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Characterization of *Bacillus anthracis* from anthrax outbreaks in Kruger National Park (2014-2016) and the role of vultures in dissemination

By

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Submitted in fulfilment of the requirements for the degree

Master of Science Veterinary Science Tropical Diseases

in the

Faculty of Veterinary Science

University of Pretoria

Supervisor

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Animal Ethics Committee

PROJECT TITLE	**Temporal and spatial dynamics at anthrax carcass sites in Kruger National Park, South Africa**
PROJECT NUMBER	**V073-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**M Tsireledzo**

STUDENT NUMBER (where applicable)	**U_13166540**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

SAMPLES	**Carcass, Faeces, Bone marrow, Soil**	
NUMBER OF ANIMALS	**n/a**	
Approval period to use animals for research/testing purposes		**July 2017- July 2018**
SUPERVISOR	**Dr. H van Heerden**	

KINDLY NOTE:

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

APPROVED	Date 31 July 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

Declaration

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

Tsireledzo Goodwill Makwarela

December 2018

Onderstepoort, Pretoria, South Africa

Department of Agriculture Fisheries and Forestry (DAFF) approval 12/11/1/1/6 according to Section 20 of the Animal Disease Act 1984 (Act 35 of 1984), South Africa

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List of abbreviations

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

Km	Kilometres
LF	Lethal factor
MAPK	Mitogen activated protein kinase
MLVA	Multiple locus VNTR analysis
MST	Minimum Spanning Tree
n	Statistical symbol representing the sample size
NCP	Northern Cape Province
OIE	Office International des Epizooties
PCR	Polymerase chain reaction
PA	Protective antigen
PET	polymyxin-EDTA-thallium acetate
qPCR	Quantitative PCR
rRNA	Ribosomal ribonucleic acid
SNP	Single Nucleotide Polymorphism
TBE	Tris-Borate EDTA
UP	University of Pretoria
UPGMA	Unweighted pair group method using arithmetic averages
UV	Ultraviolet
VNTR	Variable number tandem repeats

WHO	World Health Organization

Thesis summary

Characterization of *Bacillus anthracis* from anthrax outbreaks in Kruger National Park (2014-2016) and the role of vultures in dissemination

Supervisor	Prof Henriette van Heerden
Co-supervisor	Dr Ayesha Hassim
Department	Veterinary Tropical Diseases
Degree	Master of Science Veterinary Science Tropical Diseases

Summary

Background: *Bacillus anthracis* is the causal agent of anthrax. Dissemination aspects of this well-known zoonotic diseases are poorly understood. In South Africa, anthrax is endemic in the Ghaap Plateau and Northern Kruger National Park (KNP). The aim of this study was to use multiloci variable number of tandem repeat (VNTR) analysis (MLVA) to determine genetic diversity and track the distribution of *B. anthracis* isolated during the 2014 - 2018 outbreaks. In addition to the genotyping; camera traps and GPS collars were used on vultures to investigate their dissemination role in the, environment. **Material and Methods:** *Bacillus anthracis* isolates (n=62) were sampled from carcasses and the environment (n=38) during outbreaks in the Pafuri and Singita regions of KNP and from 24 vultures. DNA profiles of each *B. anthracis* strain were established using 31 VNTR markers (MLVA-31). Amplicons were used to generate Minimum Spanning Trees (MST) and unweighted pair group method with arithmetic mean (UPGMA) phylogenetic data using Bionumeric v6.6.5. The genotyping data was coupled with observed activity at carcass sites from camera images and video, pre-existing isolates (n=107), as well as GPS movement maps for the vultures. **Results:** MST and UPGMA cluster analysis of MLVA-31 revealed 3 dominant clonal genotypes with a further 29 unique genotypes out of 169 isolates (i.e. 32 genotypes out of 169 isolates). With regards to the role of vultures, , a spore diluting role was observed from the data as at carcass sites that vultures fed on, low soil spore counts were observed. In contrast untouched carcass sites demonstrated higher soil spore contamination. For vultures *B. anthracis* was isolated from beaks, talons, feathers and cloacal swabs. Bacterial genotyping could link vultures to anthrax

outbreaks. Beaks and feathers had clonal genotype suggesting that vultures fed during clonal outbreaks and the unique genotypes were from cloacal swabs (digested meals) representing diverse isolates from carcass sites not sampled during this study. MLVA has proven to be a useful tool to distinguish and determine genetic diversity of *B. anthracis* strains in KNP and vultures played the role of diminishing the pathogen load in the environment.

1 Chapter 1

1.1 INTRODUCTION AND LITERATURE REVIEW

1.1.1 INTRODUCTION

Bacillus anthracis is a spore-forming soil bacterium that forms Gram-positive rods and causes an infectious disease called anthrax (Coulson *et al.*, 1994, Jacotot and Virat, 1954). It is a zoonotic disease of mammals as many outbreaks have been observed in humans and livestock over the centuries (Siqueira *et al.*, 2018, Klietmann and Ruoff, 2001). The virulent *B. anthracis* strains have two plasmids namely, pX01 and pX02, that are responsible for toxin production and encapsulation respectively (Fouet *et al.*, 2002, Okinaka *et al.*, 1999). The presence of the two plasmids determines the virulence of *B. anthracis* strains (Okinaka *et al.*, 1999, Green *et al.*, 1985, Mikesell *et al.*, 1983). When one or both plasmids are lost , the bacterium lack virulence (Mock and Fouet, 2001).

Bacillus anthracis is considered to be enzootic in Kruger National Park (KNP) and endemic in Northern Cape Province (NCP), South Africa (Hugh-Jones and De Vos, 2002). Evidence suggests that various ecological and animal behavioural factors play a role in preferential host and distribution during outbreaks (Turnbull, 2008). In South Africa, Hugh-Jones and De Vos (2002) reported that blowflies (*Chrysomyia albiceps* and *C. marginalis*) contaminate the leaves of nearby plants after feeding on infected carcasses, which affects mainly browsing kudus. Scavengers such as the spotted hyena (*Crocuta crocuta*) and vultures such as the white-backed vulture (*Gyps africanus*), Cape vulture (*G. coprotheres*) and Lappet-faced vultures (*Aegypius tracheliotus*) are also a factor speculated to play a role in spreading the pathogen to the environment and water sources (Turnbull, 2008). *Bacillus anthracis* consumed from infected carcasses does not affect vultures but can be spread to water holes when vultures bathe and drink at the water points. The vegetative form of *B. anthracis* is killed in the vulture's digestive system while spores are shed in the faeces (Turnbull, 2008, Hugh-Jones and De Vos, 2002, De Vos, 1990).

The most diverse strains of *B. anthracis* can be found on the African continent where it remains endemic/enzootic in wildlife reserves and transfrontier areas (Hassim *et al.*, 2017, Antonation *et al.*, 2016, Blackburn *et al.*, 2015, Hampson *et al.*, 2011, Hugh-Jones and De Vos, 2002) such as KNP where it is endemic in South Africa (Hugh-

Jones and De Vos, 2002). *Bacillus anthracis* being endemic in KNP provided an ideal study area to investigate factors that influence outbreak patterns. There is a need to investigate anthrax bacterial genotypes and their diversity to better understand outbreaks patterns within the park; as well as for more experimental information relating to affected hosts and the magnitude of the role vultures play in dissemination of spores during outbreaks in KNP. This study was aimed at determining *B. anthracis* genetic diversity and the role vultures play in disease dissemination at positive anthrax carcass sites during 2014-2016 within enzootic and non-enzootic regions of KNP, South Africa.

Objectives:

(i) To track vulture movement and determine the distance a vulture can travel between anthrax carcasses and their nesting sites.

(ii) To observe the activities and behaviour of scavengers at positive anthrax carcass sites to gain insight into their possible roles in disseminating the disease.

(iii) To genotype the isolates obtained from swabs of the different parts of vultures and to compare MLVA-31 genotypes of *B. anthracis* isolates of the year 2014 to 2016.

1.2 LITERATURE REVIEW

1.2.1 Aetiology of *Bacillus anthracis*

The vegetative cells of *B. anthracis* are Gram-positive rods that are square-ended and ~1-4 µm x 5-8 µm in size (Dixon *et al.*, 2000, Leppla, 1982). Long serpentine chains are formed in the exponential phase of batch culture and single cells can be observed in infected tissues/blood (Hodges *et al.*, 1965). The capsules can be observed after 24 hours of incubation at 5% CO_2 atmospheric condition with 7% sodium bicarbonate medium. Capsules can be observed microscopically using India ink. Capsulated colonies appear mucoid on bicarbonate medium incubated overnight under CO_2 (Chu, 1952, Thorne *et al.*, 1952). In liquid media, this bacterium grows as non-motile aggregating cells; however, the formation of a turbid suspension can occur upon static incubation (Charlton *et al.*, 2007, Lee *et al.*, 2007). *Bacillus anthracis* form matt, grey-white, flat colonies with uniform edges and a round shape when incubated in conditions that do not lead to capsule formation (Parry *et al.*, 1983). Endospores of *B.*

anthracis initiate anthrax infection when they enter the host (Dixon *et al.*, 1999, Hanna and Ireland, 1999, Turnbull, 1986). Endospores enter the host either by abrasion, inhalation, or ingestion. The vegetative form of *B. anthracis* kill the macrophage after the spore germination and are released into the blood stream (Hanna, 1998). After vegetative cells are released from the macrophage, they show virulent factors, toxins and capsules (Hanna, 1998). The effects of toxins on the host cells lead to death. The endospores have no measurable metabolism, this form of *Bacillus anthracis* is resistant to heat, radiation and ultraviolet light and remains stable for decades (Hanna and Ireland, 1999, Watson and Keir, 1994).

1.2.2 Mode of Action of *Bacillus anthracis*

The virulent factors of *B. anthracis* are expressed by two plasmids, pX01 (181 kb) and pX02 (95 kb), that are accountable for toxin production and encapsulation respectively (Van Schaik *et al.*, 2007, Green *et al.*, 1985). The pX01 and pX02, are reliant on the action of the tripartite protein and an antiphagocytic poly-D-glutamic acid capsule (Dixon *et al.*, 2000).

Bacillus anthracis produces typical symptoms linked to exotoxin synthesis. An example of one of these symptoms is oedema caused by the (EF) enzyme-linked toxin and is responsible for cell lysis which presents as non-clotting blood and haemorrhaging (Turnbull, 2008). The protective antigen (PA) forms a tripartite protein on the cell surface (Figure 1.1). This protein serves as the channel through which the bound oedema factor (EF) and lethal factor (LF) each endocytose into the cell. The EF is a calmodulin-activated adenylate cyclase which ramps up the production of cAMP and thus attracts fluid to the area, resulting in signalling pathway disruption, cell lysis and swelling (Leppla *et al.*, 1982). The LF helps the bacterium to evade the immune system through the killing of macrophages. In macrophages, LF acts as an endoprotease that removes the N-terminus of the mitogen-activated protein kinase (MAPKK) (Duesbery *et al.*, 1998).

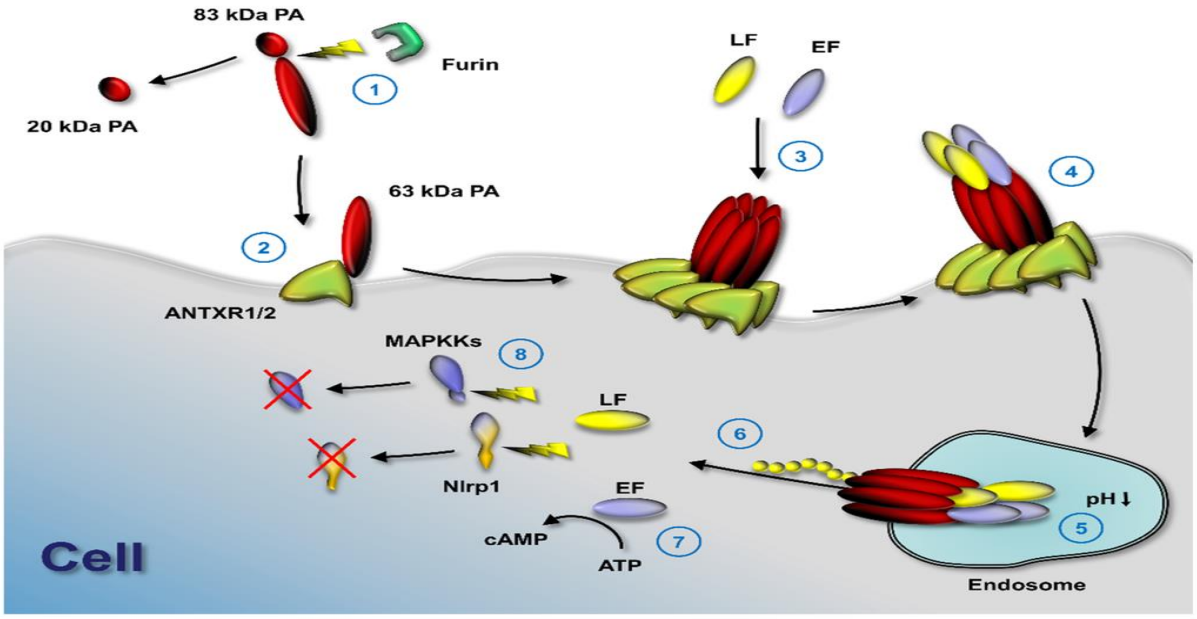

Figure 1.1 (1) Protective antigen (PA) is cleaved by Furin which enables it to bind to cell surface receptors (2) forming a tripartite protein (3) to which lethal factor (LF) and edema factor (EF) bind. (4) The PA complex gains entry into the cell by receptor-mediated endocytosis. (5) The process of acidification in the endosome results in the formation of pores which pull LF out of the endosome to the cytosol. while in the cytosol, the LF unfold (6) Unfolded LF and EF refold in the cytosol where (7) EF acts as a catalysis for the formation of cAMP from ATP and (8) LF cleaves several mitogens activated protein kinase kinases and N1rp1. Adapted from (Bachran and Leppla, 2016)

1.2.3 Classification of *Bacillus anthracis*

Taxonomically and phylogenetically, *B. anthracis* belongs to the phylum Firmicutes and is known as a member of *B. cereus* group. The group consists of *B. cereus, B. weihenstephanensis, B. pseudomycoides, B. mycoides, and B. thuringiensis* (Tourasse *et al.*, 2006, Rasko *et al.*, 2005, Helgason *et al.*, 2004, Helgason *et al.*, 2000, Turnbull, 1999, Ash and Collins, 1992, Ash *et al.*, 1991). The group has a similar cell structure, natural genetic exchange, physiology and look alike (Koehler, 2009).

Characteristics which set *B. anthracis* apart from its close relatives are that, (i) it is non-haemolytic, (ii) non-motile, (iii) sensitive to penicillin and (iv) sensitive to gamma (γ) phage (Turnbull, 2008). Most (but not all) *B. cereus and B. thuringiensis* strains are resistant to the phage, hence gamma phage is used to differentiate *B. anthracis* from the other *Bacillus* group members (Davison *et al.*, 2005, Slamti *et al.*, 2004, Chen *et al.*, 2003, Agaisse *et al.*, 1999). The characteristics which are the most distinctive of this bacterium are encoded on its virulence plasmids, pX01 and pX02, and can be detected using molecular methods (Candela *et al.*, 2005, Okinaka *et al.*, 1999, Makino *et al.*, 1989).

1.2.4 Diagnostic

Drops of blood collected from a dead carcass can be used for staining and culture (Turnbull, 2008). The rapid and easiest method to determine if an animal might have died from anthrax infection is through a stained blood smear .The number of *B. anthracis* cells in blood depends on the animal species (Turnbull, 2008). This was confirmed by Stockman (1911) when they noticed that the number of *B. anthracis* rods in pig blood smears was very low. Tubbesing (1997) did not detect the pathogen in the blood of lions and leopards that died due to anthrax. If the characteristic Gram–positive bamboo-like rods are observed in the blood smear, the suspected anthrax case can be confirmed with culture by a veterinary reference laboratory. Cultures can be established from tissue material of an infected animal or environmental samples such as soil below the carcass (Turnbull, 2008). Selective media for *B. anthracis* allow germination of *B. anthracis* spores and allow cells to grow and multiply while inhibiting growth of *B. cereus* strains (Knisely, 1966, Morris, 1955). As previously mentioned, *B anthracis* is (i) non-motile and (ii) non-haemolytic, unlike other members of *Bacillus cereus spp.* (Spencer, 2003). This enables *B. anthracis* strains to be distinguished with ease from related species on a blood agar. Cultures identified as *B. anthracis* are confirmed using PCR methods to detect the virulence genes namely the protective antigen gene (*pag*) and the capsule genes (*cap* genes) on the plasmids (Turnbull, 2008).

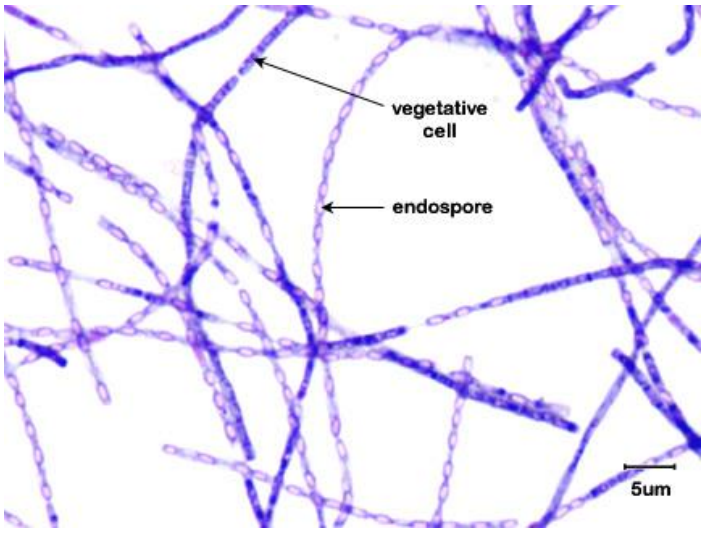

Figure 1. 2.: Morphology *of Bacillus anthracis* under the microscope. The cells have characteristic squared ends. The endospores are ellipsoidal shaped and located centrally in the sporangium. The spores are highly retractile to light and resistant to staining.http://faculty.ccbcmd.edu/courses/bio141/lecguide/unit1/prostruct/endobant.html.

1.2.5 Molecular characterization of *Bacillus anthracis*

Molecular methods have been utilized to establish the phylogenetic relatedness of pathogens (Olsen *et al.*, 1986) as well as epidemiological dispersal (Keim *et al.*, 2004, Hoffmaster *et al.*, 2002, Keim *et al.*, 2000, Smith *et al.*, 2000, Keim *et al.*, 1999, Smith *et al.*, 1999). Molecular methods such as multi-locus variable number tandem repeat (VNTR) analysis (MLVA) established by Keim et al (2000) consisting of 8 VNTR markers, was used to distinguish *B. anthracis* strains from one another using gel electrophoresis (Keim *et al.*, 2000, Smith *et al.*, 2000). MLVA typing is achieved by the aid of PCR amplification and fragment sizing to mark length polymorphisms in various VNTR regions (Keim *et al.*, 2004, Keim *et al.*, 2000). The reason for using VNTRs is that they contain greater diversity and, hence, greater discriminatory capacity than other molecular methdos (Van Belkum *et al.*, 1998, Richards and Sutherland, 1997). MLVA genotypes can be saved in a numeric code form in a database for differentiation (Grissa *et al.*, 2008, Denœud and Vergnaud, 2004). Le Flèche *et al.* (2001) developed the MLVA20 that could be differentiated using agarose gel electrophoresis. Van Ert *et al.* (2007) enhanced the MLVA8 to MLVA15 and Lista *et al.* (2006) developed a MLVA25 from the MLVA20 that could be differentiated using capillary electrophoresis expanding the discriminatory power by adding more markers. Based on genotyping data, *B. anthracis* can be clustered into 3 major lineages, A, B and C Van Ert *et al.* (2007). The full set of 31 VNTR markers (MLVA-31) has been used to evaluate the genotypic diversity of *B. anthracis* in Namibia (Beyer *et al.*, 2012). MLVA-31 enables trace back and genotyping *B. anthracis* strains (Keim et al., 2004). The discriminatory power of MLVA31 is greater as compare of MLVA8,MLVA15 and MLVA25 (Thierry *et al.*, 2014). This full set of 31 VNTR markers (MLVA-31) was employed to investigate *Bacillus anthracis* diversity found in Namibia (Beyer *et al.*, 2012).

Next generation sequencing (NGS) methods provide a revolutionary instrument for several applications and are able to generate several gigabases of sequence data in a solitary experimental run (Patel and Jain, 2012). This technology can be used to sequence whole genomes or constrained to specific regions of interest, with all 22 000 coding genes or small numbers of individual genes included (Behjati and Tarpey, 2013). Next generation sequencing enables the study of the entire *B. anthracis* genome (Pearson *et al.*, 2004). these technologies are used at a large scale because of their (i) speed, (ii) cost-effectiveness and (iii) high-throughput nature (Wang *et al.*, 2009, Mardis, 2008).

1.2.6 Anthrax as zoonosis

Bacillus anthracis causes anthrax, which is a fatal infectious disease that affects mammals and has a nearly worldwide distribution (Hugh-Jones and De Vos, 2002). It is regarded as a disease with a high mortality rate in livestock due to its pathogenicity and invasiveness (Turnbull, 2002). There is no proof of *B. anthracis* being contagious (Turnbull, 2008), and humans get infected when they directly touch infected animals or consume their products (Turnbull, 2008, Vilas-Boas *et al.*, 2007). Historically, people working in the processing industry such as wool factories and tanneries were infected by contaminated animal by-products leading to the names wool sorters disease and Cumberland disease. Since the development of the Sterne $34F_2$ vaccine, the incidence of the disease has exponentially decreased in commercially managed livestock across the globe (Gilfoyle, 2006). In disadvantaged/resource-poor communities, the disease still has a devastating effect on people and their economy.

