Diagnosis of anthrax and the roles of host and environment in the transmission of anthrax

in Kruger National Park

By

Sunday Ochonu Ochai Jr.

Submitted in fulfillment of the requirements for the degree of Doctor of Philosophy Veterinary Science Tropical Diseases in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria

Supervisor:

Prof. Henriette van Heerden

Co-supervisor:

Dr. Wendy Turner

Prof. Emma Archer

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Declaration

I, Sunday Ochonu Ochai, do solemnly declare that this work which I submitted for the degree of Doctor of Philosophy in Veterinary Tropical Diseases is my original work and has not been submitted by me anywhere else for the purpose of a degree.

_____ 01/10/2023

Sunday Ochonu Ochai **Date**

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List of Abbreviations

µL	Mcrolitre
µM	Micromolar
Ab	Antibody
Ag	Antigen
AI	Avidity index
ATP	Adenotriphosphate
ATR	Anthrax toxin receptor
cAMP	Cyclic adenosine monophosphate
BAPA	*Bacillus anthracis* protective antigen
CO2	Carbon dioxide
CVL	Central Veterinary Laboratory
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DNA	Doexyribonucleic acid
DVTD	Department of Veterinary Tropical Diseases
ECACC	European Collection of Authenticated Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
EEI	Etosha Ecological Institute
EF	Oedema factor

ELISA	Enzyme-linked immunosorbent assay
ENP	Etosha National Park
ET	Oedema toxin
FCS	Fetal calf serum
HCl	Hydrogen chloride
IFAT	Immunoflourescence antibody test
IgG	Immunoglobulin G
IMHA	Indirect microhemagglutination test
kDa	Kilodalton
Km	Kilometres
KNP	Kruger National Park
LD50	Lethal dose 50%
LF	Lethal factor
LT	Lethal toxin
LT	Lethal toxin
MAPKK	Mitogen-activated protein kinase kinases
mm	Milimetre
MTT	3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaHCO	Sodium bicarbonate

NT50	50 % neutralization
NDVI	Normalised Difference Vegetation Index
OBP	Onderstepoort Biological Company
^{o}C	Degree celsius
OD	Optical density
PAG	Protein A/G
PBS	Phosphate buffered saline
PBST	PBS with Tween
PBSTM	PBST with skimmed milk powder
PCR	Polymerase chain reaction
PGDA	Poly-gamma-D-glutamic acid
ProtA	Protein A
ProtG	Protein G
p-value	Probability values
pXO1	Plasmid XO 1
pXO2	Plasmid XO 2
qPCR	Quantitative PCR
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism

Spp	Species
SSVS	Skukuza State Veterinary Services
SPI	Standardised Precipitation Index

Dedication

I dedicate this work to God Almighty and to my Late parents; Mr and Mrs Samuel Ochonu Ochai. I will eternally be grateful and always make you proud.

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Summary

Diagnoses and the roles of host and environment in the transmission of anthrax in

Kruger National Park

By

Sunday Ochonu Ochai

Supervisor: Prof. Henriette van Heerden

Department of Veterinary Tropical Diseases, University of Pretoria

Co-supervisor: Dr. Wendy Turner, Prof. Emma Archer

U.S. Geological Survey, Wisconsin Cooperative Wildlife Research Unit, Department of Forest and Wildlife Ecology, University of Wisconsin-Madison.

Emma Archer

Centre for Environmental Studies, Department of Geography, Geoinformatics and Meteorology, University of Pretoria, Hatfield, Pretoria 0083, South Africa

Degree: Doctor of Philosophy Veterinary Science Tropical Diseases

Over the years, various techniques have been employed to diagnose infectious zoonoses, such as anthrax caused by *Bacillus anthracis*. These methods encompass microscopic examination of blood smears, bacterial culture, molecular diagnostics targeting genetic markers of the pathogen, and serological tests to

identify antibodies against pathogen antigens. The accuracy of these techniques largely depends on the specificity and sensitivity of the tests used. Disease monitoring in free-roaming wild animals is challenging, often relying on passive surveillance. However, proactive surveillance, which involves detecting specific antibodies, can provide more reliable and timely insights into disease presence and prevalence within populations, especially when disease signs are below passive surveillance detection thresholds. Nevertheless, primary binding assays, such as the indirect enzyme-linked immunosorbent assay (ELISA) used for detecting antibodies in wildlife, face challenges due to the absence of species-specific conjugates. Also, the diagnosis of anthrax, remains a matter of concern due to the challenges posed by the identification of closely related species that carry regions on plasmids (pXO1 and pXO2) and chromosome that highly resembling those found in *B. anthracis*. As mentioned, traditional methods for diagnosing anthrax include microscopy, identifying isolates through culture, and using genetic markers such as *B. anthracis* protective antigen (*pag*A also known as BAPA on pXO1), lethal factor (*lef* on pXO1), chromosomal (Ba-1), and capsule (*cap*B on pXO2) genes for molecular detection. Because anthrax is not contagious, the exposure of herbivorous mammalian hosts to *B. anthracis* is greatly influenced by environmental and climatic factors, as well as host demographics and behaviour. In Kruger National Park (KNP), the most impacted host species used to be kudu (*Tragelaphus strepsiceros*) until the early 1990s, and outbreaks were more common during the dry season. However, there has been a shift in this pattern, and impala (*Aepyceros melampus*) is now the most affected species, with outbreaks occurring more frequently during the wet season. In this study, we first developed anti-kudu and anti-impala immunoglobulin-specific conjugates in chickens to compare their binding efficiency with that of commercially available protein-G and protein-AG conjugates. This was done using an ELISA-based avidity index to enhance the serological diagnosis of anthrax. Second, we investigated the complications posed by the presence of atypical B. cereus and other closely related species in diagnosing anthrax with genetic markers and qPCR. For this purpose, we analyzed blood smears from wildlife mortalities in Kruger National Park (KNP), South Africa, comparing the outcomes of anthrax diagnostics using qPCR, microscopy, and culture methods. Finally, we explored the transition of the primary anthrax host from kudu to impala within KNP. Our focus was on identifying potential links between environmental factors—such as precipitation, soil moisture, temperature, and the Normalized Difference Vegetation Index (NDVI)—and the patterns of anthrax mortality occurrences and frequencies. Additionally, we examined the variations in environmental factors and the population densities of various host species over time, aiming to identify any correlations between the densities of host species and the rates of anthrax mortalities. The developed conjugates had a high avidity of >70% against kudu and impala sera. The commercial conjugates (protein-G and protein-AG) had significantly low relative avidity (<20%) against these species. Eighteen additional wildlife species exhibited cross-reactivity, showing a mean

relative avidity of over 50% with the developed impala and kudu conjugates, compared to less than 40% with the commercial conjugates. This study underscores the value of species-specific conjugates as crucial tools for developing and validating immunoassays in wildlife, and for monitoring zoonotic diseases across the livestock-wildlife-human interface. In our analysis of 1,706 blood smears from wildlife mortalities, 890 samples were positive for *B. anthracis*, detected either through genetic markers or microscopy. Specifically, 15.2% of these samples tested positive for the *lef* marker, and 12.6% for BAPA. The use of both BAPA and *lef* markers together identified 24.4% of samples as positive, which increased to 44.4% when combined with microscopy, indicating strong concordance between molecular and microscopic methods ($p<0.0001$). Out of 506 cultured isolates, 24.7% tested positive by either genetic markers or microscopy, but only 4 samples were definitively confirmed as *B. anthracis* through culture, microscopy, and sensitivity testing to penicillin and gamma-phage. The *lef* marker was found to have the lowest specificity and accuracy. Conversely, combinations such as Ba-1/*capB*, BAPA/*capB*, Ba-1/BAPA/*capB*/*lef*, and BAPA/*lef*/*capB* achieved 100% specificity and accuracy, with a sensitivity of 75%. The combination of BAPA/*lef*/Ba-1 also reached 100% in specificity, sensitivity, and accuracy. The findings emphasize the need to identify precise markers for *B. anthracis* in southern Africa to enhance anthrax diagnosis. The strategic use of both microscopy and multiple markers can significantly reduce false positives. The study also noted distinct trends in anthrax mortality over different years and regions, with a notable shift in the primary host species from kudu to impala. Furthermore, significant correlations were found between anthrax mortality in kudu and environmental factors such as NDVI, average temperature, and standardized precipitation indexes (SPI-6 and SPI-12). In contrast, impala mortality was associated with changes in SPI-3, temperature increases, and higher mortality rates during the rainy season. Interestingly, elephant density was negatively correlated with kudu mortality but positively correlated with impala mortality and density. These observations suggest that environmental conditions and the density of species play significant roles in determining the frequency and variety of hosts exposed to *B. anthracis*. The study concludes that over time, climate extremes could amplify the severity of anthrax outbreaks by affecting species susceptibility and exposure chances.

Chapter 1. Background, Literature Review, Aim and Objectives

1.0. Background and Literature Review

The increasing occurrence of both new and recurring diseases in human and veterinary fields highlights the critical necessity for methods grounded in solid evidence to assess the rates of infection and its widespread presence. (Lambert et al., 2022). Effective intervention strategies for diseases impacting both livestock and wildlife are contingent on a profound understanding of their epidemiology (Artois et al., 2009). Wild animals, serving as hosts and reservoirs for potentially cross-species transmissible pathogens, necessitate a comprehensive understanding of their epidemiology and ecology to inform robust policies and interventions for disease control.

Anticipating the challenges posed by anthrax, an ancient zoonotic disease with documented historical roots, requires a multi-faceted approach. Anthrax, primarily affecting herbivorous mammals, including wildlife and livestock, also poses a risk to human health through the handling and consumption of infected carcasses, meat, and hides (Kamal et al., 2011, W.H.O 2015). *Bacillus anthracis,* the bacterium responsible for anthrax, manifests in two forms—spore and vegetative—each carrying distinctive virulence factors encoded on plasmids. Understanding these factors is pivotal for comprehending the mechanisms of anthrax pathogenesis (Makino et al., 1989, Okinaka, 1999b).

The dynamics of anthrax transmission extend beyond biological factors, involving environmental and climate variables as well as the behaviours and densities of host species. Host activities such as foraging and movement influence exposure risks, highlighting the intricate interplay between host ecology and pathogen transmission (Huang et al., 2022, Ochai et al., 2022, Anderson et al., 2016, Moe et al., 2015). Disease outbreaks evolve over time due to a myriad of factors impacting hosts, pathogens, and environmental conditions. Recognizing these underlying mechanisms is essential for forecasting and managing future

epidemic events. (Lloyd-Smith et al., 2009). Shifts in disease patterns over time are interconnected with the behaviours of host species, the persistence of environmentally transmitted pathogens, and the populations of disease vectors (Altizer et al., 2006). Environmental variations, including precipitation, temperature, and plant cover, contribute significantly to the occurrence and modifications of diseases like anthrax (Chikerema et al., 2012, De-Vos and Bryden, 1996, Nsoh et al., 2016, Eisenberg et al., 2013, Steenkamp et al., 2018, Lisovski et al., 2017)

This study aims to contribute to the knowledge base surrounding anthrax epidemiology by employing a comprehensive approach that includes active surveillance, species-specific diagnostics, molecular characterization, and an exploration of the ecological dynamics influencing disease transmission. By unravelling the complexities of anthrax dynamics in the context of both host ecology and environmental factors, we aim to offer perspectives that can guide targeted interventions and enhance disease control strategies in diverse ecosystems.

1.1 Anthrax Aetiology

Anthrax, a disease resulting from the bacterium *Bacillus anthracis*, is a zoonotic disease, primarily affecting herbivorous mammals. Although it mostly causes fatalities among wildlife and farm animals, humans can also contract anthrax, typically through contact with infected animal products like carcasses and hides (Kamal et al., 2011, W.H.O 2015). Human infection can occur through several pathways, including the cutaneous form leading to eschars (Turnbull, 1999, Vilas-Bôas et al., 2007), and through transmission via contaminated heroin injections (Ringertz et al., 2000). Additionally, inhaling infected animal materials, such as drum skins, can lead to infection, especially during animal outbreaks(Bennett et al., 2018), and there is also a risk of anthrax being used as a bioweapon weapon (Jernigan et al., 2002).

1.2 Taxonomy

Bacillus anthracis, a member of the Firmicutes phylum and Bacillaceae family, is included in the *B. cereus sensu lato* group. This group encompasses eight related *Bacillus* species: *B. thuringiensis*, *B. anthracis*, B. mycoides, B. *cereus*, *B. weihenstephanensis*, *B. pseudomycoides*, *B. toyonensis*, and B. cytotoxicus, as identified (Radnedge et al., 2003, Rasko et al., 2005). Although *B. anthracis* genetically resembles species such as *B. cereus sensu stricto*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanesis*, and *B. thuringiensis*, they differ in phenotype, etiology, pathogenicity, clinical effects, and preferred hosts (Drobniewski, 1993b, Rasko et al., 2005, Pilo and Frey, 2011) .

Classified as a Gram-positive, rod-shaped bacterium*, Bacillus anthracis* exists in two primary forms: the spore and the vegetative state (Vilas-Bôas et al., 2007). The spore form is predominantly found in oxygen-rich environments outside the host (Leppla, 1982) and is capable of enduring harsh conditions due to its structural features (Turnbull et al., 1998). Upon contact with a host, these spores germinate at the infection site and migrate to nearby lymph nodes. Depending on the anthrax type, the vegetative cells proliferate at the entry point of the spores, which could be in the Peyer's patches or respiratory-linked lymphoid organs (Glomski et al., 2007).They then disperse via the lymphatic system to the bloodstream, causing bacteremia as the bacilli multiply (Glomski et al., 2007). The vegetative form of B. anthracis is known for producing potent toxins that lead to the characteristic symptoms and lesions of anthrax (Hoover et al., 1994).

1.3 Anthrax and atypical anthrax-like *Bacillus cereus*

In investigations focusing on *B. anthracis*, *B. cereus senso stricto* is often disregarded as a mere contaminant, especially when genes from both pXO1 and pXO2 plasmids are absent, leading to a potential oversight of significant samples (Logan and Turnbull, 1999). This approach can result in missing crucial samples containing pXO1 plasmids not originating from *B. anthracis*.

Recent discoveries have highlighted atypical *B. cereus* strains capable of causing anthrax-like diseases in humans and animals, possessing virulence plasmids similar to *B. anthracis*'s pXO1 and pXO2 (Baldwin, 2020). Specifically, the pXO1 plasmid in B. anthracis, spanning 181,667 bp and housing the tripartite toxin (Okinaka, 1999a), shows a striking resemblance to the pBCXO1 plasmid in B. cereus biovar anthracis (Bcbva), which is 181,907 bp (Klee et al., 2006a) in length and exhibits about 99.6% similarity to pXO1 (Rasko et al., 2005)

Although atypical *B. cereus* strains have the capability to induce anthrax-like illness, they share a closer genetic relationship with other *B. cereus* variants than with *B. anthracis* (Antonation et al., 2016). There consist categories of *B. cereus* strains that cause anthrax-like symptoms, as categorized Baldwin (2020); the atypical strains causing anthrax pneumonia which include G9241 (Avashia et al., 2007b), FL2013 (Hoffmaster et al., 2006) and 03BB102 and *B. cereus* biovar *anthracis* (Hoffmaster et al., 2004). Hoffmaster et al. (2004) documented cases of inhalational anthrax-like illness caused by atypical *B. cereus* strains that carry the pBCXO1 plasmid, which shares a 99.6% similarity with the pXO1 plasmid found in *B. anthracis*. It's worth noting that while these atypical *B. cereus* strains do possess a capsule, it is not composed of poly-γ-D-glutamic acid; instead, it is suggested to consist of a polysaccharide capsule (Hoffmaster et al., 2004).

In 2020, the U.S. Center for Disease Control and Prevention reported two instances of pneumonia among welders, one resulting in death (Dawson et al., 2021).These incidents were linked to unusual strains of *B. cereus* group bacteria, which harbored anthrax toxin genes typically found in *B. anthracis*. Known as Gram-positive facultative anaerobes proficient in toxin production, B. cereus group bacteria are commonly found in soil, dust, and food (Turnbull, 1996). Infections by *B. cereus* typically present gastrointestinal symptoms like diarrhoea and vomiting, but can also lead to more serious conditions affecting

the lungs, eyes, or skin (Dawson et al., 2021, Hoffmaster et al., 2006). Since 1994, seven cases of pneumonia caused by these bacteria with anthrax toxin genes have emerged, mainly among welders and metalworkers in Louisiana and Texas, resulting in five deaths and two critical cases requiring extensive hospitalization (Hoffmaster et al., 2004, Avashia et al., 2007a, Wright et al., 2011). Moreover, there have been reports of human skin infections mimicking cutaneous anthrax, marked by eschars, caused by these atypical *B. cereus* strains (Marston et al., 2016, Kaiser, 2011). The scope and distribution of these *Bacillus* species carrying anthrax toxin genes are not well understood and demand greater research attention.

All recorded cases of this anthrax-like pneumonia did not involve individuals with preexisting health conditions. Similar anthrax-like illnesses have been observed in animals across West Africa. In Taï National Park, Côte d'Ivoire, and the Dja Reserve's northern periphery in Cameroon, several primates, including chimpanzees and a gorilla, succumbed to suspected anthrax infections (Leendertz et al., 2004, Leendertz et al., 2006, Klee et al., 2006b). Interestingly, three of the six genomic VNTRs in these outbreaks were unique, forming a new cluster termed "forest anthrax cluster" or "F." These strains from Côte d'Ivoire and Cameroon diverged from the known group A and B strains of *B. anthracis*, indicating a greater diversity within the species (Klee et al., 2006b). Later, these were reclassified as *B. cereus* biovar *anthracis*, carrying both pBCXO1 and pBCXO2 plasmids, affecting various animals like apes (Klee et al., 2006a, Hoffmann et al., 2017). Similar cases of atypical *B. cereus* and Bcbva with these plasmids have been identified in cattle in Cameroon (Pilo et al., 2011, Somerville and Jones, 1972), elephants and goats (Antonation et al., 2016). Zimmermann et al. (2017b) examined the virulence of these *B. cereus* strains compared to the well-known *B. anthracis* Sterne vaccine strain, suggesting that while their virulence may not match that of the *B. anthracis* Ames strain, it could exceed that of the Sterne strain.

The close genetic and symptomatic links observed underline the imperative for a more in-depth retrospective investigation. This investigation aims to elucidate the roles played by these B. cereus strains in the outbreaks in KNP and, concurrently, understand their implications for complicating the diagnosis of anthrax. The sequences of the 16S rRNA genes in B. anthracis, B. cereus, and B. thuringensis show significant resemblance, supporting the concept of a close relationship (Ash et al., 2008, Somerville and Jones, 1972)

Marston et al. (2016) noted the presence of anti-PA antibodies and LF-neutralizing antibodies in patients recovering from anthrax-like cutaneous illness caused by atypical strains of *B. cereus*. Additionally, a separate study utilized the B. anthracis-specific PA antigen to identify Bcbva in wildlife in West Africa (Zimmermann et al., 2017b). Further corroborating this, Brézillon et al. (2015) confirmed the existence of anti-PA and anti-LF in Bcbva through western blot analysis. These findings collectively underscore the need for a comprehensive understanding of the relationships between these closely related strains and their potential impact on both serological and molecular diagnosis of anthrax.It is suggested that these genes of *B. cereus* biovar *anthracis* origin may be able to produce toxins or their components that are homologous to what is found in *B. anthracis* except in the presence of some fundamental alterations that may occur after the translational phase (Baldwin, 2020). The identification of *Bacillus* species that contain the virulence genes of *B. anthracis* greatly complicate both clinical and laboratory diagnosis (Hoffmaster et al., 2004). This is largely because most diagnoses of *B. anthracis* focus on the identification of the pXO1 and pXO2 plasmids and this may lead to false negatives. With the emergence of more evidence that supports the close relatedness of *B. anthracis* and other *B. cereus* strains there is a need to explore the possibility of *B. cereus* being the cause of some of the anthrax-like infections in southern Africa. While *B. anthracis* is generally considered clonal, the possibility for horizontal gene transfer (HGT) exists and may contribute to the acquisition of novel virulence factors (Saile and Koehler, 2006). Investigating the genomic plasticity of *B.*

anthracis through HGT is essential for understanding its evolution and adaptation.

1.3.1 How Bcbva ecology differ from B. anthracis

The ecological niches of *B. anthracis* and Bcbva demonstrate significant differences, with *B. anthracis* found in southern Africa's dry savannas, including wildlife reserves known for anthrax outbreaks (Romero-Alvarez et al., 2020). Bcbva, however, has a more restricted distribution in humid tropical forests, suggesting unique ecological requirements (Klee et al., 2006a, Klee et al., 2010). Models indicate B. anthracis's suitability across various parks in southern Africa, while Bcbva's potential habitats are confined to the Congo Basin and West Africa's coastal areas (Steenkamp et al., 2018, Romero-Alvarez et al., 2020, Pittiglio et al., 2022). These distinctions underline the importance of tailored surveillance and research to understand each pathogen's environmental preferences and impacts.

Soil pH and humidity significantly differentiate their habitats, with B. anthracis thriving in alkaline soils and Bcbva in more humid conditions (Romero-Alvarez et al., 2020). However, NDVI, a measure of vegetation, does not distinguish between their environments, suggesting both can exist in areas with similar vegetation indices but differing soil and humidity conditions (Romero-Alvarez et al., 2020).

1.4 Ecology of Anthrax: A Complex Interplay in Natural Environments

Bacillus anthracis is not only a significant threat to animal and human health but also exhibits a fascinating ecology deeply intertwined with environmental factors. The bacterium's lifecycle involves a delicate balance between the spore and vegetative forms, interacting with both biotic and abiotic components of ecosystems.

Since anthrax is not contagious, its spread depends on different mechanical vectors and environmental factors, including soil, water (Lindeque and Turnbull, 1994b, De-Vos, 1990, W.H.O., 2008b), vegetation, and hosts behaviour (Turner et al., 2014a). Mechanical vectors implicated in the transmission of *B. anthracis* include flies and vertebrate scavengers such as vultures, jackals, and hyenas (De-Vos, 1990, Blackburn et al., 2010a, Fasanella et al., 2010, Beyer et al., 2018, Basson et al., 2018a, Lindeque and Turnbull, 1994b, Hugh-Jones and De Vos, 2002, Bellan et al., 2012). This bacterium follows a lifecycle that includes the endospores with the soil/environment as its habitat, vectors and hosts disseminating it and the vegetative cells producing toxins once in the host. In the natural system, host populations comprise of individuals with a range of susceptibility, facing the pressure of this disease as well as other factors like climate change (influencing temperature, forage availability and water) which was implicated in the anthrax outbreak in the Artic circle (Minett and Dhanda, 1941a, Walsh et al., 2018).

One key ecological aspect of anthrax is the ability of *B. anthracis* to form highly resistant spores, allowing it to persist in soil for extended periods due to particular soil and meteorological factors that create an environment that is favourable for its survival (Van Ness, 1971, De-Vos, 1990, Smith et al., 2000). Spores, the infectious stage of the bacterium, serve as a reservoir, awaiting the opportunity for transmission to a new host (Barandongo et al., 2023). The longevity of spores in soil contributes to the endemic nature of anthrax in certain regions (Van Ness, 1971, Barandongo et al., 2023).

Spore persistence is influenced by factors such as soil type, soil chemistry and pH (Turnbull, 2008b, Van Ness and Stein, 1956, Barandongo et al., 2023). This extended spore viability plays a crucial role in the recurring nature of anthrax outbreaks, particularly in areas with a history of the disease (Barandongo et al., 2023). One key trigger is the presence of alkaline soils, as *B. anthracis* spores have a preference for such environments (Van Ness and Stein, 1956, Van Ness,

1971). Alkaline conditions promote the stability and viability of the spores, allowing them to persist in the soil for extended periods (Van Ness, 1971, Barandongo et al., 2023). Studies have shown that higher alkaline pH levels are associated with higher anthrax occurrence, creating a conducive environment for the activation of spores (Dragon and Rennie, 1995a).

Anthrax spores in soil serve as a source of infection for herbivores, which acquire the bacterium through grazing or ingesting contaminated vegetation (Hugh-Jones and Blackburn, 2009). The spatial distribution of spores in the soil contributes to the localized nature of anthrax outbreaks (Hugh-Jones and De Vos, 2002). Understanding the patterns of spore contamination in soil is crucial for predicting transmission dynamics and implementing targeted control measures in areas with a history of anthrax incidence (Mullins et al., 2013).

The availability of moisture is another critical factor influencing anthrax outbreaks. Drought conditions followed by heavy rainfall can disturb the spore's dormancy, leading to their activation and subsequent infection of grazing animals (De-Vos, 1990, W.H.O., 2008b). Study by Hugh-Jones and De Vos (2002) highlights the role of environmental moisture in triggering anthrax outbreaks, emphasizing the significance of understanding precipitation patterns in predicting and managing the disease.

Optimal temperature conditions in the soil, typically ranging from 15°C to 30°C, play a role in spore activation (Minett and Dhanda, 1941a). Warmer temperatures support the metabolic processes involved in germination. Anthrax is commonly found in tropical areas with warmer temperatures, with exception such as the outbreaks close to the Arctic circle which was attributed toward increase in summer temperatures (Minett and Dhanda, 1941a, Walsh et al., 2018). These outbreaks in the arctic often happen during summers with extended daylight hours leading to increase in temperature, which increase the survival of spores in alkaline soils (Minett and Dhanda, 1941a) with an abundance of calcium and

moderately high moisture content (Van Ness and Stein, 1956, Van Ness, 1971). Also, as temperatures rise due to the impacts of climate change, anthrax outbreaks have been reported in Siberia following the melting of permafrost (Ezhova et al., 2021).

Human interventions which include farming activities and change in grazing patterns and corridors, land use change, livestock and wildlife trade, waste disposal and carcass management play significant roles in the dissemination of *B. anthracis* (W.H.O., 2008b, Dragon and Rennie, 1995b, Blackburn et al., 2007b). For instance, the excavation of soil during construction or the movement of livestock across different regions can disturb the spores, making them more accessible to susceptible hosts (Turnbull, 1996, Turnbull, 2008b, Hugh-Jones and Blackburn, 2009). These activities can create hotspots for anthrax transmission, highlighting the need for comprehensive risk assessments in areas prone to such disturbances.

The existent, emerging and predicted effects of climate change include but are not restricted to global warming, change in rainfall events, storms, floods and droughts (Kurane, 2010, Engelbrecht et al., 2015, Lian et al., 2021, IPCC, 2023, IPCC, 2022). These climate events can cause increased contamination of water and food sources, the surge or decrease in activities and numbers of vectors and increase of infected areas of the pathogen (Anwar et al., 2019, Kurane, 2010, De Vos et al., 2016, Turner et al., 2022). It is thus important to analyse the roles climate change has played and is playing in the exposure of hosts to *B. anthracis*, the frequency of exposure, exacerbation of anthrax severity owing to climate extremes and the shift in the main anthrax host species overtime. Climate change may exacerbate the risk of anthrax outbreaks by altering temperatures, precipitation patterns, and shifts in vegetation dynamics which can collectively impact the ecology of *B. anthracis* spores.

The ecology of anthrax is therefore closely linked to its interactions with wildlife, particularly herbivores. In natural ecosystems, herbivores such as ungulates can become infected by ingesting soil or vegetation contaminated with *B. anthracis* spores (W.H.O., 2008b, De-Vos, 1990). Wildlife, therefore, serves as both victims and vectors in the anthrax lifecycle. Studies have shown that certain species of herbivores, such as ungulates and scavengers, are more susceptible to anthrax , contributing to the maintenance of the bacterium in specific ecological niches (De-Vos, 1990, De-Vos and Bryden, 1996). The dynamics of herbivore populations, their migratory patterns, and the availability of suitable habitats play crucial roles in shaping the ecology of anthrax (Turnbull et al., 1991, W.H.O., 2008b, Huang et al., 2022). Modifications of wildlife habitat, foraging behaviour and the effects of other wildlife species on vegetation play significant roles in the spread and transmission of *B. anthracis* (Huang et al., 2021, Huang et al., 2022, Hugh-Jones and Blackburn, 2009, Hugh-Jones and De Vos, 2002).

1.5 Disease progression and transmission

Animals can contract *B. anthracis* through wounds in their skin, gastrointestinal tract, or respiratory tract. The bacilli are typically shed from the body orifices of the affected animals or following when scavengers rip open infected carcasses post-death (W.H.O., 2008b, Hambleton et al., 1984). Although Bellan et al. (2013) demonstrated that vertebrate scavengers are not always necessary as putrifying organisms and arthropod scavengers can also play a role in the release of the bacilli into the environment. At 20 to 30 °C, the shed bacilli easily sporulate in soil. In times of drought or overgrazing, there is an increase in the likelihood of host species to inhale or ingest dust ladened with spores (Hambleton et al., 1984). However in later studies were shown that more soil ingestion occurs during the wet seasons (Turner et al., 2013) or following rain terminating a long phase of drought (W.H.O., 2008b). Animals typically contract anthrax in natural settings by ingesting contaminated materials (feed, grass, water, or carcasses), making the ingestional type of the disease the most common (Bhatnagar and Batra, 2001,

Turner et al., 2016, Turner et al., 2014a). When blowflies ingest the bodily fluids of anthrax infected carcasses and subsequently spread highly infectious feaces or vomit on the nearby plants, they are implicated in the disease transmission. Infection of browsing herbivores have been suggested to occur through the consumption of *B. anthracis* spores ladened leaves (Braack and De Vos, 1990b, De-Vos, 1990, De-Vos and Bryden, 1996). According to a study by Palazzo et al. (2012), blood-sucking flies like tabanids can bite mammals, cause cutaneous anthrax.

1.6 Animal Susceptibility, Clinical Signs and Pathology

The infection is severe and progresses quickly in highly vulnerable species, including ruminants (Bhatnagar and Batra, 2001) but will depend on the infectious dose. It is difficult to assess the infectious dose of *B. anthracis* spores in nature in ruminants. For sheep, horses, and cattle, an oral minimum infectious dose (MID) of 5108 spores has been determined (W.H.O., 2008b) which could be dependent on the strain of the bacteria. For some small ruminants, sheep, rodents, non-human primates, pigs, and dogs, the published LD50 of anthrax spores when administered subcutaneously were 100, 3000, 5000, 106, 109, and 51010. It is crucial to note that these LD50 or MIDs only offer a basic indication of the possible or true infectious dose in natural settings because the epidemiology of this disease is heavily influenced by variables like the strain of *B. anthracis*, the route(s) of infection, the species, breed, and health status of the animal(s) (W.H.O., 2008b)

Clinical manifestations of anthrax vary depending on the type of the disease, which is determined by the entry route into the body. However, post-mortem identification of lesions makes it challenging to distinguish between the forms once the disease is established (Glomski et al., 2007, Easterday et al., 2020). The common three types of anthrax are gastrointestinal (ingestional), cutaneous, and inhalational. In the cutaneous form, when spores are introduced into the skin,

oedema-like lessions that contain capsulated *B. anthracis* can develop within 2-4 hours (Cromartie et al., 1947). Turnbull (2008b) reported extensive oedema and shock as clinical appearances of cutaneous form of anthrax in humans.

The inhalational form is characterized by abrasions in the lungs and enlargement of regional lymph nodes, leading to signs like dysphagia, hemoptysis, and dyspnea (Gleiser et al., 1963). Splenomegaly and oozing of blood from orifices (with ruined clotting factors) shortly after death are also mostly observed in American Cervidae species (Gleiser et al., 1963, Hugh-Jones and De Vos, 2002). For the gastrointestinal form, gross pathological lesions consist of necrosis, haemorrhage, and intestinal inflammation primarily affecting the small intestine (Nieberle and Cohrs, 1967).

Ruminants are typically regarded as the most anthrax-susceptible animals following ingestion. After an incubation period the illness often manifests as peracute or acute septicemia, with variations ranging from 1 to 14 days depending on the infectious dosage (W.H.O., 2008b, Beyer and Turnbull, 2009a). Clinical indications sometimes arise unexpectedly, with staggering, recumbency, and, most importantly, the absence of antecedent visual indicators. Convulsions or spasms accompany death within minutes. Fever (>40°C), excitement or lethargy, dyspnoea, cyanotic mucosae, and oedematous swellings in diverse areas such as the neck, chest, belly, or flanks are common symptoms (Beyer and Turnbull, 2009a). Death usually occurs within 12-13 hours of the onset of these symptoms (W.H.O., 2008b, Beyer and Turnbull, 2009a). Additionally, blisters or sores on the tongue or other regions of the oral cavity may be detected, and dying animals frequently exhibit bleeding from the nose and anus. While subacute and chronic infections have been reported, they appear to be uncommon (Beyer and Turnbull, 2009a).

The acute form of anthrax in horses typically presents with elevated body temperature (40-41°C), difficulty breathing, bluish discoloration of mucous

membranes, abdominal pain (colic), bloody diarrhoea, local swellings with areas of tissue death, and the release of bloody fluids from body openings (Beyer and Turnbull, 2009a).

Pigs, exhibiting a degree of resistance to anthrax, rarely experience severe septicaemia. Instead, they are predisposed to developing a prolonged, inconspicuous infection with Bacillus anthracis, often becoming apparent only during post-mortem examinations at the time of slaughter (Redmond et al., 1997, W.H.O., 2008b). Clinical indications may involve pharyngitis, painful swelling in the head and chest, and black necrotizing papulae on the skin and mucous membranes (Redmond et al., 1997). Upon slaughter, identifiable features such as swelling of the tissue around the pharyngeal region and lymph nodes, haemorrhagic lymphangitis, and, after persistent infections, dry, encapsulated, grey-yellowish areas within regional lymph nodes may be observed (Beyer and Turnbull, 2009a).

Carnivores, particularly wild scavengers, exhibit a relative resistance to infection with Bacillus anthracis, and the possibility of chronic infection exists (W.H.O., 2008b). Mink and cheetahs represent exceptions as they appear to be more susceptible than other carnivores (Turnbull et al., 1992a, Lembo et al., 2011a, Portas et al., 2021). Nevertheless, no animal is entirely immune to the disease, and instances have been recorded in various carnivores over the years (Hugh-Jones and De Vos, 2002)(Hugh-Jones and de Vos, 2002). After an incubation period of 3-5 days, affected animals may develop symptoms such as fever (>40°C), intestinal issues with anorexia and lethargy, and inflammation accompanied by oedematous swelling in the neck region (Hugh-Jones and Blackburn, 2009, Beyer and Turnbull, 2009a). Those that do not recover may succumb to death within hours of exhibiting clinical signs, sometimes experiencing fatal bleeding from bodily openings (Gleiser et al., 1968).

The susceptibility of different animal species to anthrax can vary, and several factors contribute to these differences. Very few literatures are available about species' susceptibility to anthrax and factors responsible for variations in susceptibility. Variations in the innate immune responses among species contribute to differences in susceptibility (Turnbull et al., 1986). Some species may have more robust innate defence mechanisms against *B. anthracis*. Also, differences in the adaptive immune response, including variations in antibody production and T-cell responses, can influence susceptibility to anthrax (Turnbull et al., 1986, Ochai et al., 2022). The presence and specificity of receptors on the host cells that *B. anthracis* targets can vary among species (Turnbull et al., 1992a). Receptor availability influences the ability of anthrax toxins to bind and enter host cells (Passalacqua and Bergman, 2006). Another factor that influences susceptibility is the environment where the animals inhabit. Animals that inhabit environments with a history of anthrax contamination may develop some level of immunity or resistance over time (Ochai et al., 2022, Kanankege et al., 2019, Cizauskas et al., 2014). Conversely, species in areas with no historical exposure may be more susceptible (Kanankege et al., 2019). Animals that have survived previous anthrax infections may develop partial immunity, influencing their susceptibility to subsequent exposures (Cizauskas et al., 2014, Turnbull, 1991). Lastly, genetic variations among animal populations can influence their susceptibility to anthrax. Some species may have inherent genetic factors that confer resistance or susceptibility (Abalakin and Cherkasskiĭ, 1986).

1.7 Anthrax Toxins, Regulation of Virulence Factor Expression and Pathogenesis

Bacillus anthracis utilizes two large plasmids as carriers of its most critical virulence factors. These plasmids, denoted as pXO1 and pXO2, play a pivotal role in the pathogenesis of the bacterium (Moayeri et al., 2015, Leppla, 1982, Okinaka, 1999a). Notably, the 183-kb pXO1 plasmid encodes the anthrax toxins, while the 96-kb pXO2 plasmid is responsible for the synthesis of the poly-γ-D-

glutamic acid capsule, another key virulence factor (Makino et al., 1989). The replication mechanisms of both plasmids have been elucidated, with pXO2 belonging to the pAMβ1 family of θ-replicating conjugative plasmids (Tinsley et al., 2004), and pXO1 sharing attributes with the origin of θ-replicating plasmids, conserved among *B. cereus* group plasmids (Pomerantsev et al., 2009).

The virulence plasmids are maintained at a low copy number, a feature attributed to the presence of three genes essential for pXO1 plasmid partitioning (Pomerantsev et al., 2014). Whole-genome sequencing has revealed a molecular ratio of 3:2:1 for the levels of pXO1:pXO2:chromosome in *B. anthracis* cells (Read et al., 2002). Intriguingly, plasmids homologous to pXO1 or pXO2 have been identified in a subset of *B. cereus* isolates, challenging the specificity of these plasmids as distinguishing features of *B. anthracis* (Rasko et al., 2005).

The production of virulence factors encoded by pXO1 is tightly regulated by multiple factors. The pXO1-encoded transcriptional regulator protein AtxA (Anthrax toxin activator) (Uchida et al., 1993) and elevated levels of carbon dioxide (CO_2) and bicarbonate ($HCO3^-$) upon transition from the external environment to the host environment play pivotal roles (Koehler et al., 1994). AtxA, containing two N-terminal binding domains, regulates toxin gene expression both transcriptionally and post-transcriptionally, with its activity linked to temperature and cell nutritional status (Tsvetanova et al., 2007, Chiang et al., 2011). CO_2 increases the fraction of dimerized AtxA, providing a potential model for the CO_2-mediated activation of toxin gene production (Hammerstrom et al., 2011). Recent transcriptomic studies have identified genes regulated by AtxA and CO_2, shedding light on the intricate regulatory network controlling virulence factor production (McKenzie et al., 2014). On the pXO2 plasmid, AtxA also regulates transcription of the capsule biosynthesis operon indirectly (Uchida et al., 1993, Drysdale et al., 2004b). A *B. anthracis* atxA null mutant has been shown to be highly defective in virulence in a mouse model, emphasizing the critical role of AtxA in the pathogenicity of *B. anthracis* (Dai et al., 1995).

The pXO1 harbours several other regulatory elements influencing virulence factor expression (Koehler et al., 1994). The transcriptional activator AcpA positively regulates the expression of pagA, contributing to toxin production. Furthermore, the pXO1-encoded global regulator Ati (anthrax toxin inhibitory factor) modulates the expression of both the anthrax toxin and capsule genes (Saile and Koehler, 2002, Koehler et al., 1994).

1.7.1 Anthrax Toxins: Molecular Architecture and Cellular Interactions

The pXO1 plasmid contains genes that encode proteins such as protective antigen (PA), lethal factor (LF), and oedema factor (EF). These proteins come in two forms, A and B. Form A (EF or LF) possesses enzymatic activity (Leppla, 1982, Moayeri and Leppla, 2004, Moayeri and Leppla, 2009). The three polypeptides converge to form two distinct toxins: Lethal Toxin (LT) and Edema Toxin (ET) (Figure 1-1) (Moayeri and Leppla, 2004, Leppla, 1982). These toxins play a crucial role in subverting host defenses, hindering the host response, and ultimately causing lethality. The common receptor-binding component for both toxins is PA, an 83-kDa protein with four domains (Lacy et al., 2002, Moayeri and Leppla, 2004, Moayeri and Leppla, 2009).

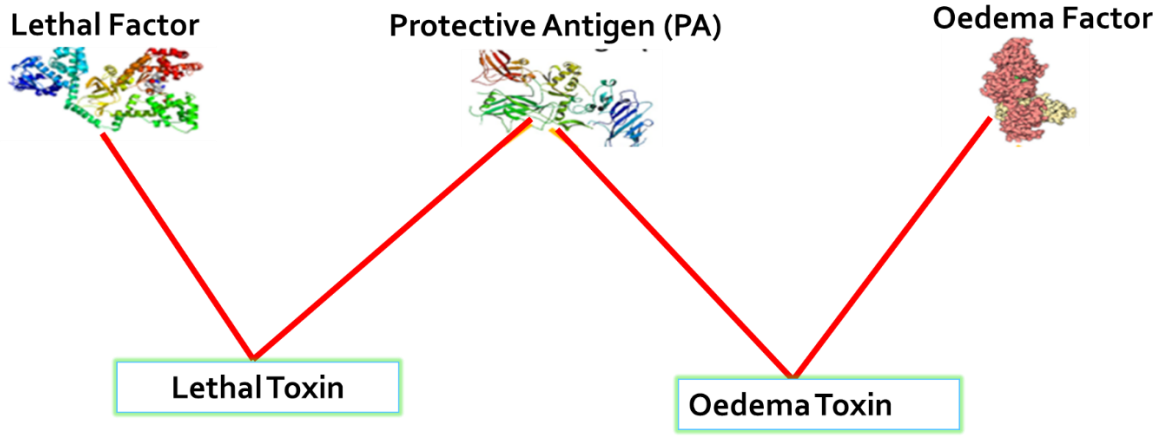

Figure 1-1: Diagram Depicting the Assembly of Proteins to Create Anthrax Exotoxins.

The process of anthrax toxicity begins with the protective antigen (PA) attaching to cell receptors like Tumor Endothelial Marker 8 (TEM8) and Capillary Morphogenesis Gene-2 (CMG2), exhibiting a high affinity in the nanomolar range (Bradley et al., 2003). These critical receptors for in vivo activity feature a metal ion-dependent adhesion site (MIDAS) within their von Willebrand domain, characteristic of integrins (Rosovitz et al., 2003, Bradley et al., 2003). After binding to these receptors, PA is cleaved by cell surface proteases, resulting in the creation of a PA63 oligomer. This oligomer is the only form of the toxin capable of undergoing endocytosis (Collier, 2009).

After being absorbed by the cell, PA undergoes a structural transformation in the endosomal acidic setting, evolving from a prepore into a pore configuration (Miller et al., 1999).This alteration aids in moving the enzymatic elements, LF and EF, into the cytosol of the host cell (Abrami et al., 2004).The lethal factor, functioning as a zinc metalloprotease, targets and splits specific members of the mitogen-activated protein kinase kinases (MAPKK) family, thereby disrupting essential cell processes (Vitale et al., 1998). The oedema factor, operating as a calmodulin-dependent adenylate cyclase, catalyzes the swift conversion of ATP into cAMP. This leads to the stimulation of various signaling channels via protein kinase A (PKA) and the exchange protein activated by cAMP (Shen et al., 2002).

