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MOLECULAR CHARACTERIZATION OF *BACILLUS ANTHRACIS* AND *BRUCELLA* SPECIES FROM SOUTHERN AFRICA

by

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A dissertation submitted in partial fulfilment of the requirements for the degree of

Master of Veterinary Science

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DEDICATION

I dedicate this thesis to my late father Tlou Joseph Ledwaba, my late brother Phuti Piet Ledwaba and the rest of my family (my mum Asina, my son Khutjo, my brothers Simon and Jeremia and my sisters Joyce and Maria).

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DECLARATION

With the exception of the assistance obtained and stated in the acknowledgement and in appropriate places in the document, this dissertation represents the original work of the author.

The investigations and findings in this dissertation have not been presented for any degree in this or any other University.

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THESIS SUMMARY

MOLECULAR CHARACTERIZATION OF *BACILLUS ANTHRACIS* AND *BRUCELLA* SPECIES FROM SOUTHERN AFRICA.

MSc Degree of: Maphuti Betty Ledwaba

Promoter: Henriette Van Heerden

Co-promoter: Ayesha Hassim

Department: Veterinary Tropical Diseases

The main focus of this project was to characterise *Brucella* and *Bacillus anthracis* strains from certain areas of southern Africa using molecular techniques. Multiplex PCR assays (AMOS-PCR and Bruce-ladder PCR) were used to identify *Brucella* strains isolated from different hosts in Zimbabwe and selected strains were further characterised by whole genome sequencing using next generation sequencing (NGS). *Bacillus anthracis* strains from wildlife in the KNP region in South Africa were typed using 31 multiple locus variable number of tandem repeats (VNTR) analysis (31-MLVA) to monitor the spread, distribution and diversity of the strains.

In this study we successfully identified *Brucella abortus, B. canis* and *B. suis* from the 16 *Brucella* isolates from Zimbabwe using AMOS and Bruce-ladder PCR assays. This is the first formal report of *B. canis* and *B. suis* in Zimbabwe, since it is documented that only *B. abortus, B. melitensis* and *B. ovis* have been reported in Zimbabwe. On the basis of the origin (sample source) and the results when characterising *Brucella* isolates with AMOS and Bruce-ladder PCR, three (ZW043, ZW046 and ZW053) strains from bovines, identified as *B. suis* (ZW043 and 046) and *B. abortus* (ZW053), were selected and sequenced to verify their accurate identity. Despite the variants observed in our genome sequences, whole-genome sequencing of these three isolates using the Illumina Miseq® (Illumina®) and their comparison to *Brucella* reference whole genome sequences have proven that the isolates

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were *B. suis* (ZW043 and ZW046) and *B. abortus* (ZW053) respectively, thus corresponding with the data obtained with the Bruce-ladder, AMOS PCR assays. As most laboratories in Africa lack adequate resources, expertise and infrastructure to do biotyping of pathogens under proper biosafety and biosecurity conditions, multiplex-PCR assays, and especially Bruce-ladder could be used in the identification of *Brucella* species.

Bacillus anthracis isolates (n=72) collected from various anthrax outbreaks and regions in KNP during 2012 and 2013 anthrax outbreaks were analysed using MLVA. The 72 *B. anthracis* isolates in KNP belonged to 23 *B. anthracis* genotypes clonal genotypes were observed in each outbreak, while five animals appeared to be infected by multiple genotypes. Clonal genotypes were observed in each outbreak and from the spread of the genotypes over large distances vultures seem to be the primary vector transmitting the anthrax strains from one region to another. Eighteen out of the 23 genotypes obtained from all the isolates were circulating during the 2012 outbreak spanning 7 regions and 5 catchment areas (a basin shaped area where rivers drained their water) compared to 6 genotypes circulating from 2013 cases and environmental samples, from sites where animals were suspected to die of anthrax during 2012 to early 2013, that were confined mostly to 1 region and 1 catchment area with 2 outlying cases. Cluster analysis of 30-MLVA data grouped 72 isolates in the major genetic A-clade. No genotype grouped in the clade B in this study although B-clade genotypes played an important role in KNP during 1970-1981 but played a minor role from the major outbreak in the 1990 onwards. MLVA is an important epidemiological tool and was proven to be an efficient method to characterize the spread as well as identify possible vectors of anthrax in KNP this study.

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CHAPTER 1

1. GENERAL INTRODUCTION

Zoonoses are infectious diseases which can be transmitted between vertebrate species directly or indirectly, through environment, food, water or vectors. Zoonoses mainly occur amongst people living in close proximity to animals as they can be transmitted from animals to humans. The occurrence of zoonosis in humans is mainly determined by the prevalence of infection in animals; hence, control and eradication of animal diseases will markedly reduce the incidence of the disease in humans (WHO/DFID-AHP meeting, 2005). Zoonotic diseases affect the health of people in farming communities and cause severe losses in productivity of their livestock (Maudlin *et al*., 2009). Bovine tuberculosis, brucellosis and anthrax have emerged to be amongst the most concerning examples of zoonotic diseases and they are of major significance to wildlife, livestock and people, especially in areas surrounding or in national park (WHO, 2005).

Bacillus anthracis and *Brucella* species are causative agents of the widespread zoonotic diseases anthrax (Bailie & Read, 2001; Sternbach, 2003; Moayeri & Leppla, 2009) and brucellosis (Corbel & Brinley-Morgan, 1984; Glynn & Lynn, 2008) respectively. Both these organisms have been used as biological weapons in bioterrorism since they are zoonoses and easily distributed (Robinson-Dunn, 2002). These zoonotic diseases can be easily misdiagnosed especially in human since their symptoms resembles other diseases like influenza. Despite this threat, anthrax and brucellosis are still ignored and continue being a concern in poor livestock-keeping communities (WHO/DFID-AHP meeting, 2005).

Anthrax is primarily a disease of herbivores as wild ungulates but the disease may also affect other species of domestic and wild animals, and people. Cases of anthrax among agricultural animals decreased after the introduction of Sterne vaccine in 1937 (Hambleton *et al.*, 1984; Ivins & Welkos, 1988), but the Office International des Epizooties (OIE) reports that the disease is still enzootic in many countries of the world. It is common in the Middle East, Africa, Asia, the Caribbean and central and southern part of the United States of America

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(USA) (WHO, 2008). Anthrax in humans can develop when the organism enters through a wound in the skin, eating undercooked meat from infected animals or through inhalation of spores during bioterrorism or from infected hides (Turnbull, 1991). Brucellosis is also widespread in Africa and is another zoonotic disease that is of great concern to livestock-owning communities. Its infections in humans may result from drinking or eating unpasteurized milk products and handling infected aborted foetuses or the afterbirth (Young, 1995; Fugier *et al.,* 2007). Factors and challenges faced globally when dealing with brucellosis include husbandry strategies, production systems and management factors like the control of the growing reservoir in wildlife, wildlife-livestock interaction as well as the development of control practices that will protect the public (Marcotty *et al*., 2009; Matope *et al*., 2010). Therefore, it is critical for the society to have knowledge to and understand the epidemiology of these diseases, as they are a threat in public health, economy and wildlife conservation. The genetic diversity of agents remains a central issue of the epidemiology of both brucellosis and anthrax.

The main focus of this project was to characterise strains of *Brucella* spp. and *B. anthracis* in southern Africa using molecular techniques. Multiplex PCR assays (AMOS-PCR and Bruce-ladder PCR) were used to identify *Brucella* strains isolated from different hosts in Zimbabwe and selected strains were further characterised by whole genome sequencing using next generation sequencing (NGS). AMOS-PCR, the first species-specific assay that differentiated *Brucella abortus, B. melitensis, B. ovis* and *B. suis* was developed by Bricker & Halling (1994). Despite improvements it still failed to characterize all *Brucella* isolates and biovars. The Bruce-ladder multiplex assay was developed by Garcia-Yoldi *et al*. (2006) and after improvements (Lopez-Goni *et al.,* 2008; Mayer-Scholl *et al.,* 2010) it allows for the identification of all *Brucella* species including vaccine strains. Based on results of Bruce-ladder and AMOS-PCR assays as well as the sample sources, 3 *Brucella* isolates (ZW043, ZW046 andZW053) were selected and sequenced with NGS.

Bacillus anthracis strains from wildlife in the endemic Kruger National Park region in South Africa were typed using 31 multiple locus variable number of tandem repeats (VNTR) analysis (MLVA-31; Beyer *et al*., 2012) to monitor the spread, distribution and diversity of the strains. MLVA includes amplification of VNTR loci and sizing of amplified fragments to

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detect length polymorphism (Keim *et al.,* 2004). These first 8 VNTR loci (MLVA-8) were developed by Keim *et al*. (1999; 2000) and it enables the identification of distinct *B. anthracis* genotypes (strain types) worldwide. Additional VNTR markers were added by various researchers (Le Fleche *et al*., 2001; van Ert *et al*., 2007, Beyer *et al.,* 2012) making use of MLVA-15, MLVA-25 or MLVA-31 system. MLVA has been adopted globally as it is a powerful tool in addressing the genetic diversity of closely related species. Unfortunately different numbers of VNTR loci is used namely MLVA-15, MLVA-25 or MLVA-31, limiting global comparison. MLVA enables the distinction of outbreaks caused by different strains from those caused by the spread of a single strain, tracing an outbreak strain back to its possible origin and to track the routes of transmission of an outbreak strain within and between animal populations. It is thus possible to study genotypic diversity in relation to the spatial dynamics behind the spread of the disease and possible relationships between genotype and host species (Beyer and Turnbull, 2009).

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CHAPTER 2

2. LITERATURE REVIEW

2.1 BACILLUS ANTHRACIS

2.1.1 AETIOLOGY

Bacillus anthracis is a Gram-positive, non-motile, spore-forming, aerobic and rod-shaped pathogen causing the disease anthrax (Turnbull & Kramer, 1995; Leppla *et al*., 2002). The organism belongs to the genus *Bacillus* which includes many species with a great diversity of properties, but all of them being aerobic and spore-forming (De Vos & Turnbull, 2004). *Bacillus anthracis* is a member of the *B. cereus sensu lato* group which also includes *B. thuringiensis, B. mycoides, B. cereus, B. pseudomycoides* and *B. weihenstephanens* (Henderson *et al.*, 1994; Jensen *et al.,* 2003).

Bacillus anthracis is able to form spores that are highly resistant to harsh conditions like chemical disinfection, heat, cold etc. and has the ability to survive in the soil for a long period. The spores are present in abundance in soil at sites where infected animals had died or been buried (Leppla *et al*., 2002). De Vos (1990) isolated *B. anthracis* spores from bones retrieved at an archaeological site in Kruger National Park (KNP), estimated to be 200 ±50 years old. This resilience of spores to harsh environmental conditions is regarded as the main cause of anthrax persistence in an area (Dragon & Rennie, 1995).

2.1.2 EPIDEMIOLOGY

Anthrax has been a global problem since ancient times and it is linked to the fifth and six plagues in the Bible (Turnbull, 1991; Hugh- Jones & De Vos, 2002). In 1875, Cohn in collaboration with Robert Koch named the species "*anthracis*" from the Greek word "anthrakis" meaning coal, referring to the large, black lesions that develop when infected by cutaneous anthrax (De Vos & Turnbull, 1994). *Bacillus anthracis*'modus operandi was established by Robert Koch in 1876 who described its ability to form resistant spores and *in vitro* cultivation. This was further supported by the findings of Louis Pasteur who established the relationship between *B. anthracis* and the disease (Van Ness, 1971; Dragon & Rennie,

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1995; Farrar & Reboli, 2006). The *B. anthracis* infection is dependent on host susceptibility, environmental factors, certain human activities and the bacterium itself (Hugh-Jones & De Vos, 2002).

Infection of the pathogen can occur through the entry of spores into the host through insect bites or abrasions (cutaneous), consumption of contaminated animal products or vegetation (gastrointestinal) or the inhalation of the spores (pulmonary) (Turnbull, 1991). Soil sample surveys conducted previously and in the KNP have established that *B. anthracis* spores cannot grow or multiply in the soil or water, the soil and water serve only as a reservoir to convey the spores to the host where they become reactivated and infect the host with the disease (De Vos, 1990). Once in the host the vegetative bacilli produce the toxin, infect and kill the host (Leppla, 1982; Mock & Fouet, 2001). Animals that die of anthrax usually discharge large amount of anthrax bacilli, returning the organism to the soil and if opened, endospores will form triggered by exposure to oxygen (McKendrick, 1980; Quinn *et al.,* 1994). Therefore opening of carcasses where anthrax is suspected is prohibited by law and confirmed anthrax cases must be reported to the appropriate authorities and OIE (Office International Epizooties) (OIE, 2008).

The disease is not directly transmitted from host to host but rather indirectly through scavengers, water and insects. The life cycle of *B. anthracis* (Figure 1) involves infection of herbivores grazing/browsing on contaminated vegetation and thereafter the animal dies. Scavengers like vultures and carnivores will feed on the infected carcass and spread the organism to waterholes or drinking holes (Dixon *et al*., 1999). *Bacillus anthracis* can also be mechanically transmitted by some bird species, house flies and biting flies (Greenberg, 1973; Hambleton *et al*., 1984; Turell & Knudson, 1987). Spores are dispersed over short distances by the flies and over a long distance by vultures (McKendrick, 1980). Blowflies feed on body fluids of infected carcasses and deposit the faecal or vomit droplets on the leaves of trees near the carcass, where bowsers eat the contaminated leaves (Hugh- Jones & De Vos, 2002). Run-off water during rainy season will also disperse the spores and contaminate the grazing in the vicinity (Hugh-Jones & De Vos, 2002). Humans mainly acquire anthrax either through consumption of infected animal products or through occupational exposure to contaminated raw products like bones, hides, hairs, etc. during manufacturing,

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processing or testing and through direct or indirect contact with sick animals (e.g. during slaughtering) (Turnbull, 2008).

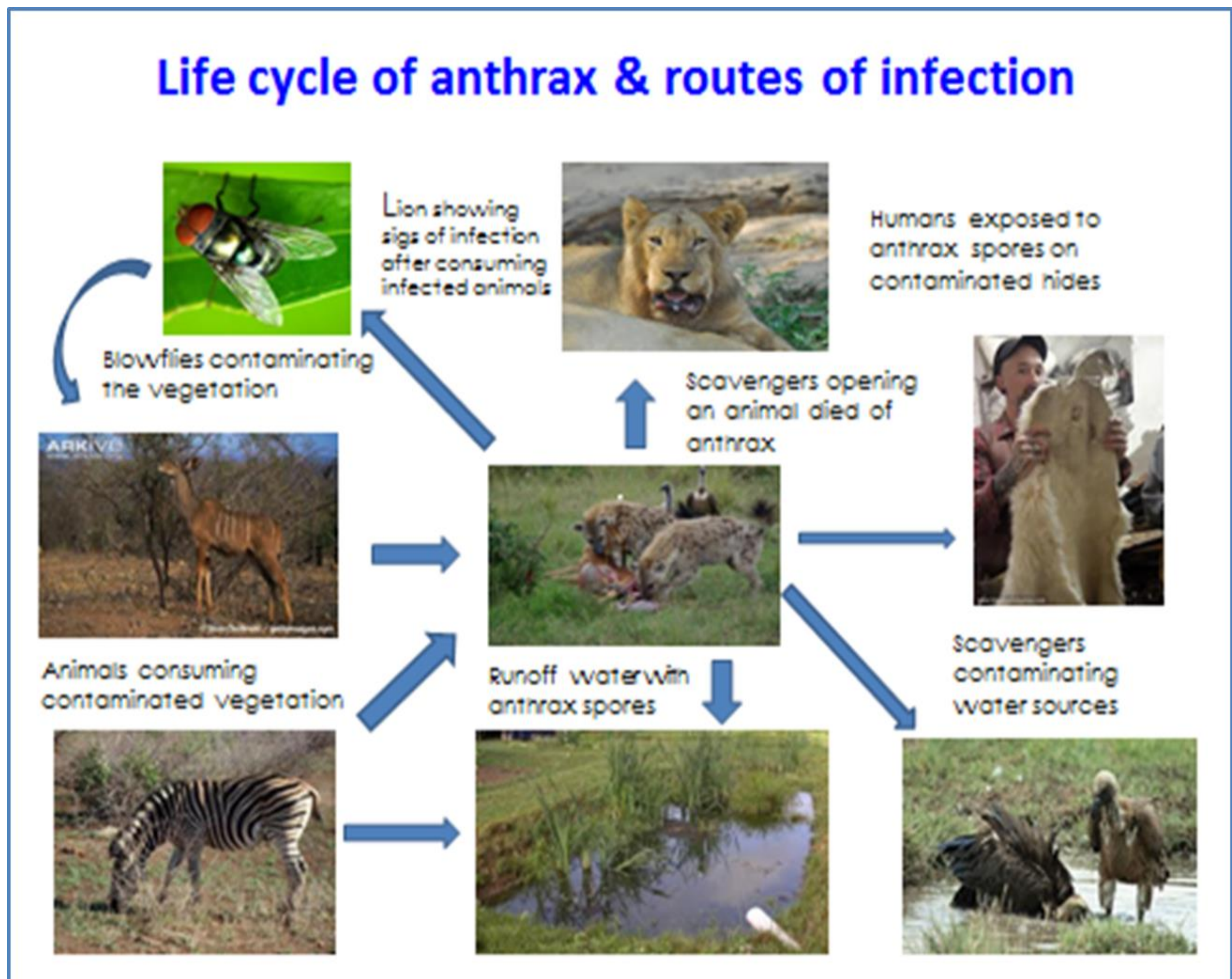

Figure 2.1: Life cycle of *Bacillus anthracis* indicating different vectors and routes of infection.

2.1.3 PATHOGENESIS

Anthrax disease manifests itself in three different ways depending on the route in which the spore entered the host. Cutaneous anthrax (most common) develop when infection occurs through the skin, gastrointestinal anthrax occurs when the spores are ingested with contaminated animal products and pulmonary anthrax which is the more severe but rare, occurs through inhalation of spores (Dixon *et al*., 1999) mainly during manufacturing and processing. Post-entry into the host, especially humans, the vegetative form of *B. anthracis* will germinate in the macrophages where the lethal and oedema toxin will also be produced (Ascenzi *et al.*, 2002; Spencer, 2003).

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The high lethality of anthrax depends on two virulent factors namely the tripartite protein toxin encoded by a large plasmid (182kb) pX01 and the capsule encoded by a smaller plasmid (95kb) pX02 (Farrar, 1994; Koehler, 2002). The organism must produce both the antiphagocytic capsule and the tripartite toxin to be fully virulent (Quinn *et al*., 1994). If one of the plasmids is lost, there will be a decrease in virulence and the organism will be attenuated (Welkos, 1991; Turnbull, 1999). The tripartite toxin of *B. anthracis* is composed of a protective antigen (PA), a lethal factor (LF) and an oedema factor (EF) which are not toxic individually. These proteins constitute a variation of the A-B enzyme model, where the A and B components work together to effectuate a change in cell conformation (Dixon *et al.*, 1999; Ascenzi *et al.*, 2002; Collier & Young, 2003).

PA is the common cell-binding domain (B) with the ability to collaborate with the other two domains (A), EF and LF which provoke cell damage (Leppla, 1995). It binds to cell receptors and stick to a single site to produce a binding site for both LF and EF, which will competitively bind to gain access to the cytoplasm. Once inside the cell, the acidic pH in the endosomes changes the pre-pore form by releasing LF and EF into the cytoplasm and inserts only PA fragments (Collier & Young, 2003). In the cytoplasm, EF got activated into a cyclase which cleave two phosphates from ATP (adenosine triphosphate) to form cAMP (cyclic adenosine monophosphate). Higher levels of cAMP in the cell will inhibit water homeostasis resulting in severe oedema (Dixon *et al*., 1999). LF, on the other hand, inactivates the mitogen-activated protein kinase kinases (MAPKKs), thereby disrupting cell cycle regulation and kills the cell (Pannifer *et al.*, 2001; Collier & Young, 2003).