1.2.7 Factors determining the infection of *Bacillus anthracis* in animals

The manifestation of anthrax in animals depends on various factors such as the amount of spore inoculum, host species susceptibility and host immunological state (Szyfres and Acha, 2003). Despite the lack of reliable evidence, most anthrax cases in wild animals are by all accounts marginally one-sided towards age (adulthood) and sex (males) (De Vos, 1998, Lindeque and Turnbull, 1994, Ebedes, 1976, Brunsdon, 1968, Pienaar, 1967). In an outbreak that happened in 1993, it was observed that bull-bias in death was real and it was not because calves carcasses were hard to find (Gates *et al.*, 1995). Females hardly wallow and calf did not graze much as they depend on their mothers for milk (Turnbull, 2008). With regards to age, research has offered based explanation that calves grazed less than adults because they were suckling on their mothers instead of grazing (Fox *et al.*, 1973).

1.2.8 Epidemiology of anthrax with emphasis on Kruger National Park

A high genetic diversity of *B. anthracis* isolates in KNP suggested that *B. anthracis* emerged in the sub-Saharan African continent (Smith *et al.*, 2000, Keim *et al.*, 1999, Smith *et al.*, 1999). Primarily, the anthrax life cycle involves replication within a susceptible host, death, and finally, reintroduction into the environment. In KNP, a mutually beneficial relationship between *B. anthracis* and the soil habitat exist (De Vos, 1998, Sterne, 1959). The *B. anthracis* B clade strains were isolated in KNP regions with higher calcium content in pH-neutral soils. The A clade strains, in contrast, were

isolated from a broad band of soils (Smith *et al.*, 1999). The survival of *B. anthracis* spores in the environment is subject to their initial numbers and the environment (Bellan *et al.*, 2013). The vegetative cells are unable to emulate along with competitive decomposers/anaerobic organisms and die in an unopened carcass (Stein and Van Ness, 1955). The bacterium has been hypothesized to survive in soil with cycles of germination, development, and sporulation, without the aid of a host, when sufficient nutrients are available (Fasanella *et al.*, 2010, Van Ness, 1971).

Transmission and dissemination of spores are significant factors of infection and outbreak triggers. The stages of *B. anthracis* consist of three various physiological processes, namely (i) vegetative growth, (ii) sporulation and (iii) germination, and are changed based on nutrient availability in the environment (Pilo and Frey, 2011, Bergman *et al.*, 2006). Key phases in the pathogenesis are represented by the transitions between germination and sporulation. The regulation of these stages accomplishes the establishment of the disease (Bergman *et al.*, 2006) . There have been many theories on the dissemination and delivery of an infective dose in the environment. Since the success of *B. anthracis* genetic markers, genotypes and molecular diversity can be used to resolve some questions regarding the dissemination of outbreak *B. anthracis* strains (Turnbull, 2008). Despite available tools, the epidemiology of anthrax is complex as there are variables such as multiple host species, as well as climate and ecological interactions. *Bacillus anthracis* replication occurs during a short period of infection and can be terminated when the host dies or when the bacteria is taken out of the host's system (Kim *et al.*, 2005, Helgason *et al.*, 2004, Keim *et al.*, 1997). Once a susceptible animal dies of anthrax, scavengers feeding on the anthrax-infected carcass open up the carcass, and blood with vegetative cells/spores are introduced to microenvironments (Bellan *et al.*, 2013, Turnbull, 2002, Dragon and Rennie, 1995) (Figure 1.3). Production of spores is triggered by nutrient deprivation and through competitive inhibition such as between *Clostridium* and *Bacillus spp.* (Phillips and Strauch, 2002). The spore form of *B. anthracis*, allows it to survive in the environment (Hanna and Ireland, 1999, Watson and Keir, 1994), and specific soil factors such as high levels of calcium reflect crucial ecological conditions that facilitate anthrax spore survival over prolonged time periods (Dragon and Rennie, 1995).

With the death of susceptible animals, scavengers also play a role, however the nature of the role is unclear as scavengers could partake in spatial distribution, dilution and/or concentration of spores (Turnbull, 2008). Dispersal can also be facilitated through faeces of scavengers such as *Crocuta* (spotted hyena) and *Gyps spp.* (vultures) (Lindeque and Turnbull, 1994, De Vos, 1990, Turnbull *et al.*, 1989). Research has shown that spores are resistant to the stomach acids of *Gyps africanus* (white-backed vulture) and passes through their systems (Houston and Cooper, 1975).

Blowflies may disperse spores over short distances after feeding on the bodily fluids of infected carcasses and contaminate the environment through faeces and emesis (Basson *et al.*, 2018, von Terzi *et al.*, 2014, Braack and De, 1990, Pienaar, 1961). *Bacillus anthracis* has been reported to be mechanically transmitted by biting flies including *Hippoboscidae*, and *Tabanidae* (Turell and Knudson, 1987, Mitzmain, 1914). Davies (1983) suggested that tabanids (*Tabanidae*) were involved in the extension of the outbreak in Zimbabwe in the year 1978–1979 as heavy rains were encouraging hatching of tabanids. Non-biting blowflies *spp.* such *Chrysoma albiceps* and *C. marginalis* transmit anthrax in browsing wild herbivores in KNP (De Vos, 1996, De Vos and Bryden, 1996, Braack and De, 1990). The isolation of *B. anthracis* bacterium in both vultures and different biting insects suggest that they might play a role in the anthrax life cycle (Turnbull, 2008, Jensen *et al.*, 2003, De Vos, 1990, Budd, 1863) (Figure 1.3).

Differences in foraging behaviour could also play a role such as kudu (*Tragelaphus strepsiceros*) browsing on leaves that were contaminated by blowflies feeding on infected carcasses in KNP (Hugh-Jones and De Vos, 2002) whereas plains zebra (*Equus quagga*) in the Etosha National Park 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). Infection in herbivores develop when they graze on spore-contaminated pastures (Klietmann and Ruoff, 2001). Germination of spores occur within the host's system and transforms into vegetative cells that are responsible for infection, and macrophage evasion and destruction (Hugh-Jones and De Vos, 2002, Minett, 1950).

Other variables such as animal behaviour is also involved in anthrax infection. In the KNP, outbreaks occur in a cyclical pattern every 10 years, most notably after successive seasons of above average rainfall, (De Vos, 1990). Run-off water during

the rainy season acts to disperse the spores which, mostly likely, settle in low lying areas (Dragon and Rennie, 1995). During the dry period, animals congregate near these scarce and possibly contaminated water sources. Water holes, running water and airborne spread of spores are experimented to take part in spores distribution (Lindeque and Turnbull, 1994). *Bacillus anthracis* spores are hydrophobic (Doyle *et al.*, 1984) and water takes part in collection and concentration of cells in suitable deposition areas, resulting in being carried with run-off (Dragon and Rennie, 1995). Studies shown that anthrax outbreaks normally occur in dry summer months following extended times of heavy rains (Acha and Szyfres, 2003, Dragon and Rennie, 1995). Association between seasonal cycles and drainage of cells from infected carcass sites to water holes has been postulated (De Vos, 1998, Ebedes, 1976). Water and wind movement has also been speculated to spread spores from infected carcasses to other substances (Steele *et al.*, 1979, Ebedes, 1976). Other factors speculated to play a role include the saprophytic interaction of *B. anhtracis* with plant roots and symbiotic relationship with earth worms and amoebas (Dey *et al.*, 2012, Schuch and Fischetti, 2009, Saile and Koehler, 2006).

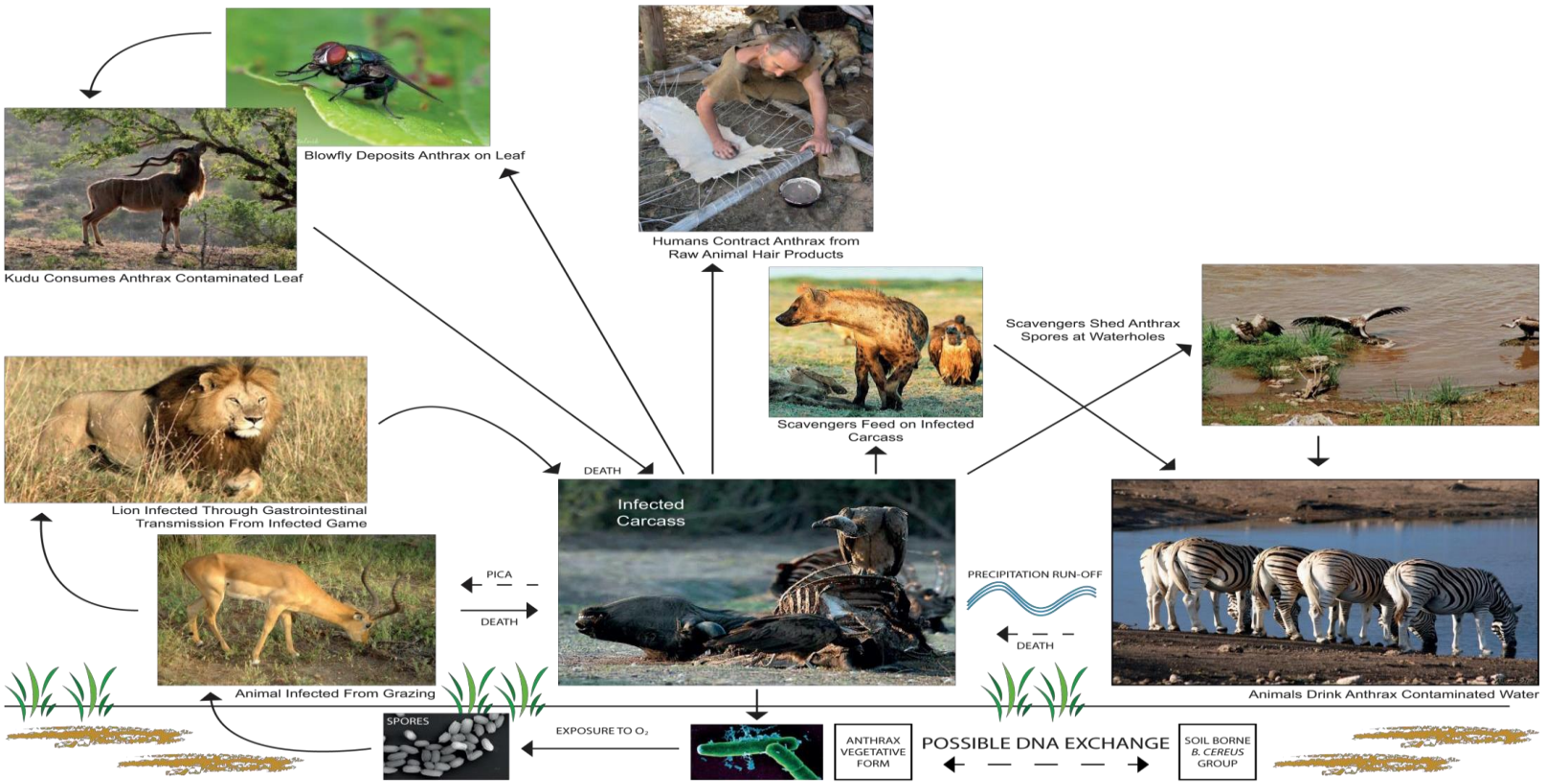

Figure 1. 3 The anthrax Life Cycle demonstrating the various routes of infection in southern Africa resulting in host death. Arrows indicate the trace of ecological and genetic factors that occur in the enzootic soil region. Adapted from (Schuch and Fischetti, 2006, Hugh-Jones and De Vos, 2002). © Hassim 2012. Infection of *Bacillus anthracis* is initiated when scavengers feed on infected carcasses. Spores are shed in the environment or inculcate the soil. Grazers that feeding on an exposed area ingest grass with spores and carnivores that feed on those herbivores may show symptoms. Blowflies that are blood feeding on the carcasses carry the spores to the leaves resulting in browsers ingesting contaminated leaves.

1.2.9 Decontamination.

For the deactivation of spores in the environment formalin is used to treat anthrax infected carcasses (Turnbull, 2008); however, Clegg *et al.* (2007) reported that the treatment of using formalin did not prevent scavengers or flies from opening the carcasses. Turnbull (1996) stated that periodic reports revealed that burial method is not reliable for long term control as spores find their way out in the soil to come to the surface and initiate other outbreaks by infecting animals. Incineration is another process used to control *B. anthracis* load, in this process carcasses are burned. Isolation of *B. anthracis* spores at incineration sites is rare , however , spores that have already inoculated the soil can survive the fire (Turnbull, 2008). Rendering is a process that involves heat treating of animal carcasses that will be used for commercial purpose. This process has three stages, namely collection, transportation and treatment of the carcass (Riedinger, 1980, Riedinger *et al.*, 1975). These decontamination practices are only practical on farms with livestock and wildlife where the wildlife is not protected through vaccination. These practices are impractical, unfeasible and logistically impossible in endemic regions in national parks like KNP where the disease is allowed to continue without intervention as a natural ecological cycle (Pienaar, 1961). In this study we explore aspects of this natural cycle.

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2 Chapter 2

Investigating the potential role of vultures in the dissemination of *Bacillus anthracis* during the 2014 anthrax outbreaks in Kruger National Park.

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Abstract

Bacillus anthracis is the causal agent of anthrax that can survive in the soil for extended periods. Anthrax is enzootic in the northern Kruger National Park (KNP) in South Africa where it mainly affects herbivores. This national park is an ideal environment to investigate this bacterium and its life cycle, especially aspects of dissemination. The aim of this study was to determine the genetic diversity of *B. anthracis* isolated during the 2014 outbreak in KNP using multi-loci variable number of tandem repeat (VNTR) analysis (MLVA-31). In addition to the genotyping; environmental factors and the role of vultures during outbreaks were investigated. *Bacillus anthracis* isolates (n=62) were isolated from carcasses (n=43) and vultures (n=24) in the Pafuri region of KNP. DNA profiles of each *B. anthracis* strain were established using 31 VNTR markers and analysed. The genotyping data was coupled with observed activity at carcass sites from camera images/video traps and GPS movement maps for collared vultures. Phylogeny analysis using MLVA-31 showed 10 genotypes consisting of one dominant clonal genotype and 9 unique genotypes of 24 *B. anthracis* strains isolated from vultures. 26 genotypes of the 62 strains were characterized from the 2014 outbreaks. Feeding behaviour observed by camera images and spore counts revealed vultures had a spore diluting role at sites at which they fed on carcasses. Low soil spore counts were recorded at the latter sites, whereas untouched carcass sites demonstrated higher soil spore contamination. Maps of GPS collared vultures indicated that vultures could spread or dilute spores over vast distances but could not deduce further information as the GPS units had a limited lifespan restricting data collection to the 2014 anthrax outbreaks in KNP. This study was able to describe the similarity in bacterial genetic fingerprints which could connect the two regions should more data become available.

2.1 Introduction

Bacillus anthracis is a bacterial species that is found in the environment in the form of spores that can persist in the soil for a very long time (Coulson *et al.*, 1994, Jacotot and Virat, 1954). Soil act as a machinal carrier of spores and the spores remain inactive in the soil and can be isolated from the soil 200 years after the animal has died (De Vos, 1998). for an example spores were detected in the soil after 36 years after trials of anthrax spore dispersion from 1942-43 in Gruinard Island, Scotland (Cherkasskiy, 1999). It is the aetiological agent of the disease anthrax (Turnbull, 2008). Anthrax is mainly a disease of ungulates; and humans incidentally contract the infection from diseased animals or their by-products (Sweeney *et al.*, 2011, Turnbull, 2008, Vilas-Boas *et al.*, 2007). Spores of *B. anthracis* are found in soil and spread to susceptible animals through contaminated dust, water, plant and animal materials (Turnbull, 2008).

A history of periodic anthrax epidemics have been recorded in the Kruger National Park (KNP), South Africa (Hugh-Jones and De Vos, 2002, De Vos, 1961). De Vos *et al.* (1973) indicated by the consistency of anthrax outbreaks, that the Pafuri region of the KNP is an enzootic anthrax region. The diversity of strains recorded in KNP led Keim *et al.* (1999) and Smith *et al.* (2000) to believe *B. anthracis* originated from southern Africa. Despite decades of observations of this robust organism and disease, it is poorly understood how an outbreak starts and ends, as well as the role that scavengers play and the magnitude of their epidemiological contribution (Turnbull, 2008). The occurrence of the *B. anthracis* bacteria in vultures and different biting insects shows possible ways in which life cycles of *B. anthracis* is maintained (Jensen *et al.*, 2003).

Differential host species susceptibility has been to be infected by *B. anthracis* (Turnbull, 2008) and different animals present with different levels of immunity (Fox *et al.*, 1977, Robertson, 1976). For example, eland (*Taurotragus oryx*) are not often affected whereas bushbuck (*Tragelaphus Scriptus*) are considered susceptible (Prins and Weyerhaeuser, 1987). During outbreaks, sable antelope (*Hippotragus niger*) showed less losses while roan antelope (*Hippotragus equinus*) showed a higher death rate (Pienaar, 1967, Pienaar, 1961). Pienaar (1961) observed cases of anthrax in impala (*Aepyceros melampus*) within KNP as well.

Minett (1950) stated that unless the carcass is opened, vegetative cells within the carcass will eventually die within 4 days when anaerobic decomposition and acidification takes place. Scavengers are believed to play a role in dissemination of spores by opening carcasses allowing sporulation to occur (De Vos, 1990). When scavengers open the carcass, bodily fluids containing vegetative cells are introduced into the environment, resulting in sporulation (Bellan *et al*., 2013). Globally, Avian scavengers, such as different species of vultures that include hooded vultures (*Necrosyrtes monachus spp.)*, white-backed vultures (*Gyps africanus spp.)*, Lappet-faced vultures (*Torgos tracheliotos spp.)*, white-headed vultures (*Aegypius occipitali spp.*), herring gulls (*Larus argentatus*), ravens (*Corvus albicollis, Corvus albus* and *Corvus capensis spp.*), and Marabou stork (*Leptoptilos crumeniferus spp.*) have the ability to transport spores over distances (Ebedes, 1976). In southern Africa, *B. anthracis* spores have been detected in scavenger's faeces (Hugh-Jones and De Vos, 2002) as well as from black-jackal (*Canis mesomelas spp.*) and spotted hyena (*Crocuta spp.*) faeces at anthrax-infected carcass sites during an outbreak that occurred in Etosha National Park (ENP), Namibia and in Luangwa Valley, Zambia (Lindeque and Turnbull, 1994, Turnbull *et al.*, 1991, Turnbull *et al.*, 1989). For vultures to transmit the disease from one area to the other, Houston and Cooper (1975) suggested that (i) *B. anthracis* must cause clinical infection in vultures, (ii) the organism must be transferred mechanically on vulture's external body structures such as talons and feathers, the pathogen is discharged in the water hole by vultures when they bath and preen, resulting in water holes being contaminated, (iii) *Bacillus* vegetative cells remain viable in the vulture's crop as the pH is almost neutral and the organism must be regurgitated, (iv) organism must tolerate exposure to very low pH in vultures stomach, pass through digestive track and be discharged with faeces. It is speculated that vultures could either act to 'concentrate' *B. anthracis* spores in an area like shared waterholes or that they could serve as environmental disinfectors by consuming carcasses containing vegetative cells and /or spores (Turnbull, 1999). Various studies hypothesise that vultures spread *B. anthracis* because when vultures and Marabou stork visit waterholes to bath and drink after feeding on infected carcasses, spores from the feathers and beaks are shed in water thereby in contaminating the water with *B. anthracis* spores (Ebedes, 1976, De Vos *et al.*, 1973, Young *et al.*, 1970, Pienaar, 1967). Vegetative cells contained in blood that contaminate the feathers are speculated to be shed in water and die within 15-68 hours

(Lindeque and Turnbull, 1994) but spores will remain at carcass sites or be disseminated by vultures and/or other vectors. In contrast, when vultures consume soft tissues of infected carcasses before vegetative cells start the process of sporulation, they minimize contamination because germination cannot occur within digestive tracks of vultures. Although *B. anthracis* anthrax spores are found in vulture faeces (Lindeque and Turnbull, 1994, Hambleton and Turnbull, 1990), spore counts were found to be very low in the droppings of the 18 vultures at the area of anthrax carcasses and the number of spores was below the minimal dose ($1.5–5x10^8$ spores) needed to initiate infection in grazing herbivores (Turnbull, 2008, Lindeque and Turnbull, 1994).

The role of vultures in dissemination of anthrax is a sensitive and controversial topic due to the endangered status of the birds. The population of vultures is declining due to poisoning by poachers (Ogada *et al*., 2015) as part of trade for traditional medicine (McKean *et al.*, 2013). Poachers intentionally poison vultures in order to hide the locations of their kills and accidental poisoning occurs on farms using strychnine and carbofuran for predator control (Kendall and Virani, 2012, Otieno *et al.*, 2010, Hancock, 2009, Brown, 1986). In this study the aim was to elucidate the role of vultures during anthrax outbreaks in the endemic region by (i) tracking vulture movement and determining the distance vultures travel, (ii) observing the activities and behaviour of scavengers at positive anthrax carcass sites during the 2014 outbreak to gain insight into their possible roles in disseminating the disease, and (iii) to genotype the isolates obtained from swabs of the different parts of vultures as well as from carcasses during the outbreak to determine epidemiological association.