The process of endocytosis is initiated by the swift transportation of the receptor-toxin complex towards lipid rafts, which triggers the activation of src-like kinases and leads to clathrin-mediated endocytosis (Moayeri and Leppla, 2004, Miller et al., 1999). Modifications such as palmitoylation and ubiquitination of the receptor play a role in this mechanism (Abrami et al., 2004). In the acidic milieu of endosomes, PA undergoes additional structural alterations, resulting in the formation of a pore structure resistant to SDS and heat. This structure facilitates

the passage of LF and EF into the cell's cytosol (Figure 1-2) (Miller et al., 1999). The transfer of these components is aided by the merging of internal vesicles with outer membranes, a process driven by a Brownian ratchet mechanism that is sensitive to charge states (Abrami et al., 2004).

This complex biochemical system highlights the elaborate method through which anthrax toxins infiltrate host cells. Understanding the dynamic interaction between PA, cell receptors, and the endocytic pathway sheds light on new opportunities for diagnostics and prevention strategies.

1.7.2 Poly-γ-D-glutamic acid Capsule

The capsule made of poly-γ-D-glutamic acid (PGDA), encoded by the pX02 plasmid, serves as a defense for the bacteria against the host's immune defenses, leading to a reduced immune reaction and evading phagocytosis (Makino et al., 1989, Little and Ivins, 1999, Moayeri et al., 2015). This PGDA capsule adheres to the peptidoglycan layer on the surface of host cells, potentially enhancing the potency of the lethal toxin (Scorpio et al., 2007, Jang et al., 2011). Contemporary research has established a connection between PGDA and the progression of anthrax, illustrating its role in targeting cytokine pathways and impairing the host's immune response (Smith et al., 1955b, Makino et al., 2002, Jelacic et al., 2014, Jeon et al., 2015). The number of pXO2 plasmids in a cell is closely linked to its virulence level (Pannifer et al., 2001). It has been observed that animals resistant to LT (lethal toxin) show increased vulnerability to spore challenges, and the inverse is also true (Lincoln et al., 1967, Welkos et al., 1989, Terra et al., 2010). Additionally, another report by Newman et al. (2010b) demonstrated a positive correlation between LT-caused deaths in rats and how the macrophages are susceptible to the effect of LT.

Capsule synthesis in *B. anthracis* is primarily regulated by the pXO2-encoded regulatory protein AtxA2 (Uchida et al., 1985). The presence of CO2 has been identified as a crucial environmental cue influencing capsule synthesis, with

elevated levels enhancing the expression of capsule genes(Makino et al., 1989, Uchida et al., 1993). The interplay between pXO1 and pXO2 regulatory elements further underscores the complexity of virulence factor regulation.

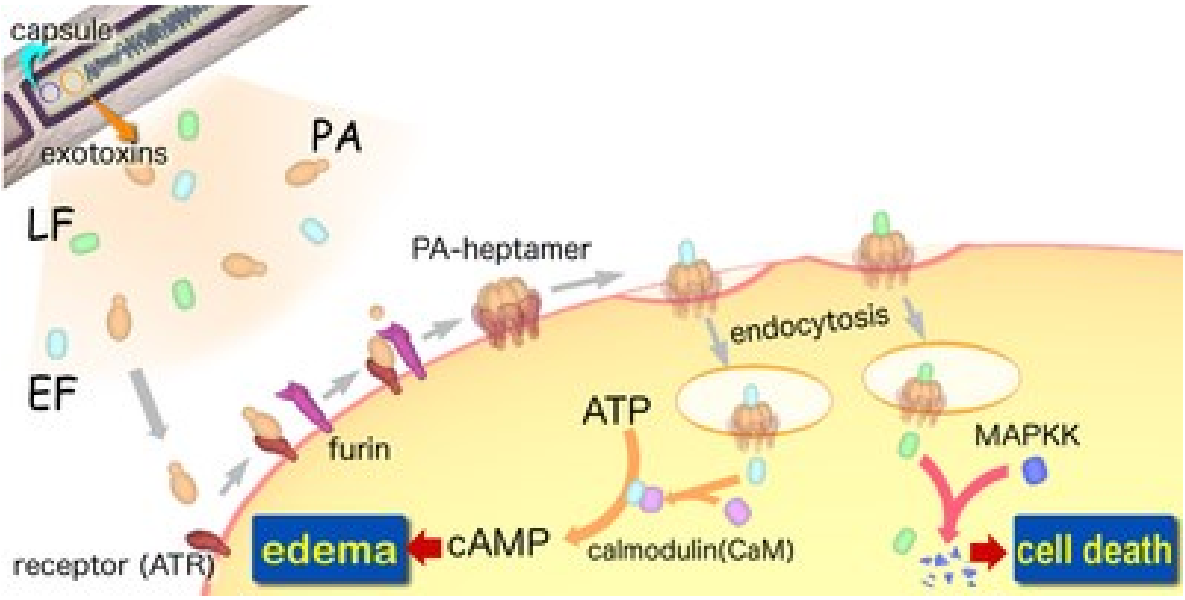

Figure 1-2: Diagram Illustrating the Mechanism of Anthrax Toxin Action in the Host: The figure depicts the sequential steps of anthrax toxin's effect within the host. It begins with the protective antigen (PA) binding to anthrax toxin receptors (ATR), followed by its proteolytic activation and formation into a heptamer. This structure can bind to either the lethal factor (LF), disrupting the host's MAPKK pathways and leading to macrophage death, or to the oedema factor (EF), which converts ATP to cAMP, deactivating host phagocytes and causing edema. The toxins are then internalized by endocytosis and translocate into host cells, undermining the host's immune defenses.Obtained from https://en.wikipedia.org/wiki/Anthrax_toxin.

1.8 Chromosomal Virulence Factors

Beyond the virulence factors encoded by plasmids, *B. anthracis* also expresses certain chromosomal factors, including phospholipases, metalloproteases (InhA1, NprB, and HtrA), siderophores, and cholesterol-dependent cytolysin O (Heffernan et al., 2007, Chung et al., 2008, Koppisch et al., 2005, Shannon et al., 2003, Drobniewski, 1993a). In contrast to *Bacillus* species like *B. cereus senso stricto* and *B. thuringiensis*, where the PlcR transcriptional regulator controls

chromosomally located virulence factors, *B. anthracis* displays a unique pattern due to a plcR gene nonsense mutation (Drobniewski, 1993a). This mutation eliminates functional PlcR and deactivates the PlcR regulon (Agaisse et al., 1999). When PlcR is artificially activated in *B. anthracis*, there is an increase in the expression of phospholipases, proteases, and hemolysins, but this does not affect toxin production or sporulation in strains with the pXO1 plasmid (Sastalla et al., 2010). This highlights the intricate and specific regulatory mechanisms governing virulence in *B. anthracis*.

1.8.1 Roles in Pathogenesis

Anthrax toxins play crucial roles in two main phases of infection. Initially, they modulate the host's immune response to ensure the pathogen's survival and spread. In later stages, they target specific tissues, leading to fatal systemic disease (Moayeri and Leppla, 2004).

Research using mice lacking PA receptors in certain tissues has illuminated anthrax toxins' significant functions in infection's early and advanced stages (Liu et al., 2010). Mice without myeloid cell receptors show anthrax resistance, highlighting the toxins' critical role in targeting innate immune cells for *B. anthracis* proliferation (Liu et al., 2010). The broad impact of LF and EF on immune cell pathways affects nearly all cell types (Guichard et al., 2012, Moayeri and Leppla, 2009).The toxins' acute effects on innate immune cells like neutrophils, macrophages, and dendritic cells are crucial (Wright and Mandell, 1986, Szarowicz et al., 2009, Agrawal et al., 2003). They influence neutrophil activation, chemotaxis, chemokine production, and recruitment inhibition in some mouse strains (Szarowicz et al., 2009).Toxins cause cell cycle arrest and death in macrophages and dendritic cells, especially following MEK signaling inhibition, and disrupt proinflammatory cytokine responses (Tournier et al., 2005, Agrawal et al., 2003). They also hinder chemotaxis, potentially by affecting the cytoskeleton, impairing macrophage phagocytosis (Yeager et al., 2009, Szarowicz et al., 2009).

LT and ET disable the innate immune response at the infection site, facilitating bacterial persistence, growth, and spread (Molin et al., 2008). Regardless of infection route, systemic anthrax involves significant bacilli proliferation in lymph nodes and blood (Molin et al., 2008). with high toxin levels contributing to late-stage lethality (Moayeri and Leppla, 2009).

Early rodent studies with toxins that were purified indicated that LT and ET cause vascular shock with distinct features (Moayeri et al., 2005). LT triggers cytokine-independent vascular collapse with hypoxic necrosis, while ET causes widespread vascular dysfunction and bleeding (Firoved et al., 2005). Tissue-specific receptor-knockout mice have helped identify the organs targeted for lethal shock (Firoved et al., 2005, Moayeri et al., 2015). LT primarily targets cardiomyocytes and smooth muscle cells (Moayeri et al., 2015). Despite previous assumptions about endothelial cells, studies show LT's impact on these cells' cytoskeletal changes and barrier function (D'Agnillo et al., 2013). Mice lacking endothelial PA receptors show the same LT susceptibility level, while those with endothelial-exclusive receptors resist toxin and spore infection (Cui et al., 2006, Newman et al., 2010a)., suggesting endothelial cells might not be the primary LT-induced vascular collapse contributors.

Apart from cardiovascular effects, toxins exhibit diverse in vivo effects related to MEK signaling inhibition and cAMP induction (Webster and Sternberg, 2005, Moayeri et al., 2005, Langer et al., 2012). Webster and Sternberg (2005). LT impedes glucocorticoid receptor-mediated gene activation via the MEK 3/6-p38 pathway, affecting early immune responses and LT susceptibility (Moayeri et al., 2005). It causes lung epithelial injury through MEK3 inhibition (Lehmann et al., 2009), and disrupts human epithelial barrier function (Langer et al., 2012). LT might also target the nervous (van Sorge et al., 2008) and gastrointestinal systems (Okugawa et al., 2011), and affects megakaryopoiesis and erythropoiesis (Chen et al., 2013).

While ET's broad effects through cAMP are presumed, its in vivo impact is less studied (Sastalla et al., 2012). It upregulates PA receptors in mice and alters circulatory protein pharmacokinetics, influencing Bacillus anthracis virulence factor concentrations in the bloodstream (Sastalla et al., 2012).

1.9 Host immunity against *B. anthracis*

The enzyme-linked immunosorbent assay (ELISA) has been utilized to detect antibodies produced as a result of either experimental or natural infections involving PA, LF, and EF proteins derived from the pXO1 plasmid of *B. anthracis* (Ivins and Welkos, 1988, Turnbull et al., 1992a, Hanson et al., 2006, Cizauskas et al., 2014, Lembo et al., 2011a, Ochai et al., 2022). Among these proteins, PA holds particular significance because *B. anthracis* strains that are missing the pXO1 plasmid do not provide immunity and are nonpathogenic to most species, except for mice (Leppla et al., 2002).). In order to establish immunity against anthrax, the host needs to either resist the development of the disease or slow down its progression (Mahlandt et al., 1966).

To hinder the advancement of the anthrax, it's vital for the animal to possess antibodies that specifically target PA. The harmful effects and pathogenesis of *B. anthracis* are linked to PA binding to LF and EF, facilitating their entry into the internal environment of host cells (Leppla et al., 2002). The most crucial immunogen in the majority of anthrax vaccines is PA (Ivins et al., 1994), and antibodies specific against PA have been shown to impede spore germination, inhibit that action of the toxin and potentiates the effects of macrophages against the pathogen (Welkos et al., 2001).

Various wildlife species have been found to produce anti-PA antibodies after exposure to sublethal doses of *B. anthacis* spores (Turnbull et al., 1992a, Cizauskas et al., 2014, Lembo et al., 2011a, Ochai et al., 2022). Although antibodies against EF and LF have been observed in specific animal species (Turnbull et al., 1992a), the degree to which EF and/or LF offer immunity and

confer protection remains poorly understood (Taft and Weiss, 2008, Dumas et al., 2020). Several studies have shown that passively transferring a neutralizing polyclonal antibody or monoclonal antibody (MAb) can protect cells against anthrax toxin or bacterial challenge (Beedham et al., 2001, Kobiler et al., 2002, Brossier et al., 2004, Dumas et al., 2017). When a combination of PA-neutralizing antibody and LF-neutralizing antibody is utilized, the efficiency of neutralizing antibodies in protection rises dramatically (Brossier et al., 2004). However, because the current licensed anthrax vaccine is mostly composed of PA, PA has been the key emphasis for passive protection. Only a few LF-neutralizing MAbs, on the other hand, have been discovered (Zhao et al., 2003, Little et al., 1990, Lim et al., 2005)(13, 24). Although their epitope specificities and mode of neutralization have not been completely investigated, these MAbs have shown interference with LF binding to PA (Lim et al., 2005). Mahlandt et al. (1966) have described that both EF and LF can augment the ability of anti-PA antibodies to protect some experimental animals. Immunization with antigens that contain LF and PA binding regions has shown more effective and wider protection compared to using only PA (Baillie et al., 2010), and in mice immunized with PA, using EF as an adjuvant has been observed to heighten the anti-PA IgG response (Quesnel-Hellmann et al., 2006).

1.10 Vaccine and Control

In the 1850s, Robert Koch discovered that anthrax was caused by bacteria (Hambleton et al., 1984). In 1881, Pasteur utilized *B. anthracis* as the first bacterial vaccine (Turnbull et al., 1991, Leppla, 1982, Leppla et al., 2002). Capsules could form in the heat-attenuated *B. anthracis* culture, but not toxins (Sterne, 1959, Farrar, 1994). In comparison to toxigenic vaccine strains created years later, such as the Sterne strain 34F2 which lacked a capsule but expressed the toxin, the Pasteur vaccination offered a lesser level of protective immunity (Sterne, 1945). The attenuated strain of Sterne, which was used in widespread immunization beginning in the 1930s, nearly eradicated anthrax in highly

developed nations (Leppla et al., 2002). By 1939, the Sterne vaccine already established its efficacy and safety in the treatment of domesticated animals, and it appeared to offer full immunity against challenge with extremely virulent *B. anthracis* strains (Sterne, 1937, Sterne, 1939). As vaccination is not long lasting, there is therefore need for repeated vaccinations. Live attenuated strains are not recommended for use in human vaccines, despite their success with animals (Hambleton et al., 1984). Although, live attenuated vaccines have been used in China (Zai et al., 2016, Clark and Wolfe, 2020) and Russia (Shlyakhov and Rubinstein, 1994, Firstova et al., 2021). Vaccines devoid of cells and containing anthrax protective antigen have been manufactured and authorized for human use in both the United Kingdom and the United States (W.H.O., 2008b). A study discovered that after two doses of Sterne live spore vaccine given 4-5 weeks apart, with the first dosage given at 5-9 weeks of age, antibody titres had dropped to barely detectable levels by 5-6 months after the second treatment (W.H.O., 2008b). An assessment of protective antigen (PA) antibody levels in zebras vaccinated with Sterne Live Spore vaccine in Namibia's Etosha National Park revealed the necessity for two initial injections roughly 8 weeks apart to develop consistently quantifiable antibody titers (Lindeque and Turnbull, 1994a, W.H.O., 2008b). The drop in titers one year after the second booster indicates that the third booster should not be used. Anthrax management in industrialized nations has mostly been achieved through vaccine campaigns and sound agricultural practices in livestock, but it remains a significant issue in many underdeveloped nations (Farrar, 1994). Furthermore, anthrax management is complicated by outbreaks in herbivores in wildlife which mostly cannot be protected by vaccination. A good example is the anthrax outbreak in the Arctic that led to the death of 2000 reindeer and hospitalization of 90 residents and the death of one child was attributed to increasing temperature (climate change) as well as cancellation of reindeer vaccination (Liskova et al., 2021).

Carbosap vaccine strain (Adone et al., 2002) has both plasmids pXO1/pXO2. Although whole-genome sequencing of the strain indicated three chromosomal

deletions of 29, 24, and 3.5 kb in comparison to completely virulent strains (Adone et al., 2002), the precise reason of the Carbosap strain's attenuation remains unexplained, although it is still widely used.

1.11 Anthrax Distribution

Anthrax is present endemically in regions spanning Asia, Sub-Saharan Africa, certain areas of Australia, Southern Europe, northwest Canada, Central and South America, the United States, Russia, and Haiti, however, sporadically occurs in other regions (Smith et al., 2000, Keim et al., 1997). In some other countries, occasional outbreaks may occur, typically linked to the importation of diseased animal materials (Bales et al., 2002). OIE Handistatus II lists southern Africa as one of the areas where anthrax is frequently endemic or epidemic. And southern Africa is, further hypothesised as the geographic source of anthrax (Keim et al., 1997). In South Africa, there are currently two areas where anthrax is consistently present: the Kruger National Park (KNP) and the Ghaap area in the Northern Cape Province. Annual reports submitted to the OIE indicate that anthrax outbreaks affect both domestic and wild animals in South Africa. It is believed that there is an issue of underreporting in this region, especially concerning animals, primarily because of inadequate surveillance and challenges in monitoring animals that roam freely. Consequently, in many cases, the official reports significantly underestimate the actual occurrence of anthrax (OIE handistatus II).

Figure 1-3: A map showing the sites where carcasses tested positive for anthrax, revealing the spread of vulnerable species, were identified on farms during the anthrax outbreak in the Ghaap area in the Northern Cape Province, South Africa, in 2008. Adopted from (Hassim et al., 2017)

1.12 Anthrax distribution in Kruger National Park

In the northernmost region of South Africa's KNP, anthrax is endemic. Among the various host species, greater kudu has historically shown the highest susceptibility, accounting for approximately 75% of documented cases from the 1960s to the 1990s (De-Vos and Bryden, 1996, Hugh-Jones and De Vos, 2002, Ochai et al., 2022). Outbreaks in KNP appear to follow almost ten-year cycles, often linked with dry seasons or droughts (Pienaar, 1960, Pienaar, 1961, De-Vos, 1990, De-Vos and Bryden, 1996, Hugh-Jones and De Vos, 2002). However, this pattern has somewhat shifted, and impala, a herbivore known for both grazing and browsing, now experiences a higher impact during annual outbreaks, especially in the rainy season (Basson et al., 2018a). Zebra and wildebeest which are both grazers account for only 10% of anthrax-related deaths in KNP as

against 90% in Etosha National Park (ENP) (Ochai et al., 2022). It is suggested that browsers, such as kudu, are exposed to *B. anthracis* spores when foraging on leaves contaminated by blowflies (Basson et al., 2018a, Hugh-Jones and De Vos, 2002). There are no available studies on the variation in susceptibility across kudu, impala, zebra and wildebeest. However, studies have demonstrated that kudu and impala in KNP are the most affected anthrax as compared to their conspecifics in Etosha National Park (ENP), Namibia (Turner et al., 2013, Havarua et al., 2014, Ochai et al., 2022). These findings suggest that different host species exhibit variations in their exposure to and the development of adaptive immunity against *Bacillus anthracis* in the two mentioned parks.

Anthrax transmission in wild herbivores occurs when an infected animal dies, releasing into the environment *B. anthracis* spores that can persist for a long time, which are then ingested by other animals. The transmission of anthrax occurs through various factors and vectors. In herbivorous wild animals and domestic stock, the primary mode of transmission is through plants contaminated with anthrax spores which could be attributed to ulcers along the mucosal layer of the gastrointestinal tract (Turnbull, 2008a, Turner et al., 2014a, Turner et al., 2016). Additionally, scavengers like hyenas (*Crocuta crocuta*), vultures (*Gyps africanus*), lions (*Panthera leo*), ravens (*Corvus albicollis*), wild dogs (*Lycaon pictus*), and wild cats (*Felis lybica*) can contribute to the dissemination of anthrax when they access carcasses (Pienaar, 1967, Ebedes, 1976a, Turnbull and Snoeyenbos, 1989, W.H.O., 2008b). The scavenging activity exposes the vegetative bacilli to oxygen, which promotes sporulation and makes the spores accessible in the soil, leading to further ingestion and dispersion (Pienaar, 1967, Hugh-Jones and De Vos, 2002). It is of interest to note that even in the absence of vertebrate scavengers, vegetative cells can still escape into the environment (Bellan et al., 2013). Moreover, insects, particularly blowflies, play a role in the spread of anthrax, most especially within the browsing population of wildlife species. Blowflies feed on carcasses and subsequently deposit *B. anthracis* on nearby tree and shrub leaves(Braack and De Vos, 1990b),, which are then

consumed by animals like kudu (Braack and De Vos, 1990b, Basson et al., 2018a). This pathway of infection via blowflies has been suggested as the primary mode of infection for browsers (Blackburn et al., 2010a, Hampson et al., 2011b, Basson et al., 2018a, Hugh-Jones and De Vos, 2002). As mentioned in the introduction, various environmental factors affect the transmission and spread of *B. anthracis*.

1.13 Environmental factors and climate events that influence persistence of anthrax spores and susceptibility of host species

A study carried out in temperate, boreal, and arctic environments found a clear connection between increasing temperatures and heightened suitability for anthrax, along with a correlation between this adaptability and a balance of water and soil that results in mild to moderate water stress (Walsh, de Smalen et al. 2018). Further research has demonstrated that seasonal fluctuations significantly influence the occurrence of anthrax in different regions (Chikerema, Pfukenyi et al. 2012, Turner, Imologhome et al. 2013, Nsoh, Kenu et al. 2016, Barandongo, Dolfi et al. 2023).

Numerous studies have been conducted to investigate the association between environmental factors and anthrax encompassing soil composition, pH, temperature, and humidity. Further research has also highlighted the significant influence of seasonal variations on anthrax occurrences in various regions (Chikerema et al., 2012, Nsoh et al., 2016, Turner et al., 2013, Barandongo et al., 2023). As per a study, most anthrax outbreaks tend to occur as the dry season nears its end and the rainy season begins. This pattern presumably compels animals to graze at lower heights, thereby increasing the risk of anthrax transmission (Nsoh et al., 2016). Nevertheless, contrasting findings from Etosha suggest that close grazing can also take place during the wet season, as there is higher soil contact at that time, higher temperature and soil moisture (Havarua et al., 2014, Davies, 1960). Alterations in habitat selection driven by forage conditions can contribute to a decreased likelihood of animal exposure to anthrax,

particularly during dry or drought conditions (Huang et al., 2021).. In periods of reduced forage availability, herbivores may modify their grazing patterns and move to areas with better vegetation or water sources, potentially avoiding regions where anthrax spores are concentrated.

The current rainfall patterns and hydroclimatic conditions seem to have an influence on anthrax outbreaks in the KNP regions (De-Vos and Bryden, 1996). According to this study, it is suggested that the disease typically emerges towards the conclusion of the winter season, allowing *B. anthracis* spores to persist in the KNP due to the dry climatic conditions (Steenkamp et al., 2018). However, outbreaks have also been linked to floods, that may be associated with the disturbance and exposure of spores from the ground (Lewerin et al., 2010).

Several studies have investigated the impact of precipitation, temperature, and soil type on anthrax occurrences. Annual precipitation of 100 mm and above, as well as temperatures over 15.5°C, are favorable for the existence of the spores (Van Ness, 1971, Blackburn et al., 2007a, Steenkamp et al., 2018) (Table 2-1). Furthermore, soil pH higher than 6.1 has been recognized as a significant determinant in spore survival and persistence (Van Ness and Stein, 1956, Nsoh et al., 2016).

Table 1-1: Summary of the effect of environmental factors and climate events on *B. anthracis* spore survival and disease outbreak

Environmental factors/Climate parameters	**Environmental/Climate conditions**	**Elements of Anthrax**	**References**
Temperature	Warmer temperatures	Anthrax suitability	(Walsh et al., 2018, Steenkamp et al., 2018)

Rainfall	Late dry season/early rainy season, drought, flood	Anthrax outbreaks	(Chikerema et al., 2012, Nsoh et al., 2016, Stears et al., 2021)
Soil type	Rich in organic matter	Spore survival	(Hugh-Jones and Blackburn, 2009)
Soil chemistry	Calcium, pH	Spore germination	(Minett and Dhanda, 1941a, Van Ness and Stein, 1956, Nsoh et al., 2016)

1.14 Climate Events in Kruger National Park

Climate change events pose a very considerable threat to the South Africa's biodiversity, health, food security and safety, water resources and the entire ecosystem (Ziervogel et al., 2014). It has also been reported that climate change the country is already leading to alteration in temperature (Davis and Vincent, 2017), in the patterns of rainfall, increased level of flood and aridity as well as changes in the hydrological cycles and these may have effects on biodiversity and agricultural activities (Serdeczny et al., 2017). Increase in CO_2 promotes woody plant growth, which could affect habitats for browsers (García Criado et al., 2020) and these changes can alter animal/animal as well as animal and plants interactions, which would be relevant to this system (McCluney et al., 2012). These events in turn result in an increase in pollutants that affect water and food sources, increase vectors' activities and increase in the impact of pathogens (Anwar et al., 2019, Kurane, 2010). Increased temperature and decreased rainfall

were described to possess deleterious impacts on overall health (Thompson et al., 2012, Ziervogel et al., 2022, IPBES, 2023).

The most significant climate events that require substantial attention worldwide include drought, increase in temperature, change in rainfall events, storms and floods (Kurane, 2010). Global warming has become an important concern worldwide and most importantly in southern Africa. This is with the attendant effects of increase in mean annual temperatures by 1.5 times more than the global average of 0.6˚C over the last 50 years (Engelbrecht et al., 2015). This is increasingly becoming a concern as reported by Jury (2012) that unlike what was reported by the IPCC Assessment Report Four (AR4) consensus was for the fast heating of the Kalahari plateau, the coastal plains will warm even faster. Also, noted occurrence of exceedingly high maximum temperatures have been on the increase across South Africa (Kruger and Sekele, 2013), this trend also reflects in both the mean minimum and maximum temperatures (van Wilgen et al., 2016).

In KNP, Zambatis and Biggs (1995) studied the increase in temperature by considering the mean daily maximum, minimum and average temperatures for all the months. For each month they reported a significant increase in some months and also for the number of the days (up to 15 days) for some of maximum temperature categories. In Skukuza section of KNP, it was reported that an analysis of the temperature data from 1960-2001 showed that the minimum temperature had more positive trends than the maximum temperature and this agreed with the findings of Easterling et al. (2000).

Droughts are projected to increase in frequency under climate change, and they already have significant impacts on the KNP (Zambatis and Biggs, 1995). Droughts are known to have devastating effect on animals such as the large herbivores. This is as a result of reduced biomass of grass which in turn results to increased death (Ferreira et al., 2019, Smit et al., 2020), although droughts are considered to be a necessary and natural phenomenon in regions that are arid

or semi-arid in nature but an it increase in the frequency and severity is of concern (Malherbe et al., 2020). Long and multiple periods of drought tend to exacerbate a wide number of factors such as the survival of herbivores, birth rate (Ferreira et al., 2019) and also biomass (Wigley-Coetsee and Staver, 2020). Drought also leads to increased fire risk (Littell et al., 2016), and fires can have important impacts on grass-tree relationships in savannas (Neary and Leonard, 2020), which would in turn affect browsers, their behaviour, and thus disease.

In KNP, there has been a noticeable trend over the decades showing a decrease in rainfall leading to periods of drought - these trends include increase in temperature accompanied by heat waves which increase the effect of decreased rainfall (Zambatis and Biggs, 1995). Contingent upon the drought measuring criteria utilized, this results in certain indicators, showing expansions in droughts universally (Dai, 2013). Both noticed and anticipated temperature patterns indicate an increasing trend in the lowveld area (Kruger and Shongwe, 2004, Engelbrecht et al., 2015). Kruger and Shongwe (2004) noticed a consistent increase of 1.2°C for each century in the early highest temperature in the Skukuza area of KNP. These extreme temperatures serve to exacerbate the impact of the drought (Yuan et al., 2018) and in certain environments it has been shown that high temperatures can affect certain organic cycles, for example decreasing seedling endurance in zones lying between plains and forests (Will et al., 2013) and restricting the movement and activity time of herbivores such as antelopes due to hyperthermia (Shrestha et al., 2014). A number of devastating droughts have been reported in literature for KNP; some of which include the 1982/1983, 1991/1992 and the 2015/2016 (Malherbe et al., 2020). The 2015/2016 drought was linked with extremely high temperatures and heat waves in the summer rainfall areas and, most importantly, in the Lowveld (Swemmer, 2016, Swemmer et al., 2018, Malherbe et al., 2020). For the 1991/1992 drought, the most affected sections of KNP were the Lower-Sabie and the Pafuri sections, while the least affected were the Crocodile Bridge-Malelane area (Zambatis and Biggs, 1995).

Swemmer (2016) reiterated the negative impacts of droughts in the KNP where he observed dwindling vegetation, and the drying of the rivers, which in turn is leading to the death of hippopotamuses (*Hippopotamus amphibius*). He also reported on the impact of reduced vegetation, which also leads to the death of herbivorous grazers which include cattle in the rural grazelands and a number of large species of antelope in KNP. Also, a study conducted on the impact of drought on herbivores by foraging type revealed grazers and mixed feeders were more affected than browsers (Abraham et al., 2019). These impacts further elucidate the need to investigate the impact of drought on wildlife mortality and how this exacerbates anthrax mortality in particular.

1.15 Roles of hosts on vegetation and possible implication on anthrax transmission.

Ecosystems remain a complex network of interdependent living organisms and the common environment they inhabit (Wyk and Fairall, 1969). Host species play an important role in influencing the vegetation that compose the foundation of the ecosystem (Gaston and Fuller, 2008). Herbivores that include browsers, grazers and mixed feeders have significant influence on the composition, structure and dynamics of plant communities and their survival (Skinner and Chimimba, 2005, Gaston and Fuller, 2008). Herbivores, the primary consumers of plant matter, are key actors in the intricate ecological theater. Herbivores have evolved diverse feeding strategies that impact vegetation at multiple scales (Gaston and Fuller, 2008). Browsers, exemplified by, elephants, kudu, giraffes and deer, focus on shrubs and trees, shaping canopy structure and contributing to plant regeneration by seed dispersal (Gaston and Fuller, 2008, Wyk and Fairall, 1969). Grazers or mixed feeders, such as antelopes, African buffalo (*Syncerus caffer*), white rhinoceros (*Ceratotherium simum*), hippopotamus and bison, predominantly feed on grasses, regulating grass height and promoting a mosaic of habitats (Prins and Weyerhaeuser, 1987). However, unchecked herbivore populations can lead

to overgrazing, altering plant species composition and reducing vegetation cover (Wyk and Fairall, 1969).

Elephants are considered keystone species and have a significant impact on the ecosystems they inhabit, including national parks (Trollope et al., 1998, Coetzee et al., 1979). Their browsing and foraging behaviors can greatly influence vegetation dynamics. Elephants feed on a variety of plant species, including grasses, shrubs, and trees, often stripping bark, breaking branches, and uprooting trees in the process (Coetzee et al., 1979). While this behavior can lead to the creation of open spaces and provide opportunities for other species, it can also result in overbrowsing and habitat degradation in some cases (Trollope et al., 1998). Elephants play a crucial role in seed dispersal, which is fundamental to plant regeneration and maintaining genetic diversity. Large-bodied animals like elephants consume a wide variety of fruits and plant materials, effectively serving as "ecosystem gardeners" (Poulsen et al., 2021, Levin et al., 2003). Seeds that are ingested are often transported over considerable distances before being excreted (Poulsen et al., 2021). This process benefits plants by helping them colonize new areas, escape competition with parent plants, and evade seed predators (Levin et al., 2003). While elephants' herbivorous nature involves the consumption of plants, their browsing behaviors extend beyond mere feeding. Elephants have a tendency to strip bark and break branches, which can lead to both negative and positive effects on vegetation (Midgley et al., 2005, Thompson et al., 2022). Bark stripping can cause injury or even death to trees, but it can also create cavities that other animals use for shelter, affecting the habitat structure (Thompson et al., 2022). Elephants also influence fire dynamics within ecosystems. By creating open spaces and breaking down vegetation, they can hinder the spread of wildfires (Dublin et al., 1990, Ben-Shahar, 1996). In savannas, for example, elephants help maintain a mosaic of grassland and shrubland areas by limiting the encroachment of woody plants, which are more susceptible to burning (Moe et al., 2014).

Kudus are known for their dietary flexibility, an adaptation that enables them to thrive in diverse habitats (du Plessis and Skinner, 1987). This adaptability allows kudus to capitalize on the fluctuating availability of food resources, which can vary based on factors such as rainfall and habitat type (du Plessis and Skinner, 1987). Kudus are browsers, primarily consuming leaves, shoots, and fruits of shrubs and trees. However, their browsing preferences are far from indiscriminate (Owen-Smith and Cooper, 1985). Studies by Owen-Smith and Cooper (1985) also revealed that kudus exhibit selective feeding behaviors, often favoring certain plant species over others. This selectivity has implications for plant community dynamics and the distribution of plant species within the KNP (Owen-smith, 1990). In KNP, the majority of kudu's diet comprises mopane (Hooimeijer et al., 2005), which has suffered severe decline and damage from elephants. In KNP, kudus share their habitat with a diverse range of herbivores, each occupying a unique ecological niche (Owen-smith, 1990). Kudus' browsing behaviors often differ from those of other herbivores like elephants, impalas, and giraffes (Owen-Smith and Cooper, 1985). Understanding the interplay between these different foraging strategies is essential for comprehending the complex dynamics of vegetation in the park.

Impalas are mixed feeders but primarily grazers, feeding on a wide range of grasses and low-lying vegetation (Meissner et al., 1996). Their feeding behaviors influence the structure of plant communities by regulating grass height and density (Moe et al., 2014). In the Serengeti National Park it was shown that impalas, as bulk grazers, contribute to maintaining grassland ecosystems by controlling grass height, which in turn affects the abundance and diversity of other plant species (Sinclair et al., 2007, Sinclair and Norton-Griffiths, 1981). Although impalas are predominantly grazers, they also engage in browsing behaviors, consuming shrubs and young tree shoots (Meissner et al., 1996). These browsing activities can shape vegetation composition and structure. A study by Prins and Weyerhaeuser (1987) indicated that impalas' selective browsing on certain plant species can influence plant community composition, potentially affecting the success of tree regeneration and woody plant dynamics. Although impalas are

predominantly grazers, they also engage in browsing behaviors, consuming shrubs and young tree shoots (Moe et al., 2014). These browsing activities can shape vegetation composition and structure.

The impact of elephants and impalas on vegetation in national parks can vary based on factors such as population density, park size, and the availability of alternative food sources. In some cases, high elephant populations have led to habitat degradation and shifts in plant species composition, while in other cases, their activities have been found to enhance ecosystem diversity (Trollope et al., 1998).

Previous research in KNP has reported a notable reduction in the number of big trees, such as *Combretum apiculatum, Terminalia sericea, Senegalia nigrescens*, *Sclerocarya birrea* (marula), and *Colophospermum mopane* (mopane), accounting for approximately 80% of the trees (Wyk and Fairall, 1969). However, other studies have suggested that elephants might not pose a significant threat to trees (Coetzee et al., 1979). Nonetheless, a recent study noted considerable declines in woody vegetation within the park between 1960 and 1989 (Trollope et al., 1998) and suggested that this decline could be due to the combination of an increase in elephant density and a fixed triennial fire policy.

While the decline in woody cover in KNP is not solely attributed to elephants, as mentioned earlier, other species such as impala have been identified as agents of change. Previous research has indicated that impala and elephants play critical roles in the transition and transformation from woodland to shrubland (Moe et al., 2015). This interplay between species becomes especially significant for browsers like kudu, which depend on woodlands for foraging, and for the transmission pathway of anthrax-blowfly (De-Vos, 1990, Basson et al., 2018b).

We therefore hypothesize that elephants' influence on browse during the dry season may further restrict the food available to kudus and/or lower blowfly distribution as vegetation cover has been described to play a part in the distribution of *Chrysoma spp* (Basson et al., 2018a) that may increase the danger

of impala exposure during drier seasons. Also, the impala's mix-feeding behavior may have had a role in the observed shift from kudu to impala.

1.16 Diagnosis and Identification of *Bacillus anthracis*

Definitive diagnosis of anthrax is often done through the use of bacterial culture and the presence of pathognomonic signs associated with this disease (Bagamian et al., 2013). For the confirmatory diagnosis of an anthrax suspected carcass the initial step is the examination of blood smears stained with either Giemsa or Gram stains. For the capsule, the existence of a square-ended capsule that reacts to the polychrome methylene blue stain indicates the presence of *B. anthracis* and requires that a sample to be delivered to a accredited laboratory for confirmation (Turnbull, 1999). To confirm the existence of *B. anthracis* in the reference laboratories, the samples are inoculated onto sheep blood agar to check for the morphology of the colony, the absence of hemolysis and sensitivity to penicillin and bacteriophages (Turnbull et al., 1998, Turnbull, 1999). Additional testing by means of PCR for the presence of both the capsular and protective antigen genes that encodes for the toxins is necessary for confirmation (Turnbull, 2008a). The recommended test for the confirmation of this pathogen is by the use of the assay based on qPCR fluorescence resonance energy transfer (FRET) that targets the PA on the pXO1 plasmid, and the capsule coding region (*cap*C) on the pXO2 plasmid and the chromosomal small acid soluble proteins markers (W.H.O., 2008b). However with the identification of *B. cereus* var *anthracis* and pneumonia anthrax caused by *B. cereus* with pXO1, real time PCR (qPCR) using genomic island GI4 region that differentiate *B. cereus* var *anthracis* from *B. anthracis* has been used together with BA-1 and *lef* by (Blackburn et al., 2014, Zincke et al., 2020).

1.16.1 Overview of sample collection from host

To diagnose anthrax, the isolation of *B. anthracis* is performed using clinical samples like blood, tissue from organs, samples of fluids from skin lesions, and aspirates from lymph nodes or the spleen. It's important to take precautions when collecting samples from anthrax-infected carcasses to prevent both infection of humans and contamination of the environment, which can occur due to bacterial sporulation upon exposure to air (W.H.O., 2008b). Blood smears are a method for examining blood from various parts of the animal's body, such as limbs, mammary glands, ears, and other peripheral veins, without the need to dissect the carcass (Hugh-Jones and De Vos, 2002). If blood collection is difficult, blood may be drawn from the nasal cavity. Microscopy is used to inspect the morphology of *B. anthracis* in the blood smear.

1.16.2 Microscopy

Blood smear from the host or haemorrhagic blood from orifices can be used for microscopy as well as culturing (W.H.O., 2008b). Microscopic identification of *B. anthracis* that is Gram-stained reveals a Gram-positive bacilli (rod-shaped) that can be observed alone, in pairs, or chains (W.H.O., 2008b). Gram stain becomes increasingly difficult following further decay of the carcass hence leading to inaccurate results (W.H.O., 2008b). Polychrome methylene blue (MacFadyean stain) is the preferred stain for visualising smears emanating from decayed samples. The smear is deemed positive when the bacilli look blue and are encompassed by a pinkish-red capsule while the capsule appears reddish-mauve with Giemsa stain (W.H.O., 2008b). To visualise the capsule of *B. anthracis* culture media used for growing it must include 0.7% sodium bicarbonate and be incubated in the presence of carbon dioxide (Fasanella et al., 2010, W.H.O., 2008b).

1.16.3 Culture

Bacillus anthracis thrives under laboratory conditions in artificial media. *B. anthracis* can be isolated from uncontaminated fresh specimens, serum, or blood media using nutrient agar (Turnbull et al., 1998). It is crucial to use selective medium for isolating *Bacillus* species that are closely related. The recommended selective medium for *B. anthracis* is the PLET agar (polymyxin-lysozyme-EDTA-thallous acetate) for isolation from soil and other environmental samples with may contain other bacteria (Knisely, 1966). PLET agar plates are incubated at 37 °C for up to 48 hours, whereas other agar cultures such as serum, blood, or nutrient agar are incubated for 12 to 24 hours (Knisely, 1966).

Typically, when *B. anthracis* is identified at reference laboratories, certain characteristics are examined, such as colony morphology, the presence of a capsule, the absence of hemolysis activity, sensitivity to penicillin, and sensitivity to bacteriophages (Turnbull et al., 1998). However, it should be noted that certain strains of *B. anthracis* may exhibit resistance to penicillin or bacteriophages, as well as hemolytic activity (Drysdale et al., 2004a, Odendaal et al., 1991, Coker et al., 2002).

1.16.4 Biochemical tests

Bacillus species can be identified using a variety of commercial biochemical assays. Biochemical features such as catalase, citrate, oxidase, urease, indole, Voges-Proskauer, and nitrate are evaluated in these assays. The API 50CHB technique, which is based on the acidification of 49 carbohydrates, is also used to identify *Bacillus* species. The Biolog system is effective for identifying *Bacillus* species and producing data for comparison with *B. anthracis* (Baillie et al., 1995).

1.16.5 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction is employed as a means to detect the presence of virulence factors, specifically the genes encoding capsule and toxins, found on plasmid genes, as well as chromosomal gene markers (Turnbull et al., 1998). To confirm the presence of *Bacillus anthracis*, specific genes such as the protective antigen (*pag* gene also refer to as BAPA), edema factor (*cya*), lethal factor (*lef*), and various capsule genes (*capB*, *capC*, and *capA*) are utilized (Beyer et al., 1995, W.H.O., 2008b). However, it is worth noting that the primers used for diagnostics for the capsular genes were observed to generate non-specific reactions with different *Bacillus* species (Beyer and Böhm, 2003).

The identification of *B. anthracis* involves the use of several chromosomal markers, such as Ba813, *sasp*B, *rpo*B, *gyr*A, *plc*R, *adk*, and Ba-1 (Ramisse et al., 1999, Qi et al., 2001, Hurtle et al., 2004, Easterday et al., 2005, Blackburn et al., 2015, Blackburn et al., 2014). However, it should be noted that the markers *rpo*B, Ba813, and genes such as of the S-layer chromosomal proteins often produce inconsistent false positive outcomes when analyzing samples of environmental origin (Ramisse et al., 1999, Papaparaskevas et al., 2004).

Quantitative Polymerase Chain Reaction (qPCR) is a valuable molecular biology technique utilized for diagnosing and detecting various pathogens, including *B. anthracis*. Additional qPCR tests are required to confirm the presence of specific virulence-related genes in *B. anthracis*. These tests target genes such as pagA/BAPA (located on pXO1), *lef* (located on pXO1), *capB* (located on pXO2), and Ba-1, encoding factors contributing to virulence, including toxin production, capsule formation, and chromosomal content specific to *B. anthracis*.

In Lekota (2018) previous work, qPCR assays focused on the BAPA (located on pXO1) and capC (located on pXO2) regions. However, it was observed that the capC target did not effectively distinguish closely related Bacillus species

from B. anthracis, unlike the capB target reported by Zincke et al. (2020), emphasizing the need for more precise markers. While the Ba-1 marker appears distinctive to *B. anthracis*, its validation has been limited to *B. cereus sensu stricto* strains, similar to *lef* validation (Blackburn et al., 2015, Zincke et al., 2020).

1.17 Serological techniques

Relying merely on mortality statistics or the detection of dead animals in the wild can frequently result in a distorted view of the most affected wildlife host and the level of exposure (Bellan et al., 2012). Except during severe outbreaks, wildlife anthrax investigations are unusual (Bagamian et al., 2013). Serological techniques, which are based on antigen-antibody interaction, are seldom employed for antemortem diagnosis of virulent zoonoses such as anthrax (Jacobson, 2007). Antibodies to the protective antigen are thought to be the most reliable approach for detecting prior anthrax exposure status (Turnbull et al., 1992a).

