis* strain used in Pasteur's anthrax vaccine is confirmed to be associated with this loss of pXO1 (Liang et al., 2016, Mikesell et al., 1983).

Even though Pasteur's duplex anthrax vaccine gained quick acceptance and was widely used for livestock vaccination, it had many issues that in the end lead to its disuse. . In order to increase the longevity of the spore, 50-60 % glycerine was added to Pasteur's duplex vaccine (Turnbull, 1991). Ten years later saponin was added to boost the immunogenicity of the vaccine, however, this increased the risk of violent inflammatory reactions at the vaccination site (Tournier et al., 2009, Turnbull, 1991). Studies showed remnant virulent pXO1 in some vaccine preparations (Liang et al., 2016, Mikesell et al., 1983, Xudong et al., 1995) that lead to toxicity in some vaccinated animals, resulting in $\geq$3 % mortality of vaccinated animals. This ultimately led to the cessation of the Pasteur's duplex vaccine (Hambleton et al., 1984, WHO, 2008, Turnbull, 1991). These limitations prompted Max Sterne to review Pasteur's duplex vaccine and led to the development of SLSV (Hambleton et al., 1984, Turnbull, 1991). Yet Pasteur duplex vaccine which is non-toxigenic and encapsulated is still manufactured for use in goats and Horses (WHO 2008)

2.5.2 Sterne vaccine (toxigenic and non-encapsulated pXO1+/pXO2-)

The *B. anthracis* Sterne vaccine strain was developed and produced in 1939 to curb the limitations experienced with Pasteur's vaccine and is still in use today throughout the world. The SLSV was developed by sub-culturing an isolate of rough colonies of avirulent *B. anthracis* from a case of anthrax in bovine (Sterne, 1939). The *B. anthracis* isolate was cultured on 50% horse serum nutrient agar overnight with 30% CO_2 at 37°C. The attenuation resulting from the culture condition led to the loss of the encapsulation plasmid (pXO2) and

the creation of the *B. anthracis* Sterne strain (designated *B. anthracis* 34F2 strain) (Mikesell et al., 1983, Sterne, 1937). SLSV consist of suspension 6 x 10^5 to 1.2 x 10^6 spores per ml in 0.5% saponin and 50% glycerine (Sterne, 1937, Sterne, 1939, Sterne, 1945). Recently OIE recommends the following doses for small ruminants and cattle to be used 1-5 x 10^6 and 2 x 10^6 spores per ml respectively. However, some countries recommend the use of 0.5 ml and 1.0 ml of 1-5 x 10^6 and 2 x 10^6 spores per ml for small ruminants (goat and sheep) and cattle, respectively. Whereas, for horses the OIE recommends two initial doses of vaccine with a 4-8 week interval, to allow sufficient protection (OIE, 2018). Two vaccinations with SLSV provides hyper-immunity to animals as reported by Ndumnego et al. (2016). In a study by Ndumnego et al. (2016) different groups of goats were vaccinated once and twice with SLSV and challenged with a virulent strain of *B. anthracis*. The group that was vaccinated once showed that the lower immune response and level protective after the first vaccination 60% goats vaccinated with SLSV and challenged 6 weeks after the vaccination survived compared to the group vaccinated twice with SLSV and challenged 62 weeks after the vaccination 80% of the vaccinated goats survived (Ndumnego et al., 2016). Goats vaccinated twice with a 3-month interval between the Sterne vaccination all survived *B. anthracis* lethal challenged (100%) (Ndumnego et al., 2016). This could be associated with an anamnestic response of the humoral immune response.

The immunity in animals is produced by germination of spores following the vaccination. The germinated spores produce the toxins that are predisposed to the macrophage for phagocytosis and thus neutralising the toxic elements (Guidi-Rontani et al., 1999). The SLSV has some shortcomings such as limited duration of protection it offers to vaccinated animals, and therefore, an annual booster is required (WHO, 2008). The limited duration of protection is a challenge for the wildlife industry where the annual booster is costly, impractical or impossible (Turnbull, 1991). Residual virulence has been reported in some animal species (like goats and llamas) (WHO, 2008, Cartwright et al., 1987, Turnbull, 1991). Hence the dosage for goat is recommended to be 0.5 ml of 1.0 ml of 1-5 x 10^6 and 2 x 10^6 spores per ml by OIE (OIE, 2018). Most significantly the live spore vaccine is not compatible

with the simultaneous antibiotic treatment required in an outbreak situation or at feedlots as earlier mentioned (Fasanella et al., 2008, Stepanov et al., 1996, Webster, 1973). It is still the vaccine of choice by the OIE since its introduction in the 1930s.

2.5.3 Carbosap/carbozoo vaccine (toxigenic and encapsulated pXO1+/pXO2+)

The Carbosap vaccine strain was procured from Professor Cilli's research group in the Instituto Vaccinogeno of Asmara, Country during World War II (Fasanella et al., 2010). The process of producing the Carbosap vaccine remains unknown. However, it is known that the Carbosap spores are suspended in 10 % saponin, which is considered adverse to the vaccinated animals (Turnbull, 1991). Carbosap vaccine was developed in 1949 and used in various countries but mainly Italy until 2006 (La et al., 2006). Carbosap vaccine was shown to be effective in vaccinated cattle and sheep (Fasanella et al., 2001), even though it contains both plasmids (pXO1+/pXO2+) (Uchida et al., 1985). The Carbosap strain is less virulent when compared to fully virulent *B. anthracis* strains (La et al., 2006, Uchida et al., 1985).

2.6 Experimental approaches of anthrax vaccine

SLSV has been very effective against anthrax in animals since its development in the 1930s and is still recommended as the veterinary anthrax vaccine of choice today. Efforts to develop or improve the present veterinary vaccine are ongoing. The characterisation of the PA, EF and LF of pXO1 in the 1950-1960s (Stanley and Smith, 1963, Stanley et al., 1961, Stanley et al., 1960) enabled researchers to develop assays to monitor immunogenic response to the vaccine and relate their findings back to protective immunity. Stanley and Smith indicated PA as the main immunogenic component of *B. anthracis* in 1963 (Stanley and Smith, 1963) which led to the advancement in anthrax human vaccine research by changing the approach to use a specific bacterial component to develop anthrax vaccine. The human anthrax vaccines consist of PA harvested from the supernatant of the unencapsulated *B. anthracis* V770 strain culture used to formulate anthrax vaccine adsorbed (AVA) in the USA. The PA used in anthrax vaccine precipitate (AVP) was prepared

from the culture supernatant of Sterne strain 34F2 in the UK (Wright et al., 1954). The AVP and AVA was development because it was considered improper to used attenuated *B. anthracis* spores as human vaccine due to the limitations recorded in some vaccinated animals. However, the culture supernatant used in human non-living anthrax vaccines, AVP in the UK and AVA in the USA were later discovered to contain remnants of LF and EF (Knop and Abalakin, 1986, Turnbull, 1991). Thus, the main shortcoming of AVA and AVP vaccines is the residual virulence due to these trace complementary toxin components (Turnbull et al., 1986, Turnbull et al., 1988), coupled with the number of boosters required to achieve adequate protective immunity (Brachman et al., 1962). The limitations necessitate the exploration of novel experimental approaches to design and investigate new and robust anthrax vaccines that provoke a sufficient protective immune response that either require no or minimum booster immunisation. These experimental vaccines are further discussed below.

2.6.1 Recombinant protein anthrax vaccine

As indicated the purification of the various *B. anthracis* toxin components PA, EF and LF (Stanley and Smith, 1963, Stanley et al., 1961) and the discovery that the immunogenic component (PA) provide sufficient protection in the absence of LF and EF (Ivins et al., 1986, Ivins et al., 1990, Ivins and Welkos, 1988, Turnbull et al., 1990), opens the door for second-generation vaccine investigations. Recombinant PA83 (rPA83) was expressed in *B. subtillis* (Ivins et al., 1990, Ivins et al., 1992) and used to vaccinate guinea pigs and the sera of the guinea pigs showed significant high anti-PA antibody titre with little to no anti-LF and anti-EF antibody titres (Turnbull et al., 1986, Turnbull et al., 1988, Turnbull et al., 1990). The rPA83 was expressed in various other organisms such as baculovirus, vaccinia virus and *Salmonella* with a good immunogenic response following the initial success (Baillie et al., 2008, Iacono-Connors et al., 1994). Little et al., (1988) evaluated the immunogenic effect of PA in mice using ELISA and *in vivo* toxin neutralisation assay (TNA). Which measure the ability of the antibodies generated in vaccinated animal to neutralised *B. anthracis* LT/ET cytotoxic effect. Much later an *in vitro* TNA was developed by Pitt et al. (2001) and Reuveny

et al. (2001) thereby minimising the use of laboratory animals for the assay. The rPA83 was also expressed in avirulent, sporulation deficient and nontoxigenic *B. anthracis* and *Escherichia coli* cells with good immunogenic responses in vaccinated humans using anti-PA IgG ELISA and *in vitro* TNA titre (Bellanti et al., 2012, Brown et al., 2010, Campbell et al., 2007, Gorse et al., 2006). The rPA serological result obtained from the human volunteers in these studies were not significantly different to serological results from the group that received AVA or AVP (Bellanti et al., 2012, Brown et al., 2010, Campbell et al., 2007, Gorse et al., 2006). The expression of active rPA83 in a medium with avirulent, sporulation deficient and nontoxigenic *B. anthracis* is a marked improvement in terms of the safety and risk associated with vaccine production (Farchaus et al., 1998). At this stage, the expression of rPA for recombinant anthrax vaccine used prokaryotic host for expression. The use of prokaryotic cells for recombinant protein expression offer benefits such as simple culture conditions and easy purification procedure. However, the disadvantages are the accumulation of protein forming inclusion bodies, mammalian proteins are usually difficult to express, contamination with host protease that lead to the degradation of the expressed protein, the production of endotoxin from hosts such as *E. coli* and *B. subtilis* as well as the codon usage differences between prokaryote organisms (require codon optimisation of expressed gene for prokaryotic host cell).

Eukaryotic systems like yeast and plants have also been used for the expression of rPA. The expressed rPA63 in *Saccharomyces cerevisiae* were not excreted in the culture media and the rPA63 was obtained under the denatured condition from rPA83. The immunogenicity of the rPA63 expressed in yeast was compared to rPA83 expressed in *E. coli.* The yeast expressed rPA63 showed a reduced effect in toxicity assay (Hepler et al., 2006). Despite the unsatisfactory expression of rPA83 in *S. cerevisiae* resulting in denatured rPA63 and reduced toxin neutralisation effect, the denatured rPA63 was evaluated for protective immunity in rabbit and rhesus macaques. The denatured rPA63 protected 100% (3 of 3) rhesus macaques and 89% (8 out of 9) rabbits against inhalational challenge with virulent *B. anthracis* spore (Hepler et al., 2006). Most studies use rPA83, but this study indicates that

rPA63 is sufficient for protection against inhalation challenge. However, most scientists use rPA83 as vaccine candidate due to the complex, laborious and expensive procedure for recombinant protein production (cleavage) and tests required to obtain rPA63 (Hepler et al., 2006, Tao et al., 2017).

Plants have been used to express rPA, despite the prokaryotic nature of rPA. Prokaryotic proteins are not glycosylated and lack all the sites for glycosylation, unlike eukaryotic proteins. The rPA expressed in *Nicotiana benthamiana* was able to induce good anti-PA ELISA and TNA titre and protected New Zealand white rabbit from *B. anthracis* spore following inhalational challenge (Chichester et al., 2013). Even though the use of eukaryotic cells for protein expression has the following benefit; good scalability, highly soluble protein, carry out post-translation modifications such as glycosylation hence, the eukaryotic cell is more suitable for the expression of eukaryotic cells. However, the eukaryotic system is deficient in the following important protein expression attributes only present in prokaryotic protein expression system such as easy to modify genetically; easy to cultivate, grow and harvest. Also, it is cost-effective and give high protein yield for rPA range from 50 to 400 mg/ml (Chauhan et al., 2001, Laird et al., 2004, Shiloach et al., 2010) compared to eukaryotic cells as seen in the yield of rPA express in eukaryotic cells range from 50 to 150 mg/ml (Hepler et al., 2006, Ramirez et al., 2002). The large-scale protein expression (up to $\geq$100 L) application of the prokaryotic system in addition to other features makes it suitable for industrial use to produce vaccine antigens, antibodies, drugs and conjugates.

The advancement in vaccine research, especially the rPA vaccine, has shown a significant improvement in terms of knowledge and skills in a vaccine study. This approach is well-marked in terms of safety of handling during production. Still a lot needs to be done because the rPA vaccine has some disadvantages such large scale production and the cost of production in comparison with the present human anthrax vaccine (AVA and AVP). The rPA vaccine did not show any significant edge over AVA and AVP in terms of dosage required to establish protective immunity in laboratory and host species (Williamson et al., 2005). as it

still requires three or more immunisations (Cybulski Jr et al., 2009, Little et al., 2006, Pittman et al., 2002) for protection. But the live spore vaccine used in veterinary practice that requires one immunisation and two to be hyperimmune (Little and Knudson, 1986, Turnbull et al., 1986). Studies have shown limited lifespan of rPA formulated with aluminium hydroxide adjuvant which may be associated with reports from some studies indicating that aluminium hydroxide adjuvant lacks ability to stimulate cell mediated immune response, as it has been shown to have lower toxin neutralising antibodies following storage (D'Souza et al., 2013, Wagner et al., 2012). Therefore, the longevity and duration of immune response needs to be improved by selecting adjuvants that can stimulate both humoral and cell mediated immune response or combining two or more adjuvants to complement the limitations of each other. Alternatively, this could also be achieved either by revamping the design and composition of the recombinant vaccine by adding more antigen such as FIS, BclA, BxpB, to rPA as previously shown to improve the immune response and level of protection (Brossier et al., 2002, Hahn et al., 2006, Little and Knudson, 1986, Majumder et al., 2018, Majumder et al., 2019, Vergis et al., 2013, Koehler et al., 2017, Ndumnego et al., 2018, Vance et al., 2015).

2.6.2 Deoxyribonucleic acid (DNA) anthrax vaccine

Instead of using the whole antigen as in the case of an attenuated live vaccine, inactivated or killed vaccine, the simple approach of utilising DNA encoding the gene for the immunogenic component of the antigen are novel and safe. Ito (1960) first reported mammalian cells can be transfected with DNA as a property of naked DNA in an *in vivo* study. Thirty years later Wolff et al. (1990) conceived the idea of a DNA vaccine by expressing β-galactosidase gene in a bacterial plasmid to obtain recombinant β-galactosidase gene which he administered to mice. Following the introduction of the genetic material encoding the immunogen into the host cell which could be by intradermal, subcutaneous or intramuscular route. There is a belief that the possibility of maintaining the vaccine antigen in the host last longer than protein-based vaccine (Cybulski Jr et al., 2009). Hahn et al. (2006) reported that PA-encoding DNA vaccine administered in sheep is

slow to initiate an antibody response when compared to the protein-based immunised sheep (each vaccine formulation was administered three times). Still, the DNA vaccine immunological response in sheep lasted longer. Even though some studies reported that the host protection attained with DNA vaccine encoding PA alone have been finite (Midha and Bhatnagar, 2009, Riemenschneider et al., 2003). In a study by Hahn et al. (2004), DNA vaccine encoding pSecTag PA83 DNA at the dose of 1 μg injected to mice was able to protect 86% of the mice against challenge with 10 LD_{50} of *B. anthracis* STI spores. Whereas, 83% and 73% survival rate were noticed among mice that were vaccinated with 1 μg and 5 μg of pSecTag PA83 DNA and challenged with 100 LD_{50} of *B. anthracis* STI spores. Some researchers have reported the augmenting immunogenicity of PA-based anthrax DNA vaccine when combined with other components of the antigen. In a study by Williamson et al. (1999), the result showed that the secondary immune response *in vivo* was augmented when the vaccination schedule is encompassing priming with plasmid DNA, and free protein was used for boosting. In another study by Köehler et al. (2015) demonstrated partial protection of mice against *B. anthracis* Ames strain when BclA-encoding DNA was combined with PA, compared to when used individually. Some studies have indicated a significant increase in the toxin-neutralisation antibody and anti-PA IgG titre through antigen combination and boosting (Hermanson et al., 2004, Liu et al., 2009, Price et al., 2001). Hence confirming some important features of DNA vaccine which includes; antigen-specific immune response, the vaccine is heat stable and can be stable for storage, it stimulates both a humoral and cellular immune response and is cost-effective to produce (Khan, 2009, Kim et al., 2010, Kindt et al., 2007). However, the limitations of DNA vaccines include weak immunogenicity due to poor coverage, it stimulates an immune response against proteinaceous immunogens only and the transfection of foreign DNA to host genome may be carcinogenic (Fowler and Barnett, 2012, Sasaki et al., 2003, Sun et al., 2010, Khan, 2009). As a result, more advanced research needs to be carried out to increase the coverage of the antigen (DNA) in the vaccinated host. and purification methods need improvement to remove the carcinogens.

2.6.3 Subunit anthrax vaccine

The molecular understanding of the pathogenesis and immune mechanism of *B. anthracis*, coupled with the deep understanding of the anthrax toxins structure have led to the exploration of PA and its domains for subunit vaccines (Bramwell et al., 2005). It has been postulated that *B. anthracis* proteins or PA domain (PA-D4) enhance the effectiveness of the subunit vaccine. Flick-Smith et al. (2002) reported the use of glutathione s-transferase fused to PA domains 1-3 was used to vaccinate A/J mice. Their finding reveals that PA-D4 proved to be immunogenic by protecting 5 of the 5 A/J mice from *B. anthracis* STI spore after challenge. The low level of protection recorded by other domains (1-3) may be associated with the lack of protective epitopes on the domains (Little et al., 1996). Baillie et al. (2010) showed 100% protection in mice (8 of 8) vaccinated with subunit vaccine consisting of PA-D4 fused to LF domain 1 (LF-D1) following challenge with a virulent strain of *B. anthracis* spores. The mice group vaccinated with only PA-D4, 88% (7 of 8) mice survived the challenge with virulent *B. anthracis* spores (Baillie et al., 2010, Chichester et al., 2007). PA-D4 vaccine was formulated with nano-emulsion and poly-lactide-co-glycolide (PLGA) in another study. The nano-emulsion PA-D4 vaccine could not significantly protect (1 out of 8) Swiss Webster mice challenged with virulent *B. anthracis* strain (Manish et al., 2013). The subunit vaccine has shown the ability to protect vaccinated laboratory animals and can be used even in an immunocompromised host. However, the subunit vaccine downside is that it does not confer protection following single vaccination, hence, it requires multiple boosters to confer protection. Therefore, the subunit vaccine requires augmenting antigen and conjugate to confer protection (Baillie et al., 2010).

2.6.4 PA/subunit combined with *B. anthracis* antigenic components

The live spore attenuated vaccine with it batch to batch variations, adverse reactions, biosafety issues and inability to use concurrently with antibiotics still outcompeted the immunogenic protection conferred by the new generation anthrax vaccines (Little and Knudson, 1986, Welkos and Friedlander, 1988). Therefore, the formulation of new generation vaccines was adapted to include the two major antigen phases of *B. anthracis*

pathogenesis namely, the spore during early infection stage and the vegetative stage strictly represented by the extracellular capsule (Brossier et al., 2002, Enkhtuya et al., 2006). Some researchers such as Brossier et al. (2002) and Gauthier et al. (2009) evaluated the protective potential of two major *B. anthracis* antigens. In their studies, they both demonstrated the combination of rPA with formaldehyde inactivated spores (FIS) was able to stimulate stronger immune response and protection against inhalation/cutaneous guinea pig and mice anthrax challenge model (Brossier et al., 2002, Gauthier et al., 2009). The protection offered by either FIS or PA alone was very poor compared to the robust immune response and complete protection shown by the combination of the two antigens (Brossier et al., 2002, Gauthier et al., 2009).

There is a notion that BclA is the immunodominant glycoprotein of the hair-like outer layer of the spore. Some researchers predicted BclA as the main target of an immune response against the spore (Bozue et al., 2007, Hachisuka et al., 1966, Steichen et al., 2003, Steichen et al., 2005, Sylvestre et al., 2002). The immunogenicity of rBclA and rPA was evaluated in A/J mice, where the mice were first vaccinated with PA and two weeks later received BclA dose as a booster followed by cutaneous challenge with Sterne spores. Individually either BclA or PA only could render insufficient protection to the mice, but in combination, conferred full protection to mice (Brahmbhatt et al., 2007). The same research group reported sera from rabbits vaccinated multiple times with BclA at monthly interval partially protected naïve mice in passive protection test by delaying the time of death. Nonetheless, the antibodies generated against BclA exposes the spores to be phagocytosed by macrophages and decreases its germination in macrophages as seen in *in vitro* (Brahmbhatt et al., 2007). The combination of BclA with PA-D4 demonstrated high IgG ELISA and TNA titre in mice and also provided 100% (6 out of 6) mice protection after challenge with the crude anthrax toxin (Majumder et al., 2018).

Similarly, goats were immunised thrice with non-living vaccine formulated with FIS, BclA, and rPA adjuvanted with lipopeptide. The group of goats vaccinated with FIS, BclA, and rPA

formulated vaccine stimulates an immune response against the antigens and showed significant toxin neutralising antibodies. The serum from the vaccinated goat was able to protect A/J mice following challenge with *B. anthracis* Sterne 34F4 spore using passive mouse protection model. The survival rate of 73% was recorded among groups vaccinated with FIS, BclA, and rPA formulated vaccine and 68% survived from the group vaccinated with BclA and rPA formulated vaccine, these findings correlated with the IgG ELISA and TNA titre (Ndumnego et al., 2018). The same research group also tested the protection in goats using rPA, BclA, and FIS vaccine and rPA and BclA vaccine against a virulence *B. anthracis* challenge. The rPA, BclA and FIS conjugated vaccine was able to protect 80% (8 out of 10) of the challenged goats, whereas rPA and BclA conjugated vaccine protected 50% (4 out of 8) of the challenged goats (Koehler et al., 2017). In a different study, the same research group evaluated the immunogenicity of BclA and rPA formulated vaccine either alone or combined adjuvanted with lipopeptide in mice. There was a significant immune response against both BclA and rPA in all vaccine groups. However, only 10% survival rate was recorded among the group of mice vaccinated with either BclA or rPA alone compared to 70% survival of the mice vaccinated with the combination of BclA and rPA following challenge with virulent *B. anthracis* Ames strain (Köhler et al., 2015). Bozue et al. (2007) noted that BclA is not required for pathogenesis of fully virulent *B. anthracis* but it is a significance immunodominant component of *B. anthracis.* This was discovered when a group of guinea pigs were injected via i.m. with BclA and another also were exposed to BclA via intranasal route. Then challenged with the wild *B. anthracis* Ames strain spore or *bclA* mutant *B. anthracis* (Bozue et al., 2007). However, previous studies in goat by Koehler et al. (2017) and Ndumnego et al. (2018) showed that BclA failed to stimulate significant immune response when compare to FIS. Hence there finding entails our the decision to use FIS as the complementary antigen to rPA.

The PDGA capsule was reported to be one of the components of *B. anthracis* virulence factors, and it is the first component *B. anthracis* that emerges to the immune system following germination of spore during infection (Harrison et al., 1989, Sirisanthana et al.,

1988). Even though the PDGA was reported to be poorly immunogenic (Little and Ivins, 1999). However, research has shown that when PDGA is fused to a carrier antigen, it results in an improved immunogenic response (Goodman and Nitecki, 1966). Studies have shown that when PA is combined with PDGA they provide better protection by augmenting the immunogenicity of each other and the immunogenicity observed is better than either PA or PDGA alone. This has been proofed where mice vaccinated with PA and PDGA conjugated vaccines showed increased survival to protect mice challenged with virulent *B. anthracis* strain compared to PA, or PDGA vaccines alone (Chabot et al., 2004, Joyce et al., 2006, Rhie et al., 2003, Schneerson et al., 2003, Sloat and Cui, 2006, Wang et al., 2004, Wimer-Mackin et al., 2006). However, majority of the studies on PDGA reported PDGA to be poorly immunogenic (Little and Ivins, 1999, Leppla et al., 2002). Based on these reports we decided to use antigens (rPA and FIS) that was reported to be immunogenic in ruminant (goat) (Koehler et al., 2017, Ndumnego et al., 2018)

The PDGA is reported to be weakly immunogenic yet recognised as a virulence determinant (Goodman and Nitecki, 1967). The PDGA enable *B. anthracis* to evade the immune system of the host which was tested assessing the anti-PDGA antibodies ability to bind to *B. anthracis* spores and deny the spores ability to evade the innate immune attack using opsonophagocytosis (Schneerson et al., 2003, Wang et al., 2004). Some studies evaluated the ability of PDGA to stimulate opsonising antibodies. Schneerson et al. (2003) injected Swiss-Webster mice with 2.5 µg of PDGA adjuvanted with Freund's complete adjuvants (FCA) while Wang et al. (2004) vaccinated female BALB/c mice with 25 µg PDGA adjuvanted with Freud (Wang et al., 2004). Interestingly the *in vitro* analysis of anti-PDGA antibodies from all the vaccine groups have displayed the capacity to actively opsonise *B. anthracis* spores and prevent it from evading phagocytosis by macrophages (Schneerson et al., 2003, Wang et al., 2004). In addition, Rhie et al. (2003) further reported the ability of anti-PDGA antibodies to induce killing of bacilli mediated by complement-dependent mechanisms.

2.6.5 Virus nanoparticle (VNP) and virus-like particle (VLPs) vaccines

So many means have been explored to develop new generation anthrax vaccine experimentally, amongst them is a novel approach where virus' structural makeup and antigenic potential is harnessed to enhance PA antigen's immunogenicity. The VNP/VLP vaccine development approach takes advantage of the size of the viruses (10-300 nm) which suit the antigen size range 10-200 nm that is the antigenic size required by antigen-presenting cells such as macrophages and dendritic cells for uptakes (Bachmann and Jennings, 2010). The antigen presenting cells (APCs) plays a vital role by inducing the adaptive immune response by taking up the antigens and initiate stimulation of CD4+ helper T-cells by presenting the antigen on MCH class II receptors of APC (Bachmann et al., 1993). The mobilised CD4+ helper T-cells provide essential B-cells activation resulting in antibody class switching and differentiation into memory B-cells and antibody generation (Bachmann et al., 1993, Hong et al., 2018, Roche and Furuta, 2015). Following the evaluation of the structural makeup of native PA and lack of long lasting immune response. It was discovered that PA is not suitable for uptake by APCs due to its size and PA lacks the repetitive structural pattern that is obligatory for the initiation of robust B-cells response (Plummer and Manchester, 2011). Therefore, virus nanoparticle (VNP) and virus-like particle (VLP) vaccine platforms have been explored to take advantage of the exclusive size and structural attributes of viruses for manifesting viral or non-viral antigens, such as bacterial toxins, by conjugating neutralising peptide sequences or protein domains to diverse types of viral capsids (Lee et al., 2016).

Several VNP and VLPs have been used to construct anthrax vaccine experimentally by utilising VNP and VLPs to present whole PA or PA subunit (single domains) (Manayani et al., 2007, Schwarz et al., 2017). Viruses such as Venezuelan equine encephalitis virus have been used by Lee et al. (2003) to develop Venezuelan equine encephalitis virus-vectored based anthrax vaccine using full-length PA83 expressed in a eukaryotic cell. The vaccine was able to produce a protective immune response that protects 90% of the A/J mice that received booster four times followed by challenge with Sterne strain of *B. anthracis* (Lee et al., 2003).

Virus particles such as the capsid of Flock house virus (FHV) was utilised to express von Willebrand A domain of anthrax toxin receptor (CMG2) on its surface (Manayani et al., 2007). Then PA83 was loaded in the capsid of the icosahedral insect virus Flock House virus in a repetitive pattern, though the interaction is not covalent but is stable. This was able to protect all 5 Harlan Sprague Dawley rats against lethal dose compared to the vaccine group with monomeric PA as control where none of the 5 Harlan Sprague Dawley rats survived the lethal toxin challenge (Manayani et al., 2007). This indicates that the multivalent frame-up of PA on the surface of VLP stimulated rapid and even more robust immune response capable of protection, contrary to monovalent PA83 thereby, rendering VLP vaccine research prototype. Studies have shown that PA-D4 has been utilised as a vaccine candidate in so many viruses (HA of influenza, Hepatitis B core, Parvovirus B19, and Rabies virus). These viruses surface proteins and capsid such as glycoprotein-G in rabies virus used to load PA-D4, and were able to stimulate strong neutralising antibodies (Bandurska et al., 2008, Li et al., 2005, Ogasawara et al., 2006, Smith et al., 2006). Notwithstanding, their protective effectiveness remains unknown since animal challenges were not carried out. In a study conducted by Zhang et al. (2006), an antibody neutralising epitope of PA was identified to be 2β2-2β3 loop region located on PA domain II (PA-D2) consisting of 302 to 323 aa. This epitope has been explored to develop VNP and VLPs vaccines against anthrax. Hepatitis B particles were genetically expressed while carrying this epitope. The vaccine was examined against anthrax as an epitope-based vaccine. Guinea pigs that were immunised with this epitope-based vaccine without adjuvant, which was able to protect 4 out of 7 guinea pigs challenged with virulent *B. anthracis* spores whereas 2 of the 3 guinea pigs vaccinated with the formulation containing adjuvant survived (Yin et al., 2008, Yin et al., 2014). This indicates that neutralising epitope-based VNP/VLPs anthrax vaccine can be utilised for the anthrax vaccine.

VNP/VLPs based anthrax vaccines are the emerging field in anthrax vaccine research of which the performance and especially protection of the vaccines developed need to be investigated to acquire a deep understanding. Researchers believed that this approach has

other advantages over the current PA vaccine used in human and animals. VNP/VLP are believed to be highly immunogenic even without adjuvant been added to the vaccine formulation it still stimulates adequate immunity due to their ability to mimic a real virus (Lee et al., 2016). They are regarded as very safe because they are just virus-like and stronger immunogenically than inactivated viruses or subunit virus vaccine (Goldman and Lambert, 2004, Vogel, 2000). Nonetheless, VNP and VLP have some limitations which include the expression and purification process. In addition, the expression, detection and quantification in the broth culture suitable for the host cells are difficult to detect /follow as VNP/VLP are surrounded by the host cell proteins and not assembled structural proteins and exosomes. VLPs are visualised using a transmission electron microscope (TEM), however, and they cannot be quantified (Lua et al., 2014).

2.7 Adjuvants

The achievement of a protective immune response from most vaccines is usually enhanced by adjuvants. Adjuvants are the association of compounds that are heterogeneous in their makeup and can enhance an immune response or revamp the immunogenicity of a peptide or protein vaccine that is weakly immunogenic (Gupta et al., 1993, Vogel, 1995). The term adjuvant was derived from a Latin word *adjuvere*; the word means “to aid or support”. In the 1920s Ramon first described adjuvants when he and his colleagues accidentally detected that an abscess, which developed at the site of infection of diphtheria toxoid in a horse, developed a higher titre of specific antibodies (Ramon, 1925, Ramon, 1926), They later injected horse with diphtheria toxoid along with a substance that is not related to diphtheria toxoid. They discovered that higher immune response from abscess generated at the injection site (Ramon, 1925, Ramon, 1926, Ramon, 1959). The selection of the most befitting adjuvant when formulating vaccine is very important as the immunogenicity and duration of the immunity especially in the case of killed vaccine are augmented by adjuvants. An adjuvant can be a relevant and potent factor to immunogenicity as well as harmful to the vaccinated host. The selection of an adjuvant should be guided by features such as the stimulation of wider immune response to various serotype, early immune

response stimulation, longevity, improve immunogenically weak antigens and provide a good delivery of antigen for uptake by APC as well as being safe to administer to the animal host (Aiyer-Harini et al., 2013, Douce et al., 1995, Marx et al., 1993, McElrath, 1995).

