Diversity of *Bacillus anthracis* and *Clostridium* species in livestock and wildlife in selected regions in South Africa

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A thesis submitted for the degree

Magister Scientiae (Veterinary Science)

Department of Veterinary Tropical Diseases

Faculty of Veterinary Science

University of Pretoria

Supervisor: Prof Henriette van Heerden

(University of Pretoria)

Co-supervisor: Dr Ayesha Hassim

(University of Pretoria)

2019

Dedication

This thesis is dedicated to my family for all their love and support during the time it took me to complete this Master's degree.

Declaration

I, Hermanus De Wet Geyer, hereby declare that this thesis submitted for the degree Magister Scientiae (Veterinary Science) at the University of Pretoria, is my own work. The work contained herein has not been submitted previously, by me or another person, for a degree at any other tertiary institution.

Hermanus De Wet Geyer **Date:** 10/10/2019

Funding and Ethical Approval

Funding was provided by the National Research Foundation (NRF) of South Africa and AgriSETA.

The Department of Agriculture Fisheries and Forestry (DAFF) approval according to Section 20 of the Animal Disease Act 1984 (Act 35 of 1984), South Africa.

Ethical and project approvals were obtained from the South African National Park's scientific services and the University of Pretoria, Faculty of Veterinary Science, Research Committee approval, South Africa.

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Animal Ethics Committee

PROJECT TITLE	**Characterization of *Bacillus anthracis* and Clostridium species to aid in the development of combined vaccine**
PROJECT NUMBER	**V144-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**H de Wet Geyer**

STUDENT NUMBER (where applicable)	**UP_163 969 62**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL ANIMALS	**No animals involved**	
NUMBER OF ANIMALS	**No animals involved**	
Approval period to use animals for research/testing purposes		**November 2016-November 2017**
SUPERVISOR	**Dr. H van Heerden**	

KINDLY NOTE:

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

APPROVED	Date 28 November 2016
CHAIRMAN: UP Animal Ethics Committee	Signature

S*4285-15*

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

Dear Mr Geyer,

Your application sent with the email on 20 April 2017 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. Only samples sent for diagnostics to Bacteriology at the Department of Veterinary Tropical Diseases, University of Pretoria, may be utilised for Clostridia culturing for this study. No active sampling may take place and samples may not originate from the Foot and Mouth Disease Infected Zone and Protection Zone;
4. Samples that are received from Skukuza State Veterinary Laboratory may only include heat inactivated samples, extracted DNA and smears that have been formalin fixed; and all must travel under the cover of Red Cross Permit issued by the

State Veterinarian of the area of origin and in full compliance with all the conditions of such a permit;

5. Work at the Skukuza State Veterinary Laboratory may only be conducted once the laboratory's anthrax culturing status has been clarified and audited by DAFF;
6. All potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study using the specified waste contractor. Records must be kept for five years for auditing purposes.
7. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval;
8. A dispensation for the storage of the cultured samples and DNA samples is attached.

Title of research/study: "*Characterization of Bacillus anthracis and Clostridium species to aid in the development of a combined vaccine*"
Researcher: Mr Hermanus de Wet Geyer
Institution: Department of Veterinary Tropical Diseases
University of Pretoria
Our ref Number: 12/11/1/1/6
Your ref: V144-16
Expiry date: Dec 2018

Kind regards,

Maja.

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2017 -05- 19

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

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Mr Hermanus de Wet Geyer
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RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "CHARACTERIZATION OF BACILLUS ANTHRACIS AND CLOSTRIDIUM SPECIES TO AID IN THE DEVELOPMENT OF A COMBINED VACCINE"

A dispensation is hereby granted for the Section 20 approval that was issued for the above mentioned study (attached):

i) Inactivated anthrax cultures as specified in the section 20 application process may be stored in the access controlled BSL-3 facility in the Center for Emerging and Zoonotic Diseases at the National Institute for Communicable Diseases;

ii) Clostridia cultures as specified in the section 20 application process may be stored in the access controlled BSL2+ facility at Bacteriology in the Department of Veterinary Tropical Diseases;

iii) Extracted DNA from anthrax and clostridia samples may be stored stored in the access controlled BSL2+ facility at Bacteriology in the Department of Veterinary Tropical Diseases;

iv) Stored samples as stated in (i), (ii) and (iii) above, may not be outsourced or used for further research without prior written approval from DAFF.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2017 -05- 19

– 1 –

Acknowledgements

I want to thank and bring praises to the Almighty God who has given me the opportunity, strength, courage and perseverance to be able to study and complete my master's degree. He was always by my side whenever I needed Him, and He guided me throughout.

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This project would not have been possible without the funding provided by the National Research Foundation (NRF South Africa) and AgriSETA, as well as the facilities and expertise provided by both the staff of the Department of Veterinary Tropical Diseases, University of Pretoria and the staff at the office of the state veterinarian at Skukuza while I was there for sample collection and training.

I offer my kindest thanks to all the postgraduate colleagues and staff of the Department of Veterinary Tropical Diseases for all their support, and for making my days filled with positivity.

For my colleagues at the National Institute for Communicable Diseases (NICD), I thank you for all the encouragement that you provided.

The people that played the biggest part in supporting me thought out this master's degree are my family. Dad and Mom, thank you for every encouraging message, e-mail and phone call that kept me going. To my grandparents, thank you for all the support that you gave me.

Summary / Abstract

Diversity of *Bacillus anthracis* and *Clostridium* species in livestock and wildlife in selected regions in South Africa

Candidate: Hermanus De Wet Geyer
Promoter: Prof Henriette van Heerden
Co-promoter: Dr Ayesha Hassim
Department: Veterinary Tropical Diseases
Degree: Magister Scientiae (Veterinary Science)

Bacteria from the genera *Clostridium* and *Bacillus* form part of the phylum *Firmicutes* and produce endospores. Both are soil-borne, toxin producing bacteria with the ability of causing diseases in animals. One of the most effective ways to combat these diseases is through vaccination. In this study, the characterisation of both the *Bacillus anthracis* and *Clostridium* species have led to a better understanding of the different species (*Clostridium*) and genotypes (*B. anthracis*) that are circulating in the country, both in livestock and wildlife.

Through data mining, *Clostridium* species responsible for animal death in South Africa for the past four years (2013–2016) have been identified using the Universal Veterinary Information System (UVIS) of the Faculty of Veterinary Science, University of Pretoria. *Clostridium* species responsible for deaths in wildlife and livestock recorded in the UVIS database were identified from a combination of culture, fluorescent antibody technique as well as pathology results. A variety of *Clostridium* species causing diseases in both livestock and wildlife were identified, of which the most common were *Clostridium perfringens* and *Clostridium. septicum*. The mined data revealed that the percentage of confirmed *Clostridium* cases between livestock and wildlife were similar, namely 53.8% for livestock versus 50% for wildlife. These results identified prominent *Clostridium* species causing disease in animals. The results could indicate that there is a lack of vaccination as clostridial disease was found equally in livestock and wildlife since vaccination is not possible in wildlife due to costs and practicality. The incorrect usage of clostridial vaccine and not maintaining cold chain for vaccines could also contribute towards disease in livestock.

Multiplex polymerase chain reaction (PCR) assays for the detection of *Clostridium* species (*Clostridium botulinum*, *Clostridium tetani*, *Clostridium perfringens*, *Clostridium novyi*, *Clostridium chauvoei*, *Clostridium septicum* and *Clostridium sordellii*) were developed and used to screen both animals and the environment of the Kruger National Park using passive

surveillance samples. The screening revealed numerous *Clostridium* species in the environment with *C. perfringens* type A, *C. sordellii* and *C. septicum* being the most prominent species, while *C. novyi, C. chauvoei* and *C. tetani* were less prominent and no *C. botulinum* detected in the tested samples. This assay can be used to monitor Clostridia in environments or to confirm culture, histopathology and/or fluorescent antibody technique results.

Bacillus anthracis was isolated and characterised (phenotypically and genotypically) into lineages, clonal groups, and genotypes from two separate outbreaks (Pafuri and Singita) in the northern parts of the Kruger National Park (KNP) in 2014–2016 and compared with data from previous years. An existing panel (MLVA7) was expanded to MLVA10 for *B. anthracis* genotyping as the MLVA7 panel was developed using *B. anthracis* strains belonging to A-clade while KNP contains both A and B-clade strains. The MLVA10 could differentiate 28 genotypes among the 79 strains *B. anthracis* from Pafuri and Singita all belonging to the A-clade and include animal species such as greater kudu *(Tragelaphus strepsiceros)*, nyala *(Tragelaphus angassi)*, impala *(Aepyceros melampus)* and African elephant *(Loxodonta africana).*

The information generated from this project has exhibited the different species of *Clostridium* and genotypes of *B. anthracis* that were located and circulating in the KNP in both the animals and the environment. This information can be used for epidemiological studies and to improve clostridial vaccination practices, verify *Clostridium* species identification, as well as, evaluating clostridial and anthrax vaccine combinations by determining the occurrence of the diseases in livestock and wildlife.

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List of Acronyms and Abbreviations

ATP	Adenosine triphosphate
ATR	Anthrax toxin receptor
cAMP	Cyclic adenosine monophosphate
BLAST	Basic Local Alignment Search Tool
BSL	Biosafety level
canSNP	Canonical SNP assay
CapC	Capsule gene region C
CPA	*Clostridium perfringens* Alpha toxin
CPB	*Clostridium perfringens* Beta toxin
CPE	*Clostridium perfringens* Epsilon toxin
CPI	*Clostridium perfringens* Iota toxin
DNA	Deoxyribonucleic acid
EF	Edema factor
ELISA	Enzyme-linked immunosorbent assay
FAT	Florescent antibody technique
GPI	Glycosylphosphatidylinositol
KNP	Kruger National Park
LD50	50% lethal dose
LF	Lethal factor
MALDI-TOF	Matrix-assisted laser desorption/ionisation (MALDI) time-of-flight (TOF) analyser
MLVA	Multi-loci variable number analysis
MST	Minimum spanning tree
OIE	World Organisation of Animal Health
PA	Protective antigen
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
PEP	Post-exposure chemoprophylaxis
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SASPs	Small acid-soluble spore proteins
SNP	Single nucleotide polymorphisms
TcsH	Haemorrhagic toxin
TcsL	Lethal toxin
UPGMA	Unweighted pair group method using arithmetic mean
UVIS	Universal Veterinary Information System
VNTR	Variable number of tandem repeats

Chapter 1
Introduction and Literature Review

1.1 Introduction

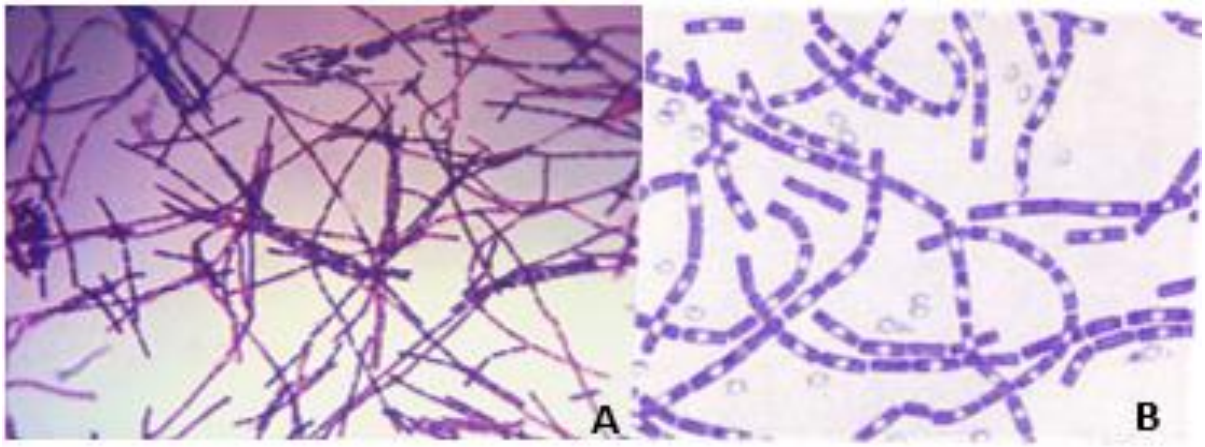
Clostridium or Clostridia (the collective name for the genus) and *Bacillus* are Gram-positive bacilli that form part of the phylum *Firmicutes* and form/produce endospores. Both are soil-borne pathogens, which persist in the environment for years. Bacteria belonging to the genus *Clostridium* cause disease in animals, but some species are known to cause zoonotic diseases. *Clostridium perfringens* (*C. perfringens*), *Clostridium difficile* (*C. difficile*), *Clostridium botulinum* (*C.botulinum*) and *Clostridium tetani* (*C. tetani*) (Num and Useh, 2014), are known to pose a threat to health in communities in specific endemic areas (Songer, 2010). The most notorious for its use as a bioweapon and zoonotic *Bacillus* species (spp), is *Bacillus anthracis (B. anthracis)* that is the causal agent of anthrax. In this study, the focus will be on *B. anthracis* and pathogenic *Clostridium* species, which are environmental bacteria of concern in the livestock industry and prone human communities.

1.2 *Bacillus anthracis*

Anthrax is primarily a disease of herbivores but has also been found in omnivores and carnivores that have fed on the carcasses of infected animals (Hugh-Jones, 2015; Turnbull, 2008). Humans become infected either through accidental exposure (occupational hazard) or by deliberate exposure (biological warfare). This infection has resulted in the disease being known around the world as woolsorters' disease, Cumberland's disease, Siberian plague, ragpicker's disease and malignant oedema. Disease presentation can be both acute and chronic, depending on the host and route of infection (Turnbull, 2008) which can include inhalation, cutaneous, gastrointestinal and injection (Centers for Disease Control and Prevention [CDC], 2015).

Anthrax is the disease caused by the bacterium *B. anthracis*, which is an aerobic or facultative anaerobic, Gram-positive rod (approximately 4 µm by 1 µm) that has the ability to form endospores (Turnbull, 2008). The *B. anthracis* endospores have a very characteristic square/blunt-ended (described as 'box-car' shaped) appearance (Figure 1.1) (Theiler, 1912; Hugh-Jones and De Vos, 2002; Jayachandran, 2002; Turnbull, 2008). Upon exposure to oxygen, as well as at the end of the exponential growth phase of the bacterium, the formation of a single ellipsoidal endospore (approximately 2 µm by 1 µm in size) can be observed centrally or subterminally (Carrera et al., 2007). The survival of *Bacillus* spp in nature is aided by the

formation of spores, which are resistant to conditions to which vegetative cells are intolerant (Nicholson et al. 2000; Turnbull, 2008; Mahon et al. 2011).



Note: The white elliptical spheres in the centre of the cell are the central/subterminal endospores Source: CDC (2017)

Figure 1.1: (A) *Bacillus anthracis* in metabolically active form (vegetative cells); (B) *B. anthracis* in sporulating form

B. anthracis is taxonomically classified in the phylum *Firmicutes*, family *Bacillaceae*, genus *Bacillus* and the *Bacillus cereus* (*B. cereus*) group (Pilo and Frey, 2011). The genus *Bacillus* (and all the species within) is divided into groups based on genetic identity and morphologic features. The *B. cereus* group consists of the following species: *B. anthracis*, *B. cereus*, *Bacillus thuringiensis* (*B. thuringiensis)*, *Bacillus mycoides* (*B. mycoides)*, *Bacillus pseudomycoides* (*B. pseudomycoides)* and *Bacillus weihenstephanensis* (*B. weihenstephanensis)* of which the first four are the most relevant groups known to cause health-related issues or disease (Liu et al., 2015). These four species in the *B. cereus* group are genetically closely related that they are considered variants of a single species (Rasko et al., 2005).

B. anthracis exists in two well-described forms: the metabolic active cells within the host (known as vegetative cells, Figure 1.1A) and the spore form (Figure 1.1B) (Goel, 2015; Turnbull, 1999). The spore form is known to persist within the soil and is considered the inactive or dormant form of the bacterium (Santelli et al., 2004; Goel, 2015). In this form, the bacterium can remain latent but viable for decades, as described by Titball et al. (1991). When conditions are not conducive or favourable for the development and proliferation of the vegetative form of *B. anthracis*, the production of endospores (sporulation) takes place (Turnbull, 2008). In order for the bacterium to undergo sporulation, free oxygen around the cell is necessary (Turnbull, 2008).

In the bacterium's natural cycle of growth, an environment with low oxygen content is needed to produce the vegetative form within the host. This form is found exclusively inside the infected host organism (Turnbull, 2008). When the vegetative form comes into contact with the

environment outside of the host, sporulation is initiated upon exposure to the free oxygen, and the spores formed are essentially the main form in which the bacteria are found in the environment (Bellan et al., 2013). Endospores are extremely resistant to many biological extremes of temperature, dehydration, chemicals (and thus to disinfection), pH, radiation, and other hostile conditions (Fox and Eder, 1969; Koike et al., 1992; Wuytack et al., 1998; Knott et al., 1995; Nicholson et al., 2000; Setlow, 2001). *B. anthracis* can persist in the endospore form for long periods waiting for the correct moment when favourable conditions will initiate germination, which will then lead to multiplication (Kaufmann, 1989).

It is most commonly thought that *B. anthracis* is contracted through the uptake of bacterial spores from the environment (Turner et al., 2016). Once the spores have resided within the infected host organism, the spores start to germinate in the presence of optimal growth requirements, to produce the vegetative forms inside the host, which multiply exponentially and secrete toxins, leading to the eventual death of the host (Turnbull et al., 1999). The uptake of the endospores by another animal (next host) may vary from less than one hour to as much as decades later (Turnbull, 2008).

The genomic precursors of the vegetative cells are suspended in a dehydrated state and consist of small acid-soluble proteins (SASPs) that bind and protect the deoxyribonucleic acid (DNA) (Liu et al., 2004). SASPs are known to act as an amino acid source during germination, while other specific organic acids act as the energy source for the initiation of the germination process (Turnbull, 2008). The vegetative cell form has a central area that is known as the ‘core’ which is surrounded by a thick protective cortex, as well as spore coats and a proteinaceous exosporium. It is known that the inner layer of the cortex comprises of the initial cell component/ precursor of the vegetative cell wall of *B. anthracis* (Huang et al., 2004). The receptor that is used to trigger germination is located between the cortex and the spore coat of the endospore (Setlow, 2014). A component that is very important in the heat resistance of the endospore is the cortex (Nicholson et al., 2000). The component that is responsible for the resistance of chemicals and physical interference of the bacterium is the endospore coat which represents roughly 50% of the volume of the endospore (Anderson et al., 2008). The role or function that the loose-fitting exosporium plays in the endospore is yet unknown but many researchers have speculated that it has a role in the way that the endospore adheres to surfaces (Turnbull, 2008).

The rate, as well as degree of sporulation by vegetative cells that are shed from infected animals is largely affected by the environmental conditions into which the bacterial endospores are exposed to (Turnbull, 2008). The role of calcium and the effect it has on spore survival and the growth of *B. anthracis* have been documented and recognised across certain ‘favourable sites’ across the globe (Turnbull, 2008). These “favourable sites” contain soils that are called

chernozem soils. Chernozem soils are defined as calcium-rich, neutral-to-alkaline soil are found to have a strong link and connection with disease in animals at the county level as described by Smith et al. (2000).

1.2.1 Distribution

Anthrax is most commonly found in the agricultural regions of central and southern America, Canada, the Caribbean, southern and eastern Europe, central and southwestern Asia, and sub-Saharan Africa (CDC, 2015). Anthrax is also found in the United States of America but in much lower numbers (mostly in the western parts) where sporadic outbreaks occur in wildlife and domestic grazing animals (Blackburn, 2006). Anthrax is known to be more commonly present in developing countries that generally do not have public veterinary health programmes that routinely vaccinate or screen animals against anthrax or where anthrax persists among wildlife (Penn and Klotz, 1997; Pile et al., 1998).

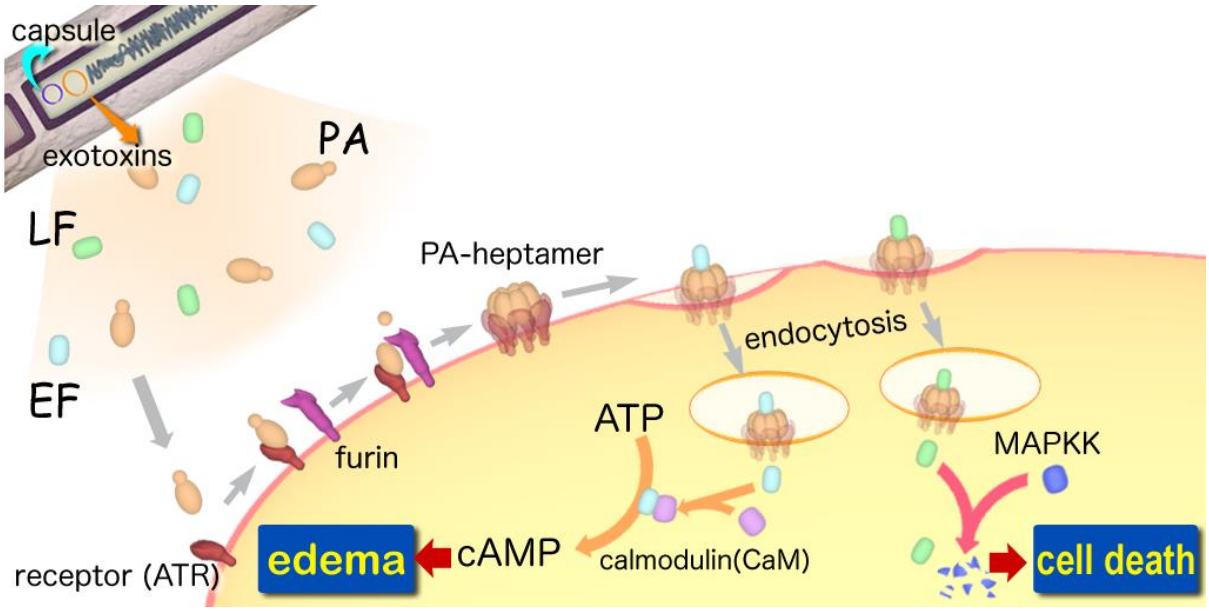
1.2.2 Virulence factors and pathogenic presentation

The virulence of *B. anthracis* depends on a glutamic acid capsule and a tripartite protein exotoxin complex. The genes that code these virulence factors are located on plasmids carried by the bacterium. *B. anthracis* isolates can only be classified as being fully virulent if both plasmids, pXO1 and pXO2, are present. The pX01 plasmid (182 kb) encodes a tripartite protein exotoxin complex (anthrax toxin complex) and the smaller pX02 plasmid (95 kb) encodes genes that are responsible for the capsule formation/generation (Dixon et al., 1999; Mock & Fouet, 2001). Repeated sub-culturing of virulent isolates *in vitro* can result in loss of plasmid(s) rendering the isolate avirulent. The capsule, which protects the organism from phagocytosis, is a polypeptide of D-glutamic acid (Makino et al., 1989). This particular isomer of glutamic acid is resistant to hydrolysis by host proteolytic enzymes because it is the 'unnatural' form of the amino acid. The capsule is thought to function as a virulent determinant through anti-phagocytic properties (Makino et al., 1989).

Anthrax toxin complex consist of three proteins called protective antigen (PA), edema factor (EF), and lethal factor (LF). Individually these proteins are nontoxic but together they act synergistically to produce damaging effects (Mock & Fouet, 2001).

PA serves as a necessary binding molecule for EF and LF, permitting their attachment to specific receptors on the host cell's surface (Brossier et al. 2000). Edema results from the combination of PA with EF, whereas death occurs when PA and LF are combined. EF is an adenylate cyclase that increases the concentration of cyclic adenosine monophosphate (cAMP) in host cells. LF is

a protease that kills host cells by disrupting the transduction of extracellular regulatory signals (Bhatnagar and Batra, 2001). The effect of the antigens on the cell is illustrated in Figure 1.2.



Note: The protective antigen (PA) attaches to an anthrax toxin receptor (ATR) and the PA is then cleaved in two by furin. As more PA-ATR complexes form together they form a heptamer. Once the heptamer is formed, lethal factor (LF) and/or edema factor (EF) binds to the heptamer. The complex that has formed is phagocytosed into the cell. In the endosome, the factors are then released to cause pathogenesis, depending on wat factor is released. LF will destroy the mitogen-activated protein kinase and cause cytolysis. If EF is released, it will bind to calmodulin and that complex will then bind to Adenosine triphosphate (ATP) which will increase cyclic adenosine monophosphate (cAMP) level and cause edema (Wikipedia.org 2016)

Figure 1.2: The actions of the secreted anthrax toxins

B. anthracis occurs mainly as latent endospores in the environment. The endospores are known to survive for long periods (more than 250 years) and are highly resistant to environmental changes (Turnbull, 2008; Goel, 2015). When an animal's host has taken up or acquired the *B. anthracis* endospores either through inhalation, cutaneous or gastrointestinal infections the spore of the bacterium starts to germinate within the host and multiplies into the vegetative form of the bacteria (Turnbull, 2008). During the rapid multiplication or reproduction of the vegetative form of the bacteria, the bacteria swiftly reproduce and start to produce both virulence factors (for example, anthrax toxin complex) and capsule (Liu et al., 2014). The reproducing bacteria need the action of the toxins to avoid the host immune system and distribute systemically (Lui et al., 2014). The dissemination is achieved through regional lymph nodes connected to infection site. A few early studies have suggested that replication of the bacterium is strictly required to take place within the cells (Dixon et al., 2000), but more recent studies have indicated that the bacterium is an extracellular pathogen rather than an intercellular pathogen that can rapidly germinate and cause pathology outside of the cell (Moayeri et al., 2010; Corre et al., 2012). Once the bacteria have disseminated, they reproduce in high quantities, including the anthrax

toxin, which will lead to the mobility or mortality to the host. While the cutaneous disease presentation is usually associated with edema and necrosis, followed by the formation of the black eschar (historically linked with anthrax), the inhalation and gastrointestinal disease ultimately leads to sepsis and shock-like vascular collapse (Moayeri et al., 2015).

1.2.3 Pathology in animals

The pathogenic presentation can be broad in different types of animals, with ruminants being the most at risk (Turnbull, 2008). Species susceptible to *B. anthracis* infections include nearly all mammals and some bird species (Schmid and Kaufmann, 2002). Disease can develop into three different forms or stages, namely pre-acute form which often develops and affects ruminants (cattle, sheep, and goats); the acute form occurs or develops in ruminants and horses; and the subacute or chronic form, which affects and develops in swine, cats and dogs (American Veterinary Medical Foundation, 2001). Disease characteristics of the pre-acute form include staggering trembling, breathing difficulties, which then leads to rapid collapse, terminal convulsions, and sudden death of the animal (Lucey, 2005). In ruminants, the pre-acute form becomes a systemic disease and is one of the most common occurrences of the disease. Sudden death is mostly known to be the only sign of the pre-acute form. The disease characteristics of the acute form include high fevers (up to 42℃), excitement of the animal, increased heart rate, laboured breathing or respiration (hyperpnea), followed by depression, incoordination, cessation of rumination, reduction in milk production, discoloured milk (blood-tinged or deep yellow), bloody discharges, respiratory distress, convulsions, abortion, and death within 48 to 72 hours of the animal (American Veterinary Medical Foundation, 2001). The disease characteristics of the chronic form include subcutaneous swelling and edema, usually involving the ventral aspect of the neck (brisket), thorax, shoulders, perineum and flank areas (American Veterinary Medical Foundation, 2001).

Incubation periods are known to vary according to the different stages, as well as the animal that it affects, and varies between 1 to 20 days. Disease signs in herbivores (ruminants) occur within three to seven days, whereas in pigs signs occur one to two weeks after exposure (Spickler, 2017).

1.2.4 Aetiology

Anthrax is a disease caused by *B. anthracis* and is a seasonal disease with outbreaks in enzootic areas after lengthy hot or dry conditions, preceded by heavy rainfalls or flooding in the specific area, or with rainfall that is ending a period of drought (Turnbull, 2008). Although, as conditions and circumstances that influence outbreaks vary from location to location and site to

site, seasonality shows consistent deviations (outbreaks taking place out of seasonality and not following the predicted pattern of when the disease will occur) from this pattern in different locations as observed by Quinn and Turnbull (1998). Much work and hypothesising has been done on the effects of vegetation, rainfall, temperature, soil, season, host conditions, and population density on the epidemiology of anthrax, yet few concurrences exist on the roles conveyed by these environmental and ecological factors in the occurrence of disease (Dragon and Rennie, 1995; Hugh-Jones and De Vos, 2002). Nevertheless, the main conditions affecting the seasonality of anthrax in its location or site would appear to be temperature, rainfall, or droughts, and humidity that are associated with seasonality (Minet, 1952; Maksimović et al., 2017; Walsh et al., 2018).

B. anthracis has been reported as a monomorphic species (Henderson et al., 1994; Sue et al. 2007). During spore dormant phases the bacterium is static, and therefore the genetic integrity of the bacterium stays the same over prolonged periods. Bacterial variation only occurs during replication and therefore during infection of a host. Phenotypically, the strain differences are only apparent in non-quantifiable or semi-quantifiable characteristics, such as colony morphology, flocculation in broth cultures, cell size, multiplication rate, sporulation efficiency, and 50% lethal dose (LD50) in animal tests (Turnbull, 2008; Kolstø et al., 2009). The basis for these genetic differences has not been established yet (Turnbull, 2008). The biochemical, serological or phage typing methods that are normally used for the identification and typing of other pathogens can be used as conformation tests. However, some strains of *B. anthracis* are known to be phage and penicillin resistant and can result in the misidentification of *B. anthracis* (Klee et al., 2006, Turnbull, 2008, Ross et al., 2009, Beesley et al., 2010).

1.2.5 Anthrax in animals

Numerous countries are still impacted by severe anthrax outbreak or epidemics, despite the availability of the avirulent live-spore vaccine (*B. anthracis* (Sterne strain)) for livestock to control the disease in animals (Turnbull, 2008). Countries with poor socio-economic conditions are especially afflicted with anthrax. Anthrax outbreaks are under-reported in low- and middle-income countries. Control programmes are complicated as immunising free-living wild animals is impractical and anthrax cases are not monitored in wildlife in many national parks and reserves (Hugh-Jones and De Vos, 2002). National parks, like KNP, are therefore ideal to study *B. anthracis* and its role in the environment, providing vital and critical baseline information on the epidemiology of the disease.

Research showed that major disease epidemics tend to take place periodically in the following African wildlife conservation regions:

- The Kgalagadi Transfrontier Park in South Africa and Botswana (Ebedes, 1981; Turnbull, 1996, Hassim, 2017).
- The Mokala (Vaalbos) National Park and the Kruger National Park (KNP) in South Africa (Pienaar, 1967; De Vos, 1990; De Vos and Bryden, 1995; Hassim, 2017; Steenkamp et al., 2018).
- The Etosha National Park in Namibia (Ebedes, 1976; Beyer et al., 2012; Turner et al., 2013; 2016).
- The Queen Elizabeth National Park in Uganda (Gainer, 1987).
- The Omo-Mago National Park in Ethiopia (Gainer, 1987).
- The Luangwa Valley in Zambia (Turnbull et al., 1991; Hang'ombe et al., 2012).
- Mana Pools National Park in Zimbabwe (Beyer and Turnbull, 2009).
- The Selous Nature Reserve in Tanzania (Gainer, 1987).

KNP in South Africa is a wildlife conservation park/nature reserve that is two million hectares in size with anthrax occurring endemically in the Pafuri area in the northern part of KNP (Steenkamp et al., 2018). Studying this disease in KNP and other African conservation areas with multiple animal species, carcass counts indicate that specific herbivores are either over- or under-represented in animal deaths relative to their population figures and/or densities (Hugh-Jones and De Vos, 2002). This finding would indicate that there are variable degrees of susceptibility and/or behavioural vulnerability to infection in specific species (Turner et al., 2013). Species that are generally found to be over-represented in KNP include the spiral horned antelope species, for example kudu (*Tragelaphus strepsiceros*), and buffalo (*Syncerus caffer*), while animals that are under-represented include the alcelaphines, zebra (*Equus* spp.) and impala (*Aepyceros melampus*) (Hugh-Jones and De Vos, 2002). Hugh-Jones and De Vos (2002) also reported that the species most susceptible to anthrax infections were herbivores, and reported suids, carnivores and ostriches were far less susceptible to anthrax, although outbreaks in these species are known to have occurred.

In Northern America (United States of America and Canada), anthrax is considered to be a big problem, especially in bison (*Bison bison*), moos (*Alces alces*) and deer (family *Cervidae*) (Hugh-Jones and De Vos, 2002; Blackburn, 2006; Blackburn et al., 2014). In North America, anthrax outbreaks occur after abundant or heavy rainfall in springtime, followed by a very hot and dry summer. Anthrax outbreak patterns in southern Africa, and specifically in the KNP, occurs in dry, winter months, and in early spring (De Vos, 1990) but since 2008 have been reported in summer months following heavy rainfall (Basson et al., 2018). Furthermore, residual heard immunity of animals with prior exposures have been speculated to provide protection, while another outbreak can occur with the weaning of the immunity against anthrax of the animals (De

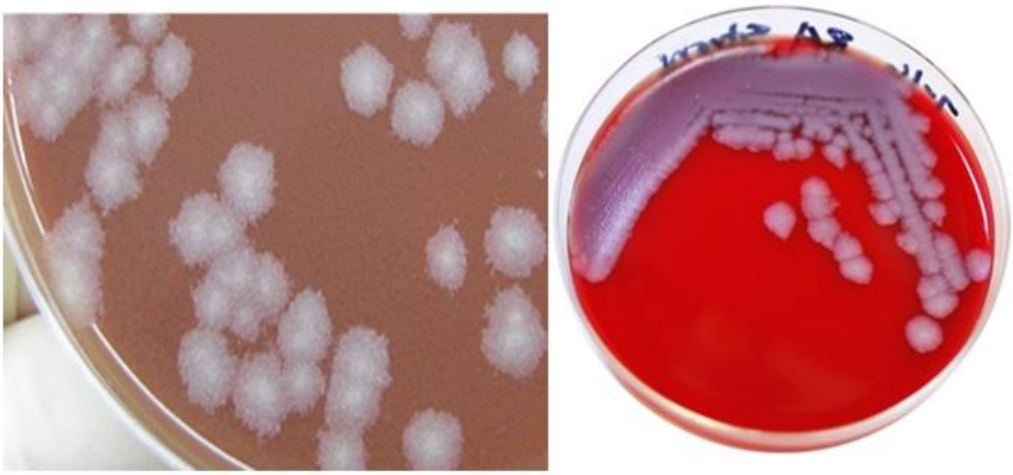
Vos and Scheepers, 1996; Hugh-Jones and De Vos, 2002). The role of climatological patterns together with exposure should be further investigated.

1.2.6 Laboratory diagnosis

The identification of *B. anthracis* can be achieved both directly and indirectly from the samples provided but care must be taken when samples arrive from suspect anthrax case(s). With some geographical variation regarding the definitions of safety levels and requirements or recommendations involving the handling of *B. anthracis*, the most general used practices involve biosafety level (BSL) facilities and their criteria must be followed (Turnbull, 2008; Johnson and Casagrande, 2016).

1.2.6.1 Phenotypical characterisation

Colony characteristics of *Bacillus* species vary considerably and are often influenced by the type of media used for isolation and proliferation. Most species form non-pigmented colonies. They are catalase positive and form endospores under aerobic and anaerobic conditions (Public Health England, 2015a). *B. anthracis* can easily be identified and differentiated from other *Bacillus* species, with rare exceptions (Turnbull, 1999). Parry et al. (1983) described the characteristic colony morphology of *B. anthracis* on nutrient agar, as well as on 5-7% blood agar (Figure 1.3), as being matted and flat in appearance, which is similar to *B. cereus* (Turnbull, 2008). *B. cereus* is generally smaller, waxier and white or grey-white on blood agar and haemolytic (weak to strong β-haemolytic) (Drobniewski, 1993; Public Health England, 2015a). *B. cereus* often has curly tailing at the edges that may look similar to the Medusa head-like morphology seen with *B. anthracis*. Colonies of *B. anthracis* are non-haemolytic, but in some occasions, very weak haemolytic colonies have been observed on sheep or horse blood agar (Turnbull, 2008). Other morphological properties of the bacterium include non-motile bacterial cells and the organisms' sensitivity to penicillin and 'gamma'-phage testing (Marston et al., 2006; Turnbull, 2008). *B. anthracis* is known for capsule formation when exposed to blood or when the organism is cultured on bicarbonate-serum agar under CO_2 conditions (Turnbull, 2008). Validation of *B. anthracis* should be accomplished by being able to display a non-motile, capsulated, spore-forming, box-shaped, Gram-positive rod in chains from culture (Turnbull, 2008; Public Health England, 2015a).



Source: CDC (2015)

Figure 1.3: The matted and flat appearance of the colonies of *Bacillus anthracis* on a 5% sheep blood agar

A wide range of different smears or stains that can be used for identification and conformation of *B. anthracis* and include Gram stain, modified Ziehl-Neelsen stain, malachite green stain, polychrome methylene blue stain (M'Fadyean reaction) for spores, and India ink for capsule visual identification (Turnbull, 2008, Public Health England, 2015a). Biochemical tests or commercial identification kits do not always identify *B. anthracis* accurately, although they may identify the isolate initially with culture. *B. anthracis* is part of the *B. cereus* group stating that, except from the morphological identification characteristics (non-haemolytic, non-motile and pathogenic), *B. anthracis* displays similar physiological and biochemical identification features that have also been described for *B. cereus* (Koneman, 1983; Turnbull, 2008). Bacteriophages or better known as phages, specific for *B. anthracis*, were isolated for the first time in 1950, but was first reported in 1955 by Brown and Cherry who specifically named the bacteriophage 'gamma-phage'. The gamma-phage has become one of the most valuable tools used for standard diagnostics in the identification of *B. anthracis* as well as susceptibility to penicillin (Klee et al., 2006).