Over time, various serological methods have been utilized to determine previous disease exposure in African animals. These methods include the complement fixation test (CFT) and agar gel immunodiffusion (AGID) test (Blackburn and Swanepoel, 1988), as well as the enzyme-linked immunosorbent assay (ELISA) employed in previous studies (Turnbull et al., 1992a, Lembo et al., 2011b, Kock et al., 1992). The enzyme-linked immunosorbent assay (ELISA) was invented by Engvall and Perlmann (1971) and relies on an antigen-antibody (Ag-Ab) very specific interaction for detection.

1.17.1.1 Agar-gel difussion test

Organ Ouchterlony invented the agar-gel diffusion method in 1953. Thorne and Belton (1957) used it to measure the concentration of antigen and antibody in culture filtrates and antisera from animals that were vaccinated with what ?. Using

cork borers in Petri plates, circular wells are created in solidified agar. Following that, the innermost wells are filled with undiluted antiserum that are specific to the target antigen. Outer reservoirs holding diluted test antigens in saline are introduced after an overnight incubation at 2°C. After another 20-24 hours of incubation, the plates are inspected for precipitation lines, which are produced when these immune complexes precipitate as a result of the diffusion and interaction of antigen and the antiserum.

The endpoint refers to the highest dilution level that results in a discernible line of precipitation. The test serum is mixed with a standardized antigen previously prepared in order to establish the antibody titre of test serum. The ability of the test serum to inhibit the formation of a precipitation line, when compared to a standard antiserum, is assessed using agar diffusion plates. In essence, this assay employs an inhibitory format. While this method is relatively simple and cost-effective, it lacks sensitivity and often yields low titres, such as 1/16, even for supposedly hyper-immune antiserum preparations. Moreover, visually determining endpoints of the same sera can lead to inconsistencies and lack of reproducibility when monitored by various technicians (Ray and Kadull, 1964).

1.17.1.2 Indirect microhaemagglutination test

Buchanan et al. (1971) developed a novel approach for identifying anthrax antibodies in serum samples in 1971. This method, known as anthrax indirect microhaemagglutination (IMHA), was a major advancement over the old AGID assay. IMHA provided better sensitivity and significantly quicker testing capabilities (Johnson-Winegar, 1984). IMHA required screening inactive complement test sera, which led in the agglutination of PA-sensitized sheep red blood cells (Buchanan et al., 1971). The existence of a smooth layer of red blood cells covering the whole bottom of the microtitre wells during haemagglutination characterized the responses as complete, partial, or negative. If a serum sample exhibited a four-fold greater titre of haemagglutination in antigen-absorbed red blood cells than any control, it was thought to indicate anthrax PA reactivity.

Despite being the favoured test in the United States in the 1970s (Turnbull et al., 1986), the IMHA encountered severe obstacles that hampered its worldwide acceptance as the standard assay for anthrax antibodies in animal serum. The advantages of this test were lessened by issues such as inability to reproduce results, variations in erythrocytes of sheep, and the instability of antigen-erythrocyte mixes (Johnson-Winegar, 1984).

1.17.1.3 Toxin neutralisation assay

Antibodies that neutralize toxins also are critical in providing protection to counter anthrax within the host (Ngundi et al., 2012). The toxin neutralisation assay (TNA) is employed to assess the serum's ability to counteract the harmful effects of LT and ET on cells in a laboratory setting (Ngundi et al., 2010, Ngundi et al., 2012). Unlike ELISA, which detects the overall anti-PA IgG antibody levels, TNA specifically measures the functional subunit of these antibodies (Ngundi et al., 2012). The toxin neutralisation assay has become increasingly important, especially in the context of *B. anthracis*, as a way to measure the protective level of antibodies against its toxins. This *in vitro* assay, described by Hering et al. (2004), aims to determine the ability of specific anthrax antibodies to safeguard macrophage cells (J774A.1) of mice from the harmful effects of LT. In this test, a colorimetric agent, such as 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), is often employed, which turns into a dye signaling either cell proliferation or cell death (Figure 1-4). This approach is extensively used (Hansen et al., 1989). Previous studies have indicated that the lethal toxin is responsible for animal mortality and can cause the lysis of certain cell lines in vitro, as observed in mice used to assess the neutralizing effects of monoclonal and polyclonal antibodies against anthrax PA (Friedlander, 1986).

This assay has further been validated in various forms (Hering et al., 2004). Different cell lines sensitive to the lethal toxin have been used in this assay. Hanna et al. (1993), for example, used the RAW264 cell line to highlight the sensitivity and involvement of macrophages in anthrax, although the J774A.1

macrophage cell line has also been widely used owing to its sensitivity to the LT(Friedlander, 1986, Hering et al., 2004).

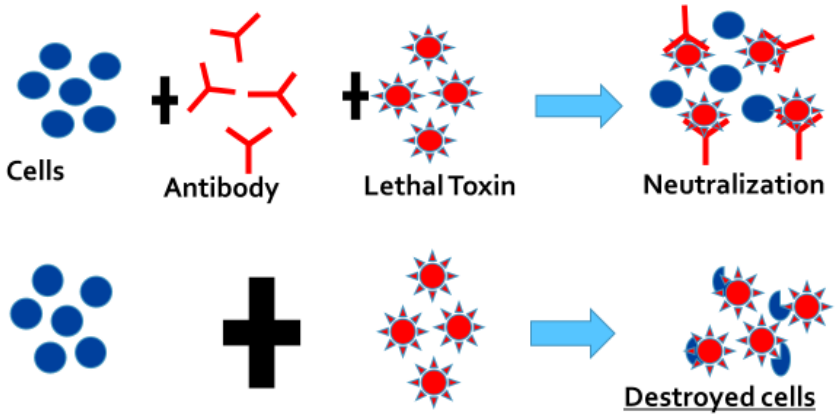

Figure 1-4:Explaining the Toxin Neutralization Assay: In this method, antibodies pre-incubated with lethal toxin are introduced to pre-seeded cells. The subsequent measurement of cell neutralization or death is conducted using a colorimetric agent. The presence of antibodies capable of neutralizing the lethal toxin is revealed by the colorimetric signal observed in the cells.

1.18 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immonosorbent assay also known as enzyme immunoassay (EIA), uses specialized plates to detect and quantify antibodies and other substances (Voller et al., 1974). Engvall and Perlmann introduced this technique for detecting antigen-antibody (Ag-Ab) interactions as an alternative to the radioisotope method (Engvall and Perlmann, 1971). For recognizing antibodies to PA, Anna Johnson-Winegar tailored and adopted the ELISA (Johnson-Winegar, 1984, Turnbull et al., 1992a).

A single target antigen, such as PA, is attached to a solid plate surface in the ELISA procedure. The antibody is then introduced, and its presence is detected by the addition of a second species specific antibody (known as a secondary antibody) that is linked to an enzyme. Incubating the plate with a chromogenic substrate measures the enzyme-linked reaction (ThermoFisher Scientific 2019). Two enzymes that are frequently associated with secondary antibodies are

horseradish peroxidase (HRP) and alkaline phosphatase (ALP) (Voller et al., 1974, Payment and Descoteaux, 1978, Rennard et al., 1980). ELISA has evolved into various forms, the most prevalent of which being the sandwich ELISA, direct ELISA, and the indirect ELISA.

Although sandwich ELISA is less often used, it is extremely successful in detecting antigens in a sample. The antigen is trapped between two antibody layers termed as capture antibody and detection antibody in this approach (Claire, 2020). While this method provides for the quick and specific detection of concentration of antigen in an unknown sample, it requires at least two antigenic epitopes within the antigen to interact with both antibodies in the sandwich (Verma et al., 2013).

A conjugated specific antibody (Ab) is used to directly detect the antigen (Ag) that is already bound on the polystyrene plate in the direct ELISA technique for identifying *B. anthracis* (Van Weemen and Schuurs, 1971, Engvall and Perlmann, 1971, Crowther, 2000). The presence and extent of this complex is then tested by adding a chromogenic material, which produces variable degrees of color depending on the amounts of Ag and Ab present (Engvall, 2010). This method is the easiest and quickest ELISA method, and it is especially useful when employing monoclonal antibodies or well-defined antigens (Crowther 2000). Nonetheless, it has limitations, including poor sensitivity, limited variety in application, and optimal performance with samples containing high antigen concentrations (Aydin, 2015).

The indirect ELISA was created to detect the presence of particular antibodies by including a secondary antibody coupled to an enzyme (Lindström and Wager, 1978). The generated Ag-Ab complex is recognized using an enzyme-linked secondary antibody in this approach, giving birth to the phrase "indirect ELISA." The intensity of the hue rises with increasing complex concentrations and diminishes with lower concentrations (Lindström and Wager, 1978, Crowther, 2000).

This ELISA approach has the benefit of evaluating several antibodies with a single non-species specific conjugate, such as Protein A/G (pAG) or its individual components, protein A (protA) and protein G (protG) (Eliasson et al., 1988, Crowther, 2000). The possibility for cross-reactivity due to different amounts of non-specific binding to antigens is a key disadvantage of this strategy (Crowther, 2000, Gan and Patel, 2013). A conjugate in ELISA is an anti-species immunoglobulin coupled to an enzyme that aids detection by color vision. To effectively measure immune responses in ELISA, high-avidity and specific conjugates are required (Smit, 2017). Given the diversity of African animal species, it is impractical to produce species-specific conjugates for all of them; however, producing conjugates for select common host species might improve present disease surveillance efforts (Figure 1-5).

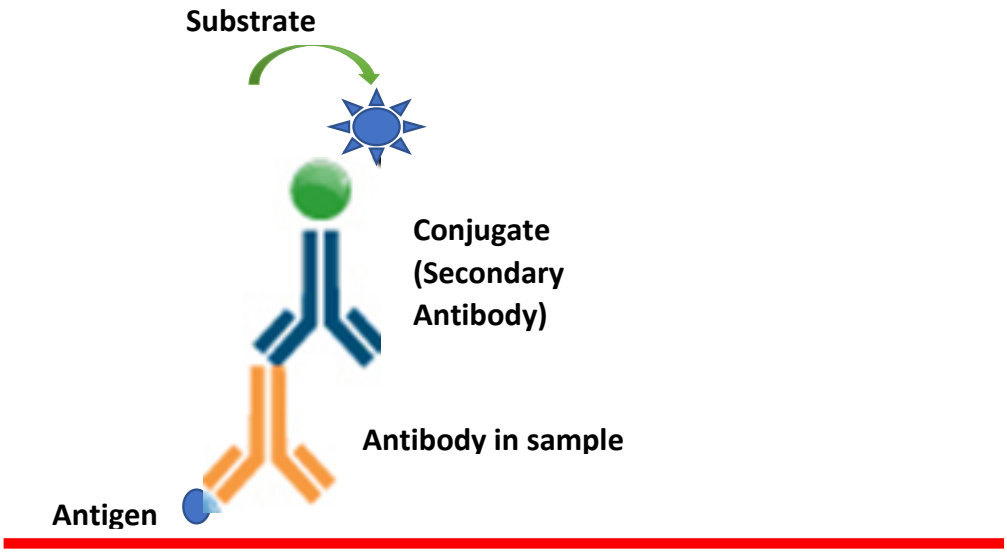

Figure 1-5: Illustration depicting the steps involved in conducting an indirect Enzyme-Linked Immunosorbent Assay (ELISA). The microtitre plate is initially coated with the antigen. Subsequently, a sample containing antibodies is introduced, allowing them to bind with the antigen. Following this, a secondary

antibody, labeled for detection, is introduced and allowed to bind. The final step involves the addition of a substrate, facilitating colour detection in the assay.

1.19 Avidity and Affinity

The connections between an antibody and an antigen are known for their robustness. The strength and reversibility of this bond depend on various forces, such as electrostatic, van der Waals, or hydrogen bonding (van Oss et al., 1987). Because some of these binding pressures are inversely proportional to the distance between molecules, binding effectiveness at the precise binding site is critical (BioRad, 2021). The strength or affinity of antibodies binding to the hapten (specific binding site) determines how effectively the antibodies attach to the antigen (Hudson et al., 1989). However, avidity on the other hand is the total and cross-dependent binding strength of all the binding sites of an antibody to the multivalent antigen (Hudson et al., 1989).

A number of techniques exist to measure the binding strength of both univalent and multivalent antibodies and antigens. Some of these methods include plotting the ODs of the conjugates against the dilution of the serum and determining the area under the curve and this becomes the binding affinity index (Pruvot et al., 2013). Another more expensive technique involves the use of a biosensor (Biospecific interaction analysis: Biacore) as described by Dauner et al. (2012). In recent times, determining the affinity or avidity for both monoclonal antibodies and univalent antigens and polyclonal and multivalent antigens respectively has been done by means of ELISA in the presence of a disassociating chaotropic agent (Hudson et al., 1989, Dimitrov et al., 2011).

Chaotropic agents are very important in determing the functional importance of antibodies (Dimitrov et al., 2011). A number of agents have been used as chaotropes some of which include thiocyanates (ammonium and potassium) (MacDonald et al., 1988), guanidine hydrochloride (Dauner et al., 2012), diethylamine (Westerlund et al., 2005) and urea (Hedman and Seppälä, 1988). Because of their ionic properties, thiocyanates can influence electrostatic

interactions, making them more generally accepted (Smit, 2017, Almanzar et al., 2013). There have been a few reports on the use of various diluents for the chaotrope, including PBS (Dimitrov et al., 2011, Ferreira and Katzin, 1995) and PBS+Tween (Smit, 2017, Dauner et al., 2012).

Disease surveillance is only as good as the methods available for detecting infections. The typical methods for diagnosing anthrax commonly involve bacterial culture, blood smears, and post-mortem identification of characteristic signs and lesions associated with the disease (Bagamian et al., 2013). Depending solely on opportunistic mortality data and biases in carcass detection can distort the accurate representation of which species are most affected by anthrax and the degree of their exposure to it (Bellan et al., 2012). Wildlife disease surveillance is seldom conducted except during outbreaks (Bagamian et al., 2013). The use of serology for diagnosing pathogenic and progressive zoonotic diseases like anthrax antemortem is not a common practice. To determine previous exposure to anthrax, measuring antibodies to PA is considered the most reliable approach (Turnbull et al., 1992a). Turnbull and Snoeyenbos (1989) indicated that some carnivorous wildlife species are frequently exposed to *B. anthracis* and exhibit signs of recovery. While anthrax infection can affect lions, such occurrences are rare (Pienaar, 1967). Our knowledge about natural immunity to anthrax and the extent to which mammalian herbivores contract and recover from the infection remains limited. A number of studies have utilised enzyme linked immunosorbent assay (ELISA) to ascertain exposure to PA, (Turnbull et al., 1992a, Cizauskas et al., 2014, Lembo et al., 2011a).

The limited research on employing ELISA for wildlife disease monitoring may be attributed to the absence of secondary antibodies specifically tailored to individual species. Some studies have investigated the utilization of commercially available non-species-specific conjugates like pAG, protA, and protG, for serological research involving wildlife (Kelly et al., 1993, Feir et al., 1993, Smit, 2017, Stöbel

et al., 2002, Ochai et al., 2023). While pAG has been found to have a broader reactivity spectrum across a wide range of wildlife species (Kelly et al., 1993), some studies have described poor binding reactivity of kudu with pAG, as demonstrated by Smit (2017). Interestingly, none of the mentioned studies reported good binding reactivity for impala with protA, protG, or pAG (Kelly et al., 1993, Feir et al., 1993, Smit, 2017, Stöbel et al., 2002, Kramsky et al., 2003). There are a number of commercially available conjugated secondary antibodies that are available for several domestic stocks (BioRad, 2021) and a number of wildlife species, primarily from Europe (Rossi et al., 2014). Because the binding affinity of these commercial conjugates varies among hosts, the creation of species-specific conjugates may improve wildlife disease surveillance. However, the discrepancies in methodology utilized in these investigations, as well as variances in data interpretation, make synthesis of their conclusions difficult. Given that kudu and impala are significant species in KNP (De-Vos, 1990, Basson et al., 2018a), the exploration of conjugates that are specific for both kudu and impala becomes crucial, as it would enhance the specificity of the currently employed anti-PA ELISA.

For many years it was assumed that the laboratory identification of anthrax and its causal agent (*B. anthracis*) was well understood (this included culture, microscopy, and biochemistry). However, novel concepts about the disease's presentation, prophylaxis, and infective organisms have been revealed on the African continent in recent decades (Klee et al., 2006b, Antonation et al., 2016, Norris et al., 2021, Tamborrini et al., 2011). Serological cross-reactivity has been documented in the anthrax endemic zone of northern KNP due to a strong interaction between pathogenic and non-pathogenic *Bacillus spp.* (Marston et al., 2016, Zimmermann et al., 2017a). Anti-PA and LT neutralizing antibodies, on the other hand, were found in animals from the non-endemic (low incidence area) of KNP (southern region) (Ochai et al., 2022). We hypothesised that the animals were responding to "anthrax-like" infections in the environment, which could include genes that are comparable to *B. anthracis* (Ochai et al., 2022). These

findings, together with the identification of anthrax cases caused by *B. cereus* biovar *anthracis* strains in west and central Africa (Hoffmann et al., 2017) motivated us to reconsider the robustness of diagnostic methods presently utilized in anthrax surveillance in southern Africa. False positivity will impact on the sensitivity and specificity of the current anti-PA-ELISA and molecular techniques. This, therefore, calls for further study to investigate the presence of *other* bacterial isolates with similar *B. anthracis* genes from archival samples.

1.20 Aim

To better understand the environmental and microbial drivers of anthrax-like disease and to improve the antemortem and postmortem diagnostic techniques available for key species in the KNP system (Figure 1-1).

1.21 Objectives and research questions

1) To develop chicken anti-kudu and chicken anti-impala specific secondary antibodies and to evaluate the binding avidity of our newly developed conjugates, in comparison to commercially available conjugates (Figure 1-6).
    a. do developed novel species-specific conjugates for kudu and impala have better avidity than commercial conjugates?
    b. do these developed conjugates cross-react with antibodies from other wild herbivores?
2) To investigate the presence of atypical *B. cereus* and other closely related microbes that may complicate the molecular diagnosis of anthrax. Specifically, we examined (Figure 1-6):
    a. how the microscopy technique that is currently used in the passive surveillance of anthrax in KNP compares to molecular methods of diagnosis,?
    b. if other *Bacillus* species contain *cap*, *lef* and *pag* genes that could complicate molecular diagnosis and

c. how closely similar are the virulence plasmids of *B anthracis* and the other *Bacillus* species?

3) To investigate the possible drivers of the change in main anthrax host species from kudu to impala, including roles of climate change, variation in environmental factors and density of other host species in KNP. Specifically, we evaluated (Figure 1-6):

   a. if changes in environmental conditions (precipitation, temperature, soil moisture, vegetation greenness and drought) were responsible or exacerbated the shift?

   b. if animal densities correlate with anthrax mortality, and the changes in trends over time?

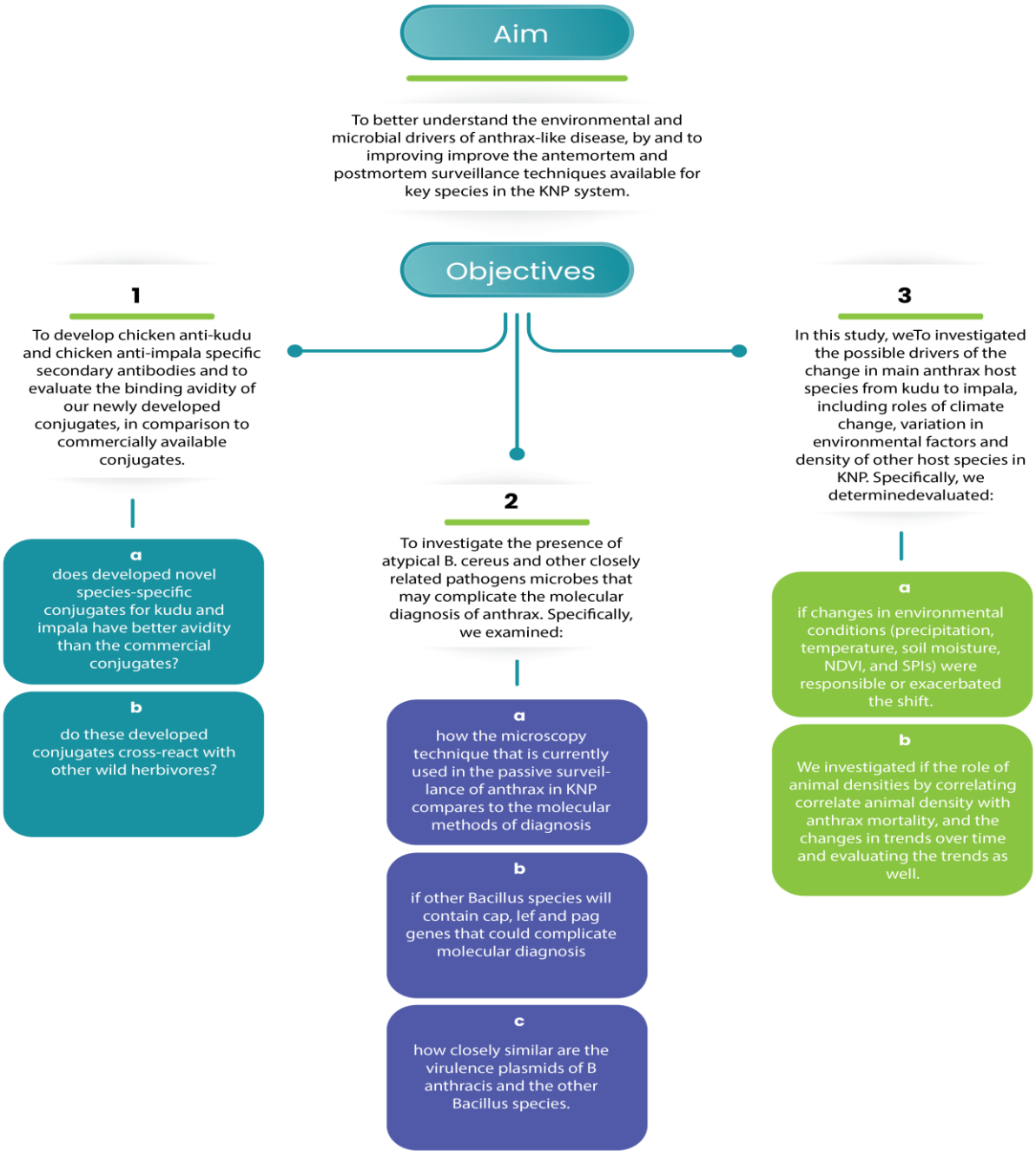

Figure 1-6: A flowchart giving an overview of the entire aim and objectives of this study

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Frontiers in Immunology

Chapter 2. Development of conjugated secondary antibodies for wildlife disease surveillance

Sunday O. Ochai[1*], Jan E. Crafford[1], Pauline L. Kamath [2,] Wendy C. Turner[3], Henriette van Heerden[1]

[1] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa.

[2] School of Food and Agriculture, University of Maine, Orono, Maine, U.S.A.

[3] U.S. Geological Survey, Wisconsin Cooperative Wildlife Research Unit, Department of Forest and Wildlife Ecology, University of Wisconsin-Madison, Madison, Wisconsin, U.S.A

* Correspondence: Sunday O. Ochai; s.o.ochaijr@gmail.com

2.0. Abstract

Disease monitoring in free-ranging wildlife is a challenge and often relies on passive surveillance. Alternatively, proactive surveillance that relies on the detection of specific antibodies could give more reliable and timely insight into disease presence and prevalence in a population, especially if the evidence of disease occurs below detection thresholds for passive surveillance. Primary binding assays, like the indirect ELISA for antibody detection in wildlife are hampered by a lack of species-specific conjugates. In this study, we developed anti-kudu and anti-impala immunoglobulin-specific conjugates in chickens and compared them to the binding of commercially available protein-G and protein-AG conjugates, using an ELISA-based avidity index. The conjugates were evaluated

for cross-reaction with sera from other wild herbivores to assess future use in ELISAs.

The developed conjugates had a high relative avidity of > 70% against kudu and impala sera. The commercial conjugates (protein-G and protein-AG) had significantly low relative avidity (< 20%) against these species. Eighteen other wildlife species demonstrated cross-reactivity with a mean relative avidity of > 50% with the impala and kudu conjugates and < 40% with the commercial conjugates.

These results demonstrate that species-specific conjugates are important tools for the development and validation of immunoassays in wildlife, and for the surveillance of zoonotic agents along the livestock-wildlife-human interface.

Keywords: Wildlife species, Adaptive immunity, Avidity, Conjugates, Diagnostics, Enzyme-linked immunosorbent Assay (ELISA), Disease surveillance, Serology

2.1. **Introduction**

With the current increase in emerging and re-emerging disease outbreaks of both veterinary and human importance, there has been an urgent need for evidence-based methods for measuring both infection incidence and prevalence (Lambert et al., 2022). Several techniques and interventions have been employed to mitigate the debilitating effects of disease-causing organisms on livestock and wildlife. However, what determines the choice of intervention to be implemented is the knowledge of the epidemiology governing or influencing these diseases (Artois et al., 2009). Wild animals are known to be hosts and/or reservoirs for pathogens that are of concern for cross-species transmission risk to humans and livestock. Therefore, an understanding of the epidemiology and ecology of pathogens in wildlife will better inform policies and interventions for control. Passive surveillance

is currently used in most wildlife settings and is largely dependent on the detection of clinical cases or case mortalities. However, opportunistic collection of mortality data and biases in the detection of carcasses and clinical signs can lead to a distortion of the true incidence, therefore, more active form of surveillance is needed (Garnier et al., 2017). The detection of antibodies against pathogens can provide insights into prior exposure as well as information on the prevalence of a pathogen in an environment and the risk of pathogen spill over (Garnier et al., 2017, Gardner et al., 1996). This approach may be especially useful for diseases with a short infection period like anthrax or those that do not cause mortality like brucellosis.

Several serological techniques have been used to detect exposure to pathogens in African wildlife. These include primary binding assays like the enzyme-linked immunosorbent assay (ELISA) (Kock et al., 1992, Turnbull et al., 1992a, Lembo et al., 2011b) as well as more historic secondary binding assays like the agar gel immunodiffusion (AGID) test and complement fixation test (CFT)(Blackburn and Swanepoel, 1988). Assays like the indirect ELISA can be highly sensitive and specific for the detection of pathogen specific antibodies in the serum of a host, but they rely on a host-specific enzyme conjugate that limits the cross-species use of the assay. Most commercially available indirect ELISA kits are only validated for use in domestic ruminants. The enzyme-linked detection technique involves a highly specific antigen-antibody (Ag-Ab) interaction and was developed by Engvall and Perlmann (Engvall and Perlmann, 1971). First, an antigen is restricted on a firm surface of a plate, followed by the addition of the sample antibody (if present) which then binds with a secondary antibody that is linked to an enzyme; next, this conjugated enzyme reaction is measured by incubating with a chromogen substrate (ThermoFisher Scientific 2019). Horseradish peroxidase and alkaline phosphatase are the most used enzymes conjugated with secondary antibodies (Voller et al., 1974, Payment and Descoteaux, 1978, Rennard et al., 1980). These conjugates in a simpler sense, refer to an anti-species immunoglobulin that is linked to an enzyme that facilitates

the detection through colour visualization. The ability to use conjugates of high avidity and specificity is therefore very important in measuring immune response through the use of ELISA (Smit, 2017). The interaction and bond that exist between an antibody and an antigen is one that is quite robust. The ability to be reversed and the strength of this bond are often dependent on the nature of the force that exists which could be electrostatic, van der Waals' or hydrogen (van Oss et al., 1987). Some of the binding forces are negatively associated with distance and this makes them highly reliant on how well the molecules bind at the binding site (BioRad, 2021). It is known that the measure of strength (affinity) of hapten-antibody binding (specific binding site) determines how well an antigen binds with an antibody (Hudson et al., 1989). Avidity on the other hand is the total and cross-dependent binding strength of all the binding sites of an antibody to the multivalent antigen (Hudson et al., 1989). It is therefore important to develop secondary antibodies that are of both high affinity and avidity. Species-specific conjugates for wildlife are often not available and the generic conjugates that are used in these assays can vary significantly in binding to wildlife antibodies and results from these unvalidated assays should always be interpreted with caution(Kelly et al., 1993, Pruvot et al., 2013, Stöbel et al., 2002, Kramsky et al., 2003).

Antibody avidity can be evaluated by means of ELISA in the presence of an immune-complex disruptive or disassociating compound like a chaotropic agent (Hedman and Seppälä, 1988, MacDonald et al., 1988, Hudson et al., 1989, Westerlund et al., 2005, Dimitrov et al., 2011, Dauner et al., 2012). The thiocyanates can impact electrostatic interactions owing to their ionic characteristics making them more widely acceptable (Almanzar et al., 2013, Smit, 2017). There are a few reports about the use of different diluents for the chaotrope, including phosphate buffered saline (PBS) (Ferreira and Katzin, 1995, Dimitrov et al., 2011) and PBS+Tween (Dauner et al., 2012, Smit, 2017).

Only a few studies (Turnbull and Snoeyenbos, 1989, Cizauskas et al., 2014, Lembo et al., 2011a, Ochai et al., 2022) are available regarding the use of ELISA for surveillance of diseases such as anthrax (caused by *Bacillus anthracis*) in herbivorous wildlife species. Anthrax is a zoonosis, but the most susceptible hosts are herbivorous mammals. While primarily a fatal disease in herbivorous wildlife and livestock, humans are susceptible to anthrax infections and human cases occur largely due to the handling of carcasses, infected meat, hides and skin (Kamal et al., 2011, W.H.O 2015). Most serological diagnoses of anthrax depend on the detection of PA as it is considered the most important immunogen in most anthrax vaccines (Ivins et al., 1994) and antibodies against PA have been shown to inhibit the germination of spores, and the effect of the toxins and enhance the activities of macrophages against the spores (Welkos et al., 2001). Anti-PA antibodies have been detected in several wildlife species following sublethal exposures to anthrax spores (Turnbull et al., 1992b, Cizauskas et al., 2014, Lembo et al., 2011a, Ochai et al., 2022). Antibodies to EF and LF have been reported in some species of animals (Turnbull et al., 1992b) but the extent to which the EF and/or LF confer immunity and how they provide protection is not fully understood (Taft and Weiss, 2008). Mahlandt et al. (1966) showed that both EF and LF have an augmenting effect on the protective ability of anti-PA antibodies in some laboratory animals.

The paucity of studies around the use of ELISA for surveillance of wildlife diseases is perhaps due to the lack or scarcity of species-specific conjugated secondary antibodies. There are various studies around the use of non-species-specific commercial conjugates such as protein A (protA), protein G (protG) and protein AG (pAG) for wildlife serological studies (Kelly et al., 1993, Feir et al., 1993, Stöbel et al., 2002, Kramsky et al., 2003, Smit, 2017) (Table S1). Some commercial conjugates are available for domestic species (BioRad, 2021) and some wildlife species, predominantly those from Europe (Rossi et al., 2014). The variation in binding affinity for the commercial conjugates among various hosts show that developing species-specific conjugates could be important to

improving wildlife disease surveillance. Furthermore, the different methods used in these studies and differences in data interpretation further complicate the synthesis of the results. Thus, it is important to develop conjugates that are specific to African wildlife and not entirely rely on commercial multispecies conjugates.

Because wildlife hosts of pathogens of both veterinary and zoonotic importance are quite diverse globally, manufacturing species-specific conjugates for all host species seems impracticable; however, developing these for a few common hosts could improve disease surveillance efforts. In this study, we developed species-specific conjugates for kudu and impala respectively. These two species have been implicated as hosts for diseases like brucellosis (Godfroid, 2017, Simpson et al., 2021), anthrax (De-Vos, 1990, Basson et al., 2018b) and foot and mouth disease (Wittmann, 1990, Vosloo et al., 2005, Letshwenyo et al., 2006). We evaluated the binding avidity of these conjugates to several wildlife species and compared them to commercially available conjugates. We addressed the following questions: (1) do developed novel species-specific conjugates for kudu and impala have better avidity than the commercial conjugates? (2) do these developed conjugates perform better across a range of related wildlife species? The validation of ELISA assays using conjugates specifically developed for pathogen detection in wildlife, rather than commercially available conjugates, is critical for improving wildlife disease surveillance and research.

2.2. Materials and Methods

2.2.1. Experimental design and samples

Species-specific immunoglobulin conjugates for kudu and impala were developed by vaccinating Highland brown, Specific Pathogen Free (SPF) chickens (Avi-farms, Centurion, South Africa) with immunoglobulin (Ig) from kudu and impala (4 animals per species), respectively. Anti-species immunoglobulin Y (IgY) were

purified from egg yolks and conjugated to horseradish peroxidase. Cross-reactivity and avidity of the new conjugates were evaluated and compared to commercially available protein G (protG) and protein AG (pAG) conjugates using different herbivore species by means of an ELISA-based avidity index (AI). Serum samples from a variety of species (10 samples per species Table 3-1) were collected from South African National Parks (SANParks) biobanks and from samples banked in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa. Species were classified into subfamilies and tribes based on Hassanin and Emmanuel (1999) and Gatesy et al. (1997) to give an indication of phylogenetic relatedness. Goat, sheep, and cattle samples were also included. Animal and research ethics from the University of Pretoria was obtained (REC063-19, REC041-19) and permits were obtained from the Department of Agriculture, Land Reform and Rural Development.

Table 2-1:List of species used for avidity and cross-reactivity tests. Species subfamily and tribe are as described by Hassanin and Emmanuel (1999) and Gatesy et al. (1997)

Common Name	Species	Subfamily	Tribe
Greater kudu	*Tragelaphus strepsiceros*	Bovinae	Tragelaphini
Impala	*Aepyceros melampus*	Antilopinae	Aepycerotini
Burchell's zebra	*Equus quagga burchellii*	Equinae	Equini
Black wildebeest	*Connochaetes gnou*	Alcelaphinae	Alcelaphini
African buffalo	*Syncerus caffer*	Bovinae	Bovini
Giraffe	*Giraffa camelopardalis*	Giraffinae	Giraffini
Blue wildebeest	*Connochaetes taurinus*	Alcelaphinae	Alcelaphini
Nyala	*Tragelaphus angasii*	Bovinae	Tragelaphini
Sable antelope	*Hippotragus niger*	Antilopinae	Hippotragini
Waterbuck	*Kobus ellipsiprymnus*	Hippotraginae	Hippotragini
Gemsbok	*Oryx gazella*	Antilopinae	Hippotragini
Springbok	*Antidorcas marsupialis*	Antilopinae	Antilopini
Hartebeest	*Alcelaphus buselaphus*	Antilopinae	Alcelaphini

Roan antelope	*Hippotragus equinus*	Antilopinae	Hippotragini
Common eland	*Taurotragus oryx*	Bovinae	Tragelaphini
Common tsessebe	*Damaliscus lunatus*	Antilopinae	Alcelaphini
Blesbok	*Damaliscus pygargus phillipsi*	Antilopinae	Alcelaphini
Bushbuck	*Tragelaphus scriptus*	Bovinae	Tragelaphini
Bontebok	*Damaliscus pygargus*	Antilopinae	Alcelaphini
Goat	*Capra hircus*	Caprinae	Caprini
Sheep	*Ovis aries*	Caprinae	Caprini
Domestic cattle	*Bos taurus*	Bovinae	Bovini

2.2.2. Precipitation of kudu and impala immunoglobulins

Immunoglobulin was extracted from kudu and impala by ammonium sulphate precipitation using the method described by Staak et al. (2001). Briefly, respective sera were diluted 1:4 with PBS (total volume 80 ml), while constantly stirring, 40 ml of saturated ammonium sulphate (Merck, Darmstadt, Germany) was slowly added to achieve a 33% saturation and the pH of the suspension was adjusted to 7.8 using a 2N NaOH (Associated Chemical Enterprises, Johannesburg, South Africa). The suspension was stirred continuously for 3 hours on a magnetic stirrer (Bibby Sterilin LTD, Staffordshire, England) and then centrifuged at room temperature for 30 minutes at 1400 × g using Eppendorf centrifuge 5810R (Eppendorf, Hamburg, Germany) and the supernatant was discarded. The pellet was resuspended to a total volume of 80 ml in PBS and further purified by two additional cycles of precipitations, as described above. The final precipitate was dissolved in PBS in a volume half of the initial serum sample. Ammonium sulphate was removed by desalting spin columns (Thermo Scientific, Rockford, USA). IgG heavy and light chains were confirmed by SDS-PAGE gel electrophoresis (Figure 3-1).

The total protein concentration of the precipitated immunoglobulins (Ig) was determined using the spectrophotometer (Xpose™ Trinean Spectrophotometer, Trinean, Burladingen, Germany). The SDS-PAGE gel electrophoresis was performed as described by Laemmli (1970) with a few modifications. Samples were diluted with the protein solvent buffer to a final concentration of 2 µg/µl. To determine the molecular size of the Ig, the protein was loaded into the wells of the SDS-PAGE at a concentration of 2 µg/µl. Samples were placed in Eppendorf tubes and put into digital dry bath (Labnet Accublock Digital Dry Bath, Labnet International Inc, Woodbridge, USA) for 10 minutes at 100 ˚C after which they were spun using the mini centrifuge (Wealtec E-centrifuge, Wealtec corporation, Sparks, USA) for 10 seconds at 1400 × g. Gel reagents were mixed in volumes indicated in Table S2 and the solution was added between the clamped glass slides. The gel was allowed to polymerize for 30 minutes and then the stacking gel (Table S2) was added and incubated for 30 minutes. The gel was run at 100 V for 2 hours after which it was stained with blue stain (GelCodeTM Blue stain, Themo Scientific™, Massachusetts, USA). After washing steps, the gel was viewed on the transilluminator (Univetec Cambridge transilluminator, Univetec, Cambridge, UK) for the presence of bands. Subsequently, the gel was transferred to the molecular image gel document system (Bio-rad molecular image gel document system, Bio-rad, California, USA) using the Image Lab software for analysis.

2.2.3. Immunisation of chickens and extraction of IgY from eggs

Preparation of vaccines for immunizing chickens and extraction of IgY from egg yolk was adapted with modifications from Staak et al. (2001). Preparations of purified Ig from kudu and impala were made up to 200 µg/ml (w/v) in PBS. One ml of vaccine (100 µg/ml) was prepared by emulsifying equal volumes (0.5 ml) of protein and Montanide ISA 50 V 2 adjuvant (SEPPIC, Paris, France) and injected into both sides of the breast muscles. Inoculation was performed on Days 0, 23 and 42 (Figure S 2-1). During this period development of specific IgY was

monitored by testing the yolks in an ELISA (see antibody tires and method in Supplementary methodology 1 and Figure S 2-2).

Egg yolks representing peak levels of anti-kudu or anti-impala IgY were harvested by separating the yolk from the albumin and diluting the yolk to 1:5 in distilled water before freezing at -20 ºC for 72 hours. The suspension was thawed slowly at 4 °C and centrifuged at 2800 x g for 20 minutes and the supernatant was collected. Ammonium sulphate was added in a concentration of 0.27g per ml of the supernatant and stirred for two hours at room temperature. Afterwards, it was centrifuged at 2800 x g for 20 minutes and the supernatant was discarded. The pellet was resuspended in 24 ml of 2 M ammonium sulphate per egg yolk and stirred for 40 minutes at room temperature, this was followed by centrifugation as before. The precipitate was resuspended in 2.5 ml of PBS for each egg yolk and dialysed against PBS at 4 °C for 48 hours. Finally, the concentration of the immunoglobulin solution was measured and stored at -20 °C (Figure S 2-1).

Affinity chromatography using the polystyrene granulate method as described by Staak et al. (2001) was used to further purify the recovered IgY. Briefly, 150mg of impala and kudu IgG were immobilised separately on the granulated polystyrene using 0.05M carbonate buffer (pH 9.6) and free binding sites on the matrix were blocked using the blocking buffer (PBS; 0.005% Tween 20, PBST). Subsequently, the packed columns were equilibrated using PBST and the chicken IgY were run through the columns using very slow rates to allow for optimal binding. Specific IgY were eluted by means of a glycine/hydrochloric acid elution buffer with a pH of 2.5. The affinity purified IgY were used for the final conjugation.

A western blot was used to confirm the specificity of IgY produced against the respective Ig of kudu and impala. The western blot was performed as described by Howell et al. (2002). The western blots were performed before and after affinity purification.

2.2.4. Horseradish peroxidase conjugation to IgY

The periodate method as described by Wilson and Nakane (1978) and adapted by Staak et al. (2001) was used to conjugate the horseradish peroxidase (HRPO) to IgY. The activity of the conjugate was tested using a checkerboard titration between the kudo or impala serum respectively (Supplementary methodology 2 and Figure S 2-2, Figure 2-1).

2.2.5. Avidity index for cross-reactions between different conjugates and wildlife sera

The respective AIs for the binding of anti-kudu IgY and anti-impala IgY conjugates to kudu and impala sera as well as to the sera of the species listed in Table 2-1 were compared. The binding of all the sera to protG- and pAG conjugates were also compared as described by Smit (2017).

Briefly, a direct ELISA was employed by coating each microtiter plate (Thermo Scientific™ Pierce 96-well Plates-Corner, USA) with 10 sera samples per species at a dilution of 1:2000. Each plate was coated by adding 50 µl of the serum diluted in PBS in rows A-D of columns 1-10 for the 10 individual animals of the same species. Rows E-H of columns 1-10 were similarly filled with 50 µl of the next 10 sera of the second species. Columns 11 and 12 were filled with 50 µl of the control serum (kudu serum for anti-kudu conjugates, impala serum for anti-impala conjugates, cattle serum for pAG (Inoshima et al., 1999, Smit, 2017) and goat serum for protG (ThermoFisher, 2023)) at a concentration of 1:2000. Following incubation at 37 ˚C for 1 hour on an orbital shaker, the plates were washed twice with PBS supplemented with 0.05% Tween-20 (PBST; Thermo Fisher Scientific, Waltham, MA USA) using a plate washer (Bio-Rad PW40, Mamesla-Coquette, France). Subsequently, all wells were loaded with PBST supplemented with 5% skimmed milk powder as a blocking step for 30 minutes at 37 ˚C and afterwards, the wells were washed twice. The conjugates were diluted with PBSTM at a final

concentration of 1:400 (as determined in Supplementary methodology 2 and Figure S 2-2) for anti-kudu IgY and anti-impala IgY HRPO, 1:10000 for protein A/G and protein G as prescribed by the manufacturer (Thermo Fisher Scientific, Waltham, MA USA). Afterwards, 50 µl of PBS was added into the wells of rows A, B, E and F, and rows C, D, G and H were loaded with potassium thiocyanate as a chaotropic agent (CT) at a final concentration of 0,25 M. The plates were incubated for 1 hour at 37 ˚C on the shaker and followed by a wash step. Colour was developed by the addition of the ABTS substrate (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt; Thermo Scientific 1-Step ABTS, USA) and incubated in the dark for 30 minutes. The absorbance was read at 405 nm using the plate reader (Biotek Powerwave XS2,Vermont, USA) (Figure 3-1). The avidity between the conjugate and serum was calculated as the reduction in colour between wells without CT and those with CT and presented as the AI for each serum. AI was calculated as the mean ELISA absorbance values (ODs) from wells treated with the dissociating chaotrope (NH_4SCN) divided by the mean ODs from wells without chaotrope and multiplied by 100.

