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Genotypic diversity of *Bacillus anthracis* from 2014 to 2015 in the Kruger National Park

by

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Submitted in partial fulfilment of the degree:

MSc (Animal/Human/Ecosystem Health)

Department of Veterinary Tropical Diseases

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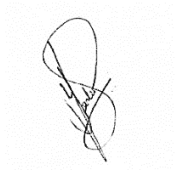
Co-supervisor: Ms Ayesha Hassim

DECLARATION

I, Frank Venter, hereby declare that this thesis submitted for the degree, MSc (Animal/Human/Ecosystem Health) 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 tertiary institution.



2016-08-05

SIGNATURE **DATE**

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Animal Ethics Committee

PROJECT TITLE	Distribution of Bacillus anthracis genotypes in the Kruger National Park, South Africa
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STUDENT NUMBER (where applicable)	UP_04403843
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KINDLY NOTE:

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

APPROVED	Date	30 October 2015
CHAIRMAN: UP Animal Ethics Committee	Signature	

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LIST OF ABBREVIATIONS

°C……...……Degrees Celsius

µL…….Microliter

µM…….Micromolar

AIDS...…….....Acquired immune deficiency syndrome

BSL……..Biosafety level

cAMP…….....Cyclic adenosine monophosphate

canSNP…….....Canonical single nucleotide polymorphism

CFU……Colony forming units

CFU/ml…….Colony forming units per millilitre

DALY……..Disability-adjusted life year

DNA…….Deoxyribonucleic acid

EF……..Oedema factor

ENP……Etosha National Park

FAO……..Food and Agriculture Organization of the United Nations

FRET…….Fluorescence resonance energy transfer

GI……Gastrointestinal

GLTP……...Great Limpopo Transfrontier Conservation Park

GPS……Global Positioning System

HIV…..……..Human immunodeficiency virus

KNP……...Kruger National Park

km……....kilometres

LF…….Lethal factor

LSU……Louisiana State University

MAPKK……...Mitogen-activated protein kinase kinase

mg/ml……..Milligram per millilitre

min…….minute

MLVA…….Multi-locus variable number of tandem repeats assay

mM……..Millimolar

MST…….Minimum Spanning Tree

n…….Sample size

NZD……..Neglected zoonotic disease

OIE…….World Organisation for Animal Health

PA……Protective antigen

PCR……..Polymerase Chain Reaction

sec…….Second

SNP……..Single nucleotide polymorphism

SNR……...Single nucleotide repeat

spp……Species

TFCA……..Transfrontier conservation area

UPGMA…….Unweighted pair group method using arithmetic mean

USA…….United States of America

US$.....United States Dollar

VNTR……...Variable number of tandem repeat regions

WHA…..……World Health Assembly

WAHID……..World Animal Health Information Database

WHO…….World Health Organization

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THESIS SUMMARY

Genotypic diversity of *Bacillus anthracis* from 2014 to 2015 in the Kruger National Park

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Anthrax is one of the most well-known, yet underestimated zoonotic diseases in the world, remaining endemic on the African continent. Here the disease not only impacts upon the health of animals and humans, but also on the livelihoods of rural communities. Even so, the disease remains neglected in terms of funding and attention, which results in the under-reported of anthrax in Africa. This study aims to discuss the reasons for under-reporting of anthrax, and the factors impeding anthrax control in Africa. We provide an updated distribution map of anthrax-endemic regions across the continent to investigate under-reporting. Furthermore, we highlight the benefits which could be gained by researchers, in Africa and abroad, through enhancing the diagnostic and research capabilities on the continent. Special attention is given to multi-locus variable number of tandem repeat assay (MLVA), a genotyping technique which has already delivered much insight into the dynamics of anthrax disease, and the diversity of *Bacillus anthracis*.

This study employed MLVA to provide insight into the genotypic diversity of anthrax in Kruger National Park (KNP), and the potential mechanisms which drive such outbreaks.

To achieve this goal, we utilised MLVA, employing 31 markers, to investigate anthrax outbreaks from 2014-2015 in KNP. Briefly, isolates were confirmed to be *B. anthracis* using classical microbiology and real-time PCR investigation. Extracted *B. anthracis* DNA was subjected to multiplex PCR, with the resultant fragments being separated by size using capillary electrophoresis. These data were then utilised to cluster *B. anthracis* into lineages, and genotypes. *Bacillus anthracis* isolates (n=81) were obtained from carcasses and the environment, in the Pafuri, Houtboschrand and Mahlangeni regions of the KNP, between 2014 and 2015. All isolates belonged to the A-clade, consistent with findings in the KNP since 1990. Phylogenetic analysis indicated that the isolates belonged to five distinct genotypes, with genotypes 1 and 2 (GT1 and GT2) dominating, present in both 2014 and 2015. GT1 and GT2 belonged to the A1 sub-clade, and the A.Br.005/006 single nucleotide polymorphism (SNP) sub-group. GT3, belonging to the A3 (SNP A.Br.Aust94) sub-clade, has been reported in the KNP in previous outbreaks, and was isolated from vulture faeces in 2015. GT4 (P15-53) and GT5 (P15-54), each representing a single isolate, presented as atypical *B. anthracis*. Three VNTR markers, including the virulence factor on pX01, could not be detected in P15-53, while two VNTR markers could not be detected in P15-54. An additional investigation, into the potential of using an MLVA7 protocol as a first line assay, was performed. The results indicated that while such a protocol was capable of distinguishing between the 5 genotypes present in this study, it could not differentiate between A and B-clade isolates.

Investigation of *B. anthracis* isolates from the environment, in the vicinity of anthrax-infected carcasses, provided insight into the complex epidemiology of the disease. Firstly, we provided evidence supporting necrophagous fly transmission of anthrax, since isolates collected from carcasses, blowflies, and vegetation in the vicinity, were of the same genotype. Secondly, we investigated the distribution of GT1, with two isolates (H15-01 & M15-01) occurring in Mahlangeni, and Houtboschrand, between 150 km and 200 km away from of the main outbreak location in Pafuri. Dissemination of GT1, by for example water-related dispersal of *B. anthracis*, was eliminated due to the topography of Pafuri, Mahlangeni, and Houtboschrand, as well as the distance involved. Alternative explanations are either that GT1 was dispersed over this distance by vultures, or that GT1 had been present in all three areas and had not been detected previously. Finally, we report here the first evidence of *B. anthracis* being shed in the milk of two *Aepyceros melampus* (impala) females.

CHAPTER 1

1.1 GENERAL INTRODUCTION

Neglected zoonotic diseases (NZD), which includes bovine tuberculosis, brucellosis and anthrax, disproportionately affects the developing world. These diseases do not receive adequate attention, or funding, even though they present a significant burden on the health of human and animal populations (WHO, 2006; Hotez et al., 2007). In addition to their healthcare burden, NZD also impedes upon the sustainable livelihoods of rural populations who depend on livestock as a source of food and financial security (Molyneux et al., 2011). This problem is exacerbated in Africa, were public-and-veterinary healthcare services are often strained by a range of factors. As a consequence, the true burden NZD is often underestimated on the continent, which promotes the idea that such diseases have a negligible impact. Given the changing landscape of Africa, either through human population growth or the establishment of transfrontier conservation areas (TCFA), NZD are likely to become increasingly important in future (Woolhouse & Gowtage-Sequeria, 2005; Thomson et al., 2013). This is indeed the case for anthrax, an NZD which remains endemic across much of Africa.

Anthrax is a disease of humans and animals, which is caused by the Gram-positive bacterium *Bacillus anthracis*. The organism has the ability to form spores when presented with unfavourable conditions, and can persist in the environment for extended periods (de Vos & Bryden, 1998; Driks, 2000). Animals become infected through the ingestion or inhalation of spore-contaminated vegetation or water sources (WHO, 2008). Humans, however, are primarily infected through handling of infected carcasses or animal products (WHO, 2008). Much of complex epidemiology of anthrax remains poorly understood, with evidence implicating a blowfly-transmission pathway, spore-dispersal by scavengers, and *B. anthracis* interactions with plants and amoebae (Pienaar 1961; Turnbull et al., 1989; Braack & de Vos, 1990; Saile & Koehler, 2006; Schuch & Fischetti, 2009; Dey et al., 2012). Bacterial genotyping, using multi-locus variable number of tandem repeat analysis (MLVA), has the potential to answer many of the question regarding *B. anthracis* epidemiology. A number of MLVA protocols have been used to investigate the genotypic diversity of *B. anthracis*, and includes systems targeting eight, 15 and 20 different marker regions (Keim et al., 2000; Le Fleche et al., 2001; Lista et al., 2006; Van Ert et al., 2007). Recently, a highly

sensitive 31 markers (MLVA31) system was utilised to investigate *B. anthracis* diversity and epidemiology in Namibia (Beyer et al., 2012).

Anthrax is endemic in the Pafuri region of the Kruger National Park (KNP), and parts of the Northern Cape, South Africa (Steenkamp, 2013). Genotyping of *B. anthracis* isolates in Pafuri, had previously been performed using MLVA targeting eight markers (Smith et al., 2000). The results indicated significant level of diversity, with isolates from both the A-clade and the B-clade being implicated (Smith et al., 2000). The drivers of anthrax outbreaks in the park, however, remain unknown. Blowflies have been hypothesised to contribute to the temporal dispersal of *B. anthracis*, given the high number of *Tragelaphus strepsiceros* (greater kudu) deaths related to anthrax in KNP (Pienaar, 1961; Braack & de Vos, 1990; de Vos & Bryden, 1995). Scavenging species have also been implicated in the contamination of water sources with anthrax, following feeding on infected carcasses (Dixon et al., 1999).

1.2 RESEARCH OBJECTIVES AND OUTLINE

The primary focus of this project, was to utilise the MLVA31 protocol to investigate the *B. anthracis* isolates implicated in anthrax outbreaks, between 2014 and 2015, in the KNP. A total of 81 *B. anthracis* isolates were obtained from carcasses, and the environment, from outbreaks in the Pafuri, Houtboschrand and Mahlangeni regions of the KNP. The dissertation first provides background and justification, with research objectives and outline in Chapter 1. Chapter 2 provides a literature review of anthrax, focussing on the challenges faced in controlling the disease in Africa, and the use of MLVA in the study of *B. anthracis*. Chapter 3 relates to the genotypic and epidemiological investigation of *B. anthracis* isolates obtained from outbreaks in the KNP, between 2014 and 2015. The specific aims addressed in the latter two chapters were as follows:

1) To provide an updated map of anthrax endemicity on the African continent;
2) review the challenges faced in controlling anthrax in Africa, and to tease apart the factors contributing to the under-reporting of anthrax;
3) provide a review of MLVA and the potential to aid in anthrax research in Africa;
4) characterise *B. anthracis* isolates obtained in this study using MLVA31;

5) investigate the mechanisms behind anthrax outbreaks in the KNP, between 2014 and 2015.

1.3 JUSTIFICATION

The KNP is bordered, to the west, by a growing rural population. Here livestock and wildlife can interact, bringing about the potential for disease transmission. Anthrax not only poses a risk to the health of humans and their livestock, but also threatens the sustainable livelihoods of these communities. In addition, the KNP houses several valuable wildlife species, such as *Hippotragus equinus* (roan antelope), which have in the past been affected by anthrax outbreaks (de Vos & Bryden, 1998). Of growing concern, is the establishment of a transfrontier conservation area (TFCA), spanning the borders of Mozambique, South Africa and Zimbabwe, which has unknown consequences for anthrax management in the area. The management and surveillance of a disease is impossible without a thorough understanding of the etiological agent involved. There is thus a need to investigate the mechanisms behind anthrax outbreaks in the KNP, and assess the risk to wildlife, livestock and humans. Data generated from MLVA31 genotyping will contribute significantly to answering many of these questions.

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CHAPTER 2

ANTHRAX IN AFRICA – A REVIEW OF THE CHALLENGES FACED IN DIAGNOSTICS AND CONTROL

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2.1 INTRODUCTION

The African continent continues to bear the brunt of the global infectious disease burden, where healthcare services are severely hampered by a combination of socioeconomic, political, and environmental factors. Complicating matters, is the close association between humans, domestic animals, and wildlife, leading to many calling for a 'One Health' approach to healthcare (Zinsstag et al., 2005; Coker et al., 2011). Even so, much of the international effort focuses on the "big three" diseases, namely HIV/AIDS, malaria and tuberculosis, with many important diseases being left neglected (WHO, 2006). Neglected zoonotic diseases (NZD), which includes rabies, bovine tuberculosis and brucellosis, are largely ignored by policy makers and funding agencies, even though they represent a disease burden similar to that of malaria, (WHO, 2006; Hotez et al., 2007; Marcotty et al., 2010; Sarnak et al., 2014). In addition to causing significant morbidity and mortality among animals and humans, NZD have been recognised for impacting upon human livelihoods (Molyneux, 2008; Maudlin et al., 2009). Rural and peri-urban African populations depend heavily upon their livestock for financial and food security (Molyneux et al., 2011). In fact, it is estimated that one third of Africa's agricultural gross-domestic profit is derived from livestock production (Jones et al., 2011). Reduced productivity, or death of an animal, increases the financial pressure on these communities which, in turn, exacerbates the health risks already present (Shwabe, 1984; Molyneux et al., 2011).

Another neglected disease includes anthrax. The developed world now perceives anthrax as a bioterrorist threat, largely ignoring the major veterinary and public health threat

in developing countries where 60% of cases occur (WHO, 2006; Ehizibolo et al., 2011). This neglect is so pervasive that the recent "Resolution WHA66.12" of the World Health Assembly (WHA) omits anthrax, along with brucellosis and bovine tuberculosis (Welburn et al., 2015). Due to the fact that many of the mechanisms behind the epidemiology and pathology of anthrax is still poorly understood, we argue that the disease is grossly under-reported in Africa. Coupled with the growing human population, interaction at the wildlife-livestock-human interface, and the presence of a large immunocompromised population, the incidence of this disease is likely to increase in future. There is thus a pressing need to accurately quantify the burden of anthrax in Africa, and to improve surveillance and diagnostic techniques. In this review, we investigate the extent, and possible causes of such under-reporting. In addition we discuss the benefits of genotyping and epidemiological investigations.

2.2 ANTHRAX IN PERSPECTIVE

2.2.1 Distribution

Anthrax has a global distribution, being found on every continent apart from Antarctica (Van Ert et al., 2007). Though most of the developed world has achieved some level of control, sporadic outbreaks still occur across Europe and North America (WHO, 2008; Beyer & Turnbull, 2009). In the developing world however, where control programmes are limited or absent, anthrax remains endemic (WHO, 2008). A global map, which plots the endemic regions across countries, has been created previously (LSU, 2003). In an effort to update this information, outbreak data from the OIE, FAO, WAHID and ProMED, for the period 2006-2014, was collated, and used to generate a new map of endemic regions on the African continent (Fig. 2.1).

These data shows that anthrax is still a major problem, with so-called 'hyper-endemic' countries being found across the continent (Fig. 2.1). An example is Zimbabwe, where the civil war of period of 1979-1980 saw 10 000 human cases, and an innumerable number of afflicted cattle (Lawrence et al., 1980; Nass, 1992). Other countries, such as South Africa, are affected to a lesser extent with 'sporadic' outbreaks occurring (Fig. 2.1). That being the case, it should be mentioned that endemic pockets exist, within South Africa, such as the northern parts of the Kruger National Park (KNP), Vaalbos National Park, and Ghaap region of the Northern Cape Province.

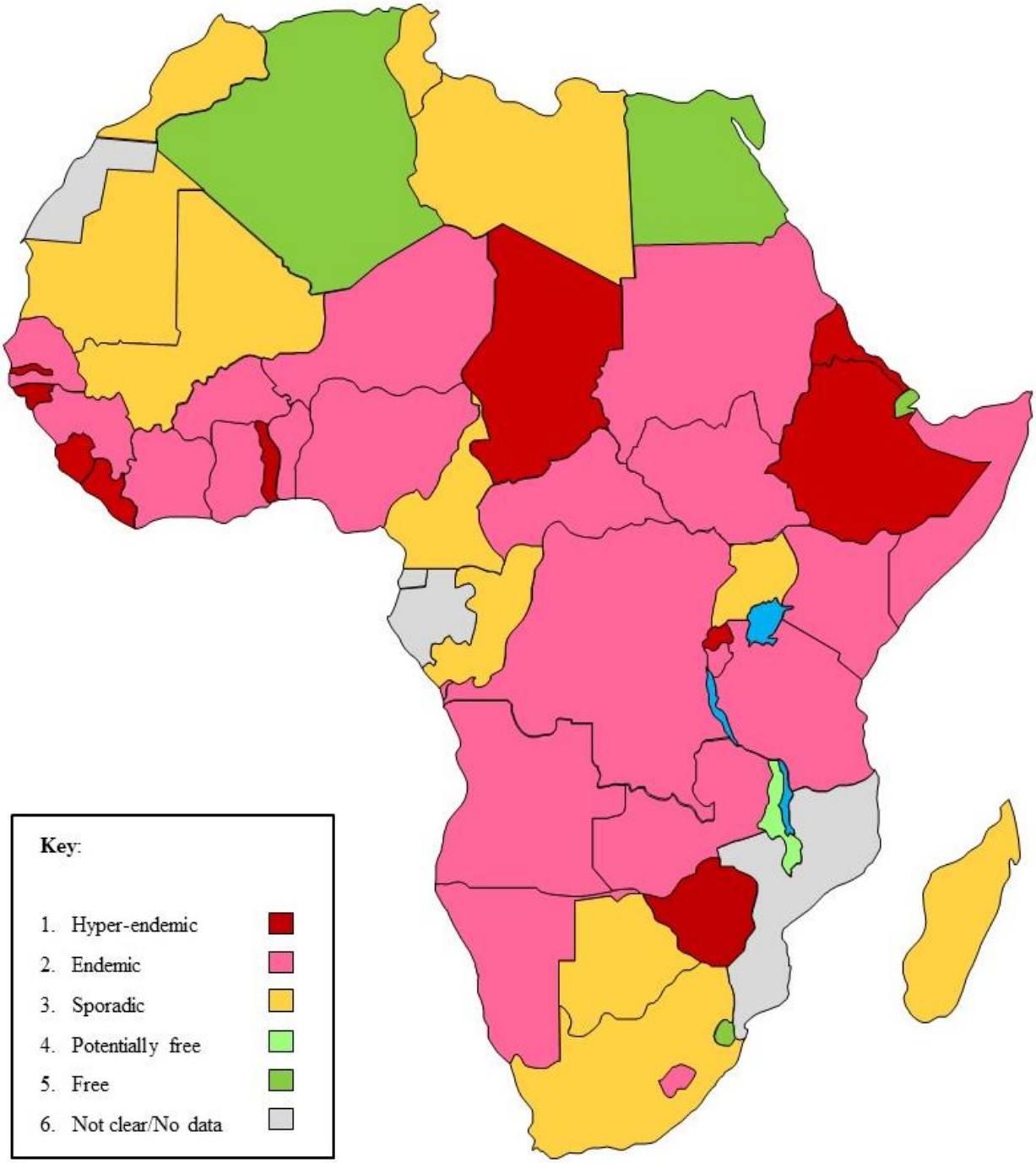

Figure 2.1: Distribution of anthrax across the Africa. Countries vary according to their level of endemicity, ranging from hyperendemic (red), to anthrax-free (green). Countries for which no data is available, or the status is unclear, are shaded grey. Island nations not visible on this map, namely (1) Cape Verde; (2) Comoros; (3) Mauritius; (4) Reunion; (5) Sao Tome & Principe; and (6) Seychelles, are all designated anthrax-free. Data courtesy of Martin Hugh-Jones, Louisiana State University, Baton Rouge, Unites States of America, (collated from OIE/FAO/ProMED/WAHID outbreak data: 2006-2014).