2.2 Material and Methods

2.2.1 Study site

Bacillus anthracis is endemic in northern KNP in the Pafuri region. Due to a constant supply of carcasses during anthrax outbreaks, in Pafuri (indicated as the topmost purple section in Figure 2.1), vultures are aplenty and therefore this region served as an ideal capture site for the study conducted in 2014. For this study, a total of 31 vultures were captured (Appendix Table 1) by the Endangered Wildlife Trust (EWT) and Skukuza State Veterinary Services during an anthrax outbreak in 2014. As a result of cost limitations, sixteen of the thirty-one captured vultures were fitted with GPS units

during anthrax outbreak periods in 2014. Ethical approval was sought from the University of Pretoria (V073-17), SANparks Scientific Committee approval (VHEH1422) and Department of Agriculture, Forestry and Fisheries (DAFF). The approval number 12/11/1/1/6 according to Section 20 of the Animal Disease Act 1984 (Act 35 of 1984), South Africa was granted.

Table 2.1 Samples collected from vultures which include serum and swabs from beaks, feathers, talons and cloaca in Pafuri by the Endangered Wildlife Trust (EWT) and Skukuza State Veterinary Services during an anthrax outbreak in 2014. The sample numbers represent vultures and laboratory processing numbers indicate the swabs used for bacterial culture and molecular diagnostics.

Serial #	Sample #	Lab #	Serial #	Sample #	Lab #	Serial #	Sample #	Lab #	Serial #	Sample #	Lab #
1	AM2014/02/20		23	A195 BEAK	A38	45	A196 CLOACA	A43	67	A212 TALON	A55
2	AM02/26		24	A196 BEAK	A41	46	G33726 TALON	3	68	A212 CLOACA	A56
3	AM01/16		25	G33726 FEATHER	A13	47	G33727 TALON	6	69	A212 FEATHER	A57
4	AM2/19	38	26	G33727 FEATHER	A14	48	G33728 TALON	9	70	A198 BEAK	A58
5	AM14/02/19	39	27	G33729 FEATHER	A15	49	G33729 TALON	12	71	A198 TALON	A59
6	AM14/02/18	40	28	G7434 FEATHER	A34	50	G7432 TALON	A17	72	A198 CLOACA	A60
7	AM2/16	54	29	G7435 FEATHER	A35	51	G7433 TALON	A20	73	A198 FEATHER	A61
8	AM2/13/2	56	30	G7436 FEATHER	A36	52	G7434 TALON	A23	74	A209 BEAK	A62
9	AM2/16/2	59	31	G7437 FEATHER	A37	53	G7435 TALON	A26	75	A209 TALON	A63
10	AM2/13	53	32	A195 FEATHER	A44	54	G7436 TALON	A29	76	A209 CLOACA	A64
11	AM2/23	35	33	A196 FEATHER	A45	55	G7437 TALON	A32	77	A209 FEATHER	A65
12	AM2/20	33	34	G33726 CLOACA	1	56	A195 TALON	A39	78	A199 BEAK	A66
13	G33726 BEAK	2	35	G33727 CLOACA	4	57	A196 TALON	A42	79	A199 TALON	A67

14	G33727 Beak	5	36	G33728 CLOACA	7	58	A213 BEAK	A46	80	A199 CLOACA	A68
15	G33728 BEAK	8	37	G33729 CLOACA	10	59	A213 TALON	A47	81	A199 FEATHER	A69
16	G33729 BEAK	11	38	G7432 CLOACA	A18	60	A213 CLOACA	A48	82	A210 BEAK	A70
17	G7432 BEAK	A16	39	G7433 CLOACA	A21	61	A213 FEATHER	A49	83	A210 TALON	A71
18	G7433 BEAK	A19	40	G7434 CLOACA	A24	62	A214 BEAK	A50	84	A210 CLOACA	A72
19	G7434 BEAK	A22	41	G7435 CLOACA	A27	63	A214 TALON	A51	85	A210 FEATHER	A73
20	G7435 BEAK	A25	42	G7436 CLOACA	A30	64	A214 CLOACA	A52	86	A226 BEAK	A74
21	G7436 BEAK	A28	43	G7437 CLOACA	A33	65	A214 FEATHER	A53	87	A226 TALON	A75
22	G7437 BEAK	A31	44	A195 CLOACA	A40	66	A212 BEAK	A54	88	A226 CLOACA	A76

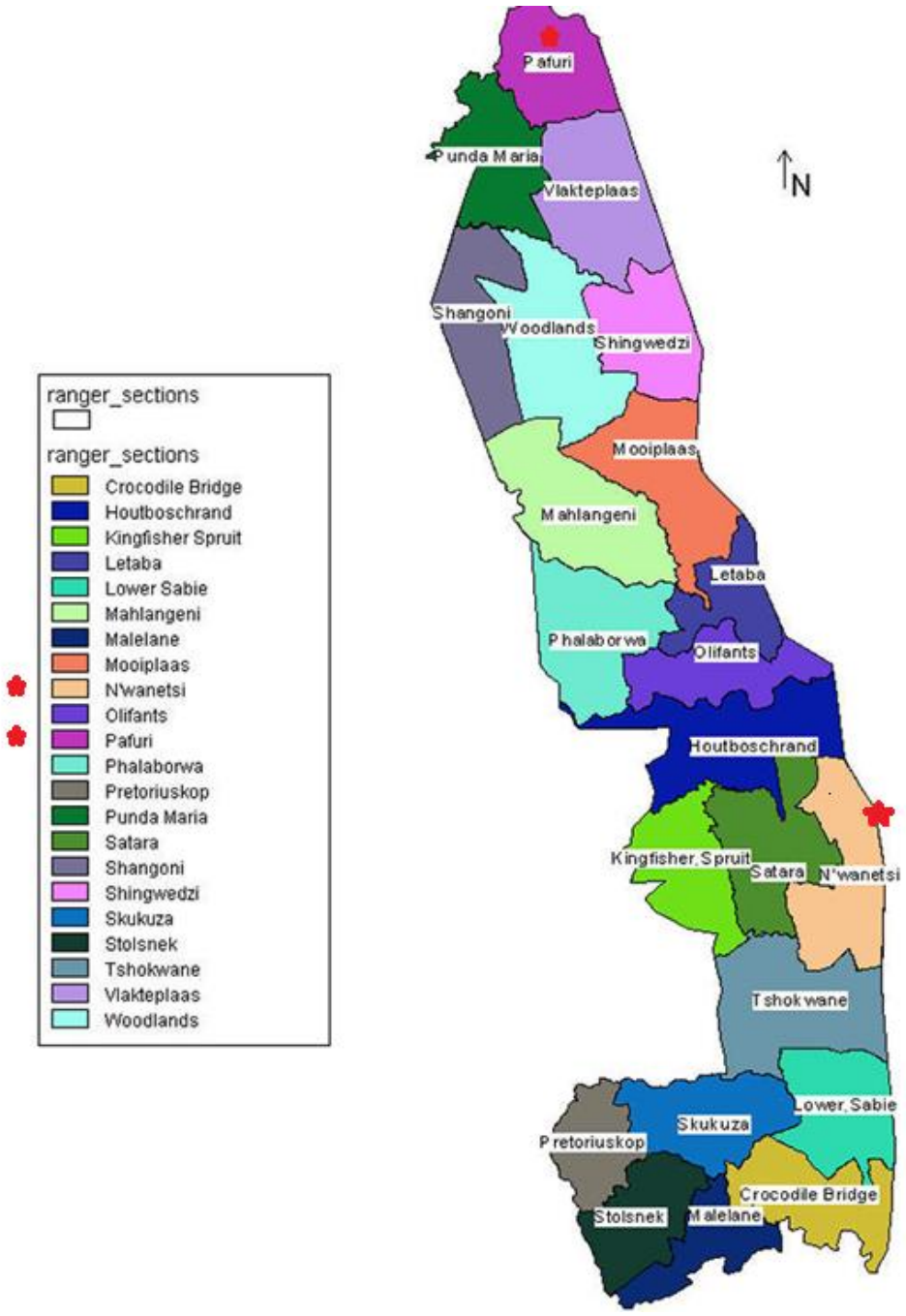


Figure 2.1: Map of Kruger National Park (KNP) indicating anthrax epidemic region in 2014-2016 namely, Pafuri and Nwanetsi (red *), where isolates of *B. anthracis* were obtained for this genetic study. The actual collection sites are indicated by stars. Different range sections are highlighted in different colours as indicated on the left side of the map adapted from http://www.thekruger.com/sections.htm

2.2.2 Vulture capture methods and vulture tracking

Vultures (88 swabs from n=31 vultures: Table 2.1) were captured in April 2014 and September 2014 by the EWT and Skukuza Veterinary Services using two methods; (i) a selective snare method, where a single, baited noose is monitored from a distance and (ii) a dead Zebra (*Equus quagga burchellii*) was caged with an opening for scavengers to enter and vultures were trapped inside the cage when they were feeding

on the carcass. Fifteen white-backed vultures (*Gyps africanus*) and one hooded vulture (*Necrosyrtes monachus*) were fitted with lightweight cell phone GPS transmitters to determine the possible distance that a vulture can cover. The GPS fitment was done after swab samples for bacterial culture and PCR diagnosis were collected from the beaks, feathers, talons, and cloacae of the vultures.

2.2.3 Camera Traps at anthrax carcass sites

The use of cameras to obtain information from the field is currently a common method for conservational and ecological study. The 12 megapixels (MP) scouting cameras (ACORN LTL-5210MM) were used to identify scavenger activity at six positive anthrax carcass sites. The cameras used had highly sensitive pyroelectric Infrared Radial (PIR) sensors that detect the sudden change of ambient temperature caused by moving game in the region of interest (ROI) which then triggers photo capture with 10 photos at 1-minute intervals and video after sustained activity. The camera also inserts the GPS co-ordinates in the file properties. A unique specification from other models is that the camera has; 5/12MP picture resolution, 1920 x 1080/1280 x 720/640 x 480/320 x 240 video resolution, excellent quality of the audio recordings, 44-piece LED that improve the quality of night pictures, as a wide-angle lens camera with a field of view of 100 degrees. The camera and video mode took both pictures and video at trigger events.

Due to logistic and hardware issues, the activities of scavengers at six carcass sites only were documented for this study (Figure 2.2). The six anthrax positive carcass sites were in the endemic (Pafuri) region of KNP. Samples from carcasses and the environment were collected to identify anthrax positive carcass sites by means of field Giemsa smear diagnostics which was performed on site by the Skukuza State Veterinary Services.

Figure 2. 2 Images taken from the six anthrax positive carcass sites in endemic Pafuri, Kruger National Park from camera traps (ACORN LTL-5210MM) used to observe scavenger activity at fresh carcasses during the 2014 outbreaks. Carcasses A, B, C and D were zebra carcasses while E and F were those of impala antelope.

2.2.4 Bacterial culture and diagnostic methods

Bacillus anthracis isolation from swabs and carcasses during anthrax outbreaks was achieved by the State Veterinary Services using protocols laid out in the WHO/OIE anthrax manual (Turnbull, 2008). Samples from carcasses (Appendix Table 1) were collected as part of the State Veterinary Services anthrax surveillance program in Kruger National Park.

2.2.5 Genotyping of *Bacillus anthracis*

2.2.5.1 DNA extraction

Bacillus anthracis cultures were grown on 5% sheep blood agar (Manufacturer) for 16 hours incubated at 37ºC using the BSL2+ Bacteriology facilities of the University of Pretoria Faculty of Veterinary Science. DNA extractions of culture isolates were performed using the Gram-positive bacterial protocol from ISOLATE II Genomic DNA Kit – (Bioline). An amount of 20 mg/ml lysozyme was used to rupture the peptidoglycan layer of *B. anthracis* and it was incubated for an hour at 37°C. Using a 100 µl pipette, 25 µl of Proteinase K solution was added with 200 µl Lysis Buffer G3. The samples were incubated at a temperature of 70°C for 15 minutes. Then 210 µl ethanol (100%) was added to the tube and vortexed. Bacterial DNA was bound by loading the lysed sample to the Genomic DNA Spin Column in a 2 ml collection tubes and following the washing process. The DNA was eluted in a 100 µl of elution buffer before storage at -20°C.

2.2.5.2 Polymerase chain reaction and gel electrophoresis

MyTaq™ Red Mix (Bioline) was used as it is a ready-to-use 2x, PCR mix. A set of 31 VNTR markers were used in individual reactions (Appendix Table 2) with a final concentration of 5 ng DNA (Beyer *et al.*, 2012, Lista *et al.*, 2006, Le Flèche *et al.*, 2001, Keim *et al.*, 2000). Every reaction mixture comprised 2.5 µl DNA template, 1 x myTaq red 2 mix, and 1.0 µM forward and reverse primers with a final reaction volume of 15 µl. PCR condition 1 was set as follows; initial denaturation at 95°C for 1 minute and 35 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 15 seconds, followed by a final extension at 72°C for 10 minutes and PCR condition 2 was set as follows; initial denaturation at 95°C for 1 minute and 35 cycles of denaturation at 95°C for 15 seconds, annealing at 56°C for 15 seconds, and extension at 72°C for 15 seconds, followed by a final extension at 72°C for 10 minutes. The PCR was conducted using an ABI-Veriti thermalcycler with DNA products loaded on Inultraflux® 96- well PCR plates semi-skirted (Lasec). PCR amplicons were analysed by gel electrophoresis for 3 hours at a voltage of 120 V. Sterne and Vollum strains were used as controls and they were loaded after every 8th well before and after the Generuler 100 base pair (bp) DNA Ladder (Thermo Scientific™) respectively to normalise for gel distortions. Ethidium bromide in Tris-borate buffer (TBE) of 2% and 3% gel (Appendix Table 2) was used to stain and differentiate the concentration and migration of the DNA amplicons. Images were taken electronically by Molecular imager ®ChemiDoc^{tm}XRS+with image Lab^{tm} BioRad software.

2.2.6 Copy Number Determination

In this study, PCR amplicons were detected using agarose gel electrophoresis to determine fragment sizes. Copy numbers of fragment sizes for every allele were generated using the copy code convention proposed by (Thierry *et al.*, 2014) as indicated in (Appendix Table 3). The copy number describes the allele for that VNTR target as it symbolizes the number of times the repeat unit is represented in each locus.

2.2.7 Analysis: UPGMA and MST

Copy number fingerprint profiles were examined by means of BioNumerics software, version 6.6.5 (Applied Maths, Belgium). Three strains were used as reference strains, namely Ames, Sterne and Vollum. Cluster analyses were generated based on

categorical data in the form of Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Minimum Spanning Tree (MST) (See Appendix Table 4).

2.3 Results

2.3.1 Vulture captures and vulture tracking

The observed distance and location covered by captured vultures is indicated in Figure 2.3. Each colour represents an individual vulture. The signal from the GPS units were lost or failed to initiate on eleven vultures shortly after capture in 2014. The distances travelled by the remaining five vultures fitted with GPS units can be seen in Figure 2.3. Over the 8 months period, the vultures travelled extensive distances from anthrax positive carcass sites (Pafuri), Kruger National Park (KNP). Four vultures flew from KNP to Botswana, Zambia and Zimbabwe. One of the vultures (represented by the light blue colour in Figure 2.3) flew from KNP to KwaZulu-Natal (KZN) before flying to the northern part of Zimbabwe. All vultures with their GPS units that returned to the park were recaptured at their nesting sites after 8 months. Nesting sites were identified by prolonged and consistent periods of signal from a single set of co-ordinates.

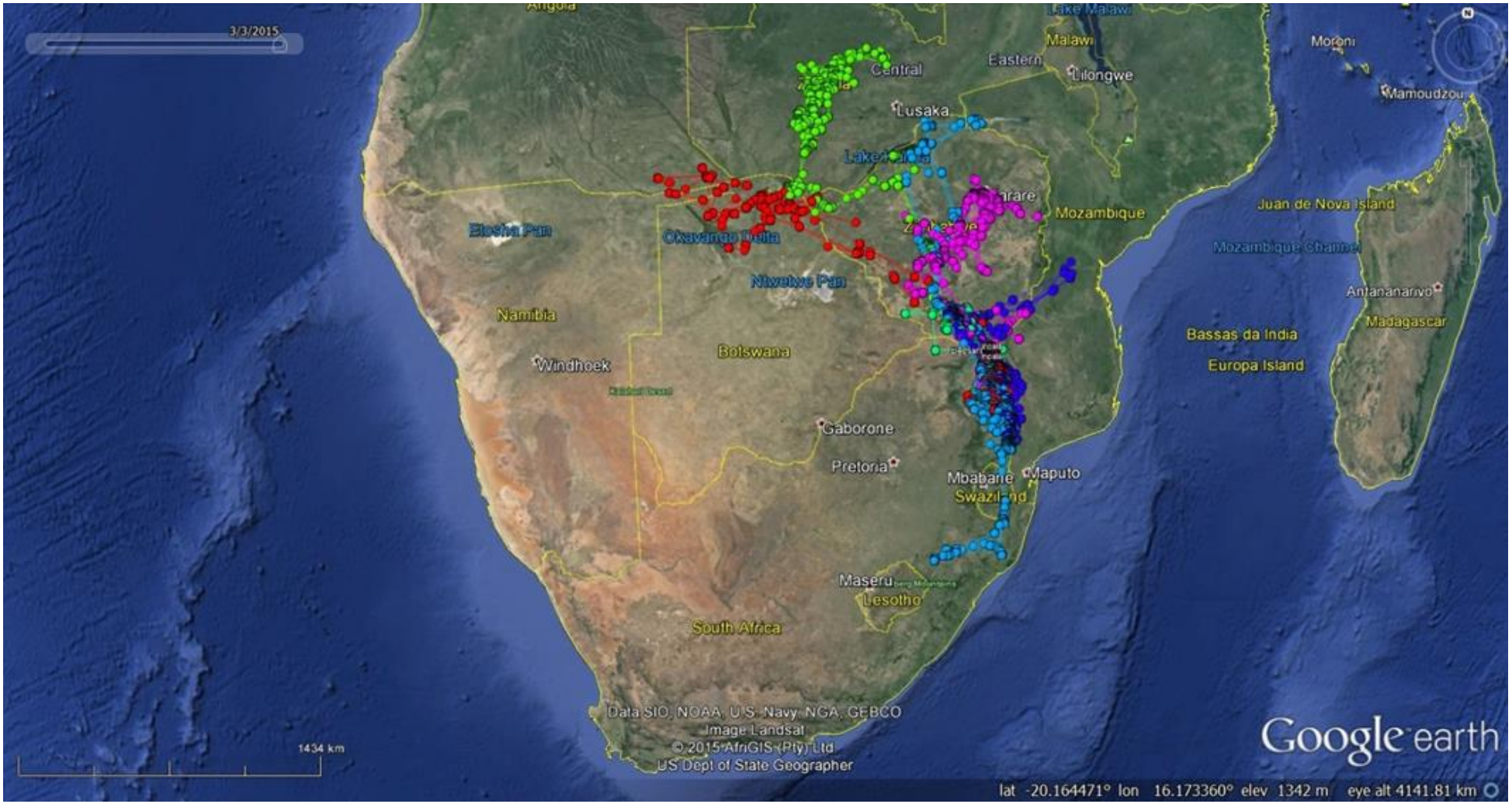


Figure 2. 3: Map of Africa showing distances covered by vultures (each colour represents an individual vulture) captured in Pafuri region in the north of Kruger National Park during anthrax outbreaks in 2014. The most extensive distance covered by a vulture is represented by the turquoise flight pattern as an example, this vulture flies from Kruger National park down to KwaZulu-Natal (KZN) close to Lesotho and all the way up to northern border of Zambia and back to Kruger National Park again

2.3.2 Camera traps

Results for scavenger activities at positive anthrax carcass sites were recorded and are summarized in Table 2.2. The activities at each of the cameras (A-F) were:

At site A (2014, January), a zebra (*Equus quagga burchellii.*) carcass seemed to have been there for a couple of hours. The environment consisted of tall grasslands and the weather was sunny. The carcass had a swarm of blowflies (*Calliphoridae spp.*) feeding on the carcass's blood, but their number were too many to count. In the afternoon, twelve vultures visited the carcass site; however, they did not feed on the carcass. A few minutes later a Marabou stork (*Leptoptilos crumenifer*) appeared, and an additional eight vultures landed at the area increasing the number to twenty. After 30 minutes, these vultures left the carcass. After 10 minutes 23 individuals returned and started feeding on the carcass until the whole carcass was consumed. Marabou storks were waiting for vultures to tear up the carcass, and as the vultures fed, the Marabou stork was stealing meat directly from vultures and eating pieces of meat dropped by the vultures on the periphery. It took them 45 minutes to consume the carcass. A hyena (*Hyaena brunnea*) visited the carcass site and sniffed at the carcass while a few vultures remained at the site.

At site B (2014, April), a zebra (*Equus quagga burchellii spp.*) carcass that was infected with *B. anthracis* was not consumed. The carcass was fresh, and it was still intact. Three warthogs (*Phacochoerus africanus*) and blowflies (*Calliphoridae spp.*) were filmed at this site. Nothing was observed to consume any part of the carcass at this site except the blowflies which were sucking the blood from the carcass around the orifices. Vegetation at this site comprised of mixed Bushveld (grass and *Acacia spp.*).

Same applies to site C; where a Zebra (*Equus quagga burchellii spp.*) carcass remained untouched and animals that visited the area were warthogs (*Phacochoerus africanus*).

At site D (2014, March), on a Zebra (*Equus quagga burchellii spp.*) carcass that was in a dry open environment was not consumed, thirty vultures and two Marabou storks (*Leptoptilos crumenifer*) visited the carcass. The birds did not feed on the carcass at all. After a couple of hours of moving around the carcass, they left the site. On the

following day, numerous vultures returned to the carcass site but still did not feed on it.