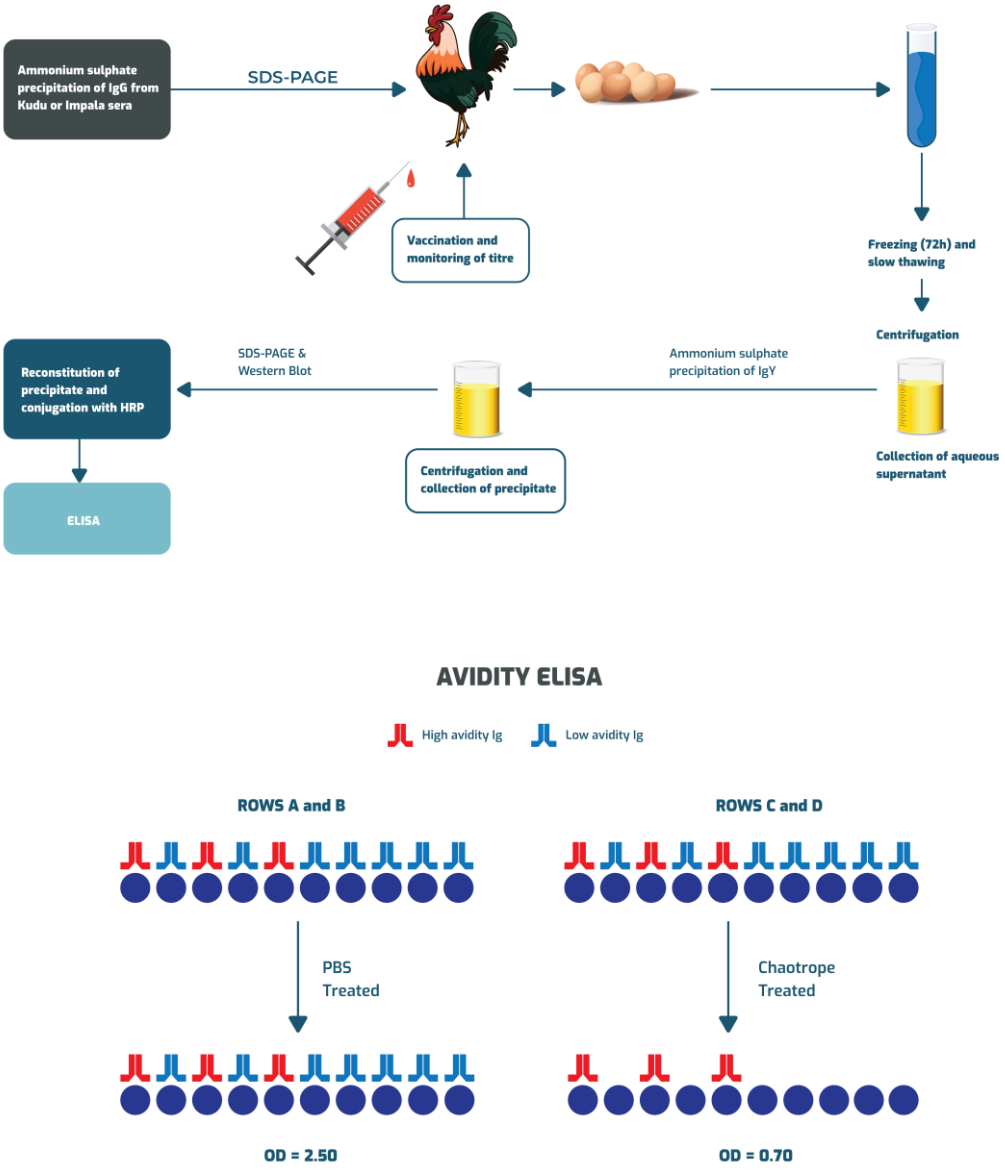

Figure 2-1:Image summarising the methodology captured in this study from the vaccination of the chickens to the testing of the avidity of the different wildlife and domestic species tested in this study.

2.3. Statistical analysis

To present differences between the developed species-specific conjugates for kudu and impala and the commercial conjugates, we calculated the mean, standard deviation for kudu and impala. A t-test was performed to measure the differences in the means of the ODs and AI for both the test samples and the controls. The AI was defined as the ratio of both the OD of the CT-treated wells and the PBS-treated wells; the AI was calculated for each species and conjugate. The AI values for all species were normalised by subtracting them from the AI of their corresponding controls in order to measure how they differed from the respective control. A multivariate generalised linear model coupled with the Tukey's Honestly Significance Difference (HSD) test for multiple mean comparisons was performed to compare the relationships between the AIs of the conjugates for the subfamily and tribes of the different species. The predictor variables included an interaction between conjugates and the subfamily and also between conjugates and tribes while the response variable was proportion (0-1) of the AI. All statistical analyses were done in R Console version 3.2.1 (R Core Team, 2017) with significance assessed when alpha was <0.05.

2.4. Results

2.4.1. Ammonium sulphate precipitation of IgG from Kudu and Impala Sera

The SDS-PAGE analysis confirmed the presence of two protein bands with molecular weights of around 50 and 25 KDa (for both kudu and impala) representing the heavy and light chains of IgG. (Figure 2-1A).

2.4.2. Western Blot

The western blot analysis confirmed the specificity of the IgY against the IgG of impala (Figure 2-1 B) and kudu (Figure 2-1C). Figures 2-1B and C (before affinity

chromatography) and 2C (after affinity chromatography) show the specificity of the immunoglobulins produced. Only binding to the 50 KDa heavy chain was observed to confirm the specificity of the secondary antibodies.

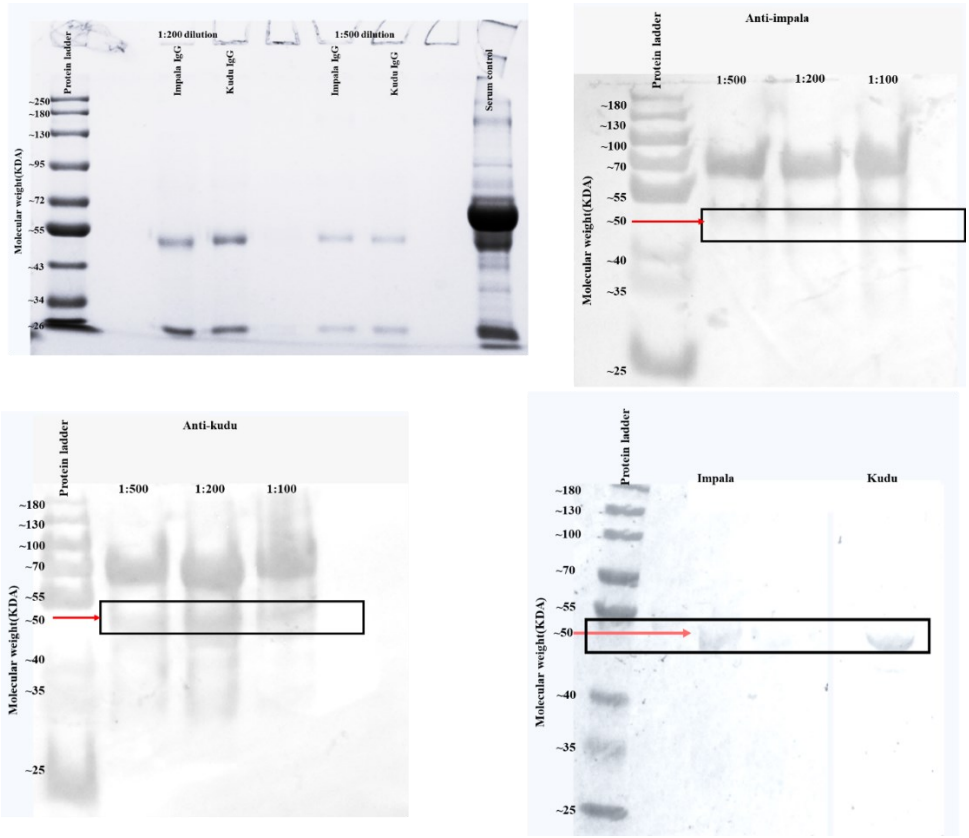

Figure 2-2: (A) Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) image from the ammonium sulphate precipitated immunoglobulin fractions from kudu and impala sera. The protein bands at 50 and 25 KDa correspond to the heavy and light chains of IgG. Kudu serum was used as the serum control. (B) Western blot image indicating the binding of impala immunoglobulin G (IgG) to the chicken anti-impala IgY directly from the ammonium sulphate precipitated egg yolk without affinity chromatography before conjugation. (C) Western blot image indicating the binding of kudu IgG to the chicken anti-kudu IgY directly from the ammonium sulphate precipitated egg yolk without affinity chromatography before conjugation. (D) Western blot image showing binding of impala (left) and kudu (right) IgG against the corresponding chicken affinity-purified IgY before conjugation. Red arrows with solid rectangles highlight the molecular weight of interest.

2.4.3. Binding activities of anti-kudu IgY, anti-impala IgY and commercial conjugates on kudu and impala sera

Kudu and impala sera bound significantly better with their respective conjugates compared to the commercial conjugates (p<0.0001). There was also a significant drop in optical densities for the commercial conjugates in the presence of the chaotrope (p<0.0001) but not the developed conjugates (p>0.05; Figure 2-2). For the anti-kudu IgY conjugate on kudu serum, the mean AI was 72.36 ± 1.13 SD compared to 15.23 ± 1.1 SD for pAG and 23.61 ± 0.99 SD for protG. For anti-impala IgY conjugate on impala serum the mean AI was 72.09 ± 0.89 SD, compared to 21.47 ± 0.66 SD for pAG and 23.52 ± 0.56 SD for protG..

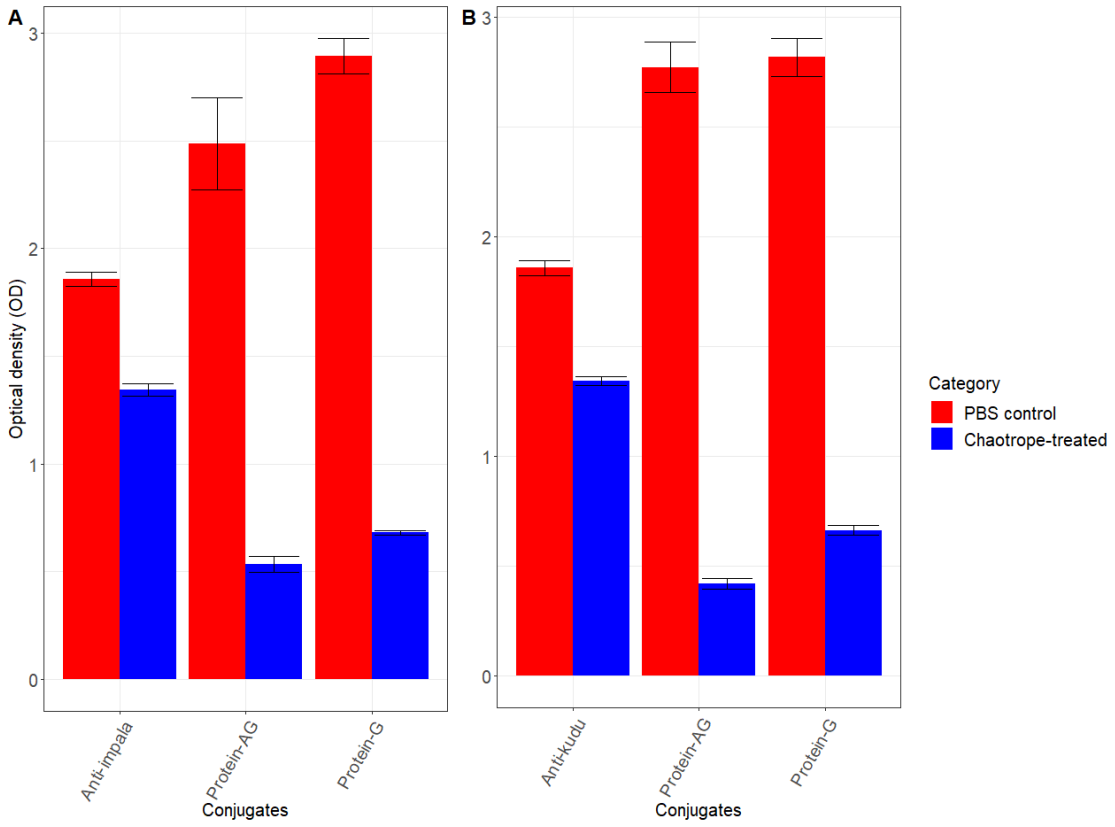

Figure 2-3: Bar charts with error bars (standard deviation) showing the differences in mean optical densities (OD) for the developed and commercial conjugates, A) impala sera against anti-impala IgY, protein AG and protein G conjugates, and B) kudu sera against anti-kudu IgY, protein AG and protein G conjugates. Red bars

represent wells without the chaotrope and the blue bars represent wells that received dissociating chaotrope. For each species, 10 replicates were used for the experiments.

Comparing the outcome of the antiPA ELISA using the developed anti-impala and commercial conjugates, showed a significant (p=0.01) difference between the mean OD of anti-impala (0.24 ± 0.04); and protG (0.48 ± 0.22). A similar trend was noticed with anti-kudu (0.72 ± 0.24), having a statistically (p<0.0001) lower OD than protG (1.58 ± 0.58) (Figure 2-4)

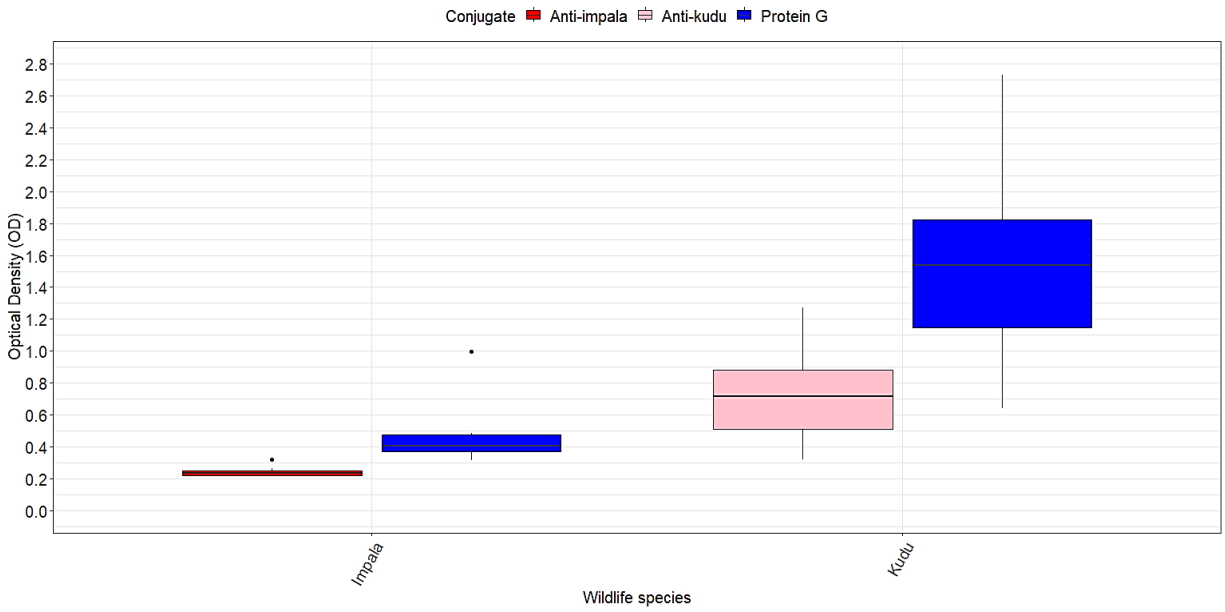

Figure 2-4: Boxplot showing differences between the optical densities (ODs) of the antiPA ELISA used in the presence of different conjugates (protein G (blue), anti-kudu (pink) and red for anti-impala).

2.4.4. Binding activities of anti-kudu IgY, anti-impala IgY and commercial conjugates on kudu and impala sera

When comparing how each host species reacted to the conjugates, we found that kudu serum had mean AIs of 72.36 ± 1.07 with anti-kudu IgY, 66.67 ± 1.17 with anti-impala IgY. There was a significant difference between the anti-kudu IgY, anti-

impala IgY, pAG and protG conjugates for kudu sera (p<0.0001; Figure 2-5). Similarly, impala serum had AIs of 72.08 ± 0.88 with anti-impala IgY, 70.20 ± 0.99 with anti-kudu IgY, 21.47 ± 0.62 with pAG and 23.52 ± 0.53 with protG conjugates, respectively (Figure 2-5). There was also a significant difference among all the conjugates for impala sera (p<0.05).

Our developed IgY conjugates out-performed the commercial conjugates for all wildlife species except for zebra specifically with an AI of less than 50 (anti-kudu IgY=30.54 ± 1.04; anti-impala IgY= 35.97 ± 0.37). The average AI for anti-impala IgY across all the species was 61.73 ± 11.25 (Table S 2-1), for anti-kudu IgY was 63.25 ± 11.51 (Table S2-4), pAG was 37.71 ± 17.25 (Table S 2-5) and protG was 36.08 ± 15.78 (Table S 2-6). All wildlife sera tested with the protG conjugate had an AI of less than 50, except for black wildebeest (57.24 ± 0.88) and tsessebe (50.38 ± 0.64) (Figure 2-5). Also, all the wildlife sera that were tested for pAG conjugate, demonstrated an AI of less than 50%, except for plains zebra (51.35 ± 0.48). The individual AIs for the wildlife sera are captured in Figure 2-5.

There were significant differences (p<0.05) between each species and its respective controls, except for impala and blesbok (p=0.088; Table S 2-3). All the animals had avidity index below the respective controls, except for gemsbok and nyala which were higher than kudu (anti-kudu), goat which was higher than cattle (pAG); and springbok which was higher than impala (Figure 2-5). Details of all the normalised AIs, are shown in Table S 2-3. Values above the zero threshold indicate higher avidity than the respective control while negative values indicate lower avidity compared to the control. Comparing the differences in avidity of the developed conjugates to the different wildlife species, there was a significant interaction between the developed conjugates (anti-impala IgY and anti-kudu Ig conjugates) and the subfamily of the wildlife species (p<0.0001; Figure 2-6A and B). Antilopinae and Caprinae subfamilies did significantly better with anti-impala, while the Bovinae, Alcelaphinae and Hippotraginae subfamilies did better with anti-kudu (p<0.0001; Figure 2-6 A and B). Tribes and subfamilies more closely related

to kudu performed better with anti-kudu conjugate than anti-impala. And wildlife species more closely related to impala performed better with anti-impala. There was a wider variation in tribes than in subfamilies as in Figure 2-6 A. Animals that share the same tribe such as the domestic cattle and the African buffalo demonstrated significant variation (p<0.0001) in their avidity to both the commercial and developed conjugate. Domestic cattle performed significantly better with pAG and protG than the African buffalo while the African buffalo demonstrated significantly better avidity than domestic cattle (p<0.0001).

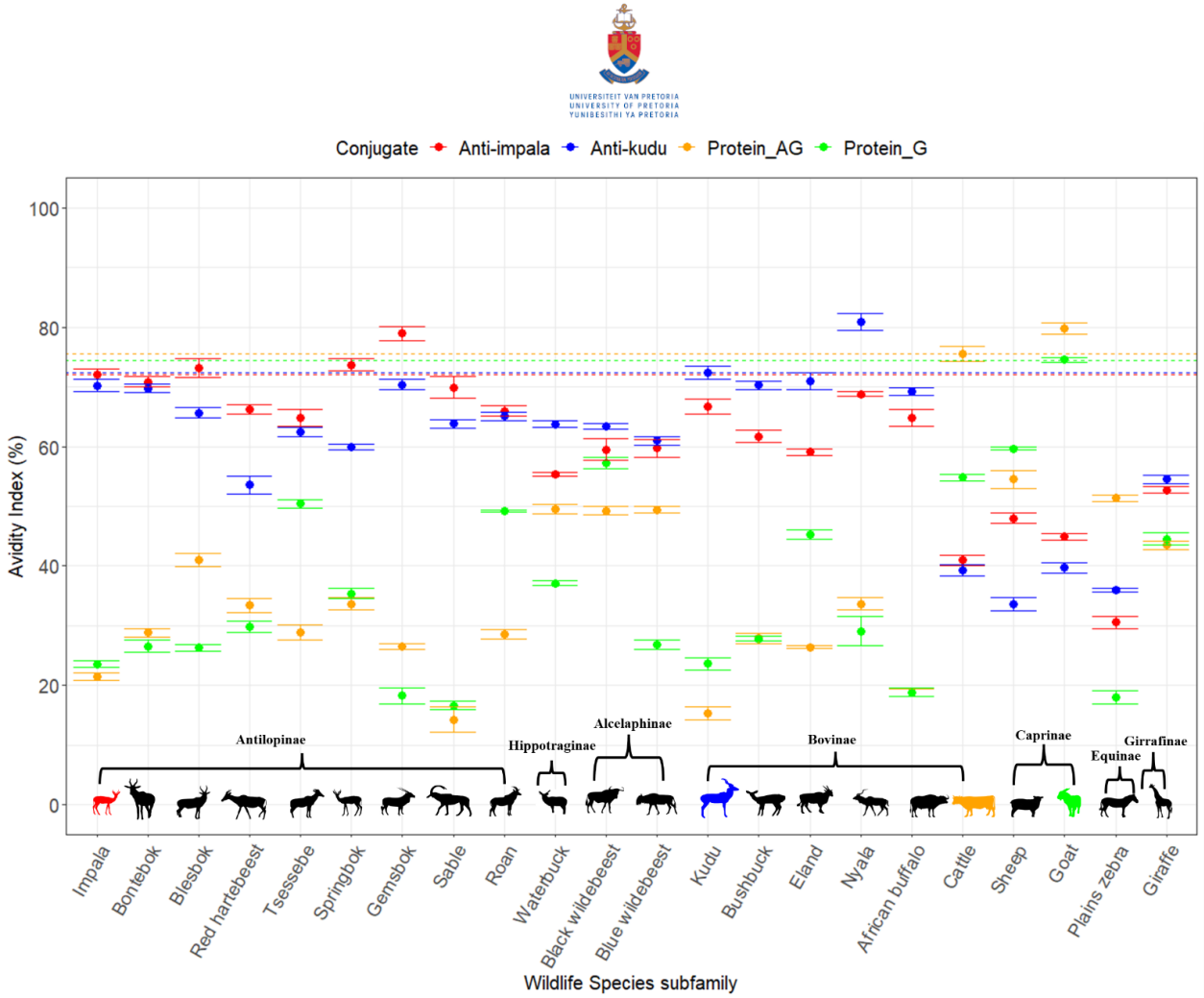

Figure 2-5: (A)Scatter plot with error bars (standard deviation) showing the avidity index for each of the conjugates (red anti-impala, blue antikudu, yellow protein AG and green protein G) determined for different wildlife species. The avidity between the conjugate and different sera was calculated as the reduction in colour between wells without a chaotropic agent (CT) and those with CT and presented as the AI for each serum. The silhouettes in colour connect species and conjugate colours to denote the species used as control for each conjugate: impala for anti-impala IgY, kudu for anti-kudu IgY, cattle for protein AG and goat for protein G. The horizontal dotted lines indicate the avidity index of the respective controls, and the colours correspond to the conjugates. Species were grouped into subfamilies as described by Hassanin and Emmanuel (1999), however, ordering of the species was not done by phylogenetic relationships.

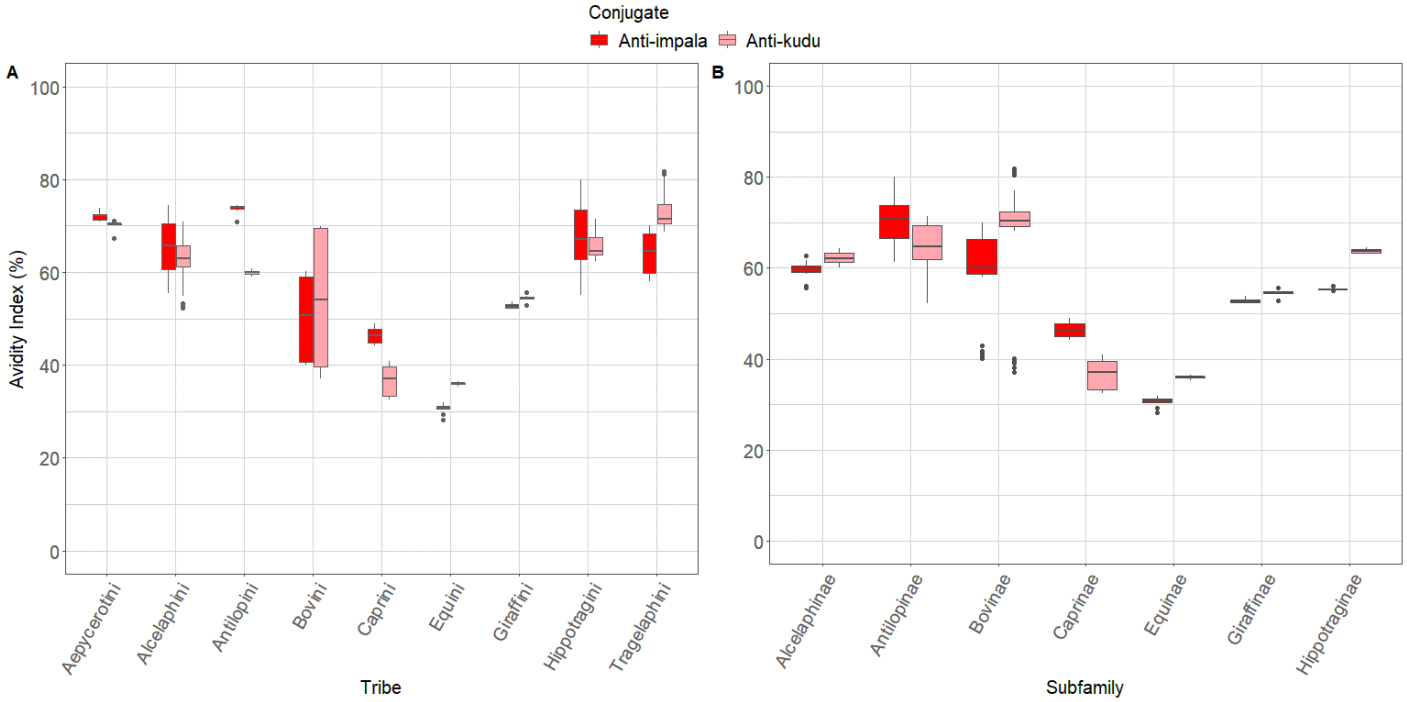

Figure 2-6: Box plots showing the avidity index for the wildlife species grouped by A) the tribe they belong to and B) their subfamilies. These species were classified based on the work described by Hassanin and Emmanuel (1999) and Gatesy et al. (1997). Red indicates anti-impala IgY and pink is anti-kudu IgY conjugate.

2.5. Discussion

We developed conjugates for kudu (IgY anti-kudu) and impala (IgY anti-impala), two important hosts in disease transmission in wildlife in South Africa. We show that the conjugates are specific to their respective species and have better avidity than the commercially available protG and pAG conjugates. This is the first study to develop species-specific conjugates for antibody detection in kudu and impala with quantitative evidence of cross-reactions with antibodies of other species of African wildlife, providing the tools for the development and validation of primary binding assays like the indirect ELISA. These assays can improve sero-surveillance for infectious diseases in wildlife.

Chicken anti-kudu and chicken anti-impala conjugates developed in this study confirm the importance of using IgY in developing secondary antibodies against mammalian sera IgY from eggs is that it is cheap to produce in large volumes and ethically preferable as no blood collection from animals is required (Amro et al., 2018).This study showed that the developed anti-kudu IgY and anti-impala IgY conjugates had higher AIs (>70%) as compared to commercial pAG and protG conjugates with AIs less than 30%. This confirms stronger binding of the secondary IgY antibodies which is an important parameter in the development of primary binding assays like the indirect ELISA (Dimitrov et al., 2011). The weak binding observed for protG and pAG conjugates to impala sera in this study was also observed in other studies(Feir et al., 1993, Stöbel et al., 2002, Kramsky et al., 2003, Smit, 2017), and is also in agreement with the findings of Smit (2017). However, it contradicts other findings that reported strong reactivity with either protG or pAG (Feir et al., 1993, Kelly et al., 1993, Stöbel et al., 2002, Kramsky et al., 2003).This could be due to the differences in the methods used. In this study, we measured the binding strength of the antibodies in the presence of a dissociative agent while other studies only compared binding of conjugates under normal physiological conditions. Similar to this study Smit (2017) also recorded high OD values for protA and pAG but showed that the avidity was weak and binding could easily be disrupted under stringent binding conditions, like in the presence of the chaotropic agent.

Sera from the different species reacted differently with the two developed IgY conjugates and the two commercial conjugates. Wildlife species had stronger binding to the IgY conjugates than to the commercial conjugates, except for the plains zebra. Although the wildlife species demonstrated good avidity with both anti-kudu IgY and anti-impala IgY conjugates, there appeared to be a phylogenetic preference between the two IgY conjugates. The antelopes more closely related to kudu had better avidity to the anti-kudu IgY conjugate and the ones more closely related to impala had better avidity to the anti-impala IgY conjugate (Figure 2-5 A and B). Species-specific conjugates can also bind with good avidity to closely related species (Smit, 2017). This means that the more distantly related they are to the species for which the conjugate was developed, the less avidity. For example, sable, roan, tsessebe, blesbok and bontebok had better avidity with anti-impala IgY as they all belong to the a*ntilopinae* family as described by Hassanin and Emmanuel (1999). Similarly, the members of the Tragelaphini tribe such as nyala, eland, and bushbuck (Hassanin and Emmanuel, 1999) had better avidity with the anti-kudu IgY conjugate (Figure 2-4 and Figure 2-5 A and B). A weaker avidity was seen in more distant related species like cattle, goat, plains zebra and giraffe (Figure 2-5 and Figures 2-6 A and B).

There are reports in the literature where assays developed for livestock were used for antibody detection in wildlife. These include studies were assays that have been developed for horses were used for zebra (Abdelgawad et al., 2015), domestic dogs for African wild dog(Kat et al., 1995), domestic cats for lions (Gumbo et al., 2022) and domestic cattle for African buffalo (Sarangi et al., 2022). However, in this study we report a significant variation between domestic cattle and African buffalo within the bovini tribe. African buffalo reacted strongly with anti-kudu and anti-impala conjugates with an avidity of greater than 60% but had a poor avidity of less than 20% with pAG and protG conjugates. Whereas domestic cattle on other hand had a stronger avidity with pAG and protG conjugates but demonstrated poor avidity index with anti-kudu and anti-impala conjugates (≤40%). These results emphasise the need to develop and validate serological assays that are specific for wildlife species and caution against interspecies use of assays without proper validation even if they belong to the same tribes.

The conjugates developed here are important tools for the development of validated assays for the surveillance of emerging and re-emerging diseases of veterinary and human importance. And the concept of a diagnostic test being fit and validated for specific host species is one that is critical and promoted by the World Organization for Animal Health (OIE) (Gardner et al., 2019). Wildlife diseases are often understudied, and little is known about the accuracy of the diagnostic techniques employed (Jia et al., 2020). One pertinent question that has remained is about the accuracy of the of diagnostic tests validated in domestic stocks when used in wildlife species. Majority of the wild animals tested in this study are important host to various pathogens responsible for a range of diseases in the wild. And these animals demonstrated strong avidity with either ant-impala or anti-kudu, this is therefore an indication that developing multi-species polyclonal conjugate consisting of a cocktail of immunoglobulins could further improve active surveillance and facilitate the validation of immunoassays in these species.

The pAG conjugate tested in this study demonstrated an avidity index of less than 40% with most wildlife species, with the exception of the plains zebra, black and blue wildebeest and waterbuck. These results corroborate the findings of Smit (2017) who reported similar AIs in these species. The poor reactivity seen in the majority of the wildlife species could be attributed to a genetic predisposition that could make pAG bind weakly with the IgG of the wildlife species. Except for black wildebeest and tsessebe, all the wildlife species had an AI of less than 50% with the protG conjugate used in this study. Factors that could influence the binding of conjugates in primary binding assays could include variation in antibody structure between species, a limited amount of IgG in the original serum, as seen in immunocompromised individuals or the presence of inhibitors(Kelly et al., 1993, Kramsky et al., 2003). Also, pAG and protG could selectively bind to the subclasses or isotypes of IgG as seen in mice, where IgG2 is bound more strongly to protG, while IgG1 binds very weakly (Björck and Kronvall, 1984). Therefore, when an immune response is predominantly of a different subclass, these subclasses may not be detected in an immunoassay that is utilizing these conjugates. The variation in the avidity of conjugates to the immunoglobulins of different species emphasises the importance of proper species-specific validation of diagnostic assays.

The level of avidity of the conjugates impacted the outcome of the antiPA ELISA significantly. The antiPA ELISA ODs for developed conjugates were about 50% less than that of the commercial conjugates. This agrees with the assertion that the conjugate will only optimally bind specifically to the species for which it was developed for or for most closely related species (Smit, 2017, Feir et al., 1993). The samples tested with protG showed higher ODs than those tested with the developed conjugates. As this study is a continuation of a previous study where we looked at antiPA antibodies in wildlife species in the Kruger and Etosha National Parks (Ochai et al., 2022), we noticed that only the animals (kudu and impala in KNP) that were positive for both ELISA and Toxin Neutralisation Assay (TNA) still remained positive following the drop in the OD values.

2.6. Conclusion

Results of this study demonstrate the need to develop conjugates for immunoassays that are specific to African wildlife, as they are important hosts to many pathogens of human, animal, and zoonotic importance in KNP and parks like it. Kudu and impala sera demonstrated better avidity to their corresponding conjugates than to the commercial conjugates. The wildlife species tested in this study showed stronger avidity to the developed conjugates than to the commercial conjugates. This could also be achieved through a multi-species polyclonal conjugate consisting of a cocktail of immunoglobulins from various wildlife species. Such evidence-based methods could allow for more accurate validation of diagnostic assays for the detection of incidence and prevalence of wildlife and zoonotic diseases.

2.7. Suggestions for Future Research

Future studies to examine the development of polyclonal cocktail conjugated secondary antibodies for other African wildlife could establish immunodiagnostic assays that would be more specific to identify pathogens of veterinary and human diseases. Secondly, owing to the varying reports of avidity and binding ability of commercial conjugates, we suggest studies that evaluate these conjugates on a wider selection of wildlife species

beyond what is covered in this study. Finally, we advocate more studies focused on how the use of different conjugates affects the outcome of disease surveillance and screening.

2.8. Data Availability Statement

At the time of publication, data were not publicly available from the authors. The raw data supporting the conclusions of this article will be made available by the corresponding author, without undue reservation.

2.9. Ethics Statement

This study was reviewed and approved by University of Pretoria Research Ethics Committee, Animal Ethics Committee (REC 049-21), Department of Agriculture, Forestry and Fisheries (DAFF) in South Africa (Ref 12/11/1/1/6 (2382SR)) in South Africa, South African National Parks (SANParks), South Africa (Ref: BMTA 006/22).

2.10. Author Contributions

SO, and HH conceived the ideas of the study. SO, HH, and JC designed the study. SO collected the data. SO, JC and HH designed the methodology. SO analysed the data. SO, and HH wrote the first draft of the manuscript. All authors contributed significantly to manuscript revision, read, and gave approval for publication.

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2.12. Acknowledgments

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2.13. Supplementary information

Table S 2-1:Summary of results obtained from publications that reported the use of commercially available Protein A, Protein G, and Protein AG in some African wildlife species and domestic stock. The table contains references of publications, methods used in parenthesis, and different interpretations of results. NA here stands Not Applicable

		Kelly et al. (1993) (Direct ELISA)		**Stöbel et al. (2002) (Indirect ELISA)**		**Feir et al. (1993) (Indirect ELISA)**		**Kramsky et al. (2003)* (Direct ELISA)**	**Smit (2017) (Direct Avidity ELISA)**
Species	**Common name**	**Protein A**	**Protein AG**	**Protein A**	**Protein G**	**Protein A**	**Protein G**	**Protein G**	**Protein AG**
Loxodonta africana	African elephant	Weak	Weak	Medium	Low	NA	NA		Weak
Giraffa camelopardalis	Giraffe	Weak	Weak	Low	High	NA	NA		Weak
Aepyceros melampus	Impala	Weak	Strong	None	Low	None	None	Lesser to control	No reaction
Connochaetes taurinus	Blue wildebeest	Weak	Strong	NA	NA	NA	NA		Moderate
Syncerus caffer	African buffalo	Weak	Strong	None	Low	NA	NA		Strong
Tragelaphus angasii	Nyala	Weak	Strong	None	Low	NA	NA		Weak
Taurotragus oryx	Common eland	Weak	Strong	NA	NA	NA	NA		Weak

Tragelaphus scriptus	Bushbuck	Weak	Strong	NA	NA	NA	NA		Weak
Hippotragus niger	Sable antelope	Weak	Strong	None	High	None	Reacted	Equivalent to control	Moderate
Kobus ellipsiprymnus	Waterbuck	Weak	Strong	None	High	NA	NA		Moderate
Equus quagga burchellii	Burchell's zebra	NA	NA	NA	NA	NA	NA		Strong
Alcelaphus buselaphus	Red hartebeest	NA	NA	NA	NA	NA	NA		Weak
Connochaetes gnou	Black wildebeest	NA	NA	None	low	NA	NA		Strong
Damaliscus lunatus	Common tsessebe	NA	NA	NA	NA	NA	NA		Weak
Damaliscus pygargus phillipsi	Blesbok	NA	NA	None	Medium	NA	NA	Equivalent to control	
Antidorcas marsupialis	Springbok	NA	NA	High	High	None	Reacted		Strong
Hippotragus equinus	Roan antelope	NA	NA	None	Low	NA	NA	Equivalent to control	Moderate
Oryx gazella	Gemsbok	NA	NA	None	Low	NA	NA		Moderate
Tragelaphus strepsiceros	Greater kudu	Weak	Strong	Medium	Medium	None	Reacted	Equivalent to control	No reaction

Bos taurus	Domestic cattle	Weak	Strong	NA	NA	NA	NA	Control	
Capra hircus	Goat	Weak	Strong	NA	NA	NA	NA		
Ovis aries	Sheep	Weak	Strong	NA	NA	NA	NA	Lesser to control	
Damaliscus pygargus	Bontebok							Equivalent to control	

Table S 2-2:SDS-PAGE gel (8%) reagents and volume of separating and stacking gel (Laemmli, 1970)

Reagents	**Separating gel (ml)**	**Stacking gel (ml)**
Distilled water	7	2.1
30% Acrylamide	4	0.5
1.5 M Tris (pH 8.8)	3.8	0.380
10% SDS	0.150	0.030
10%APS	0.150	0.030
TEMED	0.009	0.003

Supplementary Methodology 1: Briefly, the microtiter plates (Thermo Scientific™ Pierce 96-well Plates-Corner, USA) were coated overnight with 25 µg/ml of the extracted IgG from the respective species (impala and kudu) as described by Staak et al. (2001). Plates were washed twice with Phosphate Buffered Saline (PBS) supplemented with 0.05% Tween-20 (Thermo Fisher Scientific, Waltham, MA USA) (PBST) using a plate washer (Biorad PW40, Mamesla-Coquette, France). The plates were blocked blocked with PBST supplemented with 5% skimmed milk powder (PBSTM) and then incubated for 1 hour at room temperature. This was followed by washing the plates twice. A 100 µL of the the egg yolk from each chicken was added

into the plate with a starting dilution of 1:20 in PBSTM starting from the first column of each plate. This was followed by 30 minutes incubation on a rotatory incubator (Environmental Shaker-Incubator ES-20, Biosan Ltd, Germany). Afterwards, the plates were washed five times and a 100 µL of a 1:10000 dilution of goat anti-chicken horseradish peroxidase conjugate (Invitrogen goat anti-chicken, USA) was added to each well and incubated for 30 minutes on the rotary incubator. This was followed by a wash step and subsequently, the substrate 2,2'-Azinobis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) (Thermo Scientific 1-Step ABTS, USA) was added and incubated in the dark for 45 minutes. The absorbance was read at 405 nm using the plate reader(Biotek Powerwave XS2 reader, Vermont, USA).

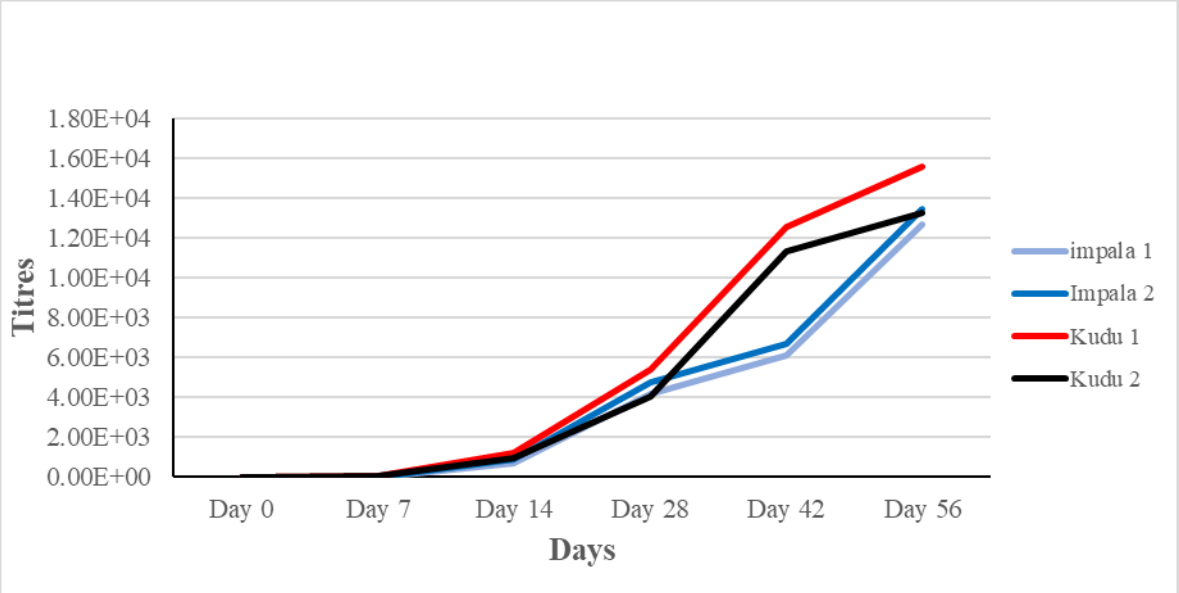

Figure S 2-1: Line chart showing the increase in titres over days of antibodies against impala and kudu Immunoglobulin G (IgG). The first vaccination was given on “Day 0”, the second dose was given on “Day 23” and the last dose was given on “Day 42”

Supplementary Methodology 2: A pooled sera for each species (impala and kudu) were coated to microtiter plates with a starting dilution of 1:1000 from column 1 to 11 in coating buffer (bicarbonate buffer) left overnight at 4°C to incubate. This was followed by a blocking step with the blocking buffer (200 µL) containing PBST and 5% skimmed milk powder (PBSTM) and then incubated at room temperature for 1 hour. The developed conjugates were tested against each species with a starting dilution of 1:200 row A to row G. The blanked wells are row H and column 11. The plates were incubated at room temperature for 30 minutes. Subsequently, the plates were washed and after which the ABTS substrate (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt; Thermo Scientific 1-Step ABTS, USA) was added and allowed in the dark for 45 minutes. The absorbance was read at 405 nm using the plate reader (Biotek Powerwave XS2 reader, Vermont, USA).