In South Africa, along with other national parks across southern Africa, wildlife outbreaks are regularly noted (WHO, 2008). Egypt is purportedly anthrax-free, along with Swaziland (Fig. 2.1). It is, however, inconceivable that these nations are not affected, when bordering countries are designated as 'endemic' and 'hyper-endemic'.

Special mention should also be given to Mozambique, which is reported to experience sporadic outbreaks by the OIE. Given the fact, however, that no cases of anthrax have been reported between 2007 and 2014, designating the country 'sporadic' may be inaccurate. As such, we indicate Mozambique's status as being 'unclear' (Fig. 2.1). The abovementioned examples are most likely as a result of under-reporting, which will be discussed elsewhere in this review.

2.2.2 Biology and genomics

Bacillus anthracis is a Gram-positive, rod shaped, aerobic bacterium which can efficiently resist harsh environments by means of sporulation (Grunow et al., 2013). Spore formation is initiated when nutrients are limited, and is facilitated by a complex signal transduction network (Hoch, 1993; Grosman, 1995). Reports exist of viable anthrax spores surviving in soil for up to 200 years (de Vos & Bryden, 1998). In this state, spores do not undergo evolutionary change, which is thought to contribute to the low genetic diversity of anthrax (Van Ert et al., 2007). It is important to note that spores cannot grow or multiply in soil or water, as shown through surveys from KNP (de Vos, 1990). The ability to sporulate also helps in the dispersal of anthrax, which will be discussed later (Hugh-Jones & Blackburn, 2009). The genome of *B. anthracis* is highly conserved, with only 3% containing any appreciable polymorphisms (Keim et al., 1997). The virulence genes of *B. anthracis* are located on two plasmids. Firstly pX01, which encodes three genes for the production of toxins, and secondly pX02, which encodes the capsule (Farrar, 1994; Koehler, 2002). Virulence is only achieved when the bacteria can produce both toxin, and the capsule (Pezard et al., 1991; Quinn et al., 1994).

Based on 15 marker multi-locus variable number of tandem repeats analysis (MLVA15) data, *B. anthracis* can be clustered into 3 major clades, A, B and C, and further into 12 sub-groups, and 221 genotypes (Van Ert et al., 2007). The A-clade is found globally, while the B-and-C clades are restricted in terms of their geographical distribution (Van Ert et al., 2007). Southern Africa has been shown to have the greatest genetic diversity of anthrax

globally, with samples from KNP belonging to both the A-and-B clades (Smith et al., 2000; Van Ert et al., 2007). Genotypes belonging to the prior, is considered to be more adaptive, being found in a wider range of environments, such as those with calcium rich soils and a higher pH (Smith et al., 2000). Genotypes belonging to the latter, on the other hand, are thought to be less adaptive, offering an explanation for the limited distribution of those organisms (Smith et al., 2000).

2.2.3 Host species

Anthrax has a wide host range, in fact, mortalities have been recorded in 52 different species in Southern Africa (Hugh-Jones & de Vos, 2002). Among these, herbivores are the most susceptible, with epidemics occurring in *Aepyceros melampus* (impala), *Tragelaphus strepticeros* (greater kudu), *Equus quagga* (plains zebra), and *Syncerus caffer* (African buffalo) (Table 2.1) (de Vos & Bryden, 1995). Though mortalities are rare, carnivores too, are susceptible with deaths being recorded in *Panthera leo* (lions), *Acinonyx jubatus* (cheetahs) and *Lycoan pictus* (wild dogs) (de Vos, 1990; Hugh-Jones & de Vos, 2002).

While anthrax certainly poses a risk to the continent's wildlife resources, it also poses a significant threat to humans and their domesticated livestock. In fact, estimates place human anthrax cases between 2000 and 20000 per annum, with human outbreaks occurring regularly on the continent (Hugh-Jones 1999). This number is most likely grossly underestimated, since non-fatal infections in humans often go unreported.

2.2.4 Epidemiology

The disease cycle of anthrax is ecologically complex and understudied, with a myriad of factors being involved (Hugh-Jones & de Vos, 2002; Hugh-Jones & Blackburn, 2009; Schuch & Fischetti, 2009). Primarily, the disease network of anthrax involves replication within a susceptible host, death, and finally, reintroduction into the environment (Fig. 2.2). Herbivores, which are the most susceptible, acquire infection indirectly from the environment, either during foraging, or from contaminated water sources (Fig. 2.2). Carnivores can acquire anthrax from infected carcasses, as illustrated by the high seroprevalence in lions of the Serengeti, and the isolation of anthrax from the faeces of *Canis mesomalis* (black-backed jackal) (Turnbull et al, 1989; Hampson et al., 2011). While animals

primarily acquire anthrax through ingestion, human infection is mostly the result of handling contaminated animal products (WHO, 2008). Interestingly, terrorism facilitated inhalation, and drug use, have also been reported to cause human anthrax infections (Cole, 2003; Grunow et al., 2013).

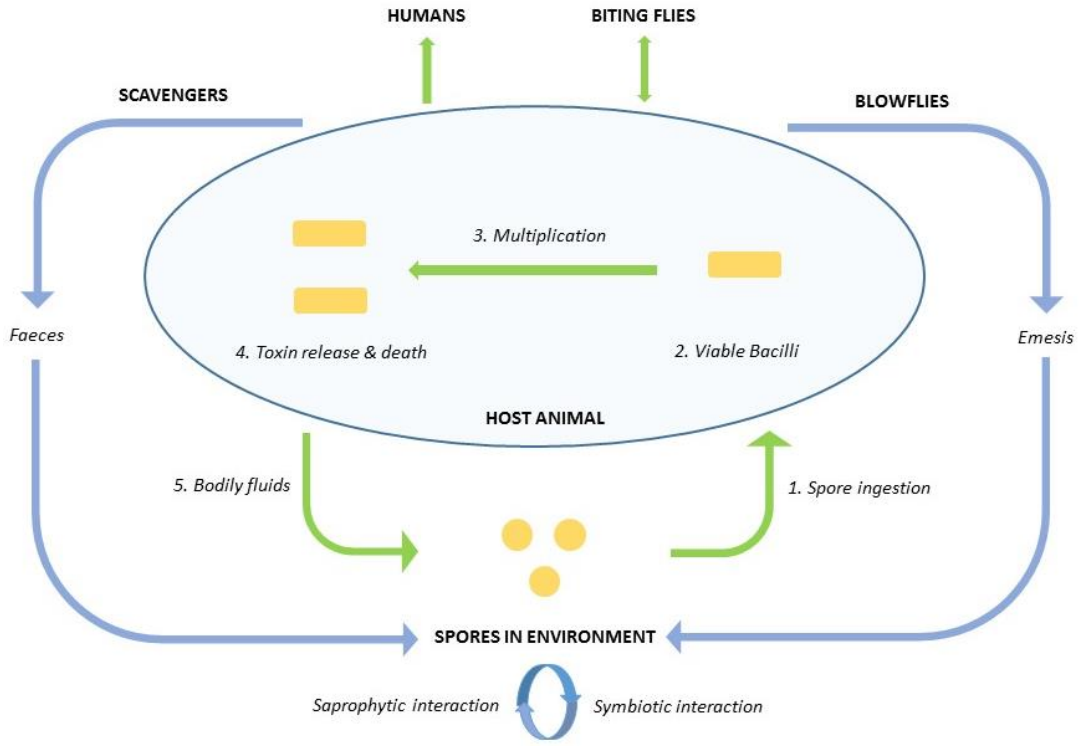

Figure 2.2: Basic disease cycle of anthrax in a natural environment. Direct dissemination (primary) of *Bacillus anthracis* is denoted by green arrows, while indirect (secondary) dissemination is shown in blue Note that the role of biting flies in mechanical transmission of anthrax is debated (Image: Frank Venter).

Further complicating the nature of anthrax epidemiology, are variables such as other animals, climate, and ecological interaction (Fig. 2.2). Firstly, scavengers may themselves play a part in the spatial distribution of spores. Dispersal, over short distances, may be facilitated by blowflies, which after feeding on the bodily fluids of infected carcasses, contaminate the environment through faeces and emesis (Pienaar 1961; Braack & de Vos, 1990; von Terzi et al., 2014). It is also worth mentioning here, that *B. anthracis* has been reported to be mechanically transmitted, under controlled conditions, by biting flies, notably Hippoboscidae, and Tabanidae (Mitzmain, 1914; Kraneveld & Djaenodin, 1940; Turrell &

Knudson, 1987). Geographical dispersal over short and extended distances is also facilitated through faeces but, in this case, by scavengers such as *Crocuta crocuta* (spotted hyena) and *Gyps spp.* (vultures) (Turnbull et al., 1989; de Vos, 1990; Lindeque & Turnbull, 1994). Research has shown that spores are resistant to the stomach acids of *Gyps africanus* (white-backed vulture), and as such, passes through their systems (Housten & Cooper, 1975).

Secondly, stochastic events and animal behaviour are also involved in exacerbating anthrax infection. For example, successive years of anthrax surveillance in the Etosha National Park (ENP), has indicated that ungulate deaths peak late in the rainy season (Ebedes, 1976; Lindeque & Turnbull, 1994; Beyer et al., 2012). In the KNP, outbreaks occur in a cyclical pattern every 10 years, most notably after successive seasons of above average rainfall, followed by a dry-spell in the winter months (de Vos, 1990). Run-off water during the rainy season acts to disperse spores which, mostly likely, settle in low lying areas (Dragon & Rennie, 1995). During the ensuing dry period, animals not only congregate near scarce water sources which may be contaminated, but come into close contact with infected carcasses, spore containing soil, and contaminated grazing in the vicinity. It should be noted, however, that the majority of anthrax cases in the KNP, for the period 2008 to 2014, where reported following rainfall in the summer months (rainy season) (pers. comm, Office of the State Veterinarian, Skukuza). It is not only the gregarious behaviour of animals which influence the transmissibility of anthrax, but also differences in foraging behaviour. For instance, *Equus quagga* (plains zebra) in the ENP were found to consume short grasses during the wet season, facilitating greater soil ingestion, and in turn experienced an increased risk of ingesting anthrax spores (Turner et al., 2013; Havarua et al., 2014).

Finally, recent studies have shown that a complex ecological network may be involved in facilitating the persistance of spores in the environment. While more research is needed, it has been shown that *B. anthracis* may, in fact, not be dormant in the soils as previously thought. Instead, other methods of replication such as saprophytic interaction with plant roots, and symbiotic relationships with soil worms and amoebas have been implicated (Fig. 2.2) (Saile & Koehler, 2006; Schuch & Fischetti, 2009; Dey et al., 2012). These studies may help to explain how anthrax can persist in the environment for extended periods of time.

2.2.5 Pathogenesis and clinical presentation

Once acquired by a suitable host, the previously dormant spore undergoes germination inside the host macrophages (Ascenzi et al., 2002; Driks 2009). This process is initiated in the presence of germinants, which include amino acids and ribonucleosides, and is detected by receptors on the spore itself (Weiner et al., 2003; Fisher & Hanna, 2005). Thereafter, water is absorbed by the spore, leading to swelling of the core, and finally, disassembly of the cortex and coat (Foster & Johnstone, 1990; Moir, 2006). Finally, the metabolic processes of the viable bacilli are initiated, with vegetative growth and toxin release occurring *in vivo* (Little & Ivins, 1999; Ascenzi et al., 2002; Hugh-Jones & Blackburn, 2009).

The virulence factors of *B. anthracis* are located on two plasmids, namely pX01 which encodes the tripartite toxin, and pX02 which contains the gene operon for capsule production (Farrar, 1994; Koehler, 2002). The tripartite toxin is composed of lethal factor (LF), oedema factor (EF) and a protective antigen (PA). It is important to note that virulence can only be achieved if both plasmids are present (Quinn et al., 1994). To ensure replication, the virulence factors of *B. anthracis* targets cells of the host's innate immunity (Hudson et al., 2008; Tournier et al., 2009). Entry of the toxin into cells is facilitated by PA binding to the cell membrane, and forming a so-called heptamer (Leppla, 1995; Collier & Young, 2003). Thereafter, LF and EF inserts into cell, where the prior inactivates mitogen-activated protein kinase kinases (MAPKKs), and the latter leads to elevated levels of cyclic adenosine monophosphate (cAMP) (Dixon et al., 1999; Collier & Young, 2003). Together, these effects lead to severe oedema and/or cell death.

Susceptibility to anthrax disease, and clinical manifestation, varies depending on feeding behaviour, host species, spore dose and route of infection (Lincoln, 1967; de Vos & Bryden, 1998; WHO, 2008). For the purpose of this discussion, only the most common clinical presentations of anthrax, in humans and animals, will be discussed. Cutaneous anthrax, which accounts for the majority of human cases worldwide, has an incubation period of 1-21 days (Kaya et al., 2002; WHO, 2008). Disease initiates as a small, painless, pruritic papule (Kaya et al., 2002). Thereafter, the lesion enlarges, vesiculates, and becomes surrounded by an area of erythematous, non-pitting oedema (Kaya et al., 2002). Finally, the lesion becomes necrotic and covered by eschar (Kaya et al., 2002). In contrast, animals are primarily infected via inhaltion (pulmonary anthrax), or ingestion (gastrointestinal anthrax) (WHO, 2008). The incubation periods vary greatly, depending on the susceptibility of the

particular species (WHO, 2008). In fact, the period between onset of disease, and death, may be hours instead of days (WHO, 2008). In herbivores, which are the most susceptible to infection, sudden death, exudation of blood from the orifices, and a lack of rigor mortis, are the most evident clinical signs (WHO, 2008). In less susceptible animals, symptoms are usually non-specific, and include fever, bloody diarrhoea and lethargy (WHO, 2008). Such non-specific symptoms, as is also the case for human infections, may complicate the already challenging diagnosis of anthrax, possibly exacerbating the phenomenon of under-reporting. For example, human anthrax cases can be confused with psittacosis, Q-fever and sarcoidosis to name but a few (Jamie, 2002). In animals, lightning strikes, poisoning and even Rift-Valley Fever, have been confused for anthrax (WHO, 2008).

2.2.6 Diagnostics and control

Diagnosing anthrax may involve blood smears, culture, serology and molecular detection (WHO, 2008). However, a definitive diagnosis may require a combination of methods, as will become clear. In general capsulated bacilli can be visualised on blood smears taken from animals having succumbed to anthrax (WHO, 2008). In fact, many African laboratories rely solely on microscopy for diagnosis. The problem with this technique, however, is the fact that the number of bacilli, on a smear, differs between animal species (WHO, 2008). A notable example is *Sus domesticus* (domesticated pig) which, even when terminal, yields very low bacilli counts on a blood smear (WHO, 2008). Low numbers of bacilli in blood smears, which is also typical with the African buffalo, can complicate diagnosis. The same is true for carnivores, as illustrated by Tubbesing (1997), who was not able to find capsulated bacilli in big cats which had died of anthrax. Even when capsulated bacilli are observed on blood smears, the identification of *B. anthracis* still requires bacterial culture.

Culturing of anthrax usually involves a combination of selective medium and blood agar. In general, anthrax is non-haemolytic, phage-sensitive and susceptible to penicillin. However, such diagnostic techniques seem to vary between isolates, with examples of atypical *B. anthracis* being previously reported (Lalitha & Thomas, 1997; Klee et al., 2006; Marston et al., 2006). As such, the confirmation of virulence genes, using molecular methods, is essential to obtain a conclusive diagnosis. PCR is a powerful technique which can confirm anthrax infection through amplification of the virulence genes located on plasmids pX01 and

pX02. However, there have been reports of *B. anthracis* strains that lost one or more of their virulence factors (Turnbull et al., 1992). Furthermore, the occurrence of horizontal gene transfer between *Bacillus spp.* complicates identification (e.g. *B. cereus* strains with pX01) (Hoffmaster et al., 2004; Oh et al., 2011).

Anthrax is effectively controlled in the developed world by adhering to the World Organisation for Animal Health's (OIE) anthrax guidelines, and the introduction of the *B. anthracis* Sterne vaccine in livestock (WHO, 2008). The vaccine, developed in 1939, is based on a living, attenuated and avirulent *B. anthracis* that does not contain p0X2 (Hambleton et al., 1984). Recently, Blackburn et al. (2015), identified a lineage (MLVA sub-clade Aβ) of anthrose-deficient anthrax strains in West Africa, using MLVA genotyping. These strains may represent vaccine escape mutants, given the fact that the Sterne vaccine ellicits a strong immunological response to anthrose in cattle (Tamborrini et al., 2011). This example highlights the potential of genotyping techniques, such as MLVA, to study the complex nature of anthrax in Africa.

2.2.7. Genotyping and epidemiological investigation

High resolution genotyping is a powerful tool for investigating the diversity, risk to livestock-and-public health, epidemiology and pathology of a bacterial pathogen. Such methods include single nucleotide polymorphisms (SNP), single nucleotide repeats (SNR) and MLVA (Keim et al., 2004). MLVA works on the premise of variable number of tandem repeat regions (VNTRs) within bacterial genes (van Belkum, 2007). A VNTR is a locus, of a particular gene, which contains repetitive DNA sequences, which vary in number between strains of the same bacterial species (Fig. 2.3). The differences in copy number of a given repeat sequence, allows investigators to differentiate between bacterial genotypes (van Belkum, 2007). MLVA then, is a system utilizing multiple VNTR regions. For example, different genotypes of *Mycobacterium tuberculosis* can be identified by investigating six to nine VNTR regions of the bacterial genome (van Belkum, 2007). In the case of anthrax, 31 VNTR markers are available to discriminate between different genotypes. The current assay was developed by combining three earlier protocols, utilizing eight, 15, and 25 loci respectively (Keim et al., 2000; Keim et al., 2004; Lista et al., 2006). The assay involves using genomic material from anthrax samples (DNA extractions or lysate), PCR, electrophoresis (using capillaries or agarose gel) to size fragments, and data analysis to

cluster samples into genotypes.

