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VALUABLE TOOLS FOR THE SURVEILLANCE OF ANTHRAX AND EQUINE PIROPLASMOSIS IN ZEBRA

MSc dissertation

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SUMMARY

Approximately 60% of all emerging infectious diseases in humans are zoonoses, with nearly 70% being linked with a wildlife origin. Due to movement of animals and people worldwide, environmental and climate change, human encroachment into natural habitats and domestication of wildlife species, the rate of wildlife-emerging infectious diseases is increasing globally in human and animal species.

There is currently a lack of adequate epidemiological reports from wildlife animals (*e.g.* zebra) compared to domestic animals. Improvement of surveillance tools is mandatory in understanding the epidemiological characteristics of wildlife infectious diseases, useful for timely decision-making and response during epidemic events.

Anthrax and equine piroplasmosis represent different important aspects of infectious diseases associated with zebra: anthrax is a highly deadly disease that can affect also humans, while equine piroplasmosis affects almost esclusively the equine industry and represents a threat for international movement of valuable horses.

Working in the Kruger National Park, a total of 40 serum samples were collected from wild plains zebras (*Equus quagga*) in the period October 2018 - February 2019. Then, an Enzyme Linked Immunosorbent Assay (ELISA) was adapted to find antibodies against protective antigen of anthrax toxin, while Reverse Line Blot (RLB) hybridization assays were performed for the search of equine piroplasmosis protozoans in zebra blood. Environmental data were acquired from the Geographic Information System (GIS) using qGIS software and finally analysed with ELISA and RLB results.

Anti-PA antibody titres were measurable in 62.5% of zebra samples, while 13% tested positive to *Theileria equi* and 0% to *Babesia caballi.*

Comparison with literature studies allowed to conclude that anti-PA ELISAs can reveal real epidemiological features of anthrax in zebra from KNP, whether the classic passive surveillance approach (microscopy on blood smears or bacterial culture from bone samples of animal carcasses suspected for anthrax) cannot. RLB species-specific probes may not be sensitive enough to catch all EP protozoans really present in samples analysed. GIS permitted to acquire a wide range of environmental variables although they were not found to be statistically significant mainly because of the paucity of samples collected.

RIASSUNTO

Circa il 60% delle malattie infettive emergenti che coinvolgono la popolazione umana sono zoonosi, delle quali più di un terzo si è originato da animali selvatici. A causa del movimento di animali e persone in tutto il mondo, del cambiamento climatico e ambientale, dell'espansione delle attività umane in habitat naturali e della domesticazione di specie selvatiche, il tasso di malattie infettive emergenti derivanti da animali selvatici sta aumentando globalmente nelle specie umane ed animali. Attualmente si registra una mancanza di studi epidemiologici adeguati su animali selvatici, come la zebra, diversamente da quello che accade per gli animali domestici. E' necessario implementare gli strumenti di sorveglianza al fine di comprendere le caratteristiche epidemiologiche delle malattie infettive legate agli animali selvatici, utili per formulare decisioni e risposte tempestive durante eventi epidemici. L'antrace e la piroplasmosi equina sono malattie infettive associate alla zebra, ognuna aventi aspetti caratteristici e distinti: l'antrace è una malattia altamente letale che può contagiare anche l'uomo, mentre la piroplasmosi equina colpisce quasi esclusivamente l'industria equina e costituisce una minaccia per lo spostamento internazionale di cavalli di valore.

All'interno del Kruger National Park sono stati raccolti 40 campioni ematici da popolazioni selvatiche di zebra della pianura (*Equus quagga*) nel periodo ottobre 2018 - febbraio 2019. In seguito, un ELISA (Enzyme Linked Immunosorbent Assay) è stato adattato per la ricerca di anticorpi contro l'antigene protettivo (PA) della tossina di antrace, mentre saggi di ibridazione RLB (Reverse Line Blot) sono stato eseguiti per trovare i parassiti protozoi della piroplasmosi equina nel siero di zebra. Alcuni dati ambientali sono stati estratti da sistemi informativi geografici (GIS) usando il software qGIS e analizzati infine con i risultati dei test ELISA e RLB. Il 62,5% dei campioni di zebra è risultato avere titoli anticorpali anti-PA misurabili, mentre il 13% è risultato positivo alla presenza di *Theileria equi* e lo 0% alla presenza di *Babesia caballi.* Il confronto con gli studi di letteratura ha permesso di concludere che la metodica ELISA per la ricerca di anticorpi anti-PA, al contrario del classico approccio di sorveglianza passiva (che prevede la microscopia su strisci ematici o la coltura batterica su campioni di ossa provenienti da carcasse animali sospettate di antrace), può svelare le reali caratteristiche epidemiologiche dell'antrace in zebre del Kruger National Park. Le sonde specie-specifiche della metodica RLB potrebbero non essere abbastanza sensibili per il rilevamento di tutti i parassiti della piroplasmosi equina realmente presenti nei campioni analizzati. Il numero limitato di campioni raccolti non ha permesso di evidenziare correlazioni statisticamente significative con i dati ambientali estratti dal GIS.

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1 PREFACE

Wildlife associated pathogens are capable of affecting directly or indirectly both domestic animals (livestock) and human health (Rhyan & Spraker, 2010). Approximately 60% of all emerging infectious diseases in humans are zoonoses, with nearly 70% being linked with a wildlife origin (Jones et al., 2008). Due to movement of animals and people worldwide, environmental and climate change, human encroachment into natural habitats and domestication of wildlife species, the rate of wildlife-emerging infectious diseases is increasing globally in human and animal species (Jones et al., 2008; King et al., 2006; Cohen, 2000).

Viral pathogens (such as West Nile virus, avian influenza virus, Hendra virus, Nipah virus, Hantavirus, Ebolavirus and Marburg virus), bacterial pathogens (like *Borrelia burgdoferi* of Lyme disease) and protozoan pathogens (such as *Trypanosoma* spp*)* have been demonstrated to infect humans starting from wildlife reservoirs (Cleaveland et al., 2005). Indeed, Human Immunodeficiency Virus 1 (HIV-1) (Gao et al., 1999) and 2 (HIV-2) (Hirsch et al., 1989) are also thought to have derived from wildlife hosts, in addition to the more recent Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (Markotter et al., 2020) and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) (Zhou et al., 2020). Emerging wildlife diseases such as rinderpest, foot and mouth disease, African swine fever, theileriosis, brucellosis and bovine tuberculosis (BTB), etc. have affected livestock animals, causing huge economic losses and restrictions in national and international trade (Cleaveland et al., 2005). Furthermore, wildlife infectious diseases such as canine distemper killing the highly endangered African wild dog (*Lycaon pictus*) populations (van de Bildt et al., 2002) may elicit a decline in already endangered wildlife populations, worsening their conservation status (Grogan et al., 2014).

Unfortunately, there is currently a lack of adequate epidemiological reports from wildlife animals (*e.g.* zebra) compared to domestic animals, which are of greater agricultural and recreational importance. It is much easier to obtain samples from domestic animals than wildlife, mainly due to complications encountered in securing permits to handle wild animals as they have to be darted (which is also an expensive exercise), and to the paucity of experienced and adequately-funded veterinary staff (Onyiche et al., 2019; Cleaveland et al., 2005). Therefore, investigating infectious diseases that occur in domesticated animals with closely related wildlife species might provide insight into possible wildlife infectious diseases. Improvement of a systematic and standardized surveillance, both passive and active, is mandatory in understanding the epidemiological characteristics of wildlife infectious diseases,

useful for timely decision-making and response during epidemic events (Grogan et al., 2014; Vrbova et al., 2010).

Zebras are members of the African equids/horse family (genus *Equus spp.,* family *Equidae,* order *Perissodactyla)* identified by their distinctive black-and-white striped coats and, unlike their closest relatives horses and donkeys, zebras have never been domesticated. Spatial and taxonomic representation of zebra species is needed to better understand the ecology of these animals and related infectious diseases. The taxonomy of zebra, proposed by Groves and Bell (2004) and later revised by Moodley and Harley (2005), describes three species of zebra that all live in sub-Saharan Africa: Grevy's zebra (*Equus grevy,* subgenus *Dolichohippus*), the plains or Burchell's zebra (*E. quagga,* subgenus *Hippotigris*), and the Mountain zebra (*E. zebra,* subgenus *Hippotigris*) that includes the subspecies Cape Mountain zebra (*E. zebra zebra*) and Hartmann's zebra (*E. zebra hartmannae*). Grevy's zebra (~2000 mature individuals) lives in arid and semiarid grasslands and shrublands of northern Kenya and a few areas of southern Ethiopia (Rubenstein et al 2016). The plains zebra counts 150.000-250.000 mature individuals distributed in Somalia, Tanzania, Zambia, Mozambique, southern Angola, Katanga Province of Zaire and South Africa (King & Moehlman, 2016). The Mountain zebra population consists of nearly 35.000 mature individuals: Hartmann's zebra subpopulation has a distribution range in Namibia and Angola regions (Gosling et al., 2018), while the Cape mountain zebra subspecies lives in the Eastern and Western Cape Province of South Africa (Van Dyk et al., 2009) (Figure 1).

The World Conservation Union (IUCN) Red List classifies Mountain zebra as vulnerable (Gosling et al., 2018) and Grevy's zebra as endangered (Rubenstein et al., 2016). The Convention on International Trade in Endangered Species (CITES) lists these species in Appendix II and I, respectively.

Few diseases have been demonstrated to affect wild zebras. These include: five viral diseases (African horse sickness, equine encephalosis, equine herpesvirus diseases, equine viral arteritis and equine sarcoid), one bacterial disease (anthrax) and two protozoan diseases (equine piroplasmosis and equine trypanosomosis).

This Thesis, which has been developed with the support of the University of Pretoria, focuses on anthrax and equine piroplasmosis, both representing different important aspects of wildlife infectious diseases associated with zebra. Indeed, while anthrax concerns more for the public health issue, equine piroplasmosis is highly dangerous for the equine industry and a threat for international movement of valuable horses. Thus, it results mandatory to monitor these diseases in wildlife and related reservoirs, in order to prevent outbreaks from the source. In this pursuit,

systematic and focused surveillance systems represent the most useful instrument to track epidemiology progress of relevant infectious diseases in wildlife.

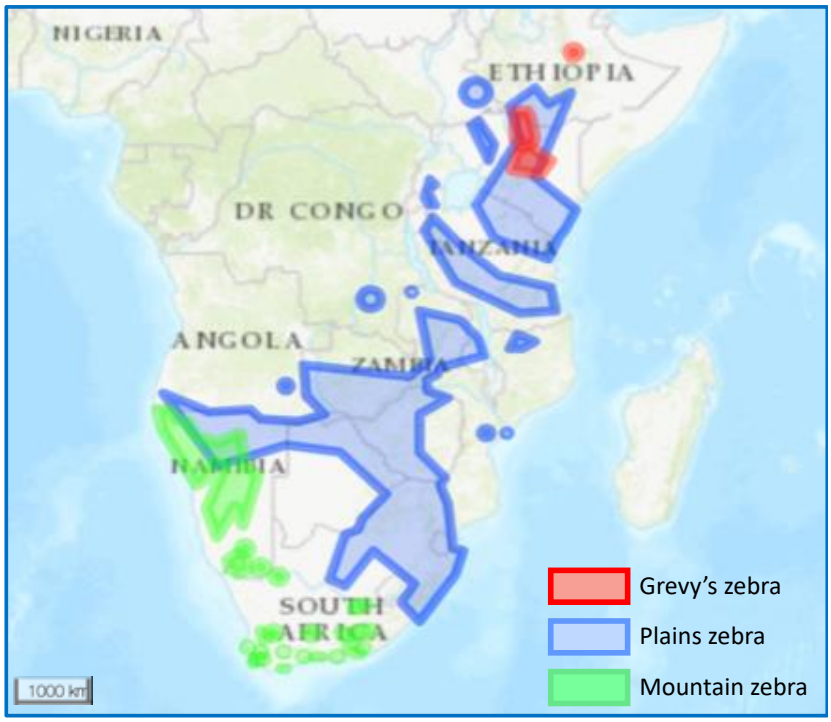

Figure 1. Geographic distribution of zebra populations of the Grevy's zebra (Equus grevy), plains or Burchell's zebra (*E. quagga*), and the mountain zebra (*E. zebra*) in central and southern Africa, modified from "IUCN (International Union for Conservation of Nature) 2016. *Equus quagga*. The IUCN Red List of Threatened Species. Version 2020-1". MapMaker NatGeo interactive: (https://mapmaker.nationalgeographic.org/dJQMXpxywvTPGlW8w82YMQ//?edit=grBhEZt0kESLYPoWGmdq1).

Therefore, activities have been carried out at two different levels:

I. Literature review regarding general aspects of anthrax and equine piroplasmosis: approximately 100+ papers from international literature have been thoroughly scrutined.

II. Experimental activities that have been organized in three subsequent steps: elaboration of the literature studies conducted on zebra; field activity regarding sampling and laboratory diagnosis; data analysis to elaborate laboratory data results and to correlate them with intrinsic/extrinsic factors. A further experimental activity planned at The Department of Veterinary Tropical Diseases Molecular Laboratory (University of Pretoria), consisting on sequencing of the 18S rRNA gene of *Theileria equi*, did not occur due to the pandemic COVID-19 travel restrictions.

FIRST PART: OVERVIEW

2 CHARACTERISTICS OF ANTHRAX

2.1 Etiology

Anthrax is caused by a Gram-positive, soil-borne, non-motile aerobic bacterium known as *Bacillus anthracis*. All bacteria of the genus *Bacillus* are aerobic and spore-bearing, although each species features markedly different properties. The vegetative form of *B. anthracis* has a rod-shaped morphology, is 1.0-1.5 x 3.0-10.0 µm in size, and is surrounded by a well-developed capsule. The spores are oval, approximately 1 x 2 µm in size, and are formed equatorially without causing sporangium deflection (Turnbull, 2008). These latter characteristics indicate *B. anthracis* as belonging to the morphological group 1 of *Bacillus* species (Gordon et al., 1973). *Bacillus anthracis* has two plasmids, pXO1 and pXO2 which contains the virulent factors. The virulence factors on pX01 and pX02 encodes the exotoxins and the antiphagocytic poly-D-glutamic acid capsule, respectively (Read et al., 2003). The cell binding protein named "protective antigen" is the main component of the anthrax exotoxin. It is cleaved and activated after binding with a receptor of the host cell, thus permitting the translocation of either lethal factor (LF, lef gene, 90 kDa) and oedema factor (EF, cya gene, 89 kDa) into the cytosol (Petosa et al., 1997).