1.2.6.2 Molecular characterisation

A number of polymerase chain reaction (PCR) assays have been described for the identification of *B. anthracis* isolates (Ramisse et al., 1996; Qi et al., 2001; Ellerbrok et al., 2002; Ko et al., 2003; Bode et al., 2004) and detection of *B. anthracis* directly in tissues (Makino et al., 1993; Cheun et al., 2001), from animal fibres (Levi et al., 2003) and in environmental samples (Beyer et al., 1996; Makino et al., 2001; Ryu et al., 2003). PCR assays differentiate *B. anthracis* strains from closely related species of the *B. cereus* group. The most important distinctive features of *B. anthracis* strains are the two plasmids and their related genes (pX01 [*lef, cya, pag*] and pX02

[*cap*]) that codes for the anthrax toxin complex (pX01) and the capsule (pX02) (Beyer et al., 1995; Ellerbrok et al., 2002).

Genetic characterisation of *B. anthracis* makes use of a multi-loci variable number (MLVA) tandem repeat analysis and single nucleotide polymorphisms (SNP) analyses, to establish the phylogenetic relationships among *B. anthracis* isolates (Keim et al., 2000; Pearson et al. 2004). The mapping of the SNPs (of the genetic population structure) allows for the identification of canonical SNP (canSNPs) which in turn can be used in molecular diagnostics for defining major genetic lineages in a species, or more to the point, define specific strains as described by Keim et al. (2004). Variable number tandem repeat (VNTR) is used to characterise monomorphic bacterial species such as *B. anthracis* (Keim et al., 2000; Le Fleche et al., 2001; Lista et al., 2006; Beyer et al., 2012). The phylogenetic relationships between *B. anthracis* isolates globally are determined using MLVA (Keim et al., 2000; Lista et al., 2006; Beyer et al., 2012; Thierry et al., 2014). MLVA targets the varying number of mini- and microsatellite repeats (VNTRs) at different loci in the genome of *B. anthracis,* which includes the hypervariable chromosome regions and plasmid regions. The copy number of each VNTR enables characterisation of each isolate resulting in a specific DNA fingerprint or genotype. The number of loci included in the assay increases the discrimination. MLVA based on 8, 15, 25 and 31 VNTR markers have been described and used to genotype strains (Keim et al., 2000; Lista et al., 2006; Van Ert et al., 2007a; Beyer et al., 2012; Thierry et al., 2014).

The first MLVA scheme for *B. anthracis* was described by Keim et al. (2000) consisting of an MLVA8 panel, targeting *vrrA, vrrB1, vrrB2, vrrC, vrrC2, CG3, pXO1-aat* and *pXO2-at* VNTR markers (Table 1.1). The MLVA8, di- and trinucleotide tandem repeats of pXO1-AAT and pXO2-AT are diverse loci (Keim et al., 2000). The MLVA8 markers pose variable levels of diversity and contribute significantly to the analysis of *B. anthracis*. The MLVA8 grouped global *B. anthracis* strains into four A-clades (A1, A2, A3 and A4) and two B-clades (B1 and B2). The A1 cluster can then be subdivided to form two distinguishing sub-clusters or groups, the A1.a branch (mainly found in North America) and A1.b branch (primarily found in Africa). The A3 cluster consists of strains distributed across numerous continents; the B1 cluster is known to be restricted again to most parts of southern Africa (Keim et al., 2000). While most of the biological significance regarding VNTR variations of *B. anthracis* is unknown, some VNTR variations have distinct effects on the biology of the pathogen as described by Van Belkum et al. (1998). Five of the MLVA8 loci are located in open reading frames. The variations identified in these VNTRs could affect the bacterial phenotype of *B. anthracis* due to the altered translational products that are produced.

Le Flèche et al. (2001) selected 20 VNTR markers of which the copy number could be determined using agarose gel electrophoresis and not capillary electrophoresis. The MLVA20 consisted of six of the MLVA8 panel (vrrA, vrrB1, vrrB2, vrrC1, vrrC2, and CG3) as well as 14 additional markers (Bams 1, 3, 5, 7,13, 15, 21, 22, 23, 24, 25, 28, 30, 31) (Table 1.1), which resulted in the significant improvement of the resolution power of the assay (Le Flèche et al., 2001). This technology (agarose gel electrophoresis) is more demanding and labour-intensive than capillary electrophoresis and restricts the VNTR markers than can be selected.

Keim et al. (2004) expanded the MLVA8 and developed MLVA15. Phylogenetic analysis using MLVA15, subdivides a global set of *B. anthracis* isolates into three major lineages (A, B and C) (Van Ert et al., 2007a, b). Furthermore, a small number of canSNPs located at key phylogenetic junctions were used to identify major and sub-lineages (Keim et al., 2004; Van Ert et al., 2007a, b). Van Ert et al. (2007a, b) indicated that the *B. anthracis* A-lineage are more widely dispersed globally, whereas the B- and C-lineages occur in more specific geographical and restricted spatial scales.

MLVA broadly separates *B. anthracis* isolates from southern Africa into two major clonal groups, namely A and B (Smith et al., 2000; Turnbull, 2008). Van Ert et al. (2007a) found that the A branch is most common worldwide, with the B1 (Kruger B) sub-branch found only in southern Africa and the B2 sub-branch is considered important to specific geographical areas in central Europe. Smith et al. (2000) observed that *B. anthracis* B1 strains in KNP were isolated in higher-calcium and pH soils than A strains.

Lista et al. (2006) developed an MLVA25 panel for the detection of *B. anthracis* using capillary electrophoresis to increase the turnaround time and accuracy of the genotyping system (Table 1.1). This panel was used to analyse global *B. anthracis* strains, and Lista et al. (2006) reported two new *B. anthracis* lineages, namely D and E. The MLVA25 panel further improved the genotyping methods for *B. anthracis* and allowed the typing of a larger collection of strains worldwide.

Beyer et al. (2012) combined the MLVA15 and 25, which shared 9 VNTR's, resulting in the MLVA31 panel for the genotyping of *B. anthracis* (Table 1.1). This panel was used to analyse *B. anthracis* strains from southern Africa, specifically Namibia. All the genotypes identified belonged to the A-branch in the MLVA and canSNP analyses. Genetic distances among *B. anthracis* strains were significantly greater for strains from different host species. Beyer et al. (2012) suggested that while species-specific ecological factors may affect exposure processes, the transmission cycles in different host species remain highly consistent. The MLVA31 data was further used to reveal single outbreak events within endemic occurrence regions of anthrax in animals. The MLVA31 panel has an application for use in routine molecular characterisation,

as well as being used as a conventional epidemiological method for the surveillance and prevention of this neglected disease.

Table 1.1: Comparison of multi-loci variable number of tandem repeat (VNTR) assay (MLVA) panels with their VNTR markers

Marker loci	MLVA panels/systems					
	MLVA7	MLVA8	MLVA15	MLVA20	MLVA25	MLVA31
vrrA_12bp_10U	●	●	●	●	●	●
vrrB1_9bp_16U		●	●	●	●	●
vrrB2_9bp_6U		●	●	●	●	●
vrrC1_9bp_53U		●	●	●	●	●
vrrC2_18bp_17U		●	●	●	●	●
CG3_5bp_2U		●	●	●	●	●
pXO1_aat_3bp_7U		●	●		●	●
pXO2_at_2bp_10U		●	●		●	●
Bavntr12_2bp_6U			●			●
Bavntr16_8bp_20U			●			●
Bavntr17_8bp_4U			●			●
Bavntr19_3bp_4U			●			●
Bavntr23_12bp_4U	●		●			●
BAMS01 (alias Bavntr32)_21bp_16U			●	●	●	●
Bavntr35_6bp_5U			●			●
BAMS03_15bp_26U	●				●	●
BAMS05_39bp_5U	●				●	●
BAMS07_ 18bp_49U				●		
BAMS13_9bp_70U				●	●	●
BAMS15_9bp_24U				●	●	●
BAMS21_45bp_10U				●	●	●
BAMS22_36bp_16U	●			●	●	●
BAMS23_42bp_11U				●	●	●
BAMS24_42bp_11U				●	●	●
BAMS25_15bp_13U				●	●	●
BAMS28_24bp_14U				●	●	●
BAMS30_9bp_57U				●	●	●
BAMS31_9bp_64U				●	●	●
BAMS34_39bp_11U	●				●	●
BAMS44_39bp_8U	●				●	●
BAMS51_45bp_9U					●	●
BAMS53_12bp_8U					●	●

Note: The marker loci identify indicate the VNTR marker name with the length of the tandem repeat and copy number of *Bacillus anthracis* Ames strain.

Thierry et al. (2014) evaluated all the panels and the MLVA7 panel was proposed to characterise *B. anthracis*. The MLVA7 panel (vrrA, Bams03, Bams05, Bams22, Bams34, Bams44, and vntr2) comprised of markers that already existed in the extensive MLVA31 panel (Table 1.1) with none

located on the virulence plasmids present in *B. anthracis* (Thierry et al., 2014). The MLVA7 panel was used to characterise *B. anthracis* strains with a limited global representation consisting of A-clade strains mainly from France.

1.2.7 Disease management in animals

As discussed in Section 1.2.5, ruminants such as cattle, sheep and goats, are among the most commonly susceptible farm animals to be affected by anthrax (Turnbull, 2008). The most common route of infection is usually through the ingestion of the *B. anthracis* spores. The animal may experience any of a number of the disease stages (Section 1.2.3), but most often the animal will succumb due to anthrax (Lucey, 2005). Disease outbreaks can be classified depending largely on the source of infection. These could include classic sporadic outbreaks, atypical outbreaks and epidemic outbreaks (Fasanella et al., 2014).

The classic sporadic outbreaks commonly take place in areas where anthrax is known to be enzootic (Fasanella et al., 2014), usually due to pastures that are contaminated by *B. anthracis* spores with animals becoming infected by grazing. Resulting outbreaks are sporadic and only involve a small number of animals (Fasanella et al., 2014). Contamination of the area can be due to numerous reasons including infected carcasses, introduction of unvaccinated susceptible species, burial site of infected anthrax carcasses, faeces of infected omnivores and carnivores in the area etc. (Lindeque and Turnbull, 1994; Blackburn et al., 2010; Fasanella et al., 2014). Outbreaks tend to take place during dry periods (summer) followed by brief rainfalls and are mostly limited to alkaline calciferous soils. Taking the above-mentioned into account, it is hypothesised that woody forage may result in the formation of small lesions and the scarceness of pasture may force animals to consume vegetation that is close to the ground (Hugh-Jones and Blackburn, 2009). Atypical outbreaks are usually associated with the climate change, different feeds, for example hay or silage, that may be contaminated due to production on contaminated soil/land, as well as a product of animal origin such as inadequately sterilised meat and bone meals derived from the infected carcasses. These outbreaks are characterised by unexpected anthrax outbreaks in conditions and situations epidemiologically distinct from the classic episodes of ground/ soil origin (Fasanella et al., 2014).

The epidemic outbreak is described as being an evolution of the classic sporadic form due to the activities of haemophagic flies around dead animals (Fasanella et al., 2014). Tabanids feed on diseased animals, especially during the bacteraemic phase of the disease, and transfer the pathogen to other animals from the same herd or a neighbouring herd (Fasanella et al., 2014). Animals are known to display a distinct extensive oedema. Cases can be separated from one

another by as much as 15 km from the outbreak source (Fasanella et al., 2014). These observations and deductions were made from European outbreaks.

Management and prevention of anthrax can be achieved by following specific guidelines or disease prevention programmes. These guidelines or programmes must be adapted for each country that is known to be endemic for anthrax. Guidelines from the World Organisation for Animal Health (OIE, 2007a), together with those from Turnbull (2008), have been used as the most complete guide for the management of anthrax. This includes management of the animal carcasses, disinfection of the animal and area, disposal of infectious sources and other means of animal carcass management.

In most anthrax outbreaks (classic sporadic outbreaks), that affects livestock and/or humans in the surrounding area (Fasanella et al., 2014), the following management procedures must be adhered to:

- Avoid the dispersion of biological fluids during sample collection.
- Correct handling (postmortem) and burial/disposal of carcasses.
- Checking of the temperatures of all other living livestock and vaccinating livestock with normal temperatures quickly that is at risk of infection.
- Delay the vaccination of animals with suspected illness and initiate antibiotic treatment in those cases; once antibiotic treatment has been completed and suspended, vaccinate these animals. Animals will be protected only after two or more weeks after inoculation of the vaccine.
- Determine the genotype of the isolated strain(s) that could be used to track the origin of infection.
- Suspend the sale of milk and meat from the animals on the infected farm for 10 days after vaccination or the last known animal death.

In atypical outbreaks the same management can be used as suggested above, but the following conditions must also be applied (OIE, 2007a; Turnbull, 2008; Fasanella et al., 2014):

- Immediate suspension of the feeds suspected to be the source of the outbreak.
- Perform laboratory tests for *B. anthracis* on the suspected feeds.
- Sanitary authorities must be informed, to suspend the sale of the suspect feed until sterilisation certification has been presented in relation to meat and bone meals.

For epidemic outbreaks (Fasanella et al., 2014), the same management guidelines as the classic sporadic outbreak should be utilised, with the following extra measures that must be taken:

- Mass vaccination of all livestock in a 15 km radius of the index cases.
- Quarantine all livestock suspected to have septicaemia in an enclosed location to avoid contact with bloodsucking insects or sprinkle/rinse the body of animals with insect repellents.
- Vaccinate normal temperature animals a second time two weeks after the initial/first vaccination.
- Suspend animal handling.
- Better and more intense attention by physicians/ veterinarians to look for an increase in skin lesions in the exposed population.

As mentioned, vaccination is very important in any disease management programme to prevent an outbreak. The Stern vaccine strain (34F2) of is one of several different avirulent strains of *B. anthracis* that are known to be used for the vaccination of animals against anthrax. The Stern vaccine strain (34F2) is a *B. anthracis* strain that lacks the PX02 plasmid which is important in the production of the capsule but retains the PX01 which is important in the production of the anthrax toxin (Stern, 1939) The loss of the PX02 was due to natural causes. Stern (1939) tested a number of avirulent strains to be used for the vaccination of animals and was successful in finding a candidate that could be manufactured for commercial use. When comparing the wild type strains (PX01+/PX02+) with the Sterne strain (PX01+/PX02–), the Sterne strain is able to enlist a protective immune response against *B. anthracis*.

1.2.8 Surveillance of anthrax in animals

Surveillance is one of the most essential components to prevent and control any disease and serves two functions: First, it is an early warning system for potential threats to public health, and second, monitoring of disease control programmes, which may be either disease-specific or multi-disease specific in nature. The term ‘surveillance’ is defined by Turnbull (2008) as “the collection, organisation and analysis of health data that enables the quick dissemination of the information to those who need to know, in order to take the appropriate actions needed”. Veterinary surveillance systems are of crucial importance to establish or maintain effective management of diseases, especially of anthrax (Fasanella et al., 2014). The monitoring of changes in any aspect of the disease is vital in order to effectively modify prevention and control strategies and activities. Reporting and conformation of report results are vital in any surveillance programme and thus communications need to be streamlined for easy reporting or results (Turnbull, 2008). This detection, confirmation and reporting should be followed by a strong response from the veterinary health system to control the disease. General guidelines for animal health surveillance are available in the OIE manual (2007b).

Surveillance systems vary regarding objectives and methodology. The design of a surveillance programme for anthrax is largely dependent on the objectives set. Most anthrax surveillance programmes tend to have the prevention or reduction of animal losses and to prevent any human disease to take place. By education of the farmers/producers and veterinarians in the detection, confirmation and reporting of cases, this objective can be achieved. A secondary objective is usually the data generation of risk areas in the country or geographical area. Other objectives can include the identification of specific characteristics of the disease, and the assessment of control and prevention activities by monitoring the frequency of the disease in both animals and humans.

In KNP a passive surveillance program has been in place for at least 40 years where smear with relevant information (species, age of carcass, coordinates, scavenger activity etc.) is made of each carcass by SANParks and State Veterinary Services personnel. The smear and sample from carcass are sent to Skukuza State Veterinary Services for microscopic examination. Suspect *B. anthracis* samples will then be confirmed with culturing.

1.3 Clostridia

Clostridium species, or better known as Clostridia, belong to the family *Clostridiaceae* and are the most widely studied anaerobes that cause diseases in humans and animals (Songer, 1996; Public Health England, 2015b). The genus consists of more than 203 species and five subspecies that are known to cause an array of diseases in both humans and animals (Euzéby, 2013; Public Health England, 2015b). The most typical Clostridia are opportunistic pathogens, and thus take advantage of a breach in a host's natural defences to infect the host. Clostridia are well known to cause a wide range of diseases in numerous animals, of which some are acute. Accurate diagnose is difficult due to similarity of the clinical disease presentations with other diseases and Clostridia ambiguous nature requiring more than one confirmation tests (Ortiz, 2000; Ortega et al., 2012).

Clinically important *Clostridium* species are well known to be Gram-positive rods (although some isolates tend to stain Gram variable) of 0.3–2.0 × 1.5–20.0 µm in size. Clostridia are known to be arranged in a variety of ways, ranging from pairs to short chains, with rounded or sometimes pointed or square ends (Figure 1.4) (Oakley, 1956; Public Health England, 2015b). Clostridia are known to be pleomorphic in morphology, Clostridia are known to be pleomorphic in morphology, and species are known to have differences or variability in their tolerance to oxygen. Other strict requirements are that some species (*Clostridium novyi* type A (*C. novyi*) and *Clostridium haemolyticum (C. haemolyticum*)) need to be included in extended incubation on pre-reduced or freshly prepared plates, and the complete handling be done in an anaerobic

chamber (Public Health England, 2015b). In contrast to the before-mentioned, *Clostridium tertium (C.tertium)*, *C. histolyticum* and *Clostridium carnis (C. canis)* are known to be aerotolerant and can form colonies on blood agar media incubated at O_2 and 5–10% CO_2 atmosphere combination (Oakley, 1956; Koneman, 1983).

Nearly all the members of the *Clostridium* genus, with the exception of *C. perfringens*, share the following morphological features: motility due to peritrichous flagellae form characteristic oval or spherical endospores that may expand the cell (Markey, 2013). Biochemical features of these species are generally catalase-negative with either saccharolytic or proteolytic nutrient requirements (Rood, 2016). Many species are known to produce potent exotoxins as virulence factors (Johnson, 1999). The genus *Clostridium* is known to be very phylogenetically heterogeneous and is generally considered to be Gram-positive but has also demonstrated to be decolourised easily and may appear to be Gram-negative, or as sometimes seen on microscope slides, to be Gram-variable (Figure 1.4). Clostridia can be seen as spore formers and non-spore formers, bacilli and cocci-bacilli, and anaerobic and facultative anaerobic (Finegold et al., 2002). Clostridia species mostly know to be Gram-positive; however, a few species are known to be Gram-negative. *Clostridium* are usually considered anaerobic, with the exception of some species to be aerotolerant, which including *C. carnis*, *C. histolyticum*, and *C. tertium* (Wells and Wilkins, 1996). Clostridia are well known to form endospores, with the exception of a few *C. perfringens* strains (Paredes-Sabja et al., 2008; Public Health England, 2015b). Just like *B. anthracis*, Clostridia are known to form spores (sporulation). These spores are extremely resistant to disinfection and drying out. This will allow Clostridia to persist in the environment for years, similar to *B. anthracis* (Gould, 2006). These features indicate the diversity and adaptability of the genus.

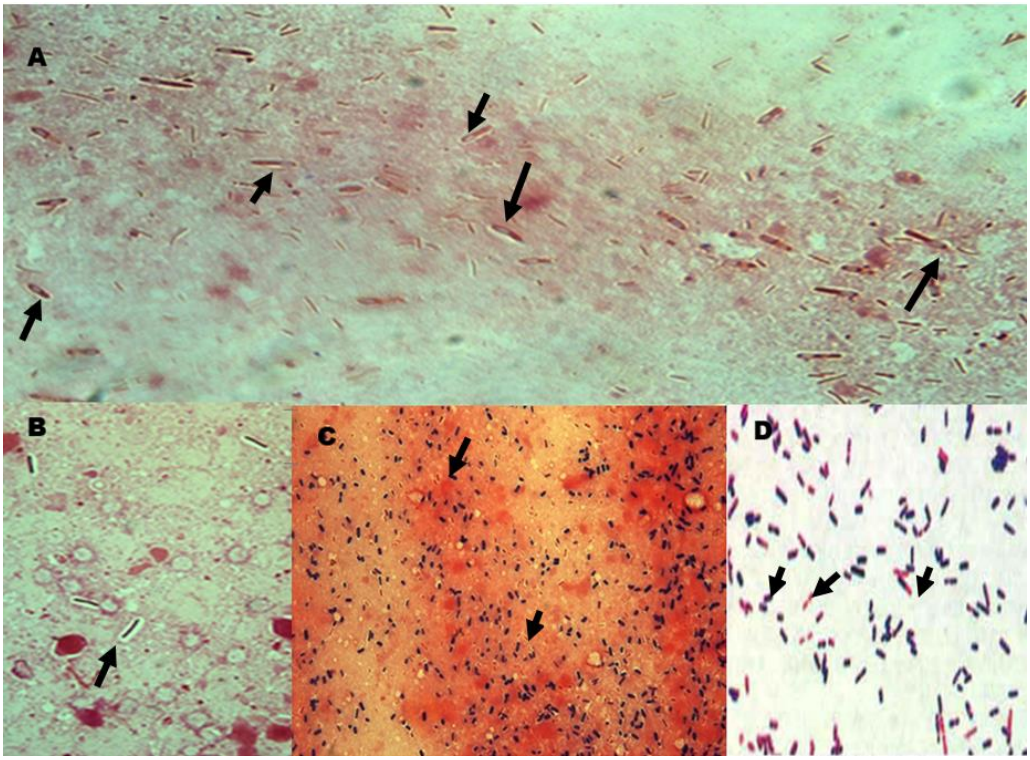


Figure 1.4: (A) A mixed anaerobic infection; at least three different Clostridia can be seen; (B) Capsule of *Clostridium perfringens*; (C) A Gram stain of *C. perfringens* and *Escherichia coli*; (D) Gram stain variability of Clostridia

Studies regarding anaerobic spore-forming Clostridia are of the utmost importance due to factors that include ubiquity in the natural environment (Hungate, 1950; Petit et al., 1999; Myllykoski et al., 2006) as well as their important applications in both medical and industrial industries. Sporulation of endospore-forming bacteria (e.g. *Bacillus* and *Clostridium*) is initiated when environmental extremes are unfavourable for growth (Nicholson and Setlow, 1990; Sonenshein, 2000). This mechanism is initiated to readily kill vegetative cells as well as survive for long periods (Nicholson et al., 2000). Endospore formation facilitates the persistence of Clostridia in the environment. Spores are resistant to chemical disinfectants and may withstand ultraviolet irradiation or boiling temperatures for some time, although not standard autoclaving conditions (121°C for 30 minutes at increased pressure) (Joslyn, 2001). Clostridia may be grouped per the location of the endospore within the cell. Spores are described as terminal when they are located at the end of the bacterial cell and subterminal when they are found at a location other than the end of the cell. Terminal spores typically cause swelling of the cell. Spores located in the centre of the cell are called central spores (Mahon et al. 2011).

When determining the significance of a Clostridia isolated from a clinical sample, it is imperative to consider the isolation frequency of the specific species, the occurrence of other microbes that share or have the same pathogenic potential, and the clinical manifestations or symptoms that the patient or animal may present with (Louisiana Office of Public Health, 2015). The easiest

way to understand Clostridia is to separate them into different disease-causing groups. *Clostridium* bacteria can be divided into three groups as seen in Table 1.2.

Table 1.2: Clostridia disease-causing groups, cause of disease and species associated with disease

Disease-causing group	Cause of disease	Species associated with disease-causing group
Neurotoxic group	Most cause disease via toxin ingestion, but toxin production can also occur in the body with spore ingestion.	*Clostridium botulinum* *Clostridium tetani*
Enterotoxic group	Normal commensal organisms living in the gut, but do not cause disease until they overgrow in favourable conditions and start producing toxins in large quantities, causing disease.	*Clostridium perfringens* *Clostridium difficile* *Clostridium septicum* *Clostridium sordellii* *Clostridium spiroforme* *Clostridium botulinum* Toxic *Clostridium baratii* Toxic *Clostridium butyricum*
Histo-toxogenic group	Spores can be ingested, or wounds may become infected with spores in this group. The bacteria reproduce in the tissue with resultant toxin production, causing clinical signs of disease.	*Clostridium perfringens* *Clostridium septicum* *Clostridium histolyticum* *Clostridium novyi* *Clostridium sordellii* *Clostridium chauvoei* *Clostridium tetani* *Clostridium botulinum*

Source: Adapted from Markey et al. (2013)

1.3.1 Distribution

Throughout the world, Clostridia have a wide distribution in soil, earth and dust, sewage, freshwater and in marine/aquatic settings, particularly those with high organic content (e.g. ponds/lakes), although some species or types are only present in localised geographical areas (Haagsma, 1991). Many of the pathogenic Clostridia are normal inhabitants of the intestinal tract of animals and humans, and often cause endogenous, destructive, and invasive infections when introduced into tissue by a break in the skin, resulting from surgery or trauma or any other deep traumatic wounds, or by changes in the gastrointestinal tract due to change in feed or use of antimicrobials (Willis, 1990; Wells and Wilkins, 1996; Rood, 2016). Their presence in infectious processes is opportunistic. Because of the abundance of Clostridia found in nature, numerous isolates from clinical specimens tend to be considered as contaminants that play no role in the association with disease (Liu, 2011; Markey et al., 2013).

1.3.2 Virulence factors and pathogenicity

Characteristic disease presentations or features due to toxin production of Clostridia especially tetanus and botulism, can be observed in animals or with post-mortem examination (Hatheway, 1990; Markey et al. 2013; Uzal et al., 2016). Botulism is characterized by a descending paralysis

while muscle rigidity and spasm occur in tetanus (Hatheway, 1990; Markey et al. 2013; Uzal et al., 2016). Tissue-destructive exotoxins that are produced by pathogenic species of *Clostridium* are responsible for the clinical as well as pathological disease manifestations in both animals and humans. A summary of the host and diseases caused by pathogenic Clostridia can be seen in Table 1.3. As mentioned earlier, Clostridia are classified according to different disease-causing groups (Rood, 2016). Each group, and sometimes each species, has its own specific virulence factors and pathogenesis that are unique to the type of diseases (Markey et al., 2013).

Table 1.3: Summary of host and diseases caused by pathogenic Clostridia

Clostridium species	Host	Diseases	Spore morphology and location situated in cell
Neurotoxic Clostridia			
Clostridium tetani	Horses, ruminants, humans and other animals	Tetanus	Round, terminal (giving a drumstick appearance)
***Clostridium botulinum* (types A–F)**	Many animal species and humans	Botulism	Oval, subterminal
***Clostridium argentinense* (*Clostridium botulinum* type G)**	Humans	Botulism	
Histotoxic Clostridia			
Clostridium chauvoei	Cattle sheep and pigs	Blackleg (black quarter)	
Clostridium septicum	Cattle, sheep pigs Sheep Chickens	Malignant oedema Braxy Necrotic dermatitis	Oval, subterminal
Clostridium novyi			Oval, central or subterminal
Type A	Sheep Cattle and sheep	Big head of rams Gas gangrene	
Type B	Cattle and sheep	Black disease (necrotic hepatitis)	
Clostridium sordellii	Cattle, sheep and horses	Gas gangrene	Oval, subterminal
Enterotoxigenic and enteropathogenic Clostridia			
Clostridium perfringens			Oval, subterminal
Type A	Humans	Food poisoning, gas, gangrene	
	Lambs	Enterotoxaemia jaundice	
	Dogs	Haemorrhagic gastroenteritis	
	Pigs	Necrotising enterocolitis	
	Chickens	Necrotic enteritis	

Clostridium species	Host	Diseases	Spore morphology and location situated in cell
Type B	Lambs (under 3 weeks old)	Lamb dysentery	Oval, subterminal
Type C	Neonatal calves and foals Piglets, lambs, calves, foals Adult sheep Chickens	Enterotoxaemia Haemorrhagic enterotoxaemia Struck Necrotic enteritis	
Type D	Sheep, goats and calves Calves	Pulpy kidney disease Haemorrhagic enteritis	
Type E	Rabbits	Enteritis	
Clostridium spiroforme	Rabbits and guinea pigs	Spontaneous and antimicrobial-induced diarrhoea	
Clostridium difficile	Foals, pigs, dogs, hamsters, rabbits and calves Humans	Spontaneous and antimicrobial-induced diarrhoea Antimicrobial-induced diarrhoea, notifiable nosocomial disease	Oval, subterminal
Clostridium colinum	Game birds, young chickens and turkey poults	Quail disease (ulcerative enteritis)	

Source: Adapted from Markey et al. (2013)

1.3.3 Neurotoxic group

In the neurotoxic group, *C. tetani* and *C. botulism* are the primary pathogens. Endospores enter wounds (traumatic or surgical) and germinate in the muscle or tissue in anaerobic conditions allowing *C. tetani* to grow and thrive (Popoff, 2016). *C. tetani* produces exotoxins, tetanolysin (a haemolysin which enhances tissue invasion and breakdown), and tetanospasmin (a neurotoxin which is plasmid encoded and responsible for the symptoms of tetanus [the disease]) (Markey et al., 2013). *C. botulinum* is the causative agent of botulism. Botulism is caused by the consumption or ingestion of *C. botulinum* spores in food for humans or animal feed. Once the organism replicates and produces toxins, the toxins spread through the body from the intestinal tract via the bloodstream (Markey et al., 2013).

Botulism caused by wound infections is less common and are caused when spores enter and germinate in a wound (Le Marechal et al., 2016).

1.3.4 Histotoxic group

Histotoxic *Clostridium* species are most commonly known to cause gas gangrene and myonecrosis, but other clinical disease presentations can also be observed within the group (Popoff, 2016). Disease symptoms vary from simple wound infections and anaerobic cellulitis to severe and fatal gas gangrene. Infections can be endogenous or exogenous in origin (Brook, 2007). The toxins produced by the Clostridia in this group are not as potent or harmful as the neurotoxic group but are much more invasive in causing disease (Markey et al., 2013). Endogenous infections are often caused by *Clostridium chauvoei* (*C. chauvoei)*, which results in the disease known as blackleg (Popoff, 2016). Spores are ingested and pass through the intestinal tract. These spores sometimes pass through the intestines via the lymphatic and bloodstream system and are transported to muscle tissue such as the hindquarters and the cardiac muscle (Markey et al., 2013). Trauma to these areas causes the spores to be lodged in the tissue where they start to geminate, which in turn cause tissue neurosis. The toxins that are produced result in localised damage and finally bacteraemia and toxaemia. With endogenous infections, spores are introduced into wounds where they germinate in anaerobic necrotic tissue and produce toxins, followed by the clinical disease presentation (Rood, 2016). It is known that histotoxic Clostridia produce a wide selection and variety of toxins (α, β, γ, δ, θ, NanA, NetB, CCtA, TcsL, TcsH ect.) but the exact function and role that these toxins play have not been established and demonstrated yet (Markey et al., 2013).

In the disease known as braxy, caused by *Clostridium septicum* (*C. septicum),* the mucosa of the abomasum could be damaged (Songer, 2010), followed by the germination and replication of *C. septicum* spores which are, in turn, followed by toxin production, resulting in the clinical signs of toxaemia and rapid death (Markey et al. 2013). *C. septicum* produces secreted proteins: α-toxin (a pore-forming, hemolytic, necrotising toxin); β-toxin (a DNase); γ-toxin (a hyaluronidase); and δ-toxin (an oxygen-labile hemolysin) (Moussa 1958; Gadalla and Collee, 1968; Swiatek et al., 1987; Stevens, 1997).

In black disease caused by *C. novyi* type A and B, and bacillary haemoglobinuria (*C. haemolyticum)*, spores must be present in the intestine. The bacteria then produce a wide variety of different toxins (α and β toxins) that travel to the liver and remain dormant in the Kupffer cells (Markey et al., 2013). Damage to the liver is a result of the germination and growth of the spore in an anaerobic environment, resulting in toxemia, bacteremia, and death due to tissue damage (Markey et al., 2013).

Pathogenic strains of *Clostridium sordellii* (*C. sordellii)* produce up to seven identified exotoxins of which the lethal toxin (TcsL) and haemorrhagic toxin (TcsH) are regarded as the major virulence factors. Virulent strains can express either TcsL or TcsH or sometimes both. These

virulent strains most often express TcsL due to the TcsH mostly being absence or a truncation of the TcsH gene responsible for TcsH expression took place. To date, no field isolates have been identified that only expresses TcsH (Walk et al., 2011; Genth et al., 2014).

C. sordellii encodes numerous virulence factors, which are likely to play a role in infection and disease. These factors include TcsL – also called edema-producing toxin – that are mostly responsible for the fatal outcome after *C. sordellii* infections, TcsH, sordellilysin, phospholipases, extracellular proteases, hemlysins, DNase, cytotoxin, and neuraminidase (Aldape et al., 2006; Voth et al., 2006; Aldape et al., 2007; Geny et al., 2007). Pathogenic strains of *C. sordellii* produce up to seven identified exotoxins of which TcsL and TcsH are regarded as the major virulence factors. Virulent strains can express both TcsL and TcsH only express TcsL, or express neither of the two toxins. To date, no field isolates have been identified that only expresses TcsH (Walk et al., 2011; Genth et al., 2007). Although the roles of these virulence factors of *C. sordellii* have been described, much is yet unknown or have not yet been described, as well as their disease role or mechanism in hosts (Carter et al., 2014).

1.3.5 Enterotoxigenic/Enteropathogenic group

In the enterotoxigenic or enteropathogenic group of which *C. perfringens* is the most important, the Clostridia produce a wide variety of toxins categorised as major and minor toxins (McDonel, 1980). Clostridial enterotoxemias are known to affect a wide variety of young animals and present in a very acute manner, which is fatal (Markey et al., 2013). More than 17 toxins and extracellular enzymes have been reported for *C. perfringens*; however, no single strain has been shown to produce the entire spectrum of toxins (Songer, 1996), while the roles of *C. perfringens* toxins in the pathogenesis of *C. perfringens*-associated disease are still under investigation (Keyburn et al., 2008; Gohari et al., 2015). *C. perfringens* toxins are classified as membrane damaging toxins, with the four major toxins including alpha (lecithinase/phospholipase), beta (pore-forming toxin), epsilon (prototoxin) and iota (prototoxin). The minor toxins are theta (haemolysin), kappa (collagenase), mu (hyaluronidase) and nu (DNase) (Markey et al., 2013). The major toxins play the most vital role in pathogenesis, while the minor toxins aid in tissue damage and destruction. Numerous methods and products are available for use as immunoprophylaxis, yet clostridial enteric infections are still a familiar presentation in most veterinary diagnostic laboratories (Songer, 1996).

1.3.6 Specimen collection

Specimen collection must be from live or recently dead animals. Interpretation of laboratory results from specimens collected from not recently dead animals are difficult due to post-mortem

Clostridia invaders that spread rapidly from the intestine into tissues. Specimens are collected aseptically and transported to the laboratory anaerobically; and therefore, blocks of tissue or fluids from affected animals collected are placed in anaerobic transport media for transfer to the laboratory. These specimens are cultured promptly after collection for successful isolation of the causative *Clostridium* responsible for the disease (Quinn et al., 2011).

1.3.7 Laboratory identification

Some species of Clostridia produce certain 'clues' to the species identity. These 'clues' include the specific characteristics of the unidentified species and can be obtained by Gram stain morphology and staining appearance, colonial morphology on cultured media, and the occurrence or absence of haemolysis on blood agar media plates (Pradhan, 2017). Additional phenotypic tests can be used and applied for the presumptive identification of Clostridia (Jousimies-Somer et al., 2002). One of the most important and essential observations for a phenotypic identification of Clostridia is to ensure that the culture isolated is pure and not mixed with other organisms, as the fine spreading growth of some Clostridia has the ability to mask other contaminating organisms also on the media plate (Public Health England, 2015b). Aside from cultural techniques, Clostridia can be identified with PCR (detecting DNA) and fluorescent antibody technique for rapid identification in tissues.

First, a smear from a colony or a specimen is performed, and Gram stained if *Clostridium* is suspected (Markey et al., 2013). Clostridia are large Gram-positive bacilli, and produce endospores, but some species may be Gram-variable in their Gram stain reaction. Spores can also be seen with Gram staining but not as prominent as with specific spore stains such as malachite green staining (Markey et al., 2013). Specimens suspected of *Clostridium* species can be streaked out on 5% blood agar and incubated anaerobically at 35–37°C for 40–48 hours. Egg yolk agar can also be used and must be incubated anaerobically at 35–37°C for 16–24 hours (Public Health England, 2015b). Due to the wide range of *Clostridium* spp. and varying levels of anaerobic stringency, there is no specific selective media for routine clinical diagnostics, although many formulations are available in the literature (George et al., 1979; Karasawa et al., 1995; Barbirato et al., 1998; Biebl and Spöer 2002). These different formulations of the selective media have mostly been used for the isolation of environmental isolates (Leja et al., 2011). It is very important that the incubation must take place in strict anaerobic conditions. Suspected *Clostridium* will be investigated using Gram stain. Other techniques used to identify Clostridia is biochemical reactions such as the Nagler, a plate neutralisation test that identifies the α toxin of *C. perfringens*, which has lecithinase activity; the reverse Christie–Atkins–Munch-Peterson test (Hansen and Elliott, 1980); and the indole, urease and lipase test that is the most crucial for the identification of Clostridia (Quinn et al., 2011; Public Health England, 2015b). Clostridia are

catalase and oxidase negative and motile except for *C. perfringens* which is immotile (Public Health England, 2015b).