MLVA has already proven invaluable in terms of forensic and epidemiological investigation of anthrax. In Europe, for example, the technique allowed scientists to identify a geographically unique genetic cluster, and to examine the source of anthrax in heroin addicts (Antwerpen et al., 2011; Grunow et al., 2013). Research on the African continent can also benefit from utilising MLVA, as was the case in Namibia, where investigation of outbreaks in the ENP, and on farms, delivered insights into the causal factors of anthrax epidemics (Beyer et al., 2012). As already mentioned, MLVA also identified a unique West African lineage, which may contain important vaccine escape mutants (Blackburn et al., 2015). Improved diagnostics, coupled with genotyping techniques, have the potential to revolutionise the study of anthrax and other NZD in Africa. However, as mentioned earlier, factors such as failing veterinary-and-public health systems, and a lack of resources, hinders the wide scale implementation of such techniques (Sarnak et al., 2014; Smith et al., 2015).

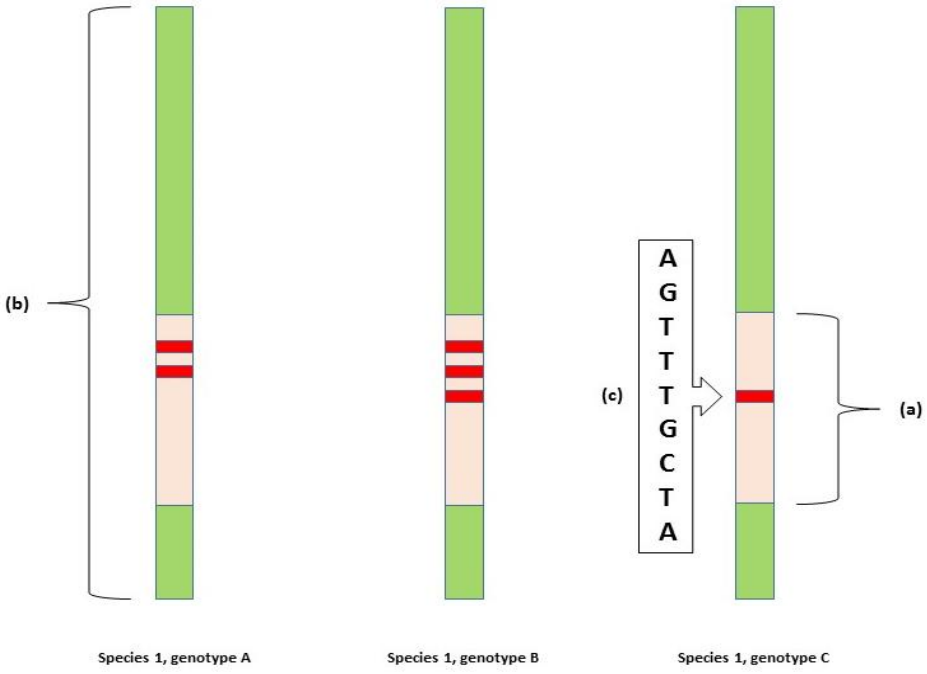

Figure 2.3: Variable number of tandem repeats (VNTR's) are regions or loci (a), of a particular gene (b), which contain differential numbers of DNA sequence repeats (c). Genotypes of the same species may have variable numbers of these repeats: Species 1, genotype A (two VNTR copies); species 1, genotype B (three VNTR copies); species 1, genotype C (one VNTR copy) (Image: F Venter).

2.3. THE DIFFICULTY OF CONTROLLING ANTHRAX IN AFRICA

2.3.1 The African Landscape

Anthrax remains endemic across much of Africa (Fig. 2.1). Here, a combination of natural and anthropogenic factors promotes the persistence, and transmission, of anthrax. Firstly, it should be mentioned that the greatest genetic diversity of *B. anthracis* is found in southern Africa (Smith et al., 2000; Van Ert et al., 2007). In fact, the organism is thought to have originated on the continent, later diverging into a number of environmentally adapted lineages (Keim et al., 1997). In general, suitable environments for anthrax transmission are characterised by calcium rich soil, and with a pH above 7 (Van Ness & Stein, 1956; Van Ness, 1971; Dragon & Rennie, 1995; Smith et al., 2000).

As mentioned earlier, infectious diseases exert a tremendous burden on the animal and human populations of Africa. In future, that burden is likely to increase due to the effects of climate change, through altered rainfall patterns, and expansion of vector ranges. Already, models have shown that diseases such malaria and dengue fever are likely to increase their geographical range on the continent (Hales et al., 2002; Hay et al., 2002). The changing distribution of anthrax due to climate change has been investigated in, for example, Kazakhstan (Joyner et al., 2010). While such studies are lacking for Africa, there is already anecdotal evidence that the previously excepted idea of seasonality of anthrax outbreaks is changing. In the KNP of South Africa for instance, anthrax outbreaks has been noted as occurring in cyclical patterns, especially in the dry season (de Vos, 1990). From our own experience, outbreaks now occur in the endemic regions of KNP throughout the year, with larger outbreaks mainly after heavy rains, which may be due to climate change. This observation remains speculative, however, and needs to be investigated further.

Another factor, which may lead to increased disease incidences in Africa, is the practice of establishing transfrontier conservation areas (TFCA) (Bengis et al., 2002; Thomson et al., 2013). The practice was initiated under the premise of re-establishing traditional migratory routes of animals, and involves the dropping of fences, often between national boundaries. One such TFCA, the Great Limpopo Transfrontier Conservation Area (GLTA), was established by creating movement corridors between Mozambique, South Africa and Zimbabwe (Ferguson & Hanks, 2010). While such measures make sense in terms of conservation, it presents a risk for the spread of disease (Bengis et al., 2002). Animals can now range freely within the GLTA, with anthrax being endemic in the northern KNP in South Africa, hyper-endemic in Zimbabwe, and of unclear status in Mozambique (Fig. 2.1),

bringing about gene-flow between previously isolated anthrax strains. A number of rural populations, and their livestock, are known to live along the fringes of the GLTA. It is here, at the wildlife-livestock-human interface, where the risk for zoonotic disease transmission is greatest.

2.3.2 Demographics and community practices

The most commonly cited driver of disease emergence and transmission, is changing human demographics, and altered land-use practices (Woolhouse & Gowtage-Sequira, 2005). Africa is experiencing rapid population growth, with the largest population of people under the age of 25 now living on this continent. Such growth is accompanied by an increased demand for meat products, and in turn, leads to agricultural intensification and incursions into areas where humans, livestock and wildlife can mix (Acha & Szyfres, 2003; Steinfeld et al., 2006). In fact, one third of the world's rural livestock keepers live in Africa, with many of these communities grazing their cattle in the vicinity of conservation areas (Lembo et al., 2011; Chikerema et al., 2013; Coffin et al., 2015). Livestock are of immense value to rural, poor communities. These animals provide humans with food, fertiliser, draught power, and in many cases, act as an investment which can be utilised to pay for school fees, or medical care. In Zimbabwe, recent estimates place the value of one head of cattle between US$ 300-500 (Chikerema et al., 2013). Considering that 51% of sub-Saharan Africa's population survive on less than US$ 1.25 per day, the value of cattle cannot be overstated (Chen & Ravallion, 2008). As such, it is not only human population growth, and a close association with animals which hamper the control of anthrax, but also human behaviour.

Recent studies in Uganda and Zimbabwe, found that there is awareness of anthrax, and its zoonotic potential, among rural communities (Chikerema et al., 2013; Coffin et al., 2015). Even so, poverty leads to these communities ignoring the risks associated with disease. Risk factors for contracting anthrax include a lack of protective measures, handling of infected carcasses, and eating contaminated meat (Mwenye et al., 1996, Chirundu et al., 2009, Gombe et al., 2010). There is also the perception that, anthrax is somehow 'contained' within the spleen, and that removal of the organ would render the rest of an infected animal safe for consumption. Another perception is that dried, overcooked, and herb treated meat, would prevent anthrax infection (Opare et al., 2000; Gombe et al., 2010). In areas where the incidences are low, communities may not take anthrax in account, with infections of livestock

often being followed by human outbreaks (WHO, 2008). Furthermore, cases where carcasses do not present with typical anthrax symptoms, may lead to community members considering the meat safe for consumption.

More studies of the socio-anthropological factors behind anthrax in Africa are needed, and can provide valuable information regarding the true burden the disease. There is also a need for improved awareness campaigns to educate rural communities on the risk of anthrax infection. However, without some measure of compensation for disposing of valuable livestock, awareness campaigns are likely to fail.

2.3.3 Politics and lack of resources

Rural, poor communities are underserviced by healthcare facilities, and isolated from education and political processes (Molyneux et al., 2011; Welburn et al., 2015). Furthermore, economic instability, or civil strife, can lead to the migration of communities into areas with a higher disease incidence (Toole, 1995; Kalipeni & Oppong, 1998). Such political phenomena have also lead to a collapse of previously functioning veterinary-and-healthcare facilities (Toole, 1995; Kalipeni & Oppong, 1998). In Zimbabwe, for example, anthrax cases have been gradually increasing following the government's suspension of state funded, mandatory vaccination (Chikerema et al., 2012). The lack of government-sponsored vaccination is wide-spread in Africa, even among economically stable countries such as South Africa, where the onus to vaccinate against anthrax, and various other controlled diseases, rests on animal owners (WHO, 2008). The problem herein lies with the fact that some farmers may neglect to vaccinate their livestock, whereas poor communities cannot afford to vaccinate.

Controlling zoonotic diseases places significant pressure on veterinary-and-healthcare systems, especially those which are already strained (Alexander et al., 2012). Such control relies on accurate surveillance and diagnostic methods (Shears, 2000). While clear anthrax surveillance guidelines exist, such programmes are often not implemented, and may suffer from defects or poor quality data (WHO, 2008; OIE, 2015). In addition, passive surveillance, as practiced across Africa, does not take into account the unique risk factors which may be present across the continent's diverse endemic regions. Where surveillance systems are in place, data is often collected independently by different role-players, and as such, may fail to identify disease outbreaks (Molyneux et al., 2011).

Furthermore, surveillance efforts are hampered by a lack of accurate and accessible

diagnostics. As mentioned earlier, the conclusive diagnosis of anthrax relies on a combination of techniques, not necessarily possible in all African countries, where there is a lack of trained personnel and biosafety laboratories (Kunda, 2006). Even when such facilities are available, the non-specific symptoms and differential diagnosis of anthrax, present further complications (Jamie et al., 2002; Owen et al., 2015). Coupled with inadequate zoonotic disease training among clinicians, misdiagnosis may in fact be a regular occurrence (Reyburn et al., 2004; John et al., 2008).

The abovementioned lack of surveillance and diagnostic facilities are major factors impeding the control of anthrax and other zoonotic diseases in Africa (Shears, 2000). To mitigate against such effects, African countries must consider implementing a 'One Health' approach, which relies on the cooperation between the veterinary-and-healthcare sectors, as well as the involvement community-led surveillance programmes (Molyneux et al., 2011). Failing to do so, exacerbates the phenomenon of under-reporting, and thus underestimation of anthrax on the continent.

2.3.4 Under-reporting

Anthrax has largely been ignored by policy-makers in the developing world (WHO, 2011). This is partly due to the fact that the burden of anthrax on the rural poor, and their livestock, has not been quantified. In general, decision-makers and scientists rely heavily on the disability-adjusted life year (DALY) as a measurement of disease burden (Mathers et al., 2007). The problem herein, lies with the fact that such a measurement undervalues the true impact of a disease (Mathers et al., 2007). To secure attention, and adequate funding, the burden of anthrax needs to be determined with a measurement beyond the DALY. Such a measurement should take into account the livelihood impacts incurred by rural livestock keepers through reduced productivity, or the death of an animal. Alternatively, policy-makers should be made aware of the benefits of controlling anthrax through so-called 'cost-benefit' approaches (Canning, 2006). Currently, there is no means of calculating the economic impact of anthrax in Africa, which is due to a lack of information regarding the disease on the continent. This dearth of information, stems from the gross under-reporting of anthrax, and is contributed to by the following factors:

(i) Fragmentary reporting - As mentioned before, the control of anthrax needs a "One Health" approach. Presently, the veterinary, public health, and wildlife sectors function as

separate entities. As such, outbreaks are often not identified, which contributes to an under-estimation of disease burden in Africa (Molyneux et al., 2011). Illustrating this issue, is the fact that anthrax outbreaks are regularly noted among wildlife in Western Uganda, while outbreaks are infrequently reported in livestock from the same area (Coffin et al., 2015). Additionally, there are notable issues with regard to reporting anthrax to global authorities. For example, four human anthrax cases, resulting in one death, occurred in the Northern Cape, South Africa, in 2005. These human cases, which were the result of consuming infected cattle meat, were not reported to the OIE (data courtesy of Hugh-Jones, M. E.). When cases are reported, discrepancies often occur between the numbers listed by global authorities. In Ghana, a single human death was recorded by the OIE in 2013, while ProMED lists four deaths for that same period (data courtesy of Hugh-Jones, M. E.);

(ii) Misdiagnosis - It has been proposed that cases of gastrointestinal (GI) anthrax, in humans, are misdiagnosed and under-reported (Sirisanthana & Brown, 2002). This is partly due to the fact that the majority of such infections occur in rural, underserviced areas of the developing world, where access to medical services are limited. In severe cases, the onset of disease is rapid, often resulting in death before those afflicted can seek medical attention. Furthermore, mild cases of GI anthrax present with non-specific symptoms, and are often disregarded (Sirisanthana & Brown, 2002). Misdiagnosis of anthrax may also be common in animals, where reporters often rely on accepted clinical manifestations as a sign of disease. Hugh-Jones et al. (1999) noted deaths among cattle and goats, in Kimberley, South Africa, with a lack of post-mortem bleeding from the orifices. Such cases may lead to investigators suspecting other causes of death;

(iii) *Deliberate under-reporting* - In some countries, the reporting of anthrax may be actively suppressed (WHO, 2008). This may be due to a variety of factors, ranging from unwillingness of governments to cooperate with the WHO, OIE and other authorities, or simply due to a lack of infrastructure in those countries. This fact is illustrated by a number of countries in Africa where there is no available data regarding the incidence of anthrax (Fig. 2.1). Furthermore, it is suspicious that some nations such as Egypt and Swaziland, are ‘anthrax-free’, especially when one considers the endemic, or sporadic nature of that disease in neighbouring countries (Fig. 2.1).

2.3.5 The isolation of African research groups

To our knowledge, little exchange of information regarding anthrax exists among African researchers. Instead, such groups work in isolation in their respective countries, occasionally collaborating with partners from Asia, Europe, and North America. While such collaborations are encouraged, we believe that much can be gained by advocating dialogue and cooperation between African researchers themselves. An unintended consequence of working in isolation, is that it promotes the understanding of 'local *B. anthracis* diversity.' To understand anthrax in Africa, we need to promote cooperation between African researchers, to instead develop a 'continent-wide' picture of diversity.

2.4 TOWARDS A PAN-AFRICAN ANTHRAX NETWORK

The continent can benefit from skilled, African researchers, trained in diagnosing and investigating NZD. Often, those on the frontlines are inadequately trained, and ill-prepared for dealing with such outbreaks. Furthermore, diagnostic laboratory services continue to be neglected in Africa, which suffers from a lack of resources. As such, accurate surveillance and diagnostics aimed at zoonotic diseases, including anthrax, are severely hampered, contributing towards under-reporting. This problem can be addressed by establishing a network of African anthrax researchers. Such a network should facilitate technical training across the continent, while promoting the sharing of information and resources. Additionally, the network can be supported through creating a central, shared e-resource for anthrax researchers across the continent. Such a data-base could facilitate the rapid dissemination of information between interested parties, in a safe and secure manner. Additional resources can be made available for researchers to pose questions to others in the field, or to help one another interpret confounding results. A united front can bring more attention to anthrax in Africa, and in so doing, garner the support from policy-makers, both on the continent, and abroad.

Strengthening the capacity of anthrax research, in Africa, will also benefit the international research community. The genotypic diversity of anthrax has yet to be investigated in the majority of Africa's endemic regions. Consequently, gaps exist in our knowledge of the global anthrax population structure which, in turn, complicates the discovery of markers for subtyping and diagnostic purposes (Blackburn et al., 2007; Van Ert et al., 2007). As such, a better understanding of the evolution of *B. anthracis* here, will aid

international researchers in launching epidemiological and forensic investigations of anthrax in future (Blackburn et al., 2015).

2.5 CONCLUSION

It is unlikely that anthrax will be eradicated, in Africa, in the near future. The endemic nature of the disease, coupled with the environmental, socioeconomic, and political factors present here, all but ensures this. The problem herein, is that anthrax is already underestimated, and will undoubtedly be a growing problem in future. As such, the focus should be on controlling the disease to mitigate against the economic, and health risks, associated with anthrax. Here, we argue the merits of MLVA as a tool to investigate the diversity of *B. anthracis*. Such endeavours have already yielded considerable insight into to epidemiology, and biology, of the bacterium, which is integral to the control of anthrax. While such techniques are indeed powerful, the diagnostic capability of African laboratories needs to be enhanced first. This basic failure is due to anthrax being grossly under-reported, and as such, being underfunded. Concerted efforts between veterinarians, physicians, scientists and politicians are needed if the true burden of anthrax is to be determined, and in so doing, provide African communities with a basic human right - protection against zoonotic diseases.

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CHAPTER 3

GENOTYPIC AND EPIDEMIOLOGICAL INVESTIGATION OF ANTHRAX STRAINS FROM 2014 - 2015 IN THE KRUGER NATIONAL PARK.

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ABSTRACT

Anthrax, caused by the bacterium *Bacillus anthracis*, is a disease affecting primarily herbivores, and is endemic in the Kruger National Park (KNP) of South Africa. In this study we investigated anthrax outbreaks in three regions of KNP, between 2014 and 2015, using multi-locus variable number of tandem repeats analysis (MLVA) employing 31 markers (MLVA31). The genotypes of 81 *B. anthracis* isolates belonged to five distinct genotypes in the A-clade. Two genotypes dominated, with most of the isolates (n=78) grouped into the A1 sub-clade of *B. anthracis*, which is commonly found in southern Africa. A single isolate belonged to the less frequently reported A3 sub-clade. Two isolates, which also grouped into the A1 sub-clade, warrants further investigation, and may represent previously unidentified *B. anthracis* strains. These data also yielded insight into the complex epidemiology of anthrax, a subject which remains understudied. We provide some evidence regarding the dissemination of *B. anthracis* by blowflies and vultures, as well as milk. In the case of the latter *B. anthracis* was isolated from the milk of *Aepyceros melampus* (impala). The potential of using an MLVA7 protocol as a first line assay, was also investigated. The results indicated that while such a protocol was capable of distinguishing between the 5 genotypes present in this study, it could neither differentiate between A and B-clade isolates, nor adequately differentiate between A sub-clades.