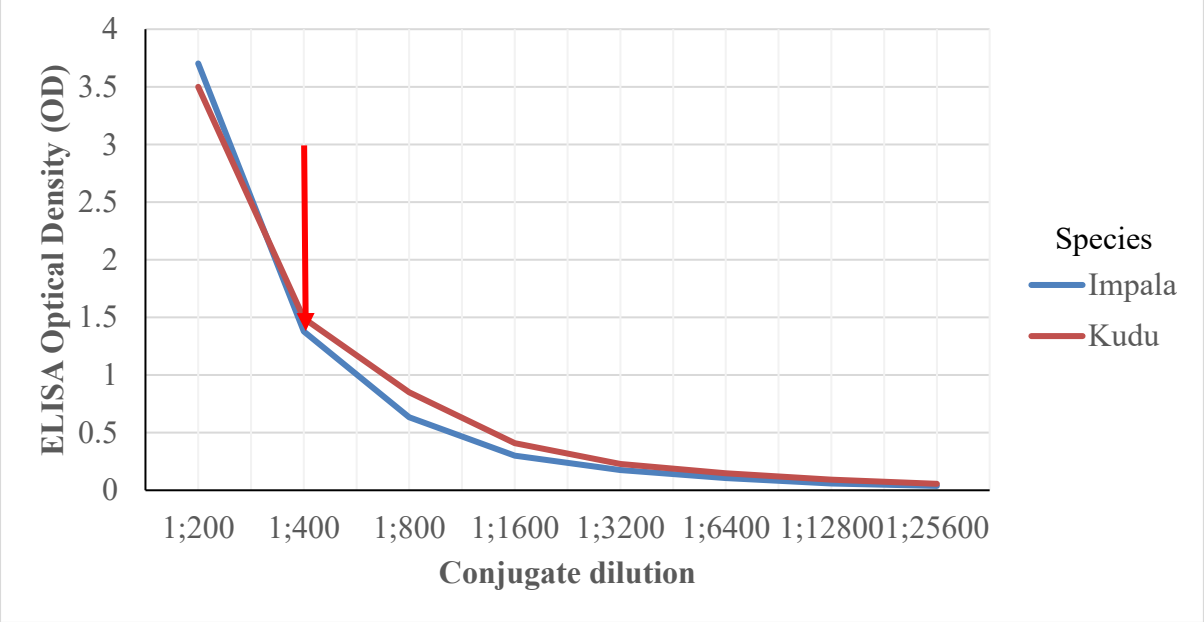

Figure S 2-2:Line graph showing the concentration (Optical Density) of chicken anti-kudu and chicken anti-impala conjugates at different dilutions. The blue line represents anti-impala conjugate while the orange line represents anti-kudu conjugate. The red arrow depicts the optimal dilution (1:400) for the conjugates.

Table S 2-3: Anti-impala summary statistics showing mean OD of PBS and potassium thiocyanate treated wells, mean OD of PBS and chaotrope treated wells for the goat control, percentage reduction in OD, and the mean avidity index and standard deviation (SD) and the p-value of the independent T-test comparing avidity index of each species and the impala control.

Species	Mean OD ± SD PBS	Mean OD ± SD Chaotrope	Mean OD impala Control	Mean OD control chaotrope	Percentage reduction	Mean Avidity index ± SD	P- value
African buffalo	1.64 ± 0.03	1.06 ± 0.02	1.67	1.03	35.18	64.82 ± 1.37	4.58E-10
Black wildebeest	1.37 ± 0.05	0.81 ± 0.05	1.62	1.34	40.50	59.5 ± 1.86	9.77E-11
Blesbok	1.46 ± 0.04	1.07 ± 0.02	1.54	1.15	26.82	73.18 ± 1.62	0.088907
Blue wildebeest	1.8 ± 0.04	1.07 ± 0.04	1.86	1.35	40.30	59.7 ± 1.55	3.10E-12
Bontebok	1.16 ± 0.02	0.82 ± 0.01	1.86	1.35	29.12	70.88 ± 0.87	0.007503
Bushbuck	1.16 ± 0.02	0.78 ± 0.02	1.68	1.18	32.93	67.07 ± 0.39	1.48E-09
Cattle	0.82 ± 0.02	0.34 ± 0.01	1.84	1.35	59.05	40.95 ± 0.9	5.17E-23
Eland	1.26 ± 0.03	0.74 ± 0.02	1.72	1.21	40.94	59.06 ± 0.51	1.07E-15
Gemsbok	1.24 ± 0.02	0.87 ± 0.02	1.86	1.35	29.66	70.34 ± 0.86	0.000329
Giraffe	1.41 ± 0.05	0.74 ± 0.02	1.62	1.34	47.29	52.71 ± 0.52	3.85E-18
Goat	0.94 ± 0.03	0.42 ± 0.01	1.84	1.35	55.11	44.89 ± 0.5	8.84E-20
Impala	1.96 ± 0.03	1.41 ± 0.02	1.96	1.36	27.91	72.09 ± 0.89	NA
Kudu	1.52 ± 0.03	1.01 ± 0.02	1.86	1.35	33.33	66.67 ± 1.23	5.30E-09
Nyala	1.64 ± 0.03	1.13 ± 0.01	1.84	1.35	31.19	68.81 ± 0.4	1.71E-07
Plains zebra	0.36 ± 0.03	0.11 ± 0.01	1.86	1.35	69.45	30.55 ± 1.04	5.32E-24
Red hartebeest	1.42 ± 0.04	0.76 ± 0.02	1.54	1.15	46.44	53.56 ± 1.46	3.41E-15
Roan	1.47 ± 0.02	0.96 ± 0.02	1.72	1.21	34.92	65.08 ± 0.72	7.79E-13
Sable	1.08 ± 0.02	0.76 ± 0.02	1.84	1.35	30.14	69.86 ± 1.81	0.004318
Sheep	0.41 ± 0.05	0.24 ± 0.03	1.68	1.18	41.47	58.53 ± 2.14	4.74E-10
Springbok	1.55 ± 0.02	1.14 ± 0.02	1.85	1.36	26.31	73.69 ± 1.04	0.002179
Tsessebe	1.64 ± 0.03	1.06 ± 0.02	1.67	1.03	35.18	64.82 ± 1.37	4.58E-10
Waterbuck	1.84 ± 0.04	1.02 ± 0.03	1.67	1.03	44.68	55.32 ± 0.29	2.16E-14

Table S 2-4: Anti-kudu summary statistics showing mean OD of PBS and potassium thiocyanate treated wells, mean OD of PBS and chaotrope treated wells for the goat control, percentage reduction in OD, and the mean avidity index and standard deviation (SD) and the p-value of the independent T-test comparing avidity index of each species and the kudu control. Abbreviation such as NA means Not Applicable.

Species	Mean OD ± SD PBS	Mean OD ± SD chaotrope	Mean OD kudu Control	Mean OD control chaotrope	Percentage reduction	Mean Avidity index ± SD	P- value
African buffalo	1.72 ± 0.06	1.19 ± 0.04	1.83	1.33	30.73	69.27 ± 0.64	2.91E-06
Black wildebeest	1.75 ± 0.03	1.11 ± 0.01	2.47	1.80	36.59	63.41 ± 0.49	2.80E-11
Blesbok	1.76 ± 0.06	1.15 ± 0.03	1.76	1.31	34.37	65.63 ± 0.86	5.77E-11
Blue wildebeest	0.85 ± 0.02	0.52 ± 0.01	1.43	1.09	39.03	60.97 ± 0.71	6.58E-14
Bontebok	0.74 ± 0.02	0.52 ± 0.02	1.46	1.20	30.23	69.77 ± 0.71	2.07E-05
Bushbuck	0.87 ± 0.04	0.61 ± 0.02	1.65	1.21	29.74	70.26 ± 0.72	0.000188
Cattle	0.66 ± 0.03	0.26 ± 0.01	1.65	1.18	60.72	39.28 ± 0.99	4.67E-22
Eland	2.15 ± 0.06	1.53 ± 0.03	2.18	1.59	29.07	70.93 ± 1.38	0.022406
Gemsbok	1.02 ± 0.05	0.81 ± 0.04	1.86	1.34	20.78	78.92 ± 1.18	3.03E-10
Giraffe	1.34 ± 0.04	0.73 ± 0.02	2.47	1.80	45.49	54.51 ± 0.69	1.47E-16
Goat	0.51 ± 0.06	0.2 ± 0.02	1.64	1.18	60.28	39.72 ± 0.87	1.39E-21
Impala	1.61 ± 0.04	1.13 ± 0.02	1.78	1.30	29.80	70.2 ± 1.05	0.000364
Kudu	1.86 ± 0.03	1.34 ± 0.02	1.86	1.35	27.64	72.36 ± 1.13	NA
Nyala	1.85 ± 0.04	1.5 ± 0.03	1.86	1.34	18.68	80.82 ± 1.42	3.76E-11
Plains zebra	0.56 ± 0.02	0.2 ± 0.01	1.43	1.09	64.03	35.97 ± 0.38	1.81E-17
Red hartebeest	1.51 ± 0.05	1 ± 0.03	1.76	1.31	33.77	66.23 ± 0.83	1.63E-10
Roan	2.14 ± 0.06	1.41 ± 0.03	2.18	1.59	34.03	65.97 ± 0.82	8.90E-11
Sable	1.76 ± 0.02	1.12 ± 0.01	1.89	1.67	36.16	63.84 ± 0.69	2.73E-12
Sheep	0.67 ± 0.04	0.45 ± 0.02	1.65	1.21	33.49	66.51 ± 1.72	1.53E-07
Springbok	1.6 ± 0.04	0.96 ± 0.03	1.80	1.31	40.07	59.93 ± 0.46	5.74E-13
Tsessebe	1.32 ± 0.04	0.83 ± 0.02	1.63	1.18	37.55	62.45 ± 0.72	2.08E-13
Waterbuck	1.26 ± 0.02	1.63 ± 0	1.18	1.18	36.28	63.72 ± 0.54	1.43E-11

Table S 2-5: Protein AG summary statistics showing mean OD of PBS and potassium thiocyanate treated wells, mean OD of PBS and chaotrope treated wells for the goat control, percentage reduction in OD, and the mean avidity index and standard deviation (SD) and the p-value of the independent T-test comparing avidity index of each species and the cattle control. Abbreviation such as NA means Not Applicable.

Species	Mean OD ± SD PBS	Mean OD ± SD chaotrope	Mean OD cattle Control	Mean OD control chaotrope	Percentage reduction	Mean Avidity index ± SD	P- value
African buffalo	3.64 ± 0.05	0.68 ± 0.02	2.46	1.83	81.28	18.72 ± 0.63	1.14E-20
Black wildebeest	2.87 ± 0.06	1.41 ± 0.04	2.26	1.46	50.78	49.22 ± 0.69	2.96E-17
Blesbok	2.49 ± 0.1	1.02 ± 0.06	2.33	1.75	59.00	41 ± 1.04	2.76E-21
Blue wildebeest	2.53 ± 0.05	1.25 ± 0.03	2.66	1.93	50.58	49.42 ± 0.53	1.07E-15
Bontebok	3.36 ± 0.08	0.97 ± 0.02	2.43	1.83	71.20	28.8 ± 0.68	2.63E-20
Bushbuck	1.86 ± 0.03	0.52 ± 0.02	2.12	1.56	72.16	27.84 ± 0.94	7.27E-23
Cattle	2.43 ± 0.04	1.83 ± 0.03	2.43	1.83	24.49	75.51 ± 1.29	NA
Eland	2.53 ± 0.03	0.67 ± 0.01	2.63	1.92	73.59	26.41 ± 0.31	1.39E-16
Gemsbok	3.59 ± 0.02	0.95 ± 0.02	2.43	1.83	73.49	26.51 ± 0.49	2.36E-18
Giraffe	2.4 ± 0.07	1.05 ± 0.03	2.46	1.83	56.50	43.5 ± 0.71	1.88E-18
Goat	2.21 ± 0.04	1.77 ± 0.04	2.43	1.83	20.20	79.8 ± 0.9	2.43E-07
Impala	2.49 ± 0.21	0.53 ± 0.04	2.21	1.77	78.53	21.47 ± 0.66	9.31E-21
Kudu	2.77 ± 0.11	0.42 ± 0.02	2.21	1.77	84.77	15.23 ± 1.1	5.96E-25
Nyala	2.52 ± 0.09	0.85 ± 0.05	3.70	2.74	66.34	33.66 ± 1.06	1.31E-22
Plains zebra	2.66 ± 0.05	1.37 ± 0.03	2.66	1.93	48.67	51.33 ± 0.48	6.22E-15
Red hartebeest	2.19 ± 0.05	0.73 ± 0.04	2.33	1.75	66.62	33.38 ± 1.12	9.05E-23
Roan	2.78 ± 0.13	0.79 ± 0.05	2.63	1.92	71.45	28.55 ± 0.72	8.83E-21
Sable	2.46 ± 0.16	0.35 ± 0.04	2.43	1.83	85.72	14.28 ± 2.15	6.41E-20
Sheep	1.93 ± 0.05	1.05 ± 0.03	2.12	1.56	45.50	54.5 ± 1.45	3.76E-17
Springbok	2.52 ± 0.09	0.85 ± 0.05	3.70	2.74	66.34	33.66 ± 1.06	1.31E-22
Tsessebe	2.81 ± 0.03	0.81 ± 0.03	2.66	1.96	71.09	28.91 ± 1.26	2.41E-23

Waterbuck	1.89 ± 0.06	0.93 ± 0.03	2.66	1.96	50.48	49.52 ± 0.8	4.66E-18

Table S 2-6: Protein G summary statistics showing mean OD of PBS and potassium thiocyanate treated wells, mean OD of PBS and chaotrope treated wells for the goat control, percentage reduction in OD, and the mean avidity index and standard deviation (SD) and the p-value of the independent T-test comparing avidity index of each species and the goat control. Abbreviation such as NA means Not Applicable.

Species	Mean OD ± SD PBS	Mean OD ± SD chaotrope	Mean OD goat Control	Mean OD control chaotrope	Percentage reduction	Mean Avidity index ± SD	P- value
African buffalo	1.3 ± 0.04	0.24 ± 0.01	2.58	1.90	81.19	18.81 ± 0.75	2.85E-24
Black wildebeest	2.45 ± 0.06	1.4 ± 0.03	2.58	1.90	42.76	57.24 ± 0.88	4.11E-16
Blesbok	2.53 ± 0.03	0.66 ± 0.01	2.63	1.93	73.70	26.3 ± 0.59	7.91E-27
Blue wildebeest	1.7 ± 0.02	0.46 ± 0.02	2.14	1.59	73.21	26.79 ± 0.78	5.41E-23
Bontebok	1.64 ± 0.09	0.43 ± 0.03	2.14	1.59	73.46	26.54 ± 1.03	1.77E-19
Bushbuck	1.94 ± 0.02	0.54 ± 0.01	2.16	1.58	72.19	27.81 ± 0.38	1.13E-30
Cattle	1.36 ± 0.03	0.75 ± 0.02	2.14	1.59	45.14	54.86 ± 0.55	8.68E-23
Eland	2.15 ± 0.03	0.98 ± 0.02	2.42	1.75	54.71	45.29 ± 0.82	8.02E-20
Gemsbok	1.52 ± 0.11	0.28 ± 0.02	2.14	1.59	81.76	18.24 ± 1.37	1.48E-17
Giraffe	2.62 ± 0.06	1.16 ± 0.04	2.58	1.90	55.50	44.5 ± 1.03	2.87E-17
Goat	2.14 ± 0.03	1.59 ± 0.02	2.14	1.59	25.47	74.53 ± 0.41	NA
Impala	2.89 ± 0.08	0.68 ± 0.01	2.14	1.59	76.48	23.52 ± 0.56	7.61E-28
Kudu	2.82 ± 0.09	0.66 ± 0.02	2.14	1.59	76.39	23.61 ± 0.99	3.19E-20
Nyala	1.61 ± 0.08	0.47 ± 0.04	2.16	1.58	70.94	29.06 ± 2.45	3.85E-13
Plains zebra	1.08 ± 0.09	0.19 ± 0.01	2.14	1.59	82.01	17.99 ± 1.09	1.14E-19
Red hartebeest	2.54 ± 0.04	0.76 ± 0.03	2.63	1.93	70.20	29.8 ± 1.01	3.36E-17
Roan	2.06 ± 0.03	1.02 ± 0.02	2.42	1.75	50.80	49.2 ± 0.23	1.66E-23
Sable	1.16 ± 0.08	0.19 ± 0.01	2.16	1.58	83.42	16.58 ± 0.71	3.17E-25
Sheep	2.15 ± 0.03	1.28 ± 0.01	2.16	1.58	40.32	59.68 ± 0.26	8.46E-22
Springbok	1.56 ± 0.03	0.55 ± 0.01	2.14	1.59	64.62	35.38 ± 0.87	1.66E-20
Tsessebe	2.41 ± 0.03	1.21 ± 0.02	2.47	1.85	49.62	50.38 ± 0.65	1.05E-21

Waterbuck	2.32 ± 0.08	0.86 ± 0.03	2.47	1.85	62.86	37.14 ± 0.47	6.05E-28

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Chapter 3. Comparing microbiological and molecular diagnostic tools for the surveillance of anthrax.

Sunday O. Ochai[1*], Ayesha Hassim[1], Edgar H. Dekker[2], Thuto Magome[3], Kgaugelo E. Lekota[3], S. Marcus Makgabo[1,4], Lin-Mari de Klerk-Loris[2], O. Louis van Schalkwyk[1,2,5] Pauline L. Kamath[6] , Wendy C. Turner[7], Henriette van Heerden[1]

[1] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa.

[2] Office of the State Veterinarian, Department of Agriculture, Forestry and Fisheries, Government of South Africa, Skukuza, South Africa

[3] Unit for Environmental Sciences and Management, Microbiology, North West University, Potchefstroom, South Africa

[4] Department of Life and Consumer Sciences, College of Agriculture and Environmental Sciences, University of South Africa (UNISA), Florida Campus, Roodepoort 1709, South Africa.

[5] Department of Migration, Max Planck Institute of Animal Behavior, Radolfzell, Germany

[6] School of Food and Agriculture, University of Maine, Orono, Maine, U.S.A.

[7] U.S. Geological Survey, Wisconsin Cooperative Wildlife Research Unit, Department of Forest and Wildlife Ecology, University of Wisconsin-Madison, Madison, Wisconsin, U.S.A

* Correspondence: Sunday O. Ochai; s.o.ochaijr@gmail.com

3.0. Abstract

The diagnosis of anthrax, a zoonotic disease caused by *Bacillus anthracis* can be complicated by detection of closely related species. Conventional diagnosis of anthrax involves microscopy, culture identification of bacterial colonies and molecular detection. Genetic markers used are often virulence gene targets such as *Bacillus anthracis* protective antigen (*pagA,* as also called BAPA, occurring on plasmid pXO1), lethal factor (*lef,* on pXO1), as well as chromosomal (Ba-1) and plasmid (capsule-encoding *cap*B/C, located on pXO2). Combinations of genetic markers using real-time/quantitative polymerase chain reaction (qPCR) are used to confirm *B. anthracis* from culture but can also be used directly on diagnostic samples to avoid propagation and its associated biorisks and for faster identification. We investigated how the presence of closely related species could complicate anthrax diagnoses with and without culture to standardise the use of genetic markers using qPCR for accurate anthrax diagnosis. Using blood smears from 2012-2020 from wildlife mortalities (n=1708) in Kruger National Park in South Africa where anthrax is endemic, we contrasted anthrax diagnostic results based on qPCR, microscopy, and culture. From smears, 113/1708 grew bacteria in culture, from which 506 isolates were obtained. Of these isolates, only 24.7% (125 isolates) were positive for *B. anthracis* based on genetic markers or microscopy. However, among these, merely 4/125 (3.2%) were confirmed *B. anthracis* isolates (based on morphology, microscopy, and sensitivity testing to penicillin and gamma-phage) from the blood smear, likely due to poor survival of spores on stored smears. This study identified *B. cereus sensu lato*, which included *B. cereus* and *B. anthracis, Peribacillus spp*., and *Priestia spp.* clusters using *gyrB* gene in selected bacterial isolates positive for *pagA* region using BAPA probe. Using qPCR on blood smears, 52.1% (890 samples) tested positive for *B. anthracis* based on one or a combination of genetic markers which included the 25 positive controls. Notably, the standard *lef* primer set displayed the lowest specificity and accuracy. The BAPA+*lef*+Ba-1 combination showed 100% specificity, sensitivity, and accuracy. Various marker combinations, such as Ba-1+*capB*, BAPA+*capB*, Ba-1+BAPA+*capB*+*lef*, and BAPA+*lef*+*capB*, all demonstrated 100.0% specificity and 98.7% accuracy, while maintaining a sensitivity of 96.6%. Using Ba-1+BAPA+*lef*+*capB*, as well as Ba-1+BAPA+*lef* with molecular diagnosis accurately detects *B. anthracis* in the absence of bacterial culture. Systematically combining microscopy and molecular markers holds promise for notably reducing false positives. This significantly enhances the detection and surveillance of diseases like anthrax in southern Africa and beyond and reducing the need for propagation of the bacteria in culture.

Keywords: Wildlife disease, *Bacillus*, specificity, genetic markers, molecular diagnostics, , Passive disease surveillance, Archival blood smears, Microscopy

3.1. Introduction

Anthrax is an ancient zoonotic disease with a documented history dating back to the Bible (Ben-Noun, 2002). While the disease affects many host species, herbivorous mammals are most susceptible, with fatalities often observed in wildlife and livestock. In addition, humans are susceptible to anthrax infections, and cases occur largely due to the handling or consumption of carcasses, infected meat, and hides (Kamal et al., 2011, W.H.O., 2008). Anthrax is generally known to be caused by *Bacillus anthracis,* which is an aerobic or facultative anaerobic, non-motile, Gram-positive, rod-shaped bacterium that produces endospores. This bacterium occurs in two forms, the spore form and the vegetative form (Vilas-Bôas et al., 2007). The virulence factors of *B. anthracis* are encoded on two plasmids: pXO1, which is responsible for the production of the toxins, and pXO2, which synthesizes the poly-ɣ-D-glutamic acid capsule (Makino et al., 1989, Okinaka, 1999). The pXO1 plasmid contains genes responsible for the production of protective antigen (PA, also referred to as BAPA; W.H.O, 2008), lethal factor (LF) and edema factor (EF) proteins. These proteins are grouped as A_2B-toxins. The A components, which consist of the EF or LF, bears the enzymatic activity (Moayeri and Leppla, 2004, Moayeri and Leppla, 2009, Leppla, 1982). The B component consists of PA, which is the receptor-binding component of the lethal toxin (LT) and edema toxin (ET), and the courier of LF and EF respectively, into the host cells (Smith et al., 1955, Barth et al., 2004, Moayeri and Leppla, 2009, Leppla, 1982).

For a century, identifying anthrax and its causative agent, *B. anthracis*, relied on microbiological culture, microscopy, and biochemistry. Recently, new hypotheses about the disease's presentation, prevention, and infective organisms have emerged in Africa (Antonation et al., 2016, Norris et al., 2021, Tamborrini et al., 2011, Klee et al., 2006). There have been reports of serological cross-reactivity between pathogenic and non-pathogenic *Bacillus spp*. (Marston et al., 2016, Zimmermann et al., 2017), including the high-incidence northern Kruger National Park (Steenkamp et al., 2018, Ochai et al., 2022). Anti-PA and LT-neutralizing antibodies were also detected at higher rates than expected in animals from southern KNP, a low-

incidence area (Ochai et al., 2022). We hypothesized that animals might be reacting to “anthrax-like” microbes with genes similar to *B. anthracis* (Ochai et al., 2022). Additionally, the discovery of anthrax cases caused by *B. cereus* biovar *anthracis* (Bcbva) in West and Central Africa (Hoffmann et al., 2017) prompted us to reassess the robustness of diagnostic tools currently used for anthrax surveillance in southern Africa. Furthermore, anthrax-like illnesses attributed to atypical strains of *B. cereus* and Bcbva. have been reported in animals, some of which include chimpanzees (*Pan troglodytes*), gorilla (*Gorilla gorilla*), elephants (*Loxodonta africana*), cattle (*Bos taurus*), and goats (*Capra hircus*) (Leendertz et al., 2006, Leendertz et al., 2004, Klee et al., 2006, Hoffmann et al., 2017, Pilo et al., 2011, Somerville and Jones, 1972, Antonation et al., 2016) in West and Central Africa. Norris et al. (2023) also reported Bcbva in archival bones and teeth of monkeys from Côte d’Ivoire.

Different methods have been employed in the diagnosis of bacterial zoonoses such as *B. anthracis* over the years. These methods include the identification of bacterial culture isolates, microscopic examination of blood smears, molecular diagnosis targeting pathogen genetic markers and serological identification employing antibodies targeting antigens produced by the pathogen. The success of these techniques, however, depends largely on the specificity and sensitivity of the test being employed (Trevethan, 2017). *Bacillus anthracis* is in the phylum Firmicutes, family Bacillaceae, and belongs to the group referred to as the *B. cereus* group. The *B. cereus* group consists of 11 *Bacillus* species (*B. anthracis, B. cereus, B. thuringiensis, B. mycoides, B. pseudomycoides, B. weihenstephanensis, B. cytotoxicus, B. toyonensis, B. gaemokensis, “B. manliponensis”* and *“B. bingmayongensis*) that have closely related phylogenies (Radnedge et al., 2003, Rasko et al., 2005, Liu et al., 2015) as reflected by high similarities in 16S rRNA gene sequences (Ash et al., 1991, Somerville and Jones, 1972) and other genetic markers such as *gyrB* within the *B. cereus* group (Liu et al., 2021). These species also differ in their aetiology, pathogenesis, clinical manifestations and host preferences (Drobniewski, 1993, Rasko et al., 2005, Pilo and Frey, 2011, Helgason et al., 2000, Ehling-Schulz et al., 2019). The *gyrB* gene encodes the B subunit of DNA gyrase, an

enzyme critical for DNA replication, transcription, and repair in bacteria. The *gyrB* gene sequence is highly conserved among bacterial species but varies enough to distinguish between them (Yamamoto and Harayama, 1995, Liu et al., 2021). Studies have shown that *gyrB* sequencing can offer higher resolution than the more commonly used 16S rRNA gene sequencing in differentiating closely related bacterial species (La Duc et al., 2004, Fox et al., 1992).

The initial step in the confirmation of *B. anthracis* in an anthrax-suspected carcass is the examination of blood smears stained with either Gram or Giemsa stain to view the rod-shaped bacterium (W.H.O., 2008). The presence of encapsulated square-ended rod-shaped bacteria that react to the polychrome methylene blue stain indicates the presence of *B. anthracis* and warrants a sample to be sent to a reference laboratory for confirmation (W.H.O., 2008). To confirm the presence of *B. anthracis* in the reference laboratories, the samples are cultured on blood agar to check for colony morphology (W.H.O., 2008), and the absence of haemolysis and sensitivity to penicillin and bacteriophages (Turnbull et al., 1998, Turnbull, 1999). For additional verification, real-time/quantitative PCR (qPCR) is conducted for the presence of *pagA* with BAPA probe, *lef* (W.H.O., 2008), *capB* (pXO2 (W.H.O., 2008)) and/or *Ba*-1 (Zincke et al., 2020) genes that encode for virulence factors including the PA and capsule as well as *B. anthracis* chromosome, respectively. The qPCR targeting *pagA* with BAPA probe (pXO1) (W.H.O., 2008) and *capC* (pXO2) regions (W.H.O., 2008) used by Lekota et al. (2016) reported that presence of *capC* to be inadequate for distinguishing closely related *Bacillus* species from anthrax outbreaks, while Zincke et al. (2020) used *capB, lef* and Ba-1 targets to differentiate *B. anthracis* from *B. cereus sensu stricto.* Although the Ba-1 marker seems distinctive to *B. anthracis*, its validation has been limited to *B. anthracis* strains of *B. cereus sensu stricto*, similar to the case of *lef* (Zincke et al., 2020, Blackburn et al., 2010). Typically, molecular targets involve the use of specific chromosomal regions unique to *B. anthracis* together with virulence factors situated on either pXO1 or pXO2 plasmids serving as markers (W.H.O., 2008, Lekota et al., 2016, Blackburn et al., 2014), due to the genome similarity amongst closely related *Bacillus* spp. (Léonard et al., 1997,

Lechner et al., 1998) and *B. anthracis* virulence plasmids or their parts detected in closely related species (Baldwin, 2020, Klee et al., 2006). One of the most common diagnostic markers used in the detection of Bcbva is the genomic island IV (GI4) which is unique to Bcbva (Zincke et al., 2020). Over the last decades, there have been calls to move away from culture identification of *B. anthracis* in a bid to reduce biosafety risk and avoid proliferation (Riedel, 2005). Thus, the ultimate goal of this study was to investigate the best practices using culture-free methods for the diagnosis of anthrax.

In a typical *B. anthracis* investigation, the presence of *B. cereus* group species that are not *B. anthracis* have been viewed as contaminants, and the absence of the genes linked to both pXO1 and pXO2 further leads the investigator to view the closely related species as not of importance are associated with less-severe disease (Logan and Turnbull, 1999). However, Toxigenic *B. cereus* are known to have pXO1-like plasmids and other capsule-encoding plasmids that are not pXO2, and *B. cereus* can cause foodborne infections without either pXO1 or pXO2 plasmids (Dietrich et al., 2021). As a result, microbes that lack the *B. anthracis*-specific chromosomal gene (Ba-1) or the pXO1 and pXO2 plasmids can be readily overlooked. In recent years, there have been reports of atypical *B. cereus* strains that are known to cause anthrax-like infections in both humans and animals (Klee et al., 2010) with very similar genes to those found on pXO1 and pXO2 plasmids found in *B. anthracis* (Baldwin, 2020).

Anthrax is endemic in KNP, and park personnel employ a passive surveillance system where blood smears are collected from any deceased animal and stored in an archival collection. We utilised blood smears from the collection, covering the years 2012-2020. This period encompassed known anthrax outbreaks from 2012 to 2015 (Hassim, 2017a, Ochai et al., 2022). From these outbreaks, *B. anthracis* bacilli were initially identified using the microscopic evaluation of blood smears from wildlife carcasses in KNP with follow-up collection of bone, hair, and tissue samples from positive carcass sites in previous study (Hassim, 2017b). In this study, 25 *B. anthracis* isolates previously confirmed using microbiology and PCR from tissue samples linked to positive blood smears served as positive controls. Our

investigation focused on employing microscopy, culture, and molecular markers, including real-time/quantitative polymerase chain reaction (qPCR), to identify *B. anthracis* and distinguish it from *B. cereus* or other closely related microbes. Specifically, we examined: 1) the performance of five molecular markers currently in use (*pagA* with BAPA probe, Ba-1, *lef, capB*, GI4) to identify *B. anthracis* from other bacteria using cultures of blood smears; (2) the performance of five molecular markers to identify *B. anthracis* from *B. cereus* and other closely related bacteria; and 3) we evaluated the agreement between anthrax diagnoses based on blood smear microscopy versus molecular techniques.

3.2. Materials and Methods

3.2.1. Study Area

The Kruger National Park (19,485 km^2; Figure 3-1) is situated in the northeastern part of South Africa, bordering Mozambique and Zimbabwe. The northern half of KNP (Figure 3-1) is considered the anthrax endemic region, where most of the anthrax mortalities have been reported (21, 54); this region is classified as semi-arid and is highly wooded with some grassland savannah (55). KNP has variable elevations, with Pafuri (found in the northernmost part of KNP; 22.4206° S, 31.2296° E) having lower elevation floodplains and mountains towards the northwestern part of the park. In KNP, the high anthrax incidence (endemic) area extends from Pafuri to Shingwedzi (23.1167° S, 31.4333° E) in the north, and the low incidence area extends from Skukuza (24.9948° S, 31.5969° E) to Crocodile Bridge (25.3584° S, 31.8935° E) in the south (Figure 1).

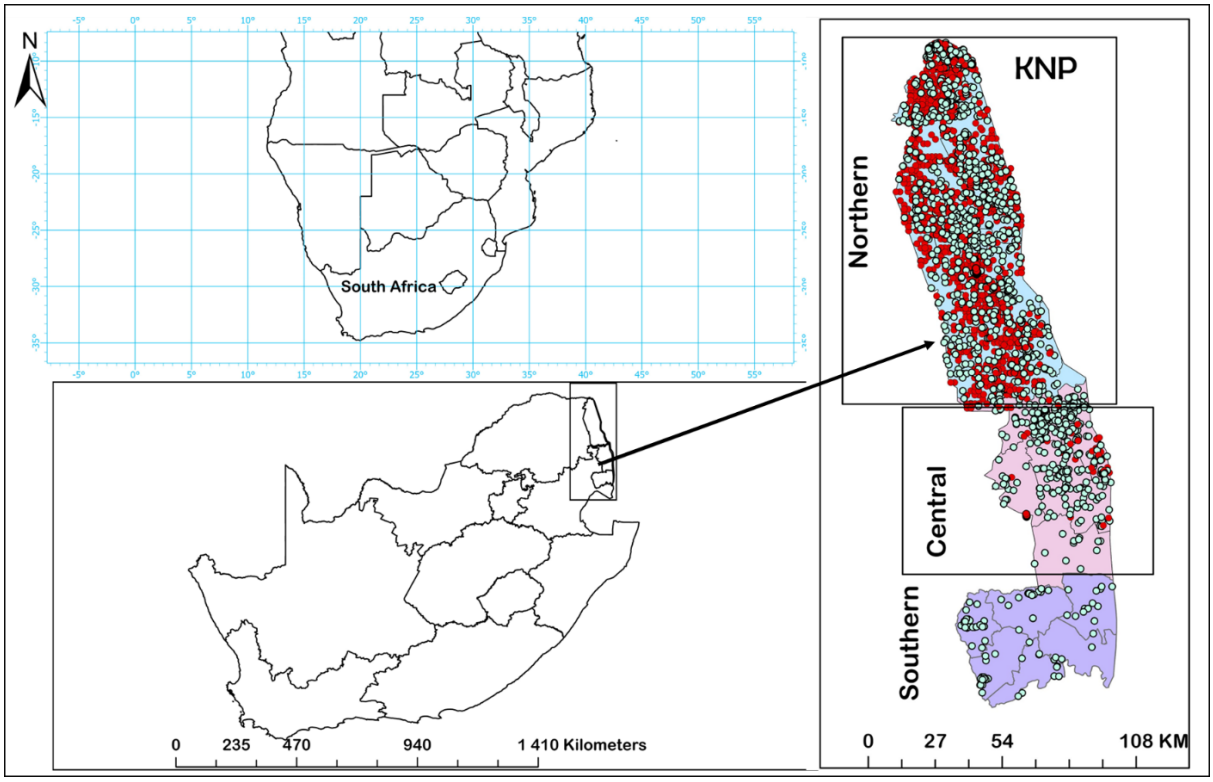

Figure 3-1:Kruger National Park (KNP) in South Africa located in southern Africa is depicted in the map, illustrating the regions where anthrax outbreaks are endemic. The left side of the KNP map displays the distribution of anthrax mortality throughout the park, delineating the administrative regions within. Anthrax positive cases, identified through microscopic examination, are marked with red dots, while green dots indicate anthrax-negative mortalities.

3.2.2. Sample preparation and DNA extraction

Archival blood smears can be an important resource for retrospective studies and for retrieving pathogens like *B. anthracis* that can remain viable for years (Vince et al., 1998, Hassim, 2017a). In KNP, as part of the passive surveillance by the Skukuza State Veterinary Services, blood smears have been collected from all carcasses discovered during field surveys. Two smears were collected per carcass, one of which is stained (with Giemsa), while the other remains unstained. Metadata captured at the

carcass sites include the date, Global Positioning System (GPS) coordinates, locality, species, and sex. These smears were first examined at the time of collection and then stored at room temperature since collection. Aminu et al. (2020) demonstrated that Azure B staining is more robust, consistent and has a higher sensitivity compared to Giemsa only, without Azure B and Polychrome Methylene Blue (PMB) stains. The Giemsa stain used in this study contained Azure B.

A total of 1708 Giemsa-stained blood smear slides (from wildlife mortalities recorded 2012-2020; Table S1) were examined by microscopy at 1000X magnification for the presence of square-ended cells indicative of *B. anthracis*. All phenotypic confirmation of *B. anthracis* by microscopy and plate assays were performed as described by the World Health Organization (W.H.O., 2008). Each slide was examined by two examiners. The selection of smears from this time period (2012-2020) was based on the findings of Hassim (2017a) who demonstrated that isolate recovery reduced with age of the smears. We used the selected unstained smears for additional genetic and microbiological work.

The unstained blood smears from each mortality were scraped into a collection plate and transferred into a 1.5 mL centrifuge tube using a sterile scalpel. The smear scrapings were added to 200 µL of phosphate buffered saline (PBS; Thermo Scientific, MA, USA) and divided into two aliquots. The first aliquot was subjected to automated DNA extraction (QIAcube, QIAGEN GmbH, Hilden, Germany) using the DNA Blood Mini kit (QIAGEN QIAmp, QIAGEN GmbH, Hilden, Germany) and the manufacturer's instructions for DNA extraction from blood were followed. The second aliquot was inoculated on 5% Sheep Blood Agar (SBA) and incubated overnight at 37 °C for use in the morphological identification of bacterial colonies, as described by Parry et al. (1983). On each plate, all bacterial colonies demonstrating different colony morphology were selected and treated as different isolates. All isolates identified were further sub-cultured onto 5% Sheep Blood Agar (SBA) to obtain pure cultures and check for the presence of haemolysis and colony morphology. The purified isolates were further subjected to gamma-phage and penicillin sensitivity tests. Isolates that did not present with a *B. anthracis* characteristic phenotype were retained and

screened using molecular methods. DNA extraction from pure isolates was performed using the Pure link Genomic DNA kit (Thermo Fisher Scientific, MA USA) as prescribed by the manufacturer.

If a mortality was identified as positive for *B. anthracis* based on microscopy, a follow-up sample (soil, bone and/or tissue) from the carcass site was collected as soon as possible (if GPS coordinates were available). From these additional samples, 25 isolates confirmed to be *B. anthracis* based on morphology, microscopy, lack of haemolytic activity, gamma-phage and penicillin sensitivity were used in this study as internal positive controls. These controls serve as a benchmark to verify that the assays are functioning correctly and to validate the results obtained from the experimental samples. Additionally, these controls were obtained at or close to the carcass site and from other tissues or samples of the respective animals, providing a more accurate reference for comparison.

The blood smears were collected as part of passive surveillance system in KNP. Once the blood smear are identified with microscopy as positive for *B. anthracis,* follow-up sample (soil, bone and/or tissue) from the carcass site is collected (if GPS coordinates are avaible). In this study 25 *B. anhtracis* isolates confirmed to be *B. anthracis* based on morphology, microscopy, haemolysis, gamma-phage and penicillin sensitivity (55) and linked to the smears used in this study will be used as positive controls. These samples had all the meta data and GPS coordinates to link them with the current smears and datasets used for this study.

3.2.3. Microscopic examination of bacterial isolates derived from blood smears

For the blood smear scrapings that yielded bacterial growth, the colonies were subcultured (to obtain pure colonies) and transferred directly to a microscope slide, and 5 µl of saline was added, emulsified, and spread evenly on the slide. The slide was allowed to dry and fixed with 95% methanol (Merck KGaA, Darmstadt, Germany) for one minute. The methanol was allowed to dry and a Gram stain was conducted to visualise the presence of Gram positive rods. Cell morphology was observed and recorded at 1000X magnification to confirm the culture

results to identify square-ended rod-shaped *B. anthracis*. Subsequently, to determine encapsulation, polychrome methylene blue stain was performed.

3.2.4. Quantitative polymerase chain reaction (qPCR)

3.2.4.1. qPCR on bacterial isolates derived from smears

The qPCR was performed on two different sample sets. First, cultured isolates from one of the two aliquots of the blood smears (506 isolates from 113 smears) were screened, targeting 5 genetic markers for *pagA* with BAPA probe, Ba-1, *lef*, GI4 and *capB* in a stepwise manner. All isolates were screened even if they were not phenotypically *B. anthracis.* The isolates were first screened with the SYBR Green assays using primers and targets in Table 1 as described by the manufacturer (CelGREEN, Celtic Molecular Diagnostics, Cape Town, South Africa). Isolates that were positive with SYBR Green (n=125) PCR assays on all the markers were then further confirmed using the TaqMan assay for targeting the Ba-1, *lef*, *capB,* GI4 targets (Table 1) as described by Zincke et al. (2020) and the fluorescence resonance energy transfer (FRET) for BAPA (W.H.O., 2008). The inclusion of the chromosomal marker, Ba-1 and GI4 in the assay was based on the premise that it enhances the specificity of the assay, as detailed by (Ågren et al., 2013). The reaction mixtures for the SYBR Green assay, targeting the *pagA* with BAPA probe, Ba-1, *lef*, GI4, and *capB* primer sets, consisted of 0.5 µM of each primer. For Ba-1, *lef*, GI4, and *capB*, the mix included 1x SYBR Green (CelGREEN, Celtic Molecular Diagnostics,, Cape Town, South Africa), while the *pagA* assay utilized FastStart Essential Green Master (Roche, Basel, Switzerland). Each mixture also contained 2 ng of DNA, resulting in a total volume of 20 µL per reaction. Cycling conditions were: a pre-incubation at 95°C for 10 min (20°C/sec ramp), followed by 45 cycles of 95°C for 10 sec and 55°C for 20 sec (both at 20°C/sec ramp), then 72°C for 30 sec (20°C/sec ramp) with signal capture post-annealing. Denaturation involved an immediate 95°C step, cooling to 40°C for 30 sec (both at 20°C/sec ramp), then 80°C instantly with a 0.1°C/sec ramp for continuous signal reading. The process concluded with a cool-down to 40°C for 30 sec (20°C/sec ramp). The assay was performed using the QuantStudio 5 Real-time PCR system (Thermo Fisher Scientific, MA USA). Isolates that were positive for *pagA* (n=14) were selected for further identification using gyrase B (*gyrB*) gene PCR as described by (Liu et al., 2021). We selected these isolates that were *pagA*

PCR positives as it has been hypothesised that closely related bacterial species might be responsible for the anti-PA serological reaction observed in anthrax nonendemic regions (Ochai et al., 2022). The cycle threshold (CT) cutoff was established at 35 for Ba-1 and GI4, as well as for *pagA*, *lef*, and *capB* (Hassim, 2017b, Lekota et al., 2016, Zincke et al., 2020). For *B. anthracis*, we used *B. anthracis* Vollum strain as the positive control, and the 25 smear samples confirmed to be *B. anthracis* in the study of Hassim (2017a) were used as internal controls. We obtained a positive control (DNA from pure culture) for Bcbva from the Robert Kock Institute, Germany.