2.2 Geographic distribution

Anthrax is an OIE listed disease and must be reported to the OIE as indicated in its Terrestrial Animal Health Code (OIE, 2019). Anthrax outbreaks are of concern for animal conservation, wildlife management, agriculture and public health. *B. anthracis* has been found on every inhabited continent, as well as several islands including Haiti and parts of the Philippines and Indonesia. Worldwide, an estimated 20,000 to 100,000 human cases of anthrax occur annually, mostly in poor rural areas (Carlson et al., 2019). Since hervivorous livestock and wild animals are more susceptible to *B. anthracis* than humans, high-risk areas for human disease may be identified pre-emptively by review of veterinary surveillance information by veterinary or public health authorities (Turnbull, 2008). However, surveillance tools are not well developed in most countries and anthrax global distribution is considered underrepresented (Turnbull et

al., 1998). In a recent study, Carlson and coworkers, using an extensive ecological niche modelling (ENM), estimated that 1.8 billion people live within anthrax-suitable areas, the vast majority of whom live in rural areas in Africa (102 millions in sub-Saharan Africa), Europe and Asia, and that areas of anthrax risk contain 1.1 billion livestock (72,8 millions in sub-Saharan Africa) (Carlson et al., 2019). Throughout sub-Saharan Africa, especially in arid and semi-arid lands, there is an endemic pattern of anthrax, with sporadic epidemics, that is thought to remain like this in the near future. Additionally, in South Africa the northern part (Pafuri) of Kruger National Park and some areas of the Northern Cape Province, such as Kimberley and Kgalagadi Transfrontier Park areas, are considered endemic with sporadic outbreaks (De Vos & Turnbull, 2004). The same happens in other African wildlife conservation areas, such as the Queen Elizabeth National Park and Lake Mburo National Park (Uganda) (Wafula, Patrick & Charles, 2008), the Selous National Reserve and Serengeti National Park (Tanzania) (Lembo et al .,2011), the Luangwa Valley (Zambia) (Turnbull et al.,1991), the Omo-Mago National Park (Ethiopia) (Shiferaw et al, 2002) and Etosha National Park (Namibia) (Lindeque & Turnbull., 1994).

In a number of endemic areas, various susceptible exotic species are now raised in semidomesticated environments for hunting. For example, a large enzootic of anthrax occurred among domesticated white-tailed deer and exotic ungulate species in central Texas during 2001. Presumably further examples exist in Africa and probably other regions of the world where anthrax is endemic. Since these animals cannot be mass-vaccinated, they represent susceptible populations and foci of persisting disease in these regions (Yu et al., 2002).

2.3 Host range

Most warm-blooded vertebrates are susceptible to *B. anthracis* infection. Herbivores are the most affected, while carnivores and omnivores are somewhat resistant to the infection (Turnbull, 2008). Cold-blooded animals (amphibians and reptiles) must be warmed while birds may have to be cooled to become susceptible to anthrax (Sterne, 1959).

The resistance to the infection and the susceptibility to lethal toxin appears to be based on different mechanisms. Innate resistance has been attributed to the inhibition of the initial germination and/or multiplication of the bacterium (De Vos & Turnbull, 2004; Turnbull, 2008). Indeed, two groups may be listed: animals highly susceptible to the infection (*e.g.* ruminants, horse, chimpanzee, mice, guinea pig) thus presenting high septicaemia values at their death, but quite resistant to the toxin; animals resistant to the infection (*e.g.* dog, pig, rat) but highly

susceptible to the toxin effects once the infection is established. These species develop a low terminal septicaemia (Lincoln et al., 1967).

In southern Africa, at least 50 wild species have died due to anthrax (De Vos, 1990; Lindeque & Turnbull, 1994; Turnbull, 2008). Predators and scavengers , i.e. hyaenas, jackals, lions and vultures, are known to disseminate *B. anthracis* either by opening and moving carcasses from their original place to other locations or defecating spores ingested with animal tissues (De Vos & Turnbull, 2004). Vultures are thought to allow tracing back to outbreaks in neighbouring countries that fall within their flight radius (Hugh-Jones and De Vos, 2002). The high seroprevalence in buffalo demonstrated in the Serengeti by Lembo et al. (2011) suggests that this species may be a potential indicator species, thus useful for anthrax sero-surveillance (Lembo et al., 2011). Moreover, domestic dogs have been shown to be useful indicators of anthrax risk in wildlife and livestock (Lembo et al., 2011; Hampson et al., 2011; Mukarati et al., 2018).

Humans can be naturally infected through direct or indirect contact with infected animals, or by occupational exposure to infected or contaminated animal products. However, human is considered a moderately resistant host. The exceptions to this rule are biological warfare of terrorism, or laboratory settings where the spores of *B. anthracis* may be manipulated and concentrated in an unnatural way to represent a direct threat to human health through respiratory, gastrointestinal or cutaneous exposure (Turnbull, 2008).

2.4 Transmission factors

Anthrax is considered a non-contagious disease. The main source of infection is generally provided by the spores shed by animals' dead from anthrax. *B. anthracis* spores are highly resistant in soil, thus increasing the risk of inhalation/ingestion of spores by grazers such as zebra (Turnbull, 2008). Alkaline soils rich in calcium and organic matter with an ambient temperature above 15.5 °C are considered optimal for persistence of spores, and anthrax is usually endemic in these regions (Steenkamp et al, 2018).

Ingestion of *B. anthracis* spores is believed to be a frequent mode of uptake in grazing animals, but only if an existing orogastrointestinal lesion initiate the infection (Turnbull, 2008).

Spores may alternatively be inhaled by animals grazing over dry and dusty sites where other animals died of anthrax in the past. Inhaled spores may remain in ungerminated form in the lungs for months after uptake, developing a chronic pattern in infected animals (Turnbull, 2008).

Many insects have been linked to *B. anthracis* as mechanical vectors, including horse flies (*Tabanidae*), common housefly (*Musca domestica*), mosquitos (*Aedes aegypti* and *Aedes taeniorrhyncus*), bottle flies (*Calliphora vecina*) and ticks (Fasanella et al., 2010; Hugh-Jones & De Vos, 2002; Von terzi et al., 2014). Non-biting blowflies (especially *Chrysomya marginalis* and *Chrysomya albiceps*) are also able to spread spores from infected carcasses to the surrounding vegetation, increasing the risk of infection for browsing animals, especially greater kudu (*Tragelaphus strepsiceros*), through ingestion of contaminated twigs and leaves (Braack & De Vos, 1990; De Vos & Braack, 1987; Basson et al., 2018; Hugh-Jones & De Vos, 2002). Direct transmission from infected animal to others is believed to occur to an insignificant extent, excluding carnivores that feed on other victims of the disease (Turnbull, 2008).

2.5 Pathogenesis

As soon as spores enter the host they start to germinate into vegetative cells that produce toxin. Bacteria are then transported via the lymphatics to regional lymph nodes where they continue to multiplicate and finally enter into the blood stream. The antiphagocytic poly-D-glutamate capsule produced by *B. anthracis* allows the bacterium to evade the immune system of the host. Lesions are caused by two exotoxins produced by vegetative cells: the lethal toxin is made up of protective antigen (PA) and lethal factor (LF), while oedema toxin is made up of PA in combination with oedema factor (EF). Lethal factor acts by releasing shock-inducing cytokines *i.e.* tumour necrotic factor (TNF) and interleukin-1 (IL-1) (Milne et al., 1994). Oedema factor alters movements of water and ions causing the characteristic oedema of anthrax (Leppla, 1995).

2.5.1 Clinical signs

Incubation period varies considerably and depends on virulence of the organisms, resistance of the animal, the route of infection and the number of infecting organisms. In naturally-infected cattle it may range from 1 to 14 days (Hugh-Jones & De Vos, 2002). OIE incubation period for trade purposes is set to be 20 days (OIE, 2019).

Anthrax in animals occurs in three different clinical manifestations. The hyperacute or apoplectic form usually lasts less than two hours and animals are normally found dead without premonitory clinical signs. Animals close to death show opisthotonus with the forelegs rigidly extended. The acute form usually lasts less than 72 hours. Oedematous swellings may appear throughout the body, in particular tongue, throat and ventral parts of thorax, abdomen and perineum (McConnell et al., 1972; De Vos & Turnbull, 2004). The subacute to chronic form

persists for more than three days up until recovery or death. Oedematous swellings affect the face, throat and neck following primary infection of the pharynx, pharyngeal tissues and regional lymph nodes (Turnbull et al., 1992; Ferguson, 1981). Captive and wild ruminants normally develop a hyperacute form of anthrax, while equids (horses, donkeys, zebra) contract acute anthrax. Omnivores, such as pigs, carnivores and other animals resistant to the infection usually contract subacute/chronic anthrax (Turnbull, 2008).

Anthrax in humans manifests in one of three clinical manifestations: cutaneous, pulmonary or intestinal. Meningitis and septicaemia may develop during the course of anyone of these. Cutaneous form is the most observed and infection occurs through abraded skin or by insect bite (Kobuch et al., 1990; Turnbull, 2008).

2.5.2 Lesions

In ruminants, necropsy findings include rapid post-mortem decomposition of the carcass, incomplete development of *rigor mortis*, presence of dark-red poorly-clotted blood from natural body openings (nose, mouth and anus), petechiae and ecchymoses throughout the carcass, pulmonary oedema, effusions in serosal cavities and enlarged pulpy spleen hence suggested by the Afrikaans name “miltsieke” (Jackson et al., 1957; Turnbull, 2008). Pigs may contract septicemic, pharyngeal or intestinal forms of the disease (Turnbull, 2008). Equids are affected by septicemic anthrax with lesions comprising oedema, haemorrhage and necrosis. In case of insect transmission, horses also manifest localized swellings at biting sites (De Vos, 1990). Carnivores display severe oedema of soft tissues of head, tongue and throat, stomach and intestine (Turnbull, 2008).

2.6 Diagnostics

Mortality data may underestimate the occurrence and spatial extent of outbreaks (Fasanella et al, 2010; Lembo et al, 2011). Antemortem screening methods are needed in combination with post-mortem approaches in order to provide a clearer picture of exposure and survival amongst affected host populations (Bagamian et al, 2013).

2.6.1 Pathogen identification

Suspect anthrax cases are normally first detected using strained (polychrome methylene blue or Wright's and Giemsa) blood smears of recently deceased animals and examined for bamboo rod shaped bacilli and capsule microscopically. Suspect anthrax is confirmed by submitting samples (blood, mesenteric fluid, other oedematous fluid) to a laboratory for culture, isolation and identification. Polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar is the most

successful selective medium for *B. anthracis.* Inoculation in guinea pig or mouse should be avoided for the purpose of animal welfare, but is considered a sensitive method for isolation of *B. anthracis* and therefore may be required in some situations (*e.g.* animals treated with antibiotics). Further tests such as motility, gamma-phage lysis, or penicillin sensitivity tests may be performed to confirm identification of *B. anthracis* (Turnbull et al., 1998). RT-PCR is the method of choice to identify virulent *B. anthracis* strains, and for the differentiation of non-virulent strains, by confirming the presence of genes on both plasmids, pXO1 (toxins like *pag, lef* and *cya* genes) and pXO2 (capsule (*cap)* genes) (Turnbull et al., 1998).

2.6.2 Antibody identification

Anthrax ELISAs are typically designed to detect antibody to a component of anthrax toxin in serum. Four indirect ELISAs have been used on wildlife samples: the competitive inhibition indirect ELISA assay against PA and LF antigens (Turnbull et al., 1986); indirect anti-PA ELISA (Hahn et al. 2004) the QuickELISA Anthrax PA kit commercialized by Immunetics (Hampson et al., 2011; Lembo et al, 2011); and, finally, two assays specific to white-tailed (*Odocoileus virgilianus*) and red deer (*Cervus elaphus*) (Peterson et al., 1993; Fasanella et al., 2007). The competitive inhibition indirect ELISA against PA and LF and indirect ELISA against PA are not commercially available hence researchers often re-establish this assay in their laboratory and need to re-evaluate and validate the test for the wildlife species of interest. The Immunetics QuickELISA uses recombinant technology to detect PA antibody independently of Ig-subtype and host species. This is a promising approach for anthrax surveillance, particularly as the assay allows cross-species comparisons. However, the assay was designed for use on human samples, and wildlife testing is an off-label use (Bagamian et al., 2013). In contrast to sampling of suspected carcasses, serosurveillance of living animals poses no risk for anthrax infection and therefore offers an opportunity to gain a better understanding of anthrax epidemiology, particularly in relation to patterns of infection and risk factors for exposure, susceptibility, and death (Lembo et al., 2011).

3 CHARACTERISTICS OF EQUINE PIROPLASMOSIS

3.1 Etiology

The haemoprotozoan parasites, *Theileria equi* and *Babesia caballi* are responsible for the development of an infectious, tick-borne disease of equids, called equine piroplamosis (EP) (Melhorn and Schein, 1998; Nuttal and Strickland, 1910). Since its initial discovery in 1901 in South Africa (Laveran, 1901), the taxonomy of *Piroplasma equi* (later reclassified as *Babesia equi*) has remained controversial. It was subsequently discovered that two morphologically distinct parasites could be identified in horses suffering from what was then known as biliary fever and the name *Piroplasma caballi* was given to the larger parasite (Koch, 1904). Both parasites belong to the phylum Apicomplexa and are classified in the family Piroplasmida (Levine, 1985). However, only *B. caballi* is regarded as a true "*Babesia*" species, as the discovery of schizogony in the lymphocytes of horses led to the reclassification of *B. equi* as *Theileria equi* (Melhorn and Schein, 1998). Recently, whole genome sequence analysis of the *T. equi* Florida strain suggested the placement of this parasite in a new genus might be more appropriate (Kappmeyer et al., 2012).

Substantial genetic diversity within *T. equi* and *B. caballi* 18S rRNA gene sequences has also been observed globally, leading to the identification of as many as five *T. equi* parasite genotypes (A-E) and three *B. caballi* genotypes (Bhoora et al., 2020; Qablan et al., 2013). Additionally, a novel *Theileria* species, named *Theileria haneyi* was identified in an infected horse at the U.S.-Mexico border (Knowles et al., 2018). Comparative genome analysis indicated that *T. haneyi* is phylogenetically distinct from *T. equi* (Knowles et al., 2018).