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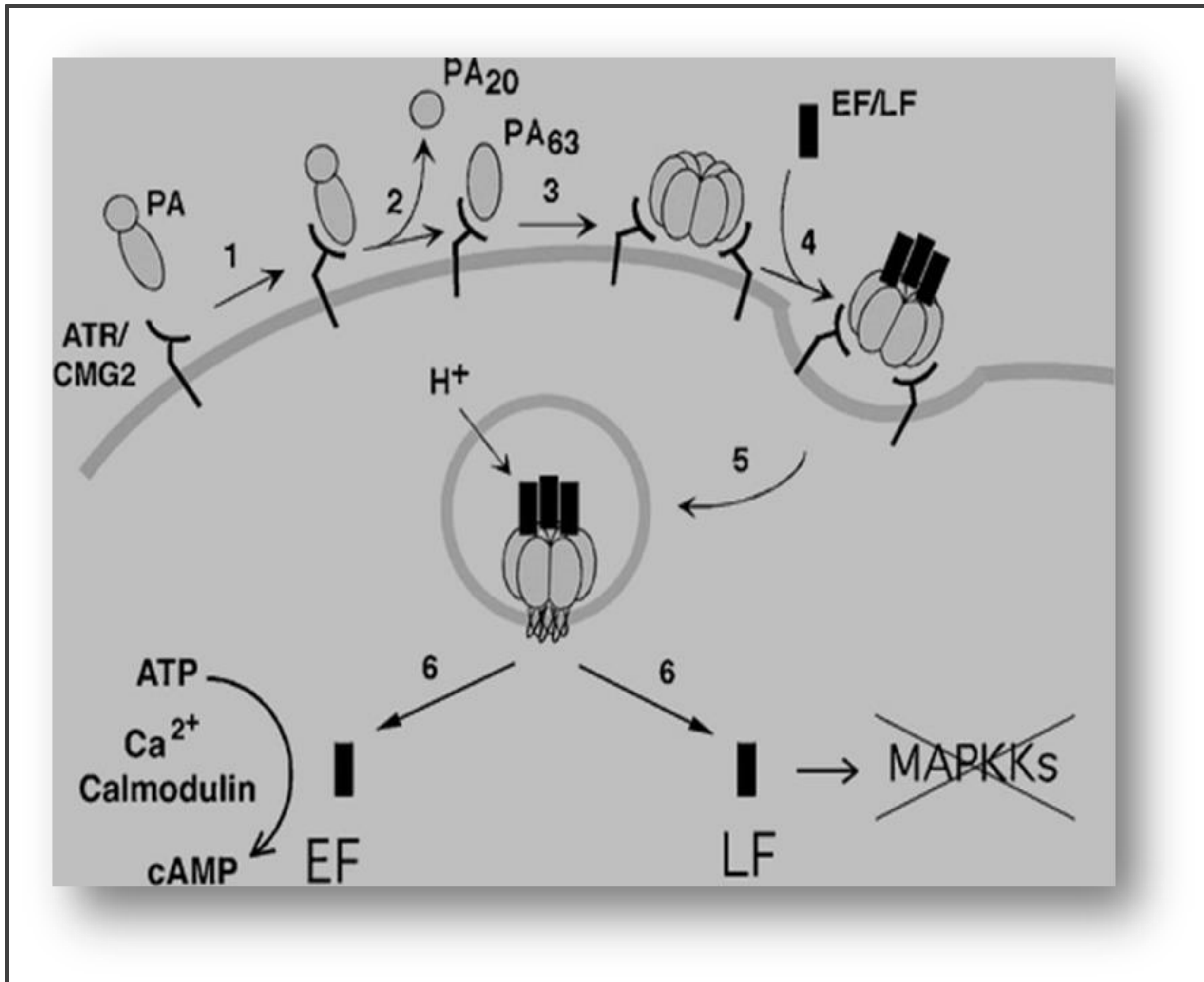

Figure 2.2: Entry mode of anthrax toxin (Collier & Young, 2003). Entry into the cell is initiated by PA_{83} (protective antigen) recognising the cell wall receptor and bind. It is then cleaved by cell wall protease into PA_{63} which oligomerise to form a heptamer/ prepore. LF and EF competitively bind on the exposed sites of the prepore to gain access to the cytoplasm. The acidic pH enables the release of lethal toxin (LT) and edema toxin (ET). In the cytoplasm, EF got activated into a cyclase and forms cAMP (cyclic adenosine monophosphate) that inhibits water homeostasis resulting in a severe edema. LF, on the other hand, inactivates the mitogen-activated protein kinase kinases (MAPKKs), thereby disrupting cell cycle regulation and killing the cell.

2.1.4 THE DISTRIBUTION AND PREVENTION OF ANTHRAX

Anthrax outbreaks occur mostly in limited regions, especially those with alkaline soil, seasonal flooding, warm environment and dry agricultural zones. It is common in Middle East, Africa, Asia, the Caribbean and the USA (most often in the central and southern part) (WHO, 2008). High mortality in livestock can occur during outbreaks due to lack of

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experience in identification of anthrax and management of the outbreak (Hugh-Jones & De Vos, 2002). Variability in host species susceptibility to anthrax is primarily determined by the feeding behaviour and infection route. Anthrax mortalities in southern Africa have been reported in 52 species with herbivores being more susceptible than other species (Hugh-Jones & De Vos, 2002). Anthrax mortalities have been reported in wild dogs, lions, leopards and cheetahs due to their feeding behaviour, despite being more resistant to anthrax (De Vos, 1990; De Vos & Bryden, 1997).

The effective and safe Sterne vaccine developed by Sterne in 1937 (Hambleton *et al.*, 1984; Ivins & Welkos, 1988) is used for prevention in animals (not for humans due to its residual virulence) (Mock & Fouet, 2001).Since its introduction and distribution by government as a compulsory vaccine to farmers, anthrax outbreak dropped steadily (Gilfoyle, 2006). Anthrax is a notifiable disease in many countries and control are established and organized by government (through state veterinarians) (Hugh-Jones & De Vos, 2002). Anthrax can be effectively controlled in developed countries through vaccination. Countries with poor socio-economic conditions are still experiencing anthrax outbreaks and/or epidemic (Hugh-Jones, 1999). Especially anthrax endemic areas in or surrounding national parks / game reserves because they remain at risk as the disease seems to be problematic to livestock due to the infections in the parks / reserves (Hugh-Jones & De Vos, 2002).

2.1.5 DIAGNOSIS AND MOLECULAR TECHNIQUES

Strain differentiation of *B. anthracis* is difficult due to the lack of molecular polymorphisms within the species (Keim *et al*., 2000). Therefore, various molecular assays like ribosomal genes (rRNA; 16S and 23S rRNAs) that are widely used to differentiate between bacterial strains have been investigated to overcome this problem. According to Ash *et al*. (1991) and Ash & Collins (1992) members of *B. cereus* group, which includes *B. anthracis,* have a difference of only one nucleotide sequence at the 16S rRNA sequences and 2 nucleotides differences at the 23S rRNA sequences. The canonical single nucleotide polymorphisms (canSNPs) are diagnostic SNPs used for identification of a particular phylogenetic point in the evolutionary history of *B. anthracis* (Keim *et al.,* 2004). The canSNPs have a low resolving power when used alone, thus it is combined with MLVA to identify main clades and key

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phylogenetic positions in major or single strain clades (Keim *et al*., 2004). Real-time PCR is mainly used for confirmation of microscopic detection and/or diagnosis made from cultures (Turnbull *et al.,* 1998).

MLVA involves the amplification of chromosomal loci carrying VNTRs and sizing of fragments to detect length polymorphism that determine the copy number of the VNTR (Keim *et al*., 2004). The development of the 8-MLVA (Keim *et al.,* 1999; 2000) and later 15-MLVA (van Ert *et al.,* 2007) enabled the identification of distinct *B. anthracis* genotypes (strain types). Le Fleche *et al*. (2001) and Lista *et al*. (2006) extended it to 25 loci (25-MLVA)) and Beyer *et al.* (2012) combined the 15-MLVA and 25-MLVA to form the 31-MLVA. The method has been adopted globally as a powerful tool in addressing the genetic diversity of closely related species including *B. anthracis* and *Brucella* species (Le Fleche *et al*., 2001; 2006). Thus, it is regarded as the assay of choice at present as it is able to group strains according to species, and also determine genetic diversity and origin.

VNTRs consists of short, multiple copies of repeated DNA sequences, classified as minisatellites, with repeat sequence consisting of 9 base pairs (9 bp) or greater, and microsatellites, which have repeat sequence of up to 8 bp (Le Fleche *et al*., 2001). Usage of VNTRs has shown to be competent in discriminating bacterial pathogens as compared to other systems. With MLVA, multiple VNTR loci are analysed simultaneously to increase the degree of investigation as a single locus provide little facts. The number of VNTRs required in an MLVA assay is determined by the bacterial species and the degree of reaction required (Vergnaud & Pourcel, 2006). The advantage of MLVA is through the analysis of a limited number of loci gives an overview of the difference within the species including the monomorphic species (Vergnaud & Pourcel, 2006).

The initial 8-MLVA assay grouped strains of *B. anthracis* in cluster A and B (Keim *et al*., 2000). The assay was later improved by adding more markers which led to the development of 15-MLVA that grouped strains into branch A, B and C (Keim *et al*., 2004).With the development of 25-MLVA a higher discrimination, which upgraded the resolution degree of the technique and added two more branches (D and E) to the cluster (Lista *et al*., 2006). All three of the above mentioned MLVA systems were independent and based on different

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protocols; therefore it was decided to combine them by forming a single common protocol of the three which led to the formation of MLVA-31 (Beyer & Turnbull, 2009; Beyer *et al*., 2012).

Beyer *et al*. (2012) used 31 MLVA (combination of 15-MLVA and 25-MLVA) to monitor the spread and distribution of anthrax outbreak in Namibia and the MLVA data obtained in their study was used to establish a model of the probable evolution of genotypes within the endemic region of the Etosha National Park in Namibia. With MLVA the authors could distinguish outbreaks caused by different strains from those caused by the spread of a single strain, trace back of strain to possible origin and the track the route of transmission.

2.2 *BRUCELLA* SPECIES

2.2.1 *BRUCELLAE* GENUS

The genus *Brucella* was named after Dr. David Bruce, who was the first person to isolate the organism in 1887 from the spleen of a soldier suffering from a disease called Malta fever (Nicoletti, 2002; Godfroid *et al.*, 2005; Banai & Corbel, 2010). It belongs to the family *Brucellaceae*, the order *Rhizobiales* and the alpha class *Proteobacteria* (O'Callaghan & Whatmore, 2011). The genus is genetically homologous and consisted of six species namely *B. melitensis* (3 biovars), *B. abortus* (8 biovars), *B. suis* (5 biovars), *B. ovis, B. canis,* and *B. neotomae* until the recent affiliation of four new species namely *B. ceti, B. pinnipedialis, B. microti* and *B. inopinata* (Table 1) (Osterman & Moriyon, 2006; Foster *et al.*, 2007; Scholz *et al.*, 2008b; 2009).

Brucella species were classified and recognized based on pathogenicity, host preference, genotypic and phenotypic properties (Alton *et al*., 1988). Little differences have been found between *Brucella* species genes, therefore, it was proposed that classical *Brucella* species should be declared biovars with *B. melitensis* as the sole species (Verger *et al*., 1987). This proposal did not gain acceptance and the classification of *Brucella* strains into the six classical species with the importance on preferred host is still preferred (Corbel & Binley-Morgan, 1984; Foster *et al*., 2009).

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Table 2.1: Recognised *Brucella* species, their biovars and possible hosts (Whatmore, 2009).

SPECIES	BIOVARS RECOGNISED	PREFFERED HOST
B. abortus	1-7 and 9	Cattle
B. melitensis	1-3	Sheep and goat
B. suis	1-5	Swine, rodents reindeer and hares
B. canis	-	Dogs
B. ovis	-	Sheep
B. neotomae	-	Rodents
B. ceti	-	Dolphins and porpoises
B. microti	-	Voles
B. inopinata	-	Unknown
B. pinnipedialis	-	Seals

2.2.2 AETIOLOGY

Brucella species are Gram-negative and intracellular pathogens that naturally do not multiply in the environment and are transmitted from host to host (Corbel & Binley-Morgan, 1984; Gorvel & Moreno, 2002). In 1905, it was demonstrated that the disease is zoonotic by isolating the organism from goat milk consumed by humans (Nicoletti, 2002). The organisms cause the disease brucellosis and most species in the genus like *B. melitensis, B. abortus, B. suis,* and *B. canis* are pathogenic to human, making *Brucella* an important zoonosis (Santos *et al*., 2005). Among the four, *B. melitensis* is the most virulent to humans whereas *B. canis* has a low zoonotic potential (Fugier *et al*., 2007).

Brucella canis and *B. ovis* occur in a rough form naturally whereas the other species including the marine species are considered smooth species even though rough mutants can occur (Dawson *et al*., 2008). Species that are naturally rough strains are less virulent as they have less or no O-polysaccharides and smooth strains with lipopolysaccharides (LPS) are more virulent (Corbel, 1990). LPS are major surface antigen and virulence factor encoded by the outer membrane of *Brucella* species (Glynn & Lynn, 2008; Zygmunt *et al*., 2009). They are composed of the O-antigen, O-side chain (lipid A) and core oligosaccharides. Lipid A is

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linked to the core oligosaccharide and it forms the most part of the outer coating of the *Brucella* outer membrane (Moreno *et al.*, 1990; Rojas *et al.*, 1994).

In recent years, new species have emerged following the unexpected isolation of *Brucella* from marine mammals in 1994 (Foster *et al*., 2007; Scholz *et al*., 2008a; 2008b; 2009; 2010). In 2007, a few strains were analysed leading to the discovery of *B. ceti* which is isolated from cetaceans and *B. pinnipedialis* isolated from pinnipeds even though its host specificity is not absolute (Foster *et al*., 2007; Dawson *et al*., 2008). The ninth species, *B. microti* was isolated in 2008 firstly from common voles and directly from the soil in the Czech Republic and later from red foxes in Austria (Scholz *et al*., 2008a; 2008b; 2009). Recently the tenth species, a novel *B. inopinata* species was isolated from a breast implant wound infection of a female patient displaying symptoms of brucellosis in Oregon, USA (De *et al*., 2008). The species was said to exhibit a unique profile and clustered with the other existing strains within the genus. *Brucella inopinata sp. nov* is different from all *Brucella* species as it displays outstanding phenotypic expression and metabolic efficiency (Scholz *et al*., 2010). Marine mammal *Brucella* species appears to have substantial variation in phenotypic characteristics within the species and they can also cause human brucellosis (Foster *et al*., 2007; Dawson *et al*., 2008).

2.2.3 EPIDEMIOLOGY

Brucellosis is endemic in most countries despite the fact that it is under-reported and it remains a worldwide zoonotic infection especially in people working in close proximity with infected animals and their products (Godfroid *et al.*, 2005). *Brucella* infections in humans occur accidentally through direct or indirect exposure to infected animals and their products (Young, 1995). Routes of infection include ingestion of infected dairy products, secretions from infected animals contaminating open cuts or abrasions and inhalation of infected dust or aerosols, which is common in laboratory personnel (Young, 1995; Fugier *et al*., 2007). Infection rate in humans depends on the disease occurrence in animals. Transmission of brucellosis from human to human through sexual contact and tissue transplants has been reported but is very rare (Mantur *et al*., 1996). Brucellosis in human is life threatening and have variable symptoms, which develop within 5-60 days of exposure, depending on the

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immunity system of the host (Young, 1995). Infected patients will develop an acute disease with flu-like symptoms such as fever, chills, headache, low back pain, joint pain, malaise, occasionally diarrhoea. Chronic brucellosis consists of complications like arthritis, spondylitis, endocarditis, kidney abscesses and psychological disturbances (Fugier *et al*., 2007).

Animals are also infected directly or indirectly depending on the species and hosts. The modes of infection involves contact with contaminated manure, young animals suckling infected milk and the licking of infected placenta after a female gave birth or aborted. Infection from these organisms can result in weight loss, reduced fertility (even in male animals), abortions and decreased milk production in female animals, indicating the economically significance of the disease (Whatmore, 2009). A large number of *Brucella* organisms are shed with vaginal discharges and foetal membranes (placentas) during parturitions and abortions, which primarily contaminate the environment (Coetzer & Tustin, 2004). Females usually abort once, develop immunity but remain infected, thus shedding the organisms in the subsequent pregnancies (Quinn *et al*., 1994). Vaginal secretions and semen are the main modes of transmission from animal to animal, especially in cattle, sheep, dogs and pigs (Alton *et al*., 1988; Whatmore, 2009). In cattle, some infected cows will not show any signs of brucellosis and also give birth to normal but infected calves. Infected bulls develop epididymitis and orchitis, which may lead to permanent or temporary infertility (Eaglesome & Garcia, 1992). When transmitted to a non-preferential host, the species will remain in the mammary glands and reticulo- endothelial tissue compared to in natural host where it locates the uterus and foetal membrane (Quinn *et al.,* 1994).

2.2.4 PATHOGENESIS

The pathogenicity of Brucella species depends on the forms they occur, the rough strains that are less virulent as they have less or no O-polysaccharides and smooth strains that are more virulent (Corbel, 1990). Unlike other bacteria, *Brucella* do not have classic virulence factors such as endotoxic LPS, plasmids, exotoxins and a capsule (Moreno and Moriyon, 2006), but invade their preferred hosts through skin cuts, the gastrointestinal tract, the respiratory tract, and the possible conjunctiva (Fugier *et al*., 2007; Xavier *et al.*, 2010). The

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effect of the disease on the host is usually determined by the species that has infected it. *Brucella* species can manifest themselves as acute, peracute or chronic infections in both animals and humans but the pathogen-host interaction appears to be different (Dornand, 2002). Their pathogenicity mainly depends on the successful entry and survival into the host cells where they multiply and infect the macrophages by inhibiting the fusion of phagosomes and lysosomes by acidifying the phagosomes (Gorvel & Moreno, 2002; Christopher *et al*., 2010).

Natural and experimental hosts have shown that *Brucella* species can survive in phagocytic cells such as trophoblasts, macrophages and dendritic cells (Ficht, 2003; Xavier *et al*., 2009), as well as the male and female reproductive organs (Adams, 2002). Most investigations regarding the pathogenicity of *Brucella* were done in the phagocytes, indicating a gap in understanding this disease and its behaviour in the host (Xavier *et al.*, 2010). An influential virulence mechanism of *Brucella* species is that they have non-classical LPS, which help the immune system in the defence of the infected cell by providing protection against foreign antigens (Moreno & Moriyon, 2006).

The development of molecular techniques used currently to characterise the genome of *Brucella* species has allowed the positive improvement in identifying the pathogen's taxonomy and virulence factors (Foster *et al.*, 2009). The recent availability of *B. abortus, B. suis* and *B. melitensis* whole genome sequences (Paulsen *et al*., 2002; DelVecchio *et al*., 2002; Halling *et al*., 2005) have also presented exceptional information in the understanding of the pathogenicity and diagnosis of brucellosis (Christopher *et al.*, 2010).