At site E, (2014, March) an anthrax infected Impala (*Aepyceros melampus spp.*) was observed to collapse suddenly and died in the late afternoon. At sunset, a flock of about 50 vultures came to the carcass site and chased off a lone eagle (a juvenile tawny eagle) attempting to penetrate the animal's hide. The vultures took 25 minutes to consume the carcass in its entirety, leaving almost nothing behind. They were competing for space during the feeding and creating a lot of dust. When the whole carcass was consumed, the bulk of the vultures left with only three remaining at the carcass site.

At site F, (2014, April) vultures were recorded feeding on another identified anthrax zebra (*Equus burchelli spp.*) carcass. The carcass was moved into a thicket by the squabbling vultures. The vegetation was quite verdant and therefore obscured the view of the carcass from camera view. We could not then discern the number of vultures and the exact time taken by the vultures to consume the whole carcass because of the obstructions. They might have remained even when there was nothing left to feed on.

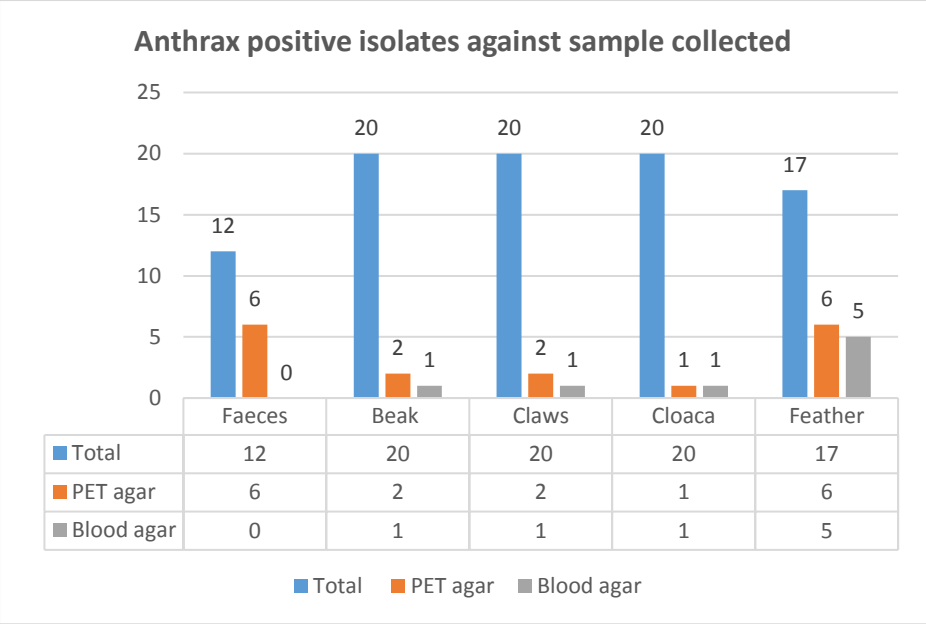
Table 1.2 Summary information regarding scavenger activities at carcasses sites (Each colour shade represents individual carcass sites.). This is a summary of 6 carcass sites where the cameras were placed. The type of scavengers that visited the carcass sites. Estimate times taken for the vultures to consume the whole carcass were recorded.

Folder #	Location	Date	Time	Animal type	Number of vultures	Number of marabou storks	Carcasses type	Carcasses condition	Estimate time taken (min)	Activities
A	Pafuri	23-March-14	1:13 PM	Vultures	20	2	Zebra	Intact	45 min	Feeding
A	Pafuri	01-Jan-14	1:40 PM	Vultures	20	2	Zebra	Ripped	45 min	Feeding
A	Pafuri	01-Jan-14	3:35 PM	Vultures	20	2	Zebra	Ripped	45 min	Feeding
A	Pafuri	01-Jan-14	7:10 PM	1 hyena	20	2	Zebra	Bones	45 min	Roaming near the carcass
B	Pafuri	11-Apr-14	09:14 - 15:46	Flies	N/A	N/A	Zebra	Intact	N/A	Laying eggs
B	Pafuri	12-Apr-14	05:53-07:04	Warthog	N/A	N/A	Zebra	Intact	N/A	Roaming near the carcass
C	Pafuri	17-Apr-14	11:50-17-16	Flies	N/A	N/A	Zebra	Intact	N/A	Laying eggs
C	Pafuri	18-Apr-14	16:33-01:43	Warthog	N/A	N/A	Zebra	Intact	N/A	Roaming near the carcass
D	Pafuri	18-Apr-14	2:55 PM	Flies	30	2	Zebra	Intact	N/A	Laying eggs
D	Pafuri	18-March-14	3:20 PM	Small live zebra	30	2	Zebra	Intact	N/A	Roaming near the carcass
D	Pafuri	18-March-14	4:48 PM	1 Vulture	30	2	Zebra	Intact	N/A	Roaming near the carcass
D	Pafuri	18-March-14	5:54 PM	Vultures	30	2	Zebra	Intact	N/A	Roaming near the carcass

D	Pafuri	18-March-14	5:56 PM	Vultures and marabou storks	30	2	Zebra	Intact	N/A	Roaming near the carcass
D	Pafuri	18-March-14	6:52 PM	Vultures and marabou storks	30	2	Zebra	Intact	N/A	Roaming near the carcass
D	Pafuri	18-March-14	10:00 PM	Hyena	30	2	Zebra	Intact	N/A	Roaming near the carcass
D	Pafuri	18-March-14	22:58-23:10	Hyena and baby zebra	30	2	Zebra	Intact	N/A	Roaming near the carcass
D	Pafuri	18-March-14	11:24 PM	Hyena	30	2	Zebra	Intact	N/A	Roaming near the carcass
D	Pafuri	18-March-14	11:29 PM	Hyena and Small live zebra	30	2	Zebra	Intact	N/A	Roaming near the carcass
D	Pafuri	18-March-14	11:55 AM	Hyena and Small live zebra	30	2	Zebra	Intact	N/A	Roaming near the carcass
D	Pafuri	19-March-14	02:06-02:39	3 Hyenas	30	2	Zebra	Intact	N/A	Roaming near the carcass
D	Pafuri	19-March-14	05:41-15:55	Vulture and Marabou stork	30	2	Zebra	Intact	N/A	Roaming near the carcass
E		11-March-14	18:20	Vultures	N/A	1	Impala	Ripped	25min	Feeding
E		11-March-14	18:22	Vultures	N/A	1	Impala	Bones	25min	Feeding
E		11-March-14	18:24-81:41	Vultures	N/A	1	Impala	Bones	25min	Feeding
E		11-March-14	18:44-18-49	2 vultures	N/A	1	Impala	Bones	25min	Feeding
F		27-Apr-14	18:27-18:54	Vultures	N/A	N/A	Zebra	Not Visible	Not visible	Feeding

2.3.3 Bacterial culture and diagnostic results

A total of 88 swabs from 31 vultures that included; faeces (n=12), Beaks (n=20), Claws (n=20), cloaca (n=20), and feathers (n=17) were cultured. Not all samples tested anthrax positive when cultured on both PET agar and 5% sheep blood agar. 15 swab samples from PET agar and 9 swabs samples from blood agar of 88 swabs were confirmed anthrax positive. Six *B. anthracis* isolates were isolated from vulture's faeces, three from beaks, three from claws, two from cloaca and ten from feathers (n=24 isolates from n=18 vultures). Feathers had a greater number of positive isolates compared to other swabs. The positive isolates were further confirmed to be *B. anthracis* using penicillin and gamma-phage tests, FRET-qPCR targeting *sasp, cap*C and *pag* virulence gene regions according to OIE guidelines and Turnbull (2008).



	Faeces	Beak	Claws	Cloaca	Feather
Total	12	20	20	20	17
PET agar	6	2	2	1	6
Blood agar	0	1	1	1	5

Figure 2. 4: Illustrates samples that were *Bacillus anthracis* positive amongst the whole collection of 89 swab samples from 31 vultures, Blue represents total sample collection, Orange represents pure isolates from PET agar and Blue represents pure isolates from blood agar. There were duplicate isolates obtained from PET and blood agar in some instances.

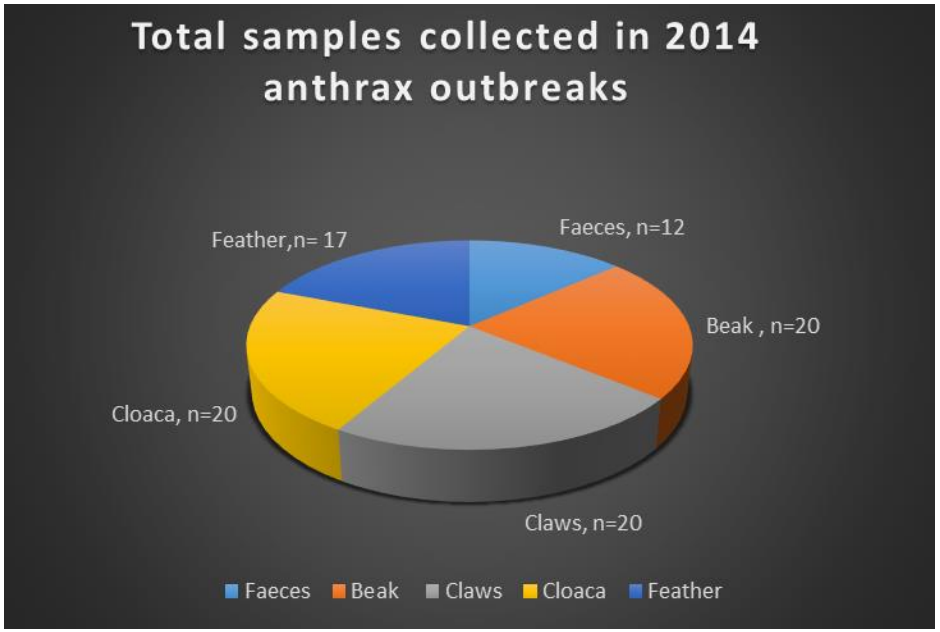


Figure 2. 5: Total samples collected during 2014 enzootics from 31 vultures by the EWT and Skukuza state veterinary services. Each colour represents swabs from different parts of a vulture.

2.3.4 MLVA assay results

Using MLVA-31, cluster analyses were generated for *B. anthracis* strains (Figure 2.6 and 2.7) isolated from vultures. Figure 2.6 represents the phylogenetic analysis of *B. anthracis* isolates using the UPGMA analysis, meanwhile Figure 2.7 represents the MST analysis. The analyses (Figure 2.6 and Figure 2.7) revealed 10 genotypes with one dominant clonal genotype. The dominant clonal genotype included *B. anthracis* strains isolated mainly from vulture beaks, claw and feathers swabs (transparent blue block, n=11; Figure 2.6). This clonal genotype suggests that vultures have fed on the same carcasses or they fed on carcasses affected by the same clonal strain (i.e. feather swab BA#010 and beak swab BA#008 were isolated from the same vulture and had the same genotype). The *B. anthracis* strains (n=12: Figure 2.6) of the vultures are mostly genetically distinct from the strains isolated from the beak and feathers (Figure 2.6)

B. anthracis strain BA#031 has 70% similarity to all the other strains from vultures based on MLVA-31. Strains BA#024 and BA#032 are clonal and have a similarity of 100% and was 95% similar with strains of the dominant clonal lineage (Figure 2.6). The *B. anthracis* strains isolated from vultures are all grouped into the A.Br005/006 (Ancient A) clade and the reference strains Sterne and Ames clustered in A.Br001/002 while Vollum clustered into A.Br007 (see Appendix Figure 1).

Cluster analyses using UPGMA (Figure 2.8) and MST (Figure 2.9) of MLVA-31 data of *B. anthracis* strains isolated during the 2014 outbreak linked *B. anthracis* genotypes that were isolated from vultures and carcasses. Using the pathogen genotype as a means of describing the outbreak, some vultures were linked to one carcass bacterial genotype for example: a strain from feather swab, BA#026, has the same bacterial fingerprint to a strain isolated from an elephant carcass, RL25014/49 (Figure 2.8). Some vultures are linked to more than one carcass (i.e. strains from vulture isolates A39/A30/A42/A13/A34/A36 have the same genotype with strains from carcasses DS2014/46/42/16/40, AD2014/02, and RL201/14/08/12) in a single clonal clade. Some vulture isolates (AM2/19 and A14#2; AM1/16 and A29 as well as A34#3) could not be linked to carcass sites. These isolates were isolated from cloacal swabs.

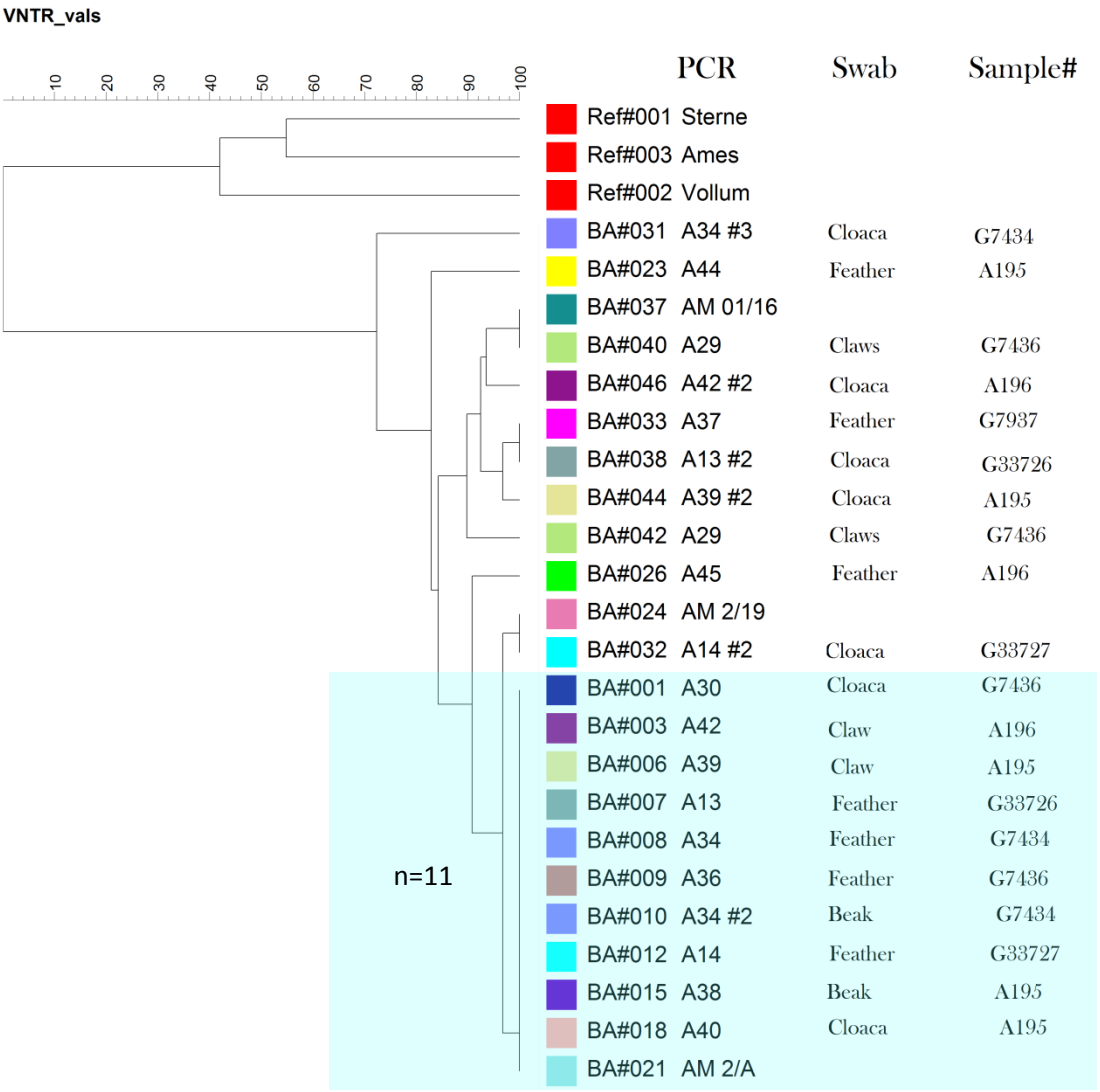


Figure 2. 6: Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram of 31-VNTR data of *B. anthracis* strains isolated from Kruger National Park (KNP) in the year 2014. MLVA-31 and MST cluster analysis was used to establish genetic relationships among the 23 *B. anthracis* isolates from (n=18 vultures). In this MST dendrogram, which was created using Bionumeric v6.6.5. Isolates were isolated from beaks, feathers, cloacal and talons swabs. Reference strains are represented by the red colour. The transparent blue block represents the dominant clonal genotype.

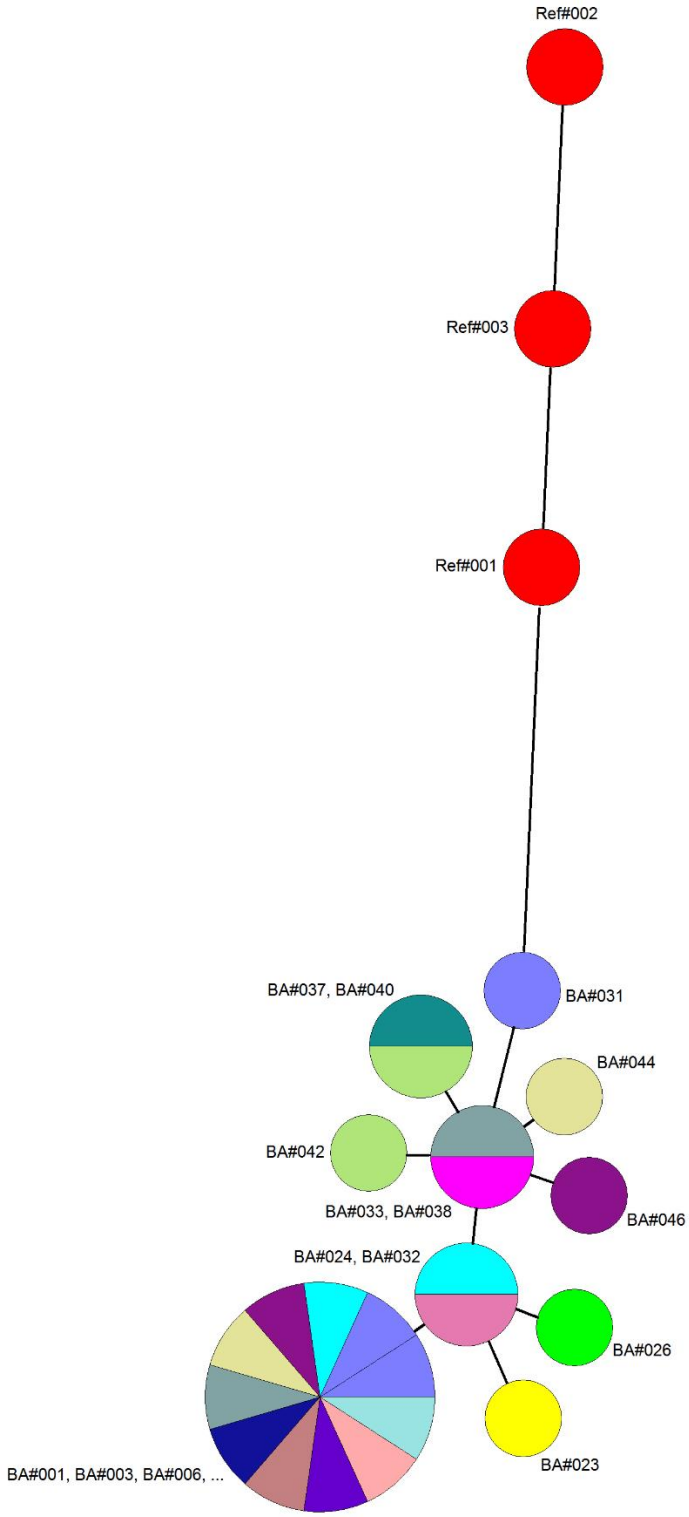


Figure 2. 7: MST dendrogram of VNTR data from Kruger National Park *B. anthracis* isolates in the year 2014: MLVA-31 and MST cluster analysis was used to establish genetic relationships among the 23 *B. anthracis* isolates. In this MST dendrogram, which was created using Bionumeric v6.6.5. Those isolates were isolated from beaks, feathers, cloacal and talons swabs.