2.7.1 Systemic ways adjuvants act

There are various ways adjuvants act, starting from a simple vehicle for antigen delivery, attracting specific immune response to the site of injection by forming a depot and decoy antigen as immune attention seeker (Awate et al., 2013, Lambrecht et al., 2009). The provocation and the enrolment of the cells of the immune system at the site of antigen deposit are usually initiated by the pro-inflammatory response induced by the adjuvant (Awate et al., 2013, Goto and Akama, 1982, Mosca et al., 2008). In most scenario, such pro-inflammation induced by the adjuvant can lead to some injury to the tissue (Calabro et al., 2011, Gerdts, 2015). This leads to detection by the immune system which immediately elicits immune cells and engages in the mechanism involving the stimulation and activation of either innate or adaptive immune response or both depending on the mechanism of action of the adjuvant which may sustain the antigen presentation activity by the adjuvant (Mosca et al., 2008, Reed et al., 2013, Seubert et al., 2008). Sometimes, necrosis or apoptosis can occur at the injection site, this leads to the mobilisation of immune cells such as dendritic cells, macrophages, and neutrophils. Swelling, local pain, and redness can be seen with localised inflammation (Calabro et al., 2011, Gerdts, 2015). The use of an adjuvanted compound to stimulate more and long-lasting immune response in anthrax vaccine have been in practice since the 19th century. Pasteur's anthrax duplex vaccine (Pasteur, 1981) was modified in the 20th century by first adding 50-60% glycerine which acted as the adjuvant by improving the immune response as well as increasing the longevity of the immunity (Sterne, 1959). Furthermore, 1-10% saponin regarded as an adjuvant was also added to the Pasteur II vaccine in the 1930s, which was believed to further attenuate the Pasteur vaccine strain. However, it turned out to stimulate severe inflammation at the injection site due to the high concentration of saponin (Sterne, 1959). Hitherto, a further study by Max Sterne reported that the combination of 50% glycerine and 0.5-1% saponin in

anthrax live spore vaccine formulation stimulated better immune response as well as increase the longevity of anthrax vaccine. Sterne finally concluded that saponin in anthrax attenuated vaccine highly enhances protective immunity with minimal virulence effect on vaccinated animals. Therefore, it should be used as an adjuvant with more attenuated strain and not more than 0.5-1% should be used in the formulation (Sterne et al., 1939, Turnbull, 1991).

2.8 Types of adjuvants for animal vaccines

There are different types of adjuvants used in the veterinary vaccine, the selection of the adjuvants is based on the types of the vaccine and vaccine formulation in question.

2.8.1 Particles adjuvants

These are adjuvants where the adjuvating components are made up of microscopic particles and nanoparticles (Gerdts, 2015, Gupta and Chaphalkar, 2015). These adjuvants deliver the vaccine to APC for immediate processing and deliver to other forms of immune cells, which also targets the mucosal surface as well as the nasal and oral route for delivery (Dalmo et al., 2016, Mutwiri et al., 2005). Most of the particles used as adjuvants range in size from 50 – 100 nm to 2-5 microns and are developed from a variety of polymer both natural and synthetics (Dalmo et al., 2016, Mutwiri et al., 2005). A synthetic group of a polymer such as polyphosphazene was optimised as a composition of adjuvant for vaccine formulation, other particles that have been used with a lot of antigens in experimental studies is poly-(DL)-lactide-coglycide (Eng et al., 2010, Magiri et al., 2018). Some researchers have explored the immunogenic effect and protective ability of microparticles as an adjuvant in the anthrax vaccine. In a study by Kachura et al. (2016) CpG-Ficoll nanoparticles were used to formulate NLAV with rPA as the antigen. The immunogenicity of the NLAV was determined in non-human primate (NHP) with aerosol *B. anthracis* Ames strain spore challenge resulting in 100 % of the NHP surviving (Kachura et al., 2016). In a different study, mucoadhesive alginate-coated chitosan microparticles (A-CHMp) was used as an adjuvant in a vaccine formulation containing rPA. The immunogenicity was determined in BALB/c mice, and the

vaccine was able to stimulate increased immune response including both IgG and toxin neutralising antibodies. However, no protective study was done to verify the protection ability of the immune response (Mangal et al., 2014). There is speculation that microparticles stimulate long-lasting immunogenicity with a single vaccination (Manish et al., 2013). However, future investigations need to ascertain the longevity of the immune response in relation to its protective capacity. In addition, the stability of the adjuvant during storage and its safety in vaccinated animals also need to be evaluated further (Sivakumar et al., 2011).

2.8.2 Emulsions

For a very long time, the emulsions have been used as adjuvants in animal vaccines. Emulsions are made up by mixing two immiscible liquids (water and oil) consisting of small droplets of either water or oil continuously dissipating with each other such that either the water-in-oil or oil-in-water and stabilised by an interfacial surfactant layer. Emulsions remain the adjuvants of choice for animal vaccines due to their potency in antibodies stimulation (Bastola et al., 2017, see review by Cox and Coulter, 1997). There are numerous types of emulsions with a different mode of action each indicated below:

2.8.2.1 Oil-in-water (O/W) emulsion

Oil-in-water (O/W) emulsions are formed by dissipating oil in the aqueous appearance. The oil droplet in O/W emulsions acts by organising macrophages and DCs through accelerating the enrolment of chemokines based immune cells (Dupuis et al., 2001, Seubert et al., 2009, Seubert et al., 2008). Some researcher used O/W emulsion such as MF59® adjuvant to evaluate the immune response enhancement ability of MF59® adjuvant by comparing its immune enhancement to calcium phosphate and aluminium hydroxide. Their findings specified that MF59® adjuvant enhances better cell-mediated immunity (CMI) against influenza virus than calcium phosphate and aluminium hydroxide (O'Hagan, 2001, Wack et al., 2008). Several O/W adjuvants are commercially available for veterinary applications including Emulsigen™ (MVP Technologies, USA), Montanide™ Incomplete SEPPIC Adjuvants (ISA) (SEPPIC, France) and Meta-Stim® (Fort Dodge Laboratories, USA). These

adjuvants have been used in vaccines formulation alone or with other products. A robust immune response is recorded in most of the veterinary vaccines where O/W emulsion adjuvants have been used in livestock (Arous et al., 2013, Barnett et al., 1996, Galliher-Beckley et al., 2015, Horohov et al., 2015, Lankester et al., 2016, Madera et al., 2016, Wallace, 1996). An rPA vaccine was formulated with O/W emulsion adjuvant (monophosphoryl lipid A; (PA+MPLA)), The immunogenic effect of the adjuvant was determined by vaccinating New Zealand White rabbit followed by *B. anthracis* Ames strain spores challenge which resulted in 100% survival of the rabbit that received the rPA vaccine formulated with MPLA (Peachman et al., 2012).

2.8.2.2 Water-in-oil (W/O) emulsion

Water-in-oil (W/O) emulsion consists of droplets of water dispersed in a steady phase of oil. The antigen is trapped in the pockets of water enveloped by the steady phase of oil. The water is unleashed gradually following the oil disintegration subsequent to injection. The clearance of the antigen by phagocytosis from the site of injection is delayed by the effect of the depot formed thereby prolonging the time required to recruit the immune cells and APC to the injection site (Cox and Coulter, 1997, Herbert, 1968). Freund's adjuvants are the known W/O adjuvants. Freund's adjuvants can be Freund's complete adjuvants (FCA) or Freund's incomplete adjuvants (FIA). FCA comprises of paraffin oil, dried heat-killed mycobacteria and a surfactant (Aracel A), whereas FIA is formulated without dried heat-killed mycobacteria. These adjuvants stimulate high antibody titres but result in localised inflammation, distress and pain in vaccinated animals and has ultimately led to the discontinuation of the use of these adjuvants in human and animals (Claassen, 1992, Freund et al., 1937, Stils Jr, 2005). Some of the commercially available W/O emulsions for veterinary vaccine use with the same level of potency as FIA with less adverse reaction are Emulsigen-D® and montanide ISA (Johnston et al., 1991, Leenaars et al., 1994). These adjuvants have been used in veterinary vaccines for both viral and bacterial agents such as porcine circovirus type 2(PCV2) vaccine, foot and mouth disease (FMD) vaccine, *Mycoplasma* vaccine and non-living anthrax vaccine with good immune enhancing effect

(Arous et al., 2013, Ibrahim et al., 2015, Ivins et al., 1995, Jorge et al., 2014, Khorasani et al., 2016, Peachman et al., 2012).

2.8.2.3 Water-in-oil-in-water (W/O/W) emulsion

To overcome the challenges of localised reaction experience at the site of injection. Multiple phase emulsion vaccine adjuvants water-in-oil-in-water (W/O/W) were developed (Bozkir and Hayta, 2004, Fukanoki et al., 2001, Leclercq et al., 2011). W/O/W comprises of pockets of oil containing internal water droplets, which are discharged into the continuous aqueous phase (Bozkir and Hayta, 2004, Fukanoki et al., 2001, Leclercq et al., 2011). Bozkir and Hayta (2004). This showed that W/O/W emulsions contribute to the prolong immune response by fast antigen release in the external water phase while the internal water droplet release is prolonged. Presently, Montanide ISA 201 and 206 are few of the commercially available W/O/W emulsion adjuvants that demonstrated efficacy against animal diseases (Bouguyon et al., 2015, Hunter, 1996, Maree et al., 2015).

2.8.3 Toll-like receptor (TLR) ligands and small molecules

TLRs are an antigen's membrane receptors that are expressed in DCs and macrophages identified peculiar components of a pathogen such as bacterial DNA and RNA, lipopolysaccharide (LPS), cytosine phosphate guanine (CpG) and flagellin are examples of TLRs and they initiate innate host defence mechanism (Gerdts, 2015, Takeda et al., 2003). TLRs activation can lead to the induction of antigen-specific immunity, thereby, initiating cytokines expression, which is involved in the differentiation of T-cell (Burakova et al., 2018). TLR4 is an agonist of gram-negative bacterial surface membrane that triggers the secretion of IL-1β, IL-12, IL-18, IL-23, and IL-27 by APCs. This activates Th1 cells and regulates CMI and humoral immunity (Burakova et al., 2018). TLRs have proved to enhance different types of an immune response in both human and animal vaccine trials (Hajam et al., 2013, Mayr et al., 2005, Qi et al., 2015, Rau et al., 2006, Xia et al., 2016). Despite the short duration of activity and their retention by non-related tissue the application of TLR agonists in the veterinary field still attracts attention with regard to immune enhancement in veterinary

vaccinology (Heegaard et al., 2011, Kanzler et al., 2007). It is paramount to understand the correlation between efficacy and safety as well as getting in-depth knowledge of the adjuvants structure in relationship to the immune response they induce in animals.

2.8.4 Saponins

Saponins are compounds that have been employed in so many pharmaceutical applications. It is a detergent-like or group glycoside derivatives from plants (Burakova et al., 2018, Gerdts, 2015). This compound's ability to stimulate an immune response in animals has rendered it well-accepted adjuvants for a long time. Saponin has been used in animal disease from the time of Pasteur. It was first used in Pasteur's duplex anthrax vaccine at the concentration 1-10%, and it was discovered to stimulate virulent inflammation at the injection site. The concentration of saponin used by Pasteur in his duplex anthrax vaccine was later review by Max Sterne, where he found that 0.5-1% is sufficient to stimulate adequate immune response with minimal to no adverse reaction (Sterne, 1945). Thereafter, more saponin adjuvants were developed, and the most common saponin adjuvant is Quil-A (Brenntag Biosector A/S, Denmark). Quil-A is used only in animals and not recommended for human use (Sun et al., 2009). Despite Quil-A's ability to enhance the immune response in animals toxicity has been reported in vaccinated animals and as such a search into an alternative source of saponin continues (Rajput et al., 2007). QS-21 is another saponin adjuvant from the same plant with Quil-A but more purified. QS-21 enhances the immune response in veterinary vaccines but is still undergoing trial in human vaccines (Zhu and Tuo, 2016). Another saponin adjuvant is immune-stimulating complexes (ISCOMs) which are composed of cholesterol, phospholipids, and purified fractions of Quil-A. ISCOMs is a safer saponin adjuvant with less toxicity and can assist in delivering the antigen to APCs (Nielsen et al., 2015, Sun et al., 2009). The antigen phagocytosis is enhanced by the adjuvant's particles size and nature complexity (Reed et al., 2009). ISCOMs have been licensed for commercial purpose as a result of its proven efficacy in the stimulation of good immune response in an array of disease (Carlsson et al., 1991, Kamstrup et al., 1992, Nordengrahn et al., 1996). More research is currently ongoing to develop more saponin adjuvant that can

be used in both human and veterinary vaccine without challenges such as species preference and the route of administration (Sun et al., 2009, Cox and Coulter, 1997). This is encouraged by the impressive ability of saponin to stimulate both CMI and humoral immune response by using a low dose of the adjuvant as indicated by Max Sterne (Rajput et al., 2007, Sterne, 1945).

2.8.5 Cytokines

Cytokines are glycoproteins, proteins or peptides produced by monocytes and lymphocytes, and they coordinate immune responses and are therefore considered as vaccine adjuvant. The immunostimulatory activities of cytokines vary, as some boost the activity of specific immune cells, while others enhance the activity of the general immune cells (Rong et al., 1995). Furthermore, some cytokines activity are induced by other cytokines (Rong et al., 1995). Cytokines such as IL-2, IL-12, and IFN-γ expedite the enhancement of Th1 cells that lead to CMI responses (Heath et al., 1991). Whereas IL-4, IL-5, and IL-10 cytokines stimulate Th2 cells that leads to humoral antibodies production (Heath et al., 1991). Cytokines also activate MHC-II molecules on APCs, which boost the uptake and the presentation of antigen (Heath et al., 1991). Fan et al. (2016) show the ability of IFN-c to develop significant anti-classical swine fever (CSF) IgG titre and enhance MHC-I and MHC-II molecules in pigs when used as an adjuvant in CSF vaccine. Cytokine adjuvants are employed in various viral diseases of livestock such as CSF (Asif et al., 2004, Babiuk et al., 1991, Hung et al., 2010, Liu et al., 2019, Tafalla et al., 2013). They are also used in DNA vaccines of anaemia virus, and fish type I interferon and have proved to stimulate antibodies and B cells and CD8+ T cell influx (Chang et al., 2015, Kotla et al., 2016).

2.8.6 Combination of adjuvants

In recent years, there has been the emergence of combination adjuvants by the combination of two or more adjuvants in a vaccine formulation. Combined adjuvant generally enhanced the potency of the vaccine and immune response. An ideal adjuvant is identified by its ability to present antigen to APCs in its original form, which triggers the

relevant cytokines secretion and activate active cell-mediated and humoral immune response. The combination of different adjuvants in a vaccine formulation with the aim to improve their performance by augmenting each other's immune stimulation synergistically is under investigation (Moingeon et al., 2001, Mount et al., 2013). Ivin and his colleagues in 1992 reported the combination of three adjuvants (Monophosphoryl lipid (MPL), Trehalose dimycolate (TDM), cell wall skeleton (CWS)) formulated with PA was able to increase the protection to 77% of vaccinated BALB/c mice and 100% of vaccinated guinea pigs following challenge with *B. anthracis* Ames strain (Ivins et al., 1992). The idea of using combinations of adjuvants was conceived because it is almost impossible to have one adjuvant that carries out every function that is required. Therefore, many combined adjuvants studies are already in the trial stage for a human vaccine against cancer and infectious diseases. Based on these outcomes, the design has now been adopted for the veterinary vaccine (Guy, 2007, Mount et al., 2013). The combination of CpG and W/O/W or O/W emulsions as combined adjuvants used in FMD and avian influenza vaccine has been evaluated in many studies (Ioannou et al., 2002, Lopez et al., 2006, Nichani et al., 2004, Ren et al., 2011). Weeratna et al. (2000) vaccinated certain groups with a formulations that contained either FIA, FCA or MPL with the antigen FIA+CpG, FCA+CpA and MPL+CpG). Their findings revealed higher neutralisation antibody levels amongst the group that received a combination of emulsion with CpG in the BALB/c mice immunised against *HAsAg*. Again, the combination of saponins and inactivated spores of *B. atrophaeus* boost the immune response in murine mice vaccinated against rabies (Oliveira-Nascimento et al., 2012). Even though combined adjuvants stimulate promising immune responses with less side effects, little information is available on the use of combined adjuvants in anthrax vaccines. It is therefore necessary, to investigate r its application further by ascertaining the efficacy and safety of combining adjuvants for livestock. In addition, the cost of combining two or more adjuvants still needs to be thoroughly investigated for commercial purpose to ensure that it is of benefit before it would be considered for adoption.

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CHAPTER THREE

General Methodology

3.1 Recombinant protein expression and purification for vaccination study

The PA83 coupled to His-tag was ligated to pStaby1.2 vector (Delphi Genetic, Belgium) to obtain the recombinant PA83-pStaby1.2, which was transformed into the *E. coli* SE1 strain (Delphi Genetic, Belgium). The detail of the rPA expression and purification is explained in the next chapter. The *E. coli* SE1 (Delphi Genetics, Belgium) harbouring the plasmid pStaby1.2 (Delphi Genetics, Belgium) encrypting the full-length protective antigen (PA83) was cultured into Luria-Bertani (LB) broth medium and incubated at 25 °C in a shaking incubator. The culture was induced with 0.3 mM of isopropyl-beta-D-thiogalactopyranoside (IPTG) concentration. The cell was further incubated overnight before harvesting by centrifuging at 3500 rpm for 35 minutes. The pellet was then washed with double distilled water to wash off the IPTG contents before proceeding to protein purification. The harvested *E. coli* SE1 strain was subject to protein purification under native condition. The protein was lysed by suspended the cells in lysis buffer (400mM/ml NaCl, 50mM/ml NaH_2PO_4 and 20mM/ml Tris) pH 7.85, then froze at -20^oC and thawed to 4^oC three times. Then followed by two cycles of probe sonication (a cycle of sonication is made up of 20 seconds on and 10 seconds off the sonicator on ice bath three times using probe sonicator (BioLogics, Manassas, VA, USA)). The lysate was centrifuged at 3500 rpm for 35 minutes, and the supernatant was collected. The supernatant was treated in two different ways, one batch was passed through the purification column (Machery-Nagel, England). The second batch was just the supernatant collected and treated with the LAL Endosafe endotoxin Quantification and removal test kit (ThermoFisher Scientific, USA). The protein was confirmed by SDS PAGE and western blot using 4-20% gel. The protein yield was quantified with the Pierce BCA protein assay kit (ThermoFisher Scientific, USA) following the manufacturer's protocol. The rPA83 for ELISA is from the batch that was passed through the purification column, and the protein will receive no further treatment. The rPA83 for

vaccination received further treatment after purification, the presence of endotoxin in the protein (rPA83) was tested and removed by filtration using LAL endosafe at Design Biologix, South Africa.

3.2 Formalin activated spores (FIS)

Bacillus anthracis 34F2 spores from Onderstepoort Biological Products batch: 863 on sporulation agar (peptone 15.0 g, yeast extract 3.0 g, NaCl 6.0 g, dextrose 1.6 g, brain heart infusion (BHI) agar 12 g and $MnSO_4.H_2O$ in 1000 ml of ddH_2O) were harvested on the 10^{th} day when it attained ≥ 90% sporulation by flushing the sporulation agar with double distilled water (ddH_2O). The spores were washed three times with (ddH_2O) then suspended the spores in 0.9% NaCl containing 0.1% gelatin and heated for 30 minutes at 65°C. The spore was then washed 4 times to get rid of the heat killed vegetative bacilli by centrifuging at 4000 x g followed by rakette spore stain to ensure purity and final spore count. The spores are re-suspended overnight in PBS with 4% formalin incubated at 37°C to inactivate the spore completely. The formalin was washed by suspending the pelleted spores in 0.9% NaCl and 0.1% gelatin and centrifuged at 4000 x g four times. Finally, the pelleted spores were re-suspended in endotoxin free PBS. An aliquot (2 ml) of the preparation was suspended in histidine (20 g/L) for 30 minutes at room temperature to neutralise the formalin and the plated-on blood agar to confirm the sterility of the preparation.

3.3 Formulation of non-living anthrax vaccine

The formulation of NLAV was produced in a large volume of 60 ml (60 doses) each for the crude and purified vaccine formulations. The antigens and PBS solution were asceptically pooled and mixed at 250 rpm with a magnetic stirrer while 50% adjuvant volume (alhydrogel®) was slowly added and stirred for 2 hours at room temperature followed by slowly adding the remaining 50% adjuvant volume (Emulsigen-D®) that was stirred for an additional 2 hours at room temperature. The vaccine formulation was transferred into sterile HDPE vaccine vials (10 ml) and retention vials were sent for quality control test at Design Biologix, South Africa.

For the vaccine formulation, the rPA for both crude and purified concentration was determined with Pierce BCA protein assay kit (ThermoFisher Scientific, USA) and the concentration of rPA for PrPA and CrPA vaccine formulation was calculated according to Appendix material 1. Then adjusted with the FIS (10^8 spores) and adjuvants to a volume of 1 ml. The aseptic vaccine formulation procedure was carried out according to the requirements for vaccines (OIE. 2012). The PrPA+FIS+adjuvants vaccine formulation constituted of 258 µl of 290 µg/ml PrPA (75 µg rPA), 400 µl of 2.5 X 10^8 spore suspension per ml (1 x 10^8 spores), 330 µl of adjuvants (Emulsigen-D®/Alhydrogel® 1:1) and 12 µl PBS in the 1 ml dose recommended (OIE. 2012). Whereas the CrPA+FIS+adjuvants vaccine formulation constituted of 517 µl of 146 µg/ml CrPA (75 µg rPA), 400 µl 2.5 X 10^8 spore suspension (1 x 10^8 spores), 330 µl of adjuvants (Emulsigen-D®/Alhydrogel® 1:1) and 3 µl PBS.

3.4 Purification and preparation immunoglobulin for passive protection

Sera were collected from each cattle in all vaccination groups, including negative control at week 5 for IgG purification. IgG was purified from each serum sample using protein G spin column (NAb™ Protein G Spin Kits, Thermo Scientific, USA) according to the manufacturer's instruction, but the elution process was repeated three times. The presence of anti-rPA specific IgG was confirmed using Enzyme-linked immunosorbent assay (ELISA) and the IgG yield concentration was measured with a Pierce BCA protein assay kit (ThermoFisher Scientific, USA) following the manufacturer's guide.

3.5 Enzyme-linked immunosorbent assay (ELISA)

Anti-rPA antibodies (IgM, IgG, IgG1 and IgG2) sera titres from vaccinated animals were determined by ELISA as previously described by Ndumnego et al., (2016). Briefly, the 96-wells of Nunc immunoplate Maxisorp were coated with 0.5 µg or 10^8 FIS in each well in 0.05M of carbonate-biocarbonate buffer (Sigma-Aldrich, USA) and incubated at 4°C overnight. This was followed by two times wash using wash buffer (PBS + 0.05% Tween 20 (PBST)) with ELISA microplate washer (Biorad PW40, France) then blocked with 200 µL of blocking buffer (PBS + 5 % skimmed milk powder (PSMP)) and incubated in a shaking

incubator at 150 rpm for 1 hour at room temperature, the plate was washed twice. The sera were serially diluted in sample dilution buffer (PBS + 0.05% Tween 20 + 5 % skimmed milk powder (PTSMP)) on the plate in duplicates across the plate, the starting concentration for IgG 1:100 and 1:50 for the immunoglobulin subclasses (IgM, IgG1 and IgG2). The plate was incubated in a shaking incubator at 160 rpm for 30 minutes at room temperature and washed five times. The various HRP conjugated secondary antibodies from ThermoFisher Scientific, USA were diluted in PTSMP and added to the plates at the following concentration: The goat anti-bovine IgG (1:10000), sheep anti-bovine IgM (1:4000), sheep anti-bovine IgG1 (1:4000) and sheep anti-bovine IgG2 (1:4000). Each plate was incubated at room temperature in a shaking incubator at 160 rpm for 30 minutes. Following the five times washing of the plates, 2,2' azino bis (3 ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich, USA) was used to develop the plates. Biotek powerwave XS2 reader (Winooski, USA) was used to read the plates at the absorbance reading of 405 nm. The endpoint of the immunoglobulin titres was calculated as the reciprocal of the highest serum dilution giving an absorbance more than the cut-off. Titres of IgM, IgG1 and IgG2 <50 and IgG <100 were ascribed an arbitrary value of 10.

3.6 Toxin Neutralisation Assay (TNA)

The ability antibody from rPA vaccinated cattle to neutralise anthrax lethal holotoxin was assessed using an *in vitro* colourimetric assay that determines the viability of J774A.1 (ECACC cat no 91051511) exposed to anthrax lethal holotoxin in the presence of antibody as previously described by Ndumnego et al. (2018). Briefly, 10^5 J774A.1 mouse macrophage cells in DMEM and 10% FBS was seeded to 96-well flat bottom tissue culture plates (Greiner bio one, Germany) then incubated overnight at 37°C and 5% CO_2. Individual animal sera were 2-fold serial diluted in duplicate in Dulbecco's Modified Eagle Media (DMEM) containing 5% Foetal Bovine Serum (FBS) containing PA (500 ng/mL) and LF (400 ng/mL) (List Biological Laboratories Inc., USA) with the starting dilution of 1:50. The plate containing the lethal holotoxin and the serum was incubated at 37°C and 5% CO_2 for 1 hr in the dark prior to transferring the lethal holotoxin and serum to J774A.1 seeded plate. This was

followed by a 3 hour incubation at 37°C and 5% CO_2. Afterwards, MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Invitrogen, USA) was added at the concentration 25 µl of 5 mg/ml to each well then incubated for 2 hours in darkness at 37°C and 5%. Afterwards, 100 µl acidified isopropanol (90% isopropyl alcohol, 0.5% SDS, 25 mM HCl) was added to each well followed by vigorously solubilise the formazan crystals dye by pipetting up and down. The plates were read with a Biotek power wave XS2 reader (Winooski, USA) at 540 nm. The neutralisation of each serum sample was calculated using the formula:

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

The reciprocal of the highest dilution at which J774A.1 cell survival yielded 50% was expressed as neutralisation (NT_{50}). This was generated by inputting the formula to Gen5 data analysis software (Winooski, USA).

3.7 Opsonophagocytic assay

The opsonophagocytic potential of rPA and FIS induced antibodies was evaluated on RAW 264.7 macrophage cells as previously carried out by Welkos et al. (2001) with few modifications. Briefly, heat-activated, refractile ungerminated *B. anthracis* spores (2.6 x 10^9 spores/ml) were pre-incubated with 10-fold serial dilutions of the immune sera and sera from the negative control (NegCtl), for 30 minutes at 4°C and then added to RAW 264.7 macrophage cells (5 x 10^5 cells/well) and incubated for 45 minutes at 37°C in 5% CO_2. The macrophage cells were washed with sterile PBS (pH 7.4 $\pm$ 1) and incubated with DMEM containing 10% FBS and 10 µg/ml gentamicin at 37°C in 5% CO_2 for 30 minutes to remove vegetative bacilli. Subsequently, the macrophage cells were washed with sterile ice-cold PBS, incubated for 5 minutes in 100 µl 0.1% Triton® X (ThermoFisher Scientific) to lyse the macrophages and plated on LB agar to count viable cfu/ml. Data are presented as percentage spore uptake by the macrophage cells. Sera from pre-vaccination screening was

used as a negative control, whereas group vaccinated with SLSV from this study were used as the positive control.

3.8 Statistical analysis

Gen 5 data analysis software (Biotek Instruments, Winooski, USA) was used to generate 4-parameter logistic curves for the ELISA and TNA titres. The data collected were log-transformed using GraphPad prism version 8.3.0 software. The antibody titres between groups at different time points on ELISA and TNA were compared using unpaired student *t*-test with a two-tailed *P*-value and Kruskal-Wallis test followed by Dunn's multiple comparisons test with adjusted *P*-value. The mean survival time of the challenged A/J mice was plotted using the Kaplan-Meier survival curve. Log-rank (Mantel-Cox) test was used to compare survival between different vaccination groups. All graphical elucidations and the analysis were done using GraphPad Prism version 8.3.0 software.

3.9 References

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CHAPTER FOUR

Cost-effective Production and Purification of *Bacillus anthracis* Recombinant Protective Antigen in *Escherichia coli*

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4.1 Abstract

Bacterial expression system for the production of recombinant protein is still the best choice in protein biotechnology. However, some of the disadvantages include the formation of inclusion bodies in over-production, protein solubilisation, plasmid instability and purification of the protein. In this study, the His-tagged *Bacillus anthracis* recombinant protective antigen 83 kDa (rPA83), an immunogen for human vaccines, was produced as a component for a veterinary vaccine. The production of rPA83 needs to be more cost-effective to compete with the current anthrax Sterne live-spore vaccine. The expression of rPA83 using pStaby1.2 bacterial expression system in *Escherichia coli* and purification was optimised using an efficacious, safe and low-cost method. Biologically active soluble rPA83 was expressed using low-temperature induction in antibiotic-free selection pStaby1.2 vector in *E. coli* SE1. The protein purification consisted of lysing the bacteria with a probe sonication in modified lysis buffer rather than using a purification kit consisting of endonuclease, lysozyme and protease inhibitors in standard lysis buffer followed by immobile metal purification. The lysis consisted of two cycles of sonication, each followed by affinity purification that produced 30.4 mg/LrPA82 compared to the 36.2 mg/L rPA83 of

the standard purification method. This purified rPA was biologically active as it exhibited cytotoxicity towards J774A.1 cells when combined with lethal factor and immunogenicity with anti-PA iELISA. The biologically active and soluble rPA83 can be up-scaled for vaccine production and serological diagnostic purpose using an antibiotic-free selection bacterial expression system and is more cost-effective using sonication to lyse the cells.

4.2 Introduction

Bacteria expression systems have been the most common and easiest means of recombinant protein production mostly in *Escherichia coli* (Saccardo et al., 2016). The *E. coli* expression systems have some shortcomings such as plasmid instability during up-scaling, antibiotic-based selection (Barroca et al., 2016, Pal et al., 2018, Reschner et al., 2013, Vandermeulen et al., 2011), low soluble protein yield, the formation of inclusion bodies during over-production, proteolytic digestion by protease (Cantu-Bustos et al., 2016, San-Miguel et al., 2013) and loss of biological activity (San-Miguel et al., 2013, Suryanarayana et al., 2016). Production of soluble protein is paramount for structural and functional analysis as well as biomedical application (San-Miguel et al., 2013, Suryanarayana et al., 2016). Many studies use lower growth temperatures, a T7 promoter and chaperons on the vector coupled with the use of lactose or an analogue isopropyl β- d-1-thiogalactopyranoside (IPTG) inducer to produce a higher yield and improve the quality of the expressed recombinant protein (Khow and Suntrarachun, 2012, Kumar et al., 2015, San-Miguel et al., 2013, Gifre et al., 2017). The reduction of the IPTG concentration induces the protein expression at the late log phase of the culture resulting in higher yields of protein in a soluble form (Masabanda and Griffin, 2003, Winograd et al., 1993).

Despite the advantages of using an *E. coli* expression system for recombinant protein production such as fast generation time, well understood biochemistry of *e. coli* and high yield as a result of the size. Yet, improvements of the downstream processes in the purification of the protein and the reduction of cost are required (Ferrer-Miralles and Villaverde, 2013, Gifre et al., 2017). The lysing of cells in large scale production, with cell

lysis reagents such as BugBuster® Protein Extraction Reagent including endonuclease, lysozyme and protease inhibitors to mildly break the *E. coli* cell wall and release the soluble protein. This enables the release of the soluble protein due to the affinity of the His-tagged to the protein to Ni column during purification even though this is costly (Fong et al., 2010). Most studies use antibiotic resistance selection marker in *E. coli* systems with different cell lysing methods (extraction kits using endonucleases and lysozyme, sonication or high-pressure homogenisation) followed by purification. However, the use of expression system free of antibiotic resistance marker for selection is highly recommended to minimise the spread of antibiotic resistance in the environment (Barroca et al., 2016).

The cost may not be a major focus for *B. anthracis* rPA83 production in human anthrax vaccines, but the cost is paramount for a veterinary vaccine as it will compete with the low-cost Sterne live-spore vaccine. Previous studies where purification column such as sepharose or a Ni^{2+} chelating column and ion exchange column was used for the purification of rPA yielded rPA ranging from 2 – 15 mg/L through small scale production (Gupta et al., 1999, Suryanarayana et al., 2016). Laird et al. (2004) and Gwinn et al. (2006) purified 370 and 270 mg/L rPA using small and large scale production, respectively. But the actual biological activity was not determined since the rPA83 was cleaved by trypsin only, with no further testing using cytotoxic and binding assays. The rPA83 has been expressed in other bacterial hosts like *Salmonella enterica* serovar Typhimurium (Hanh et al., 2004), *B. subtilus* (Singh et al., 1989) and *B. brevis* (Rhie et al., 2005, Singh et al., 1989) with 70 mg /L rPA yield from the latter (Rhie et al., 2005). Many studies approach are not cost effective and did not take antibiotic resistance free selection marker to obtain biologically active rPA as antigen for NLAV which is the major focus of this study.

In this study, production of rPA83 was optimised as a component in a NLVA for livestock using an antibiotic-free *E. coli* expression system (Jauro et al., 2020, Koehler et al., 2017, Ndumnego et al., 2018). Five purification methods were reviewed and compared to select the most cost-effective method that yield biologically active and antibiotic resistance

marker free rPA was selected because the NLAV needs to be competitive against the current SLSV used for livestock. To allow for large scale production, the StabyExpress™ system in *E. coli* (www.delphigenetics.com) was used. Expression conditions such as varying temperatures, IPTG concentrations for induction and harvesting time after induction to obtain soluble protein were optimised and the purification methods and rPA yields were compared. ELISA was used to test for the seroreactivity of the recovered protein and the biological activity assessed using a cytotoxicity test.