2.2.5 PREVENTION

Prevention of brucellosis requires the knowledge of the prevalence of the disease as vaccination alone is not enough. Like other diseases that affect animals and humans, veterinary services are critical in detecting, preventing and controlling brucellosis as it is often misdiagnosed especially in humans (Glynn & Lynn, 2008). The development of *Brucella* vaccines improved the economic consequences of the disease and since infected animals and their products are the source of human brucellosis, their treatment and control

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combined with proper heat treatment of contaminated food products will also eradicate human brucellosis (Corbel, 1997). It has been suggested in recent studies that to develop a control practice for any disease; optimum consideration should be given to the community safety, thus involving social workers and anthropologists (Marcotty *et al.,* 2009) especially where people consume raw milk products (Godfroid *et al*., 2011).

At present, the World Health Organization (WHO) recommends three vaccines for animal health in the control of livestock brucellosis. Vaccine strains include *B. abortus* strains 19 (S19), mostly used to prevent bovine brucellosis in heifers between 4-8 months (Nicoletti, 1990); *B. melitensis* Rev1 used against *B. melitensis* in goats and sheep as well as against B. *ovis* in rams (Alton, 1985) and; *B. abortus* strain RB51, which is an attenuated rough strain effective against *B. abortus* in adult cows (Lord *et al*., 1998; WHO, 1997). The use of standard full doses of *B. melitensis* Rev1 is unsafe in pregnant sheep and goats as it causes abortions. Therefore a lower dose is recommended which will also decrease the level of immunity in pregnant animals (Alton, 1970). Vaccinations with *B. abortus* S19 improve resistance to brucellosis and the use of this strain in bulls is not advisable as it can cause orchitis (Trichard *et al*., 1982). Their main disadvantage is that they are live strains and cannot be used in human (Nielsen & Ewalt, 2008). Lack of human vaccine for brucellosis led to the organism being investigated as bioterrorism agents and the discovery that the USA military has contemplated *B. suis* as a biological weapon in 1954 (Greenfield *et al*., 2002).

2.2.6 DIAGNOSIS AND MOLECULAR TECHNIQUES

Testing of products before and after importing is very important to minimise the possibilities of re-emerging (England *et al.,* 2004). Characterization of *Brucella* is based on the isolation of *Brucella* bacteria as a gold standard. However field diagnoses is made using serological techniques like the rose Bengal test (RBT), complement fixation test (CFT), serum agglutination test (SAT) and enzyme-linked immunosorbent assay (ELISAs) (indirect and competitive ELISA). The main disadvantage of serological tests is they are only genus specific, thus, further identification with molecular of cultural methods that are species specific is required. In recent years, a number of studies to discriminate *Brucella* species were conducted and different PCR methods developed.

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Differentiation of *Brucella* species is very challenging because of the high degree of genetic homology of *Brucella* (Verger *et al*., 1985). Species-specific AMOS-PCR, discriminates between strains of *B. abortus* bv. (biovar) 1, 2 and 4, *B. melitensis* (bv. 1, 2 and 3), *B. ovis* and *B. suis* bv. 1 (Bricker & Halling, 1994). The technique was later improved by the addition of vaccine strain specific primers of *B. abortus* S19 and RB51 as well as *B. melitensis* rev1 (Bricker & Halling, 1995). Ocampo-Sosa *et al*. (2005) further improved the PCR assay to distinguish between *B. abortus* bv. 3, 5, 6 and 9. Ferrao- Beck *et al*. (2006) included biovar specific primers to discriminate between *B. suis* bv. 1, 2 and 3. Garcia-Yoldi *et al.* (2006) developed a multiplex PCR assay called Bruce-ladder that identifies and differentiate most *Brucella* species and vaccine strains (*B. abortus* RB51, *B. abortus* S19 and *B. melitensis* Rev1). The only setback of this assay was that it erroneously identifies some *B. canis* isolates as *B. suis*. Lopez-Goni *et al.* (2008) enhanced Bruce-ladder to differentiate all *Brucella* species, including *B. canis* and *B. suis.*

A technique for typing *Brucella* strains using VNTRs at eight loci in the genome was developed and named hypervariable octameric oligonucleotide finger-prints (HOOF-Prints) (Bricker *et al*., 2003). HOOF printing assay was able to distinguish between all 'classical' *Brucella* species (*B. abortus, B. melitensis, B. canis, B. suis* and B. *ovis*) and field isolates to species level, but not the biovars within species (Bricker *et al*., 2003; Bricker & Ewalt, 2005). This method could not predict the biovar-specific or species-specific alleles and therefore led to the development of MLVA, an assay based on repeated DNA sequences found in all organisms (Le Fleche *et al*., 2006). The 15 VNTR loci MLVA assay is an amalgamation of panel 1 consisting of 8 moderate variable minisatellite loci and panel 2 consisting of 7 highly variable microsatellite loci (Le Fleche *et al*., 2006). Al Dahouk *et al.* (2007) added another locus to panel 2 introducing MLVA-16. Currently accurate characterization of *Brucella* species is still achieved by a combination of a number of assays, as there is no one assay that can definitely diagnose and differentiate brucellosis on its own (Poester *et al.*, 2010). The multiplex PCR assays, AMOS and Bruce-ladder only differentiate species, and MLVA can be used for intra-species discrimination.

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In the past, sequencing multiple samples using capillary electrophoresis took weeks or months to complete compared to next generation sequencing (NSG) techniques which has the ability to sequence large number of samples within two days regardless of genome size (Lo & Chiu, 2009). NSG platforms like 454 Life Science Roche, Illumina Inc. Genome Analyzer, etc. are presently used and they seem to provide hope in molecular epidemiology of pathogens. The technique is comprised of different approaches including preparation of template, sequencing and imaging, genome alignment and assembly methods. The primary advantage of this technique over conventional sequencing is that it has the ability to produce a large number of data per run at low costs. It also facilitates whole genome sequencing of closely related organisms, which were difficult in the past, therefore challenging the landscape of life science and solving previously unanswered problems/questions (Schuster, 2008).

The development of molecular techniques used currently to characterise the genome of *Brucella* species has allowed the positive improvement in identifying the pathogen's taxonomy and virulence factors (Foster *et al.*, 2009). Genome sequences of *B. suis* reference strain 1330, *B. Melitensis* bv. 1 type strain 16M and *B. abortus* field strain 9-941, were completed and proved once again that *Brucella* species are genetically homologous (DelVecchio *et al*., 2002; Paulsen *et al*., 2002; Halling *et al*., 2005). These genomes together with those that were sequenced afterwards resulted in improved understanding of the disease patterns. The above author also indicated that *Brucella* species lack plasmids and phages and this was proven by failure to purify plasmid DNA as well as the exchangeable resistance to antibiotics (Banai & Corbel, 2010). *Brucella* species have two chromosomes with chromosome 1 larger than chromosome 2 (Jumas-Bilak *et al.*, 1998). Del Vecchio *et al*. (2002) reported that chromosome 1 encodes for the processes that include protein synthesis, transcription and translation whereas chromosome 2 encode for genes responsible for membrane transport, energy metabolism and regulation. Comparison of genomes has revealed enough promising variants that can be used in distinguishing between species (Paulsen *et al*., 2002; Foster *et al.*, 2009). Thus, due to the homologous nature of *Brucella* species, discovery of these available variants will make it possible to differentiate them with available assays other than genome sequencing (Foster *et al*., 2012).

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Chapter 3

MOLECULAR CHARACTERIZATION OF *BRUCELLA* STRAIN FROM ZIMBABWE USING BRUCE-LADDER, AMOS-PCR AND WHOLE GENOME SEQUENCING

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ABSTRACT

Brucella species are genetically homogenous and mainly classified on the basis of pathogenicity, host preference and phenotypic / genotypic characteristics. Due to this reason various assays were developed in an attempt to differentiate them. In this study we used multiplex PCR assays namely, Bruce-ladder and AMOS, to characterize and distinguish 16 *Brucella* species strains collected from different regions and hosts in Zimbabwe. AMOS and Bruce-ladder PCR assays identified 8 *B. suis, 5 B. abortus* and 2 *B. canis* strains from Zimbabwe. Of the *Brucella* species identified, *B. canis* and *B. suis* have not yet been formally reported in Zimbabwe. Most of the Zimbabwean *Brucella* strains identified with AMOS-PCR, Bruce-ladder as well as Suis-ladder as *B. suis* were isolated from bovine. Whole genome sequencing was used to characterize two *B. suis* (ZW043 and ZW046) and a *B. abortus* (ZW053) strains in order to verify their true identity. Genome comparative analysis showed consistent results with the multiplex assays, thus proving that despite ZW043 and ZW046 strain being isolated from bovine, their identity are *B. suis*. Whole genome sequencing verified that Bruce-ladder, AMOS and Suis-ladder multiplex-PCR assays, enable accurate identification of *Brucella* species which is of great importance since some of the African

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laboratories lack adequate resources, expertise and infrastructure to do biotyping of *Brucella* species and biovars under proper biosafety and biosecurity conditions.

Keywords: *Brucella spp*, Brucellosis, AMOS PCR, Bruce-ladder, Next Generation Sequencing.

3.1 INTRODUCTION

Brucella species are small Gram-negative coccobacilli and causative agents of the widespread disease brucellosis. At present eight terrestrial species*: Brucella abortus, B. melitensis, B. suis, B. canis, B. ovis, B.neotomae, B. inopinata* and *B. microti* as well as two marine strains: *B. ceti* and *B. pinnipedialis* are recognized (Corbel, 1997; Foster *et al.*, 2007; OIE, 2008). Species are classified on the basis of phenotypic and genotypic properties, host preference and pathogenicity (Alton *et al*., 1988). Despite the fact that brucellosis is under-reported and mis-diagnosed (Godfroid *et al.*, 2005), it remains an economically important and endemic zoonotic infection in sub-Saharan African countries (McDermott & Arimi, 2002). It has a negative impact on global economy and public health as it causes substantial losses of livestock and affects the livelihood of communities relying on their livestock for survival. Among the *Brucella* species (*B. abortus, B. melitensis, B. suis* and *B. canis*) known to affect humans, *B. melitensis* is the most virulent. *Brucella* infections in human occur accidentally through direct or indirect exposure, especially those working in close proximity with infected animals and their products (Young, 1995), thus, infection rate in humans depends on the disease occurrence in animals.

Thus far only *B. abortus* (mainly *B. abortus* biovar (bv.) 1 and to a lesser extent *B. abortus* bv. 2) and *B. melitensis* have been reported in Zimbabwe to cause brucellosis in animals (Matope *et al*., 2009; Madsen, 1989). In wildlife, *B. abortus* bv. 1 was isolated from waterbuck and eland in Zimbabwe (Condy & Vickers, 1972). Until 1987, *B. melitensis* was believed to be absent from Zimbabwe when it was diagnosed (Madsen, 1989) and recently an isolate from a goat was identified as *B. melitensis* bv. 1 (Matope *et al.,* 2009). Corbel (1997) reported *B. abortus* in bovine, *B. melitensis* in goats and *B. ovis* in ovine from official brucellosis reports in animals in 1994 in Zimbabwe. Thus far there has been no official report of *B. suis* or *B. canis* in Zimbabwe (Corbel, 1997). Brucellosis in wildlife and livestock

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has been demonstrated in Zimbabwe mainly by serology (Swanepoel *et al.,* 1976; Bryant & Norval, 1985; Madsen, 1989; Condy & Vickers, 1972; Madsen & Anderson, 1995). Serology demonstrated the prevalence of bovine brucellosis of livestock farming in Zimbabwe that is sub-divided into commercial and smallholder groups with the latter making 60-80% of the cattle population in the country (Matope *et al.*, 2011). The prevalence of brucellosis in Zimbabwe consisting of higher prevalence in commercial farms compared to low or lack of prevalence in communal farms (Swanepoel *et al.,* 1976; Bryant & Norval, 1985; Madsen, 1989) changed after the introduction of the land reform programme in the year 2000, leading to a rise in the movement of animals from commercial to smallholder farmers (Matope *et al.*, 2010). This resulted in brucellosis being isolated in areas previously known to be free of the disease.

Brucella species are genetically homogenous and various assays were developed in an attempt to differentiate them. Isolation and identification of *Brucella* species are regarded as the gold standard (OIE, 2004). Molecular techniques currently used to characterise the genome of *Brucella* species have allowed the positive improvement in identifying the pathogen's virulence factors (Christopher *et al.*, 2010). The recent availability of *B. suis*, *B. melitensis* and *B. abortus* whole genome sequences (Paulsen *et al*., 2002; DelVecchio *et al*., 2002; Halling *et al*., 2005) have also presented exceptional information in the understanding of the pathogenicity and diagnosis of brucellosis (Christopher *et al.*, 2010).

AMOS-PCR, the first species-specific assay which could differentiate between *B. abortus, B. melitensis, B. ovis* and *B. suis* was developed by Bricker & Halling (1994) and further improved to identify *B. abortus* vaccines strains S19 and RB51 (Bricker & Halling, 1995). AMOS-PCR still fails to characterize all *Brucella* isolates and biovars, but is useful as it provides a basis for other assays like the Bruce-ladder multiplex PCR assay (Garcia-Yoldi *et al.,* 2006). Bruce-ladder was developed by Garcia-Yoldi *et al*. (2006) and can identify all of the *Brucella* species and vaccine strains; *B. abortus* S19, RB51 and *B. melitensis* Rev1. Bruce-ladder misidentifies some *B. canis* strains as *B. suis* (Lopez-Goni *et al.,* 2008). The Suis-ladder was designed to differentiate biovars of *B. suis* and *B. canis* (Lopez-Goni *et al.,* 2011). Therefore, the main focus of this project was to use multiplex PCR assays (AMOS- PCR &

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Bruce ladder- PCR) to identify *Brucella* species field isolates from Zimbabwe. Furthermore, selected strains were sequenced using next generation sequencing (NGS).

3.2 MATERIALS AND METHOD

3.2.1 SAMPLE HISTORY

Samples used in this study were collected from 1990–2009 from different hosts and regions throughout Zimbabwe. DNA was extracted by Dr. Calvin Gomo at the Central Veterinary Laboratory (CVL), Harare, Zimbabwe using Qiagen Extraction Kit and sent to the department of Veterinary Tropical Diseases, University of Pretoria, South Africa for characterization using AMOS and Bruce-ladder PCR assays. All strains used in the study are listed in Table 3.1.

3.2.2 PCR ASSAYS

Sixteen Zimbabwean field, 3 *Brucella* vaccine and 4 *Brucella* reference strains (Table 3.1) were subjected to AMOS and Bruce-ladder PCR assays. AMOS-PCR was done as described previously by Bricker & Halling (1994; 1995). The PCR reaction (25µl) composed of 0.2mM of each of the four *Brucella* species-specific primers, 1mM of IS711 reverse primer (Table 3.2), 1X MyTaq mix (Bioline), and 2 µl template DNA. PCR conditions on ABI 2720 Thermal Cycler (Applied Biosystems®)consisted of initial denaturation of 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, annealing 60°C for 2 min and extension at 72°C for 2 min and a final extension cycle of 72°C for 5 min.

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TABLE 3.1*: Brucella* strains used in the study.

Strains**	***Brucella* species**	**Origin in Zimbabwe**	**Source**
ZW011	*Brucella suis**	Shamva	Pig
ZW040	*Brucella* species (spp)	Bindura	Testis
ZW043	*Brucella* spp	Chiredzi	Cow
ZW045	*Brucella* spp	Bindura	Bull testicle
ZW046	*Brucella* spp	Norton	Bovine
ZW047	*Brucella* spp	Unknown	Unknown
ZW048	*Brucella* spp	Unknown	Unknown
ZW052	*Brucella* spp	Handerson research	Milk sample
ZW053	*Brucella* spp	Matabeland	Cow
ZW100	*B. canis**	Harare (Highlands)	Dog
ZW201	*B. suis**	Norton	Swine
ZW248	*Brucella* spp	Mazowe	Cow
ZW283	*B. abortus**	Gwanda	Cow
ZW323	*B. abortus**	Chiredzi	Cow
ZW377	*B. canis**	Harare	Dog
ZW368	*Brucella* spp	Matabeland	Cattle
Strain 19	*B. abortus* S19***		
RB51	*B. abortus* RB51***		
Rev1	*B. melitensis* rev1***		
REF16M (BCCN R1)	*B. melitensis*bv.1		Goat
REF6/66 (BCCN R18)	*B. canis*		Dog
REF1330 (BCCN R12)	*B. suis*bv.1		Swine
REF544 (BCCN R4)	*B. abortus* bv. 1		Cattle

*Identified using growth characteristics and biochemical profiles by Central Veterinary Laboratory, Harare, Zimbabwe, excluding the phage lysis which was confirmed by PCR assays in this study

** ZW indicates strains isolated from Zimbabwe and REF indicates reference DNA obtained from BCCN: *Brucella* culture collection Nouzilly, France.

*** Vaccine strains cultured and extracted at the Department of Veterinary Tropical Diseases at the University of Pretoria, South Africa.

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Bruce-ladder PCR was done as described by Garcia-Yoldi *et al.* (2006) in 25 µl PCR reactions using 0.4mM of each primer of the 8 primer pairs multiplex (Table 3.2). PCR conditions consisted of initial denaturation at 95°C for 3 min, followed by 25 cycles at 95°C for 30 sec, 64°C for 45 sec and 72°C for 3 min and a final extension of 72°C for 5 min on an ABI 2720 Thermal Cycler ((Applied Biosystems®). Suis-ladder was done as described by Lopez-Goni *et al*. (2011) in a 25 µl reaction mix using 12.5 pmol of each primer of the 4 primer pairs multiplex (Table 3.2). PCR conditions consisted of an initial denaturation of 95°C for 5 min, followed by 35 cycles of 95°C for 35 sec, 64°C for 45 sec and 72°C for 3 min and a final extension of 72°C for 5 min on an ABI 2720 Thermal Cycler (ABI). PCR products (5 µl) were separated using electrophoresis on a 2% agarose gel stained with ethidium bromide. Gel images were documented and annotated on Bio-Rad ChemiDoc™ XRS (Bio-Rad) using Imager Image Lab™ Software (version 3).

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Table 3.2: Bruce-ladder, Suis-ladder and AMOS-PCR primer information.

Primers	**Sequence (5'-3')**	**Amplicon size in base pair(bp)**
	Bruce-ladder	
BMEI0998f	ATC CTA TTG CCC CGA TAA GG	1682
BMEI0997r	GCT TCG CAT TTT CAC TGT AGC	
BMEI0535f	GCG CAT TCT TCG GTT ATG AA	450, (1320)[#]
BMEI0536r	CGC AGG CGA AAA CAG CTA TAA	
BMEII0843f	TTT ACA CAG GCA ATC CAG CA	1071
BMEII0844r	GCG TCC AGT TGT TGT TGA TG	
BMEI1436f	ACG CAG ACG ACC TTC GGT AT	794
BMEI1435r	TTT ATC CAT CGC CCT GTC AC	
BMEII0428f	GCC GCT ATT ATG TGG ACT GG	587
BMEII0428r	AAT GAC TTC ACG GTC GTT CG	
BR0953f	GGA ACA CTA CGC CAC CTT GT	272
BR0953r	GAT GGA GCA AAC GCT GAA G	
BMEI0752f	CAG GCA AAC CCT CAG AAG C	218
BMEI0752r	GAT GTG GTA ACG CAC ACC AA	
BMEII0987f	CGC AGA CAG TGA CCA TCA AA	152
BMEII0987r	GTA TTC AGC CCC CGT TAC CT	
	Suis-ladder	
BMEI1426	TCG TCG GTG GAC TGG ATG AC	774
BMEI1427	ATG GTC CGC AAG GTG CTT TT	
BR1080f	CCC TTG GTT TGT AGC GGT TG	197
BR1080r	TCA TCG TCC TCC GTC ATC CT	
BMEI1688	TCA TCG TCC TCC GTC ATC CT	278
BMEI1687	GCG GGC TCT ATC TCA AGG TC	
BMEI0205f	CGT CAA CTC GCT GGC CAA GAG	299, 425, 551, 614
BMEI0205r	GCA GGA GAA CCG CAA CCT AA	

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AMOS-PCR		
B. abortus-specific primer	GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC	495
B. melitensis-specific primer	AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA	731
B. ovis-specific primer	CGG-GTT-CTG-GCA-CCA-TCG-TCG	976
B. suis-specific primer	GCG-CGG-TTT-TCT-GAA-GGT-TCA-GG	285
*IS711 reverse primer	TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT	

[#] *Brucella* species isolates from marine mammals produce 1320 bp PCR product.

