Prevalence and genetic relatedness of *Besnoitia besnoiti* isolates from different geographical regions of South Africa

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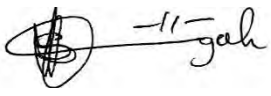
Signed:  Name: Dr. S. Chitanga Date: 30 November 2015

ABSTRACT

Bovine besnoitiosis is a protozoan disease caused by an apicomplexan parasite *Besnoitia besnoiti* and is reportedly re-emerging in Europe and occurring in many other countries including South Africa. The disease has long been neglected and has only recently started getting attention due to its increasing geographical distribution. This parasite causes significant economic losses due to reduced body condition, declined milk production, irreversible sterility in males and mortality. This study was conducted to determine the prevalence of *Besnoitia besnoiti* infection in cattle, as well as to establish the phylogenetic relationship among parasite isolates in different geographical regions of South Africa, where the disease was previously reported. A total of 688 cattle (688 blood and 376 skin samples) were randomly sampled from farms located in Limpopo, Gauteng, KwaZulu-Natal and Eastern Cape provinces of South Africa. Based on the analyses of DNA sequences of the nuclear ribosomal internal transcriber spacer 1 (ITS1), it was observed that 15.7% (108/688) of the sampled animals were positive, with 5.3% (20/376) and 14.4% (99/688) of the animals being positive on skin and blood samples, respectively. 2.9% (11/376) of the animals were positive on both blood and skin samples. The difference between in prevalence between the areas sampled were not significant, $\chi^2 = 0.263$. The parasite was more prevalent in communal farms (30.8 %) and in exotic breeds (35.3 %) than local or mixed breeds, and the difference in prevalence between farming and breed type were not significant ($\chi^2 = 0.199$, $\chi^2 = 0.227$ respectively). Aligned sequences were analysed by Maximum parsimony, neighbour joining and maximum likelihood and phylogenetic analysis of the isolates was carried out. Results showed that, based on the ITS1 region, our isolates were closely related to the wildebeest strain which is currently used for the manufacture of the vaccine, forming a clade which is separate from the European strains. One of the isolates from Gravelotte, Limpopo province, was closely related to the European strains, forming a sister clade for the European strains from GenBank. This is the first report on molecular characterisation of the parasite in South Africa and will aid in disease surveillance and control in the studied areas.

PREFACE

The research contained in this thesis was completed by the candidate, from September 2014 to October 2015, while based in the Discipline of Parasitology, School of Life Sciences, University of KwaZulu-Natal, Westville Campus, under the supervision of Professor S. Mukaratirwa and Dr S. Chitanga. The research was financially supported by National Research Foundation (NRF).

The contents of this study represent original work by the author, and have not been submitted in any form to another tertiary institution, except where the work of others is acknowledged in the text.

Signed: Prof. S. Mukaratirwa

Date: 30 November 2015

DECLARATION

I Mokgadi Pulane Malatji, declare that:

1. The research reported in this thesis, except where otherwise indicated, and is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a. Their words have been re-written but the general information attributed to them has been referenced
    b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.
5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signature: _____

Signed: Malatji M.P

Date: _____

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A special thanks to my parents and my siblings Modjadji, Mokhenete and Kabelo for their unconditional love, support, encouragement and understanding. You are the pillars of my strength

LIST OF ABBREVIATIONS

µl – Microliter

µm – Micrometre

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

H_2O – Water

IFAT – Immunofluoroscence antibody test

ITS – Internal transcribed spacer

PCR – Polymerase chain reaction

Taq - Thermus aqueous

TBE – Tris/ Borate/ EDTA

UV – Ultraviolet

MEGA – Molecular evolutionary genetics analysis

DnaSP - DNA Sequence Polymorphism

TCS – Transitive consistency score

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CHAPTER 1: INTRODUCTION

1.1 Background

Bovine besnoitiosis is a chronic and debilitating disease of cattle, caused by *Besnoitia besnoiti* (*B. besnoiti*) and reported to be endemic in sub-Saharan Africa, Middle East and France (Bigalke and Prozesky, 1994; Chatikobo *et al.*, 2013). The causative agent is a cyst-forming coccidial parasite belonging to the family Sarcocystidae, subfamily Toxoplasmatidae and phylum Apicomplexa. The disease manifests cutaneously and systemically and in its chronic form is referred to as elephant skin disease (Jacquiet *et al.*, 2010; Alvarez-Garcia *et al.*, 2013). *B. besnoiti* is regarded as a parasite of economic importance through mortality of infected animals, loss of income caused by reduced value of hides and downgrading of carcass quality, and permanent or temporary sterility in breeding bulls that survived the acute and chronic stages of the infection (Bigalke, 1968; Basson *et al.*, 1970; Sekoni *et al.*, 1992; Cortes *et al.*, 2005). The disease has recently been reported to be re-emerging in Europe (European Food Safety Authority, 2010).

The parasite affects cattle, regardless of age, sex and breed, although clinical features occur rarely in calves less than 6 months of age (Maqbool *et al.*, 2012). The cyst stage causes clinical changes in the skin, subcutis, blood vessels, and mucous membrane and during acute stage, the tachyzoites start invading the blood vessels of the skin, subcutaneous tissues, fascia and testes, causing a widespread of vasculitis and thrombosis, resulting in a severe generalized reaction accompanied by oedema of the skin and acute orchitis (EFSA, 2010). The chronic stage of the disease is characterized by progressive thickening, folding, wrinkling and necrosis of the skin resulting in chronic scleroderma (Bigalke, 1968; Alvarez-Garcia *et al.*, 2013).

Besnoitia besnoiti is thought to have a heterogeneous life cycle (Jacquiet *et al.*, 2010), with both cattle and antelopes known to be intermediate hosts (Pols, 1960; Basson *et al.*, 1970). However, the definitive host(s) has not been identified yet. Although the lifecycle of *B. besnoiti* and the routes of transmission are not yet clearly known (Diesing *et al.*, 1988; Basso *et al.*, 2011; Olias *et al.*, 2011), cattle-to-cattle transmission mechanically or via hematophageous insects, is thought to be the main transmission route (Bigalke, 1968; Castillo *et al*., 2009). According to Schofield and Torr (2002), mechanical transmission occurs when a stable fly is interrupted during blood feeding by host defensive behaviour or other flies and complete its blood meal on nearby animals (Doyle *et al.*, 2011).

In South Africa, the presence of *B. besnoiti* was first reported in Rustenburg district in the North West province (Hofmeyr, 1945). Subsequent cases were described by Bigalke (1981) in the bushveld and lowveld of the Limpopo, North West, Mpumalanga and KwaZulu-Natal provinces, with few cases reported in the Northern Cape and western Free State provinces. However, the biology and epidemiology, definitive host, intermediate and reservoir host of the parasite is still unknown (Bigalke and Prozesky, 1994).

Therefore, this study was aimed at determining the prevalence of *B. besnoiti* infection and establishing the genetic relatedness of parasite isolates from different geographical regions in South Africa.

1.2 Objectives

The specific objectives of the study are:

a. To determine the prevalence of *B. besnoiti* infection in cattle from selected regions of South Africa using molecular methods.
b. To determine the genetic relatedness of *B. besnoiti* isolates from selected regions of South Africa.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Besnoitia besnoiti (B. besnoiti) is an obligate intracellular protozoan of cattle which is widely distributed in some countries of Africa, Asia and in the south western regions of Europe. When the geographical distribution and prevalence of bovine besnoitiosis seemed to increase at the end of 20th century, the disease started receiving attention. According to Alzieu *et al*., (2007), the disease was previously encountered in the south west of France, and the clinical cases have recently been reported at a regular basis in the French Alps, the Massif Central and occasionally in the Loire region of France. Additionally, the outbreak of besnoitiosis in cattle was recently reported for the first time in Germany (Mehlhorn *et al*., 2009). The infection is responsible for severe economic losses on affected farms as well as the cattle industry as a whole (Cortes *et al*., 2006b). There is less information on the mode of transmission of the disease and the recommended measures to prevent and control the spread of this parasitic disease.

2.2 Description of pathogen

Bovine besnoitiosis is a disease of cattle caused by a cyst-forming Apicomplexan parasite *Besnoitia besnoiti* belonging to the family *Sarcocystidae* and sub-family *Toxoplasmatinae*, which is closely related to *Toxoplasma gondii*, *Neospora caninum* and *Hammondia hammondi*. To date, ten (10) species have been identified in the genus *Besnoitia* (*B. besnoiti, B. bennetti, B. jellisoni, B. wallacei, B. tarandi, B. darling, B. caprae, B. akadoni, B. neotomofelis and B. oryctofelisi*) (Nganga *et al*., 1994; Dubey and Lindsay, 2003; Dubey *et al*., 2003a, b, 2004, 2005; Oryan and Azizi, 2008; Dubey and Yabsley, 2010). Among the ten species, *B. besnoiti*, *B. caprae, B. bennetti*, and *B. tarandi*, have been observed to cause similar disease but in different host species (bovids, goats, equids and wild ruminants respectively) (Álvarez-Garcia *et al*., 2014a).

2.3 Mode(s) of transmission

Bovine besnoitiosis can be transmitted either through direct and indirect horizontal transmission via insect vectors (both hematophagous and non-biting insects) or close contact (mating or physical among animals with wounds or lacerations) and through the use of one syringe during herd health procedures (Bigalke, 1968; Pols, 1960; Bigalke and Prozesky, 1994). Papadopoulos *et al*. (2014) indicates that of the currently known mode of disease transmission, mechanical

transmission via blood-sucking insects is probably the most important one. This is thought to occur when a fly feeding on an infected host is interrupted, meaning it has to take a second feed on susceptible host, thus transmitting the parasite via its contaminated mouthparts and via regurgitation (Baldacchino *et al*., 2013). The other proposed route of infection is through ingestion of mature isosporan-type oocystes shed in faeces of definitive host (Peteshev *et al.*, 1974). Once in the host, the sporozoites enter host circulation where they multiply in endothelial cells of especially the skin, fasciae, upper respiratory tract and testes, producing more endozoites (Basson *et al.*, 1970). Whilst the genus has a wide host range, ranging from reptiles to mammals, the parasite species are highly host specific (Bigalke & Prozesky, 1994).

The clinical disease manifestation can either be viscerotropic or dermatotropic depending on the species. According to Bigalke (1968), the infection by *Besnoitia besnoiti*-like organism which has also been described in two species of antelopes (impala and blue wildebeest) appear to be viscerotropic and non-pathogenic. Cysts formation is said to commence about one week after the initial cycle of proliferation, where hypertrophic cystozoite-containing histolytic cells when activated become detectable in the same site where the endozoites were formed. The histiocytic cells are observed either within or in close association with blood vessel walls and the bradyzoites (*c. 8.4×1.9 μm*) therefore multiply by endodyogeny in vacuoles of the markedly enlarged host cells with enlarged and multiple nuclei (Rommel, 1978). The characteristics thick-walled cysts, with their conspicuous periodic-acid, Schiff-positive hyaline walls, apparently formed by the host-cell nuclei, reach a diameter of *c. 400 μm* after about six weeks (Bigalke & Prozesky, 1994). Cyst formation stage is remarkably synchronous in experimental cases; however, there is no evidence that cystozoites from disintegrating cysts give rise to further endozoites or cysts in the same animal according to Bigalke (1968). Cyst formation is associated with the chronic stage of the disease (Bigalke & Prozesky, 1994). With the *Besnoitia* species that infect rodents and lagomorphs, it has been observed that these cysts are mostly in the viscera, whilst for *B. besnoiti*, *B. benneti* and *B. tarandi*, the cysts are mostly found in the skin (Cortes *et al*., 2014).

2.4 Life cycle

Though the complete life cycle of the parasite remains unknown, it is suspected that all *Besnoitia* species have a heteroxenous (two-host) life cycle with predators (cat) as final host and prey as intermediate host in which the cysts are formed, however, the definitive host of *B. besnoiti* is still yet to be defined (Wallace and Frenkel, 1975; Dubey, 1977; Rommel, 1978) (cited by EFSA, 2010). The parasite undergoes two infective asexual stages of development (fast replicating tachyzoites and slower replicating bradyzoites) in the intermediate host where they are found in cysts within subcutaneous connective tissue (Alvarez-Garcia *et al*., 2013). Early experimental studies suggested the involvement of domestic cats or dogs as the final host(s) for *B. besnoiti* (Peteshev et al., 1975; Rommel, 1978). Peteshev *et al*. (1974) reported that the domestic and wild cats shed oocysts after ingesting cyst-containing tissue as it occurs for other *Besnoitia* species. (Figure1).