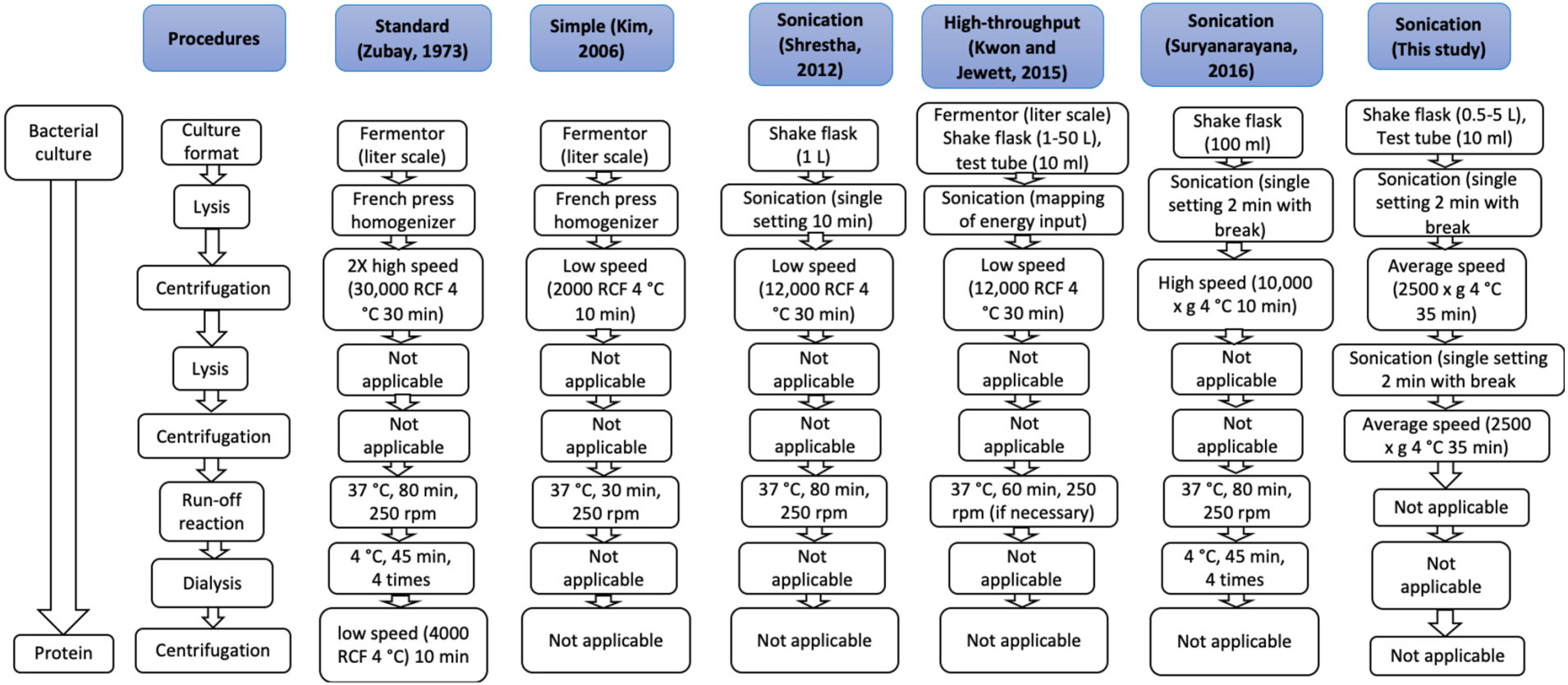

Figure 4. 1: Schematic representation of various lysing methods for Escherichia coli crude extract preparation used for recombinant protective antigen (rPA). The sonication method at the far right (Sonication this study) is the method used for this study.

4.3 Materials and methods

4.3.1 Bacterial strains and reagents

The expression vector pStaby1.2 and the *E. coli* SE1 strain used in this study were purchased from Delphin Genetics SA (Belgium). Luria-Bertani (LB) broth/agar used for the expression was obtained from Life Science (USA). Activated PA was procured from List Biological Laboratories (USA), and polyclonal anti-anthrax PA83 IgG was purchased from Innate Therapeutics (New Zealand). We purchased all other reagents from Sigma Aldrich unless specified.

4.3.2 Construction of PA expression plasmid

A synthetic PA83 using the in-frame primer sequences described by Hahn et al. (2004) and *B. anthracis* 34F2 strain PA83 sequence available in Genbank was synthesised using codons optimised for expression in *E. coli*. The recombinant construction of the synthetic PA83 in pStaby1.2 using *NdeI/PstI* in-frame to His-tag and transformation into *E. coli* SE1 strain was done by Delphin Genetics, Belgium. The full-length PA83 sequence cloned into PA83-pStaby1.2 construct was confirmed by Sanger sequencing. The full-length PA83 gene was amplified from PA83-pStaby1.2 to confirm size using the primers (5'-ATAGGTACCGAAGTTAAACAGGAGAACCG-3' and 5'-ATAGGGCCCTCCTATCTCATAGCC-3'). PCR reaction and PCR condition as described by Hahn et al. (2004) were used. The PA83-pStaby1.2 recombinant was digested with *NdeI/Pst* restriction digest products as described by the manufacturer. All PCR and restriction digest products were separated on 1.5% agarose gel electrophoresis on.

4.3.3 Optimisation of protein expression

The PA83-pStaby1.2 recombinant in *E. coli* SE1 was grown overnight in 4 mL LB broth medium at 37 ˚C in shaking incubator and sub-cultured in 10 mL LB broth. The sub-cultures were incubated at 37 ˚C, 30 ˚C and 25 ˚C and once the cultures attained the OD_{600} of 0.8 then 1.0 mM, 0.5 mM and 0.3 mM IPTG were added. The cells were harvested by centrifugation at 2500 x g for 35 minutes from each subculture hourly for 5 hours as well as overnight after induction. The pellets were washed with double distilled water. The optimised incubation

temperature, IPTG concentration and harvesting time post-induction were used in a 500 mL upscale production of the protein.

4.3.4 Purification of recombinant protein

The most cost-effective method using sonication for lysing the cells was selected and a new protocol was designed from the different methods evaluated (Figure 4.1). The first lysing method consisted of the standard method using lysis buffer consisting of BugBuster® Protein Extraction Reagent formulated to mildly break *E. coli* cells to release the soluble protein in addition to endonuclease, lysozyme and protease inhibitor. The second and more cost-effective lysing method consisted of sonication in the lysis buffer formulation by Suryanarayana et al. (2016). Briefly, the second, low-cost lysing method comprised of suspending the harvested cells in lysis buffer (400 mM/mL NaCl, 50 mM/mL NaH_2PO_4 and 20 mM/mL Tris) pH 7.85, and then freeze-thawing three times, followed by two cycles of sonication. A cycle of sonication consisted of 20 seconds on and 10 seconds off the probe sonicator in an ice bath repeated three times. The sonicated lysate from the first sonication cycle was centrifuged at 2500 x g for 35 minutes after which the supernatant was collected. An aliquot was collected from the supernatant and purified through Ni^{2+}-TED column (Machery-Nagel, England) (Figure 4.2) to determine the yield (designated as CL 1). The pellet was resuspended in lysis buffer, and the lysis process was repeated through a second sonication cycle. The supernatants of the first and second cycle of sonication (designated as CL 2) were purified using the Ni^{2+}-TED column (designated as CL 2) (Figure 4.2). The yield was quantified using Pierce BCA protein assay kit (ThermoFisher Scientific, USA) following the manufacturer's protocol. The pool of first and second cycle sonication supernatant was eluted twice through the column and indicated as first elution (elu1) and second elution (elu2). The supernatant from both methods were purified using Ni-TED column. The rPA83 yields produced with the new cost-effective sonication method and the standard method consisting of the BugBuster® protein extraction reagent, endonuclease, lysozyme and protease inhibitor followed by purification were compared. The PA83 protein expression and purification was confirmed by SDS PAGE and Western blot using 4-20% gel. The second

elution (pooled supernatant from sonicated lysate purified through the Ni^{2+}-TED column twice (elu2)) was used for further analysis using the ELISA and cytotoxicity assay.

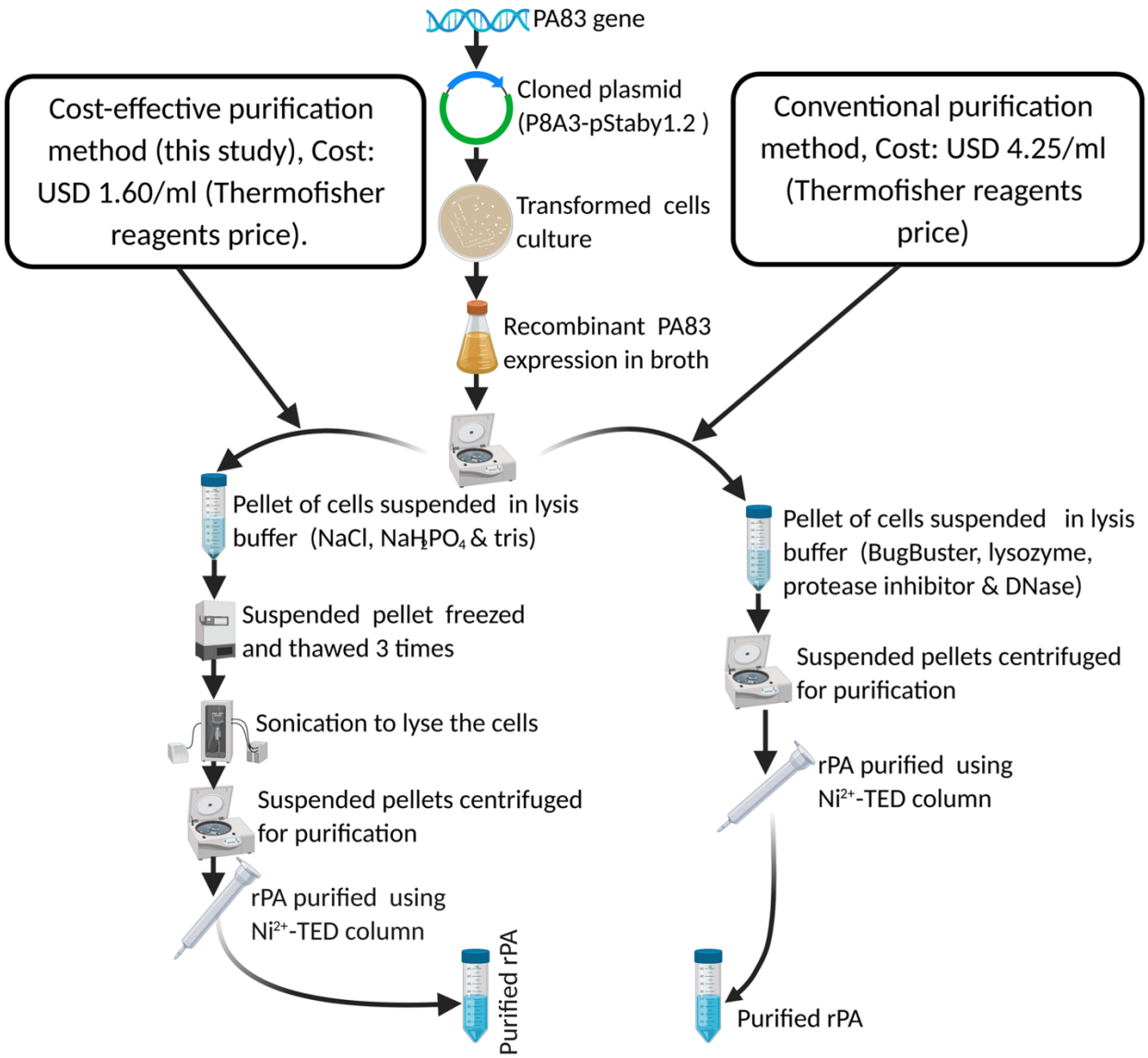

Figure 4. 2: Schematic steps of two different lysing methods for the purification of recombinant protective antigen (rPA83) and the cost implication to obtain purified protein using the different methods. The whole procedure for the cost-effective method was repeated twice.

4.3.5 SDS PAGE and western blot analysis

The SDS PAGE was performed as described by Laemmli (1970). The western blot was carried out according to Huang et al. (2005) procedure with few modifications. Briefly, the purified proteins were transferred from polyacrylamide gel onto PVDF membrane (ThermoFisher Scientific, USA) using Hoefer™ miniVE vertical electrophoresis system and transfer buffer (25 mM/L Tris, 190 mM/L glycine, 20 % Methanol, 0.1 % SDS, pH 8.3). The membrane was

blocked by incubating in 10% skim milk powder (SMP)/Tris buffer and 0.05% Tween-20 TST (blocking buffer (50 mM TRIS-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4)) with gentle shaking for 1 hour at room temperature (25 ˚C). Afterwards, the membrane was incubated in 1:100 dilution of a polyclonal anti-anthrax PA83 IgG fraction (10 mg/mL) from Innate Therapeutics (New Zealand) to confirm rPA expression in P83-pStaby1.2. The polyclonal anti-PA83 IgG fraction was diluted in TST buffer containing 10% skimmed milk powder (TSTSMP) and incubated with gentle shaking for 1 hour at room temperature, then washed three times using TST wash buffer with gentle shaking for 5 minutes. The membrane was transferred into 1:10000 dilution of a conjugated mouse anti-bovine IgG (Invitrogen, USA) in the TSTSMP and incubated for 1 hour with gentle shaking at room temperature. The membrane was washed three times with TST and stained with HRP staining solution, diaminobenzidine tetrahydrochloride hydrate (Sigma, USA) and the chromogenic reaction was halted by rinsing twice with water.

4.3.6 Protein biological activity

4.3.6.1 Enzyme linked-immunosorbent assay (ELISA)

Sera from goats vaccinated thrice with rPA, rBclA and FIS with three weeks interval between each vaccination and the negative sera were sourced from a study by Ndumnego et al. (2018) conducted at the Department of Veterinary Tropical Diseases, University of Pretoria, South Africa. The ELISA using PA as antigen was run according to the protocol previously described by Ndumnego et al. (2018) with some modifications. Briefly, Nunc immunoplate maxisorp 96-well plates were divided into three, the first segment (1-4 columns of the plate) was coated overnight with starting volume/concentration of 20 µL /ml (6.1 µg/well) of the new rPA (the cost-effective rPA) diluted in 200 µL of the diluent using two-fold dilutions (dilution started; 1:20) downward in the plate in quadruplicates and the rest of the wells were coated with 5 µg/well of rPA (standard rPA) (Ndumnego et al., 2018) used as a standard for the new rPA. After blocking with 5% SMP in PBS for 1 hour, 100 µL of 1:50 dilution of the anti-rPA sera in PTSMP sample dilution buffer was added to each well of 96-well plate, the last column wells left blank containing only PTSMP. The plate was incubated

on a shaker at 160 rpm for 30 min at room temperature and washed five times. Purified recombinant protein A/G (100 µL), peroxidase-conjugated (ThermoFisher Scientific) and diluted in PTSMP (1:10000) was added to each well. Then incubated again on a shaker at room temperature for 30 minutes and washed five times followed by an addition of 100 µL of the substrate (2,2' azino bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma, USA). The absorbance developed by new rPA and the standard rPA used as well as the positive and negative control sera for the ELISA was read at 405 nm using a Biotek Powerwave XS2 reader (USA).

4.3.6.2 Cytotoxicity assay

The biological activity of all the rPA was determined using a cytotoxicity assay on J774A.1 macrophage mouse cell line (European collection of cell cultures ECACC, cat number 91051511). The cytotoxicity assay was performed by modifying a toxin neutralisation assay, procedures previously described by Ndumnego et al. (2018). Briefly, flat-bottomed 96-well tissue culture plates (Greiner Bio One, Germany) seeded with 50000 macrophages in each well in DMEM containing 10% FCS were incubated overnight at 37 ˚C and 5% CO_2. Commercial PA 1000 ng/mL of PA (List Biological Laboratories, USA), the rPA expressed using methods 1 and 2 in this study and standard rPA previously used by Ndumnego et al. (2013) was used and serially diluted using two-fold dilution. LF was added at 300 ng/mL to each well to obtain lethal toxin (LT) (List Biological Laboratories, USA). Then followed by 1 hour incubation of the LT mixture at 37 ˚C and 5% CO_2. The LT toxin was then transferred to the plate containing overnight cultured cells (after discarding medium) and incubated for 3 hours followed by adding 25 µL MTT (5 mg/mL) (Invitrogen, USA) to each well and incubated for 2 h in the dark at 37 ˚C and 5% CO_2. Each concentration of LT was tested in triplicate. Pre-warmed (37 ˚C) acidified isopropyl alcohol (90% isopropyl alcohol, 0.5% SDS (w/v), 25 mM HCl) was used to solubilise the formazan dye and lyse the cells. The plates were read with Biotek power wave XS2 reader at 540 nm after resting for 5 minutes.

4.3.7 Statistics

The rPA yield using different lysis techniques was analysed using one-way analysis of variance (ANOVA). Spearman's correlation coefficient was used to analyse the association between cytotoxic assay results of different rPA. All analysis and graphs were carried out using GraphPad Prism 6 version 6.01. All data resulting in *P* values <0.05 are considered statistically significant.

4.4 Results

4.4.1 Construct of synthetic PA83 in pStaby1.2

The full-length of synthesised *B. anthracis* PA83 gene (2.204 kb) was successfully constructed into the PA83-pStaby1.2 recombinant as observed with PCR and restriction digest (Figure 4.3a and b). The DNA sequence analysis verified the *B. anthracis* PA83 sequence with *E. coli* codon usage incorporated in this sequence by modifying the codons at the following positions 193, 203, 286, 509, 736, 1528, 1847 and 2057 (data not shown).

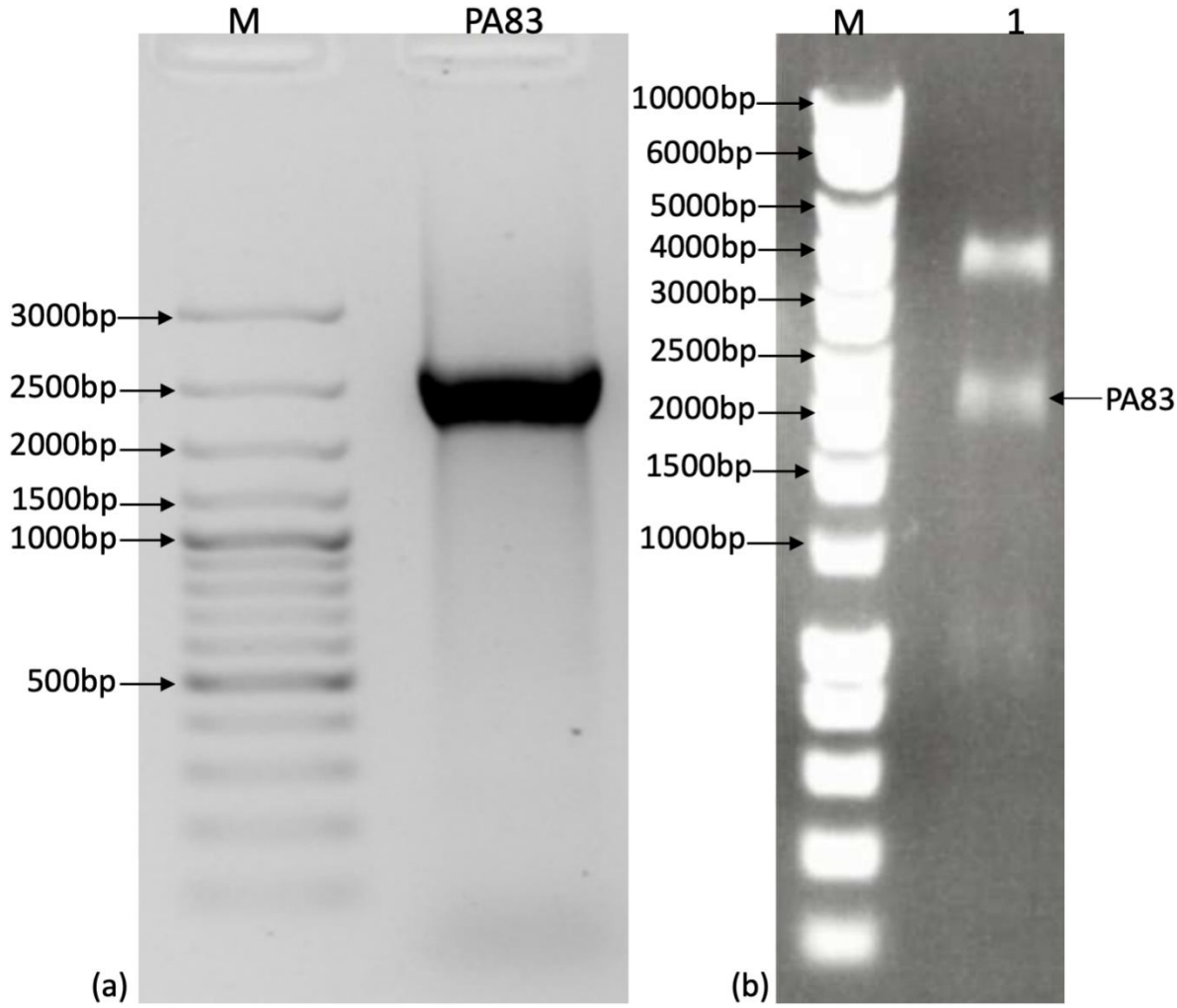

Figure 4. 3: (a) The amplified full-length synthetic *Bacillus anthracis* protective antigen 83 (PA83) gene 2204 bp using primers from Hahn et al. (2004). (b) restriction digest of recombinant PA83-pStaby1.2 yielding the PA83 2230 bp. Lane M: DNA ladder (ThermoFisher Scientific, USA).

4.4.2 rPA expression and purification

The evaluation of the effect of different temperatures, varying concentrations of IPTG and harvest time after induction of PA83-pStaby1.2 recombinant showed optimum expression at 25 °C and across the various IPTG concentrations (0.3, 0.5 and 1.0 mM) as well as different bacterial harvest time from expression culture media after induction with IPTG (Appendix Figures 1 and 2). There was no expression at different IPTG concentrations at a temperature higher than 25 °C at the optimum harvest hours after inducing with IPTG (Appendix Figure 3). The rPA expressed had relative molecular weights of 86 kDa in pStaby2.1 along with the respective His-tags (Figure 4.4a and b). The Western blot further confirmed the PA83 protein overexpression as the blot analysis with polyclonal anti-anthrax

PA83 IgG fraction as primary antibody indicated a distinct band at 86 kDa (Figure 4.4b). The rPA yield increased from the first to second sonication cycle (Figure 4.5). The rPA83 yield purified through Ni^{2+}-TED column of standard lysing method versus the sonication lysing methods using two sonication cycles showed no significant difference ($P<0.05$) between them with a yield of 36.2 mg/L and 30.4 mg/L respectively. However it was found to be significant ($P<0.0005$) when compared to the 18.9 mg/L rPA yield of one cycle sonication lysing method (Figure 4.5).. The costs of the standard and sonication lysing methods were 4.25 USD and 1.60 USD per mL of purified rPA83 which is a significant price difference for little difference in yield.

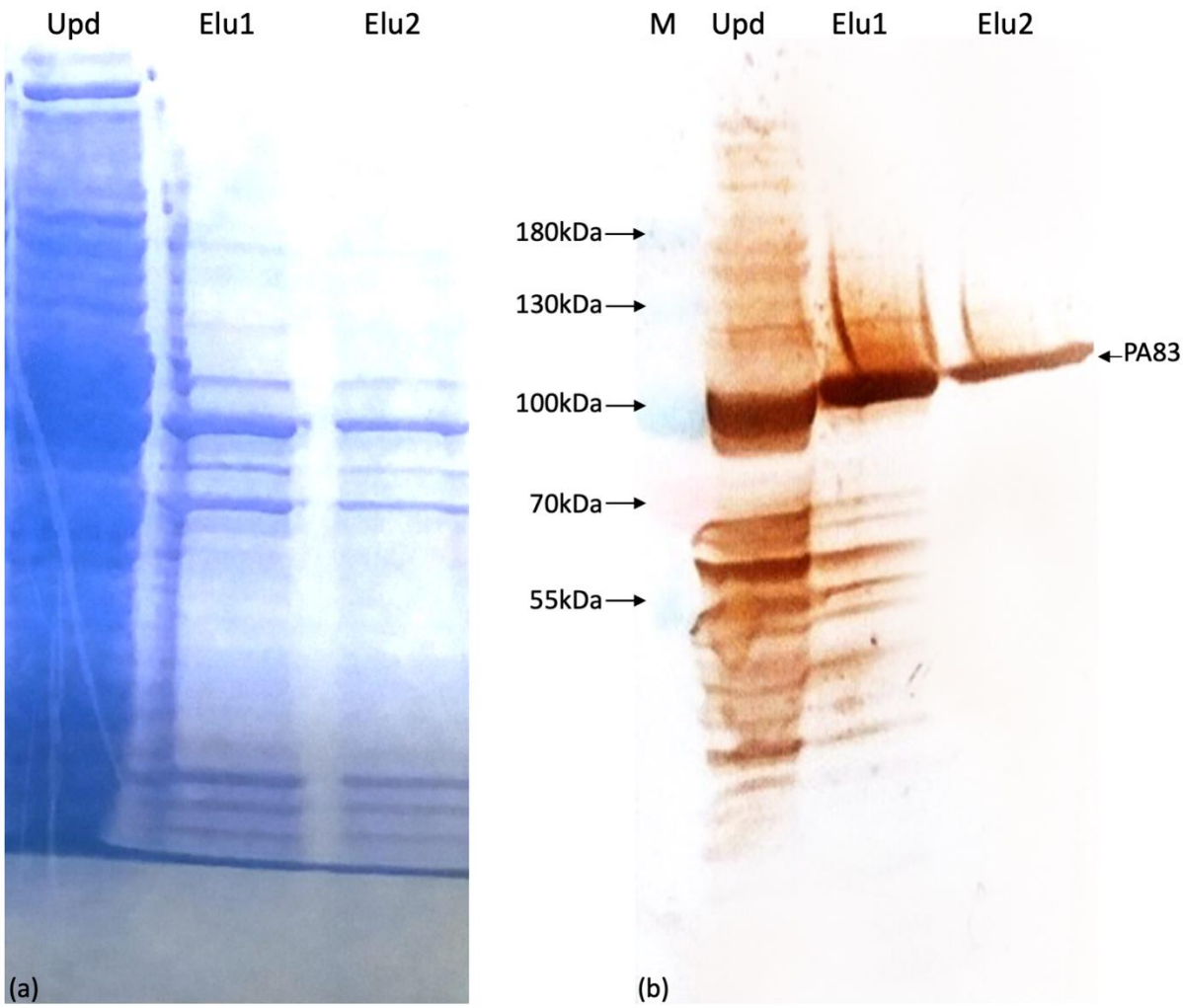

Figure 4. 4: (a) SDS-PAGE of pre-upscale analysis of full-length recombinant protective antigen (rPA83) expressed from PA83-pStaby1.2 in *Escherichia coli* SE1 at 25 °C in 500 ml culture using 0.3 mM IPTG

concentration. (b) Western blot showing expression of recombinant PA83 from PA83-pStaby1.2 in *E. coli* SE1 at 25 °C in 500 mL culture using 0.3 mM IPTG. Primary antibody used: polyclonal anti-anthrax PA83 IgG. Lane M: protein molecular weight marker, Upd lane: Unpurified protein after second sonication (not purified through the Ni^{2+}-TED column), Elu1 lane: First elution of unpurified protein passed through Ni^{2+}-TED column, Elu2 lane: First elution passed through the Ni^{2+}-TED column a second time.

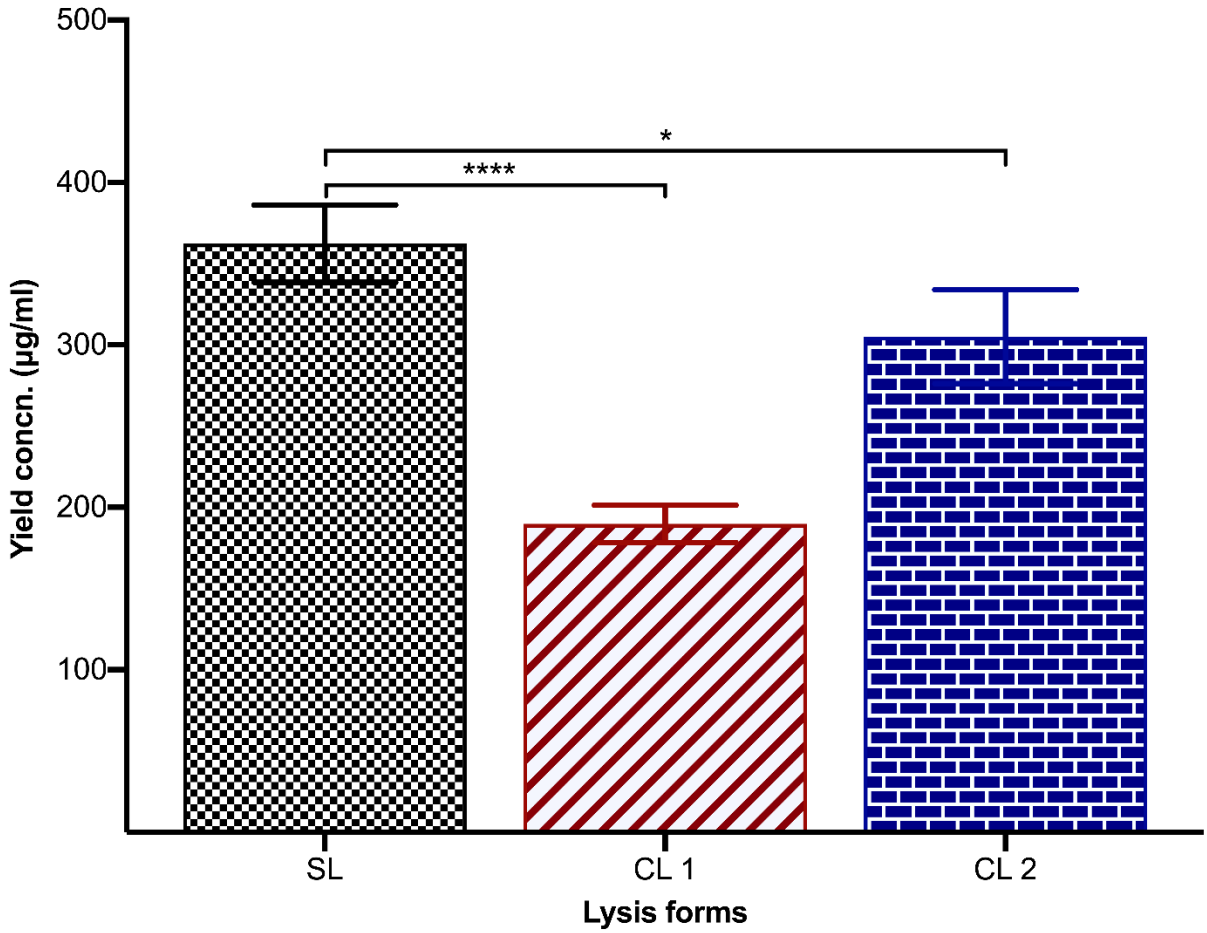

Figure 4. 5: Comparison of yield concentration for Ni^{2+}-TED column purified recombinant PA83-pStaby1.2 (rPA) yield with one (CL 1) and two (CL 2) sonication cycles with a standard lysis procedure. SL: rPA yield purified through Ni^{2+}-TED column using standard lysis method; CL 1: rPA yield after 1st sonication cycle and purified through Ni^{2+}-TED column, CL 2: rPA yield after the pool of 1st. and 2nd sonication cycle and purified through Ni^{2+}-TED column. *: P<0.05, ****: P<0.0005.

4.4.3 Biological activity.

The biological activity of recombinant soluble PA protein produced with the standard lysing method (rPA) and new sonication method (new rPA) was evaluated. The anti-rPA seroreactivity of the expressed rPA was tested by ELISA. The new rPA showed good correlation of seroreactivity as compared to the standard rPA (Figure 4.6). For cell

cytotoxicity assays the new rPA and standard rPA were lethal to the growth of macrophage cell lines with 45% and 47.5% cell death respectively at the concentration of 500 ng/mL. In comparison, the commercial rPA (List Biological laboratory) showed lethal activity of 58.44% (Figure 4.7).