These two piroplasm genera are usually distinguished from each other based on the lack of a pre-erythrocytic cycle in *Babesia* and the absence of transovarial transmission in *Theileria*. Morphologically *Theileria* (*T. equi/ T. haneyi*) merozoites divide into four pyriform parasites within the erythrocytes forming the "Maltese cross". *Theileria equi* merozoites also appear larger in mean length compared to *T. haneyi* merozoites (Knowles et al., 2018). The larger *B. caballi* merozoites form two oval or pyriform pairs that can either undergo transovarian transmission inside the tick vector or be transmitted trans-stadially. Cross-immunity between parasites does not occur. An equid can be infected with either one or all three pathogens simultaneously (Onyiche et al., 2019; Bhoora et al., 2020).

Parasitemias for *B. caballi* infections range between 0.1% to 1% (de Waal; 1992) while for *T. equi* infections, parasitaemias between 1% to 7% are more common (Friedhoff & Soule, 1996). Once infected with *T. equi*, horses remain carriers for life, regardless of whether clinical

signs resolve naturally or following drug treatment (Rothschild, 2013). Infection with *B. caballi* is self-limiting and may last up to 4 years after infection. However, many horses that recover from *B. caballi* infection later relapse, suggesting a temporary state in which organisms cannot be detected despite possible lifelong infection (Rothschild, 2013).

3.2 Geographic distribution

EP is considered to be a disease of international importance and therefore all cases are reportable to the World Organisation for Animal Health (OIE: Office International des Epizooties). The disease has a worldwide distribution that coincides with the geographic location and seasonal activity of competent tick vectors (Scoles and Ueti, 2005). Currently, only Australia, Canada, Great Britain, Ireland, Japan and New Zealand, are considered EP-free (Scoles & Ueti, 2015). Endemic regions include Africa, Asia, the Middle East, South and Central America and Southern Europe (OIE, 2009). Nearly 90% of the equine domestic population is thought to live in endemic areas (Scoles and Ueti, 2015).

In endemic countries, economic losses associated with EP include the cost of treatment, particularly in acutely infected horses, abortions, loss of performance, death, and restrictions related to the international movement for participation in equestrian sporting events (Rothschild, 2013). Factors that contribute to the spread of these parasites to regions previously considered free of infection include increased international movement of horses, development of tick populations with increased resistance to chemical acaricides, inadequate veterinary care, and climate and land usage changes that expand the habitat range of tick vectors (Scoles & Ueti, 2015). Introduction of infected ticks by wild and zoo or other exotic animals is also a threat that is even more challenging to regulate (Rothschild, 2013).

3.3 Host range

All equid species are susceptible to EP *i.e.* horses, donkeys, mules and zebras (OIE, 2009). Investigators in Europe, South Africa and Nigeria have found dogs to be carriers of *T. equi* and *B. caballi* (Beck et al. 2009; Fritz, 2010; Rosa et al., 2014; Adamu et al., 2014). *Theileria equi* nucleic acids have also been reported in sheep, goats, cows, South American tapirs (*Tapirus terrestris*) and South American rodents (*Thrichomys foster*) (Spickler, 2018). Moreover, organisms potentially related to *T. equi* have been observed in coati (*Nasua nasua*) (de Sousa et al., 2018) waterbuck (*Kobus defassa*) (Githaka et al., 2014) and a Malayan tapir (*Tapirus indicus*) (Spickler, 2018).

3.4 Transmission factors

EP is a tick-borne protozoal disease transmitted mainly via ixodid ticks (hard ticks or scale ticks). Six ixodid genera (*i.e. Hyalomma, Rhipicephalus, Dermacentor, Ixodes, Haemaphysalis* and *Amblyomma*) and 33 ixodid species have been listed as biologically vectors of EP. Important vectors in sub-Saharan Africa capable of transmitting EP to equids are: *Hyalomma truncatum* (*B. caballi)* reported in Namibia and South Africa; *Rhipicephalus evertsi evertsi* (*B. caballi* and *T. equi*) more common on the wetter eastern side of sub-Saharan Africa; *Rhipicephalus evertsi mimeticus (T. equi)* more common on the drier western side of the continent, especially Namibia; and *Rhipicephalus pulchellus* (*T. equi*), also known as zebra tick, but there is only weak microscopic evidence that this tick can be infected with *T. equi* (Scoles & Ueti, 2015; Brocklesby, 1965). Mechanical or iatrogenic transmission may occur through contaminated needles and syringes, blood transfusion, and surgical instruments (Onyiche et al., 2019). Chronically infected horses that serve as blood donors can also transmit the disease to naïve animals (Wise et al., 2013). Transplacental transmission from pregnant mares to the foetus has been reported, in most cases leading to abortion (Sant et al., 2016).

3.5 Pathogenesis

3.5.1 Life cycle

Life cycle of Apicomplexa normally includes three stages: asexual reproduction stage in tick salivary glands (sporogony), asexual reproduction stage in the vertebrate host (merogony), and sexual reproduction with the formation and fusion of gametes in the tick gut (gamogony). *Theileria equi*, however, has an additional asexual replication stage (schizogony) in the peripheral blood mononuclear cells (PBMC) of the equine host (Melhorn & Schein, 1998).

Theileria equi sporozoites are inoculated into the host via a feeding tick. Sporozoites invade the lymphocytes and develop into *Theileria*-like schizonts; merozoites released from these schizonts invade red blood cells (RBCs) and transform into trophozoites, which grow and divide into pear-shaped tetrad (‘Maltese cross’) merozoites. Haemolysis of infected erythrocytes leads to the release of merozoites that enter new red blood cells and continue the cycle of replication. Some merozoites transform into rings believed to be gamonts. Following ingestion of the gametocytes in the erythrocytes by a competent tick vector, fusion of micro and macrogametocytes lead to the formation of zygotes that invade salivary glands where sporogony starts again (OIE, 2009; Onyiche et al., 2019). *Theileria equi* is transmitted either trans-stadially or intra-stadially (Scoles & Ueti, 2015). In contrast, *B. caballi* is usually

transmitted transovarially from the midgut to the ovaries, resulting in infected offspring, but, intrastadial transmission also occurs (Scoles & Ueti, 2015).

Babesia caballi sporozoites enter the host via the tick bite. Sporozoites invade red blood cells (RBCs) where the parasite develops from a small trophozoite into a larger amoeboid sphere that divides into two round, oval or pear-shaped merozoites, which in turn, are capable of infecting new RBCs and the replication process is then repeated. When an uninfected tick subsequently feeds on the infected horse and ingests parasitized erythrocytes, some merozoites may penetrate the epithelial cells of the tick midgut and undergo gametogenesis. Zygotes develop into ookinetes that invade tick tissues like muscle fibres, haemocytes, the Malpighian tubules, and ovaries. Subsequently, secondary ookinetes invade tick salivary glands where sporogony occurs and sporozoites mature (Rothschild, 2013; Onyiche et al., 2019; OIE, 2009).

3.5.2 Clinical signs

The incubation period of equine piroplasmosis ranges from 12 to 19 days for *T. equi* infections and approximately 10 to 30 days when caused by *B. caballi* (de Waal, 1992). Both parasites cause disease in equids, which may be either acute or chronic with mortalities ranging from 5% among horses native to endemic regions, up to and above 50% in naïve horses introduced into endemic regions (Rothschild and Knowles, 2007). Infections with either *T. equi* or *B. caballi* result in the lysis of erythrocytes causing varying degrees of hemolytic anemia. *Theileria equi* tends to cause a more severe disease than *B. caballi*. Clinical signs are often non-specific and cannot be used to distinguish between *T. equi* and *B. caballi* infections, thus further complicating diagnosis. Animals that recover often remain persistently infected carriers that act as reservoirs for the spread of these protozoal pathogens by competent tick vectors.

EP is generally characterized by fever, anorexia, malaise, icterus, haemoglobinaemia, haemoglobinuria, pale mucous membranes and tachycardia. The acute form occurs frequently and is characterized by non-specific signs that include fevers ≥ 40°C, reduced appetite and malaise, elevated respiratory and pulse rates, congestion of mucous membranes, production of a dark red urine, anaemic and/or icteric animals. Subacute cases show varying degrees of anorexia and weight loss, intermittent fever, pale mucous membranes with petechiae and/or ecchymoses and increased pulse and respiratory rates. The spleen is also often enlarged. Nonspecific clinical signs such as mild inappetence, poor performance and weight loss are generally associated with chronic cases (OIE, 2009). Secondary complications that may arise from EP infections include the development of cholic, laminitis, pulmonary oedema, acute renal failure, infertility and abortion. In very rare peracute cases, animals die with no premonitory signs (OIE, 2009).

3.5.3 Lesions

Lesions observed are the result of an intravascular haemolytic condition. Necropsy findings include pale or icteric mucous membranes, thin and watery blood, hepato- and splenomegaly, pale kidneys with petechiae, subepicardial and subendocardial haemorrhages, mild oedematous swelling of the distal part of the limbs (subacute forms) and other non-specific lesions due to secondary infections (OIE, 2009).

3.6 Diagnostics

Several methods have been developed for the diagnosis of EP. The use of appropriate diagnostics is essential due to the non-specific clinical signs of EP, which may be confused with other infectious diseases such as AHS, equine infectious anaemia, equine viral arteritis virus and equine ehrlichiosis, (Rothschild, 2013).

3.6.1 Pathogen identification

Acute phases of EP may be diagnosed directly by microscopic examination of blood smears stained with a 10% Giemsa solution. In persistently infected animals, however, the parasitaemia is generally too low to allow microscopic detection, decreasing the sensitivity of this method (Friedhoff & Soule, 1996; Rothschild, 2013).

In vitro culture methods using purified equine red blood cells (RBC) have been described for both *T. equi* and *B. caballi* (Zweygarth et al., 2002). Although this test is neither practical nor economic (Rothschild, 2013), it remains useful in cases where it needs to be determined if treatment has eliminated the parasites from a carrier (Spickler, 2018).

Several PCR methods for the detection of *T. equi* and *B. caballi* have been described: conventional PCR targeting the 16S rRNA genes (Bashiruddin et al., 1999); nested PCR targeting either the 18S rRNA genes (Rampersad et al., 2003) or the equi merozoite antigen gene (*ema*-1) (Nicolaiewsky et al., 2001; Salim et al., 2013); qPCR assays targeting the *ema*-1 gene to determine the number of *T. equi* parasites in the mammalian host and in *Rhipicephalus (Boophilus) microplus* ticks (Ueti et al., 2003); a loop-mediated isothermal amplification (LAMP) assay (Alhassan et al., 2007a; Alhassan et al., 2007b); reverse line blot (RLB) hybridization assay (Nagore et al., 2004) and multiplex qPCR assays for the simultaneous detection of *T. equi* and *B. caballi* in horses as well as a molecular genotyping qPCR capable of detecting and distinguishing between the different *T. equi* parasite genotypes (Bhoora et al., 2018; Bhoora et al., 2020).

3.6.2 Antibody identification

Serological assays that include the complement fixation test (CFT), indirect fluorescence antibody test (IFAT), competitive enzyme-linked immunosorbent assay (cELISA) and very recently western blots, have been used in the diagnosis of EP (Jaffer et al., 2009; Wise et al., 2013). Cross-reactivity between *T. equi* and *B. caballi* and the lack of assay sensitivity, particularly in chronic cases has been reported for the CFT (Brüning et al., 1997). Although specific, the IFAT lacks sensitivity, but is still considered more sensitive than the CFT. The assay allows the distinction between *T. equi* and *B. caballi*, but requires the production of large amounts of antigen slides, which is not always practical (Brüning et al., 1997).

The official cELISA regulatory test for *T. equi*, which is based on the immunodominant equi merozoite antigen-1 (EMA-1), has been validated for use against multiple *T. equi* strains found globally (Knowles et al., 1992). The OIE regulated cELISA assay for *B. caballi* relies on the recognition of an epitope on BC48 (RAP-1), which lacks sequence conservation in both South African (Bhoora et al., 2010) and Israeli (Rapoport et al., 2014) isolates. Inspection of the inferred RAP-1 amino acid sequences from these isolates confirmed marked amino acid sequence differences in the carboxy-terminal repeat region, and thus the absence of the monoclonal antibody binding site, preventing the detection of parasite antibodies using this assay. Inconsistencies were also reported between infection status and RAP-1 cELISA serology results for horses in the U. S. (Awinda et al., 2013). Furthermore a high ratio of sero-positive horses detected using the RAP-1 cELISA were confirmed to be *B. caballi* negative using immunoblots, based on the observed banding patterns. Despite these observations, the cELISA remains the OIE-recognized serological assay for declaring horses free of EP.

SECOND PART: EXPERIMENTAL ACTIVITIES

This thesis is part of the research and group activities of Professor Henriette van Heerden, University of Pretoria, which I joined in November - December 2019. I have participated in research activities of Professor van Heerden and coworkers that allowed me utilize part of the research data for the realization of this thesis.

4 INTRODUCTION

Only a few infectious diseases are known to cause clinical infections in zebra and thus a highly resistant host. Surveillance systems are therefore required to study disease extent among zebra populations. Surveillance is meant here as a process including three main hierarchical steps: sampling, laboratory diagnosis and data elaboration (Figure 2). Well-developed tools are required to achieve each stage and obtain reliable results efficiently.

In this pursuit, following methods have been used:

a. Literature Review: collection and analysis of all the studies conducted on zebra for the detection of anthrax and equine piroplasmosis. The role of zebra in spreading disease is investigated.