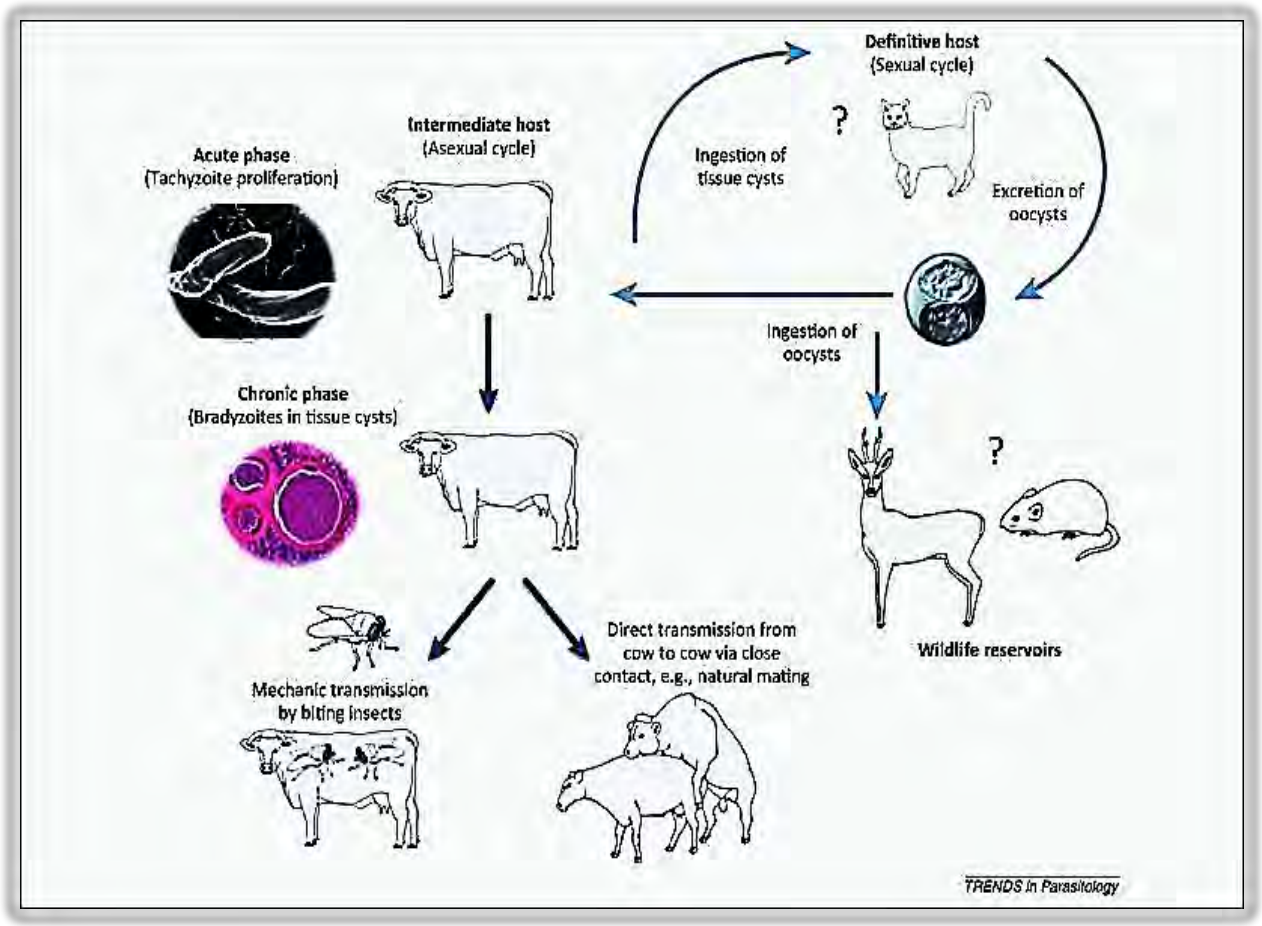

Figure 1: Life cycle and transmission of *Besnoitia besnoiti* (Álvarez-Garcia *et al*., 2013)

2.5 Pathogenesis, clinical signs and pathological findings

The transmission experiments have shown that once an animal is infected by bovine besnoitiosis, the incubation period vary from 1-13 days depending on the method of infection with an average of 13 days for natural infection (Bigalke, 1968; Basson *et al*., 1970; Bigalke and Prozesky, 1994). However, Álveraz-García *et al*. (2013) argued that the incubation period can go as far as up to two months. Bovine besnoitiosis progresses in two sequential phases: the febrile acute phase (anasarca stage) caused by the rapid replication of tachyzoites in the endothelial cells, tunica media, adventitia and mono-nuclear cells, followed by the chronic phase (scleroderma phase), during which pathognomonic tissue cysts develop and cause skin lesion (Basson *et al*., 1970). Subsequently, the affected animal remain persistently infected, and carrier of the disease (Álveraz-García *et al*., 2013).

2.5.1 Febrile acute phase

This stage may last for about 1 to 10 days. The infection is initially characterized by hyperthermia (40.8-41.6), which normally cause abortion in pregnant dams (Pols, 1960; Bigalke, 1968; Given and Marley, 2008), followed by non-specific clinical signs that may go unnoticed such as depression, swelling of the superficial lymph nodes, weight loss, photophobia, ocular and nasal discharge and increased respiratory and heart rates (Pols, 1960; Schulz, 1960). It is during this stage that the infection may be misdiagnosed as dermatophilosis, sarcoptic mange, sweating sickness, lumpy skin disease and photosensitivity **(**Bigalke & Prozesky, 1994**)**. During this acute stage, tachyzoites start invading the endothelial cells of blood vessels causing degenerative and fibrinoid necrotic vascular lesions, vasculitis, and thrombosis, which then cause congestion, hemorrhages and infarct (Pols, 1960; Basson *et al*., 1970). Based on literature, these lesions are mainly located in the smaller and medium-sized veins as well as some arteries in the skin and testes, whereby the vascular lesions play an important role in the pathogenesis of bovine besnoitiosis (McCully *et al*., 1966; Basson *et al*., 1970). The rapid multiplication of parasites have been observed up to 10-12 days post infection, with proliferative organisms only detectable from third up to the 12^{th} day of the beginning of the infection (Bigalke, 1968; Basson *et al*., 1970).

The rapid multiplication of parasite in the blood cause the increase in vascular permeability, leading to oedema which initially appears in the head and neck areas and may progress to the limbs and ventral part of the body such as the breasts and scrotum, where it more visible (Pols,

1960; Basson *et al*. 1970). According to McCully *et al*. (1970), the parasite may cause acute inflammation of the eyes, usually accompanied by oedema, increased lacrimation and photophobia. Hemorrhages, necrosis, pseudo-membranes, oedemas, and erosions may be found in the upper respiratory tract, however, oedemas in the alveolar and interstitial tissues in the lungs, accompanied by pneumonitis and emphysema occurs in severe cases of this phase and may cause severe respiratory disorders with oedemas in joints causing permanent posterior lameness (Álveraz-García *et al*., 2013).

2.5.2 Chronic or scleroderma phase

The chronic stage of the disease is generated at a slower rate by the slower replication of the bradyziotes inside the tissue cysts with tropism for connective tissues mainly on the mucous membrane, superficial skin layers and male genital tract (Pols, 1960; Bigalke, 1968; Kumi-Diaka *et al*., 1981). Although Besnoit and Robin (1912) suggested the occurrence of parasite released into the blood periodically, there is currently no evidence of parasitemia in chronically infected cattle. Based on Bigalke's findings (1968), cyst formation occurs immediately after extra-cystic proliferation stops at approximately 11 days post-infection in blood vessel wall and extravascularly as well. Young cysts may contain 1-4 bradyzoites, however, the hypertrophic cells thereof become multinucleated and rapidly enlarge until four weeks post infection when their growth declines (Basson *et al*., 1970; Dubey *et al*., 2013).

A progressive thickening, folding or wrinkling of the skin and scrotal skin, alopecia, hyperkeratosis, scleroderma, scars and nodules on udders, atrophy and induration of testes have been observed to occur as a result of oedema disappearance (Álveraz-García *et al*. 2013). In this phase, the tissue cysts are generally more likely to be found in the skin, sclera, upper respiratory tract, testes and epididymis in males and in the *vestibulum vaginae* and vagina in females. Based on Basson *et al*. (1970), the skin of the face, upper eyelid, tip of the tail and breech harboured the highest parasite load. However, Frey *et al*. (2013), using histological and molecular tools, observed that the cysts were most frequently located in the upper respiratory tract, genital tract and skin of the neck.

2.6 Diagnosis

As early as 1968, the examination and identification of cysts in the conjunctiva was the method of choice for diagnosis (Bigalke, 1968). Currently, there are a number of available diagnostic tests to augment the above mentioned methods such as cytology (Sanussi, 1991), histopathology (Bigalke, 1968), serology using either ELISA; western blot and/ or IFAT (Cortes *et. al*, 2006a; Alvarez-Garcia *et al*., 2009; Fernandez-Garcia *et al*., 2009; Fernandez-Garcia *et al*., 2010; Garcia-Lunar *et.al, 2012*) and molecular techniques using either conventional or real-time polymerase chain reaction (Cortes *et al*., 2007; Schares *et.al*, 2011). The methods vary in their sensitivity and specificity and their use is sometimes dictated by the stage of infection of the animal (Bigalke *et al*., 1974; Fernández-García *et al*., 2010; Schares *et al*., 2011). Molecular techniques, such as PCR, are useful in detecting acute infections and serological techniques like ELISA are useful aids in the detection and control of subclinical animals as well as carrier animals (Fernández-García *et al*., 2010; Schares *et al*., 2011).

During the first weeks of infection, acutely affected animals may be difficult to diagnose as clinical signs are non-specific (Kumi-Diaka *et al*., 1981). Molecular techniques based on sequence amplification of the ITS-1 of the ribosomal DNA gene of *B. besnoit*i have been advocated as the method of choice for accurate identification and diagnosis of the disease as well as indicating whether serological positive animals are infectious to the herd (Schares *et.al*, 2011). Identification of serologically positive animals is a major relevance to elaborate appropriate measure of control. While identification of clinical cases is relatively easy to carry out, the findings of subclinical forms of infection is more difficult, thus serology is considered as an appropriate diagnostic tool to detect the presence of circulating antibodies against this infection (Cortes *et al*, 2006a; Schares *et al*., 2011, Garcia-Lunar *et.al*, 2012). Although there is more information on the seroprevalence of the infection (EFSA, 2010), little has been done on parasite detection and prevalence using molecular techniques.

2.7 Economic impact and welfare

2.7.1 Effects on productivity

Based on Franco and Borges (1916) observations, the occurrence of bovine besnoitiosis frequently coincides with the introduction of animals. These are basically the males introduced specifically for reproduction (breeding males) purposes, either avoiding consanguineous situations in the herds

or promoting heterosis. By buying sub-clinically infected animals and introducing it into the farms without any appropriate diagnosis of infection for relevant parasitic diseases, owners are introducing a disease that can spread within the herd. When this occurs with *Besnoitia*-infected cattle, atleast 10% of the animals are expected to acquire the disease and to have lost their commercial value within the next 3 years (Pols, 1960; Bigalke, 1968). After three years, the intra-herd prevalence is high (frequently higher than 80%) and from time to time an animal may develop clinical symptoms. When an infected herd has a high prevalence of sub-clinically infected animals, commonly the diseased animals are the näive ones being introduced for reproduction purposes (Cortes *et al*., 2005, 2006b). The presence of the parasite in the semen reduces sperm quality causing infertility in bulls, in the alveolus reduces milk production; in pregnant dams the parasite causes abortion, reduces hides quality and market value, the animals loose body condition and in some cases, mortality occurs (Bigalke 1968; Kumi-Diaka *et al*., 1981). Overall, this poses a significant productivity burden since milk, calves, and hides production are the main basis of beef production.

2.7.2 Economic impact

The disease is characterized by both local and systematic clinical signs of varying severity. Cattle may die during the course of the infection, although low mortality rate less than 10% has been reported (Pols, 1960; Bigalke, 1968; Jacquiet *et al*., 2010). In an endemic situation, only a few animals of *B. besnoiti* infected herd develop clinical signs (Bigalke, 1968), whereas most are seropositive but remains sub-clinically infected (Bigalke, 1968; Fernandez-Garcia *et al*., 2010; Garćia-Lunar *et al*., 2013). In areas where the disease is emerging, the incidences of clinical case are approximately 15-40% per year versus 1-10% per year in areas of endemic bovine besnoitiosis (Jacquiet *et al*., 2010; Fernandez-Garcia *et al*., 2010). In many infected animals, the only sign of the disease is the presence of pathognomonic, thick-walled tissue cysts in scleral conjunctiva and vaginal mucosa. However, the disease is responsible for severe economic losses such that infected cattle may lose weight and exhibit decreased milk production, dams may abort, males may develop a transient infertility or even sterility, and the hides of affected animals are of reduced value for leather production (Jacquiet *et al*., 2010).

Although the disease causes serious illness that compromise animal welfare and great loss on market value of affected animals; the economic impacts of bovine besnoitiosis in South Africa

have not been quantified. This correlates with the scenario in Europe (EFSA, 2010). More research on the economic impact of the disease has to be done, and quantified.

2.8 Epidemiology

Due to the spread and geographical expansion of bovine besnoitiosis in Europe, considerable effort has been made to the understanding of the epidemiological aspects of *B. besnoiti* (Alzieu, 2007; Jacquiet *et al*., 2010; Liénard *et al*., 2011; Basso *et al*., 2011).

In enzootic and epizootic areas, infection is reportedly widespread with however only a small proportion of the infected animals exhibiting clinical signs (Pols, 1960; Bigalke, 1968; Legrand, 2003; Bourdeau *et al*., 2004; Cortes *et al*., 2006b; Alzieu *et al*., 2007). Such areas are typified by animals falling in either of the following sub-groups; i) only a small proportion of animals developing typical clinical signs, ii) a large subset of seropositive animals with sclera-conjunctival cysts, and iii) a large subset of sub-clinical, seropositive individuals (Pols, 1960; Bigalke, 1968; Goldman and Pipano, 1983). Typically in enzootic areas, prevalence of clinical cases ranges from 1 – 10% per annum, with incidences ranging between 2 – 5% (Legrand, 2003). In newly introduced animals, proportion of clinical cases can be as high as 20%. Even though younger animals may develop clinical disease, the disease is commonly reported in animals 2 – 4 years old (Legrand, 2003; Alzieu *et al.*, 2007).

2.8.1 Geographical distribution

Bovine besnoitiosis has been previously described in Europe, Africa and Middle East (Bigalke and Prozesky, 1994; EFSA, 2010; Cortes *et al.*, 2014). The disease was first described in in southern France in the middle nineteenth century and was first discovered by Besnoit and Robin who reported it as sarcosporodiosis. However, the first recorded incident of the disease was reported in 1884 by Cadéac as elephantiasis (Bigalke and Prozesky, 1994). Recently, it has been recognized as an emerging disease in European countries such as France, Portugal, Spain, Germany and Italy (Gottstein *et al*., 2009) and there is evidence of an increased number of cases and geographic expansion of the disease.