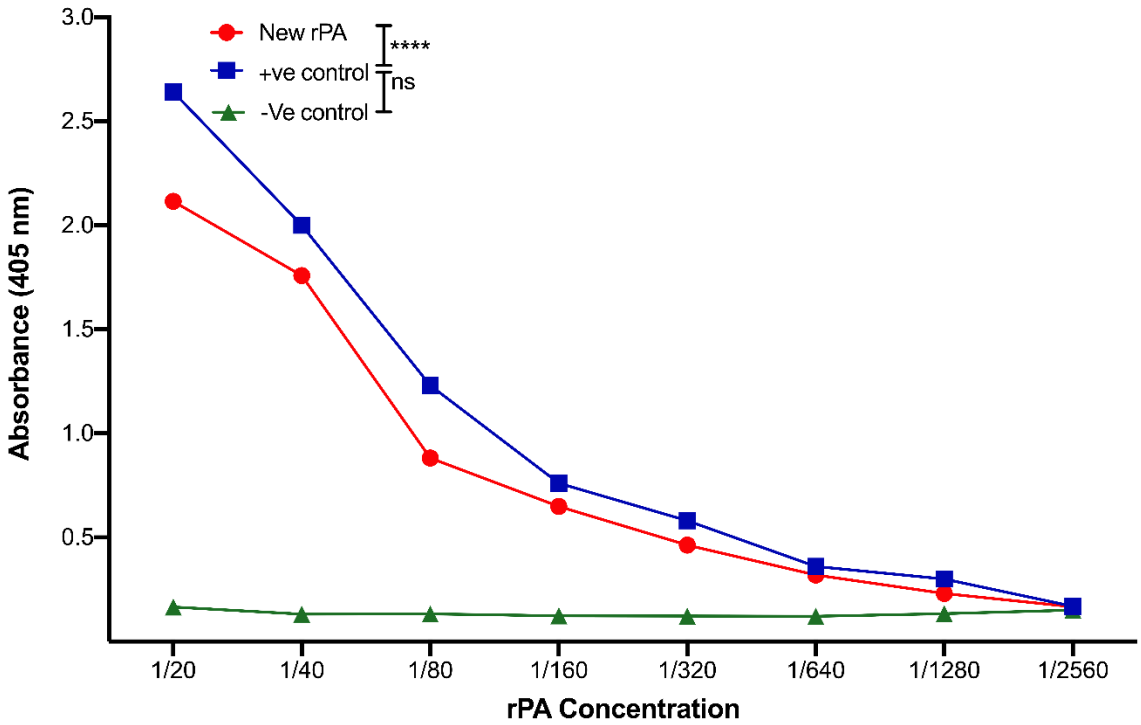

Figure 4. 6: Comparison of standard rPA (+ve control in blue) and new rPA (from P83-pStaby1.2 construct in red) using an indirect ELISA to determine seroreactivity. Twofold dilutions (1:20 to 1:2560) were made with a starting concentration of 6.1 µg/well of rPA83 alongside standard rPA (+ve control) used by Ndumnego et al. (2018). The samples included positive serum from animals vaccinated with an anthrax recombinant vaccine candidate with rPA as the main component Ndumnego et al. (2018). The relationship between new rPA and the positive control is tested by Spearman's correlation. Correlation coefficient r: New rPA vs Positive control = 1.0000, ****: Correlation is significant at level of 0.0001 P (two-tailed).

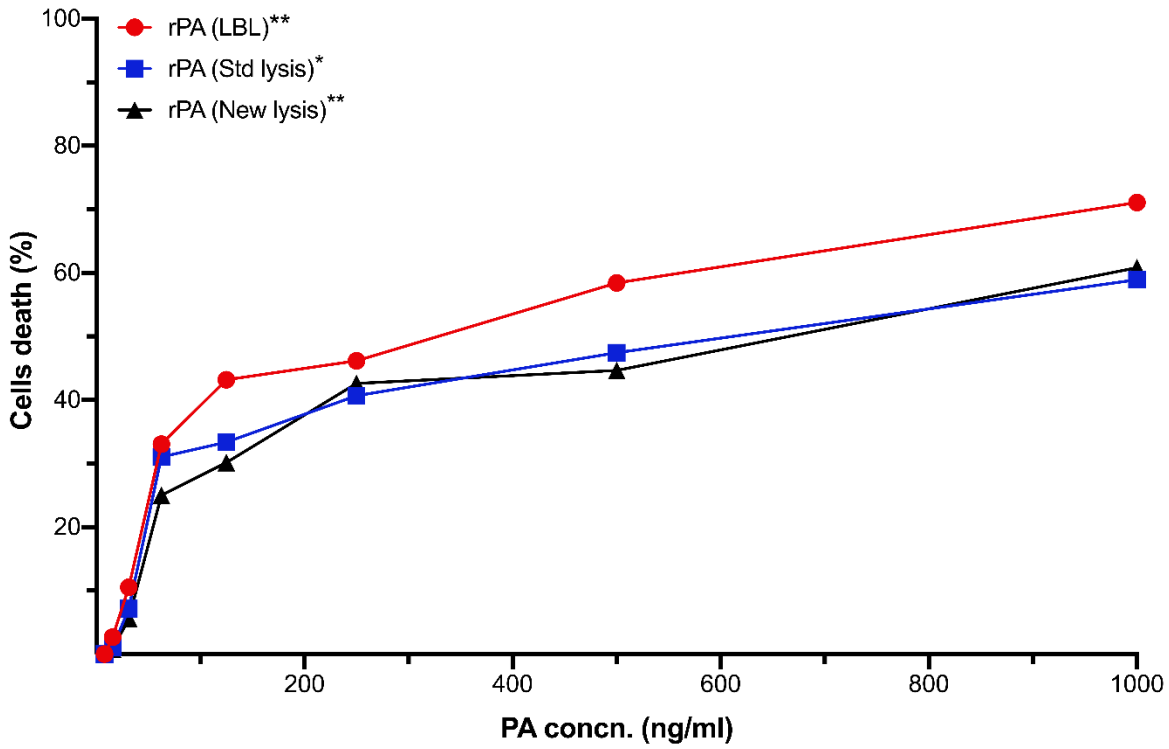

Figure 4. 7: Cell cytotoxicity assays showing the activity of recombinant protective antigen 83 (rPA83) demonstrating PA-mediated anthrax lethal toxin activity on J774A.1 cell lines. The cells were treated with different concentrations of rPA ranging from 1000 ng/mL to 7.8125 ng/mL and 200 ng/mL LF. LBL: commercial rPA from List Biological laboratory, new lysis rPA: rPA lysed using sonication method, Std lysis rPA: rPA lysed using standard lysis method. The relation of each rPA concentration (concn) and the percentage of cell death was compared by Spearman's correlation. Correlation coefficient r: PA concn. Vs rPA (LBL) = 0.8495, PA concn. Vs rPA (std lysis) = 0.9214, PA concn. Vs rPA (new lysis) = 0.9002. **: Correlation is significant at level of 0.009 P (two-tailed), *: Correlation is significant at level of 0.01 P (two-tailed), **: Correlation is significant at level of 0.006 P (two-tailed).

4.5 Discussion

PA is the main component of the tripartite protein that forms the *B. anthracis* binary toxins and the major immunogenic component of commercial human anthrax vaccine and used for detection of antibodies against *B. anthracis* either as a result of infection or vaccination (Rhie, 2011, Suryanarayana et al., 2016). The rPA83 is also used as a component in a NLAV candidate for livestock (Jauro et al., 2020, Koehler et al., 2017, Ndumnego et al., 2018). However, the cost of the veterinary NLAV needs to compete with the SLSV, the vaccine of choice used throughout the world. Currently, the most cost-effective method of producing recombinant proteins are *E. coli* expression systems (Gifre et al., 2017, Saccardo et al., 2016). In this study, purification methods that involve sonication to lyse the cells were

reviewed and a new protocol was designed (Figure 4.1). Based on the comparisons of new, standard and commercially available rPA, the newly developed purification method was equivalent in yield and biological activity with the commercially available and the standard at almost a third of the price hence can be adopted for similar applications. The standard lysis method involves the use of BugBuster®, endonuclease, lysozyme and protease inhibitor to lyse the cells. These reagents increase the cost of recombinant protein production (USD 4.25/mL) as compared with the cost of obtaining recombinant protein (USD 1.60/mL) using sonication lysis methods in the purification steps (Figure 4.2). The modified approach involves more steps and includes purification through two Ni^{2+}-TED columns but yields comparable new rPA (30.4 mg\L) to that of the standard method (36.2 mg/L) as the average of three different yields each. The new rPA is more cost effective making the approach worthwhile looking at other benefit such as antibiotic compatibility in the phase of an outbreak, eliminates batch to batch variation and residual virulence previously recorded with SLSV.

Furthermore, we demonstrated the effect of low temperature on protein solubility and importantly the cost-effective sonication lysing method during the purification of the soluble rPA83 cloned in pStaby1.2 vector, which is suitable for large scale production in *E. coli* SE1 (Barroca et al., 2016). In addition to the pStaby1.2-*E. coli* SE1 system scalability, it also offers an antibiotic-free feature and high yields (Sodoyer et al., 2012) contributing to cost reduction by stamping out antibiotics and antibiotic marker removal process for biomedical applications which reduces the spread of antibiotic resistance in the environment (Barroca et al., 2016, Pal et al., 2018, Sodoyer et al., 2012, Kim et al., 2006, Zubay, 1973).

The solubility of the protein expressed in this study was due to the effect of lower induction temperature of 25 ˚C. Cantu-Bustos et al. (2016), San-Miguel et al. (2013) and Yang and Zhang (2013) reported that higher expression temperature can lead to overexpression of the protein in the host which results in inclusion body formation. This often result in loss of

biological due to the harsh process involve during denaturing and renaturing. Thus, the inclusion body formation in our study (data not shown) was overcome by the effect of lower expression temperature while yielding a biologically active and soluble rPA83. Furthermore, lower protein expression temperatures of 10 °C – 25 °C can lead to improved protein solubility and retention of biological activity (Khow and Suntrarachun, 2012, Sahdev et al., 2008, San-Miguel et al., 2013). The solubility influenced by the lower expression temperature is a result of an increase in the level of chaperones in the *E. coli* host (Khow and Suntrarachun, 2012, Pacheco et al., 2012, San-Miguel et al., 2013, Vera et al., 2007) coupled with the contribution of the strong T7 promoter (Tegel et al., 2011) as is the case with the pStaby1.2 vector. Proper folding and biological activity of the rPA83 was confirmed by its ability to bind with LF to form LT and for its seroreactivity to be measured using ELISA and cytotoxic activity as demonstrated by its lethality on the macrophage cell line. Pavan et al. (2016) and Quintero-Ronderos et al. (2013) also reported the sero-reaction of immunodominant proteins signifies good biological activity of the protein. As clearly shown in Figure 4.7 by the positive correlation recorded between the new rPA and the standard rPA that have been used in vaccine study in goat by Ndumnego et al. (2018). This indicates there was a bond between the paratopes of the new rPA with the antibody epitopes.

The yield of rPA83 with two sonication cycles,30.8 mg/L, was significant compared with the rPA yield with one sonication cycle 18. 4 mg/L (Figure 4.5). This indicates that two sonication cycle can be used in large scale production to get more number of vaccines vials yet maintaining the 1 ml volume recommended by OIE (OIE. 2012) due to the increase in yield after second sonication. Tang et al. (2016) reported that purification of a recombinant protein with a His-Tag on the N terminal increase the yield bymore than 95% purity with sonication and Ni-affinity purification step. Ni^{2+}-NTA agarose was used after the sonication step to bind the protein and could be further investigated to replace the purification through Ni^{2+}-TED columns. Ni^{2+}-column increase the purity of the purified protein due the believe that His-tag have high affinity to the nickel in the column that remain firmly bond during the washing steps to get rid of unwanted protein.

In summary, the improved methods described have optimised and upscaled the production of the full-length rPA83 and expressed this using the pStaby1.2-SE1 system. These findings prove that the replacement of the commercial BugBuster lysing reagents with the modified lysis buffer and two sonication cycles, followed by Ni^{2+}-TED column purification, significantly reduced the protein production cost. Importantly, the up-scalable and antibiotic free expression system makes it even more cost-effective. In addition, the use of this rPA83 in the NLAV formulation would provide significant advantages that could make this vaccine competitive with the current SLSV. The pStaby1.2-*E. coli* SE1 antibiotic-free expression system can be adapted for cost-effective production of recombinant proteins for vaccine production, diagnostic purposes and pharmacotherapeutic uses in the biomedical field.

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CHAPTER FIVE

Immunogenicity of Non-Living Anthrax Vaccine Candidates in Cattle and Protective Efficacy of Immune Sera in A/J Mouse Model Compared to the Sterne Live Spore Vaccine

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5.1 Abstract

The Sterne live spore vaccine (SLSV, *Bacillus anthracis* strain 34F2) is the veterinary vaccine of choice against anthrax though contra-indicated for use with antimicrobials. However, the use of non-living anthrax vaccine (NLAV) candidates can overcome the SLSV limitation. In this study, cattle were vaccinated with either of the NLAV (purified recombinant PA (PrPA) or crude rPA (CrPA) and formaldehyde-inactivated spores (FIS of *B. anthracis* strain 34F2) and Emulsigen-D®/Alhydrogel® adjuvants) or SLSV. The immunogenicity of the NLAV and SLSV was assessed and the protective efficacies evaluated using a passive immunisation mouse model. Polyclonal IgG (including the IgG1 subset) and IgM responses increased significantly across all vaccination groups after the first vaccination. Individual IgG subsets titres peaked significantly with all vaccines used after the second vaccination at week 5 and remained significant at week 12 when compared to week 0. The toxin neutralisation (TNA)

titres of the NLAV vaccinated cattle groups showed similar trends to those observed with the ELISA titres, except that the former were lower, but still significant, when compared to week 0. The opsonophagocytic assay indicated good antibody opsonising responses with 75% (PrPA+FIS), 66% (CrPA+FIS) and 80% (SLSV) phagocytosis following spore opsonisation. In the passive protection test, A/J mice transfused with purified IgG from cattle vaccinated with PrPA+FIS+Emulsigen-D®/Alhydrogel® and SLSV had 73% and 75% protection from challenge with *B. anthracis* strain 34F2 spores, respectively, whereas IgG from cattle vaccinated with CrPA+FIS+Emulsigen-D®/Alhydrogel® offered insignificant protection of 20%. There was no difference in protective immune response in cattle vaccinated twice with either the PrPA+FIS or SLSV. Moreover, PrPA+FIS did not show any residual side effects in vaccinated cattle. These results suggest that the immunogenicity and protective efficacy induced by the NLAV (PrPA+FIS) in the cattle and passive mouse protection test, respectively, are comparable to that induced by the standard SLSV.

5.2 Introduction

Anthrax is caused by the Gram-positive bacterium *Bacillus anthracis* known to primarily infect ruminants as well as other warm-blooded mammals (Hambleton et al., 1984). It takes 3–5 days of incubation in the ruminant host for the disease to progress to a peracute or acute course (Beyer and Turnbull, 2009). The *B. anthracis* bacilli are responsible for systemic toxaemia and bacteraemia via its main virulence factors (Cote et al., 2012), which are encoded by two extrachromosomal plasmids, namely pXO1 and pXO2 (Koehler, 2002). The pXO2 encodes poly-γ-D-glutamic acid PDGA capsule, which is poorly immunogenic and prevents *B. anthracis* phagocytosis by evading immune surveillance during the early stage of anthrax disease (Guidi-Rontani et al., 1999). The aforementioned process gives the vegetative forms of the bacteria leverage to produce the tripartite toxin proteins comprising the lethal factor (LF), oedema factor (EF) and protective antigen (PA), which are encoded by the plasmid pXO1. Individually, these proteins are nontoxic until united in the binary fusion with PA as the common binding moiety, and LF and EF as the catalytic moieties. PA combines with LF and EF individually to form the binary toxins, namely lethal toxin (LT) and

oedema toxin (ET), respectively. PA facilitates the translocation of LF and EF into the cells where these toxins exert deleterious effects (Lacy et al., 2005, Leppla, 1982, Mogridge et al., 2002).

Anthrax epidemics are best controlled through vaccination (Stern et al., 2008). The Sterne live spore vaccine (SLSV) consisting of attenuated *B. anthracis* 34F2 strain (lacking the pXO2 plasmid) is used for vaccinating animals in most countries. The vaccine strain was developed by Max Sterne in the 1930s by attenuating the *B. anthracis* strain isolated from a case of bovine anthrax (Sterne, 1939). Since its development, SLSV has proved to be effective in protecting vaccinated animals. Booster immunisation with SLSV ensures hyper immunity in goats and an early booster vaccination (within 3 months) following the first immunisation has been suggested (Ndumnego et al., 2016). Nonetheless, SLSV is not devoid of some drawbacks, which include residual virulence in livestock and laboratory animals, adverse reaction in some animal species following vaccination and incompatibility with concurrent antibiotic treatment in disease outbreak situations (Fasanella et al., 2008, Sterne, 1939, Sterne, 1939, Turnbull, 1991, Turnbull, 2008). The development of a vaccine that can be administered concurrently with an antibiotic in the case of disease outbreak (Ndumnego et al., 2018), the protection of valuable wildlife or for feedlots when moving animals with unknown immune status from different locations, which require prophylactic treatment and vaccination against prevalent diseases, will be of huge benefit to the domestic/wild livestock industry. Various studies have evaluated the recombinant rPA anthrax vaccine candidate in combination with other non-living *B. anthracis* vaccine candidates in laboratory animals (Brossier et al., 2002, Cote et al., 2012, Hahn et al., 2004, Hahn et al., 2006, Little and Knudson, 1986, Parreiras et al., 2009, Ribot et al., 2006, Vance et al., 2015, Verma and Burns, 2018). Recently, goats were vaccinated thrice with non-living anthrax vaccine (NLAV) candidates comprising rPA, Bacillus collagen-like protein of anthracis (BclA) and formalin inactivated *B. anthracis* 34F2 spores (FIS) (three-step vaccination schedule), and the findings showed that rPA and FIS stimulate better immune responses compared to

BclA and that a two-step vaccination schedule may be sufficient (Koehler et al., 2017, Ndumnego et al., 2018).

In this study, rPA (crude and purified) and FIS were adjuvanted with Emulsigen-D®/Alhydrogel®. Emulsigen-D® is a unique emulsion (oil-in-water) containing dimethyl-dioctadecyl ammonium bromide (DDA), which is a good stimulator of T-cell immunity and increases the antigen surface area as well as the slow release of the antigen (Kaur et al., 2010, McGonigle et al., 2006, Shabana et al., 2018). Consequently Emulsigen-D® increase the duration of immune response. Alhydrogel® adjuvant is made up of aluminium hydroxide wet gel suspension. Alhydrogel® improves the uptake of antigens by antigen-presenting cells (APCs), induces NLRP3 inflammasome complexes as well as interleukin-1 (IL-1) and interleukin-18 (IL-18) secretion and increases Th2 antibodies response (Coffman et al., 2010, Gupta, 1998, Marrack et al., 2009). This stimulate wider range of immune response both CMI and humoral immunity In a two-step vaccination schedule, vaccine candidates were administered to cattle and the immune response and protective efficacy of the antibody-based immune responses were determined. The immune responses induced by either purified and crude rPA combined with the FIS and adjuvants were compared to the immune responses induced in SLSV-vaccinated cattle. Specific immune responses were confirmed using ELISA, in vitro toxin neutralisation assay (TNA) and opsonophagocytic assay. The protective efficacy was determined using a passive mouse protection test with purified antibodies from vaccinated cattle and lethal challenge with *B. anthracis* 34F2 spores.

5.3 Materials and methods

5.3.1 Recombinant protein expression and purification

The rPA used in this study was expressed and purified for the NLAV formulation and ELISA were carried out as stipulated in Chapter 3.

5.3.2 Formalin-inactivated spores (FIS) preparation

Bacillus anthracis 34F2 spores from Onderstepoort Biological Products, South Africa, batch 863, was cultured and sporulated as described in Chapter 3 section 3.2.

5.3.3 Formulation of non-living anthrax vaccine

The NLAV used in this study was formulated as outlined in Chapter 3 section 3.3 and the quality control were carried out by Design Biologixcc.

5.3.4 Immunisation animal experiment and passive mouse protection tests

Cattle were screened for PA-reactive antibodies using the PA-ELISA. The cattle experiment was conducted on a farm where the animals were born and raised, as approved by the Director of Animal Health, South Africa under the biosecurity section 20 of the animal disease Act 35 of 1984 (registration number: 12/11/1/1/6). After subcutaneous treatment with 4 mL Ivermectin (Ivomec injection South African Reg. No. G1142 (Act 36/1947)) and intramuscular injection with 10 mL multivitamins (Kyroligo Reg No. G3087 (Act 36/174)), the cattle were randomly allocated to four different vaccination groups, with eight animals in each group, except for the negative control group consisting of four animals, according to the animal ethics approval (protocol number; V118-17 Amendment 1) (Table 5.1). The animals were fed ad libitum and examined daily by a veterinarian. One animal died (vaccine group (C4)) after serum was collected for week 12 during the experiment. The cause of death was diarrhoea caused by *E. coli* infection, as revealed by the post-mortem examination and laboratory results.

The passive mouse protection test was conducted in the Onderstepoort Veterinary Animal Research Unit (OVARU) facility, University of Pretoria, South Africa in accordance with ethical principles and guidelines provided by the animal ethical committee of the University of Pretoria (protocol number; V118-17, Amendment 1) and section 20, Act 35 of 1984 permission granted by the Directorate of Animal Health of South Africa (registration number; 12/11/1/1/6(909). The passive protection test challenge model consisted of naïve inbred A/J mice from Jackson Laboratory, USA. The A/J mouse strain lacks the *Hc* gene that

encodes for complement component 5 (C5), which renders it vulnerable to systemic infection with the *B. anthracis* 34F2 Sterne vaccine strain spores (Ndumnego et al., 2018). The experimental design consisting of five mice per vaccinated cattle serum and three mice per negative control cattle serum is shown in Table 5.1. For the passive protection test, IgG was purified from sera collected from the vaccine groups at week 5, using the protein G spin column (Nab^{TM} Protein G Spin Kit, Thermo Scientific) according to manufacturer's instruction. The presence of anti-rPA specific IgG was confirmed using ELISA (Ndumnego et al. 2018) and the concentration of the IgG was measured with Pierce BCA protein assay kit (ThermoFisher Scientific). Purified IgG (500 µg) (Williamson et al., 2005) was injected intraperitoneally into naïve A/J mice. The lethal challenge consisted of 2.16 × 10^5 *B. anthracis* 34F2 strain spores in 200 µL injected through subcutaneous route, 24 h after the transfer of bovine polyclonal IgG. The A/J mice were monitored for clinical signs of anthrax intoxication for 14 days. Bacilli Giemsa stained smears and *B. anthracis* cultures from non-surviving A/J mice kidney, liver, and spleen on sheep blood agar were used to confirm death due to anthrax. Surviving mice were euthanised after 14 days by isoflurane overdose.

Table 5. 1. Animal trial vaccination, dosage and sampling schedules.

Vaccine Groups and Cattle Number (n).	**Vaccine and Dose**	**Cattle Vaccination and Sampling Schedule**				**A/J Mice Used in Passive Mouse Challenge (n)**
		Wk 0	**Wk 3**	**Wk 5**	**Wk 12**	
SLSV (n = 8)	SLSV vaccine with Anthravax® (10^8 spores)	±	±	+	+	5 mice/serum sample (n = 40)
PrPA+FIS (n = 8)	Purified rPA (75 µg) + FIS (10^8 spores) +Emulsigen-D®/Alhydrogel® adjuvants (33% v/v)	±	±	+	+	5 mice/serum sample (n = 40)
CrPA+FIS (n = 8)	Crude rPA (75 µg) + FIS (10^8 spores) + Emulsigen-D®/Alhydrogel® adjuvants (33% v/v)	±	±	+	+	5 mice/serum sample (n = 40)
NegCtl (n = 4)	Emulsigen-D®/Alhydrogel® adjuvants/saline (33% v/v)	±	±	+	+	3 mice/serum sample (n = 12)

The two-step vaccine schedule of the cattle included vaccination at week 0 and week 3. **±;** Blood collection before vaccination, **+;** Blood collection, SLSV; Sterne live spore vaccine, PrPA; Purified recombinant protective antigen, CrPA; Crude recombinant protective antigen, FIS; Formaldehyde inactivated spores, NegCtl: Negative control.

5.3.5 Serum immunoglobulin titre determination

ELISA was used to determine sera immunoglobulins (IgG, IgG1, IgG2 and IgM) titre for rPA and FIS as previously carried out by Ndumnego et al. (2018)

5.3.6 Toxin neutralisation assay (TNA)

An *in vitro* TNA was used to assess the neutralising antibody titres as outlined in Chapter 3 section 3.6.

5.3.7 Opsonophagocytic assay

The spore opsonising ability of the antibody generated was evaluated according to Welkos et al. (2001) with modification as described in Chapter 3 section 3.7.

5.3.8 Statistical analysis

The data analysis was carried out using the Gen 5 data analysis software as outlined in Chapter 3 section 3.8.

5.4 Results

5.4.1 Humoral immune response of living and NLAV in cattle

The immunogenicity of the NLAV was compared with SLSV. Sera from the vaccinated cattle were tested for IgG and IgM against PA and FIS, lethal toxin neutralisation as well as for immunoglobin subclasses, IgG1 and IgG2. The opsonophagocytosis of induced antibodies was also investigated.

Mean IgG titres against rPA rose significantly at week 3 for CrPA+FIS, PrPA+FIS and SLSV. The anti-rPA IgG titres were significantly higher for CrPA+FIS and PrPA+FIS at week 5 (two weeks after the second vaccination) and week 12 when compared to the titres before vaccination (Figure 5.1). Similarly, the mean IgG titres against FIS increased significantly from week 3 for CrPA+FIS and PrPA+FIS but the anti-FIS IgG titres' increase were insignificant for SLSV. At week 5 (two weeks after the second vaccination), the mean IgG titres against FIS were highly significant for CrPA+FIS and PrPA+FIS and remained significant at week 12 (Figure 5.2).

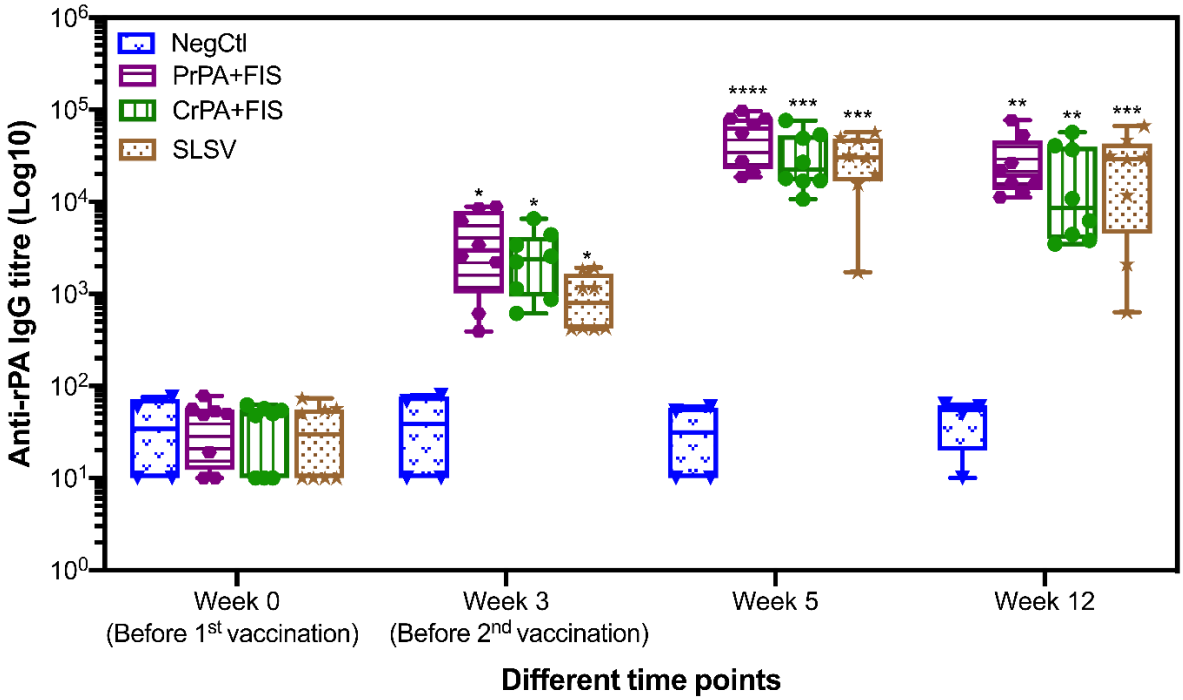

Figure 5. 1: Anti-recombinant protective antigen (rPA) IgG-titres in vaccinated cattle presented as box and whisker plots. The cattle were vaccinated twice (week 0 and 3) with PrPA+FIS+Emulsigen-D®/Alhydrogel® adjuvants (n = 8), CrPA+FIS+Emulsigen-D®/Alhydrogel® adjuvants (n = 8), Sterne live spore vaccine (SLSV) (n = 8) and NegCtl (Emulsigen-D®/Alhydrogel® adjuvants) (n = 4) with sera collected before the vaccinations at week 0 and 3 as well as samples collected at week 5 and 12. Sera dilution started at a concentration of 1:100 and values <50 were given an arbitrary value of 10. IgG titres in each vaccinated group were compared to the respective pre-immune titres. The significant values between groups are presented as **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p \leqslant 0.05$. PrPA: Purified recombinant protective antigen, CrPA: Crude recombinant protective antigen FIS: Formalin inactivated spores, SLSV: Sterne live spore vaccine, NegCtl: Negative control vaccinated with Emulsigen-D®/Alhydrogel® adjuvants.

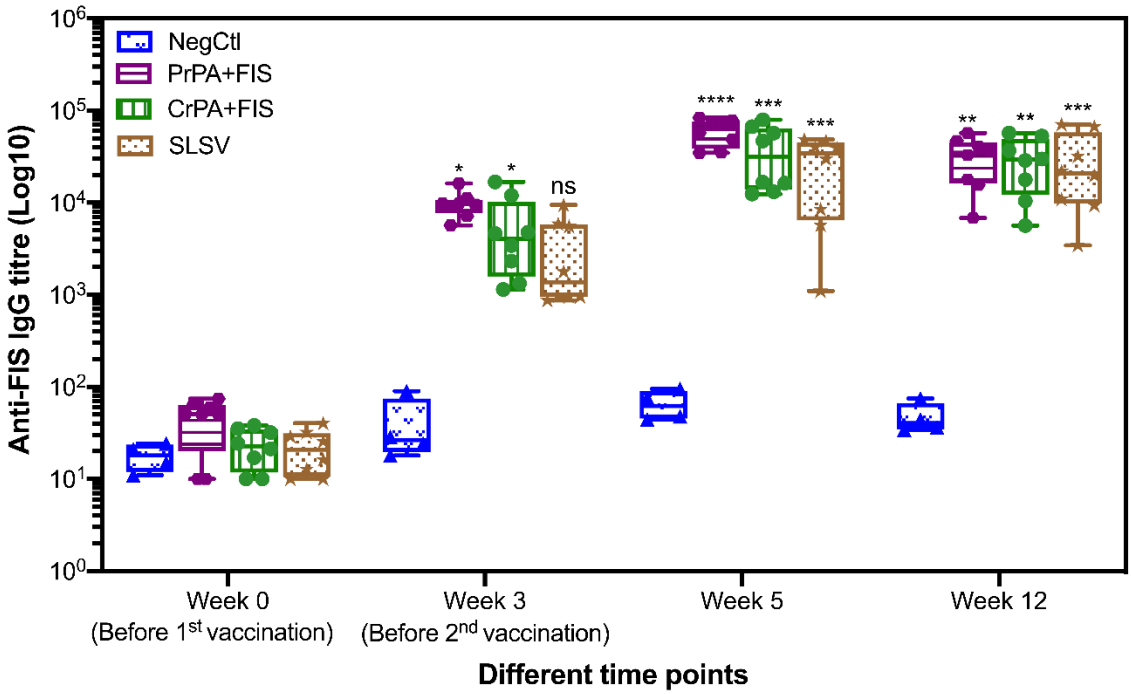

Figure 5. 2: Anti-FIS IgG-titres in vaccinated cattle presented as box and whisker plots. The cattle were vaccinated twice (week 0 and 3) with PrPA+FIS+Emulsigen-D®/Alhydrogel® adjuvants (n = 8), CrPA+FIS+Emulsigen-D®/Alhydrogel® adjuvants (n = 8), SLSV (n = 8) and NegCtl (Emulsigen-D®/Alhydrogel® adjuvants) (n = 4) and compared with sera collected before the vaccinations at week 0 and 3 as well as samples collected at week 5 and 12. Sera dilution started at a concentration of 1:100 and values <50 were given an arbitrary value of 10. IgG titres in each vaccinated group were compared to the pre-immune titres. The significant values between groups are presented as **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p \leqslant 0.05$. PrPA: Purified recombinant protective antigen, CrPA: Crude recombinant protective antigen FIS: Formalin inactivated spores, SLSV: Sterne live spore vaccine, NegCtl: Negative control vaccinated with Emulsigen-D®/Alhydrogel® adjuvants.