*IS711 reverse primer used with specific primers to obtain specific PCR size product.

3.2.3 WHOLE GENOME SEQUENCING AND ANALYSIS

In this study, three isolates, ZW043, ZW046 and ZW053 were selected on the basis of their origin and the results of the characterisation performed using AMOS and Bruce-ladder PCR. These were thus sequenced using NGS technology to understand and verify their identities. The samples were sent to Inqaba Biotec™ for sequencing using the Illumina Miseq® (Illumina®) paired-end sequencing technology. The Nextera DNA Sample Preparation kit (Illumina®) was used for the sequencing library preparation and DNA fragments in the 500 - 1000 bp range were selected. The quality of the data was checked with FastQC. Trimming with fastx_trimmer tool in fastx-toolkit (FASTX Toolkit http://hannonlab.cshl.edu/fastx_toolkit/index.html by Hannon Lab). Merging of overlapping sequence reads using the fast length adjustment of short reads to improve genome assemblies (FLASH) software (Magoc & Salzberg, 2011).

De novo sequence assembly was performed using Abyss-pe version 1.3.6 (Simpson *et al.*, 2009) with kmer length set at 64. Assembled contigs were then ordered against the respective chromosomes of the respective references (NC_006932; NC_006933 (Halling *et al.*, 2005) and NC_017251; NC_017250 (Paulsen *et al.*, 2002)) into pseudo-molecules using

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Abacas (Assefa *et al*., 2009). The ordered chromosomes were annotated using RAST (Aziz *et al.*, 2008) and aligned against reference genome respective chromosomes using Mauve 2.3.1 (Darling *et al.*, 2004). CLC Genomic Workbench version 5.5 (CLC Bio, Qiagen) was used to map the reads to references as well as performing the variants detections and comparisons.

3.3 RESULTS

Brucella abortus, *B. canis* and *B. suis* were identified using Bruceladder and AMOS- PCR assays (Figure 3.1 A and B). Bruce-ladder PCR products of 1682, 794, 587, 450 and 152 bp sizes amplified from Zimbabwean isolates ZW52, ZW53, ZW248, ZW283 and ZW323 are characteristic of *B. abortus* isolates (Figure 3.1 A). Zimbabwean isolates ZW011, ZW201, ZW040, ZW043, ZW045-048 produced 1682, 1071, 587, 450, 272 and 152 bp fragment sizes typical to *B. suis* using Bruce-ladder. Isolates ZW100 and ZW377 produced band sizes of 1682, 1071, 587, 450 and 152 bp that identified isolates as *B. canis* with Bruce-ladder multiplex PCR assay (Figure 3.1 A). Vaccine strains RB51 (lane 22), S19 (lane 23) and Rev1 (lane 15) amplified additional fragments of 2524, 587 and 218 bp as compared to other *B. abortus* and *B. melitensis* respectively.

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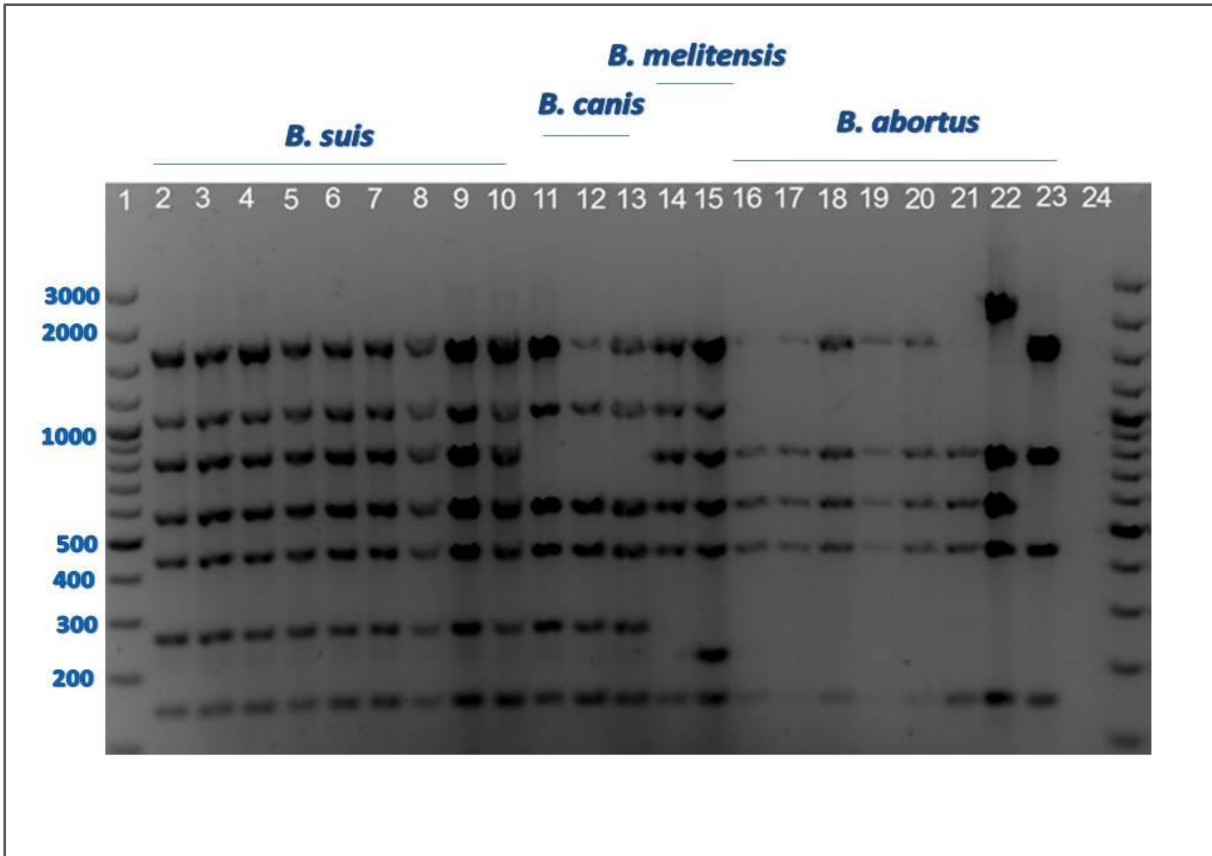
A



B

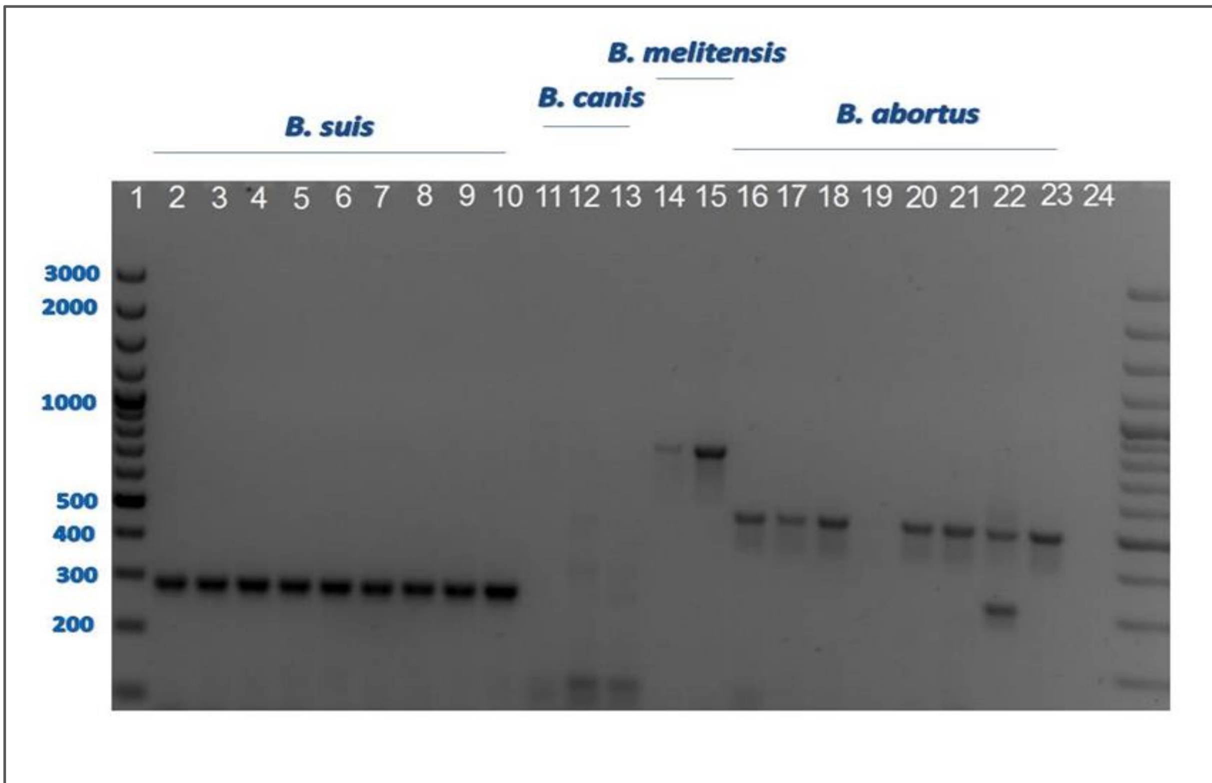


Figure 3.1: Multiplex PCR assays (A) Bruce-ladder and (B) AMOS-PCR of *Brucella* DNA from Zimbabwe, *Brucella* vaccine and reference strains. Lane 1: Fermentas 100 bp plus molecular marker,

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Lane 2-10: *B. suis* bv. 1 (REF1330, BCCN R12), ZW011, ZW040, ZW043, ZW045, ZW046, ZW047, ZW048, ZW201; lane 11-13: *B. canis* (REF6/66, BCCN R17), ZW100, ZW377; lane 14-15: *B. melitensis* bv. 1(REF16M, BCCN R1), *B. melitensis* rev 1; lane 16-23: *B. abortus* bv. 1 (REF544, BCCN R4), ZW053, ZW086, ZW248, ZW283, ZW323, *B. abortus* RB51, *B. abortus* S19; lane 24 negative (water) control.

With the AMOS-PCR, the *B. canis* isolates (ZW100 and ZW377) produced no band, while ZW201, ZW011, ZW040, ZW043, ZW045-048 produced a 285 bp fragment significant to *B. suis* whereas *B. abortus* isolates (ZW052, ZW053, ZW248, ZW283 and ZW323) produced a 498 bp fragment (Figure 3.1 B). The Suis-ladder multiplex PCR assay confirmed ZW100 and 377 to be *B. canis* since it produced amplicons of 614 and 197 bp, whereas ZW011, ZW201, ZW40, ZW043, ZW045-048 produces amplicons of 774, 425 and 192 bp typical of *B. suis* bv. 1 (Figure 3.2). Vaccine strain RB51 amplified additional 218 bp fragment size.

Figure 3.2: Suis-ladder multiplex PCR assay with *Brucella* DNA from Zimbabwe and reference strains. Lane 1: Fermentas 100 bp plus molecular marker, lane2: *B. suis* bv. 1 (REF 1330), lanes 3- 10: *B. suis* field isolates (ZW011, ZW040, ZW043, ZW045, ZW046, ZW047, ZW048, ZW201), lane 11: *B. canis* (REF6/66), lanes 12- 13: *B. canis* field isolates (ZW100, ZW377), lane 14: negative control.

The genomes of ZW043, ZW053 and ZW046 genome assemblies were in the order 3.4, 3.3 and 3.4 Mb respectively. Blastn indicated that ZW043 and 046 is 99 % similar to *B. suis* bv. 1

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strain 1330 (Paulsen *et al*., 2002) and ZW053, 99% similar to *B. abortus* strain 9-941 (Halling *et al*., 2005), thus, these genomes were used as references to perform comparative analysis of the annotated genomes. When performing *de novo* assembly, ZW043, ZW046 and ZW053 generated 89, 87 and 73 contigs respectively (Table 3.3). Just like the previously sequenced *Brucella* genomes and the reference genomes that were used, ZW043, ZW046 and ZW053 are also composed of two circular chromosomes with the average sequence lengths of 2.1 (chromosome 1) and 1.2 (chromosome 2) Mb. Comparative genome analysis showed that the ZW043 and ZW046 were most similar to *B. suis* bv. 1 strain 1330 whereas ZW053 were most similar to *B. abortus* bv. 1 strain 9-941 with an average GC content of 57 and approximately similar predicted coding sequences ranging from 3296 – 3414 (Table 3.4).

Table 3.3: Summary of the *de novo* assembly statistics of whole genome *Brucella* sequences using next generation sequencing.

Genome ID	**NO. of contigs**	**N50**	**Av. Contig length**	**Total contig bases**	**Total genome reads**
ZW043	89	11683	4026	358325	3304109
ZW046	87	12114	4558	396510	3302407
ZW053	73	11759	4904	358046	3445754

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Table 3.4: Features of *Brucella* strains ZW043, ZW046, ZW053 and the complete *Brucella* genomes of *B. suis* 1330 (Paulsen *et al*., 2002) and *B. abortus* bv1. strain 9-941(Halling *et al*., 2005).

Genomic characteristics	ZW043	ZW046	ZW053	*B.suis* strain 1330	*B. abortus* bv.1 strain 9-941
Genome size	3.31	3.31	3.44	3.31	3.28
No. of chromosomes	2	2	2	2	2
G:C content	57.2	57.2	57	57.2	57.2
Size of chromosome 1	2106309	2107702	2188994	2107792	2124242
Size of chromosome 2	1206435	1207332	1255283	1207381	1162780
Predicted genome sequences	3370	3414	3383	3388	3296
No. of CDS in chromosome 1	2219	2165	2186	2185	2158
No. of CDS in chromosome 2	1151	1249	1197	1203	1138

The genome of *B. abortus* ZW053 showed some insertions and deletions (Appendix Figure 1(chromosome 1) and Figure 3.3 (chromosome 2)) in both chromosomes. Chromosome 1 has a huge insertion encoding 104 of 136 unique hypothetical proteins in chromosome 1 ranging from 1654596 – 1852541 (Appendix Figure. 1). Chromosome 2 of ZW053 shows the inversions of 2415 bp (805 amino acids) and 975 bp (325 amino acids) DNA fragments from 1018885 – 1016471 and 974889 – 973915 regions respectively.

When comparing ZW046 to ZW043 and *B. suis* 1330, it showed the presence of a unique large subunit ribosomal RNA (IsU RNA & LSU rRNA) (1611816 - 1614549) and a small subunit ribosomal RNA (ssU RNA, SSU rRNA),which are not found in ZW043 but present and identified as hypothetical proteins in *B.suis* 1330 and *B. suis* VB122. Chromosome 1 of ZW043, features an inversion sequence (Fig/ 6666666.51002.peg.106) encoding the alkaline phosphatase (EC 3.1.3.1) that is also found in *B. abortus* bv. 1 strain 9-941 and *B. melitensis* bv. 1 strain 16M but absent in *B. suis* bv. 1 strain 1330 (Appendix Figure 4).

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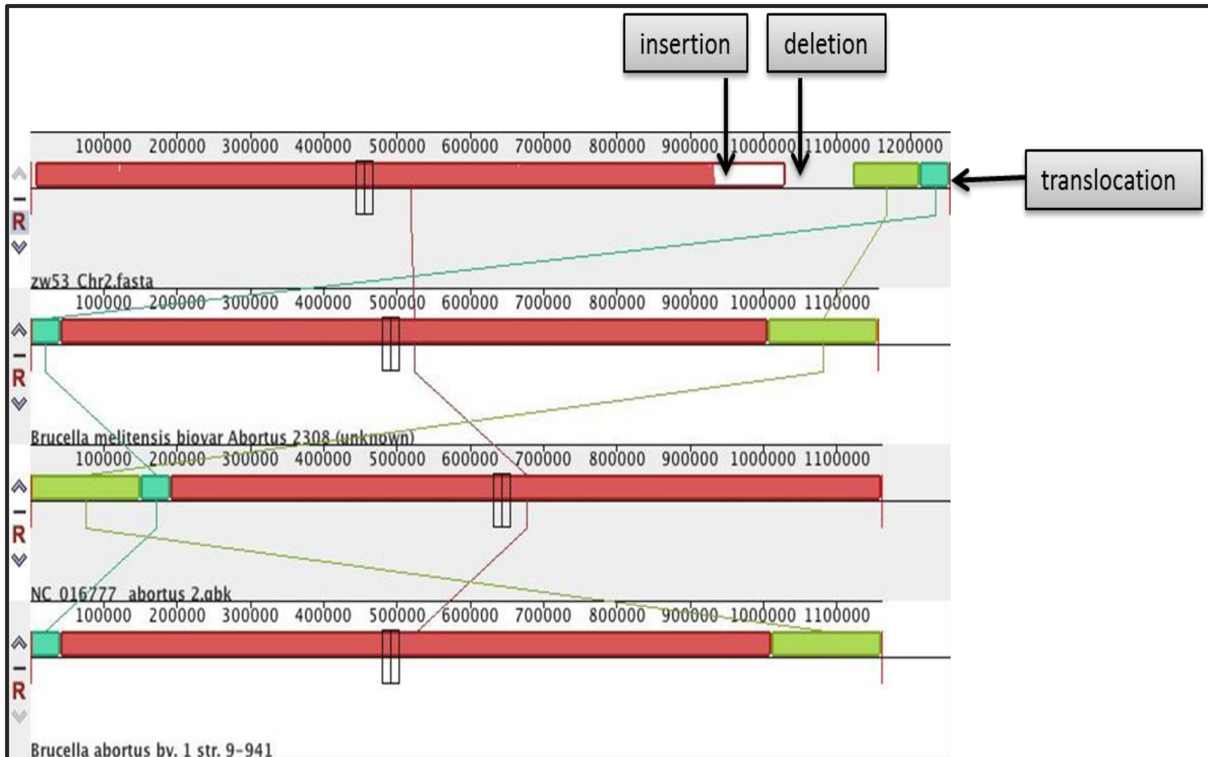

Figure 3.3: Mauve alignment image of the chromosome 2 of ZW053, *Brucella abortus* bv. 1 strain 9-941, *B. abortus* 2083 (indicated as *B. melitensis* biovar *abortus*) *and B. abortus* A13334 showing an insertion, deletion and translocation at the focus sequence ZW053.