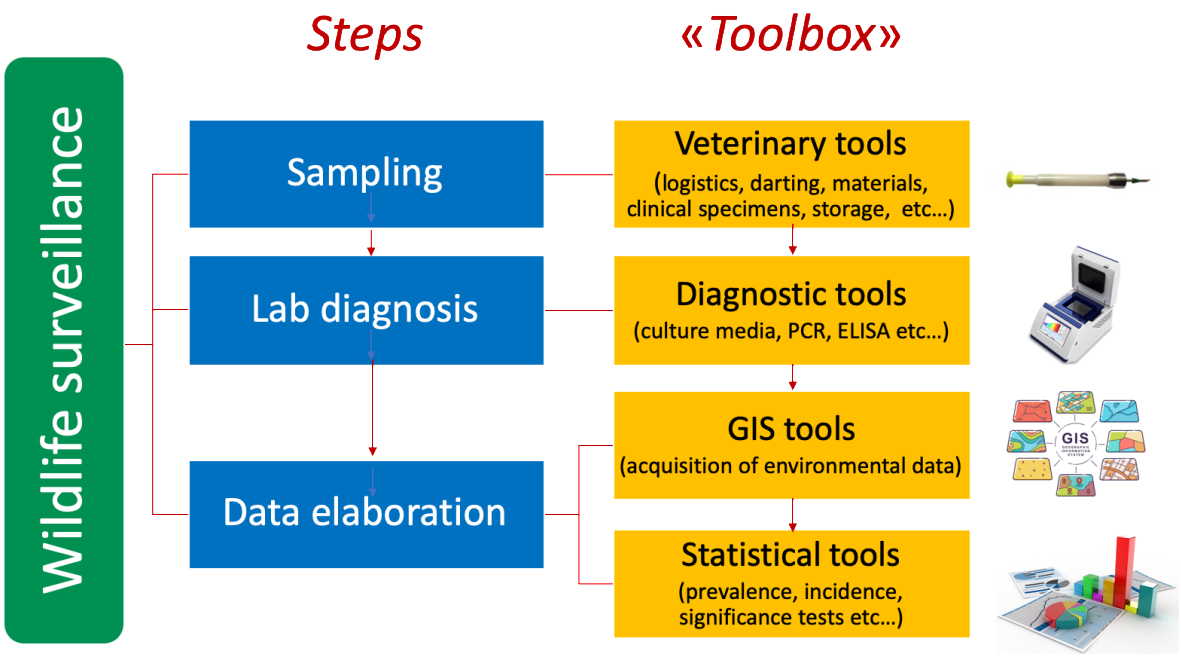

Figure 2. Surveillance planning and tools investigated.

b. Field activity: collection of blood samples from wild zebras in Kruger National Park (KNP) in the period November 2018 - February 2019. Samples were investigated for the presence of equine piroplasms and antibodies against protective antigen of *Bacillus anthracis* toxin. These activities were conducted by University of Pretoria and not first-person. Results were kindly shared for the work of this thesis. Further laboratory work should have been completed first-person genotyping *T. equi*-like piroplasms identified in this study. This activity had to be accomplished in the period March - May 2020 in the University of Pretoria. However, it could not be achieved due to COVID-19 international travel restrictions.

c. Data Analysis: elaboration of field data using statistical tools and qGIS.

Analysed tools and related outcomes are finally discussed and compared with the literature studies (Figure 3).

During the period November-December 2019 other significant activities were conducted personally in Kruger National Park, that is: collection of bone samples from animal carcasses suspected for anthrax and subsequent laboratory screening with bacterial culture; capture of live animals and collection of blood smears, blood samples and hair samples that will be available for future studies. The outcomes of these activities are not analysed in this study, but served to give a practical and personal perspective of existing surveillance and related tools.

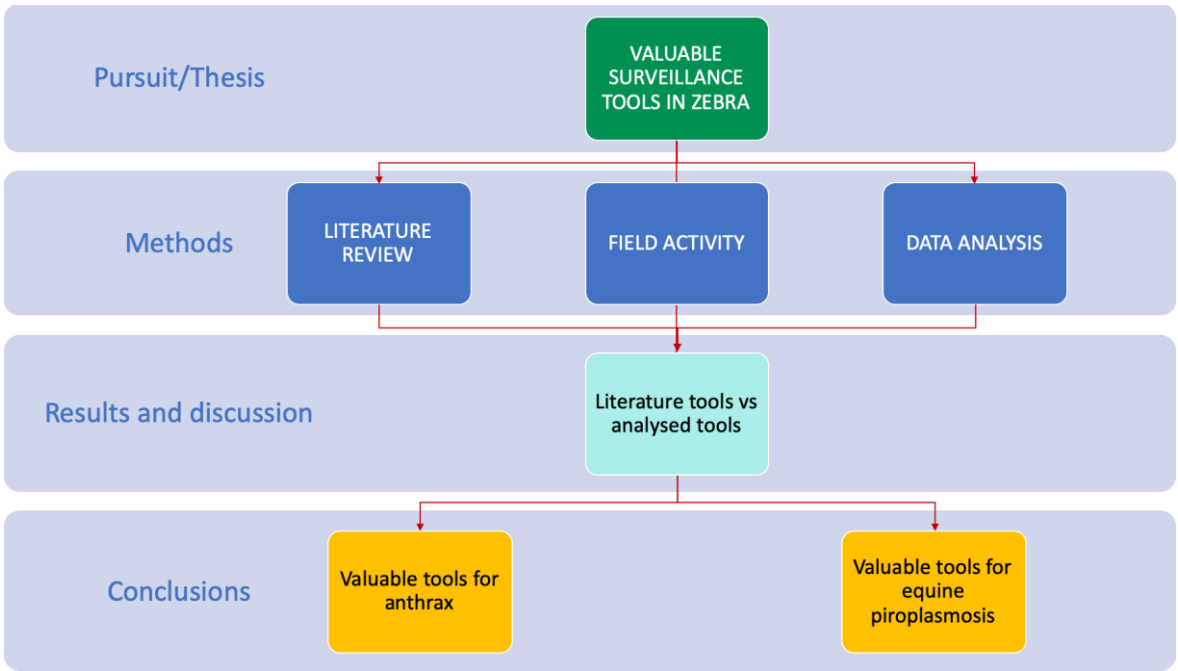

Figure 3. Organization of experimental activities and objectives.

5 MATERIALS AND METHODS

5.1 Literature review

Literature search and review was led by following questions: what is the role of zebra in spreading disease? How much is the disease entrenched in wild zebra populations?

For this purpose, we reviewed all published anthrax and equine piroplasmosis studies conducted on zebra. More specifically, we selected epidemiological studies regarding wild zebra with a minimum of 10 samples collected. Experimental studies conducted on captive zebra were included only to determine susceptibility to infection and role as a carrier and not for epidemiological purposes.

Papers reviewed were searched mainly on Web of Science™ Internet engine setting Web of Science Core Collection database for quality studies starting from 1985. Google Scholar internet engine was used to track studies back than 1985 as no temporal limit was set.

Data extracted from relevant papers are, as follows:

- susceptibility and role of zebra in spreading disease;
- main characteristics of samples collected;
- specifics of diagnostic used for laboratory confirmation and related outcome;
- intrinsic and extrinsic factors that significantly affected the results.

5.2 Field activity

5.2.1 Study area, animal capture and sampling

Samples were obtained from Kruger National Park (KNP), an almost 20 000 km^2 fenced conservation area in the provinces Limpopo and Mpumalanga in northeastern South Africa, located between 20°19′- 25°32′ S and 31°0′-32°0′ E (Smith et al., 2000). The eastern border of the park adjoins Mozambique while the northern border adjoins Zimbabwe. The entire area is surrounded by commercial farms, traditional communal grazing areas, and private nature reserves (Smith et al., 2000). The KNP contains 148 different mammalian species. Plains zebra is the second most abundant mammalian species, with population estimated around 23 700 - 35 300

(https://www.sanparks.org/parks/kruger/conservation/scientific/ff/biodiversity_statistics.php).

A total of 40 serum samples were obtained opportunistically from wild plains zebras in the period October 2018 - February 2019 (wet/rainy season). Half of the zebra sampled (20) are

from the northern regions Pafuri, Shingwedzi and Letaba, highly suitable for *B. anthracis* (Steenkamp et al., 2018), while the other half has been sampled in southern areas of KNP (non-endemic for *B. anthracis*) (Figure 4).

Zebras were immobilized through the injection of etorphine (Captivon) and xylazine via Pneu-Darts (Pneu-Dart Inc., Williamsport, PA, USA). Blood was drawn from the jugular vein and collected in EDTA tubes. Anaesthesia was finally reversed with naltrexone.

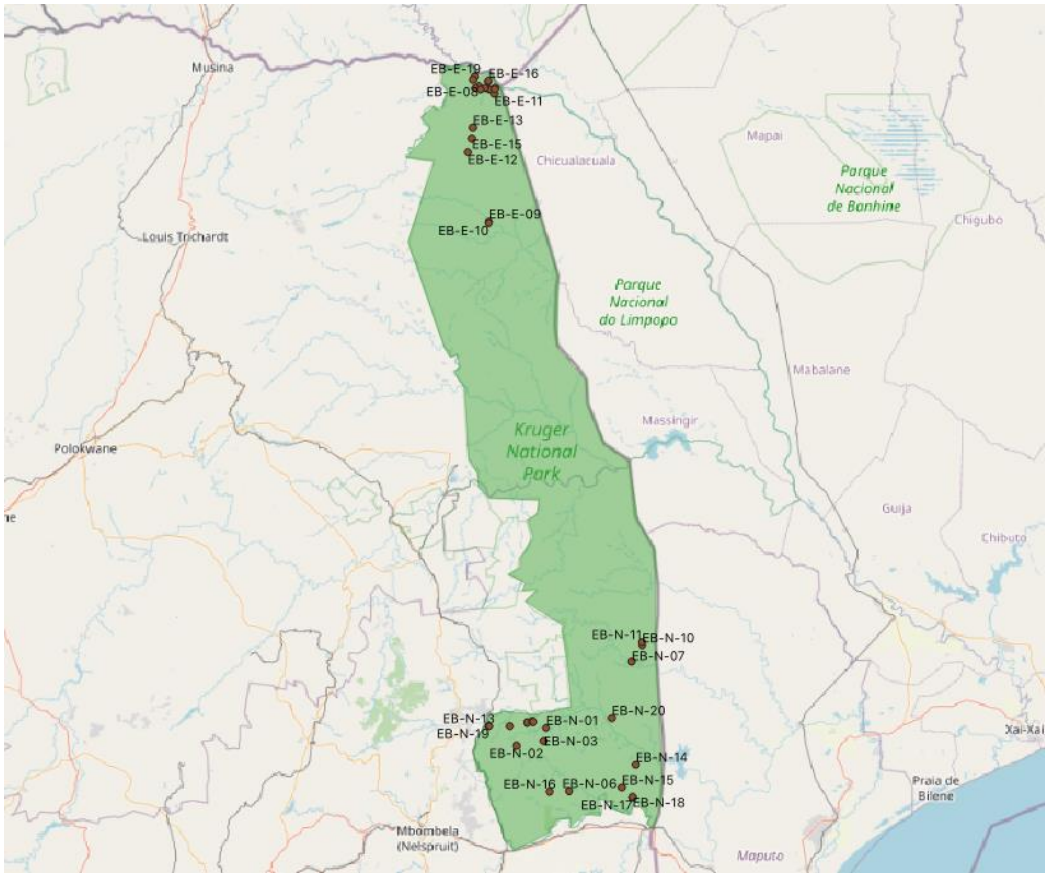

Figure 4. Individual location of zebra sampled in Kruger National Park. EB-E = *Equus burchellii*-Endemic Area; EB-N = *Equus burchellii*-non endemic area. Made with QGIS 3.14.

5.2.2 Anti-Protective Antigen Enzyme Linked Immunosorbent Assay (anti-PA ELISA)

An enzyme linked immunosorbent assay (ELISA) was performed to assess anti-PA antibodies in all the zebra sera collected, as described by Ndumnego et al., (2013) and Cizauskas et al., (2014), with some modifications.

Microtitre plates containing 96 wells (Thermo Scientific™ Pierce 96-well Plates-Corner, USA) were encrusted with 0.5 µg of antigen (PA) in the coating buffer (bicarbonate buffer) and left overnight at 4ºC to incubate. The microtitre plates coated with the antigen were washed 2 times with wash buffer (PBS + 0.05% Tween) (PBST) with Biorad PW40 washer (Mamesla-

Coquette, France). After which the coated plates were blocked with 200 µL of the blocking buffer containing PBST and 5% skimmed milk powder (PBSTM) and then incubated for 1 hour at room temperature. Subsequently, the plates were washed twice followed by adding the test sera, positive, negative controls and dilution buffer containing PBSTM to each well. These sera were put in duplicates in a dilution of 1:40 across the microtitre plates and incubate for 30 minutes on a rotatory incubator (Environmental Shaker-Incubator ES-20, Biosan Ltd, Germany). Afterwards, the plates were washed 5 times and 100 µL of recombinant protein A/G (Pierce® Protein A/G, USA and Invitrogen Protein G, USA) using the dilution of 1:10000 in PBSTM was added to each well and allowed to incubate for 30 minutes on the shaker-incubator. The plates were washed 5 more times, after which the ABTS substrate (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) (Thermo Scientific 1-Step ABTS, USA) was added, and the absorbance was read at 405 nm using the Biotek powerwave XS2 reader (USA). End point titre was determined using the OD values of the mean plus 3 times the standard deviation of known negative samples of zebra captured from the non-anthrax region in South Africa vaccinated with the Sterne Live Spore vaccine (Onderstepoort Biological Products, South Africa).

5.2.3 Reverse Line Blot hybridisation assay (RLB)

Babesia caballi and *Theileria equi* species were searched in DNA extracted from EDTA-preserved blood samples using reverse line blot hybridisation assay as previously described (Nijhof et al., 2005). *Theileria/Babesia* (T/B) specific forward primers RLB F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') (Whitehead Scientific (Pty) Ltd, South Africa) and biotin-conjugated reverse primers RLB-R2 (5'-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3') (Whitehead Scientific (Pty) Ltd, South Africa) were used in order to amplificate the V4 portion of the pathogens (Nijhof et al., 2003).

An RLB membrane (Biodyne C, Separation Scientific,Johannesburg, South Africa) was made to fit the miniblotter (Immunetics, UK). The membrane was activated for 10 minutes using a 16% solution of 1-ethyl-3-(-3-dimethylamininopropyl) carbodiimide (Separation Scientific, South Africa) and then rinsed. The membrane was placed in the miniblotter, subsequently the Oligonucleotide probes were bound to the membrane by diluting each probe to a concentration of 2 pmol/µL with a solution of 500mM NaHCO (Sigma Aldrich Pty, South Africa) at pH 8.4 and 0.2 L was added to the membrane already placed in the miniblotter and incubated for 1 minute. The specific *Theileria/Babesia* (T/B) genus and species probes are shown in Table 1. After a 2-minute incubation, inactivation of the membrane was done for 8 minutes using a solution 0.1 M NaOH (Sigma Aldrich Pty, South Africa) that was newly made.

The membrane was then washed at 60ºC for 5 minutes with a 100 mL solution of 2X SSPE (Thermo Scientific™, South Africa) and 0.1%SDS (Sigma Aldrich Pty, South Africa).