3.2.4.2. qPCR from direct scrapings of blood smears

Secondly, the 1708 DNA samples obtained from blood smear scrapings were screened for the presence of the pXO1 plasmid, with qPCR assays targeting the *pagA* and *lef*, as well as pXO2 plasmid targeting *capB*. We also screened for the chromosomal markers Ba-1 of *B. anthracis* and GI4 region for Bcbva. To determine the presence of *pagA* with BAPA probe, qPCR was conducted using the FRET on the Light Cycler Nano (Roche, Basel, Switzerland). For the TaqMan assays, the reaction conditions were standardized to a 20-µL mixture containing 1 µL of the DNA template, 1x concentration of PrimeTime Gene Expression Master Mix (IDT, Coralville, IA, USA, Cat No. 1055772), along with primers and probes as listed in Table 3.

The thermal cycling conditions for TaqMan assays were set as follows: an initial denaturation at 95°C for 3 minutes, followed by 45 cycles of denaturation at 95°C for 20 sec and annealing/extension at 60°C for 30 sec. For *lef,* Ba-1, *capB* and GI4, the quantitative PCR TaqMan assay was performed using the QuantStudio 5 Real-time PCR system (Thermo Fisher Scientific, MA USA). Two duplex assays were created for the simultaneous detection of FAM- and VIC-labeled probes. The first duplex targeted Ba-1 and GI4 markers for species identification, while the second targeted *lef* and *capB* virulence markers from pXO1 and pXO2 plasmids, respectively. To prevent spectral overlap in the QuantStudio 5 instrument, colour compensation was conducted with FAM and VIC probes, applying the results to duplex assay data. Tests included all 26 confirmed positive *B. anthracis* strains (~1 ng DNA), and specificity checks involved DNA from Bcbva, and *B. cereus* ATCC 3999. The CT cutoff for positive samples was set at 35 for all the markers (Hassim, 2017b, Lekota et al., 2016).

Table 3-1: Primers, probes and gene targets for the detection of *Bacillus anthracis* from blood smear samples from Kruger National Park, South Africa by qPCR assays.

Primer/Probe (5′–3′)	Chemistry	Target	Reference
Forward - GTACATCTTCTAGCTGTTGCAA Reverse – ACGTAGGAAGACCGTTGATTA Probe - VIC-CGTTGTTGTGTATTTG-MGB	TaqMan	Ba-1	(Zincke et al., 2020)
Forward – TAAGCCTGCGTTCTTCGTAAATG Reverse – GTTCCCAAATACGTAATGTTGATGAG Probe - NED-TTGCAGCGAATGAT-MGB	TaqMan	*capB*	
Forward – CACTATCAACACTGGAGCGATTCT Reverse – AATTATGTCATCTTTCTTTGGCTCAA Probe - Cy5-AGCTGCAGATTCC-MGB	TaqMan	*lef*	
Forward - GGAGATATTAACAAGAGATGGATTGGA Reverse - CAGTAGGCTTGTCTGCTCTAATAAAATT Probe - FAM-ACATGCCAGCGTTTTTTGCCTCTACACA-BHQ1	Taqman	*GI4*	
Forward – CGGATCAAGTATATGGGAATATAGCAA	FRET	*pagA*	(W.H.O., 2008b)

Reverse - CCGGTTT AGTCGTTT CTAATGGAT BAPA-FL - TGCGGTAACACTT CACTCCAGTTCGA-X BAPA-LCRed 640 - CCTGTATCCACCCTCACTCTT CCATTTT C-P			

3.2.5. Molecular identification and phylogenetic analysis on bacterial isolates from smears

The 14 bacterial isolates from blood smear scrapings that tested positive for *pagA* with BAPA probe by the two qPCR approaches were subjected to additional molecular and phylogenetic analysis. The gyrase B (*gyrB*) PCR product was sequenced for molecular taxonomic identification of the isolates. The PCR fragments of the *gyrB* gene of the selected isolates (n = 14), including 4 *B. anthracis* isolates based on microbiology (square-ended bacilli, colony morphology, penicillin and gamma phage sensitive), were sequenced at Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa. A BLAST search query was performed to compare the *gyrB* nucleotide sequences from the *Bacillus* isolates with publicly available GenBank sequences in NCBI (http://www.ncbi.nlm.nih.gov; accessed on 08, March, 2023). Multiple sequence alignments of the mined *gyrB* reference sequences and *Bacillus* spp. strains sequenced in this study were performed using BioEdit 7 (Hall, 1999) and using the algorithm found in Clustal W MEGA11 as described by Tamura et al. (2021). With this alignment, we inferred the phylogenetic relationships of the *Bacillus* spp. isolates with respect to other related species and *B. anthracis.* The p-distance model was used to generate a neighbour-joining tree with 1000 bootstrapped replicates, using the MEGA 11.0 software (Tamura et al., 2021), and the phylogenetic tree was visualised using ITOL 5.0 (Letunic and Bork, 2021).

The 240 bp amplicons, targeting the *pagA* gene and detected using the BAPA probe (W.H.O., 2008), were analysed on the presumptive *Bacillus* spp. isolates bearing the following sample numbers: AX2015 (1122; 1136; 1152; 1511 and 1277A) and AX2016 (1708NH and 1800) and sequenced at Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa. The BLASTn homology searches of the sequences were performed to assess homologous hits against the *pagA* region of the *B. anthracis* GenBank sequences available in NCBI (2023). Multiple sequence alignments of the *pagA* (BAPA) probe region were performed using BioEdit 7 (Hall, 1999). The isolates and/or PCR fragments that failed quality control (low-base calling during sequencing: the sequences where at least 90% of the nucleotides achieved a Phred score of less than 30) were excluded from this analysis.

3.3. Data analysis

3.3.1. Performance analysis of markers on bacterial isolates

All results for the qPCR of the isolates were presented as counts and percentages. To assess the performance of these molecular markers, we analysed 80 isolates that tested qPCR positive for individual markers or combinations of molecular markers using the probe-based approach. We used culture, microscopy, penicillin sensitivity, and gamma-phage sensitivity results as the gold standard (true positive/negative) for comparison with the assays (W.H.O., 2008). For the isolates that tested positive for any of the markers, we calculated the specificity, which detects true negative, and the sensitivity, which detects true positive (Griner et al., 1981). We also calculated the positive predictive value (PPV); probability that *B. anthracis* is present when the test is positive, the negative predictive value (NPV), probability that *B. anthracis* is absent when the test is negative, and the accuracy, which refers to the overall probability that a case is correctly classified (Griner et al., 1981). Results for specificity, sensitivity, and accuracy were presented in percentages and confidence intervals (CI) which are Clopper-Pearson CI (Clopper and Pearson, 1934) and the CI for the predictive values was calculated using the log method as described by Altman et al. (2000).

3.3.2. Analysis of smears and direct qPCR of scrapings

The outcomes of the qPCR and microscopic examination of blood smears were represented as counts and percentages of positive samples. We evaluated the extent of agreement between the binary outcomes of the molecular tests and the results of the microscopic examination of the blood smears. This was done using a Cohen's kappa (k) test (Cohen, 2003). For this analysis, kappa $\neq$ 0 implies that the extent of agreement between the two tests mentioned was significantly different from chance agreement. The measure of agreement was evaluated based on the criteria of Landis and Koch (1977), where <0 = poor; 0.01-0.20 = slight; 0.21-0.40 = fair; 0.41-0.60 = moderate; 0.61-0.80 =substantial; 0.81-1.00 = almost perfect. Statistical analyses were conducted using R version 4.1.2 (R Core Team, 2021), and significance was evaluated with a threshold of alpha < 0.05.

3.4. Results

3.4.1. Isolation and identification of cultured samples

Out of the 1708 blood smear scrapings that were cultured, only 113 samples had bacterial growth from which a total of 506 pure colonies were isolated (some smears yielded multiple different bacterial colony forming units). Only 4/506 colonies demonstrated morphological features that were consistent with those of *B. anthracis* (AX2015-1270, AX2015-1277A, AX2015-1152, and AX2015-1136). The colony morphology and structure of the four isolates on 5% SBA demonstrated non-heamolytic features, forming typical white-gray colonies with an oval, slightly granular appearance. The Gram-stained isolate smears from the 4/506 positive samples showed square-ended bacilli that are classical to *B. anthracis* (Figure 2). Upon examination of the polychrome methylene blue stained smears, the identified *B. anthracis* isolates appeared square-ended and encapsulated (with the exception of AX2015-1136, Figure 2H). The remaining 502 isolates from this study failed on all or some of the criteria (colony morphology, granularity or colour, hemolysis, capsule detection, penicillin and gammaphage sensitivity). The smear samples that failed to produce any colonies included the known positive samples from which the 25 internal control samples were established, suggesting the *B. anthracis* endospores were no longer viable to germinate on culture media.

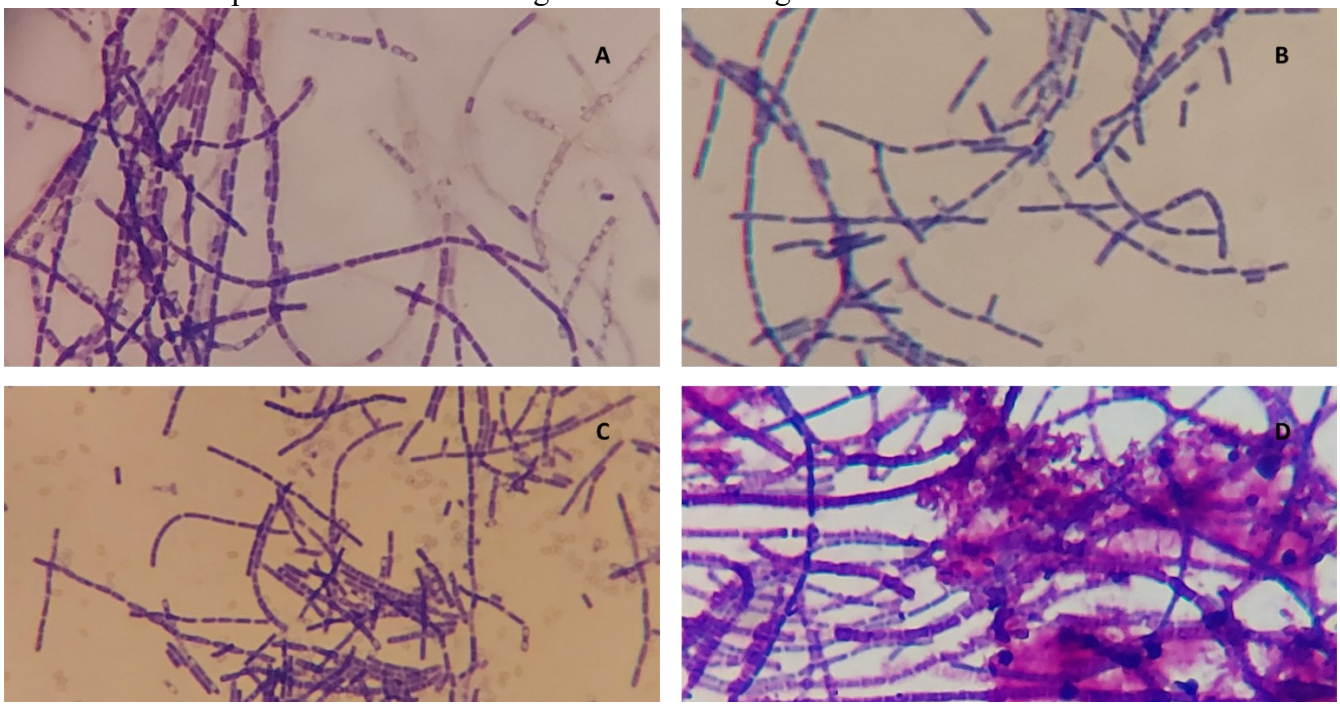

Figure 3-2:Microscopic examination of Giemsa stained cell cultures from bacterial isolates identified through morphological methods as *Bacillus anthracis* collected from blood smears of wildlife mortalities in Kruger National Park, South Africa. Images show square-ended, bacilli (A) Micrograph of isolate AX2015-1270, (B) Micrograph of

AX2015-1152, (C) Micrograph of isolate AX2015-1136 and (D) Micrograph of isolate 1277A.

3.4.2. Molecular analyses of bacterial isolates

Of the 506 bacterial isolates, only 125 (24.7%) tested positive for one or more of the molecular markers (Ba-1, *lef*, *pagA* with BAPA probe, *capB*) using SYBR Green (results not shown). Further confirmation of these 125 isolates using the probe-based approach showed that 80 isolates tested positive (Figure 3). The use of the "+" symbol in our context signifies the strategic combination of markers. The combination of BAPA + *lef* + Ba-1 successfully identified the four *B. anthracis* isolates that were confirmed through culture and microscopy. This combination yielded exclusively these four positives representing 5% (n=4) of the total.

3.4.3. Microbiological screening of the 14 bacterial isolates that were confirmed to be positive on pagA (BAPA)

The most commonly used genetic marker for the diagnosis of *B. anthracis* is *pag*A (BAPA) and the use of this marker is recommended by the W.H.O. (2008b). Following a microbiologic screening of the 14 samples that tested positive for *pagA (*BAPA) by qPCR by means of CT values (Figure 3-4) (which tested the hypothesis that serological cross-reactivity might be due to other bacterial species with similar *pagA* BAPA gene), 7/14 showed penicillin sensitivity, while only the samples that were identified as *B. anthracis* (i.e by colony morphology, capsule staining, hemolysis and molecular markers) showed gamma phage sensitivity (Table 3-2). Only two of the 14 samples that were subsequently screened out of the total were haemolytic thus, *B. anthracis* were all non-hemaolytic (Table 3-2). The 4 *B. anthracis* strains were identified by qPCR markers *Ba*-1, *lef* and *cap*B (although one tested negative for *capB*). Most combination markers of chromosome and toxin genes as well as combination of toxin genes overestimated *B. anthracis* (as most of the samples were negative following a combination of microscopy and molecular techniques) while combination of capsule and toxin genes underestimated *B. anthracis* due to the possible loss of the pXO2 (Figure 3-3).

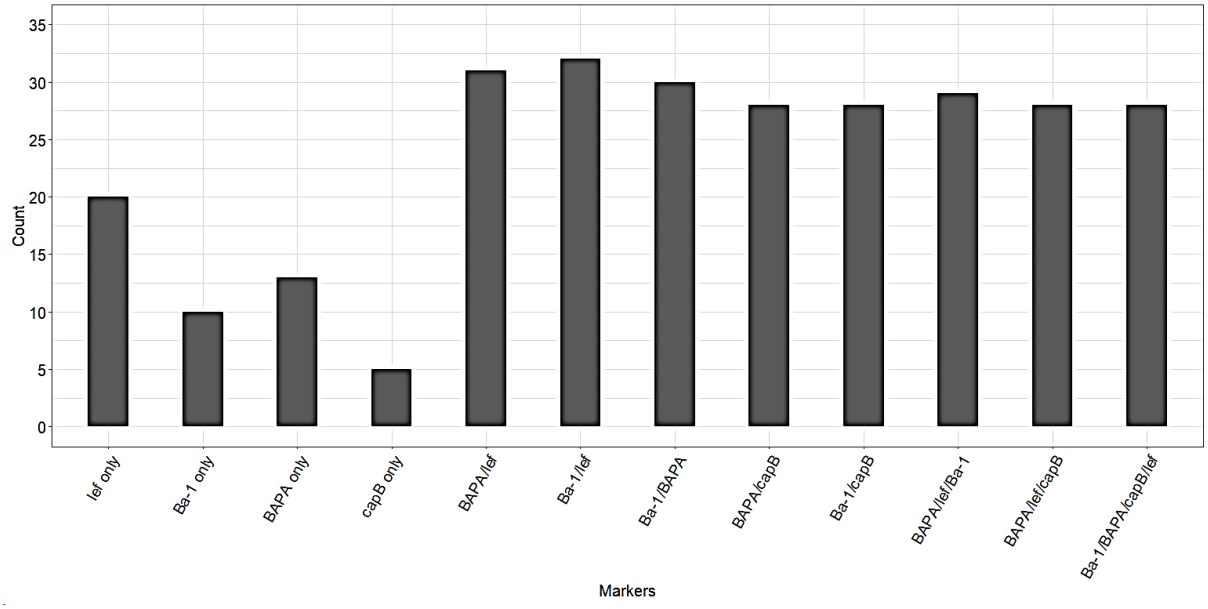

Figure 3-3: Bar plots showing counts of isolates grown from blood smear scrapings that tested positive using individual markers or combinations of molecular markers for the identification of *Bacillus anthracis*. Bars show results of qPCR of isolates positive using probe-based qPCR (total *n* = 80 positives which include the 25 internal controls). These include isolates that were negative on culture. Samples were collected from 2012-2020 in Kruger National Park, South Africa. *Bacillus anthracis* protective antigen (BAPA), lethal factor (*lef*), chromosomal marker (Ba-1) and the capsule region (*capB*) were used as markers in this study. Results for *GI4* were not included as all isolates including the internal controls tested negative. A total of 506 isolates were tested and only 80 isolates tested positive for individual markers or combinations of molecular markers using the probe-based approach.

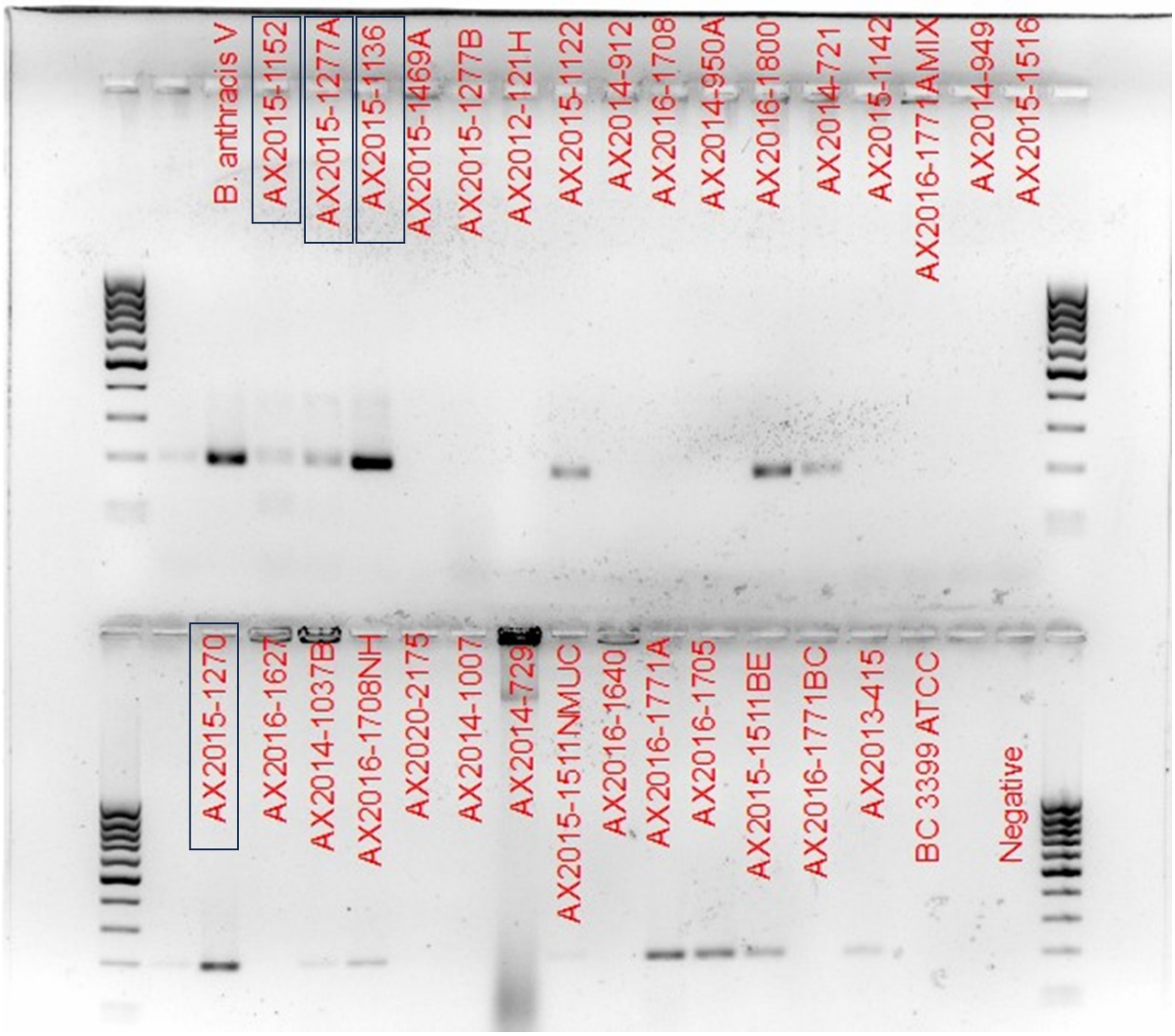

Figure 3-4: Gel image of *Bacillus anthracis* protective antigen gene region, *pagA* (BAPA), for *Bacillus anthracis* and other bacterial species isolated from cultured blood smears obtained from wildlife mortalities in Kruger National Park, South Africa. The 100 bp (Thermo Scientific™, USA) ladder was used. *B. anthracis* Vollum strain (labelled as *B. anthracis* V) served as the positive control. *Bacillus cereus* ATCC3999 and distilled water (labelled as Negative) were used as negative controls. Samples numbers highlighted with rectangles indicate *B. anthracis* confirmed samples. The assay was repeated three times.

3.4.4. Performance of the pXO1 and pXO2 gene markers

The different markers alone and in combination demonstrated varying specificity, sensitivity, PPV, NPV, and accuracy (Table 3-3). The 80 isolates identified by the qPCR/probe approach in this study which include the 4 *B. anthracis* isolates as controls were used to determine numbers for specificity / sensitivity, and accuracy. The *lef* marker demonstrated the lowest specificity and accuracy (51.2% and 72.5%, respectively; Table 3-3). Specificity and accuracy for Ba-1, BAPA and *capB*, for qPCR were all above 60.0%, with Ba-1 having the lowest and *capB* having the highest specificity and accuracy (Table 3-3). Combination of markers increased the specificity and accuracy of these markers. Combinations of Ba-1/*lef*, BAPA/*lef* and Ba-1/BAPA showed specificities and accuracies of over 95% (Table 3-3). The specificity and accuracy were 100.0% and 98.8%, respectively, for all combinations of Ba-1/*capB*, BAPA/*capB*, Ba-1/BAPA/*capB*/*lef*, and BAPA/*lef*/*capB*, however, with a sensitivity of 96.55 % (Table 3-3). The combination of BAPA/*lef*/Ba-1 showed a specificity, sensitivity, and accuracy of 100% which is the overall probability that a case is correctly classified.

Table 3-2: Results of penicillin and Gamma phage sensitivity, morphology and qPCR for the Taqman probe-based chemistry using four different molecular markers (*Bacillus anthracis* protective antigen (BAPA), lethal factor (*lef*), chromosome gene (Ba-1) and capsule (*capB*) genes) for selected bacterial isolates collected from the scrapings of carcass blood smears from 2012-2015 from Kruger National Park, South Africa. These 14 isolates were those that tested positive for the *Bacillus anthracis* protective antigen BAPA marker. Genus identity was based on the gyrB sequence data. The probe-based approach was only conducted on isolates that were positive with SYBR® Green assay.

Markers		**Ba-1**		***lef***		**BAPA**		***capB***		**Sensitivity and morphology**		
Sample ID	**Genus**	**SYBR® Green**	**Probe**	**SYBR® Green**	**Probe**	**SYBR® Green**	**Probe**	**SYBR® Green**	**Probe**	**Penicillin**	**Gammaphage**	**Haemolysis**
AX2015-1122	Peribacillus	+	-	-	+	+	+	-	-	+	-	-
AX2016-1800	Peribacillus	+	+	-	+	+	+	-	-	+	-	-

AX2015-1277A*	Bacillus	+	+	+	+	+	+	+	+	+	+	-
AX2015-1136*	Bacillus	+	+	+	+	+	+	-	-	+	+	-
AX2015-1152*	Bacillus	+	+	+	+	+	+	+	+	+	+	-
AX2015-1270*	Bacillus	+	+	+	+	+	+	+	+	+	+	-
AX2016-1771A	Bacillus	-	-	+	+	+	+	-	-	-	-	+
AX2014-1037	Bacillus	-	-	+	+	+	+	-	-	-	-	+
AX2016-1708NH1	Priestia	+	+	-	-	+	+	-	-	+	-	-

AX2015-1511Nm	Priestia	+	+	-	+	+	+	-	-	-	-	-
AX2015-1511BE	Priestia	+	-	-	-	+	+	-	-	-	-	-
AX2016-1705	Priestia	-	-	-	-	+	+	-	-	-	-	-
AX2013-415	Priestia	+	-	+	-	+	+	-	-	-	-	-
AX2014-721	Priestia	-	-	+	+	+	+	-	-	-	-	-

Asterisk (*) on sample IDs denotes *Bacillus anthracis, Priestia and Peribacillus* were previously identified as *Bacillus* and the Berkley's manual for the nomenclature of bacteria still refers to it as *Bacillus*

Table 3-3: Performance of qPCR probe-based diagnostic assays for *Bacillus anthracis,* using individual markers as well as combination of markers as assessed by their specificity, sensitivity, positive predictive value, negative predictive value and accuracy. All results are shown in percentages with confidence intervals (CI; 95%) in parentheses. The gold standard used in this analysis was culture identification, microscopy, and penicillin and Gamma phage sensitivity. Samples used here include *Bacillus anthracis* and other bacterial species (n=80) isolated from cultured blood smears obtained from wildlife mortalities in Kruger National Park, South Africa.

Markers	**Specificity (CI) %**	**Sensitivity (CI) %**	**Positive predictive value(CI) %**	**Negative predictive value (CI) %**	**Accuracy (CI) %**
Ba-1 only	75.61(59.70-87.64)	72.50 (56.11-85.40)	74.36 (62.46-83.49)	73.81 (62.33-82.76)	74.07 (63.14-83.18)
lef only	51.22(35.13-67.12)	72.50 (56.11-85.40)	59.18(50.75-67.11)	65.62 (51.54-77.41)	75.00 (64.06-84.01)
BAPA only	67.50 (50.87-81.43)	72.50 (56.11-85.40)	69.05 (57.85-78.38)	71.05 (58.68-80.93)	70.00 (58.72-79.74)
capB only	90.00 (78.19-96.67)	96.67 (82.78-99.92)	85.29 (71.58-93.03)	97.83 (86.73-99.68)	92.50 (84.39-97.20)
Ba-1/*lef*	94.12 (83.76-98.77)	100.00 (88.06-100.00)	90.62 (76.33-96.66)	100.00 (92.60-100.00)	96.25 (89.43-99.22)
BAPA/*lef*	96.08 (86.54-99.52)	100.00 (88.06-100.00)	93.55 (78.85-98.26)	100.00 (92.75-100.00)	97.50 (91.26-99.70)
Ba-1/*capB*	100.00 (93.02-100.00)	96.55 (82.24-99.91)	100.00 (87.66-100.00)	98.08 (88.14-99.72)	98.75 (93.23-99.97)

BAPA/*capB*	100.00 (93.02-100.00)	96.55 (82.24-99.91)	100.00 (87.66-100.00)	98.08 (88.14-99.72)	98.75 (93.23-99.97)
Ba-1/BAPA/*capB*/*lef*	100.00 (93.02-100.00)	96.55 (82.24-99.91)	100.00 (87.66-100.00)	98.08 (88.14-99.72)	98.75 (93.23-99.97)
BAPA/*lef*/*capB*	100.00 (93.02-100.00)	96.55 (82.24-99.91)	100.00 (87.66-100.00)	98.08 (88.14-99.72)	98.75 (93.23-99.97)
BAPA/*lef*/Ba-1	100.00 (93.02-100.00)	100.00 (88.06-100.00)	100.00 (88.06-100.00)	100.00 (93.02-100.00)	100.00 (95.49-100.00)
Ba-1/BAPA	98.04 (89.55-99.95)	100.00 (88.06-100.00)	96.67 (80.64-99.51)	100.00 (92.89-100.00)	98.75 (93.23-99.97)

3.4.5. Bacillus spp differentiation using gyrB

The BLASTn identification of the *gyrB* gene from the selected bacterial isolates (i.e., those positive for BAPA) and subsequent phylogenetic analyses identified three genetic clusters, *B. cereus sensu lato* (comprising of *B. cereus* and *B. anthracis* found in this study), *Peribacillus* spp. and *Priestia* spp (Figure 3-5). The latter two clusters were previously part of *Bacillus* and are recently proposed new genera (Gupta et al., 2020) but are still documented as *Bacillus* spp. according to the Berkley's manual (Bergey, 1923). The AX2015 strains (1152, 1277A, 1270 and 1136) grouped in the *B. cereus sensu lato* cluster with reference isolates *B. anthracis* (FDAARGOS 695 and Kanchipuram) as the closest related strains. The isolated AX2016-1771A strain clustered with *B. anthracis*, and also within a cluster including atypical *B. cereus*, although it had phenotypic characteristics with *B. cereus* as it was classified as haemolytic. AX2014-1037B; AX2015-1122 and AX2016-1800 grouped in the *Peribacillus* cluster (Figure 4). AX2015-1511BE grouped with *Priestia megaterium* reference strains, and AX2016-1708NH1 grouped closely with the *Priestia aryabhattai* reference strains (Figure 3-5). The following isolates AX2013-415, AX2014-721, AX2015-1511 Nm and AX2016-1705 were excluded from the tree as they failed to pass the quality control.

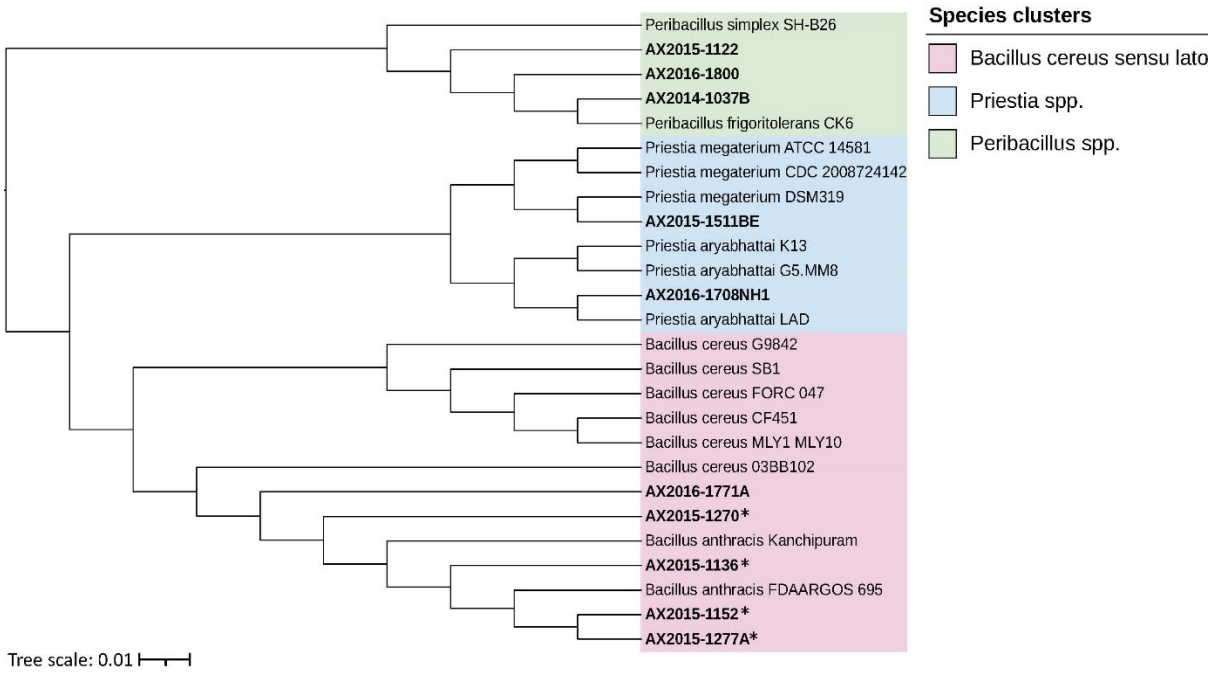

Figure 3-5:Phylogenetic tree of bacterial isolates from Kruger National Park, South Africa based on the *gyrB* gene constructed using the neighbour-joining method and p-distance model among three closely-related genera (all formerly *Bacillus* spp.). Isolates starting with AX are from this study and were matched against the closest NCBI reference isolates (through BLASTn searches) of *Bacillus cereus sensu lato*, *Priestia* spp. and *Peribacillus* spp. The scale bar represents 0.010 substitutions per nucleotide position. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Isolates classified / identified as *Bacillus anthracis* (this study) following confirmation with microscopy, culture, molecular diagnosis and penicillin and gammaphage sensitivity are denoted with an asterisk (*).

3.4.6. The pagA (BAPA) sequence alignment of the gyrB sequences of the isolates

The 14 bacterial isolates amplified 240 bp *pag*A (BAPA) region using PCR which were sequenced and the following bacterial isolates passed the quality control (high-base calling during sequencing) after sequencing of the *pagA* region (240 bp): AX2014-721, AX2015 (1122; 1136; 1152;1277A) and AX2016 (1771A; 1705; 1800; 1708NH1) out of the 14 isolates submitted (Figure 3-6). The consensus sequences were aligned against the NCBI reference strain of *B. anthracis* DFRL BHE-12 *pagA* gene region and the BAPA probes (BAPA_S and BAPA_R). The probe sequences were included to visualize the region of alignment on the consensus alignment. The results showed no difference in comparison to the reference *B. anthracis* strains. The *pagA* region of the probe was completely conserved across the isolates (Figure 3-6).

Figure 3-6: Multiple nucleotide sequence alignment of the *BAPA* probe region (targeting the *pagA* gene of *B. anthracis*) of isolates obtained from this study (starting with AX) versus the NCBI reference strain *B. anthracis* DFRL BHE-12. BAPA_S (Forward) and BAPA_R (Reverse) represent the BAPA probe sequences. Coloured blocks represent the related species clusters in which the isolates grouped in following Figure 4. The nucleotide sequences were aligned using MAFFT V7.0. Two samples

(AX2014-721 and AX 2016-1705) are missing colour blocks as their species' cluster were not assessed in the previous analysis (Figure 3-6).

3.4.7. Probe-based qPCR and microscopy results of scraped blood smears

The results of the scraped blood smear DNA analysis (n = 1708: These are DNA directly extracted from the blood smear material) revealed that 890 samples tested positive for *pag*A (BAPA), *lef,* Ba-1, *cap*B or a combination of markers (Table S 3-1). However, of the 890 samples, 165 (18.5%) samples tested positive for *lef* only and 112 (12.6%) for *pag*A (BAPA) only and negative for all other markers, and these could indicate cross-reactions. Details of other markers and combinations of markers are found in Table 3-4. A combination of Ba-1+ BAPA + *cap*B + *lef* yielded a total of 393 (44.2% of the 890 samples; Table 3-4).

Table 3-4: Showing list of species that tested positive for BAPA, *lef,* Ba-1, *cap*B or a combination of markers and count of animals positive.

Common name of species	Scientific name of species	Proportion and number (n) of smears positive for *B. anthracis with one or more markers*
Blue wildebeest	*Connochaetes taurinus*	0.4%(4)
African buffalo	*Syncerus caffer*	10.1%(90)
Burchell's zebra	*Equus quagga burchellii*	5.3%(46)
Bushbuck	*Tragelaphus scriptus*	0.3%(3)
Chacma Baboon	*Papio ursinus*	1.0%(9)
Common eland	*Taurotragus oryx*	0.1(1)
Elephant	*Loxodonta africana*	5.8%(51)
Giraffe	*Giraffa camelopardalis*	0.9%(8)

Hippopotamus	*Hippopotamus amphibius*	1.9%(17)
Impala	*Aepyceros melampus*	39.2%(349)
Greater kudu	*Tragelaphus strepsiceros*	10.4%(92)
Large-spotted genet	*Genetta tigrina*	0.1%(1)
Nile crocodile	*Crocodylus niloticus*	0.1%(1)
Nyala	*Tragelaphus angasii*	2.4%(21)
Roan antelope	*Hippotragus equinus*	0.9%(8)
Steenbok	*Raphicerus campestris*	0.3%(3)
Common tsessebe	*Damaliscus lunatus*	0.1%(1)
Vervet monkey	*Chlorocebus pygerythrus*	0.2%(2)
Warthog	*Phacochoerus africanus*	0.4%(4)
Waterbuck	*Kobus ellipsiprymnus*	1.0%(9)
White rhinoceros	*Ceratotherium simum simum*	2.4%(21)
Not labelled		16.7%(149)

Of the 1708 blood smears that were screened microscopically, 24.9% (425) demonstrated Gram positive, bamboo-shaped, square-ended bacilli indicating positive samples. However, when adding these genetic markers' combinations with microscopy, the following combinations gave the following results; BAPA + *lef* + microscopy yielded 395 (23.1% of 1708 samples) positives, Ba-1+BAPA+microscopy was 398 (23.3% of 1708 samples), BAPA+*cap*B+microscopy was 400 (44.9% of 890 samples), *lef*+*capB*+microscopy was 401(23.4% of 1708 samples), Ba-1+*cap*B+microscopy was 397 (23.2% of 1708 samples), Ba-1+BAPA,+*lef*+microscopy was 393 (23.00% of 1708 samples), while Ba-1+BAPA+*lef*+*capB*+microscopy yielded 391 (22.9% of 1708 samples) positives. All combinations included the 25 internal controls and the four *B. anthracis* isolates from this study except for AX2015-1136 which was missing in the combinations consisting of *capB*

Table 3-5: Positive results of scraped blood smears using molecular markers and marker combinations in probe-based qPCR, with "only" indicating exclusive positivity for the respective marker or combination.

Molecular Markers	**Count (%)**
Ba-1 only	13 (1.5)
lef only	165 (18.5)
BAPA only	112(12.6)
capB only	5 (0.6)
Ba-1+lef only	13(1.5)
Ba-1+capB only	3(0.3)
Ba-1 + BAPA only	45(5.1)
BAPA + lef only	131(14.7)
BAPA + capB only	10(1.1)
Ba-1+BAPA+capB+lef	393 (44.2)

There was a significant and moderate agreement between the binary outcomes of the molecular test (Ba-1/BAPA/*lef/capB*) and the result of the microscopic examination of the blood smears (kappa = 0.73, 95% CI: 0.67-0.78, p<0.0001). All samples were negative for the genomic island *GI4* gene of the *B. cereus* biovar *anthracis*.

3.5. Discussion

In this study, we investigated if closely related bacterial species can complicate anthrax molecular diagnosis in wildlife mortalities from Kruger National Park (KNP), South Africa. Based on the gyrase B sequence gene, we showed that several former *Bacillus* spp., including *Priestia* spp., *Peribacillus* spp., and *B. cereus senso lato*, are genetically distinct, yet they complicate diagnosis of *B. anthracis* by cross-reacting with molecular markers designed specifically for this species. Specifically, they cross-react with *pag*A (BAPA) or *lef* that have been used as the antigen target in serology(Brézillon et al., 2015, Marston et al., 2016). These closely related bacteria could be pathogenic or just environmental contaminants. Therefore, it is important to consider the potential for cross-reactivity and closely related species when developing and interpreting diagnostic tests for anthrax, as well as to evaluate the use of a combination of different markers as done in this study. Lastly, anthrax diagnoses agree significantly based on microscopy of blood smears versus molecular technique using array of markers. Smears in combination with array of genetic markers including virulence and chromosomal targets will allow accurate identification of anthrax from blood smears and could eliminate culture for confirmation as all the 25 controls were confirmed on both microscopy and molecular techniques.

The identification of closely related species can complicate anthrax diagnosis as these species may share similar genetic markers with *B. anthracis*, leading to false positive results. This similarity in genetic markers can lead to cross-reactivity during diagnostic tests, leading to false positive results for *B. anthracis*. In addition, other genera, such as *Peribacillus* and *Priestia* (Loong et al., 2017, Lekota, 2018), can also complicate anthrax diagnosis. These species may not share as many genetic markers with *B. anthracis* as the *B. cereus* group, but still have some similarities that could lead to false positive results as seen in this study when performing qPCR diagnostics using only *lef*, Ba-1, or *pagA* (BAPA) markers (Figure 5). For instance, *Peribacillus* and

Priestia species have been reported to have similar 16S rRNA gene sequences and protein profiles as *Bacillus* (Bhattacharjee et al., 2023), which can lead to misidentification. Specially, Lekota (2018) demonstrated that the capsular region or polyglutamate regions (*capABC*) are the ones that complicate anthrax diagnosis. Lekota et al (34) reported *capC* not to be very specific to *B. anthracis*. Thus combining capsule marker such as the *cap*B in this study with others increased the specificity. However, in this study, *capB* had a lower sensitivity that should be interpreted with caution owing to the small number of samples that were confirmed as *B. anthracis*. Because the virulence factors of *B. anthracis* occur in closely related *Bacillus* species, the combination of chromosome, toxin and capsule genes may yield the best diagnostic result as seen in this study.

Archival smears are a useful resource for retrospective studies and retrieval of environmentally persistent pathogens like *B. anthracis* (Vince et al., 1998). We were only able to culture *B. anthracis* as defined by microscopy, culture, molecular diagnosis and penicillin and gammaphage sensitivity from four samples collected during the 2015 outbreak from imapala, which did not include any of the 25 B. anhtracis internal control indicating that the endospores were not viable from these 25 smears knwoen to be *B. anthracis* cases. This agrees with the findings of Hassim et al. (2017) who reported that the longer smear is stored, the harder it is to recover *B. anthracis*, and this also affects the quality of the DNA extracted from such samples. The pXO2 plasmid was missing for 1 of the 4 *B. anthracis* isolates obtained from the smears as it was also also evident on the micrograph in Figure 3-1. This has previously been reported to occur in the long term storage of isolates (Marston et al., 2005). The mechanism of how the plasmids are lost is still not properly understood but it is hypothesized to be due to damage to the DNA or following nutrient deficiency over time (Marston et al., 2005). Of the 125 cultured isolates that tested positive for one or more of the molecular markers (Ba-1, *lef*, *pagA* (BAPA), *capB*) using SYBR® Green, a large number (n = 42; 33.6%) tested positive for Ba-1. This chromosomal marker has been previously reported to be very specific to *B. anthracis* as reported by Zincke et al. (2020). However, in this study, of those that tested positive for Ba-1, only 4/42 were confirmed to be *B. anthracis* based on morphological, microscopic and sensitivity test (gamma-phage and penicillin). The difference between this and the previous study

could be not only be due to degradation of being archived over time, but also the geographic origin of the Bcbva microbial community composition contributing to a sample pool is likely to differ. Also, Zincke et al. (2020) evaluated the Ba-1 marker using Bcbva, *B. cereus*, and *B. thuringiensis,* whereas the majority of the bacteria isolated in this study were *Priestia* spp, and *Peribacillus* spp. It is known that *Priestia* spp. and *Peribacillus* spp. are quite ubiquitous as they can be found in the soil, faeces and rhizosphere of roots (Gupta et al., 2020). Their ability to test positive for *B. anthracis* markers could complicate anthrax diagnosis in the wild. Therefore, understanding how the presence of these related organisms can complicate the diagnosis of *B. anthracis* and the use of the Ba-1 on isolates from southern Africa could greatly improve the current diagnosis. With all the samples being negative for *GI4* and with no report of *B. cereus* biovar *anthracis*, it could be that we do not have the West African Bcbva in KNP in South Africa and that *GI4* may be not a viable marker for pathogenic strains (highly pathogenic *B. cereus* causing anthrax-like disease) in southern Africa. However, it is still important to include *GI4* to in the tests to screen for Bcbva. The ecological range of Bcbva is not fully understood, especially its presence in transitional areas between humid forests and dry savannas, which are typical habitats for *B. anthracis*. It is therefore important to investigate other non-traditional regions, using new diagnostic tools like Bcbva-specific proteins for detection. Understanding Bcbva's distribution is crucial for assessing risks in these regions and guiding future surveillance and research efforts. This suggests that geographic region-specific diagnostics could be developed in the identification of anthrax-like cases, if these rare cases do exist. We also found that *lef* was less specific on the isolates, as compared to BAPA and *capB*. Penicillin sensitivity was also observed in two of the non-*anthracis* isolates, however, sensitivity to gamma phage was only seen in the *B. anthracis* isolates. The combination of Ba-1, BAPA, *capB* and *lef* was found only in the *B. anthracis* isolates with the exception of AX2015-1136, which was missing the capsule. Furthermore, the sample should test positive for BAPA/*lef*/Ba-1 or any of the combinations containing *cap*B (such as BAPA/*cap*B, Ba-1/capB, BAPA/*lef/cap*B, Ba-1/BAPA/*cap*B/*lef)*.