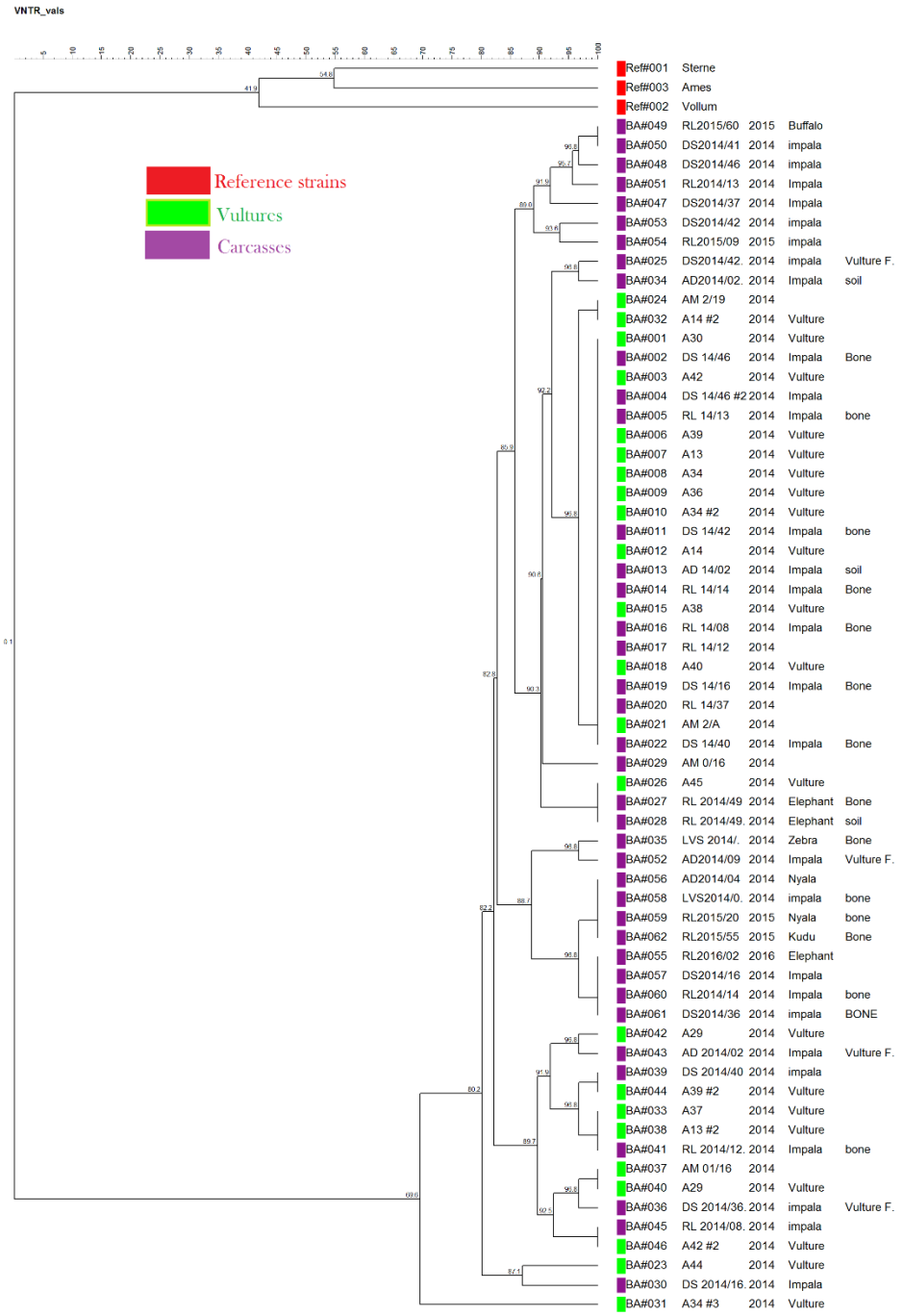


Figure 2. 8: UPGMA dendrogram of VNTR data from Kruger National Park *B. anthracis* isolates from vultures and carcass sites in the year 2014: MLVA-31 and MST cluster analysis were used to establish genetic relationships among *B. anthracis* isolates. In this MST dendrogram, which was created using Bionumeric v6.6.5. Those isolates were isolated from beaks, feathers, cloacal and talons swabs. Reference strains are highlighted by the red colour; vultures are represented by a bright green colour whereas carcass isolates are represented by the colour purple.

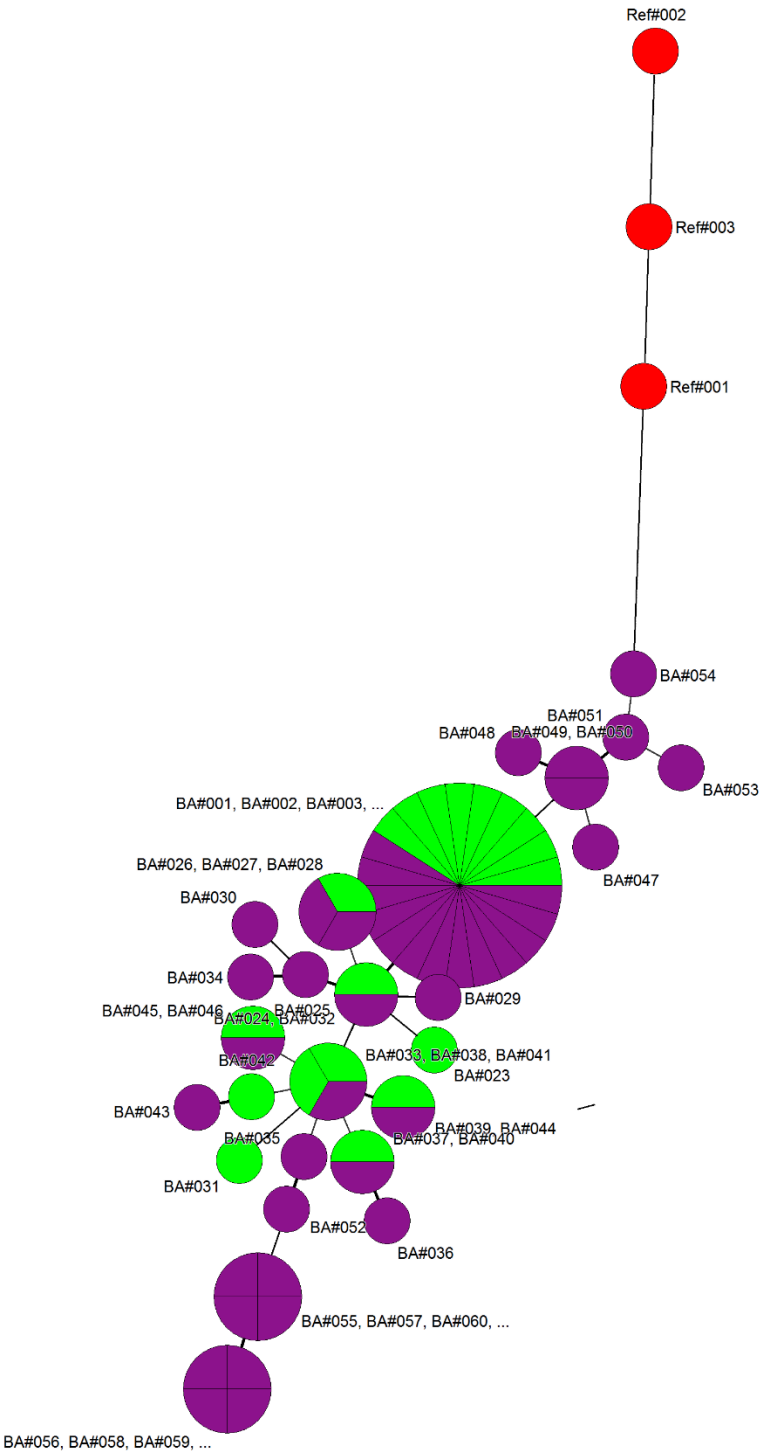


Figure 2. 9: MST dendrogram of VNTR data from Kruger National Park *B. anthracis* isolates from vultures and carcass sites in the year 2014: MLVA-31 and MST cluster analysis were used to establish genetic relationships among the *B. anthracis* isolates. In this MST dendrogram, which was created using Bionumeric v6.6.5. Those isolates were isolated from beaks, feathers, cloacal and talons swabs. Reference strains are highlighted by the red colour; Vultures are represented by a bright green colour whereas carcass isolates are represented by a purple colour.

2.4 Discussion

2.4.1 The role of vultures in spore dissemination during an outbreak

From collected swab samples (n=89) of 31 captured vultures, 23 *B. anthracis* isolates were obtained on PET and 5%SBA. A total of 3 *B. anthracis* isolates were isolated from vulture beak swabs and 10 isolates from their feathers which support a theory by Houston and Cooper (1975) that vultures spread the infection mechanically as spores could adhere to their feet and feathers. The spores also pass through vultures' digestive tract and be detected in their faeces (Ebedes, 1976, De Vos *et al.*, 1973, Young *et al.*, 1970, Pienaar, 1967). *Bacillus anthracis* was also isolated in vulture faeces two weeks after vultures fed on carcasses consisting of anthrax spores (Turnbull *et al.*, 1989). Vultures acquire spores on their feather as they feed. During consumption of carcasses, they scratch themselves with their beaks that are contaminated and fight each other for food. In some cases the access to fresh meat, normally put the whole neck inside the carcass, in this way spores adhere to their surfaces. Secretion of uropygial oil which is a preen oil that consist of mixtures formed of aliphatic monoester waxes, formed of fatty acids and monohydroxy wax-alcohols might play a role in inhibiting the spores on their feathers (Ruiz-Rodríguez *et al.*, 2009, Jacob *et al.*, 1997, Jacob and Ziswiler, 1982, Baxter and Trotter, 1969). Chemicals, mono- or diester waxes of fatty acids and alcohols, sterols and hydrocarbons (Jacob and Ziswiler, 1982) excreted by uropygial glands have antimicrobial properties that inhibit bacterial growth. However; In this study, the highest proportion of positive isolates were obtained from feather swabs. contamination of feathers with *Bacillus anthracis* might have been due to indirect contact of feathers with the positive carcass as they feed and spores that have adhered on the beaks might have been transferred to the feathers when they scratch themselves after feeding.

Various species of vultures show different feeding ecology and they have been suspected to be anthrax spore distributors as they carry spores on their external body structures (Hugh-Jones and De Vos, 2002, Bullock, 1956). The hooded vultures (*Necrosyrtes monachus*) are small and are frequently driven away by larger vultures during feeding. They normally eat the meat that has been dropped by larger birds (Datazone.birdlife.org, 2018) Hooded vultures were seen surrounding anthrax carcasses, however, due to their small body, they fed on the remains and from the meat that large vultures had dropped

during feeding. Petrides (1959) observed that the hooded vultures would quickly dash in for some limited rapidly gobbled portions, and white-backed vultures immediately shoulder them aside while also squabbling among themselves at the carcass.

The white-backed vultures *(Gyps africanus)* are more populous and account for a greater proportion of all vulture sightings in the area. This species of vultures consumed the largest meals because they overpower small vultures resulting in them feeding on the carcass first. It has been observed by Petrides (1959) that the white-backed *spp.* are fierce at the carcass and due to their body size and number, they side line other species. When other predators are not present at carcass site, the vultures are able to consume the whole carcass. In one of the observed carcass sites, an eagle which was found by a flock of vultures was out competed. vultures were squabbling to have accessibility to feed on the carcass, this led to the carcass being consumed in a very short space of time (~25 minutes).

Vultures and Marabou storks visit waterholes to clean and drink after feeding on carcasses. This activity may lead to contamination of the water points with spores on their beaks and feathers (Ebedes, 1976, De Vos *et al.*, 1973, Young *et al.*, 1970, Pienaar, 1967). Scavengers such vultures and Marabou storks have interactions during feeding. Competition between vultures and Marabou storks is not well identified; however, as Marabou storks are fewer in number compared to vultures, their presence is considered to be inconsequential (Ebedes, 1976). Marabou storks feed on small pieces that vultures drop as they feed and steal meat directly from vultures during hierarchy squabbles (Datazone.birdlife.org, 2018). At anthrax positive carcass sites for this study, meals were consumed at an average time of 20 min to 45 minutes. The amount of each carcass consumed by vultures is determined by what each vulture can take from it and the speed at which vultures consume the whole carcass differs extensively (Datazone.birdlife.org, 2018). As observed in this study, when vultures are present in large numbers, they consume carcasses in a short time period and when the carcass size is small (depending on number), they also consume it quite rapidly. When vultures rapidly consume vegetative cells before they sporulate, vegetative bacilli are destroyed in the gastric stomach of vultures therefore minimizing contamination (Turnbull *et al.*, 2008). Infected carcasses were disposed of rapidly by white-backed vultures; thus, reducing the risk of exposure to

other ungulates. Vultures have been observed to compete with terrestrial scavengers and predators (like hyena and crocodile) for carcasses, but the most known unrelated avian competitors are the Tawny Eagle (*Aquila rapax*) and the Marabou storks (*Leptoptilos crumeniferu*) (Anderson and Horwitz, 1979).

For our study, vultures consumed fresh carcasses rapidly which has implications of vegetative cells being destroyed inside the vulture gastrointestinal tract. Detection of low concentration of spores in faeces and soil from fresh and rapidly consumed anthrax carcasses suggests that vultures consumed vegetative cells before sporulation. This resulted in vegetative cells ingested with meals being killed inside the vulture stomach. Anthrax spores are shed in the soil with bodily fluids when the carcass is opened and exposed to oxygen and lack of nutrients triggers sporulation. Some grazers usually bleed from the mouth and natural orifices such as anus, ears, eyes and vagina after death which results in spores being introduced to the environment. (Hassim, 2017, Simon, 2002) reported highest bacterial spore counts from the soil under the carcasses. The discovery by Bellan *et al.* (2013) and Dragon *et al.* (2005) also revealed that there were more bacterial spore counts in the soil where bodily fluids from anthrax carcasses has soaked the ground. Since there is no evidence to show germination of the organism inside vultures' stomach, faeces from fresh carcasses are at low risk to cause infection (Turnbull, 2008). This is borne out in our results where there were low soil spore counts where vultures consumed the carcass whereas, in the untouched zebra (*Equus burchelli*) carcass, spores in the soil were found in higher concentrations (Data not shown). This study supports the hypothesis that vultures serve as environmental disinfectors by consuming carcasses containing vegetative cells and spores. Other researches have also shown that the role of vultures in dispensing anthrax is minimal (Turnbull, 2008). Clegg *et al.* (2007) did not detect anthrax spores from water-holes that were frequently visited by vultures during a major outbreak in Zimbabwe.

In contrast to the disinfector role of vulture hypothesis is the 'concentrator' hypothesis. In 2012, large numbers of white-backed vultures were observed at an elephant carcass near a fence in Mooiplaas region in KNP (Figure 2.1). These vultures cleaned themselves in the artificial waterholes in an enclosed breeding camp containing roan (*Hippotragus equinus*) and Tsessebe (*Damaliscus lunatus*). Thereafter large numbers of roan antelope

started dying from anthrax. *Bacillus anthracis* was isolated from artificial waterholes (cement troughs) within the fenced off area in non-endemic Mooiplaas during the outbreak (Ledwaba, 2014). It is speculated that vultures could have 'concentrated' *B. anthracis* spores to this area which enclosed susceptible roans and waterholes close to the large elephant carcass. According to Ledwaba (2014), this created an ideal environment for precipitating the risk of an anthrax enzootic outbreak. The dissemination of *B. anthracis* by vultures therefore depends on the host species affected as well as biotic and abiotic factors that has a determining role to each anthrax outbreak. The biotic component of anthrax is the phase in which spores are resistant dormant which happens in areas with alkaline soil with high calcium content (Damgaard, 2000).and biotic factors are occurs within the host which is believed to be the important reproducing phase (Coetzer *et al.*, 1994).

It has been observed by Pennycuick (1972) and Saggese *et al.* (2007) that vultures travel long distances while searching for food. In this study, we tracked possible distances that each vulture can travel. Vultures were captured at the northern part of KNP, South Africa and flew to other countries such as Zimbabwe, Mozambique and Zambia. The distance that they have covered is approximately 1500 km. MacAdam (1980) stated that if vultures were major carriers of anthrax, they would spread anthrax fast considering the long distances they can travel from an area of exposure to *B. anthracis* spores which might then be shed in other places during the trip, However, from our results, we suggest that because faeces (in around carcass sites) had low spore counts, that spread of the disease by this route is unlikely. Lindeque and Turnbull (1994) also found spores in vulture faeces at a very low concentrations and considered the concentration to be below the risk threshold to cause infection in grazers. The level at which spore concentration in faeces can initiate an infection in other animals is determined by (i) the number of vultures at anthrax vicinities, (ii) whether the organism had started sporulating and (iii) whether the carcass was consumed before it sporulation was initiated by exposure of *Bacillus* to oxygen. These factors will be unique to each anthrax case.

2.4.2 *Bacillus anthracis* genotypes from vulture and carcasses

In our study, the total of 24 *Bacillus anthracis* isolates generated 10 genotypes from all swabs that tested anthrax positive, with one dominant genotype. All the isolates within

the phylogeny tree clustered in A-clade and were all isolated in the northern region of KNP. The VNTR marker which the 9 genotypes differed from the dominant genotype is shown in Appendix Figure 1. Two *B. anthracis* strains, BA#001 and BA#018 were recovered from cloacal swabs and remaining strains were isolated from beaks, feathers and talons in the dominant genotype. This genotype suggests that vultures may have fed the same carcasses during clonal outbreaks. Various *B. anthracis* strains isolated from carcasses could be linked/associated with strains isolated from vultures (Figure 2.7 and 2.8). The genotypes from *B. anthracis* strains from vultures indicate that they fed on different carcasses and acquired different genotypes because cloacal genotypes did not match carcass MLVA strains identified during the 2014 outbreak.

As a result of the outbreak in 2014 there were many carcasses available and images from cameras indicated that some carcasses were not consumed by scavengers due to abundance of food. Findings by Turnbull *et al.* (1989) suggest that, positive isolates that were sampled from vulture faeces at anthrax infected zebra carcasses originated from other carcasses, They also indicated that *B. anthracis* does not colonize vulture intestines and they were passed out with vultures faeces.

2.5 Conclusion

The evidence provided in this study showed vultures playing a potential role in lowering residual contamination of the infected carcasses in the environment. The ability of vultures to spot carcasses while they are still fresh enable them to digest vegetative cells of *B. anthracis* which are then destroyed inside the vulture's gastrointestinal tract. This results in less spores being recovered from the soil at carcass sites and therefore a lower risk to infection in grazers. We can conclude that in this study that vultures were playing the role of diminishing the pathogen load in the environment of enzootic Pafuri. From the genotypes that were generated we were able to link strains collected from anthrax infected vultures to possible source of infection. These genotypes found in different carcasses within the same region suggest that the outbreaks were clonal.

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3 Chapter 3

Genotyping of *Bacillus anthracis* strains isolated from the Kruger National Park using Multi-Loci variable number tandem repeat analysis (MLVA-31)

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Abstract

Bacillus anthracis is a Gram-positive, endospore-forming bacterium that causes anthrax. Anthrax is more common in animals and incidental in humans when they handle contaminated animal products. The aim of this study was to use a multi-loci variable number of tandem repeat (VNTR) analysis (MLVA) to determine genetic diversity and track *B. anthracis* isolated during the 2014 to 2016 anthrax outbreaks in two regions of Kruger National Park (KNP), namely the Pafuri and Nwanetsi sections. *Bacillus anthracis* isolates (n=38) from the 2014-2016 were isolated from carcasses and the environment during anthrax outbreaks. Genotyping of each *B. anthracis* strain was established using 31VNTR markers. Allele copy codes were used to generate minimum spanning tree (MST) and UPGMA phylograms which included a database of strains from previous outbreaks in the region (n=107). The MST and UPGMA cluster analysis of MLVA-31 revealed 3 dominant clonal genotypes with a further 29 unique genotypes out of 169 isolates (i.e. 32 genotypes out of 169 isolates).

3.1 Introduction

Bacillus anthracis is a member of the *Bacillus cereus* group that causes a fatal infection called anthrax (Helgason *et al.,* 2000). A virulent strain of *B. anthracis* has two plasmids, namely pX01 and pX02, responsible for encoding the toxins from which the symptoms of the disease manifest (Read *et al.,* 2003). *Bacillus anthracis* is a soil bacterium capable of forming spores, which can remain inert, but stable for prolonged periods in the environment (Friedlander *et al.,* 1997). This environmental dormancy of the spores slows the bacterium's evolutionary change (Keim *et al.,* 1997). *Bacillus anthracis* is a genetically homogeneous pathogen and this makes strain differentiation difficult (Keim *et al.,* 2000). Identification of polymorphic genomic regions enables the distinction of the bacterium within genetic enclaves (Keim *et al.,* 2000).

Bacillus anthracis is considered to be a monomorphic organism (le Fleche *et al.,* 2001). Different molecular tests have been used to confirm *B. anthracis* strains in reported suspected anthrax epidemics (Turnbull, 2008). Analysis of VNTR profiles is one of the molecular typing methods popularised in the last 20 years (Keim *et al.,* 1999, Frothingham and Meeker-O'Connell, 1998, Weir, 1990). Multi-loci variable number of tandem repeat analysis (MLVA) uses polymorphism of VNTR loci to discriminate strains (Keim *et al.,* 2000). MLVA can distinguish *B. anthracis* strains and evaluate their diversity across geographically disparate regions (Van Schaik *et al.,* 2007, Keim *et al.,* 2004, Keim *et al.,* 2000). Keim *et al.,* (2000) developed the MLVA8 panel (*VrrA, vrrB1, vrrB2, vrrC1, vrrC2*, pX02 and pX01), where the markers pX02 and pX01 allow detection of the plasmids. The resolution of the MLVA8 panel was expanded to increase the resolution and discriminate between isolates and subsequently more markers were added summing to MLVA15 (Keim *et al.,* 2004), as well as the MLVA25 panel (Le Flèche *et al.,* 2001) respectively. MLVA-31 is a combination of the MLVA15, MLVA25 and the MLVA8 panels. Beyer and Turnbull (2009) reported a resolution of 158 genotypes using MLVA-31 whereas the MLVA8 had a resolution of 78 genotypes for the same number of isolates.