In Africa, the disease has been reported in South Africa (Hofmeyr, 1945; Bigalke, 1981), Swaziland (Njenga *et al*., 1999), Mozambique (Ferreira *et al*., 1983; Ferreira & Diaz, 1984) Zimbabwe (Chatikobo *et al*, 2013), Angola (Leitao, 1945; Njenga *et al.*, 1999), Congo (Njenga *et*

al., 1999), Kenya (Njagi *et al.*, 1990), Cameroon (Njenga *et al*., 1999) and Nigeria (Oduye, 1974; Kumi-Diaka *et al*., 1981; Sambo *et al*., 2007). In South Africa, the disease has been previously reported in Mpumalanga, North West, Kwa Zulu-Natal and Limpopo provinces, where they have been considered to be of economic significance, with few incidences reported from Northern Cape and Free State province (Bigalke and Prozesky, 1994). Beside the prevalence studies previously undertaken by several authors, summarized by EFSA (2010), no current prevalence studies have been conducted in South Africa.

2.8.2 Risk factors

Owing to the limited knowledge of the life cycle of *B. besnoiti*, it is difficult to clearly define the risk factors responsible for *B. besnoiti* infection at a herd level. However, key factors such as seasonality, breed, age, gender, infection route, vectors and reservoirs, and sub-clinical carriers contributes as risk factors for the transmission and incidences of the infection (EFSA, 2010).

Seasonality: According to the observation made by Bigalke (1968), majority of new cases in South Africa occurred during warmer, moist moths of the year. Based on limited observations from two outbreaks in Europe, it was noted that the emergence of clinical sings coincided with the summer period, when mixed herds shared pastures (Alzieu, 2007; Fernandez-Garcia *et al*., 2009) and when blood sucking arthropods are active.

Breed: Despite Hofmeyr (1945**)** reporting the absence of bovine besnoitiosis in dairy cattle in South Africa, the infection has been observed in both beef and dairy cattle (EFSA, 2010). Bigalke (1981) reported that most cases of the infection in South Africa occurred in the Afrikander, and the *Bos indicus* breeds, which is the most common breed in the endemic regions. There are several reports from Israel that beef cattle have been found to be more serologically positive than dairy cattle (Neuman, 1972; Gollnick *et al*., 2010), and that may mainly be due to the difference in the husbandry conditions for both production types.

Age: There is a positive relationship between age of the animal and the epidemiology of the disease (Bigalke, 1981). Reports show there is a significant increase in seroprevalence and morbidity associated with age (Fernandez-Garcia *et al*., 2010). The highest incidence of infection was detected in adult animals on a farm where the disease was present, but was rarely encountered

in calves less than 6 months of age. A similar trend was seen in a recent outbreak of the disease in central Spain (Fernandez-Garcia *et al*., 2009).

Sex: Despite the previous contradictory observations made by Goldman and Pipano (1983) on the relationship between gender and the disease prevalence, several studies have been unable to identify sex of the animal as a risk factor of the infection (Bigalke, 1981 & Bigalke 1968). According to Jacquiet *et al*. (2010), the report from France indicated that male cattle are often serologically positive than females, and clinical signs are more severe in bulls. Bigalke (1981) also did not observe sex difference with respect to clinical incidences.

Infection route: The infection route is also a critical factor of infection, mainly because not only vectors can mechanically transmit the disease, but management practices can also play a crucial role in the transmission of the disease (Álvarez-Garcia *et al*., 2014a). According to literature, incubation period of the disease in cattle is highly dependent on the route of infection, and the shortest period observed was less than two days, when tachyzoites were transmitted intravenously. Consequently, the infection route is corresponds with the time period required to develop mature tissue cysts (Bigalke, 1968; Basson *et al*., 1970). According to Álvarez-Garcia *et al*. (2014a), subcutaneous and intravenous infection appear to resemble the natural transmission of the parasite. When oral and nasal infection were experimented in cattle infection, the successful incubation period observed lasted between 9 and 14 days (Bigalke, 1968). When viscera of experimentally infected rabbits harboring most likely only tachyzoites were transmitted orally to an ox, the distinct febrile phase observed lasted for 5 days, and tissue cysts were detected 80 days post infection. However, typical bovine besnoitiosis clinical signs were absent.

Aptitude: The differences in prevalence have been detected between beef and dairy cattle. According to Álvarez-Garcia *et al*. (2014a), this dissimilarity appear to be influenced by the difference in management practices than breed susceptibilities. Seroprevalence of 10% for dairy cattle to 50% for beef cattle was reported in Israel when 1700 animals were screened, with the highest antibody titres corresponding to beef cattle (Goldman and Pipano, 1983). Similar case was observed in the mountainous areas of the traditionally endemic Northern Province in Spain (Navarra), where 16% seroprevalence was observed in beef cattle compared to 0% in dairy cattle (Álvarez-García *et al.* 2014b). This is because the management practices in beef cattle systems, such as natural mating, communal pastures outdoors, and exposure to wild ruminants (red deer

and roe deer) and blood-sucking arthropods favours parasite transmission. Hence, the observed difference in prevalence between beef and dairy cattle (Frank et al. 1977; Goldman and Pipano, 1983; Fernández-García *et al*., 2010). Furthermore, most risky management practices (e.g. natural mating and communal pastures outdoors) conducted in extensive husbandry systems are irrelevant under intensive husbandry. Because artificial insemination is primarily practiced in intensive dairy cattle herds, animal trade is considered to be the main entryway for the infection into the herds, and the most important significant outcome of the infection might be weight loss, reduced milk production and occasional abortion (Álvarez-Garcia *et al*. 2014a).

Vector and reservoirs: The existence of blood-sucking flies could be a risk factor for the rapid spread of the disease (Zacarias, 2009). However, their role in parasite transmission is highly transmitted on seasons with high density of flies (summer seasons/ wet period) (Bigalke, 1981). Mechanical transmission by *Glossina*, *Stomoxys* and tabanids has been demonstrated by Bigalke (1968) and explained earlier, and the potential role of wildlife reservoirs of disease, such as wild ruminants and rodents, has also been suggested (Bigalke, 1981; Castillo *et al*., 2009; Mehlhorn *et al*., 2009).

Sub-clinical carrier: Infection with *Besnoitia* pathogen does not always manifest as clinical disease. A higher prevalence has been observed in sub-clinical animals in some outbreaks in Spain (Fernandez-Garcia *et al*., 2010), and this also agrees with the observation made by other authors in South Africa (Bigalke, 1981). In Europe, the geographical expansion of the disease within and outside the endemic areas has been traced back to the introduction of new animals into the herds. These are animals introduced to the suggested to be healthy based on the absent of clinical signs, either for heterosis or just addition to the herd (Hornok *et al*., 2014).

2.8.3 Prevalence of *B. besnoiti* infection

Based on antibody detection, levels of exposure tend to be high, with however a few of such animals developing clinical disease. Serological surveys done in Israel and South Africa have been reported to be as high 66.9 % and 50%, respectively (Bigalke, 1981; Janitcshke *et al.*, 1984).

Few bovine besnoitiosis prevalence studies have been conducted in South Africa and Israel (Bigalke, 1981; Janitcshke *et al*., 1984). According to Bigalke (1968), the initial reported prevalence of the disease was usually less than 10 % mainly because the diagnosis only relied on

the detection of the cysts in the scleral conjunctiva and skin. The first serorevalence studies conducted Israel showed a high rate of 64.4-66.9% in beef cattle from Israel (Neuman, 1972; Frank *et al*., 1977) and, 50 % in sub-clinical animals in South Africa (Janitcshke *et al*., 1984). These studies also indicated that beef cattle normally show a higher rate of seropositivity as compared to dairy cattle, and this is probably due to their difference in husbandry (that is beef cattle are more often raised under extensive conditions)**.**

Recent work carried out in Sierra de Urbasa Andia (Navara, North Spain) area located close to Pyrenees showed individual seroprevalence varying between 44.5 % with ELISA and 48.6 % with IFAT, and it was then argued that the difference in prevalence depends on the diagnostic technique used (Zacaris, 2009). In an outbreak reported in the non-endemic areas on central Spain, 90.8 % (319 animals of the 351 animals examined) females and 71.4 (5 of the 7 animals examined) males were seropositive (Fernandez-Garcia *et al*., 2010). Nevertheless, only 43.02 % (154 from 358) of examined animals showed at least one clinical sign. In addition to that, only five out of 358 animals that showed clinical signs were seronegative by ELISA. In these animals, clinical signs only consisted of mild oedema and skin lesions in the eyes, on the udder and on the feet. According to Cortes *et al*. (2006b), these results correlate with the study on beef cattle farm in Portugal, where a 36 % seroprevalence increased to 70 % over a course of 18 months.

In the study carried out in Uganda, typical elephant skin appearance of clinical besnoitiosis was observed among 8.7 % of the cattle (Bwangamoi, 1968). Similar clinical signs were seen in 12 % of the cattle in South Korea (Hi-Suk *et al*., 1970), but may be inapparent in some cases. Based on findings by Oduye (1974), cysts of *B. besnoiti* were accidentally discovered in skin section of 4.1 % of the cattle at Ibadan in South Western Nigeria, although there were no gross lesions or clinical signs of the diseases. Further investigations indicated that 4.6 % of the cattle in Kaduna State (Sambo *et al*., 2007) and 8.7 % in Borno State (Igbokwe *et al*., 2009) harboured cysts of the parasite observed in the skin sections, with or without gross lesions of the disease. Since the absence of or failure to observe gross lesions and clinical manifestations may allow some cases of the disease to pass undiagnosed, antibody detection is recommended for the screening of the epidemiology of bovine besnoitiosis.

2.9 Control

The control of bovine besnoitiosis rely entirely on management measures coupled with clinical inspection of especially chronically ill cattle. This is because only a limited proportion of infected animals develop show clinical signs of infection. Secondly, during the acute phase of the infection, the disease can be confused with other illnesses that cause hyperthermia and anasarca (Bigalke and Prozesky, 1994). Until the animal develops cysts, especially on the scleral conjunctiva and on the vaginal mucosa, the clinical presentation alone cannot be used to conclude the presence of bovine besnoitiosis, especially during acute phase (Álveraz-García *et al*. 2013). According to Jacquiet *et al*. (2009), only up to 15 or 20 % of newly infected cattle in areas where the disease is emerging show typical signs. Since a large proportion of infected animals remain sub-clinical, the management and control of the disease calls for more sensitive and robust diagnostic tools for the implementation and monitoring of control programs.

The control of bovine besnoitiosis can be done mainly to achieve two objectives which are: avoiding the introduction of the infected animals into a herd, and avoiding the spread of the disease by reducing the prevalence of infection within a herd gradually (Figure 2). As new infections mainly occur following the introduction of carrier animals, the most feasible approach for maintaining a herd free of the disease is to implement an effective diagnostic test to all new animals prior to entry. Moreover, if the herds are located in areas of high disease prevalence, practices such as sharing of pasture and use of bull for reproduction pose risks that should be avoided. If possible, all animals should be screened for *B. besnoiti* infection at the end of the pasturing period and insect season prior to being kept indoors, as new cases are usually detected immediately after the transmission period (Bigalke, 1968). Infected animals should be removed from the herd or pastured separately in the following season.

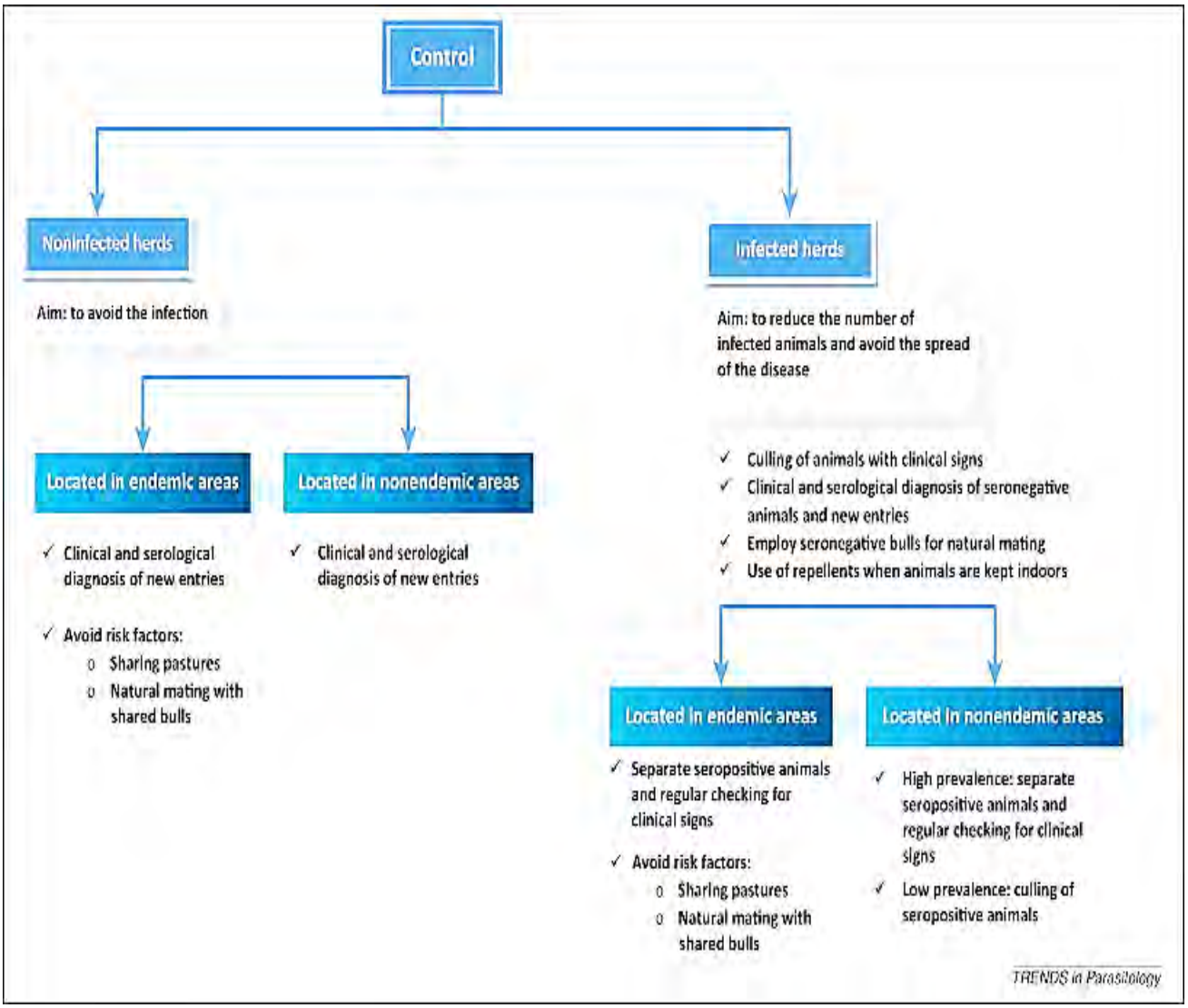

Figure 2: Schematic diagram of management measures coupled to diagnosis for the control of bovine besnoitiosis (Álvarez-Garcia *et al*., 2013).