5.4.2 Immunoglobulins IgM and IgG subclasses titres

The antibody subclasses' response against rPA in vaccinated cattle groups after the first and second vaccination are presented in Figure 5.3a–c. The anti-rPA IgM titres exhibited significant elevation against PrPA+FIS, CrPA+FIS and SLSV after the first and second vaccination, before decreasing at week 12. The IgM titres at week 12 were still significant for PrPA+FIS but not significantly higher for the CrPA+FIS and SLSV group when compared to week 0. Thus, the group vaccinated with PrPA+FIS still had significantly higher titres, despite the decline in the IgM levels (Figure 5.3a). The anti-rPA IgG1 titres also showed a significant increase for PrPA+FIS, CrPA+FIS, and SLSV at week 3 and, at week 5, the anti-rPA IgG1 titres for PrPA+FIS, CrPA+FIS, and SLSV increase were highly significant. However, the

anti-rPA IgG1 titres declined below significance at week 12 for PrPA+FIS, CrPA+FIS and SLSV (Figure 5.3b). The anti-rPA IgG2 titres at week 3 were not statistically different from the pre-vaccination titres for all vaccine groups. However, analysis at week 5 and 12 showed a highly significant increase for anti-rPA IgG2 titres in all vaccines (Figure 5.3c).

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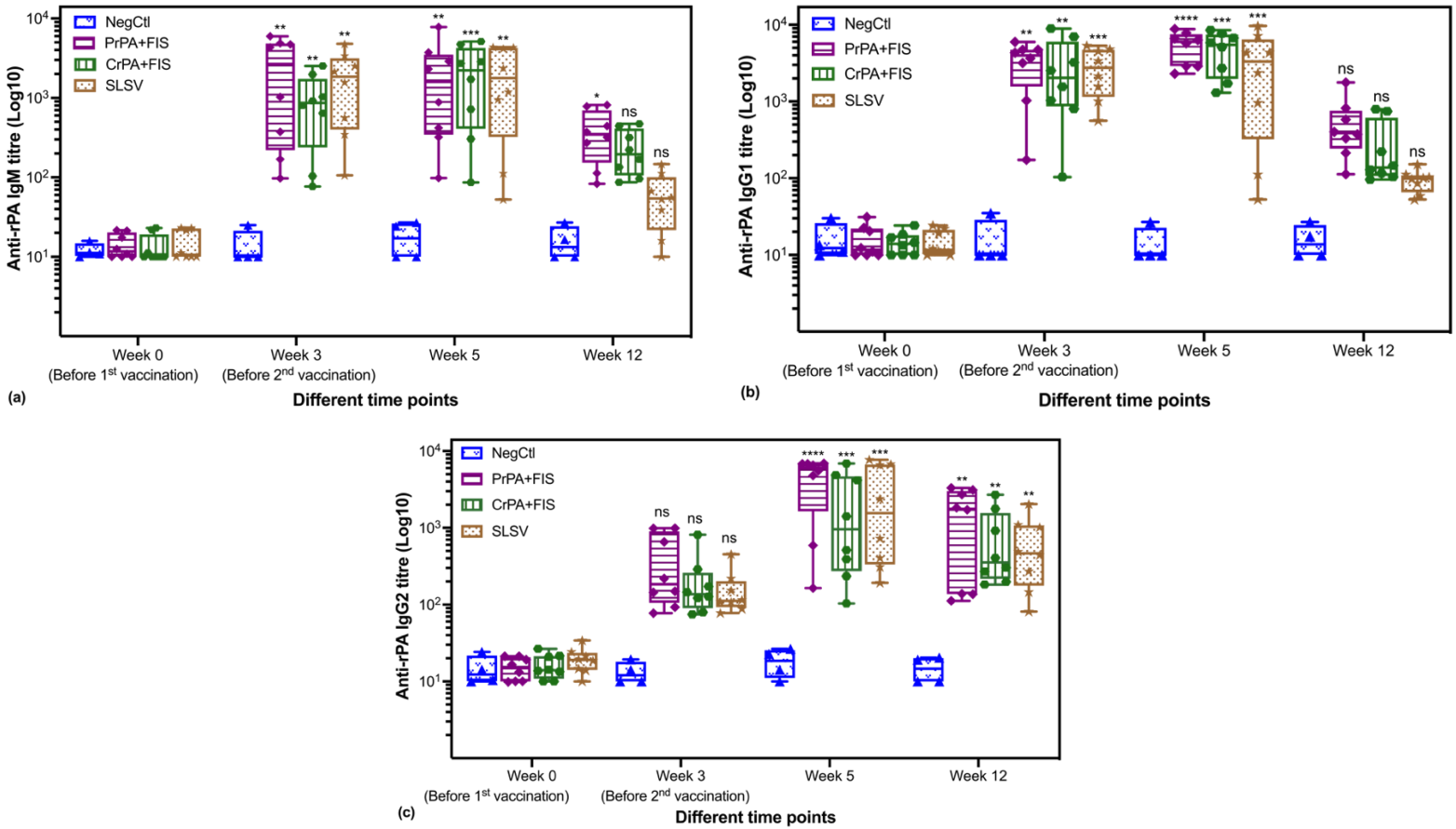

Figure 5. 3: The anti-rPA IgM and IgG (IgG1 and IgG2) ELISA titres of cattle vaccinated (week 0 and 3) with either PrPA+FIS+Emulsigen-D®/Alhydrogel® adjuvants (n = 8), CrPA+FIS+Emulsigen-D®/Alhydrogel® adjuvants (n = 8), SLSV (n = 8) and Emulsigen-D®/Alhydrogel® adjuvants (NegCtl) (n = 4) are presented as box and whisker plots. The cattle sera samples were collected before vaccination at week 0 and 3 as well as at week 5 and 12. Sera dilution started at a concentration of 1:50 and values below the (<50) were given an arbitrary value of 10. (a): Anti-rPA IgM ELISA titres, (b): Anti-rPA IgG1 ELISA titres and (c): Anti-rPA IgG2 ELISA titres. The significant values were presented as **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p \leqslant 0.05$, ns = not significant. PrPA: Purified recombinant protective antigen, CrPA: Crude recombinant protective antigen FIS: Formalin inactivated spores, SLSV: Sterne live spore vaccine, NegCtl: Negative control vaccinated with Emulsigen-D®/Alhydrogel® adjuvants.

The anti-FIS IgM and IgG response to the first and the second vaccination are shown for week 3, 5 and 12 in Figure 5.4a–c. The anti-FIS IgM titres after the first vaccination showed a significant increase for PrPA+FIS, CrPA+FIS, and SLSV. The increase in IgM titres at week 5 after the second vaccination for PrPA+FIS, CrPA+FIS and SLSV were highly significant, however these were insignificant at week 12 across all the vaccine groups when compared to week 0 (Figure 5.4a). The mean anti-FIS IgG1 titres also showed a significant increase for PrPA+FIS, SLSV and CrPA+FIS at week 3 and retained significance at week 5 across all vaccine groups. The mean anti-FIS IgG1 titres decreased but were still significant at week 12 for SLSV and PrPA+FIS but not significant for CrPA+FIS (Figure 5.4b). The mean anti-FIS IgG2 titres were significant for PrPA+FIS, CrPA+FIS and SLSV at week 3, increased further at week 5 for PrPA+FIS and SLSV, while declining (but still significantly higher than pre-vaccination titres) for CrPA+FIS. At week 12, the anti-FIS IgG2 mean titres were significant for PrPA+FIS, CrPA+FIS and SLSV (Figure 5.4c). The immunoglobulin subclasses of the NegCtl group consisting of cattle vaccinated with adjuvants only, did not change significantly across all timepoints when compared to the titres at week 0.

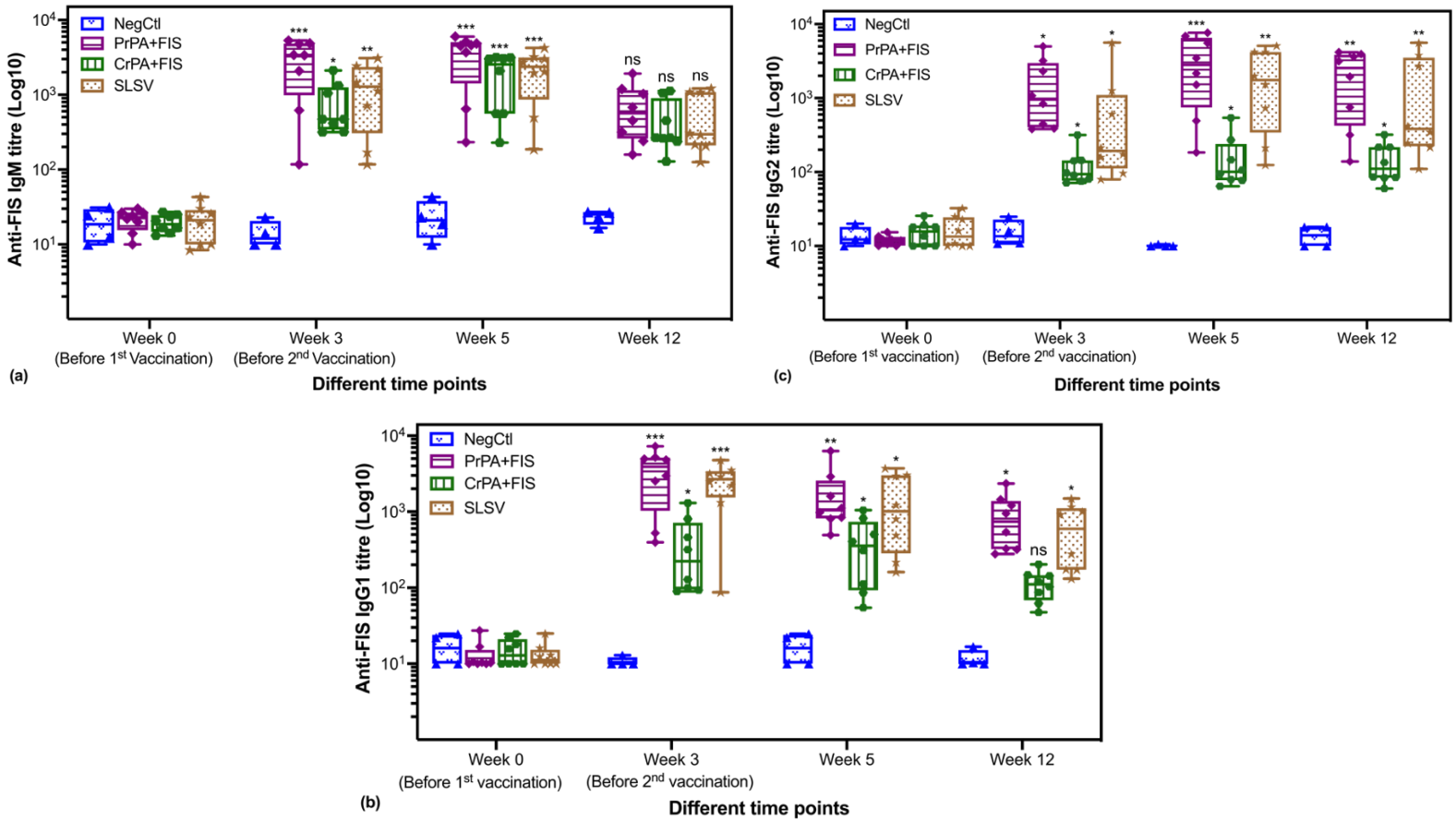

Figure 5. 4: The anti-FIS IgM and IgG (IgG1 and IgG2) ELISA titres of cattle vaccinated (week 0 and 3) with either PrPA+FIS+Emulsigen-D®/Alhydrogel® adjuvants (n = 8), CrPA+FIS+Emulsigen-D®/Alhydrogel® adjuvants (n = 8), SLSV (n = 8) and Emulsigen-D®/Alhydrogel® adjuvants (NegCtl) (n = 4) are presented as box and whisker plots. The cattle sera samples were collected before vaccination at week 0 and 3, as well as at week 5 and 12. Sera dilution started at a concentration of 1:50 and values below the (<50) were given an arbitrary value of 10. (a): Anti-FIS IgM ELISA titres, (b): Anti-FIS IgG1 ELISA titres and (c): Anti-FIS IgG2 ELISA titres. The significant values were presented as **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p \leqslant 0.05$, ns = not significant. PrPA: Purified recombinant protective antigen, CrPA: Crude recombinant protective antigen FIS: Formalin inactivated spores, SLSV: Sterne live spore vaccine, NegCtl: Negative control vaccinated with Emulsigen-D®/Alhydrogel® adjuvants

The lethal toxin neutralising antibodies (NT_{50}) titres of cattle vaccinated with NLAV (CrPA+FIS and PrPA+FIS?), SLSV and adjuvant (NegCtl) are shown in Figure 5.5. The lethal toxin neutralising antibody titres increased (though not significantly) across all vaccination groups after the first vaccination at week 3. The NT_{50} titres for all vaccine groups increased significantly at week 5 and 12 when compared to pre-vaccination titres. (Figure 5.5). The NT_{50} remained below the detection limit among the NegCtl group throughout the period of the study.

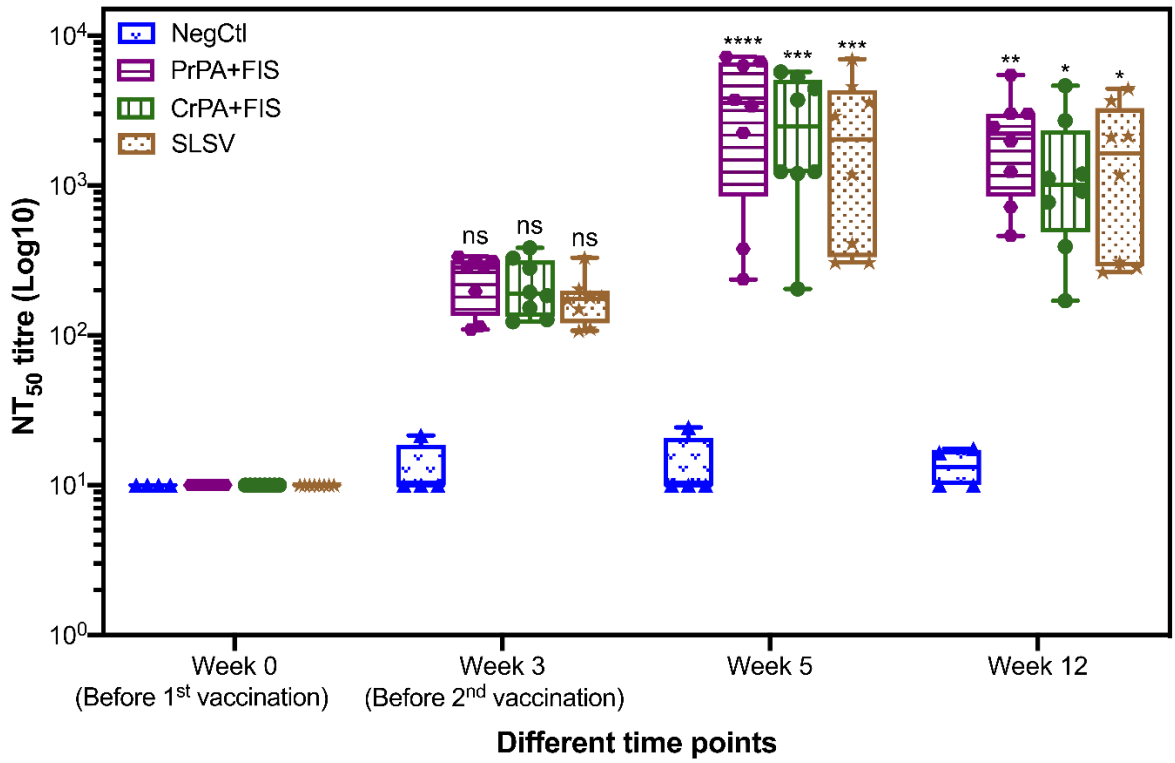

Figure 5. 5: Lethal toxin neutralising titres in vaccinated cattle. The cattle were vaccinated twice (week 0 and 3) with PrPA+FIS+Emulsigen-D®/Alhydrogel® adjuvants (n = 8), CrPA+FIS+Emulsigen-D®/Alhydrogel® adjuvants (n = 8), SLSV (n = 8) and NegCtl (Emulsigen-D®/Alhydrogel® adjuvants) (n = 4) and compared with sera collected before vaccination at week 0 and 3, as well as at week 5 and 12. Sera with no detectable toxin-neutralising titres were given an arbitrary value of 10 and sera dilution started at a concentration of 1:50. Neutralising titres in each vaccinated group were compared to the respective pre-immune titres. The significant values between groups are presented as **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p \leqslant 0.0.5$, ns = not significant. PrPA: Purified recombinant protective antigen, CrPA: Crude recombinant protective antigen FIS: Formalin inactivated spores, SLSV: Sterne live spore vaccine, NegCtl: Negative control vaccinated with Emulsigen-D®/Alhydrogel® adjuvants.

5.4.3 Opsonising ability of vaccine-induced antibodies

Sera collected at week 5 from cattle vaccinated with NLAV (CrPA+FIS and PrPA+FIS?) and SLSV were used to determine the potential of induced antibodies to opsonise *B. anthracis* spores, enabling phagocytosis by RAW 264.7 macrophage (Figure 5.6). The macrophages showed a high level of spore uptake at 1:10 sera dilution for both SLSV and NLAV vaccine groups. The RAW 264.7 macrophages showed 80%, 75% and 66% spore uptake following treatment with immune sera from SLSV, PrPA+FIS and CrPA+FIS vaccine groups, respectively. The NegCtl showed the least level of macrophage spore uptake (17%) following incubation with sera (Figure 5.6). The spore uptake at 1:100, 1:1000 and 1:10000 dilutions of sera from the SLSV and PrPA+FIS vaccine groups in the opsonophagocytosis assay remained significantly high when compared to the NegCtl sera (Figure 5.6). Sera from CrPA+FIS did not show a significant macrophage spore uptake at dilutions above 1:100.

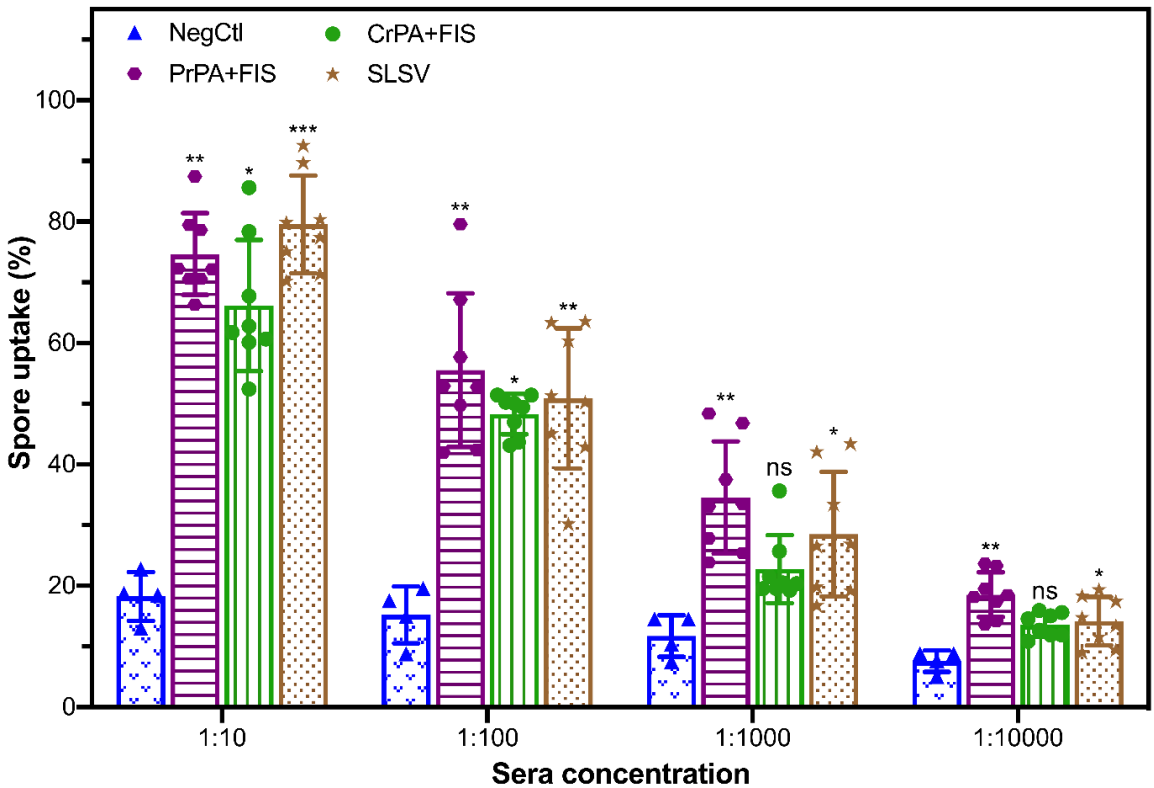

Figure 5. 6: The uptake of *Bacillus anthracis* 34F2 spores (phagocytosis) by RAW 267.7 macrophages following incubation with varying dilutions of sera taken from vaccinated and negative control cattle. The mean value of spore uptake is presented in the form of bar charts with the three standard deviations. The level of opsonophagocytosis of each group at every dilution was compared to the negative control (NegCtl) of each dilution. The significant values were presented as, *** $p < 0.001$, ** $p < 0.01$, * $p \leq 0.05$, ns = not significant. PrPA: Purified recombinant protective antigen, CrPA: Crude recombinant protective antigen FIS: Formalin

inactivated spores, SLSV: Sterne live spore vaccine, NegCtl: Negative control vaccinated with Emulsigen-D®/Alhydrogel® adjuvants.

5.4.4 Protection conferred on A/J mice by antibodies from cattle immune sera

The ability of the polyclonal IgG purified from cattle vaccinated with the two NLAV's and SLSV to protect A/J mice from challenge with toxigenic *B. anthracis* 34F2 strain spores was determined using a passive mouse protection model. The NegCtl group consisting of mice vaccinated with antibodies from cattle that had received adjuvants only, died 3–7 days following the challenge (Figure 5.7). A significant level of protection was seen among groups of A/J mice that were transfused with antibodies from PrPA+FISand SLSV-vaccinated cattle recorded 73% (29/40) and 75% (30/40) protection. However, IgG from CrPA+FIS vaccinated cattle were unable to confer significant protection to the A/J mice, with only 20% (8/40) of the A/J mice protected from the lethal effect of *B. anthracis* 34F2 strain spores.

Furthermore, we evaluated the relationship between the humoral and neutralising antibody titres from the vaccine groups and the level of protection conferred to A/J mice following lethal challenge. Our findings revealed there is a correlation between anti-rPA, anti-FIS and NT_{50} antibody titres from the group vaccinated with PrPA+FIS+Emulsigen-D®/Alhydrogel® and SLSV and the rate of survival in passively challenged A/J mice (Table 5.2). However, there was no correlation between the survival rate of the passively challenged A/J mice and the anti-rPA, anti-FIS and NT_{50} antibody titres from the CrPA+FIS+Emulsigen-D®/Alhydrogel® vaccinated group.

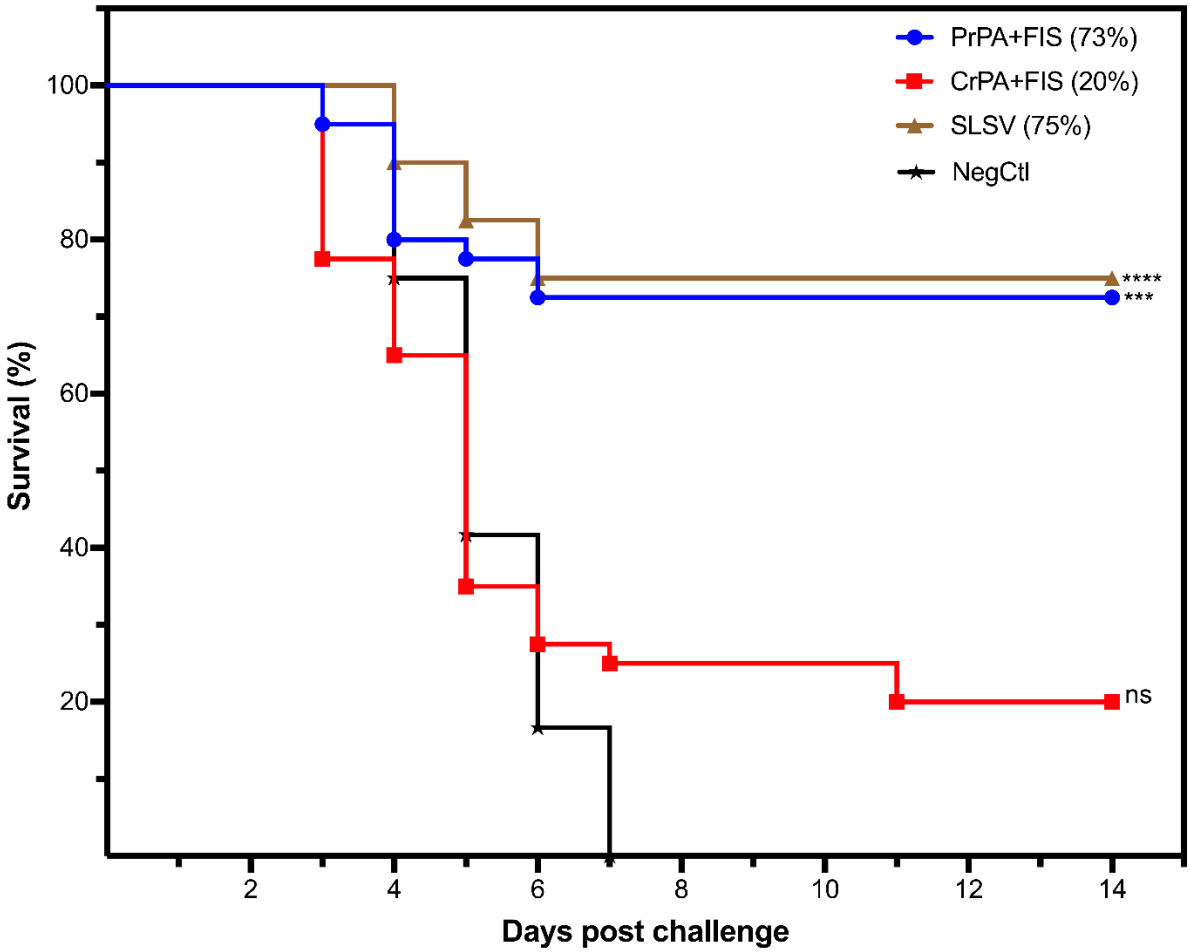

Figure 5. 7: The cumulative mice survival curve following passive *In vivo* transfer of polyclonal IgG (purified from cattle immune sera) into A/J mice. The mice were lethally challenged with 2.16 x 10^5 *B. anthracis* 34F2 spores. The sera were taken from cattle vaccinated twice (week 0 and 3) with either PrPA+FIS, CrPA+FIS, SLSV groups adjuvanted with emulsigen-D®/alhydrogel® and a NegCtl group (see Table 5.1 for the number of mice allocated to each group). The survival rate in the Log-rank (Mantel–Cox) test was compared to the NegCtl group. The significant values between groups are presented as **** $p < 0.0001$, *** $p < 0.001$, ns = not significant. PrPA: Purified recombinant protective antigen, CrPA: Crude recombinant protective antigen, FIS: Formalin inactivated spores, NegCtl: Negative control vaccinated with Emulsigen-D®/Alhydrogel® adjuvants.

Table 5. 2. The correlation between antibody titres in sera from vaccinated cattle and survival time to death of challenged mice after pa ssive transfer of purified antibodies.

	Anti-rPA (PrPA+FIS)	Anti-rPA (CrPA+FIS)	Anti-rPA (SLSV)	Anti-FIS (PrPA+FIS)	Anti-FIS (CrPA+FIS)	Anti-FIS (SLSV)	TNA (PrPA+FIS)	TNA (CrPA+FIS)	TNA (SLSV)
Pearson correlation	0.8196*	0.1237^{ns}	0.9239**	0.7883*	0.3836^{ns}	0.8518**	0.8588**	0.4564^{ns}	0.8778**
Significance (2-tailed)	0.0128	0.7704	0.0010	0.0201	0.3482	0.0073	0.0063	0.2556	0.0042

PrPA: Purified recombinant protective antigen, CrPA: Crude recombinant protective antigen, FIS: Formalin inactivated spores, NegCtl: Negative control vaccinated with Emulsigen-D®/Alhydrogel® adjuvants. **Correlation significant: 0.001 (2-tailed), *Correlation significant: 0.05 (2-tailed). ^{ns}Correlation not significant: 0.1234 (2-tailed).

5.5 Discussion

SLSV, as a live spore vaccine, is effective in the control of anthrax in livestock globally and has been the vaccine of choice in veterinary practice since the initial largescale production immunisation trials in the 1940s (Nicol and Sterne, 1942, Sterne, 1939). Despite the success achieved with the SLSV, drawbacks, such as a residual virulence in some vaccinated animals in particular goats (Turnbull 1991) and contraindications in anthrax outbreak situations due to the incompatibility of the vaccine with antibiotics, still remain (Sterne, 1939, Turnbull, 1991, Webster, 1973). NLAV can be used simultaneously with antibiotics without interfering with the immunogenicity of the vaccine. Various components of *B. anthracis,* such as PA, BclA, BxpB, LF and EF as well as the inactivated form of the whole *B. anthracis* spore, have been exhaustively studied alone or in combination for immunogenicity (Ascough et al., 2014, Brahmbhatt et al., 2007, Brossier et al., 2002, Cote et al., 2012, Duverger et al., 2010, Enkhtuya et al., 2006, Fasanella et al., 2008, Gauthier et al., 2009, Koehler et al., 2017, Majumder et al., 2018, Majumder et al., 2019, Ndumnego, 2016, Ndumnego et al., 2018). Among them, PA stands out as the major immunogenic component with the potential to stimulate toxin-neutralising antibodies (Little et al., 1997, Little et al., 1988). Other studies have reported an increase in immunogenicity and protection when PA is used in combination with other immunogenic components of *B. anthracis* such as FIS, BclA and exosporium basal layer protein (BxpB also known as ExsF) (Brossier et al., 2002, Hahn et al., 2006, Little and Knudson, 1986, Majumder et al., 2018, Majumder et al., 2019, Vergis et al., 2013, Koehler et al., 2017, Ndumnego et al., 2018, Vance et al., 2015). Most of these studies were conducted in laboratory animals except the studies by Koehler et al. (2017) and Ndumnego et al. (2018) which were both conducted using goats. These studies (Ndumnego et al., 2018, Koehler et al., 2017) reported that the combination of rPA and FIS provided a significant immune response using a three-step vaccination schedule in goats, as well as hypothesising that a two-step vaccination schedule might provide sufficient protection based on the immune response observed in goats. Therefore, in our study, cattle were vaccinated twice with NLAV (PrPA+FIS and CrPA+FIS) with an adjuvant combination

(Emulsigen-D®/Alhydrogel®) and compared to the SLSV. The timepoints of week 3, 5 and 12 were selected based on the previous reports by Koehler et al. (2017) and Ndumnego et al. (2018). Their showed that antibodies response against rPA in the vaccine groups vaccinated with rPA and SLSV all peaked around week 5-13. The PrPA+FIS and CrPA+FIS and SLSV vaccinated groups provided 73%, 20% and 75% protection, respectively, to mice that were passively immunised with purified cattle antibodies and challenged with toxigenic spores of *B. anthracis*. The negative control mice from cattle sera vaccinated twice with adjuvants only died within 7 days. Our results confirmed that a two-step vaccination schedule of PrPA+FIS in cattle provide the same level of protection in the mouse protection assay as a two-step vaccination with SLSV. Ndumnego et al. (2016) showed that a two-step vaccine schedule (three months apart) of SLSV provided maximum protection to goats and also that the thrice vaccinated NLAV (consisting of a rPA+rBclA+FIS+lipopeptide adjuvant) can probably be reduced to a two-step vaccination schedule based on the immune response in goats (Ndumnego et al., 2018, Ndumnego et al., 2016). A $Pam_3Cys\text{-}SK_4$ lipopeptide adjuvant known to enhance humoral immune response was used in these studies (Mittenbühler et al., 2003). However, this was replaced in our study by the Emulsigen-D®/Alhydrogel® adjuvants licensed to our industrial partner Design Biologix with similar results.

The immunoglobulin subclass titres exhibited a balance between Th1 and Th2-type responses. Even though the Th2-type immune response dominates the response at week 5, as seen with IgG2 in both NLAV and SLSV against FIS, a similar trend is also seen with IgG1 and IgG2 response against rPA at the same timepoint, which signifies the stimulation of Th1-type response as well. The IgG1 titres declined, but were still significant at week 12, unlike IgG2, which maintained the trends of titres from week 5 at week 12. However, the dynamics of the immune response switch between the immunoglobulin subclasses cannot be fully elucidated, as only four timepoints are reported in the 12 weeks of this study. However, this study has broaden our understanding of the trends of the immune response coupled with the pattern seen in the previous studies (Koehler et al., 2017, Ndumnego et al., 2018) along with the study time range of 10 to 13 weeks. But extended timepoint of approximately 12

month with sample collection each month need to be carried out to understand the trends of the immune response more and further work on the both CMI and HI when detail evaluation of the antibodies epitopes.