From this study, , accurate detection of *B. anthracis* in a sample may be improved with a stepwise approach based on multiple genetic markers, especially in situations where

culture is not possible. Ba-1 has been used by Blackburn et al. (2014) and Zincke et al. (2020) in the diagnosis of *B. anthracis*. However, both studies performed additional MLVA-based or WGS based efforts to confirm species or further characterize them which eliminated the possibility of an overestimation. In our study we demonstrated that using only the Ba-1 or *lef* marker produced non-specific diagnostic results, with specificities estimated as 92.1% and 79.0% for the probe-based assay, respectively. However, testing for the presence of both markers decreased the number of false positives, with a specificity 96.1% (Table 3-3). Even so, we found that 2.4% non-*anthracis* isolates were also determined to be falsely positive. More precise results were obtained while testing for the presence of BAPA*/capB,* Ba-1*/capB*, BAPA*/lef/capB*, and Ba-1*/*BAPA*/lef/capB*, with all combinations having a specificity of 100.0%, sensitivity of 75.0%, and accuracy of 98.8%. However, the problem with this combination is that there is a high chance of misdiagnosing *B. anthracis* isolates that are missing the capsule, as in the case of AX2015-1136. The only combination that demonstrated a specificity, sensitivity and accuracy of 100% was BAPA/lef/Ba-1 (Table 4-3). . Therefore, the combination of BAPA/*lef*/Ba-1 could be a useful molecular strategy to diagnose *B. anthracis* in the absence of other methods like culture and microscopy. However, the addition of *capB* is always important in identifying capsule producing *B. anthracis*. Using MLVA and genotyping, pxO2 samples are more easily identified (Mullins et al., 2013; Easterday, 2020), where these samples are integrated into phylogenetic trees alongside strains verified via qPCR, regardless of the absence of capB. Although such instances in the literature are not widespread and warrant further examination.

The genetic markers used in this study showed varying results for the 1708 samples. The most commonly used genetic marker for the diagnosis of *B. anthracis* is *pagA (*BAPA) and the use of this marker is recommended by the W.H.O. (2008b). However, 12.6% of the 890 positive (on one or more of the molecular markers and microscopy) samples were positive for for only pagA (BAPA) screened directly from the blood smear without culture and negative using other genetic markers or microscopy. This suggests the presence of other bacterial species that share similar *pagA* to that of *B. anthracis* with significant implications for *pagA*-based ELISA. The results of our study show that other closely related organisms can react to *pagA* and produce false positive

results as hypothesised in a serological study conducted in KNP (Ochai et al., 2022). It is therefore necessary to consider using other genetic markers or a combination of markers to confirm the presence of *B. anthracis*.

Of the 890 samples screened directly from the blood smear that tested positive based on at least one probe-based qPCR, 15.2% tested positive for only lef and negative for other markers and microscopy. The absence of other genetic markers and the negative result on microscopic examination suggests that *lef* is also non-specific to *B. anthracis,* present in other species. In this study, *lef* appears less specific to *B. anthracis* as compared to BAPA or other gene markers used in the diagnosis of anthrax. This report is consistent with the work of Zincke et al. (2020) who demonstrated that *lef* could also amplify *B. thuringiensis* serovar Kurstaki HD1 and *B. cereus* G9241 that also possesses a pXO1-like plasmid that carries the genes for anthrax toxin subunits. The presence of *lef* in non-*B. anthracis* pathogenic *B. cereus* has also been reported in humans (Hoffmaster et al., 2004), incorporating other markers or techniques when testing for the presence of *B. anthracis* in a sample could improve diagnosis. The combination of markers from the two plasmids or from the plasmids and the chromosome greatly improved the diagnosis of anthrax with fewer false positive results. The addition of microscopy to the various combinations increased the accuracy of the results significantly as there was very little variation on the number of positive samples when comparing all the combinations with microscopy. Also, the combination of microscopy and the genetic markers can accurately diagnose *B. anthracis* hence reducing the need for culture and proliferation.

The significant agreement between the microscopic and molecular diagnosis of anthrax in this study demonstrates the usefulness of the microscopic technique in field and onsite diagnosis of the pathogen. Microscopy has been a traditional and valuable tool in the diagnosis of anthrax. In this study, 24.9% (425) of the blood smears which included the 25b internal controls were positive for anthrax under the microscope, while an average of 23.1% were positive for combinations of markers and microscopy. This shows that molecular tests should not entirely replace microscopy as a diagnostic technique for anthrax. Microscopy allows for the visualization of anthrax-causing bacilli directly from blood smears or other clinical samples which can be a rapid and reliable means of diagnosis. It can also provide information on the

morphology and arrangement of the bacteria, which can aid in distinguishing it from other organisms. A study comparing the performance of PCR and microscopy in diagnosing cutaneous anthrax found that PCR had a higher sensitivity (100.0%) compared to microscopy (60.0%) (Berg et al., 2006). However, the authors noted that microscopy can still provide useful information on the clinical presentation and progression of the disease as a diagnostic tool. Similar studies have reported on the usefulness of microscopy as a viable and important in resource-poor or field conditions using different criteria (Aminu et al., 2020). The importance of combining different methods cannot be over emphasised especially with recent reports of Bcbva several characteristics of B. anthracis. For example, both strains are non-hemolytic and both form rods in chains that can be difficult to differentiate as reported by Antonation et al. (2016). With ongoing advancements in next-generation sequencing and decreasing costs, leveraging computational methods combined with robust bioinformatics can significantly contribute to achieving a robust diagnostics of anthrax and successful differentiation from Bcbva and other anthrax-like pathogens. This is recommended for future studies.

Some factors that can affect the quality of microscopy as a useful tool include the age of the carcass. If blood smears are made from animals that have been dead for more than six hours, the staining may become unreliable, which may result in false negative results (Berg et al., 2006). The unreliability of the staining is due to post mortem cellular degradation, bacterial proliferation and autolysis (Berg et al., 2006). Wildlife carcasses are most often detected many hours or days after the animal's death, which may explain the variation between PCR and microscopy that was seen in this study as a lot of changes can take place on the carcasses, samples and environment harboring the bacteria. It is therefore important to estimate the age of the carcass when sampling, to improve diagnosis of anthrax. The proficiency of the individual taking the smear and the examiner can also have an impact on the microscopic diagnosis of anthrax (Berg et al., 2006). Also, it has been reported that Giemsa stain only is quite limited in sensitivity to identify positive samples (Berg et al., 2006). This is not applicable to this study, however, as the Giemsa stain used contains Azure B. Additional improvement of microscopic technique currently in use could increase the sensitivity of this technique owing to its usefulness in field or resource-poor settings.

The findings of this study offer substantial implications for public health and One Health initiatives. The identification of diagnostic markers and techniques specific to *B. anthracis* addresses current challenges in detection, particularly given the increased sharing of genetic material among *B. cereus senso lato* members. The acknowledgment of microscopy as a valuable tool in confirming anthrax presence, especially in resource-limited settings, emphasizes its role as an cimportant diagnostic tool. This is crucial for efficient and rapid diagnosis, contributing to early intervention and disease management. Moreover, the accurate diagnosis achieved through microscopy and marker combinations has the potential to reduce or eliminate the reliance on culture and subsequent bacterial proliferation, streamlining the diagnostic process. The recognition of non-anthracis organisms harboring similar genes as potential complicating factors underscores the need for vigilant diagnostic strategies. The study's identification of specific marker combinations, such as BAPA/*lef*/Ba-1, and the use of additional combinations alongside microscopic analysis, not only enhance diagnostic confirmation but also reduce the risk of false positives. These insights contribute to advancing public health and One Health gains by providing more accurate, efficient, and accessible diagnostic approaches for anthrax detection, ultimately aiding in the prevention and control of the disease in livestock, wildlife and human populations.Therefore, it can be concluded that, in the absence of culture, a sample is deemed positive if it demonstrates the classical morphology of *B. anthracis* under microscopy.

3.6. Limitations of the Study

1. Despite identifying *B. anthracis* in smears from 2012-2015 outbreaks, culture success was limited during the 2015 outbreak, possibly due to challenges in the viability of *B. anthracis* endospores in blood smears over time.
2. The storage of the blood smears over time could have had an impact on the quality of the smears and possible bacterial contamination.
3. The determination of sensitivity, specificity and accuracy were based on only four positive samples plus 25 internal controls. Therefore, assessing the performance of the assays on a larger number of samples that includes more culture/gold standard-confirmed positive cases is required.

3.7. Conclusion

Results of this study demonstrate that diagnostic markers and techniques that are specific to *B. anthracis* could reduce the complications in detection that are currently experienced, especially with an increase in the sharing of genetic material amongst the *B. cereus* senso lato members. Microscopy remains a very valuable tool in confirming the presence of *B. anthracis* in the field and in resource-limited settings, as well as a confirmatory tool. Accurate diagnosis with microscopy and combination of markers can reduce or eliminate the need for culture and bacterial proliferation. The presence of non-*B. anthracis* organisms harbouring similar genes may complicate anthrax diagnosis in the field. Lastly, the study identifies that the combination of BAPA/*lef*/Ba-1 yields the most specific, sensitive, and accurate results. Additionally, employing combinations such as BAPA/*cap*B, Ba-1/*cap*B, BAPA/*lef*/*cap*B, Ba-1/BAPA/*cap*B/lef, along with microscopic analysis, can enhance diagnostic confirmation and reduce false positives, as revealed in this research.

3.8. Suggestions for Future Research

Future studies to examine the specific target gene markers for *B. anthracis* strains from southern Africa may be of immense benefit to the diagnosis of anthrax and differentiate it from other closely related organism. Secondly, whole genome analysis looking at the presence of these genes in non-*anthracis* species could inform selection of the best regions to target for primer and probe development. Owing to the varying reports as regards the specificity of these markers, studies to evaluate isolates from different geographical regions to test for the specificity of developed markers for anthrax diagnosis and reduce or eliminate cross reactivity could be informative. Lastly, exploring the variation in non-pathogenic related species could provide information about the variation that exists in nature.

3.9. Data Availability Statement

At the time of publication, data were not publicly available from the authors. The corresponding author may be contacted for questions about data availability.

3.10. Ethics Statement

This study was reviewed and approved by University of Pretoria Research Ethics Committee, Animal Ethics Committee (REC 049-21), Department of Agriculture, Forestry and Fisheries (DAFF) in South Africa (Ref 12/11/1/1/6 (2382SR)) in South Africa, South African National Parks (SANParks), South Africa (Ref: BMTA 006/22).

3.11. Author Contributions

SO, AH and HH conceived the ideas of the study. SO, HH, and AH designed the study. SO, and AH collected the data. SO, AH, KEL and HH designed the methodology. SO, SMM, TM and KEL analysed the data. SO wrote the first draft of the manuscript. All authors contributed significantly to manuscript revision, read, and gave approval for publication.

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Chapter 4. Roles of host and environment in shift of primary anthrax host species in Kruger National Park

Sunday O. Ochai[1*], Lourens Snyman[2], Amelie C. Dolfi[3], Abel Ramoelo[2], Brian K. Reilly[4], Judith M. Botha[5], O. Louis van Schalkwyk[1,6], Pauline L. Kamath [7], Wendy C. Turner[8], Emma Archer[2], Henriette van Heerden[1]

[1] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa.

[2] Centre for Environmental Studies, Department of Geography, Geoinformatics and Meteorology, University of Pretoria, Hatfield, Pretoria 0083, South Africa

[3]Wisconsin Cooperative Wildlife Research Unit, Department of Forest and Wildlife Ecology, University of Wisconsin-Madison, Madison, WI 53706, USA

[4]Department of Nature Conservation, Tshwane University of Technology, Pretoria, South Africa

[5]Savanna Research Unit, Scientific Services, South African National Parks, Skukuza, South Africa

[6] Department of Migration, Max Planck Institute of Animal Behavior, Radolfzell, Germany

[7]School of Food and Agriculture, University of Maine, Orono, Maine, U.S.A.

[8]U.S. Geological Survey, Wisconsin Cooperative Wildlife Research Unit, Department of Forest and Wildlife Ecology, University of Wisconsin-Madison, Madison, Wisconsin, U.S.A

* Correspondence: Sunday O. Ochai; s.o.ochaijr@gmail.com

2. Abstract

Environmental and climatic factors, as well as host demographics and behaviour significantly influence the exposure of herbivorous mammalian hosts to pathogens such as *Bacillus anthracis*, the causative agent of anthrax. In the Kruger National Park (KNP), kudu was the host species most affected by anthrax until the early 1990s, with outbreaks occurring in the dry season, particularly during drought cycles. This trend has, however, shifted to impala as the most affected, with more frequent wet season anthrax outbreaks. In this study, we consider the roles that environmental variation and other host species have played in shifting the primary anthrax host from kudu to impala. Specifically, we examined temporal trends in environmental variables (precipitation, soil moisture, temperature, and normalised difference vegetation index; NDVI) and their association with anthrax occurrence (presence/ absence and counts). Furthermore, we investigated potential correlations between host species' densities and anthrax mortalities over time. Anthrax cases in 1990 were concentrated in the southern part of the northern region, excluding Pafuri, and mostly affected kudus; but subsequent mortalities affected impala and were restricted to the far north, in Pafuri. Significant correlations were found between kudu anthrax mortality and a decrease in NDVI, average temperature, SPI-6 and SPI-12 (Standardised Precipitation Index). On the other hand, the occurrence of anthrax in impala and case counts were linked to a decline in SPI-3, and temperature rise, with increased mortality during the rainy season. Further, elephant density was negatively correlated with kudu mortality, but positively correlated with both impala mortality and impala density. It is therefore concluded that environmental variables and species' densities could alter the diversity and frequency of hosts exposed to *B. anthracis.* Over time, climate extremes (and alterations therein) may exacerbate anthrax severity by modifying species susceptibility and their probability of exposure.

4.0. Introduction

Environmental and climate variables (Anwar et al., 2019, Kurane, 2010, De Vos et al., 2016) as well as the behaviour and densities of host species (Prins and Weyerhaeuser, 1987) have been shown to be key transmission drivers for certain persistent pathogens in the environment. Host activities, which include foraging and movement behaviour, could play significant roles in modifying exposure risk to pathogens (Huang et al., 2022, Ochai et al., 2022, Anderson et al., 2016, Moe et al., 2015). The evolution of host and pathogens over time is modulated by a range of factors that impact disease dynamics, and seasonal and annual shifts in disease outbreaks and occurrence have been commonly observed (Kamo and Sasaki, 2005). It is thus important to understand the underlying mechanisms behind these shifts in order to predict and effectively control future outbreaks (Lloyd-Smith et al., 2009).

The changes in disease patterns over time are also linked to the behaviour of other host species in a shared ecosystem (Gaston and Fuller, 2008), the persistence of environmentally transmitted pathogens, and the populations of disease vectors (Altizer et al., 2006). Environmental variations have the potential to influence the progression of diseases by altering the susceptibility of hosts to pathogens and their level of exposure (Harvell et al., 2009). Further, biological and ecological aspects of animal hosts, along with their behaviours, could contribute to these modifications. Environmental factors, including precipitation (Chikerema et al., 2012, De-Vos and Bryden, 1996, Nsoh et al., 2016, Eisenberg et al., 2013), temperature (Steenkamp et al., 2018, Walsh et al., 2018), and plant cover (Lisovski et al., 2017), are significant drivers of the occurrence of diseases, such as anthrax, and may therefore contribute to shifts in outbreak dynamics over time.

Anthrax is a multispecies animal disease caused by the *Bacillus anthracis* bacterium that is chiefly recognized as a disease of mammals (Beyer and Turnbull, 2009b). While primarily fatal to herbivorous wildlife and livestock, humans are susceptible to anthrax infections, and human cases occur largely due to the handling of infected carcasses, meat, and hides (Kamal et al., 2011, W.H.O 2015). *Bacillus anthracis* is an aerobic, non-motile, gram-positive rod-shaped bacterium that produces endospores that persist in the environment (Barandongo et al., 2023) and are influenced by enviromental factors. Rainfall, humidity, precipitation, temperature, and soil type all influence the survival of *B. anthracis* endospores in the environment and their availability to be taken up by a host (Minett and Dhanda, 1941b). In general, anthrax endemic locations are found in warmer climates (Minett and Dhanda, 1941b, Walsh et al., 2018). Because anthrax is non-contagious, the spread of *B. anthracis* relies on various factors like soil, water, vegetation, host foraging behaviour (De-Vos, 1990, Lindeque and Turnbull, 1994a, De-Vos and Bryden, 1996, Hugh-Jones and De Vos, 2002, Saile and Koehler, 2006, W.H.O., 2008a, Hampson et al., 2011a, Turner et al., 2014b), insect mechanical vectors such as flies (De-Vos, 1990, Blackburn et al., 2010b, Fasanella et al., 2010, Beyer et al., 2018, Basson et al., 2018a), and scavengers. In a natural ecosystem, host species are exposed to a variety of pathogens and parasites and face other pressures such as climate change and interspecies competition.

Anthrax is endemic in the northernmost part of the Kruger National Park (KNP) in South Africa (Hugh-Jones and De Vos, 2002), where the most common host species has been a browser, the greater kudu (*Tragelaphus strepsiceros*), which contributed up to 75% of recorded cases from 1960s-1990s (De-Vos and Bryden, 1996). Outbreaks in KNP occurred in mostly decadal cycles, associated with dry seasons or droughts (Pienaar, 1960, Pienaar, 1961, De-Vos, 1990, De-Vos and Bryden, 1996, Hugh-Jones and De Vos, 2002). This trend has however, to have shifted to the disease predominantly affecting impala (*Aepyceros melampus*; up to 70%), a mixed grazing-browsing herbivore, with annual outbreaks, mainly in the wet season and concentrated in the Pafuri region of KNP (Basson et al., 2018a, Ochai et al., 2022, Huang et al., 2022). Grazers such as plains zebra (*Equus quagga burchellii*) and blue wildebeest (*Connochaetes taurinus*) have contributed to only 10% of anthrax related deaths in this park over the years (from 1990-2015) (Ochai et al., 2022). A linkage has been established between the dissemination of anthrax and high concentrations of blowflies (*Chrysomya spp.*) with outbreaks in southern Africa (Braack and De Vos, 1990a). Blowflies feed on carcasses and deposit *B. anthracis* on leaves of close trees and shrubs on which these animals browse (Braack and De Vos, 1990a, Basson et al., 2018a). This blowfly transmission pathway has been postulated to be the primary means of transmission for wildlife species such as kudu and other browsing animals (Blackburn et al., 2010b, Hampson et al., 2011a, Basson et al., 2018a). Impala are known to graze more in wet seasons and increase their browsing percentage in dry seasons, typically browsing below 1 metre, while kudus browse up to 3 metres (Meissner et al., 1996). We thus hypothesise that changes in vegetation structure could indirectly alter browser exposure to *B. anthracis*.

An understanding of ecosystem structure and trophic level interactions can highlight how some common species affect and influence the dynamics and resilience of the ecosystem (Gaston and Fuller, 2008) and in turn, that of disease dynamics. Large mammals such as elephants often significantly impact vegetation. Previous studies have reported a sharp drop in the number of big trees in KNP such as *Combretum apiculatum* (russet bushwillow), *Terminalia sericea* (silver cluster leaf), *Senegalia nigrescens* (knobthorn), *Sclerocarya birrea* (marula) and *Colophospermum mopane* (mopane), which constitute approximately 80% of the trees in the region (Wyk and

Fairall, 1969). Other studies have shown that elephants may not affect the abundance and diversity of tree species (Coetzee et al., 1979). However, a more recent study noted significant declines in the park's woody vegetation between 1960 and 1989 (Trollope et al., 1998), and hypothesized that this might be due to the sharp rise in elephant density. The decline in the woody cover in KNP may not be solely attributed to elephants; other species such as impala have been shown to be agents of change. Previous studies have shown that impala act as controllers for the transition from woodland to shrubland, with elephants acting as agents for the transformation from woodland to shrubland (Moe et al., 2015). This interplay becomes very important for browsers such as kudu that rely on woodlands for foraging, and for the anthrax-blowfly transmission pathway. Investigating the role of other host species on anthrax transmission dynamics with focus on the shift from kudu to impala in KNP warrants substantial further attention.

Host density may affect pathogen invasion, disease-induced population declines, and infection dynamics within individual hosts across a disease-affected landscape (Wilber et al., 2022). For a significant period of time, host density has been considered a crucial element in theoretical models due to its influence on contact rates (McCallum et al., 2001). Anthrax outbreaks in KNP have been thought to be driven by species density (De-Vos and Bryden, 1996). Most studies evaluate the influence of host density on disease transmission within a particular host species and not its effects on transmission dynamics among interacting host species at the community-level. It is therefore important to consider the influence of species' density in the shift from kudu to impala in anthrax-related mortalities.

Climate change has been linked to an increase in disease risk and exposure to pathogens (IPBES, 2023). The existent, emerging and predicted effects of climate change include but are not restricted to increased average, minimum and maximum temperatures, as well as changes in rainfall events, storms, and floods (Kurane, 2010, Engelbrecht et al., 2015, Lian et al., 2021), which can have cascading effects on vegetation, herbivore, carnivores, and scavengers. Such climate events in turn can result in increased contamination of water and food sources such as vegetation, and shifts in host and pathogen distributions (Anwar et al., 2019, Kurane, 2010, De Vos et al., 2016), especially for environmental pathogen like *B. anthracis*. In South Africa, climate change events pose a very considerable threat to the country's biodiversity, health, food security and

safety, water resources and other systems (Ziervogel et al., 2014). It has also been reported that climate change is already leading to alteration in temperature (Davis and Vincent, 2017), patterns of rainfall, increased level of flood and aridity as well as changes in the hydrological cycles and these may have effects on biodiversity and agricultural activities (Serdeczny et al., 2017). Further, increased CO_2 and nitrogen promote woody plant growth, which could affect habitats for browsers (García Criado et al., 2020). These changes can alter species distributions and interactions, which would be relevant to this system (McCluney et al., 2012). As anthrax mortality has been linked to various abiotic and biotic factors (De-Vos, 1990, Lindeque and Turnbull, 1994a, De-Vos and Bryden, 1996, Hugh-Jones and De Vos, 2002, Saile and Koehler, 2006, W.H.O., 2008a, Hampson et al., 2011a, Turner et al., 2014b), long term changes in environmental and climate parameters could modify or exacerbate host susceptibility and exposure, which in turn could alter the current disease dynamics (IPBES, 2023)(Mora et al., 2022). This, therefore, calls for an investigation into the role climate and environmental variables (such as rainfall and temperature) may play in the exposure and susceptibility of hosts to *B. anthracis*.

In this study, we investigated the possible drivers of the change in main anthrax host species from kudu to impala, including the roles of climate and environmental factors as well as the densities of other host species in KNP. Specifically, we evaluated if changes in environmental conditions (precipitation, temperature, soil moisture, NDVI, and SPIs) over time were correlated with changes in anthrax case numbers separately for each host species. Secondly, we investigated the role of host densities and host count by correlating animal density and count with anthrax mortality.

4.1. Materials and methods

4.1.1. Study area

This study was conducted in the Kruger National Park, South Africa (Figure 1). The highest incidence of anthrax mortality in KNP is in the northern section (22.4206° S, 31.2296° E) extending from Pafuri in the north to Olifants River in the south. This region of the park accounts for 88.4% of the anthrax mortalities from 1990-2015 (Ebedes, 1976b, Steenkamp et al., 2018, Ochai et al., 2022) as seen in Figure 4-1.

This area is characterised by an average rainfall of 430 mm (Huntley, 1982), heavily wooded and grassland savannas (Gertenbach, 1983). The predominant tree species are *Combretum apiculatum* (russet bushwillow), *Terminalia sericea* (silver cluster leaf), *Senegalia nigrescens* (knobthorn), *Sclerocarya birrea* (marula) and *Colophospermum mopane* (mopane) (Wyk and Fairall, 1969). The ecological seasons in KNP are classified as wet (December–March), early dry (April-July) and late dry season (August-November) (Norman, 2013, Zambatis and Biggs, 1995). Kudu, impala and African buffalo (*Syncerus caffer*) are the predominant anthrax host species, and their habitat is largely woodlands with flood plains extending along the Luvuvhu, Olifants, Shingwedzi and Limpopo rivers. Although, elephants mortalities have increased in recent years (Huang et al., 2022). .

4.1.2. Anthrax mortality data

Opportunistic passive surveillance mortality data (for kudu, impala and elephant) were obtained from the Skukuza State Veterinary services. Anthrax mortality was defined as samples confirmed positive through microscopy, culture, molecular detection of genetic markers as well as from symptoms pathognomonic of anthrax as prescribed by W.H.O. (2008b). We used anthrax mortality data from 1990-1999 and 2010-2015 (omitting data from 2000-2009 because of poor surveillance) after excluding data without georeferenced (Global Positioning System-GPS) positions to allow for standardization through time. Metadata recorded at the carcass sites include the date, georeference (GPS coordinates), locality, species, and sex. We used the number of cases in subsequent analyses and did not calculate prevalence due to the lack of consistent population estimates per species over time. The spatial extent of the analysis was the entire northern part of the park as cases occurred across the entire region even though most of the mortalities were concentrated around the Pafuri section.

4.1.3. Environmental variables

We explored the associations between different environmental variables and the number of kudu and impala anthrax mortalities. Rainfall year was defined from July to June as described by (Huang et al., 2022), data which was used to better understand

relationships between precipitation and anthrax mortalities as well as to assess whether the change in rainfall patterns has affected kudu and impala differently. The daily precipitation (rainfall) data were obtained from the Climate Hazards Group InfraRed Precipitation with Station data (CHIRPS), which is a quasi-global rainfall dataset than spans over 35 years, using a 5 x 5 square metre grid of the northern part of KNP (Funk et al., 2014). We also evaluated the relationship between the Standardized Precipitation Index (SPI) which is an indicator for characterizing and indicating droughts and probability of precipitation in any time scale and anthrax mortalities (McKee et al., 1993). The SPI is a widely used metric globally to identify and describe meteorological droughts, which are extended periods of below-average rainfall in a specific area. SPI assesses precipitation anomalies at a particular location by comparing observed total precipitation over a designated accumulation period with the long-term historical rainfall data for that same period (Adeola et al., 2021). SPI values below –1.0 indicate deficits in rainfall (drier than normal), whereas SPI values above 1.0 indicate an excess of rainfall. The magnitude of the drought increases as the SPI value decreases (Adeola et al., 2021). SPI-3 period is useful for evaluating drought on a short-term basis, while the 6- and 12-month accumulation periods are useful for evaluating drought on a long-term basis (Adeola et al., 2021). As such, SPI longer than 3 months (6-12 months) can be utilized as a predictor of decreased stream flow and reservoir storage when estimated for medium accumulation periods.

As some animal behaviour and feeding patterns are impacted at a more rapid pace than others, we hypothesized that the effect of precipitation on kudu and impala may vary depending on the time scale investigated. We calculated separate SPI values that represent different time scales (3 months; SPI-3, 6 months; SPI-6, and 12 months; SPI-12). The SPI values were calculated from the precipitation mentioned above, by using the SPEI function (Vicente-Serrano et al., 2010) in R (R Core Team, 2017). We also investigated whether environmental variability (in terms of vegetative greenness) affected kudu and impala anthrax case numbers with vegetation greenness or condition estimated using Normalized Difference Vegetation Index (NDVI), which was obtained from the remotely sensed Landsat 5/7/8/9 at30 m spatial resolution data through climate engine (Engine, 2022, Huntington et al., 2017). As temperature has also been shown to be a significant driver of anthrax outbreaks (Minett and Dhanda,

1941b, Walsh et al., 2018), we assessed the association between minimum temperature (Tmin), maximum temperature (Tmax), and average temperature (Tavg) with anthrax mortality. The dataset used for the temperature was obtained from TerraClimate, which uses climatically aided interpolation, combining high-spatial resolution climatological normals from the WorldClim database (Abatzoglou et al., 2018).

4.1.4. Host counts and density data

The survey data for impala and kudu were used to estimate the annual density and population abundance of each species. Analyses were performed using DISTANCE software in versions 4.0 and later as described by Thomas et al. (2010), with data being binned into intervals in conjunction with conventional distance sampling. To evaluate associations between anthrax mortality and host densities, animal count and density data were obtained from South African National Parks (SANParks) Scientific Services. Aerial animal count data from 1985-2015 were recorded as total area counts (sampling method details in Krueger et al. (2008)).

Initial surveys from 1998 to 2000 found that distance sampling with 15% coverage of KNP was sufficient for producing population estimates of all larger ungulates that were adequately accurate (20% CVs) (Eiselen, 1994). The east-west transect lines were deliberately spaced every 3' of latitude (about 5.6 km apart) to make surveying more representative of the region (Caughley et al., 1976). The sampling intensity was raised to 22% from 2001 through 2003 by placing transects every 2' of latitude (about 3.7 km apart). In 2004, the intensity was once more increased, this time to 27%, by flying higher and widening the transect. The 2004 data were not included in this paper since these modifications made it impossible to directly compare it to prior years. The coverage north of the Olifants River was raised to 28% (one transect per 1"36' of latitude) in 2005 and 2006 using the original height and transect width and by adding 13 transects. The precision of sampling intensities was compared with ANOVA while the coefficients of variation (%) were determined in DISTANCE.

Elephant population abundance and densities in KNP were estimated using aerial survey data, which were conducted in August and September to correspond with the

late dry season, when most animals are limited to areas near water. The survey followed the standardized technique that has been in use since 1977, with a pattern flown by helicopter along the rivers and important drainage systems, counting all elephants.

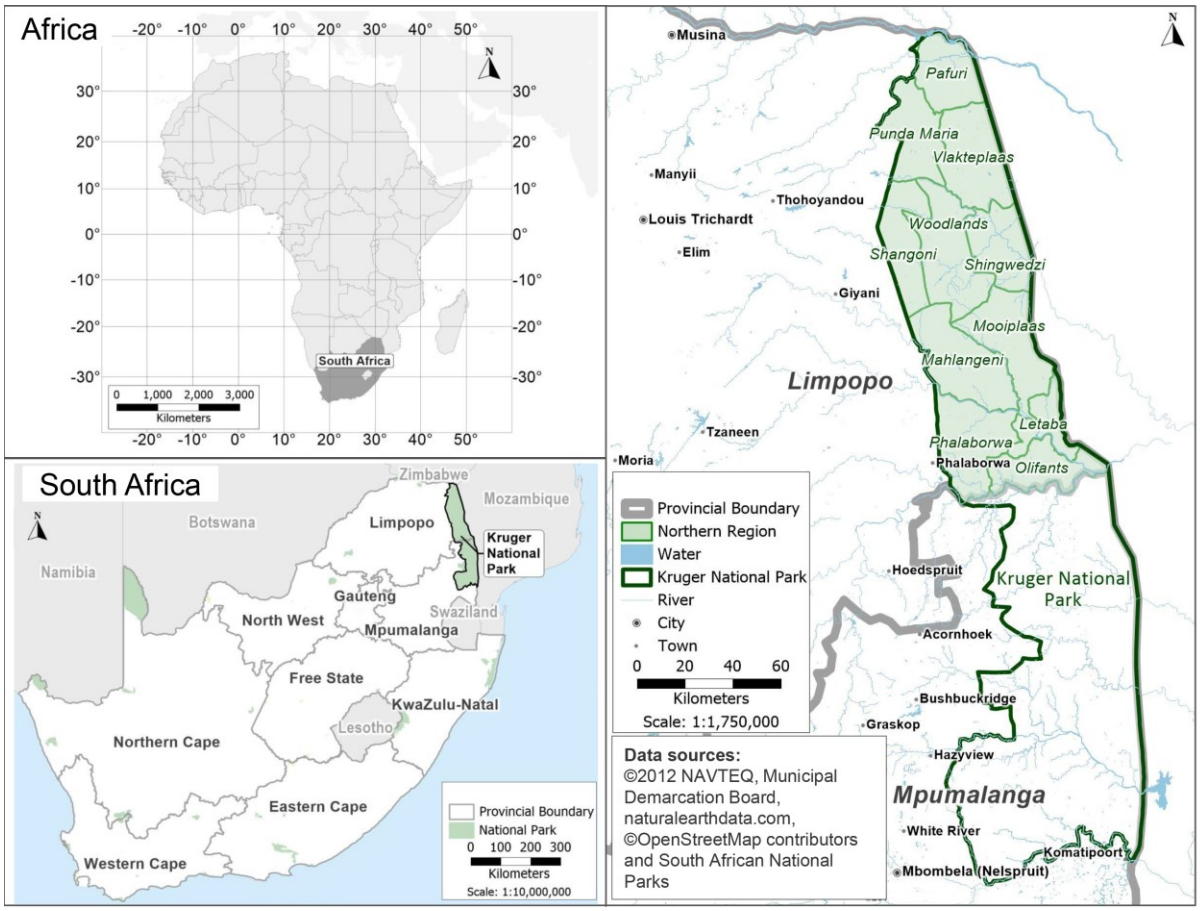

Figure 1: Map of study area in the northern region of Kruger National Park (KNP) in South Africa, showing areas where anthrax occurs endemically (shaded in green). The park management sections are labelled. Latitude and l longitude. ongitude are shown on the Africa map axis..

4.2. Statistical Analysis

4.2.1. Descriptive analysis of anthrax mortality for kudu, impala and elephants

Anthrax mortality count data for kudu, impala and elephant were plotted to understand the spatial and temporal distribution patterns of cases in northern KNP, aggregated by year between 1990-1999, the 2010-2015 periods, and according to ranger sections of KNP.

4.2.2. Trend analysis for NDVI, precipitation, SPIs, temperature, host densities and count

First, we performed time series analyses for the 1985-2015 period to understand whether there were significant temporal trends in NDVI, precipitation, SPI-3, SPI-6, SPI-12, average, minimum, maximum temperatures as well as the densities of elephant, impala and kudu. We analysed temporal trends in the monthly number of impala and kudu deaths over the 1990-1999 and 2010-2015 years. To address the issue of missing and non-linear data in population densities, we fitted a linear trend as an initial step. However, recognizing the complexity of the data, we employed the Augmented Dickey-Fuller test for a more robust analysis of these trends, incorporating a 12-month lag to reflect yearly seasonality (Dickey & Fuller, 1979). We used the tseries package in R for its comprehensive time series analysis tools, including the Augmented Dickey-Fuller test to assess stationarity (Trapletti & Hornik, 2020).

4.2.3. Generalised Linear Models for kudu and impala

Anthrax mortality data were zero-inflated, thus we developed a two models to analyse factors predicting anthrax-related deaths of kudu and impala. We utilised Generalized Linear Models (GLMs) as the foundation for our analysis due to their flexibility in handling various types of response variables (Wüthrich and Merz, 2023). The binomial GLM was chosen to model the binary outcome of anthrax mortality (presence/absence), which is a standard approach for binary data. This model assumes that the logit of the probability of mortality is linearly related to the explanatory variables. For the count data, where the number of anthrax deaths exceeded zero, we applied a negative binomial GLM. This model is particularly suited for count data that

exhibit overdispersion, a common feature in ecological and epidemiological data where the variance exceeds the mean (Enderlein, 1987). Each of the models were used to understand how environmental variables (NDVI, precipitation, SPIs, temperatures, soil moisture) and population density and count data (kudu, impala, elephant) impacted anthrax mortality within each host species. The two-step modelling approach, starting with a Bernoulli model for zeros followed by a negative binomial model for counts, was specifically designed to handle the zero-inflated nature of our data. This methodological innovation ensures a more accurate representation and analysis of the data (Lambert, 1992). For each model, we tested nine explanatory variables that had a significant trend over time: NDVI, temperature maximal, precipitation, SPI3, SPI6, SPI12, season, species' density and. We also added the variable season and year as random variables as we know anthrax deaths are seasonal utilising the lme4 package for its capabilities in fitting mixed-effects models (Bates et al., 2015). . Each variable was included as a random effect. We plotted the qqplot, residuals, and predictions to assess the model. We employed the MASS package for its implementation of the negative binomial GLM (Venables and Ripley, 2002).

The StepAIC function in R, based on the Akaike Information Criterion (AIC), was employed for model selection using the MASS package. The AIC is a widely used criterion that balances model fit with complexity, penalizing the inclusion of unnecessary variables (Akaike, 1998). Model diagnostics were performed using quantile-quantile plots (qqplots) using the ggplot2 package (Wickham, 2021), residuals analysis, and prediction accuracy checks to ensure the assumptions of GLMs were met and the models were well-fitted to the data.

4.2.4. Correlation between = host density/count and anthrax mortality

As the best models performed poorly, probably due to omission of important variables, we examined relationships between the number of anthrax deaths, and the elephant, kudu and impala densities and count. While the animal census in KNP was conducted approximately every two years, survey methodology varied over time and the census years do not always correspond to the anthrax mortality surveillance years. To further explore the relationship between anthrax mortality and the densities of various host

species, we performed Pearson correlation analyses using the base R statistical environment. This method is suitable for quantifying the linear relationship between two continuous variables (Pearson, 1895).

Hence, due to the inconsistencies and absence of density data for some years, we considered 5 time points, and treated them like independent samples, so the correlation was following individual years 1990 to 1995; 1998; 1999; 2010, and the average over the 2011-2015 years. We aggregated the years 2011-2015 because of the absence of corresponding years with population count data from impala, kudu and elephant. We used a Pearson correlation between each pair of variables to determine their relationships at 5% significance level, using R statistical software ver. 3.2.1 (R Core Team, 2017).

4.3. Results

4.3.1. Distribution of anthrax mortality in northern KNP

Distributional patterns of anthrax mortality among kudu, impala and elephant in KNP demonstrated spatial and temporal differences . Most of the earlier anthrax mortalities in 1990-1991 were comprised of kudu (Figure 4-2), and were scattered across the entire northern region (Figure 4-3). Of the total anthrax mortalities for kudu, impala and elephants (n = 1809 from 1990-2015), kudu constituted 60.47% (n = 1,094), impala contributed 35.91% (n = 650) and elephant contributed 3.62% (n = 65). In 1990, there was no kudu anthrax mortality in the northernmost part of the (Pafuri, Punda Maria, Vlakteplaas; Figure 4-3) of the park. In 1991, kudu anthrax mortality in the Pafuri region constituted 19.38% of the total mortality observed that year in kudu, impala and elephants (n = 118 out of 660 cases), and kudu anthrax mortalities that year were spread across the remaining northern areas of the park (Figure 4-3). Of the total observed kudu-only anthrax mortalities across the northern part of the park between 1990-2015, kudu in the Pafuri region constituted only 14.14% (n = 1094; Figure 4-2), showing that most of the kudu anthrax mortality over the years occurred outside the Pafuri region of the park (Figure 4-3).

The first impala anthrax mortality in Pafuri was in 1991, with mortality in Pafuri constituting 25.58% of the impala anthrax mortality for that year, but this also

represented only 1.67% (n=11) of the total anthrax mortality for the three species in that year (Figure 4-2). But from 1991-2015, 93.63% (*n* = 612) of the total impala anthrax cases in the northern region of the park were found in Pafuri (Figure 4-2 and Figure 4-3), whereas only 6.37% (*n* = 39) of impala cases occurred outside this region over the entire time period investigated (1990-2015). This shows that impala anthrax mortality in Pafuri following the reported case in 1991 has remained largely restricted to Pafuri (Figure 4-3).

For elephant, anthrax mortalities have been spread across the entire park with slight increases in case numbers from 2010-2015 in Pafuri, which accounts for 63.33% (*n* = 30) of the total elephant anthrax mortality in the northern part of KNP (Figure 4-2).

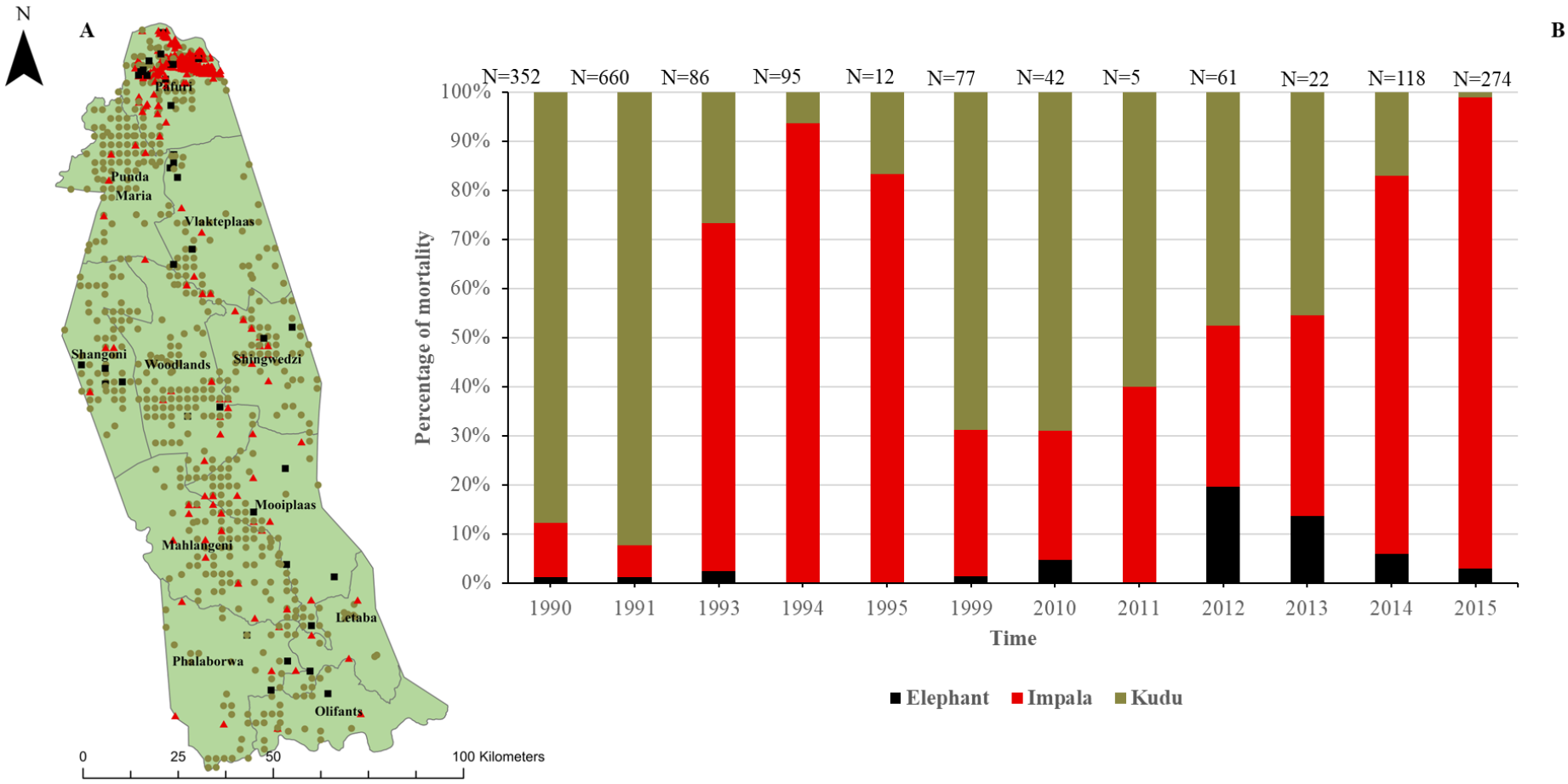

Figure 4-1: A) Map showing the distribution of anthrax mortalities across the northern part of Kruger National Park (KNP). Coloured dots represent elephant (*Loxodonta africana;* black), impala (*Aepyceros melampus*; red), and kudu (*Tragelaphus strepsiceros*; tan) anthrax mortalities found across the different ranger sections (bold font) of the park. B) Stacked bar chart showing percentage and

number of anthrax mortalities between 1990-2015 in the northern region of KNP, coloured by animal species: elephant in blue, impala in red and kudu in green. The total number of mortalities per year is shown on each stacked bar graph denoted with the letter “N”.