A biodyne® C membrane was made by cutting a piece of the membrane according to the size of the miniblotter apparatus. This membrane was then activated in 10 mL newly made 16% EDAC (1-ethyl-3-(-3-dimethylamininopropyl) carbodiimide) (DNA Thunder™, Separation Scientific, South Africa) for 10 minutes. 0.5 M NaHCO (Sigma, South Africa) was added to Individual probe with a pH 8.4 to make a final concentration of 2 pmol/µL and 200 µL was loaded onto the membrane using a miniblotter apparatus (Immunetics, UK). *Babesia bovis* species-specific probe was included in separate wells to serve as positive control for troubleshooting purposes. The membrane was inactivated at room temperature for 8 minutes with 0.1M recently prepared NaOH (Sigma, South Africa) on a shaker. The inactivated membrane was then washed in 100 mL 2X SSPE (ThermoFisher Scientific, South Africa)/0.1% SDS (Sigma, South Africa) at 60°C for 5 minutes.

Table 1. List of Oligonucleotide probes fixed on the Biodyne C membrane for the detection of protozoan DNA.

Genus/Species-specific probes	Sequences of probes (5′ to 3′)	Reference
Babesia genus-specific probe 1	ATT AGA GTG TTT CAA GCA GAC	Nijhof (unpublished)
Babesia genus-specific probe 2	ACT AGA GTG TTT CAA ACA GGC	Nijhof (unpublished)
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT	(Butler et al., 2008)
Theileria/Babesia group-specific probe	TAA TGG TTA ATA GGA RCR GTT G	(Gubbels et al., 1999)
Theileria genus-specific probe	ATT AGA GTG CTC AAA GCA GGC	Nijhof (unpublished)
Theileria equi	TTC GTT GAC TGC GYT TGG	(Butler et al., 2008)

Degenerate positions R signifies A/G while W signifies A/T

5.2.4 PCR and Hybridisation of PCR products on membrane

A final volume of 25 µL for a single reaction was made containing 0.5X Platinum Quantitative PCR SuperMix-UDG, 8pmol of each forward and reverse primer of T/B, 9.5µl of molecular grade water and 2.5 µL of the DNA sample. Two separate master mixes were made for T/B species. A Thermocycler (Gene Amp®, PCR System 9700, Life Technologies™, South Africa) was utilized.

Hybridisation of the membrane and PCR products was achieved as done by Nijhof et al., (2005). The membrane fixed with T/B specific probes were first activated for 5 minutes at 25ºC under gentle shaking in 2× SSPE/0.1% SDS.

The PCR products were diluted by the addition of 1.7× SSPE/0.08% SDS to the PCR reaction and mixture were denatured for 10 minutes in the thermocycler and immediately put to cool on ice. The activated membrane was put in the miniblotter with the slots vertical to the line design of the probes applied. The slots were filled with the diluted PCR products according to the sample list and the slots without PCR products were filled with 2X SSPE/0.1% SDS to prevent the flow of products from one lane to the next and the membrane was hybridised at 42ºC for 60 minutes on a level surface. The membrane was washed 2 times with already heated 2X SSPE/0.5% SDS at an interval of 10 minutes at 50°C between each wash. The membrane was then incubated with 1.9× SSPE/0.49% SDS (pre-heated) and 0,0016 U streptavidin-POD (peroxidase labelled) conjugate (1.25 U) for 30 minutes at 42°C under gentle shaking inside the incubator. The membrane was then washed twice in preheated 2× SSPE/0.5% SDS for 10 minutes at 42ºC incubator under gentle shaking. The membrane was washed 2 times with 2× SSPE for 5 minutes at room temperature under gentle shaking. Thereafter, the membrane was incubated for 10 minutes with 10 mL chemiluminescence detector (ECL) (5 mL ECL1 + 5 mL ECL2) under gentle shaking for 1 minute at room temperature. The membrane was exposed to an X-ray film (Kodak X-OMAT™ Blue XB-1, Separation Scientific, South Africa) and positive reactions were evident with black spots on the film. The membrane was then stripped for future use using 1% SDS heated for 30 minutes at 80ºC, after which it was washed for 15 minutes using 20 mM EDTA with a pH8 at room temperature.

5.3 Data analysis

Literature data were collected into two Microsoft Excel (Version 14.3.8., Microsoft Corp., Redmond, WA) spreadsheets, one for each disease, and correct transfer was controlled. Every relevant information reported in a paper was assigned to a line. Columns included location, period, zebra species, methods and related outcomes, and references. Outcomes were represented in ratio and percentage, and finally analysed cumulatively ("overall") in ranges from minimum to maximum values. Some values were finally represented in different graphics using Microsoft Excel.

Environmental data were extracted using Software QGIS 3.14. Data and sources information are listed in Table 2. Continuous data (*e.g.* pH, maximum elevation etc...) were then classified in two or more groups, according to each specific factor (see tables 4 and 6 for details). Statistical tools used to identify environmental factors statistically significant ($p < 0.05$) are as follows: Fisher exact test using online calculator (https://www.socscistatistics.com/tests/fisher/default2.aspx), and chi squared test especially for analysis with more than two variables.

Table 2. Specifics of environmental factors analysed.

<table>
<tr><th>Factor</th><th colspan="5">Data description</th><th>Source</th></tr>
<tr><td>Maximum elevation (m)</td><td colspan="5">Height above sea level in meters - DEM (Digital Elevation Model) derived</td><td>USGS EROS Archive; spatial resolution = 30-arc-seconds</td></tr>
<tr><td rowspan="4">Land Form = physiography</td><td>1st level</td><td colspan="2">2nd level</td><td>Gradient (%)</td><td>Relief intensity (m/km^2)</td><td rowspan="4">SOTER database; scale 1:1,000,000</td></tr>
<tr><td>Level land</td><td colspan="2">plain, plateau, valley floor etc...</td><td><10</td><td><50</td></tr>
<tr><td>Sloping land</td><td colspan="2">escarpment zone, hill, mountain etc...</td><td>10-30</td><td>100-300</td></tr>
<tr><td>Steep land</td><td colspan="2">as sloping land but high gradient</td><td>>30</td><td>>150</td></tr>
<tr><td>Soil</td><td colspan="5">FAO soil classification</td><td>SOTER database; scale 1:1,000,000</td></tr>
<tr><td rowspan="11">Lithology</td><td colspan="2">Major class</td><td>Group</td><td colspan="2">Type (sampled)</td><td rowspan="11">SOTER database; scale 1:1,000,000</td></tr>
<tr><td colspan="2" rowspan="4">Igneous rock</td><td>Acid</td><td colspan="2">Granite</td></tr>
<tr><td>Intermediate</td><td colspan="2">-</td></tr>
<tr><td>Basic</td><td colspan="2">Basalt</td></tr>
<tr><td>Ultrabasic</td><td colspan="2">-</td></tr>
<tr><td colspan="2" rowspan="2">Metamorphic rock</td><td>Acid</td><td colspan="2">Gneiss</td></tr>
<tr><td>Basic</td><td colspan="2">-</td></tr>
<tr><td colspan="2" rowspan="3">Sedimentary rock</td><td>Clastic sediments</td><td colspan="2">Sandstone; Shale</td></tr>
<tr><td>Organic</td><td colspan="2">-</td></tr>
<tr><td>Evaporites</td><td colspan="2">-</td></tr>
<tr><td colspan="2">Unconsolidated</td><td>Fluvian; Lacustrian; Marine; Colluvial; Eolian; Glacial; Pyroclastic; Organic</td><td colspan="2">-</td></tr>
<tr><td>pH in water</td><td colspan="5">pH measured in water</td><td>SOTER database; scale 1:1,000,000</td></tr>
<tr><td>Monthly precipitation (mm)</td><td colspan="5">For zebra sampled in 2018: precipitation in the respective month of sampling (October and December 2018).
For zebra sampled in 2019: mean precipitation of the years 2010-2018 in the respective month of sampling (January and February).</td><td>WorldClim; spatial resolution = 2.5 minutes</td></tr>
<tr><td>NDVI (Normalized Difference Vegetation Index)</td><td colspan="5">Band 4 (red) and Band 5 (near infrared) rasters were acquired from satellite Landsat 8. NDVI was then calculated with raster calculator function of qGIS as: (near infrared - red) / (near infrared + red).
On land values range from 0 to 1, while on water from -1 to 0.</td><td>Remotepixel.ca, Landsat 8 satellite; spatial resolution = 30 meters</td></tr>
</table>

6 RESULTS

6.1 Literature results

6.1.1 Anthrax

All zebra species have been reported susceptible to anthrax. More than this, zebra has been involved in almost all major anthrax outbreaks recorded in sub-Saharan Africa (Figure 5; Table 3).

Table 3. Zebra mortality and relative percentage in major anthrax outbreaks.

Location	*Period*	*Zebra carcasses/ total animal carcasses*			*Samples collected*	*Methods used for lab confirmation*	*References*
		Plains zebra	**Grevy's zebra**	**Mountain zebra**			
Malilangwe Wildlife Reserve (Zimbabwe)	August - November 2004	15/896 (1.7%)	-	-	Blood smears from carcasses < 7 days old	Microscopy (identification of encapsulated bacilli)	Clegg et al., 2007
Kenyan wildlife areas (Kenya)	51 outbreaks from 1999 to 2017	36/1010 (3.6%)	103/1010 (10.2%)	-	Samples from dead or visibly sick animals	Microscopy (identification of encapsulated bacilli)	Gachohi et al., 2019
Queen Elizabeth NP + Lake Mburo NP (Uganda)	July 2004 - September 2005	63/462 (13.6%)	-	-	Blood smears from ear veins, heart, spleen, lung and lymph nodes; blood samples	- Bacterial culture - Microscopy - Mice inoculation - PCR	Wafula et al., 2008
Etosha National Park (Namibia)	Outbreaks from 1968 to 2011	1586/3032 (52.3%)	-	4/3032 (0.1%)	Blood swabs, carcass exudates, soil beneath old carcasses	-Examination of visual signs at the carcass - Bacterial culture - Microscopy	Turner et al., 2013
Ghaap Plateau, Northern Cape (South Africa)	November 2007 – February 2008	6/50 (12%)	-	-	Bone samples, blood smears, soil samples, environmental samples (crow faeces) and louse flies	- Bacterial culture - Microscopy	Hassim et al., 2017
Kruger National Park (South Africa)	Outbreaks from 1990 to 2016	42/953 (4.4%)	-	-	Bone samples and blood smears	- Bacterial culture - Microscopy	Skukuza State Veterinary, KNP, unpublished
Serengeti National Park (Tanzania)	Outbreaks from 1996 to 2009	83/940* (8.8%)	-	-	Blood smears	Microscopy (identification of encapsulated bacilli)	Lembo et al., 2011; Mlengeya et al., 1998
Range (species specific)		1.7-52.3%	10.2%	0.1%	** = Probable anthrax positive cases: only some were tested in laboratory.*		
Range (all zebra species)		**1.7-52.4%**					

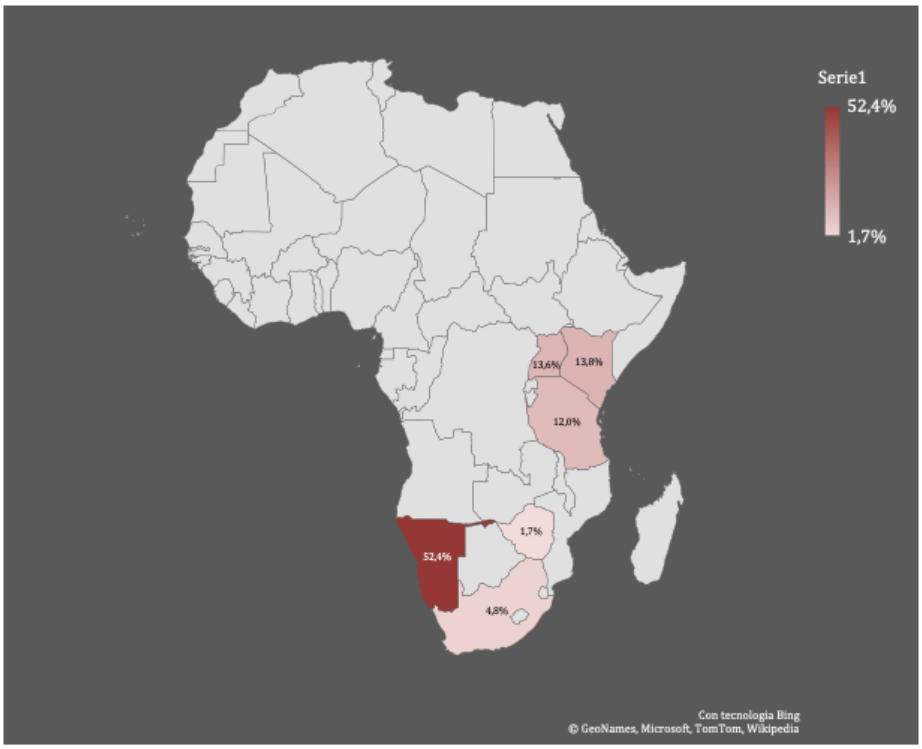

Figure 5. Relative percentage of zebra carcasses in the major anthrax outbreaks shown by country (Clegg et al., 2007; Gachohi et al., 2019; Wafula et al., 2008; Turner et al., 2013; Hassim et al., 2017; Lembo et al., 2011).

In Samburu, Lake Mburo National Park (Kenya) and Etosha National Park (Namibia), zebras are the species most widely affected by anthrax. Gender preference was described during the 2005 outbreak in Samburu, Kenya, with the researchers reporting male-biased mortality for Grevy's zebras (*E. grevyi*) and female-biased mortality for plains zebras (*E. quagga*), without reporting sex ratios for the species in the region (Muoria et al., 2007). Sex-biased mortality was also reported in multispecies outbreaks in Namibia (Lindeque and Turnbull, 1994), and Zimbabwe (Clegg et al., 2007).

Only few wildlife anthrax serology studies have been carried out. Turnbull et al. 1992 first searched for anti-PA titres in 24 zebra sera from Etosha National Park (ENP), Namibia, although no one tested positive (Turnbull et al., 1992). Such outcome may be due to the low sensitivity of the competitive inhibition ELISA used in this study to identify anti-PA antibodies. Moreover, each animal was used as its own control and comparison, and no designated control

was compared with samples (Cizauskas et al., 2014). Antibodies against *B. anthracis* were tested also by Lembo et al. (2011) on 85 zebra sera in Serengeti National Park (Tanzania) using the Immunetics Quick PA-ELISA test (QuickELISA anthrax PA kit immunoassay; Immunetics, Inc., Boston, MA, USA), but no zebra samples tested positive (Lembo et al., 2011). This study used a new, commercially available anti-PA antibody kit, which, while promising, has yet to be validated for many species. The real difference in titres, however, likely lies in the fact that anthrax is less ubiquitous in the Serengeti ecosystem than it is in ENP (Cizauskas et al., 2014).