A conservative long-term step-by-step strategy that appears to be the best option for the control of infection is to maintain the cost-benefit balance between selective culling and production, as seroprevalence might be high in an infected herd (Bigalke, 1968; Kumi-Diaka *et al*., 1981). On a regular basis, the most severely affected animals have to be replaced by healthy *B. besnoiti* seronegative animals, which, ideally, should be kept separate from the rest of the herd. The selection of replacement animals requires intensive monitoring of individual health and production data. Therefore, this approach is more effective in intensive rather than extensive management conditions, where artificial insemination and close monitoring of the production parameters are performed, and grazing is not allowed or restricted to nearby pastures. This favours the possibility

of separating healthy, tested animals from those to be replaced over time; thus, the chance of eradicating *B. besnoiti* will remain high (Liénard *et al*., 2011 & 2012). Under extensive management condition without the possibilities of repeatedly indirect transmission by biting insects, a decrease in prevalence may be achieved, but eradication may turn out to be a difficult task since there are no enough resources and or experienced people who deal with the efficient control and eradication of diseases (Álvarez-Garcia et al., 2013). However, the absence of clinical signs in the herd should not be the reasonable aim, which can be achieved by periodical visual inspection of animals to identify new clinical cases, and subsequent removal of the affected animals.

There are no effective drugs and vaccines licenced in Europe. However in South Africa and Israel a live-attenuated wildebeest strain of *B. besnoiti* vaccine has been used (Bigalke *et al*., 1974) and found 100% protective to the cattle to the chronic stage of the disease over a four year period (EFSA, 2010). Sulfonamides have been used to reduce clinical signs however, the drugs fail cure the infected cattle and relapses are not rare even if treatment is given quickly (Jacquiet *et al*., 2010). Tetracyclines have been used in the field but the efficacy has not been clearly demonstrated (Jacquiet *et al*., 2010).

CHAPTER 3: MATERIALS AND METHODS

3.1 Study site and sample collection

The study was conducted in Eastern Cape, Gauteng, Limpopo and KwaZulu-Natal provinces of South Africa where *B. besnoiti* has been reported in the past 5 years. Samples were collected from four provinces and the location names are shown in appendix 1. The sample size was determined by the following formula:

$$n = \frac{Z^2 p(1-p)}{d^2}$$

Where; n = required sample size;

z = Z value for a given confidence level (95 % = 1.96);

p = estimated prevalence, and

d^2 = allowable error (5 %).

The estimated prevalence was 15 %, which is higher than the one (< 10 %) described by Bigalke (1968) when clinical signs were examined. Therefore, the calculated sample size was $[(1.96)^2 \times 0.15 \times (1\text{-}0.15)] / (0.05)^2$ = 196 animals. However, the practical working sample was raised to a total of 688 animals to maximise the probability of finding the parasite since there is no data on molecular based prevalence of parasite on the sampled areas and South Africa.

Areas with historical cases of bovine besnoitiosis were identified, and based on their accessibility, a total of 38 farms (Eastern Cape = 140, Gauteng = 427, Limpopo = 101, Kwa Zulu-Natal = 20) were purposively selected and screened in order to increase the chances of getting positive animals for phylogenetic analysis. A total number of 688 animals (blood = 688, skin biopsy = 376), aged 24 months or more, were randomly sampled from communal, small scale and commercial farms in the above mentioned provinces (appendix 1). Blood samples were collected from the tail vein using a vacutainer and a drop on a filter paper, whilst skin biopsies were obtained from the tail or neck and preserved in absolute ethanol.

3.2 DNA extraction

DNA was extracted from blood from the filter paper and skin biopsy using the Tris EDTA protocol (Bereczky *et al.*, 2005) and DNA easy™ tissue kit system (Zymo Research Corporation) respectively.

3.3.1. Blood DNA extraction

DNA was extracted according to protocol described by Bereczky (20015). Briefly, approximately 4mm diameter of blood filter paper was cut, placed in an Eppendorf tube, and added 100µl of tris EDTA. The tubes were incubated for 15 minutes at 50°C and then another 15 minutes at 95°C. The mixture was then centrifuged for 1 minute at 14000 rpm and the supernatant were then transferred to a new tube and stored for future use.

3.3.2. Skin DNA extraction

DNA was extracted using the Zymogen DNA extracted kit (Zymo Research Corporation) according to the manufacturer's instructions. Briefly, 95µl of H_2O, 95µl of 2 × Digestion buffer and 5µl of Proteinase K solutions were added into a tissue sample in a micro centrifuged tube, mixed and incubated at 55°C for 1-3 hours. 700µl of genomic lysis buffer was added to the mixture and mixed completely by vortexing and centrifuging for one minute at 10, 000 × *g* to remove the insoluble debris. The supernatant was then transferred into a Zymo-spin IIC™ column in a collection tube and centrifuged for a minute at 10, 000 × *g*. The Zymo spin column was transferred into a new collection tube and 200 µl of DNA pre-wash buffer was added, and then centrifuged at 10, 000 ×*g* for one minute. 400 µl of genomic DNA wash buffer was added to the spin column and centrifuged for one minute at 10, 000 × *g*. The spin column was transferred to a clean micro-centrifuge tube and added 50µl of DNA elution buffer into the spin column. The mixture were incubated for 2-5 minutes at room temperature and then centrifuged at top speed for 30 seconds to elute the DNA. The eluted DNA was used for further molecular analysis (PCR).

3.3 PCR and electrophoresis

Conventional PCR was used to amplify a segment of the ITS1 region using primer sequences ITS1 F 5' – TGA CAT TTA ATA ACA ATC AAC CCT T – 3' and ITS1 R 5'-GGT TTG TAT TAA CCA ATC CGT GA- 3', which were designed on conserved sequence in the 5.8S ribosomal

gene of *B. besnoiti*. PCR amplification was performed using the above mentioned marker in a 25 µl reaction volume. Each reaction consisted of 10 µl top taq mastermix, 2.5 µl of each primer (forward and reverse) and 10 µl DNA. PCR was performed in a thermocycler (BIORAD) machine under the following conditions: 2 minutes initial denaturation step at 94°C, followed by 45 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 57°C and 1 minute polymerization at 72°C, and the program ended with a final polymerization step for 20 minutes at 72°C.

2% suspension of agarose in 1X TBE buffer was heated to dissolve the agarose. 100 µ1 of Ethidium Bromide (0.5 mg/ml) was added to the solution prior casting of the gel, in order to allow visualization of the DNA bands by the transilluminator with UV lights. 5 µl of each PCR product was mixed with 1µl loading dye prior loading into the wells. 5 µl of Gene ladder was co-electrophoresed and the samples were then electrophoresed at 100V for 40 minutes in 0.5X running buffer. An Uvitec UV transilluminator was used to visualize the DNA bands and the image was captured using an Uvitec digital camera, with the positive sampled identified by a 231 base pair band (figure 3).

Figure 3: Agarose gel- electrophoretic analysis (2%) of amplification products from conventional *Besnoitia besnoiti* ITS1 mtDNA PCR from skin samples. GeneRuler (m) and negative control (N) on the left and positive (P) control on the right

3.4 Sequencing

Unpurified PCR products and gel were despatched to Central Analytic Unit at Stellenbosch University for DNA sequencing. DNA fragments were sequenced in the forward and reverse directions using the primers used in the initial amplification.

3.5 Molecular analysis

3.6.1 Phylogenetic analyses

The sequences were manually edited to remove minor inconsistencies using BIO-Edit version 5.2 (Hall, 1999). Closest matches determined by BLAST searches of the NCBI GenBank were included in the analyses. Multiple alignment of the sequences were carried out by a computer-generated alignment Muscle with default option (Edgar, 2004), and alignments were further edited by visual inspection. Sequences were then trimmed to uniform length.

Molecular analyses included Maximum parsimony, Neighbour-joining and Maximum likelihood method using MEGA6 (Tamura *et al*., 2013). Bootstrap analysis was performed using 1000 bootstraps (100 replicates). Analyses included sequences from GenBank and their accession numbers: South Africa (AF076859.1), Israel (DQ227420.1), two isolates in Spain (DQ227418.1 and Bb-1 EU789637.1), Portugal (AY833646.1), German (Bb-Ger1 FJ797432.1) and two isolates from calf / adult *Bos taurus* cattle in Bavaria (FN257463.1 / FN257462.1) as in-groups and out-group sequences from the Sarcocystidae family *Taxoplasma gondii* (L37415.1), *Hammondia hammondi* (AF096498.1), and *Neospora caninum* (U16159.1). Individual pairwise genetic p-distances between the sequences and haplotypes were calculated using MEGA6.

3.6.2 Haplotype and population genetics analyses

DNA Sequence Polymorphism (DnaSP) version 5 (Librado and Rozas, 2009), was used to determine the number of haplotype in the data set, as well as the haplotype and nucleotide density values. TCS version 1.21 (Clement *et al*., 2000) and PopArt (Clement *et al.,* 2002) were used to create a statistical parsimony haplotype network in order to illustrate the relationship between the haplotypes.

3.6 Statistical analysis

Prevalence of *B. besnoiti* was calculated using the following formula:

$$Prevalence\ (P)\% = \frac{Total\ number\ of\ infected\ animals}{Total\ number\ of\ animals\ examined} \times 100$$

The results were categorized and summarized into tables (according to samples, region, and farming type). The differences in prevalence were tested for level of association using Chi-Square test on SPSS 23.0 for windows and values of $p < 0.05$ were considered significant.

CHAPTER 4: RESUTLS

Out of a total of 688 cattle sampled, 108 (15.7 %) were positive for PCR (table 1). It was observed that Hluhluwe, KwaZulu-Natal, had the highest molecular prevalence as compared to other areas. The differences in prevalence among regions were not significant ($p > 0.05$). It was also observed that from the 108 samples positive, 20 (5.3 %) and 99 (14.4 %) animals tested positive on skin and blood samples respectively, with 11 animals testing positive in both blood and skin. The differences in positivity by sample type were statistically significant ($p < 0.05$)

4.1.1 Prevalence of B. besnoiti by breed type

Out of the seven (7) breed types screened, cross-breeds breed showed a higher prevalence; with Nguni breed the lowest, with and without vaccination history respectively (Table 2). The differences in positivity by breed type were not statistically significant ($p > 0.05$).

4.1.2 Prevalence of B. besnoiti infection by production system

The overall molecular prevalence of the infection was higher in communal farmers, followed by commercial farmers (Table 3). However, the differences in husbandry practices were not statistically significant ($p > 0.05$).

Table 1: Prevalence of *B. besnoiti* infection in cattle from selected provinces of South Africa

Province	Region	No. of farms	No. of animals sampled		Positive (%)		Total no. of positive (%)
			blood	skin	blood	skin	
Eastern cape	Komga	2	85	-	0 (0)	-	0 (0)
	Tsolo	1	55	-	0 (0)	-	0 (0)
Gauteng	Rust de winter	27	427	362	75 (17.6)	18 (4.9)	84 (17.6)
Limpopo	Gravelotte	2	71	14	17 (23.9)	0 (0)	17 (23.9)
	Mokopane	1	30	-	0 (0)	-	0 (0)
Kwa Zulu-Natal	Hluhluwe	5	20	20	7 (35)	2 (10)	7 (35)
Total		38	688	376	99	20	108
$\chi^2 = 0.263$, $p > 0.05$							

Table 2: Prevalence of *B. besnoiti* infection in cattle by husbandry systems in selected provinces of South Africa

Farming type	No. of farms sampled	No. of animals sampled	Positive	Prevalence (%)
Commercial	6	234	37	15.8
Small scale	25	428	63	14.7
Communal	7	26	8	30.8
Total	38	688	108	15.7
$\chi^2 = 0.199,\ p > 0.05$				

Table 3: Prevalence of *B. besnoiti* infection in cattle by breed in selected provinces of South Africa

Breed	Area	No. of farms	No. of animals sampled	Positive	Prevalence (%)
Nguni	Hluhluwe	5	20	7	35
	Komga	2	65	0	0
	Mokopane	1	30	0	0
	Tsolo	1	55	0	0
Cross breed	Gravelotte	1	34	12	35.3
Brahman	Rust de Winter	7	74	15	20.3
Brahman cross	Rust de Winter	1	22	3	13.6
Bonsmara	Gravelotte	1	37	5	13.5
	Komga	1	20	0	0
	Rust de Winter	11	180	39	21.7
Bonsmara x Brahman	Rust de Winter	6	124	20	16.1
Bonsmara x Afrikander	Rust de Winter	1	27	7	25.9
Total		38	688	108	15.7

$\chi^2 = 0.227,\ p > 0.05$

4.2 Phylogenetic relationship between parasite isolates

4.2.1 Molecular phylogenetic analysis

The *B. besnoiti* from GenBank formed a well-supported clade (99.99.99) (Figure 4). Genetic p-distances among the *B. besnoiti* isolates obtained from GenBank were 0%, between *B. besnoiti* and random cattle samples ranged from 0 to 35.9% (Appendix 2). It was also observed that the isolates from Gauteng, KwaZulu-Natal and the amplified vaccine had longer sequences consisting of four repeats of 55 nucleotides. When the sequences were trimmed to one repeat (55 nucleotides), and analysed, it produced a neighbour joining tree consisting of three major groups (Group 1, 2 and 3) (Figure 6). Analyses included the highly similar *Besnoitia* sequences from Genbank database, based in ITS1. The genetic p-distances among our isolates were 0, as well as between the Genbank samples. The analysis also revealed that of the two *Besnoitia* groups formed, the study isolates are closely related to Group 2 (34 %) consisting of *B. besnoiti*, *B. bennetti*, *B. carprae*, *B. tarandi*, as compared to Group 3 (44 %), which consist of *B. jellisoni*, *B. darling*, *B. akadoni*, *B. neotomofelis* and *B. oryctofelisi*. This might lead to the assumption that Group 1 might be a new geographical group/strain, falling under the genus *Besnoitia*.