MLVA-31 is ideal for genotyping of *B. anthracis* strains. The use of VNTRs identified 3 *B. anthracis* clades namely, clade A, clade B and clade C (Keim *et al.,* 2000). Both clades A and B are found in South Africa. Clade A strains are distributed globally whereas clade B strains are restricted to southern Africa (Keim *et al.,* 2000, Keim *et al.,* 1999, Smith *et al.,* 1999) and the French Alps (Van Ert *et al*., 2007). South Africa has encounter several outbreaks in the past (Thomson and Tustin, 1994) with Kruger National Park (KNP) being one of the areas (Smith *et al.,* 2000). Due to the diversity of strains in the southern Africa, it is theorised to be the origin of *B. anthracis* (Keim *et al.,* 1997). Repeated anthrax outbreaks have been recorded in Kruger National Park (KNP) over the years (De Vos and Bryden, 1996). Anthrax outbreaks in KNP were reported to have a recurring pattern of large outbreaks every 10 years (De Vos, 1990). Anthrax outbreaks can affect the entire KNP region ; however , there are two most notable regions, namely the northernmost tip of the park and the central region, which are believed to be the focal point of anthrax (Smith *et al.,* 2000).

Disease surveillance, diagnostics and genotyping of anthrax is on-going in KNP as part of disease monitoring by state veterinary services (mandated by the Department of Agriculture Forestry and Fisheries) and SANParks (South African National Parks) (Steenkamp *et al.,* 2018, Bengis and Frean, 2014). This study aimed to compare MLVA genotypes from 2014 - 2016 outbreaks in endemic Pafuri and Nwanetsi to past outbreaks (2006,2010 and 2011) to identify *B. anthracis* outbreak trends. The isolates that are used are the same from previous study with an additional of five new isolates from the central region (Nwanetsi) of Kruger National Park. In contrast to previous study, this study focuses on genetic diversity and geographical distribution of *Bacillus anthracis*.

3.2 Material and methods

3.2.1 Sample history

In this study, *B. anthracis* isolates indicated in Appendix Table 1 were used (n=62, which consisted of *B. anthracis* isolated from 38 carcass sites (33 from Pafuri in Chapter 2 where duplicate genotypes from carcass sites have been removed plus an additional 5 from Nwanetsi outbreaks) and 24 vulture samples (Chapter 2). *Bacillus anthracis* isolates were isolated from samples collected in Pafuri and Nwanetsi sections of KNP. The collection was done by SANParks rangers together with Skukuza Veterinary services during 2014 - 2016. Samples collected from carcass sites included blood smears, bones, soil, blowflies and swabs (Appendix Table 1).

3.2.2 Bacterial culture and diagnostic methods

The sampling of environmental samples (n=38) during anthrax outbreaks was done by the State veterinary services using protocols laid out in the WHO/OIE anthrax manual. *Bacillus anthracis* isolates were confirmed at the Skukuza State Veterinary Services laboratory.

3.2.2.1 Genotyping of *Bacillus anthracis*

Bacillus anthracis isolates are listed in Appendix Table 1. Polymerase chain reaction (PCR) for MLVA-31 (Beyer *et al.,* 2012) was used as outlined in chapter 2 (Section 2.1.5.) The copy number translation was done according to the convention proposed by Thierry et al., (2014) as described in Chapter 2; Table 2.3. Unweighted pair group method using

arithmetic mean (UPGMA) tree and minimum spanning tree (MST) phylograms were generated using BioNumerics v 6.6.5 (Applied Maths; Antwerp) for categorical datasets.

3.3 Results

3.3.1 MLVA assay Results

Data of 169 *B. anthracis* isolates from 2006-2016 outbreaks and reference strains, namely 34F_2 Sterne, Ames and Vollum on the 31MLVA panel of VNTR's loci (which included the 38 *B. anthracis* carcass isolates and 24 vulture isolates) was used. The 38 *B. anthracis* were processed in this chapter by means of agarose gel electrophoresis. UPGMA cluster analysis of these 169 isolates clustered all the isolates into A-clade (Figure 3.1). These were further differentiated into 3 dominant clonal genotypes within the Ancient A (Aβ) clade with a further 28 genotypes out of 169 isolates. In this study, one isolate, Anth#001, clustered in A3 clade and it is 48.5% similar with reference strain, Sterne 34F_2. Isolate, Anth#001 is 38% similar to Ames based on MLVA-31. Vollum in A4 clade along with the isolate Anth#001 show a distinct difference to the 2014-2016 isolates (Figure 3.1).

The 3 clonal genotypes of Ancient A clade in the MST are labelled Genotype-1, Genotype-2 and Genotype-3, and 31 distinct genotypes (Figure 3.2). The largest clonal node, Genotype-1 comprised of *B. anthracis* strains with identical genotype isolated from 2006, 2010, 2011, 2014 and 2015 outbreaks. In Genotype-2, the greatest number of isolates in this clonal node were from the 2015 outbreak. This node has two *B. anthracis* strains, BA#055 and BA#059 that were isolated in the Nwanetsi section (see map in Figure 2.1). *Bacillus anthracis* BA#055 strain (2016, Elephant bone) was 100% similar to strains BA#57, BA#60 and BA#61 from Pafuri which all were isolated from impala bones (Appendix Figure 2). The BA#059 (2015, Nyala bone) strain is 100% similar with the isolate, BA#062 (Nwanetsi, kudu bone), BA#058 (2014 impala bone) and BA#056 (2014 Nyala bone) (Appendix Figure 2). The *B. anthracis* strains BA#056 and BA#058, from 2014 cases in Pafuri is 100% similar to strains BA#059 and BA#062 from 2015 in Nwanetsi. The strains are 100% similar with the rest of the strains in the same clonal genotype (Figure 3.2). In Genotype-3, only 2014 *B. anthracis* strains from Pafuri make-up the clonal node. Four strains from the 2015 outbreak in Pafuri (n=4) are similar to

genotype-3 differing at, BA#049 (BAMS23), BA#054(VrrA), Anth#052 (BAMS53), Anth#078 (BAMS13, Bam31, VrrA, BAMS3, BAMS21) markers. Various *B. anthracis* genotypes from 2014 possibly evolved from genotype-2 (Figure 3.2). Some strains like *B. anthracis* BA#50 isolated from soil under an impala carcass in 2014 is 100% similar with strain BA#049 isolated from a buffalo carcass in 2015.

A total of 12 *B. anthracis* strains (n=8 from this study and 4 from genotype database) lost either pX01 or pX02 (Table 3.1). *Bacillus anthracis* strains BA#020, BA#023, BA#31, BA#034, BA#035, BA#038, BA#053 and BA#056 lost their plasmid pX02 and therefore there are no copy numbers for VNTR's 16, 17 and marker pX02. Strain Anth#061 (2015) lost pX01 but is 96.5% similar with BA#035 (zebra, 2014) that lost pX02 (Appendix Figure 3). Strain BA#055 and BA#062 were both isolated from Nwanetsi region in 2015-2016. The genotype of strain BA#055 isolated from an elephant in 2016 was identical to strains BA#057, 060 and 062 isolated from impala in Pafuri in 2014. The genotype of strain BA#062 isolated from kudu in 2015 was identical to 2014 BA#056 and 058 from Nyala and kudu respectively as well as BA#059 isolated in 2015 from Nyala. These strains from Pafuri region from 2014-2015 outbreaks have the same genotype as outbreaks in 2015-2016 in the Nwanetsi region (See Appendix Table 1).

Table2:1 *Bacillus anthracis* strains from anthrax outbreaks that failed to amplify VNTR makers pX02, VNTR16 and VNTR17 due to plasmid loss.

Plasmid	Key	Sample No	Year	Source
Px01	Anth#061	RL 2015/17 (74)	2015	
	Anth#062	DS 2015/45 (65)	2015	
px02	BA#020	RL 14/37	2014	
	BA#023	A44	2014	Vulture
	BA#031	A34 #3	2014	Vulture
	BA#034	AD2014/02 #2	2014	Impala
	BA#035	LVS 2014/03/02	2014	Zebra
	BA#038	A13 #2	2014	Vulture
	BA#053	DS2014/42	2014	impala
	BA#056	AD2014/04	2014	Nyala
	Anth#053	LVS 028 Wespe	2014	Wasp larvae
	Anth#056	DS 2015/31 (32)	2015	

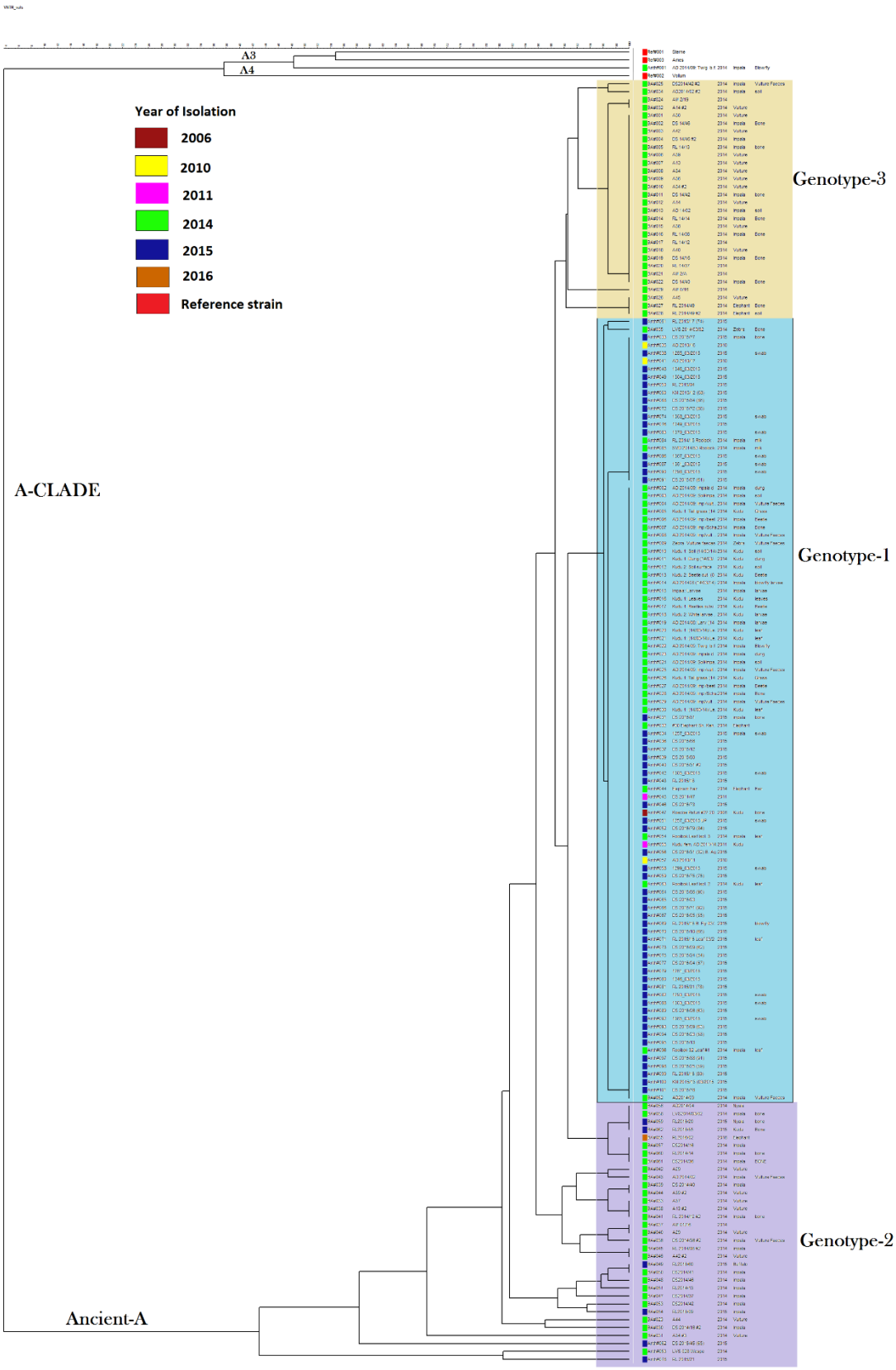

Figure 3.2: UPGMA dendrogram based on 31 VNTR loci of *Bacillus anthracis* for 169 isolates from Kruger National Park (2006-2016), South Africa as well as the 3 reference strains Sterne, Ames and Vollum in red. The dendrogram was generated using BioNumerics version 6.6.5. A colour key indicates the year of isolation.

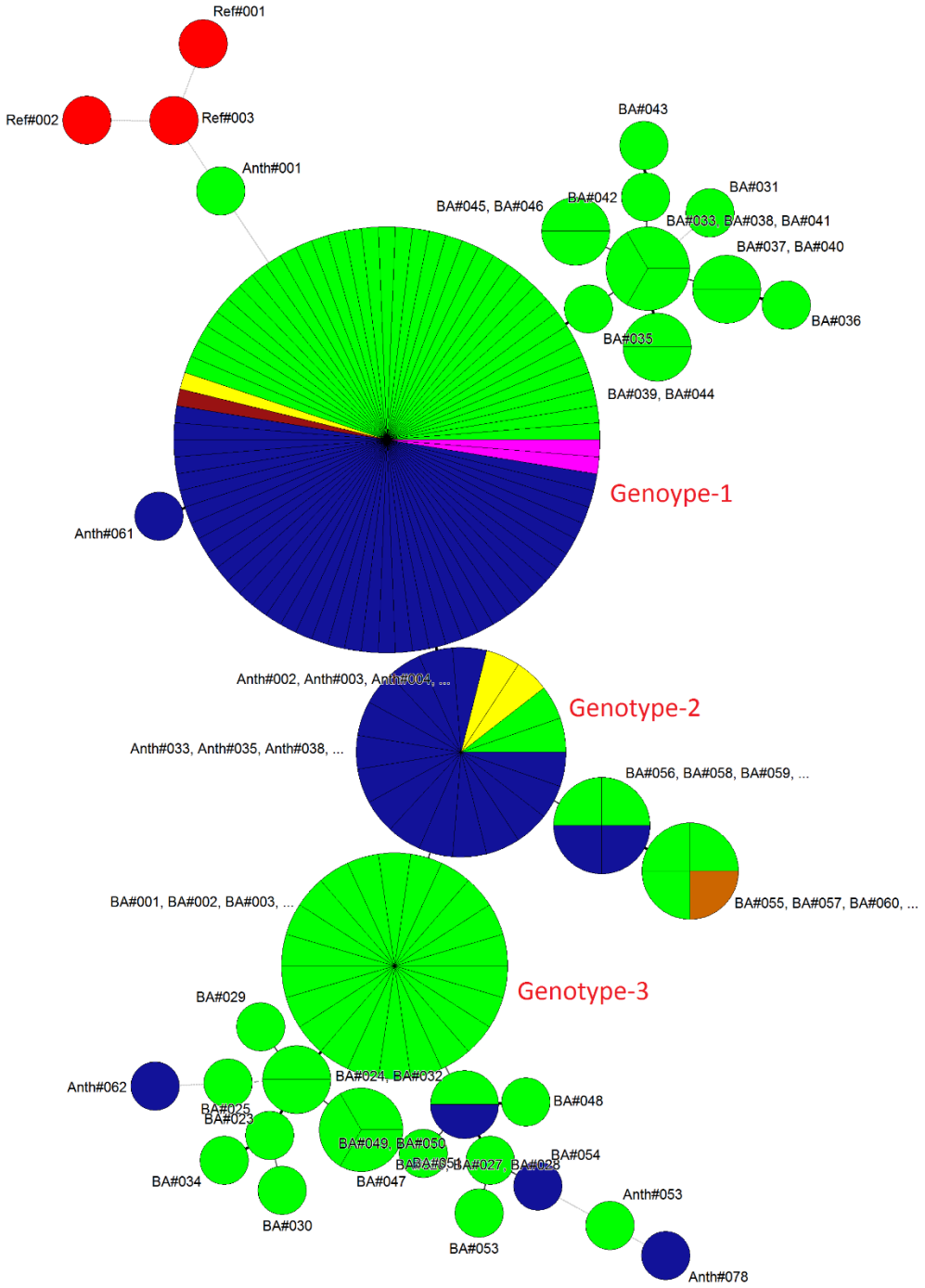

Figure 3.3: Minimum spanning tree (MST) dendrogram based on 31 VNTR loci of *Bacillus anthracis* for 169 isolates from Kruger National Park (2006-2016), South Africa. The dendrogram was generated in BioNumerics version 6.6.5 Colours indicate the year of Isolation where 2006 is maroon, 2010 is yellow, 2011 is pink, 2014 is green, 2015 is royal-blue and 2016.

3.4 Discussion

A total of 62 isolates and (n = 107 pre-existing data) differentiated *B. anthracis* isolates into 3 clonal and 32 distinct genotypes respectively. The cluster analysis of MLVA-31 (Figure 3.1) clustered all the *B. anthracis* strains in the A-clade. The A-clade consisted of strains with a worldwide distribution. All but one of the 62 *B. anthracis* strains from 2014 -2016 clustered in ancient A-clade consisting of 3 dominant genotypes. Genotype-1 and 3 in the MST (Figure 3.2) consisted of *B. anthracis* strains mainly from the endemic northern region of KNP. Genotypes of strains similar to genotype-2 from Pafuri region isolated from 2014-2015 outbreaks have the same genotypes from outbreaks in 2015-2016 in the Nwanetsi region (Figure 3.1 and 3.2). These strains differ from one another based on 1-2 VNTR markers and therefore it indicated that these strains evolved from the dominant genotype-2 strains. These genotypes differed from one another at markers BAMS3 and VNTR23 (Figure 3.1) and were linked to strains from Pafuri (endemic /enzootic area) and Nwanetsi (non-endemic) regions (Figure 3.1 and Figure 3.2). The significance of this cluster analysis is that Nwanetsi and Pafuri are approximately 420 km apart with no common watersheds or river systems. Nwanetsi borders Mozambique across a chain-link fence. Cattle died on the Mozambique side before the outbreak in Nwanetsi began. According to local rangers, the animals were purported to have been herded down from the Pafuri region along the Limpopo River. They had supposedly been herded down to a trading post before dying near the Nwanetsi fence. This could not be officially corroborated but could be a possible scenario for the commonality of the genotypes.

The Genotype-3 cluster consists of distinct genotypes almost exclusively isolated from 2014 with 4 strains isolated in 2015. A few of these distinct genotypes represent a bias in the MLVA data due to the loss of plasmids. An example is strain Anth#061 (2015) that lost pX01 but is 96.5% similar with BA#035 (zebra, 2014) that lost pX02 (Appendix Figure 3). Both these isolates are closely related to strains in Genotype-1. Price *et al.,* (1999) stated that virulent strains of *B. anthracis* contain two plasmids, namely pX01 (ca. 174 kb) and pX02 (ca. 95 kb). Theplasmid-specific genetic variations in *B. anthracis* remain poorly characterised. There isolates that have lost their plasmids were confirmed to be *B anthracis* strains when tested for bacteriophage susceptibility. They were isolated from carcass sites where anthrax was evident and confirmed through Giemsa stained blood smears. The lack of plasmid pX01 or pX02 suggests

that the strains might have lost their plasmid during isolation or storage. *Bacillus anthracis* has been known to lose the plasmids, pX02 more easily and pX01 less commonly, during processing and isolation (Coker *et al.,* 2003, Turnbull, 1991).

Clonal Genotype-1 has the most interesting composition of characterized strains in this study. It is the predominant genotype of the 2014 -2016 dataset as *B. anthracis* strains with an identical genotype were isolated from 2006, 2010, 2011, 2014 and 2015 outbreaks. This indicates that these genotypes remain in the region for decades; similar to genotypes in Etosha National Park (Beyer *et al.,* 2012). It also shows 100% fingerprint identity to isolates from outbreaks circulating in enzootic Pafuri (the northern part) region, KNP in 2006, 2010 and 2011. (Keim *et al.,* 1997) stated that environmental dormancy of spores reduces bacterial change. The clonal nature of Genotype-1 demonstrates the latency of spores in the environment and subsequent circulation of the pathogen between outbreaks in the region.

3.5 Conclusion

MLVA-31 is a useful technique in studying the temporal and geographic distribution of anthrax outbreaks. The outbreaks from 2006 – 2016 showed that some genotypes persisted in the endemic/enzootic Pafuri region resulting in outbreaks during this time. This is evidence of a clonal genotypic distribution which hints at pathogen cycling in endemic Pafuri. Genotypes occurring in the non-endemic Nwanetsi region seem to be related to dominant genotypes that occurred in the endemic region, showing diversity among *B. anthracis* strains and providing insight into strain distribution. While this study could not elucidate the possible vectors of the outbreaks between Nwanetsi and Pafuri, it was possible to demonstrate the similarity in bacterial genetic fingerprints which pending availability of more data, suggests that outbreaks in the two regions may be connected.

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4 Chapter 4

4.1 General conclusions and discussion

The anthrax endemic regions of South Africa are Ghaap region in the Northern Cape province and Kruger National Park (KNP) which spans the provinces of Limpopo and Mpumalanga (Hugh-Jones and De Vos, 2002). Pafuri region on the northern tip of KNP, is considered the anthrax enzootic zone within the Park (Hugh-Jones and De Vos, 2002). In large epidemics, anthrax cases have been recorded as far south as central KNP, but these areas do not show consistent outbreak activity and therefore are not considered to be enzootic (Smith *et al.,*, 2000). Multiple loci variable number tandem repeat analysis (MLVA) is a bacterial DNA fingerprinting technique that due to its resolution power, is increasingly used. It has proven to be a useful tool in strain trace back and distribution studies (Lista *et al.,*, 2006, Gierczyński *et al.,*, 2004, Le Flèche *et al.,*, 2001).

This study employed MLVA-31 (Beyer *et al.,*, 2012) to distinguish *B. anthracis* strains and evaluate their genetic diversity and geographical distribution. A total of 169 isolates generated 3 clonal and 31 distinct lineages which clustered in the A-clade. Multiple MLVA genotypes were isolated in Pafuri and Nwanetsi regions from outbreaks that occurred between 2014 - 2016. The isolates were collected from different animal carcasses such as kudus (*Tragelaphus*), Impala (*Aepyceros melampus*), zebras (*Equus quagga*) and elephants (*Loxodonta*) as well as environmental and vulture associated samples.