Cizauskas et al. (2014) investigated a total of 154 zebra serum samples for the presence of anti-PA antibodies in ENP, Namibia. Zebra were sampled over different season, with several individuals being sampled two to five times each. The authors adapted an ELISA to measure anti-PA antibody titres in serum and six serum samples obtained from captive plains zebras were used as negative controls. End point titres were determined following three different methods: liberal cut-off, medium-conservative cut-off, ultra-conservative cut-off. Overall seropositivity prevalence in unique zebra (*i.e.* sampled once = 69) ranged from 52% to 87% across the three endpoint titres rules and eleven resampled zebra seroconverted over the two-years study period. Zebra displayed also higher anti-PA antibody titres in wet seasons compared to dry seasons, suggesting that anthrax exposure rates differ between seasons (Cizauskas et al., 2014).

6.1.2 Equine Piroplasmosis

Piroplasmosis infections were first described in plains zebra by Koch in 1905. The inoculation of zebra blood into a susceptible horse caused fever and parasitaemia, thus providing support that zebra are carriers of piroplasms (Zweygarth et al., 2002). Piroplasmosis infections have subsequently been detected in all zebra species (Table 4) (Zweygarth et al., 2002; Bhoora et al., 2010a; Smith et al., 2019; Bhoora et al., 2020; Hawkins et al., 2015; Tirosh-Levy et al., 2020). All studies reported a higher prevalence of *T. equi* than *B. caballi*. In a recent epidemiological study in South Africa, which investigated the genotypic diversity of *T. equi* in horses and zebra, the authors showed that the level of *T. equi* 18S genetic diversity observed in zebra samples far exceeded that found in horses, suggesting that zebra are the ancestral host for this piroplasmid lineage (Figure 6) (Bhoora et al., 2020). Translocation of infected zebra to non-endemic regions could therefore potentially result in the spread of both competent tick vectors and EP to naive equids and non-equid species (Smith et al., 2019; De Waal, 1992).

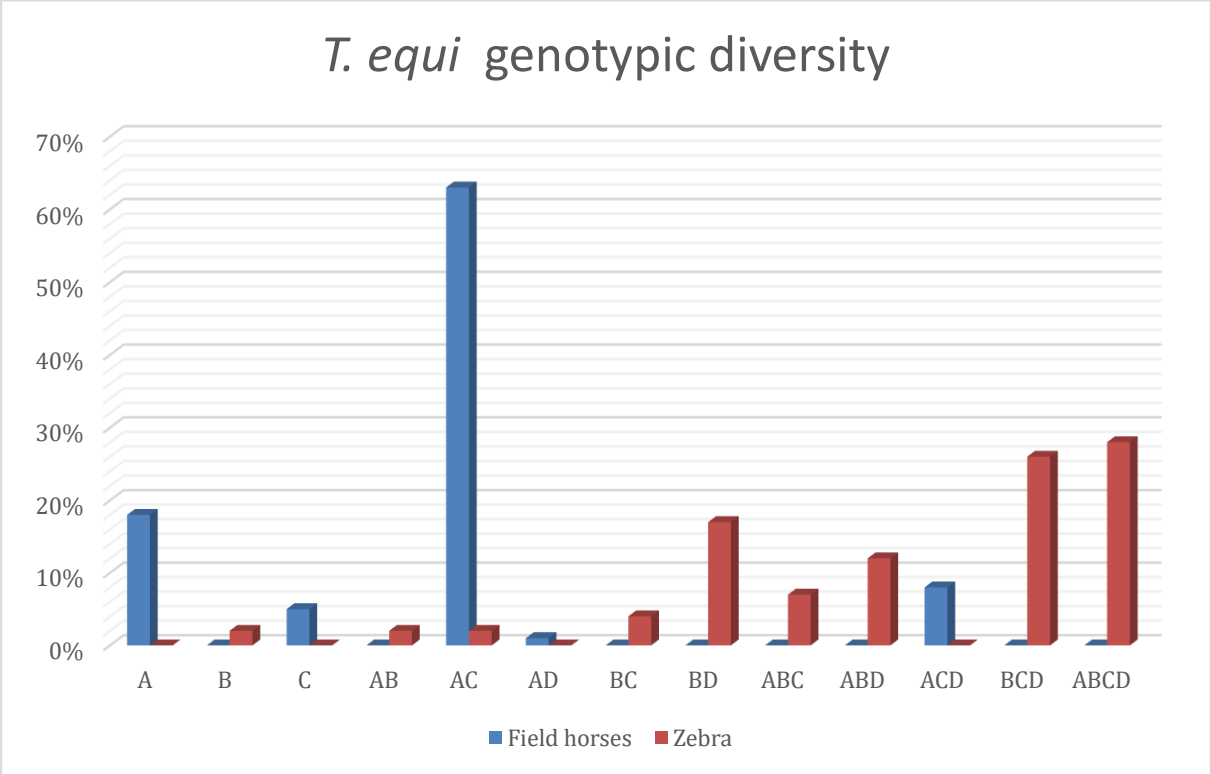

Figure 6. Genotypic diversity of *Theileria equi* parasite genotypes (A, B, C and D) in mountain zebra and field horses according to Bhoora et al., (2020).

Table 4. Prevalence of equine piroplasms in zebra populations. *NP = Natural Park, RC = Research Center, NR = Natural Reserve, PCR = Polymerase chain reaction, IFAT = Immunofluorescence Antibody Test*

		***T. equi* Prevalence**					***B. caballi* Prevalence**					**References**
Country	**Place**	***E. grevy***	***E. zebra***	***E. quagga***	**Method**	**Target**	***E. grevy***	***E. zebra***	***E. quagga***	**Method**	**Target**	
South Africa	Bontebok NP + Karoo NP	-	16/20 (80%)	-	Microscopy	-	-	0/20 (0%)	-	Microscopy	-	Zweygarth et al., 2002
	Bontebok NP + Karoo NP	-	20/20 (100%)	-	*In vitro* culture	-	-	2/20 (10%)	-	*In vitro* culture	-	Zweygarth et al., 2002
	Bontebok NP + Karoo NP	-	20/20 (100%)	-	IFAT	Antibodies against *T. equi*	-	4/20 (20%)	-	IFAT	Antibodies against *B. caballi*	Zweygarth et al., 2002
	Kruger NP + Wildlife RC + Equine RC	-	10/10 (100%)	49/60 (82%)	*T.equi* specific real-time qPCR	18S rRNA gene	-	1/10 (10%)	12/60 (20%)	TaqMan MGB^{TM} qPCR	18S rRNA gene	Bhoora et al., 2010a
	Mountain Zebra NP + De Hoop NR + Karoo NP	-	137/137 (100%)	-	Multiplex EP real-time qPCR	18S rRNA gene	-	2/137 (1.5%)	-	Multiplex EP real-time qPCR	18S rRNA gene	Smith et al., 2019
	Mountain Zebra NP + De Hoop NR + Karoo NP + Kaalplas	-	136/136 (100%)	2/2 (100%)	Multiplex EP real-time qPCR	18S rRNA gene	-	2/136 (1.4%)	-	Multiplex EP real-time qPCR	18S rRNA gene	Bhoora et al., 2020
Kenya	Samburu	3/4 (75%)	-	-	Microscopy	-	0/4 (0%)	-	-	Microscopy	-	Hawkins et al., 2015
	Samburu	16/16 (100%)	-	-	Nested PCR	18S rRNA gene	0/16 (0%)	-	-	Nested PCR	18S rRNA gene	Hawkins et al., 2015

Range (species-specific)	75-100%	80-100%	82-100%	0%	1.4-20%	20%
Range (all zebra species)	**75-100%**			**0-20%**		

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6.2 SAMPLES ANALYSED

6.2.1 Anti-PA ELISA results

Twentyfive zebra sera tested positive to anti-PA ELISA (62.5% overall seropositivity; CI (95%) = 47-78%). Difference between sampling areas seem to exist, as 15 of the positive zebras were sampled in anthrax endemic area (Pafuri). However, the difference was not statistically significant ($p < 0.05$) using the chi-squared test ($p = 0.1025$) and the Fisher exact test ($p = 0.1908$). In the same way, none of the other factors significantly affected anti-PA ELISA results (Table 5).

Table 5. Prevalence of anti-PA antibodies in wild zebras in KNP and factors associated to the collection area.

Factor	Variables	N	ELISA (pos)	ELISA (%)	p value
Prevalence	-	**40**	**25**	**62.5%**	-
Locality	Endemic area	20	15	75.0%	0.1025 ($\chi2$) 0.1908 (Fisher)
	Non endemic area	20	10	50.0%	
Sex	Male	22	13	59.1%	0.6225 ($\chi2$) 0.747 (Fisher)
	Female	18	12	66.7%	
Lithology	Basalt	21	15	71.4%	0.2336 ($\chi2$)
	Sandstone	4	3	75.0%	
	Gneiss	2	1	50.0%	
	Granite	11	5	45.5%	
	Shale	2	0	0.0%	
Landform	Level land - Valley floor	11	8	72.7%	0.6889 ($\chi2$)
	Level land - Plain	27	16	59.3%	
	Sloping land - Medium-gradient mountain	2	1	50.0%	
Soil	Eutric CAMBISOLS	13	10	76.9%	0.4169 ($\chi2$)
	Eutric LEPTOSOLS	6	4	66.7%	
	Eutric REGOSOLS	13	6	46.2%	
	Haplic PHAEOZEMS	1	1	100.0%	
	Rhodic NITOSOLS	6	4	66.7%	
	Calcic LUVISOLS	1	0	0.0%	
Maximum elevation	200m to <350m	26	8	30.8%	0.2308 ($\chi2$) 0.3101 (Fisher)
	350m to 500m	14	7	50.0%	
pH	5.5 to <6.5	8	5	62.5%	0.409 ($\chi2$) 0.6817 (Fisher)
	6.5 to 7.5	22	10	45.5%	
Monthly precipitation	0mm to <55mm	21	16	76.2%	0.0601 ($\chi2$) 0.1018 (Fisher)
	55mm to <110mm	19	9	47.4%	
NDVI	0.00 to <0.15	31	19	61.3%	0.7693 ($\chi2$) 1 (Fisher)
	0.15 to <0.30	9	6	66.7%	

6.2.2 RLB results for equine piroplasmosis

Only 5 samples tested positive for *T. equi* (13% seropositivity; CI (95%) = 2-23%) and none to *B. caballi*. However, there is high prevalence in T/B catch all (82%; CI = 70-94%), T catch all (100%) and B1 catch all (77%; CI = 64-90%) (Table 6). None of the intrinsic and extrinsic factors resulted to be statistically significant using Fisher exact test and chi squared test (Table 7).

Table 6. Reverse line blot outcomes for *Theileria* and *Babesia* genus- and species- specific probes.

	***Theileria/Babesia* catch all**	***Theileria* catch all**	***Babesia* catch all**	***T.equi***
Pos	32	39	30	5
Prev	82%	100%	77%	13%
CI (95%)	70-94%	100%	64-90%	2-23%

Table 7. Prevalence of *Theileria equi* in wild zebras in Kruger National Park and factors associated to the collection area

Factor	Variables	N	T. equi (pos)	T. equi (%)	P value
Prevalence	-	**39**	**5**	**13%**	-
Sex	Male	21	3	14.3%	0.8192 (χ2) 1.0 (Fisher)
	Female	17	2	11.8%	
Landform	Level land - Valley floor	11	3	27.3%	0.2275 (χ2)
	Level land - Plain	26	2	7.8%	
	Sloping land - Medium-gradient mountain	2	0	0.0%	
Maximum elevation	200m to <350m	25	4	16.0%	0.4274 (χ2) 0.6365 (Fisher)
	350m to 500m	14	1	7.1%	
pH	5.5 to <6.5	7	0	0.0%	0.1656 (χ2) 0.2965 (Fisher)
	6.5 to 7.5	22	5	22.7%	
Monthly precipitation	0mm to <55mm	31	4	12.9%	0.9757 (χ2) 1.0 (Fisher)
	55mm to <110mm	1	7	12.5%	
NDVI	0.00 to <0.15	30	5	16.7%	0.1896 (χ2) 0.3178 (Fisher)
	0.15 to <0.30	9	0	0.0%	

7 DISCUSSION

7.1 Literature tools vs analysed tools for anthrax

Passive surveillance is currently used in KNP to test potentially anthrax infected carcasses and monitor the extent of an outbreak (Skukuza State Veterinarian, KNP, personal communication). However, *B. anthracis* does not compete well with putrefactive bacteria and, with increasing age of the carcass, the capsulated bacilli become more difficult to visualize. Smears, as a diagnostic procedure, become unreliable about 24 hours after death, although capsular material may still be observed sometime after the bacilli themselves can no longer be seen. Blood in transit at ambient temperatures for over 72 hours may not reveal organisms on examination or culture (Turnbull, 2008). More than this, bacterial culture requires long execution times (3-4 days), demanding and labour-intensive procedures thus ability by the lab worker and availability of lab instruments. Finally, veterinary and lab staff are exposed to infection risk on field and in laboratory, respectively.

In contrast to sampling of suspected carcasses, serosurveillance of living animals poses no risk for anthrax infection and permits to gain a better understanding of anthrax epidemiology, especially in relation to patterns of infection and risk factors for exposure, susceptibility, and death. However, darting animals and blood samples collection (need of syringes, needles, EDTA tubes etc...) are an expensive and laborious burden. Therefore, veterinary staff must be expert and well-equipped in order to perform this activity. Investigation of multiple pathogens and antibodies in the same sera, like in this study, may decrease costs and workload, making easier to follow the path of an active surveillance system.

Overall seroprevalence obtained for anthrax in this study (62,5%; CI = 47-78%) is similar to results obtained by Cizauskas et al., (2014) (52-87%). Methods used were similar: both ELISA adapted to determine anti-PA antibodies (Table 8). End-titre determination used in this study is more specific than liberal cut-off rule used by Cizauskas et al., (2014).