1.3.7.1 Characterisation of neurotoxic Clostridia

This disease group of *Clostridium* includes the species *C. botulinum* and *C. tetani*. *C. tetani* is described as being an anaerobic slender Gram-positive rod. Endospores are spherical and are located at the terminal of the mother cells (Quinn et al., 2011). These bacteria then have a characteristic 'drumstick' appearance when they are stained and viewed under a microscope (Quinn et al., 2011). A swarming growth pattern can be observed on media as well as haemolysis (β-haemolytic) on blood agar. *C. botulinum* are known to produce oval endospores that are situated at the subterminal region of the mother cell (Quinn et al., 2011). Their growth pattern is irregular, large, smooth colonies that have the ability to spread. Most strains are known to produce haemolysis (β-haemolytic) on blood agar (Public Health England, 2015b). Molecular techniques can be used to identify both of these organisms by detecting the neurotoxic genes that they harbour (Akbulut et al., 2005; Prevot et al. 2007; De Medici et al., 2009).

C. botulinum is known to have a number of subtypes (types A–G) which can be detected and identified using numerous techniques (Markey et al., 2013). These techniques are used during epidemiological studies and include MLVA assays (Macdonald et al., 2008) and random amplified polymorphic DNA analysis (Hyytiä et al., 1999). Laboratory animals such as mouse bioassays can be used for the detection of toxins. In case of *C. botulinum* toxins detection, serum suspected to contain the toxin is inoculated into the mice (Quinn et al., 2011). If the toxin is present certain characteristics will develop ('wasp-waist' appearance) together with the consequence of abdominal breathing, following paralysis of the respiratory muscles (Quinn et al., 2011). Serum collected from dead animals is unsuitable for mouse inoculation (Cai and Singh, 2007). However, this method is very time-consuming, requires the correct facilities and adequate funding for up-keep (Markey et al., 2013).

1.3.7.2 Characterisation of histotoxic Clostridia

This disease group include *Clostridium* species *C. chauvoei*, *C. septicum*, *C. sordellii* and *C. novyi*. *C. sordellii* are known to be rare spore-forming anaerobic Gram-positive bacilli (Wiegel, 2009). Spores are known to be oval and develop centrally too subterminally within the cell (Public Health England, 2015b). Colony morphology is translucent to opaque with small zones of β-hemolysis on sheep or rabbit blood agar (Wiegel et al., 2006; Wiegel, 2009). *C. sordellii* are known to be very similar in morphology and biochemical profiles to *Clostridium bifermentans* (*C. bifermentans)* and is suggested to have been a virulent strain of *C. bifermentans*. However, the ability of *C. sordellii* to produce urease clearly distinguishes the species from one another

(Hall and Scott, 1931; Ellner and Green, 1963). Three animal models have been described to view the pathogenesis of *C. sordellii*. A murine intrauterine model to investigate *C. sordellii*-associated endometritis (Arseculeratne et al., 1969), intraperitoneal injections of the *C. sordellii* lethal toxin to experimentally illustrated fatal toxic shock syndrome in mice and rats (Martinez and Wilkins, 1988), and an in vivo model was developed to investigate *C. sordellii* intramuscular infections that mimicked the salient features of the infection (Aldape et al., 2006).

C. novyi are strict anaerobic, Gram-positive bacilli that produce oval central to subterminal spores inside the cell (Public Health England, 2015b). Colonies may have circular or irregular scalloped or rhizoid margins forming a spreading growth with a double zone of β-haemolysis on blood agar (Wiegel et al., 2006; Wiegel, 2009).

C. chauvoei is usually found as single cells or in pairs, but rarely in short chains. The staining properties of *C. chauvoei* may vary (Gram-positivity may be variable), especially in old cultures, where Gram-negative rods may be visible instead of the Gram-positive rods or bacilli (Markey et al., 2013). *C. chauvoei* are known to possess peritrichous flagella, and almost all strains are motile (Markey et al., 2013). The spore morphology is ovoid and can be located centrally, subterminally or terminally inside the cell. The colony morphology is slightly raised, whitish-grey with a glossy semi-transparency when incubated overnight. They are known to be β-haemolytic on blood agar (Public Health England, 2015b). The colonies are usually separated from each other and merge infrequently (Wiegel et al., 2006; Wiegel, 2009). Spore formation is characterised by the change in colony morphology from semi-transparent to opaque.

C. septicum in a spindle-shaped Gram-positive rod observed to be motile in young cultures. Colonies are known to be irregular with a rhizoidal edge, but smooth, round colonies are produced by some strains (Wiegel, 2009). When grown or isolated on blood agar, β-haemolysis can be seen. The ability to “swarm” on solid agar is one of the common morphological characteristics of *C. septicum* (Wiegel et al., 2006; Wiegel, 2009).

The florescent antibody technique/test (FAT) is routinely used in differentiating *C. chauvoei*, *C. septicum*, *C. novyi* and *C. sordellii* in disease diagnosis. Fluorescein-labelled antiserum can be obtained commercially (Assis et al., 2007). A florescent antibody is labelled with a fluorophore that the antibody binds if Clostridia antigen is present on the fixated sample material, for example a tissue smear (Lipman et al., 2005; Uzal et al., 2016). The fluorophore fixated sample material can be visualised using a florescent microscope. The florescent microscope emits an ultraviolet light that activated the fluorophore bound to the antibody, which will bind to antigen in fixed sample (Markey et al., 2013).

Molecular techniques can be used to identify many histotoxogenic species. By using PCR techniques, Kuhnert et al. (1997), Sasaki et al. (2001) and Uzal et al. (2003) have been able to

identify Clostridia in tissues, including *C. chauvoei* and *C. septicum*. By using multiplex PCR assays, numerous *Clostridium* species can be identified simultaneously from a sample where infection is suspected. Multiplex PCR assays have been developed for differentiation of a number of different histotoxogenic species. Sasaki et al. (2002) developed a multiplex PCR system, based on the flagellin gene, to use in the differentiation of *C. chauvoei*, *C. haemolyticum*, *C. novyi* types A and B, and *C. septicum*.

1.3.7.3 Characterisation of enterotoxic Clostridia

This disease group is mostly comprised of *C. perfringens* types A to E that are associated with the species (Markey et al., 2013). Clostridium perfringens is the one *Clostridium* species that can be cultured aerobically and anaerobically (facultative anaerobe) on blood agar at 37°C for 48 hours (Public Health England, 2015b). The colony morphology of *C. perfringens* is unique in the sense that the colony creates a double zone of haemolysis (Quinn et al., 2011). Colonies are known to be large, smooth and regular convex, but can be rough and flat with irregular edges. Direct smears made from the mucosa or contents of the small intestine of recently dead animals are known to contain large numbers of thick Gram-positive bacilli, which are consistent with clostridial enterotoxaemia (Quinn et al., 2011). The isolation of large numbers of *C. perfringens* types B and C, from recently dead animals, especially in pure culture, is supportive of a diagnosis (Quinn et al., 2011).

Animal testing can be used for the typing and identification for which *C. perfringens* is responsible for disease in the animal. The toxin neutralisation assay is usually performed on mice and guinea pigs (Quinn et al., 2011). The supernatant from the intestinal contents of recently dead animals are then inoculated into the mouse or guinea pig to identify the toxins. Due to the instability/lability of some of these toxins, especially the β-toxins, a negative result does not exclude a diagnosis of clostridial enterotoxaemia (Quinn et al., 2011). The supernatant is generally used when the intestinal contents has been centrifuged. Specific antitoxins for each *C. perfringens* type is added to the supernatant to produce a mixture consisting of a 3:1 ratio (three parts supernatant to one part known antitoxin) (Quinn et al., 2011). The steps involved in the procedure are as follows (Quinn et al., 2011):

- Antitoxins with specificity for each *C. perfringens* type are added to saline together with the supernatant. These are used as a positive control for the presence of toxins.
- The mixture is incubated at room temperature for one hour before inoculation of the mixture into the mice or guinea pigs.
- Inoculation of the mice takes place intravenously (0.3 ml of the mixture) and for guinea pigs intradermally (0.2 ml of the mixture).

A cross-neutralisation pattern will be observed in the mice or guinea pigs and this will indicate which specific *C. perfringens* type is the cause of the enterotoxaemia (Quinn et al., 2011). An enzyme-linked immunosorbent assay (ELISA) that detects the toxin in intestinal contents can be used as an alternative to in vivo assays (Songer, 1997). When comparing the sensitivity and specificity of the ELISA for detection of *C. perfringens* toxins to that of the mouse or guinea pig inoculation methods, they are the same. It is known that the ELISA has a greater sensitivity than other in vivo methods, which can be the cause of misdiagnosis in the absence of other diagnostic indicators. The misdiagnosis can occur because the ELISA has the ability to detect low levels of toxin in the intestinal contents of animals (Uzal and Songer, 2008).

Molecular methods can be used for the typing of *C. perfringens* in which the target is the toxin encoding genes. Ahsani et al. (2010) have used a multiplex PCR to type the different *C. perfringens* found in animals and the environment. Other molecular techniques used for the typing of Clostridia include pulsed-field gel electrophoresis, fluorescent amplified fragment length polymorphism, microarray analysis, 16S rDNA region sequencing, PCR-restriction fragment length polymorphism, ELISA for the detection of toxins and whole-genome sequencing (Public Health England, 2015b). The subtyping of different strains of organisms can be accomplished by using these methods. Each of these techniques poses different discriminatory powers, accuracy as well as reproducibility. Due to the complicity of some of the tests, it is not advised to be implemented for routine use in diagnostic laboratories and testing on clinical samples (Public Health England, 2015b).

1.3.8 Disease management in animals

Many of the pathogenic Clostridia are normal inhabitants of the intestinal tract of animals and are present in the environment. As Clostridia occurs in the intestinal tract of animals and the environment. Clostridia are opportunistic pathogens and thus takes advantage of a breach in a host's natural defences to infect the host. Due to *Clostridium*'s ambiguous nature and various factors that could lead to disease the most effective control measure to prevent clostridial diseases is through vaccination (Olivier, 2001). The various factors that can lead to clostridial diseases include:

- Changes in feed.
- Lack of care when conducting procedures when animals are handled can lead to disease, for instance castration, tail docking, wound cleaning and treatment, as well as helping animals to lamb or calve.
- Stress caused by any unusual situation.

- Eating/nibbling of carcasses or old bones, drinking water, or eating feed contaminated by dead animals.

All of these factors must be considered when suspecting clostridial diseases. Due to the acuteness of clostridial diseases, as well as the disease symptoms that start suddenly with few indications, treatment is usually administered too late and will have little impact when trying to cure or eradicate the disease from the animal. Vaccination is worldwide regarded as the most practical and best way of disease management for clostridial diseases. Vaccination must be implemented together with good animal handling practices and overall good management of feeds, number of animals per hectare to avoid the generation of stress among animals, and disposal of carcasses in the area where animals are located, for instance pastures and water sources (Olivier, 2001). Vaccines are primarily used in animals as a prevention measure against clostridial diseases but can be costly if they are administered as monovalent vaccines (Hanna et al., 2014). Thus, the best method for vaccination of clostridial diseases are polyvalent vaccines that incorporate a large number or portion of clostridium species that are known to occur or be a problem in a specific area (Hussein et al., 2000). The components of vaccines include bacterins (formalin killed/ inactivated whole bacterial cultures and their supernatants), toxoids (inactivated/killed toxin), or mixtures of both (Plotkin and Plotkin, 2011; Rappuoli et al., 2011). As previously mentioned, different animals will receive different vaccines, in different rations, and at different times. Complete vaccination usually includes an initial vaccine followed by a booster vaccine after to six weeks (Stämpfli, 2018). This is unique for each Clostridia vaccine. When farmers or animal health care officials only vaccinate with the one vaccine and not with the booster vaccine, the vaccination process is incomplete and will have to be repeated from the beginning. The initial vaccine is usually used to prime the immune system but does not completely give protection (Stämpfli, 2018). A booster vaccine is then given to complete the vaccination process and provide full protection against the disease vaccinated against (Stämpfli, 2018).

Due to the acute nature of clostridial diseases, it is sometimes not possible to treat animals with antibiotics and it is then considered better financially and is more practical to vaccinate animals than to treat animals with antibiotics (Olivier, 2001). Traditionally, *C. perfringens* type A vaccine is formulated as an inactivated toxoid vaccine as part of multi-clostridial vaccines for use in cattle, sheep and goats. Clostridial vaccines containing *C. sordellii* are traditionally formulated as inactivated toxoid and/or whole cell preparations, which include multiple species in a single formulation. *Clostridium novyi* vaccines are traditionally formulated as toxoid vaccines containing formalin inactivated alpha toxin for the prevention of malignant oedema, also called “swelled head” or “bighead”. *Clostridium chauvoei* vaccines consist of whole cell formulations.

Disease prevention is mainly achieved through vaccination with toxoid formulations for *C. septicum*.

1.3.9 Surveillance of clostridial diseases in animals

As many of the pathogenic Clostridia are normal inhabitants of the intestinal tract of animals and are present in the environment, it will therefore always be detected in surveillance programmes. Livestock can be protected through vaccination but vaccination for wildlife is not routinely done. Vaccines for the prevention of clostridial diseases are known to be very affordable and following or adhering to a set vaccination programme will not be necessary with a veterinarian in the area (Olivier, 2001). The choice of vaccines used in the programme may be influenced by the area in which the animals are kept or the prevalence of Clostridia in that region (Robertson et al., 2010). Surveillance programmes for Clostridia will only be practical if the detection of *Clostridium* species occurring in a specific environment or where there is endangered and/or valuable wildlife. This will help to select the best combination of clostridial vaccines to be used.

In South Africa, clostridial diseases do not form part of the list of notifiable or communicable animal diseases. South Africa has no general surveillance for clostridial diseases and most of the time the different diseases are just managed and not reported. Although clostridial diseases in livestock have been well documented, in wildlife there is almost no information available. Surveillance programmes for clostridial diseases in wildlife or occurrence of *Clostridium* species in the environment would provide valuable information to help veterinarians, especially to treat endangered wildlife for the different clostridial diseases.

1.4 Problem identification

Bacillus anthracis and Clostridia are both soil-borne bacteria and can rapidly spread through a wide range of different means (Titball et al., 1991; Quinn and Turnbull, 1998; De Vos and Turnbull, 2004; Setlow, 2007; Horneck et al., 2010). The fact that *B. anthracis* and Clostridia can survive for years at a time due to their spore forming properties, adds to the many ways and abilities to cause disease. Both *B. anthracis* and Clostridia are well-known to cause acute disease in animals, with most of the animals succumbing to the disease before any clinical diagnosis or laboratory confirmation could be done or completed (Turnbull, 2008; Lentino, 2015).

Both *B. anthracis* and pathogenic Clostridia are endemic in Southern Africa. As previously mentioned, both diseases can infect and affect most mammalian species, whether domestic animals or wildlife. Different avian species and are known to cause disease in humans (Turnbull, 2008; Markey et al., 2013). Although both diseases can be controlled in domesticated animals through vaccination, diseases still occur in wildlife and livestock animals.

Both diseases have similar disease presentations that can be confused with one another. Clostridia are an opportunistic pathogen and as it already occurs in the environment and host, it is difficult to make an accurate diagnosis, especially since Clostridia require specific culture conditions (Downes et al., 1990). Clostridia are therefore under-reported, and it is not known which species and toxin type predominate in livestock.

The KNP in South Africa is home to quite a large number of wildlife species, especially endangered species. KNP is also known to have one of the highest genetic diversities of *B. anthracis* described in the world (Keim et al. 2000) where new genotypes are constantly emerging. Anthrax is endemic to the Pafuri area in the northern part of the KNP (Bengis et al., 2002; Goel, 2015), which enables collection of the *B. anthracis* strains causing diseases in wildlife species, investigation of the genetic diversity as well as factors/vectors and environmental conditions that influence anthrax in an undisturbed natural environment. *B. anthracis* are known to be a genetically homogenous bacterium. In order to study the micro-evolution differences taking place in the genome within and between stains, a molecular approach is needed. While a great deal is already known about anthrax, the initiating and terminating factors of an outbreak are still poorly understood. The literature is made up of a large body of observational and anecdotal data. The continued study of the region over time enables a stronger body of data for empirical conclusions to be made.

Information on the involvement of *Clostridium* species causing diseases in animals, especially wildlife populations, is limited. Most veterinary communities report clinical findings on internet websites, such as RuralVet, but the conformation of these clinical finding using laboratory testing has not been done or completed. Thus, the conformation of these reporting is in doubt. Data regarding clostridial disease in domestic and livestock animals are not as limited as in wildlife, yet most cases are still being reported by clinical diagnosis alone. Many laboratories cannot perform laboratory cultures, identification, and/or pathology characterisation required for the polyphasic diagnosis of Clostridia. The proposed project will investigate the diversity of *Clostridium* species and *B. anthracis* in animals and the environment to improve disease surveillance and control of these diseases.

1.5 Aims and objectives

The two aims are provided with the objectives to achieve each aim.

1.5.1.1 To investigate the *Clostridium* species responsible for animal-related deaths in South Africa in both livestock and wildlife using the laboratory information system of the Faculty of Veterinary Science at the University of Pretoria (UVIS; Universal Veterinary Information System).

1.5.1.1.1 Collect and analyse data on *Clostridium* species to animal disease or death in South Africa to determine the predominant *Clostridium* species.

1.5.1.2 To investigate the occurrence of *B. anthracis* and *Clostridium* species in wildlife in the Kruger National Park using a passive surveillance system and characterisation of isolates using molecular techniques.

1.5.1.2.1 The collection and analyses of data *Clostridium* species responsible for animal disease or death in the KNP.

1.5.1.2.1.1 Developing molecular assays for the detection and grouping of pathogenic *Clostridium* species (*C. botulinum*,*C. tetani*, *C. perfringens*, *C. novyi*, *C. chauvoei*, *C. septicum*, and *C. sordellii*) and identifying these species from KNP surveillance smears from 2010.

1.5.1.2.2 The genotypically identification and characterisation of *B. anthracis* from 2016 to 2017 from KNP.

1.5.1.2.2.1 MLVA 7 and MLVA10 panels were evaluated and compared with MLVA31 results from *B. anthracis* isolates in KNP from a previous study.

1.5.1.2.2.2 Characterising the genotypes from an outbreak in the northern KNP from 2016 to 2017 using MLVA10.

1.6 Ethics and permission

- Ethical clearance was obtained from the following organisations, councils and committees:
- University of Pretoria Animal Ethics Committee – Onderstepoort campus.
- National Department of Agriculture, Forestry and Fisheries.
- South African National Parks.
- South African Council for the Non-Proliferation of Weapons of Mass Destruction.

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Chapter 2
Data Mining of the Universal Veterinary Information System for *Clostridium* Species Responsible for Animal Deaths in South Africa, 2013–2016

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2.1 Introduction

Clostridial diseases are caused by species that belong to the genus *Clostridium*. Clostridia are environmental bacteria known to be part of the phylum *Firmicutes* that produce endospores (Tille and Forbes, 2018). Some of these environmental Clostridia have the ability to become pathogenic (Wells and Wilkins, 1996), but clostridial infections require predisposing conditions in animals, either the alteration in the gastrointestinal microbiota caused by change in animal feed or treatment with antibiotic agents or breaking of the skin or intestinal barrier by deep or traumatic wounds (Uzal et al., 2016). Disease that are associated with *Clostridium* species include: botulism (*Clostridium botulinum* (*C. botulinum*)), tetanus (*Clostridium tetani (C. tetani*)), blackleg (*Clostridium chauvoei (C. chauvoei*)), malignant oedema (*Clostridium septicum (C. septicum*)), braxy (*C. septicum*), necrotic hepatitis (*Clostridium novyi (C. novyi*)), gas gangrene (*C. novyi*, *Clostridium sordellii (C. sordellii)* and *Clostridium perfringens (C. perfringens*)), lamb dysentery, necrotic enteritis in poultry, necrotising and haemorrhagic enteritis in dogs, foals and mostly neonatal individuals of mammalian species (*C. perfringens*), and spontaneous and antimicrobial-induced diarrhoea (*Clostridium difficile (C. difficile*)) (Markey et al., 2013; Uzal et al., 2016).

Clostridium species is difficult to diagnose due to the fact that the bacterium occur in the environment and/or intestine of animals therefore isolation of the bacterium does not confirm that it is responsible for disease in the animal. Some species are difficult to isolate due to their extreme anaerobic nature, whereas isolation needs to be supported by toxin detection or histopathology as the organisms commonly occur in the environment and/or intestine of animals, and appropriate sample collection is essential (Uzal et al., 2016; Wells and Wilkins, 1996). Furthermore, clostridial diseases can be confused or misinterpreted with other diseases with similar clinical signs such as anthrax, brain infection, poisoning with chemicals, cysts in the brain due to tapeworms, heartwater, rabies, toxic plant poisoning, snakebites, three-day stiff sickness, tick paralysis, twin lamb disease (domsiekte) and redwater (Olivier, 2001). Accurate diagnosis of clostridial diseases include anaerobic culturing of *Clostridium* species from the intestine or muscle tissue (appropriate sample) supported by detection of toxins in the specimen samples using mouse bioassays, immunological assays, and histopathology (Lindstrom and Korkeala, 2006; Markey et al., 2013; Grenda et al., 2014). Therefore, appropriately collected samples must be sent anaerobically to veterinary laboratories equipped to isolate and diagnose clostridial diseases.

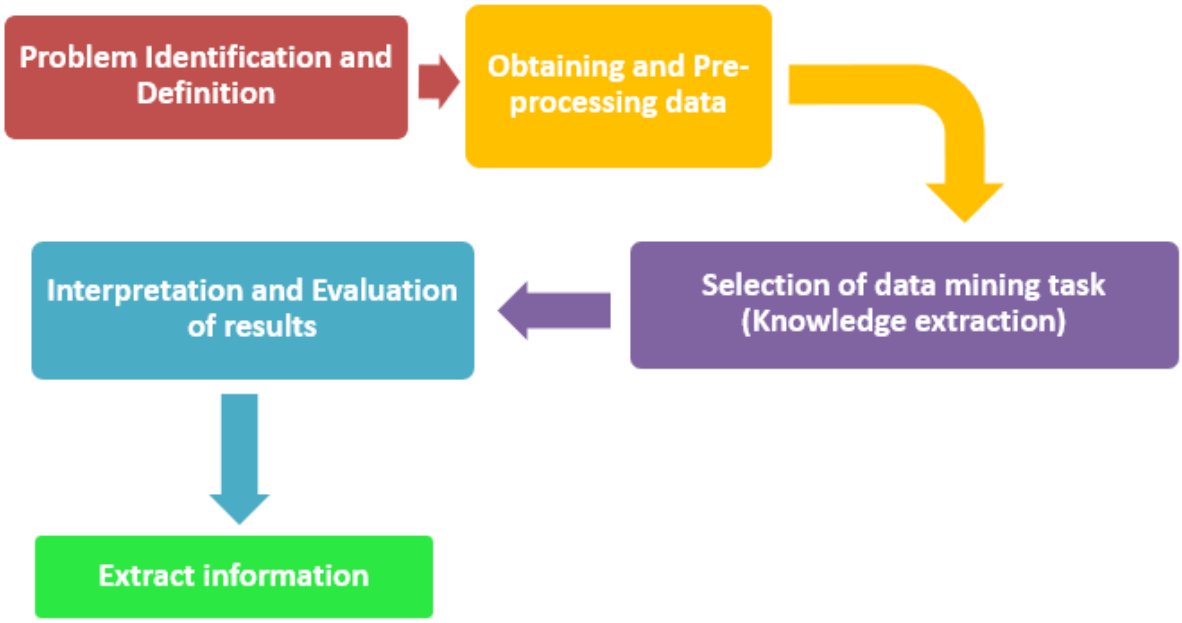
Livestock is an important income for farmers globally. The productivity of livestock can be inhibited by various factors, including diseases with socio-economic impacts for all sectors of agriculture, but especially in rural communities (Lamy et al., 2012). Clostridial diseases such as

blackleg or black quarter are some of the major bacterial infections causing economic losses to cattle herders in many parts of Africa (Rashid and Shank, 1994), together with other clostridial diseases such as pulpy kidney, swelled head, tetanus, and botulism (Mokoena et al., 2017; South African Mohair Growers' Association, 2018). Toxoid and/or bacterin clostridial vaccines are available to protect livestock. Limited data is available regarding the clostridial vaccination dosages distributed within South Africa, as well as reporting or accurate diagnosis of losses due to clostridial diseases. Furthermore, many livestock owners in South Africa do not vaccinate against clostridial diseases mainly due to economic reasons, as well as lack of knowledge (Jenjezwa and Seethal, 2014).

In the twenty-first century, the information or data retrieval method of gathering vast quantities of information from a database has become increasingly applicable in research of multiple disciplines. The process of information/data retrieval from a database or set is known as knowledge discovery in databases, or most generally known as data mining. Many consider data mining to be a combination of the fields of statistics, artificial intelligence and database research (Joseph et al., 2013). Over the last few years, data mining has been used by several scientific fields which include astronomy, biology, climate forecasting, medicine, and physics (Adams and Schubert, 2004; Jothi et al., 2015; Zhang and Zhao, 2015; Lynch and Moore, 2016). The main aim of the data mining process is to use an intensive/advance process of analysis to extract information from a datasets or databases and then turn that information into an understandable structure that is used for further analysis and interpretation. To put it simply, data mining is about solving problems by analysing data already present in databases as described by Witten and Frank (2005). Information of clostridial diseases diagnoses are based on pathology, culturing and/or the florescent antibody technique (FAT). Results of clostridial diseases of livestock and wildlife at the veterinary laboratories at the Faculty of Veterinary Science, University of Pretoria, were obtained from the Universal Veterinary Information System (UVIS) for the period of 2013 to 2016. Data mining was conducted to determine the impact of clostridial diseases on livestock and wildlife in South Africa.

2.2 Materials and methods

Information regarding each sample submitted for diagnostics at the Faculty of Veterinary Science was logged onto the UVIS system of the University of Pretoria. The sample information included the type of sample, host species, location, owner, as well as test results and diagnostic reports. No vaccination history or information was available on the UVIS. A search was conducted on the UVIS database for *Clostridium* identification from culture, FAT and pathology for the period 2013–2016. The workflow used in this study is indicated in Figure 2.1.



Source: Adapted from Asha et al. (2012)

Figure 2.1: Workflow of the data mining process

The information occurring in the UVIS system consisted of numerous identifier options or parameters that included, but was not limited to, accession number, species, breed, specimen type and tests conducted. The first step in the data mining process, namely problem identification and definition (Figure 2.1) was to select information from the UVIS record from 2013 to 2016 using the *Clostridium* keywords occurring in any of the results (called test codes in UVIS), followed by mining information from these results for livestock (ovine, bovine, camelid, caprine and equine) and wildlife species and breeds. The keyword "*Clostridium*" was used to search test codes within the UVIS system using the following search strategy: for FAT results the test code BCLFA was used; for culture results, the test codes BANA, BANA1 and BANA2 were used. These test codes were used for anaerobic bacteria with the number indicating the number of samples tested, for example, BANA was used for 1–2 samples, BANA1 for 3–4 samples and BANA2 for 4 or more samples. Within each specific test code, numerous other information was mined, namely breed information, species information, and accession/identifier numbers.

The second step was the collection and pre-processing of the data. In this step each result was extracted using the methodology described above and the data was checked for missing or incomplete information to eliminate low-quality data. Pathology results were obtained by looking at the culture-positive and FAT-positive results and then extracting the full pathology report from each case. In the third step (selection), data was extracted with *Clostridium* results in pathology, culture and FAT, pathology and FAT, pathology and culture, culture and FAT, pathology, FAT,

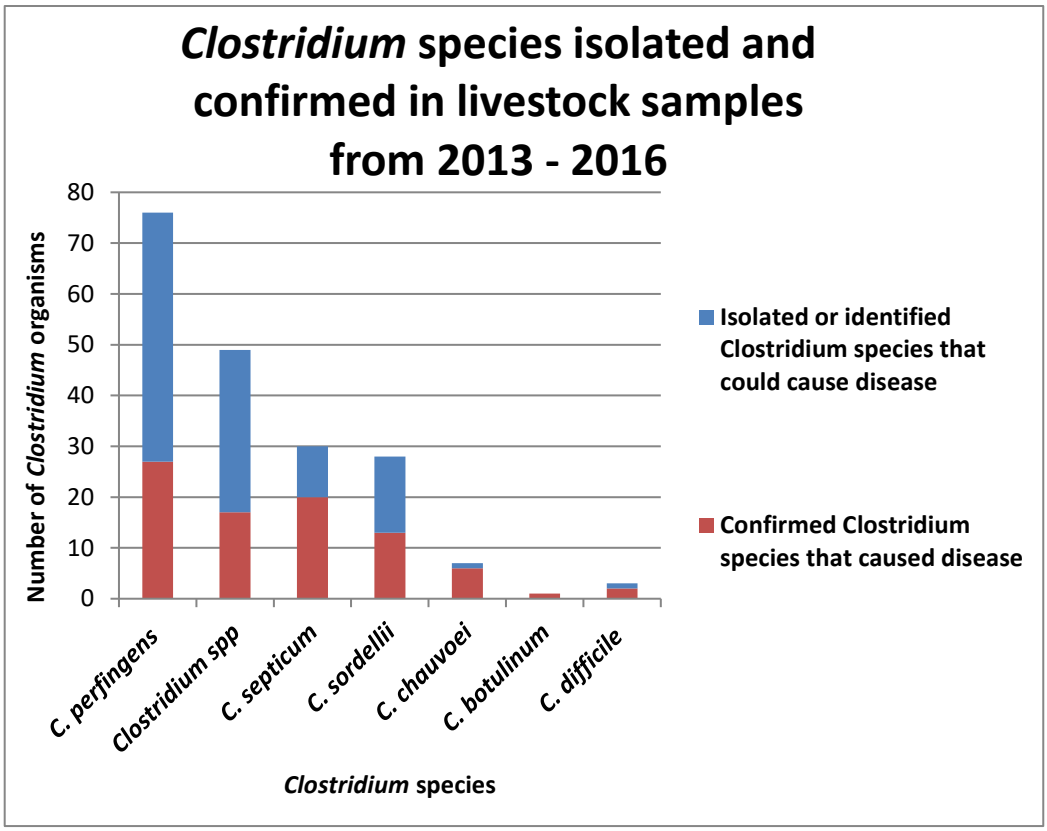
and culture. This data was further separated according to breed and species information of livestock and wildlife (Appendix 2A and 2B). This is a very important step in which intelligence models were used for the extraction of data patterns. The fourth step (interpretation and evaluation) (Figure 2.1) was to analyse and extract the relevant information to present clostridial infection (with the pathology report based on culture, pathology and/or FAT results) in livestock and wildlife reported from 2013 to 2016. Data regarding the results of the test code, the pathology and laboratory reports as well as the parameters located in the database was extracted onto Microsoft Excel software sheets and sorted into their respective parameters (Appendix 2A and 2B). Data mining took into account that numerous samples that can be submitted from a single case and isolates were defined as individual bacterial organisms that could be cultured from a specific sample, while some samples could result in the isolation of numerous isolates per sample.

In this study, a case is defined as an individual animal or animals from the same location (with a unique identifier number) for which a sample or samples were submitted for laboratory testing. Percentages were calculated for the clostridial culture results and confirmed clostridial disease using pathology and/or FAT results (Microsoft Office Excel 2010). Analysis between isolated/identified *Clostridium* species and confirmed Clostridia (with the pathology report based on culture, pathology and/or FAT results) was done using the t-test (p-value of 0.05) using Statistica® 2019, TIBCO Software to determine difference between isolated/identified *Clostridium* species in wildlife and livestock and confirmed Clostridia cases.

2.3 Results

The data analysis from UVIS indicated that from 2013 to 2016, 3 392 samples were processed using the test codes BANA, BANA1, BANA2 and BCLFA. Of these, 285 cases – livestock (n=157) and wildlife (n=128) – contained positive results for pathology, and/or culture or FAT. The analysis indicated that 82.80% (130/157) cases from livestock had pathology results, of which 53.84% (70/130) cases were confirmed to be true clostridial infections. The results also indicated that 14.61% (19/130) cases were known to be a secondary cause of infection and could not be eliminated from the diagnosis (Appendix 2C). Multiple Clostridia were isolated from single cases in both livestock and wildlife from positive Clostridia cases (Appendix 2A and Appendix 2B). In livestock the most common Clostridia species identified from the same case were *C. perfringens* together with unknown *Clostridium* spp (n=7) (Appendix 2A). In wildlife the most common Clostridia species identified were *C. septicum* and *C. perfringens* (n=9) as well as *C. perfringens* together with unknown *Clostridium* spp (n=9) (Appendix 2B). *Clostridium* species that could not be determined to species level (by either bacteriology or FAT) were termed *Clostridium* spp. in UVIS and the reports.

The livestock species that were infected with Clostridia, included bovine, equines, ovine, caprine, porcine and one camelid (Appendix 2C). The facultative anaerobe *C. perfringens* isolates (76) were the most frequent isolated species but only responsible for disease in 35.52% (27/76) confirmed cases (Figure 2.2). The obligate anaerobe Clostridia that were confirmed from isolated samples causing disease consisted of 66.66% *C. septicum* (20/30), 46.42% *C. sordellii* (13/28), 85.71% *C. chauvoei* (6/7), 100.00% *C. botulinum* (1/1) and 66.66% *C. difficile* (2/3). From that, 20 *C. septicum*, 13 *C. sordellii*, 6 *C. chauvoei*, 1 *C. botulinum* and 2 *C. difficile* were confirmed by pathology or FAT reports (Figure 2.2). Furthermore, 49 *Clostridium* spp. were isolated from livestock, of which 17 (34.69%) were confirmed as possibly caused by pathology results (Figure 2.2).

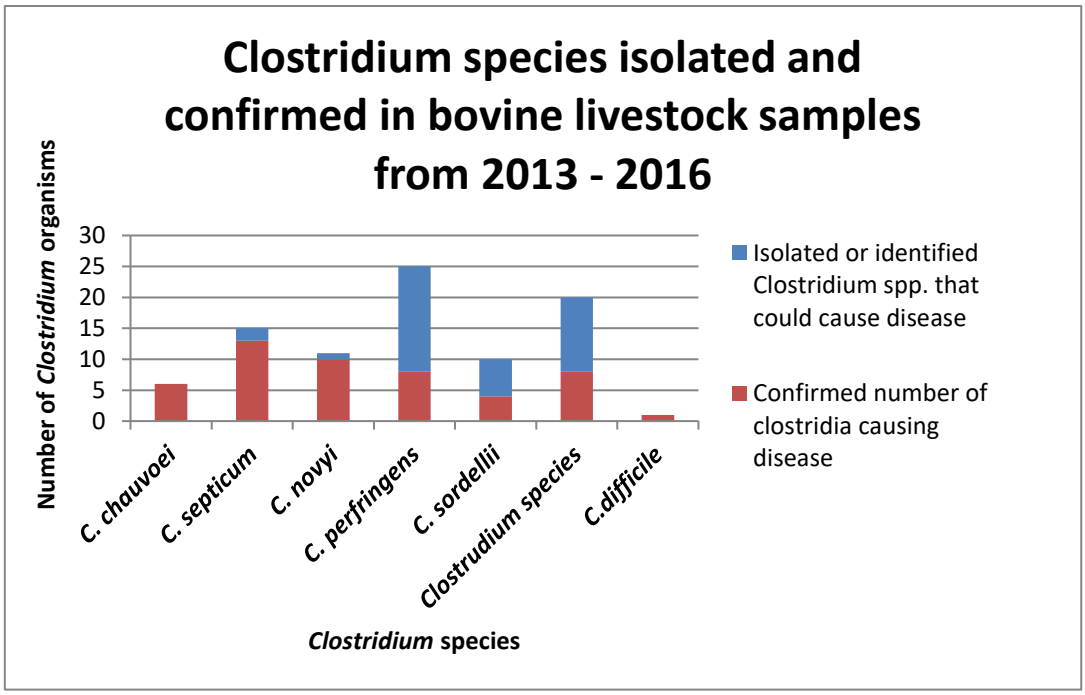


Note: Blue bars illustrate the number of *Clostridium* species isolated/diagnosed from samples for the period while the red bars indicate the confirmed *Clostridium* isolates that are responsible for disease using culture and/or FAT and pathology.

Figure 2.2: *Clostridium* species isolated and confirmed in livestock samples for the period 2013–2016

Bovine and ovine were the most affected by clostridial infections of the livestock species. Confirmed clostridial infections (pathology and isolation or FAT) from bovine samples included 100.00% *C. chauvoei* (6/6) and *C. difficile* (1/1), 90.91% *C. novyi* (10/11), 86.67% *C. septicum*

(13/15), 40.00% *C. sordellii* (4/10), 40.00% *Clostridium* spp (8/20) and 32.00% *C. perfringens* (8/25). (Figure 2.3).

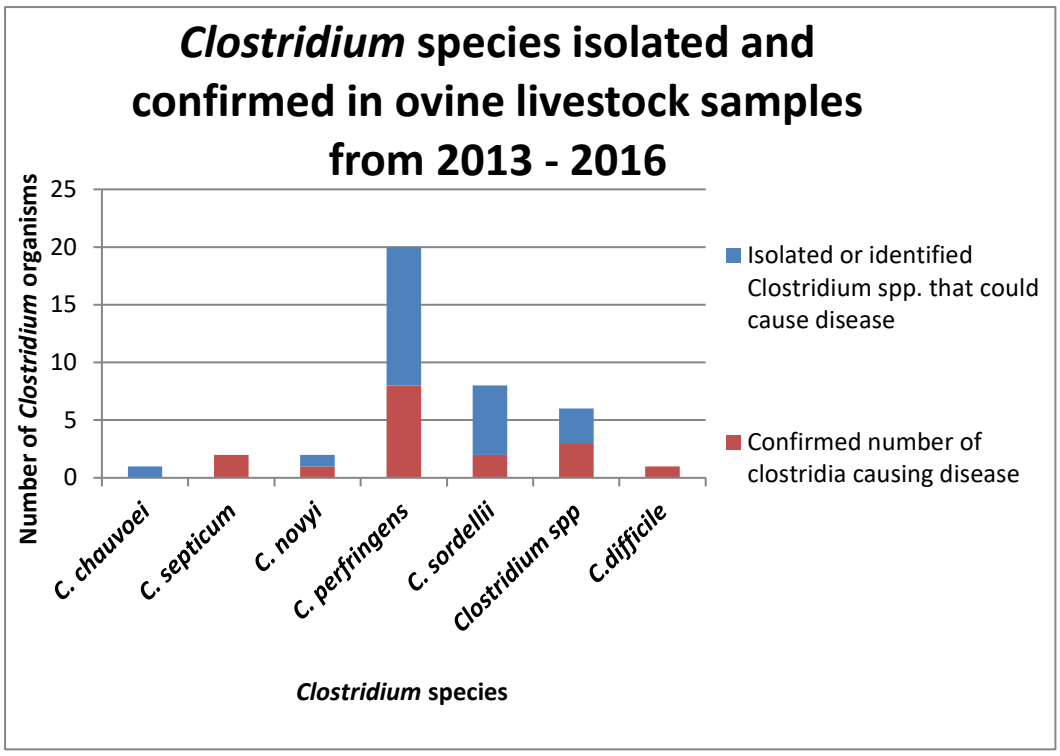


Note: Blue bars illustrate the number of *Clostridium* species isolated/diagnosed from samples for the period while the red bars indicate the confirmed *Clostridium* isolates that are responsible for disease using culture, and/or FAT and pathology.

Figure 2.3: *Clostridium* species isolated and confirmed in bovine livestock samples for the period 2013–2016

Confirmed clostridial infections (pathology and isolation or FAT) from ovine samples included 100.00% *C. septicum* (2/2) and *C. difficile* (1/1), 50.00% *C. novyi* (1/2), 25.00% *C. sordellii* (2/8), 50.00% *Clostridium* spp (3/6) and 0.00% *C. chauvoei* (0/1) (Figure 2.4).

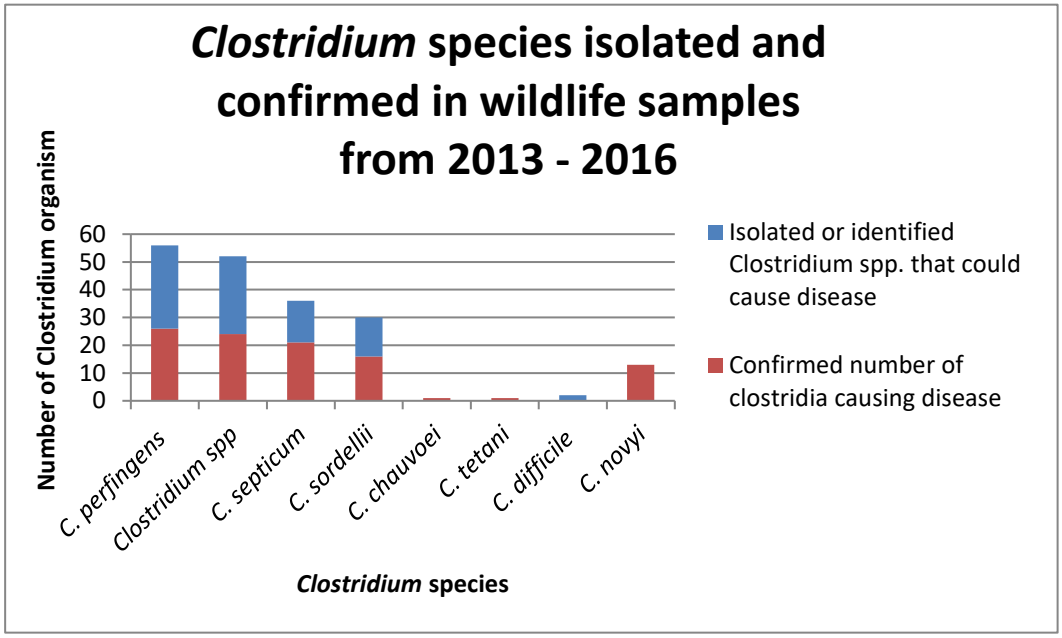
For wildlife cases, 96.87% (124/128) had pathology results, of which 50.00% (62/124) were positive clostridial infections based on the culture, and/or FAT and pathology results. Secondary infections were reported for 4.84% (6/124) cases (Appendix 2B).



Note: Blue bars illustrate the number of *Clostridium* species isolated/diagnosed from samples for the period, while the red bars indicate the confirmed *Clostridium* isolates that are responsible for disease using culture, and/or FAT and pathology.

Figure 2.4: *Clostridium* species isolated and confirmed in ovine livestock samples for the period 2013–2016

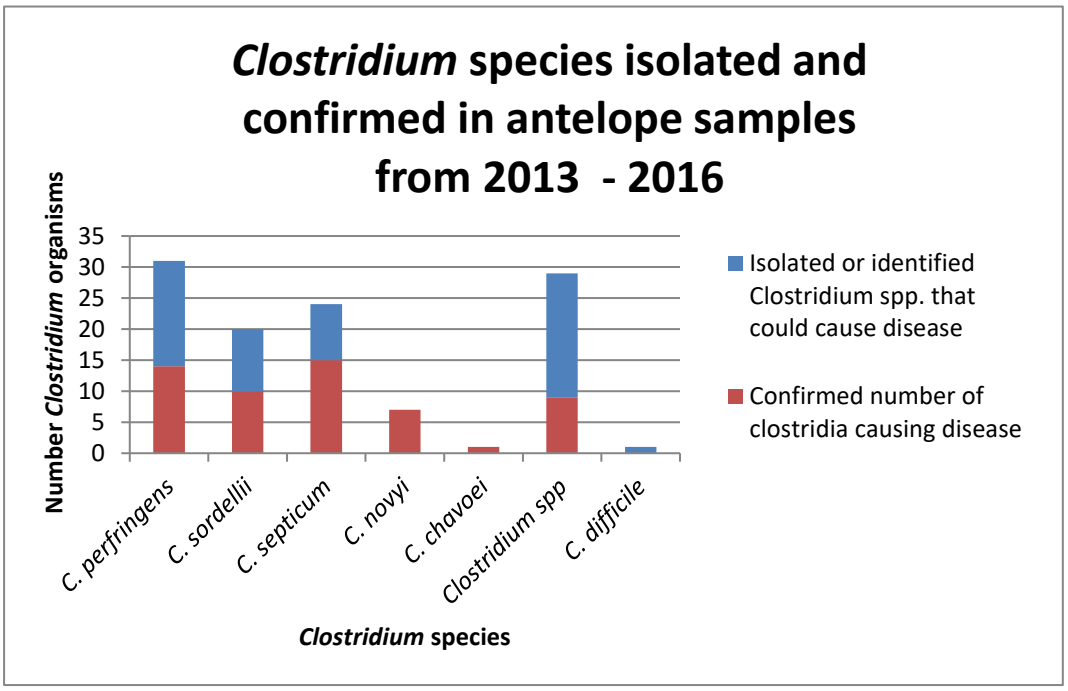
The wildlife species cases submitted included antelope, bovine (wildebeest, buffalo and eland), feline (lion and tiger), avian (vulture), equine (zebra), reptile (crocodile), rhinoceros and primate (monkey) (Appendix 2B). Confirmed clostridial infections (pathology, and isolation or FAT) from wildlife samples included 100.00% *C. chauvoei* (1/1), *C. tetani* (1/1) and *C. novyi* (13/13), 58.33% C. *septicum* (21/36), 53.33% *C. sordellii* (16/30), 46.15% *Clostridium* spp (24/52) and 46.43% (26/56) *C. perfringens*, as well as 0.00% *C. difficile* (0/2) (Figure 2.5).



Note: Blue bars illustrate the number of *Clostridium* species isolated/diagnosed from samples for the period while the red bars indicate the confirmed *Clostridium* isolates that are responsible for disease using culture, and/or FAT and pathology.

Figure 2.5: *Clostridium* species isolated and confirmed in wildlife samples for the period 2013–2016

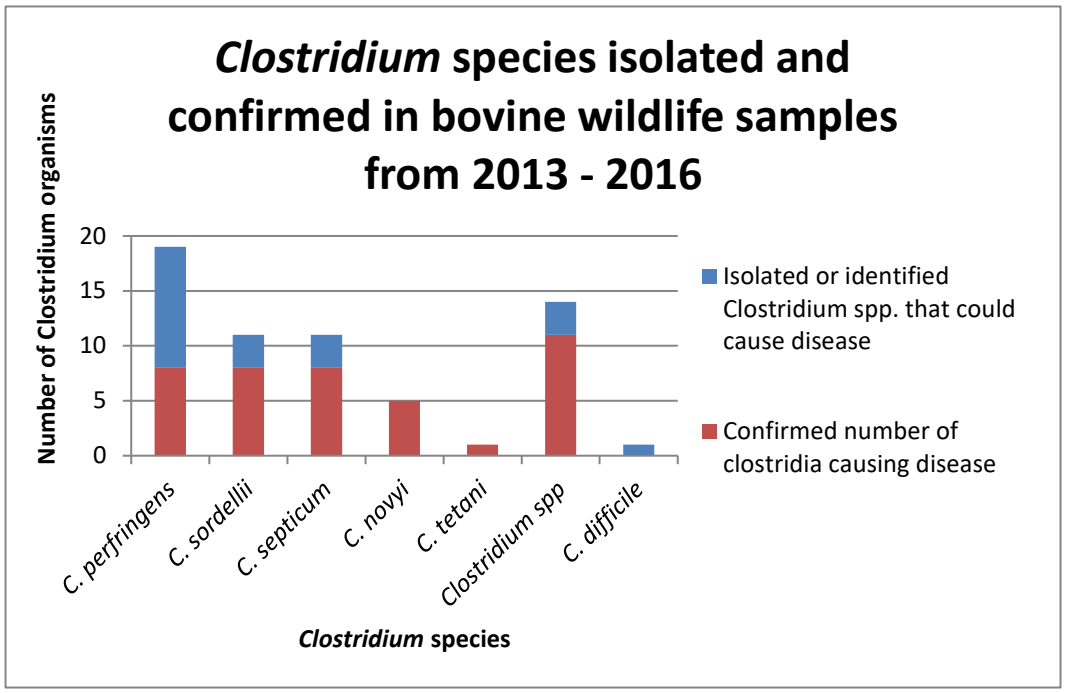
Some of the wildlife species were further analysed to determine the impact in antelope, bovine wildlife (buffalo, wildebeest and eland) and rhinoceros. Confirmed clostridial infections (pathology, and isolation or FAT) from antelope samples included 100.00% *C. chauvoei* (1/1) and *C. novyi* (7/7), 62.50% *C. septicum* (15/24), 50.00% *C. sordellii* (10/20), 45.16% *C. perfringens* (14/31), 31.03% *Clostridium* spp (9/29) and 0.00% *C. difficile* (0/1) (Figure 2.6).



Note: Blue bars illustrate the number of *Clostridium* species isolated/diagnosed from samples for the period while the red bars indicate the confirmed *Clostridium* isolates that are responsible for disease using culture, and/or FAT and pathology.

Figure 2.6: *Clostridium* species isolated and confirmed in antelope samples from the period of 2013–2016

Confirmed clostridial infections (pathology, and isolation or FAT) from bovine wildlife (buffalo, wildebeest and eland) included 100.00% *C. tetani* (1/1) and *C. novyi* (5/5), 72.73% *C. septicum* (8/11) and *C. sordellii* (8/11), 42.11% *C. perfringens* (8/19), 78.57% *Clostridium* spp (11/14) and 0.00% *C. difficile* (0/1) (Figure 2.7).

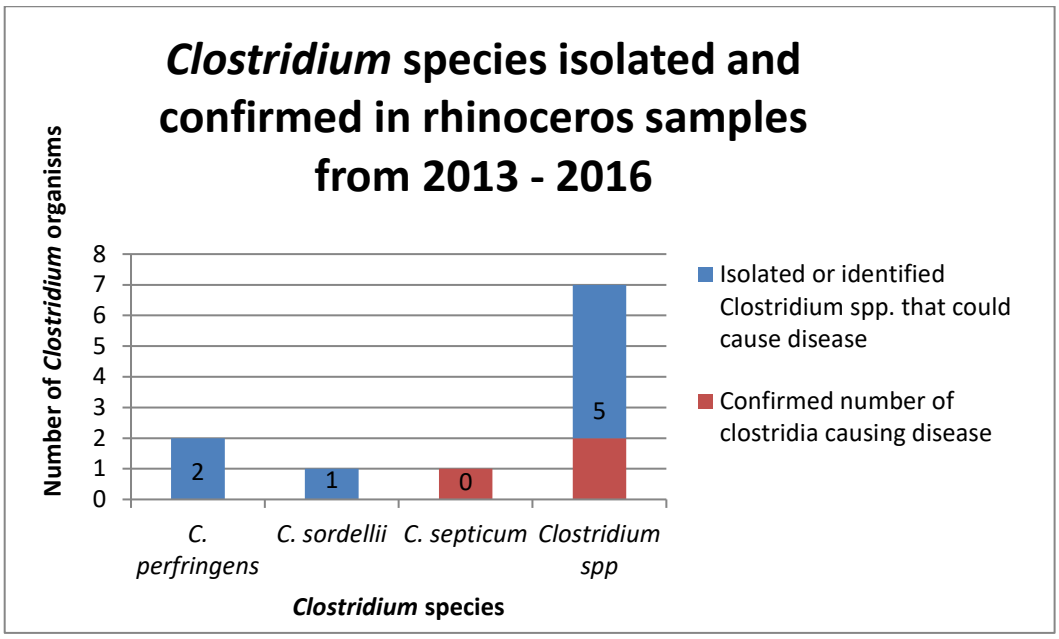


Note: Blue bars illustrate the number of *Clostridium* species isolated/diagnosed from samples for the period, while the red bars indicate the confirmed *Clostridium* isolates that are responsible for disease using culture, and/or FAT and pathology.

Figure 2.7: *Clostridium* species isolated and confirmed in samples of bovine wildlife (buffalo, wildebeest and eland) species for the period 2013–2016

Rhinoceroses are endangered and valuable wildlife species (Ferreira et al., 2017). Confirmed clostridial infections (pathology, and isolation or FAT) from rhinoceros included 100.00% *C. septicum* (1/1), 28.57% *Clostridium* spp (2/7) and 0.00% *C. sordellii* (0/1) and *C. perfringens* (0/2) (Figure 2.8).

The isolated/identified *Clostridium* species were significantly different ($p = 0.00019$) from confirmed Clostridia for livestock and domestic animals using the t-test.



Note: Blue bars illustrate the number of isolated/diagnosed Clostridium species for the period while the red bars indicate the confirmed Clostridium isolates that are responsible for disease using culture, and/or FAT and pathology.

Figure 2.8: *Clostridium* species found in rhinoceros for the period 2013–2016

2.4 Discussion and conclusion

The UVIS database can provide valuable information regarding any number of disease and/or conditions in animals, especially in livestock and wildlife. *Clostridium* species occur in the soil and intestine of animals and therefore clostridial isolation needs to be confirmed with other diagnostic methods. Various other studies reported confirmation of isolation with other techniques (Brazier et al., 2002; Lamont et al., 2018). Literature reporting on both livestock and wildlife related deaths due to Clostridia are limited. In our study we confirmed 53.80% and 50.00% of livestock and wildlife deaths from the UVIS database were due to Clostridia from submitted cases. Our study emphasise that a multiphasic diagnostic approach is essential to identify pathogenic *Clostridium*-causing disease (morbidity) or death (mortality) in animals since Clostridia are part of the normal flora of animals (Córdoba, 2011; Markey et al. 2013). The deaths due to clostridial diseases of livestock (53.80%) versus wildlife (50.00%) were similar and that might be attributed to the (i) limited or lack of vaccination in livestock clostridial disease; (ii) the incorrect use of clostridial vaccines, such as not maintaining the cold chain or not providing the booster immunisation after the initial vaccination or (iii) *Clostridium* spp, causing death not present in administered clostridial vaccination. The data indicated that bovine species (70.00%) was most affected of the livestock species with the most prominent species being *C. septicum, C. chauvoei* and *C. novyi* (Figure 2.3). Beef is the biggest individual livestock product in South Africa (Meissner et al., 2013). *C. chauvoei* causes blackleg (clostridial myositis)

in beef cattle with the most common presentation being acute death. Isolation and/or FAT are useful diagnosis but must be confirmed by typical gross and histopathological lesions. Other clostridial species, mainly *C. septicum* and *C. novyi* have been isolated either alone or with *C. chauvoei (*Niilo et al., 1969; Zeich et al., 2018). *C. septicum* might be identified instead of *C. chauvoei* and furthermore these species are difficult to differentiate base on morphology and biochemistry tests (Zeich et al., 2018). However, Barners et al. (1975) suggested that some of the *C. chauvoei* reported deaths could be caused by *C. septicum* or co-infection.

The most common disease causing Clostridia identified in both livestock and wildlife were *C. perfringens*, *C septicum*, and other unidentifiable *Clostridium* species (*Clostridium* spp.). Both *C. perfringens* and *C. septicum* can be prevented in livestock by the following vaccination programmes, which is only available in COGLAVAX, Cydectin Eweguard, MULTICLOS, MULTIVAX-P, MULTIVAX-P PLUS, One Shot Ultra 7, Tribovax 10, Ultra Choice 7 and Covexin 10 vaccines (Appendix 2D). *Clostridium* spp. can include other pathogenic Clostridia such as *Clostrdium haemolyticum* (*C. haemolyticum)*, which could be identified using other techniques (Navarro et al., 2017). This recommendation will be made to the bacteriology laboratory to include techniques that could identify *C. haemolyticum*. This disease-causing organism can also be prevented by vaccination using Covexin 10 that protects against 10 *Clostridium* species including *C. perfringens* and *C. septicum* (Appendix 2D).

In wildlife, 43.75% (56/128) of the cases were culture-positive and pathology-negative, and in livestock, 24.84% (39/157) of the cases were culture-positive and pathology-negative. The higher percentage of wildlife cases than the livestock cases might be due to the protection that is provided by vaccination of livestock, which is impractical in wildlife (Gortazar et al., 2015). The results also indicated that more livestock culture results are confirmed with pathology than in wildlife from 2013 to 2016. As indicated by the data from the UVIS database, accurate *Clostridium* diagnosis is dependent on the correct pathology presented by the animals together with culturing (culturing of *Clostridium* is still seen as the golden standard) and FAT results of submitted diagnostic samples.

The results of the FAT indicated that *C. novyi* (56.50%) in livestock was the most frequently detected, followed by *C. chauvoei* (21.70%), *C. septicum* (13.00%) and *C. sordellii* (8.70%). In wildlife, *C. novyi* (52.90%) was the most frequently detected, followed by *C. septicum* (29.40%) and *C. sordellii* (17.70%). The FAT used in the current bacteriology diagnostic laboratory of the Faculty of Veterinary Science at the University of Pretoria was only able to detect *C. novyi*, *C. septicum*, *C. sordellii* and *C. chauvoei*. FAT was developed by Assis et al. (2007), using heat labelled sera to identify each *Clostridium* species in all the tissue sections used in their study. Advantages of this technique include the ability to provide a simple and rapid method to

diagnose. This technique can be used in countries or specific geographical areas where laboratory facilities for anaerobic culturing are not available, as well as in situations where fixed tissue is submitted as a diagnostic sample (Assis et al., 2007). Disadvantages include variability of results among microscopists viewing the results by using irrelevant samples, ill-equipped laboratories and the inadequacy of the commercial FAT reagents (Cherry and Moody, 1965; Markey et al., 2013). Inconsistent results have been reported for FAT and real-time PCR (qPCR) for *C. chauvoei* and *C. septicum* (Halm et al., 2010). The most cases confirmed with the pathology report (not FAT) included 38.3% (49/128) in wildlife and 33.76% (53/157) in livestock thus more emphasis should be placed on pathology report rather than FAT. These results highlighted the polyphasic diagnostic approach necessary to confirm *Clostridium* detection linked to diseases in animals. In general, there is a lack in the agreement of diagnostic criteria for clostridial disease and caution must be taken when interpreting the results (Uzal et al., 2016).

The facultative anaerobe *C. perfringens* was most frequently isolated. Confirmed *C. perfringens* consisted of 17.83% (28/157) livestock cases and 17.97% (23/128) wildlife cases mainly from bovine and ovine (livestock) and antelope (wildlife). *C. perfringens* type A is reported to be commonly associated with enteric disease; type B causes enterotoxaemia in calves and foals, as well as lamb dysentery; type C causes haemorrhagic enterocolitis in calves, lambs and swine, as well as struck in adult sheep; type D causes enterotoxaemia in small ruminants of all ages, pulpy kidney diseases or overeating disease; while type E causes necrotic haemorrhagic enteritis that is rare in goats, calves and adult cattle (McGowen et al., 1958; Simpson et al., 2018). The limitation of our data is that the type of *C. perfringens* is unknown, due to the lack of epidemiological information, as well as evidence of predisposing factors. It is assumed that high dietary levels of digestible carbohydrates exceed the digestion and absorption capacity of the intestinal mucosa causing *C. perfringens* to proliferate which is mainly caused by types C and D in animals (Allaart et al., 2013; Uzal et al., 2016; Simpson et al., 2018). The mere isolation of these organisms is not a significant diagnostic criterion. Uzal et al. (2016) indicated that presumptive diagnosis could be made using clinical signs, pathology, as well as histology, which were used in the data mining study using culture, FAT and/or pathology. Many clinicians and pathologists found that a colony count of 10^4 to 10^7 of the intestinal content might be of diagnostic importance, although no real evidence is available to support this (Uzal et al., 2016). The presence of large numbers of *C. perfringens* organisms in animals at post-mortem must be interpreted with caution due to its ubiquitous nature (Songer, 1996; Petit et al., 1999).

C. sordellii was reported in 8.28% (13/157) of livestock cases and 14.06% (18/128) of wildlife cases, mainly from bovine, caprine (livestock), antelope, and bovine (wildlife). *C. sordellii* is rarely isolated from healthy animals (Spera et al., 1992; Vatn et al., 2000; De la Fe et al., 2006; Matten et al., 2009; Agrawal et al., 2015; Bouvet et al., 2015), but is one of the clostridial agents

of gas gangrene, gastroenteritis in mammals and abomasitis in sheep and myositis, as well as "sudden death syndrome" (Lewis and Naylor, 1998; Vatn et al., 2000; Songer, 2005). *C. sordellii* caused sudden death in captive lions (De la Fe et al., 2006) but caused death in buffalo, wildebeest, eland, impala, nyala, rhino, sable, and springbuck in this study (Appendix 2B).

C. chauvoei was identified most commonly in bovine livestock cases as mentioned. In this study, confirmed cases of *C. chauvoei* were 3.82% (6/157) in livestock and 0.78% (1/128) in wildlife. It causes blackleg in cattle between six months and two years of age (Hamaoka and Terakado, 1994; Sultana et al., 2008). A presumptive diagnosis can be made based on clinical history, clinical signs and gross and microscopic changes. A final diagnosis should be based on identification of *C. chauvoei* in affected tissues through anaerobic culture, FAT or PCR. Contamination of tissue samples from other anaerobes found in the gut may occur, thus samples should be collected immediately after death as a preventative measure (Assis et al., 2005, Assis et al., 2007; Bagge et al., 2009).

C. novyi is a soil organism implicated in wound-associated gas gangrene (MacLennan, 1962; Nishida and Nakagawara, 1964), a feature shared with *C. sordellii* and *C. chauvoei* (Ryan et al., 2001; McGuigan et al., 2002; Aronoff, 2013). The database indicated that 7.01% (11/157) cases were confirmed for livestock and 10.16% (13/128) in wildlife. Swelled head, a disease well known in domestic livestock, is caused by the toxin producing anaerobic spore forming *C. novyi* type A (Nishida and Nakagawara, 1964), with type B beta toxin causing black disease in sheep (Ardehali and Darakhshan, 1979), and bacillary haemoglobinuria caused by *C. haemolyticum* (formerly known as *C. novyi* type D) (Navarro et al., 2017). Gram stains of smears from affected subcutaneous tissue and/or muscle may provide more evidence to support disease diagnosis. Confirmation of the diagnosis should involve a combination of anaerobic culture, FAT, PCR and pathology report as previously indicated. *Clostridium novyi* type A and B have very demanding anaerobic and nutritional requirements, thus a negative culture may not necessarily rule out *C. novyi* infection (Friendship and Bilkei, 2006). No *C. novyi* have been cultured from livestock samples while three *C. novyi* have been isolated using culture in wildlife according to the database. However, in one study, FAT on liver smears was found to be more sensitive than culture for identification of *C. novyi* (Duran and Walton, 1997). This is evident seeing as all the *C. novyi* found in livestock were from FAT smears. In wildlife, nine of the confirmed *C. novyi* were from FAT smears and four were from culture (Appendix 2B).

C. septicum was confirmed together with pathology in 13.38% (21/157) of livestock cases and 19.53% (25/128) of wildlife cases. Animal species that were most commonly affected were bovine (livestock and wildlife), caprine (livestock), camelid (livestock) and antelope (wildlife). In livestock, a total of 27 *C. septicum* were isolated using culture and three were isolated using

FAT. In wildlife, 31 *C. septicum* were isolated using culture and five were isolated using FAT. This is indicative that pathology must be accompanied with the results seeing as *C. septicum* is also a frequent post-mortem invader and could cause false positive culture results for the true pathogen that is responsible for animal to succumb.

C. tetani and *C. botulinum* were only found in single animals. *C. tetani* was found in a camelid and *C. botulinum* was found in bovine wildlife. Both *C. tetani* and *C. botulinum* can be presumptively diagnosed by using clinical signs, as well as looking at the history of exposure of the animals and the area in which the animals were found. *C. tetani* can be confirmed as a diagnosis by using culture and FAT from wound tissue samples, Gram staining from blood smears (looking for terminal spores) and PCR. For *C. botulinum* a definitive diagnosis can be made by using culturing techniques, serology (detection of tetanus toxin), MALDI-TOFF, PCR (detection of toxin genes), as well as, the different chromosomal variants (mosaic variants) that are possible within the species – and mouse neutralisation assays (Uzal et al., 2016).

Most veterinary laboratories that do offer anaerobic culturing for the isolation of *Clostridium*, which restricts information on the potential, risk of clostridial diseases such as *C. chauvoei*, *C. septicum*, *C. sordellii*, *C. novyi*, *C. tetani*, *C. botulinum*, and to a lesser extent *C. perfringens*. Information on clostridial infections causing animal deaths is essential to evaluate the use of clostridial vaccines, as well as identification of most prominent *Clostridium* species involved in animal diseases. Clostridial infection reported in the study that caused death in wildlife and livestock were 49.20% and 47.40%, respectively. In South Africa, the agricultural sector includes the farming of wildlife alone or together with livestock (e.g. cattle and sheep). Many of the game farms are land where cattle or livestock farming was practised in the past (Meissner et al., 2013). Wildlife is relocated or kept in confined spaces on the farms, such as breeding camps or hunting camps, which causes predisposing circumstances for the wildlife to be infected with *Clostridium* occurring in the environment. The high occurrence of clostridial diseases are attributed to the following reasons: (i) animals not properly vaccinated or not vaccinated; (ii) change in feeding clostridial infection in the animal population (pulpy kidney); (iii) penetrating wounds (including marking wounds), dog bites, grass-seed punctures, foot trimming wounds, and dehorning (especially a cause for tetanus); (iv) muscle bruising, especially in growing animals (blackleg/black quarter); (v) liver fluke infections; (vi) wounds, especially in females associated with recent birthing (malignant oedema); (vii) use of pastures, heavy grain feeding and a sudden change in feeding (pulpy kidney); and (viii) cattle grazing on land deficient in protein and phosphorous, without adequate supplementation leading to phosphorus deficiency and bone chewing, which is commonly associated with botulism in unvaccinated cattle (Olivier, 2001; Meat & Livestock Australia, 2016). The information generated from the statistical analysis supports the fact that there is an intrinsic relationship between the number of *Clostridium*

isolated/identified and the number of confirmed *Clostridium*. The p-value (0.000019) generated by the t-test from the data mined is much lower than the norm (0.05) and thus supports the above-mentioned statement.

As mentioned, vaccines are available in numerous combinations or singly, that are made up of bacterins, toxoids or combinations of both (Langroudi, 2015). Single and combinational clostridial vaccines are available that protects against specific clostridial diseases, as well as a combination of vaccine protection against clostridial disease and others such as *Bacillus anthracis* (*B. anthracis)*. Examples of combinations of clostridial vaccines that are in combination with *B. anthracis* available in South Africa, can be viewed in Appendix 2D. Most commercial clostridial vaccines are inactivated (containing toxins or cell components as mentioned previously) and usually contain 2-, 4-, 7-, 8- or 10-way combinations of clostridial organisms or toxoids. Appropriate levels of immunity to convey protection against clostridial diseases can only be achieved if both a single vaccination and a follow-up booster dose (within three to six weeks) are given. It is recommended and advised that farmers take part in vaccination programmes for clostridial disease, as well as any other diseases that are known to be endemic in their geographical area. Individuals that are known to export/import animals, both locally and internationally, must make sure that the vaccination records of these animals are up to date before selling/purchasing animals that are known to be in a clostridial disease endemic region.

In conclusion, data mining was done using UVIS to generate data regarding disease burden of *Clostridium* spp. for the period 2013–2016. The data revealed that numerous *Clostridium* species were identified either by FAT or by culture in the four-year time, equally affecting livestock and wildlife. This might reflect incorrect vaccination practises or vaccinating with non-relevant clostridial vaccines or lack of vaccination. The data generated by using data mining will help veterinarians and farmers to adapt their management practises involving control of clostridial diseases in the different animal species that they may be working with.

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Appendix 2A

Livestock data indicating the accession number, host species and breed, specimen type and *Clostridium* species identified using FAT, culture and pathology report

Accession #	Species	Breed	Specimen Type	*Clostridium* spp identified by FAT	*Clostridium* spp identified by culture	Conformation of pathology / FAT of clostridial infection
S03834-16	Ovine		Smear	*C. novyi*		ND
S03742-15	Camalid	Alpaca	Tissue	*C. septicum*	*C. septicum*	Y
S00775-13	Bovine	Bontsmara	Exudate	*C. chauvoei*		Y
S01684-13	Bovine		Tissue	*C. septicum*	*C. septicum*	Y
S02717-13	Bovine	Bontsmara	Tissue	*C. chauvoei*	*C. chauvoei*	Y
S04312-13	Bovine	Bontsmara	Dead Animal	*C. novyi*		N
S04358-13	Bovine		Tissue	*C.sordellii*	*C. sordellii*	S
S02288-13	Bovine	Nguni	Dead Animal/Tissue		*C. perfringens*	Y
S04817-14	Bovine	Bontsmara	Tissue	*C. novyi*	*Clostridium* spp	Y
S05862-14	Bovine		Tissue	*C. novyi*		Y
S09333-14	Bovine	Bontsmara	Blood smear	*C. novyi*		Y
B03855-14	Bovine	Holstein Friesian	Synovial Fluid		*C. perfringens*	Y
S04817-14	Bovine	Bontsmara	Dead Animal/Tissue		*Clostridium* spp.	Y
S06629-14	Bovine		Dead Animal/Tissue		*Clostridium* spp.	N
S06697-14	Bovine		Dead Animal/Tissue		*Clostridium* spp.	Y
S06904-14	Bovine	Ayrshire 1	Dead Animal/Tissue		*C. perfringens*	S
S07200-14	Bovine	Ayrshire	Dead Animal/Tissue		*C. perfringens*	N
S07422-14	Bovine	Boran	Dead Animal/Tissue		*C. perfringens*	S
S08039-14	Bovine	Jersey	Dead Animal/Tissue		*C. sordellii*	Y
S08465-14	Bovine	Bontsmara	Dead Animal/Tissue		*Clostridium* spp.	N
S08962-14	Bovine		Dead Animal/Tissue		*C. sordellii*	N
S09058-14	Bovine	Tuli	Dead Animal/Tissue		*Clostridium* spp	N

Accession #	Species	Breed	Specimen Type	*Clostridium* spp identified by FAT	*Clostridium* spp identified by culture	Conformation of pathology / FAT of clostridial infection
S09603-14	Bovine	Holstein Friesian	Tissue		*Clostridium* spp.	N
S09627-14	Bovine	Friesland	Tissue		*Clostridium* spp.	ND
S00481-15	Bovine	Limousin	Tissue	*C. chauvoei*		Y
S02210-15	Bovine		Dead Animal/Blood smear	*C. novyi*		Y
S03213-15	Bovine	Dairy Swiss	Tissue	*C. chauvoei*		Y
S03290-15	Bovine	Bontsmara	Tissue	*C.novyi*	*C. septicum*	Y
S04599-15	Bovine	Nguni	Dead Animal/Tissue	*C. novyi, C. septicum*	*C. septicum*	Y
B07203-15	Bovine	Jesey	GI contents		*Clostridium* spp.	ND
B07655-15	Bovine	Sussex	Feces		*C. perfringens*	N
B07656-15	Bovine	Sussex	Feces		*C. perfringens*	N
B07667-15	Bovine	Sussex	Feces		*C. perfringens*	ND
S00201-15	Bovine		Dead Animal/Tissue		*C. sordellii,Clostridium* spp.	S
S00913-15	Bovine		Dead Animal/Tissue		*C. septicum, Clostridum* spp	Y
S02645-15	Bovine		Dead Animal/Tissue		*C. septicum, Clostridium* spp.	Y
S03080-15	Bovine	Limousin	Dead Animal/Tissue		*C. septicum*	Y
S03222-15	Bovine		Dead Animal/Tissue		*C. septicum*	Y
S03290-15	Bovine	Bonsmara	Dead Animal/Tissue		*C. septicum*	Y
S03366-15	Bovine	Holstein Friesian	Dead Animal/Tissue		*C. septicum, C. perfringens*	S
S03394-15	Bovine		Dead Animal/Tissue		*C. perfringens*	N
S03740-15	Bovine	Nguni	Dead Animal/Tissue		*C. perfringens*	Y
S03742-15	Bovine	Alpaca	Dead Animal/Tissue		*C. septicum*	Y
S04015-15	Bovine	Limousin	Dead Animal/Tissue		*C. sordellii*	Y
S04071-15	Bovine	Calf 1	Dead Animal/Tissue		*Clostridium* spp.	N
S04516-15	Bovine	Bonsmara	Dead Animal/Tissue		*C. perfringens*	ND
S04599-15	Bovine	Nguni	Dead Animal/Tissue		*C. septicum*	Y
S09911-15	Camalid	Alpaca	Dead Animal/Tissue		*C. perfringens, Clostridium* spp	Y
S00469-16	Bovine	Heifer	Tissue	*C. novyi*	*Clostridium* spp	Y
S02106-16	Bovine	Bonsmara	Dead Animal/ Blood Smear	*C. novyi*		Y
S04749-16	Bovine		Dead Animal/ Blood Smear	*C. novyi*		Y

Accession #	Species	Breed	Specimen Type	*Clostridium* spp identified by FAT	*Clostridium* spp identified by culture	Conformation of pathology / FAT of clostridial infection
B02343-16	Bovine	Friesland	Exudate		*C. perfringens*	Y
S00085-16	Bovine	Brahman	Feces		*C. perfringens, Clostridium* spp	Y
S00139-16	Bovine	Jersey	Dead Animal/Tissue		*C. sordellii*	Y
S00260-16	Bovine	Bonsmara	Dead Animal/Tissue		*C. septicum*	N
S00469-16	Bovine	Helfer	Dead Animal/Tissue		*Clostridium* spp.	Y
S01553-16	Bovine	Mixed Breed Cattle	Dead Animal/Tissue		*C. difficile, Clostridium* spp.	Y
S01638-16	Bovine	Friesland	Dead Animal/Tissue		*C. perfringens*	N
S02112-16	Bovine	Drakensberger	Dead Animal/Tissue		*C. perfringens*	N
S02392-16	Bovine	Holstein Friesian	Dead Animal/Tissue		*C. sordellii*	Y
S03363-16	Bovine		Dead Animal		*C. chauvoei*	Y
S00126-13	Bovine	Nguni	Dead Animal/Tissue		*C. perfringens*	Y
S01348-13	Bovine	Holstein Friesian	Dead Animal/Tissue		*C. perfringens*	Y
S01684-13	Bovine		Dead Animal/Tissue		*C. septicum*	Y
S01850-13	Bovine	Nguni	Dead Animal/Tissue		*C. perfringens*	S
S01986-13	Bovine	Bontsmara	Small intestine		*C.perfringens*	S
S02717-13	Bovine	Bontsmara	Dead Animal/Tissue		*C. perfringens*	Y
S03350-13	Bovine	Friesland 1	Dead Animal/Tissue		*C. perfringens, Clostridium* spp	S
S04358-13	Bovine		Dead Animal/Tissue		*C. sordellii*	S
S01584-16	Camelid	Alpaca	Dead Animal/Tissue		*C. perfringens*	N
S02242-16	Camelid	Alpaca	Dead Animal/Tissue		*C. sordelli, C. septicum, C. perfringens, Clostridium* spp*(C. botulinum)*	Y
S03386-16	Camelid	Alpaca	Dead Animal/Tissue		*C. septicum*	N
S02854-13	Caprine	Boerbok	Dead Animal/Tissue		*C. perfringens*	Y
S03624-13	Caprine	Indigenous goat 1	Dead Animal/Tissue		*C. perfringens*	N
S03641-13	Caprine	Indigenous goat	Dead Animal/Tissue		*C. perfringens*	S
S05770-14	Caprine	Boerbok 1	Dead Animal/Tissue		*C. perfringens, C. septicum*	Y
S06896-14	Caprine	Boerbok	Dead Animal/Tissue		*Clostridium* spp., *Clostridium* spp	N
S07460-14	Caprine		Dead Animal/Tissue		*C. sordellii, C. septicum*	Y
B07297-15	Caprine	Boerbok	Semen		*Clostridium* spp.	ND
S00628-15	Caprine	Boerbok	Dead Animal/Tissue		*C. perfringens, C. sordellii*	Y

Accession #	Species	Breed	Specimen Type	*Clostridium* spp identified by FAT	*Clostridium* spp identified by culture	Conformation of pathology / FAT of clostridial infection
S01132-15	Caprine	Boerbok	Dead Animal/Tissue		*C. septicum, C. sordellii, Clostridum* spp	Y
S09937-15	Caprine	Kalahari Red	Dead Animal/Tissue		*C. perfringens, Clostridium* spp	Y
S01004-16	Caprine	Boerbok	Dead Animal/Tissue		*C. perfringens*	N
S01950-16	Caprine	Saanen Milkgoat	Dead Animal/Tissue		*C.perfringens*	Y
S02224-16	Caprine	Boerbok	Dead Animal/Tissue		*C. sordellii, C. perfringens, Clostridium* spp	Y
S02409-16	Caprine	Saanen Milkgoat	Dead Animal/Tissue		*C. septicum*	N
S05040-16	Caprine		Dead Animal/Tissue		*C. perfringens*	S
S03406-16	Caprine	Boerbok	Dead Animal/Tissue		*Clostridium* spp	N
B00361-13	Equine	Thoroughbred	Vomitus		*C. perfringens*	ND
B01386-13	Equine	Friesian	Stomack Contents		*Clostridium* spp	ND
S01012-13	Equine		Dead Animal/Tissue		*C. perfringens, C. septicum*	Y
S01476-13	Equine	Arabian	Dead Animal/Tissue		*C. perfringens*	Y
S02279-13	Equine		Dead Animal/Tissue		*C. perfringens*	S
S04634-13	Equine	Appaloosa	Dead Animal/Tissue		*C. perfringens*	N
B03122-14	Equine	SA Warm Blood	Feces		*Clostridium* spp.	N
S07161-14	Equine	Friesian	Dead Animal/Tissue		*C. perfringens*	S
B06841-15	Equine		Tissue		*C. perfringens*	ND
B06988-15	Equine	Thoroughbred	Swab/Culturette		*Clostridium* spp.	ND
S00510-15	Equine	SA Warm Blood	Dead Animal/Tissue		*C. perfringens*	N
S01175-15	Equine	Arabian	Dead Animal/Tissue		*C. sordellii,Clostridium* spp.	N
S03151-15	Equine	Percheron	Dead Animal/Tissue		*C. perfringens*	N
S04387-15	Equine	Minature Horse	Dead Animal/Tissue		*C. septicum, Clostridium* spp.	Y
S09982-15	Equine	Thoroughbred	Dead Animal/Tissue		*C. perfringens*	Y
B00300-16	Equine	Percheron	Other		*C. sordellii*	ND
B00390-16	Equine	Arabian	Feces		*Clostridium* spp.	ND
B00787-16	Equine	Thoroughbred	Abscess		*C. septicum, Clostridium* spp.	Y
B00819-16	Equine	Arabian	Feces		*C. perfringens*	ND
B01323-16	Equine	Percheron	Swab/Culturette		*Clostridium* spp.	ND

Accession #	Species	Breed	Specimen Type	*Clostridium* spp identified by FAT	*Clostridium* spp identified by culture	Conformation of pathology / FAT of clostridial infection
B02085-16	Equine	Percheron	Feces		*C. perfringens*	ND
B02250-16	Equine	Warmblood Horse	Feces		*C. perfringens*	ND
B02799-16	Equine	Frieslan	Feces		*Clostridium* spp.	ND
S01403-16	Equine	Arabian	Dead Animal/Tissue		*C. sordelli, C. perfringens, Clostridium* spp.	Y
S02233-16	Equine	Nooitgedacht Pony	Dead Animal/Tissue		*C. difficile*	N
S03385-16	Equine	Percheron	Dead Animal/Tissue		*Clostridium* spp.	S
S04177-16	Equine		Dead Animal/Tissue		*Clostridium* spp.	N
S03195-16	Equine	Friesian	Dead Animal/Tissue		*C. perfringens, Clostridium* spp	N
B00174-13	Ovine	Ile De France	Tissue	C. chauvoei		ND
S00829-13	Ovine	Merino	Small intestine		*C. perfringens*	S
S01377-13	Ovine	Merino	Dead Animal/Tissue		*C. perfringens*	N
S01379-13	Ovine	Merino	Dead Animal/Tissue		*C. perfringens*	Y
S02285-13	Ovine		Dead Animal/Tissue		*C. perfringens*	Y
S02719-13	Ovine	American Merino	Dead Animal/Tissue		*C. perfringens*	Y
S06097-14	Ovine	Merino	Dead Animal/Tissue		*C. perfringens*	Y
S07895-14	Ovine	Dorper	Dead Animal/Tissue		*C. perfringens, C. sordellii, Clostridium* spp.	N
S04549-15	Ovine	Damara	Dead Animal/Tissue	C. novyi	*Clostridium* spp.	Y
S00149-15	Ovine	Merino	Dead Animal/Tissue		*C. septicum*	Y
S02236-15	Ovine	Merino	Dead Animal/Tissue		*C. perfringens, Clostridium* spp	S
S02243-15	Ovine	Merino	Dead Animal/Tissue		*C. perfringens*	S
S02244-15	Ovine	Merino	Dead Animal/Tissue		*C. perfringens*	Y
S02524-15	Ovine	Dorper 1	Dead Animal/Tissue		*C. sordellii, C. perfringens, C. difficile*	Y
S03014-15	Ovine	Merino	Dead Animal/Tissue		*C. septicum, Clostridium* spp.	Y
S03447-15	Ovine	Merino	Dead Animal/Tissue		*C. perfringens*	N
S04549-15	Ovine	Damara	Dead Animal/Tissue		*Clostridium* spp.	Y
S01278-16	Ovine	Merino	Dead Animal/ Blood Smear	C. novyi		Y
S01289-16	Ovine	Merino	Dead Animal/Tissue		*C. perfringens*	Y
S01948-16	Ovine	Merino	Dead Animal/Tissue		*C. sordellii, C. perfringens, Clostridium* spp	N

Accession #	Species	Breed	Specimen Type	*Clostridium* spp identified by FAT	*Clostridium* spp identified by culture	Conformation of pathology / FAT of clostridial infection
S02186-16	Ovine		Fecal Swab		*C. perfringens*	ND
S02411-16	Ovine	Merino	Dead Animal/Tissue		*C. sordellii, C. perfringens*	Y
S05118-16	Ovine	Sheep 2 (Dorper)	Dead Animal/Tissue		*C. sordellii, C. perfringens*	N
S03143-16	Ovine	Dorper	Dead Animal/Tissue		*C. sordellii, C. perfringens*	N
S03143-16	Ovine	Dorper	Dead Animal/Tissue		*C. sordellii*	N
S04508-15	Ovine	Sheep 2	Dead Animal/Tissue		*C. perfringens*	N
B01455-13	Porcine		Feces		*C. perfringens*	ND
S03615-13	Porcine		Dead Animal/Tissue		*Clostridium* spp	N
S01333-15	Porcine	Pig	Tissue	C. sordellii		N
S00942-15	Porcine		Dead Animal/Tissue		*C. septicum*	N
S04574-15	Porcine	Pig 1	Dead Animal/Tissue		*C. perfringens*	S
S01807-16	Porcine	Vleisvark	Dead Animal/Tissue		*C. sordellii, C. perfringens, Clostridium* spp	Y
		Friesland 2	Dead Animal/Tissue		*C. perfringens, Clostridium* spp	S
		Indigenous goat 2	Dead Animal/Tissue		*Clostridium* spp	N
		Boerbok 2	Dead Animal/Tissue		*C. perfringens, C. septicum*	ND
		Boerbok 3	Dead Animal/Tissue		*C. perfringens*	ND
		Ayrshire 3	Dead Animal/Tissue		*C. perfringens*	ND
		Dorper 2	Dead Animal/Tissue		*C. sordellii*	ND
		Calf 2	Dead Animal/Tissue		*C. sordellii*	ND
		Calf 3	Dead Animal/Tissue		*C. sordellii*	ND
		Sheep 3 (Dorper)	Dead Animal/Tissue		*C. perfringens*	ND
Total: 157						

ND: Not done

S: Secondary infection

Y: Confirmed pathology for clostridial disease

N: Negative pathology for clostridial disease

Appendix 2B

Wildlife data indicating the accession number, host species and breed, specimen type and *Clostridium* species identified using FAT, culture and pathology report

Accession #	Species	Breed	Specimen Type	*Clostridium* spp identified by FAT	*Clostridium* spp identified by culture	Confirmation of pathology report of clostridial infection
S01266-13	Antalope	Sable	Tissue	C. septicum	*C. sepricum, C. perfringens*	Y
B02022-13	Rhino		Feces		*Clostridium* spp	N
S00157-13	Bovine	Buffalo	Dead Animal/Tissue		*C. perfringens*	Y
S00826-13	Antalope	Impala	Small intestine		*C. perfringens, Clostridium* spp	N
S01266-13	Antalope	Sable	Dead Animal/Tissue		*C. perfringens, C. septicum*	Y
S01683-13	Antalope	Springbuck	Dead Animal/Tissue		*Clostridium* spp	N
S02172-13	Bovine	Buffalo	Dead Animal/Tissue		*C. perfingens*	Y
S02494-13	Antalope	Njala 1	Dead Animal/Tissue		*C. perfringens*	N
S02494-13	Antalope	Njala 2	Dead Animal/Tissue		*C. perfringens*	N
S03358-13	Rhino		Dead Animal/Tissue		*C. perfringens, Clostridium* spp	N
S03447-13	Antalope	Njala	Dead Animal/Tissue		*C. perfringens, Clostridium* spp	N
S03527-13	Bovine	Buffalo	Dead Animal/Tissue		*C. perfringens*	N
S03780-13	Lion		Dead Animal/Tissue		*C. perfringens, Clostridium* spp	N
S03824-13	Antalope	Kudu	Dead Animal/Tissue		*Clostridium* spp	N
S03924-13	Bovine	Buffalo	Dead Animal/Tissue		*C. septicum*	Y
S04621-13	Antalope	Sable	Dead Animal/Tissue		*Clostridium* spp	N
S06391-14	Rhino		Tissue	C. septicum	*Clostridium* spp	Y
S06887-14	Wildebeest		Tissue	C. novyi, C.septicum	*Clostridium* spp	Y
S07555-14	Antalope	Njala	Tissue	C. novyi, C. sordellii	*Clostridium* spp	Y
S08022-14	Antalope	Sable	Tissue	C. septicum		S
B03641-14	Rhino		Tissue		*Clostridium* spp.	N

Accession #	Species	Breed	Specimen Type	*Clostridium* spp identified by FAT	*Clostridium* spp identified by culture	Confirmation of pathology report of clostridial infection
S05484-14	Bovine	Wildebeest	Tissue		*C. perfringens*	Y
S06081-14	Bovine	Wildebeest	Dead Animal/Tissue		*C. perfringens, Clostridium* spp	Y
S06328-14	Crocodile		Dead Animal/Tissue		*C. perfringens*	N
S06391-14	Rhino		Dead Animal/Tissue		*Clostridium* spp.	Y
S06887-14	Bovine	Wildebeest	Dead Animal/Tissue		*Clostridium* spp.	Y
S06951-14	Antalope	Njala	Tissue		*Clostridium* spp	S
S07066-14	Antalope	Njala 1	Dead Animal/Tissue		*C. septicum, Clostridium* spp	S
S07066-14	Antalope	Njala 2	Dead Animal/Tissue		*C. perfringens, C. septicum*	S
S07194-14	Bovine	Eland	Dead Animal/Tissue		*C. perfringens, C. septicum*	N
S07450-14	Antalope	Njala	Dead Animal/Tissue		*C. sordellii, Clostridium* spp	N
S07493-14	Antalope	Njala 1	Dead Animal/Tissue		*C. perfringens, Clostridium* spp	N
S07493-14	Antalope	Njala 2	Dead Animal/Tissue		*Clostridium* spp	N
S07555-14	Antalope	Njala	Dead Animal/Tissue		*Clostridium* spp	Y
S07719-14	Bovine	Wildebeest	Dead Animal/Tissue		*C. perfringens, C. difficile*	N
S08000-14	Bovine	Wildebeest	Dead Animal/Tissue		*C. septicum, C.sordellii, Clostridium* spp.	Y
S08045-14	Antalope	Njala	Dead Animal/Tissue		*Clostridium* spp	N
S08113-14	Antalope	Sable	Dead Animal/Tissue		*C. septicum, Clostridium* spp	Y
S08480-14	Antalope	Impala	Dead Animal/Tissue		*Clostridium* spp	N
S08483-14	Rhino		Dead Animal/Tissue		*C. perfringens. C. sordellii*	N
S09079-14	Bovine	Buffalo	Dead Animal/Tissue		*C. sordellii*	Y
S09080-14	Bovine	Wildebeest	Fetus/Tissue		*C. sordellii*	N
S09366-14	Bovine	Wildebeest	Dead Animal/Tissue		*C. septicum*	Y
S09414-14	Bovine	Buffalo	Dead Animal/Tissue		*C. perfringens, C. sordellii, Clostridium* spp.	N
S09557-14	Bovine	Buffalo	Swab/Culturette		*C. tetani*	Y
S01197-15	Antalope	Sable	Dead Animal		*C. novyi, C.sordellii*	Y
S02310-15	Antalope	Sable	Dead Animal/Blood smear		*C. chauvoei*	Y
S03080-15	Antalope	Impala	Tissue		*C. perfringens*	Y
S03823-15	Bovine	Buffalo	Tissue		*C. novyi*	Y
S03828-15	Antalope	Sable	Dead Animal/Blood smear		*C. novyi, C.sordellii*	Y
S04286-15	Antalope	Blesbok	Dead Animal/Tissue		*C. novyi*	Y

Accession #	Species	Breed	Specimen Type	*Clostridium* spp identified by FAT	*Clostridium* spp identified by culture	Confirmation of pathology report of clostridial infection
S00067-15	Antalope	Sable	Dead Animal/Tissue/Small intestine		*Clostridium* spp.	N
S00068-15	Antalope	Impala	Dead Animal/Tissue/Intestine		*Clostridium* spp.	N
S00090-15	Antalope	Sable	Dead Animal/Tissue		*C. perfringens*	S
S00150-15	Antalope	Springbuck	Dead Animal/Tissue		*C. septicum*	N
S00700-15	Antalope	Impala	Dead Animal/Tissue		*C. septicum*	ND
S00933-15	Bovine	Wildebeest	Dead Animal/Tissue		*C. sordellii,Clostridium* spp.	N
S00984-15	Antalope	Impala	Dead Animal/Tissue		*C. septicum*	N
S01462-15	Rhino		Dead Animal/Tissue		*Clostridium* spp.	N
S01651-15	Antalope	Sable	Dead Animal/Tissue		*C. perfringens*	Y
S01919-15	Antalope	Blesbuck	Dead Animal/Tissue		*C. perfringens*	Y
S02276-15	Antalope	Sable	Dead Animal/Tissue		*Clostridium* spp.	N
S02289-15	Antalope	Sable	Dead Animal/Tissue		*Clostridium* spp.	N
S02306-15	Antalope	Springbuck	Dead Animal/Tissue		*Clostridium* spp.	Y
S02350-15	Antalope	Springbuck	Dead Animal/Tissue		*C. septicum*	N
S02436-15	Antalope	Njala	Dead Animal/Tissue		*C. septicum, C. difficile, Clostridium* spp	N
S02452-15	Antalope	Kudu	Dead Animal/Tissue		*C. septicum*	Y
S02526-15	Antalope	Sable	Dead Animal/Tissue		*C. sordellii, C. perfringens*	N
S02603-15	Antalope	Sable	Dead Animal/Tissue		*Clostridium* spp.	N
S02613-15	Antalope	Springbuck 1	Dead Animal/Tissue		*C. septicum, C. perfringens*	Y
S02613-15	Antalope	Springbuck 2	Dead Animal/Tissue		*C. sordellii, C. septicum, C. perfringens*	Y
S02688-15	Antalope	Springbuck 2	Dead Animal/Tissue		*Clostridium* spp.	Y
S02783-15	Antalope	Sable	Dead Animal/Tissue		*C. perfringens*	Y
S02810-15	Bovine	Wildebeest	Dead Animal/Tissue		*Clostridium* spp.	Y
S02948-15	Antalope	Sable	Dead Animal/Tissue		*C. septicum, C. perfringens, Clostridium* spp.	Y
S02997-15	Feline	Tiger	Dead Animal/Tissue		*Clostridium* spp.	N
S02998-15	Antalope	Impala	Dead Animal/Tissue		*C. sordellii, C.perfringens*	Y
S03125-15	Antalope	Sable	Dead Animal/Tissue		*C. septicum*	N
S03202-15	Antalope	Njala	Dead Animal/Tissue		*C. perfringens*	Y
S03368-15	Bovine	Buffalo	Dead Animal/Tissue		*C. septicum, C. perfringens*	S
S03390-15	Bovine	Buffalo	Dead Animal/Tissue		*C. perfringens*	N
S03424-15	Antalope	Blue Duiker	Dead Animal/Tissue		*C. sordellii*	N
S03425-15	Antalope	Impala	Dead Animal/Tissue		*C. septicum, C. perfringens*	Y
S03608-15	Antalope	Duiker	Dead Animal/Tissue		*C. perfringens*	N

Accession #	Species	Breed	Specimen Type	*Clostridium* spp identified by FAT	*Clostridium* spp identified by culture	Confirmation of pathology report of clostridial infection
S03687-15	Bovine	Buffalo	Dead Animal/Tissue		*C. perfringens*	Y
S03823-15	Bovine	Buffalo	Dead Animal/Tissue		*Clostridium* spp.	Y
S04286-15	Antalope	Wit Blesbuck	Dead Animal/Tissue		*C. septicum, C. perfringens, Clostridium* spp.	Y
S09839-15	Bovine	Buffalo	Dead Animal/Tissue		*C. perfringens*	N
S09958-15	Bovine	Wildebeest	Dead Animal/Tissue		*C. perfringens*	Y
S00145-16	Bovine	Wildebeest	Dead Animal/ Blood Smear	C. novyi		Y
S00185-16	Antalope	Waterbuck	Tissue	C. novyi	*C. septicum*	Y
S02100-16	Equine	Zebra	Dead Animal/ Blood Smear	C. novyi		Y
S02753-16	Bovine	Eland	Dead Animal/ Blood Smear	C. sordellii , C. novyi	*C. septicum, C.sordellii*	Y
S02871-16	Antalope	Sable	Tissue	C. novyi	*C. septicum, C.sordellii*	Y
S02969-16	Bovine	Wildebeest	Tissue	C. novyi	*Clostridium* spp, *C. perfringens*	Y
S03272-16	Antalope	Springbuck	Dead Animal/ Blood Smear	C. sordellii		Y
S03403-16	Bovine	Wildebeest	Tissue	C. septicum	*C. perfringens, C. sordellii*	Y
S00185-16	Antalope	Waterbuck	Dead Animal/Tissue		*C. septicum*	Y
S00231-16	Antalope	Njala	Dead Animal/Tissue		*C. sordellii, C. perfringens*	ND
S00270-16	Antalope	Sable	Dead Animal/Tissue		*C. sordellii*	N
S00819-16	Antalope	Impala	Dead Animal/Tissue		*Clostridium* spp.	Y
S01229-16	Antalope	Impala	Dead Animal/Tissue		*C. perfringens*	N
S01317-16	Bovine	Buffalo	Dead Animal/Tissue		*C. septicum, C. perfringens*	N
S01563-16	Antalope	Impala	Dead Animal/Tissue		*C. perfringens*	N
S01618-16	Antalope	Njala	Dead Animal/Tissue		*C. perfringens*	N
S01651-16	Avian	Vulture (White back)	Dead Animal/Tissue		*C. perfringens*	ND
S01840-16	Antalope	Sable	Dead Animal/Tissue		*C. perfringens, Clostridium* spp	N
S01998-16	Antalope	Sable	Dead Animal/Tissue		*C. sordellii, C. perfringens*	Y
S02055-16	Bovine	Wildebeest	Dead Animal/Tissue		*C. septicum, C. perfringens*	N
S02154-16	Antalope	Oryx	Dead Animal/Tissue		*C. sordellii*	N
S02356-16	Bovine	Buffalo	Dead Animal/Tissue		*Clostridium* spp.	N
S02437-16	Antalope	Black Impala	Dead Animal/Tissue		*C. sordellii, C. septicum*	Y
S02747-16	Antalope	Sable	Dead Animal/Tissue		*C. sordellii, C. perfringens*	N

Accession #	Species	Breed	Specimen Type	*Clostridium* spp identified by FAT	*Clostridium* spp identified by culture	Confirmation of pathology report of clostridial infection
S02753-16	Bovine	Eland	Dead Animal/Tissue		*C. sordellii, C. septicum*	Y
S02969-16	Bovine	Wildebeest	Dead Animal/Tissue		*C. perfringens, Clostridium* spp	ND
S03403-16	Bovine	Wildebeest	Dead Animal/Tissue		*C. sordellii, Clostridium* spp.	Y
S03907-16	Rhino		Dead Animal/Tissue		*Clostridium* spp.	N
S04030-16	Bovine	Wildebeest	Dead Animal/Tissue		*C. perfringens*	N
S04363-16	Antalope	Blesbuck	Dead Animal/Tissue		*Clostridium* spp.	Y
S02680-16	Antalope	Bushbuck	Dead Animal/Tissue		*C. sordellii*	N
S02847-16	Antalope	Njala	Dead Animal/Tissue		*C .perfringens, Clostridium* spp	N
S02848-16	Bovine	Wildebeest	Dead Animal/Tissue		*C. sordellii, Clostridium* spp	Y
S02871-16	Antalope	Sable	Dead Animal/Tissue		*C. septicum, C.sordellii*	Y
S03080-16	Antalope	Impala	Tissue		*C. perfringens*	Y
S04180-16	Primates	Capuchin Monkey	Dead Animal/Tissue		*C. perfringens*	Y
S04370-16	Bovine	Buffalo	Dead Animal/Tissue		*C. septicum, Clostridium* spp,*Clostridium* spp	Y
S01413-13	Antalope	Roan	Dead Animal/Tissue		*C. perfringens*	N
S03230-16	Antalope	Roan	Tissue	C. novyi		Y
Total: 128						

S: Secondary infection

Y: Confirmed pathology for clostridial disease

N: Negative athology for clostridical disease

Appendix 2C
Livestock and wildlife data of *Clostridium* species identified using FAT, culture and pathology report

Wildlife			
***Clostridium* species present**	**FAT**	**Culture**	**Pathology**
	C. septicum (5) C. novyi (9) C. sordellii (3)	C. septicum (31) C. perfringens (56) C. sordellii (27) C. difficile (2) C. tetani (1) C. novyi (4) C. chauvoei (1) Clostridium spp (52)	4/128 (3.1%) No patology results (ND) 124/128 (96.9%) Pathology results included in reports 62/124 (50%) Positive results from culture and/or FAT and patology for clostridial infection (y) 5/124 (4%) Clostridium could not be ruled out of diagnosis(s)
Total	17	176	
Livestock			
***Clostridium* species present**	**FAT**	**Culture**	**Pathology**
	C. chauvoei (5) C. septicum (3) C. novyi (13) C. sordellii (2)	C. septicum (27) C. perfringens (76) C. sordellii (26) C. difficile (3) C. chauvoei (2) Clostridium spp (49) C. botulinum (1)	27/157(17.2%) No patology results (ND) 130/157 (82.8%) Pathology results included in reports 70/130 (53.8%) Positive results from culture and/or FAT and patology for clostridial infection (y) 19/130 (14.6%) Clostridium could not be ruled out of diagnosis (s)
Total	23	184	

Appendix 2D
Commercial clostridial vaccine available in South Africa

Vaccines name	Manufacturing company	Protect against *Clostridium* species	Protect against other organisms	Animal species protected
BLANTHRAX®	MSD Animal Health	C. chauvoei	B. anthracis	Cattle, Sheep and Goats
BOTUVAX®	MSD Animal Health	C. botulinum types C and D	B. anthracis	Cattle, Horses, Sheep and Goats
SUPAVAX®	MSD Animal Health	C. botulinum types C1 + 2, D, and C. chauvoei	B. anthracis	Cattle and Sheep
Combined botulism black quarter vaccine	Onderstepoort Biological Products	C. botulinum types C & D and C. chauvoei		Cattle
DUOVAX	MSD Animal Health	C. botulinum types C1 + 2 ,D and C. chauvoei		Cattle, Sheep and Goats
COGLAVAX®	Ceva Santé Animale	C. perfringens type A, C, D, C. septicum, C. novyi type B, C. tetani and C. chauvoei		Cattle, Sheep and Goats
Gas Gangrene Complex Vaccine	Onderstepoort Biological Products	C. chauvoei, C. novyi type A and C. septicum		Cattle, Sheep and Goats
Cydectin Eweguard™	Zoetis	C. perfringens type D, C. chauvoei, C. septicum, C. novyi type B and C. tetani	Corynebacterium pseudotuberculosis-ovis	Sheep
MULTICLOS	MSD Animal Health	C. septicum, C. novyi type B, C. sordellii, C. perfringens type C, D, C. chauvoei and C. haemolyticum		Cattle and Sheep
MULTIVAX-P	MSD Animal Health	C. perfringens type D, C. septicum, C. tetani, and C. chauvoei	Mannheimia [Pasteurella] haemolytica	Sheep
MULTIVAX-P PLUS	MSD Animal Health	C. perfringens types B, C ,D, C. septicum, C. tetani, C. novyi type B, and C. chauvoei	Mannheimia [Pasteurella] haemolytica and Pasteurella trehalosi	Sheep
One Shot Ultra™ 7	Zoetis	C. chauvoei, C. septicum, C. novyi, C. sordellii, and C. perfringens type B, C, D,	P. haemolytica	Cattle
Tribovax 10	MSD Animal Health	C. perfringens type A, B, C, D, C. chauvoei, C. novyi, C. septicum, C. tetani, C. sordellii and C. haemolyticum		Cattle and Sheep
UltraChoice® 7	Zoetis	C. chauvoei, C. septicum, C. novyi; C. sordellii, and C. perfringens types B, C, D		Cattle and Sheep
Suiseng	LABORATORIOS HIPRA	C. perfringens type C, and C. novyi type B	E.coli serotypes, E.coli LT	Pigs
COVEXIN™ 10	MSD Animal Health	C. perfringens types A, B, C, D, C. chauvoei, C. novyi, C. septicum, C. tetani, C. sordellii, and C. haemolyticum		Cattle and Sheep
Glanvac 3®	Zoetis	C. perfringens type D, and C. tetani	Corynebactenium pseudotuberculosis	Sheep and Goats
Litterguard® LT-C	Zoetis	C. perfringens type C	E.coli [enterotoxigenic strain]	Pigs
ScourGuard 4KC	Zoetis	C. perfringens type C	Rotavirus, Coronavirus, and E. coli	Cattle
Scourmune C	MSD Animal Health	C. perfringens type C	E. coli strains	Pigs

Chapter 3

An Environmental Survey of *Clostridium* Species from Carcass Sites in the Kruger National Park, South Africa, Using Multiplex Polymerase Chain Reaction Assays

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3.1 Introduction

Clostridium or Clostridia (the collective name for the genus) are Gram-positive bacilli and are similar to the *Bacillus* genus. Bacteria from the genera *Clostridium* and *Bacillus* form part of the phylum *Firmicutes* and form or produce endospores. Both are soil-borne pathogens that persist in the environment for years. Bacteria belonging to the genus *Clostridium* cause disease in animals but some, for example *Clostridium perfringens (C.perfringens) ,Clostridium botulinum (C. botulinum), Clostridium tetani (C. tetani)* and *Clostridium difficile (C.difficile)*, are zoonotic, (Num and Useh, 2014) and pose a threat to health in communities in specific endemic areas (Songer, 2010).

Sporulation of *Clostridium* is usually, but not always, initiated under unfavourable conditions to survive for long periods in the environment (Nicholson and Setlow, 1990; Nicholson et al., 2000; Sonenshein, 2000). The main habitat of Clostridia is soils where the endospores enable long survival in the soil. Animals and humans can acquire Clostridia from the soil and vegetation through wounds or ingestion. Some Clostridia are known to inhabit the intestinal tract of animals (Hungate, 1950; Haagsma, 1979; Wells and Wilkins, 1996; Petit et al., 1999; Myllykoski et al., 2006). These Clostridia exist in the animals or environment without causing problems until there environmental conditions change, allowing proliferation and cause of disease (Haagsma, 1991).

Clostridia can be divided into three categories or groups according to the disease type and pathological presentation, namely the neurotoxic, enterotoxic and histo-toxogenic groups (Hatheway, 1990; Aldape et al., 2006; Weingart et al., 2010). The neurotoxic group includes *C. botulinum* and *C. tetani*. The enterotoxic group is composed of *C. perfringens*, *C. difficile*, *Clostridium septicum* (*C. septicum*), *Clostridium sordellii* (*C. sordellii*), *Clostridium spiroforme* (*C. spiroforme*), *C. botulinum*, toxic *Clostridium baratii* (*C. baratii*) and toxic *Clostridium butyricum* (*C. butyricum*) (Hatheway, 1990; Markey et al., 2013). The histo-toxogenic group consists primarily of *C. perfringens*, *C. septicum*, *Clostridium histolytcum* (*C. histolyticum*), *Clostridium novyi* (*C. novyi*), *C. sordellii*, *Clostridium chauvoei* (*C. chauvoei*), *C. tetani* and *C. botulinum* (Markey et al., 2013). Clostridia of clinical significance are known to produce a wide variety of toxins and enzymes.

The identification of Clostridia is complex due to the stringent requirements, as well as the obligate anaerobic nature of most of the *Clostridium* species (spp) (Bowler et al., 2001). Culturing Clostridia requires specific atmospheric conditions, media compositions and temperatures. Identification of some *Clostridium* spp with characteristic-shaped endospores can be done using stained smears of *C. tetani*. However, identification of *Clostridium* spores in samples does not indicate infection as *Clostridium* spp are natural inhabitants in the environment and the intestinal tract of animals (Haagsma, 1991, Ivanov and Honda, 2012).

Wildlife is considered as a reservoir and susceptible hosts of numerous diseases that also affect livestock, according to the wildlife group of the South African Veterinary Association (Pretorius, 2015). The diseases can be spread from domestic animals to wildlife and *vice versa*. The acute and per-acute clostridial diseases have grave economic implications for both the farmer and the country (Sterne and Batty, 1975; Pretorius, 2015; Ayele et al., 2016). These diseases affecting wildlife and domestic animals include *C. botulinum* (botulism), *C. chauvoei* (blackleg/black quarter), *C. sordellii* (gas gangrene), *C. novyi* (black disease), *C. perfringens* type C and D (enterotoxaemia), *C. septicum* (malignant oedema) and *C. tetani* (tetanus) (Pretorius, 2015). Single or multivalent clostridial vaccines are available but can only be used to control clostridial diseases in livestock.

Molecular identification of Clostridia is possible with PCR assays being the most common. The 16S ribosomal ribonucleic acid (16S rRNA) gene is generally used to identify the genus or some *Clostridium* spp, as 16S gene sequences are very homologous with a high similarity between species (Public Health England, 2015). Various single species PCRs identify non-pathogenic and pathogenic *Clostridium* spp. These PCRs target the specific toxin genes for *C. botulinum* (Prevot et al., 2007), *C. chauvoei* (Kojima et al., 2001), *C. novyi* type A (Sasakki et al., 2000), *C. septicum* (Takeuchi et al., 1997), *C. sordellii* (Bhatnagar et al., (2012), *C. tetani* (Saadatmandzadeh et al., 2011) and *C. perfringens* toxin types (Ahsani et al., 2010), as well as, the 16S rRNA region for *C. perfringens* (Wu et al., 2010). No multiplex PCR assay is available to detect these seven *Clostridium* spp. Other more specialised molecular techniques that can be used for the typing or further identification of *Clostridium* from clinical samples include: 16S rDNA gene sequencing (Brazier et al., 2002), fluorescent amplified fragment length polymorphism (AFLP) (Keto-Timonen et al., 2006), microarray analysis (Janvilisri et al. 2010), multiple-locus variable-number tandem-repeat analysis (MLVA) (Youhanna et al., 2005), pulsed-field gel electrophoresis (PFGE) (Johansson et al., 2006), and whole-genome sequencing (WGS) (Sebaihia et al.,2007). These techniques are more laborious, needs specific expertise and equipment to perform the techniques, and are thus not suited for routine diagnostics.

There is no molecular assay for the detection and speciation of *Clostridium* currently available at the veterinary laboratories of the Faculty of Veterinary Science, University of Pretoria. Furthermore, very little data regarding Clostridia in wildlife and environment are available. The aim of this chapter is to develop multiplex *Clostridium* species-specific PCR assays to identify *C. botulinum*, *C. chauvoei*, *C. novyi*, *C. perfringens*, *C. septicum*, *C. sordellii* and *C. tetani* and to test these assays to identify the species from wildlife/environment surveillance smears collected in NKP during 2010. The single and validated *Clostridium* PCRs were developed into two multiplex assays to identify and/or to confirm identification for the seven most common

pathogenic Clostridia in animals. These multiplex PCR assays were tested on a collection of DNA from wildlife carcasses in the KNP collected during surveillance programme in 2010 during an anthrax outbreak. This collection has information regarding the microscopic detection of suspect *Bacillus anthracis* (*B. anthracis)* and Clostridia bacilli, as well as, confirmation of suspect *B. anthracis* bacilli using culture and PCR.

3.2 Material and methods

3.2.1 Selection and optimisation of *Clostridium* species multiplex polymerase chain reaction assays

Multiplex *Clostridium* species PCR screening assays were developed using existing single *Clostridium* species-specific PCR that was validated (which include optimization of reaction conditions, specificity to specific species and tested using clinical samples). Each primer was investigated *in silico* using the Basic Local Alignment Search Tool (BLAST 2.0) to ensure the region was specific for *Clostridium* species. The seven primer pairs were separated into two multiplex PCR assays based on fragment size, since some amplified similar fragment sizes. *Clostridium* species-specific primers for different *Clostridium* spp were selected based on PCR amplicon size and PCR conditions (Table 3.1). The PCR annealing temperature of the two multiplex PCR assays were optimised using the gradient PCR apparatus of the Applied Biosystems® Veriti® 96-Well Thermal Cycler (Thermo Fisher Scientific) between 50°C and 60°C with 2°C increase. The PCR reaction and conditions are listed in Table 3.2. Positive controls (see section 3.2.3) and a non-template negative control were included in each PCR assay. The PCR products were visualised using a 2% agarose gel with ethidium bromide (10 µg/ml) for agarose gel electrophoresis. The electrophoresis condition consisted of 75 V at 300 mA for 90 minutes. Fragment sizes were determined by using a molecular weight marker of 100 bp sizes (according to the Thermo Scientific O'GeneRuler 100 bp DNA ladder). Once the gel electrophoresis was complete, the fragments was visualised using the GelDoc XR+ System from Bio-Rad.

Table 3.1: Summary of the DNA regions

Clostridium spp.	Primer name	Primer sequence (5'-3')	Tm (ºC)	Product size (bp)	References
C. botulinum	CB(cds2) CB(cdas3)	Forward: TTTATACGAGAATGTTCYG Reverse: CATTATATCCTGATGTATCC	55	327	Prevot et al. (2007)
C. chauvoei	CC(CCF516) CC(CCR516)	Forward: ATCGGAAACATGAGTGCTGC Reverse: AGTCTTTATGCTTCCGCTAG	54	516	Kojima et al. (2001)
C. novyi *Type A*	CNA(Nov-F) CNA(Nov-R)	Forward: CTTAAAAATTCAAGGAGGAATT Reverse: CGCCTACTTGGAAAGTTACTC	55	472	Sasakki et al. (2000)
C. perfringens	CP16S rRNA gene 1 CP16S rRNA gene 2	Forward: AAAGATGGCATCATCATTCAAC Reverse: TACCGTCATTATCTTCCCCAAA	50	279	Wu et al. (2009)
C. septicum	CSEPHF CSEPHR	Forward: AATTCAGTGTGCGGCAGTAG Reverse: CCTGCCCCAACTTCTCTTTT	55	270	Takeuchi et al. (1997)
C. sordellii	CSOR (CSP09-F) CSOR (CSP09-BR)	Forward: TGGGATGATTGGGATTATTCAG Reverse: TCAGTTCCTGCATATTCATTGT	60	176	Bhatnagar et al. (2012)
C. tetani	CT (TeT X) F CT (TeT X) R	Forward: CCGAAAGATGGAAATGCCT Reverse: GGATCGTTGCCTATTTGACC	60	203	Saadatmandzadeh et al. (2011)

Table 3.2: The polymerase chain reactions and conditions of the two *Clostridium* species polymerase chain reaction screening assays used in the study

PCR reaction 1 (Multiplex) – MyTaq™ Red HS DNA polymerase		
Mix components and concentrations	**Volume 1× (µl)**	**PCR cycling conditions**
5× MyTaq Red Master Mix (Bioline™)	13.0	Denature: • 95°C for 10 min Amplification: • 94°C for 45s • 57°C for 30s • 72°C for 90s → × 35 Extension: • 72°C for 10 min
Forward Primers (20 µM each) *C. botulinum* – CB(cds2)	1.5	
Reverse Primers (20 µM each) *C. botulinum* – CB(cdas3)	1.5	
Forward Primers (20 µM each) *C. sordellii* – CSOR(CSP09-F)	0.5	
Reverse Primers (20 µM each) *C. sordellii* – CSOR(CSP09-BR)	0.5	
Forward Primers (20 µM each) *C. novyi* – CAN(Nov-F)	1.0	
Reverse Primers (20 µM each) *C. novyi* – CAN(Nov-R)	1.0	
Forward Primers (20 µM each) *C. perfringens* – CP 16S rRNA gene 1	0.5	
Reverse Primers (20 µM each) *C. perfringens* – CP 16S rRNA gene 2	0.5	
Template DNA	5.0	
Total volume	**25**	
PCR reaction 2 (Multiplex) – MyTaq™ Red HS DNA Polymerase		
Mix components and concentrations	**Volume 1× (µl)**	**PCR cycling conditions**
5× MyTaq Red Master Mix (Bioline™)	13.0	Denature: • 95°C for 10 min Amplification: • 94°C for 45s • 53°C for 30s • 72°C for 90s → × 35 Extension: 72°C for 10 min
Forward primers (20 µM each) *C. chauvoei* – CC(CCF516)	0.5	
Reverse primers (20 µM each) *C. chauvoei* – CC(CCR516)	0.5	
Forward primers (20 µM each) *C. septicum* – CSEPHF	0.5	
Reverse primers (20 µM each) *C. septicum* – CSEPHR	0.5	
Forward primers (20 µM each) *C. tetani* – CT(TeT X)F	0.5	
Reverse primers (20 µM each) C. tetani – CT(TeT X)R	0.5	
Template DNA	5.0	
Water (ddH_2O)	4.0	
Total volume	**25**	

3.2.2 Clostridium *perfringens* toxin typing multiplex polymerase chain reaction assay

The samples positive for the *C. perfringens* PCR, using a 16S rRNA target region, were typed using the *C. perfringens* toxin typing multiplex PCR assay to identify the different toxigenic types of *C. perfringens*. The assay was adopted from Ahsani et al. (2010) and the PCR reaction and condition are indicated in Table 3.3. The fragment sizes for each of the primer sets are as follows: *Clostridium* toxin type A (CPA) is 324 bp (alpha toxin shared by all types); CPB is 196 bp (types B and C), CPE is 655 bp (type D) and CPI is 446 bp (type E) (Ahsani et al. 2010). The PCR was optimised using a *C. perfringens* type A positive control from the bacteriology laboratory of the University of Pretoria at Onderstepoort, as well as a non-template negative control. The annealing temperatures of PCR was optimised using the gradient PCR apparatus of the Applied Biosystems® Veriti® 96-Well Thermal Cycler (Thermo Fisher Scientific). The PCR products were visualised using a 2% agarose gel with ethidium bromide (10ug/ml) for agarose gel electrophoresis. The electrophoresis condition consisted of 75 V at 300 mA for 90 minutes. Fragment sizes were determined by using a molecular weight marker of 100 bp sizes (Thermo Scientific O'GeneRuler 100 bp DNA Ladder). Once the gel electrophoresis was complete, the fragments was visualised using the GelDoc XR+ System from Bio-Rad.

Table 3.3: The polymerase chain reaction and condition of the *Clostridium perfringens* toxin typing multiplex polymerase chain reaction assay

***Clostridium perfringens* toxin typing multiplex PCR assay**		
Mix components and concentrations	**Volume 1×(µl)**	**PCR cycling conditions**
5× MyTaq Red Master Mix (Bioline™)	13	Denature: • 95°C for 10min Amplification: • 94°C for 1min • 55°C 1 min • 72°C for 1 min × 35 Extension: • 72°C for 10 min
Forward Primers (20 µM each) CPA 1	0.5	
Reverse Primers (20 µM each) CPA 2	0.5	
Forward Primers (20 µM each) CPB 1	0.5	
Reverse Primers (20 µM each) CPB 2	0.5	
Forward Primers (20 µM each) CPE(etx) 1	0.5	
Reverse Primers (20 µM each) CPE(etx) 2	0.5	
Forward Primers (20 µM each) CPI(iap) 1	0.5	
Reverse Primers (20 µM each) CPI(iap) 2	0.5	
Template DNA	5	
Water (ddH_2O)	3	
Total volume	**25**	

Source: Ahsani et al. (2010)

3.2.3 *Clostridium* positive controls

C. perfringens and *C. sordellii* cultures were obtained from the bacteriology laboratory, of the University of Pretoria at Onderstepoort. *C. perfringens* and *C. sordellii* isolates were cultured on blood agar plates at 37°C in an anaerobic cabinet for 48 hours. The *C. perfringens* and *C. sordellii* plates were flooded each with 500 µl 1× phosphate-buffered saline (PBS), and the PBS-culture mixture were transferred to 1.5 ml Eppendorf tubes. Lysozyme (20 mg/ml concentration, Fuka) was also added and the tubes were incubated for 30 minutes at 110°C in a heating block. After incubation, tubes were centrifuged at 14 600 x g and the supernatant was collected. The crude boiled lysed *C. perfringens* and *C. sordellii* DNA were stored at –20°C until needed. For *C. botulinum*, *C. chauvoei*, *C. novyi*, *C. septicum*, and *C. tetani* no cultures were available and therefore the target sequences of specific *Clostridium* spp obtained from GenBank were synthesised and cloned into pBME (BiomatikTM) (see Appendix 3A for cloned control sequences and target regions). The recombinant plasmids DNA with target regions were used as positive controls.

3.2.4 Screening 2010 smear DNA collection of the Kruger National Park and data analysis

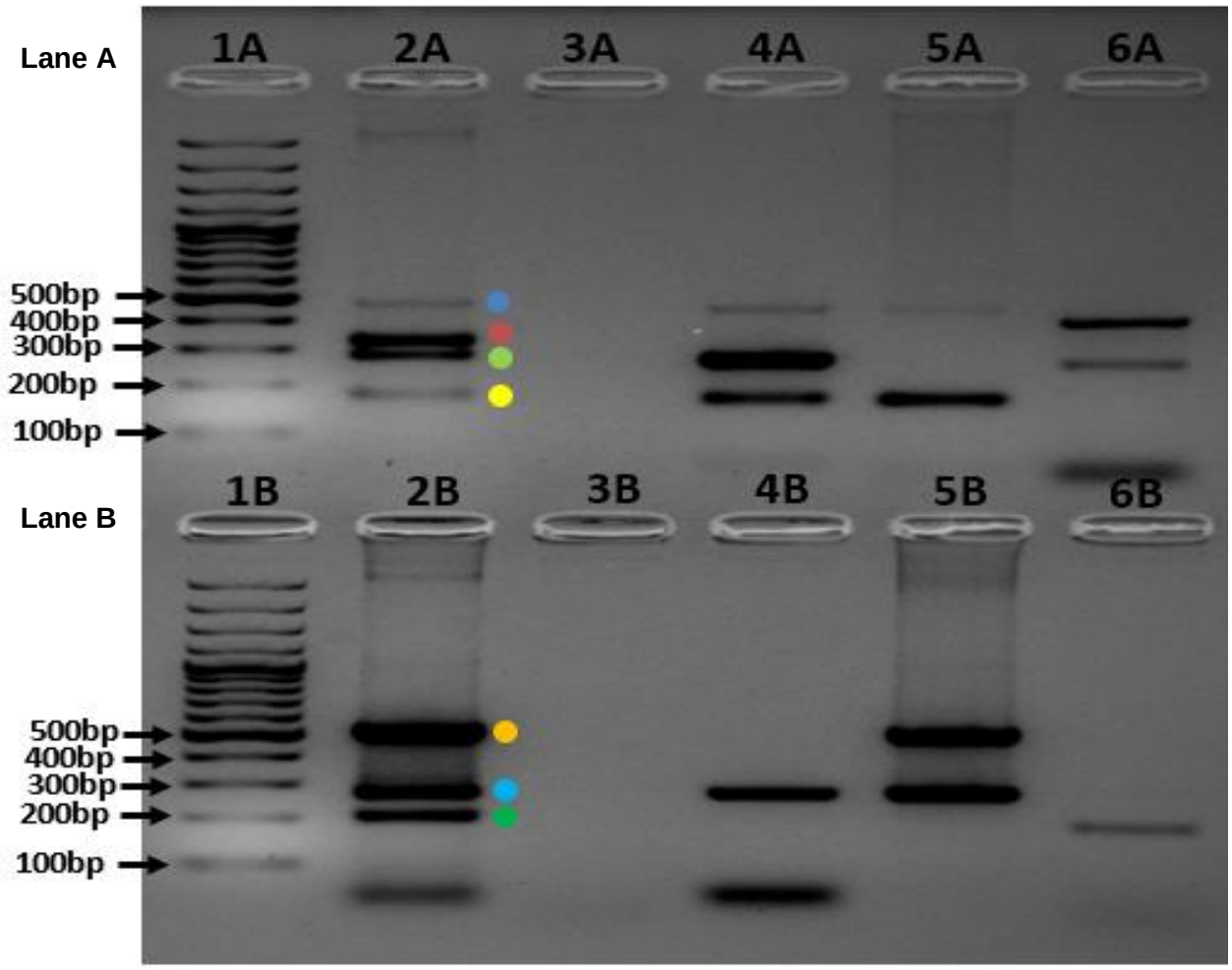
DNA extracted from smear on a microscope slide (n=95, Table 3.5) collected during passive surveillance of carcasses in KNP during a *Bacillus anthracis (B. anthracis)* outbreak in 2010, were used. *B. anthracis* information regarding the microscopy and virulence PCR assay were available for each slide/sample. DNA was extracted using a crude boil method (*C. perfringens* and *C. sordellii* positive controls), as well as a QIAamp DNA Blood Mini Kit (Qiagen^{TM}) according to the manufacturer's instructions. The 2010 KNP smear collection was screened for *Clostridium* spp using the two *Clostridium* species-specific multiplex PCR assays as described in Section 3.2.1. The smears were subjected to microscopy and molecular analyses for the detection of *Clostridium* spp.

3.3 Results

3.3.1 Selection and optimisation of *Clostridium* species multiplex polymerase chain reaction assays

C. botulinum control produced a PCR product of 327 bp of the neurotoxin gene region, *C. perfringens* control produced 279 bp PCR product of the 16S rDNA gene region and *C. sordellii* control amplified 176 bp of the phospholipase C gene region (Figure 3.1, Lane/Row A). *Clostridium* multiplex PCR assay 2 amplified 516 bp of the A flagellin gene region of the

C. chauvoei control, 270 bp of the alpha toxin hemolysin gene region of the *C. septicum* control, and 203 bp of the tetatnus toxin gene region of the *C. tetani* (Figure 3.1, Lane/Row B). The optimum annealing temperature for *Clostridium* multiplex PCR assay 1 and 2 was 57°C and 53°C, respectively. This was determined by selecting the annealing temperature with the most intense PCR amplification of all the positive controls for each multiplex PCR assay.



The optimum annealing temperature for *Clostridium* multiplex PCR assay 1 (which screens for *C. botulinum*, *C. novyi*, *C. sordellii* and *C. perfringens*) was 53°C, and for *Clostridium* multiplex PCR assay 2 (*C. chauvoei*, *C. septicum* and *C. tetani*) it was 57°C using gradient PCR (results not shown). *Clostridium* multiplex PCR assay 1 amplified 472 bp of the *C. novyi* type A flagellin gene region, 327 bp of the Note: Lanes marked with A consist of samples that have been subjected to *Clostridium* multiplex PCR assay 1 (C. botulinum, C. novyi, C. sordellii and C. perfringens) and lanes marked B consist of samples that have been subjected to *Clostridium* multiplex PCR assay 2 (*C. chauvoei, C. septicum* and *C. tetani*). Lanes marked 1A and 1B contain Bioline 100 bp molecular weight marker. Lanes 2A and 2B contain the positive controls for each assay. Lane 2A contain the positive controls for *Clostridium* multiplex PCR assay 1 (*C. novyi* – 472 bp (blue dot); C. botulinum – 327 bp (red dot); *C. perfringens* – 279 bp (lime green); and *C. sordellii* – 176 bp (yellow dot). Lane 2B contains the positive controls for *Clostridium* multiplex PCR assay 2 (*C. chauvoei* – 516 bp (orange dot); *C. septicum* – 270 bp (turquoise dot); *C. tetani* – 203 bp (green dot). Lanes 3A and 3B are non-template controls (negative control). Lane 4A contains C. *novyi, C. perfringens* and *C. sordellii*. Lane 5A contains *C. novyi* and *C. sordellii*. Lane 6A contains *C. novyi* and *C. perfringens*. Lane 4B contains *C. septicum*. Lane 5B contains *C. chauvoei* and *C. septicum*. Lane 6B contains *C. tetani*.

Figure 3.1: Polymerase chain reaction products of *Clostridium* multiplex polymerase chain reaction assays on 2% agarose

3.3.2 *Clostridium perfringens* toxin typing multiplex polymerase chain reaction assay

The *C. perfringens* type A positive control produced a PCR product of 324 bp, which identified it as *C. perfringens* toxin type A (CPA) (alpha toxin shared by all types). Samples that were tested and found to be positive for *C. perfringens* were then subjected to the *C. perfringens* toxin typing multiplex PCR assay (see section 3.3.3).

3.3.3 Screening 2010 smear DNA collection of Kruger National Park and data analysis

All the 2010 smear samples from KNP included in this study were subjected to the Giemsa stain, and the morphology of each slide/sample is indicated in Table 3.4. The samples all had Gram-positive rods with blunt or rounded ends, while some samples had endospores (Table 3.1). All the smears were collected during an anthrax outbreak in KNP in 2010. The samples positive for *B. anthracis* were already known and are indicated in Table 3.4. The DNA collection included smears from kudu, impala, baboon, warthog, buffalo, nyala, elephant, zebra, leopard, and white rhinoceros and was collected mostly from the northern regions of KNP.

Table 3.4: Information of Kruger National Park smear collection from 2010 indicating microscopy and polymerase chain reaction results regarding *Bacillus anthracis* and *Clostridium* species

Sample Lab no.	Animal species	Carcass age	Microscopy (Giemsa stain)	*Bacillus anthracis* PCR Results	Clostridium PCR Result	*Clostridium* species detected
AH001	Kudu	2 days	Positive - Bacilli observed	Positive	Negative	
AH002	Impala	1 week	Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. sordellii*
AH003	Impala	Same day	Positive - Bacilli spores observed	Positive	Negative	
AH004	Impala	Same day	Positive - Bacilli observed	Positive	Negative	
AH005	Kudu	2 days	Positive - Bacilli spores observed	Positive	Negative	
AH006	Impala	Same day	Positive - Bacilli observed	Positive	Not tested	
AH007	Baboon	Same day	Positive - Bacilli observed	Positive	Negative	
AH008	Impala	2 days	Negative	Negative	Not tested	
AH009	Warthog	2 days	Positive - Bacilli observed	Negative	Not tested	
AH010	Kudu	2 days	Positive - Bacilli and spores observed	Positive	Positive	*C. perfringens; C. sordellii; C. septicum*
AH011	Buffalo		Positive - Bacilli observed	Positive	Negative	
AH012	Buffalo		Positive	Positive	Negative	
AH013	Buffalo		Positive - Bacilli and spores observed	Positive	Negative	
AH014	Buffalo		Positive - Bacilli and spores observed	Positive	Positive	*C. perfringens; C.sordellii*
AH015	Kudu		Positive	Negative	Positive	*C. perfringens; C. sordellii; C. septicum; C. novyi*
AH016	Buffalo		Positive - Bacilli spores observed	Positive	Positive	*C. perfringens*
AH017	Kudu		Positive - Bacilli spores observed	Positive	Positive	*C. perfringens; C. tetani*
AH018	Nyala		Positive - Bacilli observed	Positive	Positive	*C. septicum*
AH019	Buffalo	3 days	Positive - Bacilli and spores observed	Negative	Positive	*C. septicum*
AH020	Buffalo	3-5 days	Positive - Bacilli and spores observed	Positive	Positive	*C. perfringens*

Table 3.4 (Continued)

Sample Lab no.	Animal species	Carcass age	Microscopy (Giemsa stain)	Bacillus anthracis PCR Results	Clostridium PCR Result	Clostridium species detected
AH021	Buffalo		Positive - Bacilli and spores observed	Negative	Not tested	
AH022	Buffalo		Positive - Bacilli and spores observed	Negative	Negative	
AH023	Buffalo		Positive - Bacilli spores observed	Positive	Positive	*C. perfringens; C. sordellii*
AH024	Buffalo		Positive - Bacilli and spores observed	Positive	Positive	*C. perfringens; C. sordellii; C. septicum; C. tetani*
AH025	Buffalo		Negative	Negative	Negative	
AH026	Nyala		Positive - Bacilli and spores observed	Positive	Positive	*C. sordellii*
AH027	Kudu		Positive - Bacilli observed	Positive	Negative	
AH028	Buffalo		Positive - Bacilli spores observed	Positive	Positive	*C. sordellii*
AH029	Buffalo		Positive - Bacilli and spores observed	Negative	Negative	
AH030	Buffalo		Positive - Bacilli observed	Negative	Negative	
AH031	Buffalo		Positive - Bacilli observed	Negative	Negative	
AH032	Kudu	1 week	Positive - Bacilli observed	Positive	Not tested	
AH033	Buffalo		Positive - Bacilli observed	Positive	Negative	
AH034	Impala		Positive	Positive	Positive	*C. perfringens; C. septicum*
AH035	Buffalo		Negative	Positive	Positive	*C. perfringens*
AH036	Buffalo		Positive - Bacilli observed	Negative	Negative	
AH037	Nyala		Positive - Bacilli and spores observed	Positive	Positive	*C. perfringens*
AH038	Impala		Positive - Bacilli and spores observed	Positive	Positive	*C. perfringens; C. septicum*
AH039	Buffalo		Positive - Bacilli observed	Positive	Negative	
AH040	Buffalo		Positive - Bacilli and spores observed	Positive	Positive	*C. sordellii; C. septicum*
AH041	Buffalo		Positive - Bacilli observed	Positive	Negative	

Table 3.4 (Continued)

Sample Lab no.	Animal species	Carcass age	Microscopy (Giemsa stain)	Bacillus anthracis PCR Results	Clostridium PCR Result	Clostridium species detected
AH042	Buffalo		Positive - Bacilli and spores observed	Negative	Positive	*C. perfringens; C. septicum; C. sordellii*
AH043	Buffalo		Positive - Bacilli and spores observed	Positive	Negative	
AH044	Buffalo	3 days	Positive - Bacilli observed	Positive	Negative	
AH045	Kudu	3 days	Positive - Bacilli observed	Positive	Positive	*C. septicum*
AH046	Kudu		Positive - Bacilli and spores observed	Positive	Positive	*C. septicum; C.sordellii*
AH047	Buffalo	2 days	Positive - Bacilli observed	Positive	Negative	
AH048	Elephant	1 week	Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. septicum; C. sordellii; C. chauvoei*
AH049	Buffalo		Positive - Bacilli observed	Positive	Positive	*C. sordellii*
AH050	Impala	2 days	Negative – no evidence of bacteria or spores	Positive	Positive	*C. sordellii*
AH051	Elephant	1 week	Positive - Bacilli and spores observed	Positive	Negative	
AH052	Elephant	5 days	Positive - Bacilli and spores observed	Positive	Positive	*C. septicum; C. tetani*
AH053	Buffalo		Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. sordellii; C. septicum; C.novyi*
AH054	Kudu		Positive - Bacilli spores observed	Positive	not tested	
AH055	Buffalo		Positive - Bacilli observed	Negative	Positive	*C. perfringens*
AH056	Buffalo		Positive - Bacilli and spores observed	Positive	Negative	
AH057	Kudu	2 days	Positive - Bacilli observed	Positive	Positive	*C. septicum*
AH058	Kudu	fresh	Positive - Bacilli and spores observed	Positive	Positive	*C. sordellii; C. septicum*
AH059	Buffalo	1 week	Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. sordellii*
AH060	Kudu	fresh	Positive - Bacilli observed	Positive	Positive	*C. septicum; C. chauvoei*

Table 3.4 (Continued)

Sample Lab no.	Animal species	Carcass age	Microscopy (Giemsa stain)	Bacillus anthracis PCR Results	Clostridium PCR Result	Clostridium species detected
AH061	Buffalo	3 days	Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. sordellii; C. septicum; C.novyi; C. chauvoei*
AH062	Buffalo	4 days	Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. sordellii; C. septicum*
AH063	Buffalo	3 days	Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. septicum*
AH064	Buffalo	1 day	Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. sordellii; C. novyi*
AH065	Kudu	1 week	Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. sordellii; C. septicum*
AH066	Buffalo	2 days	Positive - Bacilli and spores observed	Negative	Positive	*C. perfringens; C. sordellii; C. septicum*
AH067	Buffalo	2 days	Negative – no evidence of bacteria or spores	Negative	Negative	
AH068	Impala	2 days	Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. sordellii; C. septicum; C. novyi*
AH069	Buffalo		Positive - Bacilli observed	Negative	Positive	*C. perfringens; C. sordellii*
AH070	Kudu		Positive - Bacilli observed	Positive	Negative	
AH071	Kudu		Positive - Bacilli observed	Positive	Positive	*C. perfringens*
AH072	Buffalo		Negative	Positive	Positive	*C. perfringens; C. sordellii*
AH073	Buffalo		Positive - Bacilli observed	Positive	Positive	*C. perfringems; C. sordellii; C. septicum*
AH074	Kudu		Negative	Positive	Positive	*C. perfringems; C. sordellii; C. septicum*
AH075	Buffalo		Negative	Positive	Positive	*C. perfringens; C. sordellii; C. septicum; C. novyi*
AH076	Impala		Positive - Bacilli observed	Positive	Positive	*C. sordellii; C. septicum*
AH077	Kudu		Negative	Positive	Positive	*C. perfringens; C. sordellii; C. septicum*
AH078	Elephant		Positive - Bacilli and spores observed	Positive	Positive	*C. perfringens; C. sordellii; C. septicum; C. novyi*
AH079	Impala		Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. sordellii; C. septicum; C. novyi*
AH080	Kudu		Positive - Bacilli observed	Negative	Negative	
AH081	Kudu		Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. sordellii; C. septicum*
AH082	Buffalo		Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. sordellii*
AH083	Nyala		Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. sordellii*
AH084	Nyala		Negative	Positive	Negative	

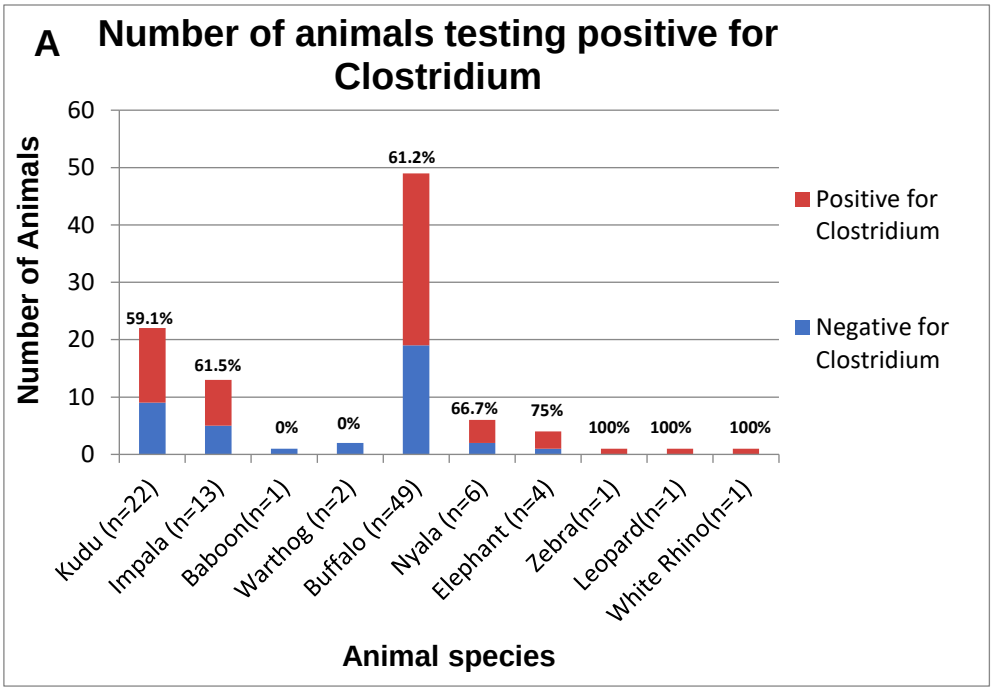
Table 3.4 (Continued)

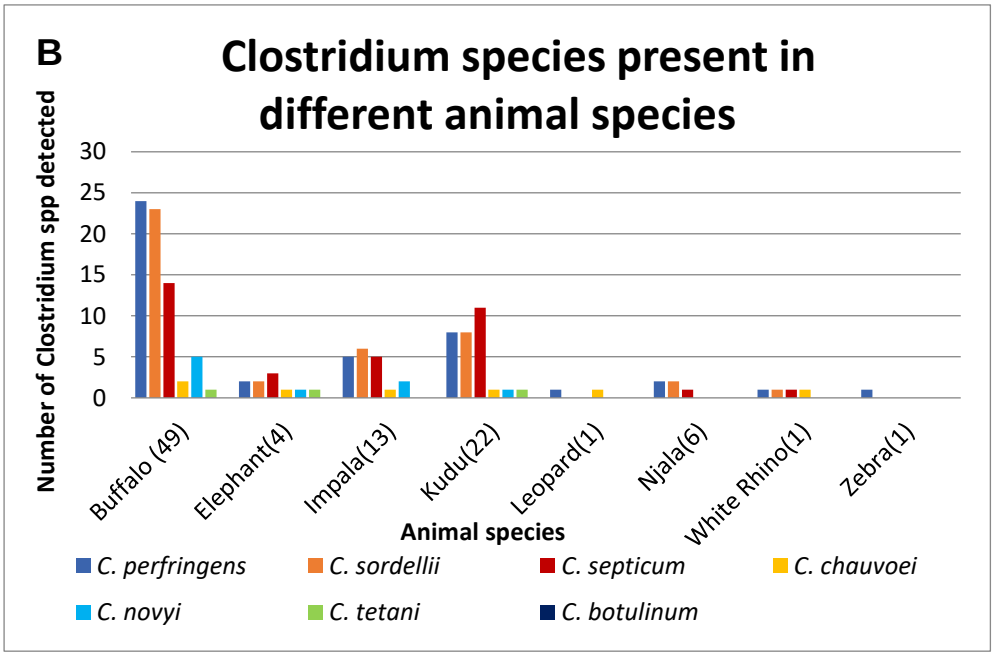
Sample Lab no.	Animal species	Carcass age	Microscopy (Giemsa stain)	Bacillus anthracis PCR Results	Clostridium PCR Result	Clostridium species detected
AH085	Buffalo		Negative – no evidence of bacteria or spores	Positive	Negative	
AH086	Kudu		Positive - Bacilli observed	Positive	Negative	
AH087	Kudu		Positive - Bacilli observed	Positive	Negative	
AH088	Buffalo		Positive - Bacilli observed	Positive	Positive	*C. perfringens*
AH089	Buffalo		Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. sordellii; C. septicum*
AH090	Buffalo		Positive - Bacilli observed	Positive	Positive	*C. sordellii; C. septicum*
AH091	Buffalo		Positive - Bacilli observed	Negative	Positive	*C. perfringens; C. sordellii; C. chauvoei*
AH092	Buffalo		Negative	Positive	Positive	*C. perfrinegns; C. sordellii*
AH093	Impala		Positive - Bacilli observed	Positive	Positive	*C. sordellii; C. septicum; C. chauvoei*
AH094	Nyala		Negative	Positive	Negative	
AH095	Impala		Positive - Bacilli observed	Positive	Negative	
AH096	Warthog		Positive - Bacilli observed	Positive	Negative	
AH097	Zebra		Positive - Bacilli and spores observed	Positive	Positive	*C. perfringens*
AH098	Leopard		Positive - Bacilli observed	Positive	Positive	*C. perfringens; C. chauvoei*
AH099	Buffalo		Positive - Bacilli observed	Negative	Positive	*C. perfringens; C. sordellii*
AH100	White Rhino		Positive - Bacilli observed	Negative	Positive	*C. perfringens; C. septicum; C. sordellii; C. chauvoei*

Note: Rows marked in red tested negative for *Clostridium* organisms, Blue tested positive for *Clostridium* organisms and rows marked with green could not be tested due to a lack of DNA needed to perform the test.

Multiplex PCR assay analysis of the 95 KNP smear samples indicated that 61 samples (64.2%) were positive for Clostridia, while 34 samples (35.8%) were negative. The samples were collected from the central to the northern parts of KNP. The host species affected are indicated in Figure 3.2A, which mainly includes host species susceptible to anthrax as the samples were collected during an anthrax outbreak. The 61 *Clostridium* positive samples were further analysed, and 45/61 were identified as *C. perfringens*, 42/61 *C. sordellii*, 35/61 *C. septicum*, 8/61 *C. novyi,* 7/ 61 *C. chauvoei*, and 3/61 *C.tetani*. Therefore, various samples had multiple *Clostridium* spp. detected. No *C. botulinium* was detected in any of the samples tested (Figure 3.2B).

Figure 3.2A indicated that of the 49 buffalo samples tested, 30/49 (61.2%) were positive for Clostridia which most samples being positive for more than one *Clostridium* spp. Buffalo was also the animal species which was found to be the most affected by *B. anthracis* of which 49/95 were positive (Figure 3.2B). From the positive buffalo smears various *Clostridium* spp were detected which included 6/7 screened *Clostridium* spp. This can be seen by the 24/30 positives for *C. perfringens*, 23/30 for *C. sordellii,* 14/30 for *C. septicum*, 5/30 for *C. novyi,* 2/30 for *C. chauvoei* and 1/30 for *C. tetani*. From the 22 kudu samples, 59.1% (13/22) tested positive for Clostridia. *B. anthracis* infections were found in 19/22 of the kudu smears. Clostridia found in the kudu smears consisted of *C. perfringens* (8/13), *C. sordellii* (8/13), *C. septicum* (11/13), *C. novyi* (1/13), *C. chauvoei* (1/13), and *C. tetani* (1/13). Impala samples (61.5%; 8/13) were positive for Clostridia and 100% for *B. anthracis*. The *Clostridium* spp detected in the impala samples consisted of *C. perfringens* (5/8), *C. sordellii* (6/8), *C. septicum* (5/8), *C. novyi* (2/8) and *C. chauvoei* (1/8).





Note: The distribution includes the animal species and the different pathogenic *Clostridium* species identified in each animal species.

Figure 3.2: (A) Number of *Clostridium* positive samples detected with multiplex Clostridium PCR assay in different host species (Sample number of each host species indicated in x-axis with Clostridium PCR positive percentage above each host species) (B) Number of different pathogenic *Clostridium* species that were detected using the developed multiplex PCR screening assays with number of samples of each animal species indicated in brackets on x-axis

A breakdown of all the different pathogenic *Clostridium* spp found in each animal species can is seen in Figure 3.2B. The multiplex PCR assays can only be used to determine the *Clostridium* spp present in the environment since the samples were collected from animals that died during an anthrax outbreak in 2010 in the northern part of KNP. All of the samples that were 16S rRNA PCR-positive for *C. perfringens* were *C. perfringens* type A.

3.4 Discussion

In this study, multiplex *Clostridium* PCR assays were developed to detect *C. perfringens* and their toxin types, *C. novyi*, *C. chauvoei*, *C. tetani*, *C. septicum*, *C. sordellii* and *C. botulinum* from smears made of carcasses during an anthrax outbreak in 2010 in KNP. When the animal succumbs to the disease, the endospores of the *Clostridium* allow the organism to remain in the environment. Most pathogenic Clostridia are obligate anaerobes (Cato et al., 1986) and are present in the environment and animal, which makes identification and diagnosis difficult. The multiplex PCR assays detected *C. perfringens* type A, *C. sordellii*, *C. septicum*, *C. novyi* and *C. chauvoei*. These species are expected since Clostridia are soil-borne bacteria persisting in the environment. Yang et al. (2017) described that most of the bacteria from the phylum *Firmicutes* (of which *Clostridium* is a part) can be isolated and identified in all carcasses and surrounding areas, especially in soil (Yang et al., 2017) and poses a potential risk for susceptible animals as the endospores remain in this environment (Haagsma, 1991). This study indicated that the PCR assays can be used to determine the *Clostridium* spp present in the environment to which animals are exposed or will be exposed to during translocation/relocation to a specific environment.

Accurate diagnosis of clostridial disease includes anaerobic culturing from the intestine or tissue of an animal where these organisms are commonly found supported by detection of toxins and or histopathological which make use of FAT. PCR can be used in diagnostics as a clinical conformation test, as well as being used for the detection of Clostridia DNA in the environment (Keto-Timonen et al., 2006; Bagge et al., 2009; De Medici et al., 2009). The multiplex PCR assays detect the different *Clostridium* DNA in the environment and determine the toxin type of *C. perfringens.* PCR can furthermore confirm culture identification, FAT or pathology results, allowing for better and more definitive diagnosis.

Validated *Clostridium* specific PCRs were used to develop the multiplex PCR assays. The multiplex PCR assays were used on a DNA collection from carcasses during an anthrax outbreak with microscopic *Clostridium* detection. The multiplex PCR assays detected Clostridia which is expected as most of these Clostridia can be identified from carcasses and surrounding areas (Yang et al., 2017). The *C. botulinum* primers (created by Prevot et al. (2007)) included in

the multiplex PCR was designed to detect both *C. botulinum* types C and D. Prevot et al. (2007) confirmed his PCR results by comparing PCR results with mouse toxin neutralisation assay results. In the study, no *C. botulinum* was detected in the samples tested. The *C. chauvoei* PCR specificity and sensitivity was tested and Kojima et al. (2001) reported that the PCR detect as little as 200 *C. chauvoei* cells in a sample. From the smear collection, seven *C. chauvoei* were detected. The *C. novyi* PCR's specificity was tested by Sasaki et al. (2002) against a panel of *Clostridium* and *Bacillus* species as well as clinical samples. In our study, eight *C. novyi* were detected mainly from buffalo. Wu et al. (2009) developed and used *C. perfringens* specific primers to detect and differentiate the organism from different bacteria (11 *Clostridium* spp as well as 38 other clinically important bacteria) in samples. This is very important seeing as the most clinical veterinary samples could be contaminated with other bacteria. In our study, a total of 74% (45/61) *C. perfringens* were detected amongst all the different bacteria in the samples. The C*. perfringens* primers target the 16S rRNA gene and the specific PCR can detect 50 copies of DNA (Wu et al., 2009). However, all of the *C. perfringens* were type A, which is not associated with animals diseases (Labbe, 2003). Takeuchi et al. (1997) reported that the *C. septicum*-specific PCR detect as little as 3.8×10^{1} cells per 10 µl PCR reaction and also tested the specificity against a large panel of other organisms. In our study, 57% (35/61) *C. septicum* were detected. The *C. sordellii* PCR was developed by Bhatnagar et al. (2012) and the primers were specifically tested against 32 other *Clostridium* spp for specificity and detect 2 ng/µl of *C. sordellii* DNA. In the current study, a total of 69% (42/61) *C. sordellii* was detected. *Clostridium tetani* specific primers was developed and tested by Saadatmandzadeh et al. (2011). Primers were tested using a panel of microorganisms and could detected 1.96×10^{2} copies of the target DNA. In the study a total of 2 *C. tetani* were detected.

Most of the studies on Clostridia are done on domestic animals with little known of the impact of Clostridia on wildlife. The smear samples from wildlife in the northern parts of KNP indicated that a large number of Clostridia were present in the environment. The samples that were tested indicated that *C. perfringens* type A (74%) is the most prominent species found, followed by *C. sordellii* (69%) and *C. septicum* (57%). Multiplex PCR assays indicated the least prominent Clostridia in decreasing order 13% *C. novyi,* 12% *C. chauvoei*, 5% *C. tetani* and no *C. botulinium*. As mentioned, these diseases are well described in livestock, but with little information in wildlife, and might pose a risk to wildlife in environments where it occurs (Kriek, 2015). Although many veterinarians report that they do clinically see clostridial diseases in animals, these observations are not supported by culturing, detection of the toxin and/or histopathology using FAT. This is due to the anaerobic nature of Clostridia as well as other factors such as the sampling period after death, the period of the sample at the laboratory, location of samples (where it is relevant or not), where the smear is taken to be tested, as already

mentioned (Uzal et al., 2016). Uzal et al. (2016) has described the clinical signs for each of the pathogenic Clostridia and many of the signs are common among most of the species but there are no specific general sights for clostridial diseases. *Clostridium perfringens* has been reported to cause death in black rhinoceros in Kenya (Ndeereh et al., 2012), without indicating exact evidence of diagnosis. De la Fe (2006) identified *C. sordellii* to cause acute clostridiosis in captive bred lions (*Panthera leo*) based on the fulminant course of the disease, isolation and gross and histological findings. In a case report, *C. novyi* was reported in big horn sheep (*Ovis candensis)* with myocardial empysema and necrosis diagnosed through necropsy, histopathology and FAT (Redford, 2017). The use of the developed multiplex *Clostridium* PCR assays could confirm *Clostridium* spp and aid diagnosis or could be used to detect *Clostridium* spp present in specific environment.

3.5 Summary

In this study two PCR multiplex assays were successfully optimised for the detection of the seven most commonly known pathogenic *Clostridium* species, which included *C. perfringens* (enterotoxaemia), *C. septicum* (malignant oedema), *C. sordellii* (gas gangrene), *C. chauvoei* (blackleg/black quarter), *C. botulinum* (botulism), *C. novyi* (black disease), and *C. tetani* (tetanus). The PCR multiplex assays were tested on samples collected in 2010 in the northern parts of the Kruger National Park during anthrax outbreaks. The screening revealed numerous *Clostridium* species in the environment with *C. perfringens* type A, *C. sordellii,* and *C. septicum* to be the most prominent species, while *C. novyi, C. chauvoei*, and *C. tetani* were less prominent with no *C. botulinium* detected in the tested samples. This assay can be used to monitor Clostridia in environments or to confirm culture and FAT results.

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Appendix 3A
Cloned control sequences and target regions

Clostridium chauvoei: Gene for flagellin protein FliB(C), complete cds (NCBI accession no. AB058932.1)

ATCGGAAACATGAGTGCTGCAAGTTTAGGATTAACTACAACTAACTCACTTGAAAGCAAAGCATTAG
CATTAAATAGTAACTTAGCTGATGGAAGTTATAAAATATCAGGAACTAATTTAGTAGATACAAATGGG
AATACTGTTGGTACATTTAATAGTGGAGCTAAAAAGATAGTAGTTAATGGACAAGATACAGTATTTAC
TAAAGCAGCATTAGCTGATGGTGCTGTTTTAACTGTTAAAGGTGGAATTGCTGATATTAAAAATACA
ATGACAGGAGCAGCTAAAAAATTATCATCAGGAAGTTATGAAATTTCAGGAACTAATGTAATAAAAG
ATGGTAAATTAGTAGGAACATTTGCAACAGGTGCTAAGAAGTTAACTATTGATGGCGTTGGAGATGT
TACAGAAGCGGAATTAGGATTTATAACAGACCAAATGAAAGATGGAGTTAAGTTTACAATAAATGGT
TCAGATGTTTCAACTAGAGAATTAGCTAGCGGAAGCATAAAGACT

Clostridium novyi: Gene for flagellin protein FliA(A), complete cds (NCBI accession no. AB058936.1)

CTTAAAAATTCAAGGAGGAATTTTATAATGATTATTAATCACAATTTAAATGCAATGAATGCTCACAG
AATGATGGGCCAAAACATTACTGGTGCTGGAAAAGCAATGGAAAAATTATCTTCAGGATTAAGAATA
AACAGAGCTGGAGATGATGCAGCAGGACTAGCAATCTCAGAAAAAATGAGAGGACAAATCAGAGG
ATTAAACCAAGCATCAAGAAATGCACAAGATGGTATATCATTAATCCAAACAGCTGAAGGAGCTTTA
AATGAAACTCATGCAATCCTTCAAAGAATGAGAGAATTAGCAGTTCAAACATCTAATGATACAAACA
CAACTGCAGATAGAACAAATGTACAAAAAGAAATAGCTCAATTAAAAGAAGAAATCACAAGAATTTC
AACAGATACAGAATTCAATACTAAAAAGTTATTAAATGGATCAGCAAAAGGAGTAACTTTCCAAGTA
GGCG

Clostridium tetani: Tetanus toxin gene, complete cds (NCBI accession no. AF154828.1)

CCGAAAGATGGAAATGCCTTTAATAATCTTGATAGAATTCTAAGAGTAGGTTATAATGCCCCAGGTA
TCCCTCTTTATAAAAAAATGGAAGCAGTAAAATTGCGTGATTTAAAAACCTATTCTGTACAACTTAAA
TTATATGATGATAAAGATGCATCTTTAGGATTAGTAGGTACCCATAATGGTCAAATAGGCAACGATC
C

Clostridium septicum: Gene for alpha-toxin, complete cds (NCBI accession no. D17668.1)

AATTCAGTGTGCGGCAGTAGTACCACATGTACAAGCTTATGCACTTACAAATCTTGAAGAGGGGGG
ATATGCAAATCATAATAATGCTTCTTCAATTAAAATATTTGGATATGAAGACAATGAAGATTTAAAAG
CTAAAATTATTCAAGATCCAGAGTTTATAAGAAATTGGGCAAATGTAGCTCATTCATTAGGATTTGGA
TGGTGCGGTGGAACGGCTAATCCAAATGTTGGACAAGGTTTTGAATTTAAAAGAGAAGTTGGGGCA
GG

Clostridium botulinum type D: phage gene for ORF-22, HA3, HA2, HA1, NTNH, NTX, complete and partial sequence (NCBI accession no. AB012112.1:10089-10416)

TTTATACGAGAATGTTCTGTAACATACTTATTTAAAAATATGCTCCCTAAAGTAATTGACGAATTAAAT
AAGTTTGATTTAAGAACTAAAACAGAATTAATTAATCTTATAGATAGTCATAATATTATTCTAGTTGGT
GAAGTAGATAGATTAAAAGCAAAAGTAAATGAGAGTTTTGAAAATACAATGCCTTTTAATATTTTTTC
ATATACTAATAATTCTTTATTAAAAGATATAATTAATGAATATTTCAATAGTATTAATGATTCAAAAATT
TTGAGCTTACAAAACAAAAAAAATGCTTTAGTGGATACATCAGGATATAATG

Clostridium perfringens strain ATCC 13124 16S ribosomal RNA, complete sequence (NCBI accession no. NR_121697)*

AGCAATCCGCTATGAGATGGACCCGCGGCGCATTAGCTAGTTGGTGGGGAACGGCCTACCAAGG
CGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAGACT
CCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGCCGCG
TGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTCTTTGGGGAAGATAATGACGGTACCCAAGGA
GGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC

Clostridium sordellii strain AIP82 phospholipase C gene, complete (NCBI accession no. KM657127)*

AAACAATTTAACAAATGAATTTAATGTAGTTTTAAAAACTGCAGACAATGAATATGCAGGAACTGACG
ATAATGTATATTTTGGATTTGAAACTAAAGATGGAAGTAAGTATGAATGGAATTTAGATAATGCAGGA
AATGATTTTGAAAAAAATCAAACAGATAATTATATACTAAA

*The positive controls were from the bacteriology diagnostic laboratory, Ondertepoort campus, University of Pretoria. These isolates were from field samples and were confirmed to be the correct organisms by amplifying and sequencing the amplicons that were generated from the PCR. Sequences where then confirmed using the Blast tool from the National Centre for Biotechnology Information.

Chapter 4

Genotyping of South African *Bacillus anthracis* from the Kruger National Park Using 10 Multi-Loci Variable Number Tandem Repeat Analysis

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4.1 Introduction

Bacillus anthracis (*B. anthracis*) is a genetically homogenous/ monomorphic bacterial species due to its ability to produce spores and remain dormant for decades and even centuries (Keim et al., 2000). This ability (spore formation) may play a role in the greatly reduced rate of evolutionary change in *B. anthracis*, which may contribute to the extremely homogeneous nature of the bacterium (Keim et al., 1997). The lack of molecular polymorphism within *B. anthracis* is well documented by numerous studies (Harrel et al.1995; Henderson et al. 1995; Keim et al., 1997). In a study conducted by Keim et al. (1997), amplified fragment length polymorphisms (AFLP) indicated that there were only 30 differences among >1,000 DNA fragments of *B. anthracis*. The study also indicated that, many of these AFLP markers have low diversity values and little discriminatory power.

To study the lineages and strains of *B. anthracis*, numerous molecular methods/approaches can be use but the two most commonly and widely used include, multi-locus variable number tandem repeat assay (MLVA) and whole genome single nucleotide polymorphism (SNP) discovery and analysis. These methods have greatly enhanced the ability to study *B. anthracis* (Van Ert et al. 2007).

Due to *B. anthracis'* near monomorphic molecular nature, the MLVA may be the only reasonable method with which to study the diversity, evolution, and molecular epidemiology of this bacterium (Keim et al., 1997). The analysis of variable number tandem repeat (VNTR) sequences is one of the more accessible methods in molecular genotyping of bacteria (Frothingham and Meeker-O'Connell, 1998; Keim et al., 1999; van Belkum et al., 1998). Short nucleotide sequences which are repeated multiple times (often vary in copy number), create lengthy polymorphisms which in turn can easily be detected with flanking primers in PCR. VNTRs are known to contain a greater molecular diversity than other methods, resulting in greater discriminatory capacity than any other molecular typing system (Richards and Sutherland, 1997). While it can be carried out on a genetic analyser using fluorophores, capillary electrophoresis is costly and not available in all laboratories (Hassim, 2011). If repeat unit sizes greater than 9 bp are employed and the appropriate known controls are used consistently, then MLVA is applicable in most laboratories using agarose electrophoresis (Vergnaud and Pourcell, 2006). MLVA is primarily used in cluster analysis of species within a genus, as well as amongst/between serovars for many pathogenic bacteria (e.g. Salmonella species/serotypes, *Escherichia coli*, *Mycobacterium tuberculosis*, *Klebsiella* species and *Brucella* species (Vignaud et al., 2017; Hill et al., 2012; Helldal et al., 2017; Derakhshan et al., 2017). Thus, the tolerance and weights for clustering analysis differs for each organism to the genetic structure and behaviour of the pathogen of interest (Al Dahouk et al., 2007).

For *B. anthracis*, multi-locus variable number tandem repeat assays based on 8, 15, 25 and 31 VNTR panels have been described and used to genotype strains (Keim et al., 2000; Lista et al., 2006; Van Ert et al., 2007a,b; Beyer et al., 2012). *B. anthracis*, clustering/differentiation is taking place within a single species of bacterium that has the ability to be dormant and hence genetically stable for prolonged periods in the environment. The tolerance is thus set at 0 for cluster analysis. Phylogenetic trees, using MLVA, cluster the *B. anthracis* isolates in A-, B- and C-clades, with most of the *B. anthracis* isolates clustering in the A-clade, while clades B and C are located and restricted to more specific geographical areas and are less abundant (Van Ert et al. 2007).

On their own individual SNP panels have very limited resolving power relative to MLVA yet; researchers have used several phylogenetic approaches to identify numerous SNPs that are used to efficiently group bacterial strains into distinct genetic clusters/lineages consistent with their known population structure (Keim et al., 2004; Van Ert et al., 2007; Stephens et al., 2006). Whole genome sequencing of *B. anthracis* strains discovered approximately 3,500 SNPs. The conserved distribution of SNPs within the *B. anthracis* phylogenetic tree was revealed in the observation that only a single character conflict (homoplasy) was detected from more than 25,000 data points. These results indicated thatt a select number of SNPs (representative of specific branches and nodes in the *B. anthracis* SNP-derived tree) would be sufficient to accurately determine the current phylogenetic position of any *B. anthracis* isolates. By using this information, a hypothesis was formulated (Keim et al., 2004). It was suggested that a small number of canonical SNPs (canSNPs)/genetic markers located at key phylogenetic junctions along the *B. anthracis* SNP tree could replace a tedious genome-wide SNP analysis.

However, canSNP analysis, have greatly enhanced the genotyping and phylogenetic analyses among *B. anthracis* isolates (Keim et al., 2009; Pilo & Frey, 2011). *B. anthracis* is divided into three clades, namely A, B and C with further subdivisions into 13 canonical lineages and genetic groups (Van Ert et al., 2007a; Van Ert et al., 2007b; Marston et al., 2011). These genetic markers have allowed better understanding of how isolates fit into regional and global phylogeographic patterns (Pearson et al., 2004; Van Ert et al., 2007a; Van Ert et al., 2007b; Simonson et al., 2009). The added advantage of canSNPs is that they corroborate MLVA cluster analyses very well when diverse lineages are included to highlight strain differences (Vergnaud and Pourcel, 2006).

The A-clade of *B. anthracis* is internationally distributed and has the most abundantly populated clade and number of subclades (Van Ert et al. 2007; Derzelle and Thierry, 2013).The rare B-clade is the most diverse in southern Africa and KNP in particular, but was thought to have become an extinct lineage (Smith et al., 2000). This clade is known as the KrugerB clade due

to its distinct genetic make-up and niche environmental requirements (Keim et al. 2000, Smith et al. 2000). Due to the large diversity of *B. anthracis* in South Africa, Keim et al. (2000) hypothesised southern Africa to be the origin of the disease anthrax. Thierry et al. (2014) determined the "international" allelic diversity of the MLVA31 to differentiate *B. anthracis* strains and proposed the use of seven agarose friendly VNTR markers (MLVA7) to establish a cost effective first-line assay. In Thierry et al. (2014) the international database of isolates is made up almost exclusively of A-clade isolates (predominantly from Europe and Namibia) and includes the French B clade isolates unique to the Alps. The database used by Thierry et al. (2014) is therefore biased toward the Aα and B2 genotypic clades as described by Pilo and Frey (2011). The South African isolate collection is made up of a small collection of the Aα clade as well as a larger representation of the Aβ (Ancient A) and the B1 (KrugerB) clades (Keim et al. 2001, Van Ert et al. 2007, Pilo and Frey 2011). The discovery of a B-clade isolate in Limpopo in 2011 demonstrated that the B-clade lineage, while rare, was still circulating in the environment in southern Africa (Lekota et al. 2018). The South African copy number database for archival isolates was evaluated for allelic frequencies in a previous research study (Hassim 2011: Appendix 4B). The allelic frequencies identified VNTR markers (Bam 13, 30 and 31) integral to the differentiation between A and B clade isolates and thus included as additional markers to the MLVA7 panel (Hassim 2011; Venter 2017). These specific markers (Bams13, Bams30 and Bams31) were added based on previous findings when studying the South African *B. anthracis* database (Hassim, 2011), but they also code for the *Bcl* genes (*Bacillus* collagen-like proteins) (Lista et al. 2006). These genes have been linked to environmental survival as well as differentiation of *B. anthracis* strains (Leski et al., 2009). The aim of this study was to use the MLVA10 genotyping panel (as proposed by Thierry et al., 2014 and the additional 3 VNTR markers) as a first-line assay to identify *B. anthracis* from suspected and confirmed cases from outbreaks 2014 -2016 in KNP.

4.2 Material and methods

4.2.1 *Bacillus anthracis* isolates from 2014-2016

Samples were collected (bones, hair/hide, soil, leaves, faeces and vultures) from animal carcass sites during 2014 to 2016 and tested and confirmed for *B. anthracis* at the Skukuza State Veterinary Laboratory in Skukuza, South Africa, and the BSL2 Plus laboratory at the Faculty of Veterinary Science, University of Pretoria, South Africa. The outbreaks were concentrated in two regions during this time period, endemic Pafuri on the northern tip of KNP and Singita on the central eastern border of KNP. Both regions border Mozambique. The samples were collected by rangers at the South African National Parks and Skukuza State Veterinary Services

in KNP. Samples were processed, plated out on 5% sheep blood agar and Polymyxin-B-EDTA-Thallous acetate agar, followed by incubation. Sheep blood agar was incubated overnight and Polymyxin-B-EDTA-Thallous acetate agar for 36 hours at 37°C in an aerobic atmosphere, as recommended by Turnbull (2008). Suspect colonies were sub-cultured onto 5% sheep blood agar to determine haemolytic activity and subjected to penicillin and gamma-phage sensitivity testing according to OIE (2012) and Turnbull (2008). The virulence factors of *B. anthracis* identified isolates were confirmed using real-time PCR (section 4.2.3). A final total of 37 isolates were isolated successfully as *B. anthracis*.

4.2.2 DNA isolation and extraction

DNA was extracted from pure *B. anthracis* isolates using the ISOLATE II Genomic DNA Kit (Bioline™, United Kingdom) according to the manufacturer's instruction, which included initial treatment of bacterial cells with 180 µl lysozyme (20 mg/ml, Fluka) that were incubated for 45 min at 37°C. Once DNA was extracted, the samples were stored at –20°C.

4.2.3 Confirmation of *Bacillus anthracis* isolates using quantitative polymerase chain reaction

The conformation of *B. anthracis* isolates were conducted by screening each isolate for the virulence factors that are required for the bacterium to be classified as *B. anthracis.* These targeted virulence factors include the *pag* gene (located on plasmid pX01), the *cap*C gene (located on plasmid pX02) and the *SASP* gene (chromosomal marker). A quantitative polymerase chain reaction (qPCR) Fluorescence Resonance Energy Transfer probe assay was used, using LCRed as a marker dye (TibMolBiol, Germany) (Ellerbrok et al., 2002).

The LightCycler® Nano instrument was used to amplify each sample under the following conditions: Pre-incubation at 95°C for 10 minutes, 45 cycles of denaturation at 95°C for 10 seconds, annealing at 57°C for 20 seconds, with the single acquisition of signals at annealing followed by extension at 72°C for 30 seconds. The melt curve conditions are as follows: 95°C, 0 seconds; slope 20°C per second; 40°C for 30 seconds; slope 20°C per second; 80°C, 0 seconds; slope 0.1 per second, with continuous acquisition of signals. Post-amplification analysis was performed for each sample using automatic quantification and melt curve analysis software from Roche Life Science (2015).

4.2.4 The MLVA7 and MLVA10 schemes

A total of 37 *B. anthracis* isolates DNA were included in the study, as well as positive control *B. anthracis* Vollum, Ames and Sterne strains. MLVA31 data of *B. anthracis* strains (n=42) from

South Africa representing the A and B-clade of Hassim (2017) dating from 1970 to 2013 together with MLVA data generated from the 37 *B. anthracis* from 2014 to 2015 (this study) was evaluated with the first-line assay proposed by Thierry et al. (2014). The MLVA data of the 42 *B. anhtracis* isolates have been included because they have a known MLVA genotypic fingerprints and SNP lineages, which have been validated on whole genome sequencing by Lekota (2018). The MLVA7 is agarose-friendly (with repeat units ranging from 12 bp to 39 bp). The additional VNTR's of the MLVA10 panel (Bams13, Bams30 and Bams31) have 9 bp repeat units which can also be differentiated on agarose, but with longer gel migrations on 3% agarose. All primers and PCRs used in both the MLVA7 and MLVA10 were optimised and validated by Venter (2016) in a previous study.

4.2.5 MLVA panels

The MLVA10 panel consisting of the ten VNTR markers included the MLVA7 panel identified by Thierry et al. (2014), namely Bams03, Bams05, Bams22, Bams34, Bams44, VNTR23 and vrrA as well as Bams13, Bams30 and Bams31. Single PCRs was performed for each marker using the Veriti® 96-Well Thermal Cycler (Applied Biosystems®) in 15 µl reaction volumes, using 7 µl 1× MyTaq™ HS Red Master Mix (Bioline™), 2 µl of 20 µM primer (2 µl forward and 2 µl reverse), 1 µl water and 3 µl template DNA (Table 4.1).

Table 4.1: PCR reaction used for each VNTR marker in the MLVA10 panel

Anthrax PCR reaction (Monoplex) – MyTaq™ Red DNA Polymerase	
Mix component:	**Volume (µl) 1×**
5× MyTaq Red Master Mix	7
Forward primers (20 µM each)	2
Reverse primers (20 µM each)	2
Template DNA	3
Water (ddH_2O)	1
Total volume	15

The PCR conditions consisted of initial denaturation at 95°C for 10 minutes, followed by 35 cycles of 95°C for 45 seconds, 60°C for 30 seconds, 72°C for 30 minutes and a final elongation at 72°C for 10 minutes. *B. anthracis* Vollum strain (A60) was included as positive control where Vollum was loaded at every eighth lane to correct for gel distortions. The amplicons were visualised using a 2% agarose gel (Agarose Molecular Grade, Bioline™) with ethidium bromide (10 ug/ml) under ultraviolet light. The electrophoresis condition consisted of 120 V for 150 minutes. Fragment sizes where determined by using O'GeneRuler 100 bp DNA Ladder (Thermo

Scientific). Once the gel electrophoresis was complete, fragments was visualised using the GelDoc XR+ System from Bio-Rad.

Base-pairs for each VNTR allele were converted into copy numbers using the described algorithm that was suggested by Lista et al. (2006) and Beyer et al. (2012) but using the copy code conversion that was proposed by Thierry et al. (2014). This is achieved by looking at the amplicon length generated for each VNTR locus. The fragment size evaluations are done in relation to the controls Vollum, Sterne and Ames (with known amplicon size as well as repeat unit copy number) which are interspersed among the unknown samples at every 8 lanes on the gel. The amplicon size is then translated to the copy code using the conversion table (Thierry et al., 2014) which denotes the number of repeats present in the expected fragment size range.

4.2.6 Data analysis

Analysis of results was conducted using the Bionumerics software version 6.6 (Applied-Maths) (Belgium) employing the Unweighted pair group method using arithmetic mean (UPGMA) to construct a dendogram (phylogenetic tree) (tolerance set at 0) and minimum spanning tree (MST) using categorical data based on MLVA7 and MLVA10 of *B. anthracis* datasets were used. Tolerance was set at 0 due to clustering taking place within a monomorphic species of bacterium.

4.2.7 Phylogenetic cluster analyses

The reference strains, Ames, Sterne and Vollum, used as internal controls are indicated in red in all the proceeding figures and analyses. Ames and Sterne represent the A3 MLVA cluster which forms the A.Br.001/002 SNP lineage, while Vollum forms part of the A.Br.007 lineage, known as the A4 MLVA clade. The 42 isolates used to evaluate clustering are separated into the Aβ (Ancient A group) which are indicated as dark green (KNP, South Africa) and dark blue (other southern African country isolates). The Aβ cluster belong to the A.Br.005/006 SNP lineage, which is the most represented clade in Africa (Van Ert et al. 2007; Pilo and Frey, 2011; Lekota (2018). The B-clade isolates are indicated in purple (the “KrugerB” lineage) and brown (B.br.001/002 lineage) (Lekota et al. 2018). The yellow and light green labelled genotypes are also A3 MLVA cluster isolates but represent the A.Br.003/004 lineage (Northern Cape Province, South Africa) and A.Br.001/002 lineages (KNP, South Africa) respectively. These isolates (light green) belong to the same lineage as Ames and Sterne, albeit different genotypes. The 37 isolates described in this study were designated by the teal colour for ease of identification.

4.3 Results

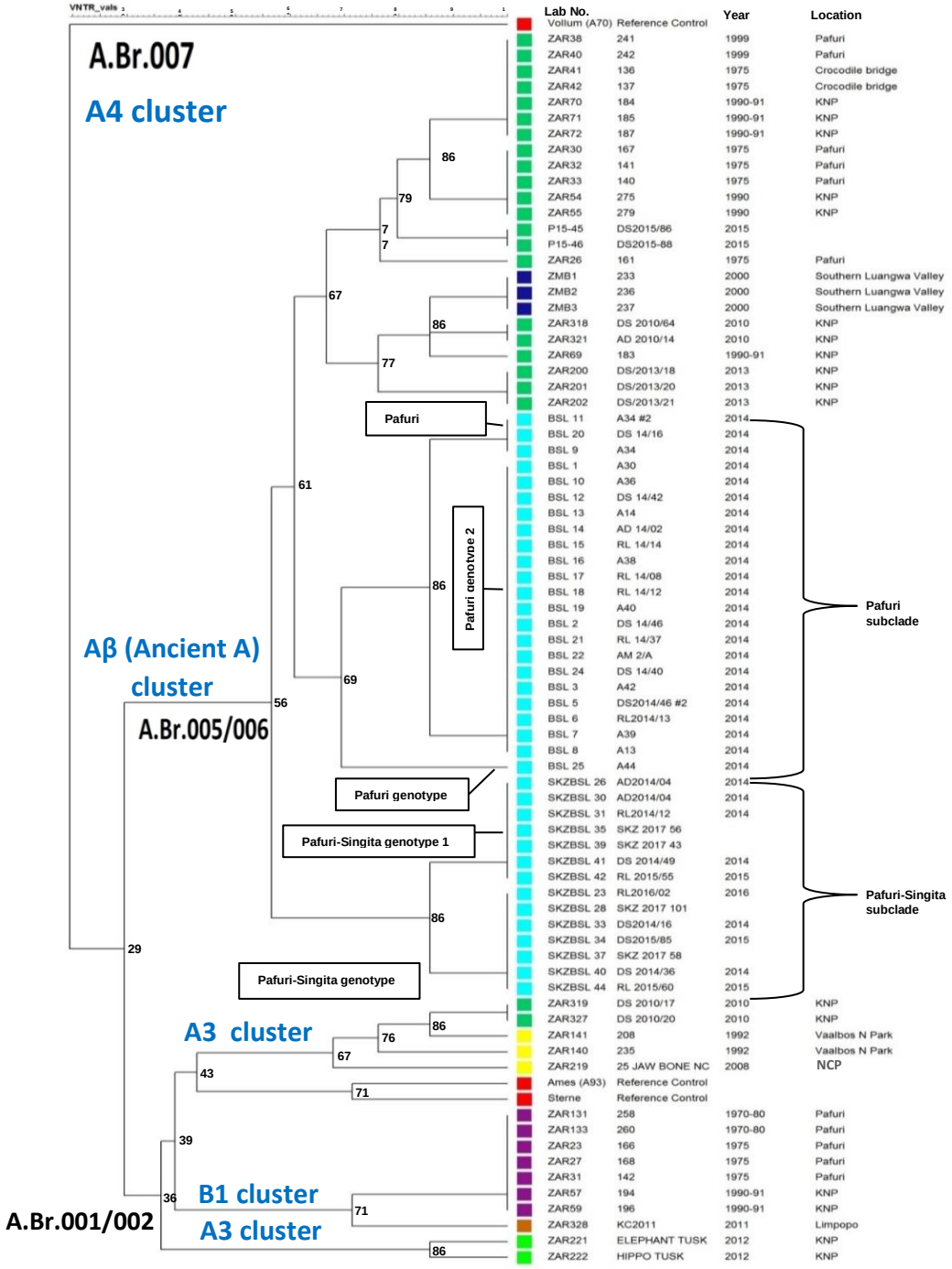
4.3.1 *Bacillus anthracis* isolates from 2014 to 2016

From the samples submitted, 37 were phenotypically identified as *B. anthracis* and confirmed by qPCR (Appendix 4A). All 37 of the isolates harboured both the pX01 and pX02 plasmids that codes for the major virulence factors making it pathogenic. The *B. anthracis* were isolated during the 2014 anthrax outbreak in Pafuri affected mainly impala (*Aepyceros melampus*) as well as two nyala (*Tragelaphus angasii*)). During this outbreak, *B. anthracis* was isolated from swabs of the beak and cloaca of vultures that were captured during the outbreak while feeding on the carcasses. *Bacillus anthracis* outbreaks occurred in the Pafuri region in 2014–2015 and in Singita in the south-eastern KNP on the border between KNP and Mozambique in 2015–2016 where it affected kudu (*Tragelaphus strepsiceros*) and African elephant (*Loxodonta africana*).

4.3.2 The MLVA7 panel

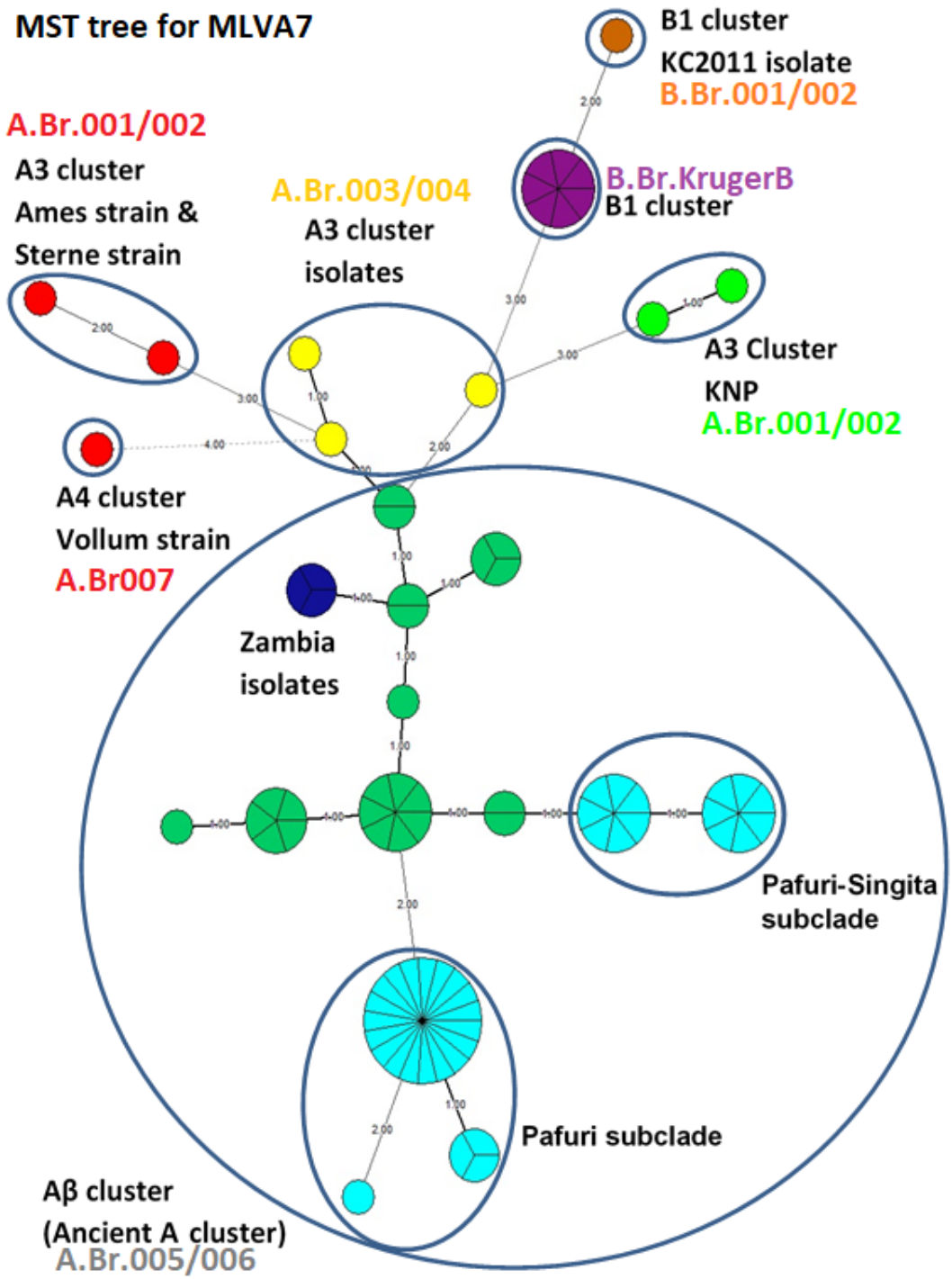
The MLVA7 first-line assay proposed by Thierry et al. (2014) was done on MLVA data of southern African *B. anthracis* strains (n=42 were used as well as Ames, Vollum and Sterne control strains) (Hassim, 2017), as presented in Figure 4.1, 4.2, 4.3, and 4.4. The MLVA7 is considered a first line assay because it is a more cost effective method to identify *B. anthracis* broad lineages. The premise being that isolates can be selected for further typing or whole genome sequencing based on this cluster analysis.

In the UPGMA cluster analysis shown in Figure 4.1, the KNP isolates have grouped into 2 broad clades with Vollum forming an outgroup on its own. The cluster names indicated in light blue (Figure 4.1) are the MLVA clades, while the “A.Br” and “B.Br” cluster nomenclature in black are the corresponding SNP lineages as described by Pilo and Frey (2011 and 2019). The numbers at the branch nodes represent clade similarity in percentages. In the MST tree shown in Figure 4.2 nodes with multiple segments represent clonal genotypes while individual/ unsegmented circles represent unique genotypes (i.e. not previously isolated in KNP). The isolates are coloured according to their known lineages with corresponding labels as determined by WGS. The isolates typed during this study are teal in colour and labelled Pafuri and Pafuri-Singita subclade, respectively.



Note: The blue, green, yellow, lime-green, brown, and purple-pink coloured isolates were from a previous study by Hassim (2017). The red-coloured isolates were controls that were used. The purple and brown isolates include the *B. anthracis* B-clade. The teal-coloured isolates were the 37 isolates from 2014 to 2016. The six genotypes from teal coloured isolates (used in the study) were named according to its genotype and indicated on the figure. MLVA cluster names were indicated in blue and SNP cluster names are indicated in black.

Figure 4.1: UPGMA tree of *Bacillus anthracis* strains based on MLVA7 isolated from 2010 to 2016 in the Kruger National Park



Note: The blue, green, yellow, lime-green, brown, and purple-pink coloured isolates were from a previous study by Hassim (2017). The red-coloured isolates were controls that were used. The purple and brown isolates include the *B. anthracis* B-clade. The teal-coloured isolates were the 37 isolates from 2014 to 2016. The distance numbers indicate the number of markers by which isolates differ from one another.

Figure 4.2: Minimum spanning tree of *Bacillus anthracis* strains based on MLVA7 isolated from 2010 to 2016 in the Kruger National Park

The branches connecting the nodes represent genetic distance where the smaller the branch value, the closer the isolate relatedness (i.e. the fewer VNTR markers it differs at).

The MLVA7 panel identified 24 genotypes among the 79 isolates (Figures 4.1 and 4.2). With the UPGMA cluster analysis of the MLVA7, the B-clade isolates (Figure 4.1, purple and brown) clustered with two groups of A3-clade isolates (Figure 4.1, yellow, red control strains and dark green as well as a separate cluster of light green 2012 isolates). The Aβ clade is made up of the isolates from this study (teal, 2014 & 2015 outbreaks), Zambian isolates (dark blue) and KNP isolates (dark green; 1975-2013). In the UPGMA MLVA7 cluster analysis, the MLVA groupings do not correspond well to the SNP lineages and published MLVA clade nomenclature for southern African isolates nor for Vollum.

In the MST tree (Figure 4.2) there are two divergent branches connecting the various clusters to the clonal genotype of 2010 KNP isolates (ZAR 319 and ZAR 327). A third branch connects this 2010 genotype to the remainder of KNP and Zambian isolates. The seven VNTR markers restricted the resolution as seen with the MST analysis where the KNP B-clade isolates (Figure 4.2, purple) differed only by three markers from the Vaalbos, Northern Cape A-clade isolate (ZAR 141) (Figure 4.2, yellow). Within the Pafuri subclade, the large clonal node is made up of 19 isolates (Pafuri genotype 2 as indicated in Figure 4.1). A branch with a value of 1 connects this larger node to a clonal node consisting of three 2014 isolates. The branch value indicates that they differ at one VNTR marker (VNTR23) by one repeat unit (i.e. 12 base pairs). A single, unique isolate (BSL25) makes up the third member of the Pafuri subclade. This isolate differs at two VNTR markers from the Pafuri genotype 2 node. At Bams22 there is a four repeat unit difference between BSL25 and “Pafuri genotype 2” which constitutes a 144 base pair difference at this marker (Appendix 4A) At the vrrA marker there is a 36 base pair difference between these nodes which translates to a three repeat unit difference (Appendix 4A). The closest node to the Pafuri subclade (by two VNTR marker differences) is the KNP isolates from 1975-1999 clonal genotype (Figure 4.2). The Pafuri-Singita subclade are made up of two clusters with an 88% similarity. These two nodes differ by one repeat unit at VNTR23. The closest genotype to the Pafuri-Singita subclade is a two-isolate node (P15-45 and P15-46). These isolates were previously described by Venter (2016) from a Pafuri outbreak in 2015 (where insects were collected from carcasses) and differ from the isolates in this study by two VNTR markers.

4.3.3 MLVA10 panel

The 37 *B. anthracis* strains isolated from 2014 to 2015 in KNP were analysed with 42 known MLVA fingerprints of *B. anthracis* strains isolated in KNP (Hassim, 2017) using the MLVA10 panel (Figure 4.3 and Figure 4.4). The copy numbers of the *B. anthracis* strains used in the MLVA10 panel are listed in Appendix 4A. The MLVA10 panel identified 28 genotypes among the 79 isolates and increased the resolution (Figure 4.3). While there was only a differentiation of four additional genotypes using this panel, the topology of the UPGMA dendogram better

represents the phylogenetic structure of global *B. anthracis* strains as described by Van Ert et al. (2007) and updated recently by Pilo and Frey (2019). The reference strain Vollum is no longer an outgroup (Figure 4.1 and Figure 4.3). The KNP isolates from 2010 which had clustered in the A3 clade using the MLVA7panel (Figure 4.1) are now correctly clustered in the Ancient A group (Figure 4.3). The UPGMA was not able to accurately differentiate between members of the A3 clade and B clades in terms of clusters since it represents isolate similarity.

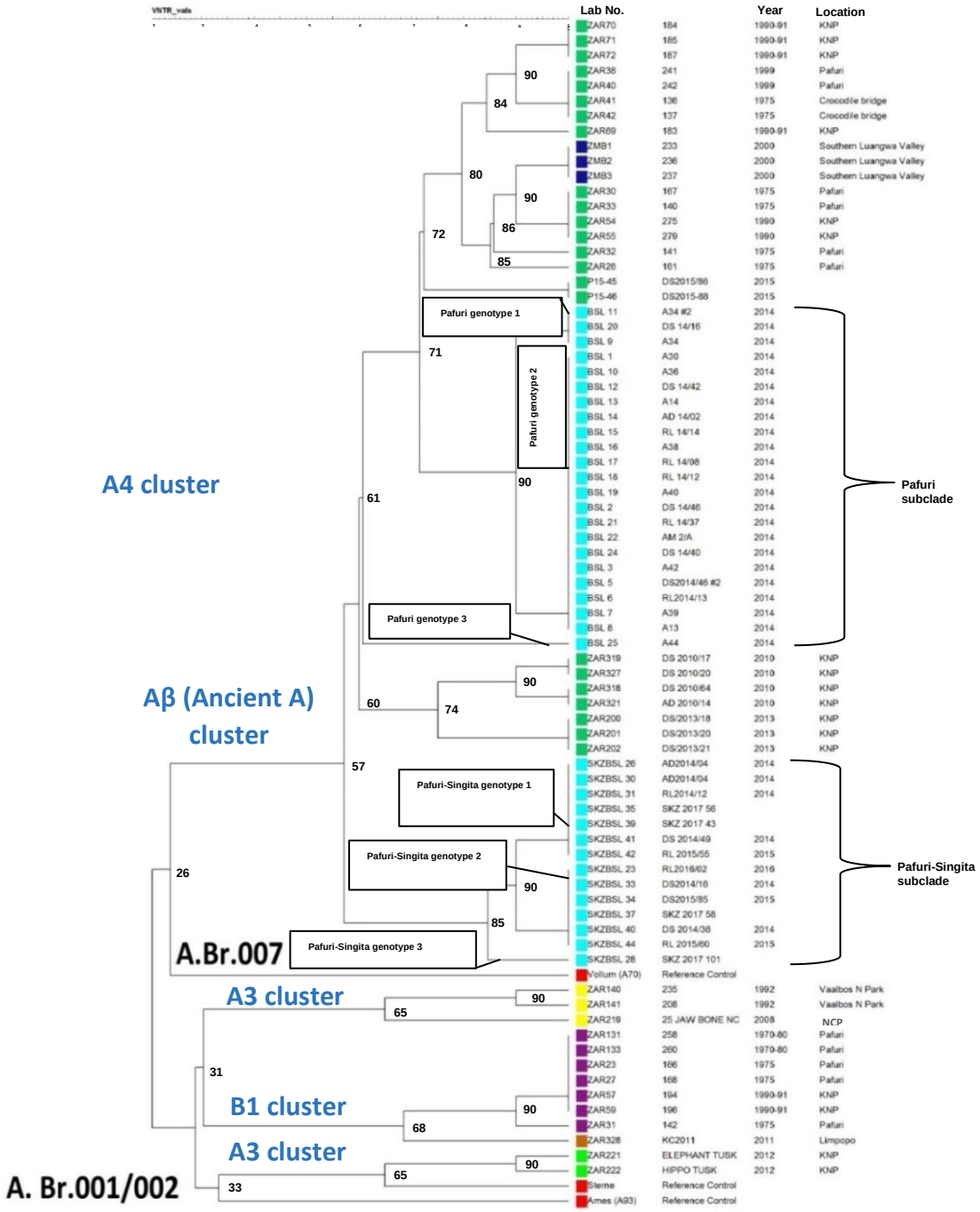
The MST tree (Figure 4.4), however was able to better demonstrate the disparity in genetic distance and geographic distribution between these clades. The additional three VNTR's enhanced the resolution as seen with the MST analysis where the KNP B-clade isolate (Figure 4.4, brown) differed by six markers from the A-clade isolates (Figure 4.4, green). This is a more accurate representation of the genetic distance between these clades. The Sterne and Ames reference strains have clustered with the KNP 2012 strains belonging to the same A.Br.001/002 lineage (Figure 4.3 and Figure 4.4). Similarly, the NCP isolates (yellow) have clustered separately and demonstrate the geographically isolated lineage which it is in the region.

The 37 *B. anthracis* strains described for the first time in this study, clustered in two subclades; the one subclade (Pafuri subclade) consisted of only 2014 isolates from Pafuri and the other subclade (Pafuri–Singita subclade) consisted of 2014–2015 isolates from Pafuri and Singita in 2015–2016 in the Aβ (Ancient A) clade (Figure 4.3). The 37 strains consisted of six genotypes in the Aβ (Ancient A) clade (Figure 4.3) with three genotypes being identified more commonly amongst the isolates (dominant genotypes). The two subclades were each clonal and deferred only by three VNTR markers from one another (Figure 4.4; Appendix 4A).

The Pafuri subclade (2014) consisted of one dominant genotype (labelled Pafuri genotype 2 and indicated in Figure 4.3) with strains isolated from vultures and impala (Appendix 4A). The Pafuri subclade (2014) (Figure 4.3, teal with BSL numbers) clustered with *B. anthracis* KNP strains from 1975 to 2013 (Figure 4.3, green and blue). The Pafuri–Singita subclade (2014–2016) (Figure 4.3, teal with SKZBSL number) consisted of two dominant genotypes (indicated in Figure 4.3) with strains from the one isolated from elephant in Singita (2016) and impala in Pafuri (2014), while the other genotype was isolated from kudu in Singita (2015), as well as nyala and impala from Pafuri (2014).

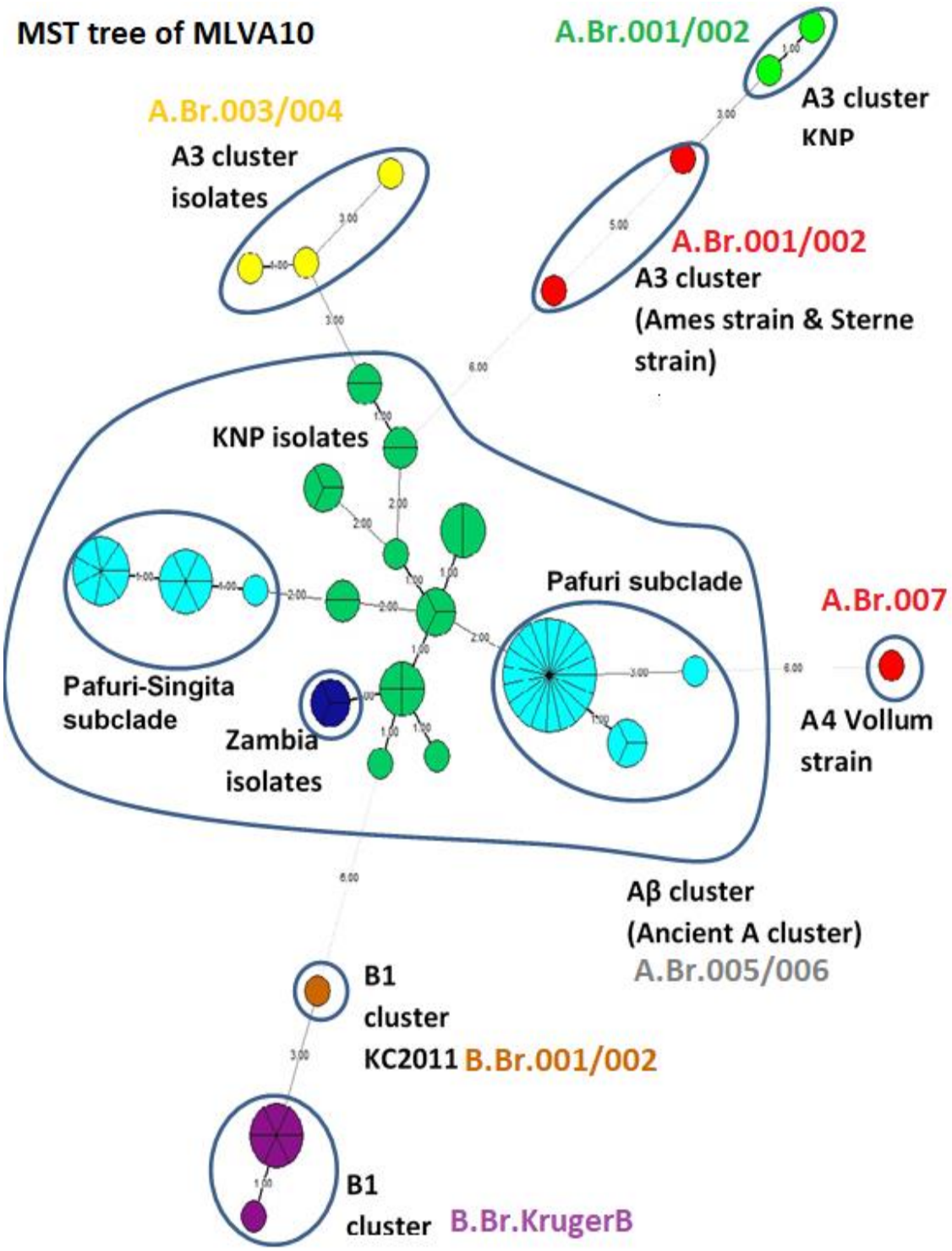
The Pafuri–Singita subclade (2014–2016) subclade clustered separately but was related to clade with the Pafuri subclade (Figure 4.3).This can be seen by looking at the VNTR markers used in the panel. When looking at Appendix 4A, the VNTR marker Bams03 is the primary marker that could be the reason that separated the Pafuri subclade from the Pafuri–Singita subclade. The difference between the two clades for this marker is one copy number/repeat unit. This is indicative of evolution taking place in the strains of that region as well as the argument

that rapid evolution is taking place in the VNTR regions. Other VNTR markers that show a different copy number/repeat unit amongst isolates include VNTR23 and vrrA as previously described for MLVA7 (Appendix 4A).



Note: The blue, green, yellow, lime-green, brown, and purple-pink coloured isolates were from a previous study by Hassim (2017). The red-coloured isolates were controls that were used. The purple and brown isolates include the B. anthracis B-clade. The teal-coloured isolates were the 37 isolates from 2014 to 2016. The six genotypes from teal coloured isolates (used in the study) were named according to their genotype and indicated on the figure. MLVA cluster names were indicated in blue and SNP cluster names are indicated in black.

Figure 4.3: UPGMA tree of *Bacillus anthracis* strains based on MLVA10 isolated from 2010 to 2016 in Kruger National Park



Note: The blue, green, yellow, lime-green, brown, and purple-pink coloured isolates were from a previous study by Hassim (2017). The red-coloured isolates were controls that were used. The purple and brown isolates include the B. anthracis B-clade. The teal-coloured isolates were the 37 isolates from 2014 to 2016. The distance numbers indicate the number of markers by which isolates differ from one another.

Figure 4.4: Minimum spanning tree of *Bacillus anthracis* strains based on MLVA10 isolated from 2010 to 2016 in the Kruger National Park

4.4 Discussion

B. anthracis is well known for being endemic in most regions of Africa, especially in southern Africa where it has a wide genotypic diversity indicating to be the hypothetical origin of the microorganism (Keim et al., 2000; Pilo and Frey, 2011). In KNP, both the A- and B-clades were reported until the 1990s, thereafter the A-clade predominated in KNP (Smith et al. 2000). Genotyping of *B. anthracis* enables researchers to determine the diversity of strains present in a specific area, region or country, as well as having the ability to trace outbreaks that are close to the borders of countries. This can be used to determine if the outbreak strain is "native" to that region.

Due to the monomorphic nature of *B. anthracis* clades and the slow rate of change, strains are commonly referred to according to lineages determined through canSNP's (canonical single nucleotide polymorphisms) (Van Ert et al. 2007; Derzelle et al. 2015). This is also the reason why isolates from previous studies (Hassim, 2011 and 2017) were included to determine the lineages for dendogram topology seeing as *B. anthracis* isolates are very region specific. *B. anthracis* can remain in the environment for centuries and genotypes can thus recur in these environments (Hugh-Jones and De Vos. 2002). The inclusion of known genotypes from the country can aid in understanding bacterial spread within the regions isolated.

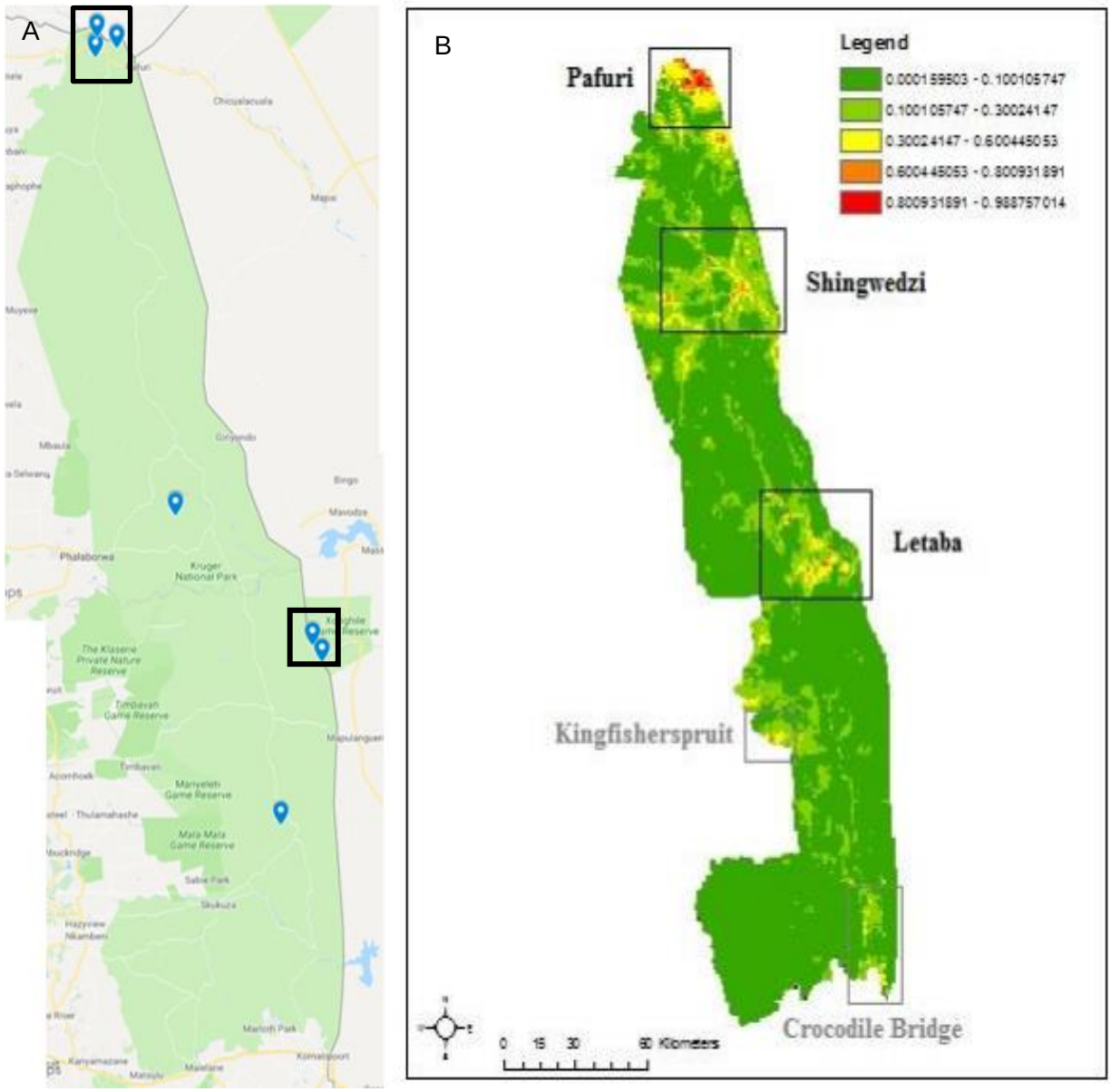
The MLVA10 panel included three additional markers, namely Bams13, Bams30 and Bams31 that Le Flèche et al. (2001) and Lista et al. (2006) identified as alleles that code for genes related to bacterial adaptability in the environment. These are the same regions that are markedly different between the A- and B- clade isolates of Africa. In this study, the proposed MLVA10 marker panel provided an indication of the phylogenetic relatedness of African *B. anthracis* strains that differentiated the A- and B-clades within a geographical context (Figure 4.4), unlike the MLVA7 panel proposed by Thierry et al. (2014) which used European and Namibian isolates (Figure 4.1 and Figure 4.2). The MLVA10 marker panel was able to genotype the *B.anthracis* strains (n=79) in the present study into 28 genotypes that included 37 strains (six genotypes with three dominant (Pafuri genotype 2, Pafuri-Singita genotype 1 and 2)) from outbreaks in KNP from 2014 to 2016, grouped in the Aβ (Ancient A) clade (Figure 4.3). Two of the dominant genotypes occurred in Pafuri during the 2014–2015 outbreaks and in Singita during the 2015–2016 outbreaks (Figure 4.3, teal SKZBSL number). The northern region, Pafuri in the KNP, is known as the endemic/enzootic region of anthrax (Figure 4.5). Singita is a concession area that lies in the central-eastern part of KNP (Figure 4.5), on South Africa's border with Mozambique situated on 33 000 acres. The Singita anthrax outbreaks were found to be uncharacteristic in the central-eastern part of KNP in 2014 and 2016 due to the region being known as a "non-enzootic" anthrax region.

B. anthracis strains of the same genotype occurred in the endemic Pafuri region in 2014–2015, as well as in the non-endemic Singita region in 2015–-2016, indicating that there was possible spread from the one region to the other. Possible dissemination of the *B. anthracis* genotype from one area to the other could include: 1) scavengers that have fed on an infected carcass and transmitted the spores to a non-endemic area which causes an outbreak in that area; 2) water run-off containing anthrax spores, but this is unlikely as areas fall in different catchment areas; 3) wind distribution of anthrax spores; and 4) bacteraemic animals (Turnbull et al., 1989; Lindeque and Turnbull, 1994). The last-mentioned reason (bacteraemic animals) will vary due the different surrounding population of animals present, home range and rate of travel the animal is capable of (Bengis, 2012).

The *B. anthracis* strains isolated from vultures belonged to three different genotypes (Pafuri genotype 1–3). Vultures have been hypothesised to contribute towards the spread of *B. anthracis* but with very little concrete evidence to prove the hypothesis correct (Bullock, 1956; Ebedes, 1976; Hugh-Jones and De Vos, 2002). In this study the vultures were caught during the Pafuri outbreak (2014–2015) and therefore the presence of *B. anthracis* on their beaks and cloacae were expected. Steenkamp et al (2018) identified areas in KNP that are ecologically suitable for the harbouring of *B. anthracis* spores and identified three main areas in Pafuri, Shingwedzi and Letaba (Figure 4.5). The suitability map identified areas where outbreaks in Pafuri and Singita (in Figure 4.5) occurred in this study.

By using the MLVA31 marker panel, Hassim (2017) characterised the KNP *B. anthracis* strains and found that new genotypes evolve from dominant genotypes over decades, unlike the genotypes that remain almost the same in the Etosha National Park for decades (Beyer et al., 2012). In this study, the same pattern (new genotypes evolve from dominant genotypes (Hassim, 2017) was observed in KNP using the MLVA10.

The Pafuri-Singita subclade (2014–2016) consisting of two dominant genotypes (Pafuri-Singita genotype 1 and 2) clustered separately but was related to the clade that included the Pafuri subclade consisting of a dominant genotype (Pafuri genotype 2) (Figure 4.3).This is seen when looking at the VNTR marker copy number/repeat unit amongst isolates in Appendix 4A. Of the 10 VNTR markers used, Bams03 is the primary marker that could be the reason that separated the Pafuri subclade from the Pafuri–Singita subclade. By looking at the copy number of the isolates for Bams03, isolates belong to the Pafuri subclade was 18 while isolates from the Pafuri–Singita subclade had a copy number of 19. The one copy number difference between the two clades for this marker is indicative of evolution taking place in the strains of that region as well as the argument that rapid evolution is taking place in the VNTR region itself.



Note: (B) Showing areas in the Kruger National Park where *B.anthracis* strains were isolated from the Pafuri in 2014 (northern region) and in Singita in 2014 and 2016 (south-eastern region). (B) Indicating Pafuri, Shinwedzi and Letaba areas as the most suitable areas in the Kruger National Park to harbour *B. anthracis* spores.

Source: Compiled from Steenkamp et al. (2018)

Figure 4.5: Map of Kruger National Park and *Bacillus anthracis* suitability map

Other VNTR markers that show a different copy number/repeat unit amongst isolates include VNTR23 and vrrA (Appendix 4A). Of the 37 isolates used from this study, 9/37 has shown to have a different copy number for the VNTR23 marker. These isolates have a copy number of four, while the rest of the isolates have a copy number of three. For the vrrA marker, two isolates

(BSL14 and BSL25) had a different copy number. BSL14 had a copy number of 5 and BSL28 had a copy number of 3.

Hyper-variability (rapid evolution) amongst VNTR markers has been described by numerous researchers (Keim et al. 2008; Fasanella et al. 2018) in numerous bacterial species in which MLVA genotyping has been used for typing isolates into their respective clusters/lineages. SNPs are known to be rare in *B. anthracis* (due to the long period of dormancy that they experience in their spore form causing very low mutation rates) and is thus not very useful in providing very stable evolutionary information. This also means that SNPs will only be able to be identified using whole genome comparison with other whole genomes of the same organism (Keim et al. 2008).

Keim et al. (2004) formulated a hypothesis where a small number of canonical SNPs (canSNPs) located at significant phylogenetic junctions along the *B.anthracis* SNP tree could replace genome-wide SNP analysis. In the current study numerous canSNPs were identified and can be observed in Figures 4.1 to 4.4. Isolates that cluster with A3 fit well with the A.Br001/002 and A.Br003/004 SNP lineage. Isolates belonging to the A4 cluster grouped with the A.Br.007 SNP lineage and isolates belonging to the Aβ (Ancient A) cluster fit well with the A.Br005/006 SNP lineage. All of these clusters belong to the A-clade. Isolates belonging to the B1 cluster belonged to the B.Br.001/002 and B.Br.Kruger SNP lineage and thus fall into the B-clade. Van Ert et al. (2007) (using the MLVA31 marker panel) described the MLVA clusters versus the SNP lineages very well and their description fitted very well with the study isolates.

Overlapping of genotypes between Singita and Pafuri was also observed for the 2014 to 2015 period. These genotypes were isolated mostly from carcasses in these regions and became two distinct sub-claces during the outbreak. This lends to the theory that genotypes develop concurrently in outbreaks and originated from a dominant genotype circulating within the environment over time. This can be seen in Figure 4.3 where these genotypes with 90% clade similarity have evolved from a dominant genotype.

When comparing the two marker panels used in the study, the MLVA7 marker panel consisted of the seven markers as proposed by Thierry et al. (2014) and was tested on French and Namibian *B. anthracis* strains that all belonged to the A-clade (A3 cluster, A1 cluster). B-clade isolates (B2 cluster) were also tested in their (Thierry et al, 2014) study, but these isolates are very different from the African B-clade strains used in the current study. The main VNTR markers that Thierry et al. (2014) identified to distinguish the B-clade (B2 cluster) were Bams01, vrrB1, Bams15 and Bams23. This is not the case because in the current study, the MLVA7 marker panel was tested on A- and B-clade southern African *B. anthracis* strains (Aβ (Ancient A) cluster) and found that this panel could not clearly differentiate the A- and B- clades from one another

as opposed to the French and Namibian *B. anthracis* strains used by Thierry et al. (2014). By adding the three proposed VNTR markers (Bams 13, Bams 30, and Bams 31, identified by Lista et al. (2006) and Le Flèche et al. (2001), which is known to be highly diverse and form part of regions coding for the exopsorium clade strains), to the MLVA7 marker panel a MLVA10 marker panel was construct. The MLVA10 panel would have the ability to differentiate between A- and B-clade African *B. anthracis* strains as well as being able to differentiate isolates/strains within the clades/clusters themselves. The MLVA10 panel that included Bams 13, Bams 30 and Bams 31 markers are known to be agarose-friendly (can be differentiated on agarose gels).

When looking at the MLVA7 marker MST tree (Figure 4.2) and MLVA10 marker MST tree (Figure 4.4) the SNP lineages for these two panels are placed quite different. The A.Br001/002 lineage (KNP isolates (indicated as yellow in figure) and Ames and Stern strain (indicated as red in figure)) is completely separate from one another in the MLVA7, while in the MLVA10 it puts the strain on the same branch as well as having them be genetically distant from one another. The MLVA10 also clusters the A.Br003/004 isolates much better than the MLVA7 (clustering isolates together (NCP) and separate from Aβ (Ancient A) cluster versus clustering isolates (NCP) separately and very close to the Aβ (Ancient A) cluster). The B1 cluster isolates are also grouped and branched much better by the MLVA10 MST tree than the MLVA7 MST tree. When looking at the genetic distances for these two panels, the MLVA10 MST tree is a much accurate representation than the MLVA7 MST tree.

When comparing the allelic frequencies (diversity index values, Appendix 4B) between the VNTR markers used by Thierry et al. (2014) and the VNTR markers used in our study, a clear difference was observed between the two VNTR panels. When looking at the Bams 13 marker, the allelic frequency observed by Thierry et al. (2014) was 0.6130 while the allelic frequency observed by Hassim (2011) was 0.9230 for the same marker. For the Bams 30 marker Thierry et al. (2014) found the allelic frequency to be 0.6739 while Hassim (2011) found it to be 0.9170 and for the Bams 31 marker Thierry et al. (2014) found the allelic frequency to be 0.6809 while Hassim (2011) found the allelic frequency to be 0.8720. The closer the allelic frequency value is to 1, the greater the allelic diversity is in that loci or marker (Keim et al., 2000; Hassim, 2011).

When taking into account the above mentioned evidence, the reasoning for adding the three VNTR markers (Bams 13, Bams 30, and Bams 31), identified by Lista et al. (2006) become clear. The information generated by the study also indicates that the data sets used by Thierry et al. (2014) is biased towards A-clade isolates used in the current study which belong to the Aβ(Ancient A) strain lineage. By adding the three VNTR markers (Bams 13, Bams 30, and Bams 31) to the MLVA7 marker panel described by Thierry et al (2014) the MLVA10 marker panel that

we used will be able to differentiate A- and B-clade African *B. anthracis* isolates from one another.

The power of the MLVA10 marker panel can be seen when viewing the MST tree (Figure 4.4) by looking at the genetic marker distances. When comparing the MST tree of the MLVA7 marker panel (Figure 4.2) with the MST tree of the MLVA10 marker panel (Figure4.4), the ability to distinguish the African A- and B-clade *B. anthracis* strains from one another is much better and stronger using the MLVA10 marker panel. The same conclusion can be said when looking at the UPGMA spanning tree for the MLVA10 marker panel (Figure 4.3) and the MLVA7 marker panel (Figure 4.1). Both the UPGMA tree and the Minimum spanning tree for the MLVA10 marker panel grouped the different SNP lineages very well in comparison with the MLVA7 marker panel. The MLVA10 clustered the 37 *B. anthracis* isolates from the 2014–2016 outbreaks in KNP into the Aβ (Ancient A) clade (Sahl et al., 2016). Furthermore, comparing the 2014–2016 *B. anthracis* strains outbreaks with historical KNP strains using the MLVA10, indicated commonality amongst the stains. The Pafuri subclade (2014) (Figure 4.3, teal with BSL number) clustered with *B. anthracis* KNP strains from 1975–2013 (Figure, 4.3 green and blue). This could be due to the limited number of genetic markers the MLVA10 has, and commonality might change should a complete 31 marker MLVA panel be used on the same samples.

4.5 Summary

In this study, a novel MLVA10 marker panel for the genotyping of African *B. anthracis* strains was reported that could differentiate A- and B-clade strains from KNP using MST cluster analyses. The panel identified 28 genotypes among the *B. anthracis* strains (n=79) isolated from KNP during anthrax outbreaks in Pafuri (northern region of the park) and Singita (south-eastern part of the park) from 2014 to 2016. Three genotypes were found in the Pafuri outbreak from 2014 to 2015 in the northern part of the KNP. Another three genotypes in the Singita outbreak from 2015 to 2016 in the south-eastern part of KNP were also found. Although the MLVA10 panel did manage to distinguish the A- and B-clade from one another, more research regarding the use of the MLVA10 is needed. Both areas (Pafuri and Singita) where outbreaks occurred from 2014 to 2016 were ecological suitability areas for *B. anthracis* spores identified in KNP by Steenkamp et al. (2018). Animal species that were affected by the outbreak included kudu and elephant in Singita and impala and nyala in Pafuri. Further studies about these animals and the role that they play in disease dissemination in KNP needs to be further investigated as well as strain-relatedness of isolates identified by Steenkamp et al. (2018).

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Appendix 4A: Sample data indicating the lab number, sample number, location, coordinates, animal species isolated, sensitivity results, qPCR results, and VNTR coding value

Lab number	Sample number	Year	Locations found/ Isolated	Latitude	Longitude	Animal species	Penicillin sensitivity	Gamma Phage sensitivity	qPCR Result	PA gene (plasmid pX01)	Cap C gene (plasmid pX02)	SASP gene	BAMS 03	BAMS 05	BAMS 13	BAMS 22	BAMS 30	BAMS 31	BAMS 34	BAMS 44	VNTR 23	VRR A
BSL 1	A30	2014	Pafuri			Vulture	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 2	DS2014/46	2014	Pafuri	-22,42354	31,17878	Impala	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 3	A42	2014	Pafuri			Vulture	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 5	DS2014/46 #2	2014	Pafuri	-22,42354	31,17878	Impala	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 6	RL2014/13	2014	Pafuri	-22,42077	31,17450	Impala	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 7	A39	2014	Pafuri			Vulture	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 8	A13	2014	Pafuri			Vulture	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 9	A34	2014	Pafuri			Vulture Beak	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	4	6
BSL 10	A36	2014	Pafuri			Vulture	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 11	A34 #2	2014	Pafuri			Vulture cloaca	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	4	6
BSL 12	DS2014/42	2014	Pafuri	-22,42614	31,17643	Impala	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 13	A14	2014	Pafuri			Vulture	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 14	AD 14/02	2014	Pafuri			Nyala bone	Sensitive	Sensitive	Positive	x	x	x	18	5	33	15	69	64	9	2	3	5
BSL 15	RL2014/14	2014	Pafuri	-22,420927	31,17440833	Impala	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 16	A38	2014	Pafuri			Vulture	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 17	RL2014/08	2014	Pafuri	-22,418475	31,176405	Impala	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 18	RL2014/12	2014	Pafuri	-22,421237	31,17428667	Impala	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 19	A40	2014	Pafuri			Vulture	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 20	DS2014/16	2014	Pafuri	-22,39904	31,25998	Impala	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	4	6
BSL 21	RL2014/37	2014	Pafuri	-22,42355	31,17852	Impala	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6

BSL 22	AM 2/A	2014	Pafuri			Vulture	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 24	DS2014/40	2014	Pafuri	-22,42502	31,17533	Impala	Sensitive	Sensitive	Positive	x	x	x	18	6	33	15	69	64	9	2	3	6
BSL 25	A44	2014	Pafuri			Vulture	Sensitive	Sensitive	Positive	x	x	x	18	6	33	19	69	64	9	2	3	3
SKZ/BSL 23	RL 2016/02	2016	Singita	-24,32566	31,90265	Elephant	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	4	6
SKZ/BSL 26	AD2014/04	2014	Pafuri			Nyala	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	3	6
SKZ/BSL 28	MTG 1	20,15	Singita			Bone	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	4	6
SKZ/BSL 30	AD2014/04	2014	Pafuri			Nyala	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	3	6
SKZ/BSL 31	RL2014/12	2014	Pafuri	-22,42124	31,17429	Impala	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	3	6
SKZ/BSL 33	DS2014/16	2014	Pafuri	-22,39904	31,25998	Impala	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	4	6
SKZ/BSL 34	DS2015/85	2015	Pafuri	-22,37576	31,18897	Impala	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	4	6
SKZ/BSL 35	LVS2014/03/02	2014	Pafuri			Impala	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	3	6
SKZ/BSL 37	RL2014/14	2014	Pafuri			Impala	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	4	6
SKZ/BSL 39	RL2015/54	2015	Singita	-24,23513	31,93647	Kudu	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	3	6
SKZ/BSL 40	DS2014/36	2014	Pafuri	-22,42354	31,17878	Impala	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	4	6
SKZ/BSL 41	DS2014/49	2014	Pafuri	-22,42292	31,17965	Impala	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	3	6
SKZ/BSL 42	RL2015/55	2015	Singita	-24,24651	31,94458	Kudu	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	3	6
SKZ/BSL 44	DS2015/85	2015	Pafuri	-22,37576	31,18897	Impala	Sensitive	Sensitive	Positive	x	x	x	19	6	33	15	69	64	9	2	4	6
Sterne							Sensitive	Sensitive	Positive	x	Absent	x	28	6	76	16	51	65	11	8	3	4
Volume (A70)							Sensitive	Sensitive	Positive	x	x	x	30	7	9	16	71	64	9	6	2	2
Ames (A93)							Sensitive	Sensitive	Positive	x	x	x	26	5	20	16	57	64	11	8	4	4

Appendix 4B
Alleles and the allelic frequency taken from Hassim (2011) and Thierry et al. (2014)

Allelic frequency				
VNTR Markers	MLVA 7 Thierry A-clade isolates	MLVA 10 Thierry A-clade isolates	MLVA 7 Hassim A- & B-clade Isolates	MLVA 10 Hassim A- & B-clade Isolates
BAMS 03	0.6382	0.6382	0.8580	0.8580
BAMS 05	0.5095	0.5095	0.1830	0.1830
BAMS 13		0.6130		0.9230
BAMS 22	0.6503	0.6503	0.7880	0.7880
BAMS 30		0.6739		0.9170
BAMS 31		0.6809		0.8720
BAMS 34	0.6549	0.6549	0.4940	0.4940
BAMS 44	0.0601	0.0601	0.5400	0.5400
VNTR 23	0.4213	0.4213	0.6780	0.6780
VRR A	0.5895	0.5895	0.7680	0.7680

Chapter 5
Discussion and Conclusion

Bacteria belonging to the genera *Bacillus* and *Clostridium* are known to be very similar in numerous ways. They are both Gram-positive bacilli, known to be soil-born, and can survive for years in harsh environments due to the ability to produce spores (Turnbull, 2008; Nicholson and Setlow, 1990; Nicholson et al., 2000; Sonenshein, 2000). Both these genera have pathogenic species that are known to cause acute disease(s) in animals and are very difficult to distinguish from one another (Olivier, 2001; Turnbull, 2008). South Africa is known to be endemic to *Bacillus anthracis* (*B. anthracis*) and numerous pathogenic Clostridia. These organisms are known to have a financial impact on small and rural farmers due to the losses of livestock that is needed to sustain their communities and families (Viljoen et al., 1928; Lamy et al., 2012).

The main aims of this study was 1) to investigate the different numbers of *Clostridium* species responsible for animal related deaths in South Africa in both livestock and wildlife by using the laboratory information system of the Faculty of Veterinary Science at the University of Pretoria, and 2) to investigate the occurrence of anthrax and clostridial diseases using a passive surveillance system and characterisation of isolates using molecular techniques in wildlife of the KNP.

In Chapter 2, the laboratory information system of the Faculty of Veterinary Science (UVIS), University of Pretoria, was used to identify the most commonly isolated pathogenic *Clostridium* species responsible for death of animals. In livestock and wildlife, *Clostridium perfringens* (*C. perfringens*) wat the most commonly identified Clostridia, followed by numerous other *Clostridium* species that the laboratory could not identify to species level and was grouped as a collective as *Clostridium* spp. Both *Clostridium septicum* (*C. septicum*) and *Clostridium sordellii* (*C.sordellii*) was the third most commonly identified *Clostridium* species causing disease in livestock and wildlife. The study indicated that equal numbers of the different pathogenic Clostridia affected livestock and wildlife in the four-year study period.

Most of these *Clostridium* species were identified by using either culture or colony morphology, as well as some biochemical analyses. Identification using FAT was mostly used on tissue of animals, but it is very labour-intensive, especially in laboratories that are short-staffed and not a reference laboratory. As mentioned, *Clostridium* that could not be identified up to species level, was then placed in a collective group called *Clostridium* spp. This just highlights the need for better identification of *Clostridium* organisms as some of the species, such as *C. septicum*, *Clostridium chauvoei* (*C. chauvoei*), *Clostridium novyi* (*C. novyi*) type A, *C. perfringens* type A,

and *C. sordellii*, can cause similar diseases (Silva et al., 2016), but could not be distinguished from one another using the methods currently being used by the diagnostic laboratory. The multiplex PCR assays developed to identify the different *Clostridium* species (*Clostridium botulinum* (*C. botulinum)*, *C. chauvoei*, *C. novyi*, *C. perfringens*, *C. septicum*, *C. sordellii* and *Clostridium tetani* (*C. tetani*) in Chapter 3 could resolve the laboratory problem and might enable speciation of *Clostridium* spp.

The high numbers of *Clostridium* might also reflect that incorrect vaccination practises or vaccinating with non-relevant clostridial vaccines or lack of vaccination, might be taking place. This information will in turn help to strengthen and identify any weaknesses and gaps in the management and control practices involving clostridial diseases in the different animal species with which veterinarians and farmers may be working or coming into contact with. The UVIS database can be used, not just as a tool surveillance tool for veterinarians and farmers, but also for epidemiologists and researchers that are studying animal diseases. Further studies involving the epidemiology and trend of clostridial diseases can be done as to determine if vaccination practices are improving or if a specific *Clostridium* species is becoming a problem.

In Chapter 3, passive surveillance was used to distinguish *Clostridium* species as well as *B. anthracis*. Both of these organisms are known to co-exist in the environment of KNP but cannot be distinguished within each genus microscopically using Gram-stained or Giemsa-stained slides. PCR assays were developed to identify the different *Clostridium* species (*C. botulinum*, *C. chauvoei*, *C. novyi*, *C. perfringens*, *C. septicum*, *C. sordellii* and *C. tetani*) present in the samples used for the passive surveillance. This assay could be used in the diagnostic laboratory to help confirm the diagnosis of suspected clostridial diseases once the organisms has been cultured. When comparing the results that were generated from Chapter 3 with those in Chapter 2, it was observed that *C. perfringens*, *C. septicum*, *C. sordellii* and *Clostridium* spp. (unidentified *Clostridium* species) most often affected wildlife. This indicates that wildlife species that are affected by clostridial disease in game ranching can also be affected in the wild.

In Chapter 4, an MLVA10 panel was used to characterise *B. anthracis* genotypes from anthrax outbreaks in KNP from 2014 to 2016. The MLVA10 panel was developed since the proposed MLVA7 panel (Thierry et al., 2014) could not differentiate *B. anthracis* A-clade genotypes from the B-clade genotypes found in South Africa (Hassim, 2017). A total of 79 samples were tested using the resent isolates from the 2014–2016 outbreaks (n=37) with southern African *B. anthracis* strains tested previously by Hassim (2017). The suggested MLVA10 could differentiate 28 genotypes among the 79 strains, while the MLVA7 identified 24 genotypes. The MLVA7 panel differentiated the B-clade strains from the closest phylogenetic A-clade strains

based on three to five VNTR markers, whereas the MLVA10 panel differentiated B-clade strains from the closest A-clade strains based on most of the VNTR markers (six to nine VNTR markers).

The anthrax outbreaks in 2014–2016 occurred in Pafuri in the northern region of the KNP, and Singita in the south-eastern part of the KNP. This is in agreement with the suitability niche for *B. anthracis* spores in KNP found by Steenkamp et al. (2018). Animal species that were affected include greater kudu (*Tragelaphus strepsiceros*), nyala (*Tragelaphus angassi*), Impala (*Aepyceros melampus*) and African elephant (*Loxodonta africana*). *Bacillus anthracis* was also isolated from vultures (n=12) that group into three genotypes but one dominate genotype. This dominant genotype accounts for the vultures feeding on the carcasses during that outbreak. Vultures have been hypothesised to contribute towards the spread of *B. anthracis* but with very little concrete proof (Bullock, 1956; Ebedes, 1976; Hugh-Jones and De Vos, 2002). Further studies about these animals and the role that they play in disease dissemination in KNP needs to be undertaken, and strain-relatedness of isolates needs to be identified.

In conclusion, both *B. anthracis* and *Clostridium* species are well known to cause disease in both domestic animals and in wildlife species. Both are spore forming organisms and can be found in the environment. Both can be controlled and managed by good vaccination practices. Wildlife, especially antelope species, can be used as models for studies regarding the impact that these organisms have on the animals in the wild. *Clostridium* species have been found in both clinical and environmental samples. The most commonly identified Clostridia in both domestic and wildlife was *C. perfringens*, *C. sordelli* and *C. septicum*, which could be prevented by vaccination. When interpreting results for clostridial disease, laboratory findings must always be matched and checked with pathology findings, as these organisms form part of the normal flora of animals. *B. anthracis* is well documented to cause large numbers of animal deaths in KNP. A MLVA10 panel was suggested that could differentiate between the A-clade and the B-clade. Animal species that were affected include a large number of antelope species and the outbreaks took place in known “hotspots” as suggested by Steenkamp et al. (2018). Further studies of both these organisms (*B. anthracis* and *Clostridium* species) are needed if we are to understand the relationship they have with the environment and the animals that are present in them.

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