4.2.2 Haplotype analysis

Analysis based on 200 nucleotides of ITS-1 region yielded 7 haplotypes (figure 5), with a haplotype diversity of 0.187. When set on 95% parsimony criterion, transitive consistency score (TCS) yielded a neighbour-joining network consisting of three major groups. Genbank isolates formed two different haplogroups, with one group consisting of the LP isolate. Haplotype 1, 2 and 3 represented the vaccine and three sequences from GP and KZN, Genbank sequences from LP, Israel, Spain and Portugal and lastly the haplogroup 3 represented by Genbank sequences form Bavaria, Spain and German.

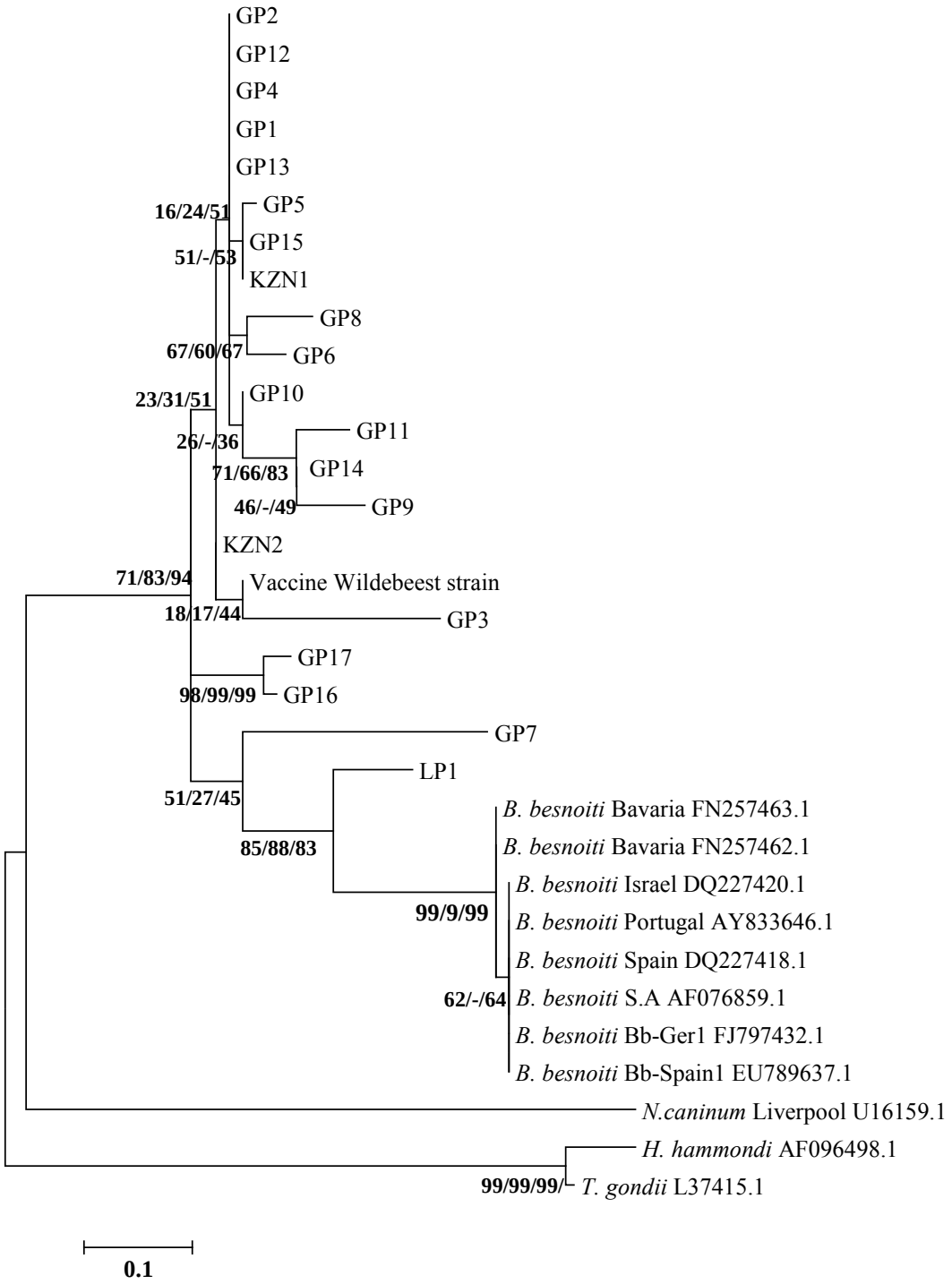

Figure 4: Phylogenetic tree based on 200 nucleotides of the nuclear ribosomal ITS-1 region of the parasite, showing the relationship between South African cattle isolates, Genbank *Besnoitia besnoiti* and outgroups. Nodal support is indicated as Neighbor-joining /Maximum parsimony/ Maximum likelihood (bootstrap %). GP = Gauteng province, LP = Limpopo province and KZN=KwaZulu-Natal.

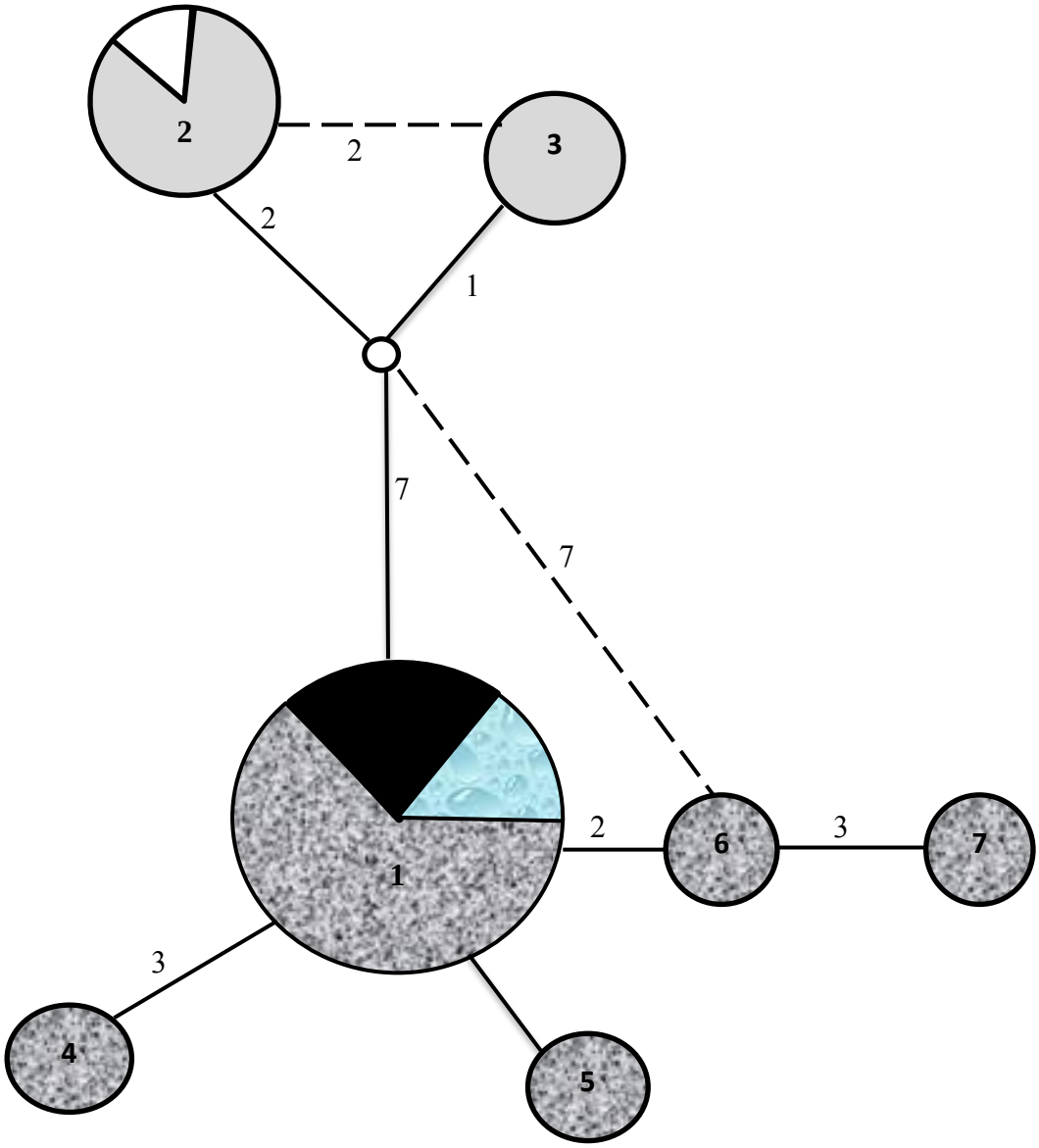

Figure 5: Statistical parsimony haplotype network on 200 nucleotides of the ITS-1 region of the cattle isolated from South Africa and Genbank *Besnoitia besnoiti* sequences. Granite = Rust de Winter (Gauteng province); White fill = Gravelotte (Limpopo province); Grey = Genbank samples; Black = Hluhluwe (Kwa-ulu-Natal province); Water droplets = vaccine. Numbers adjacent to lines connecting haplotypes represents the number of mutations

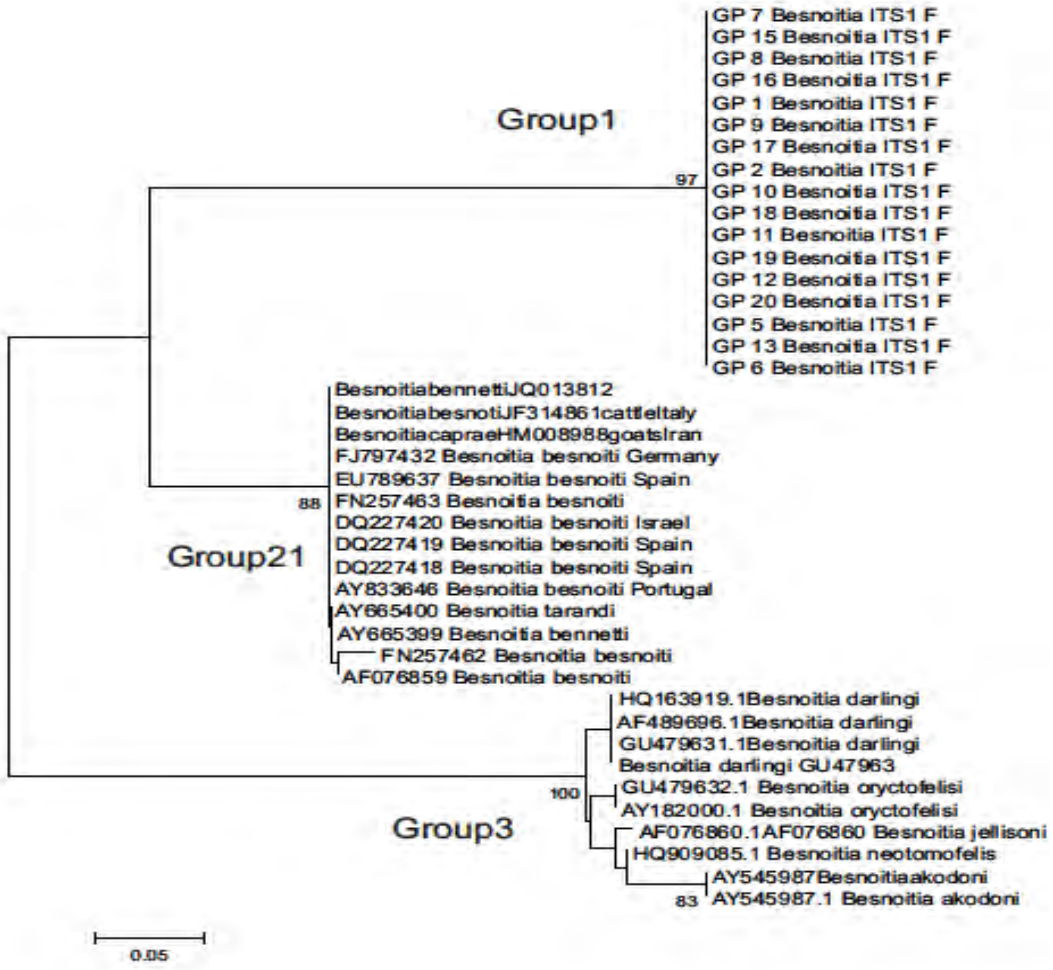

Figure 6: Phylogenetic tree based on 55 nucleotides of the nuclear ribosomal ITS-1 region showing the relationship between *Besnoitia besnoiti* isolates from cattle in South Africa, Genbank *Besnoitia besnoiti* and outgroups. Nodal support is indicated as Neighbour-joining (bootstrap%)

CHAPTER 5: DISCUSSION

Besnoitia besnoiti infections are of economic importance to cattle farmers worldwide, more especially in endemic areas (Álvarez-García *et al.* 2013). Although the disease caused by this parasite may manifest sub-clinically, the presence of the parasite in a herd causes a major impact on animal welfare and cause mortality (Bigalke, 1968; Kumi-Diaka *et al*., 1981). Prevalence studies have been conducted in South Africa and Israel, which according to Álvarez-García *et al.* (2013) were initially based on the detection of cysts in the vulva and conjunctiva of infected animals, and the effect of the parasite on the skin, which was mostly less than 10 % (Bigalke, 1968).