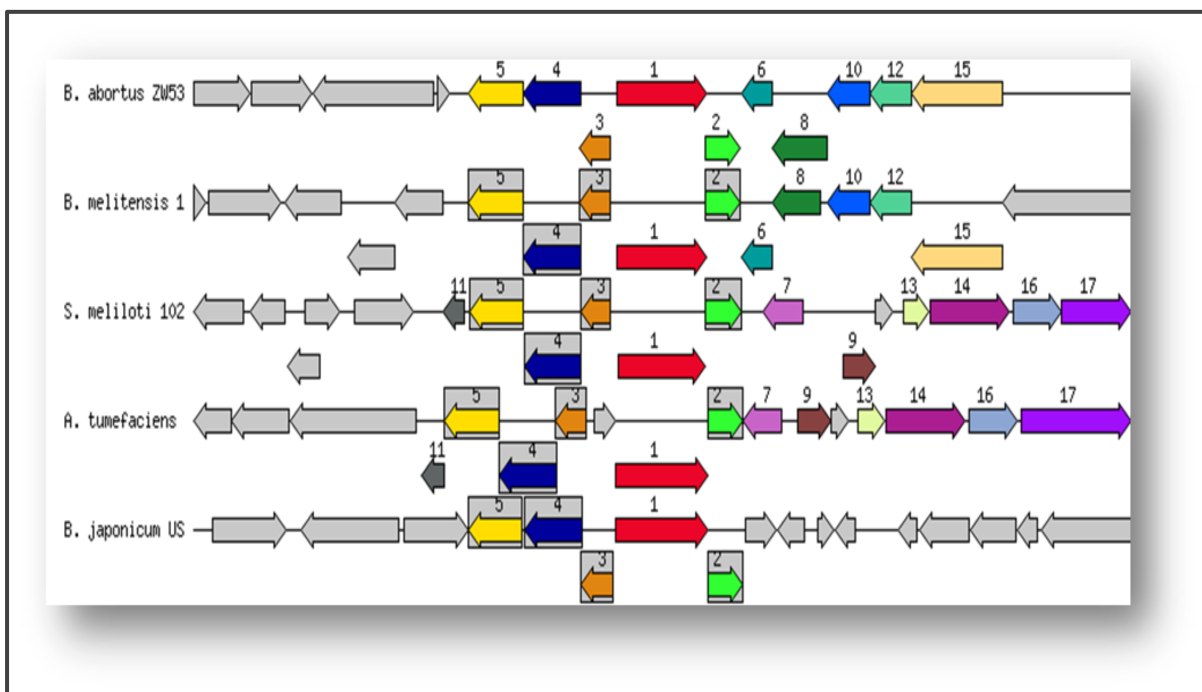

Figure 3.4: A comparison illustration from RAST Server showing a ZW053 gene, [fig/6666666.51018.peg.1947] (in red) encoding 3-polyprenyl-4-hydroxybenzoate carbolase-lyase present as an inverted sequence in *Brucella melitensis* bv. 1 strain 16M.

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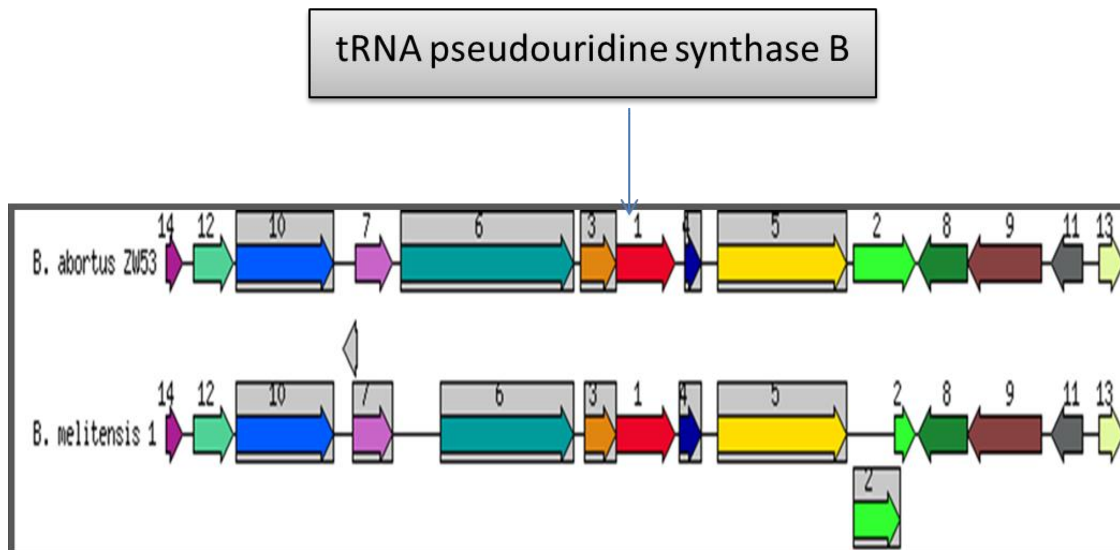

Figure 3.5: A comparison illustration from RAST Server showing a ZW053 chromosome 2 gene, [fig/6666666.51019.peg.165] (in red) encoding tRNA pseudouridine synthase B (EC 4.2.1 70).

3.4 DISCUSSIONS

Eradication of brucellosis requires fast, accurate and species-specific techniques for the diagnoses of the highly homologous genus *Brucella.* At present characterization of *Brucella* species is still achieved with a combination of a number of assays as there is no assay that accurately diagnoses and differentiates them on its own (Poester *et al.*, 2010). PCR is an important technique that supplements serological tests (Bricker & Halling, 1994). In our study, multiplex PCR assays (AMOS and Bruce-ladder) yielded corresponding results except for *B. canis* as AMOS-PCR cannot identify *B. canis* strains. Bruce-ladder is regarded as a simple, robust and specific assay to identify *Brucella* (OIE, 2008) as it allows the characterization of all *Brucella* species including vaccine and marine strains in a single run (Whatmore, 2009; Lopez-Goni *et al*., 2008; 2011; Weiner *et al*., 2011).

Out of *Brucella* isolates used in the study, *B. abortus, B.suis* and *B. canis* were characterised in this study. This is the first detailed characterisation of *B. suis* and *B. canis* from Zimbabwe since Previous studies has shown that only *B. melitensis, B. ovis* and *B. abortus* have been reported in Zimbabwe with the latter being the major cause of bovine brucellosis (Matope *et al.*, 2009). The *Brucella* reference strains (REF16M (BCCN R1), REF6/66 (BCCN R18), REF1330 (BCCN R12) and REF544 (BCCN R4)) used in the study produced PCR products as

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formerly described by Bricker & Halling (1994; 1995) and Garcia-Yoldi *et al.* (2006) for the AMOS-PCR and Bruce-ladder PCR assays, respectively (Figure 3.1 and Figure 3.2). With Bruceladder, *B. suis* isolates displayed seven amplicons (1682, 1071, 794, 587, 450, 272 & 152) as expected and described in the original Bruce-ladder multiplex PCR (Garcia-Yoldi *et al,* 2006). *Brucella canis* isolates were recognised by the absence of 794-bp fragment, *B. abortus* isolates by the absence of 1071-bp fragment and *B. melitensis* vaccine strains Rev1 identified by amplifying an additional 218-bp fragment as compared to *B. melitensis* reference strain 16M, which is distinguished by the absence of 272-bp fragment. *Brucella abortus* vaccine strains RB51 and S19 were distinguished by the absence of 1682-bp, amplifying the additional 2524-bp fragment instead and the absence of 587-bp fragment respectively (Garcia-Yoldi *et al,* 2006).

AMOS-PCR was used previously by Matope *et al.* (2009) to confirm the identity of *Brucella* isolates from Zimbabwe. In this study, when using AMOS PCR, *Brucella suis* isolates amplified 285 bp whereas 16M and Rev1 vaccine strain amplified 731 bp fragment sizes. *Brucella abortus* isolated displayed 498 bp amplicon with RB51 vaccine strain amplifying an additional 364 bp fragment as compared to other *B. abortus* isolates (Bricker & Halling, 1994). As expected, *B. canis* isolates did not amplify because AMOS PCR assay is limited and cannot identify them. Amongst field isolates used in this study, some (ZW043, ZW045 and ZW046) were from bovine and bovine products, but characterized and identified as *B. suis,* thus, due to this reason and the fact that AMOS cannot identify *B. canis* and Bruce-ladder cannot reliably resolve between B*. canis* and B*. suis* isolates, the Suis-ladder PCR assay was used to differentiate between *B. canis* as well as different biovars of *B. suis* (Lopez-Goni *et al.,* 2011). The Suis-ladder PCR assay for all *B. suis* isolates included in this assay amplified the 774, 425 and 197bpDNA fragments, displaying the same profile with the biovar 1 reference strain 1330 whereas *B. canis* isolates and the *B. canis* R6/66 reference strain, the assay amplified only 614 and 192bp fragment sizes as shown in Figure 3.2.

On the basis of the results obtained with Bruceladder, AMOS and Suisladder PCR assays, strains ZW043, ZW046 and ZW053 were selected and sequenced using NGS technologies in

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order to comfirm that data. The *B. abortus* genome of ZW053 has large insertions and deletions as described in other *B. abortus* genomes (Michaux-Charachon *et al*., 1997; Halling *et al*., 2005). ZW053 has a huge insertion in chromosome 1 encoding 104 of 136 unique hypothetical proteins ranging from 1654596 – 1852541 (Appendix Figure 1), thus explaining the difference in the genome size as compared to *B. abortus* bv. 1 strain 9-941 (Table 3.3). This insertion in ZW053 harbours genes that encode mostly hypothetical proteins. It also has a gene encoding 3-polyprenyl-4-hydroxybenzoate carbolase-lyase that is present as an inverted fragment but coding for the same function in *B. melitensis* 16M (a UbiD family decarboxylase, 3-octaprenyl-4-hydroxybenzoate carboxy-lyase [*B. melitensis* bv. 1 strain 16M]) (Figure 3.4). This gene is one of the ubiquinones which are naturally distributed in living organisms and are regarded as the elements of the respiratory chain transferring electrons from Complex I (or Complex II) to Complex III (Makoto, 2002). It is mainly regarded as the alternative eukaryotic pathway of most bacteria that lacks UbiC genes and appears in the pattern similar to that of organisms like *B. melitensis* 16M (99.21%) and *Sinorhizobium meliloti* 1021 (72.09%) as shown in Figure 3.4. Reversions occurring within the genome, incomplete functioning of the gene product as well as functional response of some of the assembled ubiquinone often lead to faulty *ubi* mutants during the interpretation of the obtained results (Soballe & Poole, 1999).

Chromosome 2 of ZW053 shows the inversions of 2415 bp (805 amino acids) and 975 bp (325 amino acids) DNA fragments encoding for genes responsible for phenylalanyl- tRNA synthetase beta chain (EC 6.1.1.20) (Appendix figure 2) and the synthesis of tRNA pseudouridine synthase B (EC 4.2.1 70) (Figure 3.5) in multiple subsystems. In a study conducted by Michaux-Charachon *et al*. (1997), physical maps of six *Brucella* genomes also showed a number of small insertions and deletions with the range of 1 – 32 kb and an inversion of 640 kb in *B. abortus* 544 chromosome 2. Both these gene are regarded as unique in the genome of ZW053 given that they are absent in the genome that was used in this study as reference (*B. abortus* bv.1 str. 9-941) but present in *B. melitensis* bv. 1 strain 16M. Phenylalanyl- tRNA synthetase beta chain (EC 6.1.1.20) is unique as compared to other aminoacyl tRNA synthetase given that it is composed of two alpha (α) and two beta (β)

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subunits (Savopoulos *et al*., 2001). Its presence activated the formation of 2′hydroxyl of tRNA (Keller *et al*., 1992; Savopoulos *et al*., 2001).

The tRNA pseudouridine synthase B (EC 4.2.1 70) (Figure 3.5) activates the formation and modification of pseudouridines in the tRNA and despite the fact that exact role of pseudouridines is still unclear; studies have shown that they are involved in the vital cellular processes mediated by RNA (Pan *et al*., 2003). Pseudouridine deposits are mainly limited and sustained in the functionally essential part of the RNA like in the peptidyl transferase center of the ribosome (Bakin *et al*., 1994; Gu *et al*., 1996) and in the spliceosome assembly (Yu *et al*., 1998). The *B.suis* genome of ZW046 also have IsU RNA and LSU rRNA and a SSU rRNA, which are not found in ZW043 but present and identified as hypothetical proteins in *B. suis* 1330 and *B. suis* VB122. Chromosome 1 of *B. suis* ZW043, features an inversion sequence encoding the alkaline phosphatase (EC 3.1.3.1) that shows a pattern similar to that of *B. abortus* bv.1 str. 9-941 (99.59%) and *B. melitensis* bv.1 strain 16M (99. 45%) but absent in *B. suis* 1330 (Appendix Figure 4). Despite the variants observed in our genome sequences, whole-genome sequencing of these three isolates using the Illumina Miseq® (Illumina®) and their comparison to reference have proven that the isolates were *B. suis* (ZW043 and ZW046) and *B. abortus* (ZW053) respectively, thus corresponding with the data obtained with the Bruce-ladder, AMOS PCR assays.

NGS technologies have made available promising data for genome comparison at a low cost rate, provided the data is analysed thoroughly (Foster *et al.*, 2012). Genome sequencing revealed that *Brucella* species have two chromosomes of which chromosome 1 is larger than chromosome 2 (Paulsen *et al*., 2002; DelVecchio *et al*., 2002; Halling *et al*., 2005), however, despite that difference in size, they have a similar GC content that ranges from 55-58% (Jumas-Bilak *et al.*, 1998). DelVecchio *et al*. (2002) reported that chromosome 1 encodes for the processes that include protein synthesis, transcription and translation whereas chromosome 2 encodes for genes responsible for membrane transport, energy metabolism and regulation. Comparison of genomes has revealed enough promising variants that can distinguish between *Brucella* species despite very little variations and conservations available (Paulsen *et al.*, 2002; Foster *et al.*, 2009). Thus, due to the

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homologous nature of *Brucella* species, discovery of these available variants will make it possible to differentiate them with available assays other than genome sequencing (Foster *et al*., 2012).

It is concluded that Bruce-ladder PCR assay can accurately identify *Brucella* species as it enables accurate identification of all *Brucella* species including vaccine and marine strains whereas AMOS-PCR is limited to *B. abortus, B. melitensis, B. ovis* and *B. suis*. This is of great importance since some of the laboratories in Africa lack adequate resources, expertise and infrastructure to do biotyping of *Brucella* species and biovars under proper biosafety and biosecurity conditions.

3.5 ACKNOWLEDGEMENTS

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Chapter 4

DISTRIBUTION OF *BACILLUS ANTHRACIS* GENOTYPES IN KRUGER NATIONAL PARK IN SOUTH AFRICA

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ABSTRACT

Bacillus anthracis, the causal agent of anthrax is endemic in the northern part of the Kruger National Park (KNP) in South Africa. Multi-loci variable number of tandem repeats (VNTR) analysis (MLVA) employing 30-VNTR markers were used to characterize the *B. anthracis* genotypes that occur among wildlife and livestock in KNP. In our study 72 *B. anthracis* isolates from outbreaks in roan, hippopotamus, buffalo, rhinoceros, kudu, impala and environmental samples in 2012 and 2013 in KNP were genotyped. These strains were isolated from various outbreaks during 2012 and 2013 from different areas in KNP. The same genotype caused outbreaks in rhinoceros during 2012 in areas approximately 250 km south of Pafuri and from 11 of 14 bone and soil samples (from sites where animals died previously in 2012 or beginning of 2013) which were collected in 2013 from Pafuri in the north of KNP. The 72 *B. anthracis* strains from various outbreaks in KNP during 2012 and 2013 consisted of 23 genotypes. In this study clonal genotypes were observed in each outbreak, however, five animals appear to be infected by multiple genotypes. According to the MLVA results obtained, all KNP genotypes from the 2012 and 2013 outbreaks group were in the A-clade with none from the B-clade. This implies that the A-clade strains isolated in the KNP and characterised in this study have substantial geographic and genetic diversity, thus, characterisation of more isolates from other sections will provide an insight in *B.anthracis* pattern of occurrence and diversity in the park.

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Key words: *Bacillus anthracis*, anthrax, Molecular characterization, MLVA31 and KNP.

4.1 INTRODUCTION

Bacillus anthracis is a Gram-positive, non-motile, spore-forming, aerobic and rod-shaped bacterium with little genetic variability and the causative agent of the disease anthrax (Turnbull & Kramer, 1995; Leppla *et al*., 2002). The disease affects herbivores such as wild ungulates and other species of domestic and wild animals, and people. This disease is endemic in the northern part (Pafuri region) of Kruger National Park (KNP) in South Africa. Anthrax has not been reported to be directly transmitted from victim to victim except through scavengers, but rather ingested by herbivores while grazing or browsing. In KNP, blowflies (*Chrysomya albiceps* and *C. marginalis*) have been indicated to contaminate browse after feeding on anthrax infected carcasses (Hugh-Jones & De Vos, 2002). In the KNP the disease exhibits two patterns of epidemiology such as: firstly; water contamination where vectors, like vultures, contaminate water after feeding on an infected carcass and secondly; vegetation contaminated by vectors like blowflies and the dung of scavengers that have fed on an infected carcass (Bengis & Erasmus, 1988). Hugh-Jones & De Vos (2002) indicated that anthrax outbreaks usually occur periodically during dry seasons in KNP when water is scarce, leading to animals sharing the remaining water points.

The incidences of anthrax among agricultural animals decreased after the introduction of Sterne vaccine in 1937 (Hambleton *et al.*, 1984; Ivins & Welkos, 1988), but the disease remains endemic in wildlife as vaccination is difficult or impossible (Hugh-Jones & De Vos, 2002). Susceptibility of individual animals to anthrax depends on the host species, the strain, age and the route of infection (Welkos *et al*., 1986; Lyons *et al*., 2004). During major outbreaks in the KNP, it has been shown that older animals are mostly affected compared to younger animals. This disease therefore acts as a natural mechanism of culling (Hugh-Jones & De Vos, 2002). Previous studies have reported browsers like kudus being the most anthrax affected species in KNP with records of over 50% of kudu population affected in the past (De Vos, 1990) compared to the Etosha National Park in Namibia where zebras are affected

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mostly (Lindeque & Turnbull, 1994; Beyer *et al*., 2012). This appears to be due to the kudu's feeding height of 1-3 m coinciding with the height where blowflies usually deposit their anthrax contaminated vomit or faecal droplets (Braack & De Vos, 1990).

The conservation of the rare roan antelope (*Hippotragus equinus*) has long been attempted in the KNP, but it still remains a low-density and rare species in the park to date due to anthrax outbreaks (Grant *et al*., 2002). The disease is regarded as the limiting factor for the roan population as the 1959, 1960 and 1970 outbreaks killed 83 roan antelope of the 250 population in the KNP but the death toll estimated might be much higher. Genotypes from South African outbreaks during 1959, 1960 and 1970 clustered in A and B-clades / lineages using MLVA, with the B1-subcluster regarded as restricted to southern Africa dominating (Smith *et al*., 2000). This led to annual immunisation of the roan antelopes by Sterne spore vaccine in disposable drop-out darts or ballistic implants (De Vos *et al*., 1973). However; this immunisation practice has fallen away in recent years leading to a large outbreak in 2012 in the Capricorn camp in KNP where this species are kept. The Capricorn camp is an enclosed camp used for the breeding of rare antelope (roan and tsessebe in particular), situated 5 km northeast of Mopani Rest camp and in the Mooiplaas ranger section of KNP (Figure 1). The enclosure is said to cover an area of 5 km^2 and is divided into 9 burn blocks. It is fenced off with an electrified fence and has a solar powered borehole, reservoir and three water points, two of which are functional, along its southern boundary (L. van Schalkwyk, personal communications).

Assays based on PCR focusing on the areas of molecular diversity within its genome such as single nucleotide polymorphism (SNPs) and variable number tandem repeats (VNTRs) improved genotypic differentiation (Van Belkum *et al*., 1998; Keim *et al*., 1999; 2000; 2004; Van Ert *et al.,* 2007). MLVA includes amplification of VNTR loci and sizing of amplified fragments to detect length polymorphism (Keim *et al*., 2004). The first MLVA used eight markers (Keim *et al*., 2000) and it was later enhanced to 15 (Van Ert *et al*., 2007) and 25 (Lista *et al*., 2006) markers leading to the identification of various new genotypes and increased discriminatory power. A combination of these assays gave rise to MLVA-31, which

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has superior resolution (Beyer & Turnbull, 2009). MLVA genotyping clearly offers a high discriminatory power and fast typing of this pathogen. The main focus of this project was to characterise *B. anthracis* strains from outbreaks during 2012 and environmental samples collected in 2013 in KNP with MLVA-31 (Beyer *et al*., 2012) to monitor the spread, distribution and diversity of the strains. Some of the *B. anthracis* strains characterized in this study included isolates collected from outbreaks occurring in 2012 amongst roan in an enclosed camp in the north of the park as well as from hippopotamus in the Sabi Sands reserve next to the south of KNP (non-endemic region).

4.2 MATERIALS AND METHODS

4.2.1 SAMPLE HISTORY

Bacillus anthracis isolates (72) were collected during 2012 and 2013 anthrax outbreaks in Pafuri, Mooiplaas, Letaba, Sabi Sands, the private game reserve near KNP, Houtboschrand, Woodlands, Satara and Capricorn camp (an enclosure where a rare species of roan are kept for breeding) in KNP as shown in Table 1 and Figure 1.

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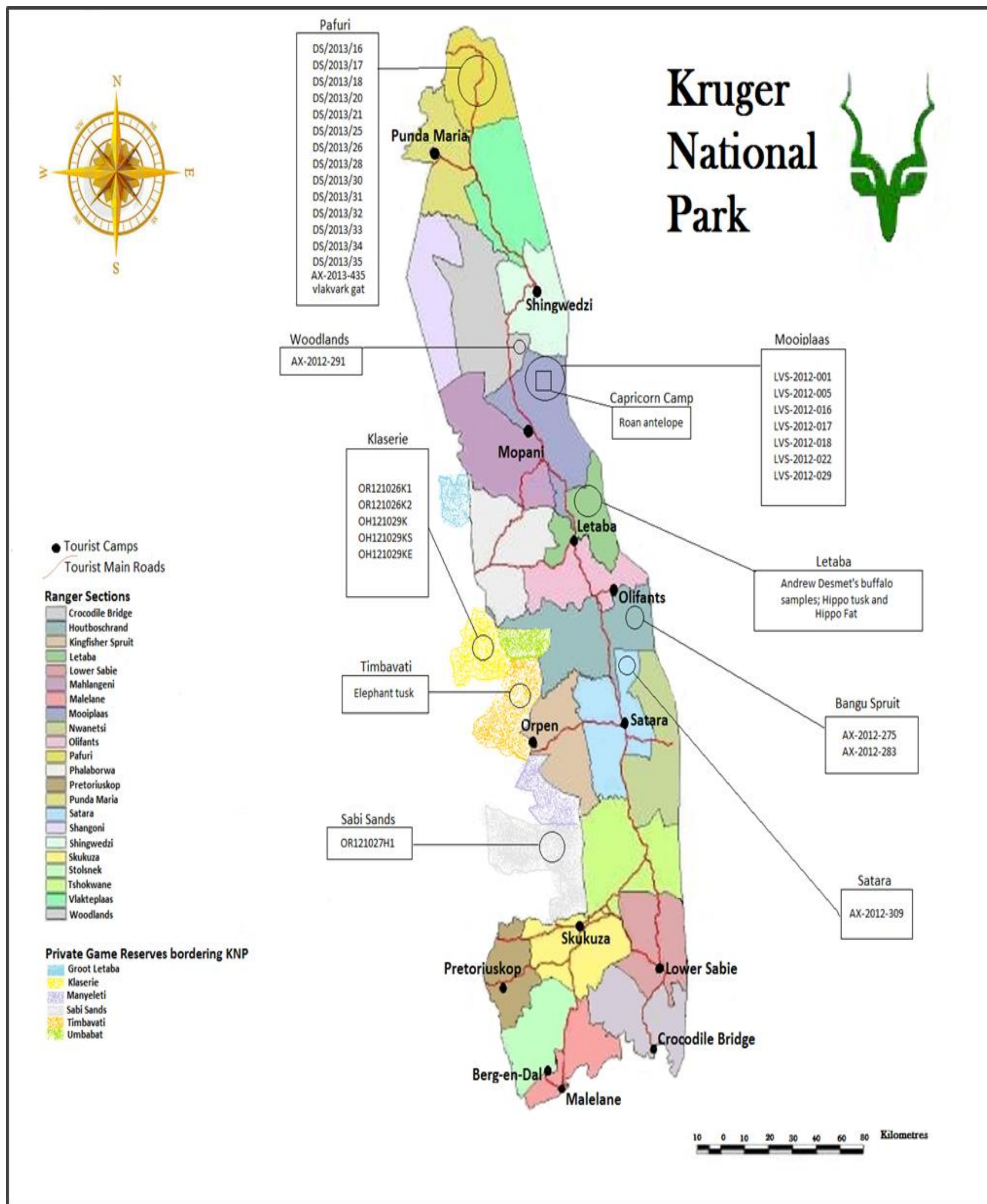

Figure 1: Map of the Kruger National Park detailing the ranger sections and private game reserves where anthrax was successfully isolated from samples submitted during the 2012-2013 outbreaks. The collector's sample identification is indicated below the regions (refer to Table 1) (Map from Sanparks website).

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Capricorn roan camp outbreak

Forty-five roan antelopes died during this outbreak from July to November 2012. Blood smears from 14 carcasses that were still fresh were Giemsa-stained and all were found to contain characteristic bacilli. Bone samples were collected from 32 carcasses and cultured as described by Turnbull (2008) on PLET (Polymyxin B - Lysozyme - EDTA - Thallous Acetate) and BTA (Blood Test Agar) of which 17 *B. anthracis* isolates (Table 1; Figure 1) were obtained.

Mooiplaas outbreak

This outbreak affected animals in the roan camp and surrounding area. Apart from the roans, other animals (waterbucks, kudus, buffaloes and an elephant outside the roan enclosure also died of anthrax-like symptoms from July 2012. *Bacillus anthracis* isolates were isolated by Ayesha Hassim at Skukuza state veterinary laboratory using Turnbull (2008) guidelines for bacterial isolation (Figure 1; Table 1).

Sabi sands outbreak

Towards the end of October 2012, Mpumalanga state veterinarian submitted a hippopotamus sample obtained from the shores of the Sabie River at Sabi sands, the private game reserve bordering KNP. *Bacillus anthracis* was isolated from this sample according to method described by Turnbull (2008).

Rhinoceros isolates

In November 2012, two white rhinoceros died of anthrax-like symptoms at Bangu spruit (Houtboschrand ranger section), south of the Olifants River approximately 80 km from Capricorn roan camp whereas the other two anthrax cases of rhinoceroses were from

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Woodlands (October 2012) and Satara (November 2012) respectively (Figure 1; Table 1). *Bacillus anthracis* was isolated from each rhinoceros.

Letaba outbreak

The *B. anthracis* from this area were isolated from buffaloes in October 2012 and hippopotamus in July 2013. Buffalo samples were isolated from bone and tissue while hippopotamus samples were from tusk and tissue according to Turnbull (2008) (Figure 1; Table 1).

Klaserie isolates

Five kudu samples were collected in October 2012 at Klaserie, a private game reserve that borders Timbavati and Umbabat private game reserves as well as KNP (Figure 1; Table 1). They were submitted by the Mpumalanga state veterinarian to Skukuza state Veterinary offices for further isolation. Anthrax was isolated from tissue, rib bones, soil under the kudu carcass and the blood soaked earth around the carcass.

Pafuri outbreak

In the beginning of 2013, anthrax cases were reported from the north of the Kruger Park in the Pafuri region. In March, fourteen soil and bone samples were collected at the sites where carcasses of animals (wild dog, nyala, 2 zebras and 10 impalas) suspected of having died of anthrax during 2012 or 2013 were found in the endemic region at Pafuri (Table 1). *Bacillus anthracis* was isolated using the guidelines by Turnbull (2008).

4.2.2 DNA EXTRACTION

Bacillus anthracis Sterne, Vollum (A70) and Ames (A93) DNA were included as references (positive controls). *Bacillus anthracis* DNA was isolated using the Gram-positive bacterial protocol of the QIAmp® DNA mini kit (Qiagen) from isolates according to the manufacturer's instructions.

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4.2.3 MULTI-LOCI VARIABLE NUMBER OF TANDEM REPEATS (VNTR) ANALYSIS (MLVA)

Genotyping of 72 *B. anthracis* isolates was done as described previously using thirty of the 31 VNTR markers (vrrA, vrrB1, vrrB2, vrrc1, VRRc2, CG3, pXO1, pXO2, Bavntr 12, Bavntr 16, Bavntr 17, Bavntr 19, Bavntr 23, Bavntr 35, BAMS 1, BAMS 3, BAMS 5, BAMS 13, BAMS 15, BAMS 21, BAMS 22, BAMS 23, BAMS 24, BAMS 25, BAMS 28, BAMS 30, BAMS 31, BAMS 44, BAMS 51 & BAMS53) that were multiplexed in seven reactions using fluorescently labelled forward primers (Keim *et al*., 2000; Le Flèche *et al*., 2001; Lista *et al*., 2006;Ciammaruconi *et al*., 2008; Beyer *et al*., 2012). Bams 34 marker was excluded due to technical difficulties.

Multiplex PCRs were prepared in 15 µl reactions consisting of 1x MyTaq HSMix (Bioline), primers (0.4 - 1mM; Appendix Table 1) and 2 µl of template DNA. Multiplex A-D and G were carried out using the following conditions: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 20 sec, annealing at 60°C for 30 sec, extension at 72°C for 2 min with a final extension at 72°C for 5 min. Multiplex E and F were carried out with the following conditions: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 20 sec, annealing at 50°C for 30 sec, extension at 72°C for 2 min with a final extension at 72°C for 5 min. The PCR amplicons were separated on a 51 cm capillary using POP 7 on an ABI 3130 Genetic Analyzer (Applied Biosystems).

4.2.4 DATA ANALYSIS

The allele sizes of multiplex PCR products were determined by comparison to the LIZ1200 size standard (Applied Biosystems) using STRand Analysis Software version 2.3.106. A database was created for *B. anthracis* using BioNumerics version 6.0 software for analysis of copy numbers of the VNTR units. *Bacillus anthracis* 34F2 Sterne (B#113), Ames (B#115) and Vollum (B#114) strains were included as reference strains to help with validating the accuracy of allele size determinations. The copy number was calculated for each marker according to the algorithm described by Lista *et al*. (2006) and Beyer *et al*. (2012) using allele sizes determined with capillary electrophoresis. Electrophoregrams were read using STRand Analysis Software version 2.3.106 and allele sizes for the 30-panel were converted into copy

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numbers (listed in Appendix Table 2: A and B). Genetic distances were calculated using similarity coefficient and cluster analysis was done by unweight pair group method using arithmetic averages (UPGMA) with BioNumerics 6.0 software.

4.3 RESULTS AND DISCUSSIONS

A collection of 72 *B. anthracis* isolates from KNP as well as three reference strains were typed with 30-MLVA using capillary electrophoresis. A total of 23 genotypes were found amongst the 72 isolates from KNP using MLVA-30 (Table 1 and 2). MLVA is a robust assay used to type bacterial pathogens globally given that it has the ability to trace back the source of infection as well as grouping the genotypes into clusters and subclusters (Keim *et al*., 2000). The substructure of *B. anthracis* is classified into three major clades namely A, B and C (Van Ert *et al*., 2007). A-clade has multiple closely related clusters that are distributed globally whereas B-clade has only two clusters with B1 restricted in southern Africa and B2 reported more frequently in Europe (Keim *et al*., 2000; Smith *et al*., 2000; Van Ert *et al*., 2007). C-clade is the base lineage and its very rare (Keim *et al*., 2009).

All the *B. anthracis* isolates from the 2012-2013 outbreak clustered in the A-clade, with subclade A1b dominating (Figure 2). Smith *et al.* (2000) indicated that two regions in KNP, the most northern tip of KNP (Pafuri) and the central regions have been most notably affected by anthrax. The northern part of KNP was affected by anthrax outbreak mostly since 1959 and the central since 1990. *Bacillus anthracis* from both the A-clade and B-clade (B1-subclade) contributed to mortality during epidemics in KNP during 1970-1981 with the B1-subclade playing an important role (Smith *et al.,* 2000). The anthrax isolates collected in the central parts of KNP were from the A-clade. With the major anthrax outbreak in 1990 most of the isolates were from the A-clade and the outbreak began in the central part of the park and progressed northwards (Smith *et al.,* 2000). The lack of B1-subclade in the northern regions since 1990 (Smith *et al.,* 2000) is also evident in this study as all the *B. anthracis* isolates clustered in the A-clade (Figure 2). Anthrax seems to spread over large

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distances in KNP based on genotype analyses indicating that vectors like water and/or vultures might play an important role in the spread of this disease.

Genotypes within A-clade have short distance phylogenetic branch length (as illustrated in Figure 3), thus, indicating that they are closely related. Van Ert *et al*. (2007) reported that even though the genotypes of A-clade are dispersed worldwide, they do have a preferable geographic region. In KNP, anthrax mortality rates are very high in regions with a soil pH of greater than 7 and the calcium content of 150 milliequivalents (Smith *et al*., 1999). Variations in distributions and occurrence among the clusters of *B. anthracis* are caused by the ability to adapt in the host or environment. This is shown in a study by Smith *et al*. (2000) where strains of the A-clade adapt to more varied environments compared to strains of B-clade which adapt to limited environments. A wide prevalence of genotypes of A-lineage subclusters was observed in this study. Keim *et al*. (2000) has reported that genotypes from cluster A3 have been responsible for the major outbreaks which previously occurred globally as well as in areas like KNP.

Woodlands isolate

There was only one isolate from this area and it shared sub-clade A1b with most of the sample from Mooiplaas, but it yielded genotype 17 which is not shared with any other isolate (Table 1). The area is in close proximity to Mooiplaas and they have a common catchment area (Table 1; Figure 1).

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Table 1: *Bacillus anthracis* isolate information indicating the collection date, sample type, area, catchment areas and clade and genotype in Kruger National Park.

Sample number	Collection Date	Sample Type	Origin	Area	Catchment Areas	Clades	Genotypes
ELEPHANT TUSK	2013/05/09	Tusk	Elephant	Timbavati	Olifants	A3.a	1
HIPPO TUSK	2013/06/12	Tusk	Hippopotamus	Letaba	Letaba	A3.a	1
HIPPO FAT	2013/06/12	Tissue/Fat	Hippopotamus	Letaba	Letaba	A3.a	1
LAB ISOLATE	2013/07/15	Swab	Biohazard cabinet	Skukuza	NA	A3.a	1
LVS-2012-006	2012/08/26	Water		Mooiplaas	Shingwedzi	A3.a	2
OR121027H1	2012/10/27	Ear tissue	Hippopotamus	Sabi Sands Game Reserve	Sabie	A3a	3
LVS-2012-007	2012/08/26	Nasal swab		Mooiplaas	Shingwedzi	A3.a	4
LVS-2012-018	2012/08/26			Mooiplaas	Shingwedzi	A3.a	4
LVS-2012-015	2012/08/26	Faeces	Vulture	Mooiplaas	Shingwedzi	A1.b	5
Andrew Desmet T	2012/10/16	Tissue	Buffalo	Letaba	Letaba	A1.b	6
LVS-2012-001	2012/08/26	Leaves	Blowflies	Mooiplaas	Shingwedzi	A1.b	7
LVS- 2012-001	2012/08/26	Bone		Mooiplaas	Shingwedzi	A1.b	7
LVS-2012-005	2012/08/26	Nasal swab		Mooiplaas	Shingwedzi	A1.b	7
LVS-2012-016	2012/08/26	Blood	Elephant	Mooiplaas	Shingwedzi	A1.b	7
LVS-2012-017	2012/08/26	Faeces	Vulture	Mooiplaas	Shingwedzi	A1.b	7
LVS-2012-029	2012/08/26	Grass	Blowflies	Mooiplaas	Shingwedzi	A1.b	7
Roan 2	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	7
OH121026K1	2012/10/26	Nose tissue	Kudu	Klaserie	Olifants	A1.b	7
OH121026K2	2012/10/26	Rib bone	Kudu	Klaserie	Olifants	A1.b	7
OH121026K2-PLET	2012/10/26	Rib bone	Kudu	Klaserie	Olifants	A1.b	7
OH121026K2-BTA	2012/10/26	Rib bone	Kudu	Klaserie	Olifants	A1.b	7
OH121029KS	2012/10/29	Soil	under Kudu	Klaserie	Olifants	A1.b	8
LVS-2012-018 SBA	2012/08/26			Mooiplaas	Shingwedzi	A1.b	9
Andrew Desmet B-BTA	2012/10/17	Bone	Buffalo	Letaba	Letaba	A1.b	10
OH121029KE	2012/10/29	Earth/mud	around Kudu	Klaserie	Olifants	A1.b	11
AX-2012-275	2012/11/17	Blood Smear	White Rhino	Houtboschrand	Olifants	A1.b	12
AX-2012-283	2012/11/30	Blood Smear	White Rhino	Houtboschrand	Olifants	A1.b	12
AX-2012-309	2012/11/30	Blood Smear	White Rhino	Satara	Nwanetsi	A1.b	12
DS/2013/16	2013/03/12	bone + soil	Zebra	Pafuri	Limpopo	A1.b	12
DS/2013/17	2013/03/12	bone + soil	Impala	Pafuri	Limpopo	A1.b	12
DS/2013/18	2013/03/12	bone + soil	Nyala	Pafuri	Limpopo	A1.b	12
DS/2013/20	2013/03/12	bone + soil	Impala	Pafuri	Limpopo	A1.b	12
DS/2013/21	2013/03/12	bone + soil	Impala	Pafuri	Limpopo	A1.b	12
DS/2013/25	2013/03/12	bone + soil	Impala	Pafuri	Limpopo	A1.b	12
DS/2013/26	2013/03/12	bone + soil	Impala	Pafuri	Limpopo	A1.b	12
DS/2013/28	2013/03/12	bone + soil	Impala	Pafuri	Limpopo	A1.b	12
DS/2013/30	2013/03/13	bone + soil	Impala	Pafuri	Limpopo	A1.b	12
DS/2013/33	2013/03/14	bone + soil	Impala	Pafuri	Limpopo	A1.b	12
DS/2013/34	2013/03/14	bone + soil	Zebra	Pafuri	Limpopo	A1.b	12
AX-2013-435	2013/04/26	Blood Smear	Elephant	Pafuri	Limpopo	A1.b	13
DS/2013/32	2013/03/13	bone + soil	Impala	Pafuri	Limpopo	A1.b	14
Roan 2-BTA	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	15
Roan 3	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	15
Roan 4	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	15
Roan 5-BTA	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	15
Roan 5-PLET	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	15
Roan 6	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	15
Roan 7	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	15
Roan 10	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	15
Roan 13-PLET	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	15
Roan 13-BTA	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	15
Roan 14-PLET	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	16
Roan 14-BTA	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	16
Roan 15	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	16
Roan 18	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	16
Roan 20	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	16
Roan 22-BTA	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	16
Roan 22-PLET	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	16
Roan 23-PLET	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	16
Roan 28	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	16
Roan 30	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	16
Roan 32	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	16
Roan 34-PLET	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	16
Roan 34-BTA	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.b	16
AX-2012-291	2012/10/27	Blood smear	White Rhino	Woodlands	Shingwedzi	A1.b	17
DS/2013/38	2013/03/23	bone + soil	Wild Dog	Pafuri	Limpopo	A1.b	18
DS/2013/31	2013/03/13	bone + soil	Impala	Pafuri	Limpopo	A1.b	19
LVS-2012-022	2012/08/26			Mooiplaas	Shingwedzi	A1.b	20
Andrew D Buffalo T1	2012/10/16	Tissue	Buffalo	Letaba	Letaba	A1.b	21
Roan 23-BTA	2012/08/26	Bone	Roan antelope	Capricorn camp; Mooiplaas	Shingwedzi	A1.a	22
Andrew Desmet B-PLET	2012/10/17	Bone	Buffalo	Letaba	Letaba	A1.a	23

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Capricorn roan camp

In this case, two elephants were shot during July 2012 for interfering with the fence surrounding the Capricorn roan camp. Vultures opened and fed on the carcasses and then drank and bathed at the water points inside the camp. Anthrax was obtained only from elephant blood where vultures opened the carcass and not from any other sample taken from the elephant carcass indicating that the vultures were already contaminated when feeding on the elephant carcass. Anthrax strains from Roan 2 (B#121), blood taken from elephant where vultures opened carcasses (B#123; LVS-2012-016) and vulture faeces (B#122; LVS-2012-017) belonged to the same genotype (genotype 7) (Table 1; Figure 2; Figure 3). Silt found at the bottom of the main trough within the enclosure was cultured by Skukuza Veterinary Services and was positive for anthrax, indicating that the water was contaminated, but the sample was not included in this study (Dr. Louis van Schalkwyk, personal correspondence). Most of the anthrax strains obtained from roans in the Capricorn roan camp, belonged to genotype 15 and 16 in subclade A1b (Table 1, Figure 3). The containment of the roan in the Capricorn camp might be the reason that genotypes 15 and 16 predominated in this camp due to the simple reason of exposure. Genotypes 7 and 15 were isolated from Roan 2 (Table 1). As indicated genotype 7 was found in samples taken from the elephant, vulture, kudu as well as from leaves from bushes that kudu feed on which will be discussed below.