3.1 INTRODUCTION

Bacillus anthracis, the causative agent of anthrax, is an aerobic, rod-shaped, Gram-positive bacterium with a broad mammalian host range. The organism is characterised by the ability to form spores and can, in so doing, resist harsh environmental conditions. While anthrax has been eradicated in the developed world, where it is now primarily perceived as a bioterrorist threat, the disease remains endemic in parts of the developing world, most notably in 'hyper-endemic' African countries (Cole, 2003; WHO, 2008). In South Africa, the Kruger National Park (KNP) is recognised as containing endemic pockets, with 543 anthrax cases being recorded between 1988 and 2011 (Steenkamp, 2013). The Pafuri region, of northern KNP, is noted as being a particular focal point for outbreaks, with the area exhibiting the perfect ecology for anthrax infection to occur (Fig. 3.1) (de Vos, 1990; Smith et al., 2000; Steenkamp, 2013).

While the primary transmission network of anthrax has been elucidated, the epidemiology of the disease remains understudied and poorly understood. In general, *B. anthracis* is transmitted to animals through the ingestion of spore contaminated soil and plant material. Animals vary with regard to susceptibility, with herbivores being recognised as particularly sensitive to infection. In fact, large outbreaks have been noted in the KNP among *Aepyceros melampus* (impala), *Syncerus caffer* (African buffalo) and *Tragelaphus strepsiceros* (greater kudu) (de Vos & Bryden, 1995). Given the fact the latter is a browsing species, unlike impala and African buffalo which are grazers, it has been suggested that kudu must acquire anthrax directly from contaminated leaves. It has been hypothesised that blowflies, having fed on anthrax infected carcasses, deposited *B. anthracis* on vegetation preferred by browsing animals such as kudu (Braack & de Vos, 1990; Hugh-Jones & de Vos, 2002). Further evidence for this hypothesis, was presented by Blackburn et al. (2014) having isolated *B. anthracis* from carcasses, blowflies and vegetation, in West Texas, USA, belonging to the same genotype. It should be noted, however, that blowflies most likely contaminate only the environment in the immediate vicinity of an anthrax infected carcass (Blackburn et al., 2010; von Terzi et al., 2014). Adding to the complex nature of anthrax epidemiology, is the role of biting flies in the mechanical transmission of anthrax, the role of scavengers in spore dispersal, the effect of stochastic phenomena, and *B. anthracis* interaction with plants and amoebae (Mitzmain, 1914; Kraneveld & Djaenodin, 1940; Turnbull et al., 1989; de Vos, 1990; Lindeque & Turnbull, 1994; Saile & Koehler, 2006; Dey et al., 2012). Many of these factors require further investigation, as the control of *B. anthracis*

necessitates a thorough understanding of the mechanisms behind anthrax outbreaks.

The level of genetic homogeneity in *B. anthracis* has, in the past, presented a major hurdle to phylogenetic studies of the bacterium. Advances in two molecular techniques, namely multi-locus variable number of tandem repeats analysis (MLVA) and single nucleotide polymorphism (SNP) analysis, has proved effective as a means of studying the evolution and epidemiology of *B. anthracis* (Keim et al., 2000; List et al 2006; Van Ert et al., 2007; Beyer et al., 2012; Thierry et al., 2014). MLVA was originally applied to differentiate between *B. anthracis* genotypes, by targeting eight markers (MLVA8), and later to include 15, 20 and 25 markers respectively (Keim et al., 2000; Le Fleche et al., 2001; Lista et al., 2006; Van Ert et al., 2007). A more sensitive, 31 marker system (MLVA31) was recently developed by combining MLVA15 and MLVA25, and used to investigate the causal factors behind anthrax outbreaks in Namibia (Beyer et al., 2012). Analysis of KNP oubreak strains, using MLVA8, previously indicated the presence of *B. anthracis* isolates representing the A-and-B clades (Smith et al., 2000). Isolates belonging to the A-clade, where distributed throughout the park, while the B-clade (Kruger B sub-clade) was restricted to northern KNP, leading the authors to hypothesise that the latter is less adaptable to ecological variables (Smith et al., 2000). MLVA is effective in its ability to subtype *B. anthracis* isolates into distinct genotypes, proving uselful in the study of outbreak patterns and their epidemiological causes. However, variable number of tandem repeats (VNTR) markers, which are targeted in MLVA, evolve rapidly and are thus evolutionarily unstable (Vogler et al., 2007). As such, MLVA is prone to homoplasy, which can bias the phylogenetic history between *B. anthracis* isolates. SNPs have limited subtyping power compared to MLVA, but are more stable, and less prone to mutations (Vogler et al., 2002; Keim et al., 2004). SNP analysis, therefore, is ideal for investigating the broader evolutionary history of *B. anthracis* which may be obscured by homoplasy (Keim et al., 2004; Van Ert et al., 2007).

Anthrax not only poses a risk to the KNP's valuable wildlife commodities, but also presents a public health risk to human communities flanking the western border of the park. Here, there is also a risk to unvaccinated domestic animals, through interaction at the wildlife/livestock interface, which subsequently threatens the sustainable livelihoods of rural communities. State-sponsored vaccination of domestic animals, with the attenuated *B. anthracis* Sterne vaccine, was suspended by the South African government, with the onus to vaccinate now lying with farmers and herders themselves (WHO, 2008). *Bacillus anthracis* strain Sterne, has been widely used in the vaccination of livestock against anthrax (Hambleton et al., 1984; Sterne, 1939). Fully virulent *B. anthracis* strains contain virulence

factors, located on two separate plasmids, which encode the capsule and tripartite toxin (Pezard et al., 1991; Farrar, 1994; Quinn et al., 1994; Koehler, 2002). The Sterne strain lacks plasmid pX02, and is thus unencapsulated, attenuated and avirulent.

In the present study, we isolated anthrax outbreak samples from the Pafuri, Mahlangeni, and Houtboschrand regions of KNP, which occurred between 2014 and 2015 (Fig. 3.1). Isolates from carcasses and the environment were subjected to MLVA31, in order to investigate the epidemiology, and genotypic diversity behind those outbreaks. The potential of an MLVA7 protocol, proposed by Thierry et al. (2014) as a first-line assay of French *B. anthracis* isolates, was also investigated. This cost-effective protocol can be visualised on agarose gel, as opposed to capillary electrophoresis which is recommended for MLVA31.

3.2 MATERIALS & METHODS

3.2.1 Sample history

Anthrax outbreaks were documented in the Pafuri region of the KNP, in March 2014, and again in Pafuri, Mahlangeni and Houtboschrand, between January and March 2015 (Fig. 3.1). Samples were collected from carcass sites (blood smears, bone, hair, milk and dung) and the environment (vulture faeces, blowflies, beetles, leaves and grass) (Table 3.1; Appendix I). Insects were manually collected using forceps, and stored in glass vials prior to identification, and processing. All samples were transported to the laboratory of the State Veterinarian, Skukuza, Mpumulanga, where microbiology and FRET-based real-time PCR were utilised to diagnose *B. anthracis* (Ellerbrok et al., 2002; WHO, 2008).

Seven animals were confirmed to have died of anthrax during the 2014 outbreak, representing impala, greater kudu and *Equus quagga* (plains zebra) (Table 3.1; Appendix I). The larger, 2015 outbreak, resulted in 55 deaths, representing five species, namely *Connochaetes taurinus* (blue wildebeest), impala, *Loxodonta africana* (African elephant), *Papio ursinus* (Chacma baboon) and plains zebra (Table 3.1; Appendix I). A total of 81 *B. anthracis* isolates were obtained from the carcass sites (Figure 3.4), and the environment (Figure 3.4), between 2014 and 2015 (Table 3.1; Appendix I).

3.2.2 Bacterial culture and DNA extraction

Arthropod isolations were performed by first washing adult insects, and larvae, in 1 ml saline (0.9% NaCl). Saline solutions were incubated on selective and non-selective media to determine external spore counts. Internal spore counts were determined by disinfecting insects, and larvae, with 0.1% peracetic acid for one hour. Samples were then washed using 100 µl thiosulphate solution (100 g/L) for 40 min. Samples were again rinsed in saline, and homogenised. Serial dilutions were performed using homogenate, on 5% SBA and PET media, and incubated at 37°C for eight hours.

Isolation from plant material was performed by taking swabs of visible blowfly spots. Swabs were soaked in 100 µl saline solution, and applied to blowfly spots in a dabbing motion, before being streaked onto 5% SBA. Plates were incubated overnight, at 37°C. Isolations from vulture faeces were performed by dissolving one gram of faecal material in 9 ml phosphate-buffered saline (PBS) solutions, followed by performing the standard processing protocol for biological samples (WHO, 2008).

Candidate colonies were selected, following isolation form the abovementioned samples, and confirmed as *B. anthracis* using the protocol described by Ellerbrok et al. (2002) and the WHO (2008). Pure *Bacillus anthracis* isolates were cultured on nutrient agar, and incubated at 37°C for 8 hours, in the BSL 2+ facility of the Department of Veterinary Tropical Diseases, University of Pretoria. Bacteria was harvested from the plates, inactivated using lysozyme (20 mg/ml), and incubated at 37°C for 30 minutes. DNA was isolated, using the High Pure PCR Template Preparation Kit © (Roche), according the to manufacturer's instructions. *Bacillus anthracis* Sterne, Vollum (A70) and Ames (A90) DNA were included in this study as positive controls.

3.2.3 Multi-locus variable number of tandem repeat analysis (MLVA)

Extracted *B. anthracis* DNA was subjected to PCR, targeting 31 VNTR markers with fluorescently labelled primers, across seven separate multiplex reactions (Keim et al., 2000; Le Fleche et al., 2001; Lista et al., 2006; 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 multiplex A-D and G (Appendix II) were as follows:

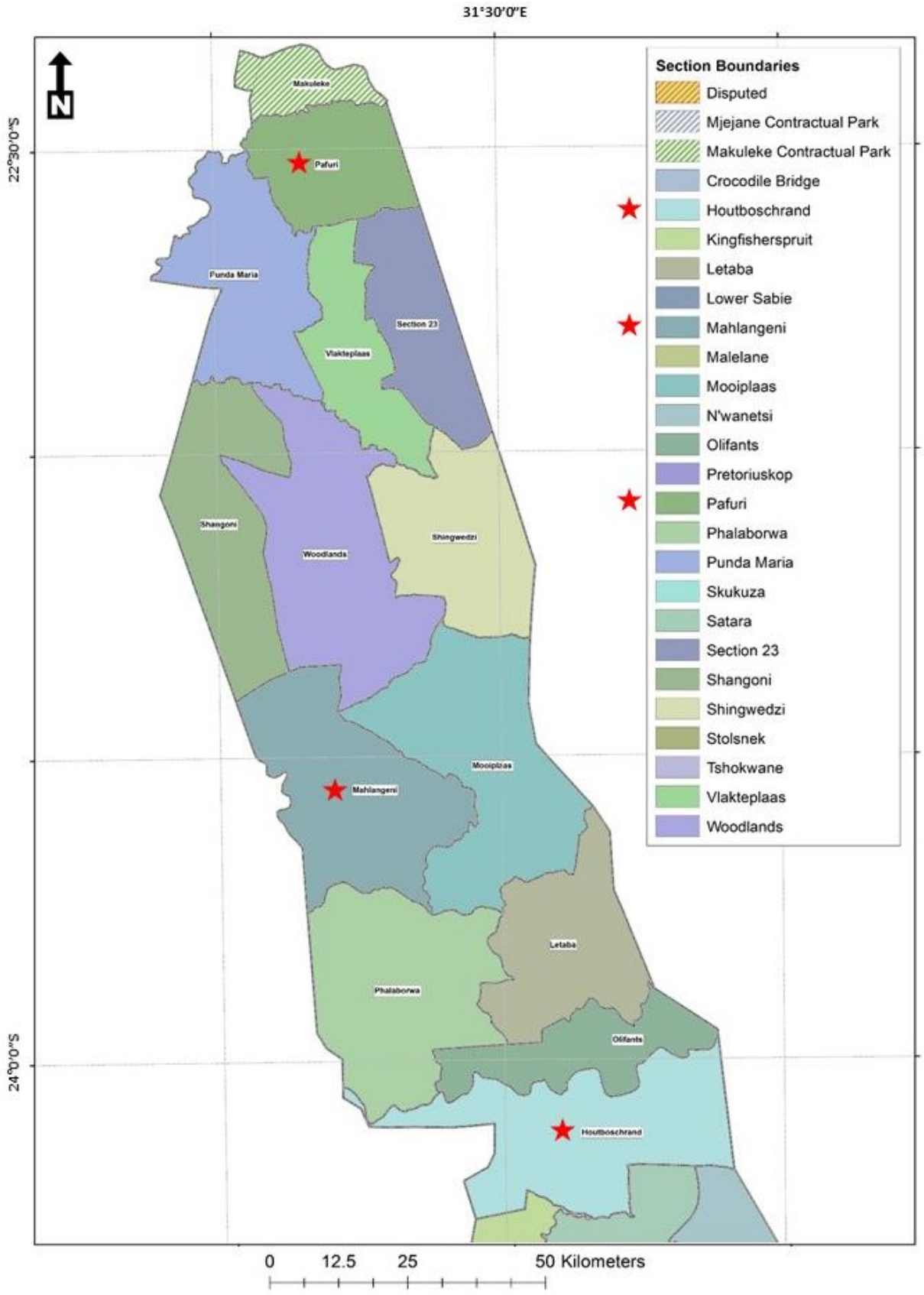

Figure 3.1: Map of central and northern Kruger National Park (KNP), indicating different regions. 2014 (Pafuri) and 2015 (Pafuri, Mahlangeni and Houtboschrand) outbreak foci are denoted by red stars (original map courtesy of SANParks).

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 multiplex E and F (Appendix II) 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.

3.2.4 Data analysis

The size of the resultant DNA fragments were determined using GeneMapper ID-X © software (Applied Biosystems), against the LIZ1200 © size standard (Applied Biosystems), and validated by including *B. anthracis* strains Sterne, Vollum (A70) and Ames (A93) as positive controls. Base-pair sizes for each allele was converted into copy numbers (Appendix III) using a previously described algorithm (Thierry et al., 2014). Genetic distance and cluster analysis was performed using unweighted pair group method using arithmetic means (UPGMA) in BioNumerics © version 6.6 (Applied Mathematics).

3.3 RESULTS & DISCUSSION

3.3.1 Genotypic diversity

A collection of 81 *B. anthracis* isolates were obtained from the Pafuri (n=79), Mahlangeni (n=1; M15-01) and Houtboschrand (n=1; H15-01) regions of the KNP, and confirmed to be *B. anthracis* at the Skukuza Veterinary Laboratory (Table 3.1). Classic *B. anthracis* morphology presented as creamy white colonies, with a rough texture and wax-like consistency following bacterial culture, and as capsulated, box-shaped, Gram-positive rods during microscopic investigation (WHO, 2008). *Bacillus anthracis* was confirmed through assessing penicillin and gamma-phage sensitivity, and testing for the presence of virulence factors using real-time PCR (Ellerbrok et al., 2002; WHO, 2008). The *B. anthracis* isolates (n=81) from 2014-2015, as well as three reference strains, were typed with MLVA31 using capillary electrophoresis (Fig. 3.2). The results indicated that five genotypes were present amongst these isolates (Table 3.1). Two genotypes (GT1 and GT2) dominated, representing 17 and 61 isolates respectively (Table 3.1). The other three genotypes each represents a single

isolate (Table 3.1).

Analysis of global *B. anthracis* isolates, using MLVA15, classified three major clades, namely A, B and C (Van Ert et al., 2007). The A-clade has a global distribution, consisting of a number of closely related, yet geographically isolated clusters. The B-clade is limited in its distribution, with the B1 lineage being found only in southern Africa, and the B2 lineage reported in Europe (Smith et al., 2000; Van Ert et al., 2007, Thierry et al., 2014). The C-clade consists of two rare isolates, and has never been reported in Africa (Van Ert et al., 2007). Analyses targeting a subset of SNP markers (canSNP) further defined the three major clades, into 12 distinct sub-groups (Van Ert et al., 2007). These sub-groups correspond to defined geographical locations, such as A.Br.005/006 and B.Br.KrugerB, which is found primarily in southern Africa (Van Ert et al., 2007). Historical data from the KNP indicated that B-clade isolates played an important role in anthrax outbreaks, in the northern regions of the park, between 1970 and 1981 (Smith et al., 2000). During those outbreaks, isolates from the northern regions belonged to both the A-and-B clades, with the latter being dominant, while outbreaks in the central KNP were dominated by A-clade isolates (Smith et al., 2000). No B-clade isolate was detected in northern KNP, during the large anthrax outbreak of 1990 (Smith et al., 2000). Smith et al. (2000) argued that torrential rains, in northern KNP in early 1990, may have contributed to spore removal from that region. Thereafter, A-clade isolates from central KNP, which are thought to be environmentally adaptive, may have re-colonised the north (Smith et al., 2000).

Results from MLVA31 of 2014-2015 outbreak isolates, revealed that all isolates except one grouped into the A1 sub-clade (SNP sub-group A.Br.005/006) (Fig. 3.2). The short branch lengths between GT1 and GT2 of the A1 sub-clade are indicative of the highly related nature of A-clade isolates (Fig. 3.2). P15-52, a sample isolated from vulture faeces in the Pafuri region of the KNP, represented a unique genotype (GT3) in the A3 sub-clade (SNP sub-group A.Br.Aust94) (Fig. 3.2). Although rare in the KNP, isolates belonging to this sub-group have been implicated in anthrax outbreaks, between 1998 and 2012 (Hassim et al., unpublished data). Conversely, genotypes from the ENP, between 1983 and 2010, belonged predominantly to the A.Br.Aust94 sub-group (Beyer et al., 2012). We did not detect B-clade isolates in this study, which is consistent with findings in the KNP since 1990 (Smith et al., 2000; Hassim et al., unpublished data).

Interestingly, two samples namely P15-53 and P15-54, did not group into any of three aforementioned genotypes, and are tentatively included in this phylogeny (Fig. 3.2). Three markers, namely vrrB2, pX01 and VNTR 16, were not detected during MLVA31 analysis of

P15-53 (Appendix III). In the case of P15-54, two markers, namely VNTR 16 and VNTR 17, could not be amplified (Appendix III). *Bacillus anthracis* isolates lacking plasmid pX01 have been reported, which may explain the absence of a detectable marker in P15-53 (Turnbull et al., 1992). While the absence of the aforementioned markers are likely to bias the genotypic relationship between P15-53, P15-54 and other isolates, it is worth mentioning that copy numbers at a number of VNTRs differs greatly from those seen in genotypes 1 to 3 (Appendix III). Following additional real-time PCR confirmation of *B. anthracis* specific gene regions, namely *Cap*, *Lef* and *PLF3* (results not shown), we hypothesise that isolates P15-53 and P15-54 represents previously unidentified *B. anthracis* genotypes (Wielinga et al., 2011; Ågren et al., 2013). These isolates will be characterised using next generation sequencing.

The genotypes in the present study, were compared to those implicated in anthrax outbreaks in Pafuri and Houtboschrand, between 1970 and 2013 (Appendix IV). None of the 2014-2015 *B. anthracis* isolates grouped into previously observed genotypes, indicating the level of *B. anthracis* diversity present in the endemic pockets of the KNP. Interestingly, GT1 and GT2 from the present study, are more closely related to three A1 sub-clade isolates obtained between 1970 and 1975 (A2, C14 and G25), than those obtained between 2012 and 2013 (Appendix IV). These three isolates may represent older progenitor strains of GT1 and GT2, which have since become ecologically established in the region.