The results in our study showed prevalence of 35%, 17.6 % and 24.2 % from animals in Hluhluwe, Rust de winter and Phalaborwa respectively. The prevalence of infection was found to be higher in the blood than in skin samples. This could be indication that most of the infections were in the acute phase of infection, indicating possible recent infection. Also none of the animals diagnosed positive exhibited clinical disease, thus showing high levels of sub-clinical infection. Such sub-clinically infected animals serve as source of infection for the naïve animals (Gollnick *et al*., 2010). The identification of such sub-clinically animals are important for successful control of the disease within a region. The results of our molecular work show that molecular methods can be effective for identification of sub-clinically infected animals and can be used for screening purposes so as to control the disease.

Although only Gravelotte cattle were exposed to vaccinated animals within the herd prior sampling, commercial farms surrounding the sampled herds in Rust de Winter had history of vaccination against *B. besnoiti* (Makgatho, personal communication). Whilst the animals with history of vaccination were not sampled in our study, according to Cortes *et al.* (2014), it is possible that the vaccinated animals could have served as source of infection for the rest of the herd. This is because the live attenuated *B. besnoiti* vaccine induces 100% protection against clinical form of the disease if vaccinated during acute phase, however, does not cure the disease but the animals become immune to re-infection. However, according to Cortes *et al.* (2014), exposure of naïve animals to live attenuated vaccinated animals also pose a risk of introducing the parasite into the herd. Therefore, the use of combination of molecular and routine diagnostic tests on valuable animals is recommended; mainly because of the time between infection and the

appearance of antibodies may be too long, hence making it hard to detect the infection at an earlier/acute stage, and/or to increase the options to either confirm the presence or absence of parasite (Álvarez-Garcia *et al*., 2013).

This study reports an overall prevalence of 15.7 %, ranging from 0 – 35 % by regions. However, lack of molecular prevalence estimate studies on *B. besnoiti* infections leads to difficulties in comparing the study results to others. Furthermore, due to purposive sampling in this study, the prevalence estimate results are limited within the study provinces; as such they cannot be extrapolated beyond those provinces. The results also show that all seven breed types screened were infected; however, cross breeds from Gravelotte had the highest prevalence as compared to the local and crossbreed especially the indigenous Nguni breed. This observation correlates with observations made by Bigalke (1968) that all cattle breeds appear to be susceptible to besnoitiosis. However, these difference detected have been suggested to be related to breed disease resistance, aptitude, and consequently, husbandry conditions that facilitate transmission (Álvarez-Garcia *et al*., 2014).

Production systems, cattle trade and movement as well as husbandry practices are considered among the major risk factors for bovine besnoitiosis (Bigalke, 1968; Cortes *et al.*, 2005; Jacquite *et al.*, 2010). Our results show that communal animals had a higher prevalence in comparison to commercial animals, and this could be attributed to the sharing of grazing lands as well as the common trade of animals amongst communities, factors which favour easy spread of infection. Lack of resource and knowledge might also contribute to the high prevalence of the parasite in the village set-ups, because most farmers trade animals among each other without screening for disease. An introduction of few sub-clinically infected animals into a naïve herd can further spread the infection within the heard through arthropod activity (Bigalke 1968).

Historically, *Besnoitia* has been considered "*Toxoplasma*-like" (Ellis *et al*., 2000). Previous studies on phylogenetic analyses of 18S rDNA sequences showed that *Besnoitia*, *Toxoplasma*, *Hommondia* and *Neospora* form a monophyletic group, with the sister clade containing *T. gondii*, *N. caninum*, and *H. hommondi* (Carreno *et al*., 1998; Ellis *et al*., 2000). In this study, phylogenetic analyses based on ITS-1 sequences confirmed the relationship with *N. caninum.* However, *T. gondii* and *H. hommondi* formed an outgroup clade.

Isolates from Gravelotte (LP1) formed a well-supported monophyletic clade to the Genbank-derived clade consisting of *B. besnoiti* isolates from Portugal, Spain, Israel, Bavaria, German and South Africa. This corresponds with TCS analysis which when 95% parsimony criterion applied, showed a large number of mutations between the Gravelotte isolate and other isolates (GP and KZN), forming a haplogroup with the Genbank samples when grouped by PopArt (figure 5) indicating that the isolates share the same ancestor. The GP and KZN isolates, besides showing strong support from *N. caninun*, formed a weakly supported group to Genbank *B. besnoiti* and Gravelotte isolates, although relatively closely related to one another. Interestingly, the amplified vaccine isolate from the wildebeest obtained from Onderstepoort, South Africa formed a haplogroup with other samples isolated during our study thus indicating the possibilities of infection from the same strain. This observation is not surprising since *Besnoitia* from cattle and wildebeest share ITS1 sequences (Ellis *et al*., 2000).

The ITS1 sequences from isolates from KwaZulu-Natal and Gauteng provinces, along with the amplified vaccine were longer, consisting of four repeats after each 55 nucleotides as compared to the isolate from Gravelotte (LP) and the Genbank sequences. Neighbour-joining analysis based on repeats consisting of 55 nucleotides produced a tree consisting of three major groups, made up of isolates from this study, and the Genbank isolates (figure 6). The p-distance matrix revealed that there was no difference between our isolates sequences, and a uniform difference between the Genbank *Besnoitia* sequences and our isolates (29 %), with exception to one isolate from Bavaria (*B. besnoiti* Bavaria FN257462.1) (appendix 3). Furthermore, the distances between the groups 1 and 2, 1 and 3, and lastly 2 and 3 are 34 %, 61 %, and 44 % respectively. Based on a study conducted on the sequence repeats of ITS1 region in brown alga, it was concluded that despite the effect of geographical structure or aptitude on species, ITS1 is most responsible for the increased length of repeated sequences (Cho *et al*., 2009). Thus, this, serves as one of the explanation to the difference in clades among the LP, vaccine, GP and KZN isolates, despite all being confirmed positive after amplification by PCR using ITS1 primers. The lower variation in sequences correlates with the findings obtained in a comparative study where sequences of the ITS – transcribed region of *B. besnoiti* from different geographical regions showed a lower degree of sequence variation (Kiehl *et al*., 2010).

CONCLUSION

This study for the first time confirmed the presence of *B. besnoiti* in South Africa using molecular techniques. Therefore, investigation on the mode(s) transmission of the parasite could be done using these techniques. This will aid in disease surveillance and control of the parasite. Further studies on epidemiology, especially the geographical distribution and prevalence of *B. besnoiti* in other provinces of South Africa, definitive host and mode of transmission, and explore the genetic diversity of *B. besnoiti* isolates from European countries, vaccine and South African strain. There is also a need to quantify the economic losses associated with the parasite in South Africa, from the time between the animal is infected and the time period the animal loose market value.

REFERENCES

1. Agosti, M., Belloni, A., Morini, M., Vacirca,G., 1994. Segnalazione di un focolaio di Besnoitiosi in bovine da came importati. Praxis 15:5-6.
2. Alvarez-García, G., Fernandez-García, A., Aguado-Martínez, A., Schares, G., Basso, W., Gollnick, N.S., Ortega-Mora, L.M., 2009. Serological diagnosis of bovine besnoitiosis: development of an indirect ELISA and a comparative study with a commercial ELISA. In: 22st International Conference WAAVP, Calgary (Canada), p.11.
3. Álvarez-Garcia, G., Fernández-Garcia, A., Gutiérrez-Expósito, D., Ruiz-Santa Quinteria, J.A., Aguado-Martinez, A. and Ortega-Mora, L.M., 2014b. Seroprevalence of *Besnoitia besnoiti* infection and associated risk-factors in cattle from an endemic region in Western Europe (Navara, Spain). Veterinary Journal 200, 328-331. Doi: 10.1016/j.tvjl.2014.02.013.
4. Álvarez-Garcia, G., Garcia-Lunar, P., Gutiérrez-Expósito, D., Shkap, V., Ortega-Mora, L.M., 2014a. Dynamics of *Besnoitia besnoiti* infection in cattle. Parasitology, 141, 1419-1435. doi: 10.1017/S0031182014000729.
5. Álveraz-Garcia, G., Frey, C.F., Mora, L.M., Schares, G., 2013. A century of bovine besnoitiosis: an unknown disease re-emerging in Europe. Trends Parasitol 29, 407-415.
6. Alzieu, J.P., 2007. Re-emerging cattle besnoitiosis (*Besnoitia besnoiti*) in France: update on clinical and epidemiological aspects. In: 21st International Conference WAAVP, Gante (Belgium), p222.
7. Baldacchino, F., Gardès, L., De Stordeur, E., Jay-Robert, P., Garros, C.0., 2014. Blood feeding patterns of horse flies in the French Pyrenees. Veterinary Parasitology 199, 282–288. doi: 10.1016/j.vetpar. 2013.10.009.
8. Basso, W., Schares, G., Gollnick, N.S., Rutten, M., Deplazes, P., 2011. Exploring the life cycle of *Besnoitia besnoiti* – experimental infection of putative definitive and intermediate host species. Vet Parasitol, 178:223-234.
9. Basson, P.A., Mc cully, R.M., Bigalke, R.D., 1970. Observations on the pathogenesis of bovine and antelope strains of *Besnoitia besnoiti* (Marotel, 1912) infection in cattle and rabbits. Onderstepoort J. Vet. Res. 37:105-126.

10. Bereczky, S; Martensson, A; Gil, P; Färnert, A., 2005. Short report: rapid DNA extraction from archive blood spots on filter paper for genotyping of Plasmodium falciparum. Am J Trop Med Hyg. 72: 249-51.
11. Besnoit, C. and Robin, V., 1912. Sarcosporidiose Cutaneè chezunevache. Reveu Veterinaire, Toulouse, 37, 649-663.
12. Bigalke, R., 1981. Besnoitiosis and Globidiosis. In Diseases of cattle in the Tropics (Ristic, M. & McIntyre, I., eds), pp. 429-442. Martinus Nijhoff.
13. Bigalke, R., Schoeman, J.H., McCully, R.M., 1974. Immunization against bovine besnoitiosis with a live vaccine prepared from a blue wildebeest strain of *Besnoitia besnoiti* grown in cell cultures. 1. Studies on rabbits. Onderstepoort J.Vet. Res. 41, 1-6.
14. Bigalke, R.D., 1968. New concepts on the epidemiology features of bovine besnoitiosis as determined by laboratory and field investigations. Onderstepoort J. Vet. Res. 35:3-138.
15. Bigalke, R.D., Prozesky, L., 1994. Bovine besnoitiosis. In: Coetzer, J.A., Thomson, G.R., Taustin, R.C. (Eds.), infectious diseases of livestock, with special reference to South Africa. Oxford Press, Cape Town, pp. 243-252.
16. Bwangamoi, O., 1968. The incidence of skin diseases in Uganda. Bulletin of Epizootic Diseases of Africa 16(11): 115-119.
17. Carreno, R.A., Schnitzler, B.E., Jeffries, A.C., Tenter, A.M., Johnson, A.M., Barta, J.R., 1998.Phylogenetic analysis of coccidia based on 18S rDNA sequence comparison indicates that *Isospora* is most closely related to *Toxoplasma* and *Neospora*. J Euk Microbiol 45: 184–188
18. Castillo, J.A., Marcein, J.M., Ortega-Mara, L.M., Alvarez-Garcia, G., 2009. La besnoitiosis bovina, presentada coma una enfermedad emergente Europa. Albeitar 127, 24-25.
19. Chatikobo, P., Choga, T., Ncube, C., Mutambara, J., 2013. Participatory diagnosis and prioritization of constraints to cattle production in some smallholder farming areas of Zimbabwe. Prev. Vet. Med 109, 327-333.
20. Cho, G.Y., Choi, D.W., Kim, M.S., Boo, S.M. Sequence repeats enlarge the internal transcribed spacer 1 region of the brown alga *Colpomenia sinuosa* (Scytosiphonaceae, Phaeophyceae). 2009. Phycological Research, 57: 242-250.
21. Clement, M., Posada, D., Crandall, K.A., 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology 9(10): 1657-1660.

22. Clement, M., Snell, Q., Walker, P., Posada, D., Crandall, K., 2002. TCS: Estimating gene genealogies. Parallel and Distributed Processing Symposium, International Preceedings. 2 (184).
23. Cortes, H., Leitão, A., Gottstein, B., Hemphill, A., 2014. A review on bovine besnoitiosis: a disease of economic impact in herd health management, caused by *Besnoitia besnoiti* (Franco and Borges). Parasitology, 141(11):1406-17.
24. Cortes, H., Leitao, A., Vidal, R., Vidal-Vicosa, M.J., Ferreira, M.L., Caeiro, V., Hjerpe, C.A., 2005. Bovine Besnoitiosis in Portugal. Vet. Rec. (In press).
25. Cortes, H.C., Nunes, S., Reis, Y., Staubli, D., Vidal, R.., Sager, H., Leitao, A., Gottstein, B., 2006a. Immunodiagnosis of *Besnoitia besnoiti* infection by ELISA and Western blot. Vet. Parasitol. 141 (3-4), 216-225.
26. Cortes, H.C., Reis, Y., Gottstein, B., Hephill, A., Leitao,A., Muller, N., 2007. Application of conventional and real-time flourencent ITS1 PRC for detection of *Besnoitia besnoiti* infections on bovine skin biopsies, Vet. Parasitol.146 (3-4).
27. Cortes, H.C., Reis, Y., Waap, H., Marcelino, E., Vaz, Y., Nunes, T., Fanzendeiro, I., Caeiro, V., Leitao, A., 2006b. Longitudinal study of *Besnoitia besnoiti* infection prevalence rates in beef cattle in Alentejo, Portugal. In: Proceedings of the COST 854 Final Conference, Liege, Belgium, 20.
28. Cortes, H.C., Vidal, R., Reis Y., 2004. Bovine besnoitiosis, one approach for a better understanding of its importance in Portugal. Proceedings on 23rd World Buiatrics Congress 2004: 35-6.
29. Diesing, I., Heydorn, A.O., Mutaschka, F.R., Bauer, C., Pipano, E., de Waal, D.T., Porgieter, F.T., 1988. Besnoita besnoti: studies on the definitive host and experimental infections in cattle. Parasite. Res. 75(2), 114-117.
30. Doyle, M.S., Swope, B.N., Hohsette, J.A., Burkhalter, K.L., Savage, H.M., Nasci, R.S., 2011. Vector competence of the stable fly (Diptera: Muscidae) for west Nile Virus. J Med Entomol 48:656-668.
31. Dubey, J. P., Lindsay,D. S., 2003. Development and ultrastructure of *Besnoitia oryctofelisi* tachyzoites, tissue cysts, bradyzoites, schizonts and merozoites. International Journal for Parasitology 33, 807–819.