Solely PA-based vaccines were reported to be less protective against virulent challenge compared to the live spore vaccine (Brossier et al., 2002, Little and Knudson, 1986, Welkos and Friedlander, 1988). The combination of PA and FIS in vaccine formulations confer better protection by protecting 100% (5/5) of guinea pig as compared to 50% for PA alone and 33% for FIS alone when challenged with *B. anthracis* strain 17JB spore (Brossier et al., 2002). Hence, the presence of FIS in our formulation may be associated with the level of protection in the passive mouse protection test. The anti-PA, anti-FIS IgG and TNA titres of cattle vaccinated with the purified rPA+FIS correlated to the rate of protection achieved in the passive mouse protection test (Table 5.2). This is similar to the report by Ndumnego et al., 2018, where the showed a a positive correlation between anti-PA, anti-FIS and TNA titres of vaccinated goats and protection observed in passive mouse protection test.

Interestingly, the rate of protection recorded in the cattle group vaccinated with CrPA+FIS did not correlate with the antibody titres obtained against CrPA and FIS as well as to the NT_{50}. Obviously, the purity of the rPA used in the PrPA+FIS vaccine was of importance for the rate of survival recorded in the mouse trial which was shown by the positive correlation in this study. The CrPA+FIS vaccine was included in this study as a lower-cost vaccine prototype, as opposed to the more expensive PrPA+FIS vaccine, which necessitates the purification of rPA by affinity chromatography. The low survival rate of mice after passive immunisation with sera of CrPA+FIS vaccinated cattle could be due to restricted access to B-cell epitopes in the crude rPA preparation. This may result in the blockage of the PA epitopes that are responsible for the inducement of protective antibodies, as previously reported by Crowe et al. (2010). Additionally, Crowe et al. (2010) observed that peptide-specific antibodies against the furin cleavage, ligand-binding and receptor binding regions of PA are responsible for neutralisation (in vitro) with the furin cleavage site mediating the best protection in vitro while displaying less protection in vivo. Antibodies against the

receptor-binding site showed the most robust protection in vivo, despite displaying lower protection in vitro. Further work will be needed to confirm this. Moreover, the presence of the array of proteins in the CrPA+FIS formulation may have stimulated non-specific antibody responses, adding to the overall titres measured in the whole-cell FIS ELISA (Casadevall, 2005). In our study, we revealed that polyclonal antibodies from cattle vaccinated with PrPA+FIS, CrPA+FIS and SLSV were able to opsonise and enable the uptake of 75%, 66% and 80% spores by macrophages. The opsonisation of spores may substantially contribute to the rate of killing of phagocytosed spores by macrophages (Enkhtuya et al., 2006).

In conclusion, further study need to be carried out to characterise the antibody epitopes of the antibodies generated in this study to ascertain the reason behind the low level protection recorded by CrPA formulated vaccine. More animal trial need to be carried out using extended timepoints to evaluate the compare immune response with SLSV.

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CHAPTER SIX

Immunogenicity and Protective Efficacy of a Non-Living Anthrax Vaccine versus a Live Spore Vaccine with Simultaneous Penicillin-G Treatment in Cattle

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6.1 Abstract

Sterne live spore vaccine (SLSV) is the current veterinary anthrax vaccine of choice. Unlike the non-living anthrax vaccine (NLAV) prototype, SLSV is incompatible with concurrent antibiotics use in an anthrax outbreak scenario. The NLAV candidates used in this study include a crude recombinant protective antigen (CrPA) and a purified recombinant protective antigen (PrPA) complemented by formalin-inactivated spores and Emulsigen-D®/Alhydrogel® adjuvants. Cattle were vaccinated twice (week 0 and 3) with NLAVs plus penicillin-G (Pen-G) treatment and compared to cattle vaccinated twice with SLSV alone and with Pen-G treatment. The immunogenicity was assessed using ELISA against rPA and FIS, toxin neutralisation assay (TNA) and opsonophagocytic assay. The protection was evaluated using an in vivo passive immunisation mouse model. The anti-rPA IgG titres for NLAVs plus

Pen-G and SLSV without Pen-G treatment showed a significant increase, whereas the titres for SLSV plus Pen-G were insignificant compared to pre-vaccination values. A similar trend was measured for IgM, IgG1, and IgG2 and TNA titres (NT50) showed similar trends to anti-rPA titres across all vaccine groups. The anti-FIS IgG and IgM titres increased significantly for all vaccination groups at week 3 and 5 when compared to week 0. The spore opsonising capacity increased significantly in the NLAV vaccinated groups including Pen-G treatment and the SLSV without Pen-G but much less in the SLSV group with Pen-G treatment. Passive immunisation of A/J mice challenged with a lethal dose of 34F2 spores indicated significant protective capacity of antibodies raised in the SLSV and the PrPA + FIS + adjuvants vaccinated and Pen-G treated groups but not for the NLAV with the CrPA + FIS + adjuvants and the SLSV vaccinated and Pen-G treated group. Our findings indicate that the PrPA + FIS + Emulsigen-D®/Alhydrogel® vaccine candidate may provide the same level of antibody responses and protective capacity as the SLSV. Advantageously, it can be used concurrently with Penicillin-G in an outbreak situation and as prophylactic treatment in feedlots and valuable breeding stocks.

6.2 Introduction

Anthrax is a bacterial disease caused by the spore-forming bacilli, *Bacillus anthracis*, which infect both animals and humans but is primarily a disease of herbivores (Hambleton et al., 1984). *B. anthracis* causes systemic bacteraemia and toxaemia in its host via virulence factors (Cote et al., 2012) which are regulated by two plasmids (Leppla, 1995). The pXO1 encodes the tripartite toxin components, namely protective antigen (PA), edema factor (EF), and lethal factor (LF). The EF and LF individually fuse with PA to form anthrax toxins, namely edema toxin (ET) and lethal toxin (LT), respectively (Leppla, 1995). The second plasmid, pXO2, encodes the poly-γ-D-glutamic acid capsule that enables the bacterium to evade host phagocytosis (Leppla, 1995).

B. anthracis endospores survive in the environment for years and are the source of natural infection through cutaneous, gastrointestinal, or inhalation routes in ruminants (Turbull, 2008, Guidi-Rontani et al., 1999). During the early phase of acute anthrax disease, endospores which are phagocytosed by macrophages retain their viability and germinate

within the macrophages while some may migrate to the regional lymph node and geminate, leading to the production of LT and ET (Welkos et al., 2002). The ability of the spores to withstand macrophages' killing ability after phagocytosis enables the bacteria to establish brief intracellular existence before lysing the macrophages to gain access to the host tissue as vegetative cells (Welkos et al., 2002). The vegetative cells produce the toxins resulting in bacteraemia and subsequently toxaemia as well as oedema in anthrax disease that eventually results in the host death (Dal Molin et al., 2008, Leppla, 1982).

The most effective preexposure prophylactic measure against anthrax, as well as curbing the continuity of the disease, is vaccination of animals following the appropriate guidelines (Turnbull, 2008). In the veterinary field, anthrax is currently controlled using attenuated *B. anthracis* vaccines. Max Sterne developed SLSV by attenuating the culture from an anthrax case in bovine resulting in the avirulent, attenuated *B. anthracis* 34F2 strain without the capsule encoded by pXO2 (Mikesell et al., 1983). The SLSV is a more effective and safer vaccine than Pasteur's duplex anthrax vaccine, which lacks the pXO1, with reports of the presence of the two plasmids in some of the isolates (Sterne, 1939, Sterne, 1945, Turnbull, 2008). The SLSV has been in use as a veterinary anthrax vaccine of choice in most countries since it was first produced for large-scale immunisation trials in the 1940s (Nicol and Sterne, 1942). Despite having been considered effective, SLSV has some shortcomings such as residual virulence resulting in the mortality of some vaccinated animals especially goats and llamas (Sterne, 1939, Sterne, 1945, Cartwright et al., 1987), variation in batches during production resulting in inconsistent immune stimulation, and risk of environmental contamination during production or vaccination campaigns. In addition, the live spore vaccine cannot be used simultaneously with antibiotic treatment during a disease outbreak or other scenarios, such as; the protection of valuable wildlife or feedlot programmes where livestock are vaccinated against various diseases and given antibiotics as a prophylactic measure against common diseases (Fasanella et al., 2008, Stepanov et al., 1996, Webster, 1973, Welkos et al., 2001).

The development of protective antibodies in livestock or animals vaccinated with SLSV has been reported to take 7-10 days (Turnbull, 2008, Fasanella et al., 2008) post-vaccination. Control measures during anthrax outbreaks consist of vaccinating animals immediately after the first case of anthrax, which may result in cessation of further cases (Turnbull, 2008). It

is advisable to use antibiotics to protect valuable or endangered animals in an anthrax outbreak, followed by vaccination with SLSV to ensure future protection after the withdrawal of antibiotics (usually 8-12 days depending on the antibiotics used) (Turnbull, 2008). This is because the vaccine is a live spore vaccine which cannot be used simultaneously with antibiotics (Turnbull, 2008). In like manner, a withdrawal period of 8-12 days is also observed to allow antibiotics to lapse from the animal's system before vaccination with SLSV in animals fed with feed containing antibiotics (Webster, 1973). Animals suspected to be at risk during anthrax outbreaks are quarantined for 21 days before vaccination (Turnbull, 2008).

The use of non-living anthrax vaccine (NLAV) candidates would be a novel approach due to the various benefits such as eliminating the danger of handling spores during production, standardising production, avoiding batch variation and environmental contamination, use of improved adjuvants, and simultaneous use with antibiotics treatment (Bellanti et al., 2012, Brown et al., 2010, Campbell et al., 2007). The human anthrax vaccines, AVP and AVA, are examples of NLAVs that were developed using protective antigen (PA) as the key antigen (Cybulski Jr et al., 2009). These older-generation human anthrax vaccines required numerous boosters to provide sufficient immunity as well as the remnant of lethal factor present with PA harvested from the culture supernatant (Fellows et al., 2001, Ivins et al., 1995, Ivins et al., 1998, Ivins et al., 1992, Welkos and Friedlander, 1988). Thus, recombinant PA (rPA) offers a pure and uncontaminated antigenic component, which can be combined with other *B. anthracis* components to enhance immunity. These components include surface layer proteins (Uchida et al., 2012), exosporium basal layer protein (BxpB also known as ExsF) (Cote et al., 2012, Majumder et al., 2019), Bacillus collagen-like anthracis (BclA) (Hahn et al., 2006, Köhler et al., 2015, Majumder et al., 2018, Steichen et al., 2003), and/or formalin-inactivated *B. anthracis* spores (FIS) (Brossier et al., 2002, Cybulski Jr et al., 2008, Gauthier et al., 2009, Koehler et al., 2017, Ndumnego, 2016, Ndumnego et al., 2018, Vergis et al., 2013). The idea was to combine rPA which produces anti-toxin antibodies to prevent the production of toxins in vivo with *B. anthracis* spore components to enhance anti-spore antibodies, thus preventing spore germination (Welkos et al., 2001). In previous studies, goats were vaccinated using rPA, BclA, and FIS adjuvanted with a lipopeptide in a three-step vaccination schedule and protection was shown using either the passive A/J

mouse challenge model or lethal virulent *B. anthracis* challenge in goats (Koehler et al., 2017, Ndumnego et al., 2018). The immunogenic findings showed that rPA and FIS stimulated a better immune response in goats compared to BclA and that a two-step vaccination schedule might be sufficient (Koehler et al., 2017, Ndumnego et al., 2018, Ndumnego et al., 2016). A three-step vaccination schedule of rPA, BclA, and FIS was also administered simultaneously with Pen-G to goats and the immune response showed similar antibodies titres against PA and FIS in NLAV plus Pen-G vaccinated group to the animal group vaccinated twice with SLSV (Ndumnego et al., 2016). However, the protective efficacy of the antibodies from goats vaccinated with PA, BclA, and FIS and simultaneous treatment with penicillin-G was not investigated. Recently, a two-step vaccination schedule of NLAV candidates (consisting of either purified and crude rPA combined with the FIS and adjuvants) and SLSV administered to cattle showed comparable immunogenicity and protective efficacy induced by the purified rPA + FIS formulation and the standard SLSV (Jauro et al., 2020).

In this study, a two-step vaccination schedule of NLAV candidates (consisting of either purified or crude rPA combined with FIS and a combination of two adjuvants) and SLSV with simultaneous antibiotic (Pen-G) treatment as well as SLSV (with no Pen-G treatment) were administered to cattle, and the immune response and protective potential of the antibodies determined. The adjuvants used were Emulsigen-D®/Alhydrogel® constituting 33% of the vaccine at the ratio of 1:1. Emulsigen-D® is an emulsion formulated using dimethyl-dioctadecyl ammonium bromide (DDA), and Alhydrogel® is a wet suspension of aluminium hydroxide. Emulsigen-D® stimulates a good T-cell response; it also slows the release of antigen from the site of injection and increases the surface area of the antigen. Alhydrogel® enhances antigen presentation cell's (APC) activity, increases Th2 response and stimulates the secretion of interleukin-1 (IL-1) and interleukin-18 (IL-18) (Coffman et al., 2010, Gupta, 1998, Kaur et al., 2010, Marrack et al., 2009, Shabana et al., 2018). The specific humoral immune responses in the vaccinated cattle were determined using ELISA, toxin neutralisation assay (TNA) and an opsonophagocytic assay. The protective efficacy was determined using the passive mouse protection test by in vivo transfer of purified antibodies from the vaccinated cattle and lethal challenge with *B. anthracis* 34F2 spores.

6.3 Materials and methods

6.3.1 Recombinant protein expression and purification

The rPA83 used for the vaccine formulation as well as for the ELISA was expressed and purified as described in chapter 3.

6.3.2 Formalin-inactivated spores (FIS) Preparation

B. anthracis 3F42 spore inactivated for vaccination and sporulated for passive mouse protection test was executed as illustrated in chapter 3.

6.3.3 Non-living anthrax vaccines

The vaccine formulation was carried out by Design Biologix following steps outline inl chapter 3.

6.3.4 Animals and approvals

The cattle study was carried out on a farm following approval under the biosecurity section 20 of the animal disease Act 35 of 1984 by the Director of Animal Health, South Africa (reference nr: 12/11/1/1/6). Based on the approval by the animal ethics committee (protocol number; V118-17 Amendment 1), seven cattle in each group were randomly allocated to the vaccination groups except the negative control groups which comprised of four cattle (Figure 6.1). The cattle were examined for rPA-reactive antibodies using anti-rPA ELISA before moving to the farm where each animal was treated with 10 ml multivitamins (Kyroligo Reg No. G3087 (Act 36/174)) via intramuscular route and 4 ml Ivermectin (Ivomec injection South African Reg. No. G1142 (Act 36/1947)) subcutaneously. The cattle were fed ad libitum and regularly monitored by a veterinarian.

The in vivo passive mouse protection experiment was conducted in a pathogen-free laboratory of the Onderstepoort Veterinary Animal Research Unit (OVARU) at the University of Pretoria, South Africa, in line with ethical procedures and principles outlined by the University of Pretoria animal ethics committee (protocol number; V118-17 Amendment 1) and section 20 of animal diseases, Act 35 of 1984 (registration number: 12/11/1/1/6(909)). The naïve inbred A/J mice, which lacks the *Hc* gene encoding for C5 and succumbs to the lethal toxin of *B. anthracis* 34F2 Sterne spores (Ndumnego et al., 2018) were procured from

the Jackson Laboratory, USA. The experiment consisted of 5 A/J mice allocated to each serum from vaccinated cattle, whereas the negative control group consisted of 3 mice per serum (Figure 6.1). For the passive protection test, IgG was purified from the serum of each vaccinated animal with protein G spin column based on the manufacturer's instruction (NAb™ Protein G Spin Kits, ThermoFisher Scientific, Rockford, IL, USA). ELISA was used to affirm the presence of IgG against PA, and a Pierce BCA protein assay kit (ThermoFisher Scientific, Rockford, IL, USA) was used to quantify the concentration of the IgG. Each A/J mouse was injected with 500 µg of the purified IgG (Williamson et al., 2005) intraperitoneally, and 24 hours later each A/J mouse was lethally challenged with 2.16×10^5 *B. anthracis* 34F2 spores (200 µL) spores subcutaneously. The A/J mice were monitored for any clinical signs related to the effect of anthrax toxins for 14 days after the challenge. Death due to anthrax was confirmed by the presence of *B. anthracis* colony morphology from the culture of A/J mouse liver, spleen, and kidney on sheep blood agar after incubation at 37 ℃ for 24 h followed by the presence of bacilli in Giemsa stained smears. Surviving mice were euthanised after 14 days using isoflurane overdose.

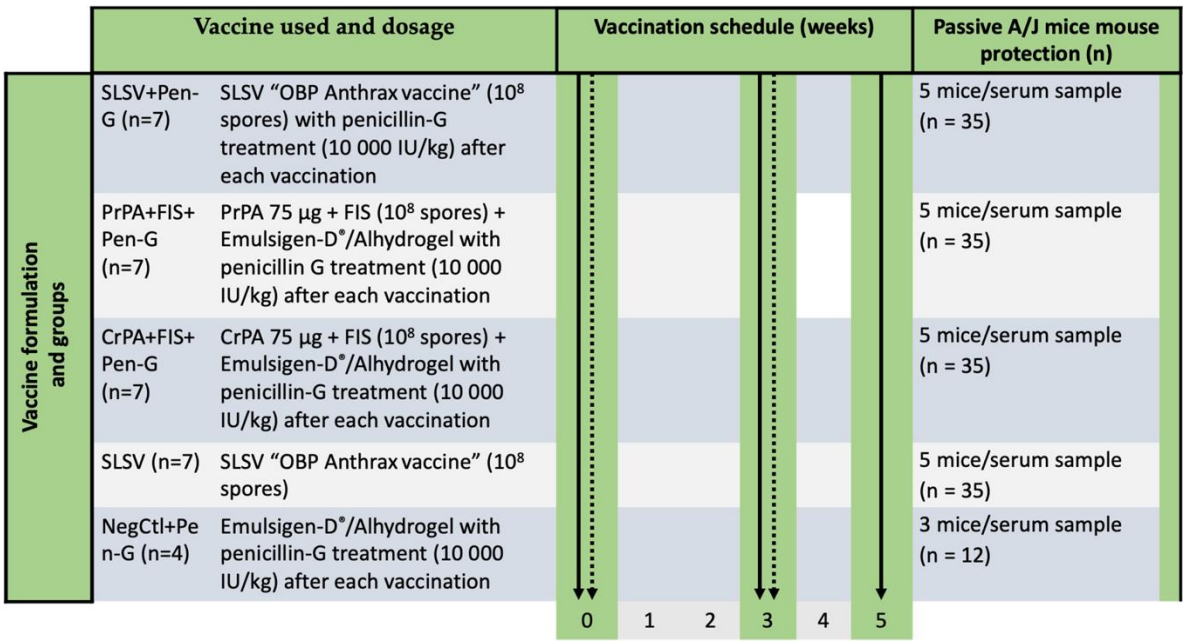

Figure 6. 1: The timeline for two-step vaccination in cattle groups consisting of Sterne live spore vaccine (SLSV) with and without penicillin-G treatment and non-living anthrax vaccines (NLAV) with penicillin-G treatment followed by sera collection and a passive mouse protection model. The passive mouse model consisted of A/J mice injected with purified polyclonal IgG taken from the sera of vaccinated cattle and challenged with *Bacillus anthracis* 34F2 spores. The thick continuous arrows show timepoints for sera collection and the dotted arrows indicate the vaccination timepoints. Sterne live spore vaccine: SLSV; Purified recombinant protective antigen:

PrPA; Crude recombinant protective antigen: CrPA; Formalin inactivated *Bacillus anthracis* spores: FIS; Negative control: NegCtl; Penicillin-G: Pen-G.

6.3.5 Enzyme-linked immunosorbent assay (ELISA) for serum immunoglobulins titre

The IgG, IgG1, IgG2 and IgM immunoglobulins titres against PA and FIS were measured as described in chapter 3.

6.3.6 Toxin neutralisation titre (TNA) for neutralising antibodies titre

The neutralising antibodies titre was determined with TNA as described in chapter 3.

6.3.7 Opsonophagocytic assay

The spore opsonising ability of the antibodies that enables spore phagocytosis by macrophages was determined as described in chapter 3.

6.3.8 Statistical analysis

All data generated from this study was statistically analysed using the same software and version stated in chapter 3 following the same trend.

6.4 Results

6.4.1 Humoral IgG titre

Both the PrPA+FIS plus Pen-G and CrPA+FIS plus Pen-G stimulated a significant IgG response against rPA, at week 3 (three weeks after the first vaccination), whereas the anti-rPA IgG titres were insignificant among the animal groups that were vaccinated with SLSV + Pen-G as well as SLSV alone at week 3 (Appendix Table 1). At week 5 (two weeks after the second vaccination), the IgG titres against rPA increased significantly for PrPA+FIS plus Pen-G, CrPA+FIS plus Pen-G and SLSV vaccinated groups, while SLSV plus Pen-G titres increased but not significantly (Appendix Table 1, Figure 6.2). In addition, the anti-FIS IgG titres for PrPA+FIS plus Pen-G, CrPA+FIS plus Pen-G, SLSV plus Pen-G and SLSV at week 3 and week 5 increased significantly (Appendix Table 1, Figure 6.3). Both anti-rPA and anti-FIS titres were compared to pre-vaccination titres (week 0).

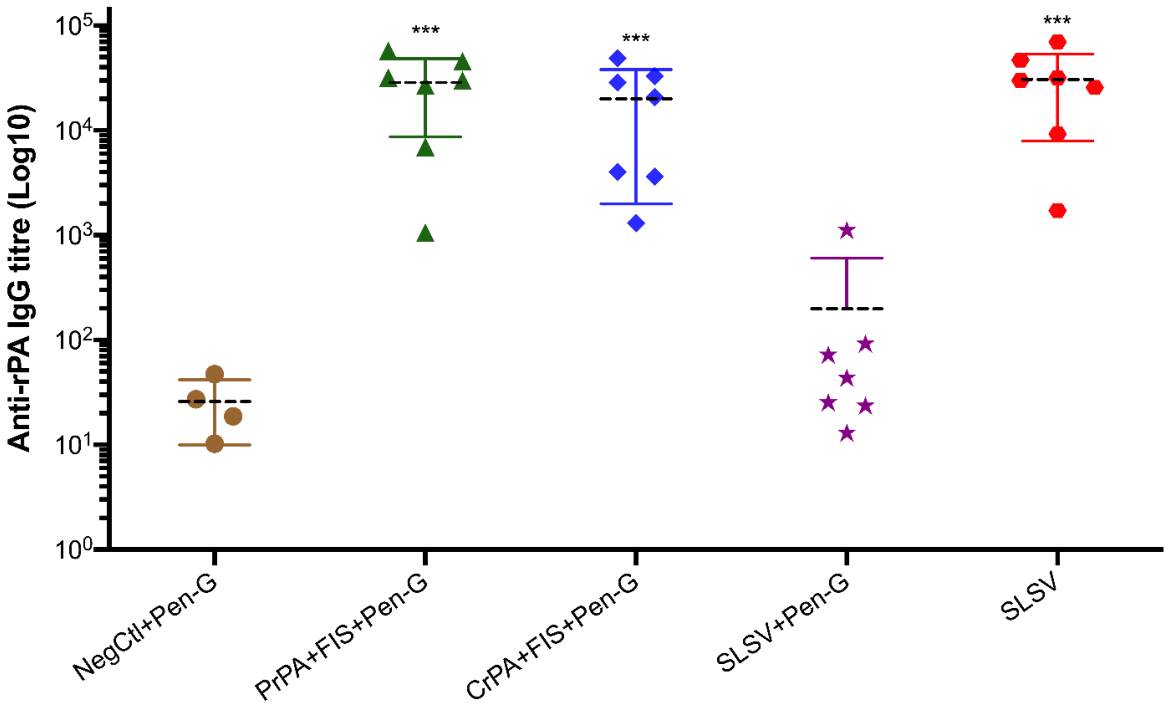

Figure 6. 2: Anti-recombinant protective antigen (rPA) IgG-titres at week 5 in cattle. The cattle were vaccinated twice (week 0 and 3) with PrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen G (n=7), CrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen G (n=7), Sterne live spore vaccine (SLSV) plus Pen-G (n=7), SLSV (n=7) and NegCtl (Emulsigen-D®/Alhydrogel® plus Pen-G) (n=4). The IgG titres of each group were compared to the respective titres at week 0 before vaccination (***$p < 0.001$, **$p < 0.01$, *$p \leq 0.0.5$). PrPA; Purified recombinant protective antigen, CrPA; Crude recombinant protective antigen, FIS; Formalin inactivated *Bacillus anthracis* spores, Pen-G: Penicillin-G, SLSV; Sterne live spore vaccine, NegCtl; Negative control

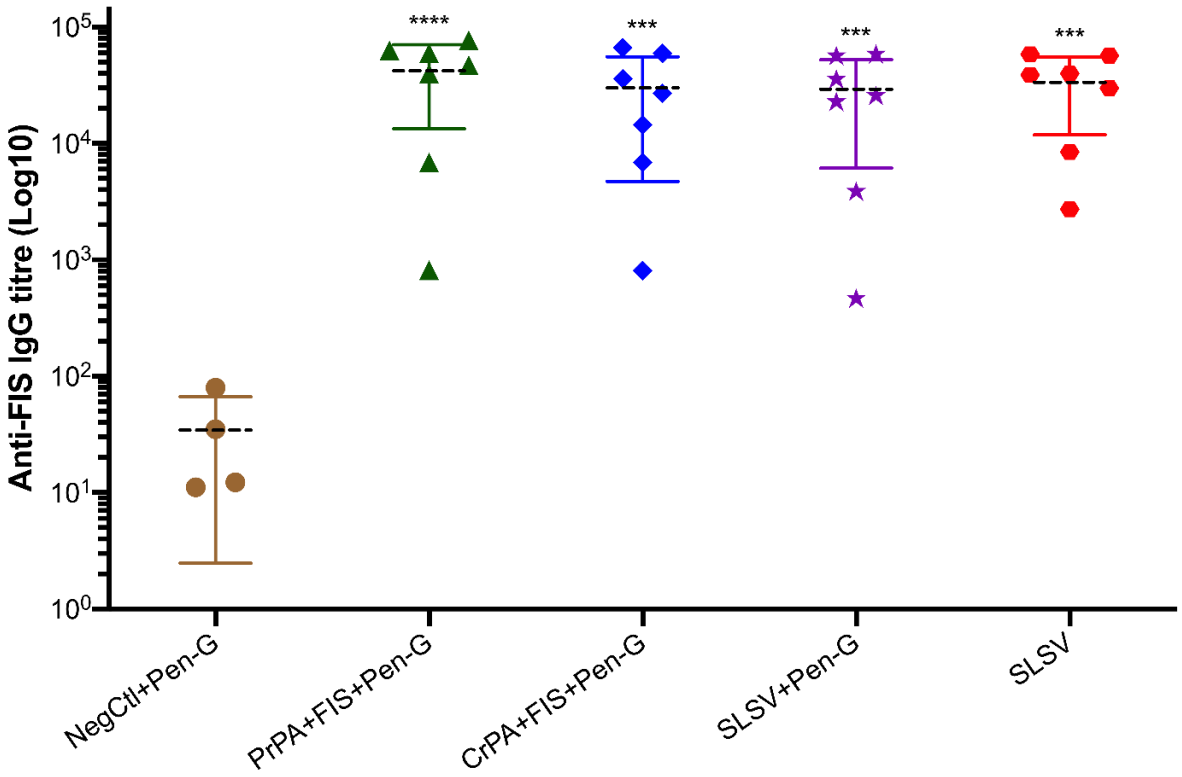

Figure 6. 3: Anti-formalin inactivated *Bacillus anthracis* 34F2 spores (FIS) IgG-titres at week 5 in cattle. -The cattle were vaccinated twice (week 0 and 3) with PrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen G (n=7), CrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen G (n=7), Sterne live spore vaccine (SLSV) plus Pen-G (n=7), SLSV (n=7) and NegCtl (Emulsigen-D®/Alhydrogel® plus Pen-G) (n=4). The IgG titres of each group were compared to the respective titres at week 0 before vaccination (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p \leq 0.0.5$). PrPA; Purified recombinant protective antigen, CrPA; Crude recombinant protective antigen, FIS; Formalin inactivated *Bacillus anthracis* spores, Pen-G: Penicillin-G, SLSV; Sterne live spore vaccine, NegCtl; Negative control.