A further finding of this study, is that sufficient diversity is present at seven loci, proposed to be used as a first line assay in the genotypic study of *B. anthracis* (Thierry et al., 2014). Thierry et al. (2014) were able to conclude that, once sufficient coverage of French isolates had been obtained, it would be possible to target a lower number of loci as a cost-effective measure of anthrax surveillance. We transposed this MLVA7 protocol, to isolates obtained in the present study, and noted appreciable differences at these loci (Figure 3.3; Appendix III). GT1 and GT2, which dominated the 2014-2015 outbreaks, were found to be highly clonal, with the only appreciable difference being found at VNTR23 (Fig. 3.3; Appendix III). GT 3-5, clustered differently using a MLVA7 protocol than the MLVA31 (Fig. 3.3; Appendix III). It should be mentioned, however, that the proposed MLVA7 protocol fails to differentiate the B-clade, when extended to Pafuri isolates since 1970 (Appendix V). We propose adding additional VNTR markers, such as Bams30 that will differentiate between A-and-B clade isolates. Given that laboratories in Africa are often resource constrained, the implementation of such a system using agarose electrophoresis,

Table 3.1: *Bacillus anthracis* isolates obtained from 2014 – 2015 outbreaks in the Kruger National Park.

ISOLATE	COLLECTION	ORIGIN	SAMPLE TYPE	SNP SUB-GROUP	CLADE/GT
P15-01	2015/02/17	Impala	Bone	A.Br.005/006	A1; GT1
P15-02	2015/02/20	Zebra	Swab	A.Br.005/006	A1; GT1
P15-03	2015/02/20	Impala	Swab	A.Br.005/006	A1; GT1
P15-04	2015/02/16	Impala	Swab	A.Br.005/006	A1; GT1
H15-01	2015/03/05	Impala	Swab	A.Br.005/006	A1; GT1
P15-05	2015/03/11	Impala	Swab	A.Br.005/006	A1; GT1
P15-06	2015/01/27	Impala	Swab	A.Br.005/006	A1; GT1
P15-07	2015/03/11	Impala	Swab	A.Br.005/006	A1; GT1
P15-08	2015/03/11	Impala	Swab	A.Br.005/006	A1; GT1
P15-09	2015/02/03	Impala	Bone	A.Br.005/006	A1; GT1
P15-10	2015/02/23	Zebra	Blood Smear	A.Br.005/006	A1; GT1
P15-11	2015/02/24	Impala	Blood Smear	A.Br.005/006	A1; GT1
P15-12	2015/02/25	Zebra	Blood Smear	A.Br.005/006	A1; GT1
P15-13	2015/02/16	Impala	Bone	A.Br.005/006	A1; GT1
P15-14	2014/03/19	Impala	Impala milk	A.Br.005/006	A1; GT1
M15-01	2015/01/15	Impala	Bone	A.Br.005/006	A1; GT1
P15-16	2014/03/19	Impala	Impala milk	A.Br.005/006	A1; GT1
P15-17	2015/03/03	Elephant	Hair	A.Br.005/006	A1; GT1
P15-18	2015/02/17	Zebra	Swab	A.Br.005/006	A1; GT2
P15-19	2015/02/17	Impala	Swab	A.Br.005/006	A1; GT2
P15-20	2015/02/17	Zebra	Swab	A.Br.005/006	A1; GT2
P15-21	2015/02/20	Impala	Swab	A.Br.005/006	A1; GT2
P15-22	2015/02/20	Impala	Swab	A.Br.005/006	A1; GT2
P15-23	2015/02/20	Impala	Swab	A.Br.005/006	A1; GT2
P15-24	2015/02/20	Baboon	Swab	A.Br.005/006	A1; GT2
P15-25	2015/03/11	Impala	Swab	A.Br.005/006	A1; GT2
P14-02-BL	2014/03/15	Impala	Blowfly larva	A.Br.005/006	A1; GT2
P14-01-B	2014/03/15	Impala	Beetle	A.Br.005/006	A1; GT2
P14-01-I	2014/03/15	Impala	Bone	A.Br.005/006	A1; GT2
P14-01-VF1	2014/03/15	Impala	Vulture faeces	A.Br.005/006	A1; GT2
P14-01-VF2	2014/03/15	Impala	Vulture faeces	A.Br.005/006	A1; GT2
P14-01-D	2014/03/15	Impala	Impala dung	A.Br.005/006	A1; GT2
P14-01-S	2014/03/15	Impala	Soil	A.Br.005/006	A1; GT2
P14-01-L	2014/03/15	Impala	Leaves	A.Br.005/006	A1; GT2
P14-02-I	2014/03/14	Impala	Blood Smear	A.Br.005/006	A1; GT2
P14-02-BL	2014/03/14	Impala	Blowfly larva	A.Br.005/006	A1; GT2
P15-26	2015/02/03	Wildebeest	Bone	A.Br.005/006	A1; GT2
P15-27	2015/02/03	Impala	Bone	A.Br.005/006	A1; GT2
P15-28	2015/02/03	Impala	Bone	A.Br.005/006	A1; GT2
P15-29	2015/02/03	Impala	Bone	A.Br.005/006	A1; GT2
P15-30	2015/02/03	Impala	Bone	A.Br.005/006	A1; GT2

Table 3.1 (continued): *Bacillus anthracis* isolates obtained from 2014 – 2015 outbreaks in the KNP.

ISOLATE	COLLECTION	ORIGIN	SAMPLE TYPE	SNP SUB-GROUP	CLADE/GT
P15-31	2015/02/03	Impala	Bone	A.Br.005/006	A1; GT2
P15-32	2015/02/03	Impala	Blood smear	A.Br.005/006	A1; GT2
P15-33	2015/02/03	Impala	Blood smear	A.Br.005/006	A1; GT2
P15-34	2015/02/04	Impala	Bone	A.Br.005/006	A1; GT2
P15-35	2015/02/04	Impala	Bone	A.Br.005/006	A1; GT2
P15-36	2015/02/04	Impala	Bone	A.Br.005/006	A1; GT2
P15-37	2015/02/04	Impala	Bone	A.Br.005/006	A1; GT2
P15-38	2015/02/23	Impala	Blood smear	A.Br.005/006	A1; GT2
P15-39	2015/02/23	Impala	Blood smear	A.Br.005/006	A1; GT2
P15-40	2015/02/23	Impala	Blood smear	A.Br.005/006	A1; GT2
P15-41	2015/02/24	Impala	Blood smear	A.Br.005/006	A1; GT2
P15-42	2015/02/24	Wildebeest	Blood smear	A.Br.005/006	A1; GT2
P15-43	2015/02/25	Impala	Bone	A.Br.005/006	A1; GT2
P15-44	2015/02/25	Impala	Bone	A.Br.005/006	A1; GT2
P15-45	2015/02/25	Zebra	Blood smear	A.Br.005/006	A1; GT2
P15-46	2015/02/25	Impala	Blood smear	A.Br.005/006	A1; GT2
P15-47	2015/02/16	Impala	Bone	A.Br.005/006	A1; GT2
P14-03-L1	2014/03/14	Kudu	Leaf	A.Br.005/006	A1; GT2
P14-03-L2	2014/03/14	Kudu	Leaf	A.Br.005/006	A1; GT2
P14-03-L3	2014/03/14	Kudu	Leaf	A.Br.005/006	A1; GT2
P14-03-B	2014/03/14	Kudu	Beetle	A.Br.005/006	A1; GT2
P14-03-D	2014/03/14	Kudu	Kudu dung	A.Br.005/006	A1; GT2
P14-03-L	2014/03/14	Kudu	Leaf	A.Br.005/006	A1; GT2
P14-03-S	2014/03/14	Kudu	Soil	A.Br.005/006	A1; GT2
P14-03-G	2014/03/14	Kudu	Grass	A.Br.005/006	A1; GT2
P14-04-B	2014/03/14	Kudu	Beetles	A.Br.005/006	A1; GT2
P14-04-S	2014/03/14	Kudu	Soil	A.Br.005/006	A1; GT2
P14-04-WL	2014/03/14	Kudu	Beetle larva	A.Br.005/006	A1; GT2
P15-48-I	2015/02/16	Impala	Soil	A.Br.005/006	A1; GT2
P15-48-BF	2015/01/28	Impala	Blowfly	A.Br.005/006	A1; GT2
P15-48-L	2015/01/28	Impala	Leaf	A.Br.005/006	A1; GT2
P15-49	2015/02/28	Impala	Bone	A.Br.005/006	A1; GT2
P15-50	2015/01/29	Impala	Bone	A.Br.005/006	A1; GT2
P15-51-L1	2015/02/15	Impala	Leaf	A.Br.005/006	A1; GT2
P15-51-L2	2015/02/15	Impala	Leaf	A.Br.005/006	A1; GT2
P15-51-L3	2015/02/15	Impala	Leaf	A.Br.005/006	A1; GT2
P14-05	2014/03/11	Zebra	Vulture faeces	A.Br.005/006	A1; GT2
P15-52	2015/02/28	Impala	Vulture faeces	A.Br.Aust94	A3; GT3
P15-53	2015/01/28	Impala	Bone	*	A1; GT4
P15-54	2015/02/26	Zebra	Bone	*	A1; GT5

would be invaluable. The value of a streamlined MLVA of *B. anthracis* should be investigated further, by assessing isolates from other regions of the KNP, and indeed South Africa as a whole.

3.3.2 Investigation of environmental isolates

Seven carcass sites were selected for further investigation, with samples being isolated from the dead animals, as well as the environment (Fig. 3.4; Table 3.1). The seven carcass sites are discussed as follows: (1) A bone sample was collected, on 15 March 2014, from an impala carcass (P14-01-I) in the Pafuri region of the KNP (Table 3.1). A number of samples were collected in the vicinity of that carcass, and included impala dung (P14-01-D), vulture faeces (P14-01-VF1/2), unidentified dung beetle species (P14-01-B), soil under the impala's head (P14-01-S), and leaves from *Acacia spp*. (P14-01-L). All isolates were genotyped, using MLVA31, and found to belong to A1 sub-clade (GT2) and SNP sub-group A.Br.005/006 (Fig. 3.2); (2) Blood smear obtained from an impala carcass (P14-02-I) in Pafuri, on 14 March 2014. Two blowfly larvae (Family: Stratiomyidae) were also obtained from the carcass (P14-02-BL1/2). The three isolates were genotyped, using MLVA31, and found to belong to A1 sub-clade (GT2) and the SNP sub-group A.Br.005/006 (Fig. 3.2); (3) Blood-soaked soil sample obtained from under a greater kudu carcass, on 14 March 2014, in Pafuri (P14-03-S). Additional samples collected from the site includes kudu dung (P14-03-D), *Dermestes maculates* (hide beetle) (P14-03-B), *Abutilon spp*. leaves (P14-03-L1 to L4) and unidentified grass species (P14-03-G). All isolates obtained were identical, belonging to A1 sub-clade (GT2) and the SNP sub-group A.Br.005/006 (Fig. 3.2); (4) Blood-soaked soil sample obtained from a second greater kudu carcass, on 14 March 2014, in Pafuri (P14-04-S). Invertebrates were collected from the carcass, namely a beetle larva (Family: Scaraboidea) (P14-04-WL) and *Necrobia rufipes* (red-legged ham beetle) (P14-04-B). Isolates from all three samples also belong to the A1 sub-clade (GT2) and SNP sub-group A.Br.005/006 (Fig. 3.2); (5) A blood-soaked soil sample was obtained from an impala carcass, in Pafuri, on 28 January 2015 (P15-48-S). *Chrysomya albiceps* (hairy maggot blowfly) (P15-48-BF) were observed on the carcass, and thereafter on *Abutilon spp*. leaves (P15-48-L) in the immediate vicinity. Isolates obtained from the carcass, blowfly and leaves, were identical, and belongs to A1 sub-clade (GT2) and the SNP sub-group A.Br.005/006 (Fig. 3.2); (6) Three leaves (P15-51-L1 to L3), from an unidentified tree species, were

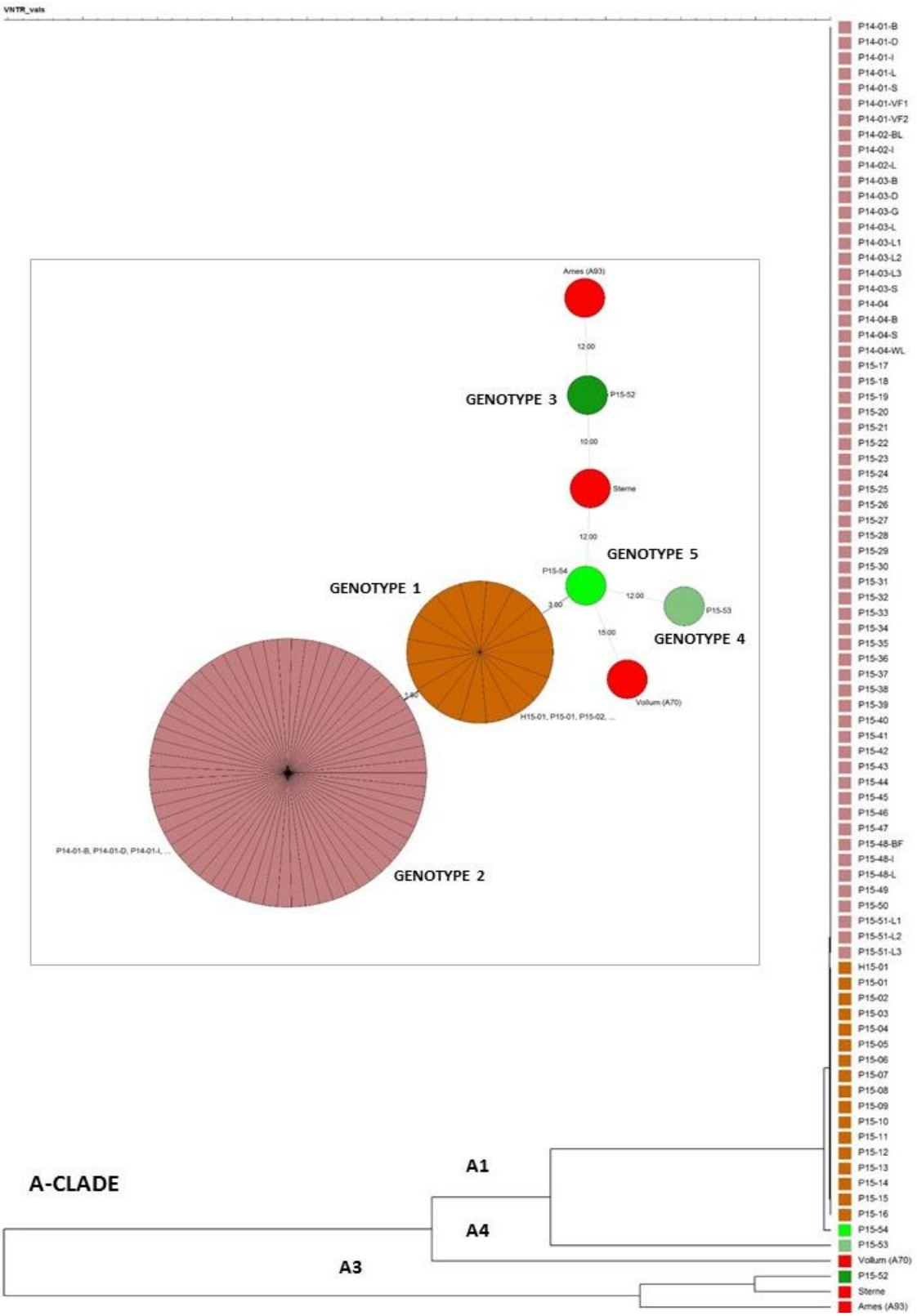

Figure 3.2: Dendrogram of 31 marker multi-locus variable number of tandem repeats assay (MLVA31) data, derived from *Bacillus anthracis* isolates from the Kruger National Park, between 2014 and 2015, and generated by means of unweighted pair group method with arithmetic means (UPGMA). The scale bar indicates genetic distance between isolates. Insert: Minimum spanning tree (MST) indicating branch lengths between genotypes.

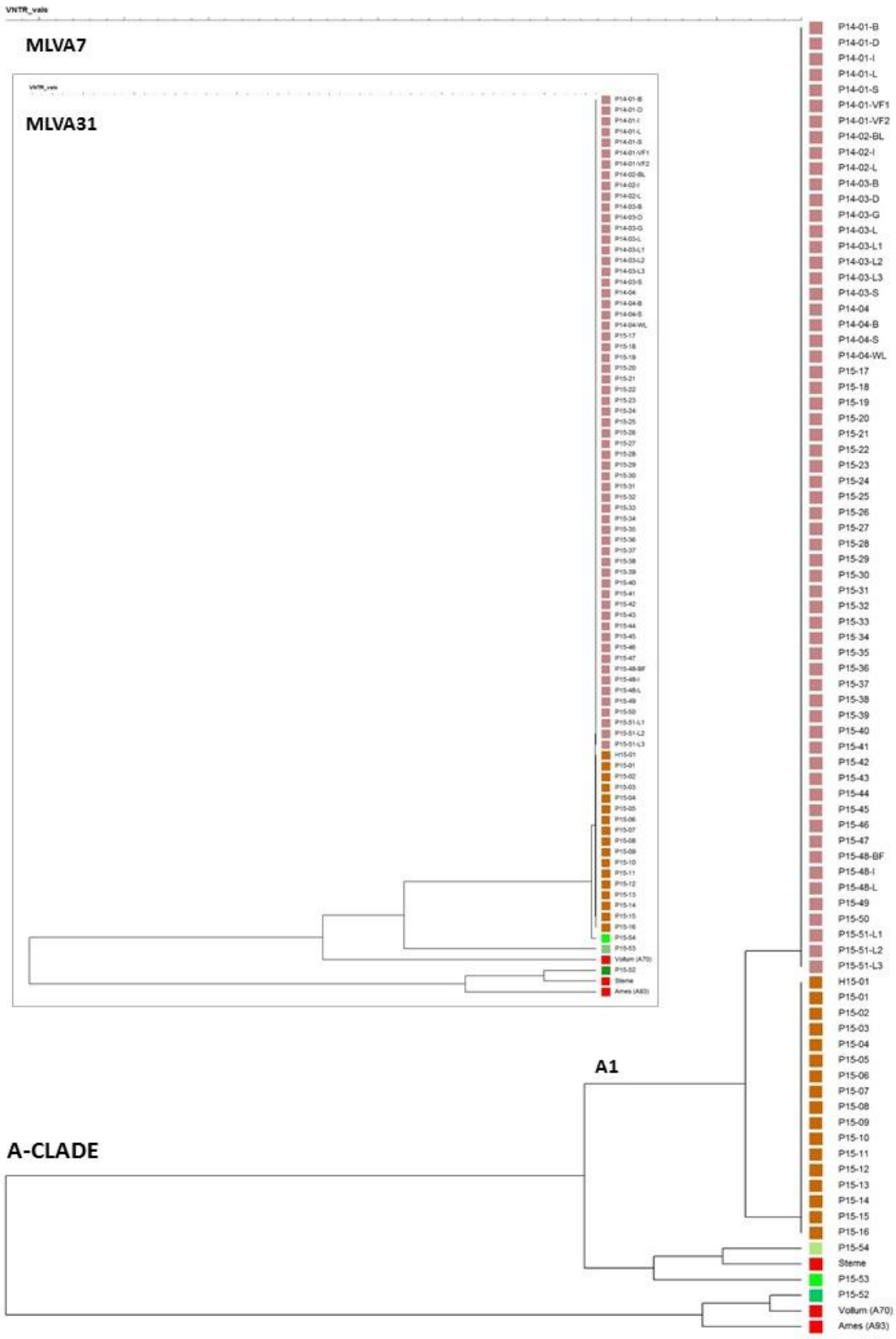

Figure 3.3: Dendrogram of seven marker multi-locus variable number of tandem repeats assay (MLVA7) data derived from *Bacillus anthracis* isolates from the Kruger National Park, between 2014 and 2015, and generated by means of unweighted pair group method with arithmetic means (UPGMA). The scale bar indicates genetic distance between isolates. Insert: Dendrogram of MLVA31 for comparison.

collected within metres of an impala carcass on 15 February 2015. The exact species could not be identified, as leaves were desiccated by the time the samples reached the laboratory in Skukuza. A sample could not be obtained from the carcass, as it was being scavenged upon by a large number of vultures. Isolates were obtained from blowfly spots on these leaves, and belonged to the A1 sub-clade (GT2) and SNP sub-group A.Br.005/006 (Fig. 3.2) and; (7) Two impala carcasses were discovered, on 19 March 2015, and found to be nursing adult females at death (P15-14 & P15-16). Milk was carefully collected from both females, and typed using MLVA31. Both isolates belong to the A1 sub-clade (GT1) and SNP sub-group A.Br.005/006 (Fig. 3.3). To our knowledge, this is the first documented case of *B. anthracis* being isolated from the milk of a wildlife species. Although such reports are scarce, evidence has been provided for *B. anthracis* being shed in the milk of infected *Bos taurus* (dairy cows) (Lenfeld & Hokl, 1941; Bowen & Turnbull, 1992).