As assessed by Cizauskas et al. (2014), zebra can experience sublethal anthrax infections in an endemic system, thus surviving and developing a short-term immunity. In this study, the high anti-PA antibody prevalence (62.5%) demonstrated on zebra samples gives a hint that also in Kruger National Park zebras can survive to sublethal anthrax infections, building up their immunity to *B. anthracis*. This finding stands in contrast with the belief that anthrax is a deadly disease for equids like zebra (Turnbull, 2008). However, duration of zebra immunity to anthrax

could not be proved in this study as zebras were sampled once and during only one season of the same year (wet season of 2018-2019 period).

Table 8. Main anti-Protective Antigen (PA) ELISA differences between Cizauskas et al. (2014) and this study.

	Anti-PA antibodies binder	OD absorbance + Reader	Negative control	End-titre determination
Cizauskas et al., (2014)	Goat anti-horse IgG heavy and light chain horseradish peroxidase (HRP) conjugate	405 nm - Biotek powerwave XS2 reader	Six serum samples obtained from captive plains zebras	liberal cut-off, medium-conservative cut-off, ultra-conservative cut-off
This study	Pierce recombinant protein A/G	450nm - SpectraMax M2 Microplate Reader using SOFTMAX PRO software v5.3	Negative zebra sera from non-anthrax regions before vaccination with *Bacillus anthracis* Sterne vaccine	OD values of the mean plus 3 times the standard deviation of known negative samples

In ENP zebra is the most representative animal in anthrax outbreaks (52,3% of total positive animal carcasses), while in KNP they are impala and kudu (50,5% and 15,2% of total positive animal carcasses, respectively) (Skukuza State Veterinarian, KNP, unpublished data) and zebra falling into a relatively small group of other affected species (4,4% of total positive animal carcasses). Differences can be explained in different population sizes: there is an estimated 13000 zebra population in ENP (Zidon et al., 2017) against 24000-35000 in KNP; estimated 1500 black-faced impala in ENP (Green et al., 1998) with only one anthrax confirmed impala carcass in all outbreaks (Turner et al., 2013) vs 132 300 - 176 400 impala in KNP (https://www.sanparks.org). Differences can be explained also by the ecological and behavioural factors. Mainly it seems to be due to blowflies in the KNP that, after feeding on anthrax carcasses, fly to nearby bushes and deposit infected blood on the leaves, which then become the source of infection for the browsing impala and kudu (Turnbull, 2008). However impala are also grazers, thus infection could depend greatly by season and soil ingestion like zebra (Turner et al., 2013).

The challenge in determining causal mechanisms for seasonally occurring infectious diseases is that many environmental, ecological and behavioral factors also vary seasonally. In order to facilitate a common understanding of environmental conditions associated with anthrax outbreaks and what impact those conditions may have on animal populations and individual behaviour, it is recommended to report in greater detail the environmental conditions associated with samples collected and the time lag in which these conditions occur. For outbreaks in grazing species like zebra, it would also be useful to know the grazing conditions during outbreaks: if animals are feeding on dry, low grasses remaining from the prior growing season or if they are feeding on lush green growth from recent rainfall (*e.g.*, Wafula et al. 2008). Untangling the many correlated seasonal environmental factors may enable us to move beyond lists of associated conditions to an understanding of specific causal factors in the temporal occurrence of outbreaks among endemic systems, like the assessment of environmental risk factors behind the spatial occurrence of anthrax (Turner et al., 2013). In this study, QGIS supplied environmental data that were missing, allowing comparison and correlation with laboratory results. This tool is not an expensive exercise and it has furnished a wide range of environmental variables, of which some cannot even be reported in field. However, no factor resulted to be statistically significant ($p < 0.05$) according to the statistical tools used for environmental data analysis.

7.2 Literature tools vs analysed tools for EP

Overall seroprevalence found in this study for *T. equi* (13%; CI = 2-23%) is surprisingly below the overall seroprevalence range of the previous studies conducted on zebra (75%-100%). However, *Theileria* catchall (100%) falls within this range. *B. caballi* prevalence (0%) was low as in literature studies (0-20%), although *Babesia* catchall prevalence resulted high (77%; CI = 64-90%). This could indicate a mixed *T. equi* and *B. caballi* infection with either parasitaemia at a level below the detection limit of the RLB species-specific probes, or the occurrence of a novel *Babesia* or *Theileria* genotype or species. *B. caballi* infections are known to occur at very low parasitaemias that rarely exceed 1%. Other reports show that it is extremely difficult to detect *B. caballi* in blood at any stage of the disease except the early acute phase. Once a carrier status is established, there may be complete absence of circulating parasites. The occurrence of such low parasitaemias could possibly explain the inability of the RLB to detect any of *B. caballi* infections. Alternatively, the presence of sequence variation, which has previously been reported to occur in the region of the 18S rRNA gene where the RLB primers and probes were designed (Bhoora et al., 2009), could explain the discrepant results.

No study exists for the comparison of RLB method in the search of equine piroplasms on zebra with other direct test. It could be interesting to perform real-time PCR on these same samples in order to identify sensibility-specificity of RLB in contrast to real-time PCR, like Bhoora et al. (2010b) did on horse samples. Standing by the outcomes of this study, either piroplasms were less present in the zebra population considered or RLB probes must be improved to gain sensibility and specificity in order to become a more reliable diagnostic tool for the surveillance of equine piroplasmosis in KNP. In contrast, real-time PCR developed by Bhoora et al., (2020), showed high sensibility, specificity and therefore suitability for the use in a surveillance system. More than this, genotyping with real-time PCR can give more useful informations of EP epidemiology in zebra, which is considered the ancestral host for this disease (Bhoora et al., 2020).

Antibody tests can also be taken into account for EP surveillance in wild zebra populations. Of the literature studies reviewed, only Zweygarth and coworkers performed an antibody test (*i.e.* an indirect fluorescence antibody test - IFAT) on zebra samples, quite successfully too (Zweygarth et al., 2002; see Table 3). However, IFAT requires the production of large amounts of antigen slides, which is not always practical (Brüning et al., 1997), and, although specific, IFAT has reported to lack sensitivity, especially when compared to real-time PCR (Bhoora et al., 2010b).

Better understanding of the risk factors for EP is crucial for the identification of the population at risk which will aid in the formulation of better control measures when equids are moved from diseased areas to a disease-free zone (Guidi et al., 2015). Risk factors could either be intrinsic host related or extrinsic/environmental related. According to the literature review, none of such investigation has been carried out on wild zebra species by far, but studies conducted on horses showed results correlation with sex, age, presence or absence of ticks, location, elevation, grazing, season of the year and other climatic factors (Ibrahim, 2018; Santos et al., 2011; Onyiche et al., 2019). In this study we analysed different intrinsic and environmental factors, even if none resulted to be statistically significant ($p < 0.05$). However, increasing the sample size may allow to unravel significant factors, not detected in this study, also because of the limited number of analysed animals

8 CONCLUSION

Our study suggests great potential for the use of an anti-PA ELISA on zebra sera as diagnostic tool in an active surveillance system, thus revealing real anthrax epidemiological features in KNP. On the contrary, in KNP is not suggested to focus passive surveillance systems on zebra as indicator, since this species is not the most representative in KNP anthrax outbreaks. Instead, impala and kudu are the best species for this purpose. Since soil is the real reservoir for *B. anhtracis*, the main goal for anthrax surveillance is not so much the understanding of the role of individual animals, which however poses interesting aspects by other ways, but the comprehension of environmental factors that facilitate anthrax outbreaks.

Since zebra shows little or no clinical signs when infected with *T.equi*/*B.caballi*, an active surveillance is the best approach to investigate and track equine piroplasms. The most suitable specimen to be sampled is represented by blood samples to be analysed then with a direct test. In this study RLB actually doesn't seem to be reliable for this purpose, due to an apparent low sensibility. On the contrary, multiplex EP real-time qPCR developed by Bhoora et al., (2020) is highly suitable for this investigation on wild zebra populations, allowing high sensibility detection and genotyping at the same time. IFAT may also be a suitable tool for EP active surveillance, but it is time consuming, highly subjective most especially in interpreting the fluorescence, and requires a large quantity of antigen (Brüning et al., 1997). The efficiency of other indirect tests (Ab detection) has not been evaluated in this study.

When possible, it is suggested to build surveillance strategies based on the search of multiple pathogens, instead of focusing on one only. This measure may allow to spare economic resources and work burden by the veterinary and laboratory staff, thus useful for further investigations.

GIS (Geographic Information System) revealed to be a useful tool for both diseases. Analysis with QGIS software permitted to acquire a wide range of environmental variables, allowing comparison with ELISA and RLB results. However, with statistical tools used (chi square and Fisher exact test) no significant correlation existed between environmental variables and diagnostic results, mainly due to the paucity of samples collected.

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APPENDICES

A. Technical sheets on zebra carcasses investigated for anthrax at KNP

Smear Date	Sex	Locality	Utilization	Result*	Section
1992/02/26	Male	Babalala		P	Vlakteplaas
1992/11/12	M	Mabyeni		P	Pafuri
1993/01/12	Female	Timhisini		P	Pafuri
1994/01/17	F	Mayingani	Lion	P	Pafuri
1994/04/04	F	Linanda	Lion	N	Tshokwane
1994/04/09	F	Mapetane	Lion	N	Satara
1994/05/02	F	Mapetane Spruit	Lion	N	Satara
1994/05/04	M	Phalaborwa	Lion	N	Phalaborwa
1994/05/14	F	Rabalais Dam	Lion	N	Kingfisherspruit
1994/06/08	F	Nyameni		N	Houtboschrand
1994/08/09	M	Tangari	Lion	N	Houtboschrand
1994/08/09	M	Tangari	Lion	N	Houtboschrand
1994/08/15	M	Kempiana	Lion	N	Kingfisherspruit
1994/08/29	M	Tixikweni	Lion	N	Lower Sabie
1994/09/07	M	Kingfisher	Lion	N	Kingfisherspruit
1994/09/20	F	Vlakteplaas	Lion	N	Vlakteplaas
1994/10/15	F	Shabila	Hyeana	N	Mahlangeni
1994/10/16	F	Welverdiend	Lion	N	Satara
1994/11/10	M	Bloupankop	Lion	N	Houtboschrand
1995/01/05	F	Kovawanwambu	Lion	N	Mahlangeni
1995/01/15	F	Mavumbye Block	Lion	N	Satara
1995/02/04	M	Bloupankop	Lion	N	Houtboschrand
1995/02/20	F	Sekelbospan	Lion	N	Lower Sabie
1995/03/01	F	Mamba Area	Lion	N	Houtboschrand
1995/03/01	M	Mananga	Lion	N	Satara
1995/03/02	F	Hbr	Lion	N	Houtboschrand
1995/03/18	F	Mananga	Lion	N	Satara
1995/03/29	F	Nwanetsi East	Lion	N	Satara
1995/03/29	F	Mathikithi Koppie	Lion	N	Satara
1995/04/03	F	Mavumbye	Sick	N	Satara
1995/04/11	F	Nwatingahla	Lion	N	Kingfisherspruit
1995/04/14	F	Mavumbye	Lion	N	Satara
1995/04/28	F	Camp	Lion	N	Shingwedzi
1995/06/01	F	Munangu	Lion	N	Mahlangeni
1995/06/24	M	Lindanda	Lion	N	Kingfisherspruit

1995/07/31	F	Larini	Lion	N	Mahlangeni
1995/08/03	F	Mavumbye	Lion	N	Satara
1995/08/04	M	Kambane	Lion	N	Satara
1995/08/21	M	Mavumbye		N	Satara
1995/08/23	F	Mavumbye		N	Satara
1995/08/27	F	Mabhakana Spruit	Lion	N	Lower Sabie
1995/09/18	F	Pretoriuskop	Lion	N	Houtboschrand
1995/10/10	F	Oukraal	Lion	N	Lower Sabie
1995/10/16	F	Mlondozi Pikithi	Ridaka'	N	Lower Sabie
1995/10/19	M	Satara	Lion	N	Satara
1995/10/21	M	N'waswitsontso	Lion	N	Kingfisherspruit
1995/10/21	F	Muntshe	Lion	N	Lower Sabie
1995/11/06	M	Mashatudrif	Lion	N	Houtboschrand
1995/11/07	F	Bekemibi	Lion	N	Crocodile Bridge
1995/11/10	F	Houtboschrand	Lion	N	Houtboschrand
1995/11/23	M	Bukweneni		N	Stolsnek
1995/12/06	M	Hbr		N	Houtboschrand
1995/12/12	F	Hans Hoheisen Gate	Lion	N	Kingfisherspruit
1995/12/22		Xitsakane Spruit	Lion	N	Satara
1995/12/23	F	Houtboschrand	Lion	N	Houtboschrand
1995/12/28		Naladzi		N	Mahlangeni
1996/01/19	M	Orpen	Lion	N	Kingfisherspruit
1996/01/23		Nkambane Spruit	Lion	N	Satara
1996/01/29	F	Monza		N	Satara
1996/02/17	F	Kovawa Nwambu	Lion	N	Mahlangeni
1996/02/20	M	Linanda	Lion	N	Tshokwane
1996/04/01	F	Hlangari	Lion	N	Houtboschrand
1996/04/09	M	Mara Blok		N	Satara
1996/04/10	F	Hangklip		N	Houtboschrand
1996/04/20	F	Houtboschrand	Lion	N	Houtboschrand
1996/07/18	M	Mamihali	Lion	N	Mahlangeni
1997/02/05	F	Mandlati	Lion	N	Houtboschrand
1997/02/18	M	Piet Grobler		N	Houtboschrand
1997/06/19	F	Satara	Lion	N	Satara
1997/08/02	F	Modzweni Block	Lion	N	Crocodile Bridge
1997/08/26	F	Nsemane	Lion	N	Satara
1997/08/29		Sweni	Lion	N	Satara
1997/09/29		Houtboschrand		N	Houtboschrand
1997/11/13	F	Muntshe	Lion	N	Lower Sabie
1997/11/22	M	Mavumbye		N	Satara