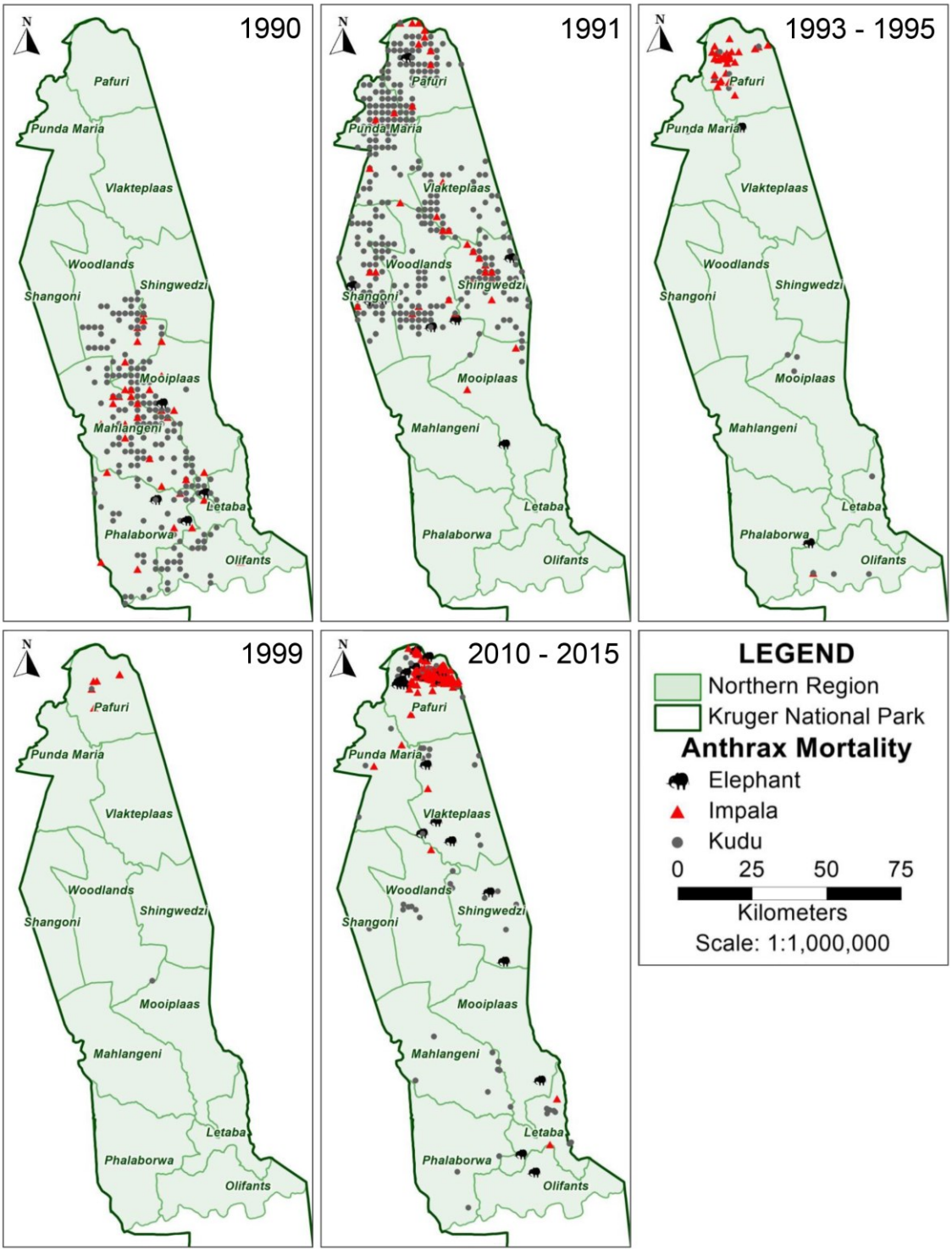

Figure 4-2: Map series showing the distribution of anthrax mortalities from 1990 to 2015. Animal species are indicated by icons, with the black animal silhouette representing elephant (*Loxodonta africana*), red triangle representing impala (*Aepyceros melampus*) and gray circle representing kudu (*Tragelaphus strepsiceros*) anthrax mortalities across ranger sections in the northern region of KNP.

4.3.2. Trend analysis and association of host mortality with environmental, climate, and host variables

The trend analysis showed a significant increase in NDVI, average and maximal temperature over time (Figure 4-4 A,B, and C), however minimal temperature and precipitation did not show significant temporal trends (Figure 4-4 D and E). There was a significant decrease in all the SPIs (Figure4- 4 F, G and H). Elephant density also significantly increased over time (Figure 4-4I).The time trend analyses showed that there was not a significant linear trend in impala (Figure 4-4J) or kudu (Figure 4-4K) densities over time.

Kudu were significantly more likely to die from anthrax during the dry season (p= 0.01) when the SPI-12 was high (p = 0.007) and NDVI low (p = 0.007; Table S1). Kudu mortality count was significantly negatively associated with NDVI (p=0.0047), average temperature (p=0.0018), and elephant density (p=0.0180), low-NDVI dry seasons are associated with more mortality than average dry seasons. On the other hand, kudu mortality count were positively associated with SPI-6 and SPI-12 (p<0.0001; Table S2), therefore, higher than average SPI in the previous wet season is associated with more kudu mortality. Presence/absence of impala mortality seemed to be linked to an increase in maximal temperature which is hotter than average condition (p=0.04), NDVI (p=0.0009), with a 12 month lag as in SPI-12 (p=0.0001), and decreased kudu density (p=0.0001; Table S3), but the model performed very poorly (Root mean square error = 4.48), thus no significant conclusion can be obtained from it. However, impala mortality count was significantly associated with a decrease in SPI-3 meaning drier than average conditions in the 3 months preceeding cases (p>0.0001), and an increase in elephant density (p<0.035), temperature (p<0.0157) and season (p<0.0009; Table S4).

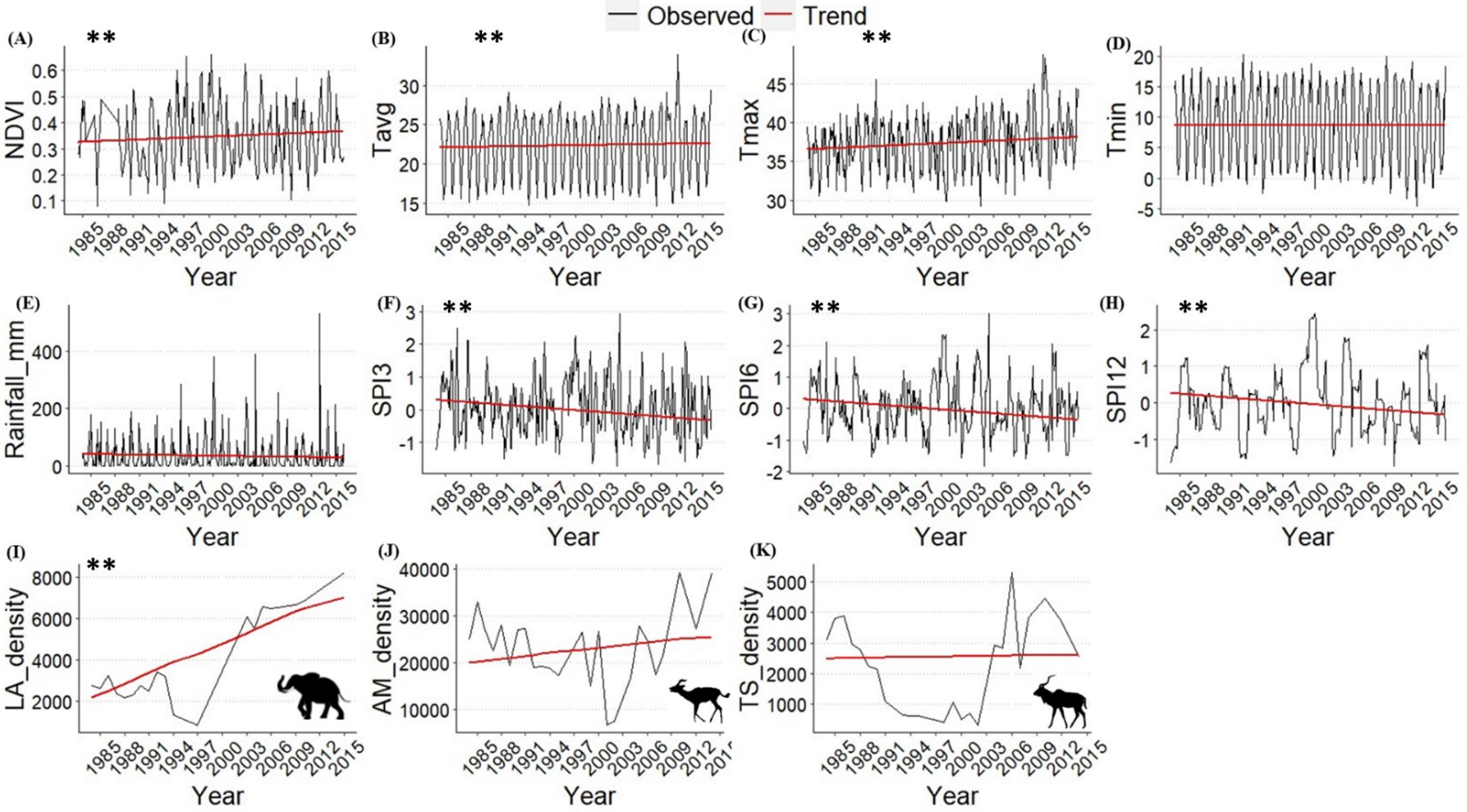

Figure 4-3: Charts showing time trend analyses of environmental,climate, and host variables (in red), against the observed data (in black) from 1980-2015. A) Normalized Difference Vegetation Index (NDVI), B) average temperature, C) maximum temperature, D) minimum temperature, E) rainfall in millimeters (mm), F) Standardized Precipitation Index (SPI) for 3 months (SPI-3), G) SPI for 6 months (SPI-6), H) SPI for 12 months (SPI-12), I) elephant (*Loxodonta africana*) density, J) impala (*Aepyceros melampus*) density, K) kudu (*Tragelaphus strepsiceros*) density. Significant trends are indicated with **

4.3.3. Correlation analysis of anthrax mortality and species density and count

Correlation analyses investigating associations between host species density and anthrax mortality for kudu, impala and elephants showed varying patterns (Figure 4-5). There was a significant negative correlation between kudu anthrax mortality and elephant density ($p<0.05$; Table 4-1), as well as a negative, but non-significant, correlation with kudu density and anthrax mortality. Also, there was a significant positive correlation between kudu density and elephant density ($p<0.01$; Table 4-1). For impala, there was a significant positive correlation between impala anthrax mortality and elephant density, but a significant negative association impala anthrax mortality and impala density. Further, there was a significant positive association between impala density and elephant density. For elephants, there was a significant positive correlation between their density and anthrax mortality ($p<0.05$; Table 4-1).

Table 4-1: Correlation table between anthrax mortality and animal density for kudu (*Tragelaphus strepsiceros*), impala (*Aepyceros melampus*) and elephant (*Loxodonta africana*). The table presents the Pearson correlation coefficients with pairwise deletion. Statistical significance is denoted with an asteriks (*) at $p<0.05$. A value of -1 represents a perfect negative correlation while a value of 1 represent perfect positive correlation.

	Kudu mortality	Impala mortality	Elephant mortality	Kudu density	Impala density	Elephant density
Kudu mortality		0.01	0.57	-0.36	0.29	-0.51*
Impala mortality			0.35	0.40	-0.41*	0.5*
Elephant mortality				0.59	-0.03	0.59*
Kudu density					-0.59*	0.81*
Impala density						0.40*

Elephant density

4.4. Discussion

Seasonal or interannual changes in resource availability affect animal habitat selection, fundamental ecological shifts that can alter pathogen exposure risks among wildlife populations over time (Ostfeld and Keesing, 2000). We explored the roles of environmental variables and host density in anthrax mortality patterns within a community of herbivorous mammals, with the goal of understanding potential mechanisms behind a change in the main anthrax host species from kudu to impala in KNP. Vegetation greenness (NDVI) and temperature both increased across the study period, while, drought measures t. Kudu and impala anthrax mortality through the years were significantly associated with changes in environmental variables and with elephant density. Changing climate trends and indirect effects of elephant density on other herbivores may contribute to changes in anthrax outbreak dynamics in the KNP ecosystem. These changes could have resulted in either a temporary or permanent shift in the KNP system to impala with wet season outbreaks. A range of factors play a role in introduction and dissemination of disease-causing pathogens in new populations, which in turn can have significant impacts on disease transmission dynamics, hosts' exposure and susceptibility (van Seventer and Hochberg, 2017).

A range of factors play a role in introduction and dissemination of disease-causing pathogens in new populations, which in turn can have significant impacts on disease transmission dynamics, hosts' exposure and susceptibility (van Seventer and Hochberg, 2017). Anthrax in KNP was historically associated with dry seasons or droughts, with explosive outbreaks occurring on a roughly decadal cycle (De-Vos, 1990, Hugh-Jones and De Vos, 2002, Pienaar, 1960, Pienaar, 1961). The earlier period of our study when kudu dominated anthrax outbreaks in the northern part of KNP (1990-1991) coincided with a major drought in KNP (Malherbe et al., 2020). The 1990 anthrax outbreak was widespread across the northern part of park and largely impacted kudu. However, in 1991, the outbreak was predominantly in the far north (Pafuri section) of the park. This outbreak also largely affected kudu, with few impalas affected. After 1991, subsequent outbreaks have been predominantly in the Pafuri

section, affecting primarily impala. Outbreaks in regions of KNP appear to be influenced by rainfall and the hydroclimatic conditions of the location (De-Vos and Bryden, 1996).

Changes in climate and environmental variables have been shown to drive or exacerbate host susceptibility to diseases and exposure, thereby affecting disease dynamics (Mora et al., 2022). Previous findings have shown that seasonal variation significantly affects anthrax occurrence in different regions (Chikerema et al., 2012, Nsoh et al., 2016, Turner et al., 2013, Hampson et al., 2011b). In line with such findings, our study confirmed that kudu mortality was higher in the dry season, especially for the earlier outbreaks (1990-1991). We found that kudu mortality was associated with a decrease in NDVI. This seasonal pattern is consistent with the hypothesis that ingesting drier leaves increases the chances of getting wounds around the oral cavity when foraging, facilitating pathogen invasion (W.H.O., 2008b). In contrast, impala (a mixed grazing-browsing herbivore) have been found previously to be affected during annual outbreaks that occur primarily in the wet season (Basson et al., 2018a). According to Nsoh et al. (2016), most of the epidemics in grazers happen at the closing of the dry season and the onset of the rainy season, which could force animals to feed closer to the ground and as such, potentially increase the likelihood of these animals acquiring anthrax. However, grazing closer to the ground can happen in the wet season too, with higher soil contact in the wet season (Havarua et al., 2014). Overall, changes in diet by season will affect exposure risk.

Impala are mixed feeders, in the rainy season they are predominantly grazers with graze constituting 91.8% and browse 8.2% of their diet, while in the dry season, their diet is comprised of 66.4% graze and 33.6% browse (Meissner et al., 1996, Codron et al., 2005, Codron et al., 2006). This means that their feeding pattern can be more affected by short- and medium-term moisture conditions. Previous studies have confirmed the correlation between soil moisture and NDVI (Zhang et al., 2018, Felegari et al., 2022). This means that impala would be grazing more during the wet-season timing of cases, since mixed feeders tend to graze more in wet seasons and browse more in dry. Our time series analysis showing a decline in SPI-3 together with the

significant negative association between impala mortality and SPI-3, suggests climatic changes may have contributed to increasing anthrax mortalities in impala over time. Most impala anthrax mortalities occur during early wet seasons which may coincide with regeneration of vegetation at carcass sites and this mortality continues for a long period of time with eventual decay of exposure dose (Turner et al., 2014a, Turner et al., 2016).

Host population density has been reported to be one of the major drivers of an anthrax outbreak in KNP (De-Vos, 1990). However, in this study, we found a negative correlation between kudu mortality and their density, suggesting that kudu mortality to some extent might not be largely influenced by their density but by other factors such as the density of other species, either directly or indirectly through effects of these species on the shared ecosystem. Although, anthrax mortality might be expected to decrease kudu density, the population counts were not frequent enough to investigate population change at the scale of an outbreak. Also, kudu anthrax mortality was correlated with an increase in elephant density. Understanding the interaction between hosts species and their ecosystem is a great determinant of understanding how their behaviour influences the survival and resilience in that system (40), which in turn influences the dynamics of an outbreak.

Impala anthrax mortality, on the other hand, was correlated with an increase in elephant density. Further, there has been a significant increase in impala population in KNP over the years. Impala has been shown to have significant influence on woody vegetation. Mature trees are felled by elephants by pushing them over or debarking them (Ben-Shahar, 1993) while impala predate tree seedlings of up to 1.5 metres in height (Barnes, 2001). Destruction of woodlands by elephants is often believed to be self-limiting as, following the relocation of the elephants from that particular site, the trees tend to regenerate (Moe et al., 2015). However Stokke and du Toit (2000) hypothesised that elephants in Chobe, who browse primarily in the 1-3 m zone above ground, cannot account for the nearly entire disappearance of regenerated knobthorn and other once numerous trees along the Chobe riverbank at the present time,

suggesting a role played by other herbivores such as impala in limiting recovery of woody species (Moe et al., 2014).

Increasing impala densities have been linked with declining densities of preferred woody recruits (O'Kane et al., 2012) and it has been hypothesised that impala may establish and maintain a dynamic mosaic of patches, acting as a major factor in determining the variability of a landscape. This hypothesis was supported by Moe et al. (2014) where they reported that elephants act as agents for the transformation from woodlands to shrublands while impalas prevent the transition from shrubland to woodlands. Elephants have been shown to create browsing “lawns” for mesoherbivores like impala and kudu (Makhabu et al., 2006); however, with impala having a higher density in the northernmost region of the park, more mortalities could be seen in their population in comparison to the kudu population. Another study reported a relationship between impala anthrax mortality and bush encroachment, where an impala population crash released seedlings from browsing pressure, allowing same-aged woody stands to establish (Herbert and van der Jeugd, 1993). This study in east Africa demonstrates how diseases such as anthrax can alter herbivore-vegetation dynamics, with rippling effects felt across the herbivore community.

Finally, this study showed that kudu anthrax mortalities were higher when elephant density was lower. For impala, there was a positive correlation between anthrax mortality and elephant density, between impala density and elephant density and also between impala anthrax mortality and density.

We therefore hypothesise that the anthrax shift from kudu to impala, and from dry to wet seasons, could be a temporary pendulum that could return back to kudu, as hinted at in what seems like cycles of kudu density and mortality (seen in Figures 2 and 4K). Secondly should the impact of elephant and impala on woody cover be abeited, the shift may return to kudu. However, with prolonged degradation of habitats, and potentially that of kudu, coupled with the forced expansion of foraging range, this shift

may become permanent. The adaptability of herbivores to changing conditions plays a crucial role in determining the permanence of these behavioral changes.

4.5. Conclusion

In conclusion, the findings of this study shed valuable light on the shifting dynamics of anthrax outbreaks in KNP. It becomes evident that comprehending the distinct roles played by various host species and the environment is crucial for unraveling the complexities of anthrax outbreaks. The temporal analysis of mortalities revealed a significant shift in the primary host species affected by anthrax, transitioning from predominantly kudu to impala over time. Temporary or permanent shifts in environmental and climate variables, as well as host-associated variables such as foraging behaviour and density, could increase or decrease the chances of exposure to *B. anthracis* which in turn may cause a temporary or permanent shift in hosts' exposure risk. Remarkably, the density of individual host species did not emerge as a significant factor influencing anthrax-related mortalities. Instead, the density of other species seemed to exert an indirect influence, which may be driven by impacts on vegetation. Such an indirect influence may contribute to increased impala exposure to *B. anthracis* and a decreased risk for kudu. In essence, this study underscores the intricate interplay between host species, environmental factors, and the evolution of anthrax dynamics in the study area.

4.6. Limitation of the Study

1. The lack of tree cover data constrained a comprehensive understanding of the impact of elephants and impalas on vegetation.
2. Inconsistencies in the sampling method for density data across all species also compromised the efficacy of the employed models.
3. The relatively sporadic population counts were insufficient to capture changes in population dynamics, given their scale mismatch with the mortality data.

4. The decrease in surveillance efforts due to the prioritization of anti-poaching activities hindered a thorough investigation of all patterns, resulting in missing data for some years.

4.7. Recommendations for Future Research

Future studies to examine the role of herbivorous host species, vegetation dynamics and animal density in wildlife disease transmission will be of immense benefit to understanding anthrax transmission dynamics in its host community. Secondly, exploring the potential effect of the shift from woodlands to shrublands on the *B. anthracis* blowfly-browse transmission pathway is necessary to better understand and predict shifts in anthrax outbreak dynamics. We also recommend that future research consider the spatial implication of the environmental variables on anthrax mortality using the geographically weighted models.

4.8. Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

4.9. Ethics Statement

This study was reviewed and approved by University of Pretoria Research Ethics Committee, Animal Ethics Committee (REC 049-21), Department of Agriculture, Forestry and Fisheries (DAFF) in South Africa (Ref 12/11/1/1/6 (2382SR)) in South Africa, South African National Parks (SANParks), South Africa (Ref: BMTA 006/22).

4.10. Author Contributions

SO, EA, WT and HH conceived the ideas of the study. SO, LS, EA, AR and HH designed the study. SO, and LS collected the data. SO, LS, EA, AR, AD and HH designed the methodology. SO, AD, BR and JB analysed the data. SO wrote the first

draft of the manuscript. All authors contributed significantly to manuscript revision, read, and gave approval for publication.

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4.13. Supplementary information

Table S 4-1: Binomial generalized linear model table for the presence/absence of anthrax mortality for only kudu with the presence/absence as the response variable and season, Normalized difference vegetation index (NDVI), standardised precipitation index (SPI), and precipitation as predictor variables.

Call:

glm(formula = Ts-Mortality ~ Season + NDVI + SPI-12 + Precipitation, family = "binomial", data = Mort_data[!is.na(Mort_data$LA_density) & !is.na(Mort_data$AM_density) & !is.na(Mort_data$NDVI),])

Deviance Residuals:

Min	1Q	Median	3Q	Max
-1.6330	-0.8844	-0.4572	0.8943	2.2572

Coefficients:

	Estimate	Standard Error	z-value	Pr(>|z|)
Intercept	-3.044e+03	1.463e+02	-2.080	0.0375
Seasondry	1.473e+02	9.917e-01	1.486	0.0100*
NDVI	-1.097e+01	4.090e+00	-2.683	0.0073**
SPI-12	1.6090e+00	5.3983e-01	2.690	0.0072**
Precipitation	1.681e-02	9.553e-03	-1.760	0.0784

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 97.074 on 71 degrees of freedom

Residual deviance: 76.933 on 63 degrees of freedom

AIC: 90.933

Number of Fisher Scoring iterations: 5

Table S 4-2: Negative binomial model table for the anthrax mortality count for only kudu with the count data as the response variable and season, Normalized difference vegetation index (NDVI), standardised precipitation index (SPI), and precipitation as predictor variables.

Call:

glm.nb(formula = Ts_Mort ~ NDVI + SPI_6 + SPI_12 + Tavg + LA_density + Precipitation, data = Mort_data[!is.na(Mort_data$LA_density) & !is.na(Mort_data$AM_density) & !is.na(Mort_data$NDVI),], init.theta = 0.2406313075, link = log)

Deviance Residuals:

Min	1Q	Median	3Q	Max
-1.5862	-1.07981	-0.50980	-0.05527	2.70088

Coefficients:

	Estimate	Standard Error	z-value	Pr(>|z|)
Intercept	5.95959	1.05307	5.659	1.52e-08***
NDVI	-8.44163	2.98379	-2.829	0.00467**
SPI_6	0.4670	0.1377	3.392	0.000014 ***

SPI_12	2.46440	0.48211	5.112	3.19e-07***
Tavg	-0.4874	0.1560	-3.125	0.001777 **
LA_density	0.4291	0.1814	2.365	0.018046 *
Precipitation	-0.02522	0.00802	-3.145	0.00166**

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for Negative Binomial(0.2406) family taken to be 1)

Null deviance: 111.529 on 71 degrees of freedom

Residual deviance: 55.221 on 68 degrees of freedom

AIC: 290.78

Number of Fisher Scoring iterations: 1

Theta: 0.2406

Std. Err.: 0.0590

2 x log-likelihood: -280.7760

Table S 4-3: Binomial generalized linear model table for the presence/absence of anthrax mortality for only impala with the presence/absence as the response variable and season, Normalized difference vegetation index (NDVI), standardised precipitation index (SPI) 3 and 12, year of mortality and kudu density (TS_density) as predictor variables.

Call:

glm(formula = AM_mort ~ NDVI +SPI_3+ SPI_12 + Year + TS_density, family = "binomial", data = Mort_data[!is.na(Mort_data$LA_density) & !is.na(Mort_data$AM_density) & !is.na(Mort_data$NDVI),])

Deviance Residuals:

Min	1Q	Median	3Q	Max
-2.5360	-0.6715	-0.2579	-0.6839	1.7639

Coefficients:

	Estimate	Standard Error	z-value	Pr(>|z|)
Intercept	-8.354e+02	2.176e+02	-3.839	0.0001***
NDVI	-1.020e+01	3.926e-00	-2.599	0.0093**
SPI_3	-7.822e+01	4.960e+01	-1.577	0.1148
SPI_12	2.268e+00	6.401e-01	3.543	0.0004***
Year	4.230e-01	1.100e-01	3.847	0.0001***
TS_density	-1.991e-03	5.402e-04	-3.684	0.0002***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 97.074 on 71 degrees of freedom

Residual deviance: 63.871 on 66 degrees of freedom

AIC: 75.871

Number of Fisher Scoring iterations: 5

Root mean square error = 4.48

Table S 4-4: Negative binomial model table for the anthrax mortality count for only impala with the count data as the response variable and season, Normalized difference vegetation index (NDVI), standardised precipitation index (SPI), maximal temperature, elephant density (LA_density), year and kudu density (TS_density) as predictor variables.

Call:

glm.nb(formula = AM_Mort ~ NDVI + SPI_3 + SPI_12 + Tmax + LA_density + Year + TS_density, data = Mort_data[!is.na(Mort_data$LA_density) & !is.na(Mort_data$AM_density) & !is.na(Mort_data$NDVI),], init.theta = 0.6831243738, link = log)

Deviance Residuals:

Min	1Q	Median	3Q	Max
-1.73716	-0.96009	-0.38733	0.1955	1.7406

Coefficients:

	Estimate	Standard Error	z-value	Pr(>|z|)
Intercept	-6.097807	1.345847	-4.531	5.88e-06 ***
NDVI	2.986190	0.838951	3.559	0.000372 ***
SPI_3	0.308030	0.151794	2.029	0.042432 *
SPI_12	1.390e+00	3.681e-01	3.775	0.00016**
Tmax	1.840e-01	7.054e-02	2.609	0.00908**
LA_density	7.149e-04	2.457e-04	2.910	0.00362**

Year	2.493e-01	5.910e-02	4.219	2.46e-05***
TS_density	-4.189e-04	2.935e-04	-1.427	0.15333

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for Negative Binomial (0.6831) family taken to be 1)

Null deviance: 106.840 on 71 degrees of freedom

Residual deviance: 70.506 on 65 degrees of freedom

AIC: 311.03

Number of Fisher Scoring iterations: 1

Theta: 0.683

Std. Err.: 0.166

2 x log-likelihood: -548.423

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Chapter 5. Synthesis, Summary, General Recommendation and Conclusion

5.0. General Summary

The overall aim of this study was to investigate the ecology of the *Bacillus anthracis* life cycle in Kruger National Park (KNP), South Africa with specific focus on in the susceptible greater kudu (*Tragelaphus strepsiceros*) and impala (*Aepyceros melampus*) hosts, together with improved diagnostic approaches. Developing diagnostic methods for anthrax in wild animals necessitates a meticulous approach to ensure both sensitivity and specificity, given the diverse range of species and potential cross-reactivity with closely related bacteria (Gumbo et al., 2022). One key strategy involves the creation of species-specific conjugates for antibody detection. This was exemplified in the first objective which focused on susceptible hosts like the greater kudu and impala in Kruger National Park, South Africa. The study successfully developed chicken anti-kudu and chicken anti-impala conjugates, offering a pioneering instance of species-specific conjugates that exhibited superior binding strength compared to commercially available ones. This development enhances the precision of diagnostic binding assays by providing a quantitative understanding of cross-reactivity with antibodies from other African wildlife species. A critical consideration in developing diagnostic methods is the avidity of secondary antibodies (Dimitrov et al., 2011). This study demonstrated that IgY anti-kudu and anti-impala conjugates displayed significantly higher avidity indices compared to commercial conjugates. High avidity is crucial for accurate primary binding assays such as the anti *B. anthracis* protective antigen (antiBAPA)-ELISA. The robust binding capacity of these conjugates underscores their potential in advancing surveillance and assay validation, ensuring a specific and reliable diagnostic tool for anthrax in the targeted wildlife species. The study's findings emphasize the importance of ethical and cost-effective production processes, as the conjugates were developed using IgY antibodies from chicken eggs.

In addition to species-specific conjugates, the diagnostic strategy must account for potential cross-reactivity with closely related bacterial species (Anahtar et al., 2020, Trevethan, 2017). Molecular characterization of anthrax in wildlife mortalities requires evaluating combinations of different markers to enhance diagnostic accuracy. Understanding genetic distinctions among *Bacillus* species are crucial, as cross-reactivity can lead to false positive results (W.H.O., 2008, Lekota et al., 2016, Blackburn et al., 2014). The second objective discussed the importance of considering cross-reactivity and closely related species when developing and interpreting diagnostic tests for anthrax by evaluating the effectiveness of molecular, microscopic and culture identification. This comprehensive approach ensures that diagnostic methods are not only sensitive but also highly specific, minimizing the risk of misdiagnosis and providing reliable results for effective disease management in wild animal populations. Employing the gyrase B sequence gene, the study analyzed various former *Bacillus* species, including *Priestia* spp., *Peribacillus* spp., and *B. cereus senso lato*. A noteworthy finding was the evident cross-reaction, particularly with the BAPA and *lef* gene regions. To enhance diagnostic accuracy, the study emphasized the importance of evaluating combinations of different markers. The comparison between microscopic analysis of blood smears and molecular techniques using an array of markers proved very relevant. This comprehensive approach that combines smear microscopy with an array of genetic markers, encompassing virulence and chromosomal targets is recommended. This integrated strategy could potentially eliminate the need for culture confirmation, streamlining the diagnostic process and reducing proliferation.

The ecology of anthrax plays a pivotal role in shaping the development of diagnostic techniques for effective disease surveillance and management. Understanding the intricate dynamics of *B. anthracis* in its natural environment, including soil persistence, wildlife reservoirs, and environmental triggers for spore activation, is essential for designing targeted diagnostic approaches. For instance, the identification of anthrax spore persistence in soil provides valuable insights into the potential hotspots for

pathogen presence, guiding the selection of sampling sites for surveillance (Dragon and Rennie, 1995, Hugh-Jones and Blackburn, 2009).

When there is a shift in the primary host species, less virulent strains of *B. anthracis* may undergo adaptive changes to enhance their virulence. Evolutionary pressures exerted by the new host environment may lead to the selection of genetic variants that confer increased pathogenicity. This process is well-documented in bacterial pathogens, where exposure to different host species can drive the evolution of virulence factors (Casadevall, 2008).

Environmental triggers for anthrax spore activation, including factors like temperature, precipitation, and vegetation dynamics (Steenkamp et al., 2018), may provide valuable information for refining diagnostic strategies. These triggers influence the timing and intensity of anthrax outbreaks (Lindeque and Turnbull, 1994), guiding the implementation of targeted surveillance during high-risk periods. The integration of climate and ecological data into diagnostic models enhances the predictive capacity of surveillance tools, informs the need for optimization and validation, aiding in early detection and response (Blackburn et al., 2007, Dragon and Rennie, 1995).

Therefore, in the final objective of the study, we delved into the transmission dynamics of anthrax within KNP and scrutinized the noteworthy shift in the primary anthrax host species from kudu to impala. Through a comprehensive analysis of the roles played by both hosts and environmental factors, the study uncovered distinct patterns in anthrax mortality. Notably, kudu mortality was prevalent across the northern part of the park, while impala mortality was concentrated in the Pafuri area. This shift in distribution was historical, with anthrax initially impacting kudu in the northern region in 1990 but later concentrating on impala, particularly in the Pafuri section. We suggested that changes in vegetation induced by elephants, including the decline of preferred woody plants, could disrupt the blowfly transmission pathway for kudu.

Wildlife reservoirs significantly impact anthrax transmission, influencing the choice of diagnostic methods. The role of herbivores in the life cycle of *B. anthracis* necessitates the development of species-specific diagnostic tools to accurately detect exposure and

infection in wildlife populations. The adaptation of diagnostic techniques to the specific ecology of wildlife hosts, such as kudu and impala, is crucial for enhancing the sensitivity and reliability of surveillance efforts in ecosystems like the KNP. In conclusion, the intricate interplay between anthrax ecology and diagnostic development underscores the need for tailored approaches that consider the pathogen's natural habitat, reservoir hosts, and environmental triggers. By aligning diagnostic techniques with the specific ecological factors influencing anthrax dynamics, researchers can optimize surveillance efforts, improve early detection capabilities, and contribute to effective disease control strategies in both wildlife and domestic settings.

5.1. General recommendations

a. Future research should focus on refining and expanding diagnostic techniques for anthrax in wildlife, considering the potential cross-reactivity with closely related bacterial species.
b. Future work should focus validating various wildlife binding immunoassays for wildlife diseases where assays developed for closely related domestic stock are used.
c. We recommend a study to test molecular markers in different anthrax diagnostic laboratories together with current diagnostic method to evaluate sensitivity and specifivity. Evaluating combinations of different markers, including virulence and chromosomal targets, can enhance diagnostic accuracy, and combining smear microscopy with genetic markers could eliminate the need for culture confirmation.
d. Further investigations into the ecological dynamics of anthrax transmission are warranted. Understanding the impact of environmental factors, such as vegetation changes induced by elephants, on the transmission pathways can contribute to a more nuanced understanding of host-pathogen interactions. Research should delve deeper into the reasons behind the shift in primary anthrax host species, exploring the

role of habitat alterations, behaviour changes, and population density dynamics on habitat and resource selection and utilisation viz-a-viz *B. anthracis* exposure.

e. Investigating the evolution of *Bacillus* species and the development of virulence in less virulent strains is crucial for anticipating potential shifts in the pathogenicity of anthrax or anthrax-like-causing bacteria. Exploring gene transfer mechanisms and evolutionary patterns can shed light on the adaptability of these bacteria to changing environments and host dynamics.

5.2. Conclusion

In summary, this study successfully developed species-specific conjugates for detecting antibodies in kudu and impala. These conjugates showed superior binding avidity compared to commercial counterparts, highlighting the importance of species-specific diagnostic assays in wildlife disease surveillance. The findings underscore the critical need for validated diagnostic tests tailored to the host species and contribute to enhancing the accuracy of wildlife disease diagnostics and surveillance techniques. The study also demonstrated the complications arising from closely related bacterial species in anthrax diagnosis, leading to false positive results. It emphasized the need for careful consideration of cross-reactivity when developing diagnostic tests and the importance of using a combination of markers to enhance accuracy. The study also highlighted the potential of combining smear microscopy with genetic markers for accurate diagnosis in resource-limited settings. Lastly, we provided insights into the intricate dynamics of anthrax transmission and the shift in host species. We emphasized the role of ecological factors, environmental variables, and animal interactions in shaping disease dynamics in a complex ecosystem like KNP.

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Appendices

UNIVERSITEIT VAN PRETORIA
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Faculty of Veterinary Science
Animal Ethics Committee

11 May 2022

Approval Certificate
Amendment 1

AEC Reference No.: REC049-21 Line 1
Title: The roles of host and environmental determinants on Bacillus anthracis exposure and mortality In Kruger National Park
Researcher: Dr SO Ochai
Student's Supervisor: Prof H van Heerden

Dear Dr SO Ochai,

The **Amendment** as supported by documents received between 2022-01-14 and 2022-05-03 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-05-03.

Please note the following about your ethics approval:

1. The additional use of samples is approved:

Samples	No Approved	Additional Required	Total Approved
Black wildebeest - Serum (Stored-Historic/retrospective) V042-16	0	10	10
Blesbok - Serum - (Stored-Historic/retrospective) V042-16	0	10	10
Bontebok - Serum - (Stored-Historic/retrospective) V042-16	0	10	10
Buffalo - Serum - (Stored-Historic/retrospective) V042-16	0	10	10
Bushbuck - Serum - (Stored-Historic/retrospective) V042-16	0	10	10
Chicken - IgY - (Stored-Historic/retrospective) REC63-19	2	0	2
Common eland - Serum - (Stored-Historic/retrospective) V042-16	0	10	10
Gemsbok - Serum -(Stored-Historic/retrospective) V042-16	0	10	10
Giraffe - Serum - (Stored-Historic/retrospective) V042-16	0	10	10
Hartebeest - Serum - (Stored-Historic/retrospective) V042-16	0	10	10
Impala - Serum - (Stored-Historic/retrospective) REC41-19	80	0	80
Kudu - Serum -(Stored-Historic/retrospective) REC41-19	80	0	80
Nyala - Serum -(Stored-Historic/retrospective) V042-16	0	10	10
Roan - Serum - (Stored-Historic/retrospective) V042-16	0	10	10
Sable – Serum-(Stored-Historic/retrospective) V042-16	0	10	10
Springbok - Serum - (Stored-Historic/retrospective) V042-16	0	10	10
Tsessebe - Serum - (Stored-Historic/retrospective) V042-16	0	10	10
Waterbuck - Serum - (Stored-Historic/retrospective) V042-16	0	10	10
Wildebeest - Serum - (Stored-Historic/retrospective) REC41-19	0	20	20
Zebra - Serum - (Stored-Historic/retrospective) REC41-19	0	80	80

2. Please note that the approved date(s) from the original application certificate / annual renewal certificate will be applicable to this amendment.

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

3. Please remember to use your protocol number (REC049-21) on any documents or correspondence with the AEC regarding your research.
4. 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.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

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

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

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Faculty of Veterinary Science
Animal Ethics Committee

6 August 2019

Approval Certificate
New Application

AEC Reference No.:	REC063-19
Title:	Development of ELISA conjugates for Kudu and Impala (Chicken anti-kudu and chicken anti-impala)
Researcher:	Dr SO Ochai
Student's Supervisor:	Prof H van Heerden

Dear Dr SO Ochai,

The **New Application** as supported by documents received between 2019-05-15 and 2019-07-29 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-07-29.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	Number
Poultry	4

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-08-06.
3. Please remember to use your protocol number (REC063-19) on any documents or correspondence with the AEC regarding your research.
4. 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

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8483
Fax +27 12 529 8321
Email aec@up.ac.za

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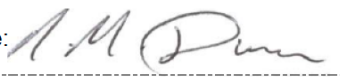
Faculty of Veterinary Science

Research Ethics Committee

Project Title	**Development of ELISA conjugates for Kudu and Impala (Chicken anti-kudu and chicken anti-impala)**
Project Number	**REC063-19**
Researcher / Principal Investigator	**Dr SO Ochai**

DIissertation / Thesis submitted for	**Masters**

Supervisor	**Prof H van Heerden**

APPROVED	Date: **2019-07-02**
CHAIRMAN: UP Research Ethics Committee	Signature: 

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Faculty of Veterinary Science
Animal Ethics Committee

3 September 2019

Approval Certificate
New Application

AEC Reference No.: REC041-19
Title: Transmission and evolution of a persistent pathogen: anthrax infection dynamics comparing two natural systems
Researcher: Prof H van Heerden
Student's Supervisor:

Dear Prof H van Heerden,

The **New Application** as supported by documents received between 2019-04-08 and 2019-08-26 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-08-26.

Please note the following about your ethics approval: The use of species is approved:

Species and Samples	Number
Zebra *(Equus burchelli)*	40
Kudu *(Tragelaphus strepsiceros)*	40
Impala *(Aepyceros melampus)*	40
Blood samples (60 ml per animal)	120 total
Hair samples (Tuft)	120 total
Tissue collection (thin slice ear) 3mm	120 total
Animals/carcasses that succumb to anthrax during the surveillance period	Opportunistic collections (to be reported on)

1. Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-09-03.
2. Please remember to use your protocol number (REC041-19) 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

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8483
Fax +27 12 529 8321
Email aec@up.ac.za
www.up.ac.za

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Faculty of Veterinary Science

Research Ethics Committee

Project Title	**Transmission and evolution of a persistent pathogen: anthrax infection dynamics comparing two natural systems**
Project Number	**REC041-19**
Researcher / Principal Investigator	**Prof H van Heerden**

DIissertation / Thesis submitted for	**Staff Research / Non Degree**

Supervisor	

APPROVED	Date: **2019-05-06**
CHAIRMAN: UP Research Ethics Committee	Signature: