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Distribution and molecular characterization of South African *Bacillus anthracis* strains and their associated bacteriophages

by

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Submitted in fulfillment of the requirements for the degree of Philosophiae Doctor in the Faculty of Veterinary Science, University of Pretoria

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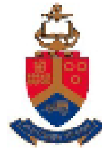

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Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

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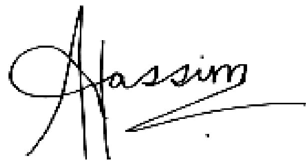
Declaration

I, Ayesha Hassim, hereby declare that this thesis submitted for the degree PhD (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.



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Dedication

To My Mum in heaven,

I'm sorry you didn't get to see me finish this one mama

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List of Abbreviations

°C	Degrees Celsius
µL	Microliter
µM	Micromolar
AFLP	Amplified fragment length polymorphisms
BSL	Biosafety Level
cAMP	Cyclic andenosine monophosphate
canSNP	Canonical single nucleotide polymorphism
CFU	Colony forming units
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleoside triphosphate
DVTD	Department of Veterinary Tropical Diseases
ENP	Etosha National Park
EDTA	Ethylenediaminetetraacetic acid
EF	Oedema factor
FRET	Fluorescence Resonance Energy Transfer
GPS	Global Positioning System

KNP	Kruger National Park
Km	Kilometres
LF	Lethal factor
MAPK	Mitogen activated protein kinase
$MgCl_2$	Magnesium chloride
MLVA	Multiple locus VNTR analysis
MLST	Multiple locus sequence typing
MS	Molecular screening
MST	Minimum Spanning Tree
MSU	Minimum spanning unit
n	Statistical symbol representing the sample size
NCP	Northern Cape Province
OIE	Office International des Epizooties
ORF	Open reading frame
PCR	Polymerase chain reaction
PA	Protective antigen
qPCR	Quantitative PCR
rRNA	Ribosomal ribonucleic acid
SNP	Single Nucleotide Polymorphism

SNR	Single nucleotide repeat
spp	Species
SSVO	Skukuza State Vetrianarian's Office
TFCA	Transfrontier conservation area
TBE	Tris-Borate EDTA
UP	University of Pretoria
UPGMA	Unweighted pair group method using arithmetic averages
UV	Ultraviolet
USA	United States of America
VNTR	Variable number tandem repeats
WHO	World Health Organization

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1. Chapter 1:

Introduction and Literature Review

1.1. Introduction

Bacillus anthracis, responsible for the disease known as anthrax, has an age-old history going as far back as 1491 BC as one of the ten plagues in the Bible (Farrar, 1995). This microbe is aerobic or facultative anaerobic, Gram-positive, non-motile rods measuring 1.0 μm wide and 3.0-5.0 μm long capable of forming central or terminal spores (endospores) (Leppla, 1982, Dixon et al., 2000). Spores are highly resistant to a variety of environmental conditions namely heat, cold, ultraviolet (UV) radiation, ionisation radiation, pressure and chemical agents compared to the vegetative forms of *B. anthracis* (Turnbull, 1999, Setlow et al., 2006, Vilas-Boas et al., 2007). These highly resistant endospores are found in soil at sites where infected animals have previously died (De Vos, 1998, Leppla et al., 2002, Bellan et al., 2013).

A host contracts anthrax through the uptake of spores or vegetative cells. Within the infected animal, the spores germinate to produce vegetative forms that multiply and which produce toxins that eventually kill the host (Leppla, 1982, Mock and Fouet, 2001, Turnbull, 2008). Endospores form during nutrient or oxygen starvation and persist for long periods in preparation for the next host, hence completing the life cycle of *B. anthracis* (Viljoen et al., 1928, Sterne, 1937b, Keim and Smith, 2002).

Anthrax is a zoonotic condition and can be transmitted to humans through contact with infected animals or animal products (Vilas-Boas et al., 2007, Turnbull, 2008). However, it is primarily a disease which affects wildlife and domesticated livestock. Despite the rare occurrence of human cases, anthrax remains a potential threat because of continued outbreaks of anthrax in animals and the importation of contaminated raw hair products (Laforce, 1978, Doganay et al., 2010). Anthrax is not transmitted directly from victim to victim, but instead is spread to susceptible host especially herbivores through various factors or vectors. Once the infected animal dies, spores are shed and distributed through watersheds and the environment (Pienaar, 1967, Dixon et al., 2000). Dissemination by stable flies, louse flies, mosquitoes and blow flies have also been reported (Viljoen et al., 1928, Turell and Knudson, 1987, Hugh-Jones and de Vos, 2002, Blackburn et al., 2014). Since endospores form upon nutrient starvation and exposure to oxygen, the opening of a carcass is prohibited by law in any circumstance where anthrax is suspected and all anthrax cases

should be reported to the OIE (Office International des Epizooties), where anthrax is a reportable disease (OIE, 2008b).

Molecular typing to differentiate isolates helps in understanding the epidemiology of anthrax, like the relationship between the disease in wild and domestic animals as well as humans. The long resting stage of the spores greatly reduces the rate of evolutionary change (i.e. it is a slowly evolving organism) (Keim et al., 2000, Sue et al., 2007). Therefore, developing molecular typing for the highly monomorphic *B. anthracis* species is challenging. Low diversity among *B. anthracis* isolates has been reported with most molecular techniques (Keim and Smith, 2002, Read et al., 2002, Hoffmaster et al., 2006). Hypervariability among short tandem repeats (indicated as variable number tandem repeats, VNTR) has been useful for strain typing (Jackson et al., 1998, Duffy et al., 2008, Hyytia-Trees et al., 2010, Lindstedt et al., 2012). Using multiple loci (MLVA) increases the resolution and discrimination of monomorphic bacteria such as *B. anthracis* (Keim et al., 2000; Van Ert et al., 2007a). Another method, normally used in conjunction with MLVA is single nucleotide polymorphisms (SNP) (Van Ert et al., 2007a).

Our current understanding of the fate of *B. anthracis* in different environments is fragmentary at best. The traditional belief is that spores remain dormant in the soil until taken up again by a mammalian host. However, this is not universally accepted and some features are not easily explained in terms of this theory. Kaufmann (1990) believed that the frequent pattern of outbreaks occurring with rain following a period of drought is best explained in terms of bursts of growth of *B. anthracis.* The persistence of spores at contaminated sites in the Etosha National Park (ENP) (Lindeque and Turnbull 1994), despite heavy rains, strong winds and hot sun with high surface temperatures raises the possibility of replication cycles maintaining contamination level. Dragon and Rennie (1995) as well as De Vos and Turnbull (2004) believed the eco-epidemiological patterns of anthrax in the northern Canada bison sanctuaries and Kruger National Park (KNP), South Africa respectively were in line with the "incubator area" hypothesis of Van Ness (1971). The hypothesis is based on depressions collecting water and dead vegetation which provide an organic medium suitable for the germination of anthrax spores and multiplication of the emergent bacilli (Van Ness 1971). Recently, Schuch and Fischetti (2009) hypothesised that *B.*

anthracis with bacteriophages in lysogenic phase dramatically altered the survival capacity as well as blocking or promoting sporulation, inducing biofilm formation and enable long-term colonisation of the soil environment and soil-borne organisms like the gut of redworm.

Key Questions

The key questions of this thesis include the following:

Does disinfection /disposal of carcass sites decrease the inoculum in the environment?

Evaluation of the most appropriate sample to collect, and can isolation be improved?

What is the distribution of anthrax and does the diversity differ in different regions of KNP and between endemic regions in South Africa?

Are there bacteriophages that impact on genetic diversity and behaviour of anthrax spores through the introduction of virus-encoded factors that alter the bacterial phenotypes through lysogenic conversion?

What is the relationship between the environment, bacteriophages and anthrax?

These research questions will be investigated with the following objectives:

Objectives

1. Isolation and enumeration of *B. anthracis* from endemic areas of South Africa.
2. Determine the distribution of anthrax strains in Kruger National Park and northern Cape using MLVA (multiple loci variable number of tandem repeats) from current and historical isolate libraries
3. To examine the value of stained blood smears (as it is a readily available sample source) and whether the possibility of retrospective studies using this resource can be conducted.
4. Establishment of a database for the spatio-temporal analyses of anthrax occurrence in relation to the environment, host and vector distribution
5. Determine the association of bacteriophages from *B. anthracis* strains and from soil where the bacterium has been isolated
6. Characterisation of bacteriophages

1.2. Literature Review

1.2.1. Taxonomy

Bacillus anthracis is a Bacillales bacterium within Firmicutes. It is related to 3 genetically similar, but phenotypically different bacteria among the broader group known as the *Bacillus cereus* group. *Bacillus cereus*, *B. thuringiensis,* and *B. anthracis* are soil borne pathogens which demonstrate similarities in their ability to sporulate, but differ in aetiology and the presence of circular DNA fragments called plasmids. The other 3 members of the *B. cereus* group are: *B. mycoides, B. pseudomycoides* and *B. weihenstephanensis* (Daffonchio et al., 1999, Radnedge et al., 2003).

The *B. cereus* group are Gram positive, rod shaped bacteria, which means that their cell surfaces are positively charged and stain dark blue or violet when Gram stained. Gram positive bacteria have a thick cell wall, which protects the cell contents from mechanical damage by external forces. *Bacillus anthracis* can be distinguished from other members of the *B. cereus* group by its characteristic square end, encapsulated bacilli, lack of motility, its absence of haemolytic activity on blood agar and penicillin sensitivity. *Bacillus anthracis* colonies are mucoid in appearance, on bicarbonate medium, with optimal growth at 37°C and show a clear zone of lysis after inoculation with gamma phage (Parry et al., 1983, Turnbull et al., 1992, Vilas-Boas et al., 2007). There are approximately 3 % - 6 % of isolates which do not conform to the penicillin and gamma phage sensitivity (Coker et al., 2002).

Anthrax spores are able to withstand harsh environmental conditions for prolonged periods (decades in length) due, in part, to its morphology. *Bacillus anthracis* spores are covered by three prominent protective layers: the cortex (composed of a thick peptidoglycan layer); the coat (covers the cortex in a large number of proteins); and the exosporium (a balloon-like layer) that serves as the primary contact surface between the spore and the environment (Beaman et al., 1972, Ezzell and Abshire, 1988, Ezzell and Welkos, 1999, Liu et al., 2008).

1.2.2. Ecology

The ability of anthrax spores to persist in the earth for extended periods is due to specific soil and climatic conditions, which provide an environment conducive to its survival (Van Ness, 1971, De Vos, 1990, Smith et al., 2000). Soils rich in organic matter, ample in calcium, prone to alkalinity and with an ambient temperature above 15.5 °C are considered optimal for persistence of spores. The exosporium of the spore is negatively charged (this charge and its strength are pH dependent) whereas humus particles are positively charged and are therefore able to chelate (i.e. collect and hold bacteria), which then provides environmental stability for the spores (Beaman et al., 1972, Baweja et al., 2008, Turnbull, 2008, Hugh-Jones and Blackburn, 2009a). This ensures that high levels of spores are available at sites where carcasses reside, levels which could be potentially lethal to the next grazer (Coker et al., 2002, Turner et al., 2013). These humus-spore clumps have the added advantage of being buoyant (Bakken and Olsen, 1983). During flooding, these clumps can be deposited or concentrated at water collection sites once the water evaporates, as the spores remain unaffected by UV radiation (Hugh-Jones and de Vos, 2002, Vilas-Boas et al., 2007). It is possible that the negatively charged anthrax spores would attract divalent cations, such as calcium, which would then aid in maintaining the spore core matrix, spore viability and germinative ability (Smith et al., 1955, Smith et al., 2000, Hugh-Jones and de Vos, 2002, Hugh-Jones and Blackburn, 2009b).

1.2.3. Routes of Infection

There are three routes to infection (in the context of this study): cutaneous, gastrointestinal and inhalation (Turnbull, 1999, Vilas-Boas et al., 2007). If left untreated all three can progress to fatal systemic anthrax (Dixon et al., 2000), although the inhalational form is considered the most lethal of the three (Turnbull et al., 1986). Incubation periods are 2-7 days for cutaneous infection, 3 hours up to 3 days for inhalational infection and generalized toxaemia during gastrointestinal infection resulting from 1 to 7 day incubation period (Hambleton and Turnbull, 1990). A fourth route to infection, observed in Europe, is injectional anthrax. It's symptoms are different due to typical infection by injection of contaminated heroin directly into the arterial system (Grunow et al., 2012).

1.2.4. Mode of Action

Bacillus anthracis strains contain two plasmids, pX01 (181 kb) and pX02 (95 kb), which are required for virulence. They are responsible for toxin production and encapsulation, respectively (Green et al., 1985, van Schaik et al., 2007). These two main virulence factors, pX01 and pX02, are dependent on the action of antiphagocytic poly-D-glutamic acid capsule and the tripartite protein (Dixon et al., 2000). Anthrax exotoxin is composed of the cell-binding protein, protective antigen (PA, *pag* gene, 83 kDa), which is cleaved after binding to a receptor on the host cell, creating a binding site for either lethal factor (LF, *lef* gene, 90 kDa) or oedema factor (EF, *cya* gene, 89 kDa) (Petosa et al., 1997, Pannifer et al., 2001).

The two exotoxins are therefore produced through an interaction of PA and LF (Figure 1.1) forming the lethal toxin and PA and EF forming the oedema toxin (Singh et al., 1994). PA is cleaved and activated by a host protease to form PA_{63}, which forms a heptamer with an exposed binding site for either of the two toxic enzymes, LF or EF (Leppla, 1982, Redmond et al., 2004). The resulting toxin complex is endocytosed into intracellular compartments called endosomes. A conformational change in PA_{63} is triggered by the natural acidification of the endosome, which then confers the toxic enzyme into the host cell interior. Once inside, the toxin is able to damage the host cell defence system (O'Brien et al., 1985, Ascenzi et al., 2002).

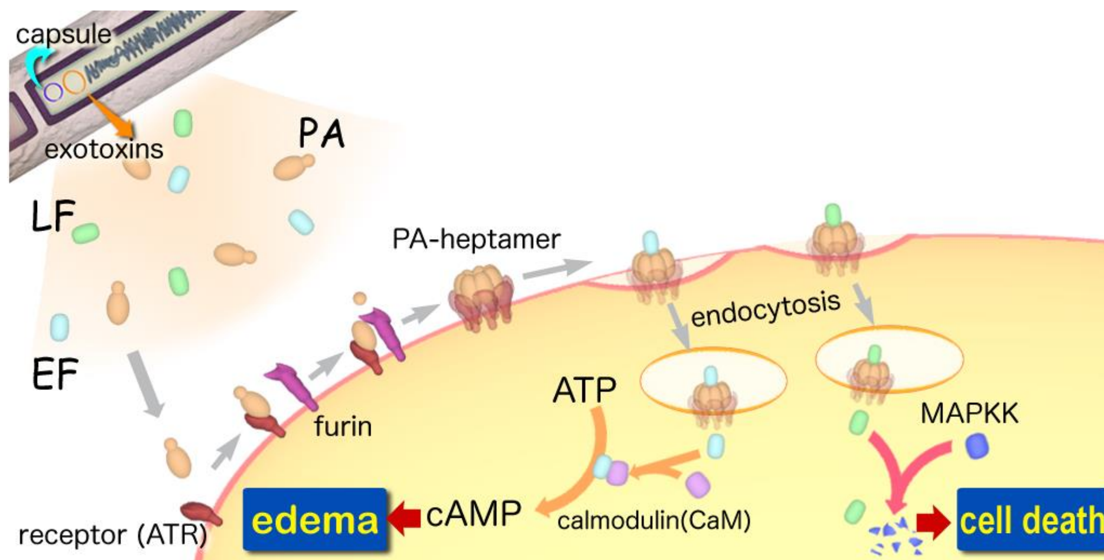


Figure 1.1: Mode of action of anthrax exotoxins; oedema factor (EF) and lethal factor (LF) leading to oedema and cell death. These toxin factors are expressed in combination with protective antigen (PA). The toxin-complex is then endocytosed into the cell interior where it damages the host cell defence system. (http://en.wikipedia.org/wiki/Image:Anthraxtoxins_diagram_en.png)

The PA and two enzyme components, EF and LF are expressed by pX01. The number of pX02 plasmids in each bacterial cell, contributes to the level of virulence associated with that isolate (Pannifer et al., 2001, Coker et al., 2003). However, a tremendous variation in pX01 and pX02 copy numbers have been observed in a genetically diverse collection of isolates using a semi-quantitative technique (Coker et al., 2003, Hoffmaster et al., 2004, Rasko et al., 2007). It is during the vegetative form of *B. anthracis* that all known anthrax virulence genes are expressed. This results from the germination of spores within the body (Dixon et al., 2000).

EF is a calmodulin-activated adenylate cyclase that generates cyclic adenosine monophosphate (cAMP) to abnormal levels, which attracts fluid within and between cells, a process known as oedema. The disruption of normal signalling pathways results in cell lysis, the sudden release of messenger molecules and toxic shock (Leppla, 1982, Pannifer et al., 2001). LF helps the pathogen to evade the immune system through killing macrophages. In macrophages LF acts as an endoprotease that removes the N-terminus of the mitogen

activated protein kinase kinase (MAPKK) (Duesbery et al., 1998). LF achieves this by removing the specific fragment from individual MAPKs that is crucial for immediate interaction with other signalling proteins. This action rapidly blocks the signals that would normally recruit other immune cells to fight the infection that results in apoptosis/ programmed cell death (O'Brien et al., 1985, Duesbery et al., 1998).

1.2.5. Symptoms/Signs and Pathology

Clinical symptoms in animals include fever, shock, multiple organ failure (Leppla et al., 2002), discharge/bleeding from various orifices, lesions which resemble black eschars/ carbuncles (Vilas-Boas et al., 2007), cardiac distress, respiratory distress and oedematous swelling of the tongue, face and throat. Often, death occurs shortly after the appearance of the first symptoms (Hambleton et al., 1984). Usually, animals that die from anthrax do not display signs of *rigor mortis.* As the names 'splenic disease' / "miltsiekte" suggest, the most obvious sign during necropsy is severe splenomegaly (Viljoen et al., 1928, Hugh-Jones and de Vos, 2002, De Vos and Turnbull, 2004).

1.2.6. Diagnostics

A Gram stain or Giemsa stain of the blood smear is the first step taken in confirming the presence of *B. anthracis* in any case where anthrax is suspected. Classically, M'Faydean reaction (polychrome methylene blue stain) can be included for confirmation of the presence of capsules. Once the presence of square ended encapsulated bacilli is confirmed microscopically, a sample (blood/tissue/bone) is sent to the reference laboratory for confirmation (Turnbull, 1999). Bacteriologic isolations are performed using heat treatment and selective media (Knisely, 1966, Marston et al., 2008, Turnbull, 2008). In human cases, a serum sample should also be included for serological assays, which involve testing for the toxin antigen (Schumann and Henry, 2001). At the reference laboratory, the bacterium is purified and thereafter cultured on blood agar and examined for colony morphology, lack of haemolytic activity, penicillin sensitivity and bacteriophage sensitivity in order to be identified as *B. anthracis* (Turnbull, 1998). A small but significant percentage of *B. anthracis* isolates demonstrate either bacteriophage or penicillin resistance. Therefore, none of these tests should be performed in isolation for the definitive identification of *B. anthracis* (Coker et al., 2002; Fulmer et al., 2003). Further testing by PCR for the *cap* and *pag* genes which code

for the plasmid encoded toxins is commonly used for verification (OIE, 2008a, Turnbull, 2008). The qPCR FRET (fluorescence resonance energy transfer) based diagnostic assay targeting the *B. anthracis* protective antigen (BAPA) on pX01, capsule coding region (Cap C) on pX02 and chromosomal SASP (small acid soluble proteins) markers are the WHO recommended tests for the confirmation of *B. anthracis* (Turnbull, 2008).

1.2.7. Vaccine and Control

Prompt and timely antibiotic treatment generally results in the recovery of the animal or human infected with anthrax. Administration of antibiotics does not guarantee patient survival once anthrax toxins are present systemically in lethal quantities, especially in the case of inhalational anthrax (Turnbull et al., 1986, Turnbull, 1991, Schumann and Henry, 2001). *Bacillus anthracis* is typically penicillin sensitive and can also be treated with tetracycline, chloramphenicol and streptomycin quite effectively (Schumann and Henry, 2001). Even if animals die after antibiotic treatment, the infective load of *B. anthracis* will be greatly reduced (Turnbull, 1998).

Robert Koch established the bacterial aetiology of the disease in the 1850's (Hambleton et al., 1984). *Bacillus anthracis* was used as the first bacterial vaccine by Pasteur in 1881 (Turnbull, 1991, Leppla et al., 2002). The heat attenuated *B. anthracis* culture was capable of forming capsules, but not toxins (Sterne, 1937a, Sterne, 1937b, Farrar, 1994). The Pasteur vaccine provided a lower level of protective immunity than toxigenic vaccine strains that was developed years later, like the Sterne strain 34F$_2$ (Sterne, 1937a). Widespread vaccination began in the 1930's, using the Sterne attenuated strain, which virtually abolished anthrax in industrialized countries (Leppla et al., 2002). By 1939 the Sterne vaccine had proven itself to be safe and effective for use in domesticated animals and seemed to provide complete protection against challenge with highly virulent strains of *B. anthracis* (Sterne, 1937a, Sterne, 1939). Although effective with animals, live attenuated strains are considered undesirable for human vaccine use (Hambleton et al., 1984, Hambleton and Turnbull, 1990). While vaccination programs and good farming practices have gained the upper hand in control of anthrax in industrialised countries, it remains a serious problem in many developing countries (Farrar, 1994).

1.2.8. Distribution and Spread

Anthrax is a disease which occurs in many parts of the world, including Asia, central and South America, southern Europe, sub-Saharan Africa and small pockets of Australia, northwest Canada, USA, Russia and Haiti (Hugh-Jones et al., 2000; Keim et al., 2000; Smith et al., 2000). Sporadic cases may occur in other countries, usually traceable to imports of contaminated animal material (Bales et al., 2002). Southern Africa is among the regions where anthrax regularly occurs endemically or epidemically (OIE handistatus II).

In South Africa there is currently two endemic regions namely the KNP and Ghaap region in NCP. Annual reports to OIE show the occurrence of outbreaks in domestic and wild animals every year in Namibia, South Africa and Zimbabwe and, at least in wild animals, most years in Botswana (OIE handistatus II). It is suspected that under-reporting occurs in this region especially in wildlife because of surveillance inadequacies and difficulties monitoring range wildlife. The official reports therefore probably fall short of the true incidence in many instances (OIE handistatus II). .

The introduction of the Sterne spore vaccine and other legislative processes influenced outbreak trends in South Africa (Figure 1.2). At the peak of the outbreaks in 1920s, anthrax killed between 30000-60000 animals in one year (1923) in South Africa (Sterne, 1967, Turnbull, 1991, Gilfoyle, 2006). During the time the vaccine was successful in reducing the incidence of outbreaks, especially once it was freely dispensed and its use became common practice. The outbreak events steadily dropped over the next 4 decades. The decrease is quite marked after 1937 when the Sterne vaccine was introduced as a compulsory annual vaccine distributed freely by the government amongst farmers and African-owned stock (Gilfoyle, 2006). Between 1968 and the mid 1980's, it became mandatory to inoculate livestock in endemic areas and the incidence of outbreaks dwindled to virtually nothing except for isolated cases (De Vos and Turnbull, 2004).

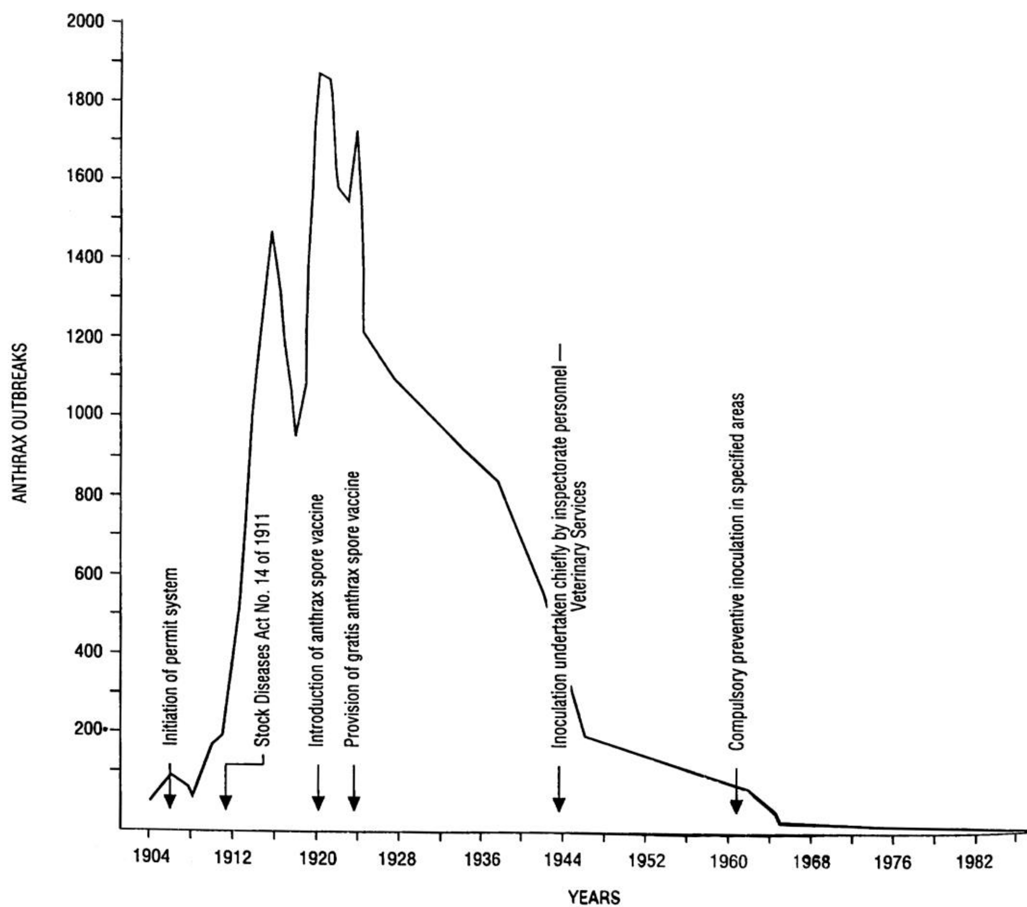


Figure 1.2: Distribution and spread of anthrax in South Africa demonstrating peak periods of the disease. Taken from De Vos & Turnbull (1994) in Infectious Diseases of Livestock, Volume 3, Chapter 176 (editors Coetzer, J.A. and Tustin, R.C.).

1.2.9. Southern African Outbreaks

1.2.9.1. Outbreaks in African Game Parks

Keim et al. (2000) believe sub-Saharan Africa to be the geographic origin of anthrax. Many of the pristine natural reserves experience cyclical/periodic epidemics. The following conservation parks experience outbreaks as part of the ecological cycle (topography and climatic conditions): the Queen Elizabeth National Park in Uganda; the Omo-Mago National Park in Ethiopia; the Selous Nature Reserve in Tanzania; the Luangwa Valley in Zambia; the ENP in northern Namibia; Vaalbos National Park; the Kgalagadi Transfrontier Park bordering South Africa and Botswana; Hwange as well as Malilangwe Wildlife Reserve in Zimbabwe and KNP in South Africa (Hugh-Jones and De Vos, 2002).

In southern Africa, anthrax does not affect all herbivorous animal species equally and there is an apparent preference by the disease for a particular species in any one region. Zebras (*Equus burchelli*), for example, are the most commonly affected species (45 % of all recorded anthrax deaths) in the ENP with kudu (*Tragelaphus strepsiceros*) only occasionally affected (0.8 % of anthrax deaths) (Lindeque and Turnbull, 1994). In the KNP, the kudu is the principal host accounting for >50% of all recorded anthrax cases with zebra falling into a relatively small group of "other affected species" (De Vos, 1990). In Malilangwe, the kudu once again seems to be the predominant host followed by the hippopotamus (*Hippopotamus amphibious*) (Clegg et al., 2007) with hippopotamus and elephant (*Loxodonta africana)* most affected in the Luangwa Valley (Turnbull et al., 1991). In the Kgalagadi, the nyala (*Tragelaphus agassi*), buffalo (*Syncerus caffer*), kudu and giraffe (*Giraffa giraffa*) are equally affected (Hugh-Jones & De Vos, 2002).

During outbreaks in the KNP in previous years, it was noted that a high stocking rate and large percentage of susceptible host species needed to be present in order for an epidemic to occur. Hugh-Jones and De Vos (2002) reported that anthrax failed to kill up to 20% of its hosts and left a great proportion of young animals to be counted among the living, which makes it an ideal means of natural culling. The authors concluded that in a natural setting, an anthrax outbreak is dependent on the stocking density and is self-limiting (Hugh-Jones and De Vos, 2002).

Anthrax is a multispecies disease that can infect (predominantly) mammals. Ruminants and hindgut digesters are most susceptible, whereas carnivores and primates (including humans) are more resistant. This is supported in serological surveys in the anthrax endemic ENP where it was discovered that naturally acquired anthrax antibodies were rare in herbivores, but common amongst carnivores (Turnbull et al., 1992, Turner et al., 2016).

1.2.9.2. Disease Vectors and Drivers

Circumstantial evidence points to certain ecological and behavioural factors as being responsible for the differences in preferential species infection and dissemination during an outbreak (Figure 1.3). Preferential species infection can be attributed, in part, to the behavioural ecology of animals in the environment (Turner et al., 2014). Hippopotamuses

are known to cannibalise their dead (Bere, 1959, Dudley, 1998) resulting in higher anthrax cases amongst hippopotamus populations during an outbreak (Hang'ombe et al., 2012, Dudley et al., 2016). Drivers such as water and feed availability, parasitic burden, nutritional stresses and climate also have an influence on disease risk and exposure (Neser et al., 2000, Steenkamp, 2013, Turner et al., 2013, Cizauskas et al., 2015). In the KNP, ecological suitability modelling highlighted the importance of water as a driver of anthrax (Steenkamp, 2013). There is a combination of manmade and natural water points for wildlife in KNP and the management thereof has an influence on animal movement and thus disease exposure (Pienaar, 1967).

Besides predators and scavengers like the spotted hyena (*Crocuta crocuta*), other carrion feeders of interest are vultures including the white backed vulture (*Gyps africanus*), Cape vulture (*G. coprotheres*) and Lappet-faced vultures (*Aegypius tracheliotus*), which are believed to open the carcasses and spread the pathogen from anthrax infected carcasses to the environment and watering holes (Turnbull *et al.*, 2008). It is believed that vultures are able to consume the bacilli laden soft-tissue of an anthrax carcass before the bacterium is exposed to air and is able to sporulate and spread spores whereas the hardy digestive system of this scavenger easily kills off the bacteria in its vegetative form (De Vos, 1990, Hugh-Jones and De Vos, 2002, Saggese et al., 2007, Turnbull et al., 2008). Vultures from the Magaliesburg Mountains in South Africa have a documented flight radius of 300 – 500 km (Saggese et al., 2007, Turnbull, 2008) and it has been hypothesised that vultures could disseminate and allow tracing back to outbreaks in neighbouring countries, like Botswana, that fall within their flight radius (Hugh-Jones and De Vos, 2002).

The link between the dissemination of *B. anthracis* from an infected carcass to the surrounding vegetation through necrophagous flies has been established diagnostically through PCR (Blackburn et al., 2014). It has been observed that blowflies (*Chrysomya albiceps* and *C. marginalis*) in the KNP, after feeding on anthrax carcasses, fly to nearby shrubbery and deposit infected blood on the leaves that are 1 – 3 m in height, which then become a potential source of infection for the browsing kudus (Braack and De Vos, 1990, De Vos and Turnbull, 2004). This is known as the case multiplier hypothesis (Blackburn et al., 2010). It has been speculated that insects spread anthrax to nearby and more distant shrubbery as *C.*

albiceps has been known to circulate up to a 32.5 km radius, while *C. marginalis* has been noted 25 km from a source (Braack and De Vos, 1990; Turnbull, 2000). In the NCP, louse flies have long been implicated as a vector of anthrax, specifically amongst equids (Viljoen et al., 1928, Henning, 1949, Howell et al., 1978). Tabinids have been described in similar terms in Italy (Fasanella et al., 2010a, Palazzo et al., 2012). The spores of *B. anthracis* have been experimentally ascertained to survive from the fly larval stage and retain virulence up to 19 days after the emergence of a fly (Graham-Smith, 1914). In contrast, a study on *Calliphora vicina* by von Terzi et al. (2014) disputes the fly's status as a disease "vector" by demonstrating an inability of *B. anthracis* to replicate and survive within the fly for mere hours to days after feeding on the contagion, although the authors concede that transmission is plausible. The magnitude of the role of fly species in the dissemination and possible transmission of *B. anthracis* has thus not been well explicated, despite the well represented anecdotal evidence.

As mentioned earlier, the soil chemistry is hypothesized to have an influence over bacterial spore survival over time (Van Ness and Stein, 1956, Smith et al., 2000). It was proposed by Van Ness (1971) that water/fluids and decomposing plant material in soil depressions could provide a nutrient rich medium conducive to bacterial replication in the environment. This is known as the incubator area hypothesis. The veracity of this hypothesis has yet to be proven. Yet there are many aspects to the soil born cycle of the bacterium which are still poorly understood. This includes the influence of other soil inhabitants (Figure 1.3). Biofilm formation increases *B. anthracis* resistance to external factors such as antibiotics and increases survivability, however its role in the ecology of the disease in the environment is unknown (Lee et al., 2007). Amoebas have been hypothesised to play a role in the germination, persistence and multiplication of *B. anthracis* in the soil environment (Dey et al., 2012). Annelids have been hypothesised to harbour bacteriophages which mediate sporulation and replication factors of *B. anthracis* (Schuch and Fischetti, 2009). In a study by Saile and Koehler (2006), it was determined that *B. anthracis* could survive as a saprophyte within plant rhizospheres. It was also demonstrated that lateral gene transfer, such as tetracycline resistance, could be exchanged between *B. cereus* group members within this environment (Saile and Koehler, 2006). This has implications for the diversification and improved fitness of the bacterium.

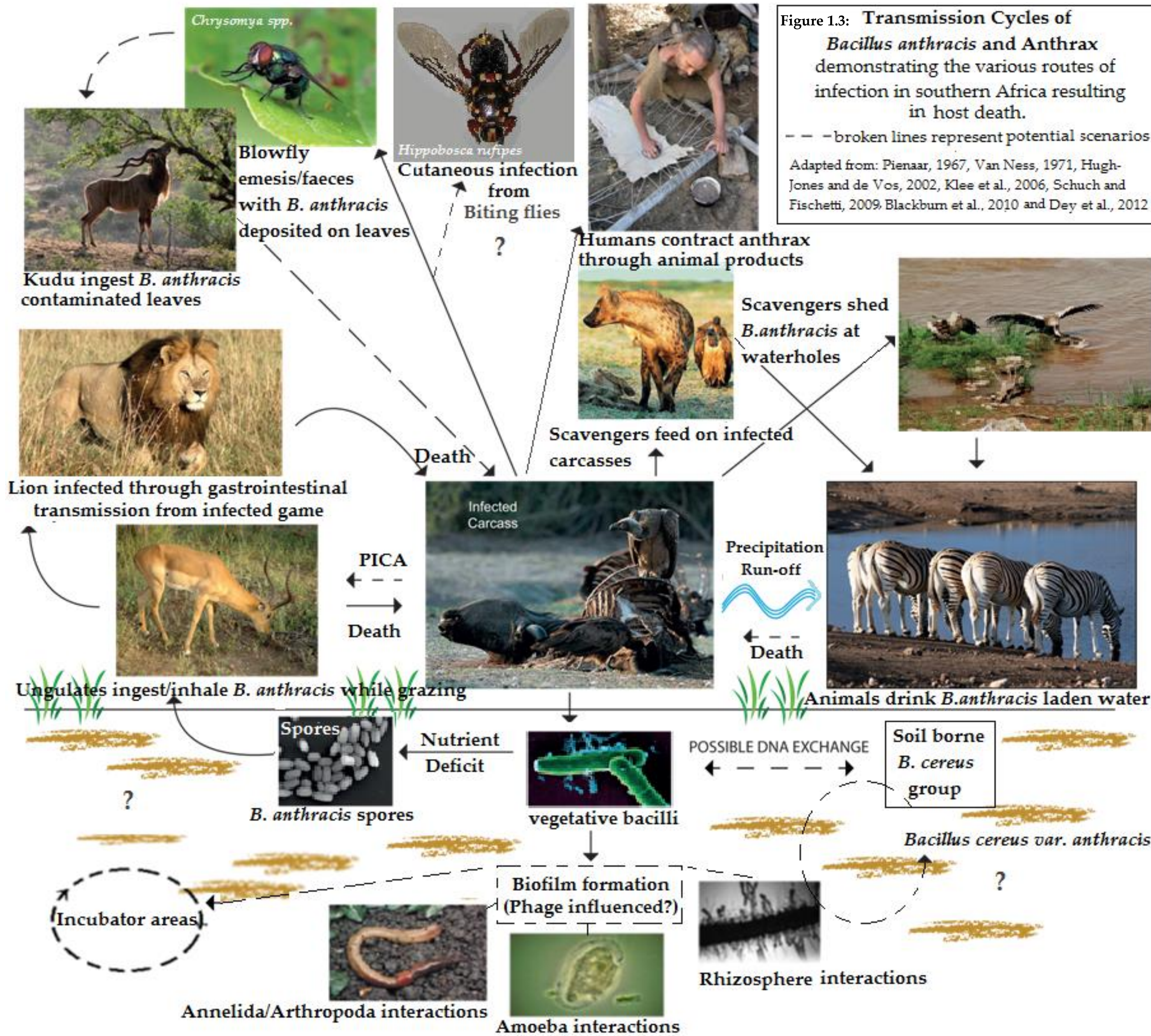


Figure 1.3:

The dissemination and transmission cycles of *Bacillus anthracis* demonstrating the various possible routes to infection in the African wildlife context

1.2.9.3. Anthrax and Anthrax-like

There are field cases which present with symptomatic indicators for anthrax, but are atypical in diagnostics. The case of a severe pneumonial condition resembling inhalational anthrax was observed in the USA. The isolate from this patient was identified as *B. cereus* containing a pBCXO1 homologous to the *B. anthracis* pX01. While homologs for pX02 were not discovered, the pBC218 coded for a polyssacharide capsule (Hoffmaster et al., 2004). Another case in the USA was published on a patient presenting with an anthrax-like black eschar, which was identified as *B. cereus* that was sero positive using the anti-PA IgG and toxin neutralizing assay and produced results equivalent to a cutaneous anthrax infection (Marston et al., 2016). Isolates belonging to the *B. cereus* group members have repeatedly produced confounding diagnostic results in such cases, which include non-haemolytic *B. cereus*, motile *B. anthracis*, antibiotic- and γ phage-resistance (Turnbull et al., 1992, Okinaka et al., 2006, Hoffmaster et al., 2008, Lekota et al., 2016, Marston et al., 2016). In west and central Africa a monophyletic group called *B. cereus* biovar *anthracis* is the causative agent of anthrax in chimpanzees, gorillas, elephant and domesticated animals. This bacterium possesses a *B. cereus* genome which includes both the *B. anthracis* plasmids (Klee et al., 2006, Klee et al., 2010, Antonation et al., 2016).

In addition to B. cereus group isolates that cause anthrax like disease, new phylogenetic groups of B. anthracis have been described with unique phenotypes. In Chad and Mali, the Aβ clade is made up of isolates that are deficient in the spore surface associated anthrose-containing oligossacharide. The Aβ clade was identified through variable number tandem repeat (VNTR) typing (Tamborrini et al., 2010, Tamborrini et al., 2011). The absence of the anthrose sugar is notable, as it forms part of the terminal structure of the *B. anthracis* collagen – like protein (BclA), which forms part of the fabric of the outermost layer of the mature spore. Anthrose has been investigated as a target for immunological identification of spores as well as for the application of therapeutic action, however, the Aβ clade isolates would in effect be excluded (Daubenspeck et al., 2004, Leski et al., 2009). These isolates have highlighted the need for detailed characterisation, which includes molecular typing, in the diagnosis of anthrax.

1.2.10. Molecular Characterization

Molecular techniques have provided valuable insight into discrimination of bacterial species. The ribosomal genes are normally worthwhile targets in differentiating bacterial strains (Woese, 1987). Ash *et al.* (1991) as well as Ash and Collins (1992) have determined that amongst the *B. cereus* group, which includes *B. anthracis*, the 16S rRNA is identical and the 23S rRNA sequences differ at only 2 nucleotides amongst the *B. cereus* group. Multi-locus sequence typing (MLST) has also been used to differentiate other pathogens, but fails to adequately discriminate *B. anthracis* strains, due to the rarity of its sequence variation (Daffonchio *et al.*, 2006; Hoffmaster *et al.*, 2008). Tandem repeats on the other hand, have proven to offer informative markers for strain genotyping (Denoeud & Vergnaud, 2004). Tandem repeats are characterized as minisatellites (repeat unit consisting of 6 – 100 base pairs (bp) and microsatellites (repeat unit consisting of 1 – 5 bp) and has been used for the identification of pathogenic bacteria (Le Fleche *et al.*, 2001). Slip strand repair mutations caused by DNA polymerase is known to occur more frequently on the short repeats and can therefore be used for strain typing in the monomorphic *B. anthracis* species (Keim et al., 1999, Achtman et al., 2004) as multiple alleles can exist for each tandem repeat locus (Lista *et al.*, 2006). Tandem repeats therefore undergo rapid evolution which then augments the phenotypic variability of the pathogen i.e. tandem repeats play an important role in the adaptation of the pathogen to the host (Le Fleche et al., 2001).

1.2.10.1. Variable Number of Tandem Repeats (VNTRs)

Tandem repeats contribute phenotypic variation in two ways: (1) Tandem repeats in the regulatory region of a gene can constitute an on/ off switch of gene expression at a transcriptional level; and (2) Tandem repeats within coding regions, with repeat unit lengths, which are not a multiple of three can induce a premature end of translation when a mutation changes the number of repeats. Loci with tandem repeats in the microsatellite class are called simple sequence contingency loci, which are believed to have limited value to epidemiological studies but are of interest for pathogenicity studies (Le Fleche et al., 2001, Fouet et al., 2002). The variability in the VNTRs of a specific VNTR locus only provides a small amount of information. Multiple loci are examined concurrently to further increase its discriminatory power, hence a MLVA system (Le Fleche et al., 2001).

The first panel of MLVA8 markers (Keim et al., 2000) were identified using the VNTR loci found in the *Vrr*A gene (Andersen et al., 1996) as well as sequenced amplified fragment

length polymorphisms (AFLP) markers of *B. anthracis* isolates (Keim et al., 1999; 2000), and VNTR loci found in the plasmids (pX01 and pX02) (Keim et al., 1999). Keim et al. (2000) used an automated fluorescent DNA sequencer (automated capillary electrophoresis) to determine the size of the PCR fragments. Cluster analysis with the MLVA8 loci identified two dissimilar cluster groups (clade A and B) (Keim et al., 2000). Isolates in clade A are found internationally and are therefore responsible for most epidemics and outbreaks globally. In contrast, Clade B strains are geographically restricted, consisting of KrugerB which is exclusively restricted to southern Africa and B "CNEVA" identified in France (Keim et al., 2000, Smith et al., 2000, Van Ert et al., 2007a). However, the resolution achieved with the MLVA8 was found to be limiting since it did not have enough resolving power to discriminate between two geographically close isolates in natural outbreaks (Keim et al., 2004, Lista et al., 2006).

Additional markers were developed and the MLVA15 assay was used in combination with canonical single nucleotide polymorphisms (canSNPs) (Keim and Smith, 2002, Van Ert et al., 2007b). The canSNPs on its own have limited resolving power when compared to MLVA. However, canSNPs can be used to define the main clades and key phylogenetic positions in the clades (Keim et al., 2004). The MLVA15 divided the *B. anthracis* isolates into three major clades, namely clade A, B and C. The Branch C isolate is also a restricted scale clade found in North America (Van Ert et al., 2007a, Pilo and Frey, 2011).

The European group extended the MLVA8 assay to MLVA25 (Le Fleche et al., 2001; Ciammaruconi et al., 2008). The PCR fragment sizes were determined using standard agarose gel, making this technique more accessible by using basic equipment. The MLVA25 loci enabled the description of two new clades (D and E). The D cluster was composed of an isolate of unknown origin and another from Italy, while the E (equivalent of Aβ) cluster was composed of isolates from Cameroon (Lista et al., 2006). The typing of strains using the MLVA markers enables distinction of outbreaks caused by different strains from those caused by the spread of a single strain, to trace an outbreak strain back to its possible origin and to track the routes of transmission of an outbreak strain within and between animal populations. It is becoming possible to study genotypic diversity in relation to the spatial and temporal dynamics behind the spread of the disease and possible relationships between genotype and host species (Beyer and Turnbull, 2009). Beyer et al. (2012) combined MLVA25

and MLVA15 to make the MLVA31 which is best able to differentiate *B. anthracis* genotypes thus far.

It is not known whether different *B. anthracis* genotypes (strain types) can account for prevalence in different host species and temporal-spatial distributions. Dominant strains can be identified with molecular techniques (Keim et al., 2000, Le Fleche et al., 2001, Lista et al., 2006, Van Ert et al., 2007a, Beyer & Turnbull, 2009) and therefore studies can be undertaken to determine the phenotypic and genotypic basis of their prevalence, including virulence, ability to sporulate, tenacity of spores and their preferential spread by living vectors.

1.2.10.2. Determining Copy Numbers for MLVA

In MLVA studies, PCR amplicons targeting VNTR's are viewed elecrophoretically, on either of two different platforms (i.e. agarose and capillary) to determine the amplicon size. Agarose gel electrophoresis is a method commonly practiced in molecular laboratories. The equipment and reagents needed for this technique are readily available. Using this platform for MLVA requires long migrations of PCR amplicons to visualise differences in amplicon size (Vergnaud and Pourcel, 2009). The copy number is then calculated using the PCR amplicon size and a comparison table (Beyer et al., 2012, Thierry et al., 2014) where the flanking sequence was subtracted from PCR product size and using size of the tandem repeat (in bp) to determine the copy number of tandem repeat unit. The copy number represents the number of times the repeat is produced for each locus and hence describes the allele for that locus. It is necessary to include both a reference strain and molecular marker for every 6 samples tested on a gel to minimise errors originating from gel distortions or poor migrations (Vergnaud and Pourcel, 2009). The accuracy of this method decreases with repeat units smaller than 8 bp as it becomes difficult to differentiate such small differences in band size.

The capillary electrophoresis technique uses capillaries loaded with a gel matrix. Fluorescently labelled amplicons are run at 500V/cm through the gel matrix and the fluorescence wavelengths are recorded. These results are displayed as an electropherogram (Buel et al., 2001). This method is clearly able to differentiate between amplicons which differ by even 2 bp; however the results are not consistent across all genetic analyzer machines (Lindstedt, 2005). The make and model as well as capillary length and diameter influence the readings (Buel et al., 2001) as well as polyacrylamide percentage within the

capillary matrix. This is further complicated by differences in the analysis software used to view the electropherograms. The results from the two platforms are therefore also not directly comparable without adjustment. The $34F_2$ vaccine, Vollum and Ames strains were used to "calibrate" the genetic analyzer and analysis software so that the copy numbers would be automatically assigned and comparable to the agarose technique. Even with these controls, different research groups have different copy number calling protocols as delineated in Thierry et al. (2014). This makes inter-laboratory comparison of datasets difficult, since adjustment is once again necessary. The international copy coding convention proposed by Thierry et al. (2014) has thus been adopted for this study.

1.2.10.3. Mismatch Amplification Mutation Assay

Single nucleotide polymorphisms (SNPs), as described above, are able to differentiate phylogenetic lineages based on point mutations traced back to ancestral strains. A necessity in this type of PCR based assay is controls from the different lineages with which to compare DNA from uncharacterised isolates (Van Ert et al., 2007a). To make this assay accessible to laboratories that do not have extensive isolate libraries, synthetic controls have been designed for each SNP marker. There are 2 synthetic controls for each allele; one for the ancestral SNP and another to represent the mutation "derived" SNP. There are two corresponding oligonucleotides to target these SNP's. A 20 bp GC - clamp forms part of the "derived" oligonucleotide in the assay to allow differentiation on size and melting tempreture. Preferential binding of the same allele in the oligonucleotide and bacterial target DNA results in amplicons with differing melt temperatures. The divergence profile of the various markers then indicates the ancestral lineage of the bacterium in question. This method is called Melt-MAMA and is applicable on either an agarose or qPCR based platform (Birdsell et al., 2012).

A major limitation with this assay is that the SNP marker panels that are already available tend to be region specific and therefore provide insufficient resolution for genotyping in Africa. With the advent of more accessible next generation sequencing technology, whole genome SNP analyses has become the more popular means of characterisation and phylogeographic analyses (Garofolo et al., 2010, Derzelle et al., 2011, Girault et al., 2014). It is also a more accurate way of tracing strains to their origin (Pullan et al., 2015).

1.2.10.4. Definitive isolate identification

Some of the SNP markers also have their place in diagnostics. The pleiotropic activator regulon (PlcR) of *B. cereus* and *B. thuringiensis* is responsible for the secretion of non-specific toxins in mammals; such as haemolysin. The presence of a nonsense mutation in the PlcR gene is specific to *B. anthracis* and renders it inactive (Mignot et al., 2001). This nonsense mutation was validated as a target in a hydrolysis qPCR based assay as a definitive diagnostic for *B. anthracis* (Easterday et al., 2005). This SNP has also been included as a Melt-MAMA marker for use in qPCR using a randomly intercalating dye or an agarose electrophoresis platform (Birdsell et al., 2012).

A contributing factor to genome diversity is the viral DNA that has incorporated itself into a host structure. This viral DNA can constitute between 10 – 20 % of a bacterial genome (Casjens, 2003). Another means of differentiating *B. anthracis* from other *B. cereus* group members are the four *B. anthracis* specific prophages within its genome (Radnedge et al., 2003). These prophages are not viable viruses even though the potential for excision from the bacterial genome exists. These prophage regions are conserved in diverse *B. anthracis* strains and make it ideal for definitive diagnostic identification of the bacterium (Klee et al., 2006, Sozhamannan et al., 2006).

1.2.11. Bacteriophages

1.2.11.1. Bacteriophage Introduction and Taxonomy

Bacteriophages are viruses that infect bacteria and are numerous and abundant with a worldwide distribution, some even within forbidding habitats like volcanic hot springs (Ackermann, 2011). The double stranded DNA tailed phages are probably the largest evolving group in the biosphere (Ackermann et al., 1995b). A smaller fraction of bacteriophages (< 3.6%) are single stranded DNA or RNA or double stranded RNA viruses (Ackermann, 2003, Ackermann, 2006). A substantial proportion (96%) of all the known ds-DNA bacteriophages belong to the order Caudovirales (Figure 1.4) of which 62% belonging to the family *Siphoviridae*that includes the Gamma phage. The other two families are *Myoviridae* with 24% and *Podoviridae* with 14% representation (King, 2011). The remaining portion of ds–DNA phages are polyhedral viruses belonging to *Microviridae*, *Tectiviridae* and *Corticoviridae* (Ackermann, 2011).

Bacteriophages can be identified based on morphology from negatively stained electron micrographs (De Rosier and Klug, 1968, Ackermann et al., 1978, Ackermann, 2011). The focus in this study will be the order Caudovirales due to the abundance of this group in the environment (Gillis and Mahillon, 2014). The three families in this group all have isometric heads. The *Siphoviridae* phages possess long non-contractile tails (100 nm -210 nm in length) with a head which is comparatively larger in width (40 nm – 80 nm). *Myoviridae* are defined by long (80 nm – 455 nm) contractile tails with either icosahedral heads (50 nm -145 nm) or elongated heads (80 nm by 110 nm). *Podoviridae* have the shortest non contractile tails (10 nm – 20 nm) and a predominating head (60 nm -70 nm) (Ackermann, 2011, Ackermann, 2006, King, 2011).

The characterisation of bacteriophages can be made on multiple levels. These include the morphology of the virion, environmental niche, nucleotide sequence identity and protein sequence identity (especially tape measure proteins) (Hendrix et al., 2003, Ackermann, 2006, Lavigne et al., 2009). The virion is composed of a major capsid head, filamentous ultrastructure surrounding a core sheath, base plate and tail fibres. The capsid and sheath houses the virion genome (packaged in the head) while the tail fibres serve as the adsorption and DNA delivery machinery (d'Hérelle and Smith, 1926, Bayer, 1968, Cumby et al., 2012, Pell et al., 2013).

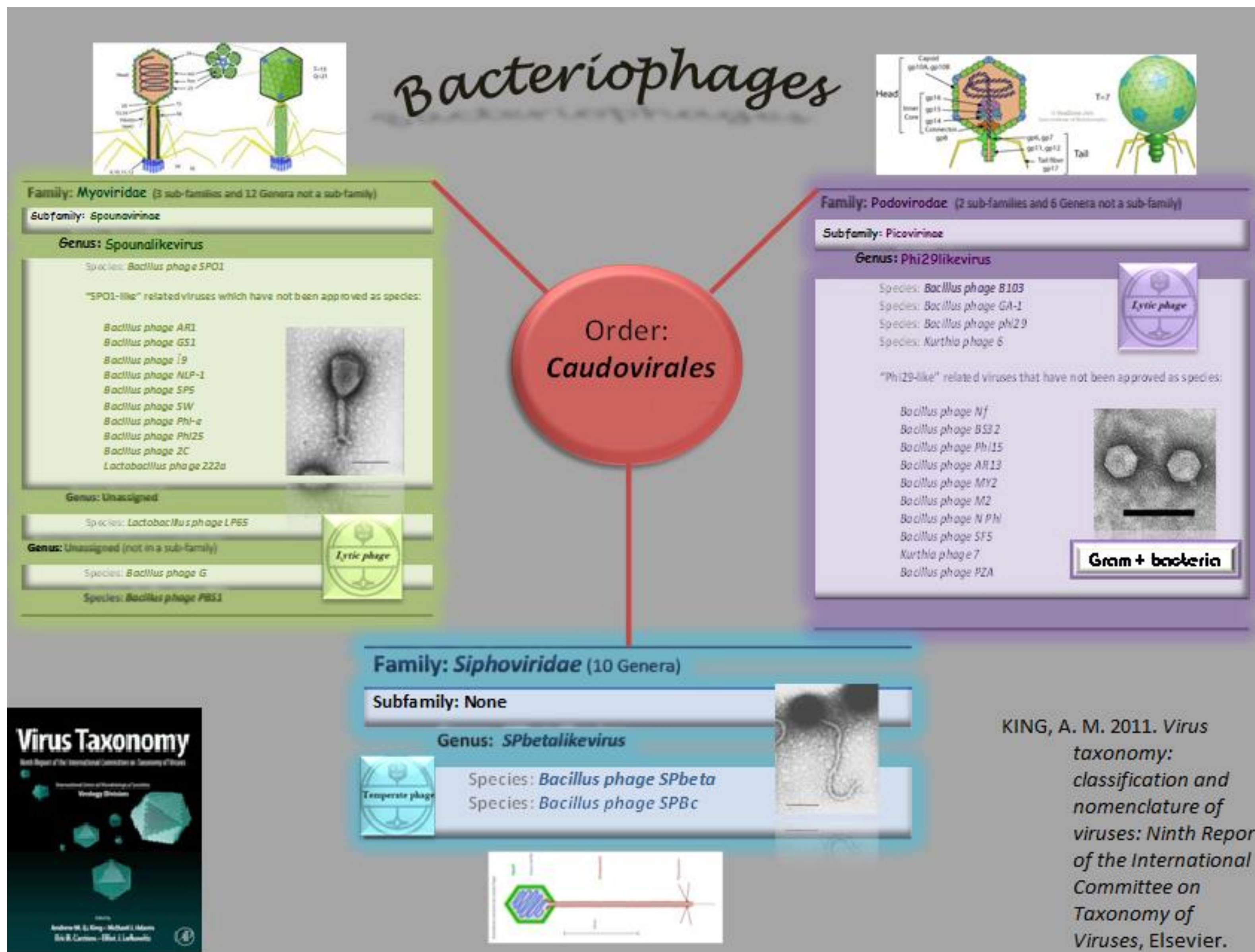


Figure 1.4: The taxonomic categorisation of bacteriophages within the order Caudovirales based on capsid and tail structure (compiled from King, 2011)

1.2.11.2. Historical perspective

The first recorded observation of the action of bacteriophages was by a famous Russian microbiologist Nikolay Gamaleya in 1898, who reported on a transmissible lytic agent specifically active against "the malignant anthrax bacteria" (Schuch + Fischetti, 2006). The same observation was later described by Frederick Twort in 1915 for micrococcus and Felix d'Herelle in 1917 on *Shigella* cultures (d'Hérelle and Smith, 1926, Ackermann, 2003). Twort went on to study other viruses, but d'Herelle was captivated by the discovery and dedicated his research toward the phage therapy of infectious diseases and he has been credited with coining the term bacteriophage. In 1933 he co-founded an institute for phage research with George Elivia in the Soviet Republic of Georgia which until today is dedicated to phage therapeutics (d'Herelle, 1930, Chanishvili, 2012). This was a discipline which lost favour in the 1940's with the advent of the application of penicillin and other antibiotics but it is once more gaining popularity for use in cases/organisms where antibiotic or drug resistance has become a problem (Liu et al., 2004, Adhya and Merril, 2006, Hausler, 2006, Chanishvili, 2012).

Since their discovery, bacteriophages have been used extensively as diagnostic aids in microbiology. They have also played a central role in pioneering discoveries in molecular biology from the identification of DNA as the genetic material; to deciphering of genetic code and biological structure. In the 1970's the field of molecular biology was forever changed by the "recombinant DNA revolution" which enabled the study of much larger genomes and eaukaryotic organisms (Marks and Sharp, 2000, Waldor et al., 2005, Clark and March, 2006, Haq et al., 2012).

1.2.11.3. Bacteriophage Infection and replication

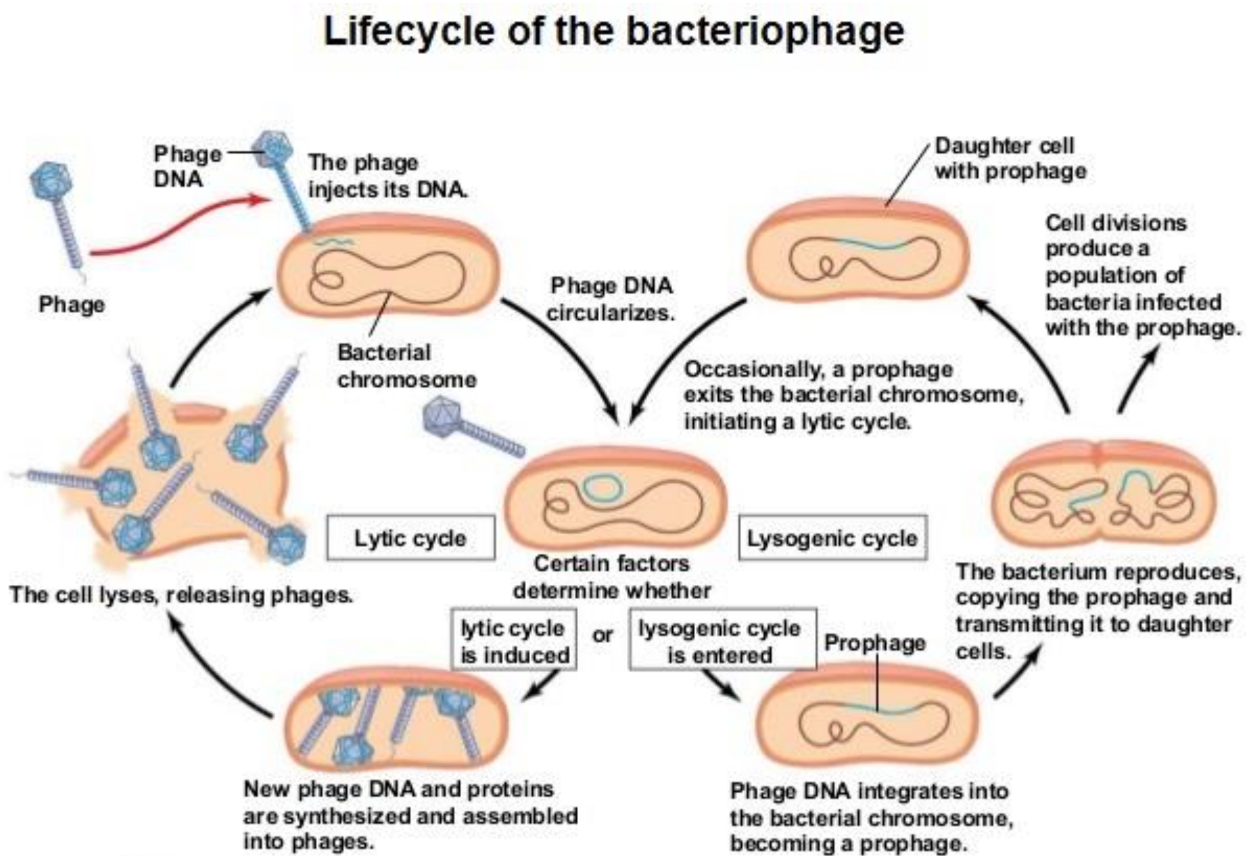
This description of the bacteriophage infection pertains to Gram positive bacteria as the *B. cereus* group is the focus of this study. The phage receptor is a peptidoglycan bound protein to which the tail fibres of the phage bind (Watanabe et al., 1975, Katsura, 1983). More than one phage can bind to a host at a time due to the dispersed distribution of receptor sites (Smith and Trevino, 2009). In a study of the gamma phage receptor, it was discovered that, once bound, there is a major conformational change in the head to tail connector. The genome of the phage is transferred to the host cytoplasm while the phage capsid and tail

remain bound to the cell surface (Lantos and Ivanovics, 1961, Davison et al., 2005). A theory by Riemer and Bloomfield (1978) as well as Earnshaw and Casjens (1980) leaned toward the idea that the genome is rapidly injected into the host cell due to pressure created in the capsid during DNA packaging. Recent studies support this hypothesis but only for some phages (Shao and Wang, 2008, Grayson and Molineux, 2007). The exact mechanisms of how genome transport occurs and the driving forces behind it still remain poorly understood for the vast majority of phages (Molineux and Panja, 2013). It is interesting to note that the rate of DNA transport (although varying from one phage to another) can reach values as high as 3 to 4 thousand base pairs (bp) per second. This rate is much higher than what is achieved during conjugation or natural transformation which is approximately a 100 bp/second (Hershey and Chase, 1952, Grayson and Molineux, 2007). The DNA strand varies in length from phage to phage from anywhere between 4 kbp to 640 kbp (Casjens et al., 1992, Lavigne et al., 2009, King, 2011). There are of course environmental influencing conditions such as the presence of calcium/ions for successful adsorption and DNA injection (Mandel and Higa, 1970, Katsura, 1983, Chhibber et al., 2014).

Once within the cytoplasm, the viral genome can go one of two ways, namely the lytic cycle or the temperate cycle (Figure 1.5). In the lytic cycle, the viral genome uses the host sigma rolling factors to replicate its own genome and uses the host proteins to build capsids and tail proteins for phage virus assembly (Dokland, 1999, Comeau and Krisch, 2008). The progeny phages then erupt from the host cell destroying it (Young, 1992, Chang et al., 1995, Young et al., 2000). Lytic phages have applications in phage therapeutics as well as environmental disinfection. A discipline which is increasing in popularity in this age of dwindling antibiotic options (Clark and March, 2006, Chanishvili, 2012). The endolysin and holin proteins responsible for bacterial cell lysis are also of interest for commercial applications (Borysowski et al., 2006, Nelson et al., 2012, Schmelcher et al., 2012, Nakonieczna et al., 2015).

In the lysogenic cycle the viral genome inserts itself into the bacterial host genome (Groth and Calos, 2004). Once inserted, it remains as a prophage being replicated under natural bacterial multiplication. When the bacterium is threatened the prophage undergoes

induction and follows the same path as the lytic cycle (Josslin, 1970, Little and Mount, 1982, Lamont et al., 1989, Young, 1992). A bacterium can contain more than one prophage at a time (Smith and Trevino, 2009). In fact 3% - 10% of the genomes of many bacterial strains sequenced to date are made up of functional or defective phage genomes (Casjens, 2003). The movement of phage DNA by lateral transfer has a significant impact on the evolution of the host genomes and that prophages greatly contribute to pathogen virulence in a process known as phage conversion or lysogenic conversion (Schuch and Fischetti, 2006, Penadés et al., 2015).



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Reece et al., 2011

Figure 1.5: Diagrammatic representation of bacteriophage infection of a bacterial host and replication via the lysogenic and lytic cycles (Reece et al., 2011).

1.2.11.4. Bacteriophages and the *Bacillus cereus* group

In 1931 Phillip Cowles described a phage which was active on *B. anthracis,* forming turbid plaques, but not specific for it (Cowles, 1931a, Cowles, 1931b). Then in 1951 Elinor McCloy described a bacteriophage designated Wα that could infect both *B. anthracis* and *B. cereus* equally. She also induced a phage from a *B. cereus* strain which would then thereafter not infect that strain but which did infect *B. anthracis.* This bacteriophage designated Wβ while fairly specific for *B. anthracis* could only infect un-encapsulated strains and was therefore not suitable as a diagnostic for fully virulent strains (McCloy, 1951). Finally, in 1955 Brown and Cherry described a phage designated Wγ (Gamma) which is still used in the confirmation of *B. anthracis* cultures (Brown and Cherry, 1955, Brown et al., 1958). The commercially available phage used extensively was called the Cherry phage (Fulmer, 2003). Fouts et al. (2006) sequenced Gamma and Cherry phage stocks from various labs around the USA as well as Pasteur institute to determine if there are any differences between them. They determined that the stock phage preparations were heterogeneous and a striking example of how diagnostic bacteriophages can evolve over several years in different laboratories (Fouts et al., 2006).

Many studies have shown how the inclusion of prophages affects the phenotype or behaviour of a bacterium through conversion (Hendrix et al., 1999, Casjens, 2003, Brüssow et al., 2004). Brown et al. (1958) demonstrated how the inclusion of phage lysates could, for example, induce motility in *B. anthracis* cultures. In more recent studies, whole genome sequencing of pathogenic bacteria has revealed the prevalence of prophages encoding proven or suspected virulence factors that are major contributors to the genetic individuality of theses strains (Brussow and Hendrix, 2002, Comeau and Krisch, 2008, Belcaid et al., 2010). Such factors may include detoxifying enzymes, hydrolytic enzymes or proteins conferring serum resistance. Such prophage encoded factors are referred to as "morons" (Cumby et al., 2012). Moron encoded functions confer a benefit to the host (example increased virulence) but not necessarily to the phage (Hendrix, 2003, Brüssow et al., 2004, Canchaya et al., 2004).

Recent efforts to characterise bacteriophages along with the technological accessibility of next generation sequencing has improved the bacteriophage databases a hundred fold. More phages are sequenced everyday (Ackermann, 2011), however, there is still a lack of

knowledge and understanding on the link between these very diverse viruses and their hosts (Gillis and Mahillon, 2014). Bacteriophages have been indicated to trigger biofilm formation during bacterial lysis. Bacteria within the biofilm are then able to take up the released DNA from lysed cells. It also creates a nutrient rich environment for replication and further infection by bacteriophages (Lee and Yin, 1996, Lee et al., 2007, Gallet et al., 2009, Schuch and Fischetti, 2009). There are over a 100 described bacteriophages infecting the *B. cereus* group. These encompass a myriad of lifestyles and encoded lysogenic factors, but are predominantly temperate in behaviour (Gillis and Mahillon, 2014). Bacteriophages are highly evolving organisms (Fouts et al., 2006, Schuch and Fischetti, 2006, Juhas et al., 2009). The phages are also able to infect multiple members of the *B. cereus* group and thus pose the potential for sharing genetic encoded advantages between the bacterial members (Minakhin et al., 2005, El-Arabi et al., 2013, Gillis and Mahillon, 2014).

A study by Schuch and Fischetti (2009) hypothesised that the presence of phages in the intestinal tract of an earthworm allowed the *B. anthracis* bacterium to persist in its vegetative state. A vegetative cell has the ability to replicate (where it would normally have sporulated over time instead) and the phage blocked sporulation. This gives credence to the hypothesis that the bacterium is able to multiply in the environment between hosts. These findings have opened a new view to the soil born lifestyle of these bacteria. In light of the anthrax-like pathologies being observed, it raises questions of the unknown factors which have yet to be quantified in the environment. It also has implications for bacterial diversity and fitness; influenced by viruses that have not been studied in relation to the anthrax endemic areas of South Africa.

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2. Chapter 2

A retrospective study of anthrax on the Ghaap plateau, Northern Cape Province of South Africa, with special reference to the 2007 - 2008 outbreaks.

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Abstract

Anthrax is a zoonotic disease caused by the Gram-positive, endospore forming and soil borne bacterium *Bacillus anthracis*. When in spore form, the organism can survive in dormancy in the environment for decades. It is a controlled disease of livestock and wild ungulates in South Africa. Vaccination of livestock is a common practice in endemic areas; however, the vaccination of wildlife can be costly and logistically difficult. In South Africa, the two endemic regions are the Kruger National Park and the Ghaap Plateau in the Northern Cape Province. Farms on the Plateau span thousands of hectares. The Ghaap region is comprised of wildlife - livestock mixed use farming. In 2008, an anthrax outbreak in the province decimated the stock numbers in the region and government officials stepped in to aid farmers in control measures against further losses. Due to the ability of the organism to persist in the environment for prolonged periods, an environmental risk/isolation survey was carried out in 2012 to determine the efficacy of control measures employed during the 2008 anthrax outbreak. No *B. anthracis* could be isolated from the old carcass sites, even when bone fragments from the carcasses were still clearly evident. This is an indication that the control measures and protocols were successful in stemming the continuity of spore deposits at previously positive carcass sites.

2.1. Introduction

The Northern Cape Province (NCP) in South Africa is situated at 30° S, 22° E. It is the largest province in South Africa spanning 372 889 km^2. A dolomitic escarpment elevates the mid-eastern border of the province, extending 275 km to the south west, known as the Ghaap Plateau (Smit, 1978, Partridge et al., 2010). The province is divided into two ecological areas, namely the Savannah biome makes up the north eastern half of the province while the south western half hosts the rare and more arid Nama Karoo biome (Fourie and Roberts, 1973). The province contains a number of state, provincial and privately owned wildlife conservancy areas. The NCP also services the Kgalagadi Transfrontier Park bordering Botswana and the Richtersveld Tranfrontier Park bordering Namibia (http://www.sanparks.co.za/conservation). Due to this predominating savannah in the eastern part of the province, the majority of the remaining land is utilized for extensive farming of sheep, cattle and mixed farming which includes wild game (www.**southafrica.info/about/geography/northern-cape.htm**).

The NCP has alkali, phosphorus deficient soils which leads to pica in grazers and browsers in the form of osteophagia and geophagia (Theiler, 1912, Boyazoglu, 1973, De Vos and Turnbull, 2004). This animal behavioural characteristic has resulted in a variety of infections with similar pathologies of which 'lamsiekte' (botulism, *Clostridium botulinum*), 'miltsiekte' (anthrax, *Bacillus anthracis*) and 'stijfsiekte' (Three Day Sickness, *rhabdovirus*) are amongst the most common (Theiler, 1912; Viljoen, 1928). Both anthrax and botulism are caused by soil-born, spore-forming and toxin-producing bacteria. Carcasses are typically observed to have an opisthotonic form with oedema or protruding of the tongue for both diseases (Theiler, 1912; Edmonds, 1922; Van der Lugt, 1995). Anthrax and botulism have been observed to occur on the same farm, even simultaneously at times and has been a cause for misdiagnosis in the past by Theiler (1912), (Theiler, 1927, Viljoen et al., 1928, Kriek and Odendaal, 1994).

Anthrax is an acute or peracute zoonotic disease predominantly affecting livestock and wild ungulates with episodic spill over to humans and carnivores. It is characterized by oedema, sudden death syndrome, black eschars and haemorrhaging from the orifices (Turnbull, 2008), which also differentiates it from botulism (Viljoen et al., 1928). The disease is caused by the Gram-positive, aerobic, endospore-forming bacterium *Bacillus anthracis*. Vegetative *B.*

anthracis cells have a distinct encapsulated, *s*quare ended "box-shaped" appearance on Giemsa stained blood smears (Hugh-Jones and de Vos, 2002, Turnbull, 2008), which is a means of distinguishing them from other Gram-positive rod shaped bacteria microscopically (Theiler, 1912). The use of selective media and morphological selection are typically employed for bacteriologic isolation of *B. anthracis* followed by testing for sensitivity to Gamma phage and penicillin as well as verification of virulence factors as added confirmation for the bacterium and differentiation from closely related *Bacillus cereus sensu lato* group organisms (Knisely, 1966, Turnbull, 1999).

Infection of a host is achieved through ingestion, inhalation or cutaneously. Once an infected animal has died, its carcass becomes a potential site of infection for the next host (Dragon et al., 2005). Anthrax is an OIE reportable disease and opening of carcasses is strictly prohibited (Turnbull, 2008). Sporulation is triggered by nutrient shortages and exposure to oxygen (Sterne, 1937b, Turnbull, 2008). Bacterial spore counts are higher where bloody discharge from the orifices and bodily fluids from the carcass soak the ground (Bellan et al., 2013). Sporulation also takes place when a carcass is opened by scavengers such as vultures (*Gyps spp. / Trigonoceps occipitalis / Torgos tracheliotos)*, crows (*Corvus spp.*), jackal (*Canis spp.)* or hyena (*Crocuta crocuta*) (Hugh-Jones and de Vos, 2002, Turnbull, 2008). Blowflies are considered as mechanical vectors of anthrax in KNP because they feed on a carcass and then deposit *B. anthracis* laden regurgitate on vegetation around the carcass. This contaminated browse may then be a potential source of infection to susceptible kudus (*Tragelaphus strepsiceros*) and other browsers (Braack and De Vos, 1990, Hugh-Jones and de Vos, 2002, De Vos and Turnbull, 2004, Blackburn et al., 2014). Spores in the environment have been recovered by De Vos (1990) from bones during archaeological excavations at a site in KNP that were estimated to be 200 ± 50 years old by carbon-dating.

To reduce such spore inoculum in the environment incineration of anthrax carcasses and burial have been the preferred method and in accordance with WHO and OIE guidelines (Turnbull, 2008). Treating anthrax carcasses with 10% formalin would kill the bacteria, deter scavengers that would open the carcass and decrease spread by flies, but remains controversial due to the negative impact of formalin on the environment. Turnbull (2008) also proposed covering or wrapping carcasses in plastic or tarpaulins to keep the skin intact

and reduce vegetative *Bacilli* through putrefaction and thus anthrax spores in the environment.

The two anthrax endemic areas in South Africa are the NCP and the Kruger National Park (KNP) (Smith et al., 2000, Bengis et al., 2002, Hugh-Jones and de Vos, 2002, De Vos and Turnbull, 2004). While mandatory vaccination schemes have virtually abolished anthrax in livestock in most of the country, these areas remain enzootic due to the predominance of wildlife conservancies and game farms (Gilfoyle, 2006; Turnbull, 2008). A large number of animals usually succumb to the disease before it is noticed due to the short incubation period and the large areas involved. Kudus are typical fatalities of anthrax, but in 2008 large numbers of antelope and equids were also affected in the NCP outbreak. The outbreak gained momentum, and by the end of March 2008, had resulted in the deaths of thousands of game and massive economic losses to the farmers of the Ghaap Plateau tallying to millions of South African rand (Visagie, 2008). It was the largest recorded outbreak in the region in recent history and the Department of Agriculture Fisheries and Forestry in South Africa mobilized national and provincial state veterinary services to aid in diagnosis, surveillance and control measures in order to stem the outbreak (Visagie, 2008, Nduli, 2009). The aims of this study were (i) to report the 2008 *B. anthracis* outbreak in the NCP, (ii) to determine the *B. anthracis* spore concentrations from bone and environmental samples during the 2008 NCP anthrax outbreak using bacteriological methods and (iii) to compare the spore concentrations from bone and soil collected at the same sites in 2012 to determine the spore endurance and/or efficacy of the control measures employed during the 2008 outbreak in reducing the inoculum in the environment.

2.2. Materials and Methods

2.2.1. Observations, sample collection and control measures during 2008 anthrax outbreak

The first cases of the 2008 anthrax outbreak in NCP were reported in November 2007 at Doringbult farm (Figure 2.1) after heavy spring rainfall during mid-August to November 2007. Later that month, the skeletal remains of 1 female Kudu (NC/17) on Grootsalmonsfontein and 2 female kudus (NC/27 and NC/28) on the adjacent farm Dikbosch were recovered, all of which were gestating (personal communication EH Dekker; Nduli, 2009). The first isolated cases of anthrax from kudu were identified in the latter farm Dikbosch (Figure 2.1). In February 2008, 5 more kudu carcasses (both males and females) were discovered on Dikbosch and Kleinsalmonsfontein farms and by the following month anthrax cases included zebra, wildebeest, impala, sheep and kudu along the length of the Ghaap escarpment (Figure 2.1).

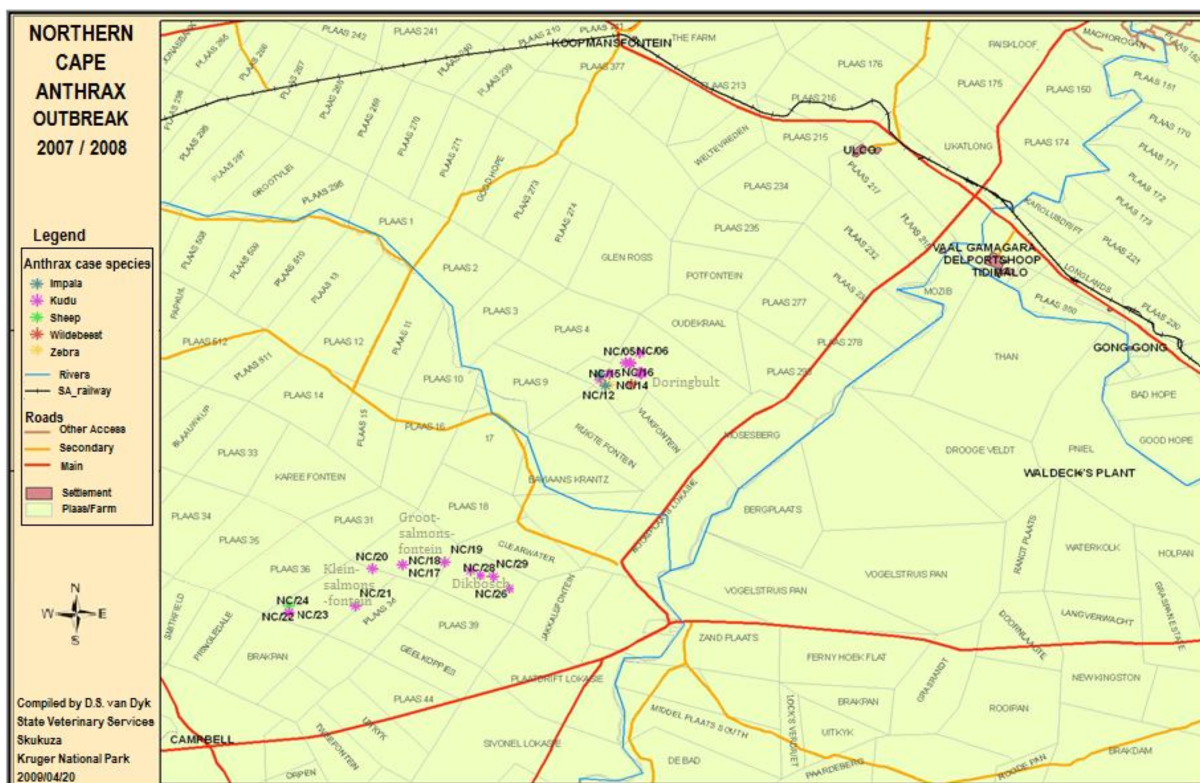


Figure 2.1: Anthrax positive carcass sites indicating the distribution of susceptible species discovered on farms during the 2008 anthrax outbreak in the Ghaap area in the Northern Cape Province, South Africa.

After the kudu at Doringbult was confirmed suspicious for anthrax from a Giemsa stained blood smear, more comprehensive samples were collected for diagnostics. KNP (Skukuza Veterinary Services) and Northern Cape Province Veterinary Services visited farms to aid farmers in the diagnosis and control of the outbreak. The condition of the carcasses were documented along with collection of blood smears and bone samples (mandibular, orbital, rib, vertebra, femur and/or pelvic bones if possible). Soil samples were collected from under the head, abdomen and tail of the carcass for diagnostic purposes. Environmental samples such as crow faeces and bone fragments were collected from carcass sites where evidence of scavenger activity was visible (Table 2.1). Louse flies *(Hippobosca rufipes)* were observed and collected from recently dead kudu carcasses at Clearwater and Klipfontein (Table 2.1, Figure 2.2A). Random soil samples were also collected from farms at the top of the plateau and pans at the base of the escarpment to determine spore counts in areas not contaminated by fresh carcasses (Table 2.1).

Table 2.1: Anthrax positive carcasses (based on Giemsa stained blood smears) and environmental samples collected from farms along the Ghaap Plateau in the Northen Cape Province, South Africa during outbreaks in 2008 indicating control measures implemented by farm owners and State Veterinary Services, as well as, the subsequent soil and bone sampling of these sites in 2012. Supplementary Data\Table 2.1.xlsx

Control measures included vaccination of livestock; treatment of the carcass sites which involved spraying either 10% chlorine or 10% formalin on the carcasses; burning of the carcasses and covering up each animal with black plastic/tarpaulin sails to increase bacterial vegetative cell death and limit blowfly and scavenger access to the carcass. Table 2.1 indicates the farms affected by the outbreak based on Giemsa stained blood smears, as well as, carcass condition and observable blowfly, louse fly and crow activity around carcasses.

Figure 2.2: (A) Louse fly (*Hippobosca rufipes)* collected from anthrax positive carcass from Clearwater during 2008 outbreak. (B) Site on anthrax positive carcass where *Hippobosca rufipes* fed.

2.2.2. Collection of soil and bone samples in 2012 from the 2008 anthrax outbreak carcass sites

In March 2012, samples were collected at each of the anthrax positive sites from farms, along the Ghaap Plateau, which suffered losses in 2008. Figure 2.3 indicates the sites visited. Soil samples were also collected from gravel pits, river and stream beds as well as dry pans where water concentrates after rain. The soil sampling followed the topography (water movement) of the land and thus included areas not sampled in 2008 in the search for concentrator sites (see Figure 2.4: using the topography of Doringbult Game Farm as an example). Bone samples were taken at sites where bones were available - for 10 out of the 48 sites visited the bone samples showed clear evidence of being from the 2008 outbreak since the black plastic tarp cover or charring from cremation sites were indicators of anthrax cases with control measures being implemented in 2008 (Figure 2.5). *Bacillus anthracis* positive controls consisted of two bone samples from the original collection in 2008 (NC/14 and NC/29) that were included as bacteriologic isolation controls.

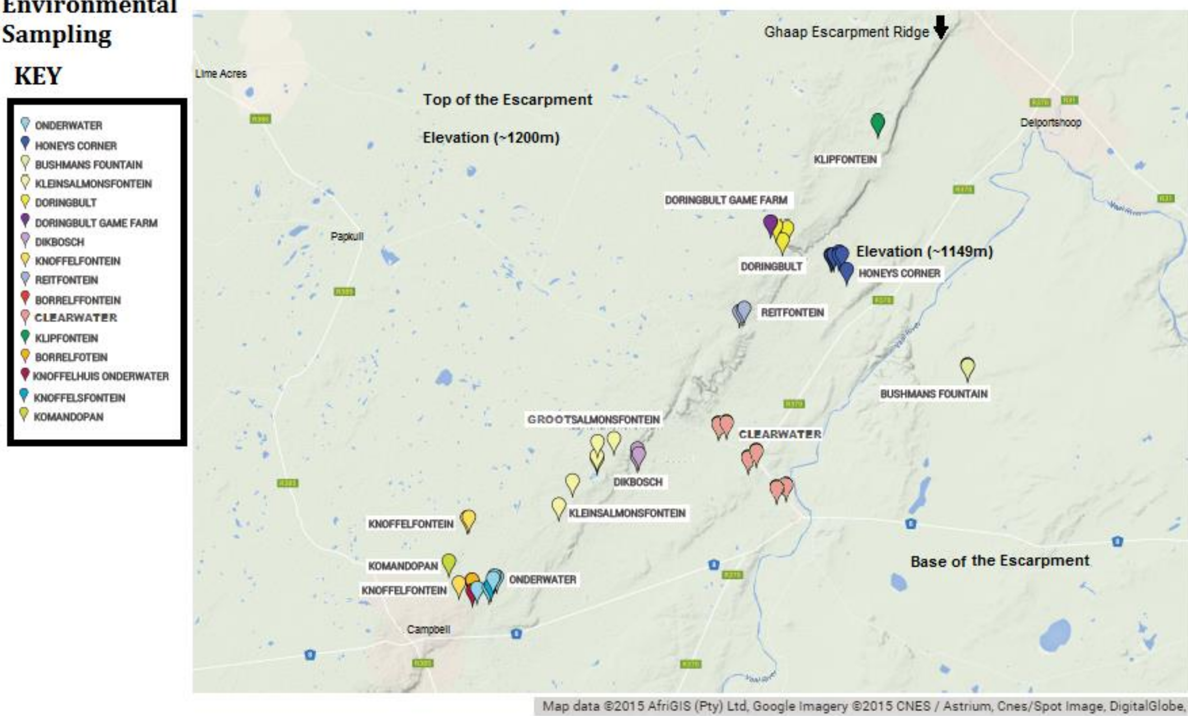


Figure 2.3: Soil and bone samples collected (n=62) in 2012 from farms and carcass sites identified as anthrax positive by farmers and State Veterinary Services in 2008. Additional soil samples were taken along water runoff paths following the Ghaap escarpment drainage topography (indicated as the diagonal ridge line across the map)

Figure 2.4: A closer view of water drainage on Doringbult and Honey's Corner farms in the Ghaap plateau in the Northern Cape Province. The water runoff moves towards Doringbult and thereafter to Honey's corner farm where mass alluvial deposition occurs (white areas). Soil samples were collected in 2012 from farms and carcass sites identified as anthrax positive by farmers and State Veterinary Services in 2008. Additional soil samples were taken along water runoff paths following the Ghaap escarpment drainage topography.

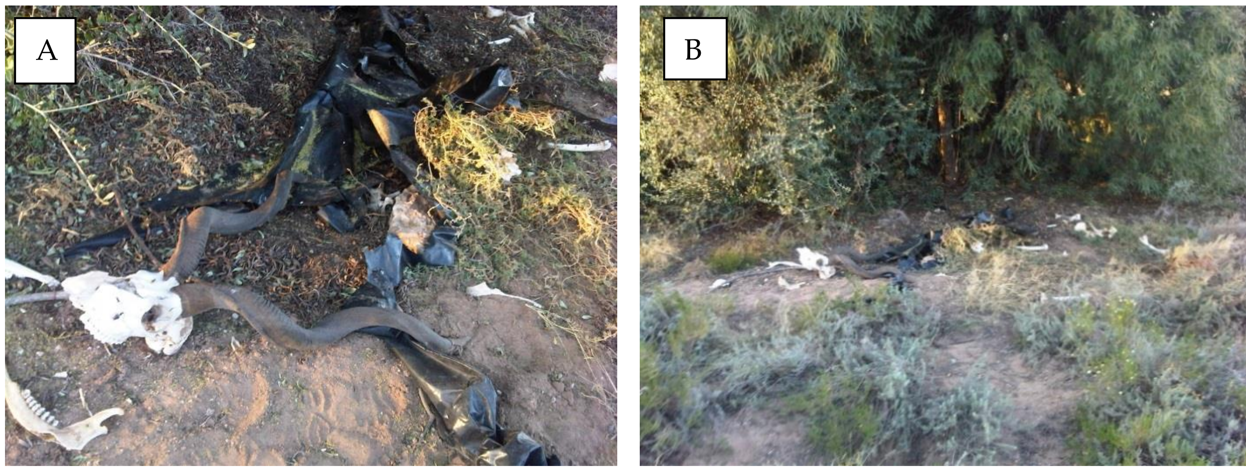


Figure 2.5: (A) Bones collected in 2012 for isolation of *Bacillus anthracis* from Doringbult game farm (Table 2.1 samples AH56 and AH57). B. The black plastic tarp covers and remaining kudu bones were clearly visible for this site, which was sprayed with 10% chlorine, before being covered up with the plastic covers in 2008 (Table 2.1 sample NC/10).

2.2.3. Bacteriologic characterization

All soil, crow faeces and ground up bone samples were weighed out to 1 gram in a McCartney bottle and then topped up with 9 mL sterile phosphate buffered saline (PBS) and shaken for at least 2 hours. Samples were heat treated at 65°C for 25 minutes to select for spores. A 100 µL of this solution was plated out onto Polymyxin EDTA Thallous acetate (PET) agar (PLET agar (Turnbull, 2008) where lysozyme was omitted; personal communication E.H. Dekker) and incubated overnight at 37°C, then further incubated for 24 hours when colonies were not apparent. Serial dilutions (by a factor of 10) for spore counts were made and plated in triplicate for $1x10^{-1}$ to $1x10^{-6}$ dilutions. The average colony forming units (CFU) of *B. anthracis* from the 3 plates was calculated. Isolates were confirmed as *B. anthracis* when sensitive to penicillin and γ-phage.

The louse flies' heads were removed and directly plated onto 5% impala blood agar followed by overnight incubation at 37°C. All white, non-haemolytic, "ground glass" domed colonies were tested with penicillin and γ-phage to confirm *B.*

anthracis. Isolation methods employed in 2012 were exactly the same as those for 2008 with the exception that trimethoprim sulphamethoxazole polymyxin blood agar (TSBPA) (Turnbull, 2008) was used in addition to PET and 5% impala blood agar to maximise isolation success.

2.2.4. Statistical Analyses

A cursory examination of the spore data appeared to highlight a disparity in isolate counts from the various bone samples collected. The spore count data from each bone sample were compared to determine if any trends were apparent. Analysis of Variance (ANOVA) was used to evaluate carcass spore counts followed by Tukey's for pairwise comparison test to compare the significance of spore yields between bone samples. Fisher's exact test was used to determine the significance of carcass effect variables such as locality, species, sex and age due to the small sample size.

2.3. Results

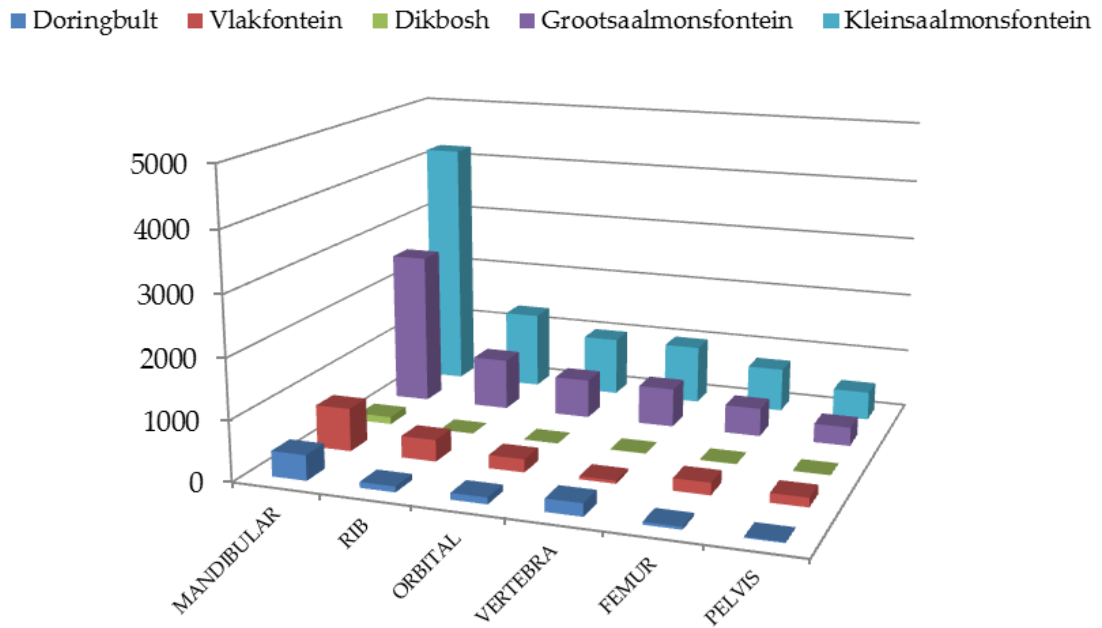
2.3.1. Isolation and spore counts from 2008 outbreak

The first reported cases, where bone samples were collected for bacteriologic diagnostics, was on Dikbosch farm (NC/27 and NC/28) with the isolation of *B. anthracis* . Thereafter, bone and soil samples were collected on 6 other farms in early 2008 (Table 2.1) where a mean of 6.87×10^3 cfu/g and 1.57×10^3 cfu/g spores from bone and soil samples, respectively were collected at all 29 carcass sites. A mean of 200 spores was isolated from the heads of the louse flies and 300 spores/g from the crow faeces (Skukuza State Veterinary Services laboratory report). The distribution of carcasses was greater on the top of the escarpment while being confined to the floodplains on the base of the escarpment (Figure 2.3). Although the first kudus discovered in 2007 were all female, by the end of the anthrax outbreak, overall twice as many male carcasses were discovered than female carcasses. The numbers of confirmed carcasses were 39 kudus, 6 zebra, 2 impala, and 2 wildebeest that died of anthrax. During the collection of samples during the 2008 outbreak various control measures were taken to decrease the inoculum of spores in the environment as indicated in Table 2.1. These control methods included spraying carcasses with disinfectants, cremation and covering the carcasses in plastic.

Average *B. anthracis* colony counts from PET and blood agar dilution series plates for the various bone samples can be seen in Table 2.2. The ANOVA evaluation of the spore counts had a significant *P*-value of 0.028 between bone types, whereas the Tukey's pairwise comparison test only showed significance when comparing the femur (p=0.029) and pelvic bones (p=0.049) with the mandibular bone. The mandibular bone generally produced higher spore counts (Table 2.2), although, no significance was evidenced by comparison to the rib, vertebrae and orbital bones.

2.3.2. Statistical Analyses

According to Fisher's scoring; the locality of the carcasses was also significant with a *P*-value of 0.022. The mean spore counts obtained from the different bone types at each locality is demonstrated in Supplementary Figure 2.2. The mandibular bone yielded the highest counts at all localities, with higher spore counts observed in skeletons/carcasses at Kleinsaalmonsfontein followed by Grootsaalmonsfontein, Vlakfontein, Doringbult and Dikbosch respectively (Supplementary Figure 2.1; Table 2.2). All of these farms are situated on the top of the Ghaap escarpment.

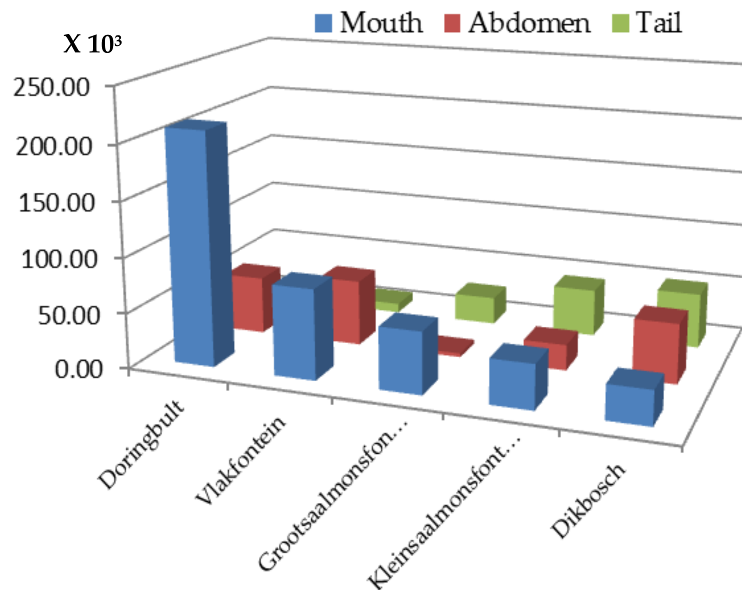


Supplementary Figure 2.1: The mean *Bacillus anthracis* spore counts obtained from different bones in the same carcass on farms afflicted with anthrax in 2008, Northern Cape, South

2.3.3. Isolation results in 2008

Isolation from the soil under the carcasses at the mouth, abdomen and tail region produced varied spore counts (Table 2.2). This is reflected in a *P*-value of 0.504 indicating that there is no significance between the sampling location under the abdomen and tail of the carcass (Supplementary Figure 2.2). *Bacillus anhtracis* spore counts from the soil under the mouth generally yielded higher spore counts accounting for 61% of the mean spores isolated from all the farms (Table 2.2). There is agreement of the *B. anthracis* spore counts between the bones and spore counts in the soil at the location of these bones (mouth, abdomen and tail) (Table 2.2). The mean spore counts under the mouth/head represented 61% of the total isolated from soil, while the mean spore counts in the mandibular (45%) and orbital (21%) bones together accounted for 66% of the total isolated. The rib (13%) with the vertebrae (9%) made up 22% of the mean spores isolated from bone versus the soil under the abdomen which represented 21% of the total soil spores isolated. The femur together with the pelvic bones comprise 12% of total spores from bone versus the soil collected from under the hind of the animal reflecting 19% of the mean (Table 2.2).

Random soil samples taken from two pans at Doringbult did not yield any *B. anthracis* spores, whereas random samples collected at the lowest points downhill on Doringbult of carcasses NC/01 and NC/04 yielded $5X10^4$ and $1.2X10^4$ spores per gram of soil on PET agar repectively (Supplementary Figure 2.1). A soil sample collected at Grootsaalmonsfontein at the lowest point below carcass NC/18 contained 400 spores per gram of soil.



Supplementary Figure 2.2: Mean *Bacillus anthracis* spore counts obtained from a gram of soil under different parts of anthrax positive carcasses on farms on the Ghaap Plateau during an outbreak in 2008, Northern Cape, South Africa.

Table 2.2: Isolation spore counts for bone samples collected from various accessible parts of a carcass, as well as soil sampled from beneath the mouth, abdomen and head of carcasses from farms along the Northern Cape Province, Ghaap Plateau during the anthrax outbreak of 2008. The mean spore counts isolated from each bone type, soil location and farm area are indicated. Supplementary Data\Table 2.2.xlsx

2.3.4. Isolations in 2012

The soil and bone samples collected in 2012, yielded a variety of *Bacillus* spp. on the different media employed including *B. subtilis, B. cereus* and *B. thuringiensis* (identified by the DVTD bacteriology section, Faculty of Veterinary Science), but contrary to expectation, no *B. anthracis* was found. The control *B. anthracis* bone samples NC/14 and NC/29 from the original 2008 outbreak were positive for the presence of *B. anthracis* spores as indicated in Table 2.3. Similar *B. anhtracis* spore counts were obtained in 2008 and 2012 from the refernce bone samples NC/14 and NC/29.

Table 2.3: *Bacillus anhtracis* spore counts obtained from different isolation media from 1 gram of ground mandibular bones collected during the 2008 anthrax outbreak in the Northern Cape Province, South Africa.

Bone Sample	*PET agar (2008)	*PET agar (2012)	**TSPBA (2012)	Blood agar (2012)
NC/14 Mandible	25500	18000	17000	26000
NC/29Mandible	900	550	500	750

*Polymyxin EDTA thallous acetate

** Trimethoprim sulphamethoxazole polymyxin blood agar

2.4. Discussion

The isolation of *B. anthracis* from samples collected in 2008 demonstrates a unique ecology at each carcass site with a variety of vectors such as water, crows, blowflies and louse flies evidencing activity. There is also a difference in *B. anthracis* spore yields between bone types and soil samples collected from the carcass sites during this outbreak. The mandibular bone yielded the highest spore counts and mean spore counts under the mouth/head of carcass yielded the highest spore counts from soil. Control measures taken during the 2008 anthrax outbreak seemed to reduce the spore counts as no *B. anthracis* could be isolated in 2012.

During the anthrax outbreak of 2008 in NCP, a disproportionate number of adult, male kudus died from the disease. This is in keeping with studies in Etosha by (Lindeque and Turnbull, 1994). This is notable because kudus are browsers and not dependent on as frequent water intake as their grazing counterparts. Kudu seemed to be the most susceptable species, which died of anthrax, followed by zebra, impala, and wildebeest; of which all except the kudu died on the game conservancies. A caveat of our findings is that carcass distribution is not well represented because State Veterinary Services only intervened on the farms and not the national parks and nature conservancies where the disease is considered part of the natural ecology. On the mixed-use game farms domestic animals and kudu died in greater numbers during the 2008 anthrax outbreak in NCP.

It has been consistently noted that spore counts in the soil appear to be dependent on the opening of a carcass and soil closest to these openings produce higher contagion concentrations (Dragon et al., 2005). This can be clearly seen for soil spore counts taken from under carcasses in Doringbult with the highest spore counts obtained from soil under the mouth of 2 female kudu carcasses (Table 2.2 samples N/C04 and NC/05). Both of these carcasses had opened heads. The 2 carcasses did differ in spore counts obtained from soil under the abdomen where NC/04 had a noticeable hole in the abdomen while no such observation was made for NC/05. According to Bellan et al. (2013), the spore density at a carcass site is dependent on the vegetative cell concentration at host death coupled with the sporulation efficiency of the bacterium, spore survival and propensity for environmental replication. Sporulation is triggered by a paucity of nutrients coupled with exposure to oxygen (Sterne, 1937b, Minett and Dhanda, 1941, van Schaik et al., 2007, Turnbull, 2008, Koehler, 2009). As such, soils contaminated by bacterial laden bodily fluids would contain more spores than an area a distance away from the carcass, which was observed by Dragon et al. (2005). The higher spore counts obtained from soil under the mouth of carcasses due to heamorrhagic discharge affirms the findings of Bellan et al., (2013), Dragon et al.

(2005) and our study where high spore count were obtained from carcass heads opened by scavangers, thus theoretically providing extravasation fluid as enrichment media to the soil for possible vegetative bacterial amplification in the environment.

Statistically the mandible, ribs, orbital bone and vertebrae produced equivalent *B. anthracis* spore counts, although the mean spore counts demonstrated better isolation success rates from the mandibular, rib and orbital bones. In the NCP, crows are often observed to peck out the eyes of a carcass (Hugh-Jones and de Vos, 2002). Due to the lack of blood clotting (Leppla, 1984) and the exposure of the haemorrhagic fluid in the orbital sockets to the elements; sporulation would be triggered along with increased contagion deposition in the environment. In KNP scavengers like vultures and hyeana are responsible for opening the carcass (Hugh-Jones and de Vos, 2002). Due to a lack or limiting numbers of scavengers in NCP, crows as well as scavengers like jackals and to a lesser extent hyena and leopards are responsible for opening the carcass.

Blowflies as well as louseflies have been observed on carcasses in NCP (Hugh-Jones and de Vos, 2002). Louse flies belong to the family *Hippoboscidae* and are blood feeding flies. These flies although strong fliers, seldom fly more than a few metres, choosing instead to move to the next closest host when disturbed. They are abundant in the summer months and can be found clustered on the perineal and pubic regions as well as the neck and sides of the animal. They have a long standing assertion for transmitting anthrax amongst cattle (Viljoen 1928, Howell et al., 1978). Cattle louse flies were observed on kudu carcasses at sites in Clearwater (Schmidtsdrif) and Dikbosch in NCP during 2008 outbreak. The louse flies were easily collected from the kudu carcasses and an average of 200 spores was isolated from the head (mouthparts) of a louse fly. As a blood feeder, it has been suggested that this makes it a potential mechanical vector to its next animal host (De Vos and

Turnbull, 2004, Howell et al., 1978), however, this requires further study to determine its verity.

The ability of anthrax spores to persist in the earth for extended periods is due to specific soil and climatic conditions, which provide an environment conducive to its survival (Smith et al., 2000, De Vos and Turnbull, 2004). Soils rich in organic matter, ample in calcium, prone to alkalinity and with an ambient temperature above 15.5 °C are considered optimal for persistence of spores. The exosporium of the spore is negatively charged (this charge and its strength are pH dependent) whereas humus particles are positively charged and are therefore able to chelate, which then provides environmental stability for the spores (Hugh-Jones and Blackburn, 2009). The spores can persist in soil and bone for decades or even centuries under such conditions (Wilson and Russell, 1964, De Vos, 1998). These humus-spore clumps have the added advantage of being buoyant (Beaman et al., 1972, Baweja et al., 2008). During flooding, as was the case of the NCP 2008 anthrax outbreak, these clumps can be deposited or concentrated at water collection sites once the water evaporates, as the spores are highly resistant to UV radiation (Hugh-Jones and de Vos, 2002, Vilas-Boas et al., 2007, Hugh-Jones and Blackburn, 2009).

(Steenkamp, 2013) illucidated the importance of the role of water and topography in the dissemination of anthrax in KNP along with various other studies (Viljoen et al., 1928, Pienaar, 1960). The Ghaap is unique in its distribution of natural springs, Kimberlite pipes and dolomitic sinkholes which serve to flush the area with groundwater during periods of heavy rainfall and after groundwater recharge (Smit, 1978). This theory is consistent with the high spore counts observed in water collection pans downhill from carcass sites during the 2008 anthrax outbreak.

The lack of *B. anthracis* spores from bone samples collected in 2012 reinforces the effectiveness of control measures employed during the 2008 outbreak. Due to the employment of a variety of control measures, the efficacy of the individual actions of

using chlorine, formalin or the black sails/tarps cannot be determined, nor whether all the measures worked synergistically to reduce the inoculum in the environment. It is not clear/evident whether the control measures of the 2008 outbreak managed to further stem the dissemination of spores through dilution in the environment. Bones treated at carcass sites were not a source of infection to animals suffering from pica in later seasons because they did not yield viable spores which could pose a threat of infection (De Vos, 1998, De Vos and Turnbull, 2004).

The reduction of inoculum in the environment and the corresponding reduced spore exposure is paramount in the control of anthrax (Watson and Keir, 1994, De Vos and Turnbull, 2004, Turnbull, 2008). This was indicated when 2012 isolations from the reference bone samples collected during the outbreak (used as postive controls) had spore counts similar to those enumerated at the time of collection. The PET agar plates used in this study had spore counts equivalent to the traditionally used TSPBA agar plates.

2.5. Conclusion

There are too many variables at each carcass site that could possibly influence the dissemination of anthrax, which serves to complicate our understanding of the disease, as seen with the 2008 anhtrax outbreak in NCP. Modelling which includes all the factors (host, bacterium, vectors and environment) will provide more insight into the unique ecology of anthrax on the Ghaap Plateau.

Carcass bones are a reliable source for the successful isolation of *B. anthracis*. The mandibular and orbital bones, as well as soil beneath the head of a carcass proved to be a prodigious source of viable *B. anthracis* spores from carcasses collected during the 2008 NCP outbreak.

The control measures of cremation or spraying carcasses with chlorine or formalin and then covering them with tarp or plastic were applied by farmers on the

recommendation of the veteterinary services during the 2008 outbreak. Since no spores could be isolated in 2012 from the same sites and bones remaining in the environment these control measures appear to have reduced the inoculum in the environment at the treated carcass sites.

Competing Interests:

The authors declare that there are no competing interests.

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3. Chapter 3

Insights gained from sample diagnostics during anthrax outbreaks in the Kruger National Park, South Africa.

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Abstract

Bacillus anthracis the causal agent of anthrax is enzootic in the northern part of the Kruger National Park (KNP) in South Africa. State Veterinary Services are actively involved in disease surveillance of anthrax in KNP. Surveillance for the disease includes Giemsa stained blood smear evaluations, molecular diagnostics and bacteriologic isolation from samples collected at wildlife carcass sites as per WHO/OIE guidelines. Isolation of *B. anthracis* from biological samples is routine in KNP, however, teasing the bacterium out of environmental samples can prove more challenging. These challenges and the observations gained from overcoming them are highlighted in this chapter. *Bacillus anthracis* cultures were established from the carcasses of hippopotamus, blood smears and environmental samples such as leaves and arthropods during outbreaks between 2012 and 2015 in KNP. This is the first documented record of *B. anthracis* cultures from hippopotamus buccal fat as well as spore counts from leaf and insect larvae obtained from anthrax carcass sites.

3.1. Introduction

Bacillus anthracis is a Gram-positive, non-motile, spore-forming, aerobic and rod-shaped bacterium with little genetic variability and the causative agent of the disease anthrax. The disease affects herbivores such as wild ungulates and other species of domestic or wild animals and people (Turnbull, 2008). This disease is enzootic in the northern tip of Kruger National Park (KNP) in South Africa. The southern half of KNP is considered a non-enzootic region as it had not produced any noticeable outbreak reports in the past (Viljoen et al., 1928, Pienaar, 1967, Smith et al., 2000, Hugh-Jones and de Vos, 2002). Studies of the *B. anthracis* life cycle indicated that no clinical evidence of direct transmission from victim to victim has occurred except through scavengers and when organisms are ingested by herbivores while grazing or browsing (Turnbull, 2008). In KNP, blowflies (*Chrysomya albiceps* and *C. marginalis*) have been indicated to contaminate browse after feeding on carcasses which have succumbed to anthrax (Braack and De Vos, 1990). The contaminated vegetation is then ingested by kudu and other susceptible hosts. The disease in the KNP exhibits various patterns of spread i.e. anthrax spores spread by water and though vectors like scavengers (Hugh-Jones and de Vos, 2002, Hugh-Jones and Blackburn, 2009, Steenkamp, 2013). In the past, anthrax outbreaks usually occurred periodically during dry seasons in KNP when water was scarce. It was hypothesised that dry seasons led to animals sharing the remaining water points which served as spore concentrator sites from where anthrax outbreaks occurred (Pienaar, 1961, Lindeque and Turnbull, 1994, Hugh-Jones and de Vos, 2002).

In South Africa the incidences of anthrax among agricultural animals decreased after the introduction of the Sterne vaccine in 1937 (Hambleton et al., 1984, Hambleton and Turnbull, 1990), but the disease remains enzootic in wildlife as vaccination is logistically difficult and costly (Gilfoyle, 2006). Susceptibility of individual animals to anthrax depends on the host species, the strain, age and the route of infection (Welkos et al., 1986, Lyons et al., 2004). During major outbreaks in the KNP, it has been shown that older animals are more easily affected in direct comparison to younger animals, which led to the hypothesis that the disease acts as a natural mechanism of culling (Hugh-Jones and de Vos, 2002). Previous studies have reported browsers like kudus (*Tragelaphus strepsiceros*) being the most anthrax affected species in KNP with records of over 50% of kudu population affected in the past

(De Vos and Turnbull, 2004) compared to the Etosha National Park in Namibia where zebras (*Equus quagga*) are most commonly affected (Lindeque and Turnbull, 1994, Beyer et al., 2012). Grazers commonly affected in South Africa are impala (*Aepyceros melampus*), roan (*Hippotragus equinus*), zebra, buffalo (*Syncerus caffer*) and white rhinoceros (*Ceratotherium simum*) (Hugh-Jones and de Vos, 2002). Other species which generally succumb to the disease in KNP are elephant (*Loxidonta africana*) and the omnivorous hippopotamus (*Hippopotamus amphibious*) (Bengis, 2000).

Diagnosis of anthrax from recently dead animals is based on the presence of encapsulated bacilli on Giemsa or M'Fadyean stained blood smears (Turnbull, 2008, Owen et al., 2013). These suspected anthrax cases are confirmed by culture from biological and environmental samples (Turnbull, 2008). Various selective media have been evaluated in the isolation of *B. anthracis* over the years, but the two most consistently used are polymyxin-B, lysozyme, ethylenediaminetetraacetate, thallous acetate agar (PLET) and trimethoprim sulphamethoxazole, polymyxin, blood agar (TSPBA) (Knisely, 1966, Turnbull, 1999, Marston et al., 2008, Turnbull, 2008, Irenge and Gala, 2012) followed by sensitivity tests of penicillin and gamma phage to differentiate *B. anthracis* isolates (Brown and Cherry, 1955, Odendaal et al., 1991a, Odendaal et al., 1991b). Further diagnostics are performed using qPCR to verify plasmid and chromosomal targets that verify isolates as *B. anthracis* (Ellerbrok et al., 2002, Turnbull, 2008). Isolation of *B. anthracis* from biological samples is routine in KNP, however, teasing the bacterium out of environmental samples can prove more difficult at times. In this chapter samples that presented a challenge to standard sample processing procedures are discussed. These samples provide some insight into the environmental factors that influence vegetative cell survival and spore germination.

3.2. Materials and Methods

3.2.1. Study Area and details

The KNP is a national game reserve located in the north eastern part of South Africa; bordering Mozambique and Zimbabwe and spanning nearly 20,000 square kilometres (Figure 3.1). It is divided into ecological niche sections/ranger sections and is thus managed according to these regions. Rangers (SANparks) and veterinarians sample accessible carcasses that they come across during routine patrols. These routine anthrax surveillance measures managed by the Skukuza State Veterinarian's Office (SSVO) have been on-going for several decades. The sampling includes collection of blood smears (in duplicate), bone and soil from carcasses as well as record keeping of site locations, animal species and sex, utilization of the carcass by scavengers or blowfly activity and the observable causes of death when clearly evident (i.e. predators versus disease). The ranger sections and private game reserves (Figure 1) are used to indicate the sample origin in and around the Kruger National Park. The blood smears and samples are sent to SSVO, where a blood smear undergoes microscopic examination, using Giemsa with additional 0.23% v/v AzureB (Owen et al., 2013).

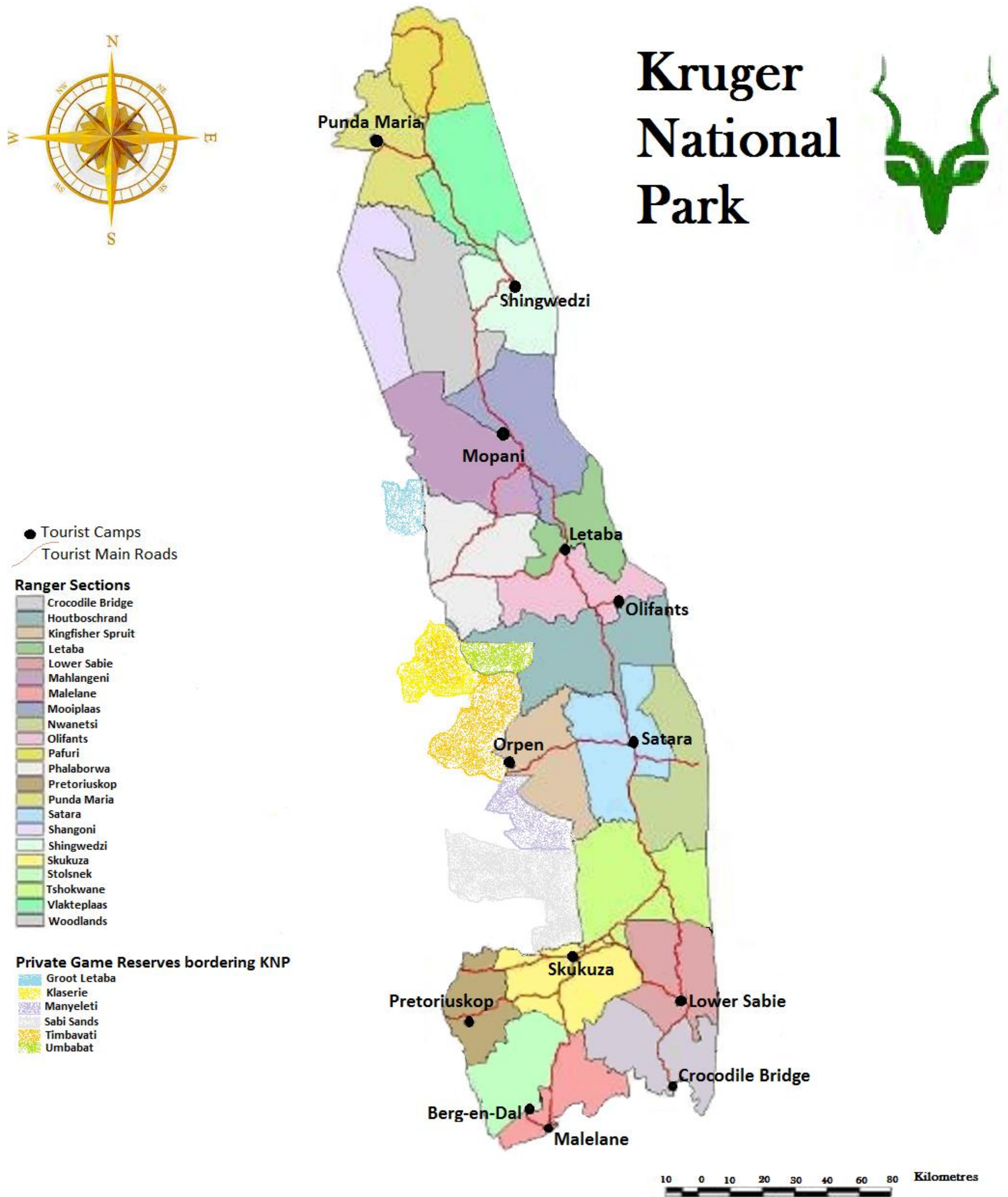

Figure 3.1: Map of the Kruger National Park, South Africa, delineating the Ranger sections which demarcate the different ecological niches/ecosystem types. Pafuri (the northern tip of the KNP in yellow) is the anthrax enzootic section.

3.2.2. Standard Sample Processing

3.2.2.1. Microscopic Analyses

Box-shaped, encapsulated Gram-positive rod samples, identified through microscopy, undergo further diagnostics at SSVO. The isolation of *B. anthracis* through bacteriologic methods then confirms the case. The qPCR is used both to screen samples at the onset of bacterial isolation and confirm the presence of both plasmids after colony purification, which then also indicates virulence (Turnbull, 2008).

3.2.2.2. Identification of *Bacillus anthracis* using bacteriologic and qPCR techniques

The usual biological (bone and swab) samples were processed according to the guidelines of WHO (Turnbull, 2008) with the exception that PET; polymyxin-B, EDTA, thallous acetate (i.e. lysozyme was excluded) was used as a selective media, along with TSPBA; trimethoprim-sulfamethoxazole polymyxin blood agar. After the primary isolation all the plates were inspected under a stereo microscope (Olympus SZ51) for typical rough textured, domed grey-white colonies (Parry et al., 1983). Suspect isolates were subcultured onto 5% sheep blood agar (5% SBA) to determine haemolytic activity and colony morphology, thereafter; purified isolates for all samples in this study were subjected to penicillin and gamma phage testing as indicated by Turnbull (2008).

For environmental samples, heat treatment at 65° C for 30 minutes and serial dilution is followed by inoculation on selective media as an enrichment step. The bacterial lawns are harvested in phosphate buffered saline (PBS) after the overnight incubation, divided in 2 and 1 half boiled at 110° C for 20 minutes then centrifuged at 30000xg for 30 minutes. The resulting supernatant was harvested as DNA lysate. The DNA lysate obtained from the bacterial lawns of all the plates (after the first enrichment step), were used in hybridization qPCR assays as published in the WHO guidelines (Turnbull 2008; Annex 1: page 135) for a LightCycler® instrument. The assay was performed on a LightCycler® Nano using Flourescence Resonance Energy Transfer (FRET) probes targeting the Cap C (capsule region C) and PA (protective antigen) genes on the plasmids as well as the SASP-B (small, acid-soluble spore proteins**)** chromosomal marker synthesized by TIB MolBiol GmbH (Germany)

(Turnbull 2008; Ellerbrok 2002). The bacterial lawn of lysates which amplify before 40 cycle thresholds (CT) are selected for serial dilution and re-plated on selective media until individual colonies of *B. anthracis* become evident for subculture and confirmation by penicillin and gamma phage.

The following samples include deviations to the standard protocol due to a failure to isolate *B. anthracis* on the first attempt using the standard sample processing procedure.

3.2.3. Sample collection history and processing

3.2.3.1. Environmental Sampling

In early 2014, sporadic suspected anthrax cases were once again confined to the Pafuri area. An impala carcass (AD2014/08), 2 kudu carcasses (LVS201403/003 & LVS201403/004) and a zebra carcass (LVS201403/001) were sampled. The carcasses were confirmed positive for *B. anthracis* through standard processing protocols at SSVO. Additional samples included maggots, dung beetle larvae (~20 cm below carcass), carrion beetles (on carcass), vulture faeces, soil beneath the remains (as an environmental control), grass and leaves with visible blowfly activity (faeces/emesis) from all 4 carcass sites (Table 3.1).

3.2.3.2. Arthropod isolations

Insects and larvae were washed in 1 mL physiological saline (0.9% NaCl), which was then used to inoculate selective and non-selective media to represent external spore counts. The exoskeleton of the insects and larvae were disinfected using 0.1% peracetic acid (C_2H_4O) for an hour. The peracetic acid solution was plated onto 5% SBA and incubated overnight to ensure microbial inactivation. The insects and larvae were then washed in 100 µL of 100 g/L sodium thiosulphate ($Na_2S_2O_3$) solution for 40 minutes to neutralize the peracetic acid. The samples were then rinsed in saline, pooled (Table 3.1) and placed in 2 mL MagNA Lyser™ Green Beads (Roche) for use in the Percellys® 24 homogeniser (Bertin Technologies). A 100 µL of homogenate was serial diluted and plated out in quadruplet on 5% SBA and PET media followed by incubation at 37° C for 12 hours. Subcultures were confirmed *B. anthracis* by penicillin, Cherry phage sensitivity and qPCR (Cap C, BAPA & SASP) amplification.

3.2.3.3. Leaf and grass isolations

Leaves belonging to a tree of the *Abutilon spp.* with blowflies visible in large numbers on each leaf (with accompanying black spots) were collected from above kudu carcass LVS201403/003 remains. The straw coloured grass around the carcass was damp and flattened from trampling (therefore unidentifiable), but was also covered in blowflies and black spots. The leaves and grass (Table 1) were processed a week after collection. For the isolation of bacteria from blowfly emesis/faecal droplets, a swab was soaked in saline (100 μL) and applied to a single spot in a dabbing motion then streaked out onto 5%SBA. This was repeated for 2 spots on each leaf. Other spots (x8 per leaf) and grass blades were cut out and placed in 1 mL of PBS then shaken for half an hour. This was followed by heat treatment at 65° C for 30 minutes before plating on PET and 5% SBA. Plates were evaluated after 12 hour incubations at 37° C for typical *B. anthracis* colony morphology (Parry et al., 1983).

3.2.3.4. Vulture faeces

Vulture faeces were scraped off a large boulder next to the well consumed remains of a zebra carcass LVS201403/001. The vultures were still visible in a baobab tree adjacent to the site. A gram of the vulture faeces was dissolved in 9 mL PBS and treated according to the standard sample processing protocol for biological samples.

Table 3.1: Environmental samples collected at carcass sites from an anthrax outbreak during March of 2014 in Pafuri, Kruger National Park, South Africa.

Site	Date	SSVO №	Carcass	Sample	*Identification	# insects homogenized (pooled)	Combined weight
Zebra	11/03/2014	LVS201403/001	±2 days old Carcass picked clean and bones scattered	Fibrous "digesta"			
				Zebra hair			
				Vulture faeces			
				Beetles	*Scarabaeinae* spp.	9x large+ 1 small	0.206g
Kudu 1	14/03/2014	LVS201403-003	±2 days old Skeleton and skin intact. Flesh and viscera removed	Beetles	*Demestes maculatus*	2x large	0.061g
				Maggots (in dung pre-pupa)	Stratiomyidae & Calliphoridae	9x	0.512g
				grass	Poaceae, unidentified		
				Leaves (with visible blowfly activity)	*Abutilon* sp.		
Kudu 2	14/03/2014	LVS201403-004	±4 days old Bones cleaned and scattered. Beetles in horns.	Red legged ham Beetles	*Necrobia rufipes*	10 x (small)	0.303g
				Beetles	Schizonycha sp.	9x large + 1x small	0.314g
				white grub	Scarabaeidae	1x large	0.206g

Impala	15/03/2014	AD2014/08	day old Animal intact. Maggots	Dung			
				Maggots	Calliphoridae	7x	0.450g

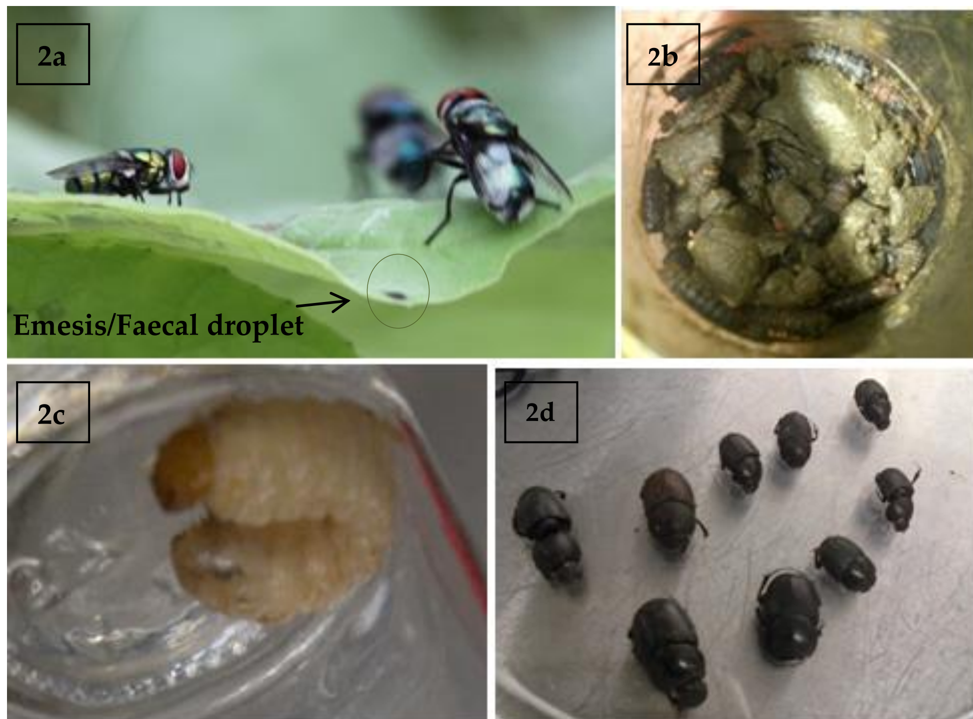

Figure 3.2: Environmental samples collected from the vicinity of anthrax carcass sites in Pafuri. 2a. Blowflies (Calliphoridae spp.) on leaf (*Abutilon angulatum)* with multiple spots indicative of emesis/faeces from blowfly activity. 2b. (Diptera: Calliphoridae and Stratiomyidae) pupae found 2 metres away from a kudu carcass burrowing into dung and soil. 2c. Scarabaeidae larva found ~15 cm beneath a kudu carcass along with dung and decomposing material. 2d. Scarabaeinae beetles found beneath the soil surface at a zebra (*Equus quagga burchellii*) carcass that had been picked clean by scavengers.

3.2.4. Pachyderm sampling and processing

3.2.4.1. Blood Smears

During passive surveillance by SSVO, 2 blood smears are taken at every carcass site, of which 1 smear is stained for microscopic analysis, while the second remains unstained, and is archived along with its associated carcass information. In 2012 - 2015, these included poached rhinoceros blood smears which required rapid diagnostics for the forensic teams involved in investigations at carcass sites during anthrax outbreaks. For this purpose, microscopy and qPCR (of the 3 above mentioned *B. anthracis* markers: (Turnbull, 2008)) of smears were used as a means of confirming anthrax within hours of the sampling.

3.2.4.2. Unstained blood smears

Culturing was done from possible anthrax cases where only smears were available. All the material on the unstained blood smear obtained from white rhinocerosceroses (Ax/2012/180, Ax/2012/275, Ax/2012/283, Ax/2012/291 and Ax/2012/309) and an elephant (Ax/2013/435) was scraped off with a scalpel and diluted in 350 µL of sterile distilled water. A 50 µL aliquot of each sample was plated onto 5% SBA and incubated overnight. The remaining volume of solutions was then vortexed and a 50 µL aliquot of each sample was heat shocked at 65° C for 20 min. The heat treated solution was then plated out onto 5% SBA and incubated at 37°C overnight. Individual colonies were subcultured onto 5% SBA for identification and confirmed using qPCR, penicillin and Gammaphage. The 250 µL of remaining solution was crude boiled at 110° C for 20 minutes and centrifuged at 14,000xg for 20 minutes. The supernatant was used in qPCR confirmation of the presence of Cap C, BAPA and SASP as indicated in the standard sample processing section (Turnbull, 2008).

3.2.4.3. Tusks

In June 2013, an elephant tusk was collected from Kingfisherspruit section as well as a hippopotamus tusk (with a thick layer of buccal fat at its root end) from Letaba section.

Scrapings off the interior and exterior surface of the elephant tusk obtained from Kingfisherspruit section and a hippopotamus tusk from Letaba were diluted separately in 15 mL of distilled water. Aliquots of 5 mL from each tusk sample was heat treated at 65°C for 20

minutes and divided into 2 samples each and plated onto PET and 5% SBA directly. The remaining bulk of the sample solutions for each animal's tusk were centrifuged at 3000xg for 40 minutes and the pellet divided between the PET and 5% SBA media. The resulting pellet was heat treated at 65°C for 20 minutes and inoculated in triplicate onto PET, TSBPA and 5% SBA. The PET and TSBPA bacterial lawns were harvested in PBS and serial diluted. An aliquot of the bacterial lawn was used in qPCR to indicate presence of plasmids (Cap C, BAPA) and the chromosomal target (SASP) of *B. anthracis* as indicated in the standard sample processing section (Turnbull, 2008).

3.2.4.4. Buccal fat from hippopotamus tusk

The hippopotamus tusk submitted from Letaba had a fat pad around the root which was solid, globular and cream to pale yellow in colour. The fat sample (0.5g) was added to 2.5 mL of PBS and vortexed. The liquid turned milky, but globules of solid fat still floated on the surface. Thereafter 2.5 mL of absolute ethanol was added and the sample vortexed at regular intervals until a uniform emulsion was obtained. A 100 µl was inoculated in triplicate onto PET and TSBPA respectively. The remaining emulsion was heat treated at 65°C for 20 min and the inoculant similarly plated out and incubated at 37°C. The bacterial lawns were harvested in PBS, divided into two and serial diluted to $1x10^{-4}$ and the first inoculated onto PET and TSBPA, then incubated overnight at 37°C, while the second was subjected to thermal lysate DNA extraction to confirm presence of *B. anthracis* using real time qPCR (BAPA, Cap C & SASP) as indicated in the standard sample processing section (Turnbull, 2008).

3.2.4.5. Hippopotamus tissue

The hippopotamus ear sample (OR121027H1) was submitted from a hippopotamus carcass discovered on the banks of the Sabie River by Mpumalanga State Veterinary Services. The hippopotamus tissue was treated in two ways: 1) using a sterile scalpel, the outer skin was cut away along the cartilage and a swab taken of the inner tissue. This swab was vortexed in 500 µL 0.9% saline and 100 µL used to inoculate a PET plate, a TSBPA plate and a 5% SBA plate. 2) one gram of tissue was placed in 9 mL of PBS, heat treated at 65°C for 20 minutes and were plated out onto PET and TSBPA in triplicate and incubated at 37°C overnight. The bacterial lawns were harvested in PBS, divided into two. The first aliquot was subjected to

thermal lysate DNA extraction. The second aliquot was serial diluted up to $1x10^{-6}$, inoculated onto PET, TSBPA and 5% SBA respectively and incubated at 37°C overnight. Subcultures were made onto 5% SBA and confirmed by penicillin and Cherry phage sensitivity.

3.3. Results

3.3.1. Environmental samples

3.3.1.1. Arthropod isolations

The isolations from the insects varied (Table 3.2), however, commonality could be found in that no *B. anthracis* isolates were observed for the "inside" of the adult beetles. The qPCR results were similarly negative for these samples. For the "exoskeleton" saline wash, only the *Dermestes maculatus* (skin beetle) at 5×10^3 spores per beetle and the *Necrobia rufipes* (red-legged ham carrion beetle) at 4.8×10^3 spores per beetle, produced *B. anthracis* isolates. This was echoed by the positive amplification of qPCR markers BAPA, Cap C and SASP of these harvested bacterial lawns. In contrast, the maggots/larvae yielded approximately 1.2×10^3 *B. anthracis* isolates per maggot from the homogenate (Table 3.2) for carcass sites AD/2014/08 and LVS201403/003. The engorged Scarabaeoidae larva (dung beetle larva) was 3.5 times the size of the average maggot. The dung beetle larva homogenate yielded 8×10^3 isolates on its own.

3.3.1.2. Leaf and grass isolations

The inoculated plates from leaves, from the *Abutilon angulatum* tree, had a sterile appearance after 12 hour incubation on all media; when using the standard sample processing protocol. Since no bacterial lawns were available for harvest, even qPCR was not possible as a confirmation. Interestingly enough, the saline swab that was used to streak a single spot produced 170 colonies with typical *B. anthracis* morphology (Table 3.2). The grass samples were contaminated by an overgrown mucoid mass on both PET and 5% SBA, therefore spore counts were not possible although a purified *B. anthracis* isolate was obtained after repeated subculture.

Table 3.2: Results for qPCR and bacteriologic diagnostics from environmental samples collected in Pafuri, during the March 2014 anthrax outbreak. The colony counts represented here are for 1:1000 dilution factor of the original sample on both selective and non-selective media. Penicillin and gamma phage sensitivity of the isolates is also indicated.

Site	Date	Sample		qPCR	PET agar Average CFU of 4 plates (12 hours)	5%SBA agar Average CFU of 4 plates (12 hours)	Overall Description of plates	subcultured	penicillin	γ Phage
Zebra LVS201403/001	11/03/2014	Fibrous digesta		+	22 colonies	overgrown	No *B. anthracis* Repeated. Overwhelmingly *B. cereus* both times.	√	X (PS)	X
		Zebra hair		-	50 colonies	overgrown	mucoid, diffuse and irregular over whole plate	X	N/A	
		Vulture faeces		+		31 colonies	30 colonies, typical *B. anthracis* morphology + tiny clear pinprick colonies + 1 *B. cereus*	√	√	√
		Beetles Schizonychia	exoskeleton	-	67 colonies	overgrown	Bacilli appear to be *B. subtilis*?" Sugary" crust with clear stringy viscous centre	X	N/A	
			homogenate	-	colonies	18 colonies	Full plate small opaque mucoid colonies	X	N/A	
Kudu 1 LVS201403-003	14/03/2014	Beetles *Demestes maculatus*	exoskeleton	+	98 colonies	98 colonies	10 typical *B. anthracis* morphology on PET agar and 12 on 5%SBA + tiny mucoid colonies	√	√	√
			homogenate	-	0	15 colonies	Full plate small irregular clear mucoid colonies	X	N/A	

		maggots (in dung)	exoskeleton	+	23 colonies	35 colonies	12 haemolytic. 23 non haemolytic *B. anthracis* morphology on 5%SBA. Pure *B. anthracis* on PET	√	√	√
			homogenate	+	6 colonies	15 colonies	all colonies haemolytic from PET subcultures. 10 *B. anthracis* on 5%SBA	√	√	√
		grass		+	28 colonies	94 colonies	Mixed culture? Subcultured and "purified"	√	√	√
		*Leaves (with visible blowfly activity)		+	0	170 colonies	60 colonies >10mm wide and typical *B. anthracis* morphology (direct streaking therefore not an average of 4 plates, all colonies counted per spot, but reflects an average for 3 spots)	√	√	√
Kudu 2 LVS201403-004	14/03/2014	Beetles metallic blue-green with red legs (skull) *Necrobia rufipes*	exoskeleton	+	9 colonies	48 colonies	almost all typical *B. anthracis* morphology	√	√	√
			homogenate	-	4 colonies	24 colonies	clear irregular mucoid colonies. No *B. anthracis*	X	N/A	
		Beetles (brown -much like zebra carcass) Schizonychia	exoskeleton	-	7 colonies	65 colonies	Repeat plated onto blood agar in dilutions. All haemolytic or mucoid dome shaped colonies	X	N/A	
			homogenate	-	3 colonies	19 colonies	pin-prick mucoid colonies	X	N/A	
		white larvae Scarabaeidae	exoskeleton	+	20 colonies	51 colonies	20 typical *B. anthracis* morphology	√	√	√
			homogenate	+	12	23	1 colony haemolytic. 8 typical *B. anthracis*	√	√	√

					colonies	colonies	morphology			
Impala AD2014/08	15/03/2014	*Dung		+	0	0	No B. anthracis on first round (6 colonies on SBA after repeating at 10 $^{-6}$ dilution factor)	√	√	√
		Maggots	exoskeleton	+	18 colonies	24 colonies	6 colonies haemolytic. 18 typical *B. anthracis* morphology	√	√	√
			homogenate	+	12 colonies	12 colonies	9 colonies flat (atypical) non haemolytic & penicillin & γ-phage sensitive. Mucoid bacteria growing over plate as single mass. Suspect colonies subcultured and purified	√	√	√

PET – Polymyxin EDTA thallous acetate agar

5% SBA – 5% Sheep blood agar

The "+" for qPCR indicates amplification on all 3 targets (BAPA, Cap C and SASP) (Turnbull, 2008)

Exoskeleton versus homogenate isolations from insects/larvae are an attempt to separate external contamination of the arthropods from the presence of *B. anthracis* due to consumption/ingestion of carcass remains.

PS – partial sensitivity. Inhibition zone ≤ 6 mm around 10 μg penicillin disc. "*" indicates samples where *B. anthracis* was isolated on agar at a different dilution factor.

3.3.1.3. Vulture faeces

A gram of vulture faeces yielded 3 x 10^4 spores of *B. anthracis* on 5% SBA, but not on PET after 12 hours incubation (Table 3.2).

3.3.2. Pachyderm samples

3.3.2.1. Blood Smears

The Giemsa stains were inconclusive for the presence of encapsulated bacilli for these blood smears. The heat treated blood solution inoculum appeared sterile on blood agar after 12 hours. For the untreated inoculum, the blood smears from the white rhinoceross (Ax/2012/283, Ax/2012/291) and elephant (Ax/2013/435) each only revealed a single colony of *B. anthracis* (≥5 mm in diameter) and tiny colourless mucoid colonies per 50 µL blood solution (Table 3.3). The smear Ax/2012/275 produced 2 large *B. anthracis* colonies and Ax/2012/309 produced 5 colonies per 50 µL blood solution (Table 3.3). This correlated well with the qPCR results which demonstrated amplification at all 3 markers for the above-mentioned blood smears while Ax/2012/180 and Ax/2013/44 failed to amplify before the 40 CT cut off. The qPCR results were ready within 3 hours of collecting the smear.

Table 3.3: Results of bacteriologic isolations from unstained blood smears obtained from rhinoceros (*Ceratotherium simum*) and elephant (*Loxidonta africana*) carcasses in Kruger National Park, South Africa.

Sample #	Animal	Gender	Age of carcass	Thickness of smear	Colony Count / Growth 5%SBA
Ax/2012/275	white rhinoceros	M	≥3 days	heavy	2 colonies *B. anthracis > 5 mm and 1 mucoid colony*
Ax/2012/309	white rhinoceros	F	Same day	heavy	~50 tiny mucoid colonies (Heat Treated); 5 *B. anthracis* colonies > 5 mm & 5 translucent mucoid colonies > 5 mm with numerous pin-prick mucoid colonies (No heat shock)
Ax /2012/283	white rhinoceros	F	< 1 week	low	1 colony *B. anthracis* < 5 mm diameter
Ax/2012/180	white rhinoceros		≤2 days	low	1 mucoid colony > 3 mm diameter
Ax/2012/291	white rhinoceros	F	< 1 week	medium	1 colony *B. anthracis* > 5 mm diameter

Ax/2013/404	rhinoceros		< 1 week	low	10 tiny mucoid colonies
Ax/2013/435	elephant		Same day	medium	1 large *B. anthracis* colony

3.3.2.2. Tusks

For the hippopotamus tusk, only the pellet (centrifuged solution) produced an isolate of *B. anthracis*. The TSPBA and 5% SBA media were overgrown with haemolytic colonies up to a dilution factor of 1:10000. The elephant tusk yielded *B. anthracis* on PET as well, not from the pellet, but from the original scrapings. Again the TSPBA and blood agar were overgrown with haemolytic colonies up to a dilution factor of 1:1000. It is important to note that the *B. anthracis* colonies were only clearly visible after 48 hour incubation on PET agar.

3.3.2.3. Buccal fat from hippopotamus tusk

The ethanol treated inoculum produced 6 *B. anthracis* colonies on 5% SBA for the fat emulsion samples. The heat treated samples appeared entirely sterile on the blood agar after 24 hours of incubation. No isolates could be observed on either PET or TSPBA media.

3.3.2.4. Hippopotamus tissue

From the hippopotamus ear tissue (OR121027H1), only the direct streaking on 5 %SBA plate revealed any *B. anthracis* isolates. The heat treated plates were almost clean in appearance for both selective and non-selective media.

3.4. Discussion

The isolation successes achieved in the Skukuza field laboratory can be attributed to the use of both semi-selective and non-selective media concurrently for every sample. Biological samples are easier to isolate, even just on non-selective media/SBA, only when heat treatment is used as reported by Fasanella et al. (2013). There has been data to show that the use of traditional PLET media can exclude germination of some *B. anthracis* spores, where the recovery on SBA is more than 2 fold better (Dragon and Rennie, 2001, Irenge and Gala, 2012). The exclusion of lysozyme in the PET agar has resulted in better yields of *B. anthracis* isolates and yield ≥ 50 % more isolates than PLET and TSBPA in the KNP (EH. Dekker

personal communication). Thallous acetate is sufficient to inhibit *B. cereus* and a great many other *Bacillus spp.* that are unable to utilize thallium instead of the chelated essential cations (Knisely, 1966). The PET agar better inhibits *B. cereus* growth compared to TSBPA which can at times be overwhelmed by *B. cereus* growth from some environmental samples like grass or leaves. On the other hand, while the lysozyme is able to inhibit many *Bacillus spp.*, it does not inhibit all *B. subtilis* (Knisely, 1966, Irenge and Gala, 2012) that are commonly present in environmental samples from this study. The lysozyme has also inhibited some *B. anthracis* growth especially in environmental samples where *B. anthracis* competes against other faster growing bacteria which are not inhibited. The choice of media is important in the enumeration of bacterial counts from a sample. Hence deference is given to non-selective media, which gives a clearer reflection of spore counts. The PET media was preferred for samples where *B. anthracis* was easily outcompeted by other bacteria.

To improve diagnostics and better understand the dissemination of *B. anthracis* at carcass sites in KNP, sampling has come to include collection and documentation of both the flora and fauna associated with animal remains at the sites. Extravasation of the carcass leads to nutrient and moisture deposition into the area surrounding the animal remains (Bellan et al., 2013). This in turn can lead to biofilm formation which aids vegetative cell longevity and multiplicity (Lee et al., 2007). Schuch and Fischetti (2009) proposed that *B. anthracis* has a dynamic lifestyle that includes multiplication in the gut of earthworms, mediated by bacteriophage lysogenic factors. No earthworms could be found at carcass sites in Pafuri, however, there are a multitude of athropods in a 2 metre radius of carcasses comprising Diptera (Stratiomyidae and Calliphoridae) and Coleoptera (Scarabaeidae, Cleridae and Dermestidae). Bearing in mind that the carcass sites discussed here were already determined to be anthrax positive by SSVO, the arthropod isolation results reported as follows: At the zebra site LVS201403/001 and kudu site LVS201403-004, the *Schizonychia* beetles collected from beneath the carcasses failed to demonstrate the presence of *B. anthracis* bacteriologically or in molecular diagnostics. In the case of site LVS201403-004, the *Necrobia rufipes*, with predatory larvae that feed on meat eating insect larvae, were swarming over the skull especially in the orbital sockets and horns. These red legged ham beetles were possibly searching for mates and ovipositioning sites. This led to spore counts of 4800 spores per

beetle as external contamination. At kudu site LVS201403-003 *Dermestes maculatus* or hide beetles, which primarily feed on carcasses ranging from a few days old to a few weeks, were collected from the wet inner hide. These beetles produced similar external spore counts of 5000 per beetle. Despite the feeding habits of the two beetle species, no isolations of *B. anthracis* could be gained from the gut of the beetles. The same was true for qPCR diagnostics. This could possibly be due to inhibition factors within the homogenate or penetration of the peracetic acid into the hemocoel through the insect's spiracles. This is nevertheless in keeping with a long held belief that these insects do not play a significant role in the dissemination of *B. anthracis* in KNP (Braack, 1987, Braack and De Vos, 1990). In contrast, the Calliphoridae and Stratiomyidae larvae/maggots for the carcass sites yielded ~1,200 *B. anthracis* colony forming units per maggot from the using gut/homogenised samples. Even though they had begun to pupate before diagnostics were carried out. There is a dearth of knowledge on the fate of *B. anthracis* thereafter. A study by Graham-Smith (1914) determined that spores fed to larvae could remain viable up to 20 days after a fly emerges. It also demonstrated that vegetative cells were not able to survive for such a period. Irrespective of the the bacterium's survival, the sheer number of maggots/larvae from a single carcass (e.g. Figure 3.3) may influence overall spore counts, whether in dissemination or decontamination needs to be determined.

Figure 3.3: Blowfly larvae/ maggots consuming an anthrax positive carcass in Pafuri, Kruger National Park, South Africa (photo credit: EH Dekker and LO van Schalkwyk)

There has been much debate over the influence of flies in *B. anthracis* spore/vegetative cell dissemination (Fasanella et al., 2010b, Blackburn et al., 2014, von Terzi et al., 2014). The SSVO have isolated *B. anthracis* from blowfly spots on leaves and grass in many sections of the KNP. The colony counts vary between 0 – 1000 when using the standard processing protocols, however, on average 500 CFU per spot is the norm for an anthrax positive site (EH Dekker personal communication). In Pafuri, there was difficulty in isolating *B. anthracis* from heat treated leaves from the *Abutilon angulatum* and lower than normal counts (170 CFU) on the 5% SBA. The leaves were collected and submitted to the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria. The plant was determined to have moderate antimicrobial activity against *B. anthracis* spores (Elisha et al., 2015), which is an indication of why the heat treated inoculum resulted in sterile plates even after 24 hour incubations. This highlights the influence of environmental factors in diagnostics from carcass sites.

The blood smears of the rhinoceros and elephant were confirmed positive for anthrax by qPCR within 4 hours of collection. It is however important to note that all 3 markers have to amplify in order for a sample to be considered positive at SSVO. This is because there has been occasions when the presence of *B. endophyticus*, *B. thuringiensis* and pathogenic *B. cereus* have caused non-specific amplification of BAPA and Cap C (Lekota et al., 2016). The qPCR results and isolation of *B. anthracis* correlated well for the blood smears evaluated in this study. The blood smear diagnostics in KNP form the greater part of the surveillance system as it is used for acid fast bacteria and rickettsial diseases as well. The unstained blood smears are an invaluable resource to explore for retrospective studies and molecular based co-infection studies of wildlife in KNP.

The investigation of *B. anthracis* isolates, obtained from hippopotamus carcasses, suggests that environmental factors may impede spore and vegetative cell viability. From the *B. anthracis* isolation from hippopotamus it seems that in some cases the *B. anthracis* spores or vegetative cells are under stress from the environment and only optimal conditions allow the bacterium to recover sufficiently to grow on the media as neither the heat treated samples nor the swabs on the selective media yielded any *B. anthracis*, but the 5% SBA did. In a study by Bellan et al. (2013) it was determined that bloating of a dead hippopotamus carcass was sufficient to rupture it without the aid of scavengers, but, given time. A possible reason for the growth of *B. anthracis* only on the blood agar from the hippopotamus ear sample, as well as the rhinoceros and elephant blood smears, is likely due to this phenomenon. Pachyderm animal skins are thick; hence bloating and putrefaction only softens the skin enough for scavengers like vultures and crocodiles to open the carcass or eventually rupturing through bloat. The delayed exposure to oxygen and microenvironment conditions would result in lower spore counts. This is because putrefaction leads to vegetative cell death as well as providing competitive growth inhibition in an environment more conducive to anaerobic bacteria (Sterne, 1937a, Turnbull, 2008). The bone and tusks of pachyderms, however, still readily yield *B. anthracis* isolates (Seideman and Wheeler, 1947) even on PET. Another possibility for the role of environmental stresses can be seen in the hippopotamus buccal fat sample.

The fatty acid composition of the adipose tissue of hippopotamuses may be the reason for the lack of *B. anthracis* growth, on either selective or nutrient media, after heat treatment of the hippopotamus fat sample. Hippopotamus fat is composed almost entirely of three fatty acids; oleic acid (39.6%), palmitic acid (27.2%) and stearic acid (23.2%). Other components include, octadecadienoic acid (linoleic acid at 3.4%), octadecatrienoic acid (linolenic acid at 1.5%), and minor proportions of other saturated fatty acids (Barker and Hilditch, 1950, Duncan and Garton, 1968). The oleic acid, linoleic acid and linolenic acid are unsaturated fatty acids with melting temperatures far below room temperature, while palmitic and stearic acids are saturated fatty acids with melting temperatures over 60°C. These fatty acids (FA) have varying levels of bactericidal activity, which also extends to bacterial spores. These FA's interact with the hydrophobic spore surface, disrupting spore germination

mechanisms, and thus bacterial growth (Yasuda et al., 1982, Johnstone, 1994). According to Lekogo et al. (2013), oleic acid, stearic acid and linoleic acid cause injury to the spore during heat treatment, thereby lowering the heat resistance of the spore with time. These FA's are used as an additive in the food industry for spore sterilization during heat treatment and food safety evaluations (Lekogo et al., 2013). The presence of these FA's in the inoculum will then further prevent germination, which is probably the reason the heat treated hippopotamus fat sample did not yield any isolates while the untreated sample did, but only on the 5% SBA. The use of ethanol does not damage *B. anthracis* spores and has been used as a means to destroy the vegetative cells of gram positive and negative bacteria that may competitively inhibit *B. anthracis* growth (Dragon and Rennie, 2001). The nutrient rich blood agar was able to provide conditions conducive to spore recovery, from the effects of the inhibitory FFA's, and thus enable the spores to germinate.

3.5. Conclusions

There are many environmental factors that may influence the dissemination of *B. anthracis* that are still poorly understood. In sampling protocols at carcass sites, it is important to document observations as well as the ecological conditions which make each site unique. The use of selective and non-selective media in anthrax diagnostics has improved SSVO isolation success rates in the KNP. There is still a need for multivariate diagnostics of anthrax due to environmental factors such as antimicrobial activity in samples. It is clear that blowflies and their larvae do play a role at carcass sites; however, the magnitude of that role and its influence on anthrax ecology still requires further study.

The passive surveillance system managed by the SSVO demonstrated itself to be an invaluable program for the monitoring of outbreaks of reportable diseases such as anthrax. It serves as an early warning system during outbreaks and results in increased vigilance in the field. It is also a resource for retrospective study that requires exploring. This in turn is promoting a better understanding of the dissemination of the disease in outbreak scenarios.

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4. Chapter 4

Through the lens: a microscopic and molecular evaluation of archival blood smears from the 2010 anthrax outbreaks in Kruger National Park, South Africa

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Abstract

Bacillus anthracis is Gram positive endospore forming bacterium responsible for the disease known as anthrax. It is enzootic in the Kruger National Park (KNP) in South Africa where annual outbreaks are commonplace. Routine surveillance for disease is performed through blood smear collection as well as tissue and bone collection where possible. The archival blood smear collection (n=99) from an anthrax outbreak in 2010 was evaluated with regards to bacterial isolation and comparison of microscopy results to qPCR based diagnostic techniques. The blood smear DNA was also subjected to single nucleotide polymorphism typing using the qPCR based melt-MAMA (melt analysis of mismatch amplification mutation assays use to identify single nucleotide polymorphisms, SNPs) technique to determine its value for retrospective epidemiological studies. Isolation of bacteria from the blood smears is worthwhile for up to a year depending on smear thickness, bacteraemia and level of spores present. As such only 10% of blood smears, ≥ 3 years old, yielded an isolate from the 2010 blood smear collection. DNA from the smears were typed on a panel of 19 Melt-MAMA markers, but belonged to only 2 SNP lineages: A.Br005/A.Br006 and Aust94. The data collected during this kind of surveillance gives context to outbreaks. With more region specific SNP marker assays, blood smear slides are a viable resource for retrospective epidemiological analysis.

4.1. Introduction

The Kruger National Park (KNP) is a game reserve located in the north eastern part of South Africa (24.0114° S, 31.4853° E); bordering Mozambique and Zimbabwe and spanning 20 000 square kilometres (https://www.sanparks.org/parks/kruger/). The KNP has a diversity of ecological types, across the length and breadth of the park, which are demarcated on the basis of topography, geography, vegetation and fauna. The KNP is thus divided and managed by SANParks according to these ecological niche sections https://www.sanparks.org/parks/kruger/conservation/scientific/exclosures/. Disease surveillance and control of the KNP sections is coordinated by the Skukuza State Veterinary Services (SSVS, Department of Agriculture Forestry and Fisheries) in collaboration with SANParks (South African National Parks) (Bengis, 2000, Bengis et al., 2003). Disease surveillance, especially in terms of the identification and management of emerging infectious diseases is of increasing importance in these ever-changing climes (Anderson et al., 2004, McCabe-Sellers and Beattie, 2004, WHO, 2000).

Bacillus anthracis an endospore forming soil bacterium is the causal agent of anthrax (Sterne, 1937c) and enzootic in the northern most part (Pafuri region) of (KNP (Smith et al., 2000, Steenkamp, 2013). Anthrax is a controlled and reportable disease that requires monitoring and management in KNP (De Vos et al., 1973, Turnbull, 2008, Bengis and Frean, 2014). While anthrax is a zoonotic disease capable of affecting a wide range of mammal hosts including humans and predators, it is preponderantly a disease of herbivores and wild ungulates. Index cases of anthrax generally occur when *B. anthracis* is ingested or inhaled by these herbivores while grazing or browsing (Turnbull, 2008).

In the KNP, blood smears of carcasses are consistently and unremittingly collected as part of the Skukuza State Veterinary Disease Surveillance System. Multiple smears are taken for carcasses discovered across KNP, one for staining (whether Gram, Giemsa or Ziehl–Neelsen: based on the observations made at the carcass site) and another unstained slide are then both archived according to the sampling date. Although the surveillance system is used for the identification of many pathogens, it is primarily used to monitor anthrax outbreaks.

On microscopic examination of a blood smear, confirmation of the presence of *Bacillus spp.* can be obtained on observing Gram-positive rods. In the case of *B. anthracis,* encapsulated cells are found in pairs or short chains, often with square ends giving them a 'box-shaped' appearance (Turnbull, 2008). As the Gram stain is not suitable to visualize the encapsulated *B. anthracis,* smears of the specimen must be stained with M'Fadyean's stain (polychrome methylene blue) that stain the capsule pink and the bacillus cells dark blue (Turnbull, 1999, Turnbull, 2008).

The diagnosis of anthrax in Africa / developing countries is predominantly achieved via microscopy to detect encapsulated *Bacillus* with M'Fadyean's stain in smears of blood or tissue from fresh carcasses. A standardized inclusion of 0.23% Azure B and 0.01% potassium hydroxide in the M'Fadyean has been suggested to improve observation of the capsule (Owen et al., 2013). The stained slides are stored (archived) and do not represent a hazard to possible infection (Amar et al., 2002).

There have been several studies on the use of archival samples as a source of DNA for use in molecular diagnostics (Poljak et al., 1995, Yokota et al., 1995, Alger et al., 1996, Poljak et al., 1996, Amar et al., 2001, Amar et al., 2002, Kirchgatter et al., 2002). These have met with differing levels of success depending on the target organism, collection conditions and storage for isolation and amplification of DNA from the samples. The use of PCR on archived material on stored slides are worth exploring in anthrax diagnoses where only stored slides are available and can be used to offer information and make retrospective studies possible.

Molecular methods have been developed to characterise *B. anthracis* strains including multilocus variable number of tandem repeats analysis (MLVA) (Keim et al., 2000, Lista et al., 2006); single nucleotide repeats (SNR) (Garofolo et al., 2010), whole genome single nucleotide polymorphism analysis (Pearson et al., 2004b, Thierry et al., 2014) and melt analysis of mismatch amplification mutation assays (Melt-MAMA) (Van Ert et al., 2007a, Derzelle et al., 2011, Birdsell et al., 2012). These DNA-based typing methods, especially

MLVA and SNP, evaluate the diversity and enable epidemiological track-back analysis of anthrax outbreaks. The objective of this study was to use the passive surveillance archival blood smears taken during an anthrax outbreak in 2010 in KNP to investigate *B. anthracis* isolation and molecular characterization (SNP genotyping) to enable a retrospective epidemiological study of the 2010 anthrax outbreak.

4.2. Materials and Methods

4.2.1. Skukuza State Veterinary Disease Surveillance System

As earlier mentioned, multiple blood smears from carcasses that were collected as part of the Skukuza State Veterinary Disease Surveillance System were used in this study. Observations from the carcass sites were stored in a database which includes data fields pertaining to the species of carcass, carcass age, possible causes of death which includes disease and notable predation or scavenging, blowfly activity, region and/or GPS co-ordinates. When only the skeleton remains, the hoof was removed and the smear was made from the drop of fluid found there. Reports were also filed by field staff of the Skukuza Office of the State Veterinarian; describing the distribution of carcasses and any unusual characteristics observed during an outbreak. Results are reported according to the requirements of the Animal Diseases Act (Act 35 of 1984). This surveillance system has been in effect for several decades.

4.2.2. Evaluating the Archival Collection

A pilot study of DNA quality and bacteriologic isolation success rates was performed on a subset of the archival blood smears (data not shown). Multiple unstained blood smears that recorded high *B. anthracis* bacteraemia and observable spores at the time of collection, were chosen from 2012 and each year going back to 1992 (n=159). The material from these slides were tested microscopically, bacteriologically and using real time quantitative PCR (FRET probes) (Turnbull, 2008) testing for the *B. anthracis* protective antigen (BAPA), encapsulation region (Cap C) and small acid soluble spore protein in the chromosome (sasp gene) regions (Ellerbrok et al., 2002, Turnbull, 2008). In the pilot study it was observed that no bacterial isolates were obtained from smears before 2006 with only 8 isolates of 159 smears established (and 6 of these were from 2010-2012). The qPCR results for the detection of *B.*

anthracis DNA from before 2002 were inconclusive due to low amplitudes and very late cycle thresholds (≥40 cycles) - if any were observed at all. The pilot study determined that smears from 2010-2012 were most suitable for study and we therefore focused on the 2010 anthrax outbreak to increase the chances of obtaining bacterial isolates from the blood smears as well as viable DNA for molecular characterization.

4.2.3. The 2010 Outbreak Year

The data and reports from the collection of the blood smears (n=291) from all regions of the KNP, submitted to the State Veterinary Research Laboratory in Skukuza between November of 2009 to December of 2010 for microscopic diagnostics (Table 4.1) was included in this study. Drought occurred during the spring and summer months of 2009 that resulted in decimation of the grasslands, followed by heavy rains which resulted in massive erosion and sediment deposits in the floodplains and pans. Rising temperatures rapidly depleted available water holes in the north of the park during the first semester of 2010. Three Giemsa blood smears positive for *B. anthracis* from Pafuri were observed in December 2009. Pafuri is enzootic for anthrax and isolated cases occur throughout the year. In total 57 impala carcasses were discovered in Pafuri between November 2009 and March 2010. Blood smears were not available for all of these carcasses due to desiccation of the carcasses, but in such cases bones were collected for bacteriologic diagnostics. There was evidence of baboons (*Papio ursinus*) scavenging on the fresher impala carcasses and subsequently, dead baboons were found with apparent haemorrhaging from the orifices and extreme diarrhoea. Large numbers of vultures were observed scavenging on the carcasses. Reports noted an inexplicable dearth of blowflies at carcass sites in Pafuri, irrespective of carcass age/freshness, which was contrary to the observations in other sections. During the second semester of 2010, anthrax infected cases were confirmed not only in the enzootic Pafuri section but also in Punda Maria, Vlakteplaas, Shingwedzi, Shangoni, Woodlands, Mooiplaas, Mahlangene and Letaba.

Table 4.1: An excerpt of the database of blood smears collected from carcasses during November 2009 - December 2010 from different regions of the Kruger National Park as part of the Skukuza State Veterinary Services Disease Surveillance System. Supplementary Data\Table 4.1.xlsx

4.2.4. Selection, evaluation, isolation and DNA extraction of blood smears

The focus will be on a blinded randomly selected subset of (n=99) blood smears for further characterization (Table 4.2). One "fresh" white rhinoceros (*Ceratotherium simum*) blood smear (AH100) was used as a non-template negative control and an impala (*Aepyceros melampus*) blood smear spiked with *B. anthracis* Sterne $34F_2$ as a positive control for molecular assays. The subset blood smear pairs were evaluated and scored for smear thickness, bacteraemia and level of putrefaction (Table 4.3), with the latter two observed on the Giemsa stained slide. The dried blood from the unstained slide was scraped off and dissolved in 180 µL of sterile physiological saline. An 80 µL aliquot of the solution was presented for DNA isolation and the remaining solution divided into three equal aliquots. The first aliquot was added to 500 µL of sterile brain heart infusion broth (Oxoid™) and incubated at 37 °C overnight. The solution was then serial diluted and plated onto both PET agar and 5% blood agar. The remaining two aliquots were plated directly onto PET and blood agar respectively with an additional 50 µL of sterile phosphate buffered saline for ease of spreading. These plates were incubated at 37 °C and evaluated every 12 hours for 48 hours. Bacterial isolates were purified through sub-culturing on blood agar and *B. anthracis* cultures were confirmed by testing for penicillin and γ-phage sensitivity and virulence factors as described in qPCR section below (Turnbull, 2008).

Table 4.2: Sample information of the evaluated subset of blood smears from the Skukuza State Veterinary Services Disease Surveillance System database. Supplementary Data\Table 4.2.xlsx

Table 4.3: The microscopic and bacteriologic evaluation of the subset of blood smears from the Skukuza State Veterinary Services Disease Surveillance System database indicating the smear thickness and bacteraemia scoring. Supplementary Data\Table 4.3.xlsx

DNA extraction from blood smear solution AH1-10 (part of the pilot trial) was done using 80 µL blood solution that was heated to 100 °C for 20 minutes followed by centrifugation at 6000xg for 20 minutes and harvesting of the supernatant using blood protocol of QIAamp® DNA blood mini kit (Qiagen). The remaining 90 blood solutions were adjusted to 200 µL

with PBS before centrifugation and using blood protocol of QIAamp® DNA blood mini kit (Qiagen). DNA extractions from bacterial culture were done from a single pure colony using the Gram-positive bacterial protocol with 20 mg/mL lysozyme L6876 (Sigma Aldrich) of the QIAamp® DNA blood mini kit (Qiagen).

4.2.5. Fluorescence Resonance Energy Transfer qPCR

The diagnostic qPCR for *B. anthracis* was performed with 2.5 µL DNA in 1x FastStart™ Taq DNA Polymerase mastermix (Roche®) and 0.5 µM of each primer (ANT-F: 5′-GC TAGTTATGGTACAGAGTTTGCGAC-3′) and (ANT-Amt: 5′-CCATAACTGACATTTGTG CTTTGAAT-3′) along with 0.2 µM of probe (ANT-Fluorescein: 5′-CAAGCAAACGCAC AATCAGAAGCTAAG-X) and 0.2 µM of probe (ANT-LC-Red640: 5′-GCGCAAGC TTCTGGTGCTAGC-P) (Tib MolBiol GmbH) in a final volume of 20 µL. The PCR conditions on a LightCycler™ Nano (Roche®) consisted of an initial cycle at 95 °C for 10 minutes, slope at 20 °C/second, followed by 40 cycles of 95 °C for 10 seconds; 57 °C for 20 second; 72 °C for 30 second, slope 20 °C/second with one single signal acquisition at the end of annealing cycle. Denaturation at 95 C for 3 seconds with a slope 20 °C/second; 40 °C for 30 second, slope 20 °C/second; 80 °C for 3 seconds at a slope of 0.1 °C/second with continuous acquisition of the signal . Cooling to 40 °C for 30 second, slope 20 °C/second (Ellerbrok et al., 2002, Turnbull, 2008).

4.2.6. Whole Genome amplification

Samples AH1-10 (due to low DNA template volume/quality) and samples with high cycle thresholds for the SASP and BAPA qPCR assay (AH9, 12, 15, 21, 23, 29, 42, 51, 56 and 98) were purified for prokaryotic DNA using NEBNext® Microbiome Enrichment kit (New England Biolabs Inc.) according to the manufacturer's instructions. The DNA yield was then amplified for the Melt-MAMA assay using rolling circle amplification through the Illustra GenomiPhi™ V2 DNA Amplification kit (GE Healthcare) with an overnight incubation at 37 °C before the 72 °C enzyme inactivation.

4.2.7. Melt-MAMA Assay

The single nucleotide polymorphism assay was performed for 20 markers (Table 4.4), as described in (Birdsell et al., 2012), where 2.5 µL DNA was diluted in 1x FastStart DNA Green Master (Roche®) with an ancestral forward and a derived forward SNP target primer (GC-clamp : no-GC-clamp) and a common reverse primer (Inqaba Biotec™) with a starting concentration of 0.2 µM depending on the ratio indicated in (Table 4) which allowed for separation of melt peaks by at least 5 °C. Thermocycling parameters on the LightCycler™ Nano (Roche®) were 95 °C for 10 minutes, and then cycled at 95 °C for 15 second and 55°C–60°C (dependent on oligonucleotides; see Table 4) for 1 min for 35 cycles. End-point PCR amplicons were subject to melt analysis using a dissociation protocol comprising 95°C for 15 seconds, followed by incremental temperature ramping (0.1 °C) from 60 °C to 95 °C. SYBR Green fluorescence intensity is measured at 530 nm at each ramp interval and plotted against temperature and observed as the separate melt peaks for each SNP. Controls included in every run were DNA from *B. anthracis* Ames, Vollum, Sterne $34F_2$ vaccine strain (OBP) and KC2011 (a known *B. anthracis* B clade isolate provided by Skukuza State Veterinary Services). The ancestral positive control was assigned a "1" designation and the derived positive control a "0." In this way, by assigning the results for each SNP melt curve, a binary fingerprint was obtained for each blood smear. This binary fingerprint was used to generate a maximum likelihood phylogenetic tree in BioNumerics® (Applied Mathematics) version 7.5.

Table 4.4: Melt analysis of mismatch amplification mutation assay oligonucleotide information as published by Birdsell et al. (2012) indicating concentrations and annealing conditions used in this study. Supplementary Data\Table 4.4.xlsx

4.3. Results

4.3.1. The 2010 Outbreak Year: Database Evaluation

Of all the samples submitted between November 2009 and December 2010, 62% were identified as positive for the presence of encapsulated *Bacillus* spp. on Giemsa stained blood smears (Figure 4.1).

The species susceptibility to infection by *B. anthracis,* as indicated by corroborating reports for 2010 samples (n=291), are represented according to carcass species in Figure 4.2. Impala carcasses were the most abundant sampled as well as identified as positive for *B. anthracis,* followed closely by buffalo and kudu carcass numbers in 2010 in KNP. Positive carcasses identified in smaller numbers (≤10 carcasses) were nyala, elephant, waterbuck, warthog, bushbuck, baboon and zebra respectively. The characterised blood smear subset, although randomly selected, does not adequately represent the database, showing a bias toward buffalo blood smears. When evaluating the 291 anthrax suspected death reports; 57% of the smears submitted were from impala and only 49% from buffalo, juxtaposed against the reported 15% impala and 41% buffalo anthrax cases from the randomly selected subset (n=99) (Figure 4.2). This has implications for determining the genotypic diversity of strains.

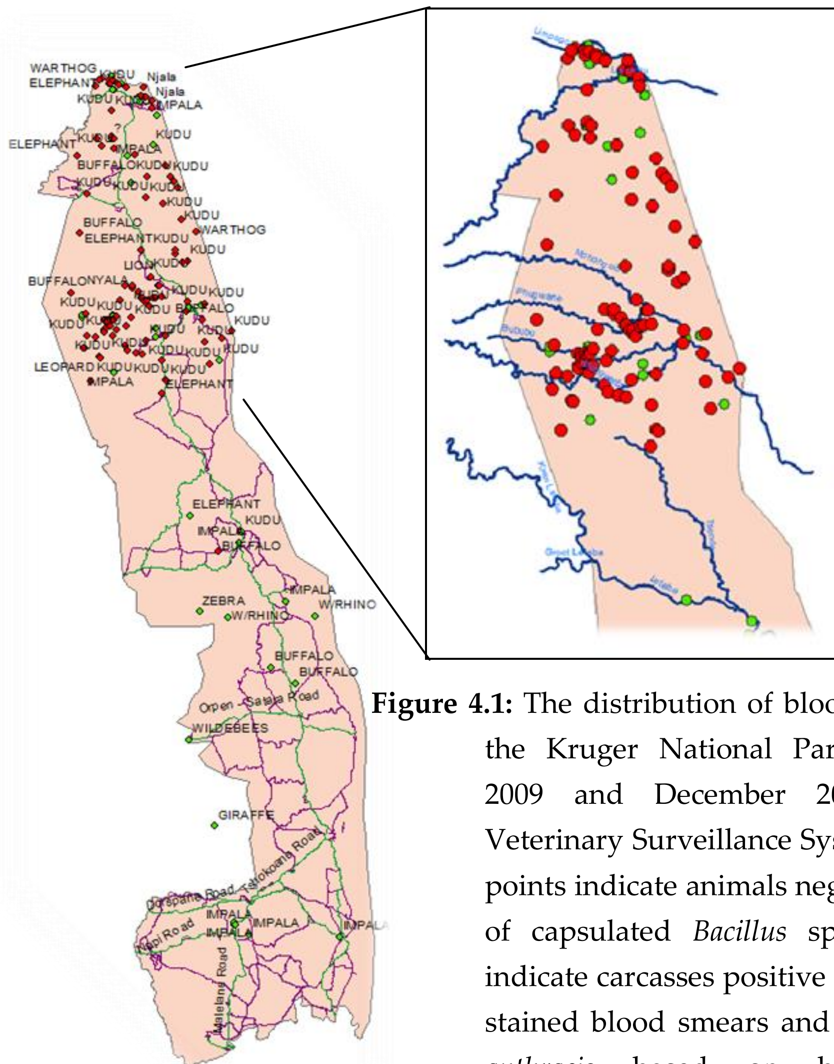

Figure 4.1: The distribution of blood smears collected in the Kruger National Park during November 2009 and December 2010 (Skukuza State Veterinary Surveillance System) database. Green points indicate animals negative for the presence of capsulated *Bacillus* spp. while red points indicate carcasses positive for *Bacillus* on Giemsa stained blood smears and later confirmed as *B. anthracis* based on bacteriologic culture, penicillin and γ-phage (representative group) and/or virulence factors using qPCR (all smears). The Pafuri region (northern most tip of the KNP) is endemic for anthrax.

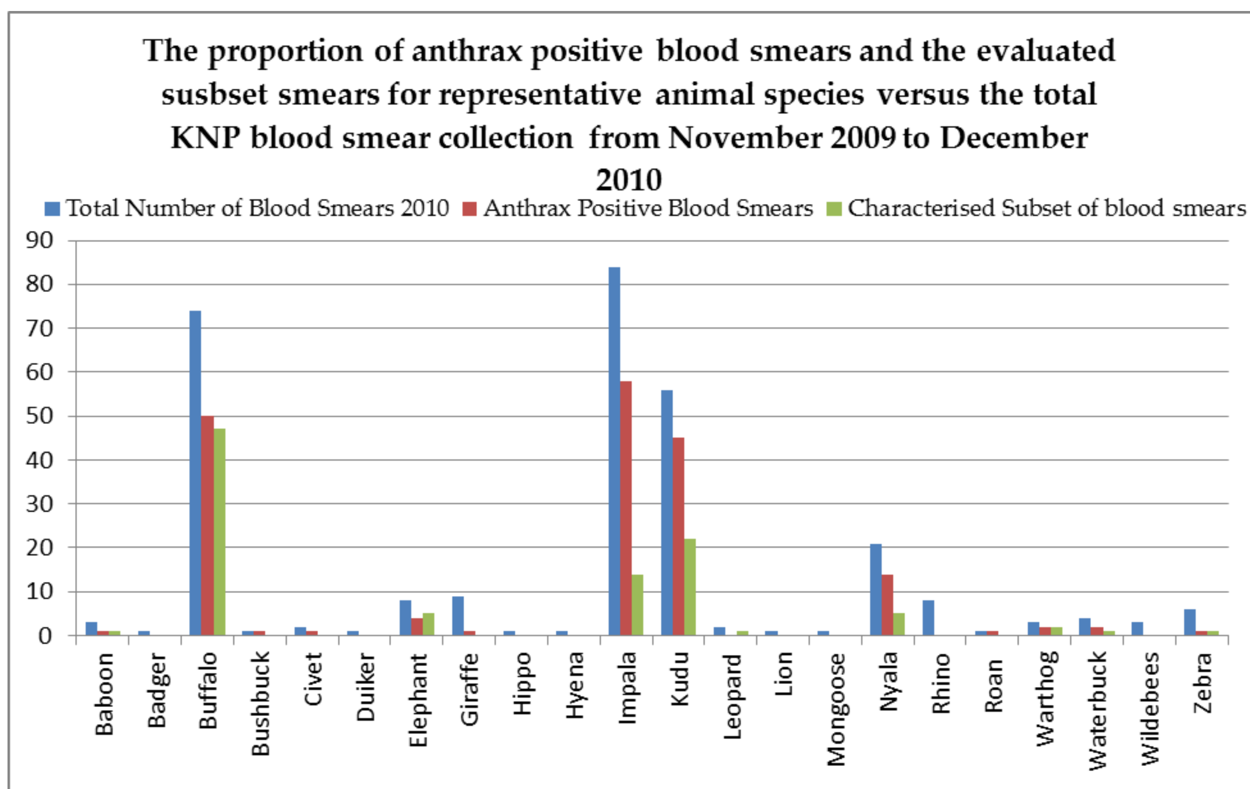

Figure 4.2: The total number of blood smears (blue) in relation to blood smears identified as positive (red) as well as a further characterised subset (green) per animal species submitted from November 2009 to December 2010 for anthrax diagnostics to the Skukuza State Veterinary Surveillance System, Kruger National Park, South Africa. The susceptible species are buffalo, kudu and impala as indicated by the carcass numbers.

The carcass distribution indicates that impala were predominantly affected (> 50) with only 3 elephant, 3 baboon, 2 zebra, a buffalo and a warthog across the enzootic Pafuri section. In addition to the grazers, the carcasses of 3 nyala and a bushbuck were clustered around a watering hole. In contrast buffalo carcasses were distributed among all the other sections in the northern half of KNP. Kudu carcasses were similarly distributed across multiple sections except for Pafuri, Mooiplaas and Woodlands (Figure 4.1 and 4.3).

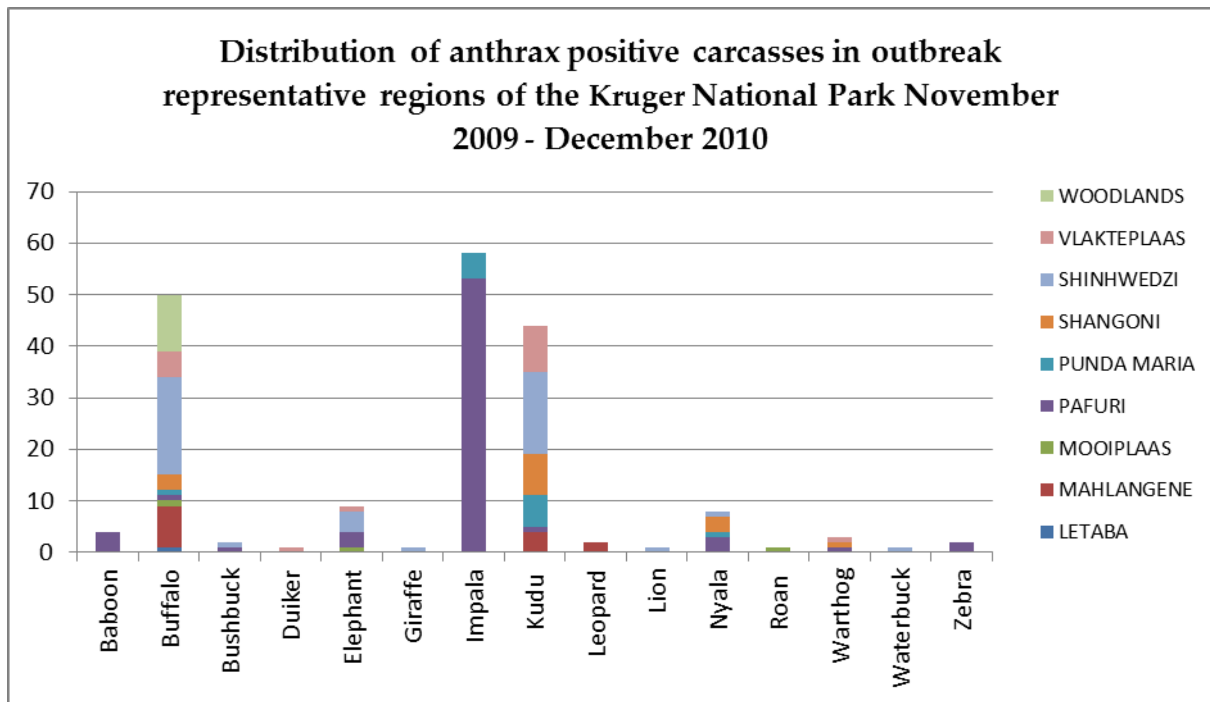

Figure 4.3: The distribution of anthrax positive carcasses in outbreak representative sections of the Kruger National Park November 2009 - December 2010 indicating the proportion of carcasses identified in each section.

The blood smear database also includes observations about predation and/or scavenging at carcass sites (Table 4.1; Figure 4.4) which provides insights into animal exposure and the possible spread of inoculum at anthrax positive carcass sites. Large numbers of vultures were recorded at carcass sites in Shingwedzi, Pafuri, Woodlands and Shangoni with fewer observed in Punda Maria, Mahlangeni, Mooiplaas and Vlakteplaas respectively. Unusually, Blowflies were only observed in the Shangoni, Shingwedzi and Vlakteplaas sections, but absent in the other sections. Baboons were noted to have scavenged on impala carcasses only in Pafuri section. As described earlier; baboons were subsequently discovered with symptomatic traits of gastrointestinal anthrax.

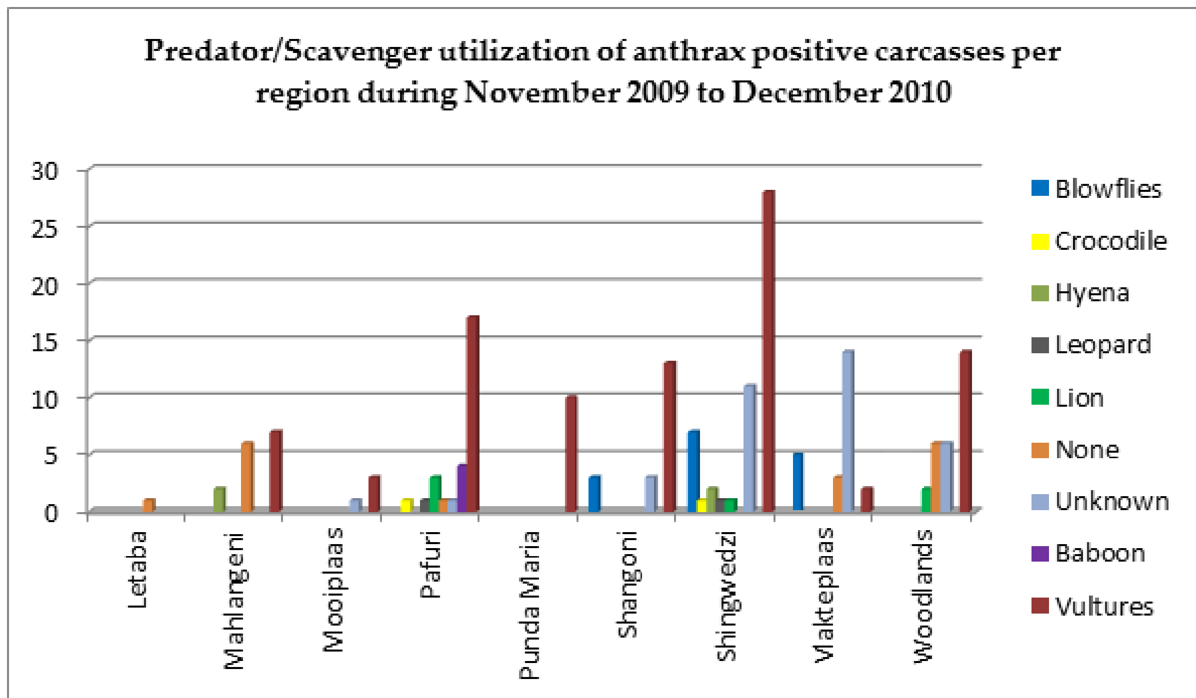

Figure 4.4: Observed predator/scavenger utilization of anthrax positive carcasses per region during November 2009 to December 2010 in the Kruger National Park, South Africa.

4.3.2. The 2010 Outbreak Year: Blood Smear Subset Evaluation

The characterised blood smear subset (Table 4.2; Figure 4.2) indicates the qPCR assay results in comparison to Giemsa blood smear and bacteriologic assays in Table 4.5. The qPCR results in relation to observations from the blood smear: True positives (n=82) and negatives (n=9) are when the qPCR and Giemsa stained blood smear results are in agreement. False negatives (n=7) are when the slide clearly demonstrates the presence of capsulated box-shaped *Bacillus* with negative qPCR results. This is demonstrated in the case of AH14 where *B. anthracis* was isolated off the slide (the colony was confirmed by qPCR, morphology, penicillin and γ-phage). Uncertain slides look 'suspicious' on blood smear but are negative on qPCR and are therefore inconclusive.

Table 4.5: Summary table of the true positives and negatives, as well as, false positives and negatives as determined by multifactorial testing (microscopic, bacteriologic and qPCR).

Uncertain	False Positives	False Negatives	True Negatives	True Positives				
AH22	none	*AH14	AH19	AH1	AH24	AH47	AH65	AH85
AH8		AH29	AH25	*AH2	*AH26	AH48	AH67	AH86
		AH9	AH31	AH3	AH27	AH49	AH68	AH87
		AH21	AH55	AH4	AH28	AH50	AH70	AH88
		AH30	AH66	AH5	AH32	AH51	AH71	AH89
		AH36	AH69	AH6	AH33	AH52	AH72	*AH90
		AH42	AH80	*AH7	*AH34	AH53	AH73	AH91
			AH99	*AH10	AH35	AH54	AH74	AH92
			AH100	AH11	AH37	AH56	AH75	AH93
				AH12	AH38	AH57	AH76	AH94
				AH13	AH39	AH58	*AH77	AH95
				AH15	AH40	AH59	AH78	AH96
				AH16	AH41	AH60	AH79	*AH97
				AH17	AH43	AH61	AH81	AH98
				AH18	AH44	AH62	AH82	
				AH20	AH45	AH63	AH83	
				AH23	*AH46	AH64	AH84	

Key	Description
*AH	smears that produced bacterial isolates in culture
(orange)	Failed DNA extraction from spores or inhibitors
(blue)	Low DNA template due to thin smear thickness at onset as well as low bacteraemia and noted putrefaction possibly resulting in DNA degradation
(green)	No stained slide for visual appraisal but report says positive for *B. anthracis*

4.3.3. Fluorescence Resonance Energy Transfer qPCR:

The qPCR FRET probe assay and Giemsa results were comparable except in the case of blood smears AH2, AH30 and AH42 which were negative for the capsule marker and had very late cycle thresholds for the chromosomal marker. The assay was repeated and results indicate that these isolates are attenuated, having lost plasmid pX02.

4.3.4. Bacteriologic Isolation

Only 10% of the 99 blood smears yielded *B. anthracis* isolates, which mainly represented sporulated bacteria on the Giemsa smears. The blood smears where cultures were established also had a higher smear thickness score which could have some bearing on its success (Table 4.3). In the case of the buffalo blood smears, which represented the majority of the sample set, the plates were generally overgrown with methicillin negative *Staphylococcus aureus.* In the case of the baboon, positive results for *B. anthracis* were almost exclusively from BHI broth enrichment media that was serial diluted.

4.3.5. Melt-MAMA Evaluation

The Melt-MAMA assay was able to amplify 19 out of the 20 SNP markers tested. The oligonucleotides for Branch 1_28 failed to produce separate melt curves for the Ancestral and Derived SNP's and had to be discarded. The remainder of the markers were used in the amplification of all of the positive blood smears. The low template of blood smears AH9, 12, 15, 21, 23, 29, 42, 51, 56 & 98 (Table 4.2) were improved through rolling circle amplification. The binary data results of the Melt-MAMA assay were used to generate a cladogram (Figure 4.5). The majority of the blood smears belonged to the A.Br.005/A.Br.006 clade of isolates. A small number of the smears grouped with the A.Br.Aust94 clade - including AH2 and AH7, which originated from Pafuri - while AH56 was collected from Letaba.

4.4. Discussion

Archival smears are a valuable resource for retrospective studies (Vince et al., 1998, Scopel et al., 2004, Karunamoorthi and Bekele, 2009). The pilot study highlighted that the older the smears are, the less reliable/stable the biological material is for experimental work. In this study, isolates could be obtained for up to 6 years of blood smear storage, but with low success rates. Fresher slides (≤1 year) on the other hand proved useful in the isolation of *B. anthracis* as well as more robust DNA stocks for molecular diagnostics and characterization. The molecular aspect of the study was dependent on good quality and quantity DNA yields which were obtained from thick blood smears and high levels of bacteraemia.

The subset of blood smears used in this study, which were randomly selected in a blind experiment, did not represent the outbreak as a whole with a greater bias for buffalo

carcasses and therefore its data is only evaluated in terms of its potential use for downstream applications.

Only 10% of the 99 blood smears in the subset yielded *B. anthracis* isolates. These mainly represented smears demonstrating sporulation on the Giemsa smear twin slide. The cultures established from blood smears were mainly from slides with a higher smear thickness score which could have some bearing on its success. In the case of the buffalo blood smears, which were in the majority in the sample set, the plates were generally overrun with MRS – *Staphylococcus aureus.* In the case of the baboon, warthog and elephant blood smears *Ralstonia picketti* dominated the plates. Isolation results were almost exclusively from BHI broth enrichment media that was serial diluted. This could indicate competitive inhibition as another possible cause for the low isolate yields. This also indicates the importance of knowing the (carcass) source and context of the sample.

The amplicons for the qPCR assay are less than 300 bp. The sensitivity of the probes coupled with the relatively small target makes this assay a viable option for blood smear diagnostics. The qPCR as a tool for diagnostics was comparable to the Giemsa results. False negatives were a result of poor DNA yields, while in the microscopic evaluation; it was due to visual uncertainty. The molecular assay demonstrates a worthwhile trait in the ability to identify the chromosomal and plasmid markers. Plasmid pX02 was missing for 3 out of the 9 isolates obtained from the smears. This is not unusual and has been described in the long term storage of isolates by (Marston et al., 2005) and (Koehler, 2009). Although the mechanism of plasmid loss is poorly understood, it is believed to be due to DNA damage or a nutrient deficit over time (Marston et al., 2005, Lekota et al., 2015).

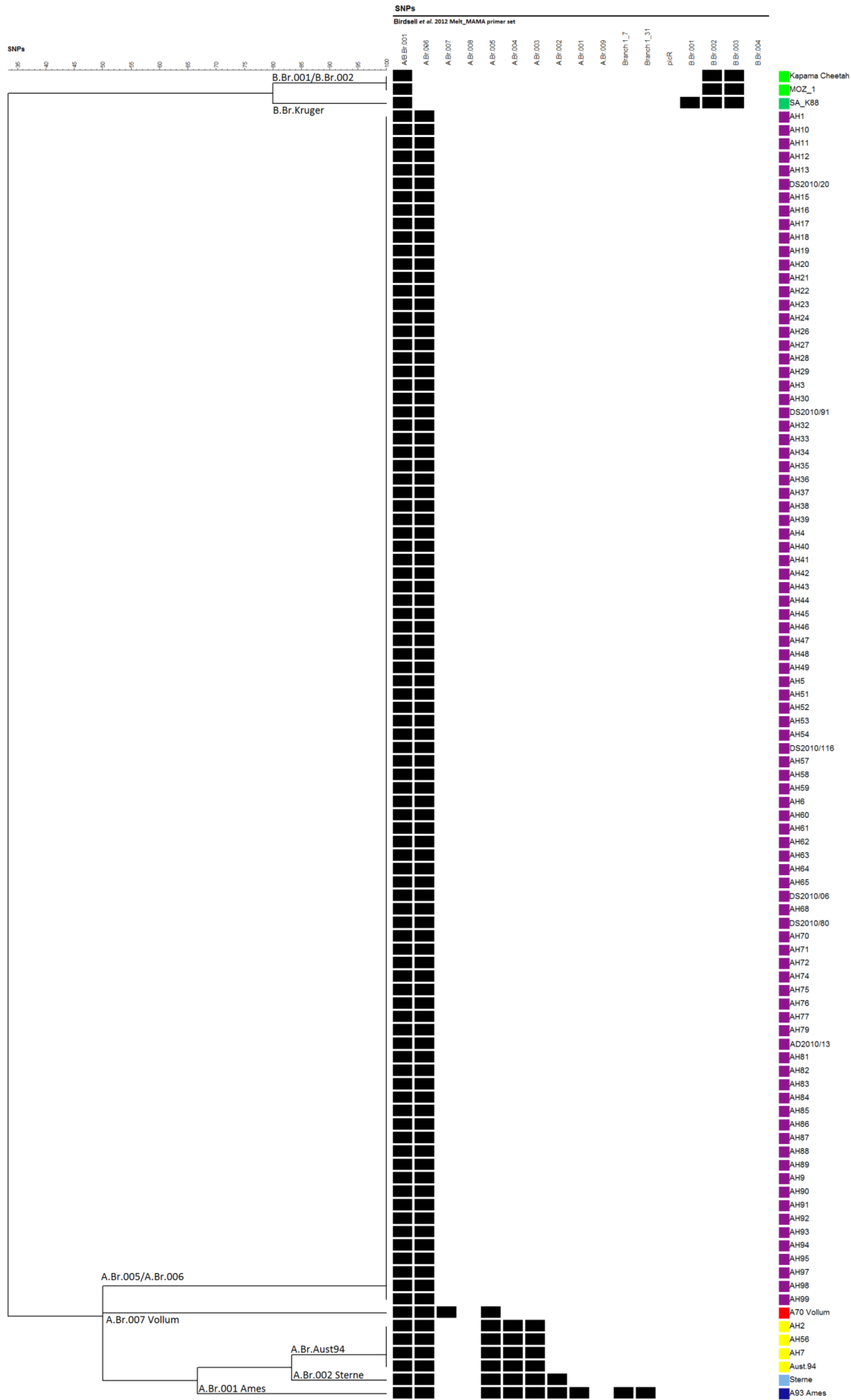

Figure 4.5: A Single Nucleotide Polymorphism cladogram generated in BioNumerics (Applied Math) v.7.5 from binary data based on Melt-MAMA for *Bacillus anthracis* obtained from blood smears collected in Kruger National Park, South Africa (B clade Moz_1 and SA_K88 from Van Ert et al. 2007 were included as *in silico* controls)

For the 10% smears that produced bacterial isolates, proper downstream characterization is possible. This is important when viewing the melt-MAMA SNP analysis as the assay is limiting in the information it provides at present. The melt-MAMA SNP analysis of the 99 smears grouped the isolates into 2 clades. The A.Br005/A.Br.006 has a global distribution (Van Ert et al., 2007a, Van Ert et al., 2007b, Birdsell et al., 2012) and is the dominant clade in KNP in recent years. The smaller clade, made up of 4 isolates, belongs to the Aust 94 clade which has also been reported in Georgia, China, India and Turkey (Van Ert et al., 2007a, Khmaladze et al., 2014). These isolates were from impala in enzootic Pafuri, of which was not well represented in the molecular subset data, as discussed earlier. MLVA would provide more resolution than is currently achieved with the SNP assays, but due to the limited amount of DNA obtained this assay is not possible. The SNP assay did demonstrate the continued prevailing of the A clade in KNP. This has been described by Smith et al (2000) where the A clade has been dominating since the 1990's while in previous decades there had been an equal quantity of the A clade and B clade cases distributed in different areas. This distribution of *B. anthracis* in Pafuri was also described by Smith et al. (2000) in relation to soil adaptability for the long term survival of spores which includes mainly A clade isolates. This trend continues to the present.

In this study, only the data from one year of the surveillance system was evaluated to determine its value as a resource. It also helps us see what insights can be gained in the disease from focusing on a single outbreak year. From that perspective, the surveillance system as a whole is a rich source of data in describing the factors and animals affected by the outbreak and their distribution in KNP.

Susceptibility of individual animals to anthrax depends on the host species, the strain, age and route to infection (Welkos et al., 1986, Lyons et al., 2004). During major outbreaks in the KNP, it has been shown that a greater proportion of older animals are affected in comparison to younger animals. This disease therefore acts as a natural mechanism of culling (Bengis, 2000, Hugh-Jones and de Vos, 2002). Previous studies have reported browsers like kudus being the most anthrax affected species in KNP with records of over 50% of the kudu population affected in past decades (De Vos et al., 1973) compared to the Etosha National Park in Namibia where zebras are predominantly affected (Lindeque and

Turnbull, 1994, Beyer et al., 2012). This appears to be due to the feeding height of 1-3 m of kudus coinciding with the height where blowflies usually deposit their anthrax contaminated regurgitate or faecal droplets (Turnbull, 2008). In KNP, blowflies (*Chrysomya albiceps* and *C. marginalis*) have been observed in large numbers to contaminate browse after feeding on anthrax infected carcasses (Braack and De Vos, 1990, Hugh-Jones and de Vos, 2002, Blackburn et al., 2014). In the KNP the disease exhibits two patterns of epidemiology: firstly; water contamination where proposed vectors, like scavengers, contaminate water after feeding on an infected carcass and secondly; vegetation contaminated by vectors such as blowflies and the dung of scavengers that have fed on an infected carcass (Bengis and Erasmus, 1988, Lindeque and Turnbull, 1994, Bengis et al., 2003). Hugh-Jones and De Vos (2002) indicated that anthrax outbreaks usually occur periodically during dry seasons in KNP when water is scarce, leading to animals sharing the remaining water points.

In enzootic Pafuri, impala were mainly infected by anthrax during the 2010 outbreak. It could be hypothesised that the drought resulted in nutritional stress on the animals as the grasslands died off. With the subsequent rains, the top soil was washed into the pans and waterholes where animals would congregate. Massive erosion was noted in reports during 2010. In the past, anthrax has been considered a disease of the dry season and also considered to be due to overstocking of animals in an area (Viljoen et al., 1928, Lindeque and Turnbull, 1994, Hugh-Jones and de Vos, 2002). This stands to reason since the first impala cases were observed during late summer in 2009 during the drought. The animals would have to graze close to the ground and competition for graze would create overstocking in areas as the water and grasslands dwindled. The rainfall does however seem to play a significant role in the spread of the disease as evidenced by the other animal species observed clustered around the water holes. The work of Steenkamp et al. (2013) adds credence to the role of water as a driver to infection and outbreaks of anthrax in KNP. According to the suitability model, outlined in that study, waterholes and river systems were identified in terms of risk assessment for the disease during outbreak periods (see Figure 6). The carcass distribution foci in 2010 follow exactly this pattern (Figure 4.1).

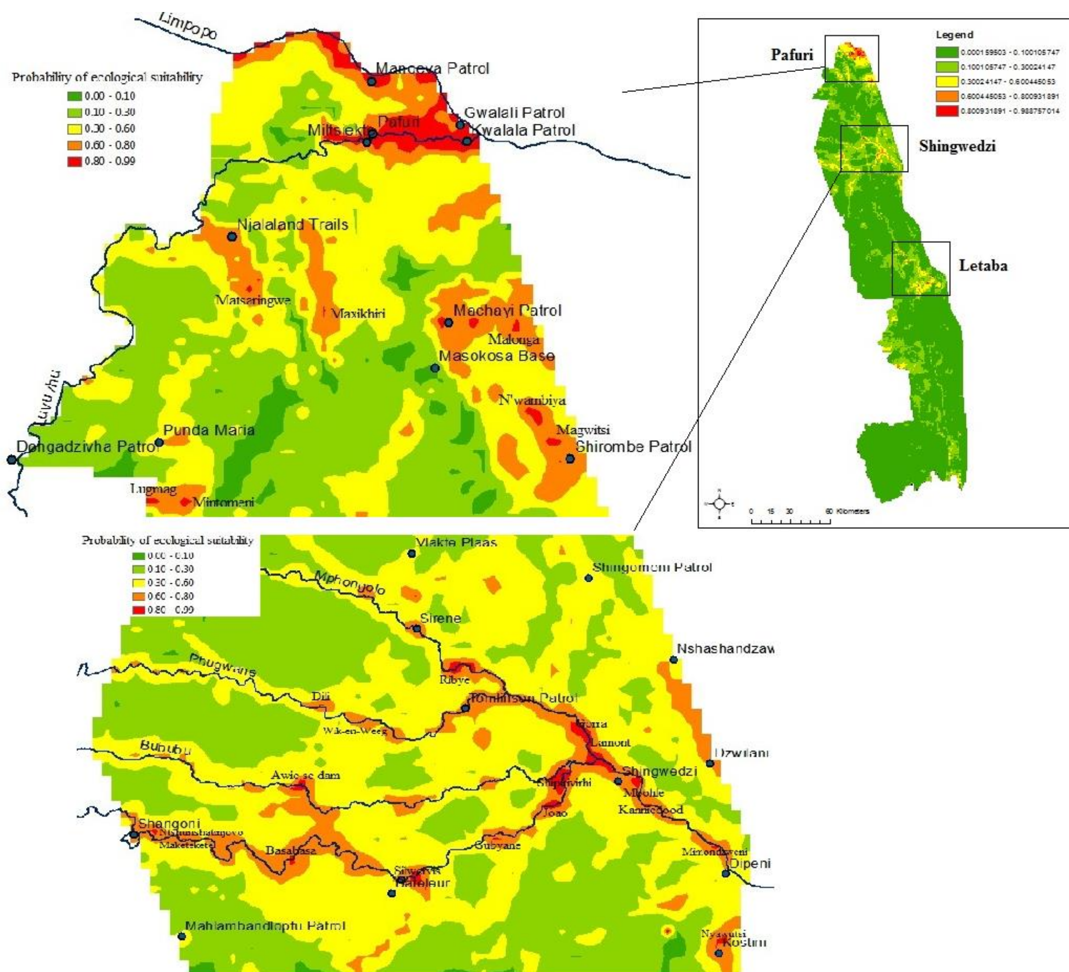

Figure 4.6: As outlined by Steenkamp (2013) the predicted ecological suitability for *Bacillus anthracis*. Red areas (greater than 80% probability) indicate areas of increased ecological suitability and black rectangles indicate the three most suitable areas for anthrax spore survival in Kruger National Park, one of which is Pafuri.

The report stating the unusual lack of blowfly activity in Pafuri was also of interest. Browsers that succumbed to the disease were only found around the dwindling waterholes and due to the low blowfly numbers, the 'multiplier hypothesis' (Van Ness, 1971, Blackburn et al., 2014) was not in effect. Water is the only other reasonable disseminator of the disease. Kudu deaths were highest in areas with high blowfly activity as witnessed in Shingwedzi and Shangoni. This is in keeping with observations made in KNP over the last few decades (Pienaar, 1967, Braack and De Vos, 1990, Smith et al., 2000, Hugh-Jones and de Vos, 2002).

4.5. Conclusion

Disease surveillance systems and databases such as those used in KNP are a veritable goldmine of information in the understanding of a disease and providing context to an outbreak. Once again, there is anecdotal evidence in the role of vectors such as vultures and blowflies in the spread of anthrax, provided that a very specific set of conditions are in effect.

Bacteriologic isolations from blood smears produce inconsistent success rates after 2 years of storage. Although isolates can be established from blood smears up to 6 years after storage, isolation success rates are inversely related to the storage time. Even with such low isolation numbers, however, it is possible to obtain at least a fraction of isolates from archival blood smear collections which could be considered worthwhile for strain typing, whole genome sequencing or other downstream applications.

The amplicons for the qPCR assay are less than 300 bp. The sensitivity of the probes coupled with the relatively small target makes this assay a viable option for blood smear diagnostics. Overall the diagnostic qPCR is an invaluable tool in the diagnostics of *B. anthracis* from archival blood smears, but subject to smear thickness and DNA quality.

The Melt-MAMA assay is a bit limiting in its resolution with the currently published panels and available controls, but the identification and development of more region specific SNP markers could make it a powerful tool in the characterisations of archival blood smears for retrospective epidemiological analyses.

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5. Chapter 5

A distribution snapshot of anthrax in South Africa: multiple locus variable number of tandem repeats analyses of *Bacillus anthracis* isolates from epizootics spanning 4 decades across southern Africa.

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Abstract

Anthrax caused by *Bacillus anthracis* has received a great deal of attention in recent years due to its association with biological warfare. For this reason there has been concerted research in typing strains using multiple locus variable number tandem repeats (VNTRs) analyses (MLVA) for the rapid and reliable differentiation of anthrax strains. In this study, 319 strains from southern Africa from archival collections were analysed using the MLVA for 31 VNTR. The 319 *B. anthracis* strain cluster analysis generated from unweighted pair group method using arithmetic averages (UPGMA) produced 2 clades. The B-clade with the rare KrugerB lineage has not been isolated since the early 1990's. The Aα-clade dominates the southern African landscape in the last 2 decades. MLVA is sufficient to type *B. anthracis* strains for the purpose of epidemiological study and is the most cost effective and appropriate technique for the average African / developing country laboratory.

5.1. Introduction

Anthrax has received considerable of attention in recent years, most commonly due to its association with biological warfare (Hoffmaster et al., 2008). Due to this negative view of *Bacillus anthracis*, the causal agent of anthrax, there has been a concerted drive in the research for the development of fast and reliable DNA fingerprinting methods to trace intentionally released strains back to their origin (Henderson et al., 1994, Keim et al., 2000, Keim et al., 2004, Van Ert et al., 2007a). MLVA is one such method and was in fact the technique of choice because of its reproducibility and feasibility. It is a PCR-based technique with few steps and adaptable for automation that relies on the polymorphism of tandem repeated DNA sequences (VNTR) that is known as micro- or minisattelites for discriminations among strains (Keim et al., 2000). MLVA uses PCR to amplify VNTR loci that differ in length due to variation in the number of times that a VNTR sequence repeats. The PCR fragment size is determined using capillary electrophoresis and the copy numbers of the VNTR unit is calculated by subtracting the size of the flanking regions from the amplicon size (Vergnaud and Pourcel, 2009). MLVA is therefore used to differentiate anthrax strains epidemiologically and thus to evaluate the diversity of *B. anthracis* from different geographical areas (Keim et al., 2000, Keim et al., 2004; Van Belkum. 2007, Van Ert et al., 2007a).

At present South Africa is considered to have sporadic anthrax endemicity (Turnbull, 2008). In South Africa, the 2 endemic regions are the Kruger National Park (KNP) and the Ghaap Plateau region of the Northern Cape Province (NCP) (Viljoen et al., 1928, Pienaar, 1960, Hugh-Jones and de Vos, 2002). South Africa shares its borders with neighbours with varying degrees of anthrax endemicity such as; hyperendemic-Zimbabwe, sporadic-Namibia, sporadic-Botswana, endemic-Mozambique as well as sporadic-Lesotho contained within South Africa (http://www.vetmed.lsu.edu/whocc/mp_world.htm). Transfrontier Conservation Areas (TCA's) are border regions where there is overlapping ecological, wildlife and

resource areas with the aforementioned neighbours of South Africa. These TCA's allow extended range to wildlife and unrestricted movement of animals within these parks (Hanks, 2003).

MLVA is thus a viable technique in evaluating strain diversity and distribution in South(ern) Africa. A study by Smith et al. (2000) demonstrated the diversity of strains in Kruger National Park using the MLVA 8 panel. Two lineages were delineated by the MLVA 8 fingerprinting. The broad A-clade lineage, which has a global distribution, and the rarer KrugerB lineage confined to the north of KNP due to specific environmental cues (Smith et al., 2000). In this study we used the 31 VNTR markers to genotype 319 isolates belonging to historical and current culture collections from South Africa to determine the diversity and distribution of strains within the country.

5.2. Materials and Methods

5.2.1. Isolates

Isolates spanning outbreaks from 1970 to 2013 were used in this study. The total (n=319) isolates are the cumulative culture collection of the National Institute for Communicable Diseases historical isolate library (isolate repository), Onderstepoort Veterinary Institute culture collection (reference laboratory) and University of Pretoria (wildlife diagnostics: Chapters 2 - 4) isolates from anthrax outbreaks in South Africa (Supplementary Data\Supplementary Data 5.1.xlsx). This includes isolates from (n=9) Zambia, (n=3) Botswana and (n=2) Richtersveld Transfrontier Park- bordering Namibia.

5.2.2. Multi-locus variable number of tandem repeat analyses (MLVA)

The DNA from single colonies of *B. anthracis* were isolated with a QIAamp Blood Mini Kit™ (Qiagen®) using 20mg/mL lysozyme L6876 (Sigma Aldrich) for the Gram positive bacteria protocol. DNA was subjected to PCR, targeting 31 VNTR markers

(Table 5.1) with fluorescently labelled forward primers, multiplexed in 7 reactions as described by Beyer et al. (2012). Multiplex PCRs were prepared in 15 µl volumes, consisting of 1x MyTaq HS Mix (Bioline), 0.4 – 1 mM primers and 2 µl of template DNA. The PCR conditions for multiplexes 1 to 4 and 7 were as follows: initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 20 sec; annealing at 60°C for 30 sec; elongation at 72°C for 2 min; followed by a final elongation step at 72°C for 5 min. The PCR conditions for multiplexes 5 and 6 were as follows: initial denaturation at 95°C for 2 min; 30 cycles of denaturation at 95°C for 20 sec; annealing at 50°C for 30 sec; elongation at 72°C for 2 min; followed by a final elongation step at 72°C for 5 min. Amplicons were separated using an ABI 3500xl Genetic Analyser © (Applied Biosystems), with a G5 filter set, and 51 cm POP 7 capillary.

The size of the resultant amplicon fragments were determined using GeneMapper ID-X© software (Applied Biosystems), with the internal LIZ1200© size standard (Applied Biosystems). Fragment size normalization was performed by including *B. anthracis* strains Sterne 34F2 vaccine strain, Vollum (A70) and Ames (A93) as positive controls

Base-pair sizes for each allele were converted into copy numbers using a previously described algorithm (Lista et al., 2006, Beyer et al., 2012), with the copy code convention proposed by Thierry et al. (2014) as seen in Table 5.1. The datasets of archival isolates from 1975 to 2000 were validated by inter-laboratory comparisons between the Department of Veterinary Tropical Diseases, University of Pretoria and the Special Pathogens Unit, National Institute for Communicable Diseases; by independent genotyping of the isolate library. Inter-laboratory comparison data for amplicon size calling is represented in (Supplementary Data\Supplementary Data 5.2.xlsx).

Genetic distance and cluster analysis was performed using unweighted pair group method using arithmetic mean (UPGMA) and minimum spanning analysis was

performed using categorical data in BioNumerics© version 6.6 (Applied Mathematics).

Table 5.1: Ames 31 VNTR amplicon fingerprints used in the translation of copy numbers as proposed by Thierry et al. (2014).

VNTR Marker and repeat unit size	Copy code (Thierry et al., 2014)	Ames MLVA31 amplicon fingerprint (University of Pretoria)	Ames MLVA31 amplicon fingerprint (Thierry et al., 2014)
vrrA_12bp	4	305.74	314
vrrB1_9bp	20	222.15	229
vrrB2_9bp	13	146.19	153
vrrC1_9bp	53	589.66	580
vrrC2_18bp	17	521.94	532
CG3_5bp	2	152.28	158
pX01-aat_3bp	7	127.98	126
pX02-at_2bp	9	135.02	141
bams01_21bp	16	485.73	485
bams03_15bp	26	543.89	549
bams05_39bp	5	298.1	307
bams13_9bp	70	806.89	814
bams15_9bp	24	414.29	418
bams21_45bp	10	632.87	676
bams22_36bp	16	714.29	735
bams23_42bp	11	632.87	651
bams24_42bp	11	591.64	595
bams25_15bp	13	392.21	391
bams28_24bp	14	491.68	493
bams30_9bp	57	723.47	727
bams31_9bp	64	761.56	772
bams34_39bp	11	502.26	503
bams44_39bp	8	422.33	417
bams51_45bp	9	490.56	493
bams53_12bp	8	232.09	236

vntr12_2bp	6	108.36	115
vntr16_8bp	20	266.82	273
vntr17_8bp	4	378.95	386
vntr19_3bp	4	90.47	96
vntr23_12bp	4	190.64	197
vntr35_6bp	5	110.42	115

5.3. Results

The UPGMA analysis of the *B. anthracis* fingerprints indicates that the delineation as described by Smith et al. (2000) holds true until present day (Figure 5.1). The isolates from 1970 – 1990 from the north of KNP predominantly cluster in the B-clade. The only other B-clade isolate outside of this time period is KC2011 from Kapama, but it groups on its own, belonging to the B.br001/002 lineage (see chapter 4). The remaining isolates clustered in the Aα clade as outlined by Pilo and Frey (2011). These include the 3 *B. anthracis* controls Ames (A3-clade), Sterne (A3-clade) and Vollum (A4-clade). Of the 319 isolates fingerprinted on 31 VNTR loci: 11 isolates were missing the plasmid pX02 related loci pX02, VNTR16 and VNTR17 (n=4 from 2010 KNP blood smear isolations and n=7 from the OVI NCP archival collections). It should be noted that these missing loci create a genotypic bias in the dendogram for these isolates (Figure 5.1; for better resolution use Supplementary Data\SuppFig5.1.png)

MST cluster analyses are arranged according to colour to better visualize trends temporally versus regionally (Figure 5.2). The larger the node, the more clonal isolates included in it. This can be seen in Figure 5.2A where there is a clear predominance of a genotype during different outbreak years.

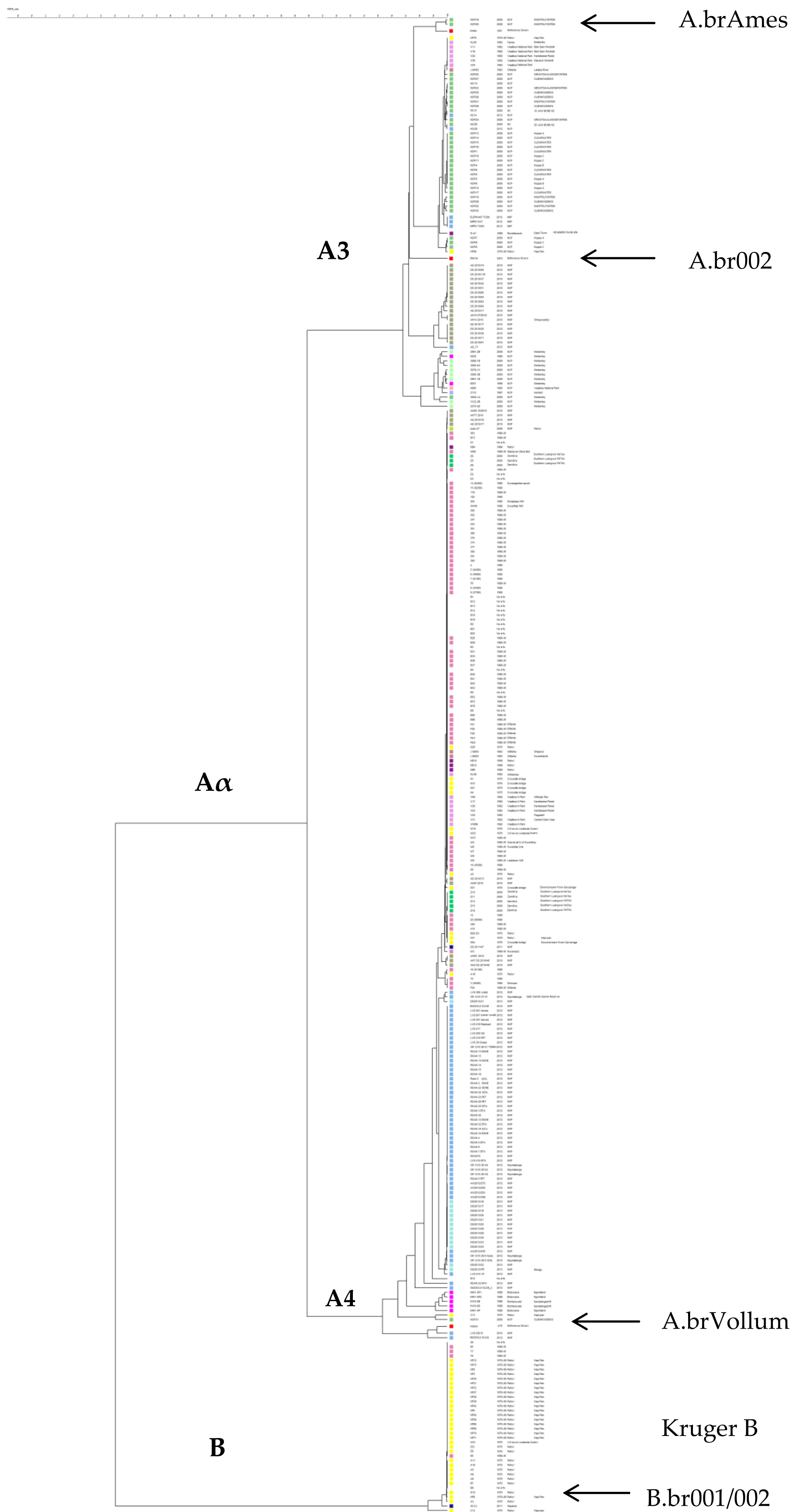

Figure 5.1: UPGMA dendogram based on 31 VNTR loci of *Bacillus anthracis* for 319 isolates from epizootics (1970 - 2013) across South Africa. The dendogram was generated in BioNumerics version 6.6. Colours indicate year of anthrax outbreak see figure 2A.

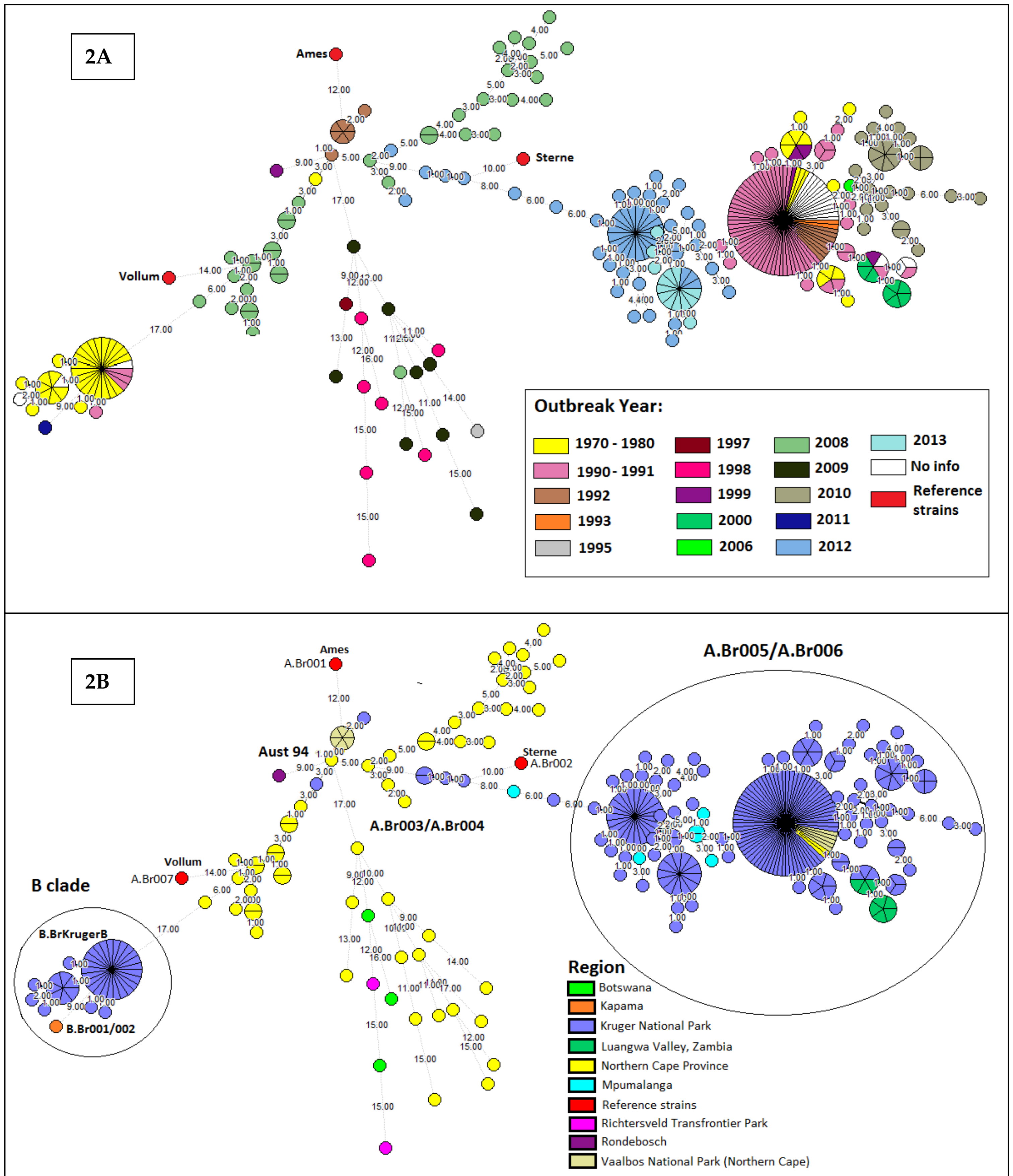

Figure 5.2: Minimum spanning trees based on categorical coefficient data of 31 VNTR loci for *Bacillus anthracis* MLVA fingerprints generated in BioNumerics (version 6.6). The MST tree is arranged visually according to 2A: the year of the anthrax outbreak, designated by colour. 2B: the region of the anthrax outbreak, designated by colour and indicating SNP lineages.

Between 1970 and 1980; the B-clade isolates dominated, however, the genotypic occurrence tapered off by the end of 1991. Between 1990 and 1993, the A-clade isolates which were only marginally represented in the 1970's and 1980's, began to dominate the outbreaks. The largest node belongs to a single/clonal genotype with clear overlap between the outbreak years of 1970 to 1999. All the outbreaks in years thereafter tend toward smaller clonal genotypes restricted temporally, except for 1998, 2008 and 2009 which demonstrate extensive diversity in genotypes. The difference between the dominant genotype in 2012 (light-blue) and the dominant genotype in 2013 (sky-blue) is 3 loci Bams15, VrrC1 and VrrA.

In Figure 5.2B it becomes clear that the larger clonal genotypic groups are within the National Parks, specifically KNP (seen in sky-blue). The isolates from Luangwa Valley in Zambia (leaf-green) group in 2 clonal genotypes, 1 of which clusters with KNP while the other is unique to Zambia differing only at the Bams44 locus. The KNP isolates are from 1990 and 1999 whereas the Zambian isolates are from the year 2000 (Siamudaala, 2005). The Vaalbos National Park (which no longer exists) had 2 dominant genotypes in 1992, 1 was unique to the NCP, while the other grouped with the dominant KNP genotype during that period. There is a diversity of genotypes in Mpumalanga, NCP, Rondebosch, Botswana and Richtersveld Transfrontier Park from this dataset. This diversity has been described earlier as the distributions from 1998, 2008 and 2009. The broad SNP lineages indicated in the figure are from whole genome sequencing analyses (Kgaugelo Edward Lekota: unpublished data).

5.4. Discussion

The 31 VNTR MLVA provides a detail rich snapshot of the spatial and temporal distribution trends of strains in South Africa. The results of the MLVA cluster analysis for KNP, which forms the largest portion of the dataset, is still in keeping with the findings of Smith et al. (2000). The KrugerB genotype has not re-emerged

since the early 1990's. The only B-clade isolate in recent years has been a single isolated wildlife case from Kapama and is nevertheless not of the KrugerB lineage.

The larger clonal genotypes tend to occur in the north of KNP which is endemic for anthrax. In endemic Pafuri, anthrax cases occur year round. Large outbreaks are considered to be when anthrax cases occur in "non-endemic" areas. These include ranger sections down south to the middle of the KNP (see chapter 4). In the past, anthrax outbreaks were common in the dry seasons with major outbreaks flaring every decade cyclically (De Vos and Turnbull, 2004). Since the advent of the new century, anthrax outbreaks are more commonplace following the rainy season, especially after flooding (Steenkamp, 2013). The change in these ecological/environmental cues could be responsible for this dominance of the A-clade isolates in recent decades, although this is merely speculative.

The difference in isolate diversity between NCP and KNP can be attributed to the restriction of animal movement and natural resources on the farms in NCP (see chapter 2). Each farm serves as its own outbreak site, perpetuating strains within the confines of the farm's borders. This echoes the findings of Beyer et al. (2012) where the greatest genotypic diversity was found outside Etosha National Park. A similar diversity is observed in the conservation areas bordering KNP in Mpumalanga (Figure 5.2B: turquoise) where animal movement is also restricted. The isolates belonging to the A.br003/004 lineage are highly diverse (Figure 5.2B). They form 3 distinct clusters made up of 1998, 2008 and 2009 isolates (Figure 5.2A). The 2008 cluster belongs to the Ghaap region. The 1998 and 2009 cluster varies between 9 and 17 VNTR loci. This genotypic disparity is remonstrative of the vast spatial distances between the areas of origin of these isolates Richtersveld TCA, Botswana and NCP (Figure 5.3).

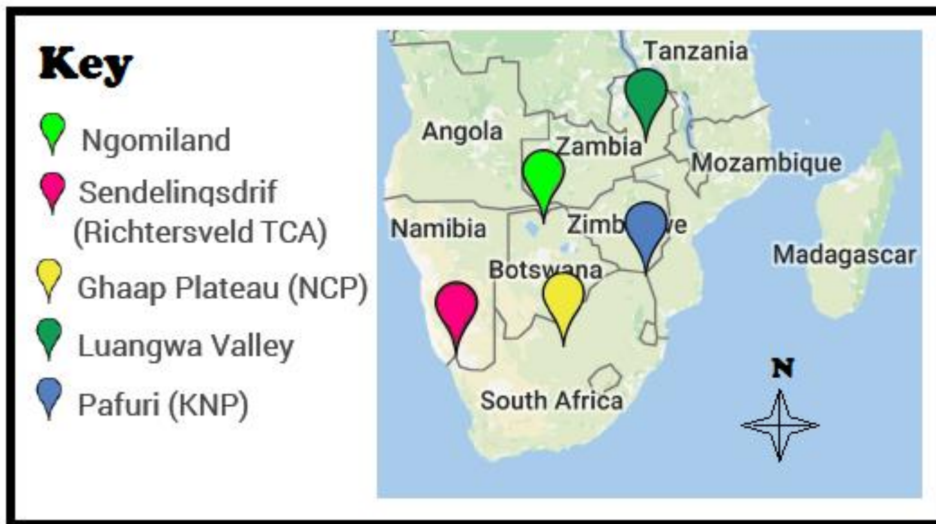

Figure 5.3: A simple map of southern Africa depicting *B. anthracis* isolate origins

The 4 isolate missing plasmid pX02 from KNP were isolated off old blood smears (chapter 4). Isolates from anthrax cases in the NCP are frequently found to be missing plasmid pX02 during routine diagnostics (Lekota et al., 2015). This phenomenon has been known to occur during storage (Marston et al., 2005) as is the case with NCP/05, 07 and 09, however, it was also observed from isolates freshly purified from diagnostic samples as was the case with 3275-2D and 3008-1A. This finding seems to be common amongst *B. anthracis* isolates obtained from NCP and could possibly be due to environmental factors related to the high calcium content of the dolomitic environment of the Ghaap Plateau which causes porosity of the bacterium and plasmid curing (Edger et al., 1981). The pX02 plasmid encodes the region responsible for encapsulation and is also needed for virulence (Leppla, 1982), therefore plasmid curing must occur after infection and fatality of an animal. This results in a genotypic bias during MLVA typing due to the 3 missing VNTR loci associated with pX02.

VNTR loci are considered to have high mutation rates, which is what makes MLVA a viable method for the differentiation of monomorphic bacteria (Hyytia-Trees et al.,

2010). The technique is also considered to have a correspondingly high homoplasy and homoplasticity. This is a drawback in assessing a definitive overview of the population structure and evolution of bacteria like *B. anthracis* (Le Fleche et al., 2001, Achtman, 2008). Despite this, MLVA typing enabled us to differentiate multiple genotypes from the same carcass as seen with Roan 5 and Roan 23 from the 2012 anthrax outbreak in Mooiplaas, KNP.

5.5. Conclusions

MLVA is a valuable yet feasible technique in the differentiation of *B. anthracis.* The UPGMA and MST analyses give us a detail rich view of anthrax in South Africa over the last 4 decades. The KrugerB lineage appears to have disappeared since the 1990's. The Aα-clade isolates now dominate the southern African landscape.

The National Parks seem to support larger, more clonal anthrax outbreaks compared to farm areas where animal movement is restricted and resources are under management. There are too many host, bacterium and ecological factors influencing anthrax outbreaks to take cognisance of in the attempt to understand this disease. Due to environmental influences on genotypic diversity and bacterial evolution, the MLVA data can form part of multivariate analyses in determining the distribution of *B. anthracis.* When combined with whole genome sequencing SNP data and prediction models such as Steenkamp (2013), this data can give us greater insight into the dissemination of the disease and the cues that culminate in an outbreak.

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Chapter 6

Isolation and Whole Genome Analysis of a Lytic Bacteriophage Infected *Bacillus anthracis* Isolate from Pafuri, South Africa.

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Abstract

Bacillus anthracis is a soil borne, Gram positive endospore forming bacteria and the causative agent of anthrax. It is endemic in Pafuri, Kruger National Park in South Africa. The bacterium is amplified in a wild ungulate host which then becomes a source of infection to the next host upon its death. While outbreaks have been documented in the area for over 30 years, the exact mechanisms involving the onset (index case) and termination of an outbreak are poorly understood due, in part, to a paucity of information about the soil based component of the bacterium's lifecycle. In this study we present an aspect of this in the form of a unique isolation of an environmental dsDNA *Myoviridae* bacteriophage from a wildebeest carcass suspected of having succumbed to anthrax. The 154,012 bp aggresively lytic bacteriophage hampered the isolation of *B. anthracis* from samples collected at the carcass site. Whole genome sequencing was employed to determine the relationship between the bacteria isolated on site and the bacteriophage dubbed Phage Crookii. The bacterium contained the usual 4 *B. anthracis* prophages described previously, but did not contain any functional temperate phages, although remnants of another bacteriophage was collected from the unamapped reads during sequence analysis. This indicates possible multiple phage infection events of the bacterial strain over time. The isolates also did not demonstrate a trend toward developing phage resistance thus making the replicating bacterium continually available to lysis by phage Crookii. The unusual isolation of this bacteriophage demonstrates the phage's role in decreasing the inoculum in the environment and impact on the life cycle of *B. anthracis* at a carcass site.

6.1. Introduction

Bacillus anthracis is a soil borne, Gram positive endospore forming bacteria and the causative agent of the disease known as anthrax (Sterne, 1937a, Turnbull, 2008). Anthrax is endemic in Kruger National Park, South Africa where outbreaks are cyclical among wildlife (Bengis, 2000, De Vos and Turnbull, 2004). Anthrax is a zoonosis, but predominantly affects wild ungulates in the KNP (De Vos et al., 1973, Odendaal et al., 1991a). *Bacillus anthracis* has a monomorphic genome which is closely related to other members of the *B. cereus sensu lato* group, specifically *B. cereus* and *B. thuringiensis.* Most of the genetic variation of these organisms occurs on their respective plasmids and pathogenicity islands which code for the toxins (Hoffmaster et al., 2004, Klee et al., 2010, Papazisi et al., 2011). The plasmids of *B. anthracis* are pX01, which codes for the lethal factor and oedema factor (Leppla, 1982, Park and Leppla, 2000), and pX02 which codes for encapsulation (Green et al., 1985). There are also four putative lamboid prophages that set *B. anthracis* apart from its close relatives (Sozhamannan et al., 2006).

While the transmission, dissemination and conditions of persistence for *B. anthracis* during anthrax outbreaks has been well described over the last few decades (Pienaar, 1960, Smith et al., 2000, Bengis et al., 2003, Turnbull, 2008), little is known about the bacterium's activity in the soil during outbreak dormancy (Saile and Koehler, 2006, Schuch and Fischetti, 2009). It is believed that the bacteria are capable of replicating within biofilms in the soil and rhizospheres of plants (Saile and Koehler, 2006, Lee et al., 2007). Schuch and Fischetti (2009) proposed an alternate cycle of the bacterium in the biofilms of the earthworm guts, mediated by bacteriophages and serving as replication sites.

Bacteriophages are viruses that infect bacteria. These viruses belong to the order *Caudovirales* with members of the *Siphoviridae, Podoviridae, Myoviridae*, and *Tectiviridae* families (Ackermann et al., 1978, Ackermann et al., 1995a, Ackermann, 2011). They have 2 pathways to replication within the bacterium; lysogeny and lysis. Lysogeny involves viral integration into the host genome, while lysis activates the host machinery to propagate its progeny. Ultimately both pathways lead to bacterial lysis (Cowles, 1931a, Bertani, 1951, Ackermann, 2011). The difference between the two phage types is marked by the phage encoded influences on the bacterium. Lysogenic bacteriophages have been theorized to impact

bacterial lifecycles through fitness traits such as antibiotic resistance, replication efficiency and sporulation rates of bacteria (Schuch and Fischetti, 2009, Hargreaves et al., 2014). Bacteriophages have been useful tools for decades as a medium for diagnostics in microbiology, vectors in molecular biology, as well as phage therapy applications (Adams, 1959, Letarov and Kulikov, 2009). The *B. anthracis* diagnostic γ-phage is but one such example (Cowles, 1931a, Fouts et al., 2006). The more popular focus for study in recent years has been in the evaluation of lytic bacteriophage endolysins as an alternative means of disinfection as well as therapy mediums in an age of dwindling antibiotic options (Sulakvelidze et al., 2004, Letarov and Kulikov, 2009, Walmagh et al., 2012). In this study, we highlight the role of a lytic bacteriophage in outbreak dynamics, as naturally occurring pathogen disinfectant agents, using the example of a wildebeest carcass site during an anthrax outbreak.

6.2. Materials and methods

The carcass of a juvenile wildebeest (carcass # DS2015/79) was discovered on the 24th of February 2015 in Crooks Corner, Pafuri, Kruger National Park, South Africa. Approximately 75% of the carcass had already been consumed by vultures. Blood smears, a swab and soil samples were collected from the carcass site for diagnostics at the Skukuza State Research Laboratory as part of the Skukuza State Veterinary Services Disease Surveillance System.

6.2.1. Isolation

The blood smear was Giemsa stained (DeMay, 1999, Turnbull, 2008) and evaluated microscopically. The smear showed low level evidence of spores and the possible remnants of encapsulated square ended *B. anthracis* vegetative cells. Since an anthrax outbreak was underway in the area at the time, the swab and soil was submitted for bacteriologic diagnostics of anthrax using selective and non-selective media. The swab was (i) directly streaked onto 5% impala blood agar (BA) and Polymyxin EDTA-Thallous acetate agar (PLET; excluding lysozyme) and then (ii) heat treated in a 100 µL phosphate buffered saline (PBS) at 65 °C for 30 minutes, thereafter it was similarly streaked onto the blood and PET agars and incubated at 37 °C overnight. A gram of soil was added to 9 mL PBS; shaken for 3

hours; heat treated at 65 °C for 30 minutes; serial diluted (to $1x10^{-8}$) and then similarly plated out onto PET and BA media in triplicate for each dilution (Turnbull, 2008).

After the incubation, only the heat treated BA plate produced 2 colonies (< 5 mm in diameter) while the 2 PET agar plates and 1 BA plate from the direct streaking still appeared sterile. The colonies were sub-cultured and demonstrated sensitivity to 10 µg penicillin discs (Oxoid) and diagnostic γ-phage. The single colonies were sub-cultured 5 more times thereafter until an opaque grey-white colony morphology was obtained (without any observed turbidity between 5 - 8 hours post incubation) and then stored in glycerol.

The 2 PET and 1 BA plate (with sterile appearance) were then each flushed with 500 µL PBS and filtered through a 0.2 µm cellulose acetate membrane (Lasec South Africa). A BA plate was divided into 2 with Sterne $34F_2$ plated onto the one half and *B. anthracis* DS2015/79 plated out onto the other. Ten microlitres of the filtrate was dropped into the centre of each half of the lawn area and the plate tilted in the dilution streak method (Adams, 1959) then incubated at 37 °C overnight.

The filtrate, dubbed "Crookii", was evaluated for plaque titres, in plaque forming units (pfu), using the double layer agar method without the use of antibiotics (Adams, 1959, Kropinski et al., 2009) with DS2015/79 as the host and a 10 fold dilution series of the filtrate with 5 replicates for each dilution.

6.2.2. The Effect of Viral Propagation on DS2015/79

To determine the effect of the bacteriophage Crookii on *B. anthracis* under different conditions (simulating carcass nutrient conditions); 1 mL of cattle blood was inoculated with 500 spores (1 set with Sterne and another with DS2015/79) and 20 µL of Crookii at $5x10^8$ pfu/mL were incubated under standard atmospheric conditions or with 8% Carbon dioxide at 37 °C overnight in a shaking incubator. Replicates included 0.8% w/v of ($NaHCO_3$) sodium bicarbonate. A third set of positive control blood tubes included Sterne and γ-phage at $5x10^8$ pfu/mL, under the same conditions, in parallel. Blood smears were made with 20 µL of blood at 8 hours, then 12 hours and at 24 hours. These smears were immediately fixed in methanol and stained with Romanowsky-Giemsa for 30 minutes (DeMay, 1999).

6.2.3. DNA Isolation

The DNA from *B. anthracis* DS2015/79 was isolated using High Pure Template Preparation Kit® (Roche) using the Gram positive bacterial protocol with 20 mg/mL Lysozyme L6876 (Sigma Aldrich). The viral DNA was isolated from propagated lysates through the phenol-chloroform isoamyl alcohol method from phage lysate at a concentration of $9x10^{11}$ pfu/mL (Wilson, 1987, Boom et al., 1990).

6.2.4. Shotgun sequencing

Shotgun libraries preparation of the *B. anthracis* and PHAGE_Crookii was performed using the Nextrera XT DNA Sample Prep Kit (Illumina, USA) by following the manufacturer's instructions. Tagmentation of the samples was performed using 1ng of the template, according to the manufacturer's instructions. Equal volumes of normalized library were diluted in hybridization buffer and heat denatured according to the Nextera XT protocol. Sequencing was performed on the Illumina MiSeq sequencing platform (Illumina) using the MiSeq Reagent Kit v3 (500 cycle).

6.2.5. Quality and Trimming

The quality sequence reads of the *B. anthracis* and PHAGE_Crookii were assessed using FASTQC software version 0:10.1 (Andrews, 2010). We obtained 3 670 390 reads of an average size of 220bp for DS2015/79 and 1 734 240 reads of an average size of 210bp reads for PHAGE_Crookii after trimming. Adaptors and ambiguous nucleotides sequences were trimmed using CLC Genomic Workbench 7.5.1. Trim settings included the removal of low quality sequence: limit 0.05, and ambiguous nucleotides: maximal 2 nucleotides allowed. Reads shorter than 50 nucleotide bases were removed.

6.2.6. De novo assembly and annotation

The *de novo* assemblies of the *B. anthracis* and PHAGE_Crookii sequence data was carried out using CLC Genomic workbench 7.5.1. *De novo* options included: minimum contig length, minimum size of assembled contig of 500bp and 1000bp respectively. *De novo* assembly of the trimmed reads was performed using the following parameters: similarity = 0.8; length fraction = 0.5; insertion cost = 3; deletion cost = 3; mismatch cost = 3; auto-detect

paired distance and perform scaffolds. The list of unmapped reads was collected for further analysis. The assembly contigs were extracted and further analyzed with BLASTn (Altschul et al., 1990) using *B. anthracis* Ames Ancestor (AE017334/5/6) (Ravel et al., 2009) as a reference genome.

The assembled contigs of *B. anthracis* DS201579 were annotated using Prokaryotic Genome Annotation Pipeline (PGAP) (Zhao et al., 2012) and Rapid Annotation using Subsystem Technology (RAST) (Aziz et al., 2008). The assembled contig of PHAGE_Crookii was also annotated using RAST annotation server for subsystems and functional annotation (Aziz et al., 2008).

6.2.7. Analysis of *de novo* assembly of unmapped reads

The collected list of unmapped reads generated from the initial *de novo* assemblies, were further assembled using CLC-Genomic workbench 7.5.1. The assembled contigs included the average minimum contig length, minimum size of assembled contig of 50bp and 500bp respectively. *De novo* assembly of the unmapped reads was performed using the following parameters: similarity = 0.8; length fraction = 0.5; insertion cost = 3; deletion cost = 3; mismatch cost = 3; auto-detect paired distance and perform scaffolds. Contigs generated from the assembly were further analyzed using BLASTn (Altschul et al., 1990).

6.2.8. Comparative sequence analyses

Whole genome alignments of *B. anthracis* and contiguation was performed using MAUVE tool (Darling et al., 2004) with *B. anthracis* Ames ancestor as a reference. Circular plots of the *B. anthracis* were generated using DNAPlotter (Carver et al., 2009). Prophage regions were predicted using PHAge Search Tool (PHAST) (Zhou et al., 2011) and confirmed using PHAGE_Finder. Circular plots of PHAGE_Crookii were generated using Geneious (Drummond et al., 2011). BLASTn was used to determine the closest sequence identity to PHAGE_Crookii (Altschul et al., 1990). Comparative sequence analysis of PHAGE_Crookii and phage W.Ph was achieved through genomic visualization employing CGview (Grant and Stothard, 2008).

6.2.9. Nucleotide sequence accession numbers

The genomes of *B. anthracis* DS2015/79 and PHAGE_Crookii have been deposited in GenBank under the accession numbers LVWF00000000 and KU847400 respectively.

6.3. Results

6.3.1. Isolation

The colonies isolated off the (heat treated swab) blood agar plate had a greyish-white, rough, domed morphology and were sensitive to penicillin and γ-phage. This confirmed the bacterial isolates to be *B. anthracis* (Brown and Cherry, 1955, Turnbull, 2008). When a whole plate of a single colony was streaked out, it took on a turbid appearance after 8 hours and thereafter developed large plaques (≥ 10 mm in diameter) in the lawn.

The soil sample failed to produce any *B. anthracis* isolates at any of the dilutions. The plates were also conspicuous for the absence of *B. cereus* colonies which are normally abundant in soil samples from Pafuri. There was however, a consistence of *B. subtilis* and Gram-negative cocci on all the plates with *B. mycoides* featuring on only the plates from the lower dilution series ($\leq 1x10^{-3}$). (*Bacillus* spp. were identified at DVTD bacteriology section, Faculty of Veterinary Science, UP).

The filtrate that was placed in the centre of the bacterial lawns demonstrated clear lytic zones at the deposit sites (Figure 6.1). The dilution streak at the edge of the plate demonstrated a smaller lysis zone for Sterne $34F_2$ than the DS2015/79 environmental strain (Figure 6.1). The DS2015/79 had also taken on a turbid appearance and the lysis zone began to spread a further 10 mm in diameter for the ensuing 4 hours. This indicated the presence of an infective agent such as a bacteriophage rather than the presence of an antimicrobial compound (which was also initially considered) in the filtrate. The viral filtrate concentration was determined to be $6.8x10^8$ pfu/mL by plaque enumeration. For this reason the *B. anthracis* isolate DS2015/79 was sub cultured until such time as single colonies remained white and opaque, after plating, without developing plaques after 6 hours at room temperature. This was to ensure that the isolates were purified of any latent bacteriophages.

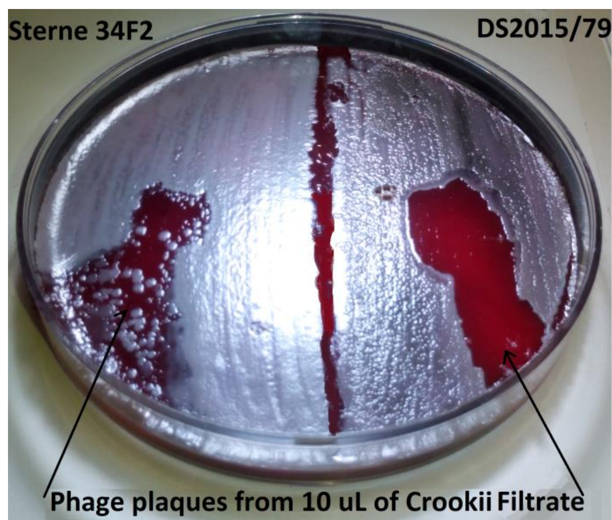

Figure 6.1: Blood agar plate with *Bacillus anthracis* bacterial lawns of Sterne (left) and pathogenic DS2015/79 (right) after 12 hour incubation at 37 °C. The plaques (indicated by the arrows) are where the bacteriophage Crookii has lysed bacterial cells. The phage demonstrates a greater affinity for DS2015/79 than Sterne; where the DS2015/79 lawn is also displaying turbidity indicating phage infection.

Phage Crookii counts were stable up to 50 °C for 15 min in buffer, however, there was no survivability after 60 °C for 15 min. This is why at least 2 spores which had escaped pre-infection could be obtained from the heat treated swab; which allowed the spores to germinate and replicate serial passage (sub culturing) of isolates cured the bacterium of the bacteriophage. The filtrate was also tested against bacterial lawns of *B. cereus, B. thuringiensis, B. subtilis* and *B.mycoides* in order to determine its specificity. The phage was only lytic against *B. anthracis, B. cereus* and *B. thuringiensis*.

6.3.2. The Effect of Viral Propagation on *B. anthracis* DS2015/79

In order to determine the activity and effect of phage Crookii on the bacterium at the carcass site, the virus was propagated under different conditions including sodium bicarbonate which is a natural trigger for encapsulation (Sirard et al., 1994, Koehler, 2009). The mean spore counts were tabulated from 100 microscopic fields of Romanowsky-Giemsa stained smears taken at 8 hours (to allow for spore germination and thereafter replication), 12hours and 24 hours (Table 6.1, Supplementary Data\Supplementary Data 6.1.docx).

The blood smear analyses is aimed at determining if sporulation is triggered as a defence mechanism against phage infection (Høyland-Kroghsbo et al., 2013, Hargreaves et al., 2014) or in response to nutrient availability (Koehler, 2009). A graphical representation of the different conditions can be seen in Figure 6.2 where typical microscopic fields were visualized for cell enumeration.

Table 6.1: To determine the Phage Crookii effects on *Bacillus anthracis* in decomposing blood, Romanowsky-Giemsa stained smears were visually appraised microscopically at three time points. The bacterial counts (vegetative cells versus endospores) were enumerated under different conditions (standard incubation, carbon dioxide incubation, sodium bicarbonate content and bacteriophage type) for comparison.

Host Bacterium	Infection by Bacteriophage	Standard Incubation	8% CO_2 Incubation	0.8% w/v $NaHCO_3$	8 hours		12 hours		24 hours	
					Vegetative cell count	Spores and Endospore count	Vegetative cell count	Spores and Endospore count	Vegetative cell count	Spores and Endospore count
Sterne	None	X			950	0	∞	0	*∞	*∞
DS2015/79	None	X			720	0	∞	0	*∞	*∞
Sterne	None		X		786	0	∞	968	*∞	*∞
DS2015/79	None		X		587	0	∞	852	*∞	*∞
Sterne	None		X	X	9	115	0	130	0	124
DS2015/79	None		X	X	6	121	0	122	0	128
Sterne	None	X		X	80	36	125	321	0	513
DS2015/79	None	X		X	46	21	112	202	0	296
Sterne	Gamma	X			551	14	118	193	0	137
Sterne	Crooki	X			410	32	157	123	0	56
DS2015/79	Crooki	X			368	24	26	32	0	11
Sterne	Gamma		X		523	182	197	215	0	168
Sterne	Crooki		X		366	162	83	151	0	89
DS2015/79	Crooki		X		168	124	15	58	0	64

Sterne	Gamma	X		X	0	45	0	32	0	35
Sterne	Crooki	X		X	0	50	0	41	0	34
DS2015/79	Crooki	X		X	0	65	0	36	0	29
Sterne	Gamma		X	X	5	78	0	79	0	70
Sterne	Crooki		X	X	5	92	0	85	0	79
DS2015/79	Crooki		X	X	12	86	0	74	0	66

∞ = cells are too numerous to count

*∞ = too numerous to count but equally
abundant

vegetative cell count = intact bacterial cells without evidence of white endospore contained within

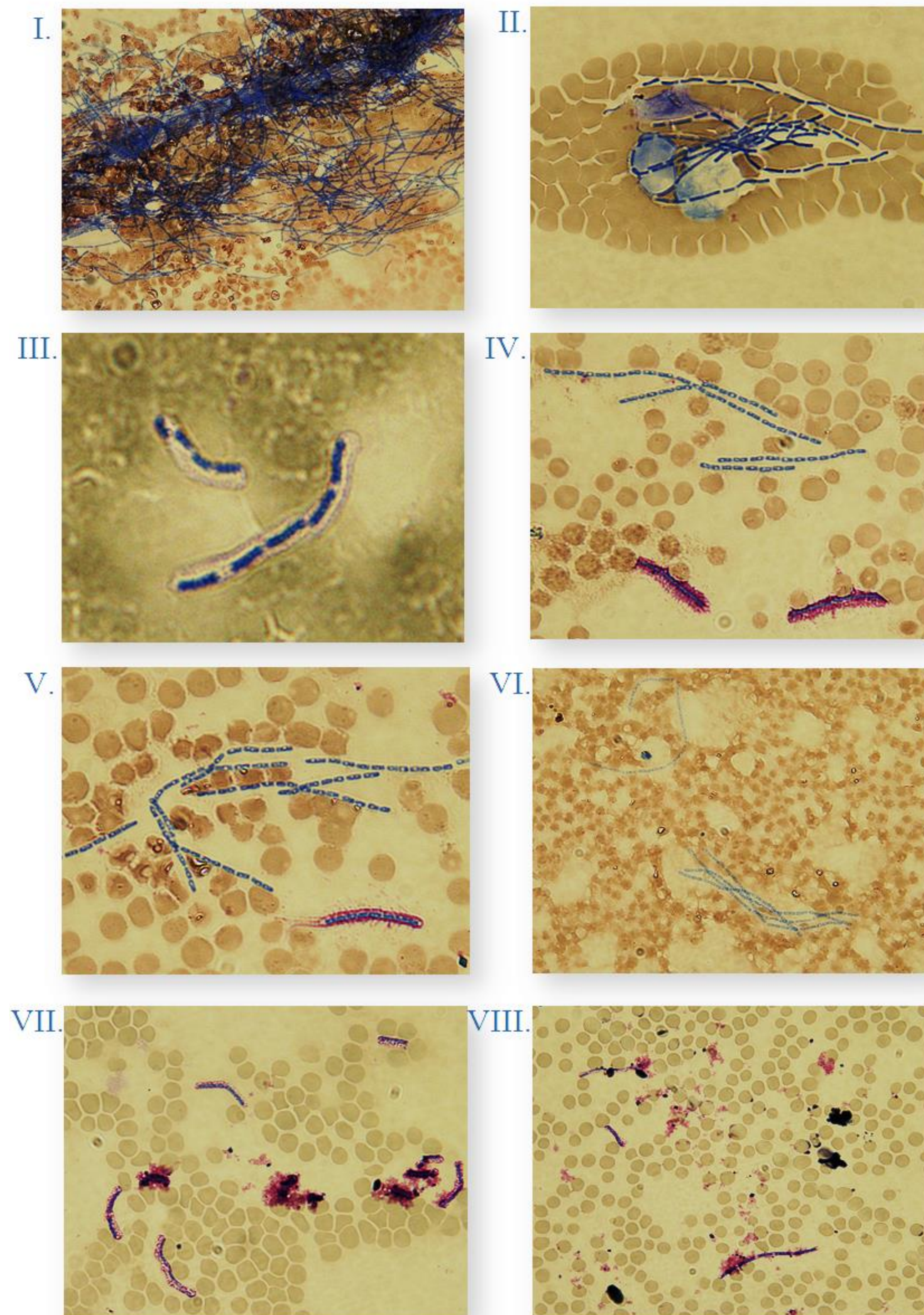

Figure 6.2: Romanowski-Giemsa stained blood smears of *Bacillus anthracis* at 100,000x magnification (I) Unencapsulated Sterne vaccine strain at 12 hours of 37 °C incubation. (II) Sterne with γ-phage at 8 hours of 37 °C incubation. (III) DS2015/79 showing encapsulation (balloon-like layer) at 8 hours of 37 °C incubation. (IV) DS2015/79 with $NaHCO_3$ in 8% CO_2 at 8 hours of 37 °C incubation showing thickened capsule (purple) and cells with endospores (blue). (V) DS2015/79 at 12 hours in 8% CO_2 37 °C incubation where all cells contain endospores (blue) and degradation of mother cell has begun (purple streaks). (VI) DS2015/79 with $NaHCO_3$ & 8% CO_2 at 12 hours of 37 °C incubation showing 100% sporulation. (VII) DS2015/79 with Phage_Crookii at 12 hours in & 8% CO_2 of 37 °C incubation showing lysed cells as well as intact endospores. (VIII) DS2015/79 with Phage_Crookii at 12 hours 37 °C incubation where all vegetative cells in view were lysed.

6.3.3. Genomic properties

6.3.3.1. Bacterium DS2015/79

About 140X sequenced reads of 280-bp paired end reads were generated for the *B. anthracis* genome. About 36 contigs were assembled for the genome of *B. anthracis* DS201579 (Table 2). The genome properties of *B. anthracis* DS201579 presented a chromosome of 26 contigs, two plasmids pX01 of 4 contigs and pX02 assembled as one contig (Figure 6.2, Table 6.2). The genome coverage of *B. anthracis* DS201579 (5.17Mb) was about 99% in relative to *B. anthracis* Ames ancestor. An average G+C content of 35% was determined in the *B. anthracis* DS201679. Annotation of the bacterial genome using RAST presented 5 469 coding sequences (CDSs). Meanwhile about 44% of the CDSs were assigned in the subsystem. Five prophages were determined in the chromosome of *B. anthracis* DS201579. This included the LambdaBa01, LambdaBa02, LambdaBa03, LambdaBa04 and PHAGE_Bacilli (Figure 6.3, Table 6.3).

6.3.3.2. Analysis of *de novo* assembly of unmapped reads

Analysis of the *de Novo* assemblies of the unmapped reads resulted in partial/minor phage contigs which had no resemblance to either of the bacterial prophages nor PHAGE_Crookii (Table 4). About 8 contigs were determined in this assembly, of which, 6 had similar sequence identities of ≤90% with Phage W.Ph. Neither of these identified contigs are similar when aligned to PHAGE_Crooki. This is indicative of multiple possible phage infection events of *B. anthracis* strain DS2015/79 by *Myoviridae* environmental phages over time (Table 4).

Table 6.2: General features of the genome sequence of *Bacillus anthracis* DS201579 and PHAGE_Crookii

Features	*B. anthracis* DS201579			PHAGE_Crookii
Domain	Bacteria			Virus
Replicon	Chromosome	Plasmid pX01	Plasmid pX02	
Sequence reads after trim	3 670 390			1 734 240
Avg. length after trim	222	222	222	143
Genome size (bp)	5 176 511	178 546	94 997	154 012
Number of contigs	26	4	1	1
Maximum length	1 768 393	6 6440	-	-
Minimum length	1 385	4 4636	-	-
G+C content (%)	35	32		37
Number of coding sequences	5 469	198	127	235
Number of RNAs	89	0	0	0
Number of tRNA	75	0	0	2
Prophages	5	-	-	-

Table 6.3: Five prophages of *B. anthracis* DS201579 in the chromosome identified using PHAST

Region	Region length (Kb)	Region position	Possible Phage	GC_Percentage	Ames ancestor Prophages
1	45.8	399829-445634	PHAGE_Bacill_virus_1NC_009737	32.2	LambdaBa04
2	19.9	1124158-1144148	PHAGE_Bacilli_NC_023693	36	PHAGE_Bacilli
3	59.6	3400955-3460564	PHAGE_Geobac_GBSV1_NC_008376	34.5	LambdaBa01
4	51.9	3687887-3739795	PHAGE_Bacill_Gamma_NC_007458	35.1	LambdaBa02
5	14.6	4784585-4799219	PHAGE_Lactob_Ld11-NC_026609	36	LamdaBa03

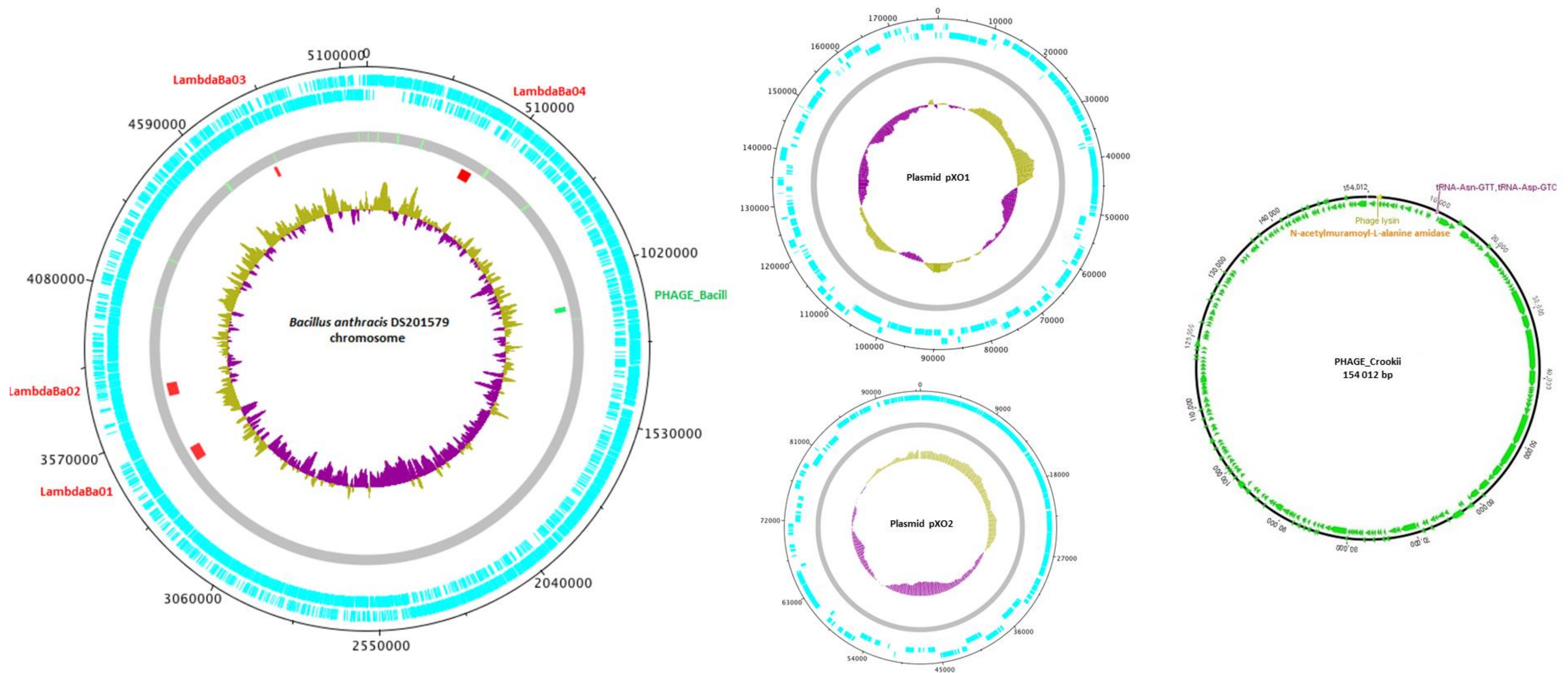

Figure 6.3: Circular DNA plots of *B. anthracis* DS201579 chromosome, plasmid pX01, plasmid pX02 and infecting bacteriophage PHAGE_Crookii using DNA Plotter and Geneious respectively. Circles display from inside to outside: GC skew (purple and gold) in the bacterial circular plots, Four regions (red) on the DS2015/79 chromosome are the known Lambda prophage regions of *B. anthracis* and fifth incomplete prophage PHAGE_Bacilli (green), Open reading frames are represented in blue. For PHAGE_Crooki genome size is represented in bp, the open reading frames indicated as green with annotations of lysine (orange) and tRNA genes (purple).

Table 6.4: *De novo* assemblies of unmapped reads collected from *B. anthracis* DS2015/79 genome illustrating sequence length

Sequence	contig size in bp	Number of matches	Max Score	Total Score	Query cover		Identitiy	Phage Name	Accession Number
CGTTTACGACGCTGAGGTTGCAGCATATAGCGAAAAAGGTGAATACCTATTCGGCGTATATTTTGGTACAT AATATGATGAGAAGCAAGCGGTTAAGGCTGCTAAAGAGATGCGTACGAAGCTACGTAAATACTTCGAAGT GAAAGGGCAGGTTTATACTTATACTTGCTAGTCGGGGCGTGAAGCCCCTTATAATTAATGCTTGACAAATAC TATTAAATAAGGCTGGGATATTATGAACGCGAATATTGAGCTTGCTATTATTGAACTTGAAACGATTGTCAA ACACGGTAATGTAATGATTGATATGTATAAGACACGCAGCACGTACTCACTTGTATTTCCAGATCGGAAAA GCAAACGTCTGAAATCCAGTAACATACAGCACTCACACATTCC	400	1	520	520	85%		94%	Bacillus phage W.Ph	HM144387.1
GAATGGAGAAAATTAAACGACTACAACCAGTGGCTCGTAGATAACACAAACTTACTAGGCGGCGCTCAAT GGAACGAGGCATCACTAGGATGGGGTAAGACTGATAAAGCTGAAGGTGAGATGTGGAGCTCTGCCCAAA CAACTCGTAGTGAGATGATTGAGTATATGCGTTCCATCGACGTTAAATTTAGAGCTGATGGTTTTGCTCCAT CCACATAGAACGGAAGATCACACGTCTGAACTCAAGACACATTCAGCAATCTCGTATGCCGTCTTCTGCTTG AAAAAAAAAATAATCGAAACCATCTCAACTCCCCCCCAACTACTCAAGA	332	5	246	246	59%	2e-61	89%	Bacillus phage BPS10C, complete genome	KC430106.1
			241	241	59%	8e-60	88%	Bacillus phage BPS13, complete genome	JN654439.1
			207	207	59%	8e-50	85%	Bacillus phage Eyuki, complete genome	KT207918.1
			207	207	59%	8e-50	85%	Bacillus phage Megatron, complete genome	KJ489401.1
			207	207	59%	8e-50	86%	Bacillus phage W.Ph., complete genome	HM144387.1
CATACAGATAGATAATAGTTTGATTAGTTTTAATGTTTCAAAGCGTGTTAACACTTGGTGTTTTGTGATTGGT TCATCATTGAAAGAAATATACATAAATATATTTGTCATACCTTTGTCATTCATTGTGACTGTTATACTATCGC TTTCTACTACATCACATACGATATGGAATTCTTTTACTTTTGTCATTATAATCCACCACCTTTTATTATTTTGTT GTACGATAACCTTGTTACTTAACGCTTAATGTTTACGCGTGCCATTGAATGTAAG	276	1	307	307	80%		92%	Bacillus phage W.Ph	HM144387.1
TCTGAATGTGACTGTAGGTAGAGTGGTTGCTCTTCCCATCTCCCTAACGTCTCATCTAGTATTTCCGGAGCCA CTAAAGGAACAGGAAACGGCAAAGTTACAGCAACAATAAACGTCAACGTAACAGGAAATGAAAAAGTAT CAAATAAACTTAGAAATAACGCGGACATGAAGAAGGCAGGAAAAGATGTTGGTAACTTACTAGGATTCTA TTCTAGGGAGATGACAATCGCTTAACTTAGGTGGTGTGGGCAAAGCTCACCCACCCTTTTTCTGTTATATTA GTAGTATACAAAGGAGGATTATAAATGACAACAATAGTTACAAGGTACCCGCGTATTGAAGT	346	6	327	327	77%	6e-86	89%	Bacillus phage W.Ph., complete genome	HM144387.1
			254	254	69%	1e-63	86%	Bacillus phage Hakuna, complete genome	KJ489399.1
			239	239	77%	3e-59	83%	Bacillus phage Eyuki, complete genome	KT207918.1
			239	239	77%	3e-59	83%	Bacillus phage Megatron, complete genome	KJ489401.1
			220	220	72%	1e-53	83%	Bacillus phage BPS10C, complete genome	KC430106.1
			220	220	72%	1e-53	83%	Bacillus phage BPS13, complete genome	JN654439.1
GATCTGCATCGTTTAACAGTGAATTTATTCTTTTCGTTAGTTTGTTCGCCGTAAATTACCTCTTCTTTATCCCAG CCTGTCCATTCTCTTGCCATAATAACCACTCCTTATTTTTGGTTAGTCCATAATCCGTGTTTTCTCCATCCCTCG CGCTTAATGTATCTACTAGGCTCATAACCTAACTTAATAACTTTACCGCTACTAAACACTCGACTGTCTACGT CCTCGACACTTGCAATTGTATCATGAAAAGCAGGTTCGCCTTCATACTCCCCATACCCGTAACCAAACGCAC TACATAGCATACTGTGACTATAATAAATTAGGTAACTATTCCCTTCAAAGGACTCCTGCTCTACGCTATACC CTTTCTCTAGTAAC	381	4	457	457	98%	8e-125	89%	Bacillus phage W.Ph., complete genome	HM144387.1
			206	206	73%	3e-49	80%	Bacillus phage BPS10C, complete genome	KC430106.1
			206	206	73%	3e-49	80%	Bacillus phage BPS13, complete genome	JN654439.1
			87.9	87.9	21%	1e-13	86%	Bacillus phage Hakuna, complete genome	KJ489399.1
GCGATGACAGCTGCTAAGGATAGAGCTGACCTAGAAGGCAAGTTTGATGGTATTGAAGGGTACAATGAAC ATCTAGAAAACGGTGGAATGACTTCCCGTGAAACAGAAGTTGCAAAGCATATAGCGAATCAGTTAGCTAA AGAAGAACAAAGAGCGAGAGAACTGGAATCAGGCGCAACATCTAGTAAAGACTTCAAGGATGACCGCGA GCGCGCAGGAGAAGTTGTCATGGATGAGGAGCATAGACTTAACAAACAGACAATGGA	266	3	337	337	100%	8e-89	89%	Bacillus phage Eyuki, complete genome	KT207918.1
			337	337	100%	8e-89	89%	Bacillus phage Megatron, complete genome	KJ489401.1
			320	320	100%	8e-84	88%	Bacillus phage Hakuna, complete genome	KJ489399.1
ATCTAGTTTACCATTAGCAGCTAATTCCACAACCTCTTCAACGAATGCTTTAGTTGGCCCTGTAACAACCGTT GTATGTTCTTTAATTAATGATACTAATTCCTTAGACTTACGGAATGCAGCGACTTTACCAAAACCGTAATCCC CACCTTCAGCTAGATTCGCCTCAATTCTCTCCTGTAATTGCTCGTGTACCTCTTGTTTAGTTAATGTGTTTGTC ATAATAAACCCTCCATTTTGTTAGTTATTAGTTTACTAGCATTTCTGCTAACTCATCAAGTTCTTCTGGAACAA ATTTGATA	302	5	431	431	100%	4e-117	92%	Bacillus phage W.Ph., complete genome	HM144387.1
			233	233	86%	1e-57	83%	Bacillus phage Eyuki, complete genome	KT207918.1
			226	226	89%	2e-55	82%	Bacillus phage Hakuna, complete genome	KJ489399.1
			154	154	69%	9e-34	80%	Bacillus phage BPS10C, complete genome	KC430106.1
			148	148	69%	4e-32	80%	Bacillus phage BPS13, complete genome	JN654439.1
GTATGTTAAATTGGTTGGTACTCAATGGCTAGTGATATTGTAACTTTACTATTCTTATCTAGTTCGGACCCAC AGCTACGAACATTACTAACTTTAATTGCATCATTTGAGACAATGCATAACGTTTCTTATGAGCATATGACAA GTTCAGTAATTGGTGTTGAACGAAAAGAGCAATACTTTGCTAAAGTTCAAGAGTTGCCATTACTTGCTAAAC GCAACTCACACATTTTGACTCACTTAGAGAACATGTCTGAC	258	2	313	313	97%	1e-81	89%	Bacillus phage BPS10C, complete genome	KC430106.1
			313	313	97%	1e-81	89%	Bacillus phage BPS13, complete genome	JN654439.1

6.3.4. **Genome of virus Crookii**

The bacteriophage identified as PHAGE_Crooki is a dsDNA *Myoviridae* virus ~154 012 bp with a G+C content of 37%. The number of coding sequences identified by RAST for this phage was 235. About 94% of the annotated CDS's are hypothetical while 6% have a known protein nomenclature. This included the lysins, head/capsid proteins, cell wall binding proteins, base plate proteins, replication and packaging machinery (Supplementary Data\Supplementary Data 6.2.xlsx). No intergrase/recombinase proteins were determined in the PHAGE_Crookii. The genome properties of phage Crookii codes for lysine N-acetyl-muramoyl-L-alanine amidase (endolysin), which is responsible for bacterial lysis. It contained multiple tRNA genes namely; tRNA-Asn-GTT (Asparagine) and tRNA-Asp-GTC (Aspartate) which denote a circular genome (Tan et al., 2007).

6.3.5. Whole Genome Viral Alignment

BLAST comparison of Crookii on NCBI revealed a quasispecies relationship to a *Myoviridae* bacteriophage W.Ph. isolated from an anthrax positive zebra carcass in Namibia *(Klumpp et al., 2012, Klumpp et al., 2014)*. The whole genome of PHAGE_Crookii was compared and aligned with phage W.Ph. (Figure 4). The genome size of phage W.Ph. is 156,897 bp. Phage_Crookii demonstrated 83% query cover and 85% sequence identity with W.Ph accession number HM144387.1. The closest blast hits after W.Ph. had a query cover of ≤65% and were excluded on that basis. The G+C content of phage W.Ph phage is 36.4%, which compares well with Phage Crookii. The same number of tRNA's were observed on the genomes of both phages.

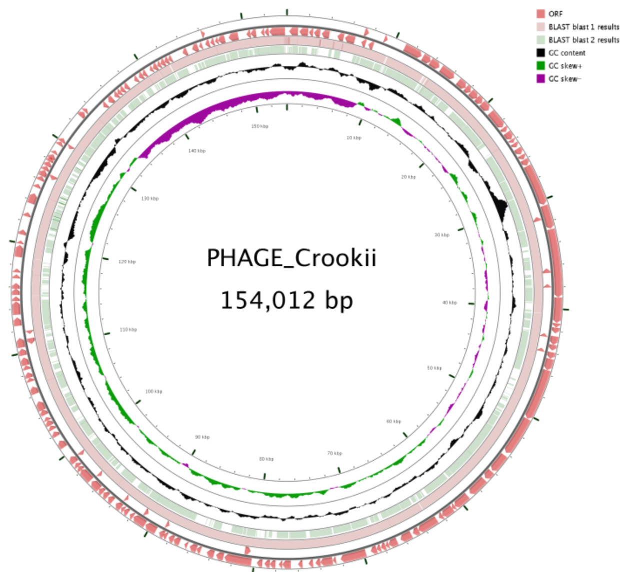

Figure 6.4: The CGView comparative tool map of PHAGE_Crookii genome sequence to phage W.Ph. Circles display from inside to outside: genomic position in kilobases, GC skew (purple and green), GC content (black). Blast 1 results show the sequence homolog between PHAGE_Crookii and phage W.Ph (blast 2). Open reading frames are indicated as pink from the outside.

6.4. Discussion

In the diagnostic evaluation of carcass site DS2015/79, the apparent "sterility" of the untreated blood agar plates drew special attention to the sample. The novelty of the identification of Phage Crookii and its inherent appetite for *B. cereus* and *B. anthracis* in culture is what sets this bacteriophage isolation apart. The heat treated samples from the carcass site yielded *B. anthracis* spores, but only 2 small colonies on enrichment media from a biological sample is unusual. When subcultured, plaques once again appeared over time, indicating that even these spores failed to escape infection.

Bacteriophages are not able to infect bacterial spores (Sonenshein and Roscoe, 1969), but they are able to harbor within spores after infection of a vegetative cell (Cowles, 1931b, Takahashi, 1964). Bacteriophages are also able to persist in biofilms, created by bacteria, in the environment (Tait et al., 2002, Schuch and Fischetti, 2009). The inability to obtain isolates from the swab before the heat treatment indicates that the bacteriophage was active in the sample. The tailed phages, of which *Myoviridae* phages form a part, tend to be the most resilient, abundant and stable to environmental factors (Ackermann et al., 1995a, Jończyk et al., 2011). This does not mean that these phages will survive for prolonged periods outside

of their ideal conditions. Phages are certainly sensitive to external environmental factors such as temperature and pH ranges (Van Elsas and Penido, 1982, Jończyk et al., 2011).

To simulate carcass conditions the spores from un-encapsulated Sterne versus the encapsulated DS2015/79 were incubated under different conditions of Oxygen concentration, pH and bicarbonate content as a control for comparison of the phage effects. The inclusion of 8% CO_2/bicarbonate was to stimulate thickening of the capsule (Sirard et al., 1994, Drysdale et al., 2005) and to mimic carcass blood conditions (Meynell and Meynell, 1964). The germination of the spores was lower in the presence of sodium bicarbonate, under standard incubation conditions, and even lower in the presence of 8% CO_2. This is expected due to the inhibitory nature of CO_2 (Enfors and Molin, 1978) and sodium bicarbonate (Hachisuka et al., 1956) in the germination of soil born spore formers. Since bacteriophages cannot infect bacterial spores (Takahashi, 1964, Sonenshein and Roscoe, 1969), sporulation before infection by the phage would ensure the bacterium's persistence in the environment. The blood smear analyses was aimed at visually appraising whether sporulation is triggered as a defense mechanism against phage infection (Høyland-Kroghsbo et al., 2013, Hargreaves et al., 2014) or in response to nutrient availability (Koehler, 2009) through cell enumeration.

Phage Crookii proved more lytic than Gamma phage to DS2015/79; however, there seemed to be no difference in the effect between Crookii and Gamma on the un-encapsulated Sterne strain. *Bacillus anthracis* DS2015/79 did not appear to develop any phage resistance characteristics over time, escaping infection only through sporulation. Sporulation and replication activity was due to the nutrient availability cues rather than phage influenced. The presence of the sodium bicarbonate and the carbon dioxide inhibited spore germination irrespective of the presence of the phages and therefore resulted in higher end point spore counts due to the inability of the phages to infect the bacteria. The lower bacterial counts from samples which included bacteriophages demonstrates the aggressive ability of environmental phages like Crookii to reduce overall bacterial counts at the endpoint. This is due to the fewer vegetative cells available for replication during the 0 to 12 hour logarithmic incubation period and its coinciding with the highest infectivity period of the phage (Luria and Human, 1950, Bertani, 1951).

While the lytic effects of the bacteriophage on *B. anthracis* are quite apparent, a genetic analysis of the bacterium and virus highlighted an alternative relationship as well. The genome of Phage Crookii included a coding region for the metal-dependent hydrolases of the metallobeta-lactamase superfamily, which was previously described in *B. cereus* for conferring resistance to carbapenems (Carfi et al., 1995, Payne, 1993) to prevent premature lysis of the bacterial cell by external factors (Jończyk et al., 2011). No holin coding region was observed in this genome; however, a large number of hypothetical proteins in the genome were specified to still require annotation. Comparative sequence analysis identified a close relative to Phage Crookii in the form of Phage W.Ph. Phage W.Ph was isolated from an anthrax positive zebra carcass in Etosha National Park, Namibia (Klumpp et al., 2012, Klumpp et al., 2014). Both phages are Myoviridae dsDNA bacteriophages based on similar GC content and sequence identity (Smith et al., 2013, Klumpp et al., 2014). These bacteriophages infect members of the *Bacillus cereus* group.

Bacillus anthracis DS201579 genome properties confirms the presence of the chromosome, pX01 and pX02. Comparative genomic analyses of the prophages described in this study are found in *B. anthracis* Ames ancestor. Different sizes of the prophage regions were also observed in the genome of *B. anthracis* DS201579. Phage Finder and PHAST analysis of the bacterial genome revealed only the 4 prophage regions that have been well described for *B. anthracis* (Rasko et al., 2005, Sozhamannan et al., 2006, Klee et al., 2010) as well as, a previously characterized partial prophage (Sozhamannan et al., 2006, Zwick et al., 2012). A separate partial phage was also identified in the analysis of the unmapped reads of the bacterial genome. This phage had similarities with Phage W.Ph but the contigs did not align with Phage Crookii. This is an indication of past infection events of the bacterium. Phage infection by virions already present in the bacterium can confer phage resistance to infection by new bacteriophages as well as through origin-derived phage-encoded resistance (Hyman and Abedon, 2010, Seed, 2015), however, this was not the case for bacterium DS2015/79 and Phage Crookii.

6.5. Conclusion

Phage Crookii is an aggressively lytic dsDNA *Myoviridae* bacteriophage infecting *B. anthracis* in the environment of Pafuri, Kruger National Park. The phage presented itself as an

unusual isolate during diagnostics from a carcass in an anthrax outbreak. Since no bacteria could be isolated from soil and only heat treatment greater than 60 °C yielded *B. anthracis* from a blood swab, it demonstrates the role of environmental bacteriophages in the natural reduction of bacterial inoculum at carcass sites.

Whole genome sequencing of DS201579 revealed the presence of the lambdaBA phages that have been previously described in Ames ancestor and commonly found amongst *B. anthracis*. No bacteriophages, other than the expected 4, were identified within the host bacterial genome as determined by whole genome sequencing.

The remnants of another partial phage within the *B. anthracis* isolate DS2015/79 is indicative of multiple possible phage infection events of the *B. anthracis* strain DS2015/79 by *Myoviridae* environmental phages over time. Despite this, DS2015/79 did not manifest phage resistance characteristics against phage Crookii even after serial passage. The initial isolation of the bacterium highlighted the bacteriophages ability to harbor within spores, thus persisting alongside the bacterium until germination.

Phage Crookii demonstrated a quasispecies sequence identity relationship to *Bacillus* Phage W.Ph. The sequence similarity of these hypervariable environmental *Myoviridae* bacteriophages is descriptive of the common ecology within African soil biomes despite geographical disparity.

6.6. References

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7. Chapter 7

Characterisation of temperate bacteriophages infecting *Bacillus cereus sensu stricto* group in the anthrax endemic regions of South Africa.

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Abstract

Bacteriophages are viruses that infect bacteria. They are ubiquitous and the most abundant and diverse set of micro-organisms on the planet, making them the largest reservoir of sequence information in the biosphere. These viruses belong to the order *Caudovirales* with tailed phages within the families of *Siphoviridae, Myoviridae* and *Podoviridae.* Phages are believed to influence fitness traits in bacteria. In this study, lysogenic phages harboured in soil borne spore formers were explored to determine their effect during peak anthrax outbreaks. The 7 bacteriophages characterized in this study infect members of the *Bacillus cereus sensu stricto* group, specifically *B. anthracis*, *B. cereus* and *B. thuringiensis.* The bacteriophages were induced, from bacterial isolates from anthrax endemic areas in South Africa, through ultraviolet irradiation and rapid cooling. The plaque morphologies differed between the bacteriophages and some showed characteristics of a temperate lifestyle. Whole genome sequencing identified these phages as lysogenic based on protein sequence identity and the presence of recombinase, Rha and Cro-CI repressor proteins. Genes encoding fosfomycin resistance and sporulation specific sigma factors were also identified. Temperate phages incorporate into the bacterial host genome and have a narrow host range, which have the potential of being vectors of horizontal gene transfer between the *Bacillus* species in the environment.

7.1. Introduction

Bacteriophages are ubiquitous and the most abundant organism on earth (Ackermann et al., 1978, Ackermann, 2011). Bacteriophages are viruses that predate on bacteria with a long history as genetic models, bacteriologic diagnostic tools, as vectors, therapeutic agents and other applications in the biotechnology industry (Cowles, 1931a, Purdy et al., 1986, Haq et al., 2012, Nelson et al., 2012). Phages have two lifecycle paths, either virulent or temperate. Virulent phages infect bacteria, use the host machinery to proliferate and lyse the bacterium by producing lysin proteins (Walmagh et al., 2012, El-Arabi et al., 2013). Temperate phages incorporate into the genome of a bacterial host (prophages), but are induced into a lytic cycle when the host cell undergoes mutagenesis or a stress response (Little and Mount, 1982, Smith et al., 2010). This merging process results in changing the genome architecture of the host and coevolution of the virus and bacterium. These prophages may also encode virulence factors conveyed through lysogenic conversion (Brussow and Hendrix, 2002). Increasingly, bacteriophages have applications in the characterization of monomorphic bacterial genomes through phage typing techniques (Sozhamannan et al., 2006).

Schuch and Fischetti (2009) described lysogenic bacteriophage-mediated ecological adaptations which included effects on sporulation of the bacterium *Bacillus anthracis,* with implications for its survival and persistence in the environment. This led to increased attention on the role of phages on the soil borne portion of bacterial life cycles (Jończyk et al., 2011). *Bacillus anthracis* is a soil borne, Gram positive bacterium belonging to the *Bacillus cereus* group and the aetiological agent of anthrax (Turnbull, 1999). *Bacillus cereus sensu lato* is composed of the endospore forming *B. anthracis, B.cereus, B. thuringiensis, B. mycoides*, *Bacillus pseudomycoides*, and *Bacillus weihenstephanensis*. The three most closely related bacteria are *B. anthracis, B. cereus* and *B. thuringiensis* which together make up the *Bacillus* ACT group or *B. anthracis sensu stricto* (Vilas-Boas et al., 2007). *Bacillus thuringiensis* is an insect pathogen (Didelot et al., 2009). *Bacillus cereus* causes emetic disease in mammals, and less typically, an anthrax-like pneumonia (Hoffmaster et al., 2006). The majority of differences, including toxin encoding factors, between these bacteria can be found on their plasmids, but there are similarities between even these regions (Turnbull et al., 1992, Hu et al., 2009). The *Bacillus* ATC bacteria are indistinguishable based on 16S rRNA sequencing

(Ash and Collins, 1992). However, phage typing has identified four putative prophages specific to the genome of *B. anthracis* which serve as a means of specific identification (Sozhamannan et al., 2006), which is an indication of the co-evolution between phages and their hosts (Brüssow et al., 2004).

A multitude of *Bacillus*-specific bacteriophages have been identified and characterised over the years (Cowles, 1931b, Brown and Cherry, 1955, Klumpp et al., 2010, Yuan et al., 2012, El-Arabi et al., 2013, Grose et al., 2014a, Grose et al., 2014b, Gillis and Mahillon, 2014). The host range of these bacteriophages differs markedly except that, DNA metabolism, cell lysis, structural, and host gene products are well-conserved in *Bacillus* phages (Grose et al., 2014b). Molecular identification of bacteriophages is based on protein sequence identity and function (Ackermann et al., 1995).

When a temperate bacteriophage infects a bacterial host it affects bacterial fitness in a few different ways. The insertion of the viral DNA can cause frame-shift gene disruptions; it protects the bacterium from premature lysis by a lytic phage and introduces new fitness traits through transduction/lysogenic conversion (Brüssow et al., 2004). One such trait is biofilm formation. Bacteriophages are known to induce lysis of a host in biofilms to increase their chances of infection of a new host (Schuch and Fischetti, 2009, Nanda et al., 2015). The high cell densities and the extracellular DNA from lysed cells in such environments make conditions ideal for horizontal gene transfer (Nanda et al., 2015). Another possibility for genetic transfer occurs during the induction process, where some of the host DNA is included during bacteriophage packaging (Merrill et al., 2014). Since bacteriophages have a host range including closely related bacterial groups (Klumpp et al., 2010, El-Arabi et al., 2013), they could be responsible for the similarities between these related bacteria (Hu et al., 2009, Gillis and Mahillon, 2014, Nanda et al., 2015). In this study we investigated and characterize bacteriophages infecting *Bacillus* ACT bacteria during anthrax outbreaks, in anthrax endemic areas of South Africa, through lysogen induction and whole genome sequencing techniques to describe their activity and role.

7.2. Materials and Methods

7.2.1. Bacteriophage identification and isolation

7.2.1.1. Bacterial isolation:

Isolates obtained during bacteriologic diagnostics of samples submitted to the Skukuza State Research Laboratory during anthrax outbreaks were used to induce bacteriophages. The host bacterium and their associated viruses were isolated from the Kruger National Park (n=7) and Northern Cape (n=1) (Table 7.1). Samples collected from anthrax outbreaks including soil, swabs, bone and insect larvae were processed according to methods described in Chapter 3. Bacillus species isolated from these samples were identified by the bacteriology laboratory of the Department of Veterinary Tropical Diseases based on morphology, biochemical tests and nutrient supplement utilization.

7.2.1.2. Bacteriophage induction:

Bacterial cultures purified on blood agar that showed evidence of plaque formation, during routine diagnsotics, were included in this study. The purified bacterium lawn was stimulated into inducing any inhabitant bacteriophages by causing cell damage to the vegetative bacterial cells i.e. a stress response reaction. Induction of the bacteriophage virus was achieved through ultra violet (UV) radiation at 312 nm wavelength with 3250 $\mu W/cm^2$ from a germicidal UV lamp (Spectroline®) (Lasec, South Africa) for 3 minutes followed by cooling to 4° C in a refrigerator for an hour (Baluch and Sussman, 1978, Berenstein, 1986). Thereafter the plates were placed in a sterile biosafety cabinet and evaluated for plaques every hour. The plate was gently flushed with 500 µL of Dulbecco’s phosphate buffered saline (DPBS) Gibco® (Thermo Fisher Scientific), which includes 100 mg/L of both calcium chloride and magnesium chloride. Without disrupting any bacterial colonies, the solution was drawn up into a 1 mL syringe and then filtered through a 0.45 µM cellulose acetate membrane (Lasec, South Africa) to obtain the viral filtrate.

7.2.1.3. Bacteriophage Propagation

Due to safety concerns (especially in the case where the original host was pathogenic *B. anthracis*) the bacteriophages were propagated using the *B. anthracis* Sterne $34F_2$ strain. *Bacillus anthracis* Sterne strain (a 5 µL bacteriologic loop full in 1 mL DPBS) was cultured in 999 mL of sterile Brain Heart Infusion Broth (BHI) Difco® along with 100 mM of calcium chloride as well as 8 mM magnesium sulfate and incubated for 8 hours until the solution went from clear golden to turbid yellow in colour. The bacteriophage filtrate was then added along with 100 µL of the *B. anthracis* Sterne strain to the surface of a nutrient agar plate (Selecta Media®) and incubated overnight at 37° C. The plates were removed from the incubator and placed at -20° C for 30 minutes and then left on the bench top until plaques became evident. A single phage colony was selected through repeat pipetting 50 µL DPBS within a plaque and using this solution to inoculate 5 mL BHI broth with *B. anthracis* Sterne strain. The liquid culture - viral filtrate stock was incubated in a shaking incubator at 60 rpm and 37° C overnight then once again placed in the freezer for 30 minutes followed by bench top incubation for 8 hours. A 100 uL of this phage-culture was plated out onto nutrient agar and incubated overnight to evaluate the success of the propagation. The plaque morphologies can be seen in Figure 1 to determine the purity of the plaque isolation, since multiple bacteriophages can be present on each bacterial lawn. If further propagation was required to increase titres, it was performed by infection of *B. anthracis* $34F_2$ bacterial lawns on nutrient agar. The liquid culture was then filtered through a 0.2 µM cellulose acetate membrane (Lasec, South Africa) to separate the propagated virus from the bacteria.

7.2.1.4. Plaque enumeration assay:

The bacteriophage filtrates were evaluated for plaque titres, in plaque forming units (pfu), using the double layer agar method without the use of antibiotics (Adams, 1959, Kropinski et al., 2009) with *B. anhtracis* Sterne $34F_2$ as the host and a 10 fold dilution series of the filtrate. A series of 5 replicates were performed for each dilution. All filtrates were propagated to approxamately 1×10^{10} pfu/mL. The Cherry phage was used as a positive control.

Table 7.1: Bacteriophages isolated from bacterial hosts during anthrax outbreaks in endemic areas of South Africa through ultraviolet irradiation induction.

Bacteriophage	**Electron Micrgraph Morphology**	***Bacillus* species host**	**Host Sample Source**	**Sample Origin in South Africa**	**Host Isolation Year**
JB25	*Siphoviridae*	*B. anthracis*	Kudu jaw bone	Dikbosch, Northern Cape Province	2008
DS2010	*Siphoviridae*	*B. anthracis*	Impala carcass	Nyala Pan, Pafuri, Kruger National Park	2010
DS2011	*Siphoviridae*	*B. anthracis*	Kudu carcass	Punda Maria, Kruger National Park	2011
Earthworm	*Siphoviridae*	*B. cereus*	Earthworm from soil	Sabie River banks, Skukuza, Kruger National Park	2012
A157	*Siphoviridae*	*B. thuringiensis*	Vulture feather swab	Pafuri, Kruger National Park	2013
A168	*Siphoviridae*	*B. thuringiensis*	Vulture beak swab	Pafuri, Kruger National Park	2013
AD2014	*Myoviridae*	*B. anthracis*	Blowfly larvae from kudu carcass	Pafuri, Kruger National Park	2014
**Crookii	*Myoviridae*	*B. anthracis*	Wildebeest carcass	Crooks Corner, Pafuri, Kruger National Park	2015

**Isolation and propagation: See chapter 6

7.2.1.5. **Viral Precipitation**:

Viral precipitation was achieved by dissolving 2.3% sodium chloride and 7% weight/volume Polyethylene Glycol 6000 (PEG_{6000}) (Merck Millipore) into the filtrate and refrigerating overnight at 4° C. The viscous solution was then centrifuged at 3000xg for 60 minutes at 4° C. The viral pellet was dissolved to a final volume of 1 mL in phage buffer containing 100 mM calcium chloride ($CaCl_2$), 8mM magnesium sulphate ($MgSO_4 \bullet 7H_2O$), 50 mM Tris-Cl (1M, pH 7.5) and distilled water. Half the precipitate was submitted for transmission electron micrograph (TEM) while the other underwent DNA extraction.

7.2.1.6. **Host Specificity Determination**

A bacterial lawn of various Gram negative and positive bacteria species (Table 2) each was grown on nutrient agar. A grid was drawn onto the back of each plate and 20 uL of each phage filtrate dilution $1x10^2$ pfu/mL; $1x10^6$ pfu/mL and $1x10^{11}$ pfu/mL were placed into a block followed by 12 hours incubation at 37° C. The host suitability range of each virus was tested against Bacillus ACT species to determine the lytic activity against each bacterium and thus determine the host suitability range of each virus.

7.2.1.7. **Transmission electron micrograph identification of bacteriophage:**

The TEM was performed at the Electron Microscope Unit of the University of Pretoria, Faculty of Veterinary Science using negative staining with 2% uranyl acetate. Identification was based on viral morphology criteria for (i) *Myoviridae* icosahedral head 60 – 145 nm; elongated heads 80 x 110 nm and tail 16 -20 x 80 – 455 nm (ii) *Siphoviridae* icosahedral head 60 -70 nm and tail 5 – 10 nm x 100 – 210 nm as described in King (2011).

7.2.2. Molecular identification of bacteriophages:

7.2.2.1. Viral DNA Isolation:

The viral DNA was isolated through the phenol-chloroform isoamyl alcohol method and 3 M sodium acetate from viral pellets at a concentration of $1x10^{10}$ pfu/mL (Wilson, 1987, Boom et al., 1990, Loparev et al., 1991).

7.2.2.2. Shotgun sequencing:

Shotgun libraries preparations of 8 bacteriophages (including Cherry as a control) were performed using the Nextrera XT DNA Sample Prep Kit (Illumina, USA) by following the manufacturer's instructions. Tagmentation of the samples was performed using 1 ng of the template, according to the manufacturer's instructions. Sequencing was performed on the Illumina MiSeq sequencing platform (Illumina) using the MiSeq Reagent Kit v3 (500 cycle).

7.2.2.3. **Quality** and Trimming:

The quality sequence data of the bacteriophages were analyzed using FASTQC software version 0:10.1 (Andrews, 2010). Different sequence paired end reads were generated for the bacteriophages (Table 7.3). Adaptors and ambiguous nucleotides sequences were trimmed using CLC Genomic Workbench 7.5.1. Trim settings included the removal of low quality sequence: limit 0.05, and ambiguous nucleotides: maximal 2 nucleotides allowed. Reads shorter than 50 nucleotide bases were removed.

7.2.2.4. De *novo* assembly and annotation:

The *de novo* assemblies of the bacteriophages sequenced data were carried out using CLC Genomic workbench 7.5.1. *De novo* options included: minimum contig length, minimum size of assembled contig of 500bp and 1000bp respectively. *De novo* assembly of the trimmed reads was performed using the following parameters: similarity = 0.8; length fraction = 0.5; insertion cost = 3; deletion cost = 3; mismatch cost = 3; auto-detect paired distance and perform scaffolds. The list of unmapped reads was collected for further analysis. The assembly contigs were extracted and further analyzed using BLASTn (Altschul et al., 1990).

Annotation of the bacteriophages was carried using RAST annotation server for subsystems and functional annotation (Aziz et al., 2008).

7.2.2.5. Comparative analysis and genome phylogeny:

BLASTn (Buhler et al., 2007, Zhao and Chu, 2014) was used to search for the closely related sequences of the bacteriophages (NCBI http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple genome alignments of bacteriophages was performed using MAFFT tool (Katoh and Standley, 2013). The MEGA file of the aligned fasta file of bacteriophages was created using MEGA 7 (Katoh and Standley, 2013, Tamura et al., 2013). Maximum likelihood analysis of the bacteriophages nucleotide sequences and related phage sequences were performed using 1000 bootstrap replication in MEGA 7 with *B. megaterium* phage Stahl (Accession number: NC028856) (Brizendine et al., 2015) as an out group.

7.3. Results

7.3.1. Bacteriophage Identification and Isolation

Bacteriophages were successfully induced from 7 *Bacillus cereus* group bacterial cultures (Table 1), isolated during anthrax outbreaks in South Africa. The bacteriophages isolated from *B. anthracis* included JB25, DS2010, DS2011 and AD2014; *B. thuringiensis* included A157 and A168 and "Earthworm" was isolated from *B. cereus* (Table 7.1). All plates included in this study showed evidence of plaque formation before 2 hours after the refrigeration step. The plaque morphology for each bacteriophage is distinct on *B. anthracis* Sterne 34F$_2$ bacterial lawns and demonstrates the purity of viral isolation from individual plaques (Figure 7.1). All the phages described in this study were included based on their ability to lyse *B. anthracis* Sterne 34F$_2$ with differing lytic efficiencies. After 6 hours, all the bacteriophages lysed ≥60% of the *B. anthracis* Sterne bacterial lawn.

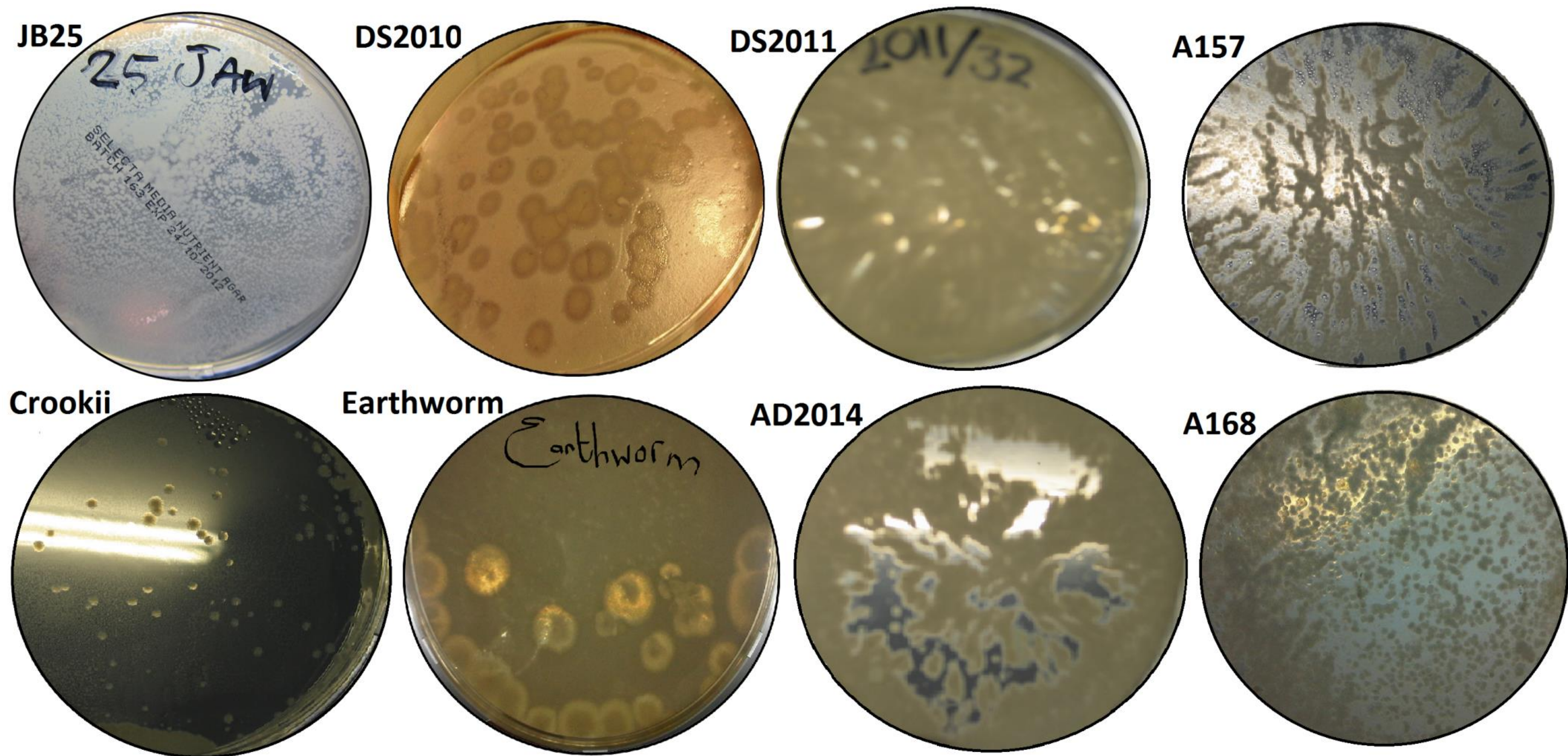

Figure 7.1: Plaque morphology of bacteriophages from *Bacillus cereus sensu stricto* group, propagated on *B. anthracis* Sterne $34F_2$ strain for 12 hours at 37° C. Phages DS2010 and Earthworm demonstrate 'donut' morphology with an outer lytic ring and inner turbid zone indicative of lysogenic bacteriophages. Phages DS2011 and A157 show 'comet' plaque morphology while phages JB25 and A168 create plaques without any discernible pattern. Phage AD2014 has a 'starburst' plaque pattern and phage Crookii is aggressively lytic.

7.3.2. Bacterial Host Specificity

The host suitability range was determined qualitatively through observation of lytic plaques on bacterial lawns from various bacilli and cocci strains (Table 7.2) at phage dilution $1x10^2$ pfu/mL, $1x10^6$ pfu/mL and $1x10^{11}$ pfu/mL concentrations. In general, the bacteriophages were lytic against *B. anthracis*, *B. cereus* and *B. thuringiensis* with the exception of phage DS2011 which was specific for *B. anthracis* (Table 7.2). At the highest concentration of phage JB25 and Earthworm ($1x10^{11}$ pfu/mL), tiny pin-prick plaques could be observed on *B. subtilis*, although no such activity could be determined at the lower concentrations. The same plaque pattern (distributed pin-prick plaques), but with larger lytic zones (1.5 mm in diameter) was observed on *Salmonella enterica* for phage AD2014. These plaques were harvested in DPBS, the DNA was then extracted and submitted for sequencing along with the other bacteriophage DNA to determine if the phage stock was contaminated. Bacteriophages A157 and A168 are Siphoviruses induced from *B. thuringiensis*, obtained from swabs of vultures during an anthrax outbreak, but which demonstrate a greater lytic activity for *B. anthracis* and *B. cereus* than their original isolation host.

7.3.3. Transmission Electron Micrographs (TEMs) Identification of bacteriophages

TEM's of the bacteriophages indicates that the majority of the phages belong to the family *Siphoviridae* with icosahedral heads, long, thin and non-contractile tails (Figure 7.2). Only Phage Crookii and phage AD2014 belong to the *Myoviridae* family, although the lytic phage Crookii was not induced experimentally (Chapter 6) and phage AD2014 was obtained from a *B. anthracis* isolate from the gut of a blowfly larva collected from an anthrax carcass (Chapter 3).

Table 7.2: Host suitability range for bacteriophages measured qualitatively through observation of lytic plaques using bacteriologic methods.