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Table 2: Summary of the *Bacillus anthracis* isolates collected in Kruger National Park in 2012 and 2013.

SAMPLE AREA	NUMBER OF ISOLATES	SAMPLE TYPE	YEAR	GENOTYPES ISOLATED
Sabi Sands	1	Clinical isolates	October 2012	1
Letaba	6	Clinical and environmental isolates	October 2012 and 2013	5
Capricorn roan camp	25	Clinical isolates	July-November 2012	4
Pafuri	15	Clinical and Environmental isolates	November 2013	5
Mooiplaas	12	Clinical and environmental isolates	July 2012	6
Houtboschrand	2	Clinical isolates	2012	1
Satara	1	Clinical isolates	November 2012	1
Woodlands	1	Clinical isolates	October 2012	1
Klaserie	6	Clinical and environmental isolates	October 2012	3
Timbavati	1	Clinical isolates	2013	1

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Mooiplaas and Klaserie isolates

Twelve clinical and environmental samples from the area surrounding the roan camp yielded 6 genotypes with genotype 7 dominating. Genotype 7 was obtained from the shot elephant's blood and the vulture faeces found on the elephant carcasses (Table 1; Figure 2), proving evidence that vultures may be the vectors responsible for the transmission of *B. anthracis* genotypes involved in these outbreaks (De Vos, 1990; Hugh-Jones & De Vos, 2002; Turnbull *et al.*, 2008). Other isolates belonging to genotype 7 include 4 kudu samples originating in Klaserie and Roan 2 from the roan enclosure, thus, linking the strains found inside and outside the fenced roan camp. Roan 2 BTA (B#140) belongs to genotype 15 together with 9 isolates from the roan camp whereas Roan 2 (B#121) belongs to genotype 7; this demonstrates that multiple strains can infect a single animal as it was also reported in Beyer & Turnbull (2013). *Bacillus anthracis g*enotype 7 was found in elephant, vulture, kudu as well as from leaves around the outbreak carcasses. In KNP blowflies have been reported as a vector in the contamination of vegetation along with scavengers like vultures that contaminate water after feeding on infected carcasses (Bengis & Erasmus, 1988; Hugh-Jones & De Vos, 2002).

LVS-2012-007 (B#120) and LVS-2012-018 (B#125) yielded clonal genotype 4 (subclade A3a), LVS-2012-016 (B#123) genotype 2 (subclade A3a), whereas LVS-2012-018 SBA (B#126) also yielded genotype 9, LVS-2012-015 (B#122) genotype 5 (all subclade A1b) once again demonstrating co-infection and strains circulating in the Mooiplaas area (Table 1; Figure 2; Figure 3). Genotypes in subclade A1 were dominant in KNP compared to the rare subclade A3 that was present in the Mooiplaas area and mainly in hippopotamus samples from Letaba and Sabi sand (Figure 2 and 3).

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Letaba and Timbavati isolates

Five genotypes were yielded from the Letaba isolates; with genotype 1 shared with an elephant tusk isolate from outside the park in Timbavati private game reserve. The other genotypes were not found in any of the other regions. Genotype 1 belongs to A3a-subclade while genotypes 6, 10, 21 and 23 are A1b-subclade (Figure 3). Although the A3-clade did not dominate in the 2012 outbreak genotypes in A3-subclade were mostly from hippopotamus. This could point to water as a vector contaminated by either run-off water or vultures that carry anthrax spores belonging to members of this clade (clade A3) from one region to the other during the 2012-2013 outbreak.

Pafuri, Houtboschrand and Satara isolates

There were 5 genotypes from the Pafuri area with genotype 12 isolated from impala, nyala and zebra dominating in this area. *Bacillus anthracis* genotype 12 was also isolated from white rhinoceros from Houtboschrand and Satara respectively (Table 2; Figure 2). These three regions were linked by genotype 12 which is dominant in Pafuri (n = 11) and the sole genotype in the other two regions. Once again this scenario might show that vultures are involved as they are the only birds that can travel such long distances inside and outside the park in the given time-frame, as Houtboschrand and Satara are approximately 250 kilometres from Pafuri.

Sabi sands isolate

There was only one isolate from Sabi sands (Table 2; Figure 2). Sabi Sands is a private game reserve outside the Park where a hippopotamus was found dead on the shore of Sabie river and no other isolates were obtained from this area. This explains the rarity of this genotype that belongs to A3a-subclade like all the other samples from hippopotamuses.

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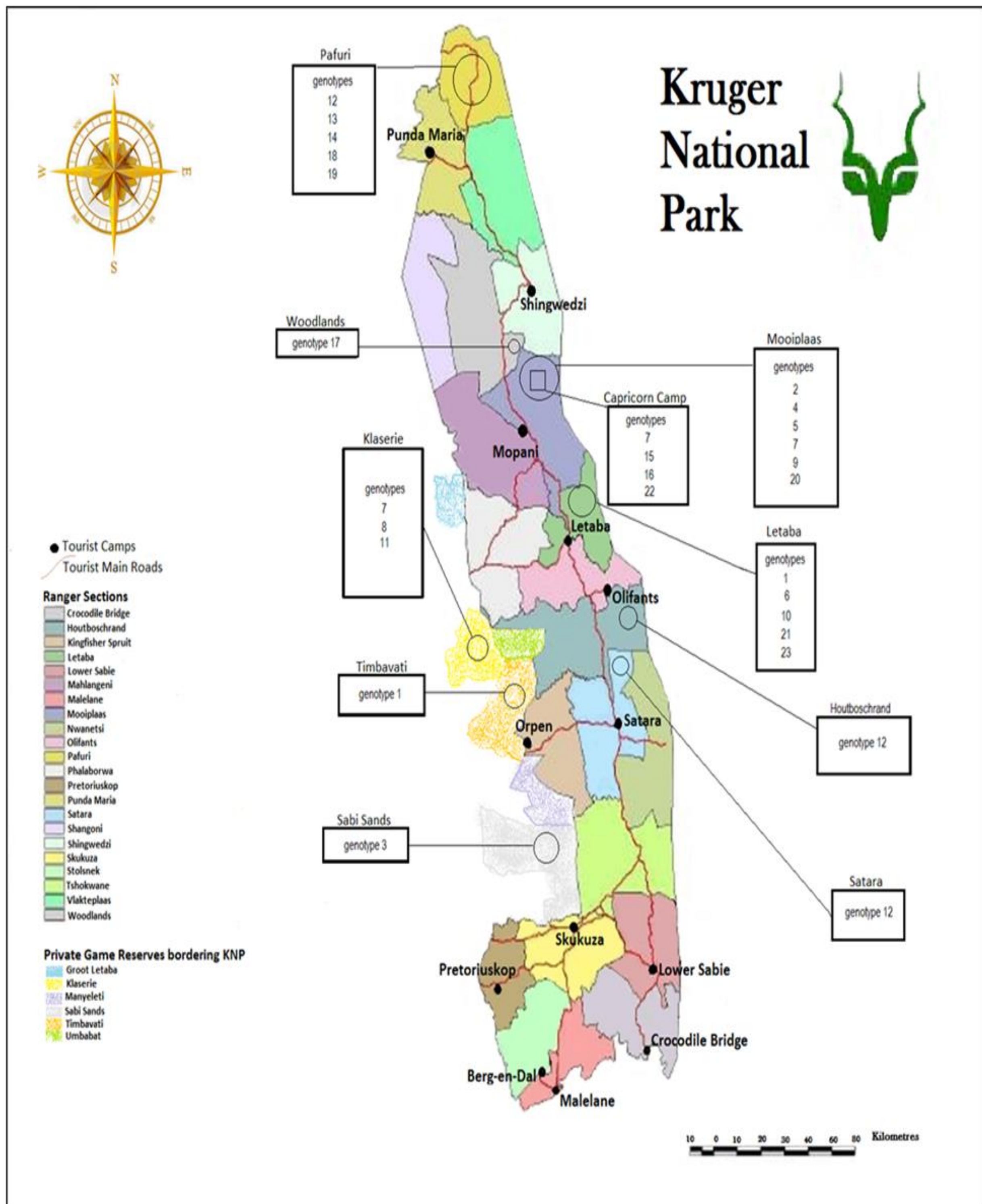

Figure 2: Map of the Kruger National Park detailing the genotypes isolated from various ranger sections in the park and private game reserves neighbouring the park.(Map from SanParks website)

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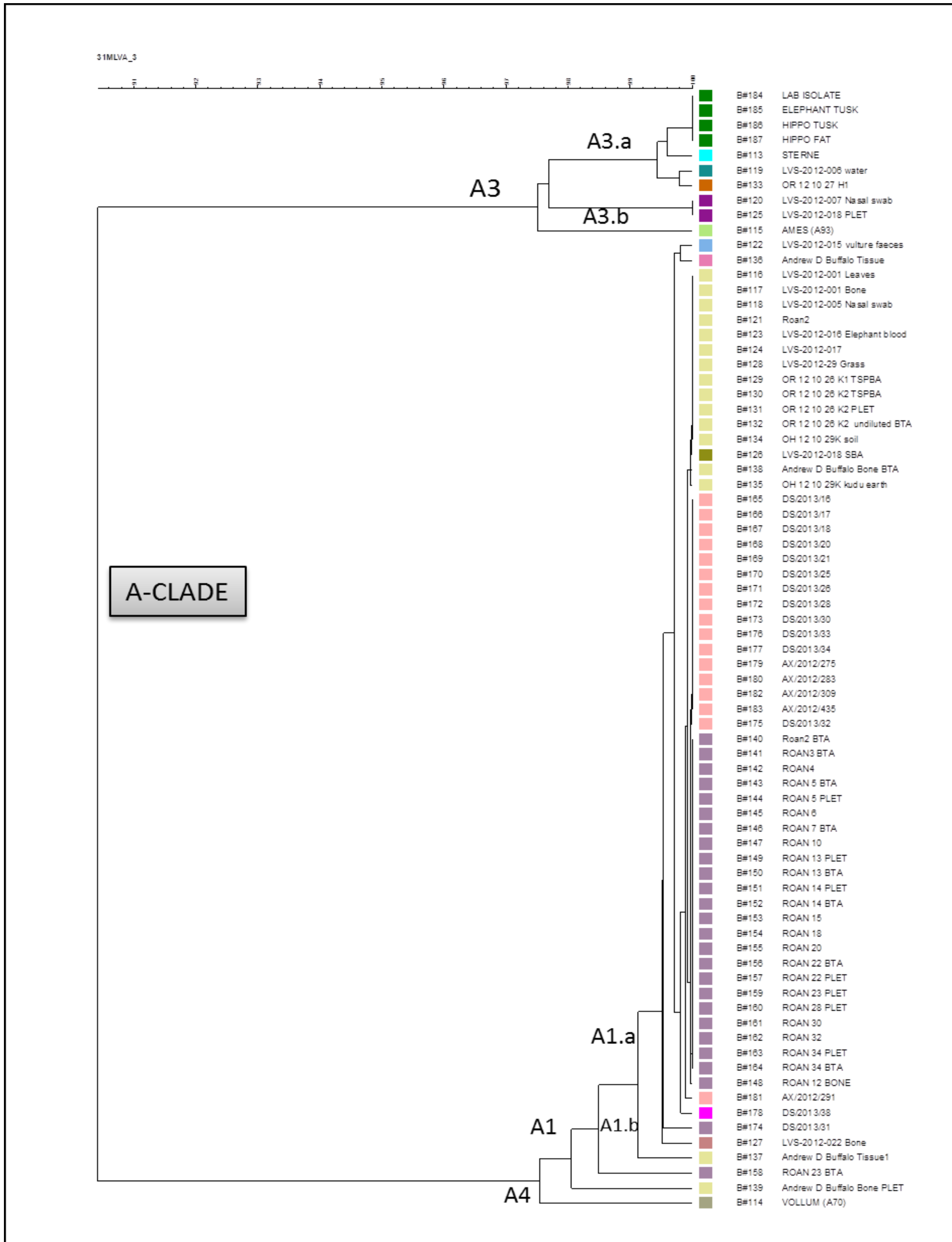

Figure 3: Dendogram of MLVA-30 data of *Bacillus anthracis* isolates from Kruger National Park, South Africa. This was generated using the unweighted pair group method with arithmetic means (UPGMA). The scale bar indicates the genetic distance among the isolates.

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4.4 CONCLUSION

MLVA genotyping has proven to be a valuable and worthwhile tool in tracing the anthrax strains involved in this outbreak study. The 30-marker MLVA has sufficient resolution to provide an interesting narrative on the outbreak scenario which allows us to draw conclusions for the dissemination of the bacterial spores in the environment. There were 23 genotypes isolated during this study. Eighteen out of the 23 genotypes obtained were circulating during the 2012 outbreak spanning 7 regions and 5 catchment areas whereas 6 genotypes were circulating through the 2013 outbreak, but confined mostly to 1 region and 1 catchment area with 2 outlying cases.

With the hippopotamus samples which point to water as the progenitor of the outbreaks, the samples belonged almost exclusively to the A3a-subclade, while the genotypes were varied. This fact, coupled with the vastness of the area affected by the anthrax outbreak across 7 distinct catchment areas, points to another vector other than water being responsible for the dissemination of spores during the initial outbreak.

The roan breeding camp provided a unique study area given that confined animals and free roaming animals were both affected by the disease. The links between these were the large number of vultures scavenging on the infected carcasses and their access to water points inside and outside of fenced areas. Vultures as the vectors for spore dissemination would also explain that clonal genotypes were found across multiple catchment areas as vultures are known to migrate along vast distances following water holes and rivers. Animals like Roan 2 and Roan 23 showed co-infections with multiple strains providing links genotypes in different areas. Strains found in the endemic region of Pafuri in 2013 linked both 2012 and 2013 anthrax cases, however the exact period that the Pafuri outbreak occurred is unknown and it might have occurred during 2012 or early 2013.

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It is also concluded that strains of A-lineage were more dominant and responsible for the major outbreaks that occurred in the past two years. The entire A-clade has also shown to have substantial geographic and genetic diversity in the park at present which continues the trend of the last few decades where previously the B-clade was dominating over the A-clade lineage.

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APPENDIX

Appendix Table 1: 31-MLVA (multi-loci variable number of tandem repeat analysis) information of primer combination in multiplex PCR reactions with PCR fragment range and repeat size for *Bacillus anthracis.*

PRIMER	RANGE OF FRAGMENT SIZE	REPEAT SIZE	
CG3	153-160	5	MULTIPLEX A
Bams 44	183-573	39	
Bams 3	429- 654	15	
vrrB2	135-198	9	
Bams 5	229-424	39	
Bams 15	409-643	9	
Bams 1	296-611	21	
vrrC1	364-688	9	
Bams 13	337-868	9	MULTIPLEX B
vrrB1	184-292	9	
Bams 28	373-505	24	
vrrC2	528-604	18	
Bams 53	322-346	12	
Bams 31	331-1087	9	
vrrA	289-338	12	MULTIPLEX C
Bams 25	376-391	12	
Bams 21	541-766	45	
Bams 34	230-581	39	
Bams 24	469-511	42	
Px01	120-144	3	
Px02	133-155	2	
Bams 51	358-538	45	MULTIPLEX D
Bams 22	519-1041	36	
Bams 23	399-693	42	
Bams 30	268-929	9	
VNTR 12	106-120	2	MULTIPLEX E
VNTR 19	91-134	3	
VNTR 35	102-126	6	
VNTR 16	137-346	8	MULTIPLEX F
VNTR 23	170-208	12	
VNTR 17	366-453	8	MULTIPLEX G

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Appendix Table 2: Copy numbers of variable number of tandem repeat markers for *Bacillus anthracis* strains used in the study according to multiplexed PCR reactions.