Cluster analyses of 2014-2016 MLVA genotypes demonstrated clonality with isolates from past outbreaks in 2006, 2010 and 2011 in endemic Pafuri region. Spore dormancy has been credited with the *B. anthracis'* slow evolution (Turnbull, 1999). This is an indication that pathogenic *B. anthracis* from these genotypes are either latent in the environment between outbreaks or circulating amongst individual infection cases until outbreaks are observed. Whole genome sequencing of isolates from these different outbreak years would give us better insight into which scenario holds truer depending on whether the strain is evolving across outbreak years.

In our study, more than one *B. anthracis* genotype was revealed in individual vultures. Strains found on the beak and feather swabs were different from those strains found in cloacal swabs. Multiplicity of genotypic strains in one animal can occur, but is rare

(Beyer and Turnbull, 2009). So, while it is possible for the vultures to have consumed multiple *B. anthracis* genotypes from a single carcass, it is unlikely for such mentioned possibility to occur. This is especially true since the beak and feather isolates had genotypes identical to strains isolated from carcasses at the time of vulture capture. Only some of the cloacal isolates (representing a digested meal) could not be linked to a carcass site. Due to the extensive distances of vulture travel observed in this study this is noteworthy. In an experimental study, spores were isolated up to 2 weeks after being fed a *B. anthracis* spore meal (Beyer and Turnbull, 2009). These unique cloacal isolate genotypes represent genotypes which are either outside the enzootic area or from carcasses that had not been identified during the outbreak.

Dissemination and transmission of *B. anthracis* by vultures is a sensitive and controversial topic in anthrax investigations. This is because white-backed vultures (*Gyps africanus*) are on the critically endangered list (Datazone.birdlife.org, 2018). This is largely due to the poisoning of vultures to prevent them from revealing poached carcasses (and consequently poachers) when they circle carrion (Ogada *et al.,*, 2016). There are two prevailing theories with regard to a vulture's role in anthrax dissemination and transmission. It is believed that vultures spread pathogens such as *B. anthracis* because after feeding on anthrax carcasses, they fly to water-holes to drink and bathe (Hugh-Jones and De Vos, 2002, Pienaar, 1967). This results in water points being contaminated with spores. As demonstrated with the cloacal swabs, and in other studies, ingested spores can survive the vulture gastrointestinal tract. The spores are then passed with the faeces which also serve as an environmental contaminant (Saggese *et al.,*, 2007).

In a study by Ledwaba (2013), white-backed vultures were observed in large numbers to be bathing in artificial/cement waterholes in a fenced off camp in Mooiplaas section of the KNP. The vultures had spent a prolonged period in the area while feeding on an elephant carcass that was negative for anthrax. These vultures could have fed before on other anthrax infected animals and faeces could have contained *B. anthracis* spores as it survives for a prolonged period in the faeces (Turnbull, 1999). A few days later roan antelopes in the camp began to die of anthrax. *Bacillus anthracis* was isolated from both the water trough and vulture faeces surrounding the waterhole. This case study is a demonstration of vultures playing a dissemination role in the anthrax cycle.

The alternative theory is that vultures play a role as pathogen reduction agents (Turnbull *et al.,* 1999). When vultures consume fresh carcasses with vegetative bacilli, they reduce contamination in the environment because the vegetative forms are destroyed in the vultures' gut. Dragon *et al.,* (2005), Bellan *et al.,* (2013) and Hassim *et al.,* (2017) revealed that spore counts were significantly lower in the soil under fresh carcasses that remained intact than when carcasses opened by scavengers were left exposed. In our study, vultures consumed an anthrax-infected fresh impala carcass in 25 minutes. The predominantly vegetative cells would have been destroyed in the gastrointestinal tract of vultures. In this way vultures served to lower pathogen contamination in the environment.

Evidence from this work and other studies may suggest that carcass sites have environmental factors that influence dissemination and transmission of anthrax. Such factors include the number of vultures, the size of carcass and the outbreak magnitude. For example, the impala carcass was consumed more rapidly compared to the zebra carcass. Both of these were consumed in a much shorter time than the elephant carcass described by Ledwaba (2013).

During this study, there were more carcasses in the region than the vultures could consume. In the camera trap work, the vultures were observed to sit around the carcass without consuming anything for up to 24 hours. There was a bias for white-backed vultures (*Gyps africanus*) in this study; and hooded vultures (*Necrosyrtes*) were not well presented due to the sheer numbers and competition with the white-backed vultures. The study was also constrained by costs which limited the number of GPS units that could be fitted, and by loss of GPS units, due either to loss of signal or loss of the unit. Nevertheless, this study is the first of its kind to describe vulture movement in relation to *B. anthracis* distribution. This study shows that there is a greater magnitude of complexity in the role that environment and scavenging animals play in the genotype of isolates during outbreaks which needs further attention and detailed investigation.

4.2 Conclusion

MLVA has proven to be a useful tool in distinguishing and determining genetic diversity of *B. anthracis* strains in Kruger National park. It also was able to demonstrate a cycling of clonal genotypes spanning outbreaks from 2006-2016. The MLVA31 was

used to genotype Kruger National Park isolates in a very fast and extremely reproducible way. This technique was very discriminatory and accurate. The genotypes found clustered in the A clade which is found in South Africa and distributed globally. Isolates from Kruger National Park, South Africa in the A clade have a wider geographic range and greater genetic diversity. This may be the results adaptation to diverse environmental condition. The data observed during this study suggest anthrax foci are heavily concentrated in the northern region and are less common in the central regions, where 35 distinct genotypes are observed. This may be due areal differences in soil composition.

For this study, vultures played a role in the lowering of the pathogen load at infected carcasses. At other carcass sites, they played no role at all by just waiting around the carcasses and not consuming anything. The evidence from other researchers and our study indicates that both theories about the role of vultures are viable.

4.3 **Recommendations for future work**

The *B. anthracis* isolates used in this study showed a greater genetic diversity in KNP, South Africa than in the study conducted by (Smith *et al.*, 2000) where they found two clonal and four distinct genotypes out of 98 isolates from Kruger National Park. As *B. anthracis* is an acute disease, it is difficult to study the life cycle in real time as well as the variables such as epidemiological, animal and environmental, and spore dissemination variables. are unique to each outbreak therefore limiting overall conclusions. It is essential to accumulate all evidence including camera data, GSP movement information, spore counts and genotyping data to have a concise understanding. Comparing spore concentration in scavenger spoor and investigating scavenger behavior independently can give more information about distribution patterns of *B. anthracis* spores. If not due to cost factors, more GPS units would be ideal on captured vultures in studies to monitor distribution and transmission of anthrax by vultures. However, fitting the GPS collars during the anthrax outbreak and its limited time span (to one outbreak and not another) limited the interpretation. It is important to capture as many vultures as possible as well as the capturing of vultures outside outbreak periods can help to examine what happens when an outbreak occurs in studies such as this one.

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5 Chapter 5

5.1 Appendix Tables and figures

Appendix Table 1: Samples collected from vultures and carcasses in Pafuri and Nwanetsi. Sample numbers represent vultures and carcasses and Key indicate samples used for bacterial culture and molecular diagnostics.

Key	Sample No	Year	Source	Sample type	Location
BA#001	A30	2014	Vulture		Pafuri
BA#002	DS 14/46	2014	Impala	Bone	Pafuri
BA#003	A42	2014	Vulture		Pafuri
BA#004	DS 14/46 #2	2014	Impala		Pafuri
BA#005	RL 14/13	2014	Impala	bone	Pafuri
BA#006	A39	2014	Vulture		Pafuri
BA#007	A13	2014	Vulture		Pafuri
BA#008	A34	2014	Vulture		Pafuri
BA#009	A36	2014	Vulture		Pafuri
BA#010	A34 #2	2014	Vulture		Pafuri
BA#011	DS 14/42	2014	Impala	bone	Pafuri
BA#012	A14	2014	Vulture		Pafuri
BA#013	AD 14/02	2014	Impala	soil	Pafuri
BA#014	RL 14/14	2014	Impala	Bone	Pafuri
BA#015	A38	2014	Vulture		Pafuri
BA#016	RL 14/08	2014	Impala	Bone	Pafuri
BA#017	RL 14/12	2014			Pafuri
BA#018	A40	2014	Vulture		Pafuri
BA#019	DS 14/16	2014	Impala	Bone	Pafuri
BA#020	RL 14/37	2014			Pafuri
BA#021	AM 2/A	2014			Pafuri
BA#022	DS 14/40	2014	Impala	Bone	Pafuri
BA#023	A44	2014	Vulture		Pafuri
BA#024	AM 2/19	2014			Pafuri
BA#025	DS2014/42 #2	2014	impala	Vulture Faeces	Pafuri
BA#026	A45	2014	Vulture		Pafuri
BA#027	RL 2014/49	2014	Elephant	Bone	Pafuri
BA#028	RL 2014/49 #2	2014	Elephant	soil	Pafuri
BA#029	AM 0/16	2014			Pafuri

BA#030	DS 2014/16 #2	2014	Impala	jaw bone	Pafuri
BA#031	A34 #3	2014	Vulture		Pafuri
BA#032	A14 #2	2014	Vulture		Pafuri
BA#033	A37	2014	Vulture		Pafuri
BA#034	AD2014/02 #2	2014	Impala	soil	Pafuri
BA#035	LVS 2014/03/02	2014	Zebra	Bone	Pafuri
BA#036	DS 2014/36 #2	2014	impala	Vulture Faeces	Pafuri
BA#037	AM 01/16	2014			Pafuri
BA#038	A13 #2	2014	Vulture		Pafuri
BA#039	DS 2014/40	2014	impala	bone	Pafuri
BA#040	A29	2014	Vulture		Pafuri
BA#041	RL 2014/12 #2	2014	Impala	bone	Pafuri
BA#042	A29	2014	Vulture		Pafuri
BA#043	AD 2014/02	2014	Impala	Vulture Faeces	Pafuri
BA#044	A39 #2	2014	Vulture		Pafuri
BA#045	RL 2014/08 #2	2014	impala	Bone	Pafuri
BA#046	A42 #2	2014	Vulture		Pafuri
BA#047	DS2014/37	2014	Impala	Bone	Pafuri
BA#048	DS2014/46	2014	impala	Bone	Pafuri
BA#049	RL2015/60	2015	Buffalo	BONE	Nwanetsi
BA#050	DS2014/41	2014	impala	Bone	Pafuri
BA#051	RL2014/13	2014	Impala	Bone	Pafuri
BA#052	AD2014/09	2014	Impala	Vulture Faeces	Pafuri
BA#053	DS2014/42	2014	impala	Bone	Pafuri
BA#054*	RL2015/09	2015	impala	Blood Smear/ Fly/ Swab	Pafuri
BA#055*	RL2016/02	2016	Elephant	Bone	Nwanetsi
BA#056	AD2014/04	2014	Nyala	Bone	Pafuri
BA#057	DS2014/16	2014	Impala	Jaw Bone	Pafuri
BA#058	LVS2014/03/02	2014	impala	bone	Pafuri
BA#059*	RL2015/20	2015	Nyala	bone	Pafuri
BA#060	RL2014/14	2014	Impala	bone	Pafuri
BA#061	DS2014/36	2014	impala	BONE	Pafuri
BA#062*	RL2015/55	2015	Kudu	Bone	Nwanetsi

* Additional samples

Appendix Table 2: List of 31 variable number tandem repeats (VNTR) for *Bacillus anthracis* used for this study that indicates the forward and reverse primers, size of the repeat unit (bp), range of fragment sizes (bp) PCR conditions and agarose gel specification (%).

VNTR locus *	Forward primer 5'-3'	Reverse primer 5'-3'	Size of repeat unit (bp)	Range of fragment sizes (bp)	PCR conditions	Percentage (w/v) Agarose gel specification **
BAMS21	TGTAGTGCCAGATTTGTCTTCTGTA	CAAATTTTGAGATGGGAGTTTTACT	45	541 - 766	1	2 %
BAMS51	ATTTCCTGAAGCAGGTTGTGTT	TGCATCTAACAATGCAGAACAA	45	358 - 538	1	
BAMS23	CCTGTTGCTCCTAGTGATTTCTTAC	CGGTCTGTCTCTATTATTCAGTGGT	42	399 - 693	1	
BAMS24	CGTCACGTACCATTTAATGTTGTTA	CTTCTACTTCCGTACTTGAAATTGG	42	469 - 511	1	
BAMS5	ATTATTAGCAGGGGCCTCTCCTGCATTACC	GCAGGAAGAACAAAAGAAACTAGAAGAGCA	39	229 - 424	1	
BAMS34	TGTGCTAAATCATCTTGCTTGG	CAGCAAAATCAATCGAATCAAA	39	230 - 581	1	
BAMS44	GCACTTGAATATTTGGCGGTAT	GCGAATTAATTGCTCCTCAAAT	39	183 - 573	1	
BAMS22	ACCGTTAATTCACGTTTAGCAGA	ATCAAAAATTCTTGGCAGACTGA	36	519 - 1041	1	
BAMS28	TATTAAACCAGGCGTTACTTACAGC	CTCTGTTGTAACAAAATTTCCGTCT	24	373 - 505	1	3 %
BAMS1	AGTTCAAGCGCCAGAAGGTTATGAGTTATC	GTTGAGCATGAGAGGTACCTTGTCCTTTTT	21	296 - 611	1	
vrrC2[a]	GTCTTTCCATTAATCGCGCTCTATC	CCAGAAGAAGTGGAACCTGTAGCAC	18	528 - 604	1	
BAMS3	TCCTCCCTGAGAACTGCTATCACCTTTAAC	GCAGCAACAGAAAACTTCTCTCCAATAACA	15	429 - 654	1	
BAMS25	TGAAAGATCTTGAAAAACAAGCATT	CCGAATACGTAAGAAATAAATCCAC	15	376 - 391	1	
VrrA	GCGCGTTTCGTTTGATTCATAC	CACAACTACCACCGATGGCACA	12	289 - 338	1	
BAMS53	CATATTTTCACCTTAATTTTGGAAG	GAGGTGTGTTAGGTGGGCTTAC	12	322 - 346	1	
VNTR23	GTAATACGTATGGTTCATTCCC	TTTAGAAACGTTATCACGCTTA	12	170 - 208	1	
vrrB1[a]	GATGAGTTTGATAAAGAATAGCCTGTG	ATAGGTGGTTTTCCGCAAGTTATTC	9	184 - 292	1	
vrrB2[a]	CCCAAGGTGAAGATTGTTGTTGA	CACAGGCTATTCTTTATCAAACTCATC	9	135 - 198	1	

vrrC1[a]	CATTTCCTCAAGTGCTACAGGTTC	GAAGCAAGAAAGTGATGTAGTGGAC	9	364 - 688	1	
BAMS13	CTAGTGCATTTGACCCTAATCTTGT	AATTGAGAAATTGCTGTACCAAACT	9	337 - 868	1	
BAMS15	GTGTACATGTTGATTCATGCTGTTT	GTATTTCCCCCAGATACAGTAATCC	9	409 - 643	1	
BAMS30	CAGAAAATATTGGACCTACCTTCC	AGCTAATCACCTACAACACCTGGTA	9	268 - 925	1	
BAMS31	GGAGTACTGTTTGTTGAATGTTGTTT	GCTGTATTTATCGAGCTTCAAAATCT	9	331 - 1087	1	
VNTR 16[b]	GAATAATAAGGGTTCTCATGGTAT	CTCTTGAAAATATAAAACGCA	8	137 - 346	2	3 % MS
VNTR17[b]	GATCGTACAACAGCAATTATCAT	TAGGTAAACAAATTTTCGTAATC	8	366 - 453	2	
VNTR35[b]	GTCCTGAAATAAATGCTGAAT	AAATAATATGTTCCTTTTGCTG	6	102 - 126	1	
CG3[a]	AAATAATATGTTCCTTTTGCTG	TGTCGTTTTACTTCTCTCTCCAATAC	5	153 - 160	1	
pXO1[a]	TCTAGAATTAGTTGCTTCATAATGGC	CAATTTATTAACGATCAGATTAAGTTCA	3	120 - 144	1	
VNTR19[b]	GAAATATTTTATTAAACATGCTTTCCATCC	GTGATGAAATCGGACAAGTTAGGAG	3	91 - 134	2	
pXO2[a]	GTGTGATGAACTCCGACGACA	TCATCCTCTTTTAAGTCTTGGGT	2	133 - 155	1	
VNTR 12[b]	GCATATAATTGCACCTCATCTAG	CGTACGAAGTAGAAGTCATTAA	2	106 - 120	2	

* Reference to VNTR markers from (a) Keim *et al.,* (2000); (b) Van Ert *et al.,* (2007) and rest from Le Fleche *et al.,* (2001).

**Monoplex PCR separated using Agarose electrophoresis with Agarose gel percentage indicated. Small tandem repeat unit loci were separated using 2% and 3% molecular screening (MS) Agarose, which improves the resolution of 500bp and fewer DNA fragments.

Appendix Table 3 Copy numbers determined for 62 *Bacillus anthracis* isolates using agarose gel electrophoresis at VNTR loci

Key	BAMS 3	BAMS 05	BAMS 13	BAMS 22	BAMS 30	BAMS 31	BAMS 34	BAMS 44	VNTR 23	VrrA	BAMS1	BAMS15	BAMS21	BAMS23	BAMS24	BAMS25	BAMS28
BA#001	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#002	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#003	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#004	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#005	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#006	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#007	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#008	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#009	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#010	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#011	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#012	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#013	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#014	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#015	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#016	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#017	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#018	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#019	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#020	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#021	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#022	18	6	32	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#023	18	6	38	24	63	65	9	2	2	5	12	46	12	11	11	13	14

BA#024	18	6	32	12	63	65	9	2	2	5	12	46	12	11	11	13	14
BA#025	18	6	32	12	63	66	9	2	2	5	12	46	12	11	11	13	14
BA#026	18	6	32	12	65	65	9	2	2	5	12	46	12	11	11	13	14
BA#027	18	6	32	12	65	65	9	2	2	5	12	46	12	11	11	13	14
BA#028	18	6	32	12	65	65	9	2	2	5	12	46	12	11	11	13	14
BA#029	18	6	32	12	63	65	9	2	4	5	12	46	12	11	11	13	14
BA#030	18	6	32	24	63	66	9	2	4	5	12	46	12	11	11	13	14
BA#031	36	6	38	12	63	65	6	8	2	5	16	46	12	11	11	13	14
BA#032	18	6	32	12	63	65	9	2	2	5	12	46	12	11	11	13	14
BA#033	18	6	33	12	63	65	6	2	2	5	12	46	12	11	11	13	14
BA#034	18	6	32	12	63	66	9	2	2	5	12	46	12	11	11	13	14
BA#035	18	6	33	12	63	65	9	2	4	5	12	46	12	11	11	13	14
BA#036	18	6	33	12	62	65	9	2	2	5	12	46	12	11	11	13	14
BA#037	18	6	33	12	62	65	6	2	2	5	12	46	12	11	11	13	14
BA#038	18	6	33	12	63	65	6	2	2	5	12	46	12	11	11	13	14
BA#039	18	6	33	12	63	66	6	2	2	5	12	46	12	11	11	13	14
BA#040	18	6	33	12	62	65	6	2	2	5	12	46	12	11	11	13	14
BA#041	18	6	33	12	63	65	6	2	2	5	12	46	12	11	11	13	14
BA#042	18	6	34	12	63	65	6	2	2	5	12	46	12	11	11	13	14
BA#043	18	6	34	12	63	66	6	2	2	5	12	46	12	11	11	13	14
BA#044	18	6	33	12	63	66	6	2	2	5	12	46	12	11	11	13	14
BA#045	18	6	33	12	61	65	6	2	2	5	12	46	12	11	11	13	14
BA#046	18	6	33	12	61	65	6	2	2	5	12	46	12	11	11	13	14
BA#047	18	5	32	12	45	65	9	2	5	6	12	46	12	11	11	13	14
BA#048	18	6	32	12	45	65	6	2	5	6	12	46	12	11	11	13	14
BA#050	18	6	32	12	45	65	9	2	5	6	12	46	12	11	11	13	14
BA#051	18	6	32	12	45	65	9	2	4	6	12	46	12	11	11	13	14
BA#052	18		33	12	63	65	9	2	4	6	12	46	12	11	11	13	14
BA#053	18	6	32	14	45	65	9	2	4	6	12	46	12	11	11	13	14

BA#055	19	6	33	12	63	65	9	2	5	6	12	46	12	11	11	13	14
BA#056	19	6	33	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#057	19	6	33	12	63	65	9	2	5	6	12	46	12	11	11	13	14
BA#058	19	6	33	12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#060	19	6	33	12	63	65	9	2	5	6	12	46	12	11	11	13	14
BA#061	19	6	33	12	63	65	9	2	5	6	12	46	12	11	11	13	14
BA#049	18	6	32	12	45	65	9	2	5	6	12	46	12	11	11	13	14
BA#054	18	6	32	13	45	65	9	2	4	6	12	46	12	11	11	13	14
BA#059				12	63	65	9	2	2	6	12	46	12	11	11	13	14
BA#062	19	6	33	12	63	65	9	2	2	6	12	46	12	11	11	13	14

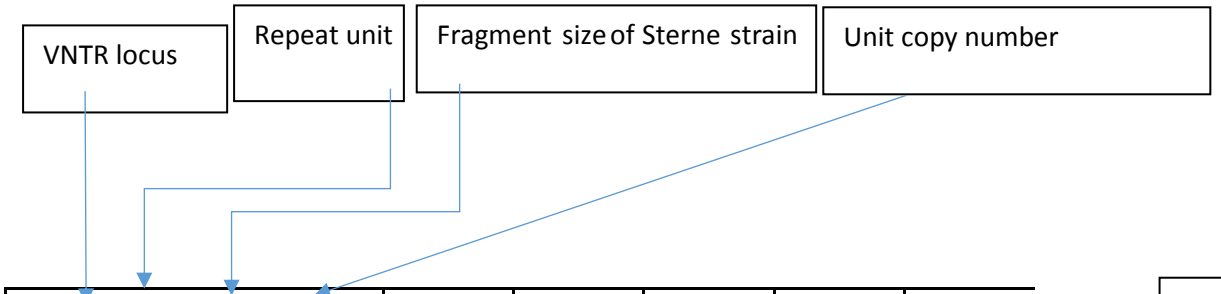
Appendix Table 3 cont.