32. Dubey, J. P., Shkap, V., Pipano, E., Fish, L., Fritz, D. L., 2003a. Ultrastructure of *Besnoitia besnoiti* tissue cysts and bradyzoites. Journal of Eukaryotic Microbiology 50, 240–244.
33. Dubey, J. P., Sreekumar, C., Donovan, T., Rozmanec, M., Rosenthal, B. M., Vianna, M. C., Davis, W. P., Belden, J. S., 2005. Redescription of *Besnoitia bennetti* (Protozoa: Apicomplexa) from the donkey (Equus asinus). International Journal for Parasitology 35, 659–672.
34. Dubey, J. P., Sreekumar, C., Rosenthal, B. M., Lindsay, D. S., Grisard, E. C., Vitor, R.W., 2003b. Biological and molecular characterization of *Besnoitia akodoni* n. sp (Protozoa: Apicomplexa) from the rodent Akodon montensis in Brazil. Parassitologia 45, 61–70.
35. Dubey, J. P., Sreekumar, C., Rosenthal, B. M., Vianna, M. C., Nylund, M., Nikander, S., Oksanen, A., 2004. Redescription of *Besnoitia tarandi* (Protozoa: Apicomplexa) from the reindeer (*Rangifer tarandus*). International Journal for Parasitology 34, 1273–1287.
36. Dubey, J. P., van Wilpe, E., Blignaut, D. J., Schares, G., Williams, J. H., 2013. Development of early tissue cysts and associated pathology of *Besnoitia besnoiti* in a naturally infected bull (*Bos taurus*) from South Africa. Journal of Parasitology 99, 459–466. doi: 10.1645/12-128.1.
37. Dubey, J. P., Yabsley, M. J., 2010. *Besnoitia neotomofelis* sp. (Protozoa: Apicomplexa) from the Southern Plains woodrat (Neotoma micropus). Parasitology 137, 1731–1747.
38. Dubey, J.P., 1977. *Toxoplasma Hammondia*, *Besnoitia*, *Sarcocystis*, and other tissue cyst-formation coccidia of man and animals. In: Parasitic protozoa, vol 3, Kreier J.P. ed New York, Academic Press.
39. Edgar, R.C. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32(5): 1792-97.
40. Ellis, J.T., Holmdahl, O.J.M., Ryce, C., Njenga, J.M., Harper, P.A.W., Morrison, D.A., 2000. Molecular phylogeny of Besnoitia and genetic relationships among Besnoitia of cattle, wildebeest and goats. Protist, 151: 329-336.
41. European Food Safety Authority, 2010. Scientific statement on Bovine besnoitiosis. EFSA 8, 1499-1514.
42. Fernández-García, A., Alvarez-Garcia, G., Risco-Castillo, V., Aguado-Martinez, A., Mareen, J.M., Rojo-Montejo, S., Catillo, J.A., Ortega-Mora, L.M., 2010. Development

and use of an indirect ELISA in an outbreak of bovine besnoitiosis in Spain. Vet. Rec. 166, 818-822.

43. Fernández-García, A., Risco-Castillo, V., Padraza-Diaz,nez,, S., Aguado-Martinez, A., Alvarez-Garcìa, G., Gomez-Bautista, M., Collantes-Fernandez, E., Ortega-Mora, L., 2009. First isolation of *Besnoitia besnoiti* from a chronically infected cow in Spain. J. Parasitol., 95(2) 474-476.
44. Ferreira, M.L., Diaz, O.H., 1984. Importance of besnoitiosis in bull fertility (in Mozambique). *Proceedings of the 10th International congress on Animal Reproduction and Artificial Insemination, Urbana Campaign*, 1:270.
45. Ferreira, M.L.P., Petisca, J.L.N., Diaz, O.H., Pires-Ferreira, M.L., Nunes Petisca, J.L., 1983. Testicular lesions in bulls in Mozambique with and without clinical signs of besnoitiosis. *Repositorio de Trabalhos do Institude Nacional de Veterinaria*, 2(14): 79-108.
46. Franco, E., Borges, I., 1915. Nota sobre a sarcosporidiose bovina (cited by Pols, 1960). Revista de Medicina veterinária, Ano XIV: 255-299.
47. Frank, M., Pipano, E., Rosenberg, A., 1977. Prevalence of antibodies against *Besnoitia besnoiti* in beef and dairy cattle in Israel. Refuah Veterinarith 34, 83-86.
48. Frenkel, J.K., 1977. Besnoitia wallacei of cats and rodents: with a reclassification of other cyst-forming isosporoid coccidian. J Parasitol, 63: 611-628.
49. Frey, C., Gutiérrez-Expósito, D., Ortega-Mora, L. M., Benavides, J., Marcén, J. M., Castillo, J. A., Casasús, I., Sanz, A., García-Lunar, P., Esteban-Gil, A., Álvarez-García, G., 2013. Chronic bovine besnoitiosis: intra-organ parasite distribution, parasite loads and parasite associated lesions in subclinical cases. Veterinary Parasitology 197, 95–103. doi: 10.1016/j.vetpar.2013.04.023.
50. Garcia-Lunar, P., Ortega-Mora, L.M., Schares, G., Gollnick, N.S., Jacquiet, P., Grisez, C., Prevot, F., Frey, C.F., Gottstein, B., Alvarez-García, G., 2012. An inter-laboratory comparative study of serological tools employed in the diagnosis of *Besnoitia besnoiti* infection in bovides. Transboundary Rmerg Dis. 60: 59-68. Doi: 10/1111/j.1865-1682.2012.01218.
51. García-Lunar, P., Ortega-Mora, L.M., Schares, G., Gollnick, N.S., Jacquiet, P., Grisez, C., Prevot, F., Frey, C.F., Gottstein, B., Alvarez-García, G., 2013. An inter-laboratory

comparative study of serological tools employed in the diagnosis of *Besnoitia besnoiti* infection in bovines. Transbound Emerg Dis 60:59–68.

52. Givens, M. D. and Marley, M. S. D., 2008. Infectious causes of embryonic and fetal mortality. *Theriogenology* 70, 270–285. doi: 10.1016/j.theriogenology.2008.04.018.
53. Goldman, M., Pipano, E., 1983. Serological studies on bovine benoitiosis in Israel. Trop. Anim. Health Prod. 15, 32-38.
54. Gollnick, N.S., Gentile, A., Schares, G., 2010. Diagnosis of bovine besnoitiosis in a bull born in Italy. En: Vet Rec. 2010. Vol.166. p. 599.
55. Gottstein, B., Jaquiet, F., Schares, G., Cortes, H., Ortega-Mora, L.M., 2009. Symposium on bovine besnoitiosis. In: 22nd International Conference WAAVP, Calgary (Canada). 38.
56. Hall, T.A., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41: 95-98.
57. Hi-Suk, L., Bak, U., Moon, M., Shin, J., 1970. Studies on bovine besnoitiosis in Korea II: A survey on incidence in enzootic region. The Korean Journal of Parasitology, 8(3): 76-80.
58. Hofmeyr, C. F. B., 1945. Globidiosis in Cattle. Journal of the South African Veterinary Medical Association, 16, 102-109.
59. Hornok, S., Fedák, A., Baska, F., Hofmann-Lehmann, R., Basso, W., 2014. Bovine besnoitiosis emerging in Central-Eastern Europe, Hungary. Parasites and Vectors, 7: 20, doi:10.1186/1756-3305-7-20.
60. Igbokwe, I.O., Abba, Y., Nwagbara, N.D., 2009. Prevalence of *Besnoitia* cysts in pachydermatous skin lesions of cattle in north-eastern Nigeria. Nigeria Journal of Experimental and Applied Biology, 10(1): 31-36.
61. Jacquiet, P., Boucher, C., Liénard, E., Grisez, C., Prévot, F., Salem, A., Beuscart, T., Bergeaud, J.P., Alzieu, J.P., 2009. Besnoitiose bovine: enquêtes épidémiologiques dans le sud de la France. Journées Nationales G.T.V., Nantes, 1213–1217.
62. Jacquiet, P., Lienard, E., Franc, M., 2010. Bovine besnoitiosis: Epidemiological and clinical aspects Vet Parasitol 174:30–36.
63. Janitshcheke, K., De Vos, A.J., Bigalke, R.D., 1984. Serodiagnosis of bovine besnoitiosis by ELISA and immunoflourescence tests. Onderstepoort J. vet. res. 51, 239-

64. Kiehl, E., Heydorn, A.O., Schein, E., Al-Rasheid, K.A., Selmair, J., Abdel-Ghaffar, F., Mehlhorn, H., 2010. Molecular biological comparison of different *Besnoitia* species and stages from different countries. Parasitology Research, 106: 899-894.
65. Kumi-Diaka, J., Wilson, S., Sannusi, A., Njoku, C.E., Osoru, D.I.K., 1981. Bovine besnoitiosis and its effect on male reproductive system. Theriogenology 16:523-530.
66. Legrand, P., 2003. La besnoitiose bovine en Ariège. Thèse d'exercice. Ecole Nationale Vétérinaire de Toulouse, Toulouse.
67. Leitao, J.L.D.S., 1949. Globidiose bovina por Globidium besnoiti. Rev. Med. Vet. (Portugal) 44(13), 152–156.
68. Librado, P., Rozas, J., 2009. DnaSP V5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451-1452.
69. Liénard, E., Salem, A., Grisez, C., Prévot, F., Bergeaud, J. P., Franc, M., Gottstein, B., Alzieu, J. P., Lagalisse, Y., Jacquiet, P., 2011. A longitudinal study of *Besnoitia besnoiti* infections and seasonal abundance of Stomoxys calcitrans in a dairy cattle farm of Southwest France. Veterinary Parasitology 177, 20–27. doi: 10.1016/j.vetpar.2010.11.030.
70. Liénard, E.,Salem, A., Jcquiet, P., Grisez, C., Prévot, F., Blanchard, B., Boushira, E., Franc, M., 2012. Development of a protocol testing the ability of Stomoxys calcitrans (Linnaeus, 1758) (Diptera: Muscidae) to transmit Besnoitia besnoiti (Henry, 1913) (Apicomplexa: Sarcocystidae). Parasitol. Res. 112, 479–486.
71. Maqbool, M.S., Bhat, S.A., Shah, S.N., Ganayi, B.A., Sheikh, T.A., 2012. Bovine Besnoitiosis- Impact on Profitable Cattle Production. Int. J. Livest. Res., 2(1): 78-81
72. McCully, R. M., Basson, P. A., Van Niekerk, J.W., Bigalke, R. D., 1966. Observations on *Besnoitia* cysts in the cardiovascular system of some wild antelopes and domestic cattle. Onderstepoort Journal of Veterinary Research 33, 245–276.
73. Mehlhorn, H., Klimpel, S., Schein, E., Heydorn, A.O., Al-Quraishy, S., Selmair, J., 2009. Another African disease in central Europe: Besnoitiosis of cattle. I. Light and electron microscopical study. Parasitol Res.
74. Neuman, M., 1972. Pathological changes causing sterility in bulls affected with *Besnoitia besnoiti.* J. Protozoal. 19(Suppl), 154.
75. Ng'ang'a, C. J., Kanyari, P.W., Munyua, W. K., 1994. Isolation of *Besnoitia wallacei* in Kenya. Veterinary Parasitology 52, 203–206.

76. Njagi, O.N., Ndarathi, C.M., Nyaga, P.N., Munga, L.K., 1998. An epidemic of besnoitiosis in cattle in Kenya. Onderstepoort Journal of Veterinary Research, 65, 133-136.
77. Njenga, J.M., Munyua, S., Bwangamoi, O., Kangethe, E.K., Mugera, G.M., Mutiga, E.R, Wamukoya, J., 1999. Prevalence of besnoitiosis in domestic ruminants in Kenya: a preliminary survey. JS Afr Vet Assoc 70:151–153.
78. Oduye, O.O., 1974. Occurrence of inapparent cutaneous besnoitiosis in cattle in Nigeria. Journal of the Nigerian Veterinary Medical Association, 3(2): 9-96.
79. Olias, P., Schade, B., Mehlohorn, H., 2011. Molecular pathology, taxonomy and epidemiology of *Besnoitia* species (Protozoa: Sarcocystidae). Infect Genet Evol 11: 1564-1576.
80. Oryan, A., Azizi, S., 2008. Ultrastructure and pathology of *Besnoitia caprae* in the naturally infected goats of Kerman, East of Iran. Parasitology Research 102, 1171–1176.
81. Papadopoulos, E., Arsenos, G., Ptochos, S., Katsoulos, P., Oikonomous, G., Karatzia, M.A., Karatzia, H., 2014. First report of *Besnoitia besnoiti* seropositive cattle in Greece. J Hellenic Vet. Med. Soc; 65(2): 115-120.
82. Peteshev, V.M., Galuzo, I.G., Polomoshnov, A.P., 1974. Cats-definitive hosts of *Besnoitia* (*Besnoita besnoiti*). (In Russian). Azu Akad. Nauk. Kazakh. SSRB, 1, 33-38.
83. Pols, J.W., 1960. Studies on Bovine besnoitiosis with special reference to the aetiology. Onderstepoort J. Vet. Res. 28:265-356.
84. Rommel, M., 1978. Comparative review of the developmental biology of the genera *Sarcocystis*, *Frenkelia*, *Isospora*, *Cystoisospora*, *Hammondia*, *Toxoplasma* and *Besnoitia*. Z. Parasitenkd 27; 57 (3): 269-283.
85. Sambo, S.J., Ibrahim, N.D.G., Esievo, K.A.N., Hambolu, J.O., Oladele, S.B., Sackey, A.K.B., Makoshi, S.M., 2007. Co-existence of besnoitiosis and dermatophilosis in cattle slaughtered at Zaria abattoir. Journal of Animal Veterinary Advances, 6(5): 617-620.
86. Sannusi, A., 1991. A simple field diagnostic smear test for bovine besnoitiosis. Vet. Parasitol. 39, 185–188.
87. Schares, G., Basso, W., Majzoub, M., Rostaher, A., Scharr, J.C., Langenmayer, M.C., Selmair, J., Bubey, J.P., Cortes, H.C., Conraths, F.J., Haupt, T., Pürro, M., Raeber, A., Bulholzer, P., Gollnick, N.S., 2011. Evaluation of a commercial ELISA for the specific detection of antibodies against *Besnoitia besnoiti*. Vet. Parasitol. 175, 52–59.