6.4.2 Analysis of humoral IgM and IgG

The anti-rPA IgM titres increased significantly at both week 3 and 5 among PrPA+FIS plus Pen-G, CrPA+FIS plus Pen-G and SLSV vaccinated groups. The anti-rPA IgM response in the group vaccinated with SLSV plus Pen-G revealed insignificant titres at weeks 3 and 5. The levels of anti-rPA IgG1 titres showed a significant increase for PrPA+FIS plus Pen-G, CrPA+FIS plus Pen-G and SLSV after the first (week 3) and second (week 5) vaccination. The anti-rPA IgG2 titres level displayed a significant response at week 3 and highly significant response at week 5 for PrPA+FIS plus Pen-G, CrPA+FIS plus Pen-G and SLSV. However, the group vaccinated with SLSV plus Pen-G showed insignificant anti-rPA titres against IgG1 and IgG2

at weeks 3 and 5 (Appendix Table 2, Figure 6.4). All titres were compared to pre-vaccination (week 0) anti-rPA IgM, IgG1 and IgG2 titres. However, the NegCtl showed insignificant IgM and IgG isotypes response against rPA among all vaccine groups (Appendix Table 2, Figure 6.4)

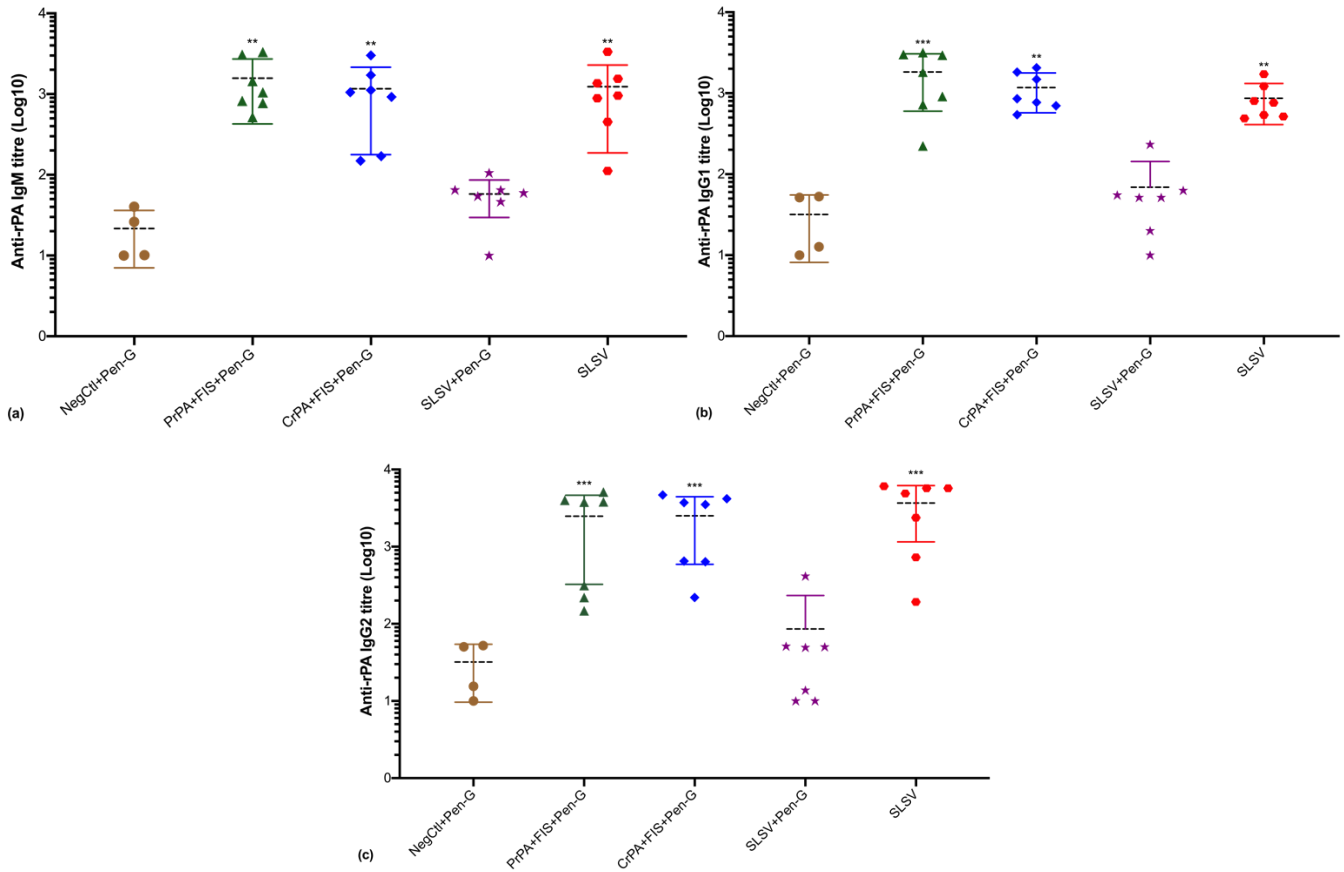

Figure 6. 4: Anti-recombinant protective antigen (rPA) IgM and IgG isotype (IgG1 and IgG2) titres at week 5 in cattle. The cattle were vaccina ted twice (week 0 and 3) with PrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen G (n=7), CrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen G (n=7), Sterne live spore vaccine (SLSV) plus Pen-G (n=7), SLSV (n=7) and NegCtl (Emulsigen-D®/Alhydrogel® plus Pen G) (n=4). The IgG titres of each group were compared to the respective titres at week 0 before vaccination (***$p < 0.001$, **$p < 0.01$, *$p \leq 0.0.5$). (a) anti-FIS IgM titres, (b) anti-FIS IgG1 titres and (c) anti-FIS IgG2 titres. PrPA; Purified recombinant protective antigen, CrPA; Crude recombinant protective antigen, FIS: Formalin inactivated *Bacillus anthracis* spores, Pen-G: Penicillin-G, SLSV; Sterne live spore vaccine, NegCtl; Negative control

The anti-FIS IgM response revealed a significant increase in titres at week 3 and week 5 among the cattle groups vaccinated with PrPA+FIS plus Pen-G, CrPA+FIS plus Pen-G, SLSV plus Pen-G and SLSV when compared to week 0 titres (Appendix Table 2, Figure 6.5). The anti-FIS IgG1 isotype response was significant among cattle groups vaccinated with PrPA+FIS plus Pen-G, CrPA+FIS plus Pen-G, SLSV plus Pen-G and SLSV after the first vaccination (Appendix Table 2). The anti-FIS IgG1 titres were higher and more significant at week 5 across all vaccination groups when compared to pre-vaccination titres (Appendix Table 2, Figure 6.5). The IgG2 isotype response against FIS showed a significant response at week 3 against PrPA+FIS plus Pen-G, CrPA+FIS plus Pen-G, SLSV plus Pen-G and SLSV (Appendix Table 2). The anti-FIS IgG2 isotype revealed higher titres that are highly significant at week 5 among all vaccine groups (Appendix Table 2, Figure 6.5). All anti-FIS IgG2 titres at weeks 3 and 5 were compared to pre-vaccination IgG2 titres. The NegCtl showed insignificant IgM and IgG isotypes response against FIS throughout the study (Appendix Table 2, Figure 6.5)

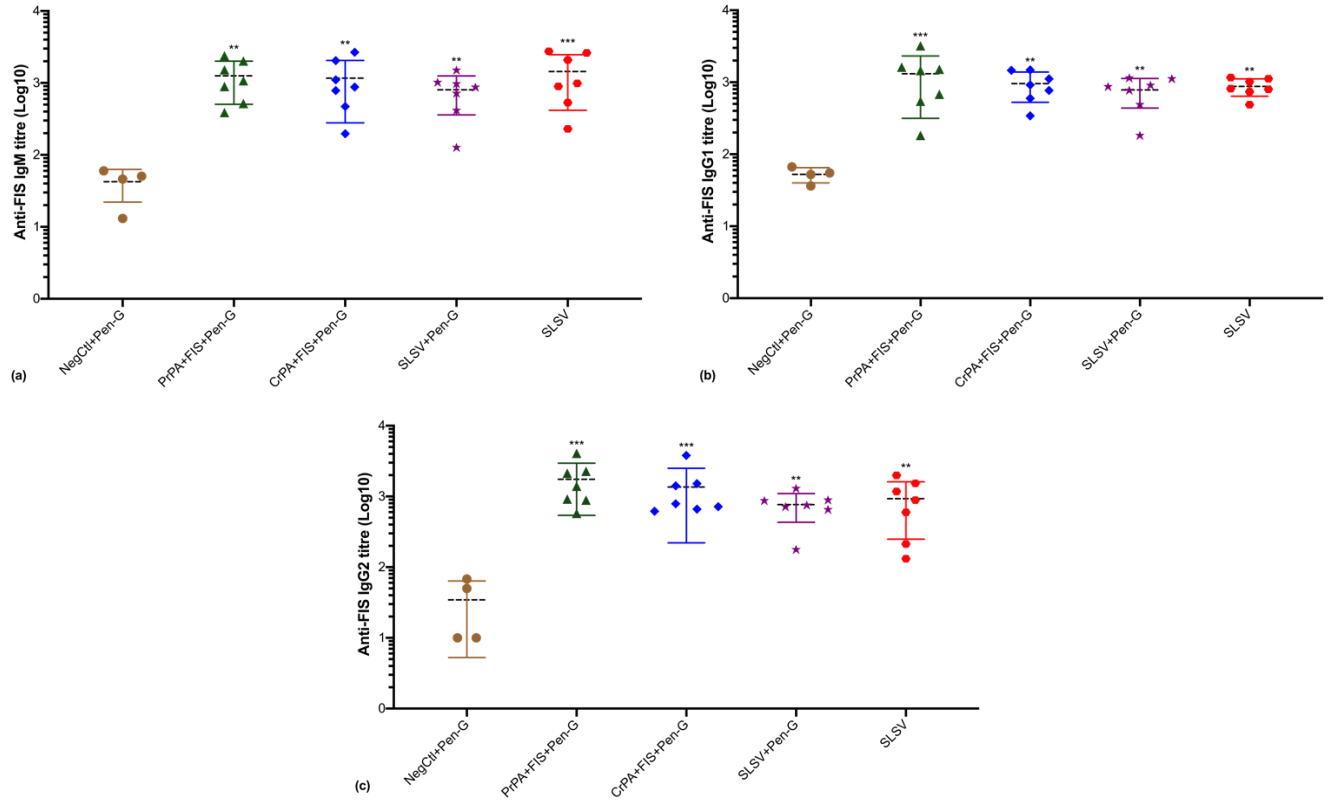

Figure 6. 5: Anti-formalin inactivated *Bacillus anthracis* 34F2 spores (FIS) IgM and IgG isotype (IgG1 and IgG2) titres at week 5 in cattle. The cattle were vaccinated twice (week 0 and 3) with PrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen G (n=7), CrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen G (n=7), Sterne live spore vaccine (SLSV) plus Pen-G (n=7), SLSV (n=7) and NegCtl (Emulsigen-D®/Alhydrogel® plus Pen G) (n=4). The IgG titres of each group were compared to the respective titres at week 0 before vaccination (***$p < 0.001$, **$p < 0.01$, *$p \leq 0.0.5$). (a) anti-FIS IgM titres, (b) anti-FIS IgG1 titres and (c) anti-FIS IgG2 titres. PrPA; Purified recombinant protective antigen, CrPA; Crude recombinant protective antigen, FIS: Formalin inactivated *B. anthracis* spores, Pen-G: Penicillin-G, SLSV; Sterne live spore vaccine, NegCtl; Negative control.

6.4.3 Toxin neutralisation antibodies titre (NT_{50})

The neutralising antibody titres (NT_{50}) were low after the first vaccination at week 3 without any significant increase for all vaccine groups, but the NT_{50} increased significantly for PrPA+FIS plus Pen-G, CrPA+FIS plus Pen-G and SLSV vaccinated groups after the second vaccination at week 5. However, there was no significant increase observed in NT_{50} titres among the vaccine groups immunised with SLSV and treated with Pen-G after the first and second vaccination (Appendix Table 1, Figure 6.6).

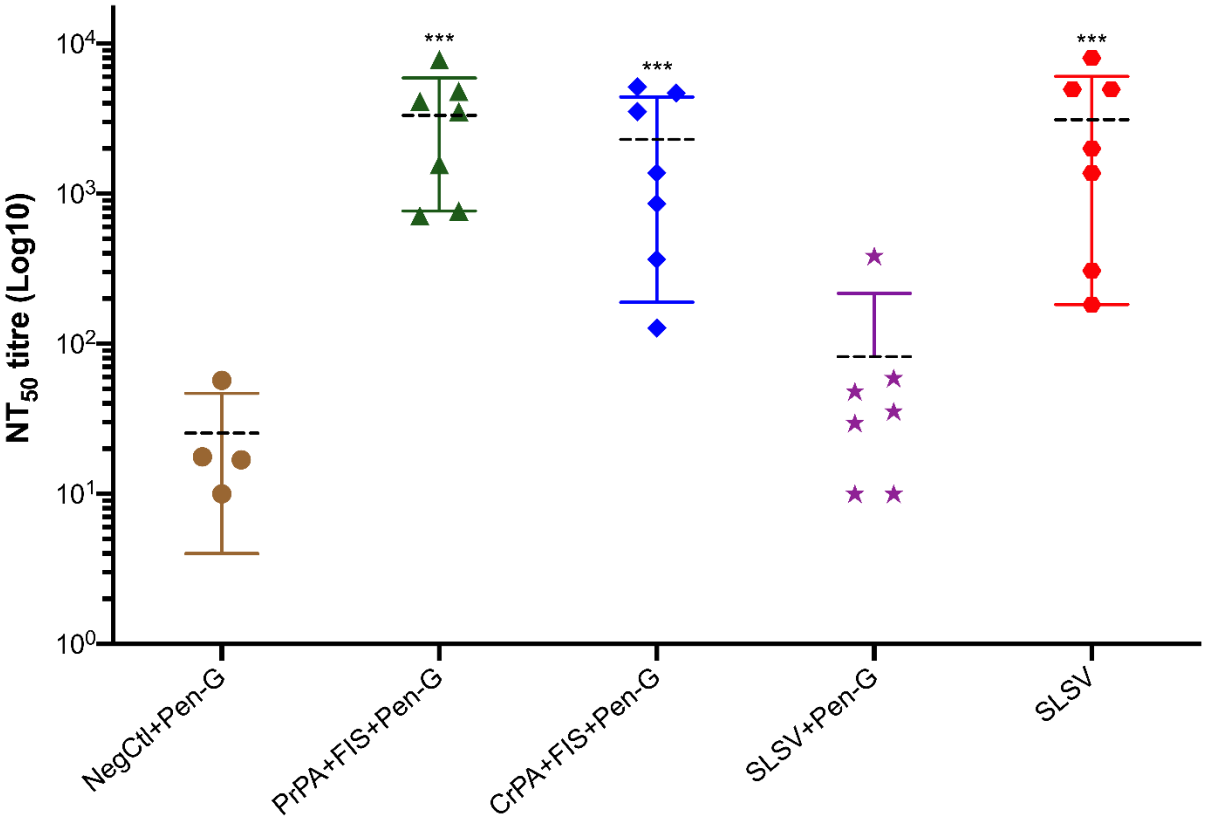

Figure 6. 6: Lethal toxin neutralising titres at week 5 in vaccinated cattle. The cattle were vaccinated twice (week 0 and 3) with PrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen-G (n =7), CrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen-G (n =7) with, SLSV plus Pen-G (n=7), and SLSV (n = 7) and negative control consisting of Emulsigen-D®/Alhydrogel® plus Pen-G (NegCtl) (n = 4). NT_{50} of each group were compared to the respective titres at week 0 before vaccination (***$p < 0.001$, **$p < 0.01$, *$p \leq 0.0.5$). PrPA; Purified recombinant protective antigen, CrPA; Crude recombinant protective antigen, FIS: Formalin inactivated *Bacillus anthracis* spores, Pen-G: Penicillin-G, SLSV; Sterne live spore vaccine, NegCtl; Negative control

6.4.4 Macrophages spore uptake induced by opsonising antibodies

The ability of the vaccine-induced antibodies collected at week 5 to opsonise and enhance phagocytosis of *B. anthracis* spores by macrophages (RAW264.7) were measured. The highest level of spore uptake by the macrophage at the sera dilution of 1:10 was 79% for SLSV, 73% for PrPA+FIS plus Pen-G and 61% for CrPA+FIS plus Pen-G whereas an insignificant level of

spore uptake by macrophages was recorded for SLSV plus Pen-G (37%) and NegCtl (20%) (Figure 6.7). A significant spore uptake by macrophages among cattle groups vaccinated with PrPA+FIS plus Pen-G and SLSV in dilutions of 1:10, 1:100, 1:1000 were observed, whereas CrPA+FIS plus Pen-G vaccine group showed significant spore uptake at the sera dilution of 1:10 and 1:100 only. The macrophage spore uptake across all vaccination groups was compared to the level of spore uptake by negative control sera.

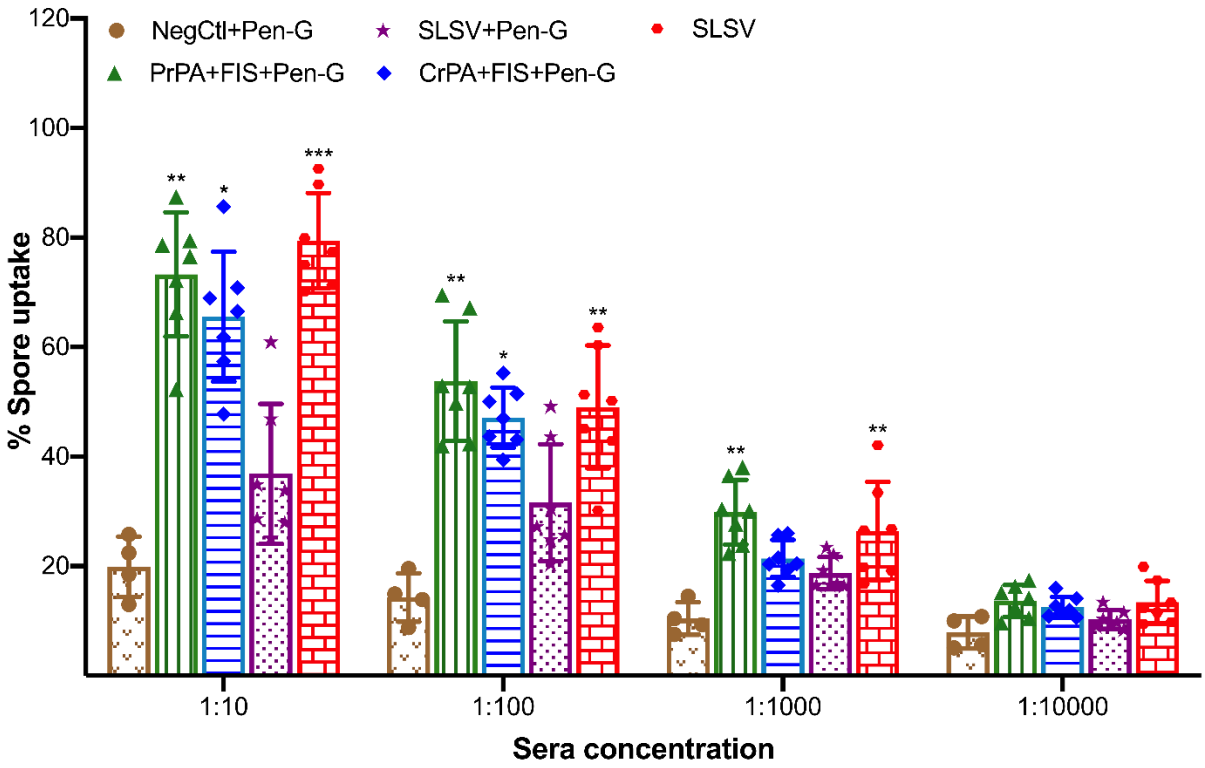

Figure 6. 7: *Bacillus anthracis* 34F2 spore phagocytosis by RAW 267.7 macrophages with varying dilutions of sera from cattle groups vaccinated with PrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen-G, CrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen-G, SLSV plus Pen-G, SLSV alone and NegCtl (Emulsigen-D®/Alhydrogel® plus Pen-G). The mean value of spore uptake is presented in the form of bar charts with the standard deviations. The significant values between groups are presented as ***p < 0.001, **p < 0.01, *p ≤ 0.0.5. PrPA: Purified recombinant protective antigen. CrPA: Crude recombinant protective antigen FIS: Formalin inactivated *Bacillus anthracis* spores, NegCtl: Negative control (Emulsigen-D®/Alhydrogel® plus Pen-G), Pen-G: Penicillin-G, SLSV; Sterne live spore vaccine.

6.4.5 Protection conferred on A/J mice by antibodies from cattle immune sera

The protection of A/J mice passively immunised with the purified polyclonal antibodies from SLSV plus Pen-G and CrPA+FIS plus Pen-G cattle groups were insignificant with 17% (6/35) and 23% (8/35) of the A/J mice surviving lethal challenge respectively (Figure 6.8). In contrast, the A/J mice immunised with the polyclonal IgG from cattle groups vaccinated with SLSV and

PrPA+FIS plus Pen-G showed a significant level of 77% (27/35) and 71% (25/35) protection of the immunised A/J mice respectively (Figure 6.8).

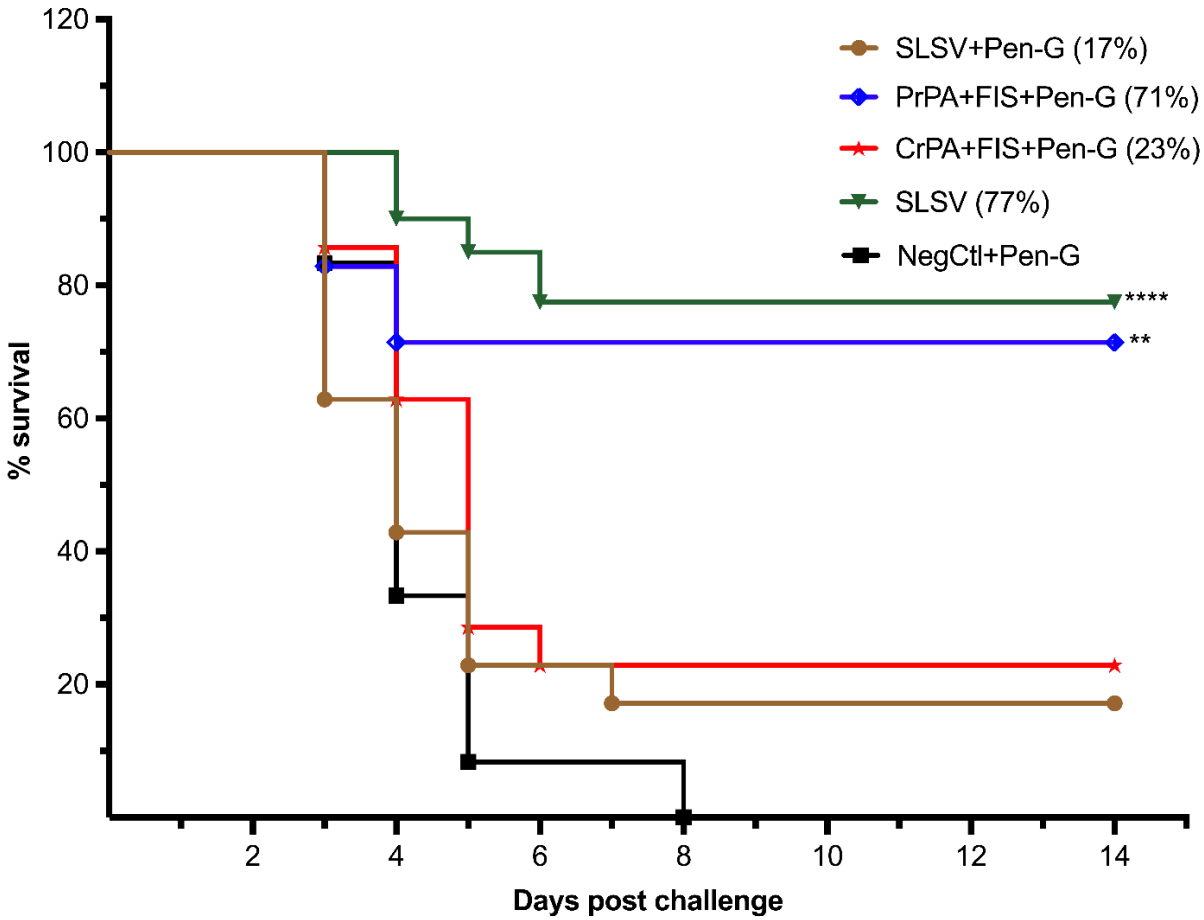

Figure 6. 8: Mice survival curve following passive *in vivo* transfer of purified IgG from sera from cattle to A/J mice. The A/J mice were lethally challenged with approximately 2.16 x 10^5 *B. anthracis* 34F2 spores. The polyclonal IgG are from sera of cattle vaccinated twice (week 0 and 3) with either PrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen-G, CrPA+FIS+Emulsigen-D®/Alhydrogel® plus Pen-G, SLSV plus Pen-G, SLSV alone groups and NegCtl groups (Emulsigen-D®/Alhydrogel® plus Pen-G). The survival rate in Log-rank (Mantel-Cox) test was compared to NegCtl group. The significant values between groups are presented as ****p < 0.0001, ***p < 0.001, **p < 0.01. PrPA; Purified recombinant protective antigen, CrPA; Crude recombinant protective antigen, Pen-G; Penicillin-G, SLSV; Sterne live spore vaccine, NegCtl; Negative control (Emulsigen-D®/Alhydrogel® plus Pen-G), FIS; Formalin inactivated *Bacillus anthracis* spores.

6.5 Discussion

SLSV is the sole vaccine available for control of anthrax in animals (Nicol and Sterne, 1942). Yet, it has shortcomings which include residual virulence in some ruminants species such as goat and llamas (Cartwright et al., 1987, Sterne, 1945). It is also incompatible with antibiotic prophylaxis. Webster (1973) reported that SLSV failed to stimulate protective antibodies in guinea pigs in the presence of antibiotics treatment. The latter implies that SLSV cannot be used concurrently with antibiotic treatment in an anthrax outbreak situation for the

protection of livestock and endangered wildlife (Stepanov et al., 1996, Webster, 1973), or in a feedlot where livestock, especially cattle, are treated and vaccinated against the common diseases as a prophylactic measure (Holman et al., 2019). In this study, both CrPA+FIS plus Pen-G and PrPA+FIS plus Pen-G vaccinated cattle groups and SLSV alone cattle group induced significant anti-rPA antibody response two weeks after the second vaccination (week 5), whereas cattle vaccinated twice with SLSV with simultaneous penicillin-G treatment were unable to induce adequate anti-rPA antibody titres (Appendix Table 1, Figure 6.2). Similar trends were observed for lethal toxin neutralising antibody titres (Appendix Table 1, Figure 6.6). The pattern of response for PrPA and CrPA was similar to anti-FIS antibody titres however significant antibody titres were detected in the group vaccinated with SLSV + Pen-G also (Appendix Table 1, Figure 6.3). Our findings indicate that the presence of penicillin-G may inhibit the PA-specific immune response without significant effects on the immunogenicity of *B. anthracis* spore. Immune data from this study showed a similar pattern to that observed in goats vaccinated thrice with either rPA + BclA + FIS + lipopeptide plus Pen-G, SLSV or SLSV + Pen-G by Ndumnego (2016). The authors reported the neutralising antibodies, and the IgG against PA was higher in two of the five goats vaccinated with rPa + BclA + FIS + lipopeptide plus Pen-G, but due to the low number of animals in each vaccination group, the results were not significant. In our study, the IgM and IgG isotype response has shown a sudden increase; dominated by IgM and IgG1 at week 3 and maintained until the fifth week when the level of IgG2 dominates week 5 across all the vaccine groups except SLSV plus Pen-G (Appendix Table 2). The stimulation of both IgG1 and IgG2 implies a balance between Th1 and Th2 response.

In this study, we used PA which is the major component of the *B. anthracis* tripartite proteins encoded by pXO1 and the principal immunogenic component. PA stimulates an early immune response against anthrax toxins; however, studies have shown that the immunity stimulated by PA is short-lived (Fellows et al., 2001, Welkos and Friedlander, 1988). To stimulate a protective and lasting immune response, several studies coupled PA with other non-living components of *B. anthracis* such as BclA, BxpB or even the whole FIS to intensify the immune response. The majority of these studies were conducted in laboratory rodents except the studies conducted by Ndumnego et al. (2018) and Koehler et al. (2017) which reported the immunogenicity of NLAV formulation comprising of rPa+BclA+FIS+lipopeptide in goats vaccinated thrice. In these studies, PA and FIS stimulated better immunity than BclA with

results indicating that two vaccinations might be sufficient to render protection. The two-step vaccination schedule was suggested based on antibody titres after second vaccination in rPA+BclA+FIS+lipopeptide in vaccinated goats, which did not differ significantly from the antibodies titres after the third vaccination and were similar to goats vaccinated twice with SLSV (Koehler et al., 2017, Ndumnego, 2016, Ndumnego et al., 2018). The two-step vaccination schedule was confirmed in cattle using the CrPA+FIS and PrPA+FIS vaccine formulations which stimulated a significant immune response with the latter significantly protecting A/J mice from the lethal challenge (Jauro et al., 2020). The immunogenic and protective ability of PrPA+FIS vaccine is thus comparable with SLSV in cattle (Jauro et al., 2020) and consequently resulted in the current study to test the simultaneous use of antibiotic treatment with NLAVs.

The level of antibodies against rPA and FIS as well as neutralising antibodies response in the two-step cattle vaccination schedule with NLAVs plus Pen-G revealed similar immunogenic trends as seen in two-step cattle SLSV vaccination. The levels of antibody titres for cattle groups vaccinated with SLSV plus Pen-G were inhibited and differed significantly from NLAVs plus Pen-G and SLSV (alone). The polyclonal IgG from PrPA+FIS plus Pen-G vaccine group demonstrated a promising level of protection by saving 71% of the A/J mice. This is comparable with the level of protection conferred to A/J mice (77%) by polyclonal IgG from the SLSV vaccine group. However, low level of protection of A/J mice (17%) was recorded by SLSV plus Pen-G polyclonal IgG against *B. anthracis* 34F2 spore challenge. Our findings revealed that the presence of antibiotic treatment in the vaccination schedule influenced the inhibition of the immunogenic ability of SLSV as previously reported by Webster (1973). The polyclonal IgG from CrPA+FIS plus Pen-G vaccinated group afforded 23% protection of A/J mice which is low considering the antibody responses in this group (Appendix Table 1, Figure 6.2 and Figure 6.3). This is similar to the polyclonal IgG from CrPA+FIS plus Pen-G vaccinated cattle that also provided only 20% protection to A/J mice in the study of Jauro et al. (2020). Based on our finding, this may be associated with the method used to obtain the crude rPA which filters out only the *E. coli* endotoxin and allows other proteins in the final products (Casadevall, 2005). In addition, it has been indicated by Welkos et al. (2002) that anti-PA antibodies play an important role in opsonising spores and, hence, increase the spore uptake by macrophages. Therefore, the anti-PA antibodies raised against the crude rPA may be less

effective in this respect; this would not be visible in ELISA or TNA, but would be apparent in the opsonophagocytic test and finally, in the passive mouse protection test.

Macrophages are primary effector cells of host innate immune response that fight bacterial infection (Kang et al., 2005). The spore opsonising ability of antibodies taken from the different vaccinated cattle in this study was evaluated. Our findings revealed that NLAVs with simultaneous penicillin-G treatment and SLSV without penicillin-G treatment induced the production of opsonising antibodies which were able to prevent 73% (PrPA+FIS plus Pen-G), 63% (CrPA+FIS plus Pen-G) and 79% (SLSV alone) of the spores from escaping phagocytosis by macrophages at the serum dilution of 1:10. Our findings are similar to the results of Jauro et al. (2020) which reported spore opsonising ability of antibodies from the NLAVs tested in cattle without Pen-G were able to prevent 75% (PrPA+FIS) and 66% (CrPA+FIS) of the spores from dodging phagocytosis by macrophage at the serum dilution of 1:10. Our data support the important role of opsonising antibodies in the immune response against infection with spores of *B. anthracis*. In addition to the test for antibodies capable of LT neutralisation, commonly accepted as a correlate for the protective immune response against infection with *B. anthracis*, testing the spore opsonising capacity of antibodies could substantially add to our methodological repertoire when testing vaccine candidates comprising cellular antigens as well. Further studies are necessary to identify which IgG isotype is primarily responsible for engaging and activating the FCγ receptors on the macrophages which results in, amongst other inflammatory responses, spore phagocytosis, following vaccination with PA and FIS.

6.6 Conclusions

Our study demonstrates the ability of a non-living vaccine candidate (NLAV) (PrPA + FIS + Emulsigen-D®/Alhydrogel®) to stimulate a protective immune response against both *B. anthracis* PA and spores under the conditions of concomitant antibiotic treatment in cattle. More importantly, after two vaccinations, the level of immune response witnessed with this vaccine was comparable to that of the SLSV . Our findings reveal the feasibility of adopting NLAV using a combination of purified rPA and FIS as an alternative animal anthrax vaccine which offers the benefit of concurrent use with antibiotic treatment in the phase of an infectious disease outbreak. Such an approach can significantly reduce economic losses, especially given the incubation period of the disease and the desire to use prophylactic

antimicrobial treatments, alongside vaccination. The novelty of our approach is in line with the recent FDA approval to simultaneously use AVA PA-based human vaccine with antimicrobial treatment in an anthrax outbreak or any disease that requires antimicrobial treatment (Food and Administration, 2016). The purified antibodies from animals vaccinated with NLAV have demonstrated significant passive protection ability in A/J mice which is comparable with the level of protection conferred by antibodies from SLSV sera. Future work will be initiated to investigate the cellular immune response and to determine the duration of the immunity.

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CHAPTER SEVEN

7.1 General Discussion

Anthrax is caused by *Bacillus anthracis* a zoonotic disease primarily of ruminants. The virulence factors of this pathogen consist of the binary toxins formed by three proteins, namely LF, EF and PA. These proteins are encoded on plasmid pXO1 (Koehler, 2002). PA is the major component of *B. anthracis* proteins and the immunogenic component used in the human vaccine as well as the catalyst of the binary toxins; ET (PA+EF) and LT (PA+LF) (Koehler, 2002). Anthrax in animals is controlled by vaccination with the attenuated *B. anthracis* Sterne live spore strain 34F2, namely SLSV. For over 70 years the SLSV has been the vaccine of choice in most countries around the world and reported to be effective in reducing anthrax in animals (Nicol and Sterne, 1942, Sterne, 1939, Turnbull and Shadomy, 2010, Turnbull, 2008). Despite the notable performance recorded by the SLSV in livestock over the years. SLSV is not without some limitations such as lingering virulence in some vaccinated animals, especially goat and llamas, variation in batches. Most of all are the unsuitability of SLSV for concurrent use with an antimicrobial agent (Turnbull, 2008, 2008, Turnbull, 1991, Webster, 1973) especially, in disease outbreak scenarios where antibiotic treatment is required concurrently with vaccination to reduce future losses within the affected herd or flock (Turnbull, 1991, Webster, 1973).

The search for an alternative veterinary anthrax vaccine that is compatible with concurrent use of antibiotics is paramount. As the use of a control strategy that is able to provide simultaneous protection and prevention of animals against future cases of anthrax is necessary. Recombinant protein production enables safer next-generation vaccines using the immunogenic PA component. Recombinant protein production circumvents the biohazards associated with direct handling of live spore vaccines and pathogenic microorganisms, batch to batch variation potential for lingering virulence and provides a platform for simultaneous use of antibiotics. The production and applications of recombinant proteins are not without challenges that need to be fixed, these include; consolidating an optimal host-vector, growth and expression conditions and ensuring that the expression is upscalable and with high yields of biologically active protein without inclusion bodies and loss of plasmids.

Even though PA is regarded as the major immunogenic component of *B. anthracis*, it does not stimulate sufficient immunity to render adequate protection and requires various booster

vaccinations (Plotkin and Grabenstein, 2008). In addition, rPA vaccine has been reported to be handicapped by the lack of stability upon long time storage (Baillie, 2009, Verma and Burns, 2018). Despite the limitations of PA as a sole vaccine candidate much research has been undertaken to enhance its abilities to provide immunity by cocktails of PA with other *B. anthracis* components such as the exosporium protein (BclA or BxpB) and inactivated *B. anthracis* spores (FIS) have been shown to increase the immune response and protection (Brahmbhatt et al., 2007, Brossier et al., 2002, Cote et al., 2012, Enkhtuya et al., 2006, Gauthier et al., 2009, Koehler et al., 2017, Majumder et al., 2018, Majumder et al., 2019, Ndumnego et al., 2018).