Pienaar (1961) and Hugh-Jones and de Vos (2002) hypothesised that blowflies contaminated leaves, preferred by browsing species such as greater kudu, with *B. anthracis*. This hypothesis was further tested by Blackburn et al. (2014) in West Texas, between 2009 and 2010. The authors provided evidence for a necrophagous fly transmission pathway, where blowflies and maggots feed on an anthrax infected carcasses (Blackburn et al., 2014). Thereafter, flies deposit faeces and emesis, which may contain *B. anthracis*, on vegetation in the vicinity of the carcass (Blackburn et al., 2014). Browsing animals then come into with the pathogen when feeding upon such vegetation, and become infected (Blackburn et al., 2014). Fasanella et al. (2010) investigated the ability of Muscidae to acquire spores from rabbit carcasses, and deposit *B. anthracis* in their emesis and faeces, in a laboratory study. Their results indicated that Muscidae were capable of depositing a concentration of 40 000 CFU/ml, up to 20 hours post feeding (Fassanella et al., 2010). Recent investigation, in Namibia, failed to isolate vegetative *B. anthracis*, or spores, from fly spots on vegetation in the area (Nalisa, 2013). The authors, however, failed to recognise the potential of certain plant species which present natural bactericidal properties (Elisha et al., 2016). The authors make no mention of the vegetation assessed in their study, and admit that their method of sampling may have contributed to the failure to isolate *B. anthracis* (Nalisa, 2013). In the present study, we were able to isolate *B. anthracis* from vegetation featuring visible blowfly activity, namely *Abutilon spp*. (54 CFU per spot), *Acacia spp*. (94 CFU per spot), and an unidentified grass species (1.0×10^3 CFU per spot) (Table 3.1). Furthermore, the genotypes in the given vegetation samples were identical to those isolated from blowflies and other

Figure 3.4: Carcass sites and corresponding samples collected from the environment: (A – B) Impala carcass (P14-01-I) and *Acacia spp*. leaves with blowfly activity (P14-01-L); (C – D) Kudu carcass (P14-03-S) and *Abutilon spp*. with blowflies and blowfly spots visible (P14-03-L1 to L4); (E – F) Kudu carcass (P14-04-S) with recovered beetle larva (P14-04-WL). All images courtesy of Ayesha Hassim.

samples at corresponding carcass sites. These data supports the hypotheses of Pienaar (1961), Hugh-Jones and de Vos (2002) and Blackburn et al. (2014) that blowflies are case multipliers in the immediate vicinity of anthrax infected carcasses.

An interesting finding of this study relates to the temporal distribution of anthrax outbreaks in 2015. The first outbreak, was documented on 15 January 2015, in the Mahlangeni region of KNP, and resulted in the death of a single impala (M15-01) (Table 3.1). Thereafter, a total of 53 anthrax deaths were recorded, between 27 January and 19 March 2015 in the Pafuri region (Table 3.1). Another isolated anthrax case (H15-01), was documented on 5 March 2015, in the Houtboschrand region of KNP (Table 3.1). MLVA31

identified both M15-01 and H15-01 as belonging to the A1 sub-clade (GT1) and SNP sub-group A.Br.005/006 (Fig. 3.2; Table 3.1). This genotype is identical to 15 other isolates obtained in 2015, all from the Pafuri region (Fig. 3.2). A review of the carcass sites, and their corresponding GPS-locations, indicates that Pafuri is located 150 km from Mahlangeni, and 200 km from Houtboschrand respectively (Appendix I). Above average rainfall was documented in Pafuri, during January 2015, but not in the other two regions (Appendix VI). A review of the Pafuri region's topography, indicates a number of drainage channels into the Levuvhu River, and a pronounced low-lying northern depression (Fig. 3.5). Furthermore, no drainage channels connect the Pafuri, Mahlangeni and Houtboschrand regions of the KNP. Given the distances involved, and the topography of the Pafuri region, it is unlikely that *B. anthracis* (A1 sub-clade; GT1) was dispersed between the three outbreak foci due to weather-related phenomena. Alternative explanations are, that *B. anthracis* was either dispersed over this distance by scavenging species or, that this genotype previously occurred in Mahlangeni and Houtboschrand and had not been detected. Spores are known to be resistant to the stomach acids of *Gyps africanus* (white-backed vultures), being deposited along with the faeces of those animals (Housten & Cooper, 1975). Furthermore, an investigation of the foraging behaviour of white-backed vultures, indicated that these animals can travel in excess of 220 km per day (Phipps et al., 2013). Vulture activity was indeed observed at a number of anthrax infected carcasses, in Pafuri, during the 2015 outbreak (Appendix I). We did isolate *B. anthracis* from vulture faeces in the present study, albeit from isolates belonging to GT2 (A1 sub-clade; SNP sub-group A.Br.005/006), and GT3 (A3 sub-clade; SNP sub-group A.Br.Aust94) (Table 3.1). Dispersal of *B. anthracis* by vultures and other scavengers, as described by Lindeque and Turnbull (1994), warrants further investigation.

3.4 CONCLUSION

MLVA31 was successfully employed, in this study, to investigate anthrax isolates obtained from the KNP between 2014 and 2015. The dominant outbreak genotypes (GT1 and GT2), belonged to the A1 sub-clade and SNP sub-group A.Br.005/006, and were detected in 2014 and 2015. A third genotype (GT3), belonging to the A3 sub-clade and SNP sub-group A.Br.Aust94, was isolated in 2015 from vulture faeces. Two further genotypes (GT4 and GT5), representing a single isolate each, are tentatively described as belonging to the A1 sub-clade and SNP sub-group A.Br.005/006. These isolates will be characterised using next

generation sequencing. These data indicates that multiple strains of *B. anthracis* were circulating in the Pafuri region of KNP, though solely of the A-clade. This is consistent with findings in the area since 1990. Analysis of a subset of 7 loci, reported to be used in a streamlined MLVA protocol, shows promise. These 7 VNTR markers were indeed capable of grouping the isolates obtained in this study, into 5 distinct genotypes. Analysis of historical data, however, indicates that the MLVA7 protocol cannot differentiate A-clade isolates from those in the B-clade. Future work will focus on adding additional markers such as Bams30, to improve the discriminatory power of a streamlined MLVA protocol. MLVA7 also lacked the ability to adequately differentiate between A sub-clades.

Data from the present study presented insightful epidemiological information, which warrants further investigation. Firstly, we isolated *B. anthracis* from the milk of two impala females. Information regarding shedding in milk is known, but scarcely documented in the literature, and holds implications for both human and animal health. Secondly, we provided evidence of a blowfly transmission pathway, with *B. anthracis* of identical genotypes being isolated from carcasses, blowflies, and vegetation. While such data supports previous observations on the subject, there remains a lack of transmission studies in the field and controlled environments. Such work needs to be performed in future, if the debate around blowfly transmission of *B. anthracis* is to be put to rest. Lastly, was the detection of two outbreak samples, from Mahlangeni and Houtboschrand. These isolates belonged to GT1 (A1 sub-clade; SNP sub-group A.Br.005/006), implicated in anthrax outbreaks during the same period, in Pafuri. The distance involved, implicates vultures in the temporal expansion of the 2015 outbreak. Future studies involving MLVA and GPS tracking of vultures can provide insight. Such studies, however, are sensitive in nature, given the conservation status of these animals. These data summarises the complicated nature of *B. anthracis* epidemiology. Failure to address this dearth of knowledge, is likely to hamper future anthrax control efforts in Africa, and the developing world as a whole.

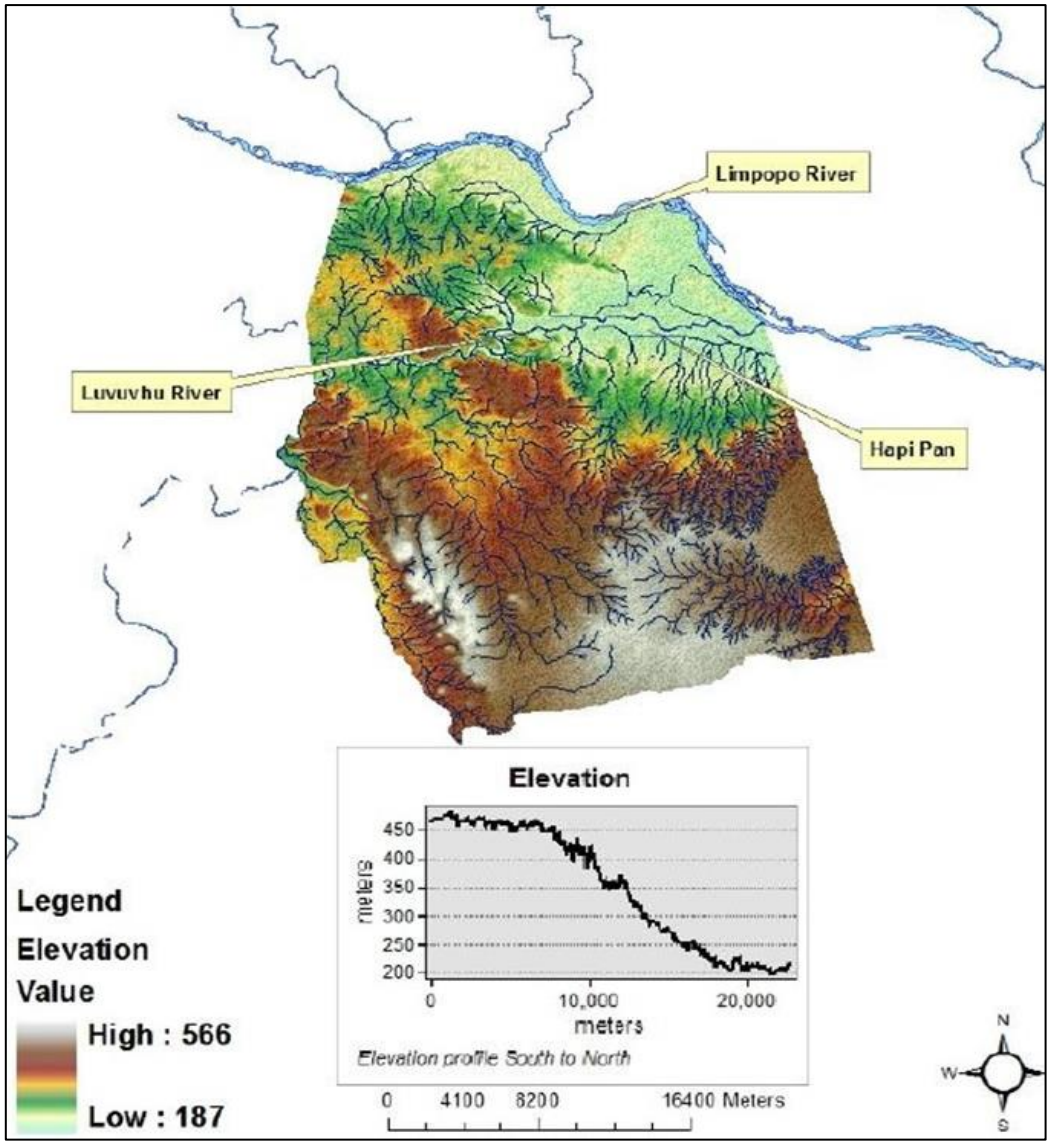

Figure 3.5: Topography of the Pafuri region, Kruger National Park (KNP). The area is characterised by a number of drainage channels into the Levuvhu River, and a low-lying northern depression (Steenkamp, 2013).

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APPENDIX I

Table: Additional information pertaining to *B. anthracis* isolates, from 2014-2015 outbreaks in the KNP. N/O = No observed or unknown scavenger activity at carcass site.

ISOLATE	SAMPLE NUMBER	SCAVENGER ACTIVITY	LATTITUDE	LONGITUDE	REGION
P15-01	1249	Vulture	-22.43261	31.27224	Pafuri
P15-02	1285	Vulture	-22.44072	31.24804	Pafuri
P15-03	1298	Vulture	-22.42723	31.21690	Pafuri
P15-04	1304	Vulture	-22.40431	31.24197	Pafuri
H15-01	1346	N/O	-24.07766	31.75223	Houtboschrand
P15-05	1361	Vulture	-22.41199	31.22353	Pafuri
P15-06	1367	Vulture	-22.41829	31.21456	Pafuri
P15-07	1368	Unopended	-22.42641	31.21784	Pafuri
P15-08	1370	Vulture	-22.41972	31.21527	Pafuri
P15-09	DS 2015/07	Vulture	-22.43225	31.28936	Pafuri
P15-10	DS 2015/72	Vulture	-22.42626	31.22610	Pafuri
P15-11	DS 2015/77	Vulture	-22.39959	31.26149	Pafuri
P15-12	DS 2015/84	Vulture	-22.37886	31.18849	Pafuri
P15-13	KM 2015/12	Vulture	-22.42009	31.22848	Pafuri
P15-14	RL 2014/15	Unopened	-22	31	Pafuri
M15-01	RL 2015/04	Vulture	-23.64268	31.32165	Mahlangeni
P15-16	SVD 2014/53	N/O	-22	31	Pafuri
P15-17	KM 2015/30	N/O	-22	31	Pafuri
P15-18	1246	Vulture	-22.42669	31.24622	Pafuri
P15-19	1257	Vulture	-22.44232	31.27533	Pafuri
P15-20	1261	Vulture	-22.43385	31.23111	Pafuri
P15-21	1293	Vulture	-22.35923	31.17737	Pafuri
P15-22	1299	Vulture	-22.44492	31.29633	Pafuri
P15-23	1303	Unopened	-22.35980	31.17723	Pafuri
P15-24	1305	Vulture	-22.44400	31.24893	Pafuri
P15-25	1365	Vulture	-22.40810	31.22578	Pafuri
P14-02-BL1	AD 2014/08	Vulture	-22	31	Pafuri
P14-01-B	AD 2014/09	Vulture	-22.25131	31.12557	Pafuri
P14-01-I	AD 2014/09	Vulture	-22.25131	31.12558	Pafuri
P14-01-VF1	AD 2014/09	Vulture	-22.25131	31.12559	Pafuri
P14-01-VF2	AD 2014/09	Vulture	-22.25132	31.12560	Pafuri
P14-01-D	AD 2014/09	Vulture	-22.25132	31.12561	Pafuri
P14-01-S	AD 2014/09	Vulture	-22.25132	31.12562	Pafuri
P14-01-L	AD 2014/09	Vulture	-22.25132	31.12563	Pafuri
P14-02-I	AD 2014/08	Vulture	-22	31	Pafuri
P14-02-BL2	AD 2014/08	Vulture	-22	31	Pafuri
P15-26	DS 2015/03	Vulture	-22.43276	31.28869	Pafuri
P15-27	DS 2015/04	Vulture	-22.43238	31.28875	Pafuri
P15-28	DS 2015/05	Vulture	-22.43252	31.28917	Pafuri
P15-29	DS 2015/06	Vulture	-22.43249	31.28924	Pafuri
P15-30	DS 2015/09	Vulture	-22.43202	31.28953	Pafuri

Table (continued): Additional information pertaining to *B. anthracis* isolates, from 2014-2015 outbreaks in the KNP. N/O = No observed or unknown scavenger activity at carcass site.