88. Schofield, S., Torr, S.J., 2002. A comparison of the feeding behaviour of tsetse and stable flies. Med Vet Entomol 14, 177-185.
89. Schulz, K. C., 1960. A report on naturally acquired besnoitiosis in bovines with special reference to its pathology. Journal of the South African Veterinary Medical Association 31, 21–35.
90. Sekoni, V.O., Senusi, A., Abata, M.O., Oyedipe, E.O., Rekwot, P.I., Eduvie, L.O. 1992. Loss of libido and terminal sterility in a Frisean bull naturally infected with *Besnoitia besnoiti* in Northern Nigeria: A case report. Theriogenology 37:533-549.
91. Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution. 30: 2725-2726.
92. Wallace, G.D. & Frenkel, J.K., 1975. Besnoitia species (Protozoa, Sporozoa, Toxoplasmatidae): recognition of cycle transmission by cats. Science. 1975 Apr. 25; 369-71.
93. Zacarias, J.A., 2009. Epidemiología (seroprevalencia y vectores) de la Besnoitiosis bovina de las Sierras de Urbasa y Andía. Doctoral Thesis. University of Zaragoza.

APPENDICES

Appendix 1: Summary of sampling and vaccination history

Province	Area	No. of farms	Farming type	No. animals sampled			Vaccinating	Outbreak history
				blood	skin	total	Yes / No	Yes / No
Eastern Cape	Komga	2	Small scale	85	N/S	85	Yes	No
	Tsolo	1	Commercial	55	N/S	55	No	No
Gauteng	Rust de Winter	27	Small scale and commercial	427	342	427	No	No
KwaZulu-Natal	Hluhluwe	5	Communal	20	20	20	No	No
Limpopo	Gravelotte	2	Commercial	71	14	71	Yes	Yes
	Mokopane	1	Small scale	30	N/S	30	No	No
Total		38		688	376	688	688	

N/S = not sampled

Appendix 2: P-distance matrix between *Besnoitia besnoiti* isolates as shown in figure 3

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	Vaccine Wildebeest strain																															
2	GP1	0,04																														
3	GP8	0,10	0,06																													
4	GP15	0,04	0,00	0,06																												
5	GP5	0,05	0,01	0,08	0,01																											
6	GP4	0,04	0,00	0,06	0,00	0,01																										
7	GP2	0,04	0,00	0,06	0,00	0,01	0,00																									
8	GP10	0,05	0,01	0,08	0,01	0,03	0,01	0,01																								
9	KZN2	0,03	0,01	0,08	0,01	0,03	0,01	0,01	0,03																							
10	GP3	0,12	0,14	0,21	0,14	0,15	0,14	0,14	0,15	0,13																						
11	GP11	0,08	0,06	0,08	0,06	0,05	0,06	0,06	0,05	0,05	0,18																					
12	GP16	0,12	0,13	0,19	0,13	0,14	0,13	0,13	0,14	0,12	0,22	0,17																				
13	GP14	0,06	0,05	0,09	0,05	0,04	0,05	0,05	0,04	0,04	0,17	0,01	0,15																			
14	GP13	0,21	0,21	0,23	0,21	0,22	0,21	0,21	0,22	0,22	0,24	0,23	0,26	0,23																		
15	GP12	0,04	0,00	0,06	0,00	0,01	0,00	0,00	0,01	0,01	0,14	0,06	0,13	0,05	0,21																	
16	GP17	0,09	0,10	0,17	0,10	0,12	0,10	0,10	0,12	0,09	0,19	0,14	0,03	0,13	0,23	0,10																
17	GP9	0,10	0,09	0,13	0,09	0,08	0,09	0,09	0,08	0,08	0,18	0,05	0,18	0,04	0,24	0,09	0,15															
18	KZN1	0,04	0,00	0,06	0,00	0,01	0,00	0,00	0,01	0,01	0,14	0,06	0,13	0,05	0,21	0,00	0,10	0,09														
19	GP6	0,08	0,05	0,08	0,05	0,06	0,05	0,05	0,06	0,06	0,17	0,10	0,18	0,09	0,23	0,05	0,15	0,13	0,05													
20	GP7	0,04	0,00	0,06	0,00	0,01	0,00	0,00	0,01	0,01	0,14	0,06	0,13	0,05	0,21	0,00	0,10	0,09	0,00	0,05												
21	LP1	0,22	0,18	0,24	0,18	0,19	0,18	0,18	0,19	0,19	0,29	0,24	0,27	0,23	0,28	0,18	0,24	0,27	0,18	0,23	0,18											
22	*B. besnoiti* Israel DQ227420.1	0,28	0,24	0,31	0,24	0,26	0,24	0,24	0,26	0,26	0,36	0,31	0,33	0,29	0,33	0,24	0,31	0,33	0,24	0,29	0,24	0,19										
23	*B. besnoiti* Portugal AY833646.1	0,28	0,24	0,31	0,24	0,26	0,24	0,24	0,26	0,26	0,36	0,31	0,33	0,29	0,33	0,24	0,31	0,33	0,24	0,29	0,24	0,19	0,00									
24	*B. besnoiti* Spain DQ227418.1	0,28	0,24	0,31	0,24	0,26	0,24	0,24	0,26	0,26	0,36	0,31	0,33	0,29	0,33	0,24	0,31	0,33	0,24	0,29	0,24	0,19	0,00	0,00								
25	*B. besnoiti* Bavaria FN257463.1	0,28	0,24	0,31	0,24	0,26	0,24	0,24	0,26	0,26	0,36	0,31	0,33	0,29	0,33	0,24	0,31	0,33	0,24	0,29	0,24	0,19	0,00	0,00	0,00							
26	*H. hammondi* AF096498.1	0,47	0,50	0,54	0,50	0,51	0,50	0,50	0,51	0,49	0,54	0,54	0,54	0,53	0,53	0,50	0,53	0,56	0,50	0,50	0,50	0,54	0,58	0,58	0,58	0,58						
27	*N. caninum* Liverpool U16159.1	0,50	0,47	0,54	0,47	0,47	0,47	0,47	0,49	0,47	0,50	0,51	0,51	0,50	0,50	0,47	0,49	0,51	0,47	0,51	0,47	0,50	0,50	0,50	0,50	0,50	0,65					
28	*T. gondii* L37415.1	0,45	0,47	0,51	0,47	0,49	0,47	0,47	0,49	0,46	0,50	0,51	0,51	0,50	0,50	0,47	0,50	0,54	0,47	0,47	0,47	0,51	0,55	0,55	0,55	0,55	0,08	0,62				
29	*B.besnoiti* S.A AF076859.1	0,28	0,24	0,31	0,24	0,26	0,24	0,24	0,26	0,26	0,36	0,31	0,33	0,29	0,33	0,24	0,31	0,33	0,24	0,29	0,24	0,19	0,00	0,00	0,00	0,00	0,58	0,50	0,55			
30	*B. besnoiti* Bb-Ger1 FJ797432.1	0,28	0,24	0,31	0,24	0,26	0,24	0,24	0,26	0,26	0,36	0,31	0,33	0,29	0,33	0,24	0,31	0,33	0,24	0,29	0,24	0,19	0,00	0,00	0,00	0,00	0,58	0,50	0,55	0,00		
31	*B. besnoiti* Bb-Spain1 EU789637.1	0,28	0,24	0,31	0,24	0,26	0,24	0,24	0,26	0,26	0,36	0,31	0,33	0,29	0,33	0,24	0,31	0,33	0,24	0,29	0,24	0,19	0,00	0,00	0,00	0,00	0,58	0,50	0,55	0,00	0,00	
32	*B. besnoiti* Bavaria FN257462.1	0,28	0,24	0,31	0,24	0,26	0,24	0,24	0,26	0,26	0,36	0,31	0,33	0,29	0,33	0,24	0,31	0,33	0,24	0,29	0,24	0,19	0,00	0,00	0,00	0,00	0,58	0,50	0,55	0,00	0,00	0,00

Appendix 3: P-distance matrix between *Besnoitia besnoiti* isolates as shown in figure 4

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	GP_7_Besnoitia_ITS1_F																														
2	GP_15_Besnoitia_ITS1_F	0,00																													
3	GP_8_Besnoitia_ITS1_F	0,00	0,00																												
4	GP_16_Besnoitia_ITS1_F	0,00	0,00	0,00																											
5	GP_1_Besnoitia_ITS1_F	0,00	0,00	0,00	0,00																										
6	GP_9_Besnoitia_ITS1_F	0,00	0,00	0,00	0,00	0,00																									
7	GP_17_Besnoitia_ITS1_F	0,00	0,00	0,00	0,00	0,00	0,00																								
8	GP_2_Besnoitia_ITS1_F	0,00	0,00	0,00	0,00	0,00	0,00	0,00																							
9	GP_10_Besnoitia_ITS1_F	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00																						
10	GP_18_Besnoitia_ITS1_F	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00																					
11	GP_11_Besnoitia_ITS1_F	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00																				
12	GP_19_Besnoitia_ITS1_F	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00																			
13	GP_12_Besnoitia_ITS1_F	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00																		
14	GP_20_Besnoitia_ITS1_F	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00																	
15	GP_5_Besnoitia_ITS1_F	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00																
16	GP_13_Besnoitia_ITS1_F	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00															
17	GP_6_Besnoitia_ITS1_F	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00														
18	BesnoitiabennettiJQ013812	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29													
19	BesnoitiabesnotiJF314861cattleItaly	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,00												
20	BesnoitiacapraeHM008988goatsIran	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,00	0,00											
21	FJ797432_Besnoitia_besnoiti_Germany	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,00	0,00	0,00										
22	EU789637_Besnoitia_besnoiti_Spain	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,00	0,00	0,00	0,00									
23	FN257463_Besnoitia_besnoiti	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,00	0,00	0,00	0,00	0,00								
24	FN257462_Besnoitia_besnoiti	0,31	0,31	0,31	0,31	0,31	0,31	0,31	0,31	0,31	0,31	0,31	0,31	0,31	0,31	0,31	0,31	0,31	0,02	0,02	0,02	0,02	0,02	0,02							
25	DQ227420_Besnoitia_besnoiti_Israel	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,00	0,00	0,00	0,00	0,00	0,00	0,02						
26	DQ227419_Besnoitia_besnoiti_Spain	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00					
27	DQ227418_Besnoitia_besnoiti_Spain	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00				
28	AY833646_Besnoitia_besnoiti_Portugal	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,00			
29	AY665400_Besnoitia_tarandi	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,00	0,00		
30	AY665399_Besnoitia_bennetti	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,00	0,00	0,00	
31	AF076859_Besnoitia_besnoiti	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,29	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,00	0,00	0,00	0,00

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

21 April 2015

Dr Simbarashe Chitanga
Biology Building
Westville Campus
schitanga@email.com

Dear Dr Chitanga

PROTOCOL: Perception of commercial farmers to the economic cost of *B. besnoitia* infection in cattle in selected regions of South Africa: Non-Degree. BREC REF: BE357/14.

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 14 July 2014.

The study was provisionally approved pending appropriate responses to queries raised. Your responses received on 08 April 2015 to queries raised on 24 March 2015 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval.

This approval is valid for one year from **21 April 2015.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2004), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its meeting taking place on 12 May 2015.

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

OFFICE USE ONLY

Ref	Date

UNIVERSITY OF KWAZULU-NATAL ETHICS COMMITTEE

ANIMAL ETHICS SUB-COMMITTEE

APPLICATION FOR APPROVAL OF RESEARCH PROTOCOLS USING ANIMALS

Please note that approval must be obtained for ALL work involving animals irrespective of the source of funding.

This form is to be completed in typescript and one signed, hard copy submitted to Animal Ethics Administration, School of Life Sciences, Rm 105, John Bews Building, Pietermaritzburg Campus AND an electronic copy submitted to animalethics@ukzn.ac.za. Please enter your surname between the marks at the top of each page.

1. TITLE OF PROJECT

Determination of the prevalence, distribution, economic impact and risk factors for bovine besnoitiosis in South Africa

2. DETAILS OF APPLICANT

2.1 Title (e.g. Dr): Dr

2.2 Surname: Ndudane

2.3 Full name: Nandipha Toyota

2.4 Qualifications: MSc Animal Pathology, BVMCh, BSc Agric

2.5 Position: PhD student

2.6 School: Biological Sciences 2.7 Campus: Westville

2.8 Internal mailing address for hard copy of approval letter: Prof Mukaratirwa's office

2.9 Tel ext.: 0724300970 2.10 Fax: 0867151481 2.11Email: ndudane@hotmail.com

3. STAFF, RESEARCH ASSOCIATES, STUDENTS AND TECHNICIANS AUTHORISED TO CARRY OUT THE PROPOSED HANDS-ON ANIMAL STUDIES.

Name (initials and surname)	Academic qualification	Animal training
S. Mukaratirwa	PhD, MSc, BVM	Veterinarian
S. Chitanga	PhD, MSc, BVSc	Veterinarian
T. Chimatira	BVSc	Veterinarian
P. Malatje	BSC	Scientist
L. Gcanga	BSc Hons (Parasitology), BSc Biol	Scientist