Strain	Cherry	JB25	DS2010	DS2011	Earthworm	AD2014	A157	A168	Crookii
Bacillus cereus ATCC® 33019™	-	+	+	-	+	+	+	+	+
Bacillus licheniformis ATCC® 12759™	-	-	-	-	-	-	-	-	-
Bacillus pumilus ATCC® 14884™	-	-	-	-	-	-	-	-	-
Bacillus subtilis ATCC® 6633™	-	x	-	-	x	-	-	-	-
Enterobacter cloacae ATCC® 13047™	-	-	-	-	-	-	-	-	-
Klebsiella pneumoniae ATCC® 13883™	-	-	-	-	-	-	-	-	-
Salmonella enterica ATCC® 13311™	-	-	-	-	-	x	-	-	-
Staphylococcus epidermidis ATCC® 12228™	-	-	-	-	-	-	-	-	-
Streptococcus mutans ATCC® 35668™	-	-	-	-	-	-	-	-	-
Sterne $34F_2$ (OBP)	+	+	+	+	+	+	+	+	+
Bacillus cereus (lab strain)	-	+	+	-	+	+	+	+	+
Bacillus mycoides (lab strain -Pafuri)	-	-	-	-	-	-	-	-	-
Bacillus anthracis (lab strain KC2011-Kapama)	+	+	+	+	+	+	+	+	+
Bacillus anthracis (lab strain Roan5 - Mooiplaas)	+	+	+	+	+	+	+	+	+
Bacillus thuringiensis (lab strain –Pafuri)	-	+	+	-	-	+	+	+	x

ATCC strains obtained from (Microbiologics) QUANTUM BIOTECHNOLOGIES PTY LTD

+ = ≥10 mm diameter lytic zone

- = no lysis observed

x = partial lysis: pin-prick-type plaques

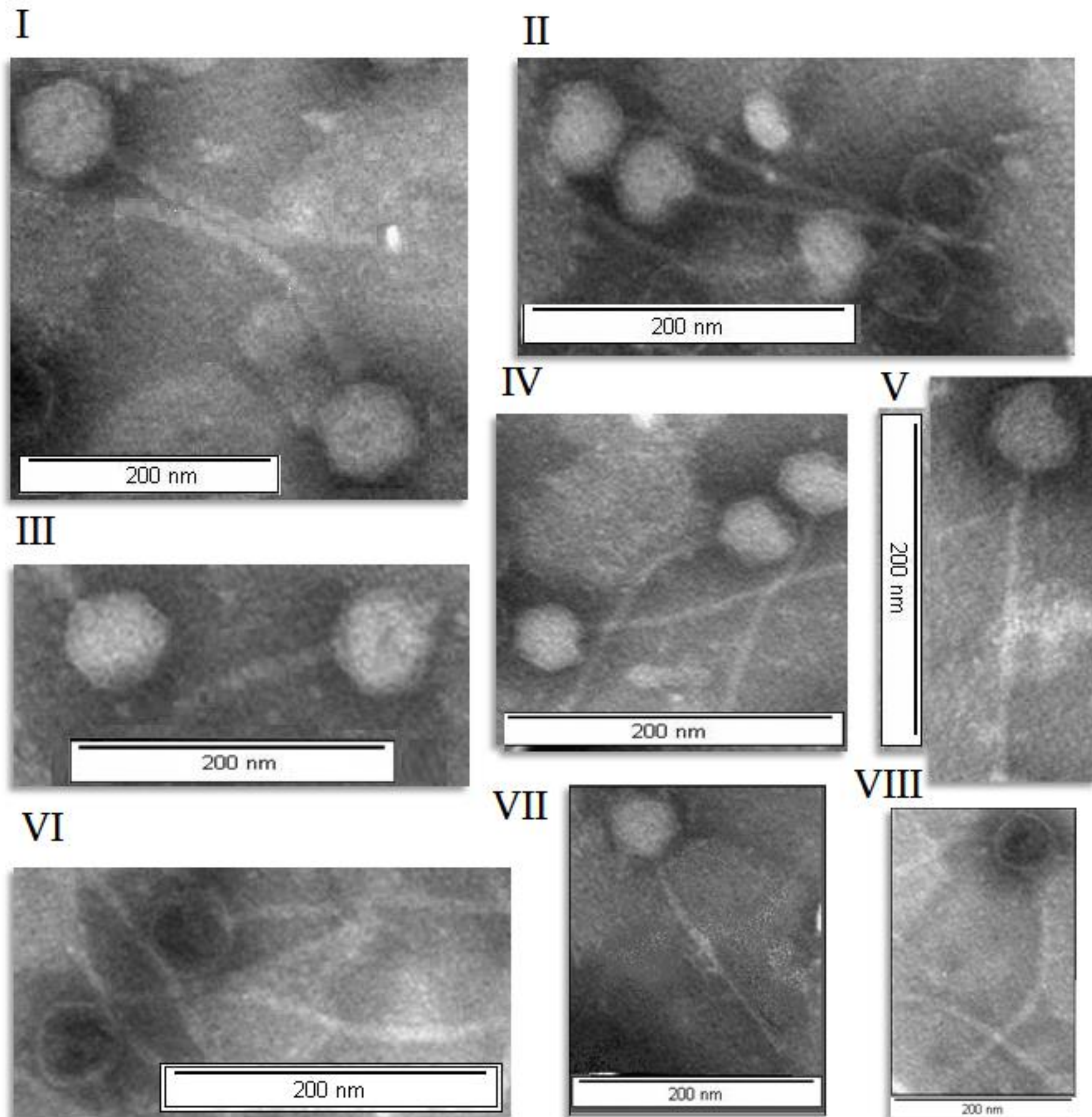

Figure 7.2: Transmission electron micrographs of bacteriophages, induced from members of the *Bacillus cereus* group, negatively stained with 2% uranyl acetate. (I) Phage Crookii and (III) AD2014 have a *Myoviridae* morphology with a thickened contractile tail in relation to head, while, all the other viruses: (II) DS2010, (IV) JB25, (V) Earthworm, (VI) A157, (VII) AD2011and (VIII)A168 have a *Siphovirus* morphology with long (≥150 nm), non-contractile, thin tails in relation to the capsid.

7.3.4. Molecular identification of bacteriophages

7.3.4.1. Whole Genome Analysis:

Genome assembly of the bacteriophages revealed different sizes of phage genomes. The genome sizes correlate with the two different families, Siphophages and myophages. The genome sizes of Siphophages reported in this study range from 30 kb to 172.5 kb. The 2 Myoviruses, phages AD2014 and Crookii, are 62 kb and 154 kb respectively (Table 7.3). The phages have an overall genomic guanine and cytosine (GC) content of ~36% with the exception of phage DS2010 at 40.3% and phage AD2014 at 44.6%. No RNA's were found in the genomes of the phages except for phages Earthworm and Crookii, both of which presented 2 RNAs namely; tRNA-Asn-GTT (Asparagine) and tRNA-Asp-GTC (Aspartate). The whole genome sequencing of the plaques isolated from *Salmonella enterica* ATCC 13311 during host range determination indicated the phage to be a *Salmonella* phage. Sequencing of the original phage AD2014 viral filtrate proved it to be a *Bacillus* phage and did not align with the *Salmonella* phage, although both belong to the family *Myoviridae*. This could indicate 2 possible occurrences: (i) the *Salmonella* phage was obtained from the original sample AD2014 and propagated as a contaminant alongside the *Bacillus* phage or (ii) the phage was present in *Samlonella enterica* ATCC® 13311. Since no *Salmonella* phage sequence reads could be found in either the mapped or unmapped reads of *Bacillus* phage AD2014, the latter is the more likely occurrence.

Table7. 3: General features of the genome sequence of the bacteriophages isolated from *Bacillus cereus* group of bacteria.

Bacteriophage	Sequence reads after trimming	Number of contigs	Genome size	G+C % content	RNAs	Number of coding sequences	Integrase or recombinase
Cherry	154 221	5	37 078	35.2	0	57	Recombinase
JB25	104 414	20	39 541	37.6	0	58	Integrase
DS2010	35 966	23	76 197	40.3	0	112	Integrase
DS2011	106 961	21	101 301	36.0	0	168	Recombinase
Earthworm	46 996	49	172 518	36.4	2	333	Recombinase
A157	92 905	7	35 813	35.2	0	50	Integrase
A168	69 488	13	30 815	36	0	57	Integrase
AD2014	56 856	9	62 189	44.6	0	78	Integrase
Crookii	3 670 390	1	154 012	37.2	2	235	None

7.3.4.2. Sequence Annotation

Annotation of whole genome sequences established commonality between the bacteriophages for functional coding regions in the form of major capsid proteins, tail fibre proteins, tape measure proteins, DNA packaging and replication proteins as well as lysis proteins (Table 7.4). Phages DS2010 and the diagnostic Cherry phage had similarities in coding regions for fosfomycin resistance as well as sporulation specific sigma factors (Sig F) (Table 7.4, Supplementary Data\Supplementary Data 7.1.xlsx). Phage DS2011 was the only other virus that possessed a SigF protein coding region. The recombinase family of proteins with site specific integrase regions were present for all the phages except lytic Phage Crookii (Table 7.3). The lytic phages Crookii and Cherry also lacked the proteins Rha, RecU, LexA and the phage transcriptional regulator, Cro/CI family, which is present in all the other phages. (Detailed annotation information is in Supplementary Data\Supplementary Data 7.1.xlsx).

7.3.4.3. Bacteriophage Genome Phylogeny:

The bacteriophage genomes grouped into the 2 families *Siphoviridae* and *Myoviridae* in the order *Caudovirales* (Figure 7.3). There is a large disparity in genome size between the viruses ranging from 30 kbp to 173 kbp which lends to the wide range of bootstrap values at the dendogram nodes. As a baseline, bootstrap values less than 30 were discarded for comparison. The topology of the dendogram shows diversity in viral strains belonging to both families. The Siphoviruses made up the majority of phages induced and are made up of 2 major subclades. Phage DS2010 is one such Siphovirus subclade forming part of the Cherry-like bacteriophages as seen by its proximity to the Cherry and Cherry branch of the tree. The second subclade is further subdivided. Phages JB25 and Earthworm form their own subgroup along with the less related *Bacillus* phage phi4B1. Phages A157, A168 and DS2011 form another subgroup with other *Bacillus* ACT phi bacteriophages. The Myoviruses which form the second broad clade are comprised of the aggressively lytic Phage Crookii and AD2014 *Bacillus* phage. The contaminating *Salmonella* phage isolated from host range suitability trials for AD2014 is also indicated for the purposes of quality control.

Table 7.4: The annotated coding regions and functions for genes found in the bacteriophages isolated in this study during whole genome sequencing analyses and when submitted for rapid server annotation (RAST).

Function	Annotated Coding Region	Description	Reference
Virion structural and assembly genes	Phage major capsid protein	The protein structure created around the scaffold which forms the head structure into which DNA is packaged	(Iannolo et al., 1995)
	Phage head maturation protease	Double-stranded DNA bacteriophages assemble their heads by scaffolding a procapsid then, the prohead undergoes a maturation process mediated by the protease which cleave the scaffold proteins and/or process the capsid proteins to expand into the final, mature head.	(Laemmli and Favre, 1973, Dokland, 1999)
	Phage tail length tape-measure protein	Conserved VNTR's which serve to anchor the auxiliary proteins used to scaffold the tail of a phage. The length of this protein correlates to phage tail length	(Belcaid et al., 2011)
	Phage tail fibers	The family of receptor binding proteins (RBP's) responsible for host cell binding sites during infection and host range determination	(Bishop et al., 1974, Le et al., 2013)
	Phage DNA packaging protein	Phage DNA packaging occurs by DNA translocation into a preformed protein capsid with the aid of this packaging enzyme or a terminase	(Fujisawa and Morita, 1997)
Genes involved in phage DNA replication	Helicase loader DnaI	DnaI proteins load the replicative ring helicase onto DNA during priming of DNA replication	(Ioannou et al., 2006)
	Phage integrase/recombinase	Phage integrases are site-specific recombinases that mediate controlled and precise DNA integration and excision.	(Smith and Thorpe, 2002, Fogg et al., 2014)
	Translation elongation factor G	EF-G is a large, five-domain GTPase that promotes the directional movement of mRNA and tRNAs on the ribosome in a GTP-dependent manner.	(Wintermeyer and Rodnina, 1999, Wintermeyer et al., 2001)

	RNA polymerase sigma-70 factor	Members of the σ70 family of sigma factors are components of the RNA polymerase holoenzyme that direct bacterial or plastid core RNA polymerase to specific promoter elements that are situated 10 and 35 base-pairs upstream of transcription-initiation points.	(Paget and Helmann, 2003)
	RNA polymerase sigma factor SigB	Replication pausing; the stationary-phase and general stress response genes are regulated by σ^B	(Kazmierczak et al., 2005)
	DNA helicase, phage-associated	DNA helicase allows the phage recombination machinery to drive branch migration through an RNA-polymerase promoter	(Salinas and Kodadek, 1994)
Genes involved in phage life cycle regulation	SOS-response repressor and protease LexA	Pleiotropic SOS response includes phenomena such as enhanced capacity for DNA repair and mutagenesis, inhibition of cell division and prophage induction. If previously induced cells are able to recover from the inducing treatment, the SOS functions disappear and the cells revert to a normal growth state. Accordingly, it is likely that the regulatory system is poised to respond in either direction, as needed, eg. Triggered by UV irradiation. i.e. LexA needed for lysogeny maintenance	(Witkin, 1976, Little et al., 1981, Little and Mount, 1982, Sozhamannan et al., 2008)
	phage transcriptional regulator, Cro/CI	the CI–Cro regulatory circuit is necessary for stable lysogeny	(Schubert et al., 2007)
	Phage Rha protein	The presence of this gene interferes with the infection of bacterial strains lacking the integration host factor that regulates the rha gene i.e. Rha is responsible for inhibiting host DNA synthesis	`(Henthorn and Friedman, 1995)

	RecU Holliday junction resolvase	RecU activity include: (i) cleaves Holliday junctions; which are cross-shaped structures that form during the process of genetic recombination, when two double-stranded DNA molecules become separated into four strands in order to exchange segments of genetic information and (ii) anneals complementary strands	(Carrasco et al., 2009)
	RNA polymerase sigma factor SigF	This sigma factor is responsible for the expression of sporulation specific genes	(Kazmierczak et al., 2005)
	Fosfomycin resistance protein FosB	FosB is a metallothiol transferase requiring Mg^{2+} and L-cysteine as cofactors. To confer resistance; expression of FosB also requires ςW to chemically inactivate fosfomycin/antibiotics	(Thompson et al., 2015)
Genes involved in lysis	Phage N-acetylmuramoyl-L-alanine amidase	This enzyme belongs to the family of hydrolases, a N-acetylmuramoyl-L-alanine amidase is an enzyme that catalyzes a chemical reaction that cleaves the link between N-acetylmuramoyl residues and L-amino acid residues in certain cell-wall glycopeptides a.k.a endolysin	(Iannolo et al., 1995, El-Arabi et al., 2013)
	Holin, toxin secretion/phage lysis	Holin oligomerization forms a pore in the host cytoplasmic membrane through which an endolysin can enter the cell	(Govind and Dupuy, 2012)

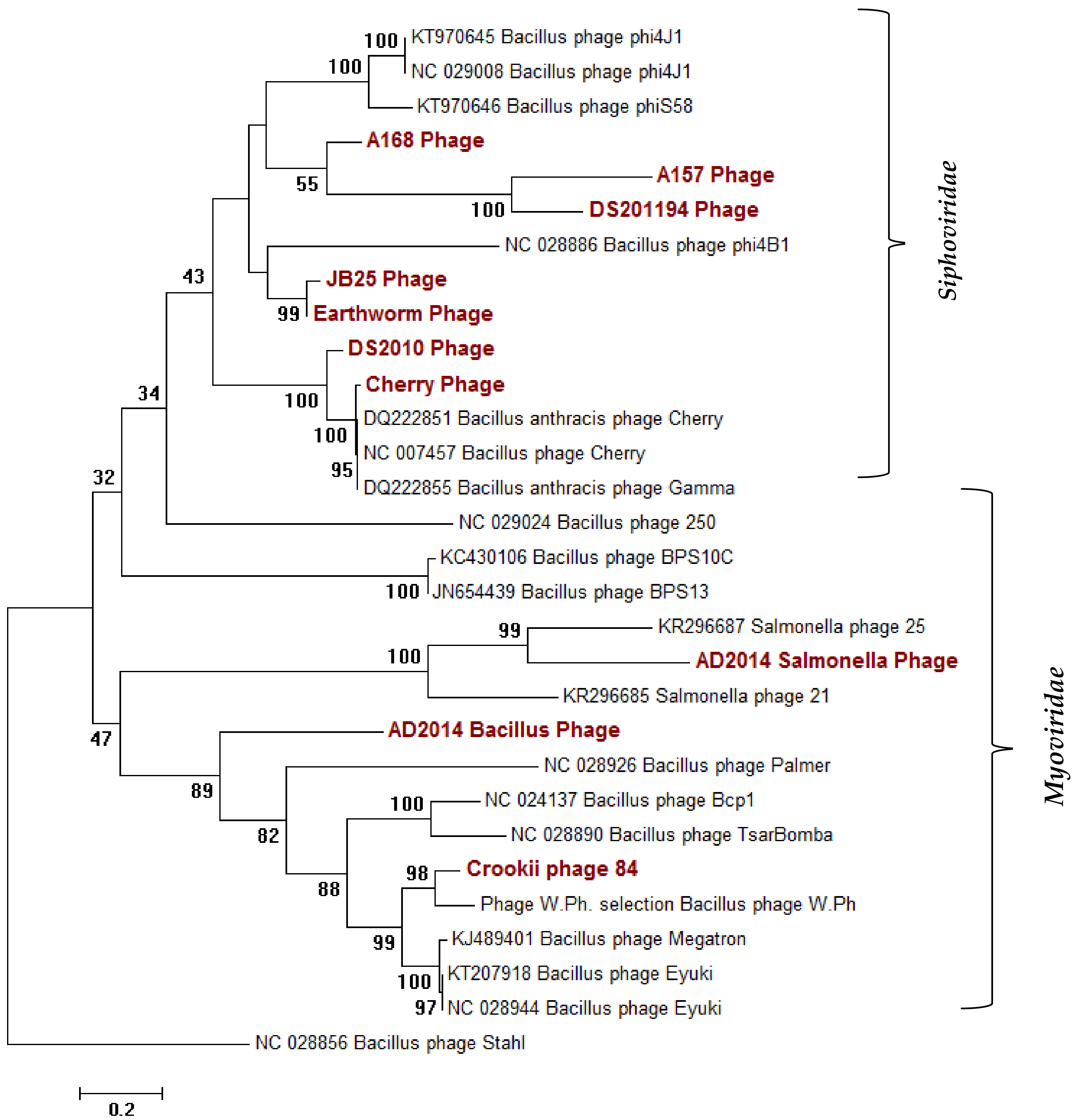

Figure 7.3: A Maximum Likelihood phylogenetic tree based on bacteriophage nucleotide sequences aligned using MAFFT tool (Katoh and Standley, 2013) with 1000 bootstrap replication in MEGA 7 including *Bacillus megaterium* phage Stahl as an out group. Due to the diversity of the sequences, bootstrap values ≤ 30 were discarded. Phages in red are isolates from this study; while those in black were included from NCBI based on Blastn similarity results.

7.4. Discussion

The bacteriophages (including the control phages namely Myovirus phage Crookii and Siphovirus phage Cherry) described in this study, represent a relationship between the members of the *Bacillus cereus sensu stricto* group. The majority of bacteriophages presented infectivity for *B. anthracis*, *B. cereus* and *B. thuringiensis* with the exception of the diagnostic phage Cherry and DS2011 which are specific for *B. anthracis* alone. Bacteriophages A157 and A168 are Siphoviruses induced from *B. thuringiensis*, obtained from swabs of vultures during an anthrax outbreak, but which demonstrate a greater lytic activity for *B. anthracis* and *B. cereus* than their original isolation host. No *B. anthracis* or *B. cereus* was isolated from the vulture swabs. Similarly, phage Earthworm induced from a *B. cereus* isolate from the gut of an earthworm also has an equal affinity for *B. anthracis* and *B. thuringiensis*. The remaining phages induced from *B. anthracis* culture also had this narrow host range. This was demonstrated in the host suitability assay, especially when *Bacillus* spp. from the same environment as the host species was isolated (Table 7. 2). The evidence of this host range is not a new phenomenon and has been well described since the 1930's (Cowles, 1931a, Brown and Cherry, 1955, Sozhamannan et al., 2008, El-Arabi et al., 2013, Gillis and Mahillon, 2014, Grose et al., 2014a, Klumpp et al., 2014).

In the identification of bacteriophages, virion morphology as well as whole genome analysis are a means of differentiating viruses from each other (Ackermann et al., 1978, Hendrix et al., 1999). The 'behaviour' of these phages also markedly differs; as can be observed from their plaque morphologies on Sterne lawns (Figure 7.1). Viruses like Phage Crookii are aggressively virulent/lytic, while phage Earthworm and DS2010 produce plaque morphologies more indicative of a lysogenic lifestyle. The plaque morphology for lysogeny is a turbid centre surrounded by a lytic ring (Sozhamannan et al., 2008). The phages also showed lytic activity 2 hours sooner in bacterial strains (*Bacillus* ACT) isolated from the same environment than the ATCC strains (Table 2).

Sequence similarities are generally weak due to the extensive diversity of tailed phages (Ackermann et al., 1995), but bacteriophages generally tend to contain a G+C content

equivalent to its host bacterium (Ackermann, 2004). The Bacillus cereus sensu stricto group contain 36.4 ± 0.6 % G+C regardless of the species (Kaneko et al., 1978). On average the bacteriophages in this study have a G+C content of 36 % which is equivalent to the host species, except for DS2010 at ~40 % and AD2014 at ~44.5 %. The phylogenetic topology of the phages used in this study grouped them according to the tailed viral families: *Siphoviridae* and *Myoviridae*. This is corroborated by the morphology on electron micrographs. More detailed comparisons of virions are based on protein sequence identity (Ackermann et al., 1995, Hendrix et al., 1999, Lavigne et al., 2009).

The genetic make-up of the two phage lifestyles, lytic versus lysogenic, differs according to machinery and mode of action required by the phage to propagate. The major difference between these 2 types of bacteriophages is the recombinase family of proteins which facilitates integration into the bacterial genome. The presence of a recombinase alone does not guarantee that the bacteriophage will employ a lysogenic lifestyle, it merely suggests that there is a potential for such an occurrence (Smith and Thorpe, 2002, Fogg et al., 2014). The transcriptional generators, repressors and recombinases work as an epistatic system in the integration of the phage genome with that of its bacterial host (Henthorn and Friedman, 1995, Sozhamannan et al., 2008). Phage Cherry is a good example of a phage which encodes a recombinase but enjoys a lytic lifestyle. Phage Cherry contains a site specific recombinase but has a virulent lifecycle as opposed to a lysogenic one. This is because phage Cherry is missing the Cro-CI regulator (Gillis and Mahillon, 2014) as well as proteins Rha, RecU and LexA (Table 4) which ensure successful insertion and maintenance of the prophage in the host genome. All the bacteriophages sequenced in this study (except phage Crookii: Chapter 6) contain recombinase or integrase coding regions. All the bacteriophages also contain protein Rha and the transcriptional regulator Cro-CI confirming these phages to be lysogenic. The majority of bacteriophages also bore the LexA protein except for phages Cherry, DS2010 and DS2011, which instead harboured SigF. This sigma factor is associated with the expression of sporulation genes (Kazmierczak et al., 2005). Even with this coding region, the bacteriophages did not appear to influence sporulation of the infected bacterial strains in any appreciable fashion (data not shown: based on malachite green staining of colonies at 1 hour time intervals over a period of 12 hours).

Prophages also confer other traits that influence the fitness of their host such as mutagenesis repair (Witkin, 1976, Little et al., 1981, Little and Mount, 1982) and antibiotic resistance (Thompson et al., 2015). The Cherry-like bacteriophages, in this case phages Cherry and DS2010, have the fosfomycin resistance gene FosB (Supplementary Table 4.1). All bacteriophages, whether employing a lytic or lysogenic infective lifestyle, eventually result in bacterial cell lysis (Ackermann et al., 1978, El-Arabi et al., 2013). As such, phages contain lysins such as N-acetylmuramoyl-L-alanine amidase that rupture the cell wall/peptidoglycan layer (Iannolo et al., 1995, El-Arabi et al., 2013). Holin proteins create the path through which these lysins can operate (Govind and Dupuy, 2012, Iannolo et al., 1995). When a lysogenic phage is induced, it can take host DNA with it from the terminal excision sites (Juhas et al., 2009). This poses a means of horizontal gene transfer between members of *Bacillus* ACT. The presence of the prophages themselves also give diversity to the bacterial strains (Sozhamannan et al., 2006).

7.5. Conclusions

There is a diversity of bacteriophages infecting *B. anthracis* and closely related *B. cereus* and *B. thuringiensis* in the anthrax endemic regions of South Africa. These phages could be induced through an SOS response by ultraviolet irradiation of the bacterial host. Phages associated with anthrax outbreaks only grouped into two families for this study: *Myoviridae* and *Siphoviridae*. The Cherry-like phage DS2010 encodes the (FosB) fosfomycin resistance gene to protect its host from premature lysis. This trait, as well as the sporulation influencing sigma factors, demonstrates the potential of phages to influence the fitness of their host bacterium. Although none of these effects were observed to be expressed in infected host bacterial strains during this study. The broad host range of these lysogenic phages also demonstrates the potential for horizontal gene transfer between the closely related bacteria of the *Bacillus cereus* group. Ultimately however, the lysis of the host is the end result and a role player in the reduction of bacterial inoculum in the environment.

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8. Chapter 8

General discussion, conclusions and recommendations

The purpose of this study was to investigate selected anthrax outbreaks and the quantifiable environmental influences at play (on *B. anthracis*) during these outbreaks. In terms of disease epidemiology, a case of a reportable zoonotic disease can be counted as an outbreak. For the purpose and framework of this discussion, isolated anthrax cases are not considered as an outbreak while large outbreaks are defined as multispecies epizootics within a region. This study has been entirely dependent on cases submitted to/through the state services and the reports generated of these suspect anthrax cases. The study of *B. anthracis* and its longevity in the environment during and after anthrax outbreaks is complicated by many external factors. These factors are discussed below.

8.1. Anthrax on the Ghaap Plateau

The two endemic regions in South Africa are the NCP and KNP. The most prominent difference between these regions is that in NCP anthrax is a controlled disease whereas in KNP it is a controlled disease but since it is in a national park it is considered part of the natural cycle in wildlife (Chapter 2). Thus anthrax outbreak trends differ between the NCP and KNP. Prior to 2008, the largest anthrax outbreak in NCP was in 1998 based on the sample registry at the state reference laboratory (Onderstepoort Veterinary Institute). The NCP has not experienced large anthrax outbreaks since 2009 (according to state annual reports) to present day (Nduli, 2009). The NCP is divided by fenced, mixed - use farms (game and livestock) that are managed using vaccination schemes for livestock and restricted livestock movement. The wildlife species most affected in NCP are kudu followed by zebra (Hugh-Jones and de Vos, 2002, De Vos and Turnbull, 2004).

The “vectors” implicated in the spread of anthrax also differ between KNP and NCP. In NCP, both blowflies and louse flies (*H. rufipes*) have been observed at anthrax carcass sites since the late 1800’s (Viljoen et al., 1928, Howell et al., 1978, De Vos and Turnbull, 2004),

which was also noted in the 2008 outbreak. Louse flies are blood sucking flies (Howell et al., 1978). The ~200 *B. anthracis* spore count per louse head, obtained during this study, is the first documented record for this arthropod species thus the potential exists for mechanical transmission. Viljoen (1928) and Henning (1949) presented circumstantial evidence to support this likelihood in horses demonstrating atypical oedema. They described swelling concentrated around the flies' bite sites and identified *B. anthracis* from both the Hippobosca and the horses. It was noted that flies swarmed on ill animals and thereafter epizootics were observed, owed in part to the gregarious nature of these animals. Anthrax associated subcutaneous oedema has been similarly described in horses in Italy where tabanids are believed to be the culprit (Fasanella et al., 2010a, Palazzo et al., 2012).

The Ghaap Plateau in NCP is a dolomitic escarpment marked by phosphorous deficient soils which leads to pica in grazers and browsers (Theiler, 1912, Theiler, 1927, Boyazoglu, 1973, Partridge et al., 2010). Pica presenting as osteophagia and geophagia are behavioural characteristics in response to nutritional deficiencies (Wilson and Hirst, 1977, de Vries, 1994). In the 2008 anthrax outbreak, the index cases on 4 of the farms were reported to be gestating kudus. There is insufficient data to conclude that pica could be the route to infection in these cases, however, it presents a reasonable hypothesis after high rainfall when these grasslands and bushveld are known to be mineral deficient. A study in Etosha National Park (ENP) suggests that soil ingestion is a seasonal behavioural characteristic which increases the risk of exposure in areas historically endemic for *B. anthracis,* but is not the only contributing factor to infection (Turner et al., 2013). This does not rule out water as a carrier of *B. anthracis*. Further investigation of vectors and other factors influencing anthrax outbreaks in the NCP is required. Statistical modelling which can account for rainfall, temperature, soil, geomorphology, stocking rates, physiological cues and vegetation along with surveillance data could give us a more objective understanding of anthrax in the Plateau region, NCP.

It has been well documented that environmental concentrations (determined through isolations of *B. anthracis*) do not compare to the experimentally determined lethal doses required for an infection. The LD_{50} for impala is 15 million spores in an oral dose (Turnbull, 2008), although the spore concentrations in soil at natural water depression sites in NCP during the 2008 outbreak were only $5x10^4$ and $1.2x10^4$ cfu/g respectively (Chapter 2). The

State Veterinary Service intervention, in terms of carcass disposal (burning, wrapping and disinfection), vaccination and heightened surveillance for anthrax cases in 2008/2009 outbreak served to stem the spread of the outbreak well into 2009 in NCP. No *B. anthracis* was isolated from the bones of disinfected carcasses in 2012 which means that these sites were not a source of infection thereafter. The 2012 survey failed to identify *B. anthracis* along the dry river beds or at sites surrounding the 2008 carcass points. The inability to isolate *B. anthracis* in the soil, carcass sites and sediment deposition sites in 2012 can be attributed to a possible dilution effect, carcass disposal and/or inability of spores to survive in environment. It has been noted in a number of studies that spore counts in the soil decrease over time (Turnbull, 1999, Bellan et al., 2013, Turner et al., 2016). While the precipitator(s) of the anthrax outbreak in 2008 are still unclear; the outbreak provided insights into site processing during an outbreak. Spore counts from carcass bone indicated the mandibular bone, orbital process and rib bones as the best source of *B. anthracis* during bacteriologic diagnostics for the disease.

With regards to vaccination as a control measure, this has a long term benefit rather than an immediate effect. Vaccinated animals require ≥ 52 days before an appropriate immune response is registered and the animal is protected (Turner et al., 1999). Antibiotic prophylaxis of staff and valuable stock within the outbreak zone is more effective in protecting the humans and animals exposed to the bacterium (Brookmeyer et al., 2003, Turnbull, 2008). In Australia the vaccination scheme was effective in stemming an outbreak over time when applied to encircle the outside of the outbreak zone as a 30km wide buffer zone against the disease's spread in livestock (Turner et al., 1999). Vaccination using the live spore Sterne vaccine was used as a control measure of livestock during the 2008/2009 NCP outbreak which prevented the use of antibiotic prophylaxis. The NCP consists of livestock and wildlife (with the latter unprotected i.e. not vaccinated or treated with antibiotics) populations, varying in density in the endemic area, which continues to evade existing control measures and has the highest economic impact in the region.

8.2. Anthrax in the Kruger National Park

8.2.1. Surveillance

Anthrax is considered a natural ecological cycle in the KNP. This study has been based on submissions from the Skukuza State Veterinary surveillance and sampling system. The existing surveillance system in the KNP is a continuous source of data from endemic and non-endemic areas and thus increases our understanding of anthrax as a disease in this region. The environment (geology, hydrology, biomass and census data) is also constantly being monitored and updated by SANparks Scientific Services (https://www.sanparks.org/parks/kruger/conservation/scientific/). There is however some bias in the data. The sampling is confined within the borders of KNP and does not include the transfrontier parks. Blood smears and samples can only be collected from accessible carcasses that are discovered. The presence of predators and logistically inaccessible carcasses (e.g. hippopotamus carcasses in crocodile infested water) introduces a sampling bias. Also, surveillance teams are more vigilant and active once anthrax cases have been confirmed in a section. Nevertheless, with over three decades of surveillance data, the KNP anthrax ecological suitability model (Steenkamp, 2013) has proved a successful and invaluable tool in predicting large outbreaks based on environmental cues. The database itself informs on the predominant species affected in each outbreak and the spread of cases over time. The observations of technologists in the field are also logged in reports and give context to each site and animal behavioural ecology in each ranger section (Chapter 4).

8.2.2. Bacteriologic Isolations

Standard sample processing isolation protocols do not always yield bacterial isolates, even when qPCR diagnostics together with microscopic analyses indicate the presence of *B. anthracis* (Chapter 3). These samples provide insights into environmental influences that affect isolation success rates. The hippopotamus samples that were negatively impacted by heat treatment during isolation are such an example. Hippopotamus samples are difficult to isolate as no *B. anthracis* isolate from hippopotamus has been reported and thus have a generally low isolation success rate (Turnbull, 2008, Lembo et al., 2011, Hang'ombe et al., 2012). Other pachyderms like elephant and rhinoceros are also difficult to isolate *B. anthracis* due to the advanced putrefaction normally observed in these samples. Alternative protocols

have been suggested in Chapter 3 to overcome germination inhibition factors mainly focussing on using different growth media. Non selective media (blood agar) aids spore germination despite the presence of inhibitory free fatty acids. Blood agar also can be successfully used for *B. anthracis* isolation from leaves. Selective media such as TSPBA and PET provide equivalent results for environmental and biological samples such as soil and putrefying tissue and are superior to the traditional PLET (Chapter 2 and Chapter 3).

8.2.3. Blood Smears

Blood smears are the cornerstone of the surveillance system. The Giemsa stained smears are an important part of the first phase of the (multifactorial) diagnostic system in KNP. For blood smears that are either positive or inconclusive for the presence of *B. anthracis* bacilli on visual appraisal, *B. anthracis* specific qPCR diagnostics targeting the SASP, CAP C and PA- are used as an additional confirmation. The use of PCR together with microscopic analysis and bacteriologic confirmation is essential for accurate diagnostics in anthrax outbreaks (Turnbull, 1999, Berg et al., 2006). The unstained smears are stored indefinitely and a resource for retrospective study. The 2010 anthrax outbreak smears (Chapter 4) were used for both qPCR based SNP profiles and bacterial isolates. The resulting isolates were further characterised using MLVA (Chapter 5), since the SNP profiles only highlighted 2 broad lineages in effect during the outbreak. Whole genome sequencing of the bacterial isolates is currently being employed to identify SNP markers unique to the region (Kgaugelo Lekota: unpublished data). A region specific panel will produce more worthwhile lineage data for future genotyping from smears and biological samples.

Turner et al. (2013b) described the role of helminth infections and ectoparasite burden in immunosuppression and the seasonality of anthrax infections. For future studies; the DNA from the 2010 anthrax positive unstained blood smears can be used in the diagnostics of rickettsial and haemoparasitic co-infections. The inclusion of diagnostics from non-endemic areas will aid in the creation of species specific stress response profiles for comparison across KNP. This may shed light on the seasonality of outbreaks in KNP. With the increased feasibility, advances in sequencing technology and analyses software, metagenomics studies can improve the host/co-infection studies and identify other stresses that may contribute to species specific anthrax infection (Pallen, 2014).

8.2.4. Molecular Characterisation of *Bacillus anthracis*

Molecular genotyping techniques are epidemiological tools to aid in tracing the origin and spread of disease outbreaks. MLVA is one such technique that has been used in monomorphic bacteria such as *B. anthracis* (Keim et al., 2004, Ciammaruconi et al., 2008, Lindstedt et al., 2012). In Chapter 5, MLVA is used to distinguish genotypes from outbreaks in relation to the distribution over time from data spanning 4 decades. It is apparent that in KNP, which has the largest genotypic variation (A clade and B clade), that there is a trend towards a predominant genotype in each outbreak section. This can be attributed to the free movement of animals within the park and thus the greater exposure risk. Singular genotypes are distributed outside the dominant foci, but differ by only 1-3 VNTR markers. This shows localised VNTR marker evolution from 1 outbreak to the next. In contrast the NCP has dispersed multiple unique genotypes confined to each farm. These findings echo a similar pattern to Namibia where clonal genotypes predominate in the ENP and diverse genotypes are isolated from farming areas (Beyer et al., 2012). In the 2012 and 2013 outbreaks, multiple MLVA genotypes were isolated from a single roan carcass in KNP. The multiplicity of genotypes within a single host is not an uncommon phenomenon (Beyer and Turnbull, 2009). The differences in marker evolution between distant regions makes MLVA data perfect for spatio-temporal analyses in the KNP and NCP context, which is the next step in modelling analyses for South Africa.

The drawback of MLVA is the homoplasy associated with phylogenetic differentiation (Achtman, 2008). Most studies include SNP's to determine lineages in combination with MLVA for genotypic differentiation (Kenefic et al., 2008, Derzelle et al., 2011, Birdsell et al., 2012, Khmaladze et al., 2014). The SNP markers are region specific and the available panels are limiting for the diversity of strains in southern Africa (Chapter 3). For this reason and the increased feasibility, SNP's from whole genome analyses is now the preferred method for genotyping *B. anthracis* (Pearson et al., 2004a, Girault et al., 2014).

8.3. *Bacillus anthracis* in the environment

8.3.1. Bacteriophages

The bacteriophage is most abundant organism on earth (Ackermann, 2011). The temperate phages are the most numerous in the *B. cereus* group phages isolated in South Africa. Only one lytic phage was isolated during this study (Chapter 6), namely phage Crookii was isolated from a carcass in endemic Pafuri. This phage was only identified due to the aggressive nature of its lysis as the agar plates consistently appeared sterile. Lytic phages could easily be overlooked during bacteriologic isolation of *B. anthracis*.

Schuch and Fischetti (2009) propounded that bacteriophages in the ground (worm gut) were able to mediate sporulation factors within the bacterium to allow persistence and multiplication of *B. anthracis* in the environment. Attempts at mimicking the carcass and natural conditions did not yield such a response from *B. anthracis* strains when infected with temperate bacteriophages (Chapter 7). Whole genome analysis of the phages did reveal the genetic potential to influence sporulation efficiencies and antibiotic resistance. The bacterium showed no phenotypic response to either the temperate or lytic phages in terms of fitness traits during this study. Even the 4 prophages within the bacterial genome (Klee et al., 2006, Sozhamannan et al., 2006) failed to elicit a defence response to the lytic Phage Crookii under carcass simulated nutrient conditions. The ability of the phages to infect multiple members of the *B. cereus* group does allow for the possibility of introducing diverse genetic material into its host. It also suggests that the phage can propagate between anthrax outbreaks (Gillis and Mahillon, 2014). A constraint in the study of *B. anthracis* is that propagation of the phages is carried out in Sterne $34F_2$ due to safety protocols. It could be a limiting factor when evaluating the bacteriophage activity that the pathogenic hosts from the phage's native environment are not employed. The end point of bacteriophage infection is lysis irrespective of the phage's lifestyle (Ackermann, 2011). The natural role of reducing the inoculum in the environment is observed and plays a vital role in the ecology of the disease.

For future work, phage propagation in liquid medium should include regular optical density readings to determine the rate of killing of host cells, especially for lytic phages. This

is a means of determining the lytic efficiencies of the phages (Young, 1992, Capparelli et al., 2007). This would be worthwhile in the selection of bacteriophages to screen for lytic proteins for the purpose of biofriendly disinfection or antimicrobial purposes (Wang et al., 2000, Young et al., 2000). For phages that include genes that code for sporulation, growth and antibiotic resistance; total nucleic acid extraction should be performed at the bacterial exponential growth phase and thereafter at the lag phase. Transcriptome analyses could then shed light on the phage-host interactions and its effect on bacterial fitness (Clokie, 2009, Leskinen et al., 2016).

8.3.2. The Arthropod Effect

Braack (1987) has described the entomological activities around carcasses in Pafuri in extensive detail. It was determined from these studies that the adult carrion beetles and such arthropods were not key disseminators of *B. anthracis*. The isolation results from this study support these findings. It has however, become apparent from the high internal bacterial/spore counts that the larvae do have a role to play. This role is unclear as the ultimate fate of the spores in the environment is unknown. The larvae could be responsible for the inoculum "dilution effect" around carcass sites, since they migrate away from the site as they mature. They could also serve as mini incubation sites as was suggested by Schuch and Fischetti (2009) or as biofilm agents (Lee et al., 2007). A large proportion of the larvae at carcass sites belong to the Calliphora family. A study by Graham-Smith (1914) on *Musca domestica* and *Calliphora erythrocephala* larvae fed *B. anthracis* spores demonstrated an exponential decay in isolation success of the bacterium over time. Colony counts were not given, although *B. anthracis* colonies were obtained up to 19 days after emerging. Also; isolates were predominantly isolated from external parts of the flies. There are physiological and behavioural differences between fly species. Blowfly activity in northern KNP during anthrax outbreaks has been well described (Braack and De Vos, 1990). The *Chrysomya* spp. are most active in Pafuri and differ in their feeding habits. The spore counts from this study will be documented along with the arthropod behavioural ecology at the individual carcass sites as a descriptive base for the dissemination of *B. anthracis*. Further studies, which include in depth sampling, are required to elucidate the fate of the bacterium thereafter.

8.3.3. Dissemination versus Transmission

There has been great debate over the topic of dissemination and transmission in the study of anthrax. As stated earlier, the *B. anthracis* concentrations in the environment do not lend toward the experimentally derived lethal doses (Turnbull, 2008). The discrepancy between these concentrations and our lack of understanding of the empirical factors that precipitate the index cases in the environment has led to conflicting views on the true drivers and vectors of this disease. The study of flies as disseminators and transmitters of *B. anthracis* is one such debate. Dissemination does not necessarily equate to transmission. Although, there is sufficient anecdotal evidence to support the role of flies in the transmission of anthrax (Viljoen et al., 1928, Henning, 1949, Howell et al., 1978, Palazzo et al., 2012, Blackburn et al., 2014) there is conflicting data (Fasanella et al., 2010b, von Terzi et al., 2014) or insufficient published empirical data to resolve the ambiguities. During this study, the aim was to produce quantifiable, empirically derived data. In reality, each carcass site has its own unique environmental influences and these factors (observable and microscopic) are too numerous to quantify. The wilderbeest carcass that produced Phage Crookii is such an example. Bacteriophage populations are numerous, ubiquitous and diverse. The influence of plant derived antimicrobials on the survival of *B. anthracis* on leaves is another example. Further studies are required with well documented observational data along with empirical data to determine which factors are merely disseminators and which result in transmission of this disease.

8.4. Conclusion

In the control of anthrax, as previously described, the disinfection/disposal of infected carcasses lowers the exposure risk and hence is an effective method to halt the dissemination of *B. anthracis* at a carcass site. The vaccination of animals within an outbreak is a long term control measure, but given time is also a worthwhile step in stemming the advance of the disease.

In the face of waning vaccination programs, the surveillance for diseases like anthrax is an important early warning system to prevent epidemics. In the diagnosis of anthrax, multifactorial diagnostics which include microscopy, molecular assays and bacteriologic

techniques are essential to the validation of results. The collection of blood smears, soil, swabs and rib/mandible/orbital bones are ideal for diagnostic purposes. The concurrent use of selective and non-selective media increases the isolation success rates of *B. anthracis*, especially for environmental samples.

The soil born ecological cycle of *B. anthracis* is still poorly understood. Bacteriophages are just one aspect of this cycle. Lytic bacteriophages serve as a natural disinfection agent at carcass sites. The temperate phages in this study did not have any observable effect on bacterial fitness traits, although the genetic potential is present.

The spore counts from blowfly studies in KNP support the hypothesis of the involvement of flies in the transmission of anthrax, however, further evidence is required to make it absolutely conclusive. The activity and bacteriologic isolation of arthropods at carcass sites needs to be explored in more detail to understand their role in the dissemination or "disinfection" at carcass sites.

In the KNP, with the aid of the suitability model (Steenkamp, 2013), observational data and empirically derived data from this study, we have come to the conclusion that a specific set of factors serves as the trigger to an anthrax outbreak within an ecosystem. No two systems are the same and a great deal of further study is required to understand the ecology of anthrax. However; from our studies we know that anthrax outbreaks in KNP belong to 4 broad SNP lineages (A.Br005/006; A.Br003/004, B.Br001/002 and B.Br.Kruger). These lineages represent diverse genotypes as determined by MLVA, but clonal genotypes have been observed within an outbreak and region. The genotypic diversity is observed across watersheds and ecological niche sections, but water presents a significant disease driver within sections. The Kruger B clade isolates have not been isolated in recent decades but the rare B.br001/002 clade has been identified in 2011. The A clade is thus maintaining its predominance in South Africa.

8.5. References

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