A

SAMPLE ID	ISOLATES	CG3	vrrB2	BAMS1	BAMS5	BAMS44	BAMS3	vrrC1	BAMS15	BAMS 13	vrrB1	BAMS 28	vrrC2	BAMS53	BAMS 31
B#113	**STERNE**	**1**	**13**	**15**	**6**	**8**	**27**	**53**	**44**	**74**	**16**	**13**	**13**	**7**	**63**
B#114	**VOLLUM (A70)**	**1**	**12**	**12**	**6**	**5**	**29**	**48**	**44**	**19**	**16**	**13**	**18**	**7**	**62**
B#115	**AMES (A93)**	**1**	**12**	**15**	**4**	**7**	**26**	**53**	**24**	**68**	**16**	**13**	**13**	**7**	**62**
B#116	LVS-2012-001 Leaves	1	14	10	6	8	17	46	43	32	17	14	15	7	64
B#117	LVS-2012-001 Bone	1	14	10	6	8	17	46	43	32	17	14	15	7	64
B#118	LVS-2012-005 Nasal swab	1	14	10	6	8	17	46	43	32	17	14	15	7	64
B#119	LVS-2012-006 water	1	14	10	6	8	27	53	43	74	17	14	15	7	64
B#120	LVS-2012-007 Nasal swab	1	14	10	6	8	17	46	43	74	17	14	15	7	64
B#121	Roan2	1	14	10	6	8	17	46	43	32	17	14	15	7	64
B#122	LVS-2012-015 vulture faeces	1	14	16	7	8	19	47	45	36	17	14	15	7	64
B#123	LVS-2012-016 Elephant blood	1	14	10	6	8	17	46	43	32	17	14	15	7	64
B#124	LVS-2012-017	1	14	10	6	8	17	46	43	32	17	14	15	7	64
B#125	LVS-2012-018 PLET	1	14	10	6	8	17	46	43	74	17	14	15	7	64
B#126	LVS-2012-018 SBA	1	14	10	6	8	17	46	43	32	17	14	15	7	64
B#127	LVS-2012-022 Bone	1	13	10	7	8	17	46	44	32	17	6	19	7	64
B#128	LVS-2012-29 Grass	1	14	10	6	8	17	46	43	32	17	14	15	7	64
B#129	OR 12 10 26 K1 TSPBA	1	14	10	6	8	17	46	43	32	17	14	15	7	64
B#130	OR 12 10 26 K2 TSPBA	1	14	10	6	8	17	46	43	32	17	14	15	7	64
B#131	OR 12 10 26 K2 PLET	1	14	10	6	8	17	46	43	32	17	14	15	7	64
B#132	OR 12 10 26 K2 undiluted BTA	1	14	10	6	8	17	46	43	32	17	14	15	7	64
B#133	OR 12 10 27 H1	1	14	10	6	8	27	47	43	74	17	14	15	7	64
B#134	OH 12 10 29K soil	1	14	10	6	8	17	46	43	32	17	14	15	7	64
B#135	OH 12 10 29K kudu earth	1	13	10	7	8	19	46	44	32	17	14	15	7	64
B#136	Andrew D Buffalo Tissue	1	14	16	7	8	17	46	45	32	17	14	19	7	64
B#137	Andrew D Buffalo Tissue1	1	14	-2	7	8	17	46	45	32	17	14	15	7	65
B#138	Andrew D Buffalo Bone BTA	1	14	10	6	8	17	46	43	32	17	14	15	7	65
B#139	Andrew D Buffalo Bone PLET	1	13	10	5	8	17	46	25	32	17	14	15	7	64
B#140	Roan2 BTA	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#141	ROAN3 BTA	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#142	ROAN4	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#143	ROAN 5 BTA	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#144	ROAN 5 PLET	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#145	ROAN 6	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#146	ROAN 7 BTA	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#147	ROAN 10	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#148	ROAN 12 BONE	1	14	10	6	9	19	46	43	32	17	13	14	7	62
B#149	ROAN 13 PLET	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#150	ROAN 13 BTA	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#151	ROAN 14 PLET	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#152	ROAN 14 BTA	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#153	ROAN 15	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#154	ROAN 18	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#155	ROAN 20	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#156	ROAN 22 BTA	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#157	ROAN 22 PLET	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#158	ROAN 23 BTA	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#159	ROAN 23 PLET	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#160	ROAN 28 PLET	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#161	ROAN 30	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#162	ROAN 32	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#163	ROAN 34 PLET	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#164	ROAN 34 BTA	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#165	DS/2013/16	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#166	DS/2013/17	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#167	DS/2013/18	1	14	10	6	9	17	46	43	32	17	13	14	7	62
B#168	DS/2013/20	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#169	DS/2013/21	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#170	DS/2013/25	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#171	DS/2013/26	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#172	DS/2013/28	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#173	DS/2013/30	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#174	DS/2013/31	1	14	10	6	8	26	46	43	32	17	13	14	7	62
B#175	DS/2013/32	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#176	DS/2013/33	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#177	DS/2013/34	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#178	DS/2013/38	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#179	AX/2012/275	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#180	AX/2012/283	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#181	AX/2012/291	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#182	AX/2012/309	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#183	AX/2012/435	1	14	10	6	8	17	46	43	32	17	13	14	7	62
B#184	LAB ISOLATE	1	14	16	7	8	27	52	45	73	17	13	14	7	63
B#185	ELEPHANT TUSK	1	14	16	7	8	27	52	45	73	17	13	14	7	63
B#186	HIPPO TUSK	1	14	16	6	8	27	52	45	73	17	13	14	7	63
B#187	HIPPO FAT	1	14	16	7	8	27	52	45	73	17	13	14	7	63
		MULTIPLEX A								MULTIPLEX B					

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B

SAMPLE ID	ISOLATES	vrrA	BAMS 25	BAMS 21	BAMS24	Px01	Px02	BAMS51	BAMS 22	BAMS 23	BAMS 30	VNTR 19	VNTR12	VNTR35	VNTR 16	VNTR23	VNTR17
B#113	**STERNE**	**3**	**3**	**8**	**8**	**7**	**0**	**8**	**12**	**9**	**49**	**5**	**2**	**2**	**0**	**2**	**0**
B#114	**VOLLUM (A70)**	**2**	**3**	**8**	**8**	**8**	**5**	**8**	**12**	**9**	**69**	**3**	**4**	**2**	**7**	**1**	**10**
B#115	**AMES (A93)**	**3**	**3**	**8**	**8**	**5**	**6**	**8**	**12**	**9**	**56**	**3**	**4**	**2**	**7**	**3**	**3**
B#116	LVS-2012-001 Leaves	6	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#117	LVS-2012-001 Bone	6	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#118	LVS-2012-005 Nasal swab	7	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#119	LVS-2012-006 water	4	3	10	9	7	6	9	12	10	51	2	3	2	8	2	3
B#120	LVS-2012-007 Nasal swab	6	3	9	9	4	6	8	11	10	68	2	3	2	8	3	3
B#121	Roan2	6	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#122	LVS-2012-015 vulture faeces	6	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#123	LVS-2012-016 Elephant blood	6	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#124	LVS-2012-017	6	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#125	LVS-2012-018 PLET	6	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#126	LVS-2012-018 SBA	6	3	10	9	4	6	8	11	10	68	1	3	3	8	3	3
B#127	LVS-2012-022 Bone	6	3	10	9	4	6	9	11	10	68	2	3	2	8	3	3
B#128	LVS-2012-29 Grass	6	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#129	OR 12 10 26 K1 TSPBA	6	3	10	9	4	6	9	11	10	68	2	3	2	8	3	3
B#130	OR 12 10 26 K2 TSPBA	6	3	10	9	4	6	9	11	10	68	2	3	2	8	3	3
B#131	OR 12 10 26 K2 PLET	6	3	10	9	4	6	9	11	10	68	2	3	2	8	3	3
B#132	OR 12 10 26 K2 undiluted BTA	6	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#133	OR 12 10 27 H1	6	3	10	9	4	6	9	12	10	51	1	3	3	8	3	3
B#134	OH 12 10 29K soil	6	3	10	9	4	6	9	11	10	68	2	3	2	8	3	3
B#135	OH 12 10 29K kudu earth	6	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#136	Andrew D Buffalo Tissue	6	3	10	9	4	6	8	11	10	68	2	3	2	8	2	3
B#137	Andrew D Buffalo Tissue1	6	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#138	Andrew D Buffalo Bone BTA	4	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#139	Andrew D Buffalo Bone PLET	6	3	10	9	4	6	8	11	10	68	2	3	2	8	3	3
B#140	Roan2 BTA	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#141	ROAN3 BTA	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#142	ROAN4	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#143	ROAN 5 BTA	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#144	ROAN 5 PLET	4	3	9	8	4	4	7	11	9	67	2	3	2	8	3	3
B#145	ROAN 6	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#146	ROAN 7 BTA	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#147	ROAN 10	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#148	ROAN 12 BONE	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#149	ROAN 13 PLET	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#150	ROAN 13 BTA	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#151	ROAN 14 PLET	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#152	ROAN 14 BTA	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#153	ROAN 15	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#154	ROAN 18	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#155	ROAN 20	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#156	ROAN 22 BTA	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#157	ROAN 22 PLET	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#158	ROAN 23 BTA	4	3	10	8	4	4	8	11	9	49	2	3	2	8	3	3
B#159	ROAN 23 PLET	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#160	ROAN 28 PLET	3	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#161	ROAN 30	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#162	ROAN 32	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#163	ROAN 34 PLET	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#164	ROAN 34 BTA	4	3	10	8	4	4	7	11	9	67	2	3	2	8	3	3
B#165	DS/2013/16	4	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#166	DS/2013/17	4	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#167	DS/2013/18	4	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#168	DS/2013/20	4	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#169	DS/2013/21	4	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#170	DS/2013/25	4	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#171	DS/2013/26	4	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#172	DS/2013/28	3	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#173	DS/2013/30	4	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#174	DS/2013/31	4	3	10	8	4	4	8	11	9	67	2	3	2	8	3	3
B#175	DS/2013/32	4	3	10	8	4	4	8	11	9	67	2	3	2	7	2	2
B#176	DS/2013/33	4	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#177	DS/2013/34	4	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#178	DS/2013/38	4	3	10	8	4	10	8	11	9	67	2	3	2	7	3	3
B#179	AX/2012/275	4	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#180	AX/2012/283	4	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#181	AX/2012/291	4	3	10	8	0	4	8	11	9	67	2	3	2	8	1	3
B#182	AX/2012/309	4	3	9	8	4	4	8	11	9	67	2	3	2	8	1	3
B#183	AX/2012/435	3	3	10	8	4	4	8	11	9	67	2	3	2	8	1	3
B#184	LAB ISOLATE	2	3	9	8	7	0	8	12	9	49	1	3	3	8	1	3
B#185	ELEPHANT TUSK	2	3	9	8	7	0	8	12	9	49	1	3	3	8	2	3
B#186	HIPPO TUSK	2	3	9	8	7	0	8	12	9	49	1	3	3	8	2	3
B#187	HIPPO FAT	2	3	9	8	7	0	8	12	9	49	1	3	3	8	2	3
		MULTIPLEX C						MULTIPLEX D				M5			M6		M7

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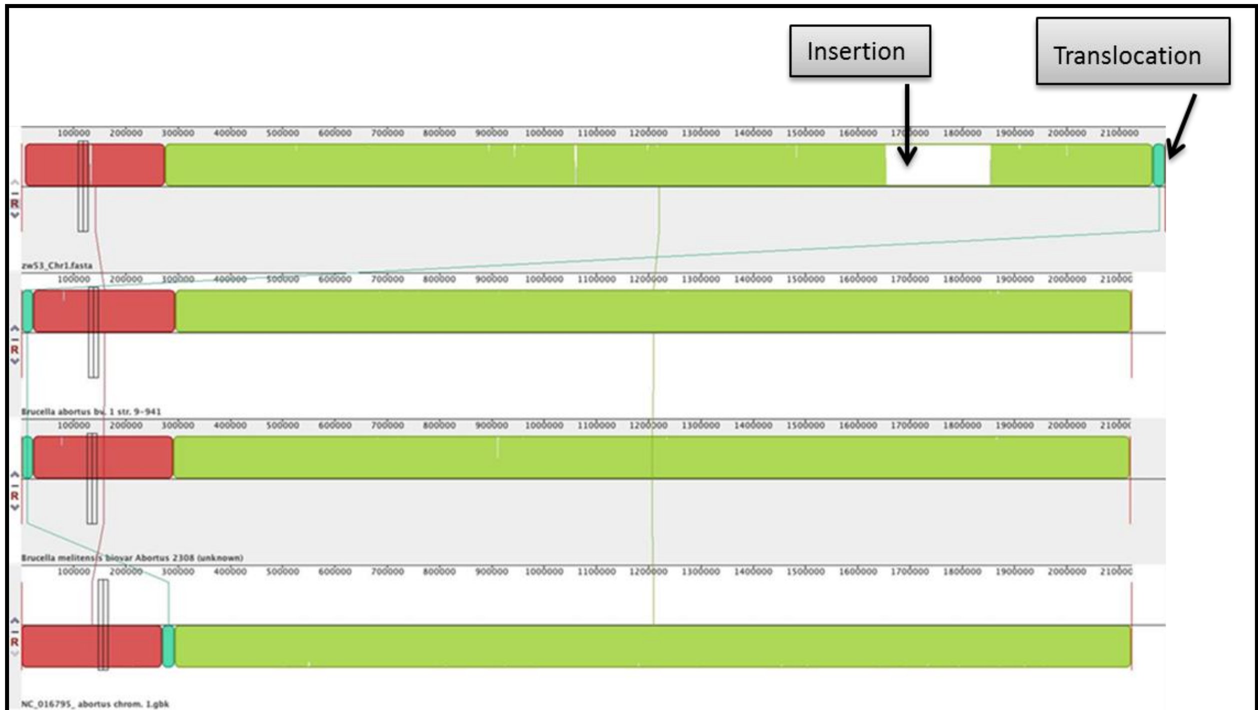
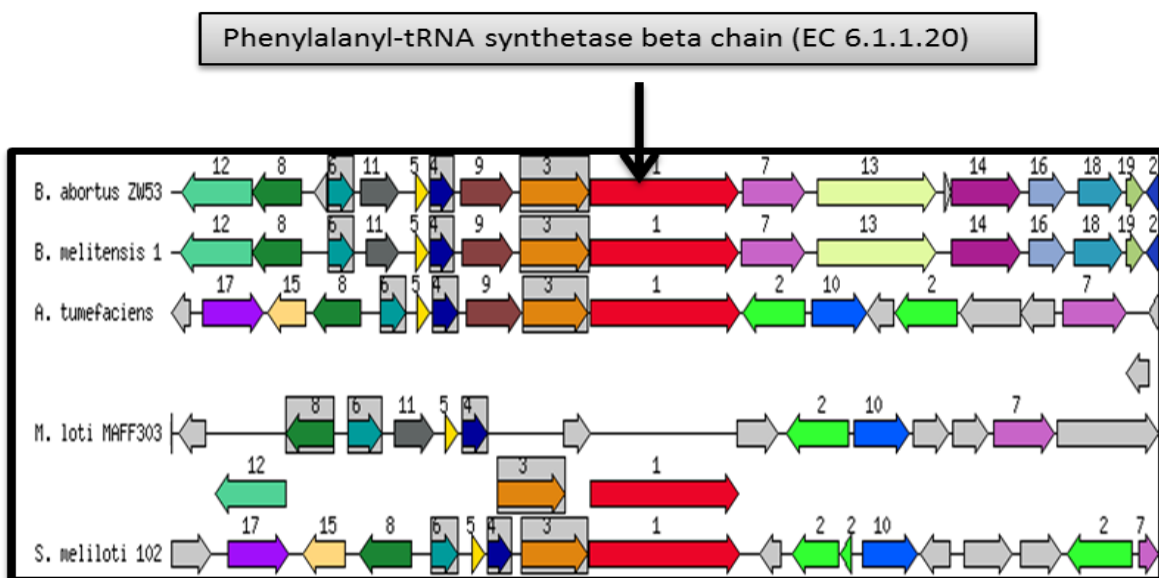


Appendix Figure 1: Mauve alignment image of the chromosome 1 of ZW053, *Brucella abortus* bv. 1 strain 9-941, *B. melitensis* bv. *abortus* 2083 *and B. abortus* A13334 showing an insertion and translocation at our focus sequence ZW053.



Appendix Figure 2: A comparison illustration from RAST Server (Aziz *et al.*, 2008) showing a ZW053 chromosome 2 gene, [fig/6666666.51019.peg.207] (in red) encoding phenylalanyl-tRNA synthetase beta chain (EC 6.1.1.20).

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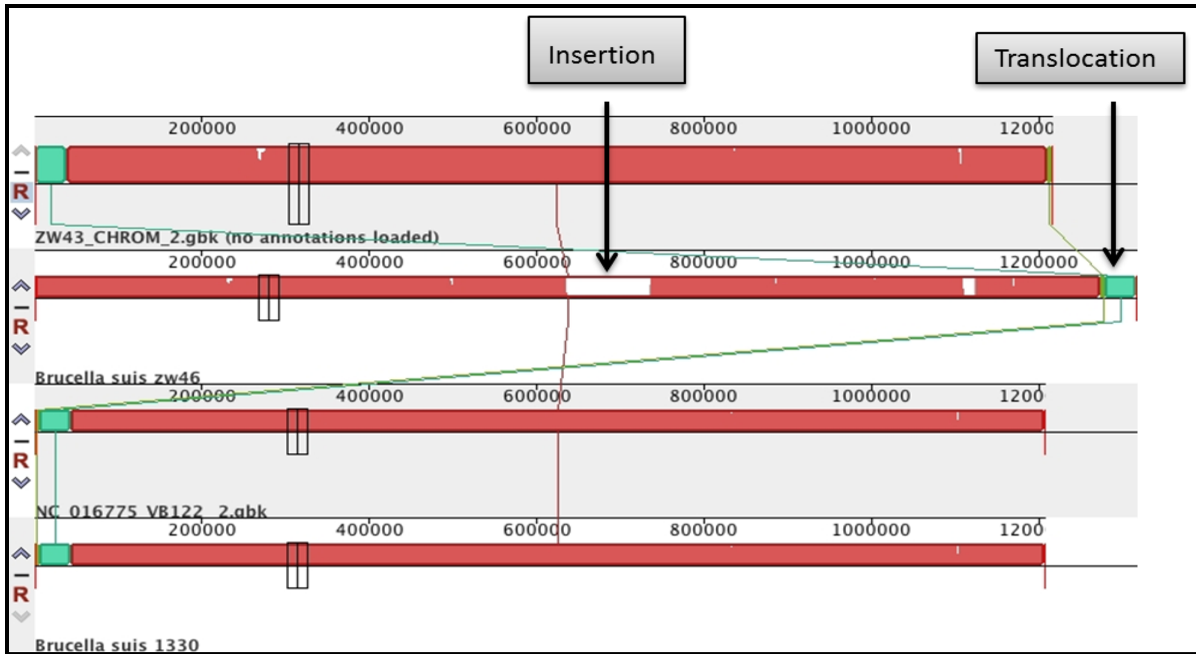
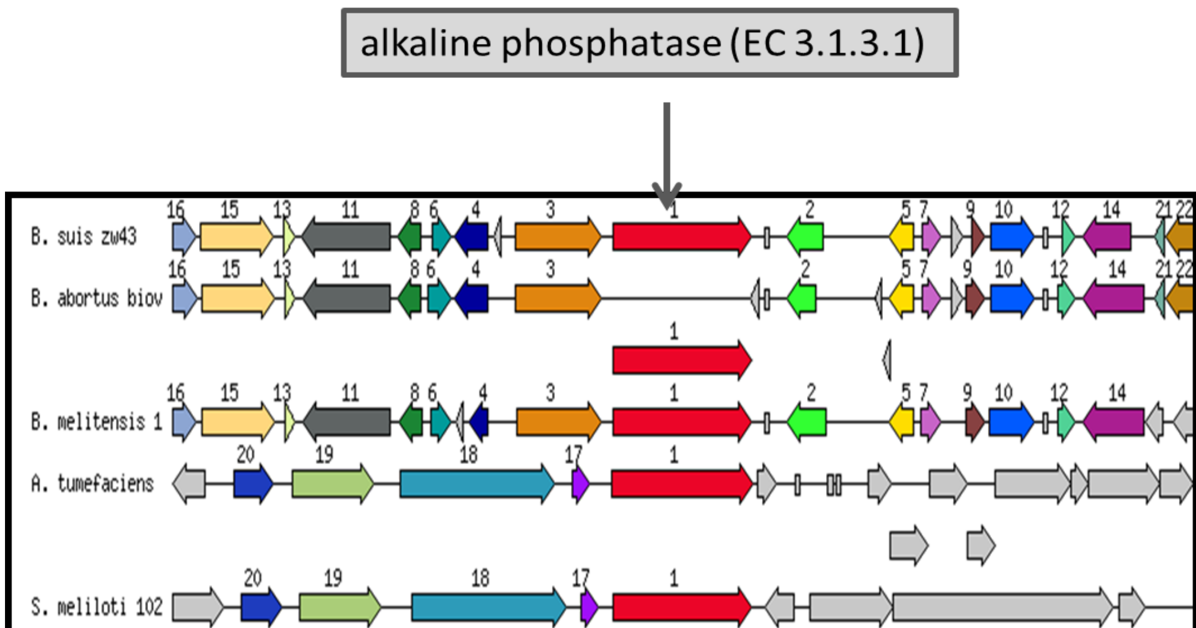


Appendix Figure 3: Mauve alignment image of ZW043, ZW046, *Brucella suis* 1330 and *B. suis* VB122 showing a translocation and insertion on ZW046 chromosome 2.



Appendix Figure 4: A comparison illustration from RAST Server (Aziz *et al.*, 2008) showing a ZW043 chromosome 1 gene, [fig/6666666.51002.peg.106] (in red) encoding alkaline phosphatase (EC 3.1.3.1).

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