Key	BAMS51	BAMS53	CG3	pX01	pX02	VNTR12	VNTR16	VNTR17	VNTR19	VNTR35	VrrB1	VrrB2	Vrrc2	VrrC1
BA#001	9	8	2	6	6	6	21	4	5	4	20	14	17	55
BA#002	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#003	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#004	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#005	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#006	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#007	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#008	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#009	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#010	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#011	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#012	9	8	2	6	6	6	21	4	5	4	20	14	17	53

BA#013	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#014	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#015	9	8	2	6	0	6	0	0	5	4	20	14	17	53
BA#016	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#017	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#018	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#019	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#020	9	8	2	6	0	6	0	0	5	4	20	14	17	53
BA#021	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#022	9	8	2	6	0	6	0	0	5	4	20	14	17	53
BA#023	9	8	2	6	0	6	0	0	5	4	20	14	17	53
BA#024	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#025	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#026	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#027	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#028	9	8	2	6	6	6	21	4	5	4	20	21	17	53
BA#029	9	8	2	6	6	6	21	0	5	4	20	14	17	53
BA#030	9	8	2	6	0	6	0	0	5	4	20	14	17	53
BA#031	9	8	2	6	0	6	0	0	5	4	20	14	17	53
BA#032	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#033	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#034	9	8	2	6	0	6	0	0	5	4	20	14	17	53
BA#035	9	8	2	6	0	6	0	0	5	4	20	14	17	53
BA#036	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#037	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#038	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#039	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#040	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#041	9	8	2	6	6	6	21	4	5	4	20	14	17	53

BA#042	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#043	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#044	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#045	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#046	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#047	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#048	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#050	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#051	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#052	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#053	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#055	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#056	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#057	9	8	2	6	0	6	0	0	5	4	20	14	17	53
BA#058	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#060	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#061	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#049	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#054	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#059	9	8	2	6	6	6	21	4	5	4	20	14	17	53
BA#062	9	8	2	6	0	6	0	0	5	4	20	14	17	53

Appendix Table 4 Comparative Appendix Table used to convert PCR product sizes to copy numbers for the MLVA-31 panel. Information includes the MLVA marker (VNTR locus) with the repeat unit length, PCR product size of Sterne reference strain and of copy numbers (tandem repeat size) for the Sterne strain with listed PCR product sizes with corresponding copy number unit obtained with 80 *B. anthracis* isolates.



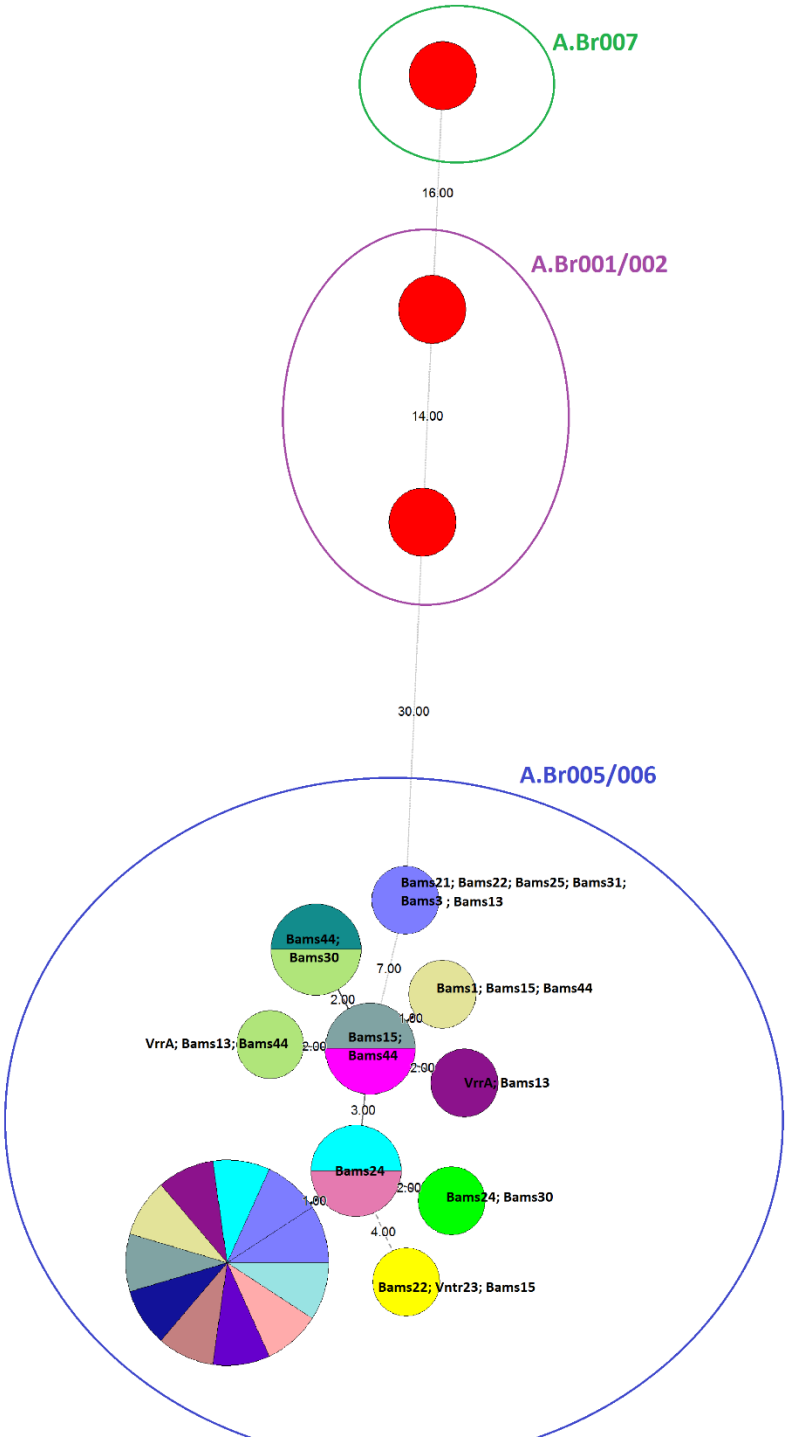
VrrA_12bp_282bp_2U	(306) 4	(294) 3	(282) 2	(270) 1	
VrrB1_9bp_225bp_17U	(246) 19	(234) 18	(225) 17	(216) 16	(204) 15
VrrB2_9bp_169bp_14U	(189) 16	(177) 15	(168) 14	(159 13	(147) 12
VrrC1_9bp_563bp_17U	(584) 19	(572) 18	(563) 17	(554) 16	(542) 15
VrrC2_18bp_525bp_17U	(555) 19	(543) 18	(525) 17	(507) 16	(495) 15
CG3_5bp_175bp_2U	(192) 4	(180) 3	(175) 2	(170) 1	
pX01(aat_3bp_125bp_8U	(141) 10	(129) 9	(126) 8	(123) 7	(111) 6
pX02(at_2bp_150bp_5U*	(164 7	(152 6	(150 5	(148) 4	(136) 3
BAMS01_21bp_488bp_16U	(518)	(506)	(485)	(464)	(452)

	18	17	16	15	14
BAMS03_15bp_500bp_28U	(527) 30	(515) 29	(500) 28	(485) 27	(473) 26
BAMS05_39bp_387bp_9U	(406 11	(394) 10	(382) 9	(370) 8	(358) 7
BAMS13_9bp_814bp_70U[a]	(832) 72	(823) 71	(814) 70	(805) 69	(796) 68
BAMS15_9bp_575bp_45U	(596) 47	(584) 46	(575) 45	(566) 44	(554) 43
BAMS21_45bp_750bp_10U	(733) 11	(721) 10	(676 9	(631) 8	(619 7
BAMS22_36bp_725bp_16U	(773) 18	(761) 17	(725) 16	(689) 15	(674) 14
BAMS23_42bp_625bp_4U	(679) 6	(667) 5	(625) 4	(583) 3	(571) 2
BAMS24_42bp_600bp_110U	(654) 13	(642) 12	(600) 11	(558) 10	(546) 9
BAMS25_15bp_400bp_13U	(427) 15	(415) 14	(400) 13	(385) 12	(370) 11
BAMS28_24bp_487bp_14U	(523 16	(511) 15	(487) 14	(463) 13	(448) 12
BAMS30_9bp_627bp_51U	(669 53	(657) 52	(627) 51	(597) 50	(582) 49
BAMS31_9bp_772bp_64U[a]	(790 66	(781) 65	(772) 64	(763) 63	(754) 62
BAMS34_39bp_500bp_11U	(551 13	(539) 12	(500) 11	(461) 10	(446) 9
BAMS44_39bp_425bp_8U	(468)	(456)	(417)	(378)	(363)

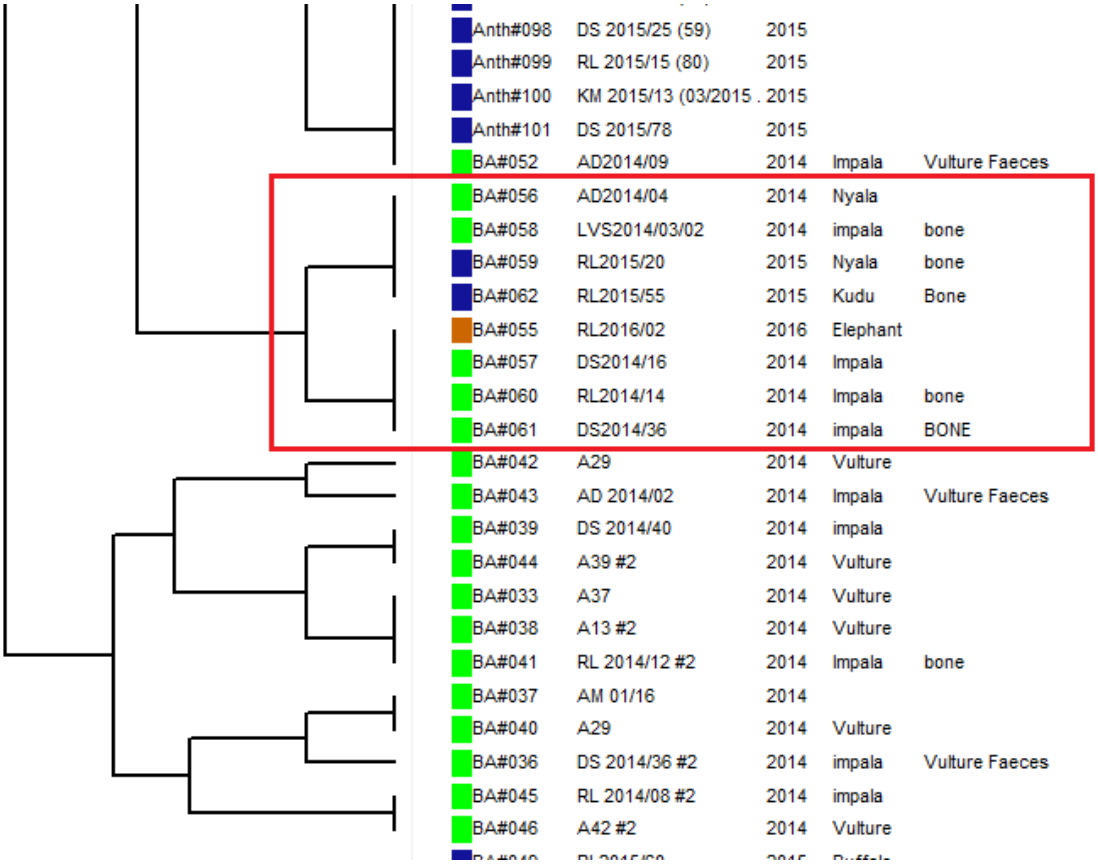
	10	9	8	7	6
BAMS51_45bp_488bp_9U	(544) 11	(532) 10	(487) 9	(442) 8	(427 7
BAMS53_12bp_250bp_9U	(272) 11	(260) 10	(248) 9	(236) 8	(224) 7
VNTR12_2bp_125bp_8U	(153 10	(141) 9	(129) 8	(117) 7	(115) 6
VNTR16_8bp_288bp_20U*	(307 22	(295) 21	(287) 20	(279) 19	(264) 18
VNTR17_8bp_388bp_4U*	(407 6	(395 5	(387) 4	(379) 3	(364) 2
VNTR19_3bp_100bp_4U	(115 6	(103 5	(100) 4	(97) 3	(82) 2
VNTR23_12bp_150bp_4U	(174) 6	(162) 5	(150) 4	(138) 3	(126) 2
VNTR35_6bp_113bp_5U	(131) 7	(119) 6	(113) 5	(107) 4	(92) 3

[a] (Thierry *et al.,* 2014)

* Vollum strain was used as a reference strain

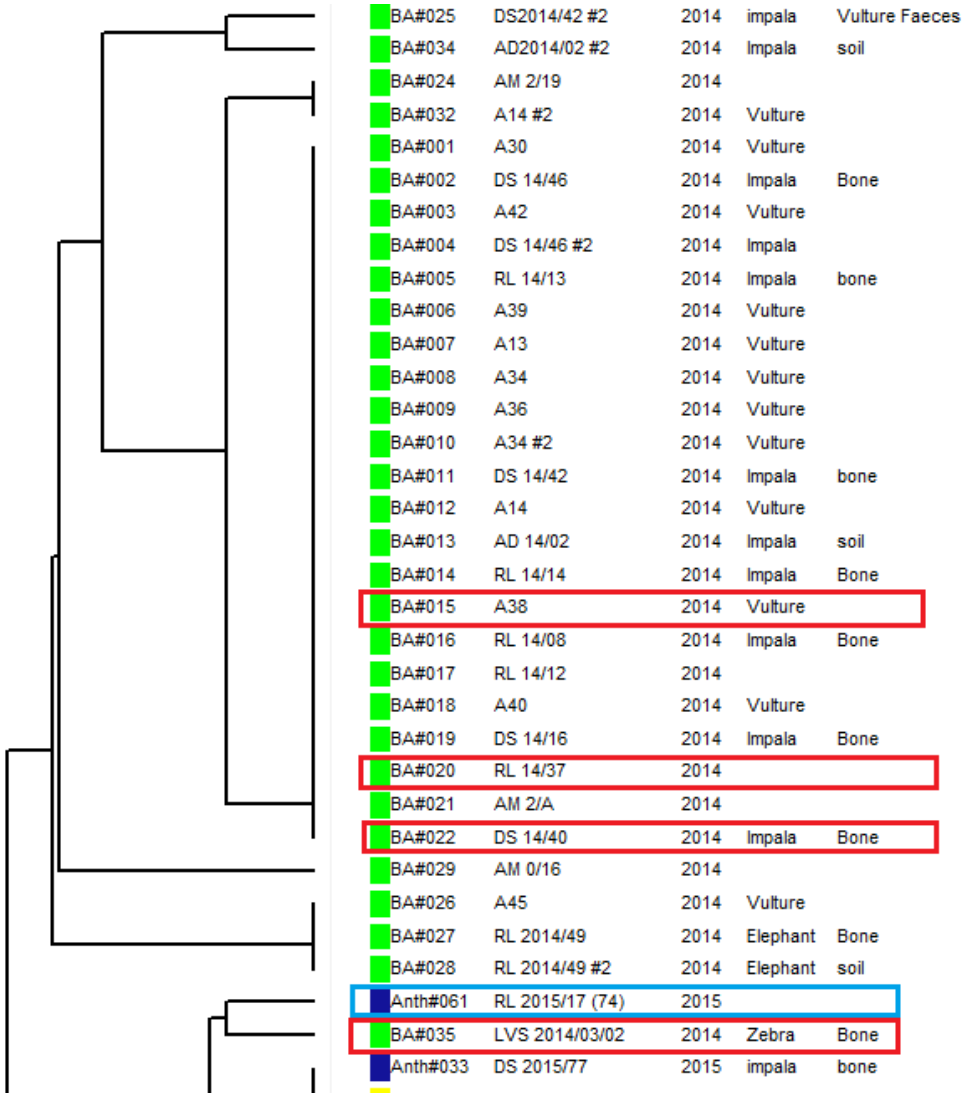


Appendix Figure 1 MST dendrogram of VNTR data from Kruger National Park. *B. anthracis* isolates in the year 2014: MLVA-31 and MST cluster analysis was used to establish genetic relationships among the 24 *B. anthracis* isolates.

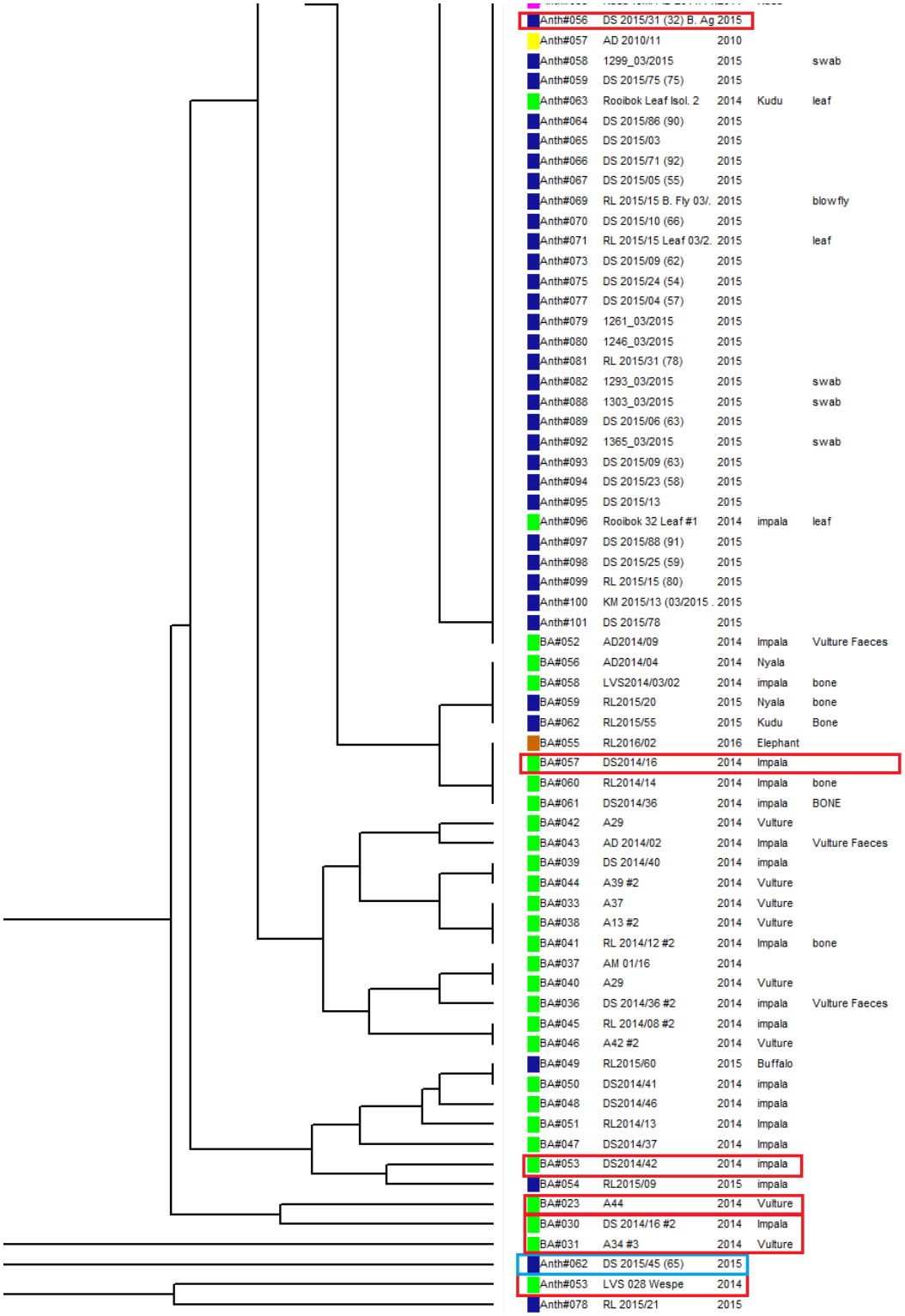


Appendix Figure 2 UPGMA dendrogram based on 31 VNTR loci of *Bacillus anthracis* for clonal genotype from Nwanetsi and Pafuri in Kruger National Park (2014-2016), South Africa. The dendrogram was generated in BioNumerics version 6.6.5 Colours indicate the year of Isolation

Figure 2:



Appendix Figure 3 UPGMA dendrogram based on 31 VNTR loci of *Bacillus anthracis* for 5 isolates that lost pX01 and/or pX02 from Kruger National Park (2014-2015), South Africa. The dendrogram was generated in BioNumerics version 6.6.5 Colours indicate the year of Isolation



Appendix Figure 4 UPGMA dendrogram based on 31 VNTR loci of *Bacillus anthracis* for 7 isolates that lost pX01 and/or pX02 from Kruger National Park (2014-2015), South Africa. The dendrogram was generated in BioNumerics version 6.6.5 Colours indicate the year of Isolation

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