ISOLATE	SAMPLE NUMBER	CARCASS ACTIVITY	LATTITUDE	LONGITUDE	REGION
P15-31	DS 2015/10	Vulture	-22.43173	31.28912	Pafuri
P15-32	DS 2015/12	Vulture	-22.43010	31.25924	Pafuri
P15-33	DS 2015/13	Vulture	-22.43135	31.25662	Pafuri
P15-34	DS 2015/23	Vulture	-22.43261	31.28962	Pafuri
P15-35	DS 2015/24	Vulture	-22.43692	31.28776	Pafuri
P15-36	DS 2015/25	Vulture	-22.43664	31.28789	Pafuri
P15-37	DS 2015/31	Vulture	-22.43118	31.26079	Pafuri
P15-38	DS 2015/66	Vulture	-22.37554	31.18941	Pafuri
P15-39	DS 2015/71	Vulture	-22.37595	31.19122	Pafuri
P15-40	DS 2015/73	Vulture	-22.43144	31.26927	Pafuri
P15-41	DS 2015/78	Vulture	-22.37988	31.20691	Pafuri
P15-42	DS 2015/79	Vulture	-22.43709	31.30692	Pafuri
P15-43	DS 2015/80	Vulture	-22.43352	31.24788	Pafuri
P15-44	DS 2015/81	Vulture	-22.43373	31.24815	Pafuri
P15-45	DS 2015/86	Vulture	-22.37447	31.18624	Pafuri
P15-46	DS 2015/88	Vulture	-22.35669	31.16783	Pafuri
P15-47	KM 2015/13	Vulture	-22.41937	31.22906	Pafuri
P14-03-L1	Kudu 1: Leaf 2	Vulture, hyena	-22.24548	31.13160	Pafuri
P14-03-L2	Kudu 1: Leaf 3	Vulture, hyena	-22.24549	31.13161	Pafuri
P14-03-L3	Kudu 1: Leaf 4	Vulture, hyena	-22.24550	31.13162	Pafuri
P14-03-B	Kudu 1: Beetles	Vulture, hyena	-22.24551	31.13163	Pafuri
P14-03-D	Kudu 1: Dung	Vulture, hyena	-22.24552	31.13164	Pafuri
P14-03-L	Kudu 1: Leaves	Vulture, hyena	-22.24553	31.13165	Pafuri
P14-03-S	Kudu 1: Soil	Vulture, hyena	-22.24554	31.13166	Pafuri
P14-03-G	Kudu 1: Tall grass	Vulture, hyena	-22.24555	31.13167	Pafuri
P14-04-B	Kudu 2: Beeltles	N/O	-22	31	Pafuri
P14-04-S	Kudu 2: Soil	N/O	-22	31	Pafuri
P14-04-WL	Kudu 2: White	N/O	-22	31	Pafuri
P15-48-I	RL 2015/15	Unopened	-22.42491	31.24721	Pafuri
P15-48-BF	RL 2015/15	Unopened	-22.42491	31.24721	Pafuri
P15-48-L	RL 2015/15	Unopened	-22.42491	31.24721	Pafuri
P15-49	RL 2015/18	Vulture	-22.42650	31.22782	Pafuri
P15-50	RL 2015/31	Vulture	-22.42069	31.21383	Pafuri
P15-51-L1	Rooibok 32 Leaf #1	N/O	-22	31	Pafuri
P15-51-L2	Rooibok Leaf #2	N/O	-22	31	Pafuri
P15-51-L3	Rooibok Leaf #3	N/O	-22	31	Pafuri
P14-05	Zebra: Vult. Faeces	Vulture	-22	31	Pafuri
P15-52	RL 2015/21	Vulture	-22.41890	31.21945	Pafuri
P15-53	DS 2015/45	Vulture	-22.43241	31.27635	Pafuri
P15-54	RL 2015/17	Vulture	-22.42671	31.22791	Pafuri

APPENDIX II

Table: Primer combinations used for multi-locus variable number of tandem repeat analysis (MLVA) 31 genotyping of *Bacillus anthracis* strains.

PRIMER	FRAGMENT SIZE (bp)	REPEAT SIZE	DYE
MULTIPLEX A			
CG3	153-160	5	VIC
Bams 44	183-573	39	VIC
Bams 3	429-654	15	VIC
vrrB2	135-198	9	NED
Bams 5	229-424	39	NED
Bams 15	409-643	9	NED
Bams 1	296-611	21	6-FAM
vrrC1	364-688	9	6-FAM
MULTIPLEX B			
Bams 13	337-868	9	VIC
vvrB1	184-292	9	6-FAM
Bams 28	373-505	24	6-FAM
vrrC2	528-604	18	6-FAM
Bams 53	322-346	12	NED
Bams 31	331-1087	9	NED
MULTIPLEX C			
vvrA	289-338	12	VIC
Bams 25	376-391	12	VIC
Bams 21	541-766	45	VIC
Bams 34	230-581	39	NED
Bams 24	469-511	42	6-FAM
pX01	120-144	3	6-FAM
pX02	133-155	2	VIC
MULTIPLEX D			
Bams 51	358-538	45	6-FAM
Bams 22	519-1041	36	6-FAM
Bams 23	399-693	42	NED
Bams 30	268-929	9	VIC
MULTIPLEX E			
VNTR 12	106-120	2	NED
VNTR 19	91-134	3	6-FAM
VNTR 35	102-126	6	VIC

Table (cont.): Primer combinations used for MLVA 31 genotyping of *Bacillus anthracis* strains.

PRIMER	FRAGMENT SIZE (bp)	REPEAT SIZE	DYE
MULTIPLEX F			
VNTR 16	137-346	8	6-FAM
VNTR 23	170-208	12	VIC
MULTIPLEX G			
VNTR 17	366	8	NED

APPENDIX III

Table: Copy numbers, following multi-locus variable number of tandem repeat analysis, MLVA31, of 2014 – 2015 *B. anthracis* isolates, from the KNP.

ISOLATE	CG3	BAMS44	BAMS3	VRRB2	BAMS5	BAMS15	BAMS1
Sterne	2	8	28	14	6	45	16
Vollum (A70)	2	6	30	13	7	44	13
Ames (A93)	2	8	26	13	5	24	16
P15-01	2	2	18	14	6	43	10
P15-02	2	2	18	14	6	43	10
P15-03	2	2	18	14	6	43	10
P15-04	2	2	18	14	6	43	10
H15-01	2	2	18	14	6	43	10
P15-05	2	2	18	14	6	43	10
P15-06	2	2	18	14	6	43	10
P15-07	2	2	18	14	6	43	10
P15-08	2	2	18	14	6	43	10
P15-09	2	2	18	14	6	43	10
P15-10	2	2	18	14	6	43	10
P15-11	2	2	18	14	6	43	10
P15-12	2	2	18	14	6	43	10
P15-13	2	2	18	14	6	43	10
P15-14	2	2	18	14	6	43	10
M15-01	2	2	18	14	6	43	10
P15-16	2	2	18	14	6	43	10
P15-17	2	2	18	14	6	43	10
P15-18	2	2	18	14	6	43	10
P15-19	2	2	18	14	6	43	10
P15-20	2	2	18	14	6	43	10
P15-21	2	2	18	14	6	43	10
P15-22	2	2	18	14	6	43	10
P15-23	2	2	18	14	6	43	10
P15-24	2	2	18	14	6	43	10
P15-25	2	2	18	14	6	43	10
P14-02-BL	2	2	18	14	6	43	10
P14-01-B	2	2	18	14	6	43	10
P14-01-I	2	2	18	14	6	43	10
P14-01-VF1	2	2	18	14	6	43	10
P14-01-VF2	2	2	18	14	6	43	10
P14-01-D	2	2	18	14	6	43	10
P14-01-S	2	2	18	14	6	43	10
P14-01-L	2	2	18	14	6	43	10
P14-02-I	2	2	18	14	6	43	10
P14-02-L	2	2	18	14	6	43	10
P15-26	2	2	18	14	6	43	10
P15-27	2	2	18	14	6	43	10
P15-28	2	2	18	14	6	43	10
P15-29	2	2	18	14	6	43	10

Table (cont.): Copy numbers, following MLVA31 of 2014 – 2015 *B. anthracis* isolates, from the KNP.

ISOLATE	CG3	BAMS44	BAMS3	VRRB2	BAMS5	BAMS15	BAMS1
Sterne	2	8	28	14	6	45	16
Vollum (A70)	2	6	30	13	7	44	13
Ames (A93)	2	8	26	13	5	24	16
P15-30	2	2	18	14	6	43	10
P15-31	2	2	18	14	6	43	10
P15-32	2	2	18	14	6	43	10
P15-33	2	2	18	14	6	43	10
P15-34	2	2	18	14	6	43	10
P15-35	2	2	18	14	6	43	10
P15-36	2	2	18	14	6	43	10
P15-37	2	2	18	14	6	43	10
P15-38	2	2	18	14	6	43	10
P15-39	2	2	18	14	6	43	10
P15-40	2	2	18	14	6	43	10
P15-41	2	2	18	14	6	43	10
P15-42	2	2	18	14	6	43	10
P15-43	2	2	18	14	6	43	10
P15-44	2	2	18	14	6	43	10
P15-45	2	2	18	14	6	43	10
P15-46	2	2	18	14	6	43	10
P15-47	2	2	18	14	6	43	10
P14-03-L1	2	2	18	14	6	43	10
P14-03-L2	2	2	18	14	6	43	10
P14-03-L3	2	2	18	14	6	43	10
P14-03-B	2	2	18	14	6	43	10
P14-03-D	2	2	18	14	6	43	10
P14-03-L	2	2	18	14	6	43	10
P14-03-S	2	2	18	14	6	43	10
P14-03-G	2	2	18	14	6	43	10
P14-04-B	2	2	18	14	6	43	10
P14-04-S	2	2	18	14	6	43	10
P14-04-WL	2	2	18	14	6	43	10
P15-48-I	2	2	18	14	6	43	10
P15-48-BF	2	2	18	14	6	43	10
P15-48-L	2	2	18	14	6	43	10
P15-49	2	2	18	14	6	43	10
P15-50	2	2	18	14	6	43	10
P15-51-L1	2	2	18	14	6	43	10
P15-51-L2	2	2	18	14	6	43	10
P15-51-L3	2	2	18	14	6	43	10
P14-05	2	2	18	14	6	43	10
P15-52	2	8	29	14	7	45	16
P15-53	2	2	18		6	43	10
P15-54	2	2	18	14	6	43	10

Table (cont.): Copy numbers, following MLVA31 of 2014 – 2015 *B. anthracis* isolates, from the KNP.

ISOLATE	VRRC1	BAMS13	VRRB1	BAMS28	VRRC2	BAMS53	BAMS31
Sterne	53	76	20	14	17	8	65
Vollum (A70)	46	9	20	14	21	8	64
Ames (A93)	53	70	20	14	17	8	64
P15-01	57	33	20	14	17	8	64
P15-02	57	33	20	14	17	8	64
P15-03	57	33	20	14	17	8	64
P15-04	57	33	20	14	17	8	64
H15-01	57	33	20	14	17	8	64
P15-05	57	33	20	14	17	8	64
P15-06	57	33	20	14	17	8	64
P15-07	57	33	20	14	17	8	64
P15-08	57	33	20	14	17	8	64
P15-09	57	33	20	14	17	8	64
P15-10	57	33	20	14	17	8	64
P15-11	57	33	20	14	17	8	64
P15-12	57	33	20	14	17	8	64
P15-13	57	33	20	14	17	8	64
P15-14	57	33	20	14	17	8	64
M15-01	57	33	20	14	17	8	64
P15-16	57	33	20	14	17	8	64
P15-17	57	33	20	14	17	8	64
P15-18	57	33	20	14	17	8	64
P15-19	57	33	20	14	17	8	64
P15-20	57	33	20	14	17	8	64
P15-21	57	33	20	14	17	8	64
P15-22	57	33	20	14	17	8	64
P15-23	57	33	20	14	17	8	64
P15-24	57	33	20	14	17	8	64
P15-25	57	33	20	14	17	8	64
P14-02-BL	57	33	20	14	17	8	64
P14-01-B	57	33	20	14	17	8	64
P14-01-I	57	33	20	14	17	8	64
P14-01-VF1	57	33	20	14	17	8	64
P14-01-VF2	57	33	20	14	17	8	64
P14-01-D	57	33	20	14	17	8	64
P14-01-S	57	33	20	14	17	8	64
P14-01-L	57	33	20	14	17	8	64
P14-02-I	57	33	20	14	17	8	64
P14-02-L	57	33	20	14	17	8	64
P15-26	57	33	20	14	17	8	64
P15-27	57	33	20	14	17	8	64
P15-28	57	33	20	14	17	8	64
P15-29	57	33	20	14	17	8	64

Table (cont.): Copy numbers, following MLVA31 of 2014 – 2015 *B. anthracis* isolates, from the KNP.

ISOLATE	VRRC1	BAMS13	VRRB1	BAMS28	VRRC2	BAMS53	BAMS31
Sterne	53	76	20	14	17	8	65
Vollum (A70)	46	9	20	14	21	8	64
Ames (A93)	53	70	20	14	17	8	64
P15-30	57	33	20	14	17	8	64
P15-31	57	33	20	14	17	8	64
P15-32	57	33	20	14	17	8	64
P15-33	57	33	20	14	17	8	64
P15-34	57	33	20	14	17	8	64
P15-35	57	33	20	14	17	8	64
P15-36	57	33	20	14	17	8	64
P15-37	57	33	20	14	17	8	64
P15-38	57	33	20	14	17	8	64
P15-39	57	33	20	14	17	8	64
P15-40	57	33	20	14	17	8	64
P15-41	57	33	20	14	17	8	64
P15-42	57	33	20	14	17	8	64
P15-43	57	33	20	14	17	8	64
P15-44	57	33	20	14	17	8	64
P15-45	57	33	20	14	17	8	64
P15-46	57	33	20	14	17	8	64
P15-47	57	33	20	14	17	8	64
P14-03-L1	57	33	20	14	17	8	64
P14-03-L2	57	33	20	14	17	8	64
P14-03-L3	57	33	20	14	17	8	64
P14-03-B	57	33	20	14	17	8	64
P14-03-D	57	33	20	14	17	8	64
P14-03-L	57	33	20	14	17	8	64
P14-03-S	57	33	20	14	17	8	64
P14-03-G	57	33	20	14	17	8	64
P14-04-B	57	33	20	14	17	8	64
P14-04-S	57	33	20	14	17	8	64
P14-04-WL	57	33	20	14	17	8	64
P15-48-I	57	33	20	14	17	8	64
P15-48-BF	57	33	20	14	17	8	64
P15-48-L	57	33	20	14	17	8	64
P15-49	57	33	20	14	17	8	64
P15-50	57	33	20	14	17	8	64
P15-51-L1	57	33	20	14	17	8	64
P15-51-L2	57	33	20	14	17	8	64
P15-51-L3	57	33	20	14	17	8	64
P14-05	57	33	20	14	17	8	64
P15-52	53	76	20	14	17	8	65
P15-53	57	37	20	14	17	10	39
P15-54	57	33	20	14	17	8	64

Table (cont.): Copy numbers, following MLVA31 of 2014 – 2015 *B. anthracis* isolates, from the KNP.

ISOLATE	VRRA	BAMS25	BAMS21	BAMS34	BAMS24	PX01	PX02
Sterne	4	13	10	11	11	9	
Vollum (A70)	2	13	10	9	11	10	6
Ames (A93)	4	13	10	11	11	7	7
P15-01	6	13	12	9	11	6	6
P15-02	6	13	12	9	11	6	6
P15-03	6	13	12	9	11	6	6
P15-04	6	13	12	9	11	6	6
H15-01	6	13	12	9	11	6	6
P15-05	6	13	12	9	11	6	6
P15-06	6	13	12	9	11	6	6
P15-07	6	13	12	9	11	6	6
P15-08	6	13	12	9	11	6	6
P15-09	6	13	12	9	11	6	6
P15-10	6	13	12	9	11	6	6
P15-11	6	13	12	9	11	6	6
P15-12	6	13	12	9	11	6	6
P15-13	6	13	12	9	11	6	6
P15-14	6	13	12	9	11	6	6
M15-01	6	13	12	9	11	6	6
P15-16	6	13	12	9	11	6	6
P15-17	6	13	12	9	11	6	6
P15-18	6	13	12	9	11	6	6
P15-19	6	13	12	9	11	6	6
P15-20	6	13	12	9	11	6	6
P15-21	6	13	12	9	11	6	6
P15-22	6	13	12	9	11	6	6
P15-23	6	13	12	9	11	6	6
P15-24	6	13	12	9	11	6	6
P15-25	6	13	12	9	11	6	6
P14-02-BL	6	13	12	9	11	6	6
P14-01-B	6	13	12	9	11	6	6
P14-01-I	6	13	12	9	11	6	6
P14-01-VF1	6	13	12	9	11	6	6
P14-01-VF2	6	13	12	9	11	6	6
P14-01-D	6	13	12	9	11	6	6
P14-01-S	6	13	12	9	11	6	6
P14-01-L	6	13	12	9	11	6	6
P14-02-I	6	13	12	9	11	6	6
P14-02-L	6	13	12	9	11	6	6
P15-26	6	13	12	9	11	6	6
P15-27	6	13	12	9	11	6	6
P15-28	6	13	12	9	11	6	6
P15-29	6	13	12	9	11	6	6

Table (cont.): Copy numbers, following MLVA31 of 2014 – 2015 *B. anthracis* isolates, from the KNP.

ISOLATE	VRRA	BAMS25	BAMS21	BAMS34	BAMS24	PX01	PX02
Sterne	4	13	10	11	11	9	
Vollum (A70)	2	13	10	9	11	10	6
Ames (A93)	4	13	10	11	11	7	7
P15-30	6	13	12	9	11	6	6
P15-31	6	13	12	9	11	6	6
P15-32	6	13	12	9	11	6	6
P15-33	6	13	12	9	11	6	6
P15-34	6	13	12	9	11	6	6
P15-35	6	13	12	9	11	6	6
P15-36	6	13	12	9	11	6	6
P15-37	6	13	12	9	11	6	6
P15-38	6	13	12	9	11	6	6
P15-39	6	13	12	9	11	6	6
P15-40	6	13	12	9	11	6	6
P15-41	6	13	12	9	11	6	6
P15-42	6	13	12	9	11	6	6
P15-43	6	13	12	9	11	6	6
P15-44	6	13	12	9	11	6	6
P15-45	6	13	12	9	11	6	6
P15-46	6	13	12	9	11	6	6
P15-47	6	13	12	9	11	6	6
P14-03-L1	6	13	12	9	11	6	6
P14-03-L2	6	13	12	9	11	6	6
P14-03-L3	6	13	12	9	11	6	6
P14-03-B	6	13	12	9	11	6	6
P14-03-D	6	13	12	9	11	6	6
P14-03-L	6	13	12	9	11	6	6
P14-03-S	6	13	12	9	11	6	6
P14-03-G	6	13	12	9	11	6	6
P14-04-B	6	13	12	9	11	6	6
P14-04-S	6	13	12	9	11	6	6
P14-04-WL	6	13	12	9	11	6	6
P15-48-I	6	13	12	9	11	6	6
P15-48-BF	6	13	12	9	11	6	6
P15-48-L	6	13	12	9	11	6	6
P15-49	6	13	12	9	11	6	6
P15-50	6	13	12	9	11	6	6
P15-51-L1	6	13	12	9	11	6	6
P15-51-L2	6	13	12	9	11	6	6
P15-51-L3	6	13	12	9	11	6	6
P14-05	6	13	12	9	11	6	6
P15-52	4	13	10	11	11	9	6
P15-53	2	17	10	8	7		6
P15-54	2	13	12	9	11	6	6

Table (cont.): Copy numbers, following MLVA31 of 2014 – 2015 *B. anthracis* isolates, from the KNP.

ISOLATE	BAMS51	BAMS22	BAMS23	BAMS30	VNTR19	VNTR35	VNTR12
Sterne	9	16	11	51	4	5	6
Vollum (A70)	9	16	11	71	5	4	6
Ames (A93)	9	16	11	57	4	5	6
P15-01	9	15	11	71	5	2	6
P15-02	9	15	11	71	5	2	6
P15-03	9	15	11	71	5	2	6
P15-04	9	15	11	71	5	2	6
H15-01	9	15	11	71	5	2	6
P15-05	9	15	11	71	5	2	6
P15-06	9	15	11	71	5	2	6
P15-07	9	15	11	71	5	2	6
P15-08	9	15	11	71	5	2	6
P15-09	9	15	11	71	5	2	6
P15-10	9	15	11	71	5	2	6
P15-11	9	15	11	71	5	2	6
P15-12	9	15	11	71	5	2	6
P15-13	9	15	11	71	5	2	6
P15-14	9	15	11	71	5	2	6
M15-01	9	15	11	71	5	2	6
P15-16	9	15	11	71	5	2	6
P15-17	9	15	11	71	5	2	6
P15-18	9	15	11	71	5	2	6
P15-19	9	15	11	71	5	2	6
P15-20	9	15	11	71	5	2	6
P15-21	9	15	11	71	5	2	6
P15-22	9	15	11	71	5	2	6
P15-23	9	15	11	71	5	2	6
P15-24	9	15	11	71	5	2	6
P15-25	9	15	11	71	5	2	6
P14-02-BL	9	15	11	71	5	2	6
P14-01-B	9	15	11	71	5	2	6
P14-01-I	9	15	11	71	5	2	6
P14-01-VF1	9	15	11	71	5	2	6
P14-01-VF2	9	15	11	71	5	2	6
P14-01-D	9	15	11	71	5	2	6
P14-01-S	9	15	11	71	5	2	6
P14-01-L	9	15	11	71	5	2	6
P14-02-I	9	15	11	71	5	2	6
P14-02-L	9	15	11	71	5	2	6
P15-26	9	15	11	71	5	2	6
P15-27	9	15	11	71	5	2	6
P15-28	9	15	11	71	5	2	6
P15-29	9	15	11	71	5	2	6

Table (cont.): Copy numbers, following MLVA31 of 2014 – 2015 *B. anthracis* isolates, from the KNP.

ISOLATE	BAMS51	BAMS22	BAMS23	BAMS30	VNTR19	VNTR35	VNTR12
Sterne	9	16	11	51	4	5	6
Vollum (A70)	9	16	11	71	5	4	6
Ames (A93)	9	16	11	57	4	5	6
P15-30	9	15	11	71	5	2	6
P15-31	9	15	11	71	5	2	6
P15-32	9	15	11	71	5	2	6
P15-33	9	15	11	71	5	2	6
P15-34	9	15	11	71	5	2	6
P15-35	9	15	11	71	5	2	6
P15-36	9	15	11	71	5	2	6
P15-37	9	15	11	71	5	2	6
P15-38	9	15	11	71	5	2	6
P15-39	9	15	11	71	5	2	6
P15-40	9	15	11	71	5	2	6
P15-41	9	15	11	71	5	2	6
P15-42	9	15	11	71	5	2	6
P15-43	9	15	11	71	5	2	6
P15-44	9	15	11	71	5	2	6
P15-45	9	15	11	71	5	2	6
P15-46	9	15	11	71	5	2	6
P15-47	9	15	11	71	5	2	6
P14-03-L1	9	15	11	71	5	2	6
P14-03-L2	9	15	11	71	5	2	6
P14-03-L3	9	15	11	71	5	2	6
P14-03-B	9	15	11	71	5	2	6
P14-03-D	9	15	11	71	5	2	6
P14-03-L	9	15	11	71	5	2	6
P14-03-S	9	15	11	71	5	2	6
P14-03-G	9	15	11	71	5	2	6
P14-04-B	9	15	11	71	5	2	6
P14-04-S	9	15	11	71	5	2	6
P14-04-WL	9	15	11	71	5	2	6
P15-48-I	9	15	11	71	5	2	6
P15-48-BF	9	15	11	71	5	2	6
P15-48-L	9	15	11	71	5	2	6
P15-49	9	15	11	71	5	2	6
P15-50	9	15	11	71	5	2	6
P15-51-L1	9	15	11	71	5	2	6
P15-51-L2	9	15	11	71	5	2	6
P15-51-L3	9	15	11	71	5	2	6
P14-05	9	15	11	71	5	2	6
P15-52	9	16	11	51	4	3	6
P15-53	9	18	4	71	5	2	6
P15-54	9	15	11	71	5	2	6

Table (cont.): Copy numbers, following MLVA31 of 2014 – 2015 *B. anthracis* isolates, from the KNP.

ISOLATE	VNTR23	VNTR16	VNTR17
Sterne	3		
Vollum (A70)	2	20	13
Ames (A93)	4	20	4
P15-01	2	21	4
P15-02	2	21	4
P15-03	2	21	4
P15-04	2	21	4
H15-01	2	21	4
P15-05	2	21	4
P15-06	2	21	4
P15-07	2	21	4
P15-08	2	21	4
P15-09	2	21	4
P15-10	2	21	4
P15-11	2	21	4
P15-12	2	21	4
P15-13	2	21	4
P15-14	2	21	4
M15-01	2	21	4
P15-16	2	21	4
P15-17	4	21	4
P15-18	4	21	4
P15-19	4	21	4
P15-20	4	21	4
P15-21	4	21	4
P15-22	4	21	4
P15-23	4	21	4
P15-24	4	21	4
P15-25	4	21	4
P14-02-BL	4	21	4
P14-01-B	4	21	4
P14-01-I	4	21	4
P14-01-VF1	4	21	4
P14-01-VF2	4	21	4
P14-01-D	4	21	4
P14-01-S	4	21	4
P14-01-L	4	21	4
P14-02-I	4	21	4
P14-02-L	4	21	4
P15-26	4	21	4
P15-27	4	21	4
P15-28	4	21	4
P15-29	4	21	4

Table (cont.): Copy numbers, following MLVA31 of 2014 – 2015 *B. anthracis* isolates, from the KNP.

ISOLATE	VNTR23	VNTR16	VNTR17
Sterne	3		
Vollum (A70)	2	20	13
Ames (A93)	4	20	4
P15-30	4	21	4
P15-31	4	21	4
P15-32	4	21	4
P15-33	4	21	4
P15-34	4	21	4
P15-35	4	21	4
P15-36	4	21	4
P15-37	4	21	4
P15-38	4	21	4
P15-39	4	21	4
P15-40	4	21	4
P15-41	4	21	4
P15-42	4	21	4
P15-43	4	21	4
P15-44	4	21	4
P15-45	4	21	4
P15-46	4	21	4
P15-47	4	21	4
P14-03-L1	4	21	4
P14-03-L2	4	21	4
P14-03-L3	4	21	4
P14-03-B	4	21	4
P14-03-D	4	21	4
P14-03-L	4	21	4
P14-03-S	4	21	4
P14-03-G	4	21	4
P14-04-B	4	21	4
P14-04-S	4	21	4
P14-04-WL	4	21	4
P15-48-I	4	21	4
P15-48-BF	4	21	4
P15-48-L	4	21	4
P15-49	4	21	4
P15-50	4	21	4
P15-51-L1	4	21	4
P15-51-L2	4	21	4
P15-51-L3	4	21	4
P14-05	4	21	4
P15-52	3	21	4
P15-53	2		4
P15-54	2		

APPENDIX IV

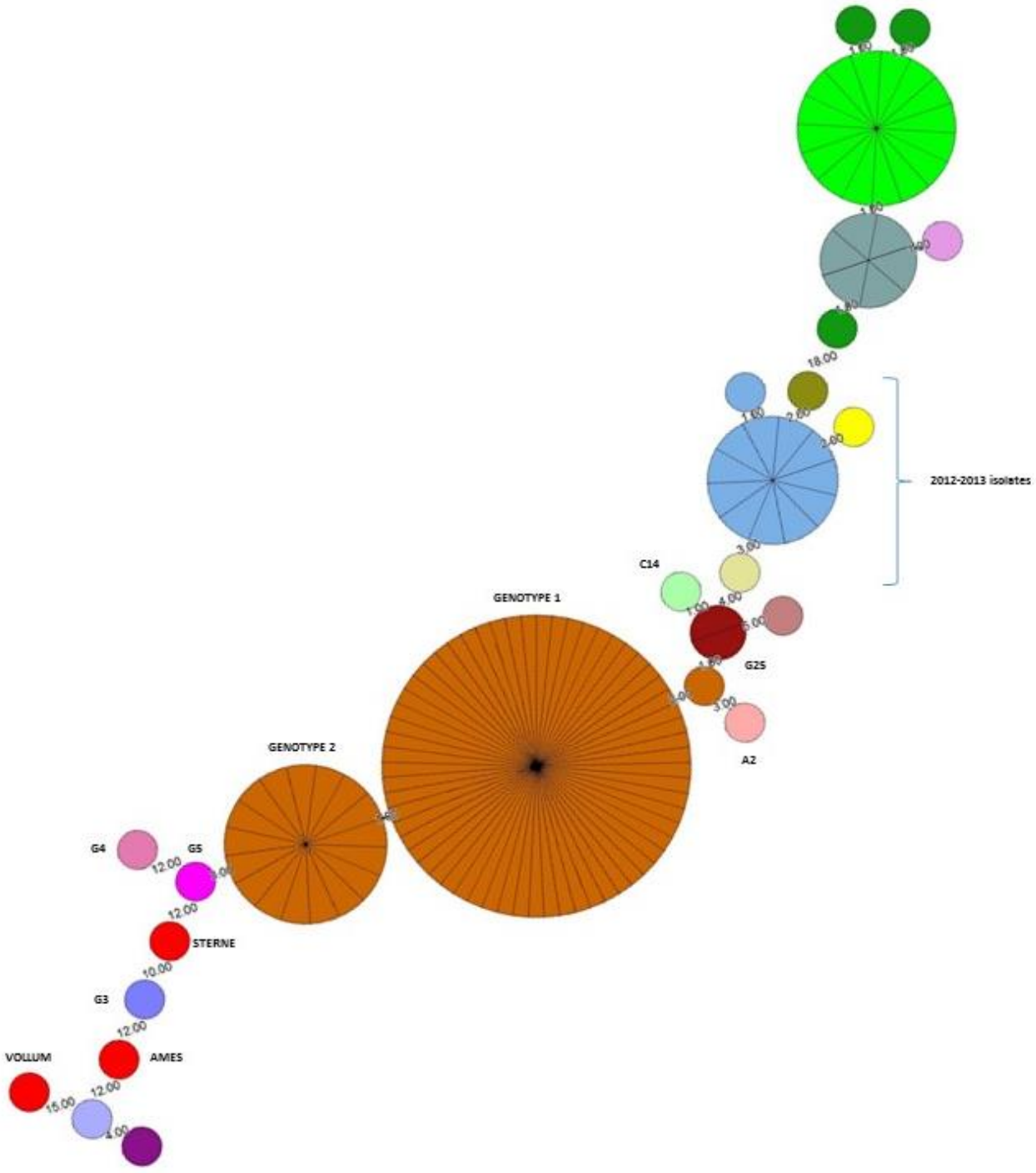

Figure: Minimum spanning tree (MST) of *B. anthracis* isolates from Pafuri and Houtboschrand in the Kruger National Park, between 1970 and 2015. Isolates from the present study are as follows: Genotypes 1 and 2 (orange); genotype 3 (violet); genotype 4 (bright pink); genotype 5 (pink). The size of each node is indicative of outbreak size. The values between nodes are indicative of the genetic distance between each genotype. Note the distance between genotypes 1 and 2, and isolates A2, C14 and G25.

APPENDIX V

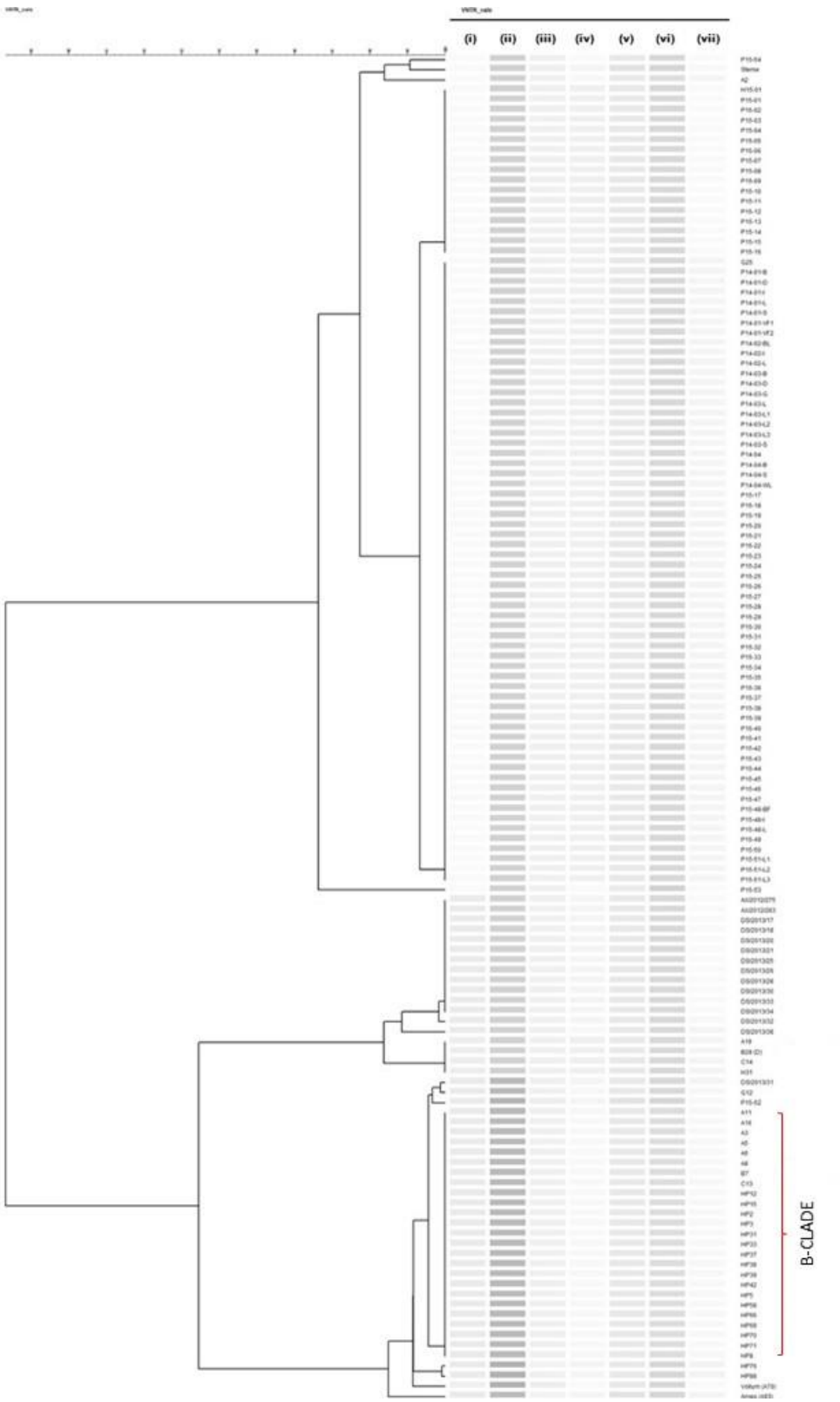

Figure: MLVA7 dendrogram of *B. anthracis* isolates from Pafuri and Houtboschrand in the KNP, from 1970 to 2015, using UPGMA. Markers are: (i) bams44; (ii) bams3; (iii) bams5; (iv) vrrA; (v) bams 34; (vi) bams22; (vii) VNTR23. Shading below each marker, represents differentiation. MLVA7 cannot differentiate between A-clade and B-clade isolates (indicated with a red bracket).

APPENDIX VI

Table: Rainfall across the Kruger National Park, during January 2015 (Scientific services, KNP).

MONITORING STATION	YRS OF DATA	# OF MISSING RETURNS	RETURN RECIEVED	RAINFALL TOTALS (mm)						ABOVE MTH AVERAGE
				LONG-TERM AVERAGE ANNUAL TOTAL	TOTALS FOR THIS MONTH			CUMULATIVE TOTAL BY END OF THIS		
					LONG-TERM AVERAGE FOR MONTH	THIS MONTH	% OF MONTHLY AVERAGE	AVERAGE CUMULATIVE TOTAL	CUMULATIVE TOTAL BY MONTH END	% OF AVERAGE CUMULATIVE TOTAL
					a	b	c	d	e	f	g
PAFURI WENELA	69	0	1	423.6	82.1	27.0	32.9	257	258.0	100.4	TRUE
PUNDA MARIA	72	1	1	530.6	111.8	53.4	47.8	322.1	204.5	63.5	FALSE
VLAKTEPLAAS	18	0	1	515.1	104.4	22.0	21.1	327.4	150.2	45.9	FALSE
SHINGWEDZI	40	0	1	504	93.3	48.0	51.4	315	238.1	75.6	FALSE
SHANGONI	43	0	1	458.2	103.4	11.5	11.1	332.2	212.3	63.9	FALSE
WOODLANDS	19	0	1	478.9	80.5	38.1	47.3	307.1	209.4	68.2	FALSE
MOOIPLAAS	28	0	1	496.3	89.4	5.1	5.7	288.7	204.8	70.9	FALSE
MAHLANGENI	42	0	1	462.7	74.4	6.3	8.5	281.4	120.9	43.0	FALSE
LETABA	45	0	1	456.7	85.1	19.4	22.8	297.4	255.9	86.0	FALSE
PHALABORWA	63	1	1	500.3	86.7	17.8	20.5	304.9	156.7	51.4	FALSE
OLIFANTS RANGER	27	0	1	496.5	88.9	22.2	25.0	320.1	140.2	43.8	FALSE
HOUTBOSCHRAND	19	0	1	438.9	79	11.0	13.9	286.4	188.6	65.9	FALSE
SATARA	54	0	1	543.7	91.6	38.8	42.4	328.5	247.2	75.3	FALSE
NWANETSI	33	1	1	541.4	99	54.1	54.6	334.6	251.6	75.2	FALSE
KINGFISHERSPRUIT	41	0	1	571.8	89.2	39.6	44.4	339.9	257.0	75.6	FALSE
TSHOKWANE	60	0	1	562.3	92.1	47.2	51.2	341.7	309.0	90.4	FALSE
SKUKUZA	76	1	1	550.4	95.6	4.8	5.0	330.9	197.4	59.7	FALSE
LOWER SABIE	33	0	1	602.6	117.1	10.0	8.5	362.9	553.1	152.4	TRUE
PRETORIUSKOP	60	0	1	737.2	129.4	68.0	52.6	453.7	386.9	85.3	FALSE
STOLZNEK	20	0	1	672.9	99.2	7.0	7.1	392.9	244.0	62.1	FALSE
CROCODILE BRIDGE	55	1	0	620	115.6	0.0	0.0	379.5	426.0	112.2	TRUE
BERG EN DAL	#	0	1	660	*	0.0	*	*	251.2	*	*
MALELANE	85	2	0	639.3	112.2	0.0	0.0	385.5	212.5	55.1	FALSE
AVERAGE KNP		**7**	**21**	**537.2**	**96.5**	**24.0**	**24.8**	**329.3**	**246.8**	**74.9**	**FALSE**