In this study, we successfully expressed soluble rPA83 in *E. coli* SE1 using pStaby1.2 vector which is an antibiotic-free system, which contributes to the cost-effective expression and allows the production of recombinant proteins without the risk of antibiotic resistance (Barroca et al., 2016, Pal et al., 2018). The solubility, and the overproduction (30.08 mg/L) of the rPA83 obtained in this study, was due to the effect of the low inducing temperature of 25 °C. Low inducing temperatures influence protein solubility and also aids in the retention of biological function by increasing the level of chaperones in the *E.coli* host, whilst reducing the expression process and subsequent formation of inclusion bodies (Khow and Suntrarachun, 2012, Sahdev et al., 2008, San-Miguel et al., 2013, Vera et al., 2007). The cost of the protein production (rPA) was significantly reduced by our novel approach of cost-effective lysing in modified lysis buffer and purification technique compared to the conventional lysis buffer and reagents (BugBuster, lysozyme, endonuclease and protease inhibitor). The lysing and purification method consisted of two cycles of sonication each followed by Ni-TED column purification which is more cost-effective (1.60 USD / mL) than the conventional technique (4.25 USD / mL). The second sonication step in our study improved the yield of rPA compared to one cycle sonication used by Shrestha et al. (2012) and Khow and Suntrarachun (2012). Furthermore, the seroreactivity and cytotoxicity assays performed on goat sera previously vaccinated with rPA (Ndumnego et al., 2018) and J774A.1 mouse cell respectively to confirmed biological activity of rPA as previously reported by Pavan et al. (2016) and Quintero-Ronderos et al. (2013). Therefore, our study has revealed a more cost-effective approach to acquire biologically active rPA83. The rPA83 can be used for anthrax diagnostic purposes, and our approach can be adopted for industrial production recombinant

proteins. Even though SLSV may be relatively cheap but SLSV's striking limitations of incompatibility with antibiotic, reversal to virulence and risk of handling live bacteria make the cost-effective approach in this study worthwhile.

Following the successful expression of the rPA83, we formulated non-living anthrax vaccines (NLAVs) using rPA83 and FIS adjuvanted with Emulsigen-D®/Alhydrogel®. The components of that made up our NLAV were selected based on previous studies (Koehler et al., 2017, Ndumnego et al., 2018). Koehler et al. (2017) and Ndumnego et al. (2018) where they formulated NLAVs with rPA, FIS and BclA and adjuvanted with lipopeptides and evaluated their immunogenicity. Their findings revealed that for goats rPA in combination with FIS stimulated the best immune response and vaccinating twice provided a sufficient immune response in comparison to immune recorded for BclA. Protection of the rPA+FIS+BclA+lipopeptide as well as rPA+BclA+lipopeptide vaccinated goats were tested in virulent *B. anthracis* challenge in goats (Koehler et al., 2017) and in passive A/J mouse model using serum from the vaccinated goats (Ndumnego et al., 2018). The A/J mice were passively immunized with the goat serum and challenged with live spore. The protection was obtained with the rPa+FIS+BclA+lipopeptide and rPA+BclA+lipopeptide using the virulent challenge in goats were 80% and 50% whereas passive protection test conferred 73%. But the group vaccinated with rPA+BclA+lipopeptide offered lower protection and lower antibody titres than the formulation containing FIS (Koehler et al., 2017, Ndumnego et al., 2018) Hence the cattle in this study were vaccinated twice based on the outcome and recommendation from previous studies by (Koehler et al., 2017) and (Ndumnego et al., 2018). The selection of the adjuvants was guided by the following features; Emulsigen-D® is an oil-in-water made of dimethyl-dioctadecyl ammonium bromide (DDA) that stimulate T-cell immunity and increases the surface area of the antigen while slow release of the antigen (Kaur et al., 2010, McGonigle et al., 2006, Shabana et al., 2018). Whereas Alhydrogel® adjuvant is a aluminium hydroxide wet gel suspension. Alhydrogel® improves the uptake of antigens by antigen-presenting cells (APCs), induces NLRP3 inflammasome complexes and interleukin-1 (IL-1) and interleukin-18 (IL-18) secretion at the same time increases Th2 antibodies response (Coffman et al., 2010, Gupta, 1998, Marrack et al., 2009).

In line with reported limitations of recorded with SLSV, there is a need for more safe livestock vaccine. Therefore, we assess the immunogenicity of the rPA coupled with FIS was evaluated in cattle in two different animal experiments. The first experiment consisted of cattle groups vaccinated twice with CrPA+FIS or PrPA+FIS adjuvanted with Emulsigen-D®/Alhydrogel®and their immunogenicity was compared to the SLSV group at different timepoints (week 0, 3, 5 and 12). The second experiment consisted of cattle groups vaccinated twice (week 0 and 3) with CrPA+FIS and PrPA+FIS adjuvanted with Emulsigen-D®/Alhydrogel® with Pen-G treatment. Their immunogenicity was compared to cattle groups vaccinated twice (week 0 and 3) with SLSV alone and with pen-G treatment at three timepoints (week 0, 3 and 5). The timepoints for the first and second experiments was selected to have wider picture of cattle immune response to our vaccine formulation. Based on our finding, we have initiated a study to evaluate the longevity of the immune response for the period of 12 months equivalent to SLSV current ongoing to analyse the trend of immune response each month for the period of the study.

The immune response analysis of the first vaccine experiment that was performed without antibiotics treatment and was carried out for 12 weeks. The anti-rPA and anti-FIS IgG ELISA titres in these study groups were significant at week 3 after the first vaccination for the NLAVs except for the SLSV vaccine group that was significant against rPA only. Both the anti-FIS and anti-rPA IgG titres of all the vaccine groups peaked two weeks after the booster at week 3. The titres remain significantly high at week 12 in the vaccine groups without antibiotic treatment. Similar trends of IgG titres against FIS and rPA were seen in goats vaccinated thrice with rPA+FIS+BclA+lipopetide and twice with SLSV (Koehler et al., 2017, Ndumnego et al., 2018). The same pattern of antibodies titres are also seen in NT_{50} in the vaccine groups without antibiotics treatment though it showed a significant increase in titres after the booster dose (second vaccination) at week 5 across all vaccine groups and retained the significant NT_{50} titres to week 12. our findings indicate the presence antibodies against PA and LT in the NLAV and SLSV vaccinated groups base on the toxins neutralisation results (Fowler et al., 1999, Reuveny et al., 2001, Weiss et al., 2006). The immune response analysis of the second vaccine experiment of cattle vaccinated with NLAVs and SLSV with simultaneous antibiotic treatment showed an impressive surge in their antibody titres (anti-rPA and NT_{50}) across all vaccination groups similar to the immune response to the cattle in

the first experiment except the group vaccinated with SLSV+Pen-G that as expected had low antibody titres. The simultaneous Pen-G antibiotic treatment as part of the vaccination schedule in the vaccine groups with simultaneous antibiotic treatment possibly inhibited the immunogenicity of SLSV in this study, as reported previously by Webster (1973). However, the anti-FIS titre showed significant surge across all the vaccination groups including the group vaccinated with SLSV+Pen-G in the second experiment groups with concurrent antibiotics treatment. This finding is similar to previous report by Ndumnego et al. (2016).

The immunoglobulin subclass titres in all vaccinated groups across experiment one and two exhibited a balance between Th1 and Th2-type responses. Even though the Th2-type immune response dominates the response at week 5, as seen with IgG2 in all the NLAVs and SLSV against FIS in all the vaccine groups across both studies. The same pattern was also seen with IgG1 and IgG2 response against rPA at the same timepoints, which signifies the stimulation of Th1 as well as Th2-type response. The IgG1 titres declined but were still significant at week 12, unlike IgG2, which maintained the trends of titres from week 5 to 12 among the vaccine groups without antibiotic treatment in the study. The balance noticed between IgG1 and IgG2 signifies the ability of the NLAVs to induce a cell-mediated immune response by regulating IFN-gamma (Th1) and IL-4 (Th2) response. The macrophages are activated by the IFN-gamma whereas, the IL4 secreted by Th2 stimulate B-cells to produce antibodies (Estes and Brown, 2002). However, the dynamics of the immune response switch between the immunoglobulin subclasses, cannot be fully elucidated due to the limited timepoints taken in experiment 1 and experiment 2, respectively. The antibodies epitopes need to characterise to determine the antibodies dynamics

The passive mouse model used by Ndumnego et al. (2018) used the whole serum from the vaccinated goats. In our study, the polyclonal IgG were purified from the sera and injected into the A/J mice. Ndumnego et al. (2018) indicated that some of the mice showed an adverse reaction after injection of serum. Thus, this was circumvented by using the purified polyclonal IgG from sera as described by other studies (Brahmbhatt et al., 2007, Caldwell et al., 2017, Williamson et al., 2005).

The passive protection in the A/J mouse model of cattle group vaccinated with CrPA+FIS+Emulsigen-D®/Alhydrogel® with and without Pen-G treatment provided low

protection 20% to mice by the polyclonal IgG. However, an adequate level of protection of 73% and 75% of the challenged A/J mice were observed after immunisation with polyclonal IgG from cattle groups vaccinated with PrPA+FIS+Emulsigen-D®/Alhydrogel® and SLSV in the first study and polyclonal IgG from PrPA+FIS+Emulsigen-D®/Alhydrogel®+Pen-G, and SLSV vaccine groups also offers 71% and 78% protection to the challenged A/J mice respectively. All the A/J mice immunised with the polyclonal antibodies from the negative control group died within 3-8 days post-challenge. The immunoglobulin subclass response in addition to the IgG titre recorded in this study suggests that the presence of FIS in the vaccine formulation has also been a contributing factor to the survival rate recorded among PrPA+FIS+Emulsigen-D®/Alhydrogel® as well as PrPA+FIS+Emulsigen-D®/Alhydrogel®+Pen-G vaccine groups and is consistent with other studies. This is thought to be because the presence of FIS in rPA vaccine formulation augments the immune response which leads to better protection (Brossier et al., 2002, Koehler et al., 2017, Ndumnego, 2016, Ndumnego et al., 2018).

The opsonophagocytosis findings revealed the ability of the IgG and IgM to aids the evasive ability of *B. anthracis* spore from being phagocytosed by macrophages (Stepanov et al., 1996, Welkos et al., 2001). Consequently, this prevents the *B. anthracis* spore germinating and propagating to cause septicaemia and toxaemia (Enkhtuya et al., 2006, Brossier et al., 2002, Cohen et al., 2000). Our findings in the cattle groups without antibiotic treatment revealed that the polyclonal antibodies from cattle vaccinated with PrPA+FIS, CrPA+FIS and SLSV were able to opsonise and enable the uptake of 75%, 66% and 80% spores by macrophages. Whereas the polyclonal antibodies from the cattle groups vaccinated with PrPA+FIS plus Pen-G, CrPA+FIS plus Pen-G and SLSV were able to prevent 73%, 63% and 79% of the *B. anthracis* spores from evading phagocytosis by macrophages at the sera dilution of 1:10. Our results are consistent with studies conducted in mice by Enkhtuya et al. (2006), Majumder et al. (2018) and Majumder et al. (2019) that reported opsonophagocytosis is prompted by the ability of the FC gamma receptor (FCγR) of macrophages to detect the FC portion of the opsonising antibodies on the opsonised spore (Abboud et al., 2010, Fitzer-Attas et al., 2000). Based on our findings there is an indication of the effect of FCγR in both CrPA+FIS and PrPA+FIS which contributed significantly in level of spore opsonisation recorded in both . The opsonisation of spores may substantially contribute to the rate of killing of phagocytosed spores by macrophages (Enkhtuya et al., 2006).

Based on the outcome from this study, our findings has confirmed that cost-effectively produced rPA+FIS+adjuvants are compatible with simultaneous antibiotic treatment when compared to SLSV+Pen-G which showed a poor immune response and protective ability.

7.2 Conclusion and recommendation

Various cost-effective expression systems and purification methods for recombinant proteins are being explored globally due to the critical role of recombinant proteins in biomedical applications such as vaccine production, diagnostics and other fields. In this study, we successfully expressed the full-length PA83 using the antibiotic-free pStaby1.2-SE1 system. The effect of low inducing temperature improved the solubility of the expressed recombinant protein. Furthermore, the modified lysis buffer and two sonication cycle that replaced the more expensive lysis reagents have reduced the cost of protein production. However, the cost of the rPA might be competitive with the current SLSV, but this is without FIS and adjuvant of the NLAV. Therefore, when the cost of FIS production and adjuvants are added the cost may be relatively higher than what is reported in the fourth chapter of this study. The expression and purification of rPA in an antibiotic-free system can be optimised for the biomedical field for purposes such as vaccine production, diagnostic applications and pharmacotherapeutic purposes.

Furthermore, our study indicates the ability of the PrPA+FIS+Emulsigen-D®/Alhydrogel® to stimulate a protective immune response against *B. anthracis* rPA83 and inactivated spore either with or without concurrent antibiotic treatment in cattle. More importantly, the level of the immune response and protection conferred by the purified polyclonal IgG from cattle vaccinated with NLAV was equivalent to the SLSV hyperimmunised group after two vaccinations. Therefore, our results indicate the possibility of NLAV formulated with purified rPA and FIS to be considered as a future veterinary anthrax vaccine due to its additional benefit of concurrent use with antibiotic treatment in a disease outbreak situation and in endangered wildlife species as well as in feedlot practice. Such an approach will significantly reduce economic loss should there be an incubating disease that requires antimicrobial treatment and vaccination against anthrax. The FDA recently approved the simultaneous use of human PA-based anthrax vaccine (AVA) with antimicrobial treatment in an anthrax outbreak or disease situation that requires antimicrobial treatment (Food and Administration,

2016). Finally, further study is ongoing in our laboratory to elucidate the duration of the antibodies stimulated by NLAVs in cattle compared to SLSV for 12 months with more timepoints.

Based on our findings, we recommend further studies to, characterise the antibody epitopes and the PA paratopes they target for their binding. This will help determine the reason for the lack of protection despite a significant immunogenic response base on ELISA and TNA seen among CrPA+FIS vaccine group. The storage stability of rPA in relation to the retention of biological activity of the vaccine needs to be ascertained for it to progress to large scale use/commercialisation. Following the guidelines by The International Cooperation on Harmonisation of Technical Requirements of Registration of Veterinary Medical Products (http://www.vichsec.org/guidelines/biologicals/bio-safety/target-animal-batch-safety.html) which specifies that the main standard requirements for the quality, safety and efficacy needed for veterinary vaccines authorisation. This would enable the determination of dose and the requirement to administer booster dose even though our study and Ndumnego et al. (2016) indicates the need of booster within from 1-3 months after the first vaccination to enables robust immune response. It is hoped that future studies will provide a definitive answer to whether the PrPA/CrPA+FIS formulation with simultaneous Pen-G administration produced in this study can compete favourably with the positive attributes of the SLSV. In addition to the added benefits of PrPA/CrPA+FIS being efficacious with the use of antibiotics.

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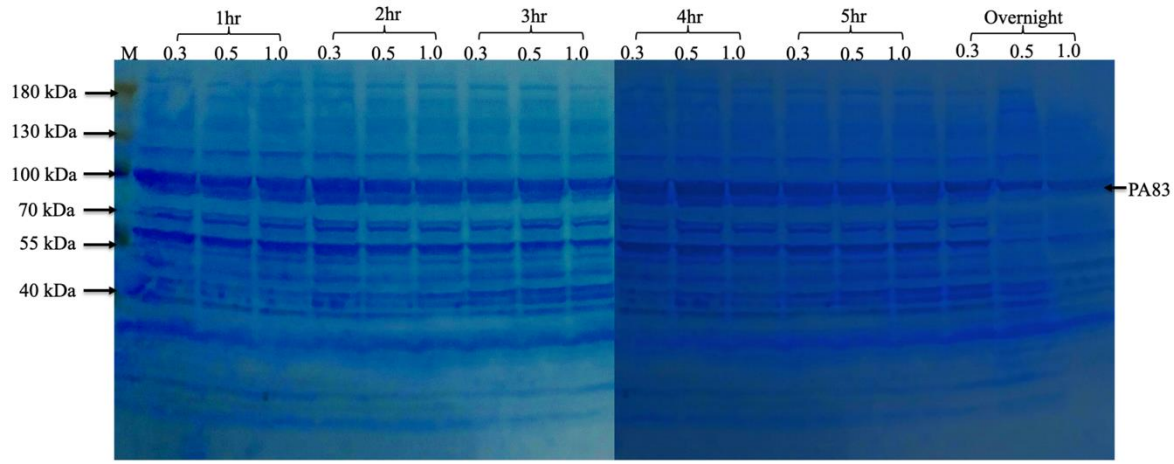
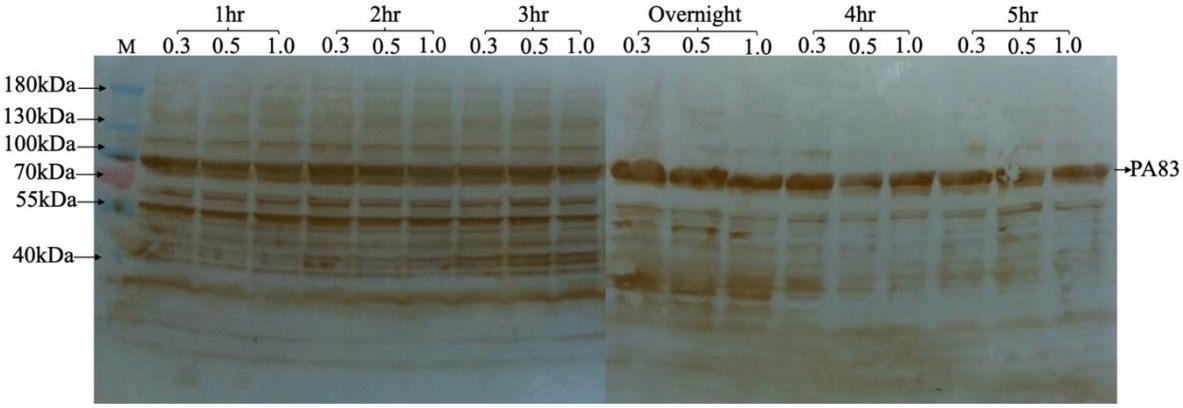
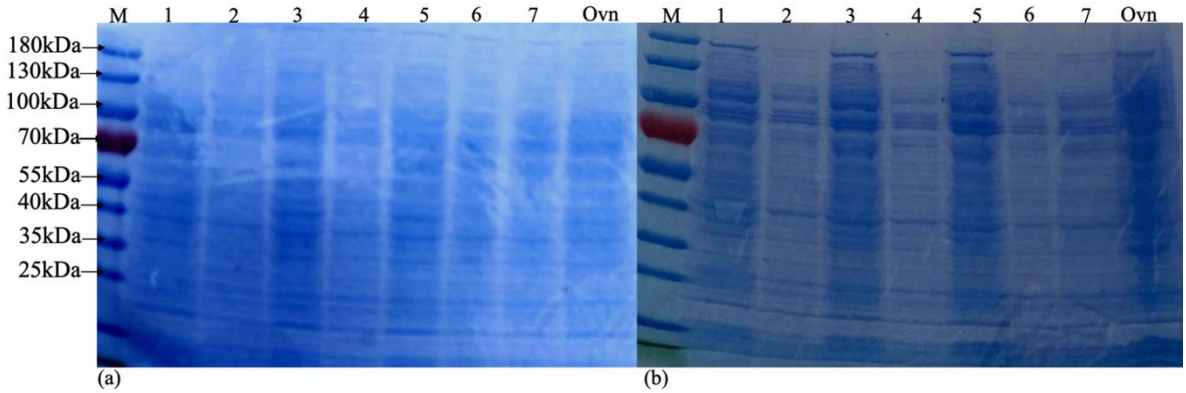
Appendices



Appendix Figure 1: SDS-PAGE of full-length PA83 expressed from PA83-pStaby1.2 in *Escherichia coli* SE1 at 25°C using different concentrations of IPTG and different time intervals. Lane M: protein molecular weight marker. 0.3, 0.5 and 1 represent the IPTG concentration in mM



Appendix Figure 2: Western blot of full-length PA83 expressed from PA83-pStaby1.2 in *Escherichia coli* SE1 at 25 ℃ using different concentrations of IPTG and different time interval. Primary antibody used; polyclonal antibody from serum collected from an animal vaccinated with rPA.. Lane M: protein molecular weight marker. 0.3, 0.5 and 1 represent the IPTG concentration in mM



Appendix figure 3: (a) SDS-PAGE of full-length PA83 expressed from PA83-pStaby1.2 in *Escherichia coli* SE1 at 37°C in 500 mL using 0.5 mM IPTG concentration. (**b)** SDS-PAGE of full-length PA83 expressed from PA83-pStaby1.2 in *E. coli* SE1 at 37°C in 500 mL using 1.0 mM IPTG concentration. Lane M: protein molecular, Lane 1- 7: 1 to 7 hour harvest for full length PA83 cloned to pStaby1.2 expression in *E. coli* SE1, Ovn: Overnight harvest for full length PA83 cloned to pStaby1.2 expression in *E. coli* SE1.

Appendix material 1: Purified and crude rPA calculation for vaccine formulation

The vaccine formulation for the purified and crude rPA was calculated using:

$$C1V1 = C2V2.$$

C1 = Primary concentration
V1 = Primary volume
C2 = Final concentration
V2 = Final concentration

The concentration of the CrPA used for the vaccine was determined using a 1 ml volume of the final lysed cell CrPA supernatant. The formulas used to determine the rPA concentration in the crude whole supernatant are:

$$tx\ concn = wl\ concn - PrPA\ concn$$
$$ubrPA\ concn = tx\ concn - ft\ concn$$
$$CrPA\ concn = ubrPA + PrPA\ concn$$

$tx\ concn$ = Total protein concentration without the purified rPA concentration

$wl\ concn$ = Whole protein concentration in supernatant after centrifugation

$PrPA\ concn$ = Purified rPA concentration after purification using Ni-TED column (Machery-Nagel, England)

$ubrPA\ concn$ = Concentration of unbound rPA present in the flow through after Ni-TED column purification (Machery-Nagel, England).
$ft\ concn$ = Concentration of all proteins present in the flow through after Ni-TED column purification.
$CrPA\ concn$ = Concentration of rPA in the whole supernatant after centrifugation.
The CrPA vaccine was formulated as described for the PrPA vaccine formulation.

Appendix Table 1. The Anti-recombinant protective antigen (rPA) and anti-formalin inactivated spores (FIS) IgG titres (log_{10}) in cattle vaccinated at week 0 and 3 and measured at week 0, 3 and 5 (with means and standard deviations). Cattle were vaccinated twice (week 0 and 3) with purified rPA (PrPA)+FIS+Pen-G (penicillin-G), crude rPA (CrPA)+FIS+Pen-G, SLSV+FIS+Pen-G, SLSV and NegCtl+Pen G (vaccinated with Emulsigen-D®/Alhydrogel® plus Pen-G).

Serology	Vaccine type	Week 0	Week 3	Week 5
Anti-rPA IgG ELISA titre (log_{10})	NegCtl+Pen-G	1,25 ± 0,32	1,36 ± 0,34	1,54 ± 0,30
	PrPA+FIS+Pen-G	1,17 ± 0,29	3,30 ± 0,34*	4,45 ± 0,33***
	CrPA+FIS+Pen-G	1,25 ± 0,40	2,93 ± 0,40*	4,30 ± 0,75***
	SLSV+Pen-G	1,17 ± 0,22	1,64 ± 0,41	2,18 ± 0,41
	SLSV	1,22 ± 0,21	2,88 ± 0,38	4,29± 0,40***
Anti-FIS IgG ELISA titre (log_{10})	NegCtl+Pen-G	1,14 ± 0,05	1,32 ± 0,34	1,53 ± 0,40
	PrPA+FIS+Pen-G	1,21 ± 0,25	3,36 ± 0,09*	4,58 ± 0,36****
	CrPA+FIS+Pen-G	1,21 ± 0,29	3,68 ± 0,38*	4,48 ± 0,45***
	SLSV+Pen-G	1,14 ± 0,18	3,11 ± 0,38*	4,46 ± 0,40***
	SLSV	1,27 ± 0,20	3,37 ± 0,35*	4,53 ± 0,29***
TNA[c] titre (NT_{50}) (log_{10})	NegCtl+Pen-G	ND	1,38 ± 0,31	1,40 ± 0,36
	PrPA+FIS+Pen-G	ND	2,43 ± 0,40	3,46 ± 0,38***
	CrPA+FIS+Pen-G	ND	2,15 ± 0,19	3,30 ± 0,49***
	SLSV+Pen-G	ND	1,48 ± 0,22	1,56 ± 0,36
	SLSV	ND	2,26 ± 0,27	3,40 ± 0,43***

[a]Mean log10 titres ± 95% confidence interval, [b]Titres were compared to the respective pre-vaccination titres (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p \leq 0.0.5$), [c]TNA; Lethal toxin neutralisation titres, rPA; Recombinant protective antigen, FIS; Formaldehyde inactivated spore, ND; Not detected, ns; Not significant, PrPA; Purified recombinant protective antigen, CrPA; Crude recombinant protective antigen, Pen-G: Penicillin-G, SLSV; Sterne live spore vaccine, NegCtl; Negative control.

Appendix Table 2. The anti-recombinant protective antigen (rPA) and anti-formalin inactivate *Bacillus anthrcis* 34F2 spore (FIS) titres (log_{10}) of different isotypes in vaccinated cattle (with means and standard deviations) of Immunoglobulin isotypes titre of vaccinated cattle measured at weeks 0, 3 (two weeks after first vaccination) and 5 (two weeks after second vaccination).

[a]Mean log10 titres ± 95% confidence interval, [b]Titres were compared to the respective pre-vaccination titres (***$p < 0.001$, **$p < 0.01$, *$p \leq 0.0.5$). rPA83;

Serology	Vaccine types	IgM			IgG1			IgG2		
		Week 0	**Week 3**	**Week 5**	**Week 0**	**Week 3**	**Week 5**	**Week 0**	**Week 3**	**Week 5**
Anti-rPA Igs ELISA titre (log_{10})	NegCtl+Pen-G	1,17±0,23	1,51±0,14	1,34±0,36	1,50±0,35	1,52±0,35	1,50±0,33	1,65±0,49	1,51±0,35	1,50±0,32
	PrPA+FIS+Pen-G	1,62±0,24	3,05±0,36**	3,19±0,35**	1,55±0,33	2,98±0,45*	3,26±0,31***	1,50±0,29	2,79±0,12**	3,39±0,48***
	CrPA+FIS+Pen-G	1,57±0,32	2,94±0,38**	3,06±0,45**	1,52±0,32	2,89±0,51**	3,07±0,22**	1,58±0,23	2.65±0,18*	3,40±0,38***
	SLSV+Pen-G	1,56±0,32	1,70±0,28	1,76±0,21	1,51±0,28	1,61±0,22	1,83±0,30	1,58±0,22	1,69±0,77	1,93±0,41
	SLSV	1,64±0,23	2,84±0,39**	3,02±0,45**	1,51±0,28	2,81±0,27**	2,93±0,23**	1,54±0,24	2,66±0,21*	3,56±0,32***
Anti-FIS Igs ELISA titre (log_{10})	NegCtl+Pen-G	1,62±0,37	1,65±0,31	1,62±0,43	1,73±0,09	1,60±0,30	1,72±0,17	1,68±0,136	1,57±0,36	1,53±0,37
	PrPA+FIS+Pen-G	1,64±0,15	2,95±0,23**	3,09±0,27**	1,62±0,26	2,98±0,38**	3,11±0,37***	1,61±0,23	2,71±0,31*	3,24±0,32***
	CrPA+FIS+Pen-G	1,52±0,30	2,89±0,23**	3,06±0,37**	1,57±0.21	2,86±0,24*	2,98±0,19**	1,60±0,23	2,75±0,30*	3,13±0,44***
	SLSV+Pen-G	1,58±0,22	2,74±0,23*	2,90±0,24**	1,62±0,21	1,85±0,37**	2,29±0,46**	1,57±0,21	1,97±0,30*	2,00±0,41**
	SLSV	1,51±0,27	2,91±0,20**	3,08±0,27***	1,63±0,23	2,74±0,29*	2,94±0,11**	1,66±0,15	2,60±0,24*	2,96±0,35**

Recombinant protective antigen 83, PrPA; Purified rPA83, CrPA; Crude rPA83, Pen-G: Penicillin-G, SLSV; Sterne live spore vacci8ne, NegCtl; Negative control (Emulsigen-D®/Alhydrogel® and Pen-G)

University of Pretoria Animal Ethics Committee approval both cattle trial and passive mouse protection test

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Evaluation of veterinary non-living anthrax vaccine candidates in target animal species**
PROJECT NUMBER	**V118-17 (Same as v134-16)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. S Jauro**

STUDENT NUMBER (where applicable)	**U_17303410**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Cattle**	**Mice**
NUMBER OF ANIMALS	**40**	**130**
Approval period to use animals for research/testing purposes		**November 2017- November 2018**
SUPERVISOR	**Dr. H van Heerden**	

Condition: Please provide a letter for the use of OTAU animals

KINDLY NOTE:

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

APPROVED	Date 27 November 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

Amended University of Pretoria Animal Ethics Committee approval both cattle trial and passive mouse protection test

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

8 July 2019

Approval Certificate
Amendment 1

AEC Reference No.:	V118-17 (AMENDMENT 1)
Title:	Evaluation of veterinary non-living anthrax vaccine candidates in target animal species
Researcher:	Dr S Jauro
Student's Supervisor:	Prof H van Heerden

Dear Dr S Jauro,

The **Amendment** as supported by documents received between 2019-05-17 and 2019-06-24 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-06-24.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	Number
Cattle and mice	80 Cattle and 265 mice

2. Please remember to use your protocol number (V118-17 (AMENDMENT 1)) on any documents or correspondence with the AEC regarding your research.

3. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Directorate of Animal Health of South Africa approval for cattle trial

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6

Dr. Solomon Jauro
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Tel: 012 529 8265
Fax: 012 529 8312
E-mail: Henriette.vanheerden@up.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Dear Dr. Jauro,

Your application sent with the email on 20 April 2017 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. All potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study. Records must be kept for five years for auditing purposes. A dispensation application may be considered by the Director Animal Health in the event that any of the above is to be stored or distributed;

4. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82);
5. Permission in terms of the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947 (Act No 36 of 1947) and the Medicines and Related Substances Control Act, 1965 (Act No 101 of 1965) must be obtained prior to the start of the study;
6. A veterinary import permit must be obtained prior to importation of samples (*Bacillus anthracis* protective antigen and lethal factor from List Biological Laboratories in the United States of America, via a distributer in the United Kingdom, and purified recombinant protective antigen protein from the University of Hohenheim in Germany);
7. Only animals currently on the property of the University of Pretoria experimental farm may be used for the study. No outside animals may be sourced without written permission from the Director: Animal Health;
8. Animals vaccinated with the candidate vaccine may not leave the property of the University of Pretoria experimental farm or enter the human or animal food chain in any way within 60 days after vaccination. Any mortalities within 60 days should be incinerated;
9. Samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National Road Traffic Act, 1996 (Act No. 93 of 1996);
10. This permit is valid up to and including 31 December 2019 and renewal of this permit is required for research to continue beyond this date. Please ensure that the renewal application is submitted timeously to DAFF

Title of research/study: Evaluation of veterinary non-living anthrax vaccine candidates in bovines
Researcher: Dr. S Jauro
Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria
Our ref Number: 12/11/1/1/6
Your ref:
Expiry date: 31 December 2019

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2017 -06- 0 6

Directorate of Animal Health of South Africa approval for passive mouse protection test

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6 (909)

Dr Solomon Jauro
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Tel: 012 526 8265
E-mail: henriette.vanheerden@up.ac.za ; anette.durand@up.ac.za

Dear Dr Jauro,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Your application sent with the email on 28 September 2018 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The study is approved as per the application form received on 28 September 2018 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82);
4. Clinical Trial approval from the Medicines Control Council/ Veterinary Clinical Committee may be required prior to the start of the study;
5. A veterinary import permit must be obtained prior to importation of the specified A/J mice from the USA and all conditions stated therein must duly be met;
6. Laboratory mice kept in IVC cages at the University of Pretoria Biomedical Research Centre may be injected with purified serum from the previous section 20 approved

study (12/11/1/1/6, signed 6 June 2017) and may be challenged with Stern strain (34F2) *Bacillus anthracis* organisms. Blood, liver and spleen samples may be collected from the mice as per specified SOP's provided;

7. All animal work must be conducted in the specified laminar flow cabinet and PPE should be utilised according to the relevant SOP;
8. A drug accountability system must be in place for the Stern strain (34F2) *Bacillus anthracis* utilised for this study. Any leftover material has to be destroyed using the specified waste contractor and relevant waste disposal SOP;
9. All samples and potentially infected material must be packaged and transported in accordance with the National Road Traffic Act, 1996 (Act No. 93 of 1996). Triple packaging and disinfection of the outermost layer is required;
10. All related microscopy and culture activities of collected samples may only be conducted within the DVTD BSL2+ facility, as specified;
11. Subsequent to the completion of the study, all study animals must be humanely euthanized and carcass material incinerated according to the SOP provided using the incinerator at the Faculty of Veterinary Science of the University of Pretoria;
12. All potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study using the specified waste contractor and relevant waste disposal SOP's provided. Records must be kept for five years for auditing purposes;
13. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: "*Evaluation of veterinary non-living anthrax vaccine candidates in bovine" (mouse study)*
Researcher: Dr Solomon Jauro
Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science University of Pretoria, in collaboration with the University of Pretoria Biomedical Research Centre
Our ref Number: 12/11/1/1/6 (909)
Your ref: V118-17
Expiry date: February 2020

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2019 -04- 15