1998/01/21	M	Byaxixi	Lion	N	Mahlangeni
1998/01/26	M	Mlondozi Picket	Lion	N	Lower Sabie
1998/01/27	M	Lower Sabie	Lion	N	Lower Sabie
1998/01/31	M	Houtboschrand	Lion	N	Houtboschrand
1998/02/02	F	Roodewal		N	Houtboschrand
1998/02/23	F	Houtboschrand		N	Houtboschrand
1998/03/09	M	Mlondozi	Lion	N	Lower Sabie
1998/03/19	M	Nwanetsi Bridge	Lion	N	Satara
1998/03/23		Nwanetsi	Lion	N	Satara
1998/05/05		Mafuyekana	Lion	N	Crocodile Bridge
1998/07/14	F	Piet Se Fontein	Lion	N	Houtboschrand
1998/08/24	M	Rockvale	Lion	N	Satara
1999/02/17	M	Tshokwane	Lion	N	Tshokwane
1999/04/16	F	Xidzidzi	Lion	N	Satara
1999/04/16	M	Xidzidzi	Lion	N	Satara
1999/04/16	F	Xidzidzi	Lion	N	Satara
2002/09/30	F	Leeubron		N	Satara
2003/05/19	M	Satara		N	Satara
2003/05/24	F	Timbavati		N	Timbavati
2005/11/26	F	Bomas	Lion	N	Satara
2006/07/10	M	Shitsakani West		N	Satara
2007/08/24	M	Xixangani Solar Pump	Shot	N	Satara
2007/10/19	M	Mananga	Lion	N	Satara
2007/11/04	M	Bangu	Lion	N	Houtboschrand
2007/11/10	M	Bangu		N	Houtboschrand
2008/02/18	M	Xidzidzi	Lion	N	Satara
2008/02/18	F	Xidzidzi	Lion	N	Satara
2008/03/10	M	Mapetane	Lion	N	Satara
2008/03/17	M	Mavumbye S190 Road		N	Satara
2008/05/08	M	Morgenson North		N	Kingfisherspruit
2008/07/05	F	Nkomgoma	Lion	N	Lower Sabie
2009/02/21	F	Maxagadzi		N	Shingwedzi
2009/04/01	M	Mapetane	Lion	N	Satara
2009/09/11	M	8 Miles	Lion	N	Houtboschrand
2009/09/20	F	Nkambana	Lion	N	Satara
2009/11/19	F	Bangu	Lion	N	Houtboschrand
2010/02/19	F	Ngotso	Lion	N	Houtboschrand
2010/03/10	F	Shakhegula Next To Road		N	

2010/10/21	F	Mansengane Pan	Lions , Vultures	P	Pafuri
2010/10/22	F	Stapelkop Dam	Lion	N	Mahlangeni
2012-01-16	Female	Shitsalaleni	Unknown	N	Houtboschrand
2012-01-16	Female	8 Miles Boundary	Unknown	N	Houtboschrand
2012-02-28	Female	Nhhlatswani	Leopard	N	Shangoni
2012-05-24	Unknown	Makuleke	Unknown	N	Pafuri
2012-05-29	Female	Swein Spruit		N	Satara
2012-09-10	Female	Gorge	Unknown	N	Houtboschrand
2012-09-10	Female	Bouvlei Block	Lion	N	Mahlangeni
2012-09-18	Male	Capricorn Inclosure	Vulture	N	Mooiplaas
2012-09-18	Male	Nyarini Block	Unopened	N	Mahlangeni
2012-09-19	Female	Gotji	Lion	N	Houtboschrand
2012-09-19	Female	Bovlei Block	Lion	N	Mooiplaas
2012-10-04	Female	Erfplaas		N	Phalaborwa
2012-10-18	Unknown	Mshatu F/Break	Lion	N	Houtboschrand
2013-02-18	Male	Sweni	Lion, Jackal	N	Satara
2013-03-18			Jackal, Vulture	N	Pafuri
2013-04-08	Female	Houtboschrand	Unknown	N	Houtboschrand
2013-04-08	Male	Xitsalaleni	Jackal, Vulture	N	Houtboschrand
2013-05-21	Female		Unopened	P	Pafuri
2013-05-21	Male	Levubu East		N	Pafuri
2013-06-07	Male	Mashikiri Block	Lion, Vulture	N	Pafuri
2013-06-07	Female	Shikuyu Block	Unopened	N	Pafuri
2013-07-16	Male	Mavumbye	Lion	N	Satara
2013-07-22	Female	Shitsalaleni	Lion	N	Houtboschrand
2013-10-15	Female	Detol Block	Lion	N	Punda Maria
2013-10-15	Male	Makhadzi	Unopened	N	Letaba
2013-11-11	Female	Mbhatsi	Lion	N	N'wanetsi
2013-11-11	Male	Pumbe	Lion, Hyaena, Jackal, Vulture	N	N'wanetsi
2013-12-02	Male	Mahubyeni Block	Vulture	N	Mahlangeni
2013-12-12	Unknown		Lion	Inconclusive	Houtboschrand
2014-01-21	Female	Punda Maria Rest Camp	Unopened	N	Punda Maria
2014-01-21	Male	Ecotraining	Other (Specify)	N	Pafuri
2014-02-18	Unknown	Mashikiri	Vulture	N	Pafuri
2014-03-08	Male	Rhino Boma	Hyaena, Vulture	P	Pafuri
2014-03-19	Unknown		Lion	N	Shingwedzi
2014-03-19	Male	Hapi Block		N	Pafuri
2014-03-19	Unknown	Barnard Graf	Jackal, Vulture	P	Pafuri
2014-05-02	Male	Kumani Block	Lion	N	N'wanetsi

2014-05-02	Female		Hyaena, Vulture	N	Letaba
2014-05-19	Female	Hutwini	Vulture	N	Pafuri
2014-06-03	Male	Rhino Boma	Vulture	P	Pafuri
2014-07-03	Male		People	P	Pafuri
2014-07-03	Male		Hyaena, Jackal, Vulture	N	Pafuri
2014-07-03	Male	Mayengani	Vulture	P	Pafuri
2014-07-23	Male	Shisalaleni Block	Lion	N	Houtboschrand
2014-07-26	Unknown	Nwatsitsumbe	Lion, Vulture	N	Vlakteplaas
2014-08-04	Female	Wildernes	Unopened	P	Pafuri
2014-08-25		Deku Block	Hyaena, Vulture	N	Pafuri
2014-08-27	Female		Vulture	P	Pafuri
2014-08-27	Female	Number 12	Lion, Vulture	N	Pafuri
2014-09-08	Female	Phery South	Lion	N	Houtboschrand
2014-09-29	Female	Tar Road Close To K/Fish Ranger	Vulture	N	Kingfisherspruit
2014-11-24	Female	Pongolo River	Lion	N	Woodlands
2014-11-24	Female	Shireni	Lion	N	Woodlands
2014-12-18	Female	Nwasvetsakani	Vulture	N	Woodlands
2014-12-18	Male	Mhala Mhala	Vulture	N	Shangoni
2015-01-26	Female	Sholuka	Vulture	P	Pafuri
2015-01-29	Male	Hapi Block	Vulture	P	Pafuri
2015-01-29	Male	Hapi Pan	Vulture	N	Pafuri
2015-02-02	Female	Xitsalaleni	Lion	N	Houtboschrand
2015-02-06	Male	Xitsaleni	Vulture	N	Houtboschrand
2015-02-09	Female	Hapi, Picniek, South	Vulture	P	Pafuri
2015-02-09	Female	Hapi	Vulture	P	Pafuri
2015-02-09	Unknown	Witklippad	Vulture	P	Pafuri
2015-02-09	Unknown	Witklippad	Hyaena, Vulture	P	Pafuri
2015-02-09	Male	Hapi Block	Vulture	P	Pafuri
2015-02-17	Male	Hapi	Vulture	P	Pafuri
2015-02-17	Female	Hapi Block	Vulture	P	Pafuri
2015-02-17	Male	Hapi	Vulture	P	Pafuri
2015-02-17	Male		Vulture	P	Pafuri
2015-02-17	Female	Hapi	Vulture	P	Pafuri
2015-02-17	Female	Hapi	Vulture	P	Pafuri
2015-02-17	Male	Hapi	Unopened	P	Pafuri
2015-02-19	Female	Mhinga Block	Vulture	P	Pafuri
2015-02-19	Male	Hapi	Vulture	N	Pafuri
2015-02-19	Male	Hapi	Vulture	P	Pafuri

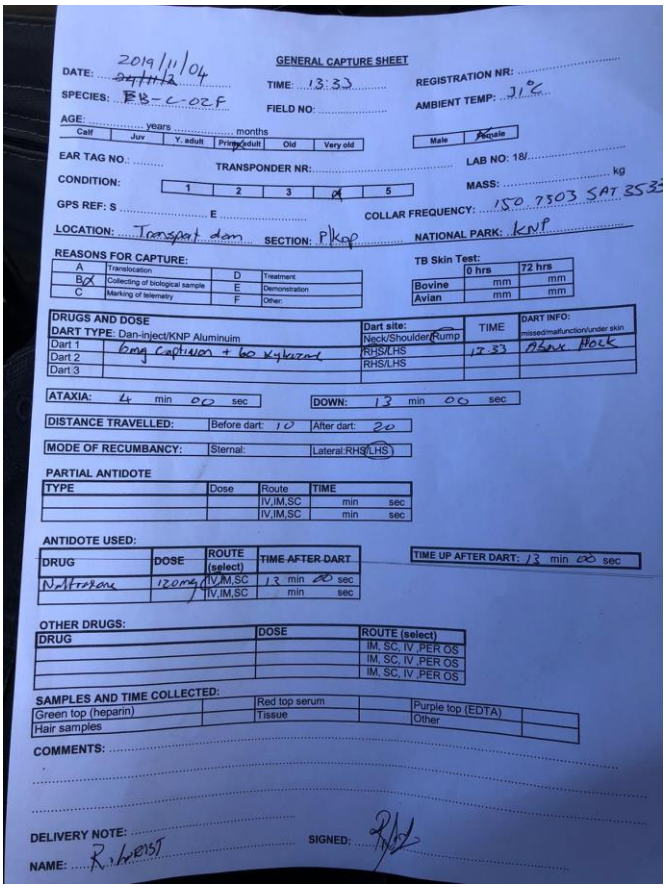
2015-02-19	Female	Hapi	Vulture	P	Pafuri
2015-02-19	Female	Thandzwi Block	Vulture	P	Pafuri
2015-02-20	Female	Pafuri	Vulture	P	Pafuri
2015-02-20	Female	Happi Pan	Vulture	P	Pafuri
2015-02-20	Female	Happi Pan	Vulture	P	Pafuri
2015-02-24	Unknown	Xinonyana	Vulture	N	Pafuri
2015-02-24	Male	Hapi Block	Unopened	P	Pafuri
2015-02-27	Female		Vulture	P	Pafuri
2015-02-27	Female	Hapi Link Road		P	Pafuri
2015-02-27	Male		Vulture	P	Pafuri
2015-02-27	Female	Palm Vlei	Vulture	P	Pafuri
2015-03-04		Ranghela	Vulture	N	Letaba
2015-03-05		Balule Block	Lion, Jackal, Vulture	N	Houtboschrand
2015-03-10	Female	Hapi	Vulture	P	Pafuri
2015-03-25	Female	Xikuyu Block	Unopened	N	Pafuri
2015-03-25			Unopened	N	Pafuri
2015-04-13	Male	Tihongoneni	Jackal, Vulture	N	Mooiplaas
2015-09-08	Female	Singita Consession	Unopened	N	N'wanetsi
2015-10-01	Male	Mahhiaghele	Vulture	P	Pafuri
2015-11-17	Female	Mbatsane	Unopened	P	N'wanetsi
2015-12-03	Male	Phiri	Unopened	N	Mahlangeni

P indicates positive Giemsa stain smears for typical bacilli of *Bacillus anthracis* collected during passive surveillance system, while N indicate negative smear.

B. Photo gallery

Monday 4/11/2019, Skukuza, Kruger National Park

Capture of a live prime adult female zebra and collection of blood samples with Louis van Schalkwyk and At Dekker.



GENERAL CAPTURE SHEET

DATE: 2019/11/04 (Day/M/Y)
SPECIES: EB-C-02F
AGE: ..... years ..... months
Calf | Juv | Y. adult | Prime adult | Old | Very old
REGISTRATION NR: .....
TIME: 13:33
FIELD NO: .....
AMBIENT TEMP: 31°C
Male | Female
EAR TAG NO.: .....
CONDITION: 1 | 2 | 3 | 4 | 5
TRANSPONDER NR: .....
LAB NO: 181 .....
MASS: ..... kg
GPS REF: S ..... E .....
COLLAR FREQUENCY: 150.7503 SAT 353
LOCATION: Transport dam
SECTION: Pkop
NATIONAL PARK: KNP

REASONS FOR CAPTURE:

A	Translocation	D	Treatment
B/X	Collecting of biological sample	E	Demonstration
C	Marking of telemetry	F	Other:

TB Skin Test:

	0 hrs	72 hrs
Bovine	mm	mm
Avian	mm	mm

DRUGS AND DOSE
DART TYPE: Dan-inject/KNP Aluminuim

		Dart site: Neck/Shoulder/Rump	TIME	DART INFO: missed/malfunction/under skin
Dart 1	6mg Captivon + 60 Kyluzol	RHS/LHS	12:33	Above Neck
Dart 2		RHS/LHS		
Dart 3				

ATAXIA: 4 min 00 sec
DOWN: 13 min 00 sec
DISTANCE TRAVELLED: Before dart: 10 After dart: 20
MODE OF RECUMBANCY: Sternal: Lateral: RHS/LHS

PARTIAL ANTIDOTE

TYPE	Dose	Route	TIME
		IV,IM,SC	min sec
		IV,IM,SC	min sec

ANTIDOTE USED:

DRUG	DOSE	ROUTE (select)	TIME AFTER DART
Naltrexone	120mg	IV,IM,SC	13 min 20 sec
		IV,IM,SC	min sec

TIME UP AFTER DART: 13 min 00 sec

OTHER DRUGS:

DRUG	DOSE	ROUTE (select)
		IM, SC, IV ,PER OS
		IM, SC, IV ,PER OS
		IM, SC, IV ,PER OS

SAMPLES AND TIME COLLECTED:

Green top (heparin)		Red top serum		Purple top (EDTA)	
Hair samples		Tissue		Other	

COMMENTS: .....

DELIVERY NOTE: .....
NAME: R. Louis
SIGNED: .....

Thursday 14/11/2019, Skukuza, Kruger National Park

Injecting naltrexone in the jugular vein of a kudu in order to reverse anaesthesia/opioids effects.

Monday 18/11/2019, Pafuri, Kruger National Park

Collecting pelvis bones from an impala carcass suspected for anthrax.

Tuesday 19/11/2019, Pafuri, Kruger National Park

Collecting rib bones from a giraffe carcass suspected for anthrax.

Monday 25/11/2019, Skukuza laboratory, Kruger National Park

Performing bacterial culture from the bone samples collected in the Pafuri area, KNP.